

Mechanisms underlying statin-induced modulation of ryanodine receptors



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Abstract

Statins, a class of HMG-CoA reductase inhibitors, are amongst the most widely prescribed drugs for the treatment of elevated cholesterol. Despite their success, their use is associated with debilitating muscle side effects such as myopathy, myalgia and in the most serious cases, rhabdomyolysis. These effects have limited the ability of many patients to tolerate statin treatment. There is mounting evidence suggesting that statins cause a dysfunction of cellular Ca^{2+} -release in skeletal muscle, which results in muscle damage and myopathy.

In this thesis, I have investigated, using single-channel techniques, how statins interact with the skeletal ryanodine receptor (RyR1), the channel which acts as the pathway for Ca^{2+} -release in skeletal muscle. I have shown that statins as a class, activate RyR1 in a concentration-dependent, reversible manner. These effects were seen at nanomolar levels, which correspond to concentrations found in the skeletal muscle during statin treatment. In contrast, statin effects on the cardiac isoform (RyR2) were more complex, causing inhibition at low concentrations, and activation at higher concentrations, a finding that may be clinically important given the reports that statins reduce instances of arrhythmia.

To investigate if the ability of statins to bind to RyR1 can be reduced without affecting their binding HMG-CoA reductase, the statin minimal pharmacophore was synthesised. It was found not to activate RyR1, indicating that the activation could be separated from the effect on their intended target. Subsequently, single-channel experiments indicated that the RyR1 ATP-site is a plausible location for statin binding, and a molecular modelling study utilising Autodock software revealed the key structural features required for binding to this site. This facilitated the identification of statin analogues with no effect on RyR1, but potent HMG-CoA reductase inhibition.

In summary, my experiments have revealed that statins can potentially alter the activity of cardiac and skeletal RyR channels by distinct gating mechanisms. I suggest that these interactions of statins with RyR channels may contribute to the statin-induced changes in muscle Ca^{2+} -release that are reported in the literature and hence may be the main underlying cause of statin-induced myopathy. In view of this hypothesis, I have screened a number of atorvastatin analogues and identified new statin compounds devoid of the ability to activate RyR1. This work will provide a firm foundation for future work investigating the usefulness of these compounds as lipid lowering agents with reduced side effects.

Acknowledgments

Well here we are.

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Chris Lindsay

1 October 2019

Authors Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated my specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED DATE.....

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Abbreviations

[Ca ²⁺] _i	intracellular Ca ²⁺ concentration
4cmc	4-chloro-m-cresol
AcOH	acetic acid
ADP	adenosine diphosphate
AF	atrial fibrillation
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMP	adenosine monophosphate
AMP-PCP	β,γ-Methyleneadenosine 5'-triphosphate disodium salt
ANOVA	analysis of variance
Ator-H	atorvastatin sodium salt
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
BzATP	(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate
cADPR	cyclic adenosine 5'-diphosphate ribose
CaM	calmodulin
CCD	central core disease
ChCl	choline chloride
CICR	Ca ²⁺ -induced-Ca ²⁺ -release
CK	creatine kinase
CPVT	catecholaminergic polymorphic ventricular tachycardia
Csol	core solenoid
CSQ	calsequestrin
CTD	C-terminal domain
CTP	cytosine triphosphate
CVD	cardiovascular disease
CYP[450]	cytochromes P[450]
DHPR	dihydropyridine receptor
DIDs	diisothiocyanostilbene-2',2'-disulfonic acid
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMAP	4-dimethylaminopyridine

DTT	dithiothreitol
EC-coupling	excitation-contraction coupling
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
EM	electron microscopy
ER	endoplasmic reticulum
FKBP	FK506-binding protein
GTP	guanosine triphosphate
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HF	heart failure
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HSR	heavy sarcoplasmic reticulum
IP ₃ R	inositol 1,4,5-triphosphate receptor
KO	knockout
LC-MS	liquid chromatography-mass spectrometry
LDL	low density lipoprotein
Log D	distribution coefficient
Log P	partition coefficient
LSR	light sarcoplasmic reticulum
MH	malignant hyperthermia
MM	mixed membrane
NCX	Na ⁺ /Ca ²⁺ exchanger
NTD	N-terminal domain
OATP	organic-anion-transporting polypeptide
PCC	pyridinium chlorochromate
PE	phosphatidylethanolamine
Pi	phosphate
PIPES	piperazine-1,4-Bis-2-ethanesulfonic acid
PKA	protein kinase A
PLE	pig liver esterase
PMSF	phenylmethanesulfonyl fluoride
Po	open probability

PPi	pyrophosphate
ppm	parts per million
PPPi	triphosphate
Pyr	pyridine
R ²	pearson correlation coefficient
RyR	ryanodine receptor
RyR1	ryanodine receptor type 1 (skeletal isoform)
RyR2	ryanodine receptor type 2 (cardiac isoform)
RyR3	ryanodine receptor type 3
SD	standard deviation
SEM	standard error of the mean
SERCA	sarcoplasmic/endoplasmic reticulum Ca ²⁺ -ATPase
Sim –H	simvastatin sodium salt
Sim-L	simvastatin lactone
SR	sarcoplasmic reticulum
TAE	(+)trifluoroanthranyl alcohol
TAST	tris(dimethylamino)sulfonium difluorotrimethylsilicate
TBAF	tetra- <i>N</i> -butylammonium fluoride
TBDPS	tert-Butyldiphenylsilyl
TBHP	<i>tert</i> -butyl hydroperoxide
Tris	tris(hydroxymethyl)methylamine
VA	ventricular arrhythmia
VT	ventricular tachycardia

List of Publications

Full papers

Quantitative RyR1 reduction and loss of calcium sensitivity of RyR1Q1970fsX16+A4329D cause cores and loss of muscle strength Elbaz M, Ruiz A, Bachmann C, Eckhardt J, Pelczar P, Venturi E, **Lindsay C**, Wilson AD, Alhussni A, Humberstone T, Pietrangelo L, Boncompagni S⁵, Sitsapesan R, Treves S, Zorzato F. *Hum Mol Genet.* 2019 Sep 15; 28(18):2987-2999

Promiscuous attraction of ligands within the ATP binding site of RyR2 promotes diverse gating behavior **Lindsay, C.** Sitsapesan, M. Mun Chan, W. Venturi, E. Welch, W. Musgaard, M. Sitsapesan, R. *Scientific Reports* **8**, 15011 (2018)

Simvastatin activates single skeletal RyR1 channels but exerts more complex regulation of the cardiac RyR2 isoform. Venturi, E*. **Lindsay, C***. Lotteau, S. Yang, Z. Steer, E. Witschas, K. Wilson, A. Wickens, J. Russell, A. Steele, D. Calaghan, S. Sitsapesan, R. *British Journal of Pharmacology* 175 (6). pp. 938-952

****equal first author contribution***

Dampened activity of ryanodine receptor channels in mutant skeletal muscle lacking TRIC-A. El-Ajouz, S., Venturi, E., Witschas, K., Beech, M., Wilson, A. D., **Lindsay, C.**, Eberhardt, D., O'Brien, F., Iida, T., Nishi, M., Takeshima, H. and Sitsapesan, R. (2017), *J Physiol*, 595: 4769–4784.

Published abstracts

Modelling the ATP Binding Site of RyR2 to Rationalise Ligand-Induced Gating Behaviour. **Lindsay, C.** Sitsapesan, M. Mun Chan, W. Venturi, E. Welch, W. Musgaard, M. Sitsapesan, R. Abstract selected for oral presentation at Biophysical Society 63rd Annual Meeting in Baltimore (March 2019) *BIOPHYSICAL JOURNAL*. 116: 42A. 15 Feb 2019

Statins bind to cardiac ryanodine receptor (RyR2) channels to alter opening frequency. Wilson, D. **Lindsay, C.*** Venturi, E. Russell, A. Sitsapesan, R. Abstract presented at 2017 British Heart Foundation Oxford CRE Annual Symposium

Atorvastatin Activates Skeletal RyR1 Channels: Towards Reducing Statin Side-Effects **Lindsay, C.*** Wilson, D. Venturi, E. Russell, A. Sitsapesan, R. *Biophysical Society Meeting Abstracts. Biophysc. J., Supplement* 114(2) pp. 470A

Statins bind to cardiac ryanodine receptor (RyR2) channels to alter opening frequency. Wilson, D. **Lindsay, C.** Venturi, E. Russell, A. Sitsapesan, R. *Biophysical Society Meeting Abstracts. Biophysc. J., Supplement* 114(2) pp. 621A-622

Atorvastatin activates skeletal ryanodine receptors: Design of next-generation statins **Lindsay, C***. Wilson, D. Venturi, E. Russell, A. Sitsapesan, R. *Proceedings of the British Pharmacological Society*. Abstract presented at British Pharmacological Society meeting 'Pharmacology Futures 2018 – exploring future drug development' in Edinburgh, UK, May 2018.

1

Introduction

1.1 Statins

1.1.1 Setting the scene

Statins are amongst the mostly widely prescribed drugs worldwide. They are the gold standard treatment for patients with elevated cholesterol levels which put them at significant risk of developing cardiovascular disease.¹ First discovered in 1970 by serendipity, they are competitive inhibitors of one of the key enzymes responsible for the synthesis of cholesterol in the body (**Figure 1.1**). With current estimates that one billion people worldwide may be taking statin treatment by 2020², they are undoubtedly one of the greatest success stories of modern medicine. Despite this efficiency at reducing plasma cholesterol, their widespread use has been plagued with muscle-related side effects, ranging from mild pains to fatal rhabdomyolysis which have limited their use for many patients.³ This chapter will introduce statins, how they came to be developed, and the side-effects which have restricted them. I will then introduce the hypothesis which has led to the work outlined in this thesis, the ryanodine receptor (RyR) and how this ion channel may hold the key to the development of the next generation of statin drugs.

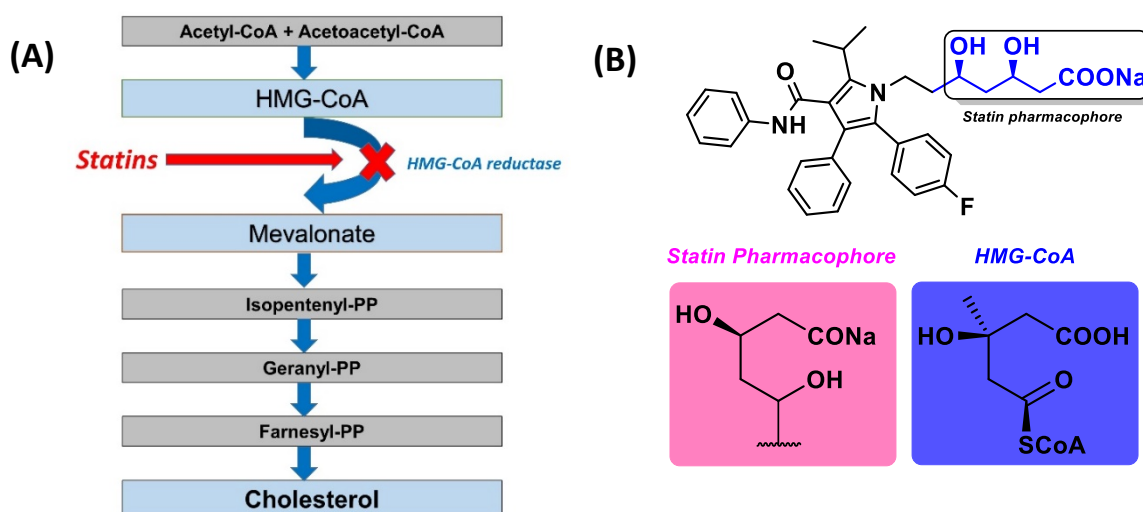


Figure 1.1 Statins are competitive inhibitors of HMG-CoA reductase

(A) Statins inhibit HMG-CoA reductase, one of the key enzymes responsible for the synthesis of precursors to cholesterol. **(B)** The statin pharmacophore mimics HMG-CoA, allowing statins to act as a competitive inhibitor.

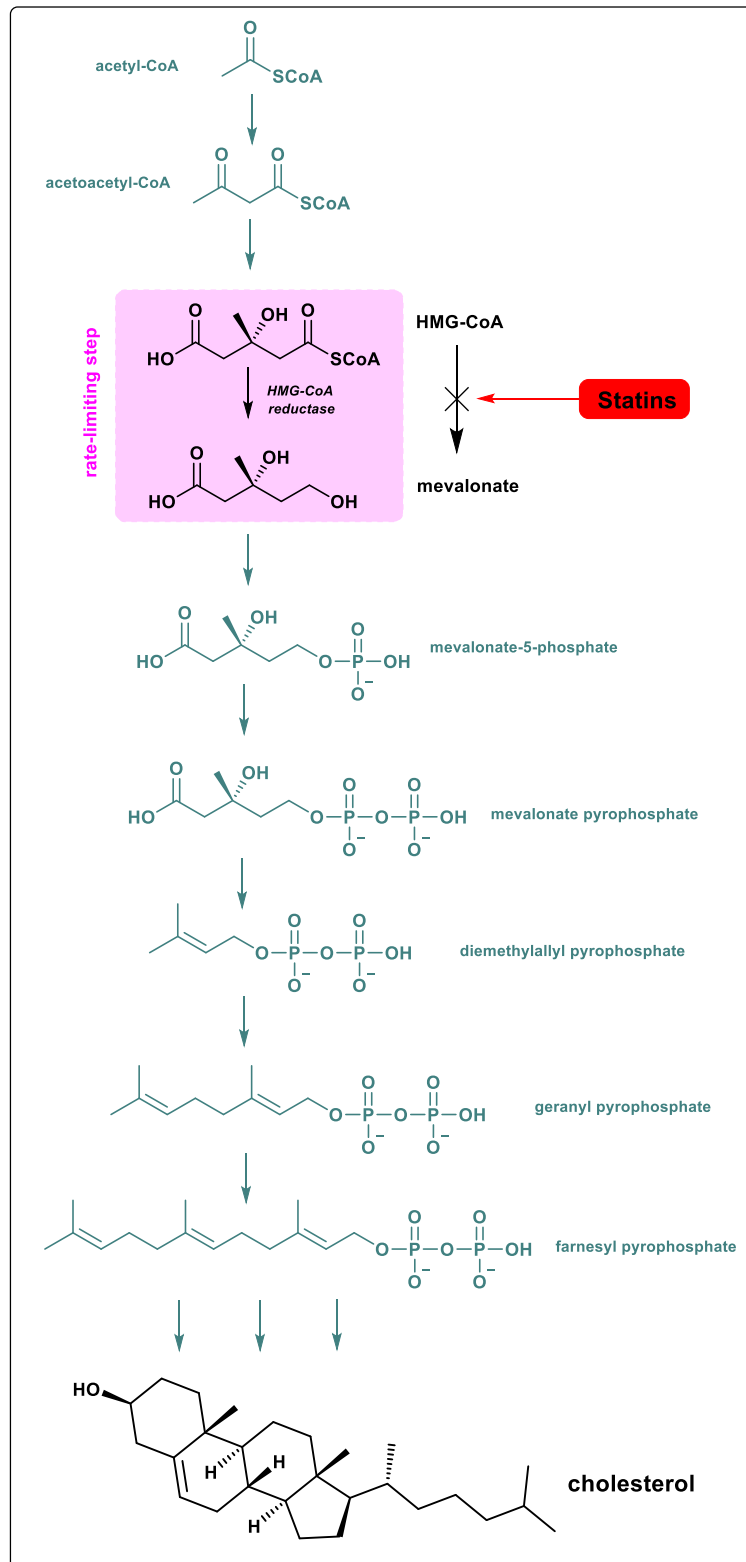
1.1.2 History and development of statins

1.1.2.1 *The Lipid Hypothesis*

In 1856, the German pathologist Rubert Virchow first observed a common feature that appeared to be present in a group of patients who had died from acute myocardial infarction. Virchow found that the artery walls of these patients were often thickened and irregular, and were coated with a thick, yellowish substance that would later be named cholesterol.⁴ It was later found that feeding rabbits with cholesterol could also lead to the formation of the same phenotype, with the rabbits developing similar arterial fatty deposits.⁵ However, it was not until much later, that the link between plasma cholesterol levels and coronary heart disease became accepted. This became known as the *Lipid Hypothesis*. It remained controversial for so long, due to a lack of clear evidence that lowering a patient's cholesterol levels resulted in a measurable clinic benefit. Some small studies had observed a decrease in cardiovascular events when cholesterol was lowered by dietary intervention.⁶ Eventually by 1984, enough evidence had accumulated from various clinical trials⁷ to persuade the National Institutes of Health to initiate a programme of education amongst physicians about the importance of treating hypercholesterolaemia.⁸ However, for many patients, dietary adjustments were not sufficient, and an alternative approach would be needed for successful treatment.⁹

1.1.2.2 *HMG-CoA reductase*

More than 30 enzymes are involved in the complex pathway known as the *mevalonate pathway* which leads to synthesis of cholesterol (**Figure 1.2**). The rate-determining steps of this process is the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) into mevalonate, which is catalysed by the enzyme HMG-CoA reductase.¹⁰ Inhibitors of this enzyme (such as statins) increase the expression of low density lipoprotein (LDL) receptors in the liver the major site of cholesterol synthesis, increasing the rate of removal of cholesterol from the bloodstream, and an overall reduction in plasma cholesterol concentration.¹¹

**Figure 1.2 The mevalonate pathway**

A simplified outline of the pathway for cholesterol synthesis is shown. Statins inhibit HMG-CoA reductase which catalyses the conversion of HMG-CoA into mevalonate as shown.

1.1.2.3 *The discovery of statins*

Given that the route to cholesterol synthesis is such an intricate and lengthy pathway, the first attempts at producing an inhibitor of this process focused on the late steps. In 1959, the first drug targeting cholesterol synthesis was patented by Richardson-Merrell, and was known as triparanol.¹² This drug was a 2,4-dehydrocholesterol reductase inhibitor, targeting the final step of the synthesis, the conversion of desmosterol into cholesterol.¹³ This proved to be nothing short of a disaster. Triparanol resulted in desmosterol accumulating in the tissue resulting in severe adverse effects, including skin disorders and cataracts.¹⁴ This approach was swiftly abandoned, and clearly an alternative strategy was needed.

The story of statins began in Tokyo, Japan in the lab of Akira Endo, a Japanese microbiologist. Endo had been working on a project in search of new antibacterial agents^{15,16}. From a fermentation broth of *Penicillium citrinum*, Endo isolated various natural product compounds which appeared to inhibit HMG-CoA reductase, and so inhibited cholesterol synthesis. Amongst them were ML236A, ML236B (later known as compactin) and ML236C. He hypothesised that since bacterial cell walls require cholesterol to develop, inhibiting this process would likely slow bacterial growth and replication. This turned out to be unsuccessful, and no HMG-CoA inhibitor had antibiotic activity in his experiments.¹⁷ However, Endo quickly realised that a drug reducing cholesterol synthesis could be very useful therapeutically. These compounds became known as *statins* and were to become the most widely used drugs in human history. In contrast to the previous drugs targeting cholesterol synthesis, statins targeted the early stages of cholesterol synthesis. In particular, the target for statins (HMG-CoA reductase) converts HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) into mevalonate (**Figure 1.3**). Unlike desmosterol and the compounds in the later steps of the pathway, hydroxymethylglutarate itself is highly water-soluble, and therefore does not accumulate in the tissue in the same way. These smaller molecules which build up, are innocuous and are easily excreted from the body.

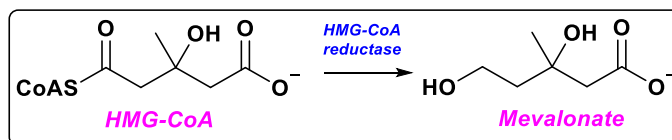


Figure 1.3 Conversion of HMG-CoA into mevalonate

1.1.3 Statin structure

Following the discovery of compactin, statins became widely marketed; Merck's Meacor (Lovastatin) made it to the market in 1987, Zocor (simvastatin) in 1991 from Pfizer, Pravachol (pravastatin) in 1991 and Lescol (fluvastatin) in March 1994. Finally, Lipitor (atorvastatin) was approved for use in 1996 and Crestor (rosuvastatin) in 2003. As of 2019, five of these statins are approved for use in the UK: atorvastatin, fluvastatin, pravastatin, rosuvastatin and simvastatin. The structures of these statins are shown in **Figure 1.4**. It is often helpful to classify statins as either type 1 or type 2 based upon their structure. This grouping was first used by Istvan et al.¹⁸ where type 1 statins are those based upon the decalin-type core (such as simvastatin and pravastatin) and type 2 are those which are not (such as atorvastatin and fluvastatin for example). All statins share a 'HMG-CoA-like' 3,5-dihydroxyhept-6-enoic acid unit which acts as the pharmacophore. Type 2 statins also have in common a fluorophenyl group. In 2001 Istvan and Deisenhofer reported crystal structures for the catalytic portion of the HMG-CoA reductase enzyme, complexed with different statin molecules¹⁸. Unsurprisingly, they found that the statins occupy the same binding site of the enzyme as the natural substrate, HMG-CoA, thus blocking its access. However, more importantly this work provided insight into the interactions between the statin molecules and HMG-CoA reductase. A figure from this work is reproduced as **Figure 1.5**, and the important binding interactions are highlighted. The pharmacophore forms a large number of hydrogen bonds with the cis loop of the HMG-CoA reductase binding site, most notably between S684, D690, K691 and K692. Additionally, the terminal carboxyl group of the pharmacophore appears to form a salt bridge with the K735 residue.¹⁸

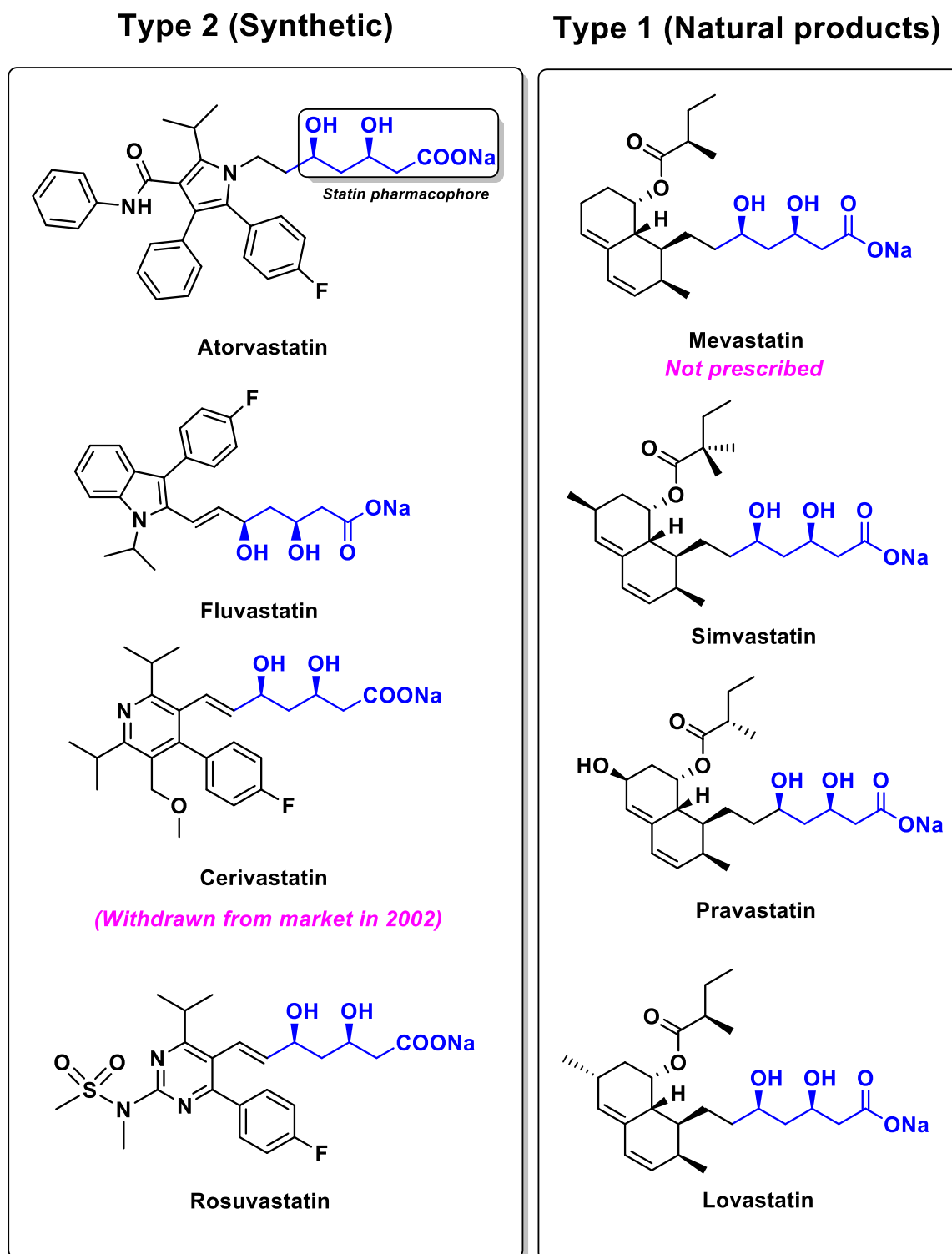


Figure 1.4 Structures of statin drugs

The structures of commonly prescribed statins can be grouped into either type 1 (based on a decalin core) or type 2 (fully synthetic). The HMG-CoA reductase pharmacophore is indicated in blue.

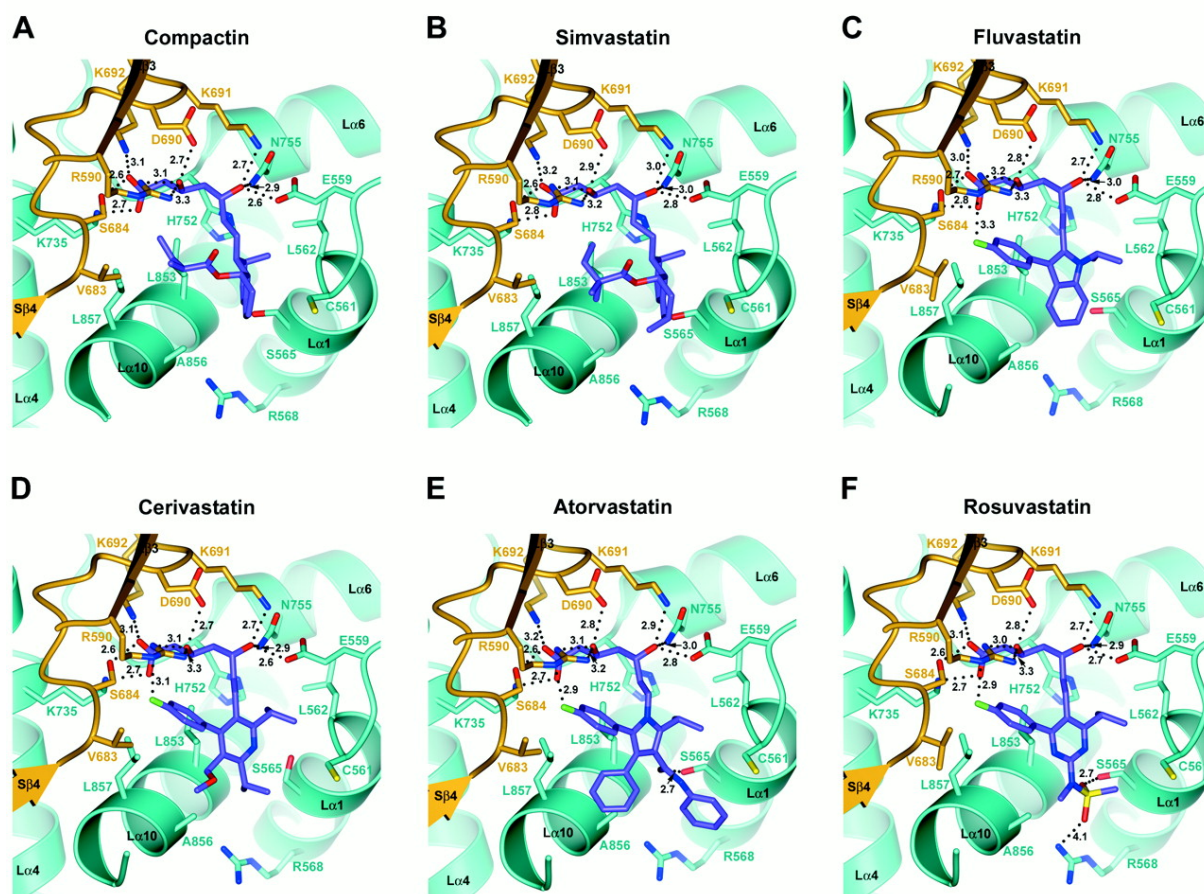


Figure 1.5 Mode of binding of commonly prescribed statin to human HMGR, adapted from Istvan *et al* (2002).

The binding modes of compactin (A), simvastatin (B), fluvastatin (C), cerivastatin (D), atorvastatin (E), and rosuvastatin (F) are shown, as determined from the relevant crystal structures by Istvan *et al* (2002). Important interactions are represented by dotted lines, and the adjacent numbers refer to the bond length in Å.

Additionally, we can see the reason for the addition of a fluorophenyl group, as when the pharmacophore is bound, this group is positioned for a stacking interaction with R590, as well as internal hydrogen bonding with the carboxylate tail of the statin itself. Rosuvastatin is alone in having an additional sulfone group, and it appears this group is ideally positioned to interact favourably with the R568 and S565. This additional interaction may explain why rosuvastatin has the highest affinity of all statins for binding to HMG-CoA reductase.¹⁹

1.1.4 Pharmacology of Statins

We have seen that statins inhibit the enzyme responsible for the synthesis of mevalonate, HMG-CoA reductase, involved in the rate limiting step in cholesterol synthesis. The affinity of statins for HMG-CoA is some 8000 times higher than that of the natural substrate, and many statins have IC_{50} values of < 5 nM.²⁰ This inhibition, not only slows the production of cholesterol, but also results in increased expression of LDL-receptors, proteins responsible for removal of cholesterol rich low-density lipoprotein (LDL) by endocytosis. This is the result of cells in the liver (where HMG-CoA reductase is extensively expressed) sensing the reduced cholesterol levels and compensating by up-regulation of LDL-receptors to remove cholesterol from blood circulation and return it to the liver. The result is a reduction in LDL plasma concentration.^{21,22}

1.1.4.1 *Statin pharmacokinetics*

The family of statin drugs are classed as either lipophilic, or hydrophilic based on their log D (**Figure 1.6**). Cerivastatin is the most lipophilic statin, and simvastatin, fluvastatin and atorvastatin are also considered relatively lipophilic while rosuvastatin and pravastatin are more hydrophilic.²⁰ Indeed, the lactone 'prodrug' form of the statins are almost three orders of magnitude more lipophilic than their respective open acid/salt forms.²³ This relatively high lipophilicity of statins results in a low bioavailability, and extensive first pass metabolism.²⁴ All of the statin drugs are orally bioavailable, and are prescribed at between 10-80 mg per day depending on whether the treatment is for primary or secondary prevention of cardiovascular events.² Pravastatin is the only statin which is not significantly bound to plasma proteins²⁵, while the other statins are generally bound to plasma proteins at high levels (e.g. simvastatin $> 95\%$, fluvastatin $> 99\%$). Lipophilic statins can easily cross cell membranes by passive diffusion, whereas the more hydrophilic statins require active transportation cross cell membranes by organic anion transporters. Pravastatin, rosuvastatin and atorvastatin have all been found to be substrates of the human OATP1B1 transporter which actively transports

them into cells^{26–28}. Cerivastatin in particular is both rapidly taken up by cells through diffusion, and can also accumulate in cells, via active transport.²⁹ Since these transporters are generally selective for the open acid forms of statins, over the lactone forms (and the forms are in equilibrium in the cell), the result is that statins accumulate inside the cells, as the lactone form cannot be transported out.³⁰

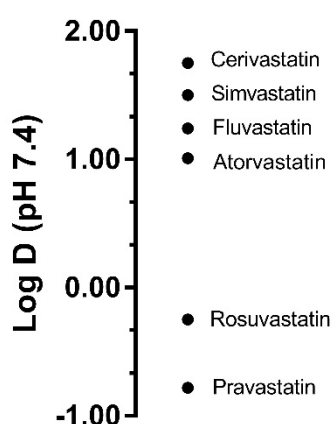


Figure 1.6 Log D of statins at pH 7.4.

Relative lipophilicity of statins expressed at log D measured at pH 7.4 (Sitori, 2014)

All statins have relatively short half-lives ($t_{1/2}$) *in vivo* of between 0.7 and 3 hours. They are metabolised by the cytochrome P450 family of enzymes, simvastatin and atorvastatin are predominantly metabolised by CYP3A4, fluvastatin by CYP2C9 and cerivastatin by both CYP2C8 and CYP2C9.^{31–35} The more hydrophilic statins pravastatin and rosuvastatin undergo relatively little metabolism, and are excreted directly.^{19,35} Metabolism by P450 enzymes, makes statins highly susceptible to drug-drug interactions, when they are administered with other drugs inhibiting these enzymes. One of the best characterised is gemfibrozil, a fibrate often prescribed in conjunction with statins. Other such examples include, clarithromycin, nelfinavir, verapamil, fluconazole and grapefruit juice.^{36–39}

1.1.4.2 *Clinical effectiveness of Statins*

There is strong evidence that statin treatment, reducing the circulating levels of LDL plasma cholesterol, is effective for reducing mortality from cardiovascular disease (CVD). In the UK statins are prescribed in general to patients who either have CVD or who have a risk greater than 10% of developing it in the next 10 years, patients diagnosed with diabetes mellitus, or those over 40 years of age with a blood LDL level of > 4.9 mmol/L. For most patients, 'low intensity treatment' with 20 mg of atorvastatin is the first line of treatment prescribed. The 'intensity' of statin treatment to be prescribed to a patient, is dependent upon the level of LDL reduction desired. Low intensity dosages achieve around 30% LDL reductions, moderate intensity (e.g. 40 mg atorvastatin) achieve around 50% and high-intensity (80 mg atorvastatin) > 50%. For all statins, doubling the dose given to patient results in a 6% decrease in LDL levels.⁴⁰ In general, the level of LDL reduction a statin produces is independent of the pre-treatment levels, and therefore the greatest absolute reductions in LDL levels are seen for patients where it is already elevated.⁴¹ In the UK, it is now recommended that atorvastatin is prescribed in the first instance, with other statins being utilised in cases of patient intolerance. The evidence for the efficacy of statins comes from an extensive history of randomised, placebo-controlled clinical trials.⁴² A meta-analysis of 26 trials, including approximately 170,000 patients in total, found that statins treatment significantly reduced major vascular events, incidences of cardiovascular disease, and total mortality.^{43–47} Trials of more intensive statin therapy have shown that further reductions in LDL appear to increase the beneficial outcomes.⁴⁸ Overall, it appears that lowering LDL levels by 1 mmol/L corresponds to a 20% reduction in cardiovascular events, and a 12% decrease in mortality rate. These risks are further reduced for each subsequent year of statin treatment.⁴⁰ However this does not appear to be case the case for all patients, particularly those suffering from heart failure with reduced injection fraction, where statin treatment did not appear effective at reducing mortality rates.^{49,50}

1.1.5 Statin side effects

While the effectiveness of statins at lowering plasma cholesterol is beyond doubt, and they have enjoyed wide success, they are associated with a number of muscular side effects which have precluded their use in certain contexts.⁵¹ Statins have been reported to result in skeletal myopathy, including myalgias and muscle pain, myositis and in very severe cases, rhabdomyolysis.⁵² The side effects of statins are often evaluated by raised levels of the protein creatine kinase (CK) which is associated with muscle damage. In general 'myopathy' refers to any disease of the muscle, myalgia refers to muscle pain without an elevation in CK and myositis refers to that with elevated CK. In the most serious cases, the damaged skeletal muscle may breakdown into the bloodstream, in a process known as rhabdomyolysis, associated with marked (> 10 times normal) CK levels.⁴⁰ This condition can lead to kidney failure and can be fatal.⁵³

Observational studies have suggested that the incidence of side-effect is as high as 30%. The occurrence of statin side effects are a matter of intense debate, and it is clear that the reports from observational trials are higher than those from placebo controlled trials, which is not surprising.⁴⁸ More controlled trials have shown that the incidence of myopathy is closer to 1%, with 1 in 10,000 suffering from rhabdomyolysis. However, co-treatment with drugs such as gemfibrozil, which increase the plasma concentration of statins, also increase the prevalence of side effects. The PRIMO trial of statin therapy found that of 7924 patients treated with atorvastatin or simvastatin, muscle symptoms were found in 10.5% of patients.⁵⁴ A similar trial including placebo (STOMP) found a similar level of incidence.⁵⁵ In the majority of patients, cessation of statin treatment and washout from tissue appears to rescue symptoms. Statin side-effects do not appear to be correlated with the potency of individual statins against HMG-CoA reductase, or with reduction in LDL cholesterol. There are many suggested hypotheses for the mechanism of action of statin side effects, including mitochondrial dysfunction, ATP depletion, oxidative stress etc.^{56–58} However more recently, statins have been shown to trigger

Ca²⁺-release in skeletal muscle fibres, a phenomenon which will be investigated in this thesis as a possible explanation for the side effects that statins generate.

1.1.5.1 *Cerivastatin*

One statin drug has been omitted from this list, cerivastatin, and for good reason. Cerivastatin is a pyridine-based statin drug, originally marketed by Bayer as a competitor against the highly successful atorvastatin. Although cerivastatin was initially well tolerated, and had shown no propensity towards causing muscle side effects during clinical trials, post-market surveillance revealed that the drug was associated with severe side-effects in some patients. By the time cerivastatin was finally withdrawn from the market, there were 52 deaths from rhabdomyolysis attributed to treatment with the drug.^{59–61} Post-market surveillance revealed that the risk of rhabdomyolysis was up to 80 times higher with cerivastatin than with other statins.⁶² The reason for this does not appear to be related to efficacy at reducing LDL cholesterol, as cerivastatin appears to have a comparable potency against HMG-CoA to other statins. It therefore appears that the enhanced side effects may be the result of cerivastatin's high lipophilicity, penetrating muscle tissue to a greater extent than other statins.⁶³

1.1.5.2 *Cardiovascular effects*

Besides their effects on plasma cholesterol, there is evidence that statins provide a variety of additional cardiovascular benefits. Since statins by definition are prescribed to individuals who are at increased risk of suffering from a cardiovascular event, it is important to understand how statins may affect cardiac function. There is increasing evidence that statins inhibit the development of ventricular arrhythmias (VA), particularly in patients who have suffered an acute myocardial infarction.⁶⁴ Further evidence comes from the fact that early withdrawal of statin therapy in these patients lead to increased rates of post-infarction arrhythmias.⁶⁵ Statins appear to be particularly effective at reducing episodes of VA in patients with diabetes, a disease commonly associated with elevated cholesterol levels, which brings with it an increased risk of cardiovascular disease.^{66,67} Colhoun and colleagues reported in a placebo

controlled trial of 2838 patients being given either 10 mg atorvastatin, or placebo, a 27% decrease in mortality rate and a 36% decrease in 'coronary heart disease events'.^{41,68} A large clinical trial of 126,128 patients who had been hospitalised following myocardial infarction, found that patients who began statin treatment within 24 hours of admission had a significantly reduced mortality rate, and a lower incidence of arrhythmia development.⁶⁵ Other reports from patients treated with statins following a cardiovascular event have shown similar beneficial endpoints.^{69,70} Therefore statins appear to be beneficial both if administered following a cardiovascular event (secondary prevention) and if given as a preventative measure (primary prevention). These results are also seen in animal models, such as reported by Lazar *et al.* (2003) which indicated that pretreatment of pigs with 40 mg of atorvastatin reduced episodes of VA following myocardial infarction.⁷¹ Similar observations have been made in other species, with other statin drugs, such as pravastatin treatment reducing the incidence of ischemia-induced VA in rats.⁷²

It is less clear whether statins are effective in reducing other forms of arrhythmia, such as atrial fibrillation (AF), due to a lack of studies. Some observational studies had suggested that statins may also provide a protective role against AF. For example, Young-Xu *et al.* (2003) followed 449 patients with coronary arterial disease for 5 years, and found that statin treatment significantly reduced risk of developing AF.⁷³ These benefits were also found to be independent of cholesterol lowering. Later, randomised clinical trials appeared to collaborate these findings, such as the ARMYDA¹ trial which investigated the effect of 40 mg atorvastatin in 200 patients undergoing cardiac elective surgery. It was found that statin treatment significantly decreased the incidence of post-operative AF relative to the placebo group.⁷⁴ Other studies found that incidences of AF that occur during statin treatment are of shorter duration than in placebo group.^{75,76} Taken together, there is compelling evidence that statin treatment

¹ Atorvastatin for Reduction of Post-operative Atrial Fibrillation in Patients Undergoing Cardiac Surgery (ARMYDA).

is beneficial for the prevention of cardiovascular events, and both NICE and the American College of Cardiology recommend that statin therapy should be prescribed to reduce the risk of VA in particular, to patients at increased risk.^{2,77}

1.1.6 The mechanism of statin side-effects

With the widespread use of statins, much effort has been dedicated to understanding the mechanism of these side-effects. The mechanisms underlying this toxicity have not yet been fully elucidated, and a complete explanation is still needed. One proposed explanation, has been linked directly to their inhibition of cholesterol synthesis, and the subsequent depletion of downstream metabolites, such as farnesyl pyrophosphate.⁵² However, although all statins are associated with muscle side effects, their occurrence does not appear to be correlated either with the potency of the individual statin for inhibition of HMG-CoA, or with the degree of success with which this is achieved in the individual patient.³ This would suggest that the side-effects are the result of some other mechanism. It would also appear to rule out the direct reduction of cholesterol content of cell membranes.⁷⁸ Other mechanisms proposed include, the inhibition of mitochondrial respiration inducing their dysfunction⁷⁹ or induction of cellular apoptosis^{80,81}, although these effects have been observed *in vitro* only at relatively high (micromolar) concentrations. A common theme amongst the explanations proposed, is impaired Ca^{2+} signalling in the skeletal muscle.⁸² It has been reported that cerivastatin can stimulate the release of Ca^{2+} from the sarcoplasmic reticulum (SR) vesicles, and this release could be inhibited by co-treatment with ryanodine.⁸³ Statin-induced SR Ca^{2+} -release has also been observed in muscle fibres.^{84,85} We therefore hypothesised that a dysregulation of skeletal muscle Ca^{2+} -release, through modulating the ion-channel that serves as the pathway for SR Ca^{2+} -release, RyR1, could be implicated in statin-induced myopathy.

1.2 Excitation-contraction coupling in skeletal and cardiac muscle

In order to fully understand the effects that statins may have in skeletal and cardiac muscle, we must first introduce the fundamental mechanism that drives muscular contraction; excitation-contraction coupling (EC coupling).

1.2.1 Ca^{2+}

Without doubt Ca^{2+} is the most common secondary messenger in the human body regulating a wide variety of cellular processes. Second messengers are molecules or ions released by a cell to facilitate or trigger a physiological process.⁸⁶ Neuronal transmission, transcription, mitochondrial function, fertilisation, and (most importantly for this work) muscle contraction are all regulated by rises and falls in Ca^{2+} .⁸⁷ In the muscle, EC coupling is the mechanism by which cardiac and skeletal myocytes allow Ca^{2+} to be released to bind to the contractile machinery of the cell, and sequestered to facilitate muscle relaxation.^{88–92} The exact mechanisms employed in skeletal and cardiac muscle respectively have some important differences, and therefore we will consider them in turn.

1.2.1.1 *Cardiac muscle*

We start by considering the role of Ca^{2+} in EC coupling in the heart. The cascade of processes is triggered by an action potential, which propagates in cardiac muscle from the sino-atrial node throughout the heart so that the atria and ventricles contract in syncytium.⁹³ Depolarisation of the plasma membrane of the muscle cells results in activation of the voltage-gated L-type Ca^{2+} -channels (LTCC) which allow Ca^{2+} influx into the cytosol of each cardiomyocyte, and into the confined space between the LTCC and nearby SR, known as the dyadic cleft.⁹⁴ The result is a rise in $[\text{Ca}^{2+}]_i$ from approximately 100 nM to 10–100 μM in this microdomain.⁹⁵ This localised rise in cytoplasmic Ca^{2+} , ($[\text{Ca}^{2+}]_i$) causes activation of the RyR2 channels located in the SR membrane, which results in the channel opening and allows larger amount of Ca^{2+} to be released from the SR Ca^{2+} -store in a graded manner.⁹⁶ This process is known as *calcium-induced-calcium-release* (CICR). The subsequent Ca^{2+} -release allows Ca^{2+} to

bind to troponin C resulting in a conformational change which results in muscle contraction.⁹⁶ To allow for muscle relaxation, the global $[Ca^{2+}]$ is reduced by Ca^{2+} being pumped into the SR by the Ca^{2+} -ATPase (SERCA), and out of the cardiomyocyte by the Na^+/Ca^{2+} exchanger (NCX) which trades the entry of three Na^+ into the cell for one Ca^{2+} leaving.⁹⁷ Approximately 70% of Ca^{2+} is taken back up into the SR and 30% leaves the cell in each cardiac cycle.⁹⁴ The overall process of EC-coupling is shown in **Figure 1.7**. This process of CICR was first suggested by Fabiato & Fabiato in 1983 by studying the contraction and Ca^{2+} -transients in skinned canine muscle cells.^{98,99} From their experiments they inferred the presence of Ca^{2+} -release channels, which would later be established to be RyR.

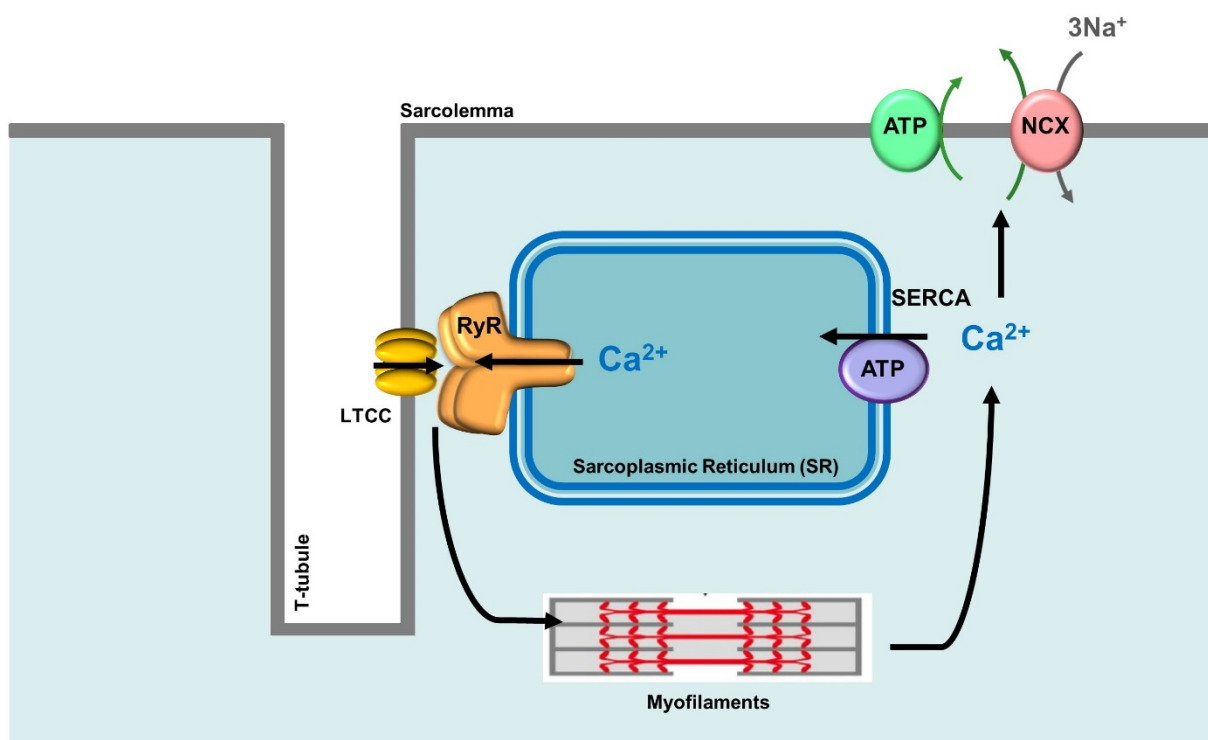


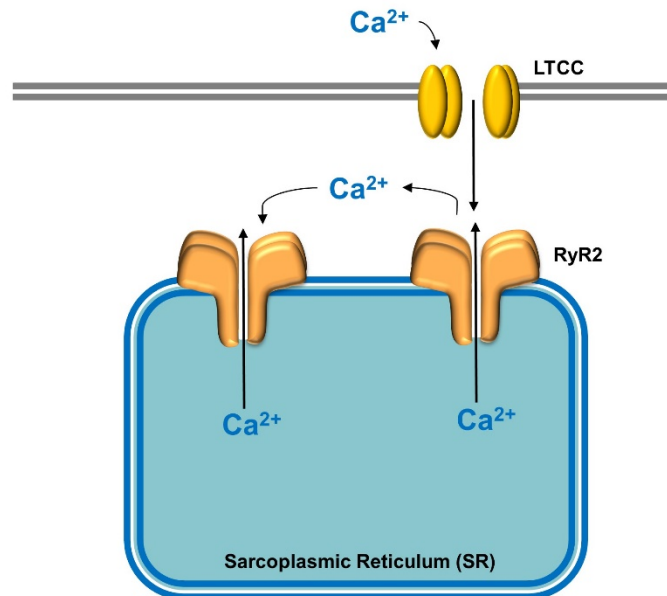
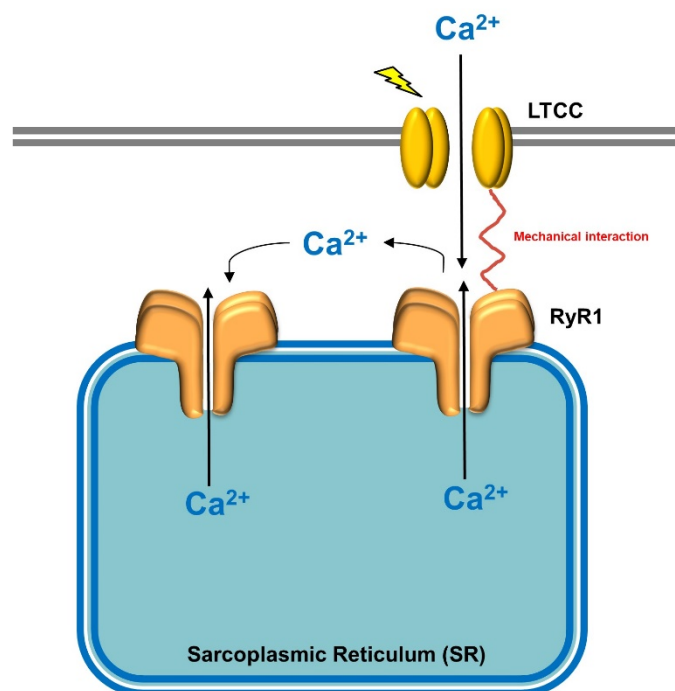
Figure 1.7 Excitation-contraction coupling

Ca^{2+} enters the cell through the LTCC, which trigger RyR2 channels to open and release Ca^{2+} from the sarcoplasmic reticulum (SR) calcium store. Ca^{2+} binds to the myofilaments and facilitates contraction. To allow relaxation, Ca^{2+} is freed and can be taken sequestered back to the SR through SERCA or pumped out of the cell via NCX and CaATPase.

1.2.1.2 *Skeletal muscle*

In skeletal muscle, the process of EC coupling has many similarities, however, the trigger in this case is depolarisation of the sarcolemmal T-tubules and voltage-activation of the LTCCs (or dihydropyridine receptors (DHPRs)) which act as 'voltage-sensors'.¹⁰⁰ This process is facilitated by the fact that the T-tubule network is much more extensive in skeletal muscle than in cardiac.¹⁰¹ In skeletal muscle a different isoform of RyR, RyR1 is involved and the entry of extracellular Ca^{2+} is not required to open the channels.¹⁰² Instead the LTCCs in the T-tubule membrane and RyR1 channels in the SR membrane are physically coupled. Voltage activation of the LTCC induces a conformational change, which allosterically modulates RyR1 and causes it to open, triggering Ca^{2+} -release.¹⁰³ Each LTCC consists of four homologous 'repeats', and these are clustered together in a diamond-like arrangement known as a tetrad.¹⁰⁴ The RyRs are arranged in a lattice where individual RyRs are closely associated to four others, one at each corner, resulting in a 'checkerboard-like' array.¹⁰⁵ Tanabe *et al.* (1988) demonstrated the essential role of the LTCC in skeletal EC coupling. Mice containing a single-point mutation, which caused the complete disruption of LTCC expression, exhibited a failure of EC coupling.¹⁰⁶ EC coupling could be restored in the muscle fibres obtained from these mice by transfecting with the cDNA for the LTCC channel. This was later confirmed using single-channel experiments, where exposure to a peptide derived from the LTCC was found to activate RyRs derived from skeletal muscle but not cardiac, thus suggesting that initial LTCC activation is the crucial initiating step in skeletal EC coupling.^{107,108}

Therefore Ca^{2+} is the trigger of EC coupling in cardiac muscle, while skeletal muscle relies upon a physical coupling of the LTCC and RyR. A comparison of these two mechanisms is shown in **Figure 1.8**. However, there is evidence that the Ca^{2+} released from RyR1 also triggers neighbouring RyR1 channels (which are not themselves coupled to LTCCs) to open following the initial triggering of the process, and therefore the sensitivity of RyR1 to cytosolic Ca^{2+} is also thought to play a role in EC coupling.¹⁰⁹

(A)**(B)****Figure 1.8 EC coupling in cardiac and skeletal muscle**

A comparison between EC coupling in **(A)** cardiac and **(B)** skeletal muscle. In cardiac muscle, an action potential causes depolarisation of the surface membrane which triggers the LTCC to open and allow Ca^{2+} influx. RyR2 is activated by this increase in $[\text{Ca}^{2+}]$. In skeletal muscle, the LTCC acts as a voltage sensor responding to depolarisation and RyR1 becomes activated through a mechanical linkage between the LTCC and RyR1. In both cases, release of Ca^{2+} through the RyR activates adjacent RyRs.

1.3 Ryanodine Receptors

As we have seen, the RyR acts as the pathway for Ca^{2+} -release from the SR Ca^{2+} -store. RyRs are homotetrameric cation channels present in the junctional SR, with a total molecular mass of approximately 2 MDa¹⁰³ (around the same mass as a bacterial ribosome) making them the largest known ion channels.¹¹⁰ There are three main isoforms, known as RyR1, RyR2 and RyR3 and their prevalence is highly tissue specific. RyR1 is known as the skeletal isoform and is expressed almost exclusively in skeletal muscle. It was also the first to be cloned.^{111,112} RyR2 is predominately expressed in cardiac muscle and therefore is known as the cardiac isoform, although it also found in brain tissue.¹¹³ RyR3 is expressed more widely, such as in neurons, Purkinje cells, skeletal muscle (most notably in the diaphragm) and the stomach/kidneys.^{114–116} All three isoforms are largely similar, sharing approximately 70% sequence identity.¹¹⁷ Ryanodine receptors derive their name from the plant alkaloid ryanodine, found in the roots and stem of South American plant *Ryania speciosa*. Ryanodine was itself discovered as a potent insecticide, causing paralysis in skeletal and cardiac muscle.¹¹⁸ Purification of the ryanodine binding protein in the muscle revealed the ion channel that we now know to be RyR.¹¹⁹ Some have suggested the existence of a fourth isoform, specific to fish ('RyR-slow').¹²⁰ It has been shown that RyR1 and RyR2 knockouts are fatal, with RyR1 deficient mice failing to develop the diaphragm muscles required for breathing post-birth, while RyR2 knockouts are embryonically lethal. However mice missing RyR3 can survive to adulthood¹²¹ indicating that the loss of RyR3 can be compensated for during development.¹²² The role of RyR3 is less well understood, however there is evidence that it is involved in the development of ocular muscles.¹²³ The work described in this thesis will investigate RyR1 and RyR2 exclusively. Despite being thought of as Ca^{2+} channels, RyRs have poor selectivity, and also easily pass ions such as Na^+ , Mg^{2+} and K^+ .¹²⁴ They have a small preference for passing divalent cations ($P_{\text{Ca}}/P_{\text{K}} = 6$)¹²⁵ with a very large conductance of RyRs for both divalent (100-150 pS for 50 mM Ca^{2+}) and monovalent (750 pS for 250 mM K^+) cations.¹²⁶ This is not surprising given that they must facilitate both a rapid and large increases in $[\text{Ca}^{2+}]$.¹²⁵ The preference of RyR for cations is the

result of 8 negatively charged residues which line the selectivity filter.¹²⁷ The selectivity of RyR is mostly crowded by these groups which results in the dominant factor determining selectivity of cations to be the size of the ion, rather than charge.^{124,128} The ability of RyR to conduct K^+ efficiently as well as Ca^{2+} , allows RyR to conduct a counter current during Ca^{2+} -release to prevent the development of large membrane potential.¹²⁴

1.3.1 RyR regulation by Ca^{2+} and Mg^{2+}

Given the central role RyR plays in controlling the release of Ca^{2+} during muscle contraction, it is important that tight control of its gating be maintained. In RyR1 this is achieved predominately through its physical linkage with the LTCC, however the gating of both RyR1 and RyR2 can be influenced by the presence of ions and small molecule ligands. Two of the most important ionic regulators of both RyR isoforms are Ca^{2+} and Mg^{2+} ions. The effect of these ions on RyR channel activity has been the subject of intense research, the effect of Ca^{2+} in particular being investigated for its effect on the cytosolic side (cytosol facing) and luminal (SR content facing) side of the receptor.^{129–133} Studies investigating a single RyR gating a lipid bilayer have shown that both RyR1 and RyR2 have a bi-modal concentration response curve to cytosolic Ca^{2+} .¹³⁴ That is, both isoforms are activated by low concentrations of Ca^{2+} and both are inhibited by high levels of Ca^{2+} , however the detailed sensitivity to Ca^{2+} appears to be different. Both are activated by micromolar Ca^{2+} however RyR2 is more sensitive to activation with a steeper dose-response relationship with a Hill coefficient of 2-4, which suggests cooperative binding of multiple Ca^{2+} ions.^{132,135} In both isoforms, Ca^{2+} causes activation predominantly through increasing the frequency of channel openings.^{132,136} Both isoforms are inhibited by high $[Ca^{2+}]$, however RyR1 can be almost completely inhibited by 1 mM Ca^{2+} , while a substantially higher $[Ca^{2+}]$ of up to 10 mM is required to full inhibit RyR2.^{125,133,137,138} Such concentrations are unlikely to be reached *in vivo* and so a physiological role for this inhibition is not clear.¹²⁵ RyR channels are also sensitive to the $[Ca^{2+}]$ surrounding the luminal portion of the channel. While luminal Ca^{2+} alone does not activate either RyR1 or RyR2, in combination with other activators such as ATP and cytosolic Ca^{2+} , luminal Ca^{2+} is capable of potentiating

RyR channel activity.^{139,140} The effects of luminal Ca^{2+} can therefore be thought of as increasing the sensitivity of RyR for cytosolic activators. Tryptic digestion of the luminal portion of RyR2 has indicated the presence of both activating and inhibitory Ca^{2+} binding sites.¹⁴¹ Given that, in a physiological context, the $[\text{Ca}^{2+}]$ which the luminal portion of RyR is exposed to, is a reflection of the SR store content, luminal activation of RyR is of considerable importance. It is well established that a rise in SR $[\text{Ca}^{2+}]$ to above a level required to trigger luminal activation of RyR, is implicated in a number of RyR disease pathologies, and is termed store-overload-induced- Ca^{2+} -release.¹⁴² Mg^{2+} on the other hand, is well established as an inhibitor of both RyR1 and RyR2.^{129,130,143–145} Cytosolic Mg^{2+} competes with Ca^{2+} for both its activating and inhibition site, and therefore reduces the ability of a given $[\text{Ca}^{2+}]$ to activate RyR.^{143,145,146} However, the affinity of Mg^{2+} appears lower than that of Ca^{2+} ($\text{EC}_{50} \sim 50 \mu\text{M}$).^{135,147} Cytosolic Mg^{2+} is capable of closing channels, even in the presence of activating levels of luminal Ca^{2+} . Mg^{2+} is present at concentrations of 0.5 - 1 mM in a physiological setting and the inhibitory effect of Mg^{2+} ensures that RyR can be regulated by the concentrations of Ca^{2+} between 100 nM and 10 μM , hence facilitating EC coupling. Luminal Mg^{2+} has also been shown to inhibit RyR2, by two complementary mechanisms. Mg^{2+} also appears to compete for the luminal Ca^{2+} activation site.¹⁴⁷

1.3.2 Action of Ryanodine

We already mentioned that both isoforms of RyR bind the plant alkaloid ryanodine (**Figure 1.9**), and they do so with high (nanomolar) affinity, although the effects of ryanodine on RyR are complex.¹⁴⁸ At low concentrations, ryanodine causes RyRs to adopt a long-lived sub-conductance state.^{149,150} High concentrations (100 μM) of ryanodine however result in irreversible channel closure.¹⁵¹ These effects are thought to be the result of RyR containing both a high and low affinity ryanodine binding sites, and that the high affinity site is only accessible when the channel is in the open conformation.¹⁵² The use of labelled

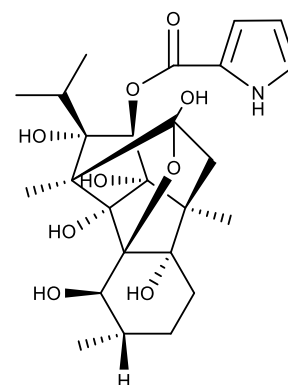


Figure 1.9 Ryanodine

[³H]ryanodine is a method used extensively as a means to assess the proportion of a population of channels that are in the open state.^{125,153}

1.3.3 RyR regulation by ATP and adenine nucleotides

Ca²⁺ is an important regulator of RyR channel behaviour, however its effects can be influenced by a wide range of other physiological ligands, including ATP. ATP is present at millimolar levels in the cell¹⁵⁴, and is thought to always be available to activate both RyR1 and RyR2. Studies in skeletal muscle have shown that ATP activates RyR1, increasing release of Ca²⁺ even in the presence of nanomolar cytosolic [Ca²⁺]_i. In cardiac muscle, ATP has a similar effect (ATP is less effective at activating RyR1 than RyR2).^{155,156} Single-channel bilayer studies have shown that ATP increases RyR Po through increasing both the frequency and duration of channel openings.¹⁵⁷ Therefore ATP works synergistically with cytosolic Ca²⁺ by potentiating the activation of Ca²⁺.^{156,158–160} This is evidenced by the fact that during periods of ischemia, ATP can be degraded into metabolites such as ADP, AMP, adenosine and inorganic phosphate and this results in marked changes to intracellular Ca²⁺ release.^{161,162} Under these conditions, the inhibitory effect of Mg²⁺ dominates and channel activity is decreased. It has also been shown in skeletal muscle that, LTCC activation of RyR is blunted in the presence of low (<0.5 mM) [ATP], indicating that ATP is vitally important for maintenance of contraction.^{163,164} Given this, the single-channel effects of ATP and adenine ligands on RyR have been extensively studied.^{139,165,166}

In 1993 Shoshan-Barmatz identified a putative binding site for ATP using a photoaffinity analogue of ATP (BzATP).¹⁶⁷ She showed that since this probe bound to RyR1 in the ratio of 1:1, there was one ATP binding site per monomer of RyR.^{168–170} This was later confirmed by cryo-EM¹⁷¹. The effect of adenine nucleotides on RyR2 and how the structure of the RyR2 ATP binding site influences their effects on gating, is considered in **Chapter 7**.

1.3.4 RyR in human disease

Given the central role of RyR in maintaining proper Ca^{2+} release from the SR, it is not surprising that dysregulation of RyR is associated with many human diseases.¹⁷² Mutations in both RyR1 and RyR2 are known to result in disease pathology, as well as post-translational modifications such as oxidation and phosphorylation are thought to be involved in impairing the function of these channels.^{173,174}

1.3.4.1 *RyR1*

The first disease reported to be due to dysregulation of RyR1 was malignant hyperthermia (MH). This is a disease caused by mutations to RyR1, which can result in a life-threatening hypermetabolic reaction to be triggered by exposure to volatile anaesthetics (e.g halothane), or heat/exercise exertion.^{103,175} Symptoms include elevated core body temperature, hypoxia, muscle rigidity and rhabdomyolysis. It has been shown that these derive from increased activation of RyR1 which results in undesired SR Ca^{2+} release, resulting from the channels becoming more sensitive to increased temperature and activators.¹⁷⁶ This increase in $[\text{Ca}^{2+}]$ triggers the muscle fibres to contract, generating excess heat and acidosis. To date, around 250 mutations to the RYR1 gene have been identified as being implicated in MH, which are clustered into three mutation ‘hotspots’ found in the N-terminal domain, central domain and C-terminal domains respectively.¹⁷⁷ These hotspots are shown in **Figure 1.10**. Dantrolene can provide a treatment for MH if administered quickly enough, and the prognosis following treatment is generally good.¹⁷⁸ Dantrolene acts as an inhibitor of RyR1, which reduces the SR Ca^{2+} -release and relieve the symptoms of MH.¹⁷⁹ Treatment with dantrolene is not ideal given the very low solubility and undesirable pharmacokinetic properties, and many research groups are working on alternative RyR1 inhibitors.^{179,180}

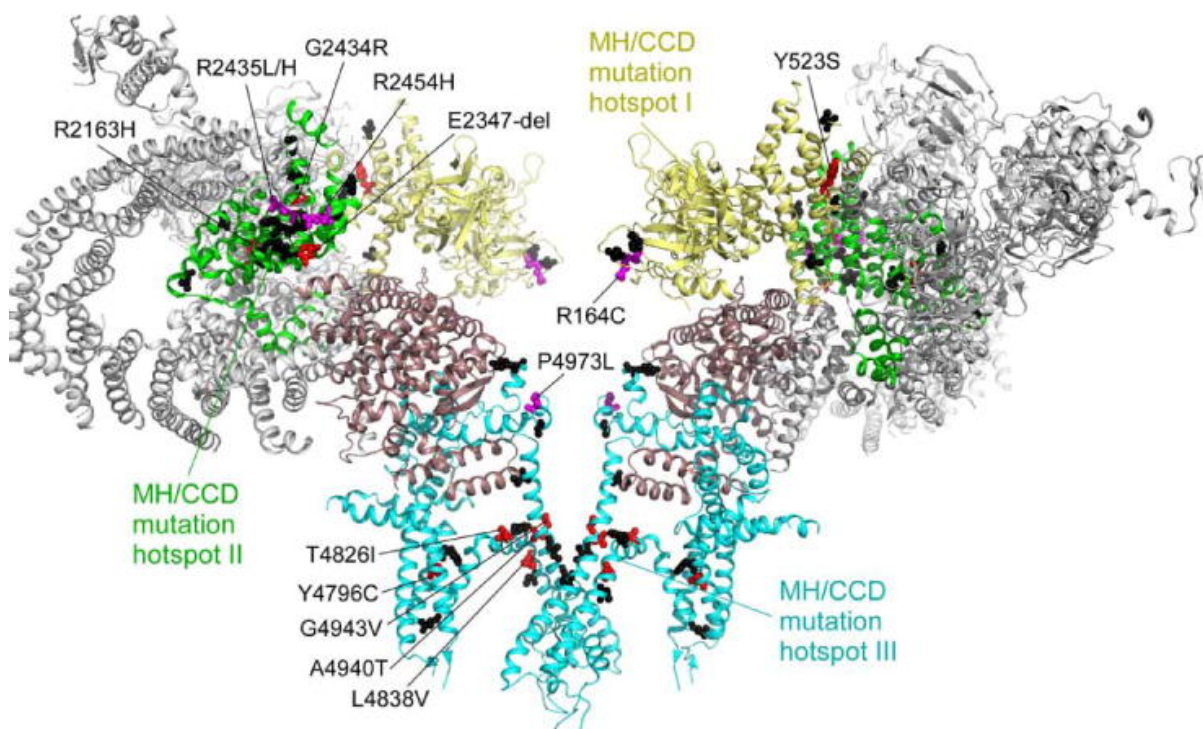


Figure 1.10 RyR1 mutation hotspots

Two monomers of RyR1 are shown with the mutation hotspots indicated. There are three MH/CCD hotspots, hotspot 1 is indicated in yellow, hotspot 2 is indicated in green and hotspot three in cyan. Individual residues contained within hotspot are also shown. This figure is adapted from Chen *et al.* Biochem J. 2017, 474: 2749-2761.

In addition to MH, mutations in the RYR1 gene have also been shown to result in an autosomal dominant congenital myopathy known as central core disease (CCD). The incidence of CCD is around 1/15000, and it results in muscle atrophy, skeletal muscle weakness and the development of cores in the muscles (areas devoid of enzyme activity).¹⁸¹ In CCD, RYR1 mutations are thought to be hyperactive, which results in excessive SR Ca^{2+} -leak. During development of the muscle, this dysregulation results in a reduction in the SR content and reduced force of contraction, resulting in decreased muscle function. There is currently no known treatment for CCD and treatment is largely supportive.^{172,182,183}

1.3.4.2 RyR2

Mutations to RyR2 are known to result in a disease known as catecholaminergic polymorphic ventricular tachycardia (CPVT), which is a familial disorder resulting in cardiac arrhythmias.^{173,184,185} It can also be caused by mutations to proteins associated to RyR such as calsequestrin, although this is less common.¹⁸⁶ In common with MH, CPVT mutations result in dysfunction of RyR2, triggering abnormal release of Ca^{2+} from the SR. In cardiac muscle, this results in the generation of ventricular arrhythmias and can lead to sudden cardiac death. In most CPVT RYR2 mutations, this occurs through an increase in the activity of RyR2, resulting in Ca^{2+} release being triggered during diastole.¹⁸⁷ As one possible mechanism, it has been suggested that this Ca^{2+} -sensitivity is caused both by 'domain-unzipping' between the N-terminal region and central domain of RyR2, which causes the closed state of RyR2 to become less stable, and through dissociation of an associated protein FBKP12.6.¹⁸⁸ Other mechanisms have been proposed and it is likely that whichever mechanism dominates, is a function of the exact mutation in question.

CPVT is treated with β -blockers which reduce the development of arrhythmias.¹⁸⁹ In cases where this is not successful, the drug flecanide is used.¹⁹⁰ Some groups have reported that flecanide is believed to act as a direct inhibitor of RyR2, however others believe it reduces arrhythmias through sodium channel block.^{191,192} Some patients do not respond to drug treatments however, and surgical intervention (such as addition of a pacemaker) becomes necessary, to reduce the levels of catecholamines supplied to the heart, to attempt to reduce the risk of further arrhythmias.

1.3.5 Pharmacological regulation of RyR

Many small molecules are capable of influencing RyR function, including both exogenous and endogenous ligands. One of the most well established ligands affecting RyR is the methylxanthine caffeine, the effect of which has been known for over 30 years.¹⁹³ Caffeine had been shown to trigger SR Ca²⁺-release in muscle fibres, and this was confirmed in detail by single-channel studies.^{194–196} Caffeine can activate both RyR1 and RyR2 at low micromolar concentrations, however millimolar concentrations are needed to see maximal activation.¹⁹⁶ Caffeine is used throughout this thesis as a positive control, at millimolar levels. An overview of a few of the many ligands known to influence RyR channel gating are shown in **Table 1.1**.

Given the association of RyR with various diseases, extensive efforts have been made towards developing a high throughput screen to identify small molecules that affect RyR function. Recent reported attempts have utilised various methods using fluorescence resonance energy transfer (FRET), or measurement of Ca²⁺-release in cells. Examples include the work reported by Rebbeck *et al.* (2016) which utilised a FRET-based approach to identify molecules which reduce Ca²⁺-leak, screening a library of 727 compounds.¹⁸⁰ Murayama *et al.* (2018) utilised a different approach using fluorescent indicators to measure the SR Ca²⁺ load, which could screen 1535 compounds. However, high-throughput (1000-10000) methods have not yet been achieved. Additionally, most ligands which modulate one isoform of RyR, generally also affect the other isoforms, therefore achieving specificity between isoforms presents a challenge to identifying drugs to target RyR.¹⁹⁷

To date, only one drug targeting RyR has been approved for use, dantrolene (the mechanism of action of flecainide has not been established as involving direct RyR inhibition).^{191,192} There have been several attempts towards the design of drugs targeting disease pathology, including several molecules which inhibit RyR (e.g. JTV519, S107 and K201) however none have yet been approved for use.^{198–200}

Drug or small molecule	RyR1	RyR2	
Caffeine	↑ mM	↑ mM	Regarded by many as the 'classic' agonist of RyR. Binding site in RyR1 identified in 2016. ¹⁷¹ Mechanism of action involves sensitisation of RyR to cytosolic Ca^{2+} and increasing the frequency of channel openings. ¹⁹⁶ High concentrations (10 mM) can activate in sub-activating cytosolic $[\text{Ca}^{2+}]$. ¹⁹⁸
Dantrolene	↓ μM	↓ μM	Dantrolene is a direct RyR1 inhibitor, and is the primary drug used for the treatment of malignant hyperthermia, although it also inhibits RyR2. ^{201,202} It functions by stabilising domain interactions in RyR. ²⁰³ Inhibition is thought to be dependent on the presence of ATP and Mg^{2+} . ²⁰⁴
Digoxin	-	↑ nM	Digoxin is a digitalis glycoside which activates RyR2 but not RyR1. ²⁰⁵ Activates RyR2 at therapeutic concentration when added to the cytosolic, but not luminal side of the channel. 10 – 20 nM digoxin increases the frequency of RyR2 openings, and appears to sensitise RyR2 to cytosolic $[\text{Ca}^{2+}]$. ²⁰⁶
Suramin	↑ μM to mM	↑ μM to mM	Originally developed as an anticancer agent. Activates both RyR1 and RyR2, but is more potent as a RyR2 activator. Suramin increases single-channel conductance of RyR1 and RyR2 and increases P_o both by increasing the duration and frequency of openings. Activation was still possible in both isoforms at sub-activating $[\text{Ca}^{2+}]$. ²⁰⁷
Diisothiocyanostilbene-2',2'-disulfonic acid (DIDS)	↑ μM	↑ μM	DIDs and structurally similar compounds are thought to bind to the suramin sites and they affect RyR channel conductance and gating in a similar manner to suramin. ²⁰⁸ Due to the isothiocyanide groups, the effects are irreversible. ^{209,210}
4-chloro-m-cresol (4cmc)	↑ μM	IA	4cmc is a polyphenol compound which activates RyR1 ($\text{EC}_{50} = 50 - 200 \mu\text{M}$) ^{211,212} and RyR2 and RyR3 only at millimolar concentration. ²¹³ Studies have indicated that the 1-hydroxyl group is critical for RyR1 activation. ²¹⁴ There are reports of RyR2 activation at lower concentrations but this remains controversial. ²¹⁴
Local anesthetics (Tetracaine/Procaine)	↓ μM	↓ μM	Tetracaine and procaine inhibit both isoforms of RyR (RyR1 EC_{50} of 180 μM) although they are selective for mammalian RyR over other species. ^{215,216} Inhibition can occur from both the luminal and cytoplasmic sides of the membrane in a reversible manner, and is a cooperative process. ²¹⁷
Ruthenium Red	↓ nM	↓ nM	Ruthenium red is a potent (nanomolar) inhibitor of both RyR1 and RyR2. Inhibition has a strong voltage dependence, and is thought to result from the binding of multiple molecules to the conduction pore of the channel. ²¹⁸
Amitriptyline	NR	↑ μM	Amitriptyline is prescribed for the treatment of depression, however it causes an increase in Ca^{2+} sparks and RyR2 P_o . High concentrations (25 μM) increase P_o to approximately 0.7, with frequent long openings. ²¹⁹

Table 1.1 Comparison of the effects of some of the many RyR modulators

The ability of various pharmacologic agents to affect RyR channel activity is indicated. Upwards arrows (↑) indicate activation, while downwards arrows (↓) indicate inhibition. NR = not reported. IA = inactive. This table is not exhaustive.

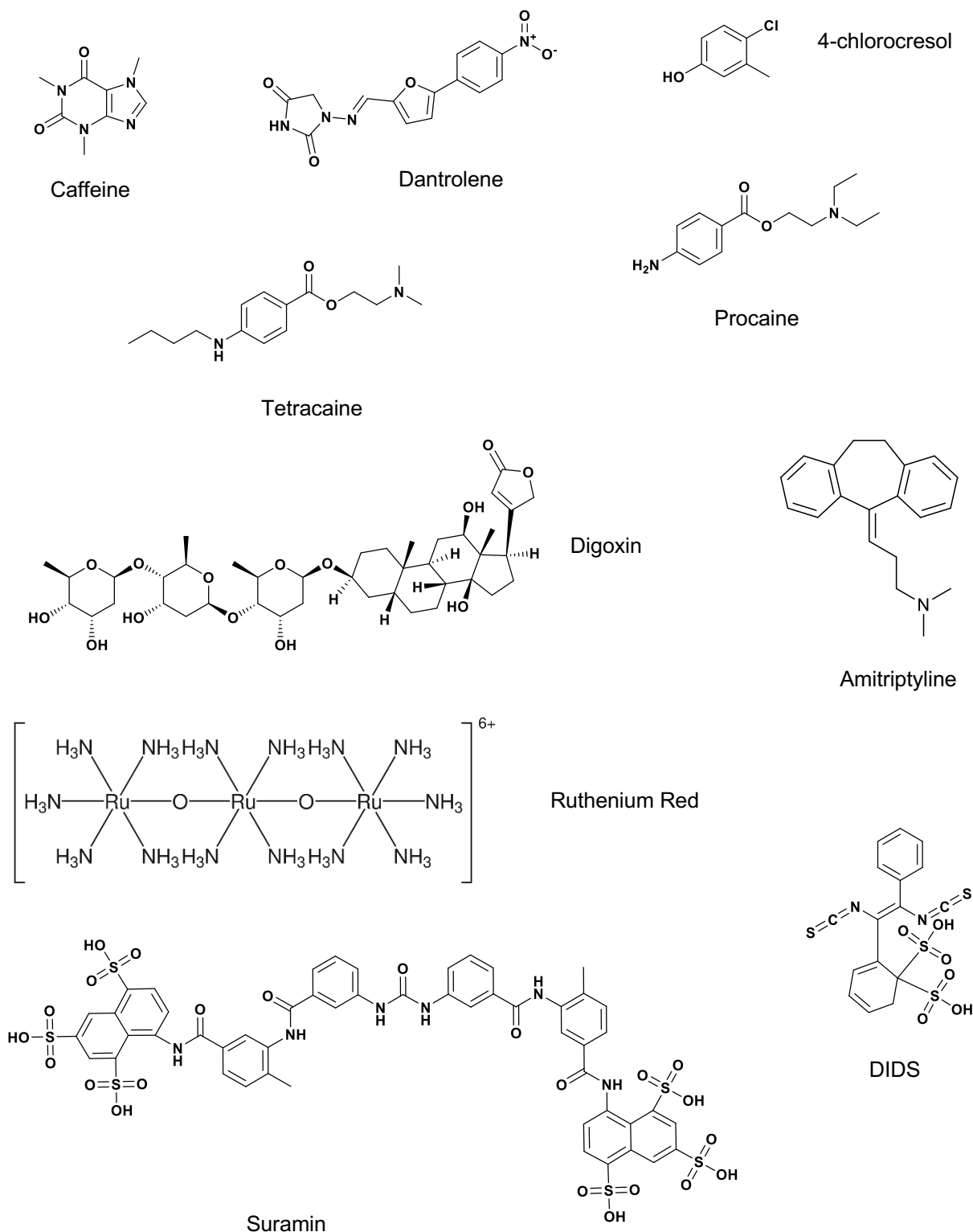


Figure 1.11 Structures of common pharmacologic regulators of RyR

1.3.6 RyR regulation by closely associated proteins

In addition to ions and small molecules, a large number of junctional SR membrane proteins can form a complex with RyR, and regulate its channel function.¹²⁶ The most important and widely studied are calmodulin, calsequestrin and FK-506 binding proteins.

1.3.6.1 *L-type Ca²⁺-channel*

We have already seen how in both skeletal and cardiac muscle, the initiation of SR Ca²⁺ release requires RyR activation via the LTCC. In skeletal muscle this is achieved by means of a direct linkage between the LTCC and RyR1, while, in contrast, in cardiac muscle the process relies on a small Ca²⁺ influx through the LTCC which then activates RyR2. In both cases, the LTCC provides the main trigger for Ca²⁺-release via RyR channels although other sources of Ca influx can add to this trigger and influence RyR2 gating, such as via Na⁺/Ca²⁺ exchange current.²²⁰ In skeletal muscle, not all RyR1 channels are physically coupled to the LTCCs, as each alternate RyR1 relies on Ca²⁺-release from its neighbour to trigger its own opening.¹⁰⁵ Indeed, in skeletal muscle the spatial arrangements of LTCCs and RyRs are highly ordered such that groups of 4 LTCC (known as a 'tetrad') are located opposite alternate RyRs. Cardiac muscle lacks this organisation, due to its less extensive t-tubular structure.²²¹ The LTCC is approximately 450 kDa in size, and consists of 5 subunits ($\alpha 1$, $\alpha 2$, δ , β and γ).²²² It has been shown that an intracellular loop between the second and third transmembrane repeats of the LTCC is one site of interaction with RyR1¹¹⁷, additionally the C-terminal tail of the LTCC also appears to bind to RyR1²²³. Conversely, the RyR1 binding site has been mapped to the region between M3201 and W3661.²²⁴ It has been shown that mutations of the LTCC can result in malignant hyperthermia in skeletal muscle, and arrhythmias in cardiac muscle.²²⁵

1.3.6.2 *Calmodulin*

Calmodulin (calcium-modulated protein, CaM) is a small(16.7 kDa), 148 residue cytoplasmic Ca²⁺ binding protein which is ubiquitously expressed, and participates in many Ca²⁺ signalling pathways, including inflammation, metabolism, neuronal transmission, and importantly,

muscle contraction.^{226–228} Structurally, CaM consists of two lobes (N-terminal and C-terminal) sometimes called ‘globular heads’ connected by a flexible helix or ‘hinge’.^{228,229} CaM is highly flexible, allowing it to find a wide variety of target proteins.²²⁹ In total CaM can bind four Ca^{2+} (two on each lobe), which induces a conformational change, influencing CaM’s ability to bind to other proteins. The Ca^{2+} -free state is known as apoCaM, and the Ca^{2+} -bound state CaCaM.²³⁰ While it is thought that the majority of proteins that bind to CaM do so to the CaCaM form, both forms are capable of binding to RyR, however regulation of RyR by CaM is isoform specific.^{231,232}

Both isoforms are thought to bind one molecule of CaM per monomer^{231,233}. CaM binding has dual effects on RyR1 and RyR3, activating the channels at low (nM) concentration while inhibiting them at higher (μM) concentration.^{233,234} It had been thought that CaM was only capable of inhibiting RyR2, however it was later discovered that in fact low physiological concentrations of CaM (50 nM) can activate RyR2, as it does for RyR1 and that inhibition occurs only at higher micromolar concentrations.^{232,235}

1.3.6.3 Calsequestrin

Calsequestrin (CSQ) is a Ca^{2+} -binding protein which acts as a buffer in the SR lumen, concentrating Ca^{2+} in the cisternae of the SR and maintaining a high $[\text{Ca}^{2+}]$ to facilitate muscle contraction. CSQ contains a large number of exposed acidic residues which allow each molecule of CSQ can bind 40 to 50 Ca^{2+} ions each.^{236,237} There are two isoforms, type 1 CSQ (CSQ1) which is found in skeletal muscle and type 2 CSQ (CSQ2) which is expressed in both cardiac muscle and slow-twitch skeletal muscle.²³⁸ The isoforms are very similar, however CSQ2 contains additional acidic residues.²³⁶ CSQ forms oligomers in the SR lumen and is anchored to RyR via the proteins junctin and triadin which are themselves embedded in the SR membrane. Both proteins have been shown to interact with both RyR and CSQ.²³⁹ Conformational changes in CSQ may also modulate RyR activity directly.²⁴⁰ Single-channel experiments have shown that addition of CSQ can activate RyR directly, although it is thought

that this plays a minor physiological role.²⁴¹ The regulation of RyR by CSQ is isoform dependent, as CSQ2 activates RyR2 (and is thought to be a luminal sensor for RyR2 channels), CSQ does not appear to regulate RyR1.^{237,241–243} Therefore, it is thought that in skeletal muscle CSQ1 functions only as a Ca^{2+} -buffer.²⁴¹

1.3.6.4 FK-506 Binding Protein

FKBP12 and FKBP12.6 are immunophilin proteins which associate with RyR1, RyR2 and RyR3, with each monomer of RyR binding one FKBP molecule.^{244–246} Both isoforms of FKBP can be found bound to RyR1 and RyR3, however the cardiac isoform RyR2 preferentially binds FKBP12.6 (even in high concentrations of FKBP12).^{244,247} These proteins are constitutively bound to RyR, and removal of FKBP has been shown to lead to a loss of Ca^{2+} -sensitivity and a reduction in Po .^{125,197,248,249} The effects of FKBP12 on RyR2 were less clear, however Galfre *et al.* (2012) showed that FKBP12 activates RyR2, and its effect can be antagonised by the additional presence of FKBP12.6, although FKBP12.6 on its own has low efficacy.¹⁹⁷ The same authors later showed that FKBP12.6 activates RyR1 while FKBP12 inhibits it.²⁵⁰ They showed that FKBP12 and FKBP12.6 likely compete for the same binding site on RyR1, as pre-addition of FKBP12.6 to RyR1 single-channels prevented subsequent inhibition by FKBP12. There is evidence therefore that both forms of FKBP can modulate both forms of RyR (at low concentrations) and therefore both are likely to be important for the regulation of RyR in both skeletal and cardiac muscle. There remains some controversy regarding this, which is likely due to differences between investigators in the various tools used to dissociate FKBP from RyR, such as FK506 and rapamycin, which have themselves independent effects on RyR even in the absence of FKBP.^{244,251,252}

1.3.7 RyR Structure

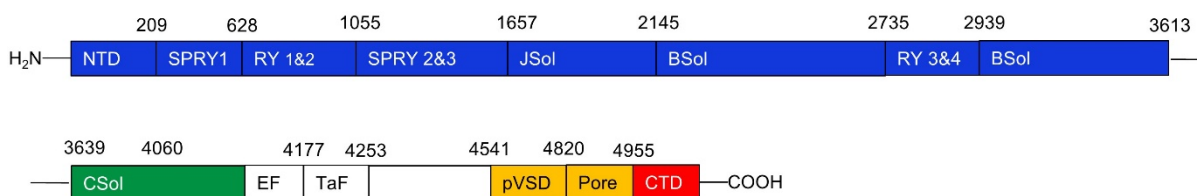
RyRs consist of a tetrameric arrangement of four monomers (each of 560 kDa), with the full channel having a mass of 2.2 MDa. The huge size of RyR, combined with the difficulty in

preparing large quantities of protein, have hampered efforts to produce x-ray crystallographic structures of the channel.²⁵³ However in the absence of this, recent advances in cryo-electron microscopy (cryo-EM) have allowed some insight into the structural make-up of RyR. Initially, cryo-EM provided low resolution structures but from these the overall structure of RyR could be visualised for the first time. The full structure of RyR takes on a 'mushroom-like' architecture which spans the SR membrane, with the majority of the channel structure residing on the cytosolic side of the membrane.²⁵⁴ Further refinements in cryo-EM techniques increased resolution to 10 Å, from which individual domains could be visualised, such as the N-terminal domain (NTD)²⁵⁵, the phosphorylation hotspot²⁵⁶ and the 'amino terminal disease' hotspot²⁵⁷, however finer details were still not clear. Mostly recently, as cryo-EM techniques were refined still further, in 2015, three groups published high-resolution structures of RyR1 from rabbit skeletal muscle.^{258–260} A schematic indicating the location of each domain and relevant residue numbers is shown in **Figure 1.12(A)**.

Wei *et al.* (2016) utilised exposure of RyR1 to activating levels of Ca²⁺ followed by treatment with ruthenium red to lock the channel in a 'blocked-open' conformation. From this they could show that upon activation by Ca²⁺, the channel undergoes a so called 'breathing motion' in which the helical domain 1 and N-terminal domain both move upward and outward, widening the aperture of the pore and facilitating ion flux through the channel.²⁶¹ In 2016, higher resolution allowed Des George *et al.* (2016) to determine the locations of the caffeine, ATP and Ca²⁺ binding site in RyR1.¹⁷¹ These reconstructions were established at a much higher resolution than had previously been obtained, of between 3.8 Å – 4.2 Å. We therefore have access to a full structural model of RyR1, at a high enough resolution to allow individual residues to be visualised. The structure determined by Des George *et al.* is shown in **Figure 1.12**. Des George and colleagues found that ATP, Ca²⁺ and caffeine all bind to interdomain interfaces of the C-terminal domain (CTD).²⁶² They suggested that the binding of these three activatory ligands initiates a rearrangement of the central core of RyR1 (a motion termed as 'priming'),

which prepares the channel for opening. In the presence of two or more ligands, the channel P_o is increased, as the channels are moved from their primed state to their open state. Des George *et al.* also found that ryanodine appears to bind to the open state of RyR1 and particularly occlude the pore, inhibiting the passage of ions through the conduction pathway.¹⁷¹ This is in keeping with the single-channel observations that ryanodine promotes RyR1 states of reduced conductance.¹⁹⁸

(A)



(B)

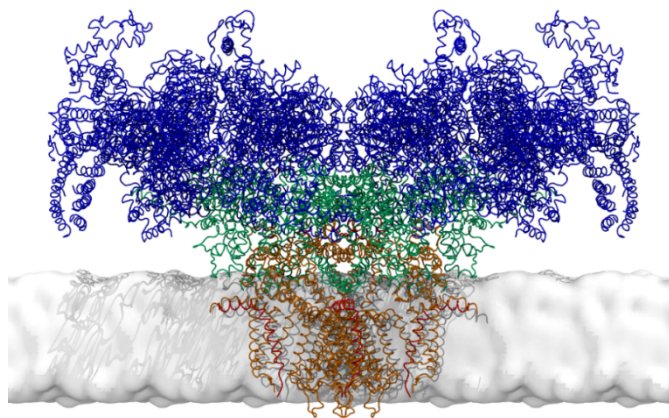


Figure 1.12 Cryo-EM structure of RyR1

(A) Domain organisation of RyR1 are shown in a 2D schematic with residue numbers indicated, the size of the boxes are not proportional to the number of residues. (B) The cryo-EM structure of RyR1 as determined by Des George and colleagues is shown. The residues as coloured as in Des George *et al.* (2016). The shell (residues 1-3666) are coloured blue, the core solenoid (residues 3667-4253) in dark green, the transmembrane domain and S6c (residues 4540-4956) in orange and the CTD (residues 4957-5037) in dark red).

Parallel work on RyR2 lead to similar reports for this isoform. Peng *et al.* (2016) reported both the closed and open forms of RyR2 in the presence of activating ligands and Ca^{2+} .²⁶³ However to date, binding site information has not been established for this isoform. Comparison of the structural changes in RyR2 between open and closed forms, reveals that the mechanism of channel opening appears to be the same in RyR1 and RyR2, although as we know, the exact response to ligands may be different.

1.3.8 Study of RyR

The first methods used to study EC-coupling and Ca^{2+} -transport in muscle, largely utilised radiolabelling methods (such as use of ^{45}Ca) to investigate how Ca^{2+} moved throughout a cell. These methods provided only a very basic level information about Ca^{2+} influx and efflux²⁶⁴. However, it had become clear that the plant alkaloid ryanodine, which was used to identify RyR channels, could become a useful tool for studying their function.

We have described the action of ryanodine on RyR already (section 1.3.2). At low concentrations (nM), ryanodine is exquisitely specific and binds only to channels in their open conformation. It should also be apparent that, the proportion of channels in their open form depends heavily on the conditions especially on the presence of activating ligands, temperature and holding potential. By adding to the ryanodine molecule, a radioisotope (most commonly tritium, ^3H) to form [^3H]ryanodine (more specifically [9, 21(n)- ^3H]ryanodine) we have access to a probe which binds (essentially specifically and irreversibly) to only open RyR channels.^{152,265} Therefore conditions which increase the level of [^3H]ryanodine binding, can be considered to increase SR Ca^{2+} -release by increasing the activity of RyR. Thus [^3H]ryanodine binding provides a powerful tool for assessing the influence of a ligand across a population of channels, in their native SR membranes.¹⁴⁸

However, there are limitations to binding techniques in that they provide solely a holistic overview of how a ligand influences the proportion of channels that are open or closed. To gain further insight into the mechanism of channel gating, native SR vesicles can be

incorporated into planar lipid bilayers under voltage-clamp conditions, and the current amplitude fluctuations measured. This is typically achieved by 'painting' a lipid bilayer across a small aperture between two solution containing compartments (typically one side being high $[Ca^{2+}]$ and the other being low $[Ca^{2+}]$). An RyR-containing SR vesicle can be incorporated into this bilayer, with the result being the RyR fixed in a known orientation. A patch-clamp amplifier can be used to record the current passing through the bilayer, which will of course be zero when the channel is closed, and only non-zero when the channel opens and is allowed to pass a permeant ion (such as Ca^{2+} or K^+). SR vesicles can be obtained by differential centrifugation of muscle homogenate, and given the tissue specific expression of RyR it is straightforward to study both RyR1 (by using skeletal muscle) and RyR2 (by using cardiac muscle). Mutations to RyR may also be studied routinely by preparation of vesicles from tissue derived from mutant animals.

This provides for a powerful technique that allows the effect of a ligand on an individual channel to be studied.²⁶⁶ Measurement of the single-channel current allows the P_o of the channel to be calculated, a quantity which refers to how likely a channel is to be in its open or closed state, where zero is fully closed and one is fully open. Dwell time analysis reveals the effects of the environment of the RyR on the duration of channel opening and closing events. This allows us to investigate whether, for example, an agonist increases P_o by increasing the frequency of channel openings (such as cytosolic Ca^{2+}) or by increasing the duration of the openings (such as suramin). This technique has been employed extensively in the Sitsapesan group to study the channel gating of RyR^{139,165,196,209,267–269}

Both [3H]ryanodine binding, and single-channel techniques have been employed in this thesis, to investigate how statins and other ligands affect RyR channel gating, and are described in detail in the methods chapter (**Chapter 2**).

1.4 Aims of this Thesis

As I have introduced, the proper regulation of muscle contraction depends intimately on SR Ca^{2+} release through RyR. We have seen that statins, drugs which are commonly prescribed for the treatment of hypercholesterolemia, cause skeletal muscle side-effects which appear to be related to their triggering of Ca^{2+} -release.

This therefore raises the question, **do statins influence or interfere with the gating of RyR?** This thesis attempts to address this question, through the use of electrophysiology, molecular modelling and medicinal chemistry approaches.

My first aim was to understand how statins may affect RyR gating. The effect of statins on the regulation of the skeletal ryanodine receptor RyR1, are investigated in **Chapter 3**, and in **Chapter 4**, I have then investigated whether statins influence the gating properties of RyR2, the cardiac isoform. My results indicate that statins are capable of causing RyR1 activation, while inducing more complex regulation of RyR2.

To establish whether these effects were general to all HMG-CoA inhibitors, my second aim was to synthesise and test the statin minimal pharmacophore, the synthesis of which is reported in **Chapter 5**. Having observed that statins can directly influence RyR activity, my third aim addressed in **Chapter 6** was to investigate a possible binding site for statins on RyR, and, using molecular modelling techniques, develop molecules capable of inhibiting HMG-CoA reductase, without affecting RyR gating. My final aim, having found that the RyR ATP-site might be a possible binding site for statins, was to use the docking model generated previously to understand how adenine nucleotides, including ATP can bind and cause RyR activation at this site. This is explored in **Chapter 7**.

2

Methods

2.1 Isolation of heavy and light sarcoplasmic reticulum membrane vesicles

2.1.1 Sheep cardiac and skeletal tissue

Fresh sheep hearts and leg muscle were collected from Long Compton Abattoir, where animals were killed by captive bolt. Sheep were 8-10 months (Suffolk breed). The muscle was immediately transported to the laboratory in cardioplegic solution at 4 °C (102 mM NaCl, 21.4 mM KCl, 16 mM MgCl₂, 28.7 mM Na-lactate, 2.4 mM CaCl₂ and 1 mM procaine). The muscle was cut into portions (approximately 200 g each). The fat and connective tissue was removed and the muscle was immediately stored in liquid N₂. For each sheep skeletal and cardiac preparation, one of these portions was used. In the case of sheep hearts, only the left ventricular tissue including septal wall was isolated.

2.1.2 Mouse skeletal and cardiac tissue

Skeletal and cardiac tissue was dissected from adult (10-14 weeks) mice of mixed sex. After dissection, the tissues were snap frozen and stored immediately in liquid N₂. For each membrane preparation, either 10 whole hearts (for cardiac preparations) or 20 hind legs (for skeletal preparations) were used, in order to collect enough tissue for successful enrichment of SR.

2.1.3 Rabbit skeletal tissue

Skeletal muscle was dissected from the hind legs of white New Zealand rabbits. The tissues were snap frozen and stored immediately in liquid N₂. For each membrane preparation, a single rabbit leg was used.

2.1.4 SR membrane preparation procedure

For all membrane preparations (regardless of species), the same protocol was used. All solutions and equipment were stored at 4 °C prior to use. The frozen tissue was ground into a

fine powder in a pestle and mortar and homogenised in 400 ml of solution A (1 mM phenylmethylsulphonic fluoride (PMSF), 300 mM sucrose, 20 mM potassium piperazine-N-N'-bis[2-ethansulphonic acid] (PIPES), 300 nM aprotinin, 1 μ M leupeptin, 1mM benzamidine and 2.5 mM dithiothreitol (DTT), pH 7.4) using a blender (Braun GmbH, Kronberg, Germany). The resulting mixture was homogenised further with the Ultra-Turrax (IKA Werke GmbH & Co. KG, Staufen, Germany). The mixture was centrifuged at 6000 g for 20 min at 4 °C in a Sorvall GSA rotor. The resulting supernatant was centrifuged at 100,000 g for 1 hour at 4 °C in a Beckman Type 45i rotor. The resulting pellet contains mixed membrane (MM) SR vesicles. The pellet was resuspended in a solution containing 400 mM KCl, 0.1 mM CaCl_2 , 0.1 mM EGTA, 5 mM PIPES and 10% w/v sucrose, pH 7.2. This mixed membrane solution was then layered on top of discontinuous sucrose gradients (containing 20%, 30% and 40% w/v sucrose respectively) in 32 ml Beckman tubes, and centrifuged at 100,000 g for 2 hours at 4 °C in a Beckman SW 32i rotor. This process produces three bands, sarcolemma (top band), light SR (LSR) at the interference of the 20-30% sucrose bands, and heavy SR (HSR) at the 30-40% interface. LSR contains many K^+/Cl^- channels, but few RyR channels, whereas HSR is rich in RyR²⁷⁰. Both LSR and HSR bands were collected and resuspended in a solution containing 400 mM KCl, 10 mM PIPES at pH 7.2. The two membrane fractions were then centrifuged at 100,000 g at 4°C for 1 hour in a Beckman 45i rotor. The final pellets were resuspended in a minimum volume of a buffer containing 400 mM sucrose, 5 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), titrated to pH 7.2 with Tris. Aliquots were made, frozen with liquid N_2 and stored at 80°C until use.

2.2 Measurement of protein concentration

The protein concentration of membrane preparations was assessed using a Bradford Assay, utilising a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin (BSA) standards were prepared containing 1 mg, 0.75 mg, 0.50 mg, 0.25 mg and 0.135 mg of BSA. 100 μ l of BSA standards were added separately to 5 eppendorf tubes and 1 ml of

1:4 Bradford reagent dye (containing alkaline copper tartrate) : deionised water, was added. The absorbance of each standard was measured using a Biomate 3 Series Spectrophotometer (wavelength 595 nm, Thermo Electron Limited, Cambridge, UK) and a standard curve plotted. The protein sample to be measured was diluted 1:1000 in deionised water and its absorbance measured. The protein concentration was then determined by extrapolation from the standard curve.

2.3 Planar phospholipid bilayer methods

2.3.1 Bilayer apparatus

Bilayers were formed from phosphatidylethanolamide lipids (PE) purchased from Avanti Polar Lipids (Birmingham, AL, USA). The solid lipids were dissolved in chloroform at a concentration of 50 mg/ml and stored at -80°C until use. When ready for use, the chloroform was removed under a stream of nitrogen and the lipids were resuspended in decane at a concentration of 35 mg/ml and stored on ice. This method was adapted from that first described by Miller in 1978²⁷¹.

The apparatus required for planar lipid bilayer experiments is shown in **Figure 2.1**. This consists of a block and a cup made from Delrin (acetyl resin) obtained from Warner Instruments (LLC, Hamden, CT, USA). The cup contains two compartments, one which houses the cup and forms the *cis* side of the bilayer, and the other forms the *trans* side. The cup contains an aperture of 150 µm on one side, which forms a connection between the two chambers. The two smaller chambers on the left-hand side of the block are filled with 3M LiCl and house the agar bridges and Ag/AgCl electrodes. The agar bridges themselves are made from capillary glass and filled with 3M LiCl and 2% agar, allowing them to make an electrical connection between the LiCl solution and recording solution in the *cis* and *trans* chambers.

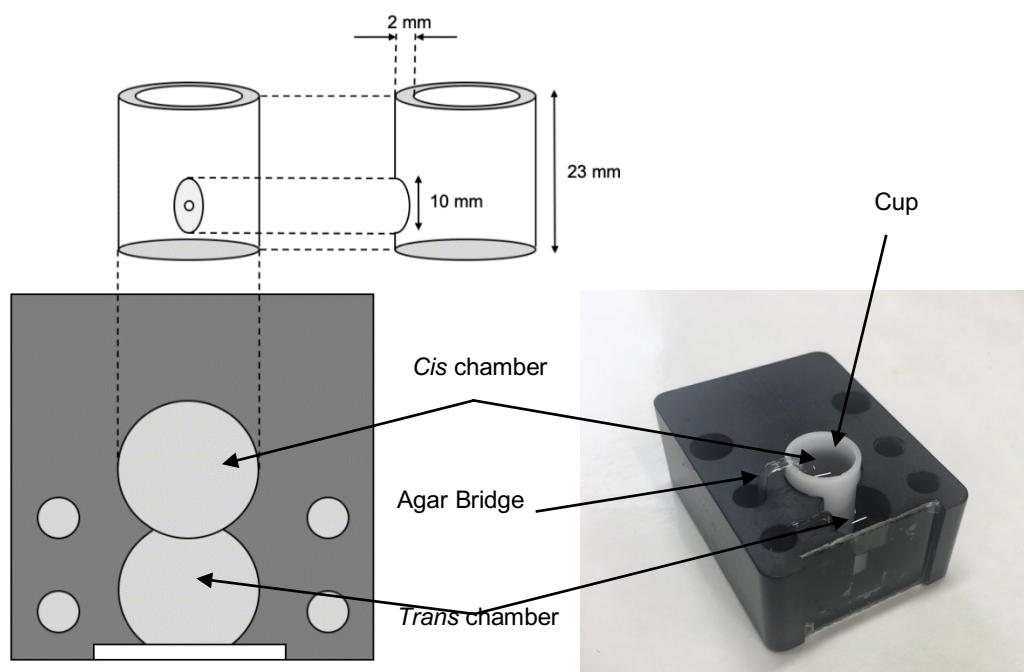


Figure 2.1 Apparatus for planar lipid bilayer experiments

The apparatus for performing planar lipid bilayer experiments is shown in schematic form (left) and in a photograph (right). The *cis* and *trans* chambers are indicated, and the dimensions of the cup which forms the cytosolic chamber are shown. The agar bridges are accommodated in two smaller chambers in the block and are filled with 3M LiCl solution.

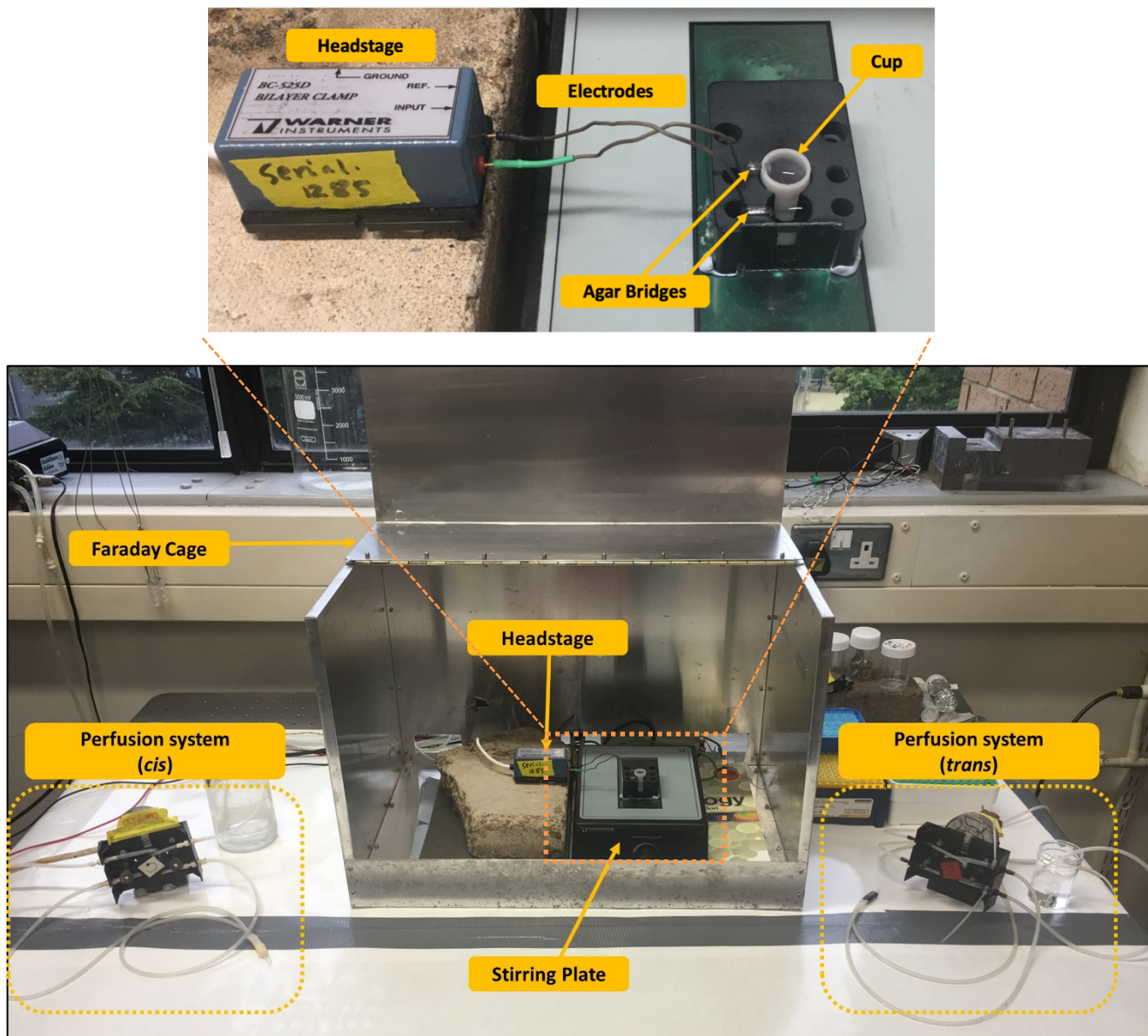


Figure 2.2 Full planar lipid bilayer set-up

The full setup for performing planar lipid bilayer experiments is shown. The block and cup are located on the stirrer plate inside a faraday cage and are connected to the recording equipment via the headstage. The inset shows an expanded view of the headstage connection to the block and cup via electrodes and the agar bridges. There are two perfusion systems, on either side of the setup for changing the recording solutions in both the *cis* and *trans* chambers. The Faraday cage surrounding the set-up shields the recordings from electrical and *acoustic* noise. The setup rests on an air table to reduce vibrational noise.

2.3.2 Electrical equipment for data acquisition

The following electrical equipment was used to record single channel traces:

- BC-535 Bilayer Clamp Amplifier (Warner Instruments, LLC, Hamden, CT, USA) which provides a voltage clamp across the lipid bilayers and records the current flowing between the *trans* and *cis* chambers. It includes a resistive feedback headstage and multi-step 4-pole Bessel filter.
- InstruTECH ITC-18 (HEKA, Elektronik Lambrecht/Pfalz, Germany), which allows the analogue signal received from the amplifier to be converted into a digital format that can be recorded by the computer. It is connected to the computer via a dedicated host interface.
- Oscilloscope (Hitachi VC-6545, TTid, Huntingdon, UK) which displayed the filtered trace in real time.
- A computer for recording the digital signal and storage using Axon™pCLAMP™ 10 Electrophysiology Data Acquisition & Analysis Software (Windows 10, 32 bit).
- A low-pass 8 pole Bessel filter (Frequency Devices Inc, Ottawa, Canada) to filter the signal at the required frequency (800 Hz) before it is displayed on the oscilloscope.
- A magnetic stirring plate (Spin-2 stirplate, Warner Instruments, LLC, Hamden, CT, USA) on top of which the block and cup are placed. A magnetic flea inside each chamber facilitates the mixing of the solutions.
- A Faraday cage which houses the bilayer set-up including the headstage, block and stirrer plate, shields the set-up from electrical noise.

A diagram of the recording set-up is shown in **Figure 2.3**. In order to achieve a good signal to noise ratio during the single-channel recordings, the BC-535 amplifier system was grounded from a point on the rear panel of the amplifier. The Faraday cage was grounded directly by a connection to the amplifier, and the head-stage ground coincided with the amplifier ground.

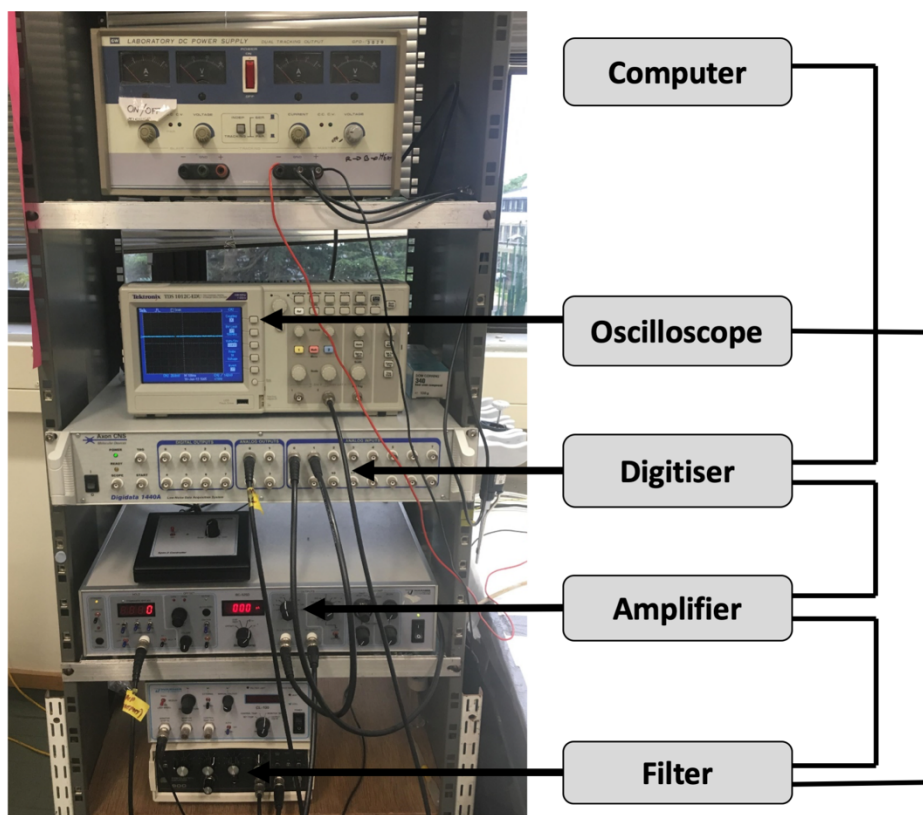


Figure 2.3 Equipment required for data acquisition and recording of single-channel traces

The principal electrical equipment for processing single channel bilayer recordings. The signal is received from the headstage to the amplifier, the signal is converted into a digital format, filtered and saved on the computer.

2.3.3 Planar phospholipid bilayer formation

The first step to forming a lipid bilayer involves 'priming' the cup. A drop of lipid solution (35 mg/ml in ice-cold decane) was applied to the interior of the aperture of the cup using a Gilson (Gilson Scientific UK, Bedfordshire, UK) pipette. After 5 min, this process was repeated. The primed cup was then allowed to dry. The cup is inserted into the block. A stirrer bar is added to both the *cis* and *trans* compartments, and each was filled with 1 mL of cold 50 mM choline chloride (ChCl) solution. The agar bridges and electrodes were then fitted to the block.

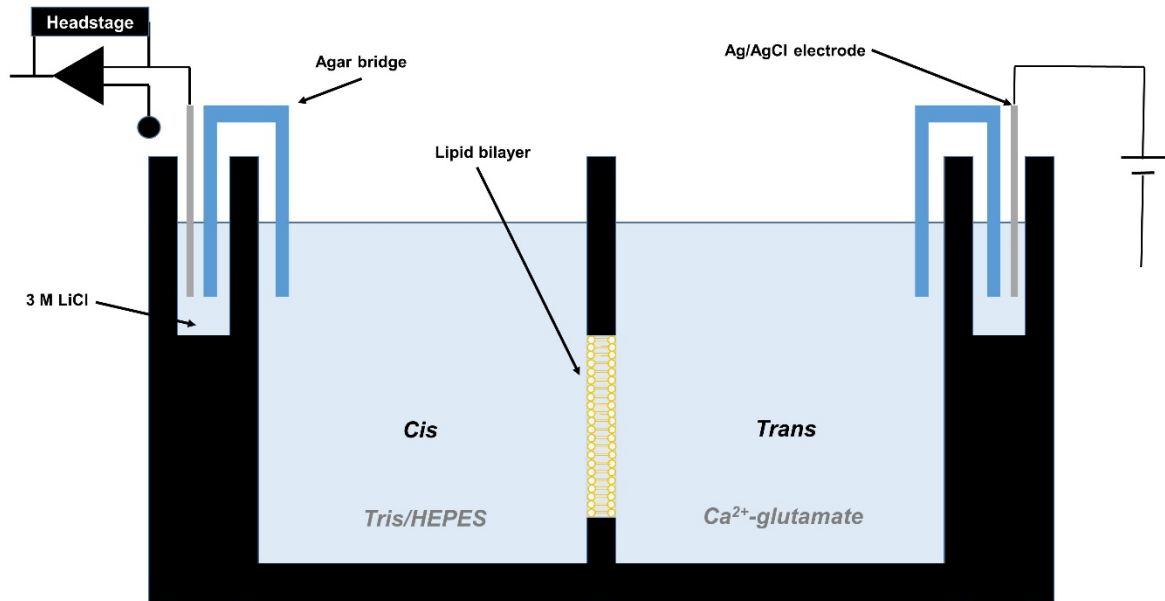


Figure 2.4 Schematic diagrams of the planar lipid bilayer system.

A lipid bilayer is painted across the aperture in the cup, which separates the *cis* and *trans* chambers. The Ag/AgCl electrodes are housed in two smaller compartments which contain a 3M LiCl solution. These compartments are linked with the *cis* and *trans* chambers, via LiCl containing agar bridges. The headstage commands the input from the amplifier, voltage-clamp and bilayer. The *trans* chamber is held at ground potential.

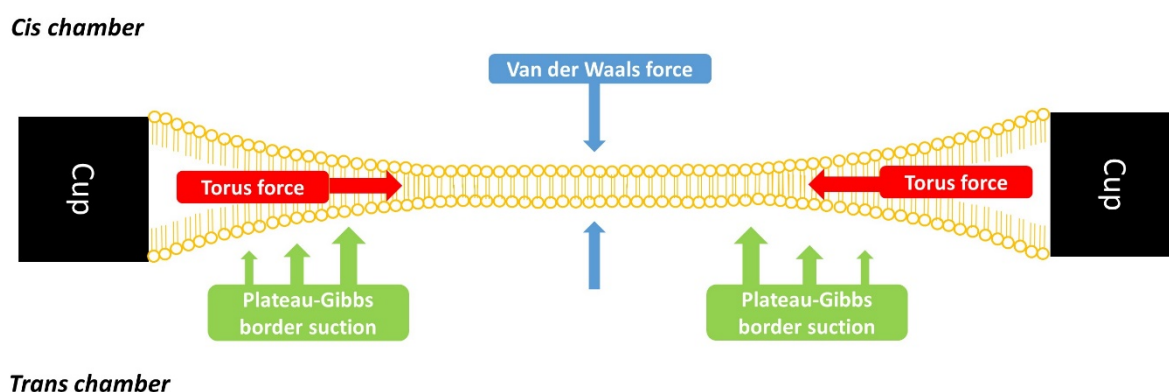
A simplified diagram indicating the main parts of the bilayer set-up is shown in **Figure 2.4**. Once symmetrical conditions (with zero offset) are established, a small amount of lipid is ‘painted’ across the aperture of the cup on the *cis* side, using a modified Pasteur pipette. This lipid forms a film of several microns in thickness which spontaneously thins into a planar bilayer due the combined effect of several forces. Due to the curvature of the bilayer as it contacts both the borders of the cup and the film, a hydrostatic pressure difference (ΔP) develops which creates a force known as the Plateau-Gibbs border suction. The pressure difference is described by LaPlace’s Law:

$$\Delta P = 2\gamma/R$$

Where γ refers to the interfacial tension, and R is the ‘radius of curvature’ (the perpendicular length of the annulus from cup border to bilayer). As ΔP has an inverse dependence on R , the pressure difference, and therefore Plateau-Gibbs force is greatest towards the middle of the

bilayer (at maximum R). Solution therefore flows from the middle of bilayer outwards towards the border, causing the bilayer to thin.²⁷² As the bilayer thins, van der Waals forces between the aqueous phases either side of the bilayer act to thin the bilayer further. These forces are balanced by an additional osmotic force, which is the result of the osmotic pressure created by the solvent trapped between the forming layers of lipid in a section known as the torus. Hence this force is also referred to as the 'torus force'.²⁷³ These forces are indicated in **Figure 2.5**. This process of 'thinning' can be monitored by following the capacitance of the bilayer, for the reasons described in the following section.

(A)



(B)



Figure 2.5 Forces involved in the formation of a planar lipid bilayer

(A) A schematic representation of the forces involved in the formation of a planar lipid bilayer are shown. The lipid bilayer forms in an aperture between the borders of the cup (shown in black). The important forces are indicated, namely the torus or osmotic force (red), Plateau-Gibbs force (green) and van der Waals force (blue). (B) A simplified diagram of lipid bilayer formation indicating the annulus.

2.3.3.1 Voltage-clamp technique

In the absence of a bilayer, the aperture of the cup allows ions to flow freely between and *cis* and *trans* chambers. Thus, a current can flow from the headstage, through the electrodes, conducting solutions and back to the headstage. When the aperture is 'blocked' by the formation of the lipid bilayer across it, current can no longer flow freely and the circuit is interrupted. To compensate, the electrical energy is stored in the lipid bilayer. Under these conditions the lipid bilayer therefore acts as a basic capacitor due to the excellent insulating properties of PE lipids and conducting properties of the surrounding salt solutions.

A capacitor stores a charge (Q) which is directly proportional to the potential difference (V) with a constant of proportionality known as the specific capacitance (C). Charge across a capacitor under steady state conditions can be expressed as:

$$Q = VC \quad \text{Equation 1}$$

A lipid bilayer can be thought of as a capacitor of fixed distance d (approximately the length of two lipid tails) between two parallel plates of area A . The capacitance (C_b) is therefore:

$$C_b = \epsilon \frac{A}{d} \quad \text{Equation 2}$$

Where ϵ is the dielectric constant which is a function of the material the capacitor is made from, in this case the lipids. When ions accumulate on one side of the bilayer, a potential difference (or voltage, V) is generated due to the build-up of charge. This potential difference generates a current I as the ions move to reduce the potential difference. Since current is defined as the rate of flow of charge in amperes:

$$I = \frac{\Delta Q}{\Delta T}$$

Therefore

$$I = \frac{\Delta Q}{\Delta T} = \frac{\Delta V}{\Delta T} C$$

For an infinitesimal change in V and T ($\Delta V = dV$ and $\Delta T = dT$), and therefore:

$$I = C \frac{dV}{dt} \quad \text{Equation 3}$$

Once an ion-channel has been incorporated into the lipid bilayer, ions are now able to follow between the compartments. However, an ion-channel acts as a filter, only passing certain ions. The ion-channel therefore acts as a form of resistor, R. The total current across the lipid bilayer (I_b) is therefore the sum of two components, the current following through the ion channel (Ionic Current, I_i) and any additional current otherwise flowing through the bilayer capacitor (C_b). The total current across the bilayer can therefore be expressed as:

$$I_b = I_i + C_b \frac{dV}{dt} \quad \text{Equation 4}$$

The voltage-clamp technique developed by Hodgkin *et al.* in 1952²⁷⁴ allows the membrane potential to be held at a constant value throughout single-channel experiments. The two-electrodes in the set-up are connected to the BC-535 bilayer amplifier. One electrode measures the voltage (i.e. input) and the other passes a feedback current which compensates (known as the reference). Therefore, $\frac{dV}{dt} = 0$ and the contribution of the capacitance C_b can be eliminated. Therefore, the current measured is solely that passing through the ion-channel under investigation.

2.3.3.2 *Bilayer formation*

During bilayer formation, close attention is paid to the capacitance of the bilayer. This is achieved through use of the CAPTEST function of the BC-535 bilayer amplifier. As can be seen from **Equation 2**, the capacitance C_b of a capacitor is related to the area between the two plates from which it is made. In our case, A is the size of the cup aperture, which is fixed. We see, therefore, that the capacitance is inversely proportional to the distance between the two plates, in this case, the thickness of the bilayer. Substituting **Equation 2** into **Equation 4**, we find that:

$$I = \varepsilon \frac{A}{d} \frac{dV}{dt} \quad \text{Equation 5}$$

During the experiments, a triangular waveform is applied to the membrane, which has a constant rate of voltage change. $\frac{dV}{dt}$ is therefore a constant. We can therefore express **Equation 5** as:

$$I = kA \quad \text{Equation 6}$$

Where k is a constant and A is the area of the lipid bilayer.

2.3.4 Fusion of native SR ion channel-containing vesicles into the lipid bilayer

Once a stable bilayer of the required capacitance (80 – 120 pF) has been achieved, the next step requires the insertion of an ion-channel-containing SR vesicle into the bilayer. A high salt gradient is created, by the addition of 200 μL of 3M salt solution (either ChCl or KCl) to the cytosolic (*cis*) chamber. This results in an osmotic gradient, causing water to flow from the *trans* to the *cis* chamber as shown in **Figure 2.6**. 1-2 μL of HSR membrane preparation, obtained as described in section **2.1.4**, is added to the cytosolic chamber and stirred. These vesicles move towards the osmotic gradient, and make contact with the lipid bilayer. Water crossing the membrane causes these vesicles to swell²⁷⁵, and to fuse with the membrane, with

the ion channel (in this case RyR) now fixed in orientation in the same way as it had been in the vesicle²⁷⁶. Incorporation always occurs with the cytosolic side of the RyR corresponding to the *cis* chamber, and the luminal side of the RyR corresponding to the *trans* chamber. This process is briefly illustrated in **Figure 2.7**.

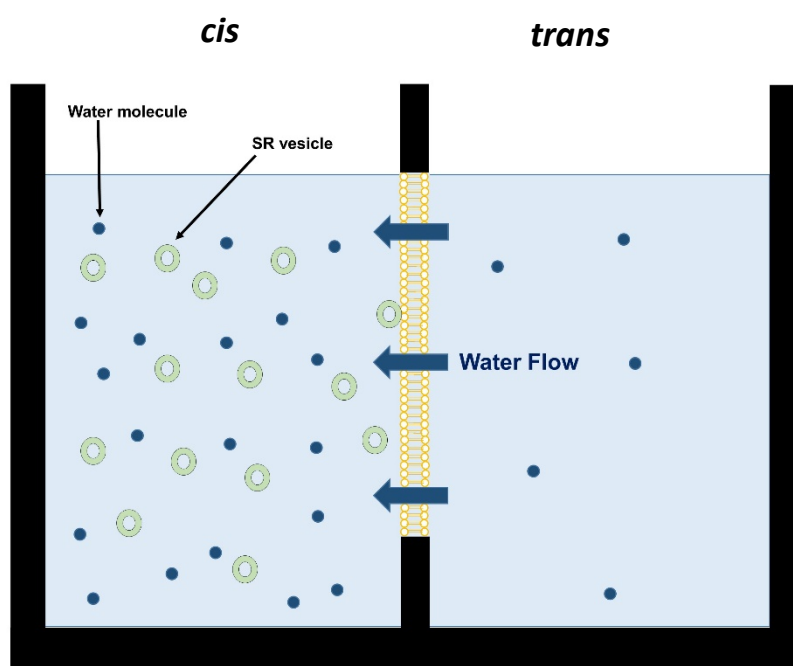


Figure 2.6 Osmotic gradient promoting fusion of SR vesicles to lipid bilayer.

Addition of a high-salt solution to the *cis* chamber causes water to move from the *trans* chamber to the *cis* chamber via osmosis.

Once the fusion process is complete, the symmetrical solutions are carefully perfused away and replaced with recording solutions, using perfusion pumps. These pumps contain an inflow and outflow system consisting of polyethylene tubing terminating in narrow glass capillary tubes. This system allows the solution in each chamber to be changed without disturbing the level of the solution. Each chamber is perfused for a sufficient time to allow 10 chamber volumes of solution (10 ml) to wash through, as this ensures removal of all unfused SR vesicles, and the addition of the required cytosolic recording solutions.

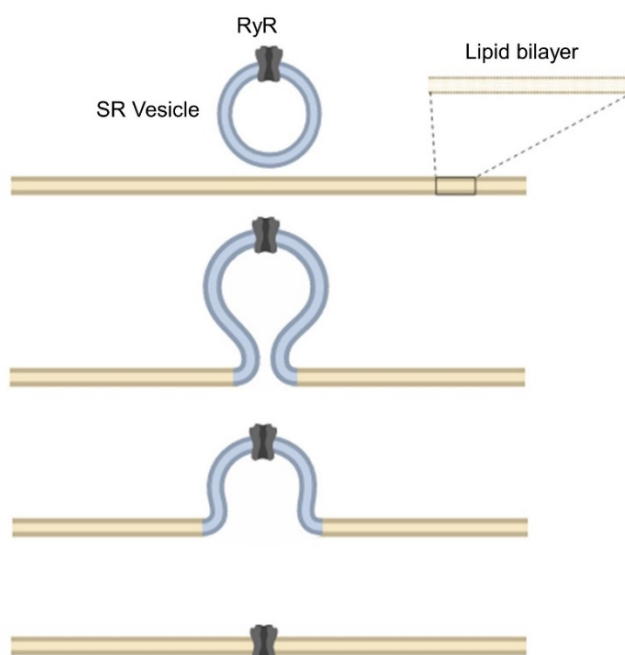


Figure 2.7 Incorporation of SR vesicles with a planar lipid bilayer.

Having added vesicles containing RyR to the *cis* compartment of the bilayer set-up, the osmotic gradient causes the vesicles to swell. As the swollen vesicles come into contact with the lipid bilayer, they begin to fuse with it. This ensures that all the RyR channels incorporated have the same fixed orientation, with the *cis* side corresponding to the cytosolic side of the channel, and the *trans* side the luminal side.

2.3.5 Recording solutions used for the single-channel study of RyR

In this thesis, the single-channel properties of RyR were studied using native SR vesicles. These vesicles contain not only RyR, but many other channels including predominately Cl^- and K^+ channels. The initial symmetrical solution used for vesicle fusion contained 50 mM ChCl, 10 mM HEPES, pH 7.2 with Tris. In the presence of these Cl^- containing solutions during the process of vesicle fusion, Cl^- channel incorporations can be visualised and used to determine when an SR vesicle has incorporated. In addition, if KCl is used as the salt solution to form the gradient, RyR channels can be visualised directly, as RyR is K^+ permeant. After SR vesicle incorporation, the *cis* chamber was perfused with 250 mM HEPES, 125 mM Tris at pH 7.2, with free $[\text{Ca}^{2+}]$ being buffered with EGTA to 10 μM Ca^{2+} . The *trans* chamber was perfused with 250

mM glutamic acid, 10 mM HEPES at pH 7.2, with free $[Ca^{2+}]$ being buffered to approximately 50 mM Ca^{2+} with $Ca(OH)_2$. The free $[Ca^{2+}]$ was measured as described in section 2.4.

2.4 Measurement of free $[Ca^{2+}]$

For all the experiments described in this thesis, the free $[Ca^{2+}]$ was maintained constant at 10 μ M unless otherwise stated. The free $[Ca^{2+}]$ was maintained by buffering with EGTA and $CaCl_2$ and measured using at 22 °C using a calcium electrode (Orion 93-20). Before use, the electrode was washed repeatedly with Milli-Q deionised water. It was then soaked for 48 hr in a low Ca^{2+} solution made up of 250 mM HEPES and 1 mM EGTA with the pH maintained at 7.2 by addition of Tris. Electrode calibration was subsequently performed using standards containing 0.10 mM, 1.0 mM and 10.0 mM $CaCl_2$ in a 250 mM HEPES solution titrated to pH 7.2 with Tris. The voltage recorded by the electrode upon immersion in the standard solution was recorded and a standard Ca^{2+} calibration curve was plotted. The voltage recorded from the *cis* and *trans* recording solutions could then be compared to this standard and the free $[Ca^{2+}]$ determined. Very low concentrations (< 10 nM) of Ca^{2+} cannot be measured using this technique, and the required concentrations of EGTA and $CaCl_2$ needed to achieve the desired free $[Ca^{2+}]$ were calculated using MAXCHELATOR (Chris Patton, Stanford, USA, maxchelator.stanford.edu)²⁷⁷. The pH and temperature of the solutions were measured using a Ross-type pH electrode (Orion 81-55).

Simvastatin sodium salt and atorvastatin sodium salt did not alter the free $[Ca^{2+}]$ solution of these buffered solutions when added at up to 1 mM concentration, nor was pH affected. Previous work from the Sitsapesan Lab had indicated that addition of Na_2ATP reduced the free $[Ca^{2+}]$. To prevent this, Na_2ATP was prepared in a 100 mM stock solution to which $CaCl_2$ was added to a $[Ca^{2+}]$ of 7.90 mM. Addition of this stock solution did not alter the free $[Ca^{2+}]$ or pH of the recording solutions. The Ca^{2+} chelating properties of ADP, AMP, Pi, PPi, PPPi, AMP-PCP

and GTP had been previously examined by the group, EGTA and CaCl_2 were added to the recording solutions to maintain the free cytosolic $[\text{Ca}^{2+}]$ at $10\ \mu\text{M}$.²⁷⁸

2.5 Analysis of single-channel recordings

2.5.1 Data acquisition and filtering

During a single-channel experiment, the analogue signal that is provided by the amplifier, is converted into a digital signal by the Instrutech ITC18 interface. This signal is then recorded by the program WinEDRv3.05 (John Dempster, Strathclyde University, UK). During the process of digitisation, the optimum sample frequency f_s must be chosen with care, to ensure that the signal is accurately reproduced and information is not lost through signal distortion (known as aliasing). In accordance with the Nyquist-Shannon Sampling Theorem, the optimal sample frequently must be at least twice the frequency B of the original signal^{279,280} (**Equation 7**).

$$f_s > 2B$$

Equation 7

To comply with this requirement, the sampling frequency chosen was 10 times greater than the filtered signal. The amplifier used an internal low-pass 4-pole Bessel filter, which filters the data at 10 kHz, the sampling frequency was therefore 100 kHz. Sampling frequency is measured as sample per unit time, therefore the sample interval in our procedure was 0.01 ms.

$$f_s = \frac{1}{t} = 10B$$

Equation 8

Due to the filtering process, some very brief events will be ‘missed’ as their amplitude is truncated and they do not therefore reach the 50% threshold level required to be considered as openings (**Section 2.5.3**). In order to prevent this from interfering with the analysis, events shorter in duration than approximately twice the ‘dead time’ (t_{min}) are excluded (stripped) from the analysis. The t_{min} value of the low-pass filter can be determined experimentally

using a signal generator which can create a square wave pulse. The amplitude of the wave over a range of frequencies can be measured, and the frequency at which the amplitude of the wave is reduced by 50% corresponds to the dead time for that level of filtration. Twice this value gives the t_{min} .²⁸¹ Due to the sampling interval, the shortest resolvable event is of 1 ms duration, and following inspection of the idealised trace, typically filtering is carried with the slightly greater value of 1.25 ms (which corresponds to 800 Hz). Therefore following digitisation, the recorded data were low pass filtered at 800 Hz using WinEDRv30.5 software prior to further analysis.

2.5.2 Single channel currents

An ion-channel that has been incorporated into a planar lipid bilayer allows the passage of certain ions from one side of the bilayer to the other, and hence acts as a conductor, allowing the passage of charge. The rate at which ions travel through a single-channel channel is known as the single-channel ionic conductance G , and is measured in units of Siemens (S). The single-channel conductance of an ion-channel is related the current I passing through it by Ohm's Law. Single-channel conductance is the reciprocal of the resistance, R ($G = \frac{1}{R}$) and therefore:

$$I = \frac{V}{R} = VG$$

Equation 9
Ohm's Law

Where I is the current flowing through the single ion-channel and V is the voltage across the bilayer. In order to measure the single-channel conductance of a RyR channel, current measurements at different holding potential V were carried out in order to construct a single-channel current-voltage plot. The gradient of the relationship calculated by linear regression corresponds to the single-channel conductance of the channel. The amplitude of the ionic current was measured manually using cursors in WinEDRv3.05. As previously described and to ensure accuracy, the cursors were positioned only at levels where a full event that was

resolved to a duration of greater than 1 ms. Linear regression was performed using GraphPad 8, La Jolla, CA, USA.

2.5.3 Open probability of a single channel

An ion-channel acts as a 'molecular gate' and in terms of current it can exist in one of two main conformations. Either fully open, where ions can pass, or fully closed where they cannot. The rapid fluctuations between these two states are known as 'gating'. When the channel opens, ion channels can pass through it and hence a current flows. This is observed as a current step in the single-channel trace. As the channel closes, we see the current return to zero as ions can no longer be conducted through the ion channel.

Analysis of how much time is spent by the channel in either the open or closed state (known as the 'dwell time') and the times at which each transition between the two states occurs, provides information about the single channel function. We can also use this information to determine the effect of a drug on how RyR channels gate. Analysis is typically carried out using commercial software packages (such as ClampFIT and WinEDR) and may be a complex process depending on the gating of the channel under investigation. Some of the complicating factors may be the presence of sub-conductance states (those that do not reach the fully open level), the incorporation of multiple channels into the bilayer, or a poor signal to noise ratio.

In order to accurately detect open/closed channels transitions, traces are analysed using the 50% threshold method²⁸², and the results of this produces an 'idealised trace'. A typical single-channel recording following filtering and digitisation is shown in **Figure 2.8**. The 50% threshold level is defined as the level of 50% of the maximum current amplitude of the channels. Any opening that reaches this level is deemed 'detected' and considered as a channel opening event. Any openings which do not reach this level are excluded. This process produces an idealised trace, which can be seen in the lower panel of **Figure 2.8**. This method has been used

extensively by our laboratory to analyse a variety of ion-channels^{136,141,269,283–285}, including RyR which is the subject of the study in this thesis.

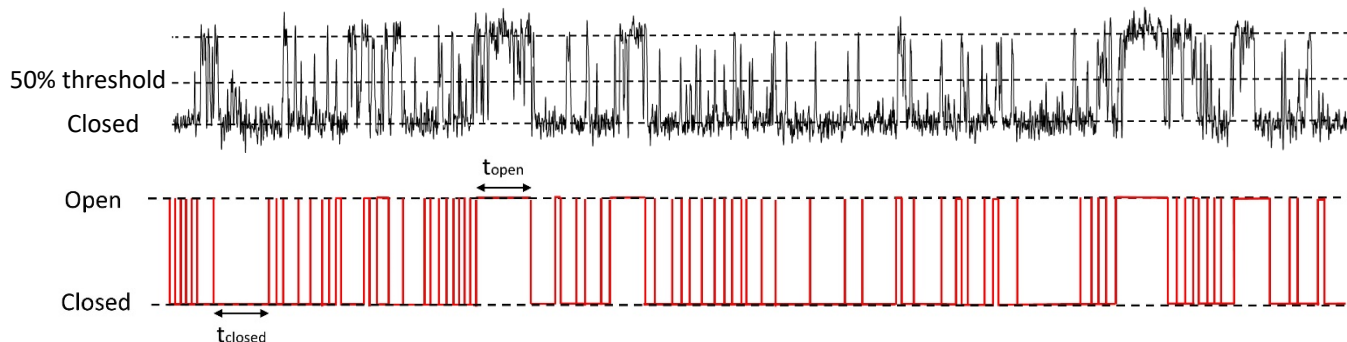


Figure 2.8 Single-channel event detection using the 50% threshold analysis method

The top trace shows a typical RyR recording after having been low-pass filtered at 800 Hz. The open and closed channel levels are indicated with dotted lines. Events which cross the 50% threshold are ordered as channel opening events, while those that don't are excluded. The result of this is the formation of an idealised trace, shown in the bottom panel. T_{open} and T_{closed} represent the time that the channel opens in either the open or closed dwell time respectively.

One of the main analyses performed on single-channel data, is the calculation of the open probability (P_o) of the channel. This is the probability that a channel will exist in the open conformation under the specific experimental conditions. The steady state P_o is calculated from all the events detected from at least 3 min of single-channel recording. The open probability can be calculated as:

$$P_o = \frac{T_{open}}{T_{closed} + T_{open}} \quad \text{Equation 10}$$

Where T_{open} and T_{closed} are the times spent by the channel in the open and closed states, respectively. In the case where multiple channels are incorporated into the bilayer, it is necessary to calculate the average P_o for N channels, where N is the number of channels incorporated into the bilayer. The total NP_o of N channels can be calculated as:

$$NP_o = \frac{T_{open1} + 2(T_{open2}) + 3(T_{open3}) + \dots n(T_{openn})}{T_{total}} \quad \text{Equation 11}$$

Where T_{open1} , T_{open2} and T_{open3} are the open dwell times for the first, second and third channel. T_{total} is the total recording time, which was 3 minutes unless specified otherwise. If the number of channels incorporated into the bilayer is known, from **Equation 11**, we can determine the average P_o of the channels in the bilayer as:

$$\text{average } P_o = \frac{NP_o}{N} = \frac{P_{o1} + P_{o2} + P_{o3} + \dots nP_{on}}{N} \quad \text{Equation 12}$$

Where P_{o1} , P_{o2} and P_{o3} are the probability of dwelling in the first, second or third channel level respectively.

2.5.4 Open and closed lifetime distributions

It is also possible to perform a more detailed analysis of single-channel recordings, by performing *lifetime analysis*.²⁸² As we have already seen, an ion-channel can exist in a number of conformations, mostly notably its open and closed states. In fact, there are an infinite number of conformations that an ion channel may exist in, between these two states, however, many are fleeting and not stable enough to be detected in single-channel recordings. Hence, a channel will gating more frequently in the more stable conformations. Of course, this is a random process, and so a mathematical approach is required to understand this process and relate it back to how the ion-channel operates physiologically. It is helpful to visualise this process by plotting a frequency histogram.

After 3 min of consecutive recording, filtering at 800 Hz and stripping of short events below the resolvable duration is carried out as described earlier (**section 2.5.1**). The data collected

are grouped into *bins* (intervals of duration) in order to obtain two distinct duration histograms, one for the open lifetimes, and one for the closed life-times. A histogram is plotted where the y axis is event duration, while the x axis represents the dwell time duration, t , for a given channel conformation.

A probability density function (pdf) can be used to fit the data, which is the sum of several exponential components.²⁸² The pdf of a single exponential is given by:

$$f_{(t)} = \frac{1}{\tau} \exp\left(\frac{-t}{\tau}\right) \quad \text{Equation 13}$$

Where $f_{(t)}$ is the probability that the channel will be in a dwell time of duration t , τ is the time constant, which is equal to the mean of the durations within the distribution. Often, more than one exponential term will be required to properly define a pdf. When the dwell time distributions require more than one exponential, the pdf is given by:

$$f_{(t)} = \sum_i^n \frac{a_i}{\tau_i} \exp\left(\frac{-t}{\tau_i}\right) \quad \text{Equation 14}$$

Where a is the relative area corresponding to a given time constant τ .

The initial fitting of a pdf is done manually by trial and improvement, and then the set of parameters (a_i and τ_i) that best fit the data are calculated by the software programme using a method known as *maximum likelihood fitting*.²⁸⁶ As the name suggests, this method generates the 'most likely' pdf for a given distribution of open or closed events for a specified number of exponentials. The method adjusts the set of parameters until the optimum value of the log of likelihood L is reached.²⁸¹ The number of exponentials that best fit the distribution is determined using the likelihood ratio test.²⁸²

2.6 [³H]ryanodine binding

[³H]ryanodine binding to HSR vesicles was performed using the method first described by Holmberg & Williams in 1990.²⁶⁵ The exact protocol used was as follows. 100-130 µg of HSR vesicles were incubated for 90 minutes at 37°C, in the presence of 5 nM 9, 21(n)-³H]ryanodine (“³H]ryanodine”) in 250 µl of a buffer containing 250 mM HEPES, 125 mM Tris at pH 7.2, with free [Ca²⁺] being buffered with EGTA to 10 µM Ca²⁺. Each experiment was performed in triplicate and averaged. Additionally, non-specific binding was measured by the addition of 5 µM unlabelled ryanodine (1000 fold excess)(Agrisystem International, Wind Gap, PA, USA) to additional tubes. After incubation and continuous shaking for 90 min, the reaction was quenched by addition of 5 ml of ice-cold buffer (as above). The samples were filtered through Whatman GF/B filters which had been pre-soaked in buffer containing polyethyleneimine (Alfa Aesar, Haverhill, MH, United States). The filters were washed 3 times with 10 mL of ice-cold buffer, and placed in scintillation vials with 10 ml of Emulsifier-Safe™ scintillation cocktail (PerkinElmer Waltham, MH, United States) and counted using a 1219 Rackbeta liquid scintillation counter (Wallac Inc, USA). The specific binding was then calculated by subtraction of the non-specific binding from that obtained from the experimental samples. [³H]ryanodine binding was carried out using the above protocol unless stated otherwise in individual chapters.

2.7 Liquid chromatography–mass spectrometry analysis

Liquid chromatography–mass spectrometry analysis (LC-MS) of both simvastatin sodium salt and simvastatin lactone was carried out using Thermo Exactive mass spectrometer equipped with Water Acquity liquid chromatography system. Instrument control was performed using Thermo Xcalibur Software. Electrospray conditions were adjusted to maximise sensitivity.

Sim-H and Sim-L were prepared as 10 mM solutions in water and DMSO respectively, frozen with liquid N₂ and stored at -80 °C until use, and warmed to room temperature immediately

before injection and analysis. 1 μL of each solution was added to 1 mL of water or Tris/HEPES (250 mM HEPES, 80 mM Tris, 10 μM free Ca^{2+} at pH 7.2) buffer and sampling begun immediately. Samples were kept at room temperature for the duration of the experiment; 3 μL of each sample was injected into the system.

The column was a 2.1 x 50 mm (3 μm) ACE equivalence C18. A mixed eluent gradient was employed as shown in **Table 2.1**. The four sample types were analysed sequentially; the 5 min analysis time for each sample provided a 20 min sampling frequency for each sample. Mass analysis was performed using MestReNova MS Software (2016, V11.0), where the peaks were integrated to determine relative percentages of Sim-H and Sim-L overtime.

Retention [min]	Flow [ml/min]	% Acetonitrile	% Water + 0.1 % Formic acid
0	0.4	5	95
0.5	0.4	5	95
3.5	0.4	99	1
4	0.4	99	1
4.1	0.4	5	95
5	0.4	5	95

Table 2.1 Conditions for LC-MS analysis of statin samples

A mixed gradient of eluent was used as described in the table, with the percentage of acetonitrile/water/formic acid being varied with retention time as indicated.

2.8 Chemistry methods

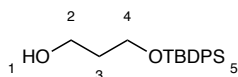
2.8.1 General Experimental

All chemicals were purchased from Sigma-Aldrich, UK, Apollo Scientific UK, Alfa Aesar UK, Fluorochem UK or Fisher Scientific UK unless otherwise stated. All anhydrous solvents were dried using a protocol modified from that reported by Pangborn *et al.*²⁸⁷ Water was purified by an Elix® UV-10 system. All other reagents were used without further purification. ‘Petrol’

refers to the 30-40 °C fraction of petroleum ether. All reactions involving moisture-sensitive reagents were carried out using oven dried glassware under an atmosphere of nitrogen using anhydrous solvents. Thin layer chromatography was performed using Merck silica gel 60 F₂₅₄ thin layer chromatography sheets, supported by aluminium backs. Plates were visualised either under UV light (254 nm), or thermal development after dipping in 1% aq. KMnO₄ or p-Anisaldehyde. Flash column chromatography was performed on Kieselgel 60 silica in a glass column or on a Biotage SP1 Purification system. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter with a water-jacketed 10 cm cell at the temperature, indicated. Specific rotations are reported in 10⁻¹ deg cm² g⁻¹ and concentrations in g/100 mL. Infrared spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer using thin film samples dissolved in CHCl₃. Selected characteristic peaks above 1500 cm⁻¹ are reported in wavenumbers (cm⁻¹). High resolution mass spectra (HRMS) were recorded on a Bruker MicroTOF. ¹H NMR spectra were recorded on a Bruker AV400 (400 MHz) spectrometer and referenced to residual solvent peaks. Chemical shifts are quoted in parts per million (ppm). Coupling constants J (Hz) are recorded to the nearest 0.5 Hz. Where peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broadened, dd = doublet of doublets, ddd = double doublet of doublets.

2.8.2 Compounds synthesised in this thesis

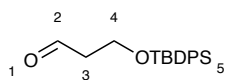
3-((*tert*-butyldiphenylsilyl)oxy)propan-1-ol, (8)



NaH (570 mg, 13.2 mmol, 1.0 eq) was suspended in THF (60 mL) under nitrogen and cooled to 0 °C. A solution of 1,3-propanediol (0.950 mL, 13.2 mmol, 1.0 eq) in THF (10 mL) was added dropwise. The reaction mixture was warmed to room temperature and allowed to stir for 16 h. The reaction mixture was again cooled to 0 °C before the dropwise addition of *tert*-butyl(chloro)diphenylsilane (3.70 mL, 13.2 mmol, 1 eq). After stirring for a further 2 h at 0 °C,

the reaction was quenched by adding crushed ice (100 g), allowed to warm to room temperature and diluted with EtOAc (25 mL). The layers were separated, and the aqueous layer extracted with EtOAc (25 mL). The combined organic layers were washed with water, brine, dried (MgSO_4) and concentrated under reduced pressure. Purification by flash column chromatography (eluent EtOAc/petrol, 25:75) gave **8** as a white solid (3.49 g, 84%); **mp** 39-42°C (lit 42-43°C, EtOAc)²⁸⁸; TLC (SiO_2): R_f = 0.40 (3:7 ethyl acetate:cyclohexane); $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 7.71-7.66 (m, 5H, PhH), 7.48-7.36 (m, 5H, PhH), 3.85 (td, J = 5.7, 1.6 Hz, 4H, H_2 , H_3), 2.38 (s, 1H, O-H), 1.82 (q, J = 5.7 Hz, 2H, H_2), 1.06 (s, 9H, $\text{C}(\text{CH}_3)_3$); $^{13}\text{C NMR}$ (101 MHz, CDCl_3): δ 135.4 (Ph), 133.1 (Ph), 129.6 (C_2), 61.8 (C_3), 34.1 (C_4), 26.7 ($\text{C}(\text{CH}_3)_3$), 18.9 ($\text{C}(\text{CH}_3)_3$); m/z (ESI^+) 337; **HRMS**: $\text{C}_{19}\text{H}_{26}\text{O}_2\text{NaSi}$ requires 337.15943; found 337.15948. Spectral data were found to be consistent with literature values²⁸⁹.

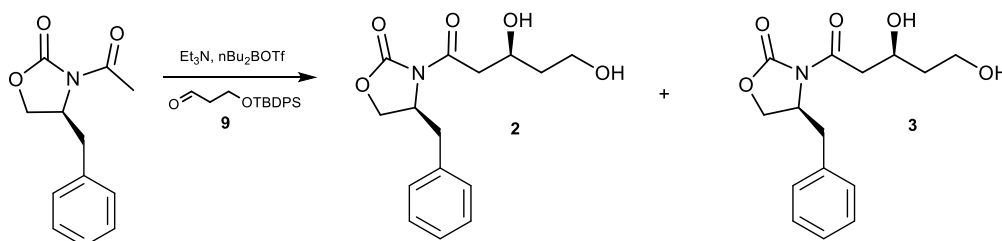
3-((tert-butyldiphenylsilyl)oxy)propanal, (**9**)



Oxalyl chloride (1.20 mL, 14.3 mmol, 1.5 eq) was added to CH_2Cl_2 (60 mL) under nitrogen and the solution was cooled to -78 °C. DMSO (2.20 mL, 30.6 mmol, 3.2 eq) was added slowly dropwise and the solution was allowed to stir for 5 min. A solution of compound **1** (3.00 g, 9.54 mmol, 1.0 eq) in CH_2Cl_2 (20 mL) was added dropwise. The reaction mixture was stirred for 15 min at -78 °C before addition of triethylamine (6.9 mL, 47.7 mmol, 5.0 eq). The reaction mixture was allowed to warm to room temperature, water (50 mL) was added and layers were separated. The aqueous layer was extracted with CH_2Cl_2 (3 x 15 mL). The combined organic layers were washed with 1M HCl (aq., 2 x 50 mL), brine and dried (MgSO_4). The solvent was removed under reduced pressure. Purification by flash column chromatography (eluent EtOAc/petrol, 10:90) gave **9** as a pale yellow oil, which solidified after drying under high-vacuum (2.69 g, 90%). TLC (SiO_2): R_f = 0.60 (3:7 EtOAc:cyclohexane); $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 9.83 (t, J = 2.2 Hz, 1H, H_2), 7.71-7.63 (m, 5H, PhH), 7.50-7.36 (m, 5H, PhH), 4.03 (t, J = 6.0 Hz,

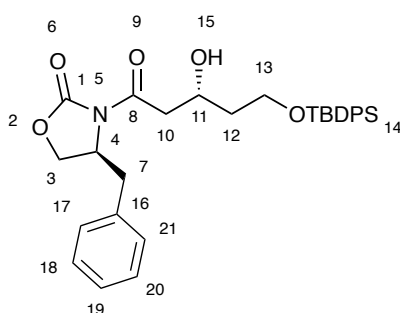
2H, H_3), 2.61 (td, $J = 6.0, 2.2$ Hz, 2H, H_4), 1.05 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (101 MHz, CDCl_3): δ 201.7 (C_2), 135.4 (Ph), 133.1 (Ph), 58.1 (C_3), 46.2 (C_4), 26.6 ($C(CH_3)_3$), 19.0 ($C(CH_3)_3$); m/z (ESI $^+$) 335; HRMS: $\text{C}_{19}\text{H}_{24}\text{O}_2\text{Na}^{28}\text{Si}$ requires 335.14378; found 335.14384. Spectral data were found to be consistent with literature values.²⁸⁹

(*R*)-4-benzoyl-3-(*R/S*)-5-((*tert*-butyldiphenylsilyl)oxy)-3-hydroxypentanoyloxazolidin-2-one (2) and (3)

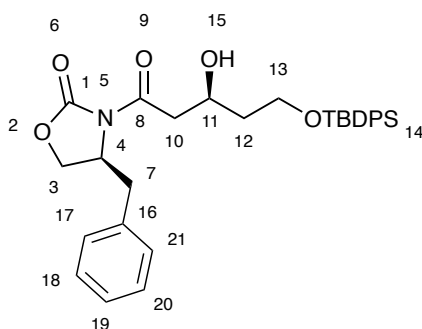


Acetyl-4-benzyl oxazolidine (1.00 g, 4.56 mmol, 1.0 eq) was added to a flame-dried flask under nitrogen and dissolved in dichloromethane (degassed (x3), 20 mL) before cooling to -78 °C. The resulting solution was treated with dibutylboron triflate (1.0 M in dichloromethane, 0.83 mL, 6.02 mmol, 1.32 eq), followed by dropwise addition of triethylamine (787 μL , 5.70 mmol, 1.32 eq.) at such a rate as to keep the internal temperature below 3 °C. The solution turned slightly yellow or green during the dibutylboron triflate addition, and then to light yellow when triethylamine is added. When the internal temperature dropped below -65 °C, a solution of aldehyde **9** (1.56 g, 5.11 mmol, 1.11 eq) in dichloromethane (20 mL) was added. The resulting solution was stirred for 5 h at -78 °C, then quenched by addition of a pH 7.4 aqueous phosphate saline buffer (5 mL) and methanol (14 mL) and allowed to warm to 0 °C. A solution of methanol and 30% aqueous hydrogen (2:1, 14 mL) was then added at such a rate as to keep the internal temperature below 10 °C. The resulting mixture was stirred for a further 1 h, the solvent was partially removed on a rotary evaporator at a bath temperature of 25 – 30 °C. The

resulting slurry was extracted diethyl ether (3 x 25 mL). The combined organic extracts were washed with aq. saturated sodium bicarbonate, brine and dried (MgSO₄). The solvent was removed under reduced pressure. Purification by flash column chromatography (eluent EtOAc/40-60°C petrol, 10:90 → 25:75) gave **2** (1.57 g, 53%) and **3** (925 mg, 31%) as colourless oils.

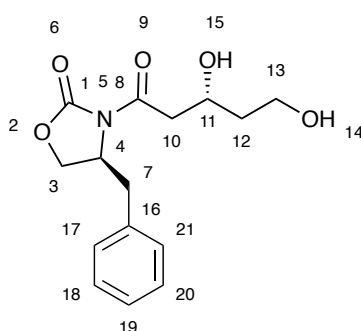


TLC (SiO₂): **R_f** = 0.65 (3:7 EtOAc:cyclohexane) **IR** ν_{max} (film): 3512 (O-H), 1781 (C=O), 1696 (C=O), 1103 (C-N); **¹H NMR** (400 MHz, CDCl₃) δ 7.75 – 7.63 (m, 4H, PhH), 7.50 – 7.42 (m, 3H, PhH), 7.41 – 7.39 (m, 2H, PhH), 7.38 – 7.31 (m, 2H, PhH), 7.33 – 7.25 (m, 2H, PhH), 7.24 – 7.18 (m, 2H, PhH), 4.76 – 4.64 (m, 1H, *H*₄), 4.45 (m, *J* = 4.1 Hz, 1H, *H*₁₁), 4.26 – 4.07 (m, 2H, *H*₃), 3.97 – 3.82 (m, 2H, *H*₁₀), 3.45 (s, 1H, O-H), 3.30 (dd, *J* = 13.5, 3.3 Hz, 1H, *H*₇), 3.21 – 3.12 (m, 2H, *H*₁₃), 2.79 (dd, *J* = 13.4, 9.5 Hz, 1H, *H*₇), 1.91 – 1.71 (m, 2H, *H*₁₂), 1.06 (s, 9H, 3 x CH₃); **¹³C NMR** (101 MHz, CDCl₃): δ 201.7 (*C*₈), 135.4 (*C*₁), 133.1 (Ph), 129.6 (Ph), 127.6 (Ph), 127.6 (Ph), 58.1 (*C*₃), 52.1 (*C*₁₁) 46.2 (*C*₁₃), 41.4 (*C*₄), 41.2 (*C*₁₀), 36.6 (*C*₇) 19.0 (*C*₁₂); ***m/z*** (ESI⁺) 532; **HRMS**: C₃₁H₃₈O₅NSi requires 532.25138; found 532.25179.



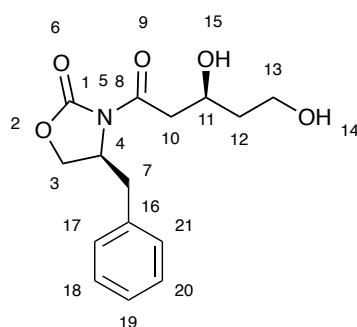
TLC (SiO₂): **R_f** = 0.60 (3:7 EtOAc:cyclohexane); **IR** ν_{max} (film): 3034 (O-H), 1826 (C=O), 1781 (C=O), 1104 (C-N); **¹H NMR** (400 MHz, CDCl₃): δ 7.73 – 7.63 (m, 4H, PhH), 7.48 – 7.41 (m, 3H, PhH), 7.41 – 7.37 (m, 2H, PhH), 7.37 – 7.31 (m, 2H, PhH), 7.31 – 7.25 (m, 2H, PhH), 7.25 – 7.18 (m, 2H, PhH), 4.76 – 4.65 (m, 1H, *H*₄), 4.48 (p, *J* = 4.2 Hz, 1H, *H*₁₁), 4.26 – 4.07 (m, 2H, *H*₃), 3.97 – 3.82 (m, 2H, *H*₁₀), 3.45 (s, 1H, O-H), 3.32 (dd, *J* = 13.4, 3.4 Hz, 1H, *H*₇), 3.21 – 3.12 (m, 2H, *H*₁₃), 2.79 (dd, *J* = 13.4, 9.6 Hz, 1H, *H*₇), 1.91 – 1.71 (m, 2H, *H*₁₂), 1.06 (s, 9H, 3 x CH₃); **¹³C NMR** (101 MHz, CDCl₃): δ 201.7 (*C*₈), 135.4 (*C*₁), 133.1 (Ph), 129.6 (Ph), 127.6 (Ph), 127.6 (Ph), 58.1 (*C*₃), 46.2 (*C*₁₃), 41.3 (*C*₄), 41.2 (*C*₁₀), 36.6 (*C*₇), 19.0 (*C*₁₂); ***m/z* (ESI⁺)** 532; **HRMS**: C₃₁H₃₈O₅NSi requires 532.25138; found 532.25269. Spectral data were found to be consistent with literature values.²⁸⁹

(*R*)-4-benzoyl-3-((*R*)-3,5-dihydroxypentanoyl)oxazolidin-2-one, (4)



HF/pyr (118 μ L, 4.13 mmol, 5.0 eq) was added slowly to an ice-cold solution of imide **2** (440 mg, 0.827 mmol, 1.0 eq) in THF (15 mL) in a falcon tube a. The resulting mixture was stirred at 0 °C for 4 h. The falcon tube was opened to air, and aq. saturated sodium bicarbonate was added dropwise until no further gas could be seen evolving. The organic layer was extracted with EtOAc (2 x 20 mL), and washed with HCl (aq., 1M), NaHCO₃ (aq. sat. sol.) and brine. The organic layer was dried (MgSO₄) and concentrated under reduced pressure. Purification by flash column chromatography (eluent 75% $\rightarrow$ 100% EtOAc; cyclohexane) gave alcohol **4** as a colourless oil (165 mg, 65%). TLC (SiO₂): R_f = 0.70 (100% EtOAc); IR $\nu_{\max}$ (film): 3396 (O-H), 2929 (O-H), 1774 (C=O), 1694 (C=O); ¹H NMR (400 MHz, CDCl₃) δ 7.43 – 7.31 (m, 2H, PhH), 7.31 – 7.24 (m, 2H, PhH), 7.24 – 7.16 (m, 1H, PhH), 4.71 (ddt, J = 9.3, 7.0, 3.3 Hz, 1H, H_4), 4.39 (dddd, J = 9.1, 8.0, 4.6, 3.6 Hz, 1H, H_3), 4.28 – 4.20 (m, 1H, H_{11}), 3.88 (td, J = 4.9, 4.4, 1.3 Hz, 2H, H_7), 3.29 (dd, J = 13.5, 3.5 Hz, 1H, H_3), 3.21 – 3.06 (m, 2H, H_{12}), 2.81 (dd, J = 13.5, 9.4 Hz, 2H, H_7), 1.92 – 1.71 (m, 2H, H_{10}), OHs were not observed; ¹³C NMR (101 MHz, CDCl₃): δ 172.8 (C₈), 135.1 (C₁), 129.6 (Ph), 129.2 (Ph), 127.6 (Ph), 77.5 (Ph), 77.2 (C₃), 76.8 (C₁₁), 68.1 (C₁₃), 66.6 (C₄), 61.3 (C₁₀), 55.2 (C₇), 42.9 (C₁₂); m/z (ESI⁺) 316; HRMS: C₁₅H₁₉O₅NNa requires 316.11554; found 316.11535; Spectral data were found to be consistent with literature values.²⁸⁹

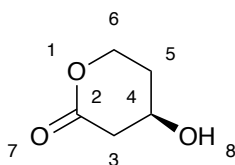
(*R*)-4-benzoyl-3-((*S*)-3,5-dihydroxypentanoyl)oxazolidin-2-one, (5**)**



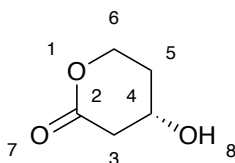
Using a similar procedure to the synthesis of alcohol **4**, from imide **3** (440 mg, 0.827 mmol) **5** was obtained as a colourless oil (147 mg, 59%). TLC (SiO₂): R_f = 0.70 (100% EtOAc); IR $\nu_{\max}$

(film): 3396 (O-H), 2929 (O-H), 1774 (C=O), 1694 (C=O). $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.45 – 7.31 (m, 2H, PhH), 7.38 – 7.24 (m, 2H, PhH), 7.23 – 7.15 (m, 1H, PhH), 4.71 (ddt, J = 9.2, 7.0, 3.3 Hz, 1H, H_4), 4.40 (m, 1H, H_3), 4.28 – 4.23 (m, 1H, H_{11}), 3.88 (td, J = 4.9, 4.5, 1.3 Hz, 2H, H_7), 3.30 (dd, J = 13.5, 3.5 Hz, 1H, H_3), 3.20 – 3.06 (m, 2H, H_{12}), 2.81 (dd, J = 13.5, 9.4 Hz, 2H, H_7), 1.92 – 1.71 (m, 2H, H_{10}), OHs were not observed; $^{13}\text{C NMR}$ (101 MHz, CDCl_3): δ 172.8 (C_8), 135.1 (C_1), 129.6 (Ph), 129.2 (Ph), 127.6 (Ph), 77.5 (Ph), 77.2 (C_3), 76.8 (C_{11}), 68.1 (C_{13}), 66.6 (C_4), 61.3 (C_{10}), 55.2 (C_7), 42.9 (C_{12}); m/z (ESI $^+$) 316; HRMS: $\text{C}_{15}\text{H}_{19}\text{O}_5\text{NNa}$ requires 316.11554; found 316.11564. Spectral data were found to be consistent with literature values.²⁸⁹

(R)-4-hydroxytetrahydro-2H-pyran-2-one, (R-6)



Triethylamine (0.28 mL, 1.36 mmol, 6.0 eq) was added dropwise to a solution of alcohol **4** (80 mg, 0.272 mmol, 1.0 eq) in CH_2Cl_2 (5 mL) and the mixture allowed to stir at room temperature for 16 h. When TLC indicated complete consumption of the starting material (R_f = 0.7, 100% EtOAc), toluene (2 mL) was added and the mixture concentrated under reduced pressure. After co-evaporation with 2 further aliquots of toluene, purification by flash column chromatography (eluent 50% $\rightarrow$ 100% EtOAc: pentane) gave **R-6** as a colourless oil (16 mg, 51%). TLC (SiO_2): R_f = 0.52 (100% EtOAc); $[\alpha]_D^{25}$ = -0.60 (CHCl_3 , c = 0.1); IR ν_{max} (film): 3386 (O-H), 1717 (C=O); $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 4.57 (ddd, J = 11.4, 8.2, 4.2 Hz, 1H, H_4), 4.41 – 4.17 (m, 2H, H_6), 2.81 (ddd, J = 17.6, 5.3, 0.9 Hz, 1H, H_3), 2.61 (m, 1H, H_3), 2.11 (ddd, J = 13.7, 8.4, 4.7 Hz, 1H, H_5), 1.89 (dtdd, J = 14.1, 6.4, 4.2, 1.2 Hz, 1H, H_5), OH was not observed; $^{13}\text{C NMR}$ (101 MHz, CDCl_3): δ 170.8 ($\text{C}_2=\text{O}$), 77.8 (C_4), 77.5 (C_6), 65.8 (C_5), 63.7 (C_3); m/z (ESI $^+$) 117; HRMS: $\text{C}_5\text{H}_9\text{O}_3$ requires 117.05462; found 117.05480. Spectral data were found to be consistent with literature values.²⁸⁹

(S)-4-hydroxytetrahydro-2H-pyran-2-one, (S-6)

Triethylamine (0.28 mL, 1.36 mmol, 6.0 eq) was added dropwise to a solution of alcohol **5** (80 mg, 0.272 mmol, 1.0 eq) in CH₂Cl₂ (5 mL) and the mixture allowed to stir at room temperature for 16 h. When TLC indicated complete consumption of the starting material ($R_f = 0.7$, 100% EtOAc), toluene (2 mL) was added, and the mixture concentrated under reduced pressure. After co-evaporation with 2 further aliquots of toluene, purification by flash column chromatography (eluent 50% → 100% EtOAc: pentane) gave **S-6** as a colourless oil (15 mg, 49%). TLC (SiO₂): $R_f = 0.54$ (100% EtOAc); $[\alpha]_D^{25} = +2.70$ (CHCl₃, $c = 0.1$); IR $\nu_{\max}$ (film): 3381 (O-H), 1716 (C=O); ¹H NMR (400 MHz, CDCl₃): δ 4.57 (ddd, $J = 11.3, 8.2, 4.2$ Hz, 1H, H_4), 4.42 – 4.16 (m, 2H, H_6), 2.81 (ddd, $J = 17.5, 5.3, 0.9$ Hz, 1H, H_3), 2.60 (m, 1H, H_3), 2.10 (ddd, $J = 13.6, 8.4, 4.7$ Hz, 1H, H_5), 1.90 (m, 1H, H_5), OH was not observed; ¹³C NMR (101 MHz, CDCl₃) δ 170.8 (C₂=O), 77.8 (C₄), 77.5 (C₆), 65.8 (C₅), 63.7 (C₃); m/z (ESI⁺) 117; HRMS: C₅H₉O₃ requires 117.05462; found 117.05473. Spectral data were found to be consistent with literature values.²⁸⁹

2.9 Molecular Modelling

In the case of RyR1, docking calculations were performed using the ATP-bound rabbit RyR1 protein structure (PDB accession number 5TAS, resolution 6.2 Å) as reported by des George *et al.*¹⁷¹ in the presence of 30 μM Ca²⁺, 2 mM ATP and 5 mM caffeine and determined in the open-state. All residues within 10 Å of the ATP site are conserved between rabbit (structural data) and sheep (single-channel experiments carried out in this chapter). The centre of the ATP-site was taken as the M4989 residue. Similarly for RyR2, docking calculations were performed using the ATP-free pig RyR2 protein structure (PDB accession number 5GOA, at a

resolution of 4.2 Å) determined in the open channel state as reported by Peng *et al*²⁶³. All residues within 10 Å of the ATP site are completely conserved between pig²⁶³ (cryo-EM structure) and sheep (functional data in this chapter). The M4885 residue was taken as the approximate centre of the ATP site, and only residues within 50 Å of this residue in chain A were retained for docking studies. Bound Zn²⁺ ions in the vicinity of the ATP site were found to have minimal effect on the docking of ATP and so were removed for further docking calculations.

The downloaded pdb-like files were combined into a single-file, and residues more than 50 Å from the centre of the ATP-site were removed. The bound ATP molecule and neighbouring Zn²⁺ ion were removed for docking calculations. All ligands were prepared in Maestro (Schrodinger, LLC, New York, 2016 academic version). Gasteiger charges were added to the ligands using the Antechamber tool in AmberTools14²⁹⁰. Gasteiger charges for the protein were added using AutodockTools. Docking calculations were performed with Autodock 4.2.3^{291,292}, using a gridbox of 40 x 40 x 40 Å³ centred around the ATP site. In RyR1, residues K4167, K4170 and R4171 were treated as flexible, as were the equivalent residues in RyR2. All other settings were in default. Structural images were prepared in either Pymol²⁹³ or VMD²⁹⁴. Binding energies were calculated using Autodock using the generic algorithm and the resulting poses were clustered using Autodock Tools 1.5.6 to a tolerance of 2.0 Å²⁹¹. The Pearson correlation coefficient (R^2) was used to assess the likely correlation between the binding energy obtained from the docking procedure and the experimentally observed activation of RyR caused by each ligand.

2.10 Statistics

Data were expressed as mean $\pm$ SEM (standard error) where $n \geq 4$ and as mean $\pm$ SD (standard deviation) where $n = 3$. Unless otherwise stated in the figure legend, where data were normally distributed, statistical comparisons were drawn using a two-tailed Student's t-test

between mean values, and differences were taken to be significant when $P < 0.05$. For comparison of larger groups (three or more), a one-way ANOVA, followed by Dunnett's tests were carried out.

2.11 Materials

Statin sodium salts were purchased from CalBioTech. [9, 21(n)-³H]ryanodine was purchased from Amersham Biosciences, Buckinghamshire, UK. All other chemicals were purchased from Sigma-Aldrich (Dorset, UK) or VMR (Poole, UK). Water was deionised (Millipore, Harrow, UK) and all single channel recording solutions were filtered through a membrane of 0.45 μ M diameter before use.

3

**Statins activate
skeletal RyR1**

3.1 Introduction

We have already discussed that statins are widely prescribed competitive inhibitors of HMG-CoA reductase, the enzyme that catalyses the primary step of cholesterol synthesis.¹⁸ Their use has been associated with a wide variety of muscle-related complaints, which appears to be common to all statins, and is not correlated with the potency of their ability to inhibit HMG-CoA reductase and therefore lowering of cholesterol.³

However, a convincing explanation for how statin side effects are triggered has not yet been established, although there is general agreement that a dysregulation of Ca^{2+} homeostasis in myocytes involved. Sirvent and colleagues, have noticed that statins can increase the resting $[\text{Ca}^{2+}]$, as well as triggering Ca^{2+} -release from the sarcoplasmic reticulum (SR) of skeletal muscle cells.^{82,85} Driven by this, our collaborators Sarah Calaghan and Sabine Lotteau (Leeds, UK) have recently shown that simvastatin sodium salt increased the frequency of Ca^{2+} sparks in permeabilised rat muscle fibres (**Figure 3.1**)²⁸⁵. These results were consistent with the previous suggestions that the impaired Ca^{2+} regulation is the result of increased Ca^{2+} release from the SR.

It is well documented that Ca^{2+} -sparks are the result of microscopic Ca^{2+} -release events from the SR^{295,296}. However, the question remains as to why the frequency of Ca^{2+} -sparks is increased by simvastatin. This effect could be readily explained by a direct effect on RyR1, In this chapter, I will investigate whether the commonly prescribed statins simvastatin, and atorvastatin can influence the gating properties of the skeletal ryanodine receptor, RyR1.

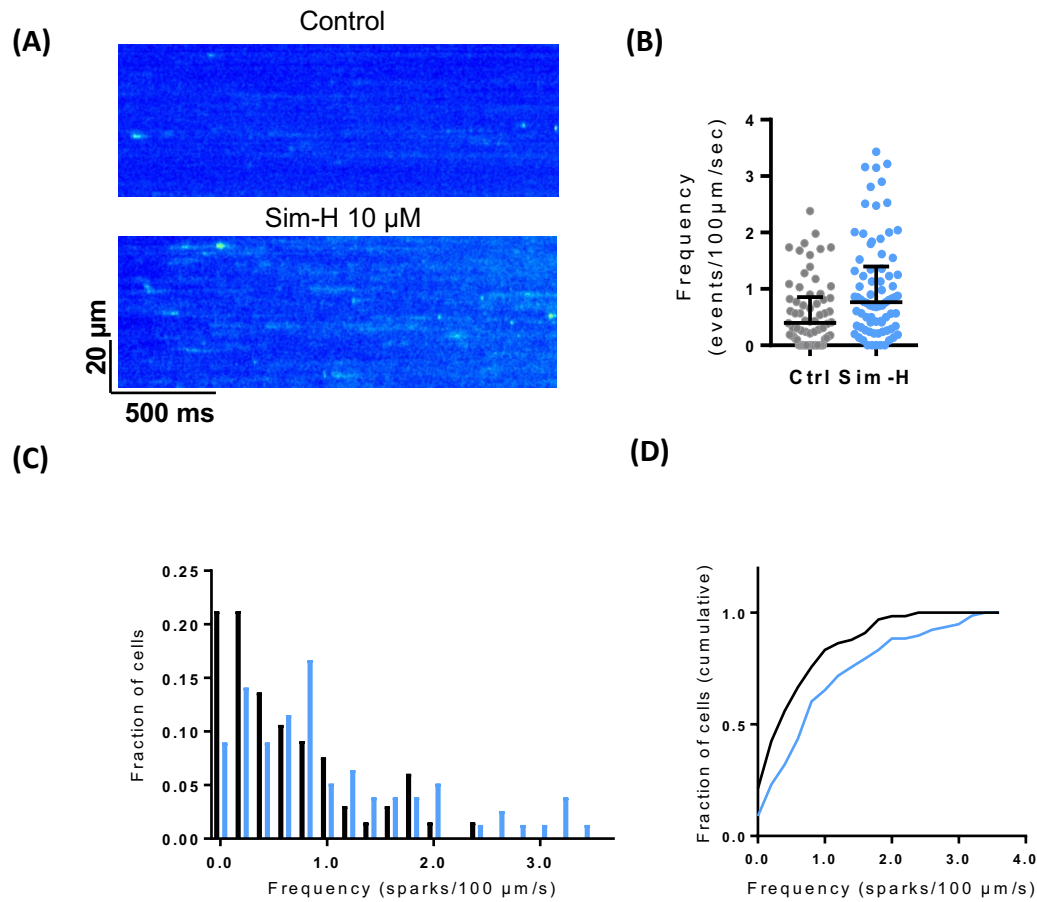


Figure 3.1 The effects of simvastatin on spontaneous SR Ca^{2+} release in permeabilised skeletal flexor digitorum brevis (FDB) fibres.

(A) Representative linescan confocal images showing Ca^{2+} sparks in saponin-permeabilised FDB fibres perfused with intracellular solution with or without 10 μM Sim-H for 5 min. **(B)** Medium frequency of Ca^{2+} spark events with interquartile range indicated. **(C)** Frequency distribution histogram for Ca^{2+} spark frequency in FDB fibres. Ca^{2+} spark frequency was significantly higher ($p < 0.05$) when analysed using each cell as an independent sample, but was not significantly different when each rat was used as the independent sample (Mann-Whitney). **(D)** Cumulative frequency distribution for Ca^{2+} spark frequency in FDB fibres. Data are from 66 control (black/grey) and 78 statin-treated cells (blue) from $n = 5$ rats. Bin size = 0.2 sparks/100 $\mu\text{m}/\text{s}$; tickmarks on X-axis denote bin centre for both groups. This figure was adapted from Venturi *et al.* (2018) and the experiments performed by Dr Sabine Lotteau.

Since statins, can exist as either a closed ring lactone form, or a ring open salt form (**Figure 3.2**), it will be important to investigate whether one form is more effective at inducing Ca^{2+} release. In this chapter, I will refer to simvastatin sodium salt as Sim-H and simvastatin lactone as Sim-L. Atorvastatin sodium salt will likewise be referred to as Ator-H.

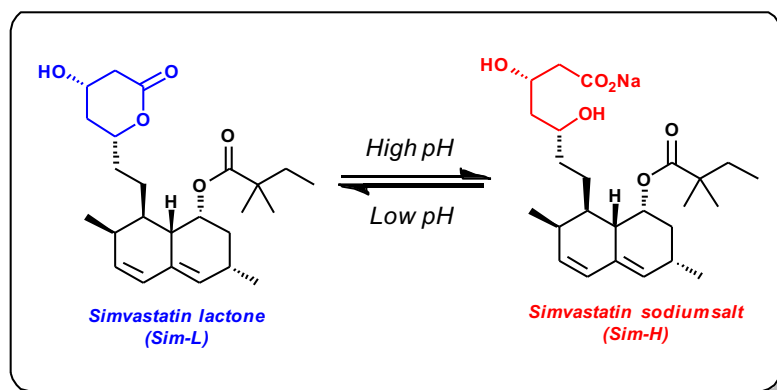


Figure 3.2 The chemical structures of Sim-L and Sim-H

Simvastatin exists in both a lactone form (Sim-L) and an open/salt form (Sim-H). the interconversion between these two forms has a strong dependence on pH.

These findings may yield important information about the target of statins and which functional groups in the statin molecule are responsible for their muscle side-effects. I have utilised functional techniques which probe RyR activity directly to address this question, with the aim of increasing our understanding of how these widely prescribed drugs function in skeletal muscle.

3.2 Results

3.2.1 Contribution by other members of the lab to the experiments described in this chapter

The results described in this chapter, where the effect of simvastatin was investigated on RyR1 were the result of a group effort to which I contributed. Dr Elisa Venturi began many of experiments described in this chapter, and following the start of my DPhil I continued with these experiments. The final results were published as Venturi, E*, Lindsay, C, *et al.* Br J Pharmacol. 2018 Mar; 175(6): 938–952 (*joint first author contribution). The figures which include data collected by other lab members are shown in **Table 3.1** and the number of experiments performed by each lab member is noted in the individual figure legends for the figure concerned. I have contributed to data to all the figures shown below.

Figure 3.4	Bar chart also includes single-channel data obtained by Dr Elisa Venturi
Figure 3.5	Bar chart also includes binding data obtained by Dr Katja Witchas
Figure 3.6	Graph also includes single-channel data obtained by Dr Elisa Venturi
Figure 3.7(B)	Bar chart also includes single-channel data obtained by Dr Elisa Venturi
Figure 3.8(B)	Bar chart also includes single-channel data obtained by Dr Elisa Venturi
Figure 3.10	Bar chart also includes single-channel data obtained by Dr Elisa Venturi
Table 3.2	Lifetime analysis also includes channels obtained by Dr Elisa Venturi
Figure 3.12(B)	Bar chart also includes single-channel data obtained by Dr Elisa Venturi
Figure 3.13	Bar chart also includes single-channel data obtained by Dr Elisa Venturi
Figure 3.14(B)	Bar chart also includes single-channel data obtained by Dr Elisa Venturi

Table 3.1 List of figures illustrating group data

The table lists the figures in this chapter to which other members of the lab have contributed data.

I have therefore shown my own data in most instances but have included data obtained by others in the mean data shown in figures. All of the results shown investigating the effect of atorvastatin were obtained by myself, and no other lab members contributed to these experiments. Cellular work performed by Dr Sabine Lotteau, Dr Zhang Yang and Miss Emma Steer in Leeds, UK under the supervision of Profs Steele and Calaghan formed part of the manuscript submitted for publication, and the relevant figure has been reproduced in the introduction of this chapter (**Figure 3.1**) but are not presented as results for this thesis.

3.2.2 Simvastatin directly modulates RyR1 function

To determine if simvastatin could directly modulate RyR1, the most effective method is to investigate how the addition of simvastatin affects RyR1 channel openings using single-channel biophysical recordings.

3.2.2.1 *Simvastatin activates RyR1 channels incorporated into lipid bilayers.*

We therefore investigated the effect of simvastatin sodium salt (Sim-H), on single native RyR1 channels that were incorporated into planar lipid bilayers. As previously discussed, this is the form which is active *in vivo* against HMG-CoA reductase.²⁹⁷ A representative single-channel recording under these conditions is shown in **Figure 3.3**. In the presence of 10 μM cytosolic Ca^{2+} , the addition of cytosolic Sim-H caused a concentration dependent increase in RyR1 Po. The addition of Sim-H appeared to increase the frequency of channel gating, as 'flickery' openings were observed, similar to the type of RyR activation caused by ATP or caffeine.^{156,196} When the cytosolic chamber was perfused with recording solution (TRIS/Hepes at 10 μM Ca^{2+}) to restore control conditions, activation was reversible (**Figure 3.3**, bottom trace). The mean data is shown in **Figure 3.4**. Higher concentrations of simvastatin were not used in the single-channel experiments as, due to simvastatin's relative lipophilicity ($\log P = 4.51$) the artificial bilayers appeared to be destabilised in the presence of high Sim-L concentration.

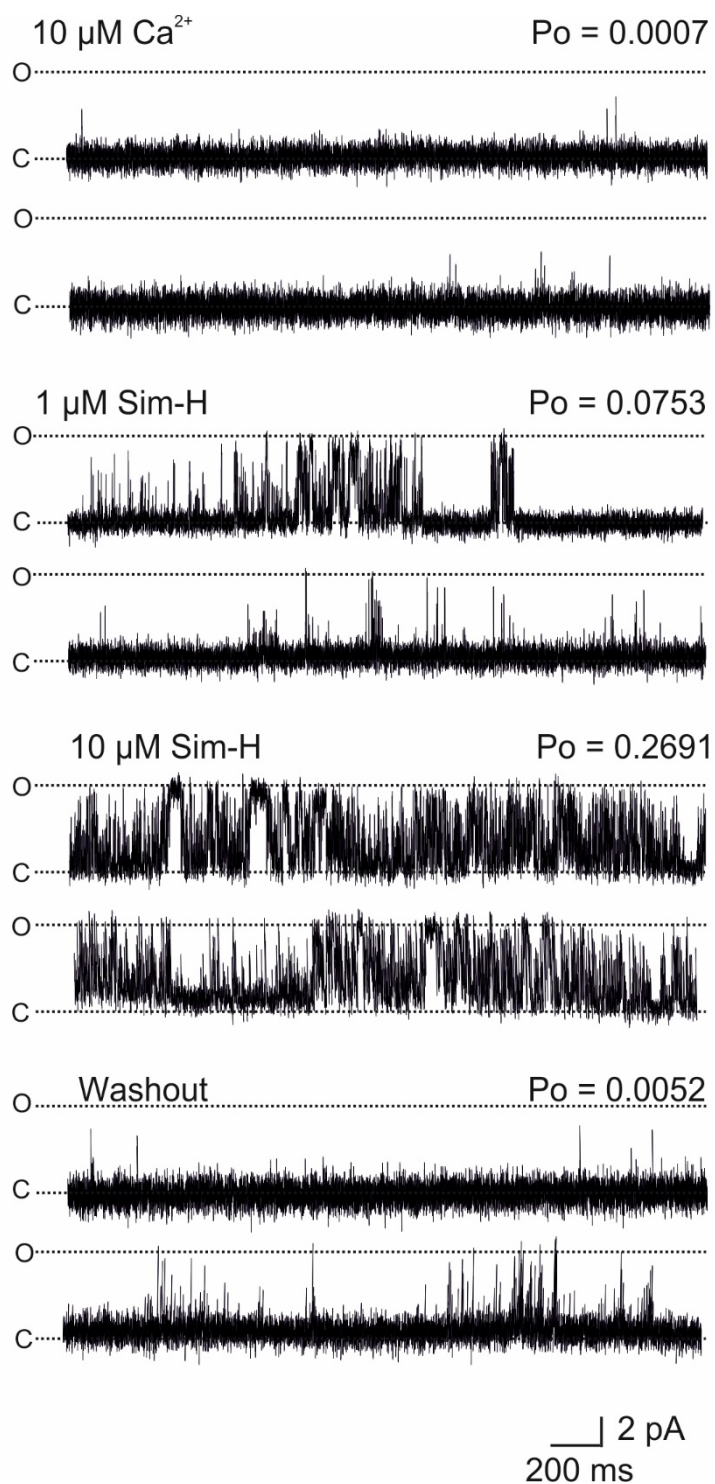


Figure 3.3 Effect of Sim-H on RyR1 channel gating.

Representative mouse skeletal RyR1 single-channel current fluctuations in the presence of 10 $\mu\text{M Ca}^{2+}$ alone (top traces), after addition of cytosolic Sim-H to 1 μM and 10 μM (as indicated), and after washout back to control conditions (10 $\mu\text{M Ca}^{2+}$, bottom traces). The P_o above the traces refers to that calculated over three minutes of recording. O and C indicate open and closed channel levels respectively.

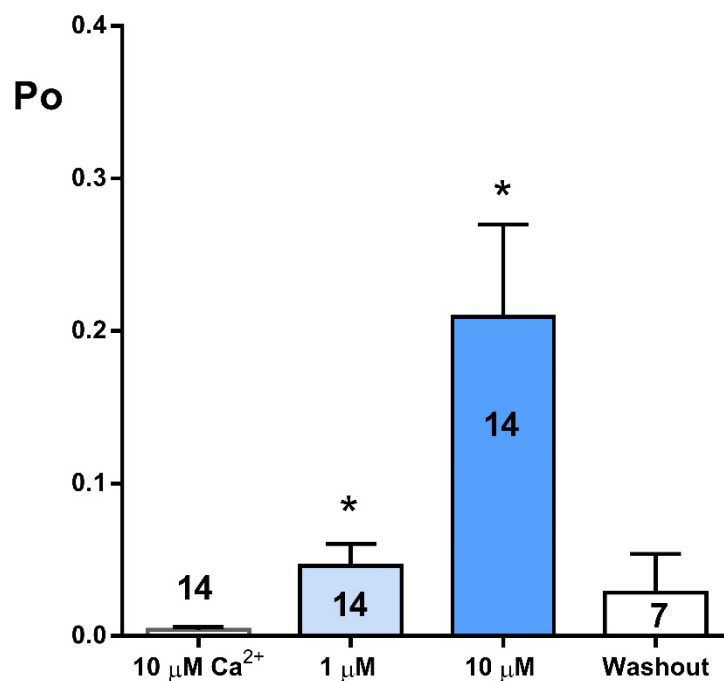


Figure 3.4 Sim-H activates RyR1

Mean RyR1 Po values under control conditions (10 μM cytosolic Ca^{2+} as sole channel activator), after addition of cytosolic Sim-H to 1 μM and 10 μM (as indicated), and after washout back to control conditions. Mean $\pm$ SEM is shown; numbers on the bars indicate the number of independent experiments. * $P < 0.05$. Data collected by myself ($n = 6$) and Dr Elisa Venturi ($n = 13$).

The mean washout Po appeared to be slightly higher than control levels although not significantly different. It is possible that the Sim-H is not completely washed away (perhaps again due to its lipophilicity) or that there is a small irreversible effect on channel openings.

3.2.2.2 *Simvastatin increases [³H]ryanodine binding to skeletal SR vesicles*

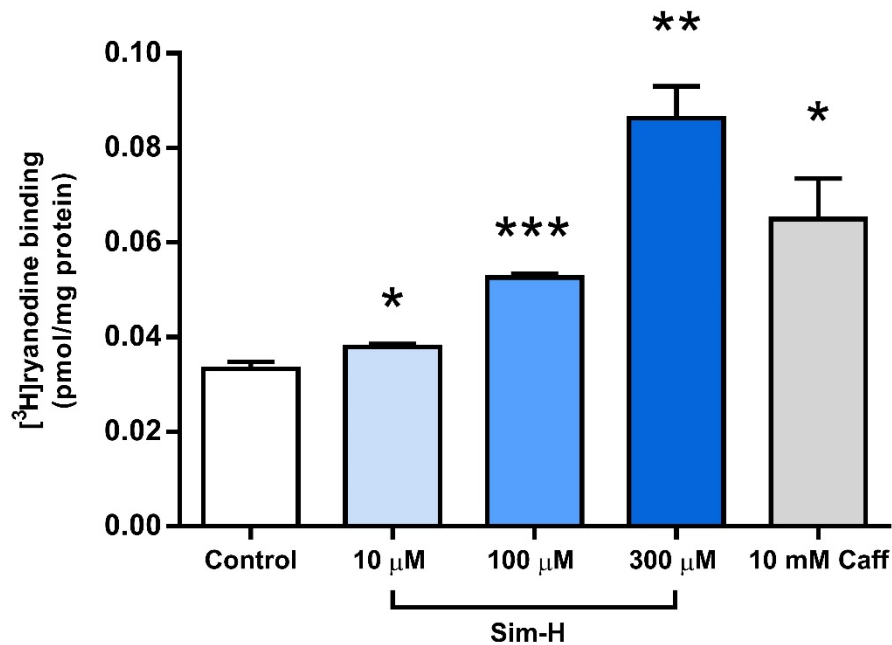


Figure 3.5 Effect of Sim-H on [³H]ryanodine binding

Stimulation of [³H]ryanodine binding to mouse skeletal SR membrane vesicles by 10 μM cytosolic Ca²⁺ alone (control conditions), and in the presence of additional Sim-H at the concentrations indicated. 10 mM caffeine (caff) is shown as a positive control under the same conditions. Mean ± SEM; n = 5, **P* < 0.05, ***P* < 0.005, ****P* < 0.001. Data collected by myself (n = 2) and Dr Katja Witschas (n = 3)

To demonstrate that the effects of Sim-H were not artefacts of the artificial bilayer membrane, the binding of [³H]ryanodine to RyR1 containing SR membrane vesicles was also assessed. Ryanodine binds only to open RyR1 channels, and does so irreversibly. Thus an increase in the binding of [³H]ryanodine to SR membranes in the presence of a drug, indicates that the drug is increasing channel opening. Therefore [³H]ryanodine binding levels are an approximate measure of *P*_o measured across a population of channels. Sim-H was found to stimulate [³H]ryanodine binding at concentrations between 10 μM and 300 μM in a dose-dependent manner (**Figure 3.5**).

3.2.2.3 *Single-channel conductance is not altered*

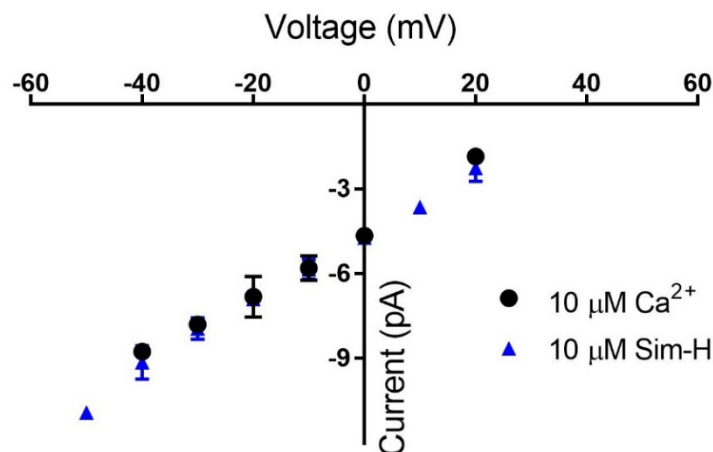


Figure 3.6 Effect of Sim-H on RyR1 single-channel conductance

Single-channel current-voltage relationship for RyR1 channels before and after addition of 10 μM Sim-H. Mean $\pm$ SEM; $n = 5$. Where error bars are not shown, they are within the symbol. Data collected by myself ($n = 2$) and Dr Elisa Venturi ($n = 3$).

It was also found that the addition of Sim-H did not affect the single-channel conductance of RyR1 (Figure 3.6).

3.2.2.4 *Luminal (trans) simvastatin has no effect on RyR1 channel gating*

Simvastatin was also added to the luminal (*trans*) side of bilayer to establish if the same effect would be observed as when added to the cytosolic side. The luminal portion of the RyR channel is the part which would be exposed to the SR lumen in a physiological setting. Addition of 10 μM Sim-H caused no significant increase in RyR1 Po (Figure 3.7). This suggests that the RyR1 activation caused by Sim-H treatment is due to a cytosolic binding site, and is not due to other effects such as Sim-H partitioning into the bilayer or other such effects. This also suggests that Sim-H is not capable of crossing the lipid bilayer over the time course of the experiment (several minutes) as we only see activation when Sim-H is added to the cytosolic side.

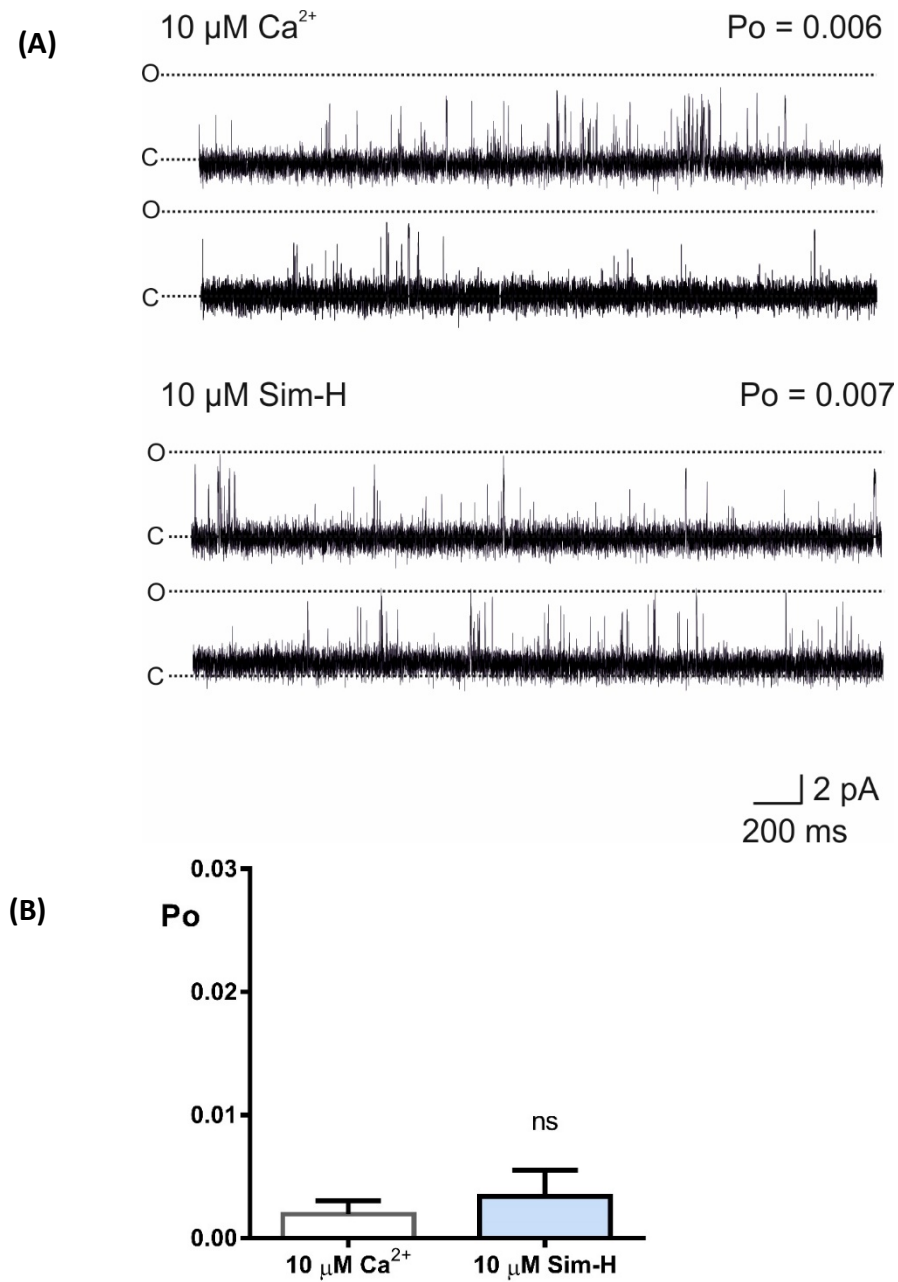


Figure 3.7 Effect of luminal Sim-H

(A) Representative mouse skeletal RyR1 single-channel current fluctuations in the presence of 10 μM Ca^{2+} alone (top traces), after addition of luminal (*trans*) 10 μM Sim-H. The P_o above the traces refers to that calculated over three minutes of recording. O and C indicate open and closed channel levels respectively. **(B)** Mean RyR1 P_o values before and after addition of 10 μM Sim-H to the luminal (*trans*) side of the bilayer (mean $\pm$ SEM are shown; $n = 6$). ns = not significant. Data collected by myself ($n = 3$) and Dr Elisa Venturi ($n = 3$).

3.2.2.5 *Sim-L also activates RyR1*

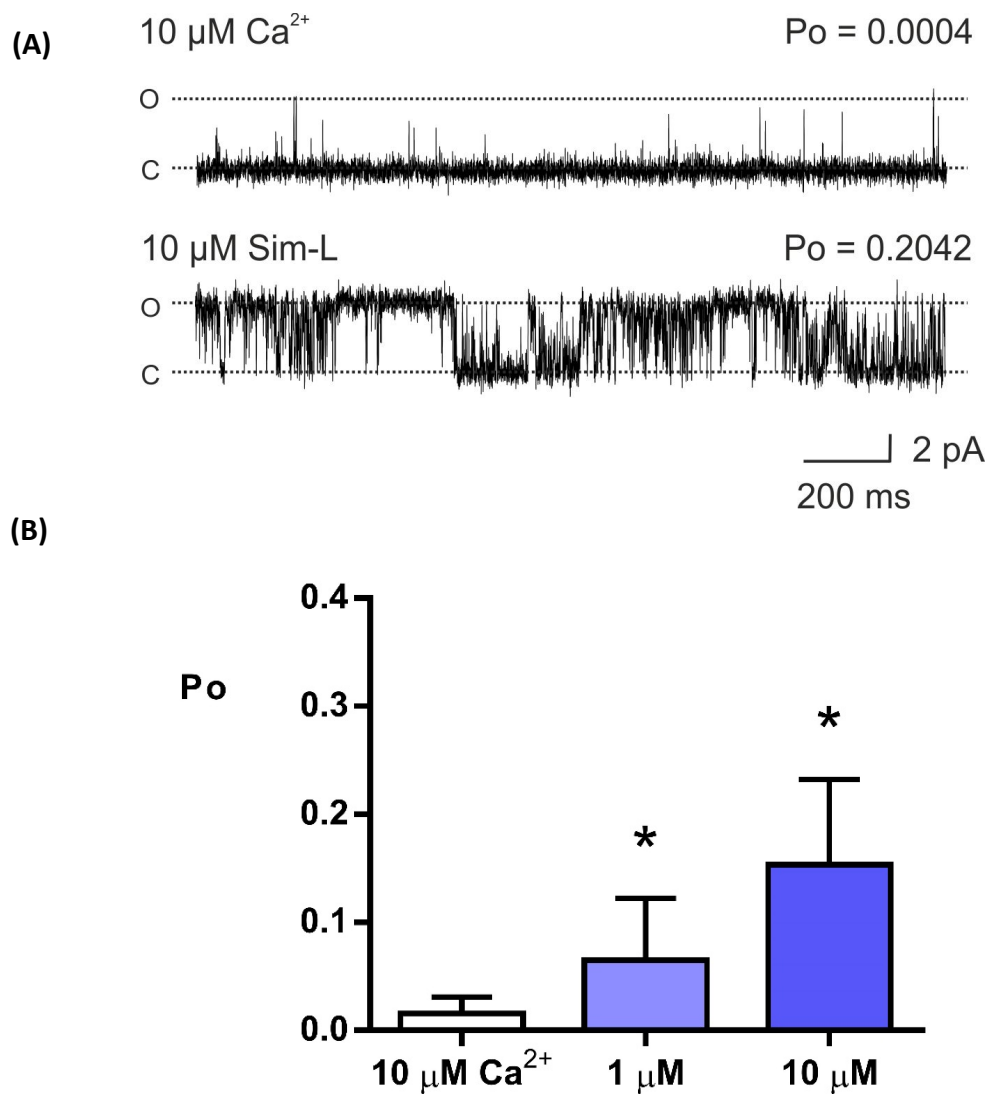


Figure 3.8 Effects of Sim-L on RyR1 channel gating

(A) A representative RyR1 channel recording in the presence of 10 μM cytosolic Ca^{2+} alone (top trace) and after addition of 10 μM cytosolic Sim-L (bottom trace) is shown. The P_o above each trace refers to that calculated over three minutes of recording. O and C indicate the open and closed channel levels respectively. (B) Mean RyR1 P_o values in the presence of 10 μM Ca^{2+} alone and after addition of 1 μM Sim-L and 10 μM Sim-L (mean $\pm$ SEM; $n = 10$; $*P < 0.05$). Data collected by myself ($n = 4$) and Dr Elisa Venturi ($n = 6$).

Given the finding that Sim-H can activate RyR1, and a possible connection between this activation and statin side effects, it is important to determine if this activation can be separated from HMG-CoA reductase inhibition. As discussed previously, the HMG-CoA reductase pharmacophore can exist in one of two forms, an open (acid/salt) form and a closed lactone form, but only the open ring form inhibits HMG-CoA reductase (**Figure 3.2**)²⁹⁸. The effect of 'HMG-CoA inactive' lactone form (Sim-L) on RyR1 channel gating was therefore examined. If Sim-L also activates RyR1, we could conclude that the statin pharmacophore is not essential to RyR1 binding and/or activation.

This was indeed the case, Sim-L activated RyR1 at both 1 μ M and 10 μ M concentrations. A representative RyR1 single channel is shown in **Figure 3.8(a)** and the mean data of multiple experiments in **Figure 3.8**. The activation with Sim-L was similar to that observed with Sim-H. It has been reported previously that the interconversion of statins has a strong pH dependence²⁹⁸, so it is therefore possible that in the buffered conditions of the single-channel experiments, Sim-L might spontaneously be hydrolysed to Sim-H. To confirm that Sim-L does not undergo hydrolysis during the time frame of the single-channel experiments, the interconversion rate of the lactone form Sim-L into Sim-H was measured using LC-MS.

The experiment was repeated for Sim-H, both in water and the TRIS/Hepes buffer used in the single-channel experiments. In the case of Sim-L, it was found that even after 1 hr, 95% of the simvastatin detected was still in the closed-ring lactone form. Hence in the experiment shown in **Figure 3.8**, at least 99% of the Sim-L added to the cytosolic chamber would remain by the end of the experiment. In the case of Sim-H, there was no apparent tendency for free acid to undergo ring-closing to form Sim-L, as no detectable levels of Sim-L were found even after 1 hr incubation (**Figure 3.9**). We can therefore conclude that in both single-channel experiments investigating Sim-H and Sim-L, interconversion between the two statin forms is not a complicating factor.

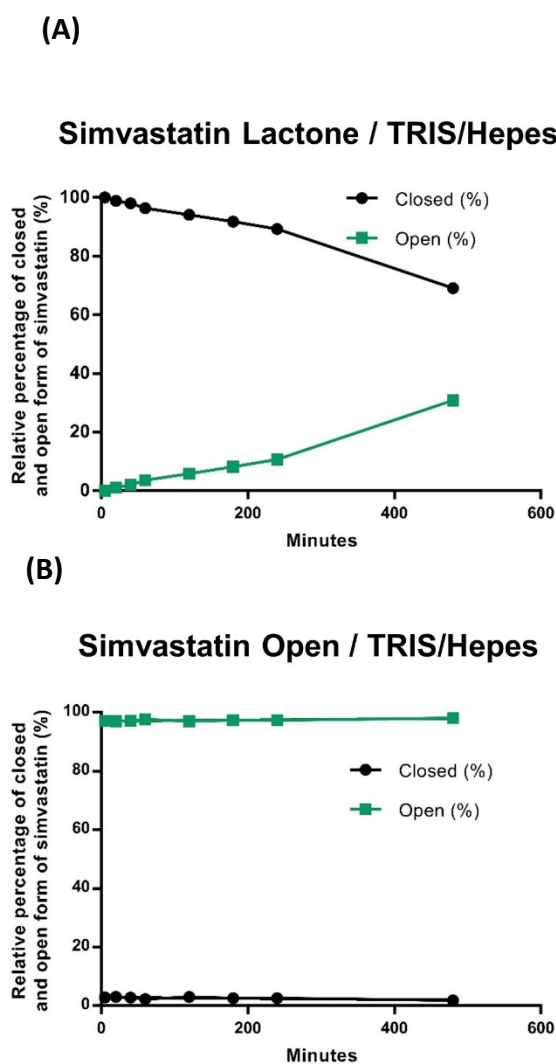


Figure 3.9 Simvastatin acid and lactone interconversion was assessed by LC-MS

The interconversion between Sim-H and Sim-L is shown for **(A)** Sim-L in TRIS/Hepes, **(B)** Sim-H in TRIS/Hepes, where the percentage of Sim-L over time is shown in black, and Sim-H in green. Mean $\pm$ SEM is shown; $n = 3$; error bars where not shown are within the symbol.

3.2.2.6 Simvastatin predominately increases RyR1 opening frequency

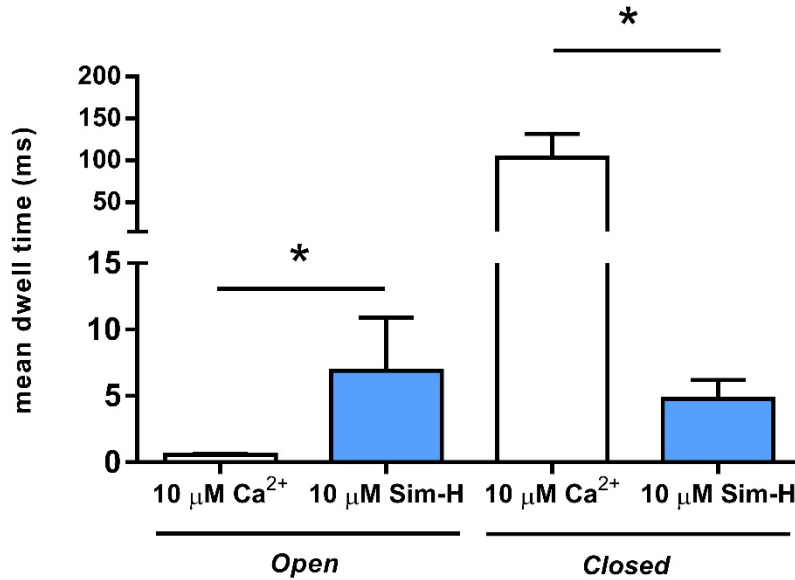


Figure 3.10 Effects of cytosolic Sim-H on mean open and closed times.

Mean $\pm$ SEM is shown; $n = 5$; $*P < 0.05$. Data collected by myself ($n = 2$) and Dr Elisa Venturi ($n = 3$).

Where only one RyR1 channel incorporated into the bilayer, further analysis was performed to determine the mechanism of how Sim-H causes an increase in P_o . Mean open and closed times are shown in **Figure 3.10**. There was a small but significant increase in mean open time in the presence of 10 μM Sim-H, as mean open time was increased from 0.577 ± 0.059 ms (Mean $\pm$ SEM, $n = 5$) in control conditions to $6.908 \text{ ms} \pm 3.977 \text{ ms}$ ($n = 5$). However, there was a much larger (20 fold) reduction in the mean closed time. Mean closed time was decreased from $103.7 \text{ ms} \pm 28.32 \text{ ms}$ ($n = 5$) in control conditions to $4.783 \text{ ms} \pm 1.415 \text{ ms}$ (10 μM Sim-H, $n = 5$). Open and closed lifetimes for a representative channel were grouped into non-cumulative histograms, and a probability density function (pdf) was fitted to the histograms using the theory of maximum likelihood²⁸², as described in detail in General Methods. **Table 3.2** shows the time constants (τ) for each exponential term (τ_1, τ_2 , etc) and the corresponding percentage areas (A1, A2, etc) for five channel recordings. The open time distribution in control conditions (10 μM Ca^{2+}) was best described by one exponential term (with one

exception, where there was a small area in a second term). Following addition of 10 μM cytosolic Sim-H, there was a trend to increase the number of exponentials as there was a small shift towards longer opening times. However, the majority of openings (between 63% and 83%) were still brief and were described by the shortest time constant τ_1 . In contrast, the closed time distributions required a more complex pdf of multiple (up to 4) terms in control conditions. Following Sim-H addition, there was a larger shift towards a reduction in mean closed time, with an approximately 50% increase in the percentage areas described by the shortest time constant, τ_1 .

Open times

	10 μM Ca^{2+}								after 10 μM Sim-H							
	τ_1 (ms)	A1 (%)	τ_2 (ms)	A2 (%)	τ_3 (ms)	A3 (%)	τ_4 (ms)	A4 (%)	τ_1 (ms)	A1 (%)	τ_2 (ms)	A2 (%)	τ_3 (ms)	A3 (%)	τ_4 (ms)	A4 (%)
channel 1	1.3	100							1	73	7	26	199	1		
channel 2	1.2	100							1	83	5.2	16	98	1		
channel 3	1	94	5	6					2	63	24	27	304	10		
channel 4	1.2	100							1.3	61	9.5	30	91	9		
channel 5	0.59	100							0.70	81	2.8	19				

Closed times

	10 μM Ca^{2+}								after 10 μM Sim-H							
	τ_1 (ms)	A1 (%)	τ_2 (ms)	A2 (%)	τ_3 (ms)	A3 (%)	τ_4 (ms)	A4 (%)	τ_1 (ms)	A1 (%)	τ_2 (ms)	A2 (%)	τ_3 (ms)	A3 (%)	τ_4 (ms)	A4 (%)
channel 1	1	30	7	33	149	31	7075	6	2	69	29	28	1765	3		
channel 2	2.5	32	23	44	123	23	3276	1	1.3	46	6	37	37	16	327	1
channel 3	2	58	18	29	200	11	11897	2	1.5	76	15	22	557	2		
channel 4	3	32	45	37	457	23	4771	8	2	67	28	29	1094	4		
channel 5	1.3	20	5	37	36	33			1	67	7.2	33				

Table 3.2 The effects of Sim-H on lifetime parameters

Time constants (T_1 , T_2 , T_3 , T_4) and percentage areas (A_1 , A_2 , A_3 , A_4) obtained from maximum likelihood fitting of probability density functions to open and close lifetime distributions of 5 independent single RyR1 channels in control conditions (10 μM cytosolic Ca^{2+}) and after addition of 10 μM Sim-H are shown. Data collected by myself ($n = 2$) and Dr Elisa Venturi ($n = 3$).

Figure 3.11 shows a typical example of the open and closed lifetime distributions for a channel in both control conditions ($10\ \mu\text{M}\ \text{Ca}^{2+}$, top histograms) and after addition of $10\ \mu\text{M}$ cytosolic Sim-H (bottom histograms). In control conditions, the best fit to open lifetimes is described adequately by τ_1 with no need for additional exponential terms. Following addition of Sim-H, a small proportion of events were shifted towards longer opening times, and now three exponential terms are required to describe this aspect of the pdf. However despite this, the majority of the openings remained very brief (approximately 1 ms). In control conditions, the closed lifetime distribution is described by four time constants with a reduction to three following addition of Sim-H. After addition of Sim-H, there was a reduction in the duration of all the time constants unless the durations were so shortened that the longest time constants were lost. Thus, the corresponding pdf is less broad in appearance, and the percentage area of time constant τ_1 was particularly increased. These results demonstrate that Sim-H increases channel P_o predominantly through increasing the frequency of channel openings.

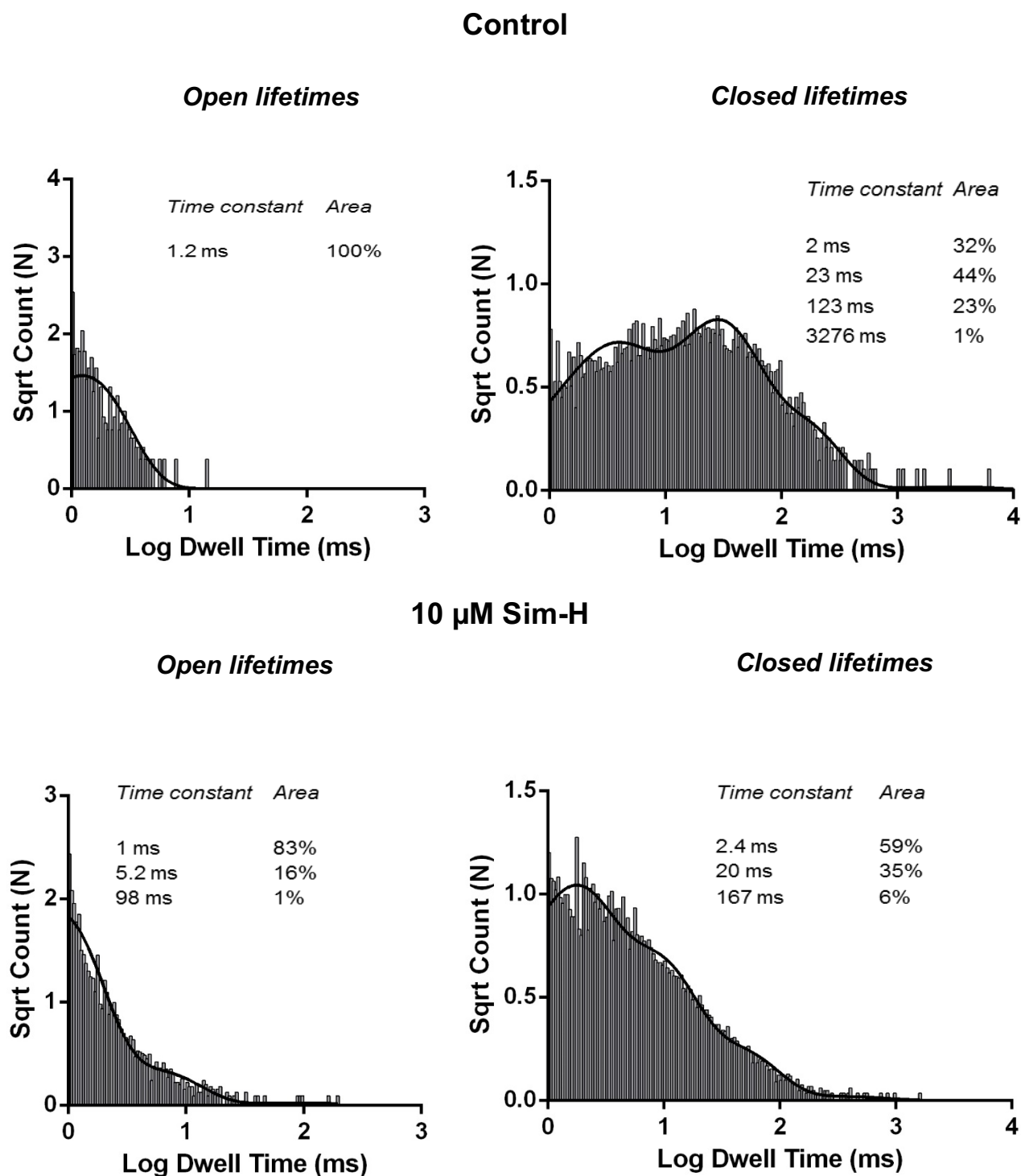


Figure 3.11 Effects of cytosolic Sim-H on lifetime distributions

Open and closed life-time distributions and probability density functions for a representative RyR1 channel in control conditions (10 μ M Ca^{2+} , top), and after addition of 10 μ M Sim-H (bottom). The time constants (τ) and percentage areas are shown.

3.2.2.7 *Simvastatin acts via both Ca²⁺-dependent and Ca²⁺-independent pathways*

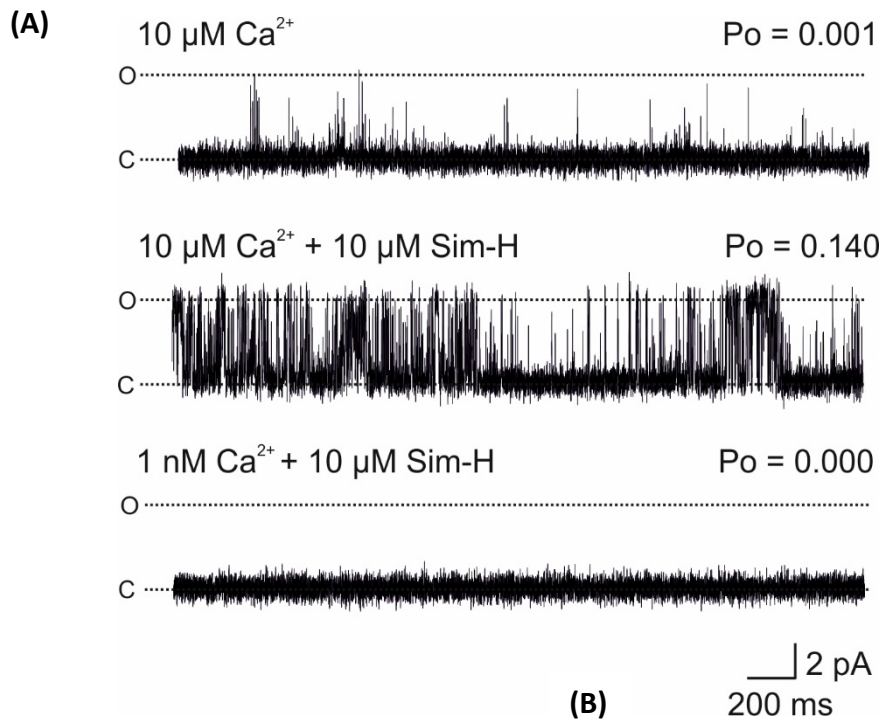
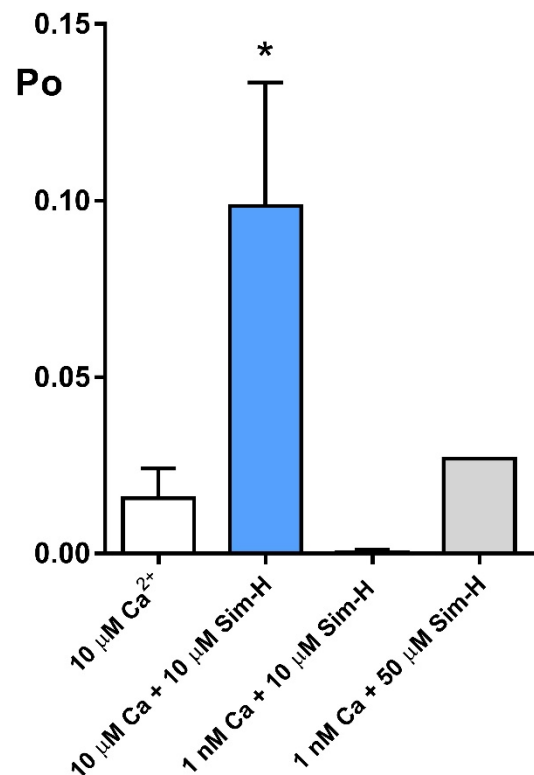


Figure 3.12 Ca²⁺ dependent openings in the presence of Sim-H

(A) Representative RyR1 channel recording in the presence of 10 μM cytosolic Ca^{2+} (top trace), and after sequential additions of 10 μM cytosolic Sim-H (second trace), and 1 mM EGTA (free cytosolic $[\text{Ca}^{2+}] \sim 1$ nM)(bottom trace). The P_o above each trace refers to that calculated over three minutes of recording. O and C indicate the open and closed channel levels respectively. (B) Mean data $\pm$ SEM from six independent experiments similar to the one shown in (A); $n = 2-6$, $*P < 0.05$. Data collected by myself ($n = 2$) and Dr Elisa Venturi ($n = 4$).



It is well established that cytosolic Ca^{2+} activates RyR1 predominately by increasing the frequency of channel openings.^{103,129} The lifetime analysis in **Section 3.2.2.6** suggested that Sim-H also modulates RyR1 through an increase in frequency of openings. One possibility therefore, is that Sim-H increases P_o through increasing the sensitivity of the channel to cytosolic Ca^{2+} . That is, that the presence of Sim-H amplifies the effect of cytosolic Ca^{2+} . In this case, we would expect that the ability of Sim-H to increase channel P_o would display an absolute dependence on the presence of cytosolic Ca^{2+} . To investigate this, the free cytosolic $[\text{Ca}^{2+}]$ was lowered to sub-activating levels ($< 1 \text{ nM}$) by treatment with EGTA following channel activation by Sim-H. A typical experiment is shown in **Figure 3.12(A)**. In the presence of $10 \text{ }\mu\text{M}$ Ca^{2+} , addition of $10 \text{ }\mu\text{M}$ Sim-H increased P_o . However, subsequent lowering of the cytosolic $[\text{Ca}^{2+}]$ to sub activating levels (**Figure 3.12**, third trace) completely abolished any channel openings, thus indicating that the activation of RyR1 seen with $10 \text{ }\mu\text{M}$ Sim-H is dependent on the simultaneous presence of activating levels of cytosolic Ca^{2+} . The mean data are also shown in **Figure 3.12(B)**. Therefore, since, cytosolic Ca^{2+} itself also acts by increasing the frequency of channel openings¹²⁹, taken together these findings suggest that Sim-H activates RyR1 through sensitising the channel to cytosolic Ca^{2+} . That is, in the presence of Sim-H, the channel is more responsive to increases in $[\text{Ca}^{2+}]$.

In a separate experiment when the concentration of Sim-H was increased further (to $50 \text{ }\mu\text{M}$), some channel openings could be induced even in the absence of activating levels of Ca^{2+} . A representative trace showing this effect is shown in **Figure 3.13**. The top trace shows that in the presence of 1 nM Ca^{2+} , $10 \text{ }\mu\text{M}$ Sim-H appears to produce no openings. However, when the $[\text{Sim-H}]$ was increased to a high concentration, the channel could open, and produced longer openings that can be seen clearly in the bottom three traces (these openings are marked with an asterisk*). These types of longer than expected openings are characteristic of certain Ca^{2+} -independent openings seen with other ligands such as caffeine and ATP^{156,196}. Lifetime analysis of a representative channel under these conditions is also shown in **Figure 3.14**. Mean open

time was significantly longer than in the presence of 10 μM Ca^{2+} alone, while mean closed time was not significantly different. The lifetime histogram shown (**Figure 3.14(A)**) also demonstrates that in comparison to Sim-H activation in the presence of 10 μM Ca^{2+} (**Figure 3.11**), there is a shift towards longer openings with an decrease in percentage areas A1, and increase in areas A2 and A3 . This suggests that Sim-H is activating RyR1 through a different mechanism when activating levels of Ca^{2+} are not present. Therefore Sim-H may activate RyR1 at low concentrations by sensitising the channel to Ca^{2+} but through a Ca^{2+} -independent mechanism at higher concentrations.

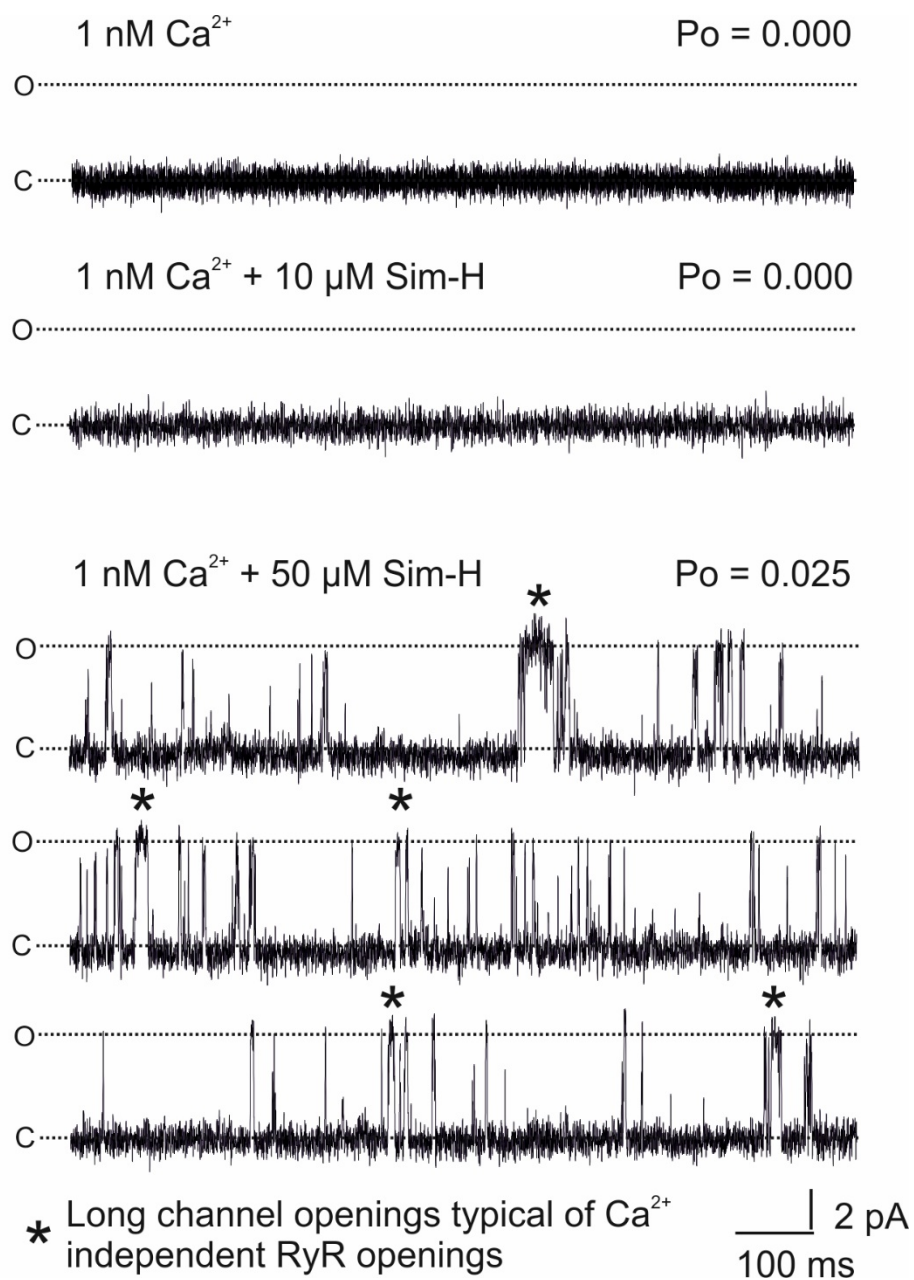


Figure 3.13 Ca^{2+} independent openings

Representative RyR1 single-channel behaviour in the presence of sub-activating cytosolic Ca^{2+} (free $[\text{Ca}^{2+}] \sim 1$ nM)(top trace) to show zero openings. There are still no openings after addition of 10 μM Sim-H (second trace). The lower traces provide examples of the longer type of openings exhibited after addition of 50 μM Sim-H. O and C indicate the open and closed channel levels respectively. The symbol (*) indicates long channel openings typical of Ca^{2+} independent RyR openings.

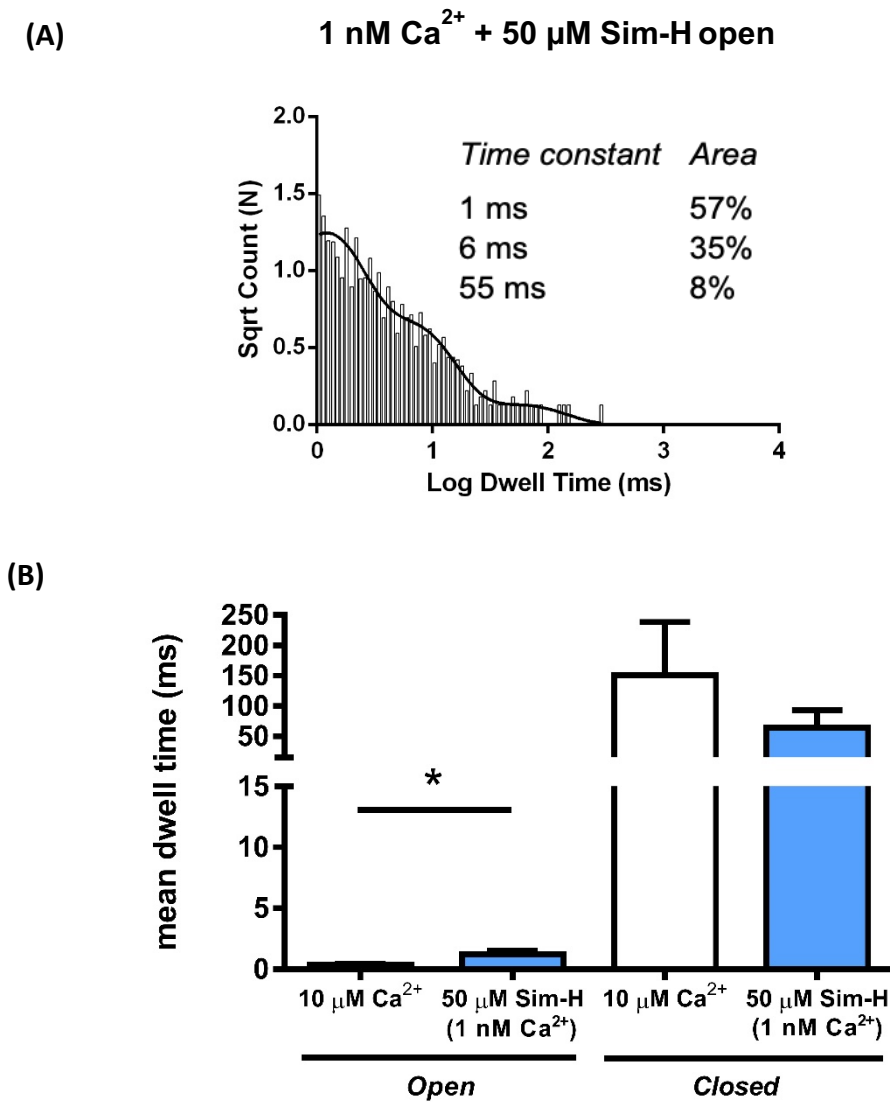


Figure 3.14 Lifetime analysis at sub-activating Ca^{2+}

(A) Open lifetime distributions and probability density function for a representative RyR1 channel activated solely by 50 μM Sim-H in the absence of activating cytosolic Ca^{2+} [~ 1 nM]. (B) Mean effects of cytosolic Sim-H on RyR1 mean open and closed times. Mean $\pm$ SEM is shown; $n = 5$; $*P < 0.05$. Data collected by myself ($n = 2$) and Dr Elisa Venturi ($n = 3$).

3.2.3 Atorvastatin activates RyR1

3.2.3.1 *Single channel activation of RyR1 by atorvastatin.*

Having seen that simvastatin (Sim-H) produced RyR1 activation, the next question was whether this activation is seen with other statins, particularly those with a different structure, such as the Type 2 statins⁸. Of the Type 2 statins available, I chose to investigate atorvastatin. This was because, of all statins it is the most widely prescribed¹⁴, its pyrrole core makes it highly synthetically accessible²⁹⁹ and there is a wealth of clinic data on its use. The structure of atorvastatin is shown in **Figure 3.15**. Like simvastatin, atorvastatin is available in both an open ring (hydroxy acid form, Ator-H) and a closed ring lactone form. However, in contrast to simvastatin, it is prescribed in its active, *in vivo*, open ring form.

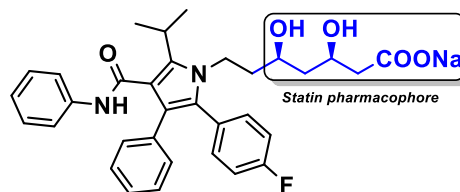


Figure 3.15 Atorvastatin (Ator-H)

Atorvastatin was investigated in a single-channel bilayer context, as previously described for simvastatin. **Figure 3.16** shows a representative RyR1 single channel trace following treatment with Ator-H. Ator-H caused a concentration dependent increase in RyR1 Po, reaching a maximum Po of around 0.1 in the presence of 10 μM Ator-H. There was significant activation at lower concentrations of Ator-H as shown in **Figure 3.17**. Before addition of Ator-H, the Po obtained with 10 μM Ca^{2+} alone was 0.0273 ± 0.0102 (mean $\pm$ SEM, $n = 22$) and following washout of Ator-H was 0.0251 ± 0.0126 (mean $\pm$ SEM, $n = 8$) indicating that the activation of Ator-H was fully reversible. This was the case in all instances where Ator-H was perfused away.

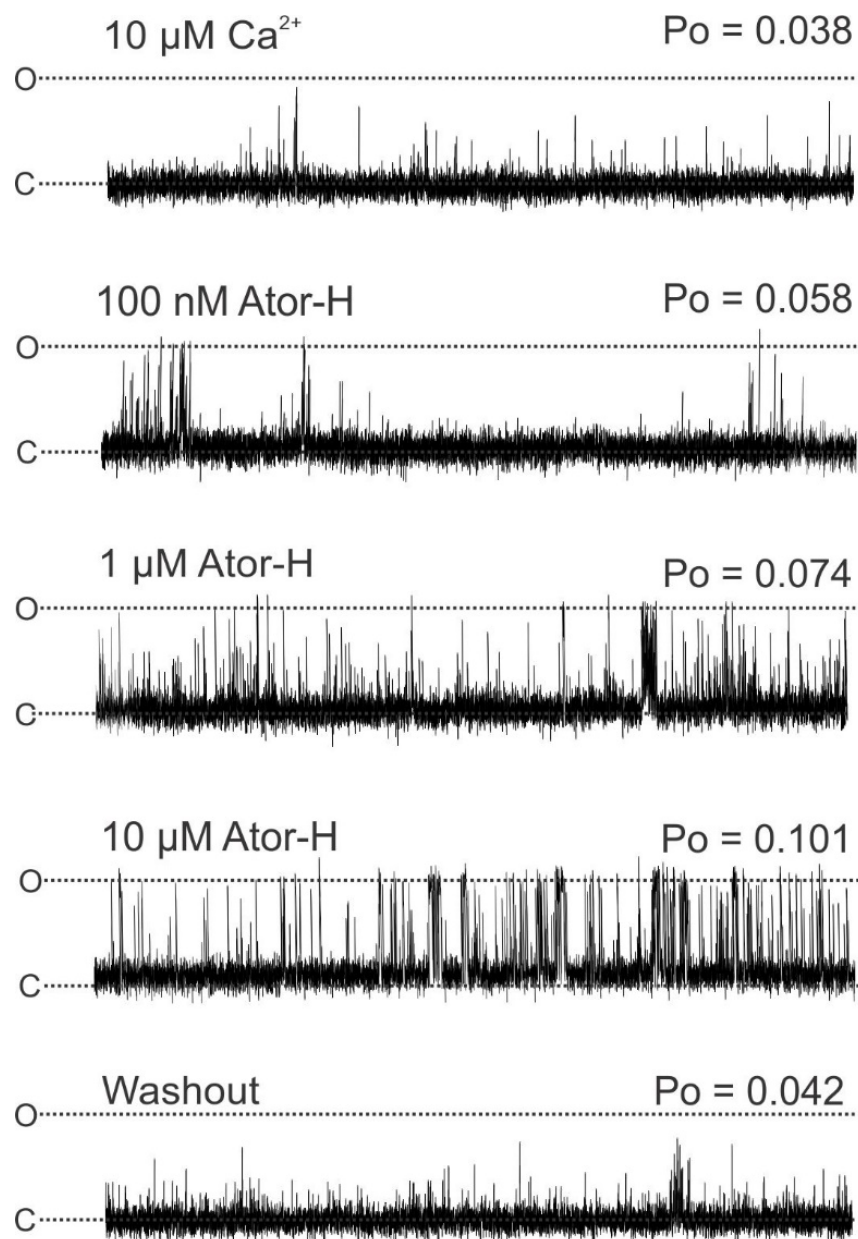


Figure 3.16 The effects of cytosolic Ator-H on RyR1 channel gating.

Representative sheep skeletal RyR1 single-channel current fluctuations in the presence of 10 μM Ca^{2+} alone (top trace), after cytosolic addition of Ator-H (at concentrations as indicated) and after washout of Ator-H back to control conditions (10 μM Ca^{2+}). The P_o above each trace refers to that calculated over three minutes of recording. O and C indicate open and closed channel levels respectively.

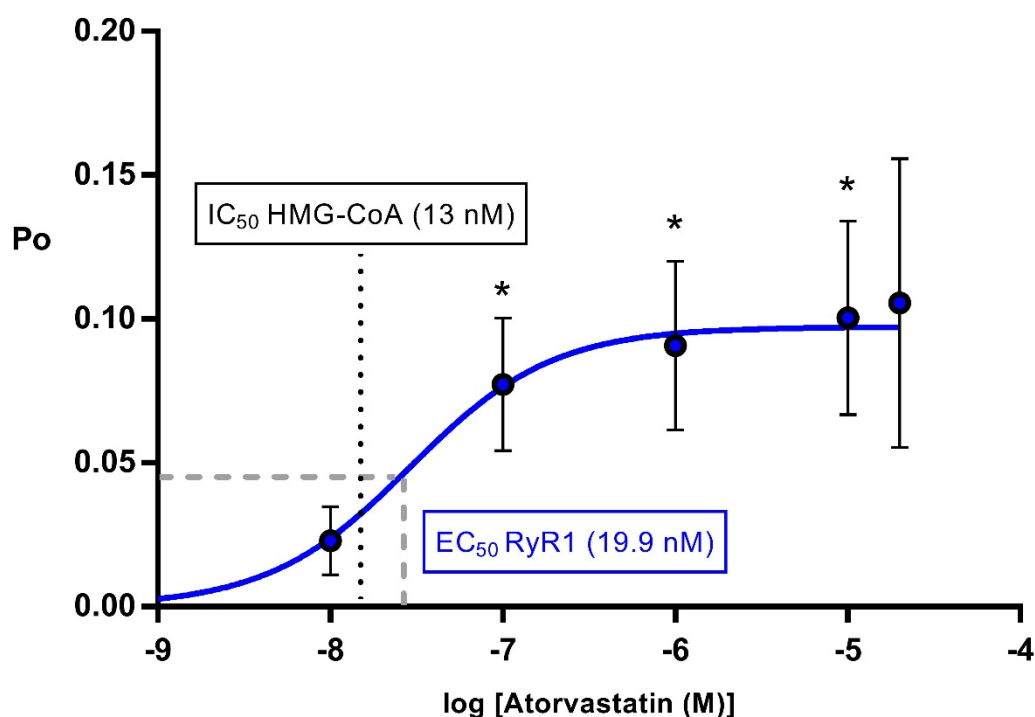


Figure 3.17 Dose response relationship for RyR1 activation by Ator-H.

Mean $\pm$ SEM are shown. The EC_{50} value for Ator-H RyR1 activation is indicated (19.9 nM). The IC_{50} for HMG-CoA reductase inhibition is shown for reference (13 nM). * $P < 0.05$ relative to control conditions; $n = 8-22$, $R^2 = 0.10$.

The EC_{50} value for RyR1 activation was 20 nM (**Figure 3.17**) which is similar to the IC_{50} value for atorvastatin against its intended target of HMG-CoA reductase.¹⁸ The effect of Ator-H appears to plateau at concentrations above 10-20 μ M, with further increases in drug concentration unable to increase P_o beyond approximately 0.1. Thus Ator-H appears to be a high affinity partial agonist of RyR1.

3.2.3.2 *Atorvastatin predominately increases RyR1 mean opening frequency*

To establish whether Ator-H activates RyR1 through the same mechanism as Sim-H, further analysis of the single channel data was conducted including lifetime analysis. Life-time histograms of open and closed states for a representative channel are shown in **Figure 3.18** and mean open and closed times are shown in **Figure 3.19**.

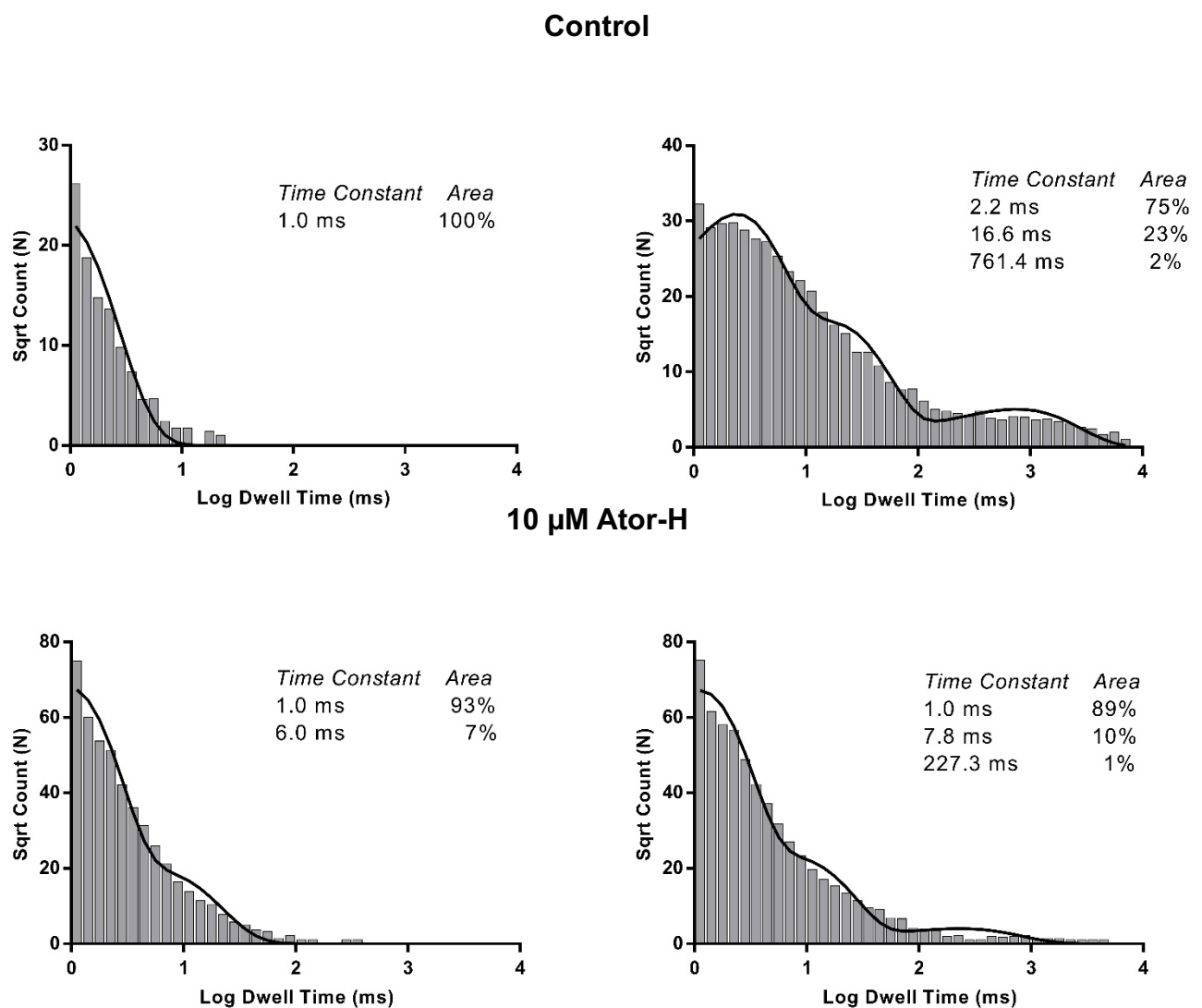


Figure 3.18 Effect of cytosolic Ator-H on lifetime distributions

Open and closed life-time distributions and probability density functions for a representative RyR1 channel in control conditions (10 μ M Ca^{2+} , top), and after addition of 10 μ M Ator-H (bottom). The time constants (τ) and percentage areas are shown.

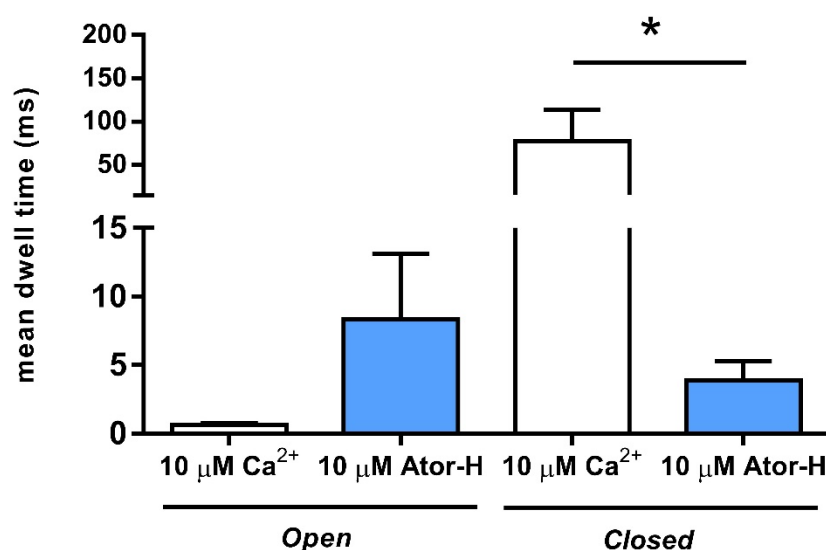


Figure 3.19 Effect of cytosolic Ator-H on mean open and closed times

Mean $\pm$ SEM is shown; $n = 5$; $*P < 0.05$.

Atorvastatin was observed to exert similar effects on both mean open and closed times, and lifetime distributions as simvastatin. It was found that 10 μM Ator-H increased mean open time from 0.619 ± 0.132 ms (mean $\pm$ SEM, $n = 5$) in control conditions to 8.334 ± 4.793 ms (mean $\pm$ SEM, $n = 5$) after addition of 10 μM cytosolic Ator-H. Mean closed time was decreased from 77.81 ± 36.03 ms (mean $\pm$ SEM, $n = 5$) to 3.879 ± 1.405 ms (mean $\pm$ SEM, $n = 5$). Looking in further depth at a representative channel (**Figure 3.18**), it was found that Ator-H appeared to cause a slight rightward shift towards longer opening times, however as for simvastatin, the majority of events were described by the first time constant. Ator-H also appeared to cause a larger shift in closed time distributions to shorter closed times. In control conditions, the majority of events are described by the second time constant (22.1 ms), however following exposure to Ator-H, the closed times are almost entirely described by the first time constant (1.2 ms) indicating shorter closing events. This is consistent with the increase in P_o caused by Ator-H treatment to be predominantly through increasing the frequency of openings, the same as previously seen for Sim-H.

3.2.3.3 Luminal atorvastatin does not increase RyR1 channel P_o

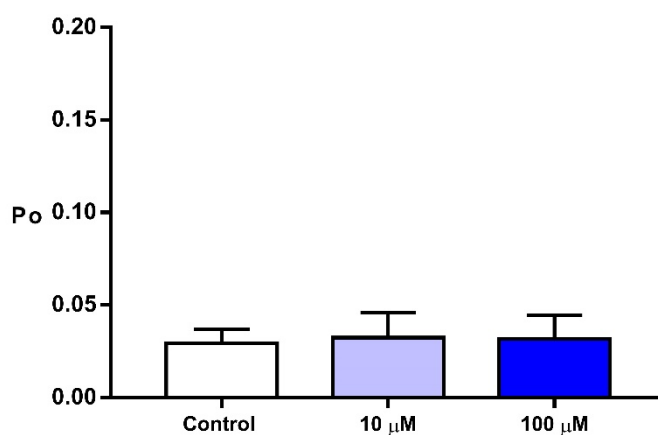


Figure 3.20 Effect of luminal Ator-H

Mean RyR1 P_o values before and after addition of 10 μ M and 100 μ M Ator-H to the luminal (*trans*) side of the bilayer (mean $\pm$ SEM are shown; $n = 5$). 'Control' conditions were 10 μ M cytosolic Ca^{2+} and approx. 50 mM luminal Ca^{2+} .

As was the case for simvastatin (ref **Figure 3.7**), addition of Ator-H to the luminal (*trans*) side of the lipid bilayer, did not affect RyR1 P_o . The mean data is shown in **Figure 3.20**. The P_o did not appear to be increased relative to control conditions (10 μ M Ca^{2+}), even at high concentrations of Ator-H (100 μ M). This indicates that the effect of cytosolic Ator-H is not a non-specific bilayer effect, and that the binding site for atorvastatin resides on the cytosolic portion of RyR1.

3.2.3.4 Species differences

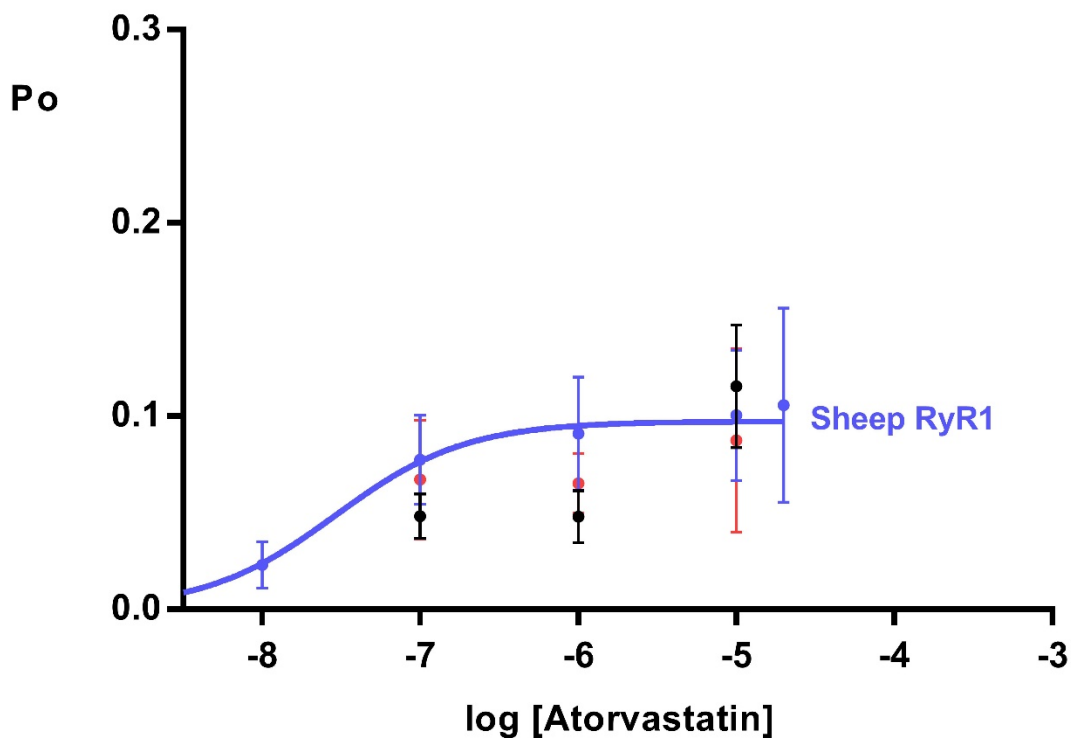


Figure 3.21 Species comparison of RyR1 activation in the presence of atorvastatin.

Mean RyR1 P_o values in the presence of increasing concentrations of Ator-H for RyR1 single channels obtained from either mouse (red), rabbit (black), or sheep (blue) skeletal tissue.; SEM; $n = 3 - 22$.

Single-channel experiments were also conducted using tissue derived from other mammalian species including mouse and rabbit to ensure that statin-induced gating changes were not specific to sheep. These species are routinely used in the Sitsapesan Lab and others in the field to assess the effect of a drug on RyR channels. **Figure 3.21** shows the mean P_o data for Ator-H activation from RyR1 channels isolated from mouse, rabbit and sheep skeletal muscle. Ator-H activated channels from all three species.

3.2.3.5 *Atorvastatin stimulates [³H]ryanodine binding to HSR vesicles*

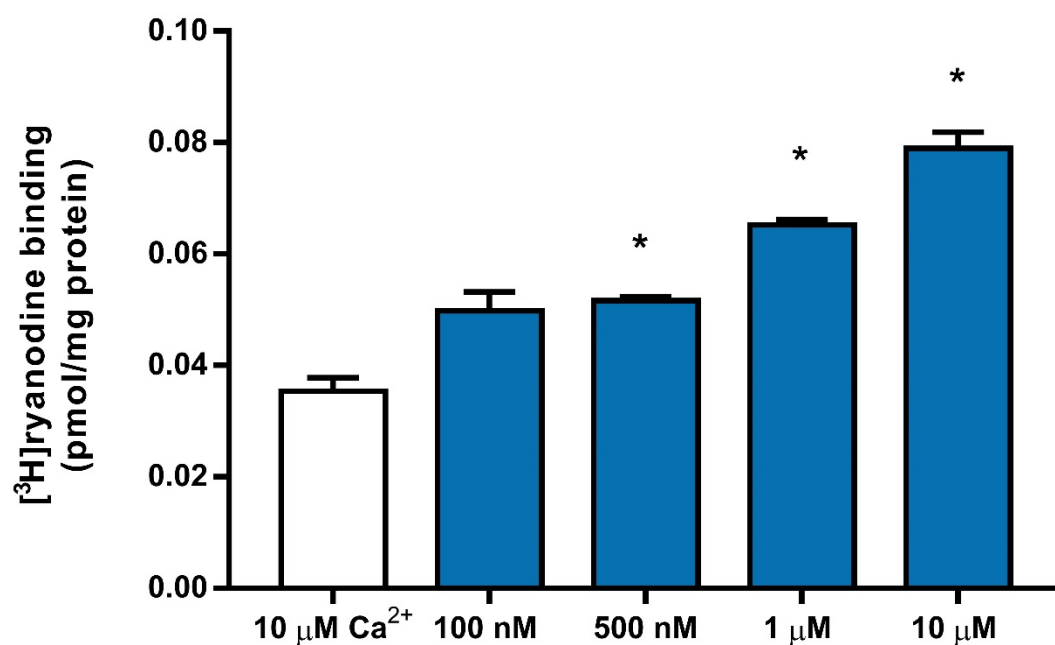


Figure 3.22 Effect of Ator-H on [³H]ryanodine binding

Stimulation of [³H]ryanodine binding to sheep skeletal HSR membrane vesicles by 10 μM Ca²⁺ alone (control conditions), and in the presence of additional Ator-H at the concentrations indicated. Mean ± SEM; n = 5, *P < 0.05.

Ator-H also stimulated [³H]ryanodine binding to sheep skeletal HSR vesicles. **Figure 3.22** illustrates the concentration dependent effects of Ator-H on [³H]ryanodine binding in the presence of 10 μM Ca²⁺. Ator-H caused a significant stimulation of [³H]ryanodine binding at 500 nM.

3.3 RyR1 activation is a class effect of statins

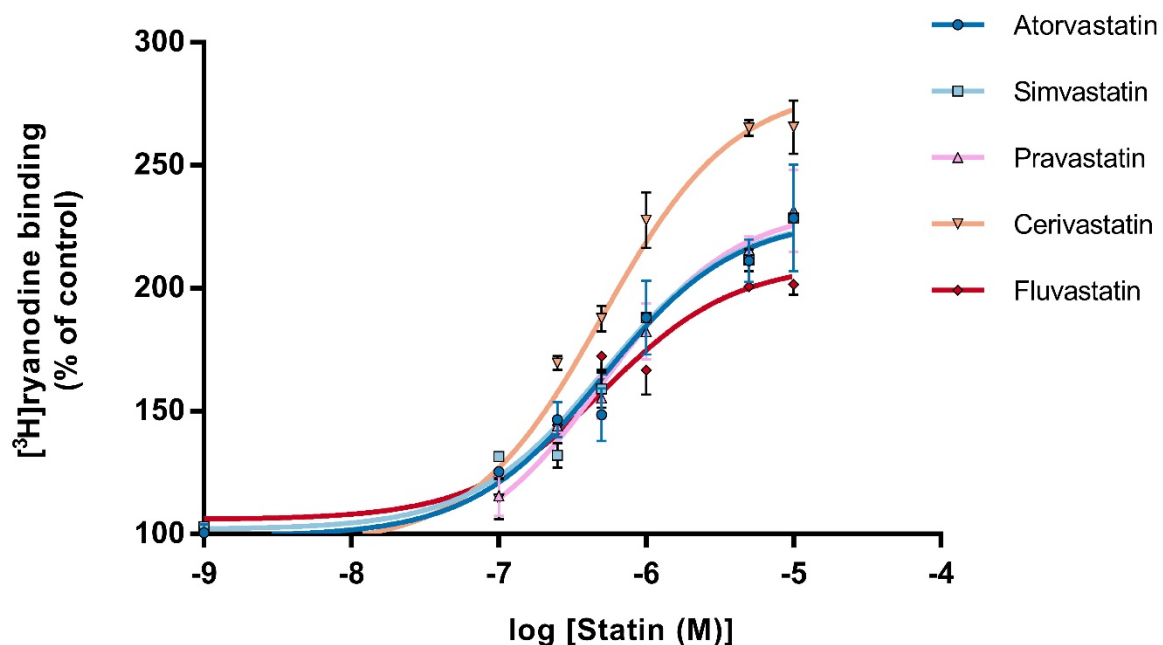


Figure 3.23 Comparison of RyR1 activation by commonly prescribed statins

Stimulation of [^3H]ryanodine binding to sheep skeletal HSR membrane vesicles by 10 μM Ca^{2+} (control value, white) and increasing concentrations of commonly prescribed statins as shown. Mean $\pm$ SEM; $n = 3-5$; $*P < 0.05$

	EC ₅₀ (μM)	Max binding at 10 μM (pmol/mg protein); Mean $\pm$ SEM	Hill Coefficient
Atorvastatin	0.76	0.079 $\pm$ 0.0028	0.81
Simvastatin	0.70	0.079 $\pm$ 0.0029	0.88
Pravastatin	0.44	0.073 $\pm$ 0.0043	0.48
Cerivastatin	0.42	0.082 $\pm$ 0.0008	1.30
Fluvastatin	0.30	0.064 $\pm$ 0.0018	0.82

Table 3.3 Dose response data for RyR1 activation of commonly prescribed statins.

The EC₅₀, maximum binding values and Hill coefficients are shown for the data presented in Figure 3.23.

Having established that simvastatin and atorvastatin both activate skeletal RyR1 channels, this raised the question of whether other statins also activate RyR1? To answer this question, the ability of other statins that are commonly prescribed in the UK, to stimulate [^3H]ryanodine binding were investigated (**Figure 3.23**). All the drugs tested were in their sodium salt form, using the same experimental conditions as used previously. Atorvastatin, pravastatin, fluvastatin, simvastatin and cerivastatin all appear to activate RyR1 to similar extents and show similar concentration-dependent relationships. The EC_{50} values for the individual statins were similar (**Table 3.3**), however, cerivastatin promoted the greatest level of [^3H]ryanodine binding at the concentrations studied, suggesting that it has the greatest efficacy for inducing RyR1 channel opening.

3.4 Discussion

Despite the prevalence of statin induced myopathies, an explanation for how they are caused, has remained elusive. In this chapter I have described how statins, including simvastatin and atorvastatin, can modulate the gating of skeletal muscle RyR1 channels which were incorporated into planar phospholipid bilayers. It was found that these statin drugs increase the Po of RyR1 channels, and therefore cause activation of the channels tasked with regulating Ca^{2+} -release from the SR. Consistent with these observations, both statins also increased the binding of [^3H]ryanodine to skeletal SR vesicles, indicating an increase in channel openings across a population of channels.

RyR1 activation by both simvastatin and atorvastatin was concentration dependent and in both cases it appeared to be reversible. However, the 'washout' Po in the case of Sim-H appeared to be higher than in control, whereas for atorvastatin washout Po was indistinguishable from control. Given that Sim-H is more lipophilic than Ator-H, it may be that Sim-H can partition into the lipid bilayer to a greater extent, and hence be more difficult to

remove by perfusion. However, this effect seems minor and for both statins the activation is largely reversible. Additionally, it was found that neither simvastatin nor atorvastatin appeared to cause any change in P_o when added to the luminal side of the bilayer, which suggests a cytosolic binding site, and rules out any non-specific bilayer interaction at the concentration used.

Lifetime analysis of single-channel events revealed that both Ator-H and Sim-H decrease the closed times of the channel, along with promoting a small increase in mean open times. Taken together, these results indicate that statins bind to the closed state of the channel and increase RyR1 P_o through increasing the frequency of channel openings. Cytosolic Ca^{2+} activates RyR1 by a similar mechanism and this suggests that statins may increase RyR1 P_o through sensitisation of the channels to cytosolic $[Ca^{2+}]$. Indeed, this has previously been reported for other ligands, such as caffeine.¹⁹⁶ This is supported by the observation that lowering the $[Ca^{2+}]$ to < 1 nM by addition of a Ca^{2+} -chelator, abolished all single-channel openings. Thus statin activation at these concentrations is absolutely dependent on cytosolic $[Ca^{2+}]$. However, we also found that even in the presence of sub-activating levels of cytosolic $[Ca^{2+}]$, if the [statin] was increased to 50 μ M, P_o could be increased (**Figure 3.13**). These openings had increased mean open time when compared with those observed with micromolar $[Ca^{2+}]$, an effect again seen with caffeine and demonstrate a second, Ca^{2+} -independent mechanism of activation.¹⁹⁶ This is consistent with previous reports that statins could cause Ca^{2+} -release in skeletal muscle fibres, in the presence of EGTA (and therefore sub-nanomolar $[Ca^{2+}]$).⁸³

Sirvent *et al.* showed that in human muscle fibres, simvastatin increased the resting $[Ca^{2+}]_i$ and hypothesised that this was due to Ca^{2+} -release from the SR.⁸⁵ Our collaborators, Lotteau and colleagues later found that simvastatin caused an increase in Ca^{2+} -sparks in permeabilised *flexor digitorum brevis* fibres (**Figure 3.1**). Our results now provide an explanation for both of these

observations. If statins increase RyR1 Po, and do so by increasing the sensitivity of the channel to cytosolic Ca^{2+} , we would expect that as $[\text{Ca}^{2+}]$ rises, RyR1 channels in the SR membrane would become activated at a lower cytosolic $[\text{Ca}^{2+}]$, thus leading to inappropriate triggering of Ca^{2+} -release from the SR. This also explains the finding that the voltage threshold for activation of EC-coupling in rat muscle fibres, is decreased in rats treated with statin.³⁰⁰ Sensitisation of RyR1 to cytosolic $[\text{Ca}^{2+}]$, or indeed increased cytosolic $[\text{Ca}^{2+}]$ due to Ca^{2+} -leak, could reduce the additional mechanical activation required to initiate Ca^{2+} -release.

We have found that this effect appears to be common to all statins, with all of those studied being capable of promoting RyR1 activation. It is notable that cerivastatin, the statin drug which was withdrawn from the market following its adverse side effects, shows the greatest efficacy as an activator of RyR1. That is, at all concentrations investigated, cerivastatin most effectively stimulated the binding of $[\text{^3H}]$ ryanodine to isolated SR membranes. Whether its propensity to trigger muscle side effects may instead be explained by its increased lipophilicity and hence muscle penetration or due to its increased ability to activate RyR1 is not yet clear, the association however is noteworthy.

However, an oft-noted concern about the cellular experiments previously conducted is their use of supraclinical concentrations of statins, for example Sirvent *et al.* employed 200 μM , and Knoblauch *et al.* upwards of 1 mM.^{85,301} Both concentrations are well in excess of the likely plasma concentration, and therefore some have questioned their relevance to the clinical use of statins.³⁰² An important consideration for this study therefore was, what is a relevant concentration of statin to investigate to provide clinically relevant results? There is no doubt that the plasma concentration of statins, even after continuous dosing during long term treatment is very low. Some reports suggest that this is around 1 nM, and the degree of binding to plasma proteins is extremely high, the actual 'free' plasma concentration is around 0.01 nM.³⁰² The IC_{50} values of statins for HMG-CoA reductase range from approximately 5 nM

to 44 nM, therefore at this low plasma concentration we would not expect significant inhibition of HMG-CoA reductase.¹⁸ It is important therefore to recognise that this is not the pharmacologically active concentration of statins, being around 100 times lower than the HMG-CoA reductase IC₅₀ of the most potent statins. Rather, it has been shown that atorvastatin and simvastatin accumulate in the muscle of human patients to 300 and 490 times the plasma concentration respectively, following the same trend as their lipophilicity.⁷⁹ The uptake of statins into skeletal muscle cells is driven largely by transporters, such as OATP2B1 and thus the plasma concentration is not reflective of the concentration inside cells. Therefore, our finding that statins can activate RyR1 at concentrations of 100 nM is indicative of an effect that could be reproduced in a clinical setting.

Moreover, many cellular studies have been limited by the fact that statin treatment is chronic rather than acute, and therefore it is a challenge to design an experiment which ensures that the cellular concentration of statins replicates that observed *in vivo*. Since transporters play such a key role in statin uptake, their expression and tissue distribution (both highly species dependent) will dictate the systemic exposure to the statins when dosed. For example, the plasma concentration of statins following dosing in guinea pigs is less than 10% of that in humans due to reduced expression of these transporters. However, through the experiments described in this chapter, we can look directly at the gating of RyR1 in a lipid bilayer, without the need to worry about whether the concentration at the RyR1 itself is the same as that being tested as it is necessarily the same as the ion channel is directly exposed to the recording solutions. We can now therefore show that activation can be seen at much lower concentrations than previously reported in cellular experiments, as we found significant increases in Po at 100 nM ator-H. We therefore can conclude that statins at a clinical concentration, can activate RyR1 by increasing the frequency of openings and therefore stimulating inappropriate Ca²⁺-release.

We have already noted that statins may exist in either an open-ring or closed-ring (lactone) form, although most statins are prescribed in the open-ring form given this is the active form *in vivo*. The lactone forms have also been reported to exhibit higher levels of myopathy, as determined by *in vitro* experiments utilising muscle fibres. However, it is not yet clear whether this is a result of their increased lipophilicity relative to the acid form, a factor that would increase their rate of diffusion into cells, or through some other mechanism. However both have been found to increase the frequency of Ca^{2+} sparks in muscle fibres.⁸² We have found that both the lactone and open-acid form of simvastatin can activate RyR1 in approximately the same dose dependent manner. This is a critically important observation given that the lactone forms of statins are incapable of inhibiting HMG-CoA reductase.²⁹⁸ That is, since the statins appear equally capable of activating RyR1 whether the hydroxyacid unit is in the lactone or open form, it suggests that this part of the statin molecule is not essential for its interaction with RyR1. Therefore, it should be possible to design a molecule capable of targeting and inhibiting HMG-CoA reductase, without also activating RyR1.

There is emerging consensus that statin-induced myopathy is the result of an alteration of Ca^{2+} -homostasis in skeletal muscle, and it is well documented that patients with RyR1 mutations which cause 'leaky' channels suffer from skeletal myopathies such as central core disease and malignant hyperthermia^{303–306}. Studies have shown that often this pathology is driven by RyR1 displaying an enhanced sensitivity to opening, as demonstrated by their increased sensitivity to activators such as caffeine and 4cmc³⁰³. Most notably for this work, Knoblauch *et al.* have previously reported that fibres obtained from mice carrying the RyR1 mutation Y542S, an MH mutation found in humans³⁰³ suffer a 'hypermetabolic response' upon exposure to simvastatin. Following the introduction of an RyR1 inhibitor, this Ca^{2+} release was abolished in both wild type and mutant fibres, directly implicating RyR1 as its source. This is consistent with my findings that statins directly activate RyR1, as an agent that increases RyR1 Po will necessarily increase Ca^{2+} -release in muscle fibres.

Given our finding that statins induce RyR1 activation, it is important to consider whether there is a connection between those patients experiencing the most severe statin side effects, and those who harbour RyR mutations. We might hypothesize that patients carrying these mutations might be more susceptible to statin-induced myopathy. Recent population studies have indicated a potential link between those patients suffering from statin-induced myopathy and those with RyR1 mutations associated with skeletal muscle disease, in particular MH.^{306,307} MH mutations can result in 'leaky' RyR channels, which results in an increased resting $[Ca^{2+}]$.³⁰⁸ If statins also increase RyR1 activity as we have found in this chapter, and increase resting $[Ca^{2+}]$ still further, then we can expect that the deleterious effects on the muscle will be exacerbated by statin treatment. Consistent with this hypothesis is the finding reported by Metterlein *et al.* that both atorvastatin and simvastatin significantly increased muscle contractions (leading to increased temperatures and muscle damage) in MH pig muscle relative to wild-type.³⁰⁵ In particular this would also explain the observation that some patients do not experience myopathy symptoms, despite having a high concentration of statins accumulating in their muscle³⁰⁹, as these patients may not possess the required RyR1 mutations.

3.4.1 Future experiments

It has been previously suggested that this SR Ca^{2+} release observed in muscle fibres with statins was a result of a direct interaction with RyR1^{85,310}. The study presented in this chapter demonstrates that statins do activate RyR1 and induce undesirable Ca^{2+} release events. It is therefore tempting to speculate that RyR1 activation is the ultimate culprit responsible for triggering the muscle side effects seen with statins. Of course, the effects of statins on skeletal muscle have been widely studied, and it is possible that statin myopathy is the result of a number of mechanisms. Further experiments will be required to determine the extent to which RyR1 activation plays a role in statin side effects, how statins influence the frequency of RyR1 openings, and how other isoforms of RyR are affected by statins. While we have found

that RyR1 incorporated into lipid bilayers can be activated by addition of statin, it is not yet clear whether this is an effect promoted by direct binding to RyR1. It is important not to forget that physiologically, RyR1 exists in a state bound with multiple associated proteins such as FK506 binding proteins, CaM, as well as small molecule ligands and ions such as ATP and Mg^{2+} .³¹¹ A simplified representation of the complexity of RyR1 bound proteins is shown in **Figure 3.24**.

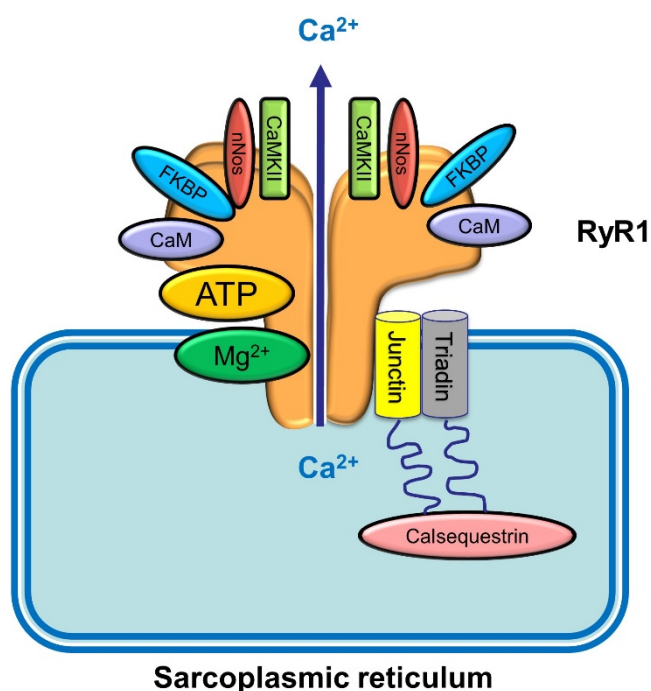


Figure 3.24 A schematic representation of RyR1 in the SR, with some of the known associated proteins and ligands bound.

The positions and relative sizes of the proteins are not to scale.

The reversibility of the RyR1 activation induced by statins excludes the possibility that it is driven by dissociation of one of these proteins. However, given the number of associated proteins bound to RyR, it is possible that statins do not bind directly to the RyR protein itself, but to one of these proteins. The use of purified RyR1 channels would allow us to gain an insight into whether, having stripped away these auxiliary proteins, statin-induced RyR1

activation is maintained. Of course, if statins do bind directly to RyR1, then where is the location of their binding site? The vast size of RyR has so far restricted our ability to investigate the binding of ligands using traditional methods such as x-ray crystallography. Alternative techniques such as cryo-EM, or the use of photoaffinity labelled statin molecules could be utilised to answer this question. In **Chapter 6**, I report experiments towards determining the location of the statin RyR1 binding site.

We have already raised the question of whether RyR1 mutations may predispose patients towards statin myopathy. Given the dormant characteristics of MH as a disease, in that it requires a trigger such as anaesthesia or physical stress, it will be important to consider whether statins may be deleterious towards this group of patients in particular. Such a finding would allow more informed prescription of statins as drugs. Future experiments should therefore include the use of tissue derived from MH mutant animals, to determine whether these mutations increase the P_o of these channels to a greater extent than wild type channels. Additionally, given that RyR is expressed elsewhere in the body, most notable in cardiac tissue (in the form of RyR2) that raises the question of whether statin-induced activation is isoform specific, or whether statins may also modulates the gating of RyR in other tissues besides skeletal muscle. In particular, since statins are prescribed to patients who are at increased risk of suffering from cardiovascular events, coupled with the fact that excess activation of the cardiac isoform RyR2 is associated with the generation of arrhythmias, it will be important to know whether statins also affect RyR2. The effects of statins on RyR2 channels, are investigated in the following chapter

4

Statins activate and inhibit RyR2

4.1 Introduction

Having found that statins can activate the skeletal ryanodine receptor (RyR1), this raises the question, do statins also affect other RyR isoforms. In particular, is the cardiac isoform (RyR2) affected?

RyR2 is commonly known as the cardiac isoform, due to it being primarily expressed in cardiac tissue, although it is also expressed elsewhere such as the brain.³¹² Sirvent *et al.* have previously noted that while statins have well documented deleterious effects in skeletal muscle, the heart appears unaffected.⁸⁴ However, there is an increasing body of evidence to suggest that the effect of statins may provide beneficial, cardioprotective effects by reducing instances of ventricular tachycardia and atrial fibrillation.^{72,76} In turn, there is broad consensus that stimulation of RyR2, that results in increased SR Ca^{2+} leak during diastole can result in the generation of life-threatening arrhythmias. Therefore, the effect of statins on RyR2 must be established in order to further understand if these protective effects are the result of RyR2 modulation.

Initially, our collaborators Lotteau & Calaghan (Leeds, UK) examined the effect of simvastatin on permeabilised rat cardiomyocytes.²⁸⁵ They found that the frequency distribution of Ca^{2+} sparks was shifted towards lower frequencies, but the amplitude, width and duration of the sparks was unaffected. Their findings are shown in **Figure 4.1**. Ca^{2+} -sparks are the result of localised SR Ca^{2+} -release events, from RyR2, and therefore these results suggested that statins may reduce the frequency of these sparks through RyR2 inhibition.

We therefore investigated the effects of Sim-H and Ator-H on the single channel behaviour of sheep cardiac RyR2, and their ability to stimulate the binding of [^3H]ryanodine to cardiac SR vesicles.

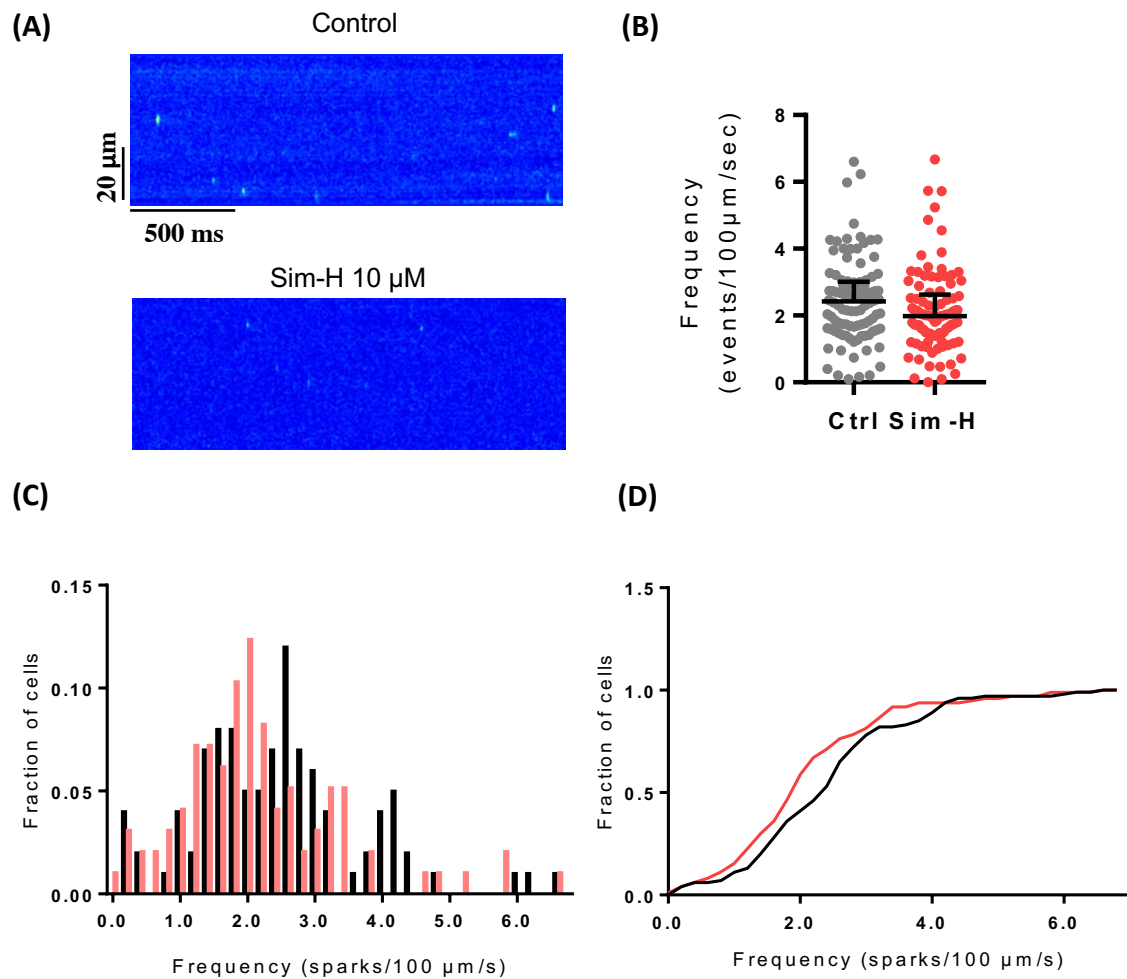


Figure 4.1 The effect of Sim-H on spontaneous SR Ca^{2+} release in permeabilised cardiac myocytes.

(A) Representative linescan confocal images showing Ca^{2+} sparks in saponin-permeabilised cardiac ventricular myocytes perfused with intracellular solution with or without 10 μM Sim-H for 5 min. **(B)** Medium frequency of Ca^{2+} spark events with interquartile range indicated. **(C)** Frequency distribution histogram for Ca^{2+} spark frequency in cardiac myocytes. Ca^{2+} spark frequency was significantly lower ($p < 0.05$) when analysed using each cell as an independent sample, but was not significantly different when each rat was used as the independent sample (Mann-Whitney). **(D)** Cumulative frequency distribution for Ca^{2+} spark frequency in cardiac myocytes. Data are from 100 control (black/grey) and 97 statin-treated cells (red) from $n = 5$ rats. Bin size = 0.2 sparks/100 μm /s; tickmarks on X-axis denote bin centre for both groups. This figure was adapted from Venturi *et al.* (2018) and the experiments performed by Dr Sabine Lotteau.

4.2 Results

4.2.1 Contribution by other members of the lab to the experiments described in this chapter

The results described in this chapter, where the effect of simvastatin was investigated on RyR2 were the result of a group effort to which I contributed. Dr Elisa Venturi began many of experiments described in this chapter, and following the start of my DPhil I continued with these experiments. The final results were published as Venturi, E*, Lindsay, C, *et al.* Br J Pharmacol. 2018 Mar; 175(6): 938–952 (*joint first author contribution).

The figures which include data collected by other lab members are shown in **Table 4.1**. I have contributed to data to all the figures shown. The number of experiments contributed by each other to the figures below is noticed in the individual figure legend.

Figure 4.3	Bar chart also includes single-channel data obtained by Dr Elisa Venturi
Figure 4.4	Bar chart also includes single-channel data obtained by Dr Elisa Venturi

Table 4.1 List of figures illustrating group data

The table lists the figures in this chapter to which other members of the lab have contributed data.

4.2.2 Simvastatin directly modulates RyR2 function

4.2.2.1 *Simvastatin activates RyR1 channels incorporated into lipid bilayers*

We first investigated how addition of simvastatin sodium salt (Sim-H) affected the gating and Po of single RyR2 channels. Sim-H was therefore added at increasing concentrations to the cytosolic side of RyR2 channels incorporated into phospholipid bilayers. A representative single-channel recording is shown in **Figure 4.2**, and the mean data are shown in **Figure 4.3**. The Po of sheep RyR2 in control conditions (10 μM Ca^{2+}) is typically higher than that of sheep RyR1^{313–315} and we observed a control Po of approximately 0.174 ± 0.060 (mean $\pm$ SEM; n = 11), consistent with previous reports.³¹⁶ In these conditions the gating of RyR2 is rapid, with the channel appearing to constantly gate. Periods of long closing or openings are rare. The detailed gating of the channel under these conditions has been extensively studied by this lab previously^{141,197,232,314,317,318}.

Surprisingly, it was found that, in contrast to the effect seen previously with the skeletal isoform (RyR1), following addition of 1 μM cytosolic Sim-H, channel Po was decreased from 0.174 ± 0.060 (mean $\pm$ SEM; n = 11) to 0.042 ± 0.022 (mean $\pm$ SEM; n = 7). This represents a decrease in Po to 22% of control conditions. However, further addition of Sim-H to 10 μM appeared to reverse this effect, as Po was restored to approximately control levels. Further increases in Sim-H concentration (to 20 μM) increased Po to 0.478 ± 0.188 (mean $\pm$ SEM; n = 5).

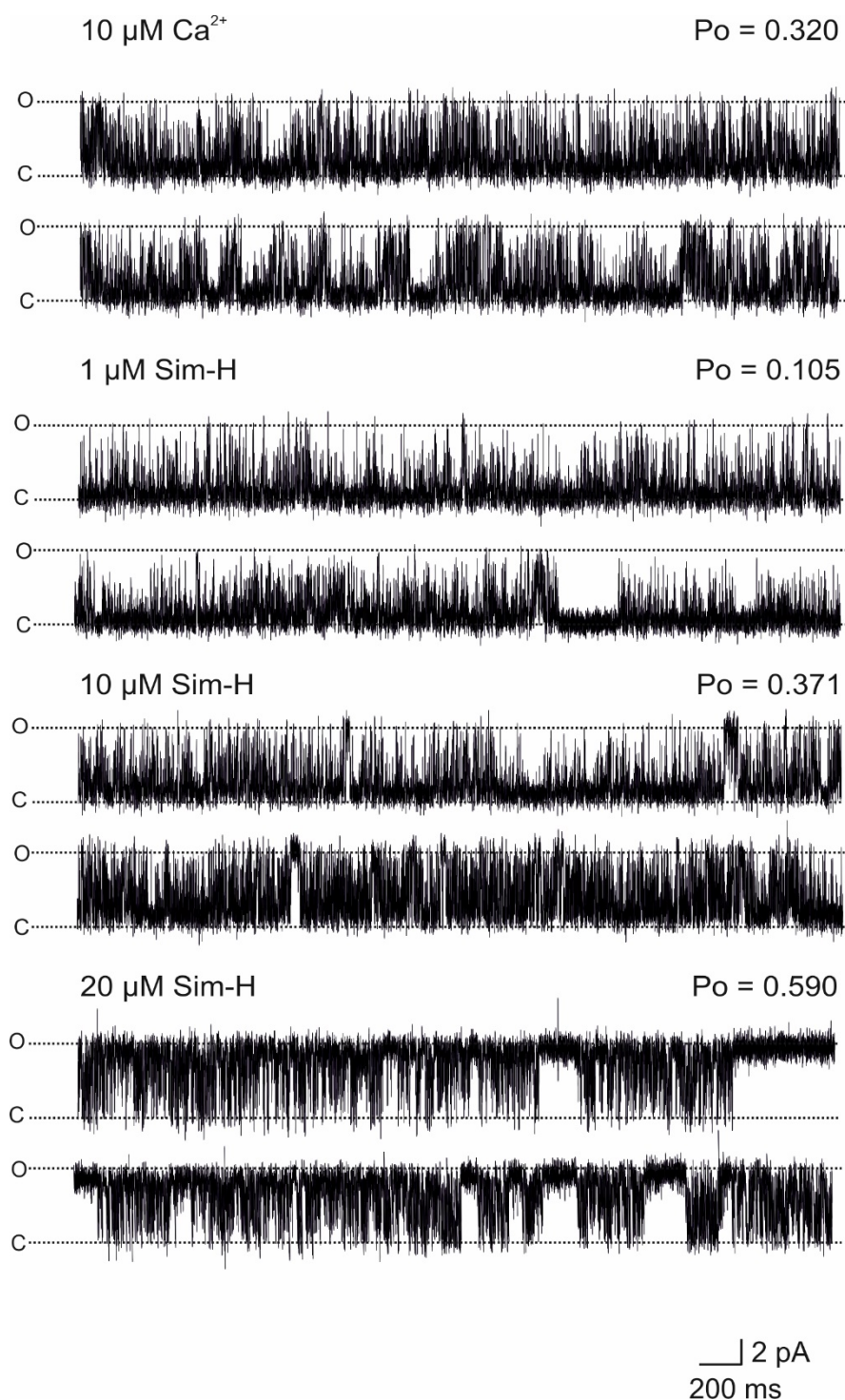


Figure 4.2 The effects of Sim-H on sheep cardiac RyR2 function

A typical recording of RyR2 in the presence of 10 μM Ca^{2+} as the sole activator (top two traces) and after addition of 1 μM Sim-H to the cytosolic chamber. The P_o above each trace refers to that obtained over three minutes of recording. O and C indicate the open and closed channel levels respectively.

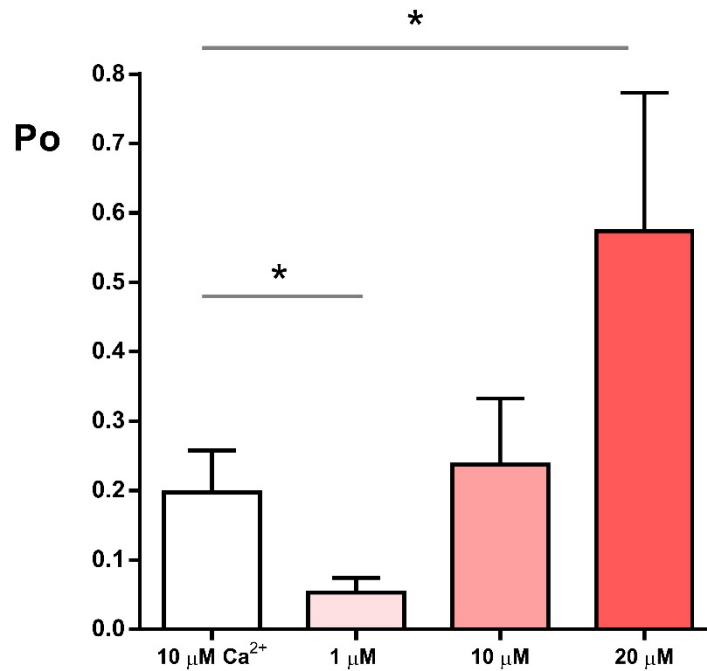


Figure 4.3 Mean effects of Sim-H on sheep cardiac RyR2 function

Mean RyR2 Po values in the presence of 10 μM Ca^{2+} as the sole activator and in the added presence of increasing concentrations of cytosolic Sim-H (1 μM , 10 μM and 20 μM as indicated). Mean $\pm$ SEM; n = 11, * P < 0.05. Data collected by myself (n = 4) and Dr Elisa Venturi (n = 7).

4.2.2.2 Effect of Simvastatin on RyR2 mean open and closed times

Further analysis of mean open and closed times, allows a greater insight into the mechanism by which Sim-H affects RyR2 gating. Mean open and closed times in both control, and following treatment with 1 μM Sim-H are shown in **Figure 4.4**. It was found that there was a trend for mean open time to decrease from 1.61 ± 0.51 ms (mean $\pm$ SEM; n = 7) to 0.79 ± 0.18 ms (mean $\pm$ SEM; n = 7) after addition of Sim-H, although the change was not statistically significant. In contrast there was a large increase in mean closed time from 18.31 ± 4.97 ms (mean $\pm$ SEM; n = 7)(control) to 8116 ± 5129 ms (mean $\pm$ SEM; n = 7) after addition of Sim-H. This shows that the predominant mechanism for RyR2 channel inhibition was a decrease in the frequency of channel openings.

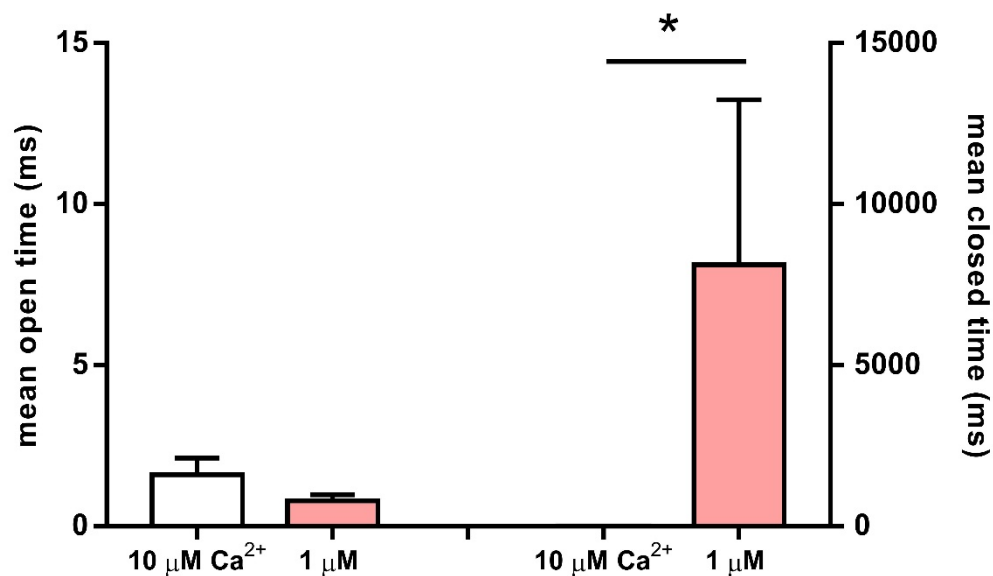


Figure 4.4 The effects of Sim-H on RyR2 mean open and closed times

Results are presented as mean $\pm$ SEM; $n = 7$; $*P < 0.05$, before (10 $\mu\text{M Ca}^{2+}$ alone) and after addition of cytosolic 1 $\mu\text{M Sim-H}$. Data collected by myself ($n = 3$) and Dr Elisa Venturi ($n = 4$).

Further analysis of lifetime distributions was also conducted, and lifetime histograms for a representative channel at increasing concentrations of Sim-H are shown in **Figure 4.5**. In control conditions, the open lifetime distribution was described by at least two time constants although most of the events occurred in the shortest time constant, and this was largely unchanged in the presence of 1 $\mu\text{M Sim-H}$. Subsequently however, there was a shift to longer open times with 10 $\mu\text{M Sim-H}$, with only 73% of events being described by the first time constant and 25% of events by the second time constant. In the case of the closed time distributions, there was a clear shift towards longer closed times in the presence of 1 $\mu\text{M Sim-H}$, consistent with the reduction in channel P_o observed under these conditions. This shift in mean closed times was reversed when Sim-H concentration was increased, with the vast majority of events now being described by the first time constant of 1.2 ms.

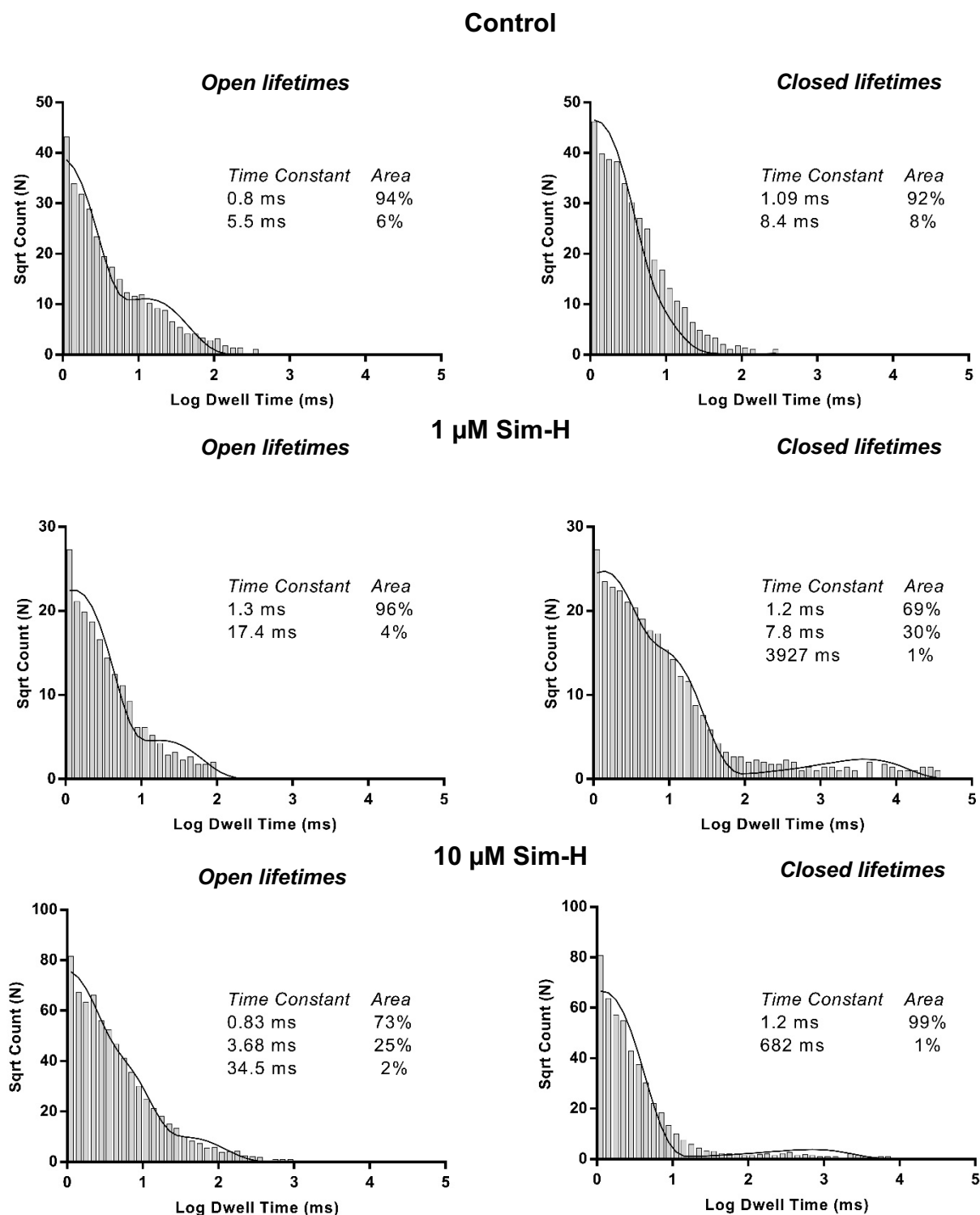


Figure 4.5 Effects of Sim-H on lifetime distributions

Open and closed life-time distributions and probability density functions for a representative RyR2 channel in control conditions (10 μM Ca^{2+} , top), and after addition of 1 μM (middle) and 10 μM Sim-H (bottom). The time constants (τ) and percentage areas are shown.

4.2.2.3 *Simvastatin has complex effects on [³H]ryanodine binding*

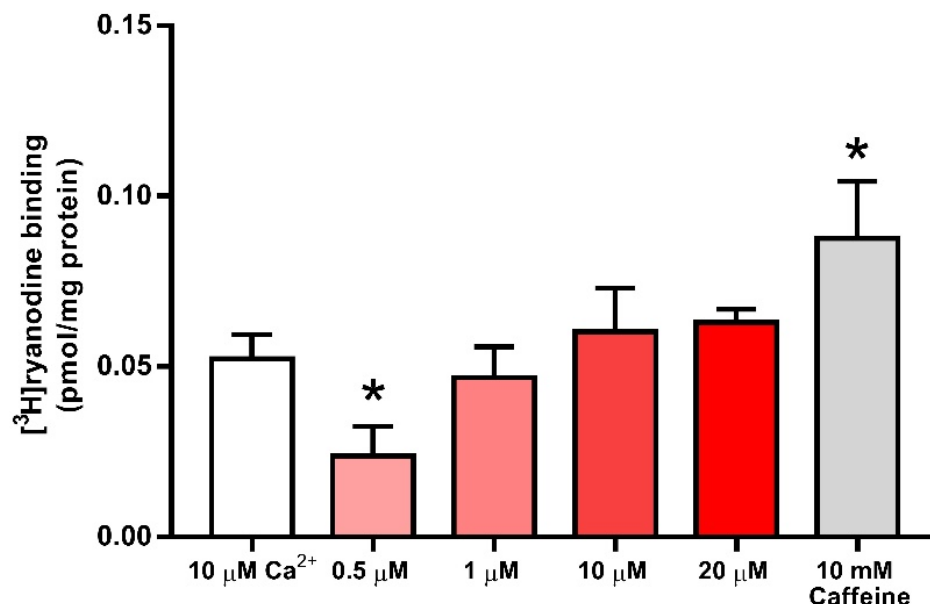


Figure 4.6 Effect of Sim-H on [³H]ryanodine binding.

Stimulation of [³H]ryanodine binding to sheep cardiac heavy SR membrane vesicles by Sim-H at increasing concentrations as indicated, or 10 mM caffeine (shown as a positive control). Data was obtained from three different membrane preparations. Mean $\pm$ SEM; n = 7; **P* < 0.05.

The effects of Sim-H on [³H]ryanodine binding to sheep cardiac heavy SR vesicles was then investigated. It was found that, consistent with the single-channel data, that low concentrations of simvastatin (500 nM) significantly inhibited [³H]ryanodine binding. The effect was reversed when concentration was increased (1–10 μ M) and appeared to plateau at about 20 μ M. The mean data is shown in **Figure 4.6** 10 mM caffeine is shown as a positive control for comparison.

4.2.3 Atorvastatin also activates and inhibits RyR2

Having observed the dual effects of simvastatin on RyR2, I then investigated whether the same effects are also seen with atorvastatin under the same experimental conditions.

4.2.3.1 *RyR2 native channels*

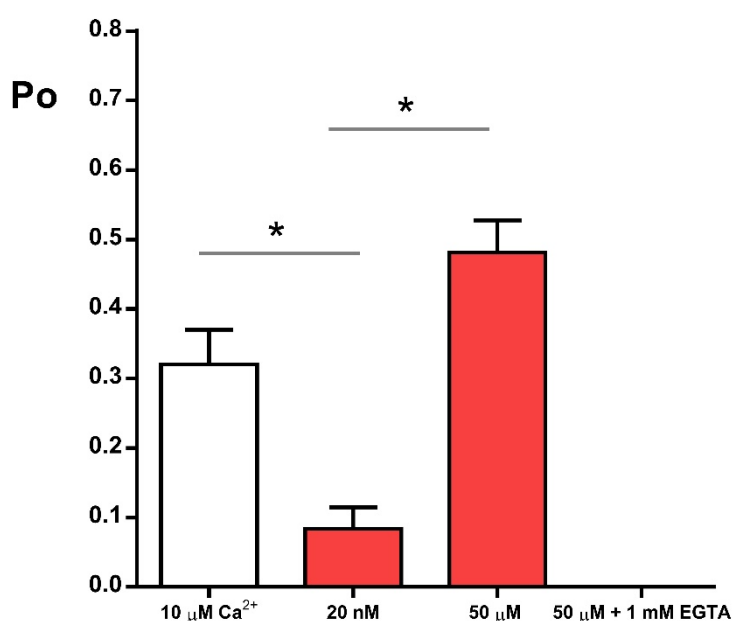


Figure 4.7 The effects of cytosolic atorvastatin on sheep native cardiac RyR2 gating.

Mean RyR2 Po values. Cytosolic atorvastatin and EGTA (free Ca^{2+} to 1 nM) additions are indicated. Mean $\pm$ SEM; n = 4

Native RyR2 channels were incorporated into planar phospholipid bilayers, and Ator-H was added at both low (20 nM) and high (50 μM) concentrations. The mean RyR2 Po data obtained is shown in **Figure 4.7**. As for simvastatin, low concentrations of atorvastatin significantly decreased RyR2 Po, and this effect is rescued upon addition of high concentrations of Ator-H (50 μM). In contrast to Sim-H I found that Ator-H was a more potent inhibitor of RyR2, with maximum inhibition occurring at 20 nM rather than 1 μM as previously observed with Sim-H.

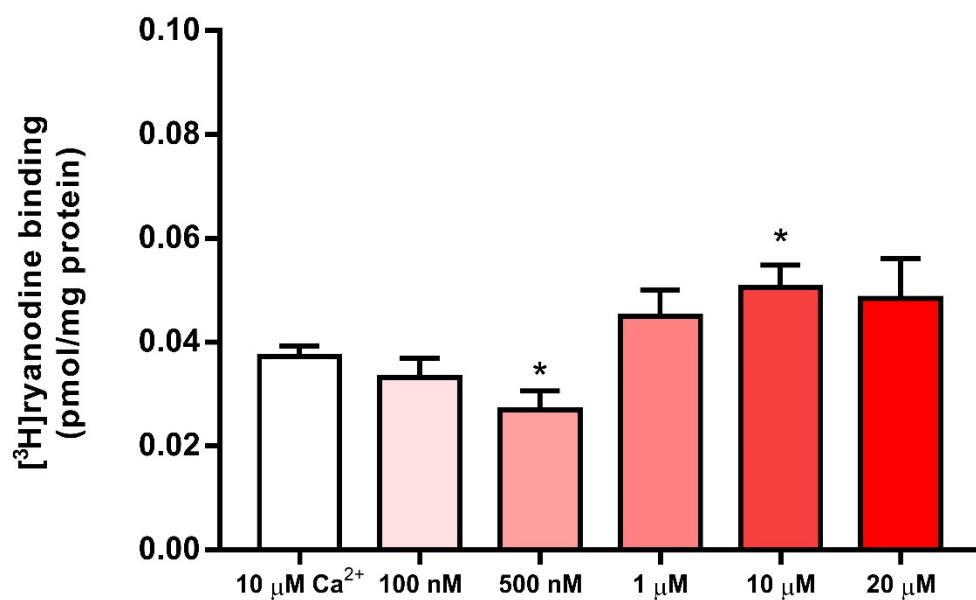
Furthermore, addition of 1 mM EGTA (free $[Ca^{2+}] < 1$ nM) completely abolished all channel openings in the presence of 50 μ M Ator-H, indicating that the activation up to this concentration was absolutely Ca^{2+} dependent.

4.2.3.2 *Effect of atorvastatin on [3 H]ryanodine binding*

Finally, Ator-H also exhibited similar effects as Sim-H on [3 H]ryanodine binding to sheep cardiac SR vesicles. Again in this case, we see that nanomolar Ator-H significantly decreases [3 H]ryanodine binding and that increasing the statin concentration to 1-10 μ M reverses the effect. Upon further increases in statin concentration to 20 μ M, it is not possible to increase the level of [3 H]ryanodine binding further. The mean [3 H]ryanodine binding data is shown in **Figure 4.8(a)**.

Figure 4.8(B) compares the relative ability of Sim-H and Ator-H to stimulate [3 H]ryanodine binding to RyR2. We can see that a similar dose-response trend is observed and the two drugs appear to exert broadly similar effects. The drugs caused a decrease in [3 H]ryanodine binding relative to control at 500 nM, and then subsequently cause an increase at higher concentrations. Atorvastatin appears to increase [3 H]ryanodine binding to higher levels than simvastatin, reaching a maximum of 135 ± 14 % of control binding at 10 μ M, compared to simvastatin reaching a maximum of 121 ± 18 % at 20 μ M.

(A)



(B)

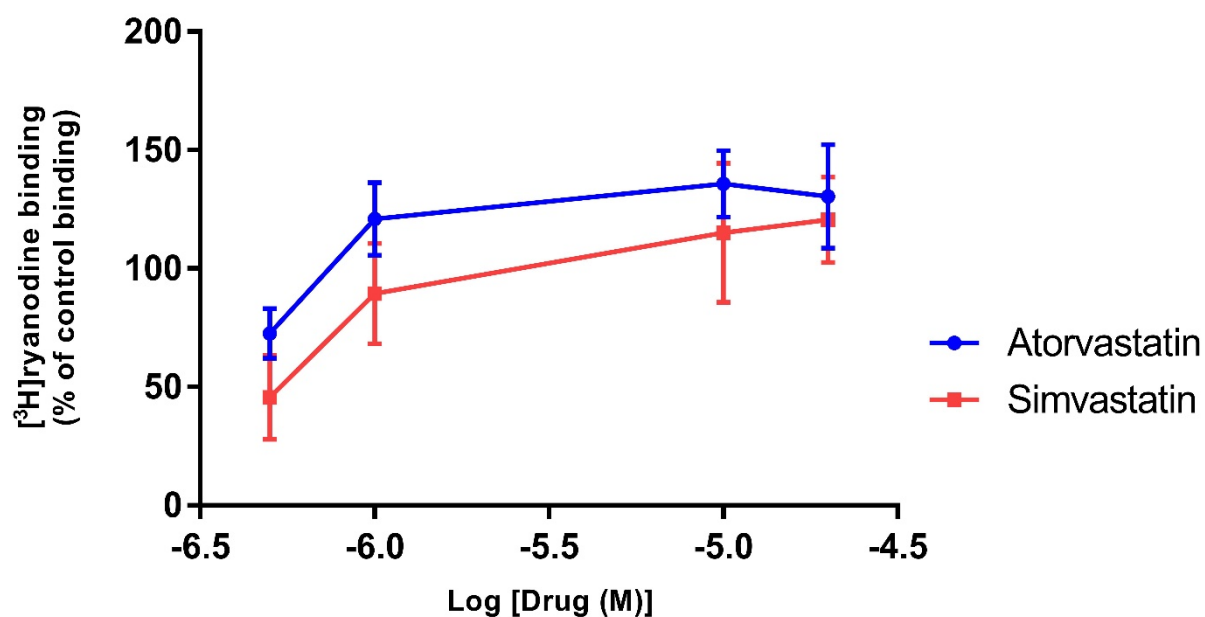


Figure 4.8 Effect of Ator-H on [³H]ryanodine binding

(A) Stimulation of [³H]ryanodine binding to sheep cardiac heavy SR membrane vesicles by Sim-H at increasing concentrations as indicated; Mean $\pm$ SEM; $n = 7$; * $P < 0.05$. Panel (B) shows a comparison between the stimulation of [³H]ryanodine binding in the presence of Sim-H (red) or Ator-H (blue).

4.3 Discussion

The study in this chapter has demonstrated that statins can also regulate the gating of the cardiac ryanodine receptor, RyR2, however the effect is more complex than the dose dependent activation seen with RyR1. It was found that 1 μM Sim-H, a concentration that activates RyR1 instead inhibited RyR2, that is, decreased the P_o relative to control. Higher concentrations (10 μM) increased P_o to control levels, and 20 μM caused significant activation. As an initial matter, the finding that Sim-H treatment can cause inhibition of RyR2 is consistent with the findings of Lotteau and colleagues (**Figure 4.1**) that Sim-H could reduce the generation of Ca^{2+} sparks in ventricular myocytes. It is interesting that at the higher concentrations of simvastatin, the inhibition effect on single channels is reversed, as this suggests there may be two binding sites for statin on RyR2; a high affinity inhibition site, and a low affinity activation site. In line with the single-channel experiments, a similar trend in the stimulation of [^3H]ryanodine binding was also observed, confirming the effect across a population of channels (**Figure 4.6**). A similar effect is seen with Ator-H, a type 2 statin, where significant inhibition of RyR2 was observed at 20 nM, an effect being reversible with increasing concentration. A notable feature of the activation of RyR2 at micromolar Ator-H, is that it appears to be highly Ca^{2+} -dependent, as in the presence of sub-activating levels of Ca^{2+} (< 1 nM) all channels openings were abolished, even in the presence of 50 μM Ator-H (**Figure 4.7**). In contrast to RyR1, no Ca^{2+} -independent openings could be observed under these conditions. More detailed lifetime analysis indicates that 1 μM Sim-H, a concentration which is inhibitory, achieves this by increasing mean closed times, without changing mean open times. A further increase in [Sim-H] restores P_o to control levels, which appears to occur through a combination of reversing the reduction in mean closed time, as well as a modest increase in mean open time (**Figure 4.5**). This suggests that statins bind to the closed state of the channel only to cause inhibition and thereby inhibit openings. However, while increases in P_o are caused primarily by statins binding to the closed channel state, at higher concentrations

statins also appear to bind to open states of the channel and prolong the duration of openings. This analysis could not be carried out for atorvastatin, due to insufficient single (as opposed to multiple) channels incorporating into the bilayer during experiments. However, the mechanism of action of both statins may be similar given their similar effects on channel Po.

Other members of the Sitsapesan Group have found the same ‘inhibition followed by activation’ effect reproduced with Ator-H when purified RyR2 single-channels have been used.³¹⁹ Purified channels lose the associated proteins that are typically bound to RyR in native preparations.³²⁰ Therefore, the observation that these channels also respond to Ator-H with the same concentration-response relationship would provide evidence that Ator-H binds directly to RyR2 to modify its gating, rather than to an associated protein. However, further experiments will be necessary to confirm this with confidence.

In **Chapter 3**, I discussed what a clinically relevant concentration of statins to investigate is, and this question is also relevant to RyR2 which is expressed predominately in cardiac muscle as opposed to skeletal muscle. Therefore we need to consider what *in vivo* concentration within cardiomyocytes might be found in a patient being treated with statins. The difficulty of obtaining human cardiac tissue makes this difficult to determine experimentally. However, while further work will be required to establish the exact concentration, there is significant evidence that similar mechanisms are employed in both tissues, with the more lipophilic statins being capable of utilising passive diffusion and more hydrophilic statins using organic anion transporting polypeptides and transporters. Indeed, the OATP2B1 transporter which is capable of transporting atorvastatin, simvastatin and cerivastatin is also expressed in the heart.³²¹ It is therefore reasonable to conclude that the concentration of statins that penetrate into cardiac muscle is at least the same as, and likely higher than the plasma concentration. Therefore our observation of inhibition of RyR2 occurring at 20 nM can be taken to represent an effect that could occur in a clinical setting.

Given the important role of RyR2 in mediating EC-coupling in the heart, it is not surprising that disruption of RyR2 regulation is associated with disease pathology, such as in heart failure (HF), CPVT and other arrhythmic diseases.^{184,322} In many cases, overactive RyR2 channels producing a diastolic Ca^{2+} -leak have been shown to be implicated.²⁰⁰ An undesired increase in Ca^{2+} -release events, in the form of sparks, which act as the trigger for the propagation of Ca^{2+} waves, has been shown to lead to delayed after-depolarisations and subsequent arrhythmias.³²³ Many have suggested that a mechanism to inhibit, or reduce the Po of these overactive RyR2 channels would provide a therapeutic benefit.³²⁴ Driven by this, the search for inhibitors of RyR2 has gained new ground, as it is hoped that being able to directly inhibit RyR2 will provide a way of rescuing these higher activity channels. Many research groups have been working to establish new screening methods to allow the identification of potent and selective RyR2 inhibitors.^{180,325} Therefore, our finding that statins, already well established and tolerated drugs, can act as RyR2 inhibitors may be a useful means for investigating the role of RyR2 inhibition for the treatment of CPVT and HF. This is particularly true for HF as many patients are already prescribed statins.

While there is no doubt that overactive RyR2 can result in arrhythmias, there is significant evidence that reducing RyR2 activity could provide a beneficial cardioprotective effect by the prevention of arrhythmias.³²⁶ For example, it has been shown that increasing the affinity of CaM for RyR2, an associated protein which acts as an inhibitor at high concentrations, reduces RyR2 Ca^{2+} leak³²⁷ and dantrolene (an RyR inhibitor) can improve the function of cardiomyocytes in HF³²⁸ or CPVT.³²⁹ Much effort recently has also focused on stabilising RyR2 by preventing CaMKII mediated phosphorylation, a process thought to increase RyR2 Po in the failing heart.³³⁰ These efforts have been so far been frustrated by the difficulty of producing CaMKII inhibitors with suitable pharmacokinetic properties (e.g. most CaMKII inhibitors are large peptides which cannot be dosed orally).³³¹ A molecule capable of inhibiting RyR2 directly,

such as simvastatin or atorvastatin could provide a powerful means to address this unmet clinical need.

These observations are therefore consistent with the findings of several large randomised clinical trials which have suggested that statin treatment provides beneficial cardioprotective effects, beyond that which can be rationalised by inhibition of cholesterol synthesis. For example, the CARE trial of 4159 patients treated with 40 mg pravastatin appeared to reduce the incidence of coronary events to a greater extent than a similar trial investigating simvastatin, despite both trials reporting the same degree of reduction in LDL cholesterol levels.^{332–334} Indeed, there is evidence that the cardioprotective effects of statins are independent of whether they are successful at lowering the patient's cholesterol levels at all, which provides further support that the mechanism is independent from their inhibition of HMG-CoA reductase in some patients.³³⁵ Their opposite effects on mitochondria derived from skeletal and cardiac muscle respectively are also another possible explanation, although these effects may occur secondarily to an increased release of SR Ca^{2+} .³³⁶ Thus, the direct inhibition of RyR2 *in vivo* may help to explain how these reductions in instances of arrhythmias are achieved independent of cholesterol lowering.

As such, the explanation for the anti-arrhythmic effects of statins is not yet clear, however it is likely to be the product of a number of mechanisms, of which RyR2 inhibition may play a crucial role. Statins have been reported to provide anti-inflammatory and anti-oxidant effects³³⁷, as well providing a stabilising effect on the development of arterial plaque which subsequently may reduce instances of ventricular tachyarrhythmias.³³⁸ Additionally, statins can dampen the impact of ischemic reperfusion injury by facilitating the regression of atheroma, increasing NO production and reducing the aggregation of platelets.^{339–341} Therefore there is no doubt that any satisfactory explanation of the cardioprotective effects of statins will be multifaceted. However many of these alternative mechanisms could be

secondary to the direct inhibition of RyR2. For example, ischemia-reperfusion injury has already been linked to oxidative damage to RyR, stimulating redox modifications to the channel and increasing its P_o .³⁴² The finding that statins can inhibit RyR2 provides an explanation for the beneficial effect of statins following myocardial infarction.

This hypothesis is further strengthened by the fact that the concentrations of statins at which we have observed RyR2 inhibition appear to be more closely aligned to the clinical situation than reports suggesting alternative mechanisms.³⁴³ Given that it is unlikely that the concentration of statins in cardiac muscle reaches tens of micromolar, it is not clear what role, if any, activation of RyR2 plays in statin treatment. However their inhibitory effect may play an important role, especially given that statins are prescribed extensively to patients at an increased risk of cardiac arrhythmias.³⁴⁴ Combined with the data from **Chapter 3**, we find that clinically relevant concentrations are likely to activate RyR1 thus potentially driving muscular side effects while providing cardioprotective properties through simultaneous inhibition of RyR2.

4.3.1 Future experiments

That statins can inhibit cardiac RyR2 at clinically relevant concentrations is an important finding given how widely prescribed these drugs are to patients at risk of developing cardiac arrhythmias. Further experiments will be required to confirm whether these effects are also observed with other clinically used statins and to further elucidate the role they may play in the clinical findings of statins providing cardiovascular benefits.

As we have previously considered for the effects of statins on RyR1, it will be important to determine (i) which functional groups of the statin molecules are required to drive RyR2 binding and (ii) the location of the RyR2 statin binding site. A further important question indeed will be, given the extensive level of sequence identity (> 70%) between RyR1 and RyR2, how is it that statins can promote differential effects in their gating? How can nanomolar

concentrations of statins activate RyR1 in skeletal muscle, while inhibiting RyR2 in cardiac muscle? There is precedent for ligands to affect these isoforms differentially, such as that seen with FK506-binding protein³⁴⁵, CaM²²⁸, suramin²¹⁰, dantrolene¹⁷⁹, and Ca²⁺¹⁰³. However if this finding regarding statins is to be fully understood, more detail on how statins bind to RyR2 will be required. Future experiments utilising techniques such as photo affinity labelling and cryo-EM will be required to establish this.

Given that undesired Ca²⁺ leak from RyR2 has been documented in both atrial and ventricular arrhythmias, it is assumed that reversal of this Ca²⁺ leak would rescue the arrhythmogenic phenotype in both tissues. However, whether statins achieve this by inhibiting both atrial and ventricular RyR2 is not yet clear. While there is consensus amongst clinical trial reports that statin treatment is protective against ventricular tachycardia (VT), whether the same is true in the case of atrial fibrillation (AT) is a matter of debate.^{346,347} In the present study, RyR2 was isolated exclusively from sheep ventricular tissue and hence these results do not necessarily indicate that atrial RyR2 would respond in the same manner to statin exposure. There are some key differences to the EC-coupling machinery in atrial cardiomyocytes, such less extensive t-tubular structure and more spatially distributed clusters of RyR.³⁴⁸ Additionally, other factors such as the level of phosphorylation of RyR2 in different tissue types may influence its response to statins. Further experiments will be required, which utilise RyR2 derived from atrial tissue, to determine whether statins promote the same effects as in ventricular RyR2 towards providing a definitive answer to the question of whether statins may protect against atrial fibrillation.

5

Statin Pharmacophore Synthesis

5.1 Introduction

5.1.1 The statin pharmacophore

As we have described in Chapter 3 and 4, both simvastatin and atorvastatin sodium salt have distinct effects on both the skeletal (RyR1) and cardiac (RyR2) ryanodine receptors, causing activation only in the former, and more complex effects in the latter. In the case of simvastatin, these effects are also observed with simvastatin lactone (Sim-L), suggesting that the effect is independent of the modified hydroxyglutaric acid HMG-CoA pharmacophore. **Figure 5.1** provides a reminder of the structure of the statin pharmacophore in both the open (hydroxyglutaric acid) and closed (lactone) forms. The statin pharmacophore is a structural analogue of the natural substrate for HMG-CoA reductase and is the part of the molecule responsible for the binding of statin molecules to this enzyme, and for their subsequent effects on cholesterol synthesis. Crystal structures determined by Istvan and Deisenhofer¹⁸ at high resolution (2.3 Å) have provided insight into how statins bind to HMG-CoA reductase.

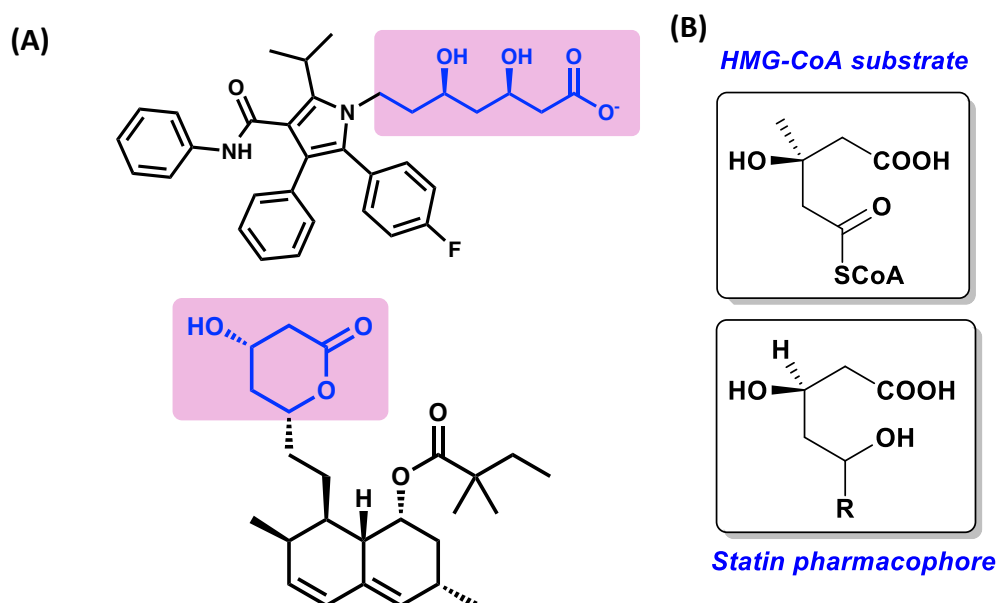


Figure 5.1 The statin pharmacophore exists in both an open (salt) and closed (lactone form).

(A) Atorvastatin sodium salt (top molecule) and simvastatin lactone (bottom molecule) are shown, with the HMG-CoA reductase pharmacophore indicated in each case. (B) Structural similarity between the physiological substrate (top) and the statin pharmacophore (bottom). The latter acts as a surrogate of the physiological substrate.

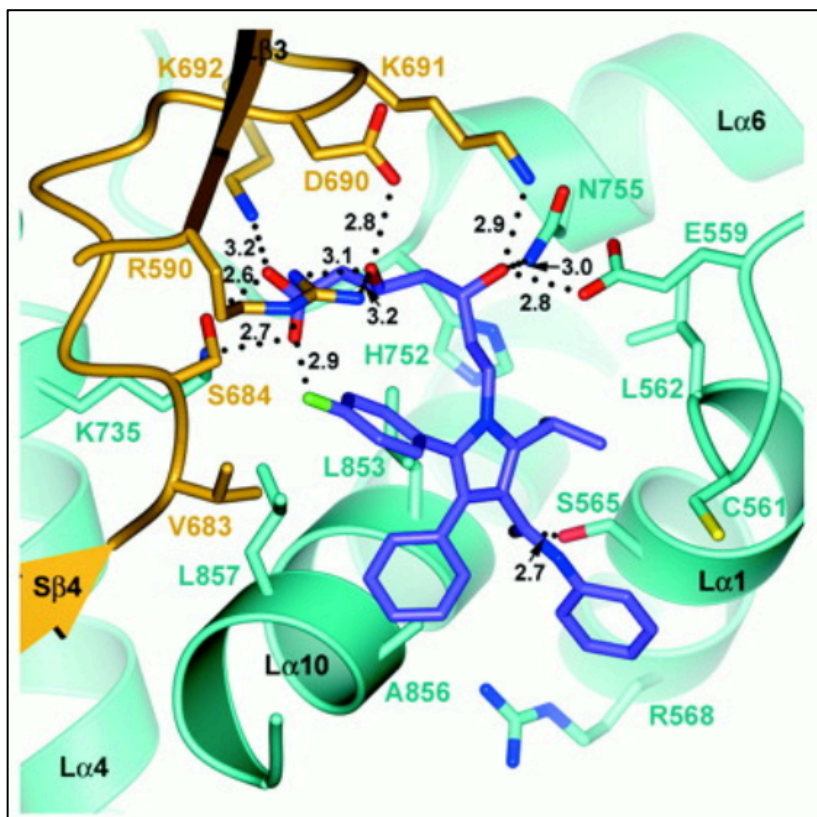


Figure 5.2 Binding mode of atorvastatin to human HMG-CoA reductase, reproduced from Istvan *et al.* (2001)

Key interactions with the HMG-CoA reductase enzyme are indicated.

An example of one (atorvastatin) is shown in **Figure 5.2**. There are a large number of interactions between the HMG-CoA reductase protein and the hydroxyglutaric acid moiety of the statin molecule, while the hydrophobic portion of the statin is positioned in a shallow groove with most of the residues at too great a distance for specific interactions ($> 2 \text{ \AA}$), which suggests a level of flexibility in how these groups are orientated. However, in the case of the acid region of the statin, there are a number of polar residues (e.g Ser684, Asp690, Lys691 etc) which appear to form hydrogen bonding interactions, and Lys735 in particular forms a salt-bridge with the terminal carboxylic acid group of the pharmacophore¹⁸. Similar interactions were seen for all other statins which were crystallised with HMG-CoA reductase, and both type 1 and type 2 statins showed a very similar binding mode (with complete conservation of

the polar interactions mentioned above)³⁴⁹. In atorvastatin (and rosuvastatin), the Ser565 residue forms a hydrogen bond with the carbonyl oxygen of the amide group (or sulfone group of rosuvastatin).¹⁸ The conclusions from this work are that the hydroxyglutaric acid moiety is the essential component for allowing a statin drug to bind to and inhibit HMG-CoA reductase.

5.1.2 Aims

Having seen how important this functional group is to statins binding to their desired target, **a key question therefore was, is this part of the statin molecule also responsible for statin effects on RyR?** If RyR activation, and the resultant undesired Ca^{2+} -release is the driving force for skeletal myopathic effects the statin family of drugs are associated with, then it will be important to establish whether undesirable binding to RyR can be separated from the desired binding to HMG-CoA reductase. First it must be determined if the statin hydroxyglutaric acid pharmacophore can itself bind to and influence the gating of RyR channels. In this chapter, I will describe how the statin pharmacophore (and variants of it) were synthesised and investigated for their effects on RyR activity, to provide an answer to this crucial question.

5.1.3 Previous Work

While no synthetic route to the hydroxyglutaric acid HMG-CoA pharmacophore itself has been reported, there have been several routes towards (-)-nor-mevalonic acid lactone, a structurally related analogue of the statin pharmacophore, formed from nor-mevalonic acid under acidic conditions (**Figure 5.3**).

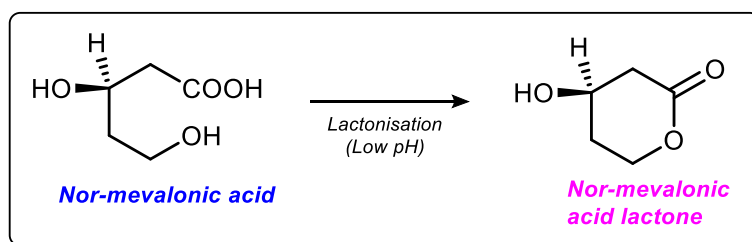
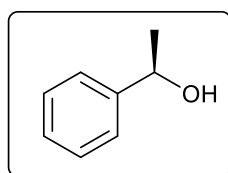


Figure 5.3

Cyclisation (or conversion) of the statin pharmacophore to the lactone form under low pH conditions.

Producing the desired stereochemistry presents a challenge, and various strategies have been reported for its installation on a hydroxyglutaric acid unit. These can be grouped into four categories, which broadly describe the synthetic methodology employed in each. These are: desymmetrisation³⁵⁰, asymmetric synthesis³⁵¹, enzyme-mediated³⁵² and Evans aldol strategies³⁵². A summary of each method 1-4 are shown in **Figure 5.4**. Each of these methods was investigated to determine their suitability for the synthesis of (-)-nor-mevalonic acid lactone. In 2015, enantioselective desymmetrisation (**method 1**) of a *meso*-dialdehyde precursor with a chiral alcohol ((+)-(*R*)-phenyl-ethanol) and molecular sieves, was reported to yield (-)-nor-mevalonic acid lactone in 2.7% yield and 79% enantiomeric excess (*e.e.*) over 6 steps³⁵⁰.



(+)-(*R*)-phenyl-ethanol

The original synthesis of (-)-nor-mevalonic lactone from anhydromevalonolactone used Sharpless epoxidation (**method 2**) with chiral (+)-diethyltartrate as its key step providing the final lactone in 24% yield and 89% e.e. The alternative stereochemistry could be obtained by instead using (-)-diethyltartrate. A disadvantage with this synthesis, despite its high yield and enantioselectivity, is that the precursor is not commercially available and must itself be synthesised from 4-methyl-5,6-dihydropyran.³⁵¹ Another strategy for producing the desired stereochemistry, is via enzymatic methods such as pig liver esterase (PLE) bound to eupergit (**method 3**) which allows large scale reactions (gram-scale) to be performed by recovering and recycling the enzyme.³⁵² This method is reported with a yield of 20% and e.e. of 75%, comparable to the asymmetric methods above.

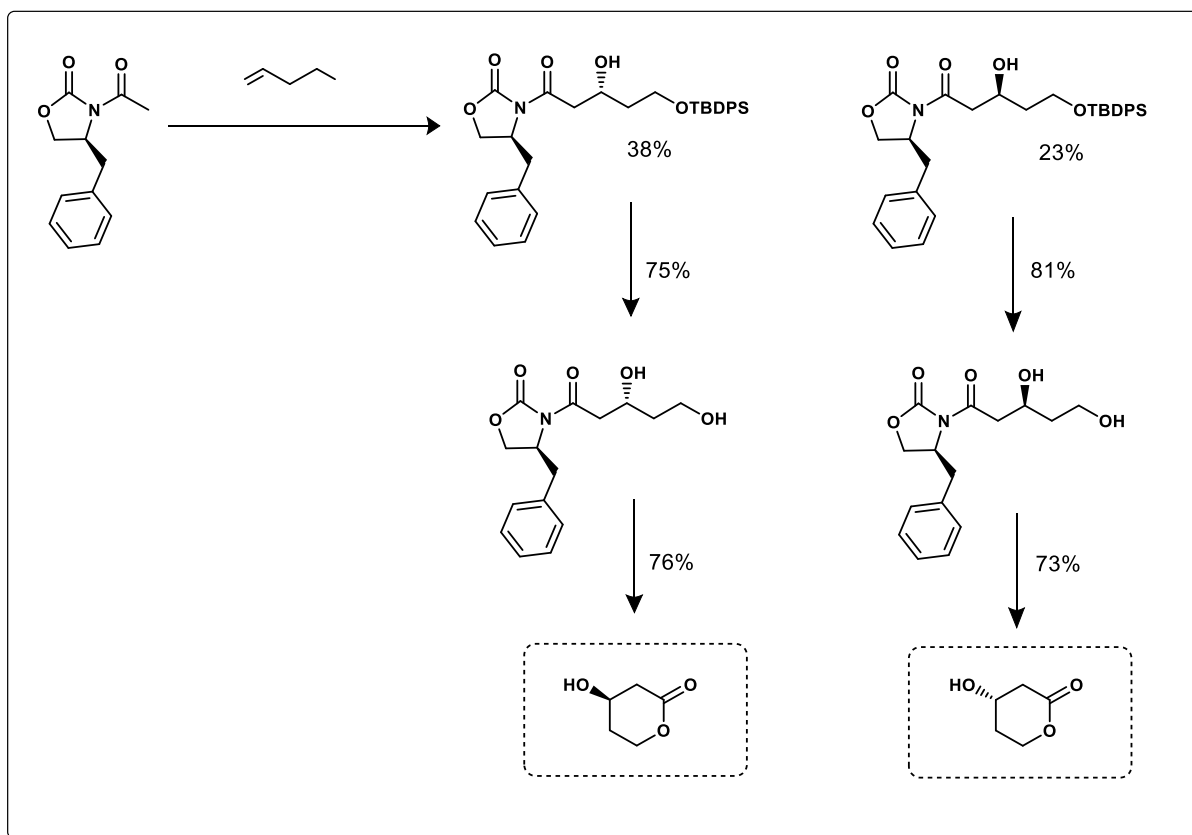
Finally, Loubinoux *et al.* reported in 1995 an alternative method utilising an Evans aldol protocol using 4*S*-Isopropyl-*N*-acetyl-oxazolidinone as the chiral auxiliary (**method 4**). The reaction affords a mixture of diastereomers in the ratio of 33:20 of the desired to undesired diastereomer. However, these are readily separable by simple column chromatography. Following removal of the chiral auxiliary and lactonisation, the final lactone was isolated in 22% yield and >95% e.e.

Having considered the available methods, a modified protocol adopted from Loubinoux *et al.* using Evans auxiliary was selected for the synthesis of the statin pharmacophore. Despite the low selectivity of this method, access could be gained to both enantiomers of the final lactone product, thus providing a suitable control. Additionally, the enhanced e.e. of the final product compared to other methods made this synthesis an attractive one.

		Ref.
1	Desymmetrisation Conditions: (i) 4 Å mol. sieves, (+)-(R)-1-phenyl-ethanol, RT, 24h; (ii) PCC, CH₂Cl₂, RT, 18h; (iii) MeONa, MeOH, -35°C, 24h; (iv) NaBH₄, MeOH, -35°C, 24h; (v) pTsOH, CH₂Cl₂, RT, 1h; (vi) TBAF, AcOH/THF, RT, 1 h.	[350]
2	Asymmetric synthesis Conditions: (i) PCC, CH₂Cl₂, 70°C, 8h; (ii) KOH 12N, reflux, 15 min; then CH₃I, DMT, RT, 24h; (iii) TBMS-Cl, DMAP, Et₃N, CH₂Cl₂, RT, 1 hr (iv) DIBALH, toluene, 0°C; 1 hr (v) Ti(OiPr)₄, (+)-L-tartrate diethyl, TBHP, CH₂Cl₂, -30°C, 12h; (vi) LiBH₄, THF, anh. Reflux, 2h, then MeOH, CO₂, 1 hr; (vii) RuCl₃, aq. NaIO₄, CCl₄, CH₃CN, H₂O, RT, 1 h; (viii) pTsOH, CH₂Cl₂, RT, 1 hr.	[351]
3	Enzyme-mediated Conditions: (i) PLE/Eupergit pH 7 phosphate buffer, RT; (ii) BH₃Me₂S, THF, RT, 20h, then MeOH; (iii) TFA, MeOH, 20h then C₆H₆; (iv) pTsOH, C₆H₆, Dean-stark conditions, 3h.	[352]
4	Evans aldol Conditions: Et₃N, nBu₂BOTf, CH₂Cl₂, -78°C to 0°C, 1 h; (ii) HF/Pyr/THF, RT, 3 h, (iii) Et₃N, CH₂Cl₂, RT, 48 h, then toluene.	[352]

Figure 5.4 Previous synthetic strategies towards (-)-nor-mevalonic acid lactone

Previous synthetic strategies to synthesise (-)-nor-mevalonic acid lactone can be grouped into four different categories by methodology: (1) desymmetrisation³⁵⁰, (2) asymmetric synthesis³⁵¹, (3) enzyme-mediated³⁵² and (4) evans aldol strategies³⁵². PCC = pyridinium chlorochromate, AcOH = acetic acid, PLE = pig liver esterase, DMAP = 4-dimethylaminopyridine, TBHP = *tert*-butyl hydroperoxide.

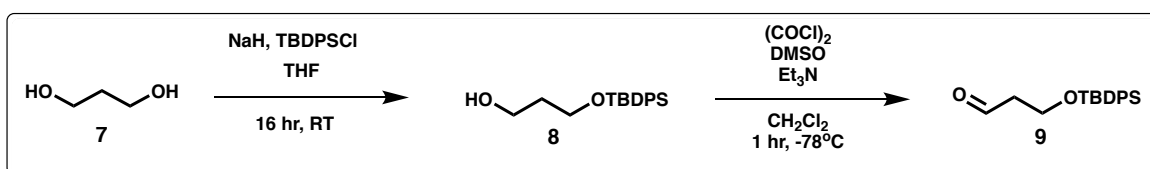
**Scheme 5.1 Synthesis reported by Loubinoux *et al.* chosen as the preferred route**

Reagents and conditions (i) Et₃N, nBu₂BOTf, CH₂Cl₂, -78°C to 0°C, 2.5 h; (ii) HF/pyr, THF, RT, 3 h; (iii) Et₃N, CH₂Cl₂, toluene, 48 h, RT.

Loubinoux *et al.* began the synthesis from (4S)-isopropyl-N-acetyl-oxazolidinone, forming the corresponding enolate in the presence of triethylamine and dibutylboron triflate. Subsequent aldol reaction with TBDPS-protected aldehyde **9** (prepared in two steps from 1,3-propanediol) yielded diastereomers **2** and **3** in a 1 : 1.65 ratio, which were subsequently isolated in 38% and 23% yield respectively. This method is unselective, affording a mixture of diastereomers, however as **2** and **3** were separable by flash column chromatography. Subsequent removal of the silyl groups with HF in pyridine, treatment with triethylamine to remove the chiral auxiliary, and finally repeated coevaporation with toluene yielded lactones (**R**)-**6** and (**S**)-**6**.³⁵² This method was employed in this chapter for the preparation of (-)-nor-mevalonic acid lactone (**R**)-**6** (Scheme 5.1).

5.2 Results

5.2.1 Preparation of starting materials



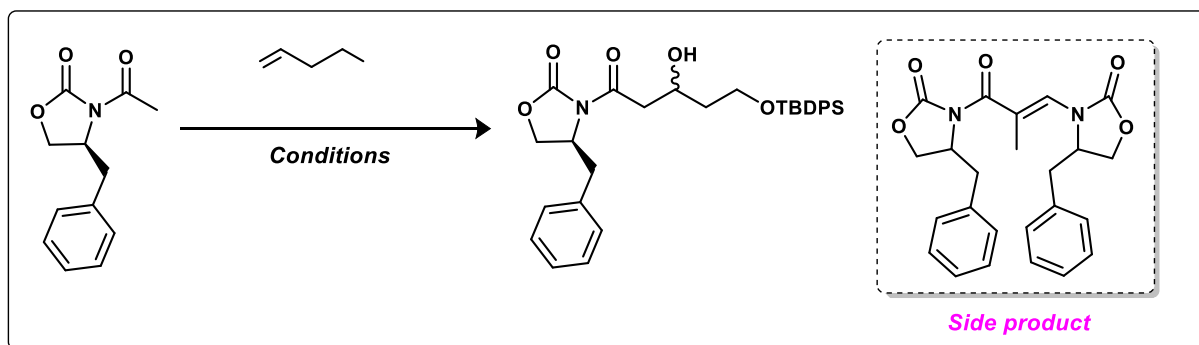
Scheme 5.2 Preparation of starting aldehyde **9**

The first requirement was to synthesise the TBDPS-protected precursor aldehyde **9** to facilitate the planned aldol reaction (**Scheme 5.2**). Treatment of 1,3-propanediol with NaH in THF for 16 h followed by addition of TBDPSCI yielded alcohol **8** in 84% yield²⁸⁹. Subsequent Swern oxidation afforded aldehyde **9** in good (90%) yield²⁸⁹. Both steps proceeded smoothly using literature conditions without modification.

5.2.2 STEP 1 Evans aldol reaction utilising chiral auxiliary

Having prepared aldehyde **9**, the aldol condensation with acetyl-4-benzyl oxazolidine could be attempted. Literature conditions³⁵² did not prove successful with no conversion being observed, and both TLC and LC-MS showing only starting materials. Various modifications to the procedure were attempted, as shown in **Table 5.1**, including alternative bases (e.g. *N,N*-diisopropylethylamine), also without signs of successful conversion. To exclude the possibility that solvent-dissolved oxygen was preventing reaction, it was decided to repeat the experiment using degassed dichloromethane. The solvent was repeatedly degassed using Schlenk-line techniques immediately before use, and under these conditions, conversion was then observed. While the need for rigorously air-free conditions has not been so far reported in the literature, it is likely that the oxygen sensitive nature of dibutylboron triflate, prevents the formation of the boron enolate in the presence of air.³⁵³

However, following the use of the reported temperature conditions (-78°C (20 min) then 0°C (3 h)), it was found that the predominant product formed was that corresponding to self-condensation of the oxazolidine ketone (**Scheme 5.3**), which was confirmed by NMR following purification. In order to prevent this side-reaction and promote the desired aldol reaction



Scheme 5.3 Aldol reaction in STEP 1

The aldol reaction towards **2** and **3**, as well as the side product observed, are indicated

between the oxazolidine and protected aldehyde, the reaction was carried out at -78°C for 5 hours. Under these conditions, the reaction proceeded in an overall yield of 84%.

	Base	Solvent	Conditions	Yield (2 + 3) %
1	$\text{Et}_3\text{N} \geq 99\%$	CH_2Cl_2	-78°C (2 hr) then 0°C (2 hr)	NC
2	$\text{Et}_3\text{N} \geq 99\%$	$\text{CH}_2\text{Cl}_2^{\#}$	-78°C (2 hr) then 0°C (2 hr)	NC
3	DIPEA	$\text{CH}_2\text{Cl}_2^{\#}$	-78°C (2 hr) then 0°C (2 hr)	NC
4	$\text{Et}_3\text{N}^{\#}$	$\text{CH}_2\text{Cl}_2^{\#}$	-78°C (2 hr) then 0°C (2 hr)	NC
5	$\text{Et}_3\text{N}^{\#}$	$\text{CH}_2\text{Cl}_2^{\#}$	-78°C (2 hr) then 0°C (2 hr) then RT (1 hr)	NC
6	$\text{Et}_3\text{N}^{\#}$	$\text{CH}_2\text{Cl}_2^{\#}$	0°C (4 hr)	NC
7	$\text{Et}_3\text{N}^{\#}$	$\text{CH}_2\text{Cl}_2^{\#}$	-78°C (20 min) then 0°C (3 hr)	12
8	$\text{Et}_3\text{N}^{\#}$	CH_2Cl_2^*	-78°C (20 min) then 0°C (3 hr)	Side product
9	$\text{Et}_3\text{N}^{\#}$	CH_2Cl_2^*	-78°C (20 min) then 0°C (3 hr)	Side product
10	$\text{Et}_3\text{N}^{\#}$	CH_2Cl_2^*	-78°C (5 hr)	84

Table 5.1 Conditions investigated for STEP 1

NC = no conversion; $^{\#}$ = anhydrous; * = degassed; the side product is as indicated in **Scheme 5.3**

Acetyl-4-benzyl oxazolidine is known to afford poor selectivity in aldol reactions, with the methyl ketone group being less able to sterically promote the favoured (Z)-transition state

that results in the desired syn-aldol product.³⁵⁴ It was found that, the desired diastereomer was formed in a ratio of 1.7 : 1 relative to the undesired diastereomer, consistent with literature reports (of 1.65 : 1)^{352,354}. Despite the relatively low selectivity of this reaction when compared to other methods, the diastereomers were readily separable by flash column chromatography on silica gel (**Figure 5.5**) providing access to both with relative ease. From the acetyl-4-benzyl oxazolidine starting material, alcohol **2** was prepared in 53% yield and dr > 95:5 following purification. Both diastereomers were found to be stable for long periods at room-temperature.

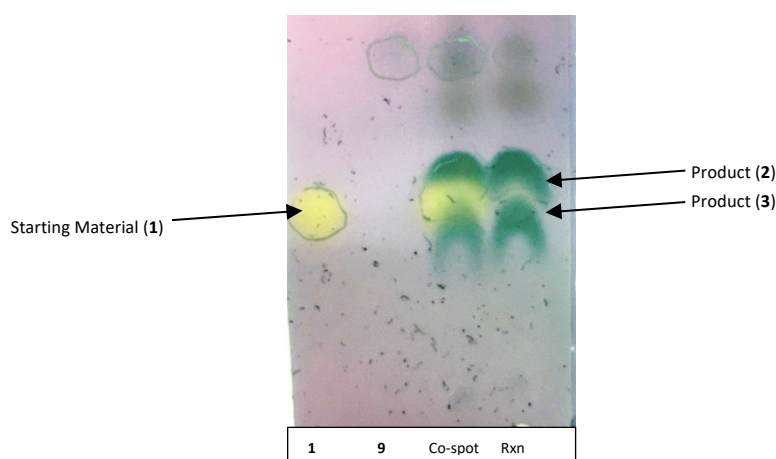


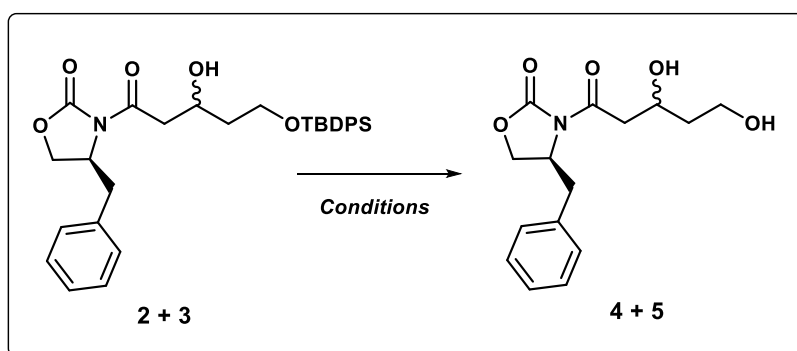
Figure 5.5 Aldol diastereomers are separable by chromatography

A thin-layer chromatogram showing separation of diastereomers **2** and **3**. The TLC plate was run in 50% EtOAc in hexane, and thermally developed following immersion in *p*-anisaldehyde. Note the disappearance of the starting oxazolidine can also be followed.

5.2.3 STEP 2 Silyl deprotection

With compounds **2** and **3** in hand, the next steps involved removing the silyl TBDPS protecting group and the chiral auxiliary. Silyl deprotection was attempted first (**Scheme 5.4**), as it was envisaged that the free terminal hydroxyl group could promote removal of the Evans auxiliary in an intramolecular fashion. Literature conditions called for the use for HF in pyridine ('Olah's reagent')³⁵², however due to the safety concerns in using HF, alternative and milder conditions were attempted first. The most common reagent for TBDPS-deprotection is tetra-*N*-butylammonium fluoride (TBAF), which provides a nucleophilic source of fluoride.³⁵⁵ However,

when either diastereomer **2** or **3** was treated with TBAF, a complex mixture of products was formed, none of which could be identified as the desired alcohols **4** and/or **5**. Further analysis revealed that these conditions promoted a side-reaction caused by deprotonation of the free hydroxyl group and subsequent intramolecular attack on the oxazolidine carbonyl group in a 6-exo-trig fashion (**Figure 5.6**). Both protected and unprotected products were observed, but the desired product was not. Although the basic character of TBAF has been previously reported³⁵⁶, this has been the reagent of choice of silyl-deprotection, even on complex substrates.³⁵⁷ This rearrangement was still observed even with the addition of water, which is reported to attenuate the basic character of the nucleophile.³⁵⁸



Scheme 5.4 Deprotection of 2 + 3 in STEP 2 to produce 3 + 4

	Reagent	Solvent	Conditions	Yield %
1	TBAF (5 eq)	THF	RT, 1 hr	Side reaction
2	TBAF (1 eq)	THF	RT, 1hr	Side reaction
3	TASF (5 eq)	THF	RT, 1 hr	Side reaction
4	HF/pyr (5 eq)	THF	RT, 1hr	15
5	HF/pyr (20 eq)	THF	0°C, then RT for 5 hr	65

Table 5.2 Conditions investigated for STEP 2, silyl deprotection

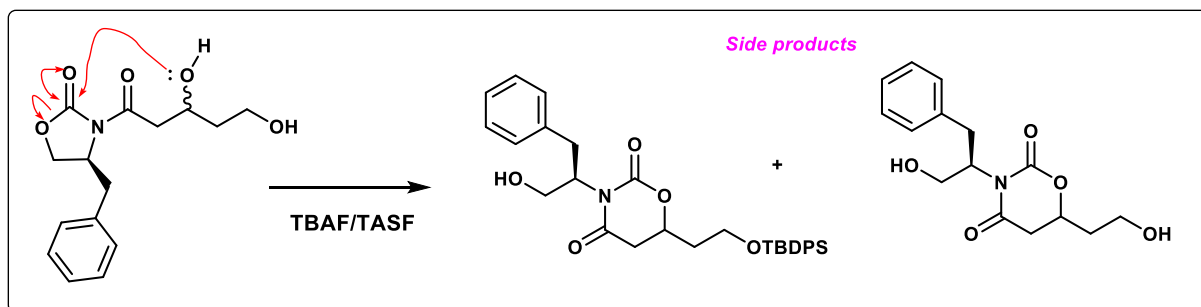


Figure 5.6 Base catalysed side-reaction promoted by treatment of **2** and **3** with TBAF.

Treatment of **2** and **3** with TBAF or TASF resulted in an undesired intramolecular nucleophilic attack of the oxazolidine carbonyl.

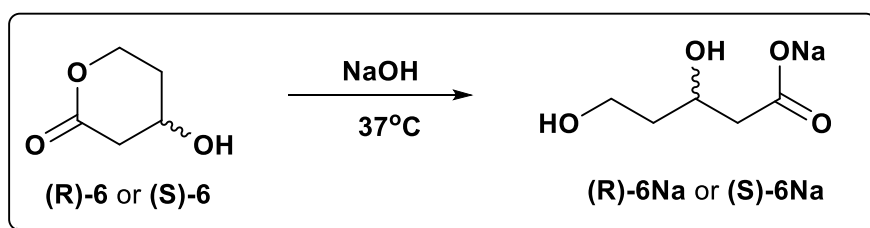
With TBAF having proved unsuccessful, we also investigated an anhydrous source of fluoride, in the form of Tris(dimethylamino)sulfonium difluorotrimethylsilicate (TASF). Due to its anhydrous nature, this reagent is also markedly less basic than TBAF and rearrangements are less common³⁵⁹. However, in spite of this the same side reaction was observed (**Figure 5.6**). A summary of the conditions attempted for silyl deprotection is shown in **Table 5.2**. With milder reagents failing to provide the necessary solution, we attempted the conditions using HF/Pyr as reported by Loubinoux *et al.*³⁵² In this case, conversion to the desired products was now observed with a single product being formed, albeit in low yields (approx 15%). However, an excess of HF/Pyr (5 eq) and longer reaction times (5 h) afforded **4** and **5** in 65% and 59% yield respectively (**Scheme 5.4**).

5.2.4 STEP 3 Removal of chiral auxiliary

The final step of the synthesis involved the removal of the Evans oxazolidine auxiliary. Following treatment of **4** and **5** with triethylamine at room temperature lead to cleavage of the auxiliary. Flash column chromatography and repeated co-evaporation with toluene afforded the target lactones (**R**)-**6** and (**S**)-**6** in 51% and 49% yield, respectively and high enantioselectivity for both was observed (*e.e* >95%). The absolute configuration of both (**R**)-**6** and (**S**)-**6** were unambiguously determined previously by X-ray crystallography.³⁵² Therefore, the *e.e* of both prepared (**R**)-**6** and (**S**)-**6** was calculated by integration of the H-C₃ signal in the

^1H NMR spectrum (at approx. 2.8 ppm in both) using a chiral shift method, and comparison to the literature. This peak was shifted to 2.4 ppm and 2.5 ppm respectively after addition of several drops of the chiral shift reagent (+)trifluoroanthranyl alcohol (TAE) to a NMR tube containing **(R)**-6 and **(S)**-6 in CDCl_3 . These values were consistent with those previously reported.³⁵²

Both **(R)**-6 and **(S)**-6 lactones were stable for long periods at -80°C , and their respective sodium salts **(R)**-6Na and **(S)**-6Na, formed by preparing stock solutions of the lactones in water, increasing the pH to 7.4 with NaOH, and overnight incubation at 37°C (Scheme 5.5). The conversion was confirmed by LC-MS.



Scheme 5.5 Base-mediated ring-opening of **(R)-6 or **(S)**-6**

Both **(R)**-6 and **(S)**-6 can be ring-opened in the presence of NaOH to form their sodium salt, **(R)**-6Na and **(S)**-6Na respectively.

5.2.5 Statin pharmacophore does not activate RyR1

Having successfully synthesised the statin minimal pharmacophore we could then examine its effect on RyR1 channels, to address our aim of establishing if this part of the statin molecule is responsible for statin induced RyR1 activation. This was assessed by examining the effect of **(R)-6** and **(S)-6** on [^3H]ryanodine binding at increasing concentrations in the presence of 10 μM Ca^{2+} buffer solution. Both the lactone and 'open' sodium salt forms were tested and the results are shown in **Figure 5.7**. The [^3H]ryanodine binding was carried out as described in Methods (**Chapter 2**) without modification. It was found that at all of concentrations tested from 100 nM to 1 mM, there was no significant increase or decrease in [^3H]ryanodine binding relative to control conditions. This was the case for all four compounds, **(R)-6** lactone and sodium salt **(R)-6Na**, and for **(S)-6** lactone and sodium salt **(S)-6Na** also. This is consistent with **(R)-6** and **(S)-6** having no effect on the probability of RyR1 channel opening.

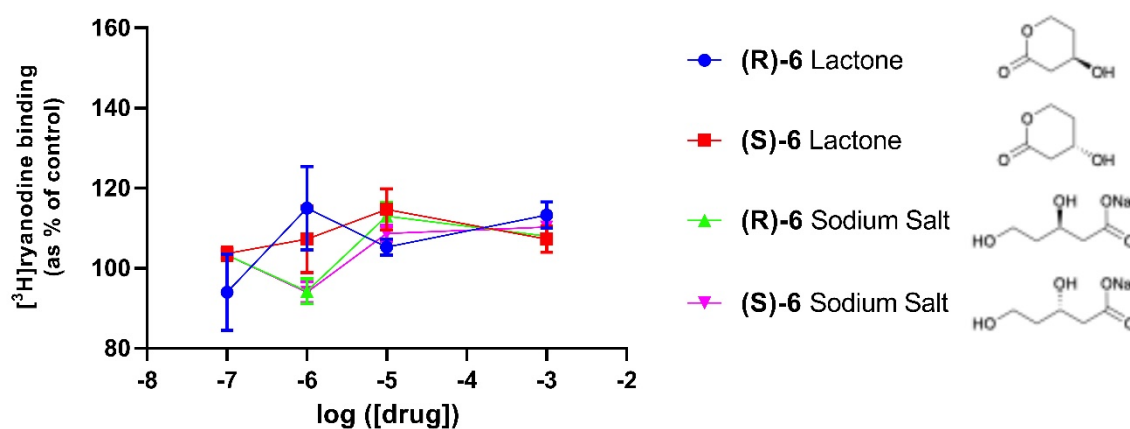


Figure 5.7 Relative ability of (R)-6 and (S)-6 to stimulate [^3H]ryanodine binding to skeletal SR vesicles

Stimulation of [^3H]ryanodine binding to sheep skeletal mixed SR membrane vesicles by **(R)-6** and **(S)-6** in both their respective lactone and sodium salt forms as indicated, in the presence of 10 μM cytosolic Ca^{2+} . Mean $\pm$ SEM; n = 5.

5.2.6 Synthesis of an extended propyl statin pharmacophore

In parallel with the work described above, and to complement the results with the minimal pharmacophores (**R**)-**6** and (**S**)-**6**, the synthesis of a more extended statin pharmacophore **16a** was carried out by Dr Carole Bataille (CB). CB had the aim of investigating the effect of the addition of a propyl substituent on the 1-position and the effect of this extended statin pharmacophore on RyR1 activity, as this substituents mimics the connecting alkyl chain between the statin pharmacophore and pyrrole core of atorvastatin.³⁶⁰

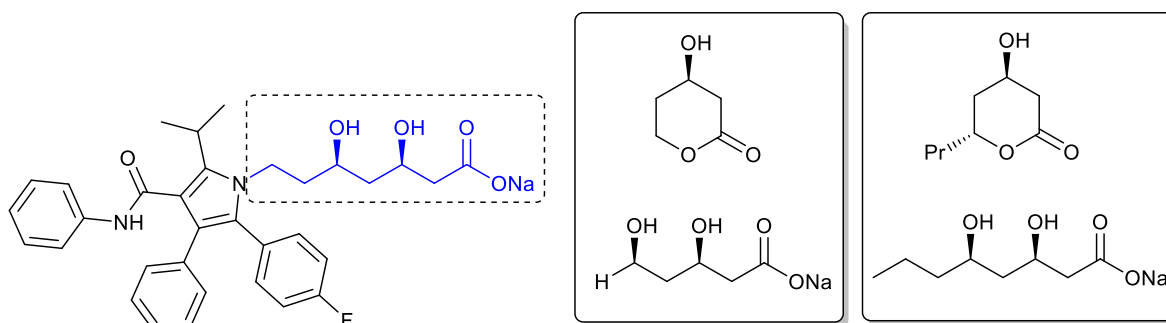
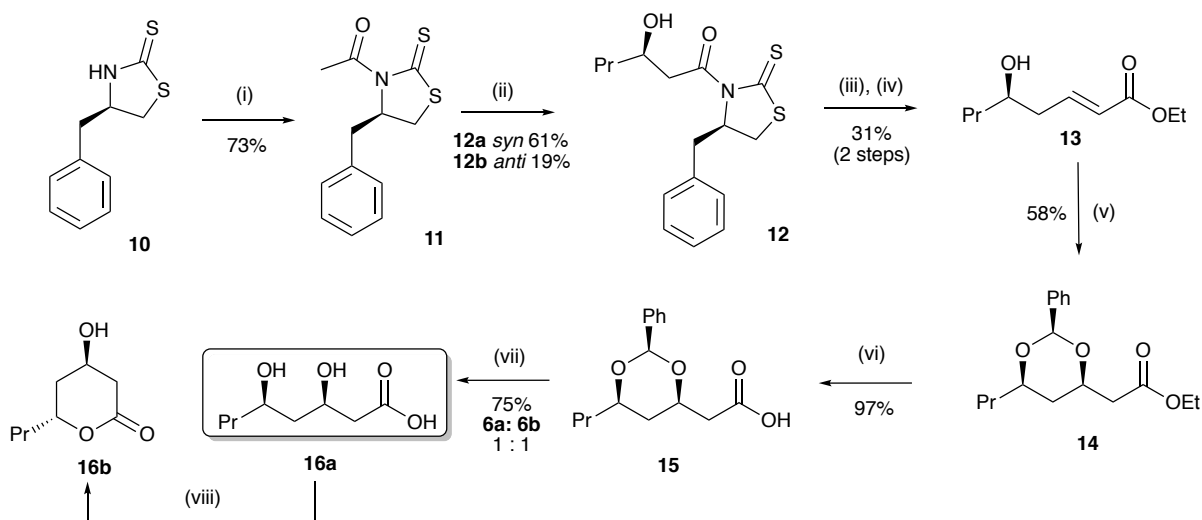


Figure 5.8 Comparison of the pharmacophore models synthesised in this chapter.

The figure shows a comparison between the minimal pharmacophore (left panel) and the extended (Pr) pharmacophore (right panel). Atorvastatin sodium salt is also shown for comparison, with the HMG-CoA reductase pharmacophore indicated in blue.

The synthetic route first reported by Venkatesham *et al.*³⁶¹ was followed with modification as described in detail by Lawrence³⁶⁰ (**Scheme 5.6**). The synthesis started with the acetylation of (*R*)-4-benzylthiazolidine-2-thione utilising *n*-BuLi and acetyl chloride, affording **11** in good (77%) yield. Subsequently a Crimmins modified Evans Aldol reaction^{362,363} between **11** and *n*-butyraldehyde gave access to **12** with 4:1 ratio of diastereomers. As in the previous case, flash column chromatography on silica gel lead to straightforward separation of **12a** and **12b**, yielding the desired *syn* (*R*, *R*)- β -hydroxyketone in good (61%) yield. The Crimmins modification was selected, due to the enhanced selectivity of this protocol on substrates such as **11** that lack an α -substituent, as compared to standard Evans Aldol methodology.³⁶⁴

Cleavage of the chiral auxiliary could be affected with DIBAL-H, leading to the corresponding aldehyde which could be taken forwards directly via Wittig reaction³⁶⁵ with (carbethoxymethylene) triphenylphosphorane to give **13** in 31% yield over two steps. Treatment with *t*-BuOK and benzaldehyde promoted an intramolecular Michael reaction, affording **14** (58% yield) which underwent ester hydrolysis with NaOH in excellent yield (97%). Standard hydrogenation conditions of H₂ on Pd/C gave the final syn-dihydroxy acid **16a** in 75% yield. For characterisation purposes, **16a** could be converted to lactone **16b** by treatment with HCl in 1,4-dioxane, which had increased solubility in organic solvent.



Scheme 5.6 Synthesis of the extended statin pharmacophore (performed by HL) (**16a**)

Reagents and conditions: (i) *n*-BuLi (1.2 eq., 1.6 M in toluene), acetyl chloride (2.4 eq.), THF, 2.5 h, 0 °C; (ii) TiCl₄ (1.1 eq., 1M in toluene), DIPEA (1.1 eq.), then *n*-butyraldehyde (2 eq.), CH₂Cl₂, 3.5 h, -78 °C; (iii) DIBAL-H (2.5 eq., 1M in toluene), CH₂Cl₂, -78 °C, 2 h then (iv) Ph₃PCHCO₂Et (1.5 eq.), CH₂Cl₂, RT, 16 h; (v) PhCHO (1.1 eq.), *t*-BuOK (1.1 eq.), RT, 2 h; (vi) NaOH (aq. sol., 1M), THF:H₂O (3:1), r.t, 20 h; (vii) H₂, Pd/C (10 mol%), THF:MeOH (4:1), RT, 12 h; (viii) HCl (4M in 1,4-dioxane), 1,4-dioxane, RT, 14 h. Data collected and synthesis performed by Dr Carole Bataille.

5.2.7 Effect of the extended statin pharmacophore on RyR1 channel activity

The extended pharmacophore was then also examined for its effect on RyR1 channel activity, under analogous conditions. [^3H]ryanodine binding was again used as described in General Methods (**Chapter 2**) without modification. The lactone **16b** was tested, as well as a 1:1 mixture of both the acid and lactone forms **16a** and **16b**. The results are shown in Figure 5.9. Lactone **16b** appeared to produce complex effects on the ability of RyR1 to bind [^3H]ryanodine (**Figure 5.9(A)**). At low concentrations (1 μM), the lactone caused a significant increase in [^3H]ryanodine binding relative to control conditions (to approximately 150%). However higher concentrations reversed this effect, with 10 μM **16b** producing a binding level comparable to control. At very high, supraphysiological concentrations (1 mM), **16b** instead promoted a significant decrease in [^3H]ryanodine binding to approximately 50% of control.

In comparison, these effects were altered when a 1:1 mixture of **16a** and **16b** was tested (**Figure 5.9(B)**). In this case, at 1 μM , the mixture appeared to have no effect on [^3H]ryanodine binding. At 10 μM there was a small (non-significant) trend towards increasing binding, and at 1 mM a trend towards a decrease in [^3H]ryanodine binding relative to control was observed. Given these experiments are presented as the mean data of 5 independent experiments further experiments would be required to draw a more definitive conclusion about the trends, and to establish their significance (or not).

In both cases above, the known RyR1 channel activator caffeine was used as a positive control to confirm that a poor SR preparation was not the reason for a negative result. In both cases, caffeine caused a significant increase in [^3H]ryanodine binding as expected.^{146,148}

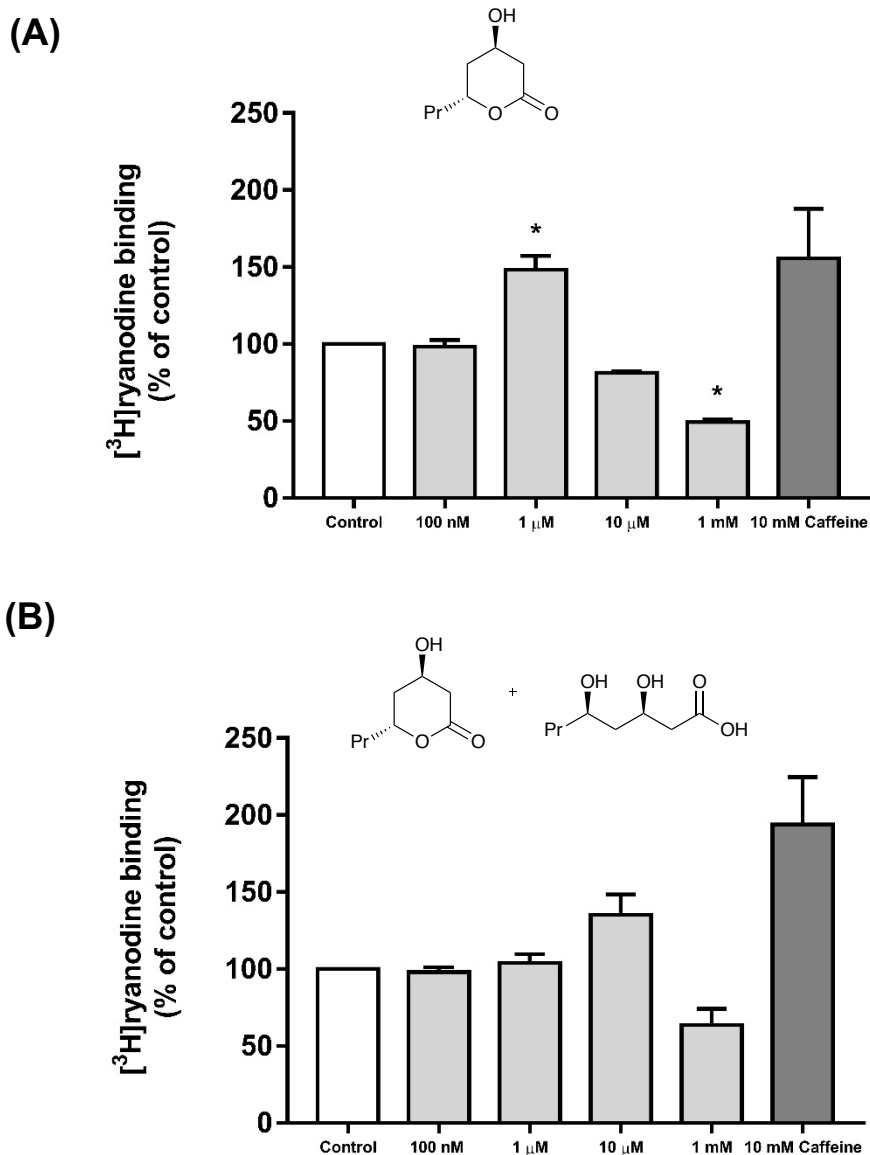


Figure 5.9 Ability of the extended statin pharmacophore (16a**) and (**16b**) to stimulate [³H]ryanodine binding to skeletal SR vesicles.**

Stimulation of [³H]ryanodine binding to sheep skeletal mixed SR membrane vesicles by (A) lactone **16b**, and (B) A 1:1 mixture of lactone **16b** and hydroxy acid **16a**, in the presence of 10 μM cytosolic Ca²⁺, presented as % of control conditions. 10 mM caffeine is also shown as a positive control for comparison. Mean ± SEM; **P* < 0.05 relative to control conditions; n = 5.

5.3 Discussion

We have seen in this chapter a description of the synthesis of both a minimal and extended (propyl derivative) statin pharmacophore, and their effect on RyR1 activity. As we saw in Chapter 3, both simvastatin sodium salt, and simvastatin lactone are capable of activating RyR1. This finding suggested that the statin pharmacophore is therefore not (at least solely) responsible for the RyR1 activation seen with statins. Following the synthesis and testing of that pharmacophore, we now confirm this is the case.

A key difference between HMG-CoA itself and both the pharmacophores described in this chapter are that in addition to the hydroxyglutaric acid unit, HMG-CoA contains an adenine-based unit and linker to a total molecular weight of 911.6 g mol^{-1} (the full structure is shown in **Figure 5.10**).

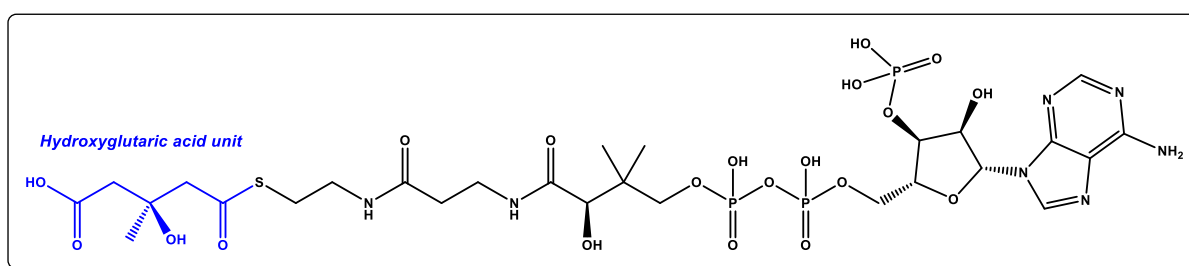


Figure 5.10 Full structure of HMG-CoA

The full structure of HMG-CoA, with the hydroxyglutaric acid unit indicated in blue.

A direct comparison between HMG-CoA and the pharmacophore synthesised in this chapter must therefore be made with care. This raises the question, *what is a relevant concentration of the model pharmacophore to investigate?* For comparison, the IC_{50} of the HMG-CoA subunit itself against HMG-CoA reductase is approximately $4 \mu\text{M}$ ¹⁸, the K_M is $1 \mu\text{M}$ ³⁶⁶ and the IC_{50} of statins against HMG-CoA are in the nanomolar range.³⁶⁷ We found in Chapter 3 that the EC_{50} of the statins investigated was in the low micromolar range. Therefore we would expect that if the statin pharmacophore is responsible for the effect of statins on RyR1, that effect should

be seen at a micromolar range. Therefore concentrations in this range up to 1 mM were investigated.

It was found that the minimal statin pharmacophore had no effect on RyR1 activity (**Figure 5.7**) with either the lactone or acid form of the pharmacophore causing a deviation of [³H]ryanodine binding from control levels at the concentrations investigated. However, the addition of a propyl-group to the 1-position in the case of the extended pharmacophore caused the lactone form of this molecule (**16b**) to activate RyR1 at 1 μ M. The effect of **16b** was complex, with no effect at 100 nM, activation at 1 μ M and inhibition at 1 mM. Further experiments may be required, at the single-channel level to confirm that this effect is replicated under those conditions.

Figure 5.9(B) shows the effect of a mixture of the extended pharmacophore in acid (**16a**) and lactone (**16b**) form. It was not possible to separate these forms effectively as the interconversion between them was rapid. However, the mixture of both has clinical relevance as it is well established that the statin pharmacophore readily undergoes interconversion *in vivo* and therefore both the acid and lactone forms will be present in different quantities in a clinical setting.^{298,368,369} The interconversion displays a higher degree of pH dependence, and in skeletal muscle both forms of statin can be found.³⁷⁰ The mixture did not appear to affect RyR1 channel activity (**Figure 5.9(B)**) and in fact it appeared to inhibit RyR1 at higher concentrations (1 mM). However given that the full statin molecules in both forms can activate RyR1, we would expect a mixture of both of these to also activate, were the statin pharmacophore the functional group responsible for binding to and/or activating RyR1. However, neither the lactone, nor mixture of lactone and acid form of the pharmacophore activated RyR1 at low concentration (100 nM).

From this we can conclude that it is highly likely that the statin pharmacophore is not the principal functional group driving interaction with RyR1, thus demonstrating that the undesired agonistic action of statins on RyR1 is separable from their desired inhibition of their target enzyme HMG-CoA. We can therefore imagine the possibility that further medicinal chemistry efforts can lead to the design of a molecule capable of lowering plasma cholesterol, but without causing RyR1 activation. Such a compound would also provide further clarification of the role of RyR1 in statin-induced myopathy.

6

Identification of the RyR1 statin binding site

6.1 Introduction

Given the finding that simvastatin and atorvastatin, can both activate RyR1, it will be essential to determine which structural features of the statin drugs are crucial for their binding to RyR1, and whether modifications to the structure can remove this effect and hence the extent of the involvement of RyR1 in statin myopathy determined. Having established in the preceding chapter that the statin HMG-CoA reductase pharmacophore is not essential, the remaining question is which functional groups are. In order to approach this in a rational manner, some knowledge or prediction of the likely interactions is helpful to guide the development of a structure-activity relationship. The particular focus of this question was driven by the fact that it was this interaction that is thought to be implicated in driving statin side effects. Therefore an understanding of the changes that could be made to the molecular architecture of statins that would disrupt this interaction is of great interest. With the publication of full cryo-EM structures for RyR1²⁵⁸ (and its sister isoform RyR2²⁶³), we now have access to the basic tools which allow us to probe whether statins may share the same binding sites as the well-established regulators of RyR. Of the ligands which have been identified as RyR1 modulators, only the binding sites of Ca²⁺, ATP, caffeine and ryanodine have been determined with near certainty by cryo-EM¹⁷¹. Putative residues for the binding of dantrolene have however also been identified.³⁷¹

6.1.1 Ligand binding sites of RyR1

The three ligands for which the RyR1 binding sites have been identified provide a starting point for considering the possible location of the statin binding sites, particularly as all three of these ligands are activators of RyR1 wherefore we can conclude that binding at these sites is associated with channel activation. These sites, as reported by des George *et al.* (2016)¹⁷¹ are shown in **Figure 6.1**. Ryanodine has been suggested to block the conduction pathway, and since its behaviour is markedly different to that observed with statins, I did not consider this to be a likely binding site to be shared by statin molecules. Hence the ryanodine binding site

was not considered further. The Ca^{2+} , caffeine and ATP binding sites were however considered in detail as potential locations for statin binding.

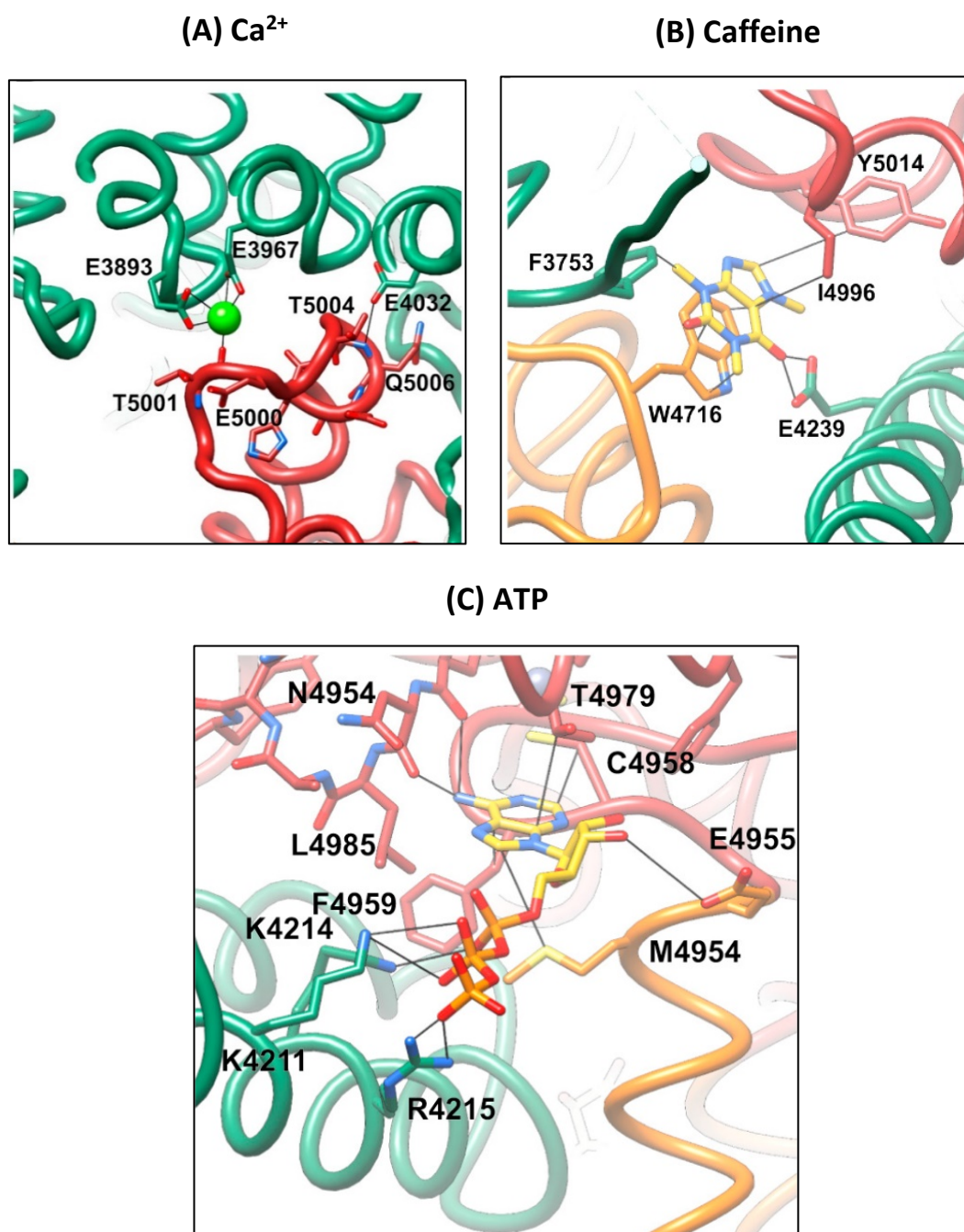


Figure 6.1 The RyR1 Ca^{2+} , caffeine and ATP binding sites as reported by des George *et al.* (2016).

The RyR1 (A) Ca^{2+} , (B) caffeine and (C) ATP binding site are shown in ribbon representation, with the relevant molecule bound and the likely interacting residues indicated in stick representation. Thin black lines indicate the likely RyR-ligand interactions. This figure was adapted from des George *et al.* (2016).

The Ca^{2+} -binding site was identified by comparing the cryo-EM structures determined in 'Ca²⁺-free' conditions versus in the presence of 30 μM Ca^{2+} . The site is located at the interface between the C-terminal domain (CTD) and core solenoid (Csol) with three key residues, E3893, E3967 and E5000 interacting directly with the bound Ca^{2+} ion.¹³⁴ Additionally two further residues (Q3970 and H3895, not included in **Figure 6.1**) are thought to interact with it indirectly, and all five residues are conserved between all RyR isoforms (**Figure 6.1(A)**).¹⁷¹ The Ca^{2+} binding site appears smaller than the coordination shell of the Ca^{2+} ion (to prevent coordination of water inhibiting the binding of the ion). It is therefore unlikely that a small molecule of the size of a statin molecule (approx. 400 Da) could bind in this site would create significant steric interactions with surrounding residues. It is therefore unlikely that the Ca^{2+} binding site could be shared by statins.

The caffeine binding site is located at the junction between the CTD and S2S3 domain, adjacent to the Ca^{2+} binding site. Several hydrophobic residues appear to be involved, namely E4239, I4996 and W4716 (**Figure 6.1(B)**). However, the resolution of the des George cryo-EM structure, coupled with the symmetrical electron distribution of the caffeine molecule makes it unclear which orientation caffeine adopts. It has been suggested that the close proximity of the caffeine and Ca^{2+} binding sites is the reason for the Ca^{2+} sensitivity of RyR1 caffeine activation, indeed, a mutation to the closely associated tryptophan residue (W4645R) in RyR2 has been shown to reduce the Ca^{2+} sensitivity of caffeine activation.^{171,372} The caffeine binding site is comparatively more spacious than the Ca^{2+} binding site, and the xanthine ring upon which caffeine is based bears similarity to decalin and pyrrole core of simvastatin and atorvastatin. However, the caffeine site appears to lack positively charged residues, which would be required to adequately bind the dihydroxyheptanoic acid unit of the statin molecule.

The final of the three sites is the ATP, or adenine nucleotide binding site. It is found between the CTD and S6c domain, and forms a hydrophobic pocket or cleft. The edge of this pocket is

lined by positively charged lysine and arginine residues (K4214, K4211, R4215). The adenine base of the ATP appears to be buried in this pocket, aligned to interact with the M4954, F4959, T4979 and L4975 residues.¹⁷¹ The phosphate tail of the ATP molecule is then positioned to interact with the positively charged residues lining the pocket, and the E4955 residue appears to interact with the ribose ring of the ATP molecule (**Figure 6.2**).¹⁷¹ A ligand interaction diagram for ATP binding in this site is shown in **Figure 6.2(B)** with these protein-ligand interactions highlighted. des George *et al.* found that characteristic changes to the channel conformation occur upon the binding of either Ca^{2+} , ATP or caffeine, which allow the channel to become 'primed' for activation. This primed state can then be driven to the open state by the binding of a second ligand. The binding of ATP in this site is therefore consistent with experimental observations that the RyR1 activation of ATP is Ca^{2+} dependent.

6.1.2 Do statins bind to the RyR1 ATP binding site?

In **Chapter 3**, it was noted that Knoblauch *et al.* (2013) had reported that simvastatin could trigger SR Ca^{2+} release in skeletal muscle fibres derived from both WT and MH mutant mice. They also found that this Ca^{2+} -release trigger by simvastatin could be inhibited by prior addition of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR, **Figure 6.2(C)**), but 4cmc activation was not similarly affected.³⁰¹ This they attributed to AICAR preventing the production of reactive oxygen species, and therefore the prevention of redox modification of RyR.³⁷³ However, given the structural similarity between AICAR and the RyR1 agonist ATP (the structure of AICAR is shown in **Figure 6.2(C)**), it is likely that AICAR could bind at the ATP sites. Indeed, AICAR also shares similarities with adenosine, which is known to compete with ATP for a common binding site on RyR.³⁷⁴ Therefore since pretreatment with AICAR appears to reduce the ability of simvastatin to activate RyR1, one may speculate that both AICAR and simvastatin bind to a common binding site. Therefore I concluded that this warranted further investigation.

Of these three ligand binding sites, the RyR1 ATP site would appear to be the most plausible for binding of statins. The ATP molecule shares several similarities with statin molecules, most notably both families of molecules have a hydrophilic portion (the phosphate tail in ATP, and the dihydroxyheptanoic acid unit of statins) and a hydrophobic portion (adenine ring vs pyrrole or decalin core) (**Figure 6.3**). This is also suggested by the use of the commercial software package Forge³⁷⁵ (Cresset, UK) which indicated a similarity between ATP and atorvastatin of 48%. Additionally, both are small molecules of approximately the same molecular weight (ATP: 507.18 g mol⁻¹, Ator-H: 558.64 g mol⁻¹, Sim-H: 458.6 g mol⁻¹).

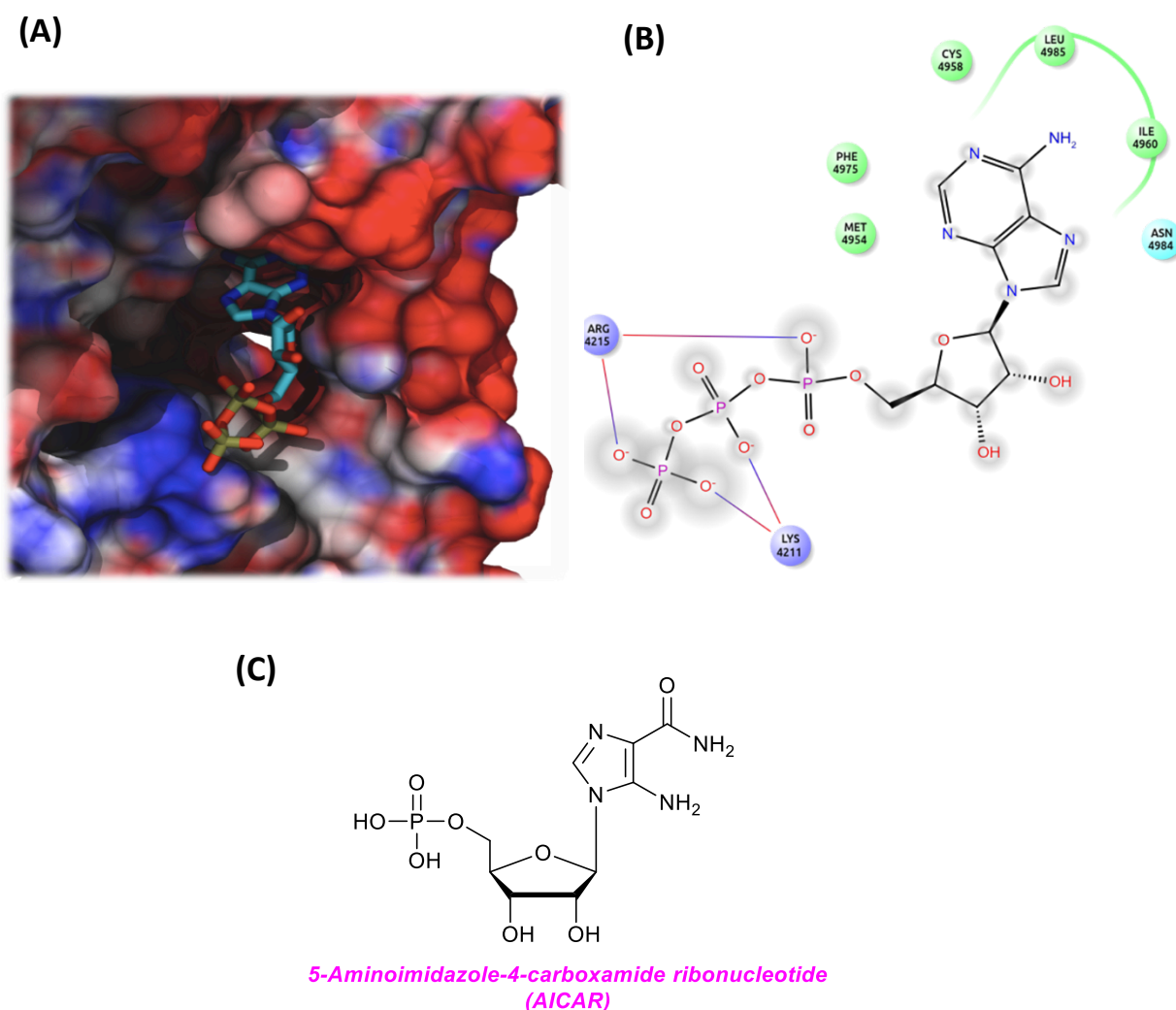


Figure 6.2 The RyR1 ATP site

The RyR1 ATP site is best described as a hydrophobic pocket lined with positively charged residues. (A) The RyR1 (PDB-ID 5TAS) ATP site shown in surface representation with an ATP molecule bound. The surface is coloured by electrostatic potential, going from dark blue (most positive), through white (neutral) to red (negative.). It can be seen that the ATP site consists of a hydrophobic pocket which binds the uncharged adenine portion of the ATP molecule, lined with a positively charged region which coordinates the negatively charged phosphate groups. (B) A ligand interaction diagram prepared in Maestro, indicating the residues thought to be critical for the binding of ATP. The phosphate residues interact primarily with the Arg 4215 and Lys 4211 residues which bear a positive charge, while hydrophobic residues such as Ile 4960 and Leu 4985 cradle the adenine core of the ATP molecule. (C) The structure of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), the ATP analogue utilised by Knoblauch *et al.* (2013).

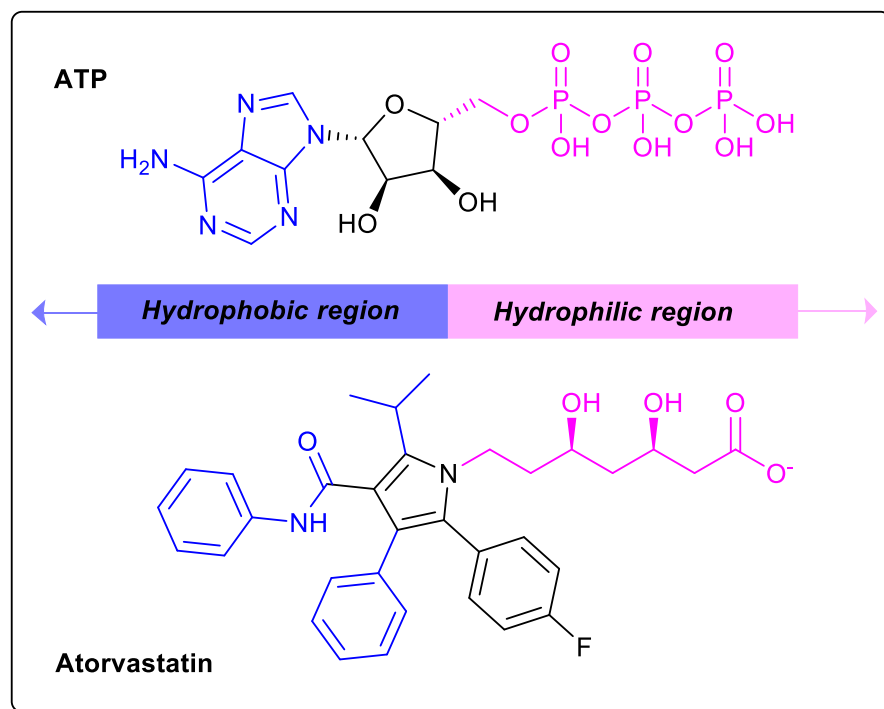


Figure 6.3 Similarity between ATP and Atorvastatin

The structures of ATP and atorvastatin are shown for comparison. The hydrophobic regions (more lipophilic) are shown in blue, while the hydrophilic (polar) regions are shown in pink.

Given the structural similarities between AICAR and ATP, and between ATP and statins, I hypothesised that the binding of ATP and statins to a shared binding site could be an explanation for why AICAR appears to inhibit statin induced Ca^{2+} -release. It is of course likely that statins may bind at none of these sites, and have their own unique binding site or share a binding site with some of the many other RyR regulators for which the binding sites are currently unknown. This is especially true given the size of RyRs, at greater than 2MDa and being made up of over 5000 individual amino acid residues per chain, the number of potential binding sites for small molecule will number many millions.¹³⁴ It is this challenge which makes more traditional cavity search algorithms aimed at computationally identifying likely binding sites both impractical and computationally intensive. Therefore I concluded that an appropriate preliminary approach was to identify whether statins may share a binding site with ligands already known.

6.1.3 Aims

In this chapter, I aimed to utilise both single-channel and [^3H]ryanodine binding experiments to further understand whether ATP may compete with statins for a common binding site. For this work I selected atorvastatin for experiments over simvastatin, for several reasons. The pyrrole core of atorvastatin makes this molecule more synthetically accessible than simvastatin, which is based on a 'natural-product-like' decalin core. In addition, atorvastatin is prescribed in the open hydroxyacid form rather than the lactone form in contrast to simvastatin.

With new access to cryo-EM maps of RyR1 with ATP bound, I then aimed to use these structures to inform a molecular modelling study with the Autodock docking software²⁹¹ to investigate which structural features of statins could be involved in their binding to the RyR1 ATP site.

Finally, a catalogue of atorvastatin analogues, from a variety of structural classes, were experimentally examined for their ability to activate RyR1. This would allow me to confirm the results predicted by the modelling approach, as well as to establish a structure-activity relationship for statin induced RyR1 activation. These findings will allow the involvement of RyR1 in statin-induced myopathy to be assessed, and could lead to the design of a molecule which retains HMG-CoA inhibition, without also causing undesirable SR Ca^{2+} -release.

6.2 Results

6.2.1 ATP activates RyR1

6.2.1.1 *ATP Activates RyR1 single-channels*

ATP is a well-established regulator of RyR1, however in order to investigate if statins modulate RyR1 P_o by binding at the ATP-binding site, it was necessary to examine the mechanisms by which ATP itself activates RyR1 at the single-channel level. A representative RyR1 channel activated by ATP is shown in **Figure 6.4** and the mean data for all experiments are shown in **Figure 6.5**.

It was found that ATP activates RyR1 in a concentration-dependent manner, as expected from previous reports.^{143,156,376} P_o was increased from 0.0836 ± 0.0374 (mean $\pm$ SEM; $n = 8$) in control conditions ($10 \mu\text{M Ca}^{2+}$), to a maximum of 0.613 ± 0.0805 (mean $\pm$ SEM; $n = 8$) in the presence of 2 mM ATP. The EC_{50} for activation was 129 μM , and the hill coefficient was 1.24 (**Figure 6.5**). The gating of these channels was rapid, with no periods of long openings readily observable. This type of rapid gating can be seen in **Figure 6.4** (third panel) where an RyR1 is activated with 1 mM ATP.

Upon washout of the highest concentration of ATP (5 mM), P_o was reduced to 0.071 ± 0.042 (mean $\pm$ SEM; $n = 4$), which being comparable to control P_o , indicates that the effect of ATP was fully reversible at this concentration. This is demonstrated in **Figure 6.4** as the first panel ($10 \mu\text{M Ca}^{2+}$, control conditions) is indistinguishable from the final panel (washout). This indicates that there is no evidence of irreversible phosphorylation under these experimental conditions, a concern noted by other researchers.³⁷⁷ These effects are also comparable with the single-channel activation of the cardiac RyR isoform, RyR2, by ATP previously reported by the Sitsapesan Lab.^{153,156,316}

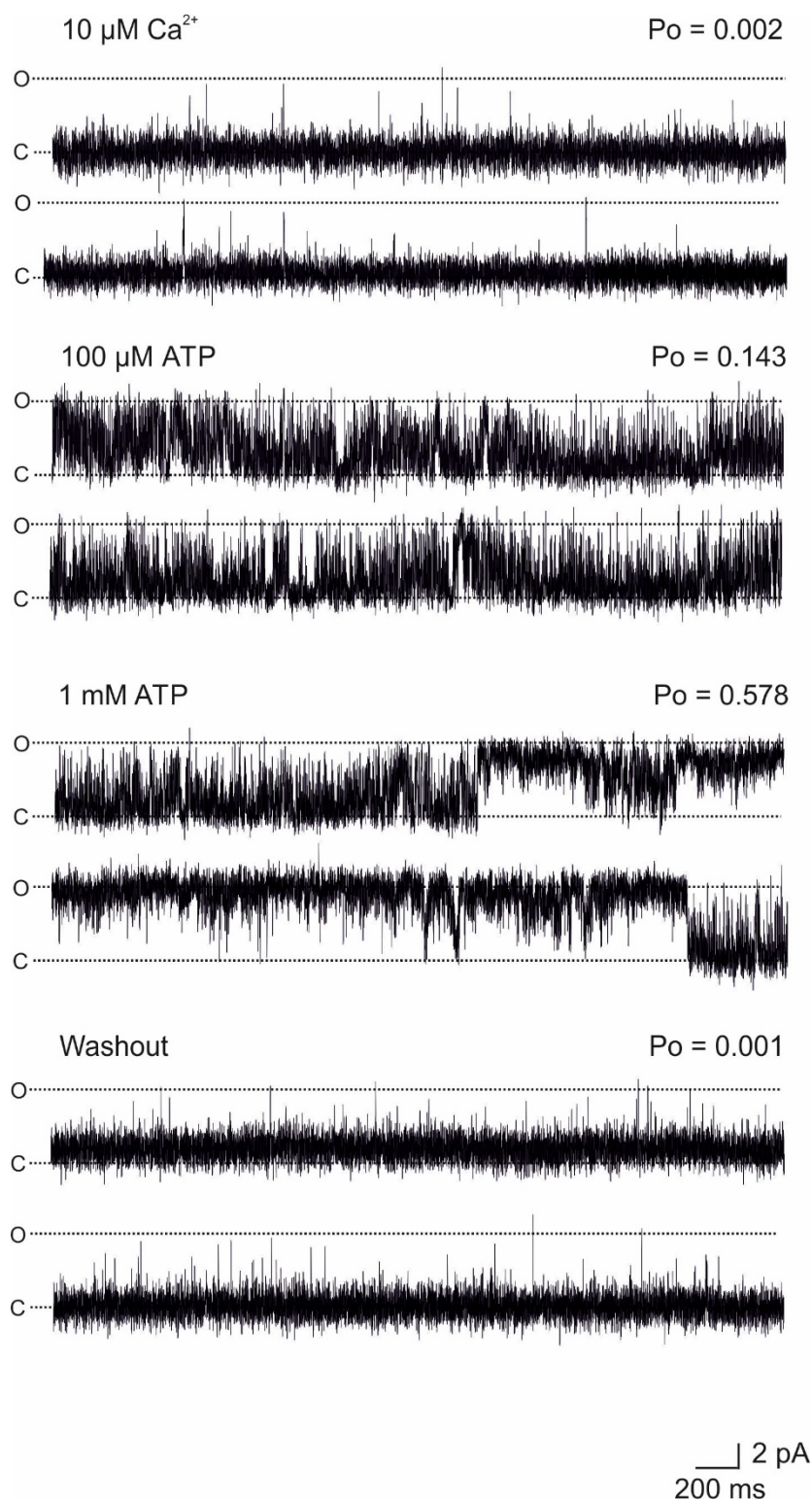


Figure 6.4 Effect of ATP on single RyR1 channel gating

Representative sheep skeletal RyR1 single-channel current fluctuations in the presence of $10\ \mu\text{M}\ \text{Ca}^{2+}$ alone (top traces), after addition of $100\ \mu\text{M}$ and $1\ \text{mM}$ ATP (second and third traces respectively) and after washout back to control conditions ($10\ \mu\text{M}\ \text{Ca}^{2+}$, bottom traces). The P_o above the traces refers to that calculated over three minutes of recording. O and C indicate open and closed channel levels respectively.

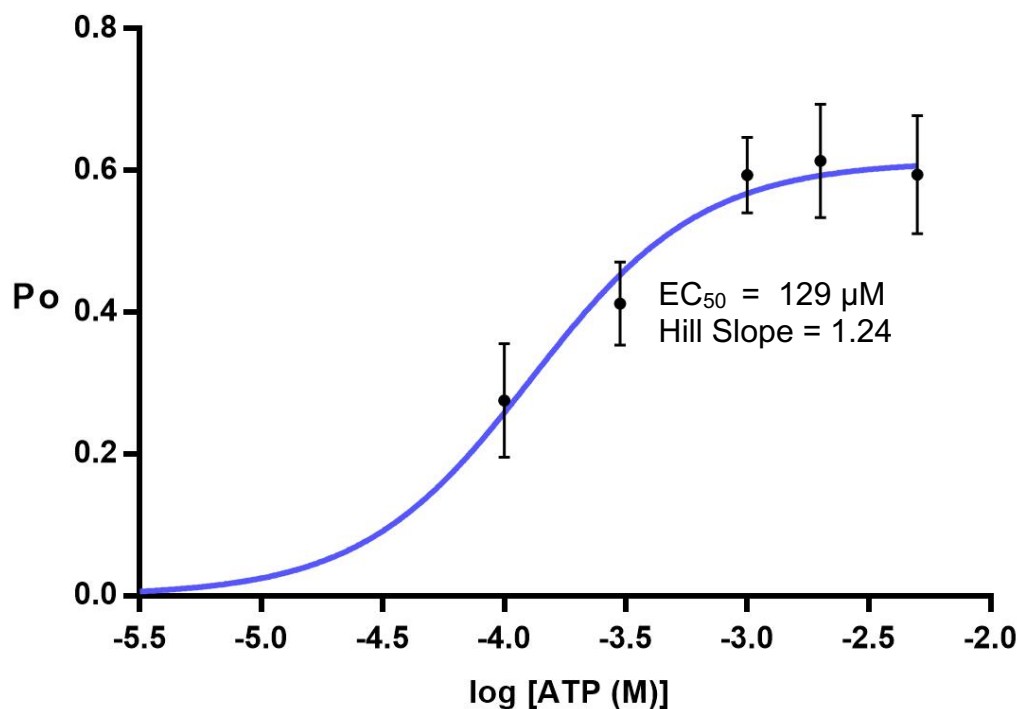


Figure 6.5 Mean effect of ATP on RyR1 Po

Concentration-response relationship for the effect of ATP on RyR1 Po, showing the effect of addition of cytosolic ATP to 100 μM , 300 μM , 1mM, 2 mM and 5 mM as indicated. Mean $\pm$ SEM is shown; $n = 4 - 8$.

6.2.1.2 ATP stimulates [3H]ryanodine binding to RyR1

The ability of ATP to stimulate [3H]ryanodine binding was also assessed, and the mean data are presented in **Figure 6.6**. The overall trend is the same as seen in the single-channel studies. ATP increased the ability of RyR1 to bind [3H]ryanodine indicating a tendency of ATP to promote RyR1 channel opening.

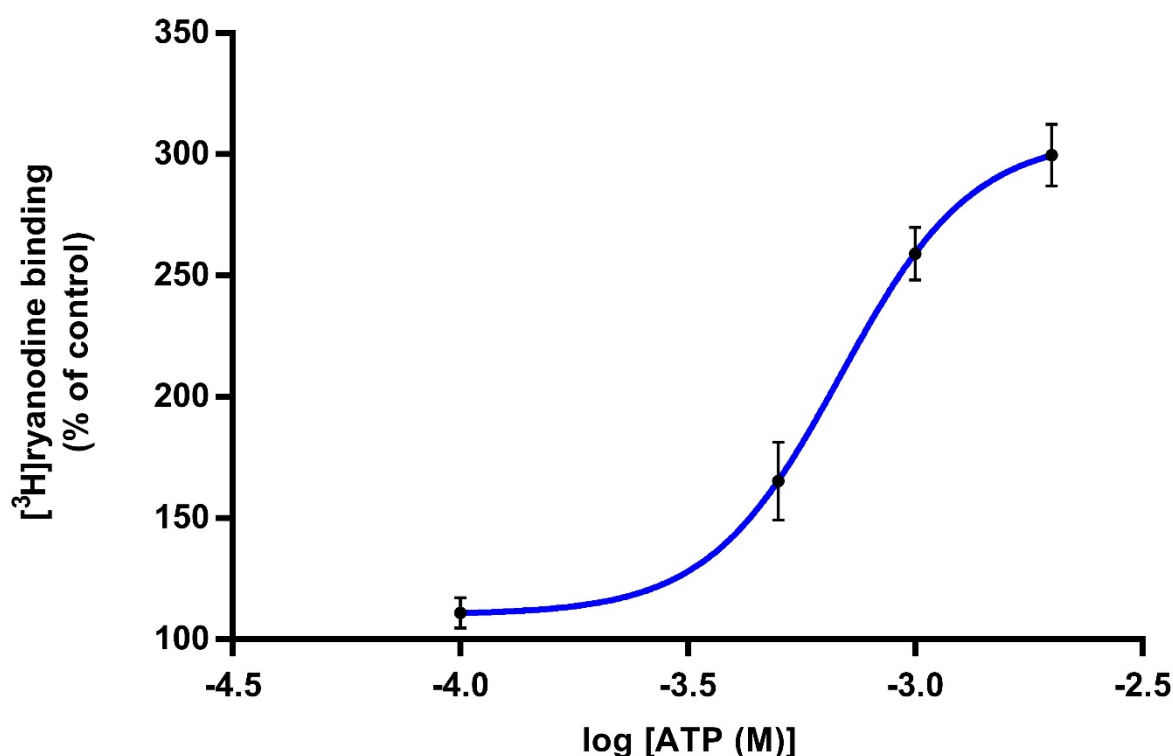


Figure 6.6 Effect of ATP on [³H]ryanodine binding

Concentration-response relationship for the stimulation of [³H]ryanodine binding to sheep skeletal HSR vesicles by ATP at the concentrations indicated. Mean ± SEM; n = 5,

6.2.2 ATP & Statin competition experiments

ATP was then added to the cytosolic side of a single RyR1 channel, in the presence of 100 nM Ator-H (and 10 μ M Ca^{2+}). A representative channel subjected to such treatment is shown in **Figure 6.7**, and mean data for a number of channels is reported in **Figure 6.8**. To enable comparison with the competition experiments in this chapter, the data collected for the effect of Ator-H on RyR1 in Chapter 3 is presented in **Figure 6.9**. It was observed that in the presence of 100 nM Ator-H, there was a small increase in channel P_o from 0.031 ± 0.020 (mean ± SEM; n = 10) to 0.056 ± 0.023 (mean ± SEM; n = 10). Following the addition of 300 μ M ATP, there was a further increase in P_o to 0.166 ± 0.082 (mean ± SEM; n = 5), which was reduced compared to the value obtained from exposure to 300 μ M ATP alone of 0.412 ± 0.059 (mean

$\pm$ SEM; $n = 5$). A further increase in ATP concentration did not increase channel openings further. The fact that Ator-H appears to dampen the ability of ATP to activate RyR1, is consistent with both ligands sharing a common binding site, since Ator-H behaves as a high affinity partial agonist as an activator of RyR1.

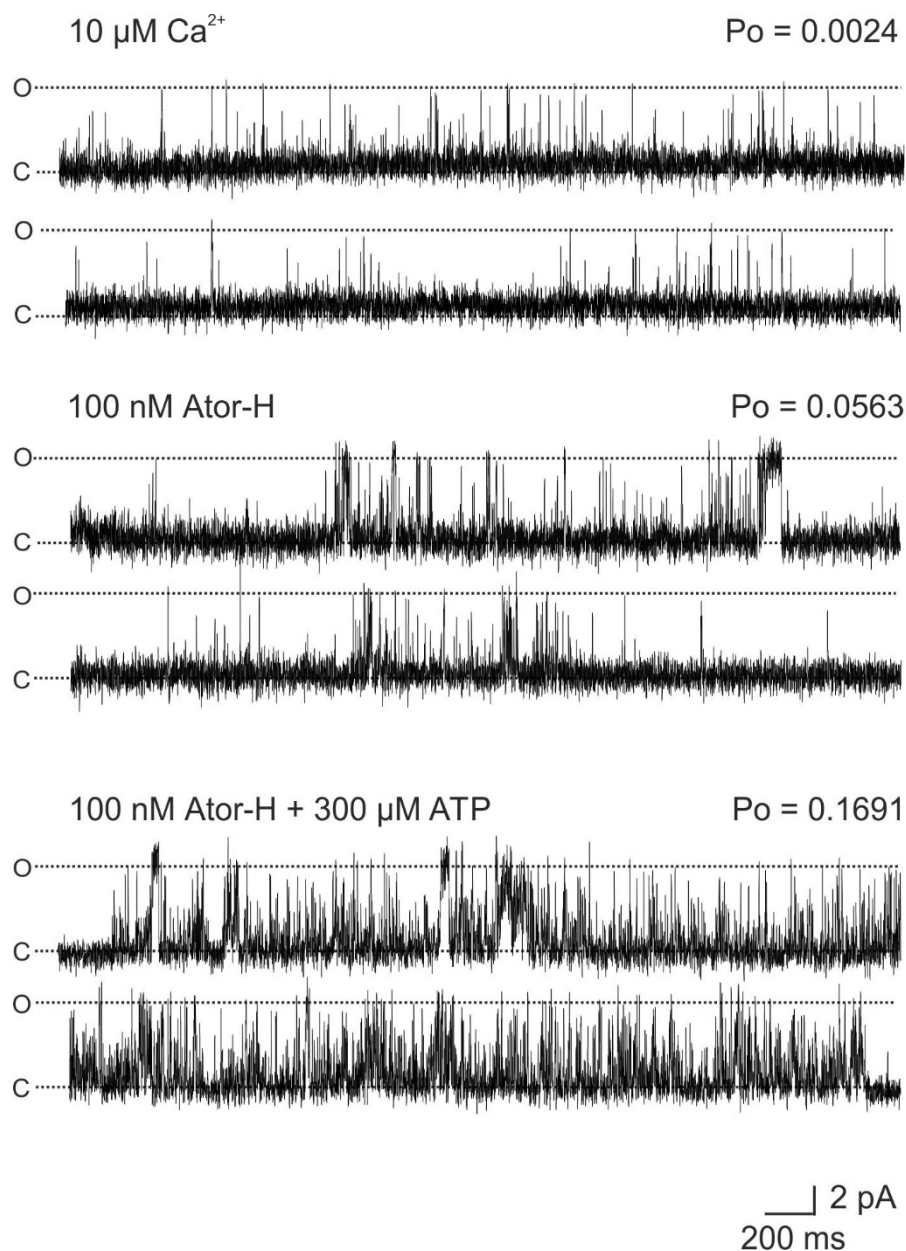


Figure 6.7 The presence of Ator-H dampens activation by ATP

Representative sheep skeletal RyR1 single-channel current fluctuations in the presence of 10 μ M Ca^{2+} alone (top traces), after addition of 100 nM Ator-H and after the subsequent addition of 300 μ M ATP. The P_o above the traces refers to that calculated over three minutes of recording. O and C indicate open and closed channel levels respectively.

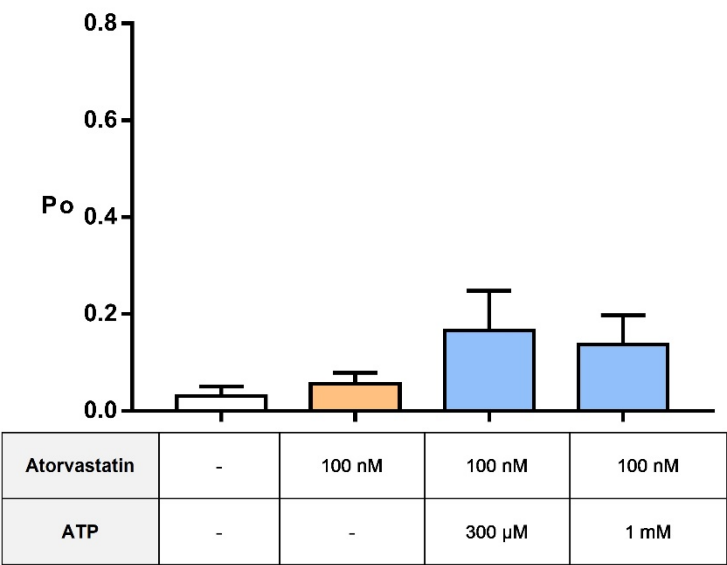


Figure 6.8 The presence of Ator-H dampens activation by ATP
Mean RyR1 Po values under control conditions, after addition of 100 nM cytosolic Ator-H and after subsequent addition of cytosolic ATP at, 300 μM, 1 mM as indicated. Mean ± SEM is shown; n = 5 – 10.

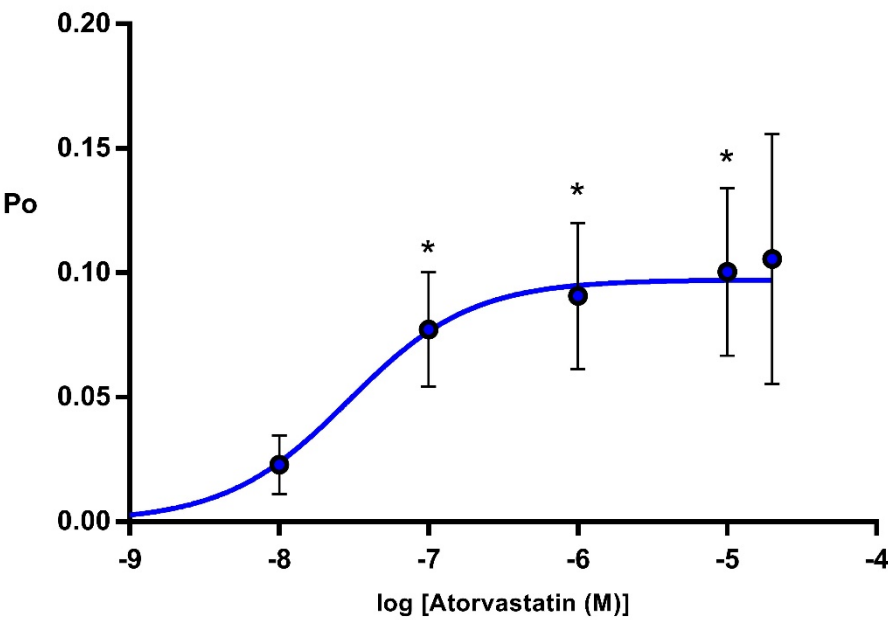


Figure 6.9 Dose response relationship for RyR1 activation by Ator-H. (Data from Figure 3.17, Chapter 3)
Data from Chapter 3 presented for reference. Mean ± SEM are shown, **P* < 0.05 relative to control conditions.

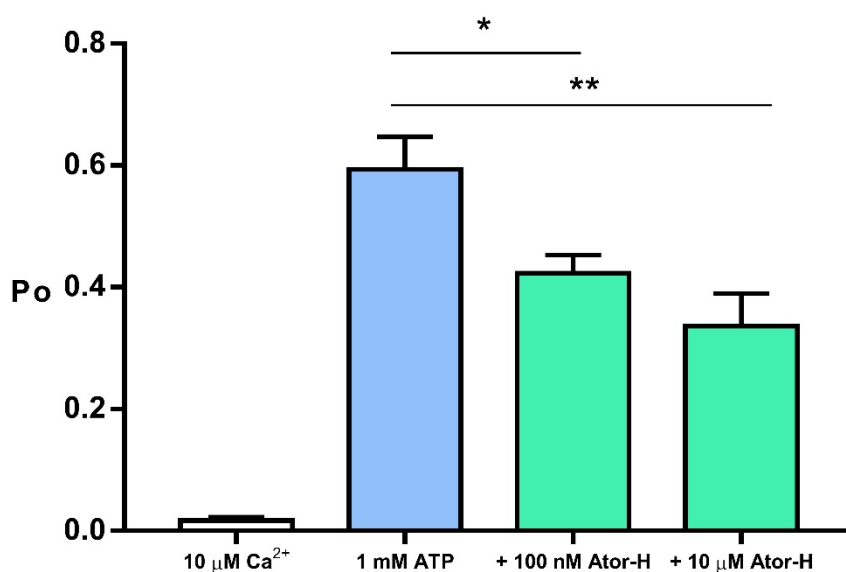


Figure 6.10 Competition studies between ATP and Ator-H

(A) Mean RyR1 P_o values under control conditions ($10\ \mu\text{M}\ \text{Ca}^{2+}$), after addition of 1 mM ATP, and after subsequent addition of both 100 nM Ator-H and 10 μM Ator-H. Mean $\pm$ SEM is shown; $n = 5 - 9$; $**P < 0.005$; $*P < 0.05$.

A further experiment examined the effect of reversing the order of the addition of ATP and Ator-H, that is, the effect of addition of Ator-H on RyR1 channels already activated by high concentrations of ATP. The mean data of these experiments are shown in **Figure 6.10**. Following addition of cytosolic 1 mM ATP, Ator-H was added sequentially at 100 nM and 10 μM concentrations. 100 nM Ator-H caused a decrease in P_o from that seen with 1 mM ATP alone, decreasing it from 0.594 ± 0.0533 (mean $\pm$ SEM; $n = 14$) to 0.4227 ± 0.03052 (mean $\pm$ SEM; $n = 13$). Further addition of 10 μM Ator-H decreased P_o still further to 0.3363 ± 0.0533 (mean $\pm$ SEM; $n = 7$). This finding is again consistent with the hypothesis that more efficacious agonist ATP, is in competition for a shared binding site with the less efficacious (but more potent) agonist Ator-H. While it must be emphasised that this is not conclusive evidence that ATP and statins compete for a common binding site, this data is consistent with that hypothesis.

6.2.3 Modelling of the binding of Atorvastatin into the RyR1 ATP site

To gain a deeper understanding of whether it is indeed feasible for statins (specifically Ator-H) to share a binding site with ATP, molecular modelling studies were undertaken using a recently reported cryo-EM structure for RyR1 with ATP bound.¹⁷¹ A molecular modelling approach, utilising docking software allows the interactions of a given ligand or small molecule with the target protein to be examined, and the predicted binding energy and preferred orientation for the binding of this ligand to be calculated. Comparisons between various ligands can then be drawn, and the likelihood of a given ligand binding in the binding site in question can be inferred. I therefore used a commercially available docking software, Autodock^{291,292}, to investigate whether statin drugs could bind at the RyR1 ATP-site.

Given that the cryo-EM structure reported by Des George *et al.* had ATP bound, the first step in this process was to attempt to use docking software to reproduce the binding of ATP as accurately as possible. We could therefore compare the docking results obtained with ATP to the orientation observed in the cryo-EM in order to increase our confidence in the ability of the docking to reproduce the binding of ligands seen experimentally. The process is described in more detail in the Methods **Chapter 2**.

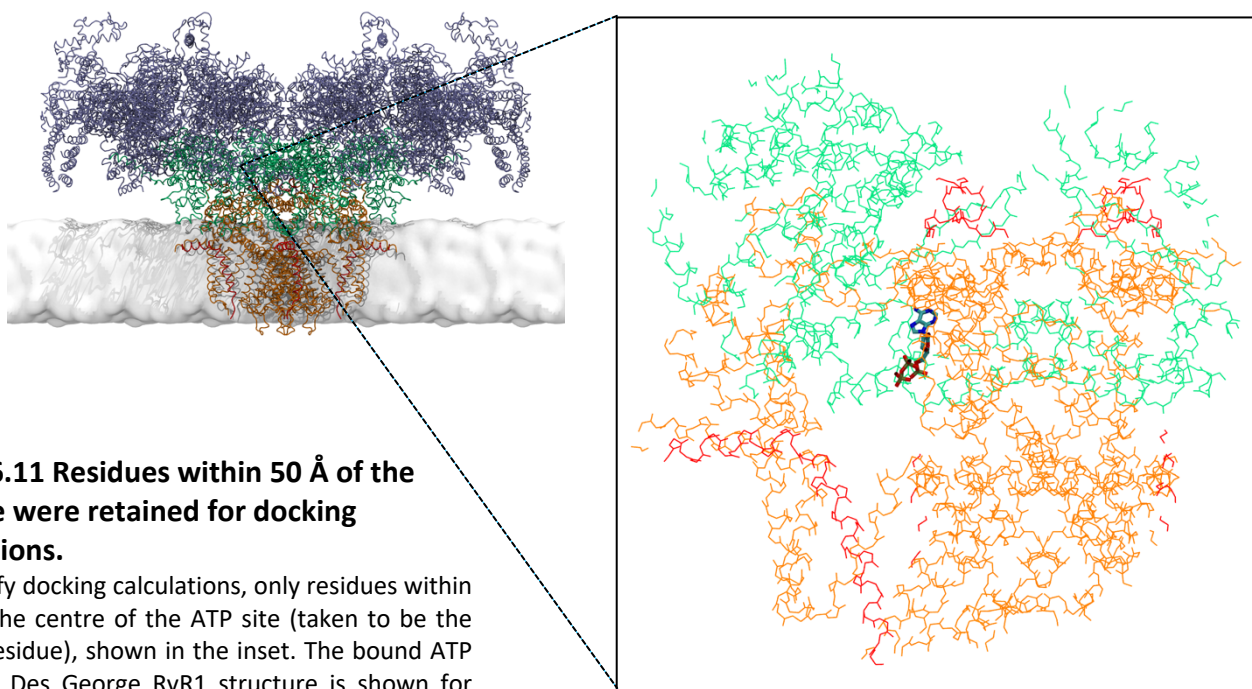


Figure 6.11 Residues within 50 Å of the ATP site were retained for docking calculations.

To simplify docking calculations, only residues within 50 Å of the centre of the ATP site (taken to be the M4989 residue), shown in the inset. The bound ATP from the Des George RyR1 structure is shown for reference.

In order to simplify the docking calculations, all residues which were more than 50 Å from the centre of the ATP binding site (as defined above) were removed. **Figure 6.11** shows the resulting truncated protein structure. Importantly, as only select residues were treated as flexible, this action has no effect on the position of the residues within the ATP site. It was found that when Autodock was used to simulate the binding of ATP itself to the RyR1 ATP-site, the predicted orientations (known as ‘poses’) appeared very similar to the orientation observed by cryo-EM (bearing in mind the resolution of the published structures at 4 Å). The lowest energy pose for the docking of ATP is shown in **Figure 6.12**. We can see that, the orientation of the bound ATP mirrors that observed by Des George *et al.* experimentally (cf **Figure 6.1**). Preliminary steps complete, our next goal was to assess the binding of statin drugs into this binding site, making use of the same docking model and settings. Five commonly prescribed statins (atorvastatin, fluvastatin, rosuvastatin, simvastatin and cerivastatin) were modelled and docked in to the RyR1 ATP-site. The mean data are shown in **Table 6.1**.

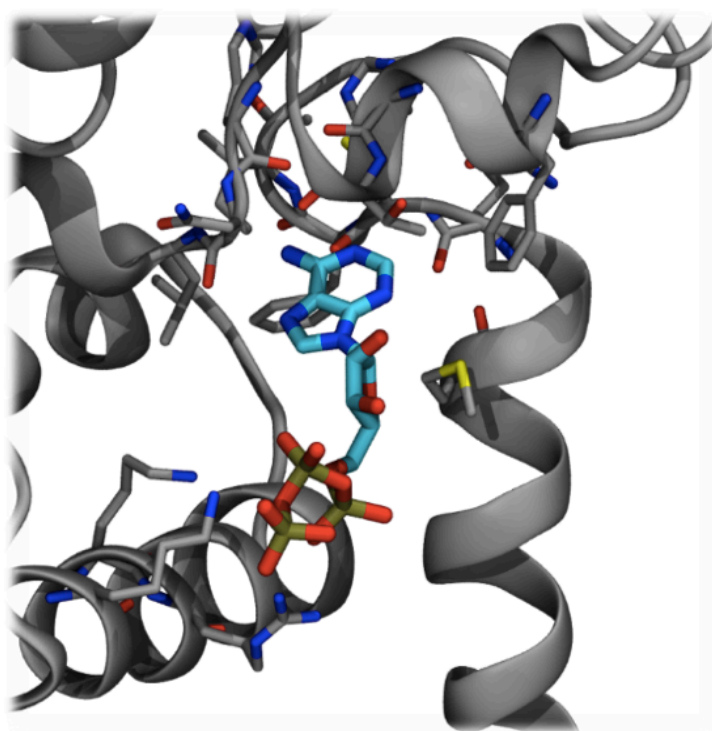


Figure 6.12 Lowest energy pose for the docking of ATP into its site on RyR1.

The lowest energy pose for the docking of ATP into the ATP-site of RyR1 as predicted by Autodock, is shown.

Statin	Predicted binding energy (kcalmol ⁻¹)	No. of poses in the lowest energy cluster
ATP	-7.40	10
Atorvastatin	-5.34	5
Cerivastatin	-5.43	4
Fluvastatin	-6.60	13
Rosuvastatin	-6.01	8
Simvastatin	-6.74	9

Table 6.1 Predicted binding energies and poses generated by Autodock for commonly prescribed statins

Autodock results for ATP, atorvastatin, cerivastatin, fluvastatin, rosuvastatin and simvastatin reporting the predicted binding energy of the lowest energy pose, and the number of poses in the lowest energy cluster. In each docking experiment, there are 50 iterations performed.

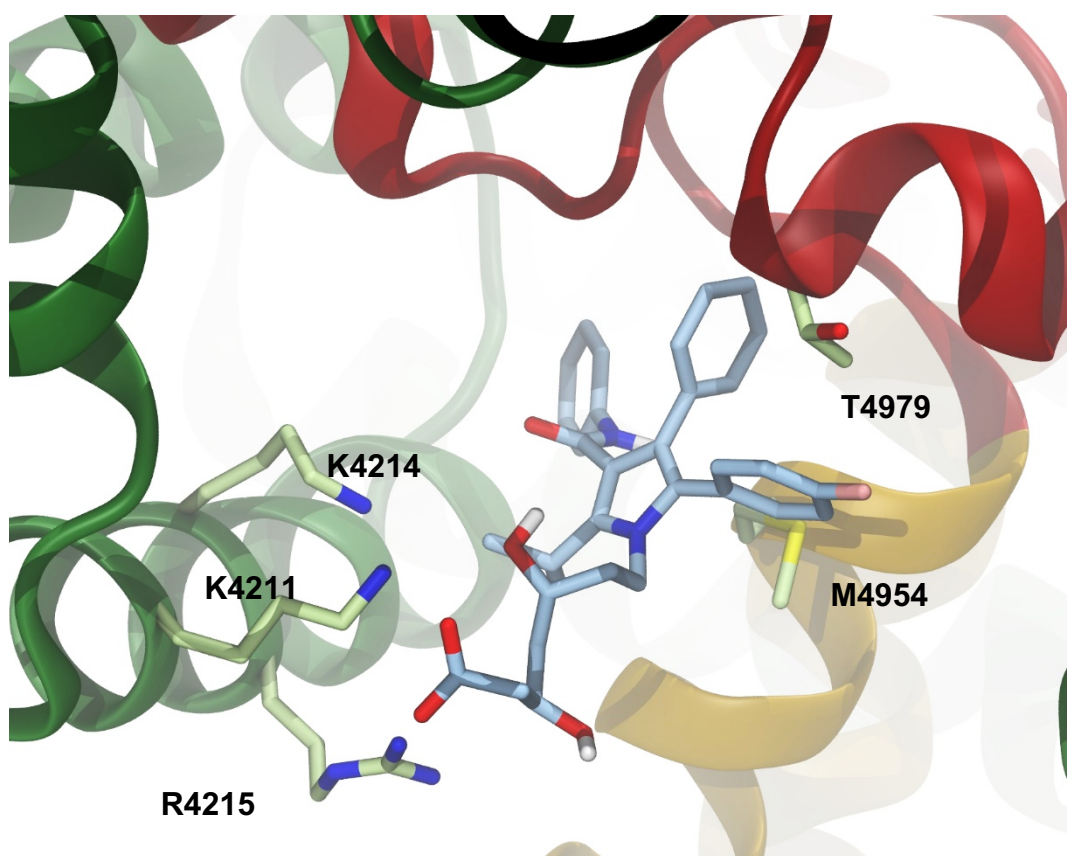


Figure 6.13 Lowest energy pose predicted by Autodock for atorvastatin.

The lowest energy pose predicted by Autodock for docking of atorvastatin is shown. Residues are coloured by type. Important residues are indicated.

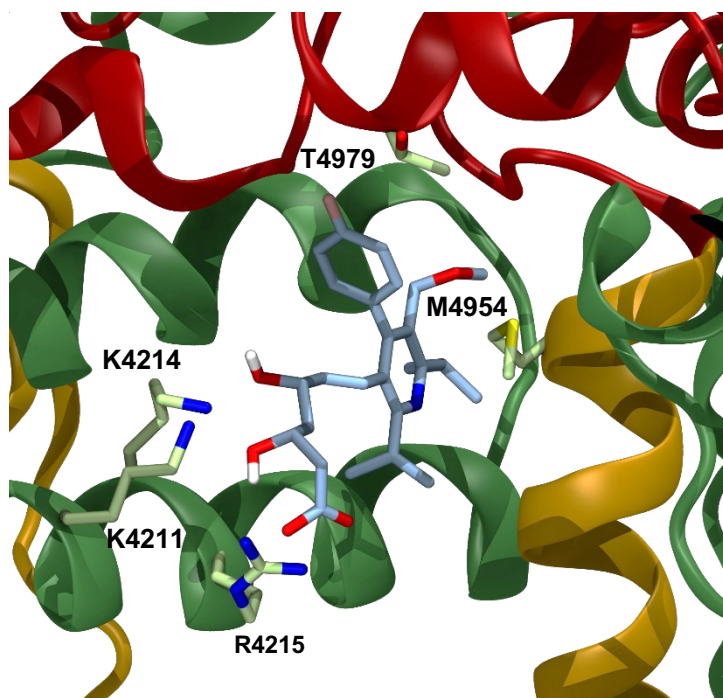


Figure 6.14 Lowest energy pose predicted by Autodock for cerivastatin.

The lowest energy predicted by Autodock for docking of cerivastatin is shown. Residues are coloured by type. Important residues are indicated.

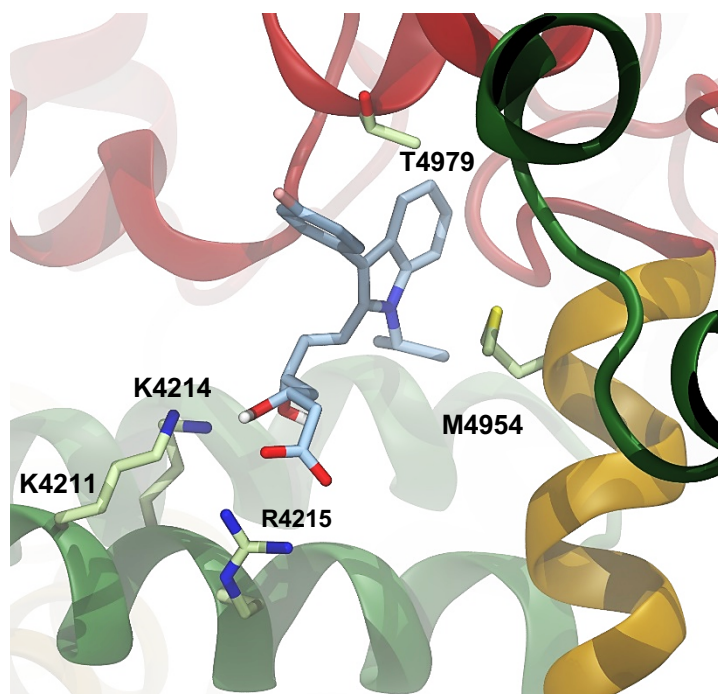


Figure 6.15 Lowest energy pose predicted by Autodock for fluvastatin.

The lowest energy predicted by Autodock for docking of fluvastatin is shown. Residues are coloured by type. Important residues are indicated.

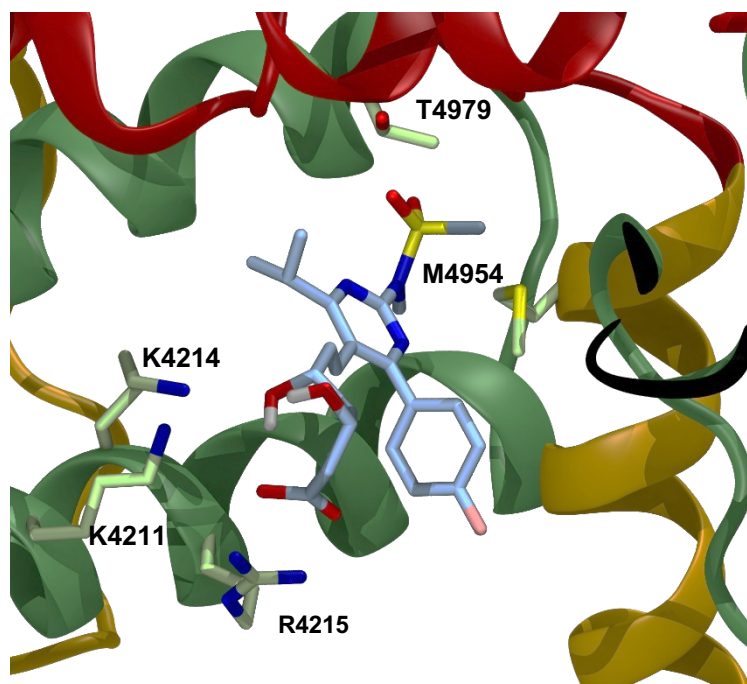


Figure 6.16 Lowest energy pose predicted by Autodock for rosuvastatin.

The lowest energy predicted by Autodock for docking of rosuvastatin is shown. Residues are coloured by type. Important residues are indicated.

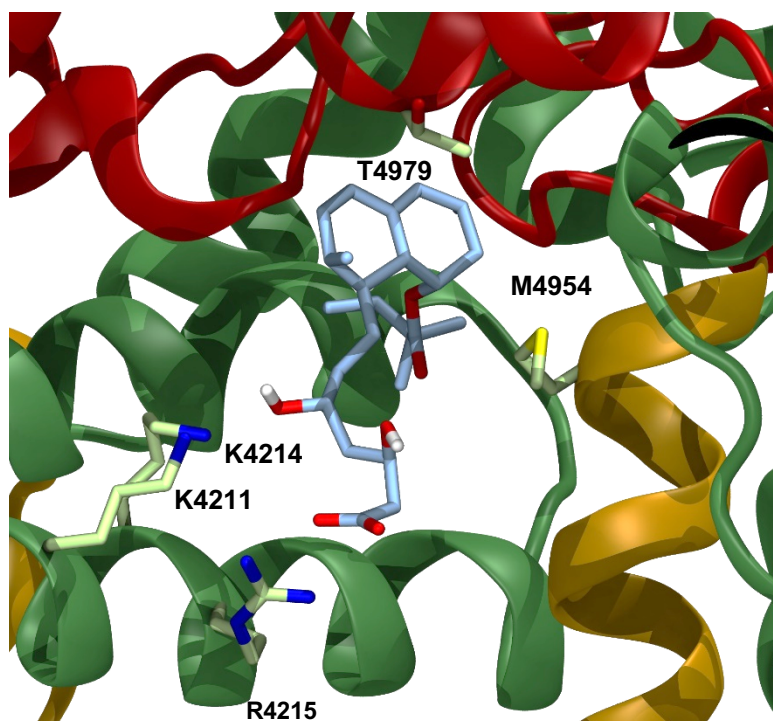


Figure 6.17 Lowest energy pose predicted by Autodock for simvastatin.

The lowest energy predicted by Autodock for docking of simvastatin is shown. Residues are coloured by type. Important residues are indicated.

Figures 6.13 – 6.17 show a selection of the resulting poses for the five statins investigated. In all cases, the two energy pose as predicted by Autodock is shown. It can be seen first, that in the case of atorvastatin, the hydroxyglutaric acid statin pharmacophore appears to be positioned to interact with the positively charged lysine and arginine residues which line the pocket, specifically K4170, K4167 and R4171. The more hydrophobic phenyl and fluorophenyl groups are accommodated in the adenine binding pocket and interacts with the M4954, F4959, T4979, and L4985 residues. **Figure 6.18** shows a comparison between the lowest energy poses for atorvastatin and ATP, allowing a direct comparison to be made. We see a similar binding mode for the other statins. In particular, considering a type 1 statin such as simvastatin, the statin hydroxyglutaric acid is again bound by arginine and lysine residues and the hydrophobic cleft binds the decalin ring of the statin. Therefore despite the differences in structure between type 1 and type 2 statins, there is significant similarity in how these molecules are predicted to bind in the RyR1 ATP site.

It can be seen from the mean binding energy data that, all of the statins have similar binding predicted binding energies within several kcalmol⁻¹. The number of poses in the cluster of lowest energy is also reported. During the docking calculations, 50 poses are generated and grouped into 'clusters' based on orientation. This value, reveals how many times the docking program produces a given orientation out of 50. Therefore, the more poses in the cluster, the more likely a given orientation is to be realistic. We can see that for the statins investigated, the lowest energy pose was repeatedly produced between 4 and 13 times between the statins. The statins investigated are structurally distinct, and it appears that simvastatin and fluvastatin would be predicted to bind most strongly to the RyR1 ATP-site, however the binding energies are broadly comparable. Having established a model for how statins would

bind to the ATP-site, the next stage of the project would require the design of potential HMG-CoA inhibitors.

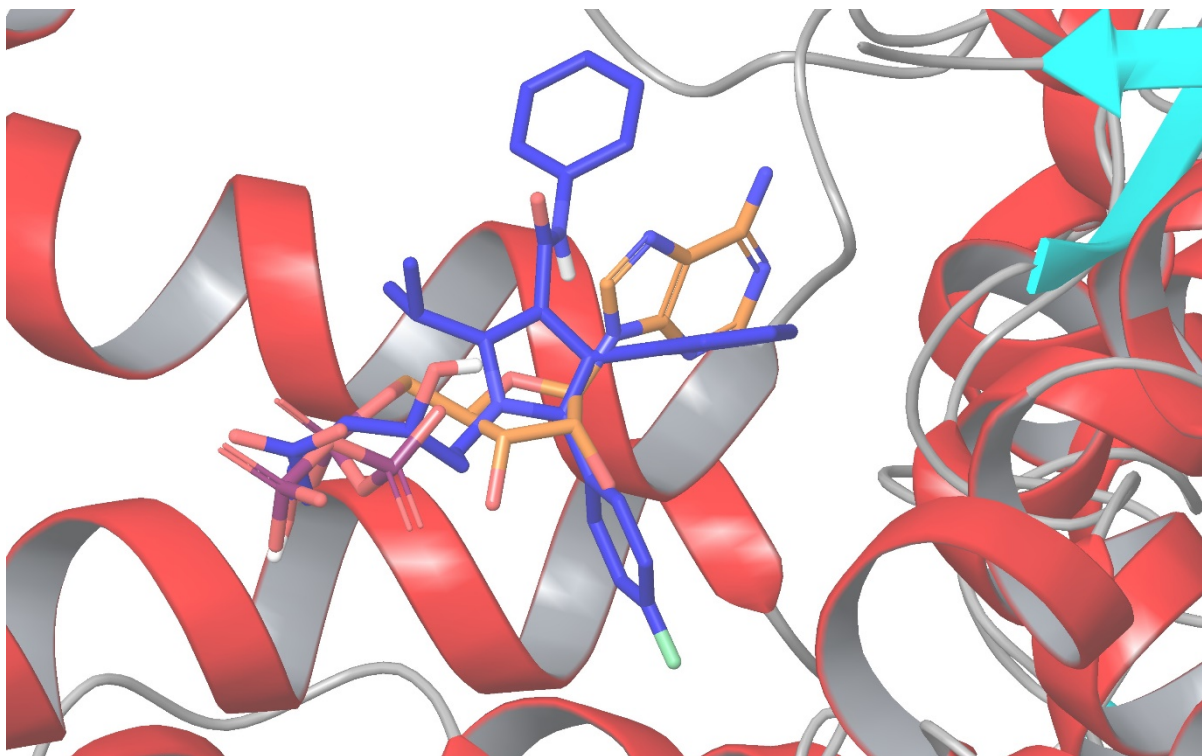


Figure 6.18 Atorvastatin's preferred binding mode in the RyR1 ATP site.

The lowest energy pose predicted for the docking of atorvastatin into the RyR1 ATP-site. Atorvastatin is shown in blue, and the bound ATP in the cryo-EM structure reported by des George *et al.* in orange and blue.

6.2.4 Atorvastatin analogues provide insight into binding to RyR

6.2.4.1 Analogues of Atorvastatin

In their search for novel HMG-CoA reductase inhibitors, *Pfizer Global Research & Development* ('Pfizer') reported the development of, synthesis towards, and pharmacological properties of a wide variety of analogues based on atorvastatin, spanning 10 publications. Many of these analogues are based around 'Inhibitor 2', an analogue of atorvastatin which is more synthetically accessible and based around a transposition of the central pyrrole ring, as well as an additional fluorophenyl group (**Figure 6.19(A)**). These reported analogues cover a wide variety of structural classes, including alternative heterocycles, 5- and 6- membered rings as

the core and a variety of appending groups. This previous work by Pfizer could therefore provide a large resource of known HMG-CoA inhibitors for molecular modelling, all with reported and experimentally determined IC_{50} values for HMG-CoA inhibition in the low nanomolar range. These analogues therefore formed the basis of docking study described in the rest of this chapter. Initially, it was found that the structural differences between atorvastatin and inhibitor 2, resulted in only a modest change in predicted binding energy and the lowest energy predicted poses shared the same orientation (**Figure 6.19(B)**), which is unsurprising given the small difference between these molecules. In particular, fluorine is also a classical bioisostere for hydrogen commonly employed in drug discovery, and so we would not have expected this change to have promoted a change in the predicted binding.³⁷⁸

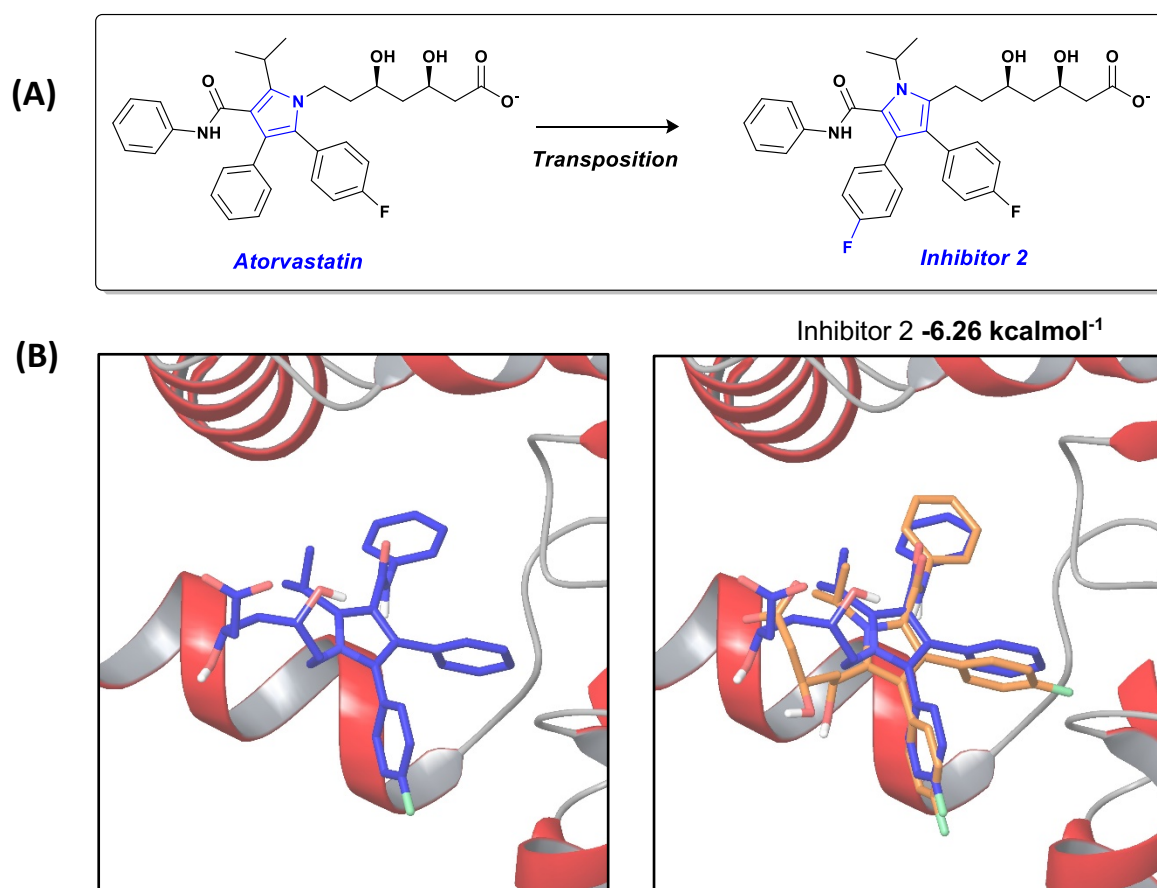
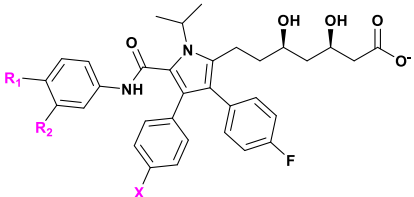
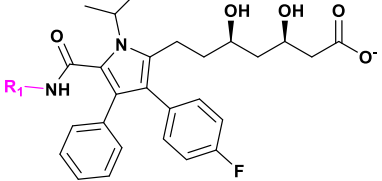
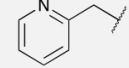
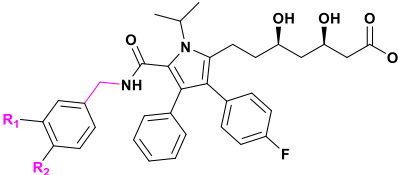
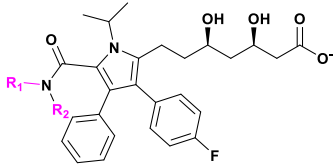
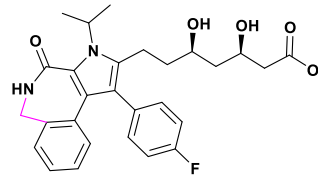
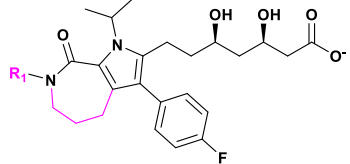
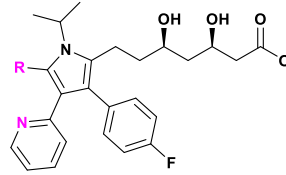
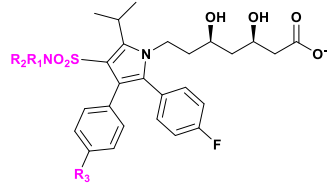


Figure 6.19 Inhibitor 2

(A) Inhibitor 2 is related to atorvastatin by a transposition of the pyrrole core, and the addition of a fluorophenyl group. (B) Lowest energy pose predicted by Autodock for atorvastatin (left-hand panel) and a superposition of the lowest energy poses predicted for both atorvastatin (blue) and inhibitor 2 (orange).

From this, I investigated the structures of the HMG-CoA inhibitors reported by Pfizer, with the aim of identifying structural features that would make RyR1 binding less favourable, and therefore lessen the ability of that molecule to bind to, and activate RyR1. From the docking model of atorvastatin, we have seen that the hydrophobic functional groups surrounding the core pyrrole ring are predicted to bind in the 'hydrophobic cleft' of the ATP binding site. We would therefore predict that bulky or polar groups in these positions could disrupt the interactions in this position, and make binding less likely.

With this in mind, I selected from the large number of reported analogues of atorvastatin those analogues which contained groups that were either bulky or heavily substituted groups on the amide-phenyl group. The analogues selected are shown in **Table 6.2**, and are grouped into several series **(A)** to **(H)**, based upon their structures, with analogues sharing a common structural basis being grouped together. These groups are named **(A)** pyrrole, **(B)** heterocyclic amide, **(C)** benzyl amide, **(D)** alkyl amide, **(E)** conformational restriction, **(F)** CN and amide, **(G)** 4-sulfamoyl pyrrole and **(H)** quinoline. For each series, several analogues were chosen that are representative of the whole series with the aim of limiting the number of analogues that need to be modelled, in order to appreciate how favourable the binding of the series as a group would be. All of these molecules had an experimentally determined IC_{50} for inhibition of HMG-CoA reductase of < 5 nM through an *in vitro* assay, and confirmed through inhibition of cholesterol synthesis in an *in vivo* mouse model.

	
(A) Anilide series 2 $R_1 = \text{H}$, $R_2 = \text{OH}$, $X = \text{H}$ 3 $R_1 = \text{H}$, $R_2 = \text{C(O)NH}_2$, $X = \text{H}$	(B) Heterocyclic amide 7 $R_1 =$  8 $R_1 =$ 
	
(C) Benzyl amide 4 $R_1 = \text{H}$, $R_2 = \text{CH}_2\text{OMe}$ 5 $R_1 = \text{H}$, $R_2 = \text{CN}$ 6 $R_1 = \text{H}$, $R_2 = \text{C(O)NMe}_2$	(D) Alkyl amide 9 $R_1 = \text{H}$, $R_2 = \text{Et}$ 10 $R_1 = \text{H}$, $R_2 = c\text{-Pr}$
	
(E) Conformational Restriction 11	12 $R_1 = \text{Ph}$ 13 $R_1 = \text{Bn}$
	
(F) CN and amide 14 $R =$  15 $R =$ 	(G) 4-sulfamoyl pyrroles $-\text{NR}_1\text{R}_2 =$ $\text{R}_3 = \text{H}$ 16  17  18 

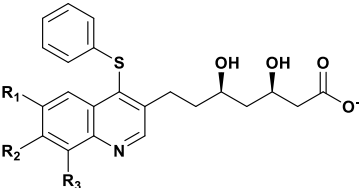

(H) Quinolines 19 R₁ = 4-CH(CH₃)₂, R₂ = F, R₃ = F, R₄ = F 20 R₁ = 3-OCH₃, R₂ = F, R₃ = SC₆H₄-3-OCH₃, R₄ = H 21 R₁ = 4-F, R₂ = F, R₃ = SC₆H₄-F, R₄ = SC₆H₄-F 22 R₁ = 4-CH(CH₃)₂, R₂ = SC₆H₄-4-CH(CH₃)₂, R₃ = SC₆H₄-4-CH(CH₃)₂, R₄ = SC₆H₄-4-CH(CH₃)₂

Table 6.2 Atorvastatin analogues reported by Pfizer grouped into series by structural similarity.

The atorvastatin analogues reported by Pfizer can be grouped into several series **(A)** to **(H)** based on their structural similarities.

6.2.4.2 Structure-activity relationships for atorvastatin analogues

All of the analogues were docked in to the RyR1 ATP-site using the same settings and conditions as for atorvastatin and inhibitor two, and the results are reported in **Table 6.3** and **Table 6.4**. It was found that many of these analogues had broadly similar predicted binding energies, taking into account the approximate error in the scoring function of $\pm 1 \text{ kcalmol}^{-1}$.³⁷⁹ However many of the analogues had a reduced number of poses in their lowest energy clusters, which indicates that there is a greater variety in the orientations predicted by Autodock, and therefore we would infer that binding is less favourable (as an 'obvious' favourable orientation is less apparent). Both of these values must therefore be taken together. Analogue **2**, modified by adding a hydroxyl group to the phenyl ring of Ator-H, would be predicted to bind more favourably than atorvastatin as the lowest energy pose had a lower energy (more favourable binding), and this pose was reproduced 4 times. Inhibitor **3** had a similar result, the modification being an additional amide group in this case. Series **(B)**, the heterocyclic amides had varied results, with analogue **7** being found to have the least negative binding energy (least favourable) of all analogues, while **8** showed more favourable binding to RyR1 with its pyridine substituent. The alkyl amide series **(D)** of **9** and **10** were both comparable to atorvastatin. The series of conformationally restricted analogues were also

uniformly predicted to bind more strongly to RyR1, and so this series was not investigated further. The CN and amide series (**F**) was more promising, with both **14** and **15** having more positive predicted binding energies combined with one only pose in the cluster of lowest energy. The sulfamoyl pyrroles (**G**) were varied, with **17** (piperidine substituent) binding favourably while **16** (morpholine) and **18** (pyrrolidine) binding less well. The quinoline series (**H**) in contrast, showed a trend towards binding more favourably, and were therefore not of further interest.

Series (A) – (D)		
Compound	Binding Energy (kcal/mol)	No. in cluster
Atorvastatin	-5.84	5
Inhibitor 2	-6.26	3
2	-6.18	4
3	-6.99	3
4	-5.48	1
5	-5.28	1
6	-5.51	2
7	-4.67	2
8	-6.27	1
9	-5.54	1
10	-5.34	2
Series (E) – Conformational Restriction		
Compound	Binding Energy (kcal/mol)	No. in cluster
Atorvastatin	-5.84	5
Inhibitor 2	-6.26	3
11	-7.45	6
12	-6.61	6
13	-7.45	12
Series (F) - CN and amide		
Compound	Binding Energy (kcal/mol)	No. in cluster
Atorvastatin	-5.84	5
Inhibitor 2	-6.26	3
14	-5.23	1
15	-5.87	1

Table 6.3 Binding energies predicted by Autodock for Series (A) to (F)

The lowest predicted binding energies generated by Autodock for the most 'ATP-like' pose for the compounds in series (**A**) to (**F**). The binding energy is reported in kcal/mol, and the no. of poses in the most ATP-like cluster is also reported.

Series (G) - Sulfamoyl Pyrrole		
Compound	Binding Energy (kcal/mol)	No. in cluster
Atorvastatin	-5.84	5
Inhibitor 2	-6.26	3
16	-5.13	2
17	-7.19	4
18	-4.86	1
Series (H) - Quinolines		
Compound	Binding Energy (kcal/mol)	No. in cluster
Atorvastatin	-5.84	5
Inhibitor 2	-6.26	3
19	-6.43	1
20	-5.95	1
21	-6.99	4
22	-8.54	2

Table 6.4 Binding energies predicted by Autodock for Series (G) and (H)

The lowest predicted binding energies generated by Autodock for the most 'ATP-like' pose for the compounds in series (G) and (H). The binding energy is reported in kcal/mol, and the no. of poses in the most ATP-like cluster is also reported.

Taken together, these results suggest that polar and bulky groups make binding less favourable, however the hydrophobic pocket does appear to be quite accommodating in terms of the functional group changes which can be tolerated. To investigate this further, the most successful series (pyrrole and 4-sulfamoyl pyrrole) were studied in more detail, with **7** and **18** being the analogues that were predicted to have the most unfavourable binding energies in those series. Two additional series of compounds were designed based on these two most promising analogues. Series (I) and series (J) which were based on **7** and **18** respectively. Series (I) therefore contains analogues with two-heteratom containing 5-membered rings as a substituent and series (J) is constructed of 4-sulfamoyl pyrrole substituents which are combination of polar amide, and heteratom substituents. It is also important to note, that Istvan *et al.* reported the crystal structures of atorvastatin (and other commonly prescribed statins) bound to HMG-CoA reductase and from this, demonstrated that

the both the hydroxyglutaric acid unit, and the fluorophenyl group α to it, were essential for binding to HMG-CoA reductase. Therefore, I concluded that only analogues containing these groups should be modelled, in order to increase the probability that analogues retain their HMG-CoA reductase inhibition. Series **(I)** and **(J)** contain analogues based on compounds **7** and **18** respectively that maintain these groups.

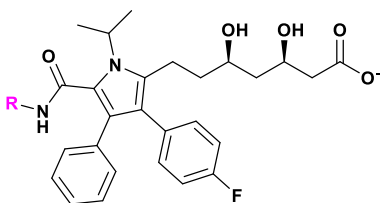
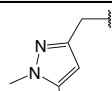
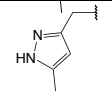
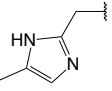
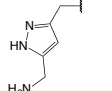
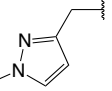
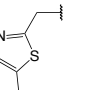
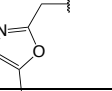
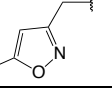
Series (I)			
			
Compound		Binding Energy (kcal/mol)	No. in cluster
Atorvastatin		-5.84	5
Inhibitor 2		-6.26	3
23	R = 	-4.67	1
24	R = 	-5.18	1
25	R = 	-4.93	4
28	R = 	-5.56*	1
29	R = 	-7.56*	1
31	R = 	-4.30*	2
32	R = 	-5.21	1
33	R = 	-5.61	4

Table 6.5 Binding energies predicted by Autodock for Series (I)

The lowest predicted binding energies generated by Autodock for the most 'ATP-like' pose for the compounds in series **(I)**. The binding energy is reported in kcal/mol, and the no. of poses in the most ATP-like cluster is also reported. *indicates an analogue where the lowest energy pose is not 'ATP-like'.

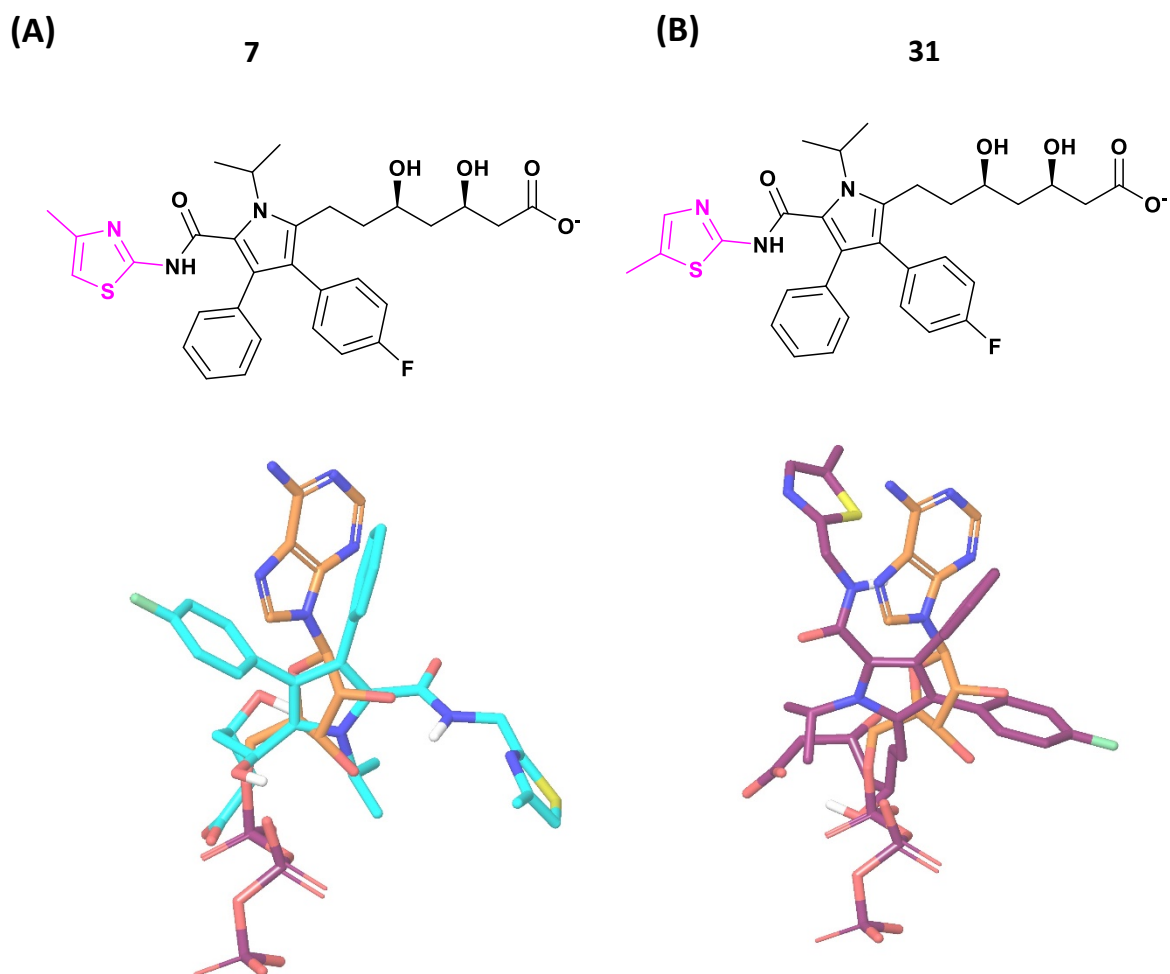


Figure 6.20 Lowest energy poses for **7** and **31**

The docking results for series **(I)** and **(J)** are shown in **Table 6.5** and **Table 6.6** respectively. In series **(I)**, it was found that analogue **23** and **31**, had less negative (and less favourable) binding energies. In particular, substitution of a methyl group in the 2-position appears to be the driving force for this. We can also observe that in the cases of analogues **28-31** the lowest energy pose is not 'ATP-like' (indicated with an asterisk*). That is, the pose does not appear to bind as we have previously described for atorvastatin, with hydroxyglutaric acid unit interacting with the lysine and arginine residues etc. The lowest energy poses for analogues **7**

and **31** are shown in **Figure 6.20**, with the cryo-EM determined ATP pose shown for comparison. It can be observed that, in both cases the lowest energy pose of both **7** and **31** do not share the same orientation as ATP, and indeed **7** and **31** do not share the same pose between them. This would indicate that the change in substituents is driving a change in how the analogues bind in the RyR1 ATP-site.

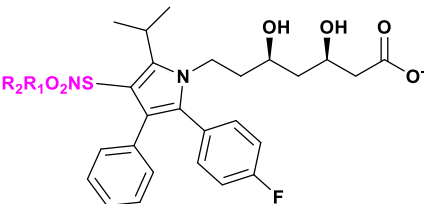
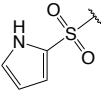
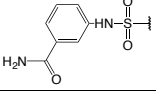
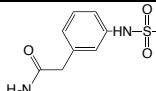
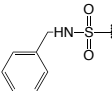
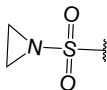
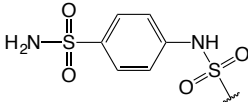
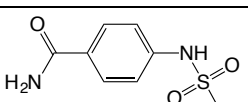
Series (J)			
			
Compound		Binding Energy (kcal/mol)	No. in cluster
Atorvastatin		-5.84	5
Inhibitor 2		-6.26	3
39	R = 	-4.51*	1
40	R = 	-7.12	1
41	R = 	-4.97	1
42	R = 	-5.39	1
43	R = 	-5.62	1
44	R = 	-5.52	1
45	R = 	-5.13	1

Table 6.6 Binding energies predicted by Autodock for Series (J)

The lowest predicted binding energies generated by Autodock for the most 'ATP-like' pose for the compounds in series (**J**). The binding energy is reported in kcal/mol, and the no. of poses in the most ATP-like cluster is also reported. *indicates an analogue where the lowest energy pose is not 'ATP-like'.

Analogues **2** to **11**, and **14** to **18** were kindly provided by Pfizer under a material transfer agreement, and so their effect on RyR gating could be studied and the data compared to the docking scores. Compounds **12** and **13** were unfortunately not available. The ability of the compounds that were accessible to stimulate [³H]ryanodine binding was therefore investigated, and the results are shown in **Figure 6.21** at 100 nM drug concentration. I observed that, the compounds tested had a variety of effects between strong activation of RyR (**2**, **7** and **15** for example), inhibition (**9-10**, **16-18**) and those which appear to have no effect on RyR relative to control levels of binding (**inhibitor 2**, **5**, **6** and **8**).

Figure 6.22 shows a comparison between the predicted binding energy produced by Autodock and the level of [³H]ryanodine binding. We can see that overall there is a broad similarity between analogues that were predicted to bind poorly and those that appeared not to influence the level of [³H]ryanodine binding to RyR, and hence channel openings. This trend is particularly apparent for compounds **10** to **16**, where the trend of increases and decreases in binding energy predicted by Autodock, appear to reproduce the same trends seen experimentally. It is important to emphasise however that the docking model predicts affinity only and gives no indication of efficacy. Therefore a direct correlation between predicted binding energy and ability to activate RyR1 would not be expected in all cases. Indeed, it is noteworthy that analogue **7**, despite having the lowest predicted binding energy of all the analogues investigated, had a relatively high level of [³H]ryanodine binding. The same is true for the trend seen between compounds **16** to **18**, where **17** was predicted to bind much more favourably than its counterparts **16** and **18**, but in fact this whole series exhibited approximately the same level of binding. Whether or not predicted by the docking model, it is nevertheless pleasing that, many of the analogues tested had no effect on RyR1, and have confirmed levels HMG-CoA inhibition. This reaffirms the conclusions of **Chapter 5** that the statin pharmacophore functionality can be retained, but the activation of RyR1 removed.

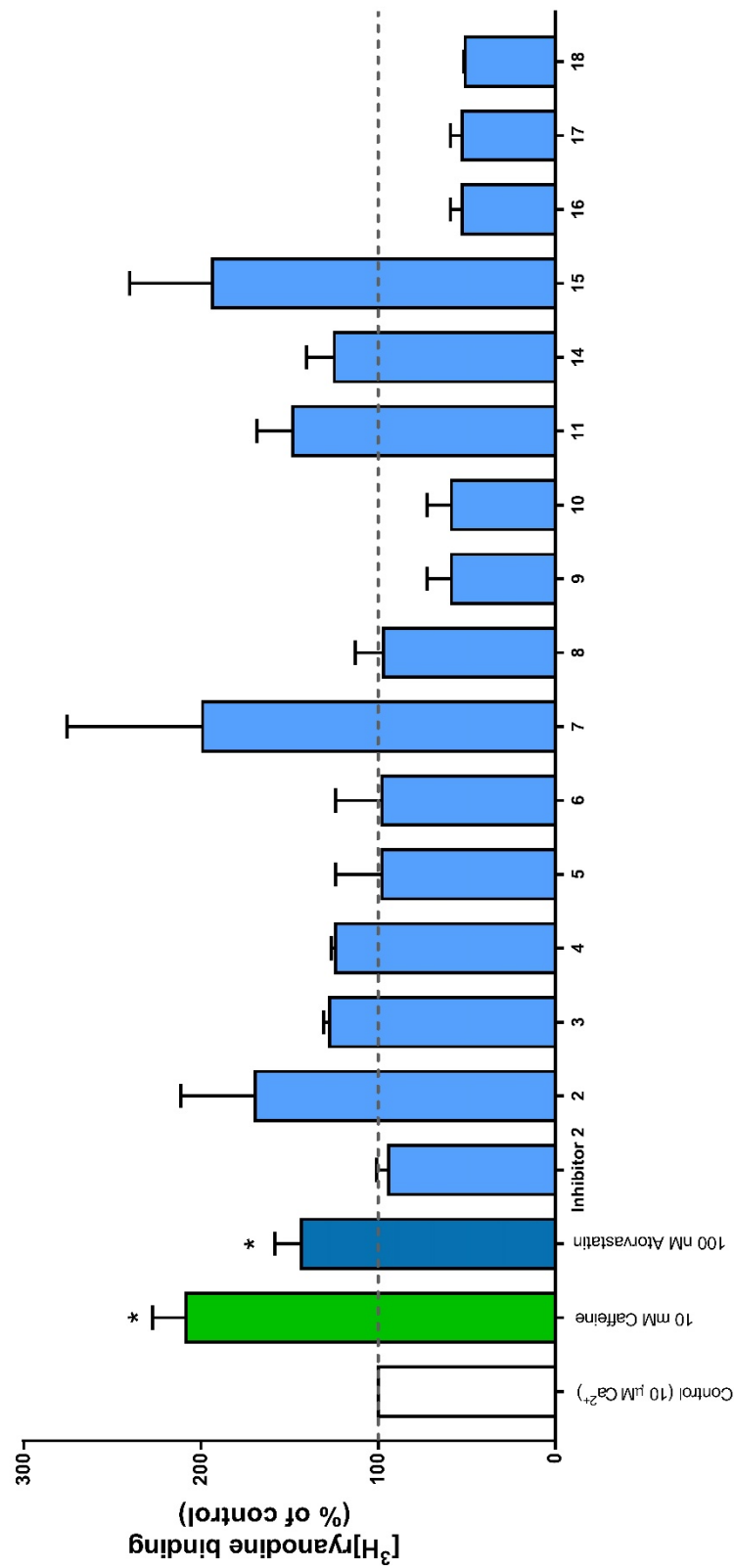


Figure 6.21 Comparison of the ability to stimulate $[^3\text{H}]$ ryanodine binding of atorvastatin analogues. Stimulation of $[^3\text{H}]$ ryanodine binding to sheep skeletal SR vesicles by 10 mM caffeine, 100 nM atorvastatin and compounds 2-11 and 14-18 at 100 nM. Mean $\pm$ SEM; $n = 3$; * $P < 0.05$

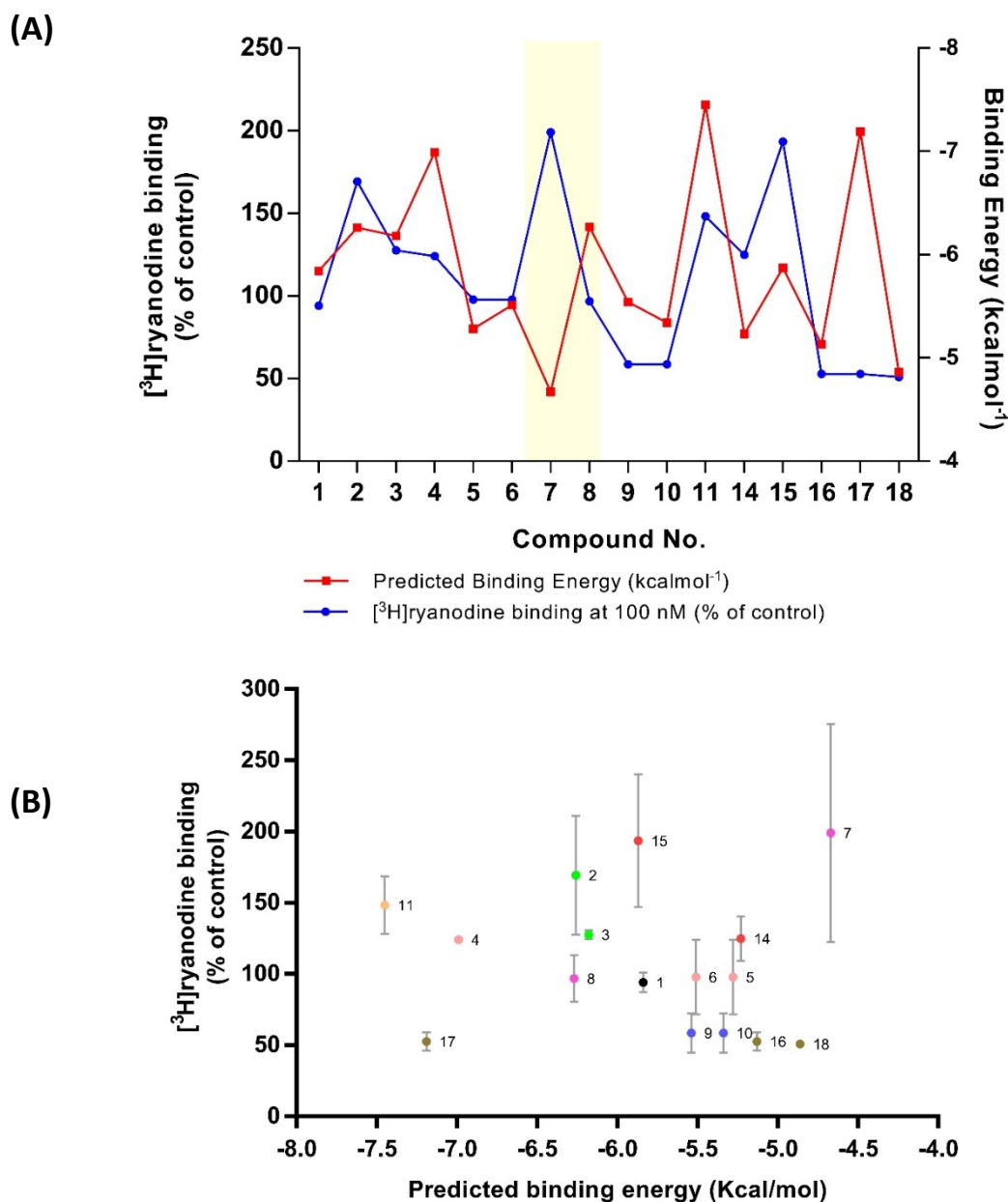


Figure 6.22 Comparison of the binding energy predicted by Autodock for atorvastatin analogues.

(A) The relative ability of compounds 1-11, 14-18 to stimulate $[^3\text{H}]$ ryanodine binding, is shown as a percentage of control binding in blue, and the binding energy predicted by Autodock (in kcalmol⁻¹) is shown in red. Compounds 7 and 8 which are discussed in the text, are highlighted with a yellow background. **(B)** The correlation between ability to stimulate $[^3\text{H}]$ ryanodine binding, vs Autodock predicted binding energies. Each series of compounds is shown as a different colour as follows: A (green), B (purple), C (pink), D (blue), E (orange), F (red), G (brown). Inhibitor 2 is shown in black.

6.3 Discussion

The ability of ATP to activate RyR1 in a Ca^{2+} -dependent fashion is well established³⁸⁰. I hypothesised that the similar effect of statins on RyR1 in single-channel experiments was a result of statins binding to the ATP site. One could therefore predict that this would promote the same change in channel conformation leading to increased P_o in the presence of activating levels of cytosolic $[\text{Ca}^{2+}]$.

In keeping with the findings of previous reports, I found that ATP increased the P_o of single RyR1 channels in a concentration dependent manner (**Figure 6.5**) and similarly stimulated the binding of [^3H]ryanodine to skeletal SR vesicles (**Figure 6.6**). Importantly, P_o could be returned to control levels following ATP washout, indicating reversible activation and hence there is no phosphorylation of channels. Although the activation of RyR1 by ATP is well established, these experiments allowed competition experiments to be performed with the knowledge that ATP was not irreversibly modifying the channels. To examine the hypothesis that atorvastatin and ATP may compete for a common binding site, the simultaneous effect of both ligands on RyR1 in a single-channel context was investigated. It was found that the addition of a low concentration of Ator-H (100 nM) reduced the ability of subsequent addition of ATP to increase P_o (**Figure 6.8**), and it was not possible to achieve a P_o of above 0.2 in this case. The converse was also true, and channels which were activated by 1 mM ATP were inhibited by the subsequent addition of Ator-H (**Figure 6.10**). These findings are consistent with both ligands sharing a common binding site, although of course, are not definitive. We previously found that atorvastatin is a partial agonist of RyR1, capable of increasing P_o at nanomolar levels. The EC_{50} values obtained for Ator-H and ATP respectively were 20 nM (**ref. Chapter 3**) and 129 μM , hence Ator-H is 6450 times more potent than ATP and we can describe ATP as a low affinity, high efficacy ligand, and Ator-H as the reverse, a relatively high affinity, low efficacy ligand. Given this, we would therefore expect that if both ligands compete for the

same site that Ator-H, at the concentrations used, would antagonise the effects of 300 μM and 1 mM ATP.

The observation that statins can induce an increase in P_o through both Ca^{2+} -dependent and Ca^{2+} -independent mechanisms, as demonstrated in **Chapter 3**, is also an effect observed with ATP, as ATP can increase P_o to high levels with low $[\text{Ca}^{2+}]$ (100 nM).¹³⁵ The changes in RyR1 Ca^{2+} -sensitivity caused by statins could also be explained by its binding at the RyR1 ATP-site. Murayama *et al.* have suggested that the Ca^{2+} -sensitivity of the caffeine activation of RyR1 is the result of the close proximity of the caffeine and Ca^{2+} -binding sites. They found that the binding of caffeine induced an interaction between a tryptophan (W4716) residue in the caffeine binding site and a phenylalanine (F3753) residue in the Ca^{2+} site, the result of which was to cause a 2 Å conformational change making the Ca^{2+} -binding site more suitable for the binding of Ca^{2+} .³⁷² In a similar way, the ATP site, which also resides in the same junction of the CTD may also interact with the Ca^{2+} -site to increase the affinity of Ca^{2+} , and therefore sensitise the channel to cytosolic $[\text{Ca}^{2+}]$.

However, the close proximity of these sites also cautions that further experiments will be required to definitively conclude that statins may compete for the same binding site as ATP, as other mechanisms must be ruled out, such as competitive allosteric inhibition. This is where one ligand inhibits the ability of the other to influence a channel or receptor, but through binding to another (allosteric) binding site. A notable example can be drawn from the development of p38 MAPK inhibitors, where kinetic studies had suggested competition between ATP (the substrate) and a small molecule inhibitor.³⁸¹ More detailed studies revealed that the inhibitor bound adjacent to the ATP binding site, inducing a movement of residues which blocked the ATP site, and created the appearance of competition with ATP.³⁸² Given the close proximity of the three ligand binding sites in RyR (Ca^{2+} , caffeine and ATP) this is a

possibility that must be considered, and further more detailed studies utilising techniques such as cryo-EM will be required.

With the competition experiments being consistent with Ator-H and ATP binding to a common binding site, I decided to carry out a molecular modelling study to investigate how atorvastatin in particular might bind to the ATP binding site. The recently published cryo-EM structures for RyR1 was utilised, and the docking of five commonly prescribed statins (**Table 6.1**) suggested that binding in the ATP-site would be favourable. All five molecules produced favourable (i.e. negative) predicted binding energies, and the lowest energy poses appear to reveal a binding mode similar to ATP (**Figure 6.13**). Taking simvastatin as an example of a type 1 statin, it was found that the decalin core could be accommodated in the hydrophobic pocket of the ATP site, while the hydroxyacid pharmacophore was then positioned ideally to interact with the positively charged lysine and arginine residues which line the site. Atorvastatin (a type 2 statin) showed a similar binding mode, this time with the pyrrole core and its associated phenyl groups cradled by the 'adenine' binding pocket'. Effectively therefore the hydrophobic core of the statin molecules provide the same positive interaction with the binding site as the adenine and ribose rings of ATP, while the charged hydroxyacid pharmacophore is a surrogate for the triphosphate group of ATP. Encouragingly, the binding of these statin drugs in the RyR1 ATP appears to be both feasible and favourable.

In order to further the original aim of developing a HMG-CoA inhibitor with reduced RyR activity, I attempted to use the model of the ATP binding site to identify statin analogues for which binding would be less favourable. A catalogue of analogues based on 'inhibitor **2**' as developed by Pfizer, was iteratively investigated for binding to the ATP-site (**Section 6.2.4.1**). While the results were varied, cumulatively the docking model suggests that addition of the polar or bulky groups to the phenyl rings of atorvastatin reduced the ability of the analogues to bind to the ATP site. **Figure 6.23** shows the proposed explanation for this. As previously

discussed, atorvastatin is simultaneously able to bind deep into the site, interacting favourably with both the hydrophobic residues forming the cavity, and with the positive residues lining it. However, following a substitution to the phenyl group to add a bulky component, such as a dimethyl benzamide group (as in compound 6, as an example), binding deep in the site is no longer possible. This is caused by the bulky and polar group creating a steric clash between the hydrophobic residues, as well as the polar nature of the residue. If a polar or charged group is to bind in a hydrophobic pocket, necessarily the coordination shell of water molecules surrounding this residue must be removed, a process requiring energy. If this energy requirement is not reimbursed by a favourable interaction with the site, the overall binding will become unfavourable. Hence the addition of polar/charged or bulky groups will decrease the favourability of the atorvastatin analogues to bind in the site.

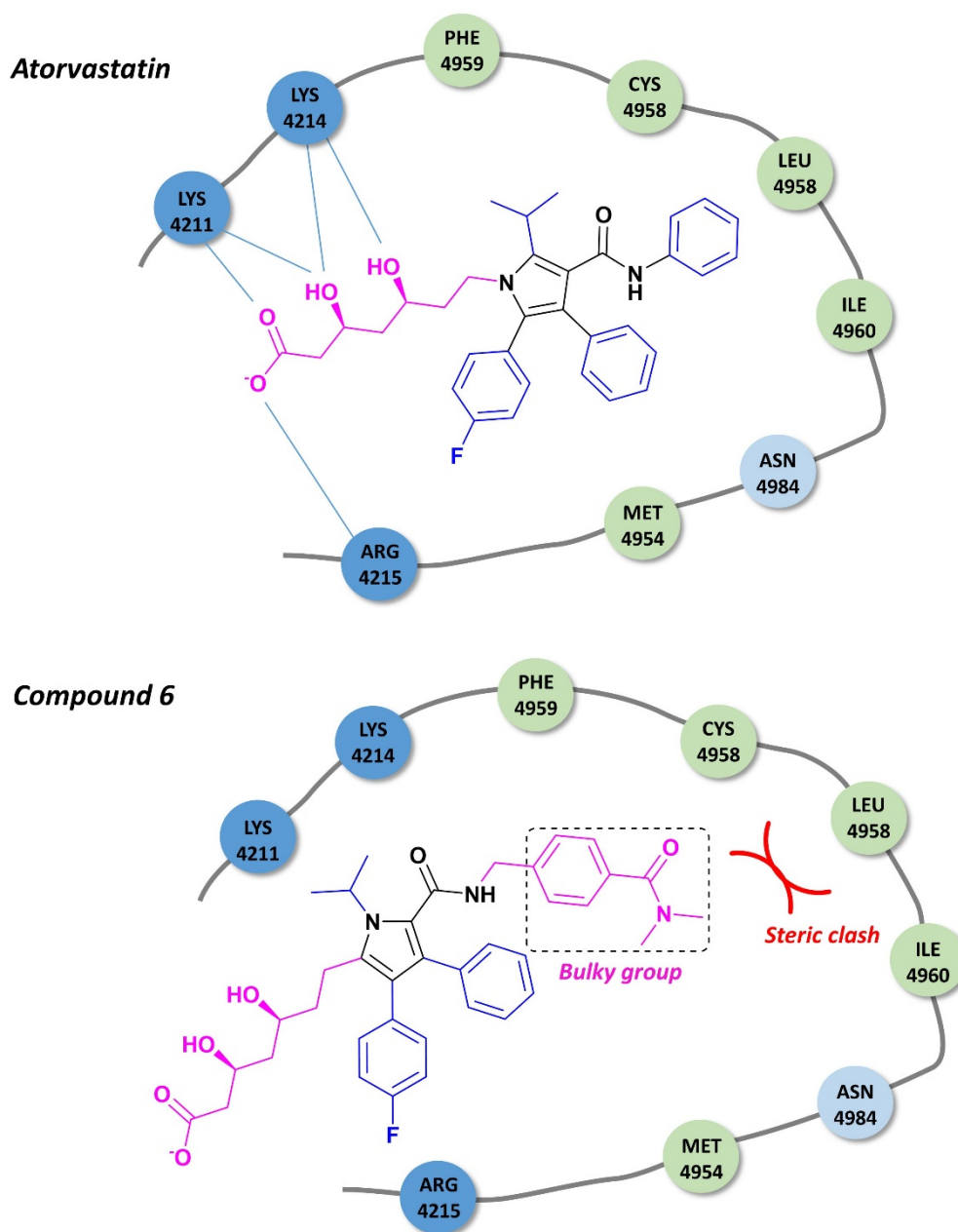


Figure 6.23 The effect of adding sterically bulky and polar groups to analogues of atorvastatin on proposed binding to the RyR1 ATP site.

The figure shows the proposed binding interactions which lead to the reduced ability of atorvastatin analogues investigated in this chapter to activate RyR1. The top panel shows the binding of atorvastatin in the RyR1 ATP site, with important residues indicated. The bottom panel shows the effect of the substitution of phenyl for a dimethyl benzamide group in **compound 6**. In both cases, charged residues are indicated in dark blue, polar uncharged residues in light blue and residues with hydrophobic side chains in green. In both atorvastatin and compound 6, hydrophobic portions are indicated in blue, and polar portions in pink.

To confirm these results experimentally, the analogues which were predicted to have the least favourable (i.e. most positive) binding energy were investigated by [^3H]ryanodine binding, and the results are shown in **Figure 6.21**. Pleasingly, this screen identified several compounds which did not have an effect on RyR1 channel activity, such as **inhibitor 2, 5, 6 and 8**, which will facilitate further investigations into the extent to which RyR1 is implicated in statin myopathy. The structural similarities of these compounds are shown in **Figure 6.24**. In particular, these compounds have in common no substitution at the 3-position of the benzylamide substituent, nor on the 3-position of the unsubstituted phenyl ring of atorvastatin. They also all have a substitution on the 2-position of the benzylamide, which appears to be the driving force for reducing their ability to bind to RyR1. Therefore a simplified structure-activity relationship can be deduced, where a substitution on the phenyl ring adjacent to the fluorophenyl group is to be avoided, while the remaining phenyl group should be substituted with either a bulky or polar group (**Figure 6.25**).

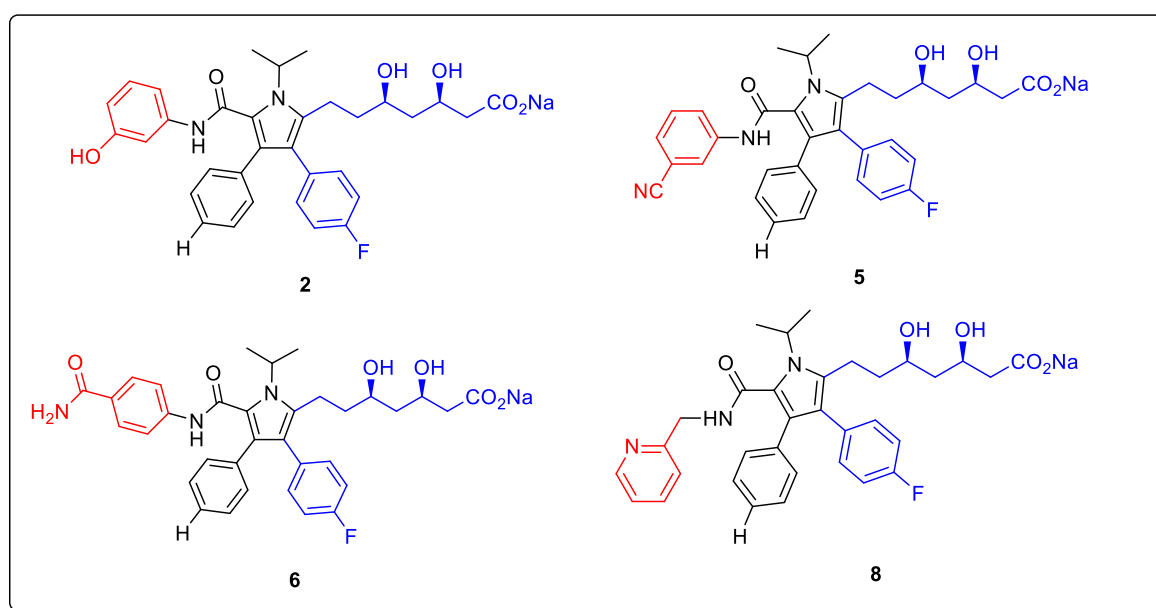


Figure 6.24 Compounds which were found to have no effect on RyR1 activity

Inhibitor **2** and compounds **5, 6** and **8** which were found to have no effect on the activity of RyR1 in a [^3H]ryanodine binding assay are shown. The substitution pattern of the atorvastatin phenyl group is shown in red, and the functional groups required for HMG-CoA inhibition are shown in blue.

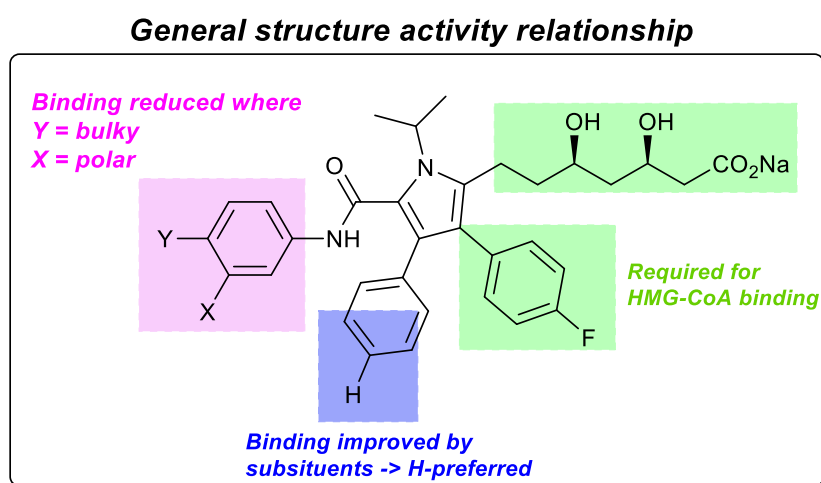


Figure 6.25 Proposed structure-activity relationship between statins and RyR1.

An important question is, to what extent the computational model was able to predict the

experimental effect of ligands on RyR1? **Figure 6.22** shows a comparison between the experimentally determined ability of each compound to increase [³H]ryanodine binding and their Autodock predicted binding energy. It can be seen that in general, both parameters follow a similar trend (**Figure 6.22(A)**), where substitutions that decrease the predicted binding energy (i.e. become more negative) largely also increase [³H]ryanodine binding. For example, from compounds **8** – **11** both values follow the same increase and decrease pattern. However, while the binding energy predictions appear to reflect the trend, they do not predict the extent of the increase or decrease to the same degree. For example, the docking model fails to predict the degree of the increase driven by the substitution from compound **14** to compound **15**.

To fully appreciate why a tight correlation would not be expected, we should consider the limitations of computational ligand docking ('docking'). Importantly, docking can only provide a prediction of the affinity of a ligand for a given binding site, it cannot provide any information on the efficacy of the ligand at providing a change in function. In other words, docking cannot predict the ability of a ligand to increase RyR1 Po, or to stimulate [³H]ryanodine binding, it only can only assess the relative ability of two ligands to bind to RyR. Therefore the docking will not take into account the subtleties of how two different ligands might differ from each other in their ability to induce conformational changes to drive channel opening. It purely considers the interaction of a ligand binding within a given site on the protein. For example, there is a large difference between the binding energy predicted for compound **7**, and it's experimental effect on RyR. While compound **8**, from the same series is more closely predicted (highlighted in **Figure 6.22(A)**). The major difference between these molecules is in the basicity of the substitution, in replacing a thiazole ring with a more basic pyridine group. This difference would not be taken into account by the docking model used.

One of most extreme simplifications of the docking software, is the use of the rigid receptor model. The protein of interest, in this case RyR, is not treated as flexible, but rather as a rigid charged body.³⁸³ This approximation is necessary due to the number of possible permutations of the structure that treating the residues as flexible would create. However, this should be kept in mind when analysing docking results. Additionally, the docking software considers each atom of the protein and ligand as a point charge, an approximation which becomes poorer with atoms of greater size, and therefore more diffuse electron clouds, such as sulfur.³⁸⁴ This is coupled with the fact that the resolution of the published structures for RyR1 (and RyR2) are relatively poor at approximately 4 Å. In sum, the docking results should be interpreted with some care. However, notwithstanding these limitations, the docking approach in this chapter has revealed several compounds that are both potent inhibitors of HMG-CoA reductase, but have no effect on RyR1 activity.

6.3.1 Future experiments

The importance of fully understanding how statins bind to, and activate RyR1 both necessitates and provides an opportunity for, substantial further investigation. Most obviously, there is a need for conclusively identifying the statin binding site of RyR1. Further development of cryo-EM techniques may allow the elucidation of the RyR1 structure with statin bound, thus answering this question more definitively.

I have already noted that the docking software provides solely a prediction of affinity, not efficacy. Therefore in order to further assess the proficiency of the docking model at predicting the affinity of a compound for RyR, an experimental approach for assessing affinity of binding to RyR will be required. This could include methods such as radio-ligand binding utilising a radiolabelled statin molecule or a comparable photo affinity approach. Further structural investigations which can be undertaken include dynamical simulations in which the receptor can be treated as flexible, to further understand which residues (of the ATP site in particular) are important for driving opening.

As discussed in **Chapter 4**, if the development of a statin molecule which does not activate RyR1 is to be successful, it is important to also consider activation of RyR2. Therefore the effect on RyR2 must also be investigated for the ligands assessed in this chapter, as compounds which activate RyR2 which may result in undesirable cardiac effects.

7

The RyR2 ATP site

7.1 Introduction

7.1.1 Ligand regulation of RyR2

It is well established that a disturbance in cardiac Ca^{2+} regulation is associated with undesirable cardiac arrhythmias and possible sudden cardiac death.^{173,385} The most well-known is catecholaminergic polymorphic ventricular tachycardia (known as CPVT), a disease caused by genetic mutations in the *RYR2* gene, which affects how the RyR2 channel senses Ca^{2+} , leading to abnormal heart rhythm and arrhythmia³⁸⁶. In cardiac muscle, the primary trigger that activates opening of RyR2 channels in the sarcoplasmic reticulum (SR) is Ca^{2+} entry through the LTCC which then causes further Ca^{2+} to be released into the cytosol, through the RyR to stimulate muscle contraction.³⁸⁷ This process is known as calcium-induced-calcium-release (CICR). Many other ligands, however are also responsible for controlling the opening of RyR2 physiologically including ATP. ATP is constitutively present in millimolar concentrations in cardiac cells³⁸⁸. It is known to act synergistically on RyR2 to potentiate the effects of cytosolic Ca^{2+} , and promote high Po levels^{156,159,389}. Although ATP can increase RyR2 Po to a limited extent in the absence of Ca^{2+} ¹⁵⁶, CICR is abolished if the concentration of ATP in cardiac myocytes is allowed to drop below 0.1 mM, demonstrating the crucial role of ATP in the maintenance of Ca^{2+} homeostasis in cardiac muscle¹⁶⁰.

7.1.2 Previous work

While ATP is typically present in millimolar concentrations in cells, ATP production is reduced in ischaemic tissue where oxygen levels are low. This can lead to the formation of ATP breakdown products of ATP, such as inorganic phosphate compounds (e.g Pi), ADP, AMP and adenosine.³⁸⁰ Fabiato & Fabiato first showed in 1977 that micromolar concentrations of AMP could result in Ca^{2+} -accumulation in rat SR vesicles and stimulated EC coupling in cardiac muscle.³⁹⁰ Similar effects were seen in skeletal muscle by Meissner where he observed that adenine nucleotides (AMP, ADP, adenosine) could promote Ca^{2+} efflux from the SR.^{144,155} Morri

and Tonomura observed the same effect, but noted that non-adenine based trinucleotides such as cytosine triphosphate (CTP) and guanosine triphosphate (GTP) had no effect, suggesting a crucial role for adenine functionality.³⁹¹ Smith and colleagues were the first to demonstrate that ATP could activate RyR directly, albeit the skeletal isoform RyR1, by increasing the Po of single-channels incorporated into phospholipid bilayers. They found that the effect was dependent on Ca^{2+} and that it was possible to achieve nearly full channel opening ($\text{Po} = 1$) in the combined presence of 2 mM ATP and 2 μM Ca^{2+} .¹²⁹ A similar effect was observed in RyR2 by Kermode *et al.* indicating that the cardiac RyR isoform could also be similarly activated by ATP¹⁵⁶. ATP has also been found to increase [^3H]ryanodine to cardiac SR vesicles, along with its non-hydrolysable analogue AMP-PCP. This indicates the effect is likely due to ATP binding directly to RyR rather than through promoting phosphorylation¹⁶⁹. Previous work in the Sitsapesan group has extended these findings and similarly indicated that these ATP and its analogues affect Ca^{2+} release via a direct effect on RyR2 single channel gating. A summary of the adenine-nucleotide single-channel data is shown in **Table 7.1**.

Ligand	EC ₅₀ (mM)	Max Po*	Hill Coefficient	Reference
ATP	0.22	0.90	1.50	Kermode <i>et al.</i> (1998) ¹⁵⁶
ADP	1.2	0.60	0.97	Kermode <i>et al.</i> (1998) ¹⁵⁶
AMP	3	0.07	0.78	L.L. Ching <i>et al.</i> (1999) ³⁹²
cADPR	ND	0.30	ND	Sitsapesan <i>et al.</i> (1994) ³⁹³
Adenine	0.31	0.20	1.8	W. M. Chan <i>et al.</i> (2000) ³⁷⁴
AMP-PCP	0.16	0.35	2.1	W. M. Chan <i>et al.</i> (2000) ³⁷⁴
Adenosine	0.27	0.11	1.7	W. M. Chan <i>et al.</i> (2000) ³⁷⁴
GTP	ND	0.04	ND	W. M. Chan <i>et al.</i> (2000) ³⁷⁴
Ap5A	0.14	0.60	1.74	Song <i>et al.</i> (2009) ²⁶⁷

Table 7.1 Data from previous work on the effect of nucleotides on RyR2

*In the presence of 10 μM Ca^{2+} . ND = not determined. Ap5A = diadenosine pentaphosphate.

AMP is a partial agonist of RyR2, increasing Po to a maximum of 0.2 at 2 mM, even in the presence of supraphysiological concentrations of Ca²⁺ (> 65 μM).³⁹² In the presence of 10 μM Ca²⁺, the maximum Po was only 0.07.³⁹² ADP was also a weak agonist, increasing RyR2 Po to a maximum of 0.6, and causing inactivation at high concentrations.¹⁵⁶ Since AMP and ADP are both less effective than ATP at activating RyR2 it has been suggested that the triphosphate group of the ATP molecule is essential for enabling ATP to drive the channel into a high open probability state.^{374,376} Conversely however inorganic phosphate alone (without its accompanying adenine base) appeared to decrease the magnitude of CICR in cardiomyocytes³⁹⁴ but slightly increased RyR2 Po in single-channel bilayers at high concentrations (40 mM).³⁹⁵ Therefore, since these changes in RyR2 open probability may have a deleterious effect in ischaemia, by promoting the generation of arrhythmias, it is important to investigate how these ligands bind to and effect RyR2 channel activity.

7.1.3 The RyR2 ATP Site

Many attempts have been made to establish the location of adenine nucleotide binding site on RyR2, and competition experiments have revealed that many nucleotide compounds compete for a common binding site. McGarry *et al.* (1994) showed that adenosine was capable of discriminating between the caffeine and adenine nucleotide site on RyR2 as caffeine could potentiate the effect of adenosine activation³⁹⁶. Further experiments established that adenosine³⁷⁴, cyclic ADP-ribose³⁹³ and diadenosine pentaphosphate²⁶⁷, all compete with ATP for the same binding site on RyR2, as in their presence the activatory effect of ATP was diminished.

While the single-channel function of RyR2 has been studied for some time, the enormous size of this ion channel has limited attempts to determine a full high-resolution crystal structure, and thus identify ligands binding sites with more certainty. Only some domains have been reported for either RyR2 or its sister channel RyR1^{397–401}. In 1993, Shoshan-Barmatz and colleagues employed the photosensitive ATP analogue 2'(3')-O-(4-Benzoylbenzoyl)adenosine-

5'-triphosphate (BzATP) to identify that ATP bound to two fragments of RyR2 of 27 and 13 kDa respectively in the C-terminal domain.¹⁶⁸ More insight did not come until the recent advances in cryo-EM²⁶² allowed the full RyR2 structure to be determined to a resolution of 4.2 Å.²⁶³ In the case of RyR1, similar work has led to identification of a putative ATP binding site in this protein.^{171,259} Given the high degree of similarity between RyR1 and RyR2, both in terms of their structure and their response to ATP, it is not surprising that this region is also highly conserved between RyR isoforms.

The Des George *et al.* structure revealed that the RyR1 ATP consists of a hydrophobic pocket located at the interface between the S6c helix and part of the C-terminal domain. This pocket consists of hydrophobic amino acids (e.g. Met, Phe, Leu etc) which cradle the adenine core of the ATP molecule (**Figure 7.1**). The pocket is lined by positively charged residues (Lys and Arg) which interact with the phosphate tail of ATP. This is consistent with previous suggestions deduced by comparative molecular field analysis (CoFMA).³⁷⁶ Des George *et al.* proposed that the role of ATP was to 'prime' the channel for opening that was then facilitated to open fully by the binding of Ca²⁺, which would be consistent with previous studies indicating the Ca²⁺ dependence of ATP action.

The aims of this chapter were to utilise a combination of single-channel and [³H]ryanodine studies to understand how nucleoside ligands bind to RyR2; and with the recent cryo-EM structures in hand, to rationalise the results using molecular modelling techniques to understand at a molecular level, how ATP regulates RyR2.

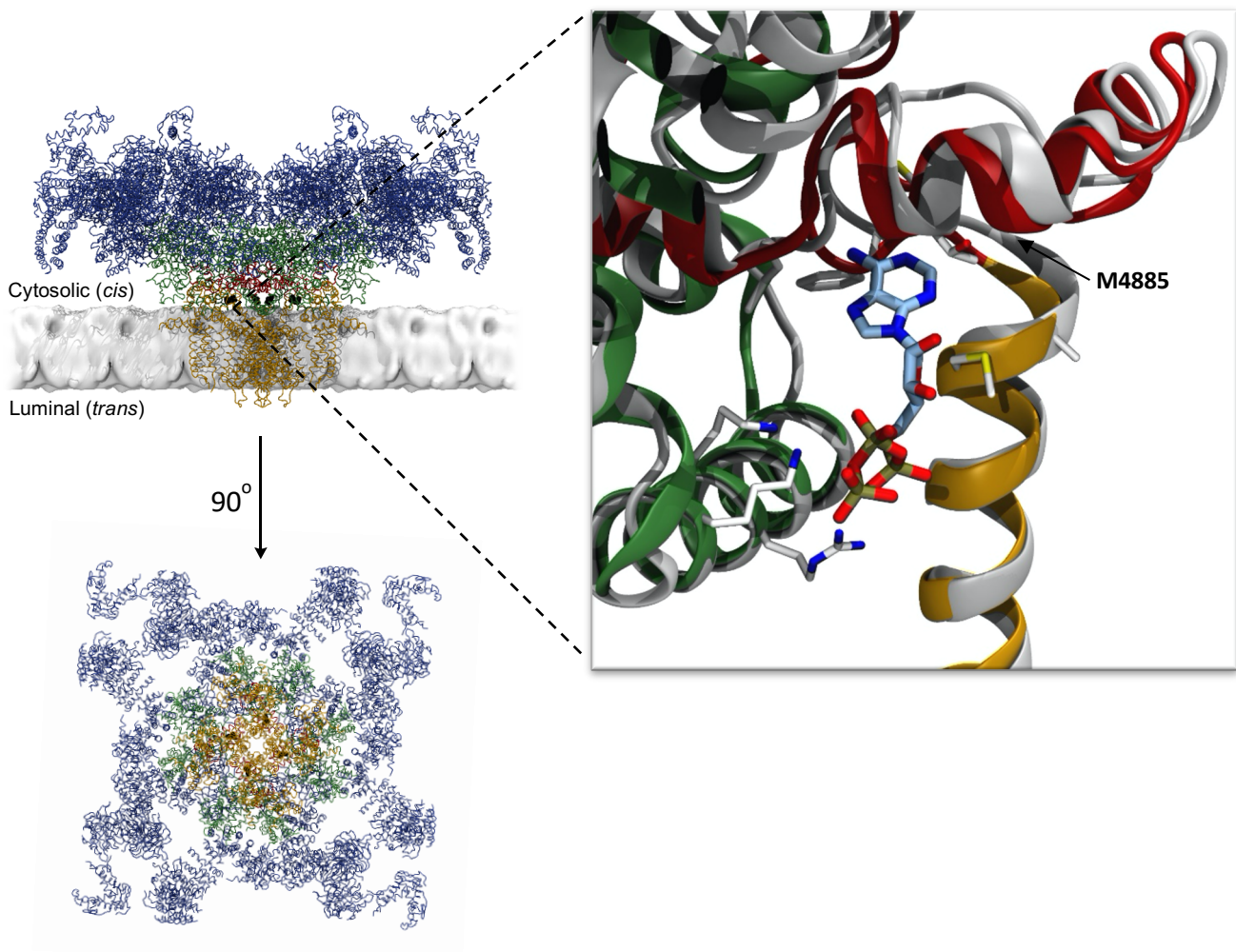


Figure 7.1 Comparison of the RyR1 and RyR2 ATP binding site.

The overall structure of RyR2 is shown (PDB-ID 5GOA) in cartoon representation. The lipid membrane is shown (top). The membrane position was taken from the Orientation of Proteins in Membranes database. Residue colours are taken from Des George *et al.* 2016. Residues 1-3636 are shown in blue, 3636-4207 in green, 4480-4888 (transmembrane domain) in orange and 4889-4967 (core terminal domain) in dark red. The ATP site in each monomer is indicated by a black dot. The inset to the right shows an expanded view of the site. Important residues are shown (including K4170, K4167 and R4171). RyR1 (PDB-ID 5TAS) is shown in grey cartoon representation for comparison, to illustrate the high degree of similarity of the ATP site of these proteins.

7.2 Results

7.2.1 Contribution by other members of the lab to the experiments described in this chapter

This chapter describes a study investigating how ATP and its analogues affect the gating of RyR2. The single channel data was collected by Dr Mano Sitsapesan, and the [^3H]ryanodine binding data was collected by Dr Wei Mun Chan, both between 2000 and 2002. These experiments are described in the results section which follows. Following the publication of full cryo-EM structures of both RyR1 and RyR2 in 2015, I then undertook a molecular modelling study to rationalise the experimental results collected by Drs Sitsapesan & Chan, and provide a structural explanation for how ATP activates RyR2 expanding upon the Sitsapesans groups previous work utilising CoFMA. The combined results of this study were published as Lindsay et al., *Promiscuous attraction of ligands with in the ATP binding site of RyR2 promotes diverse gating behaviour*, *Sci Rep*, vol. 8, no.1 (2018). The figures to which other authors contributed are listed in **Table 7.2**. The remainder of the data collecting in this chapter was collected by myself.

Figure 7.3	Single-channel data obtained by Dr Mano Sitsapesan
Figure 7.4	Single-channel data obtained by Dr Mano Sitsapesan and figures made by myself.
Figure 7.5	Single-channel data obtained by Dr Mano Sitsapesan and [^3H]ryanodine binding data collected by Dr Wei Mun Chan.
Figure 7.6	[^3H]ryanodine binding data collected by Dr Wei Mun Chan
Figure 7.7	Single-channel data obtained by Dr Mano Sitsapesan

Table 7.2 List of figures illustrating group data

The table lists the figures in this chapter to which other members of the lab contributed data.

7.2.2 Ligands investigated in this chapter

To probe the effect of adenine and phosphate based ligands on RyR2 activation, we studied the ligands shown in **Figure 7.2**. These include adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine (which lacks phosphate groups). Also shown is GTP (guanosine 5'-triphosphate) and the non-hydrolysable ATP analogue (β,γ -Methyleneadenosine 5'-triphosphate).

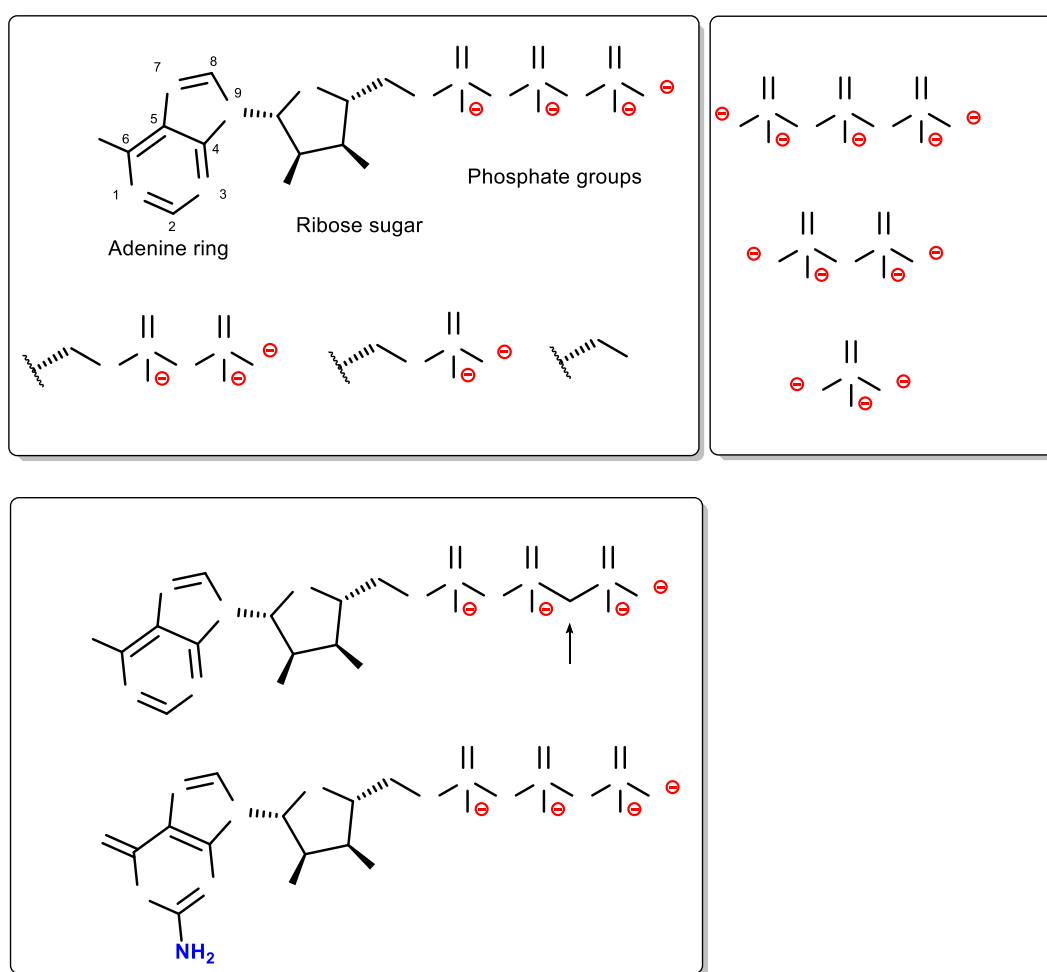


Figure 7.2 The ligands investigated for ability to activate RyR2.

A molecule of adenosine triphosphate (ATP) is shown with the adenine core, ribose ring and phosphate groups. The adenine-based ligands and phosphate ligands investigated in this chapter are shown, and their comparison to ATP are indicated

7.2.3 The dual effects of PPPi on single-channel gating

Dr M. Sitsapesan first investigated the effect of PPPi, that is, the phosphate groups alone, on RyR2 Po. This was achieved by addition of PPPi to the cytosolic side of planar lipid bilayers containing RyR2 channels in the presence of 10 μM Ca^{2+} , as shown in **Figure 7.3**.

It was found that PPPi alone, could activate RyR2 to a high Po and that the effect was reversible on washout back to control conditions. This type of activation was seen in 7 out of 15 experiments (47%) and the mean data from 7 channels are shown in **Figure 7.4**.

Figure 7.3 shows a typical RyR2 channel that was activated by PPPi. In control conditions (10 μM Ca^{2+}) only brief openings were seen. Upon addition of increasing concentrations of PPPi, an increase in channel openings was observed and a concurrent increase in the length of openings. After washout of the PPPi, the control channel behaviour is regained and the gating behaviour indistinguishable from that observed before the experiment.

The EC_{50} value for activation was calculated as 2.4 mM, with a Hill coefficient of 2.03, which suggests that two (i.e multiple) PPPi molecules must bind to RyR2 to achieve the maximum increase in Po. In all, a maximum Po of 0.791 ± 0.126 (SEM; $n = 7$) was observed when $[\text{PPPi}] = 10 \text{ mM}$. Further increases in PPPi concentration beyond 10 mM did not further increase Po. (**Figure 7.4**).

Note that when compared to previous studies, this activation effect is slightly lower than the maximum Po obtained with ATP under the same experiment conditons ($\text{Po} > 0.9$)¹⁵⁶ but that it is higher than that seen with the other adenine nucleotides containing fewer phosphate groups or less negative charge such as AMP-PCP (Po approx 0.4)³⁷⁶.

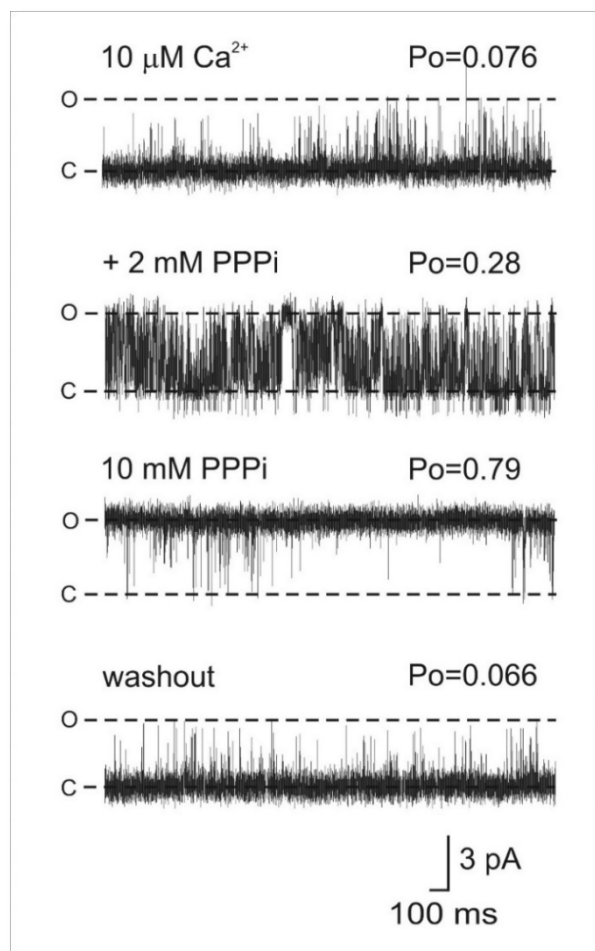
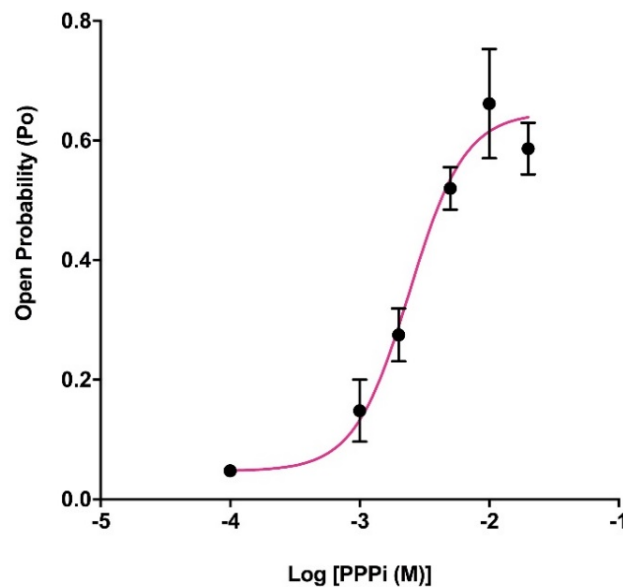


Figure 7.3 PPPi activates single cardiac RyR2 channels

Representative current fluctuations through a single RyR2 channel incorporated into a bilayer are shown. In the top trace the channel is activated by 10 μM cytosolic Ca^{2+} alone. 2 mM PPPi and 10 mM PPPi are then added sequentially to the cytosolic chamber as shown. After perfusing away the PPPi with 10 μM Ca^{2+} solution (washout), P_o returns to control levels. The holding potential was 0 mV. O and C represent open and closed channel levels respectively. The P_o above each trace refers to the value determined for the channel over 3 minutes of recording. These experiments were performed by Dr Mano Sitsapasan.

(A)



(B)

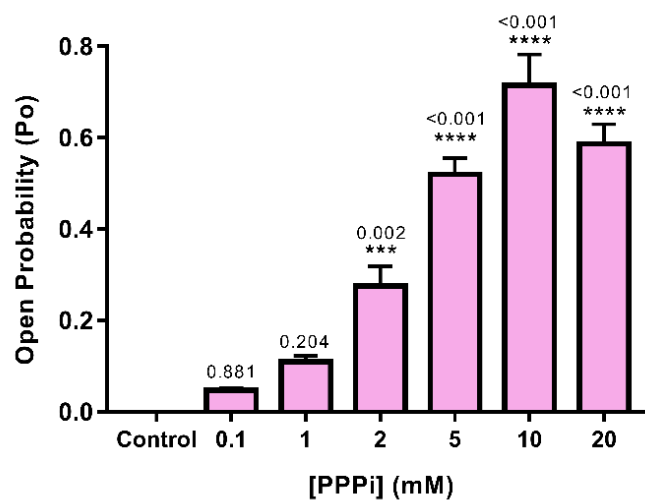


Figure 7.4 PPPi activates RyR2 in a dose dependent manner

(A) The relationship between PPPi concentration and RyR2 Po for channels where the effect of PPPi was activatory and reversible. Increasing concentrations of PPPi were added sequentially (B) The bar chart illustrates that the increases in Po in the presence of PPPi were highly significant at high concentrations. Mean values $\pm$ SEM are shown, and p values relative to control are indicated above each bar ($n = 6$, *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$). These experiments were performed by Dr Mano Sitsapesan, and the figures made by myself.

However, not all channels investigated appeared to respond in the same manner. In the other 8 experiments (53%), a different effect was seen. Irreversible inactivation was observed at high PPPi concentration. An example of this kind of inactivation is shown in **Figure 7.5A**. Treatment with 10 mM PPPi reduced the P_o to 0 with only very occasional brief openings being observed. This effect was seen after approximately 150 s (range 56 – 143 s) exposure to PPPi. Upon washout of the cytosolic chamber, the inactivation did not appear to be reversible, with no increase in P_o seen even after several minutes of washout.

To investigate how PPPi treatment affects a population of channels, [^3H]ryanodine binding was used. **Figure 7.5B** shows the effect of PPPi on [^3H]ryanodine binding, as compared to ATP. We can see that PPPi was much less effective than ATP at stimulating [^3H]ryanodine binding to cardiac SR vesicles. The maximum level of binding was lower, and the EC_{50} higher. This appears to be consistent with the dual effects of PPPi, with some channels being promoted to open, and others being irreversibly inactivated.

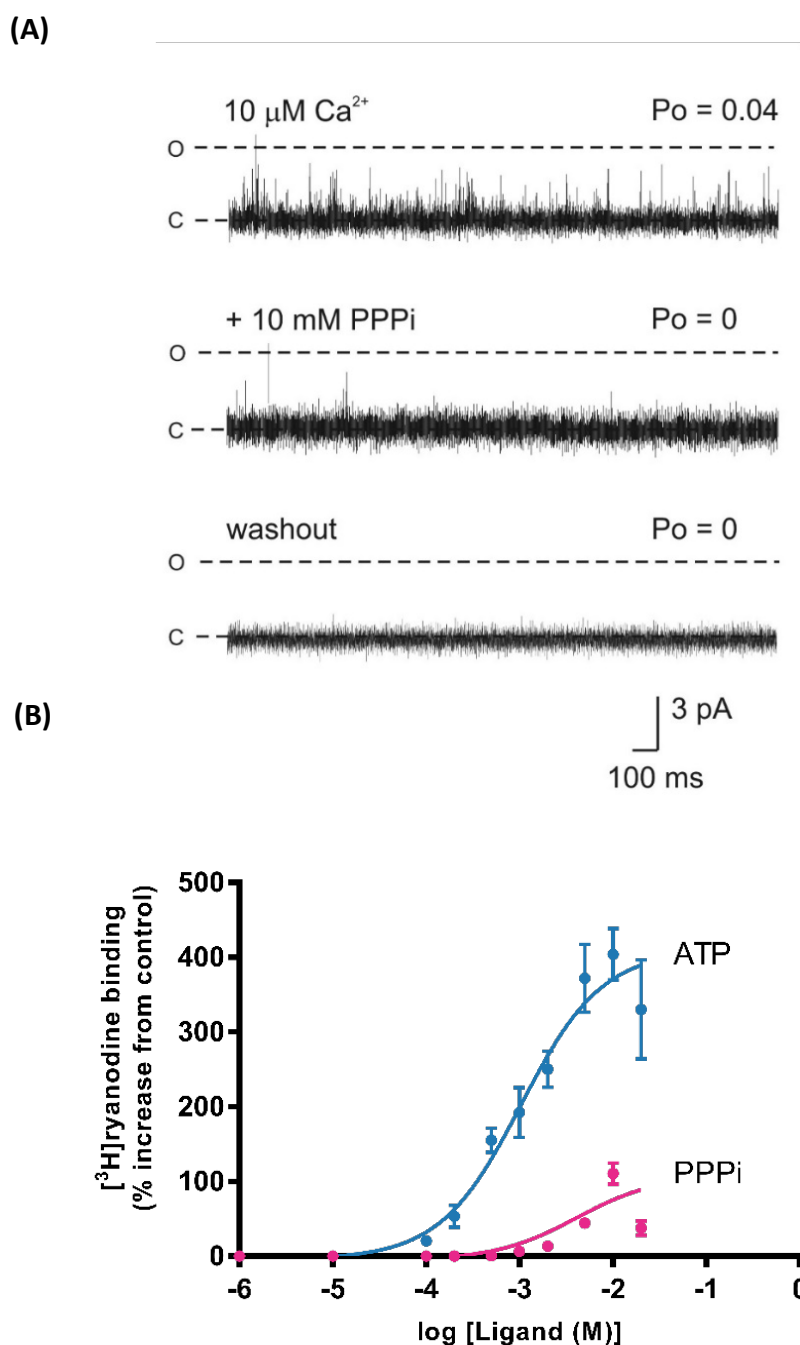


Figure 7.5 PPPi also causes RyR2 inactivation

(A) The top trace shows a typical single RyR2 channel activated by 10 μM cytosolic Ca^{2+} alone. The subsequent trace shows an example of the inactivating effects of 10 mM PPPi. Occasional, very brief openings were observed initially as shown in the middle trace but after 140 s no further openings were detected. The bottom trace shows that after perfusing away the PPPi from the cytosolic chamber, the channel remained shut (no openings during the 3 min recording period). The holding potential was 0 mV. O and C represent the open and closed channel levels respectively.

(B) Comparison of the stimulation of [^3H]ryanodine binding to vesicles of sheep cardiac heavy SR by ATP (blue) and PPPi (pink). The results are expressed as a percentage of the control binding at 10 μM cytosolic Ca^{2+} which was 0.18 ± 0.02 pmol [^3H]/mg protein (SEM; $n = 10$). Where not shown, error bars are within the symbol. The maximum stimulation of [^3H]ryanodine binding was significantly greater for ATP than for PPPi ($p = 0.0065$). These experiments were performed by Drs Mano Sitsapesan & Wei Mun Chan.

7.2.4 A fragment based approach to ATP RyR2 activation

7.2.4.1 *[³H]ryanodine binding*

Given the finding that PPi alone could activate RyR2 channel openings, but it was not as effective as ATP, M. Sitsapesan & Chan investigated whether a fragment based experimental approach would reveal which functional groups are essential for binding to RyR2. The question was, could different fragments making up the whole ATP molecule, when added together, promote the same channel behaviour as a full ATP molecule? [³H]ryanodine binding was used to investigate this, and the results are shown in **Figure 7.6**. In each case, a comparison was made between the [³H]ryanodine binding resulting from incubation of cardiac SR vesicles with either ADP, AMP or adenosine, and their respective phosphate groups (Pi, PPi and PPPi). In each case, the [³H]ryanodine binding found with ATP at the same concentration is indicated with a blue line.

It was found, consistent with previous single-channel studies^{156,402}, that the adenine ligands with fewer than three phosphate groups were not as effective as ATP in stimulating [³H]ryanodine binding. Adding the complementary phosphate group in the case of ADP + Pi, did not appear to have any effect on binding, as the maximum level of binding with ADP + Pi was indistinguishable from that of ADP alone. In the case of AMP + PPi there was a small increase in binding, which appears to be approximately additive of the effects of AMP alone and PPi alone, but this was still not comparable with ATP. However, in the case of adenosine + PPPi, the level of [³H]ryanodine binding produced by these ligands together, is broadly similar to that seen with ATP. The effect is not exactly the same however, with the maximum level of binding being slightly lower than that of ATP alone and the concentration-response relationship appears to be slightly left-shifted. Since the effect of adenosine + PPPi appears to be similar to that of ATP, this raised the question – can adenosine prevent PPi induced inactivation in a single-channel context?

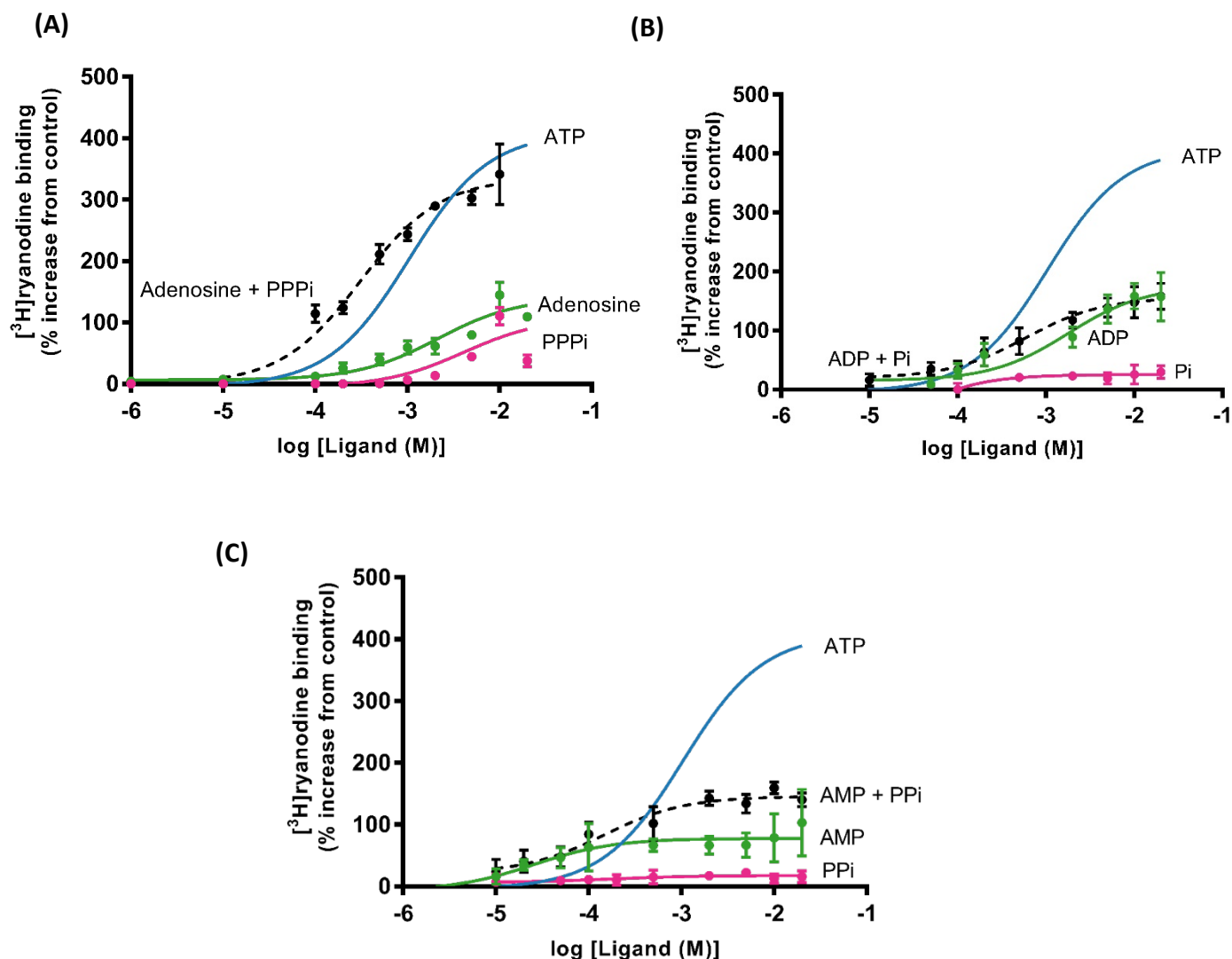


Figure 7.6 Investigating the ability of ATP fragments to stimulate $[^3\text{H}]$ ryanodine binding as effectively as ATP alone.

For clarity, in each graph, the ATP data from Figure 7.6(B) is shown as a solid blue line. **(A)** Comparison of the effects of PPPi alone (pink), adenosine alone (green) and adenosine in the presence of 10 mM PPPi (black dashed line). **(B)** Comparison of the effects of PPI alone (pink), AMP alone (green) and AMP in the presence of 10 mM PPI (black dashed line). **(C)** Comparison of the effects of Pi alone (pink), ADP alone (green) and ADP in the presence of 10 mM Pi (black dashed line). Where combinations of ligands were used, they were added simultaneously at the start of the incubation period. The addition of Pi to ADP and PPI to AMP, did not significantly increase binding above that observed with ADP ($p = 0.2101$) or AMP ($p = 0.1917$) alone whereas the addition of PPPi significantly increased binding above that observed with adenosine alone ($**p < 0.01$ (0.0065)). The error bars indicate SEM ($n = 5$). Where not shown, error bars are within the symbol. The experiments were performed by Dr Wei Mun Chan and the figures made by myself.

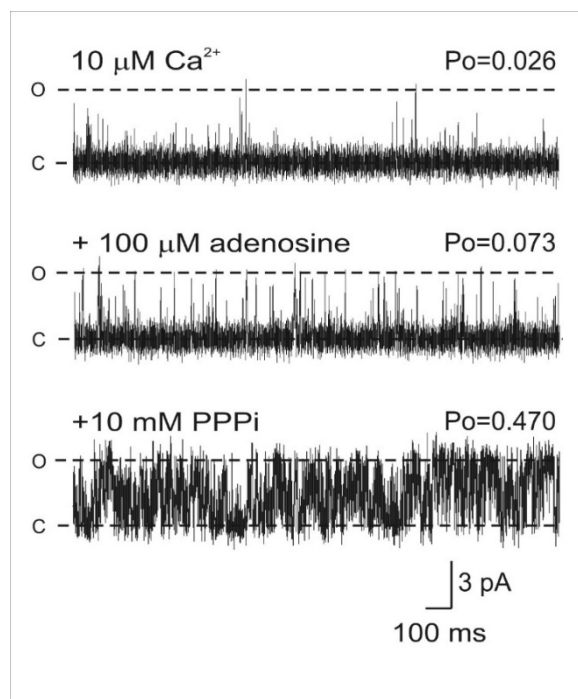


Figure 7.7 Adenosine protects against PPPi induced inactivation

Current fluctuations through a typical single RyR2 channel recording in the presence of 10 μM Ca²⁺ (top trace), after addition of 100 μM cytosolic adenosine (middle trace) and the subsequent addition of 10 mM cytosolic PPPi in the continued presence of 100 μM adenosine (bottom trace). The holding potential was 0 mV. O and C represent the open and closed channel levels, respectively. These experiments were performed by Dr Mano Sitsapesan

It was found that, consistent with previous reports³⁷⁴ adenosine causes a small increase in RyR2 Po. Po increased from control conditions (0.038 ± 0.009 ; SEM, $n = 6$) to (0.068 ± 0.014 ; SEM, $n = 6$) in the presence of 100 μM adenosine. Subsequent addition of 10 mM PPPi caused activation and an increase in Po (0.572 ± 0.107 ; SEM, $n = 5$). Higher concentrations of adenosine were also investigated (1 mM, 10 mM) and channel inactivation was not observed in any of 16 experiments (**Figure 7.7**), indicating that pre-treatment with adenosine can protect against PPPi-induced inactivation of RyR2.

Table 7.3 shows the mean RyR2 Po in the presence of 100 μM, 1 mM and 10 mM adenosine before and after subsequent addition of 10 mM PPPi.

Adenosine concentration	Mean RyR2 open probability (Po) $\pm$ SEM	
	Adenosine alone	Adenosine + 10 mM PPPi
100 μ M	0.068 $\pm$ 0.014 (n = 6)	0.374 $\pm$ 0.056 (n = 6)
1 mM	0.080 $\pm$ 0.032 (n = 5)	0.331 $\pm$ 0.099 (n = 5)
10 mM	0.155 $\pm$ 0.081 (n = 5)	0.572 $\pm$ 0.107 (n = 5)

Table 7.3 Effect of adenosine on the ability of PPPi to activate RyR2

To avoid inaccuracies in the [adenosine] due to the limits of adenosine solubility, 1 and 10 mM adenosine were perfused into the cytosolic chamber following fusions of the SR vesicles and therefore it is not possible to determine a 'control' value in the absence of adenosine (although comparison with other channels at 10 μ M cytosolic Ca²⁺ could be made). 100 μ M adenosine was obtained by addition of a 10 mM stock solution of adenosine, and in this case the control Po in the presence of 10 μ M Ca²⁺ alone was 0.038 $\pm$ 0.009 (SEM, n = 6). Inactivation was not observed in any experiment. These experiments were performed by Dr Mano Sitsapesan.

7.2.4.2 Molecular Modelling

However, does the RyR1 cryo-EM provide a basis for understanding how ATP might interact with the cardiac isoform, RyR2? An ATP-bound RyR2 structure has not yet been determined, however there are indications that the proteins share a high degree of homology in terms of ATP binding. To further understand the varied gating effects of ATP-like ligands, I conducted a molecular modelling study. The goal of this approach was to see whether the hypothesis suggested by M. Sitsapesan & Chan, namely that multiple phosphate and adenine-based nucleotides could bind together in the RyR2 ATP site, was structurally feasible. With the publication of a full cryo-EM structures for RyR2, I could use the docking software, Autodock²⁹¹, to assess both the estimated binding energies and the probable binding modes of ligands when bound in RyR2-ATP site, using the RyR1 structure as a model. The highest resolution cryo-EM structure of RyR2 in the open state (PDB ID: 5GOA) from Peng *et al.*²⁶³ was selected as the best model of RyR2. Taking the Des George *et al.* RyR1 structure as a model, I investigated all residues which are within a 10 Å radius of the ATP molecule, of which there are 50. Comparing RyR1 and RyR2, only three of these residues differ between RyR1 (rabbit) and RyR2 (pig), these are: M4207 (K4163 in RyR2), S4965 (N4896 in RyR2) and E4981 (Q4912

in RyR2). Of these three residues in RyR1, none appear to make a direct contact with the bound ATP molecule, and in fact, their side-chains are orientated away from it. Furthermore, comparing these structures (PDB IDs 5TAS and 5GOA), the root-mean-square deviation (RMSD) between the C α atoms of these residues in the ATP site was 1.84 Å (i.e. < 2 Å), confirming that the site is also structurally conserved. We can therefore conclude that the ATP binding site is highly conserved between RyR1 and RyR2, and that therefore the RyR1-ATP bound Des George *et al.* structure is also a suitable model for investigating RyR2. The first step in the docking analysis was to verify that this approach could reproduce, in RyR2, the binding mode observed in the RyR1 cryo-EM structure with ATP bound as reported by des George *et al.*¹⁷¹ (PDB ID: 5TAS). The protein and ligands were prepared for docking as described in the Methods **Section 2.9**.

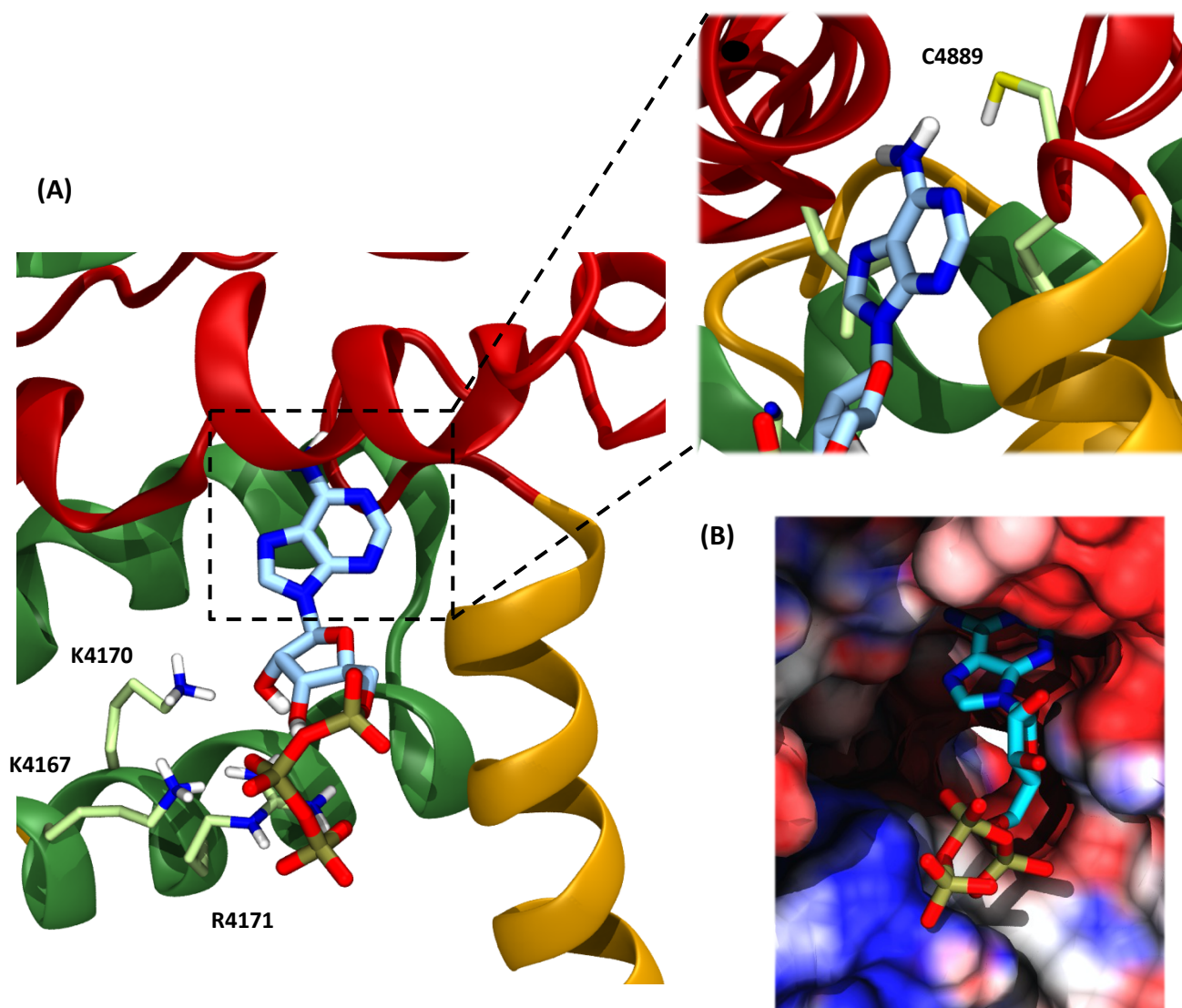


Figure 7.8 The RyR2 ATP site

(A) The RyR2 ATP site is expanded to show the Autodock-predicted pose for ATP which is most similar to the ATP binding mode observed in the RyR1 cryo-EM structure (*Des George et al. 2016*). Important residues are indicated (including K4170, K4167 and R4171). The inset shows an enlarged view of the adenine binding region with the C4889 residue shown. **(B)** The RyR1 (PDB-ID 5TAS) ATP site shown in surface representation with an ATP molecule bound. The surface is coloured by electrostatic potential, going from dark blue (most positive), through white (neutral) to red (negative.). It can be seen that the ATP site consists of a hydrophobic pocket which binds the uncharged adenine portion of the ATP molecule, lined with a positively charged region which coordinates the negatively charged phosphate groups.

Ligand	Mean binding energy of most ATP-like cluster (kcal/mol)	No. of clusters (different binding modes among the 50 generated poses)	No. of results in the most ATP-like cluster/binding mode
ATP	-2.60	38	4
ATP (+ Zn)	-3.25 [#]	33	6 [#]
PPPi (deep in site)	-3.55	11	4
PPPi (on edge of site)	-2.95 [*]	11	22 [*]
Adenosine	-1.07	28	3
AMP	-0.85	31	2
ADP	-0.73	32	2
AMP-PCP	-1.73	33	2
GTP	+3.63	34	2
Phosphate docking with nucleoside already bound			
PPPi (+ adenosine)	-5.34	11	24
PPi (+ AMP)	-3.39	5	31
Pi (+ ADP)	-2.90	8	20

Table 7.4 Docking scores of ATP-like ligands

Shown are the mean binding energies (kcal/mol) of the most ATP-like cluster of results predicted by Autodock for the various ligands listed (column 2), the total number of clusters (i.e binding modes grouped together by similarity, column 3), and the number of poses that were in the most ATP-like cluster (column 4). The table provides an indication of the extent to which the results predicted by Autodock were similar to the RyR1 ATP-bound des Georges *et al* (2016) structure.

^{*}PPPi has two different interesting binding modes. The first-mentioned is the lowest-energy one which is fairly deep in the adenine pocket. In the second-mentioned cluster, the triphosphate occupies a position in which it would overlap with the triphosphate of ATP.

[#]For ATP (with Zn²⁺ bound to the protein) none of the poses obtained were 'ATP-like' when compared with the des Georges *et al.* (2016) RyR1 ATP-bound RyR1 structure. In this case we therefore report the energy of the lowest energy cluster.

Initially, attempts to reproduce the binding mode of ATP seen in RyR1 could not be successfully reproduced in RyR2. I observed a wide variety of binding modes, none of which appeared to share a similarity with the orientation adopted in the RyR1 cryo-EM. This inspection was done visually, comparing the orientation of the adenine core and phosphate groups of the ATP molecule and the residues in the site that they appeared to interact with.

None of the binding modes seen appeared to have the adenine core of ATP bound in the hydrophobic pocket, with many binding modes appearing to be structurally unfeasible.

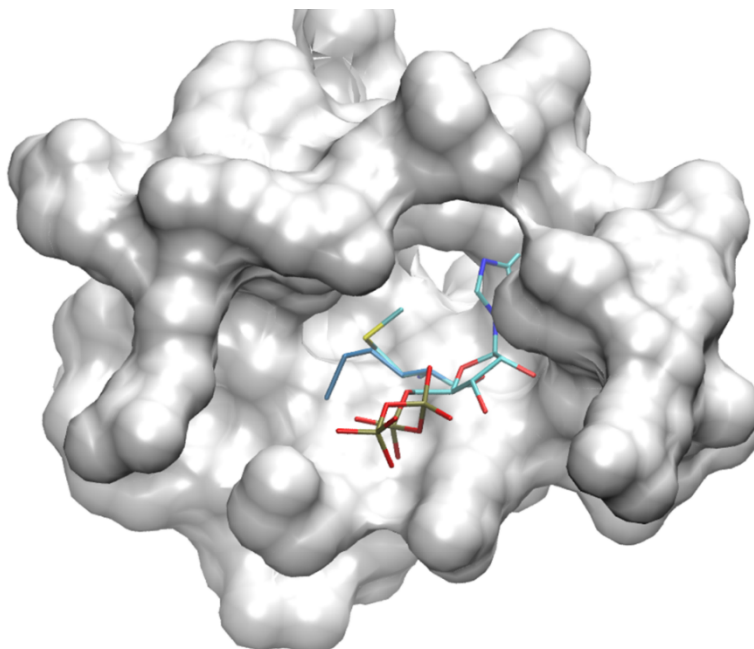


Figure 7.9 The M4885 residue was rotated to facilitate docking of ATP.

The ATP pocket is shown with the M4885 indicated as it is in the 5GOA structure (light blue) and the subsequent rotation (yellow/light-blue) following use of the Pymol 'rotamer' tool.

Closer inspection of the ATP site revealed an explanation for this initial lack of success. The RyR ATP site contains a methionine residue M4885, which in the apo-RyR2 structure appeared to be blocking access to the hydrophobic pocket. This residue can be seen in **Figure 7.1** (indicated in yellow) and in RyR1 it appears to be orientated away from the ATP molecule. To correct this, the 'rotamer toggle' functionality of Pymol²⁹³ was used to select an alternative rotamer of this residue that was more similar to that observed in the RyR1 structure. The original position of this residue and the subsequent rotation are shown in **Figure 7.9**. I found that subsequent attempts to dock ATP revealed binding modes which were much more similar to the ATP binding mode experimentally observed in RyR1¹⁷¹. In these binding modes the adenine core of the ATP molecule was buried in the hydrophobic pocket as we would expect based on the cryo-EM, and the negatively charged phosphate groups were now positioned to

interact with the positively charged residues which surround the pocket (K4167, K4170 and R4171). One of these binding modes is illustrated in **Figure 7.8**.

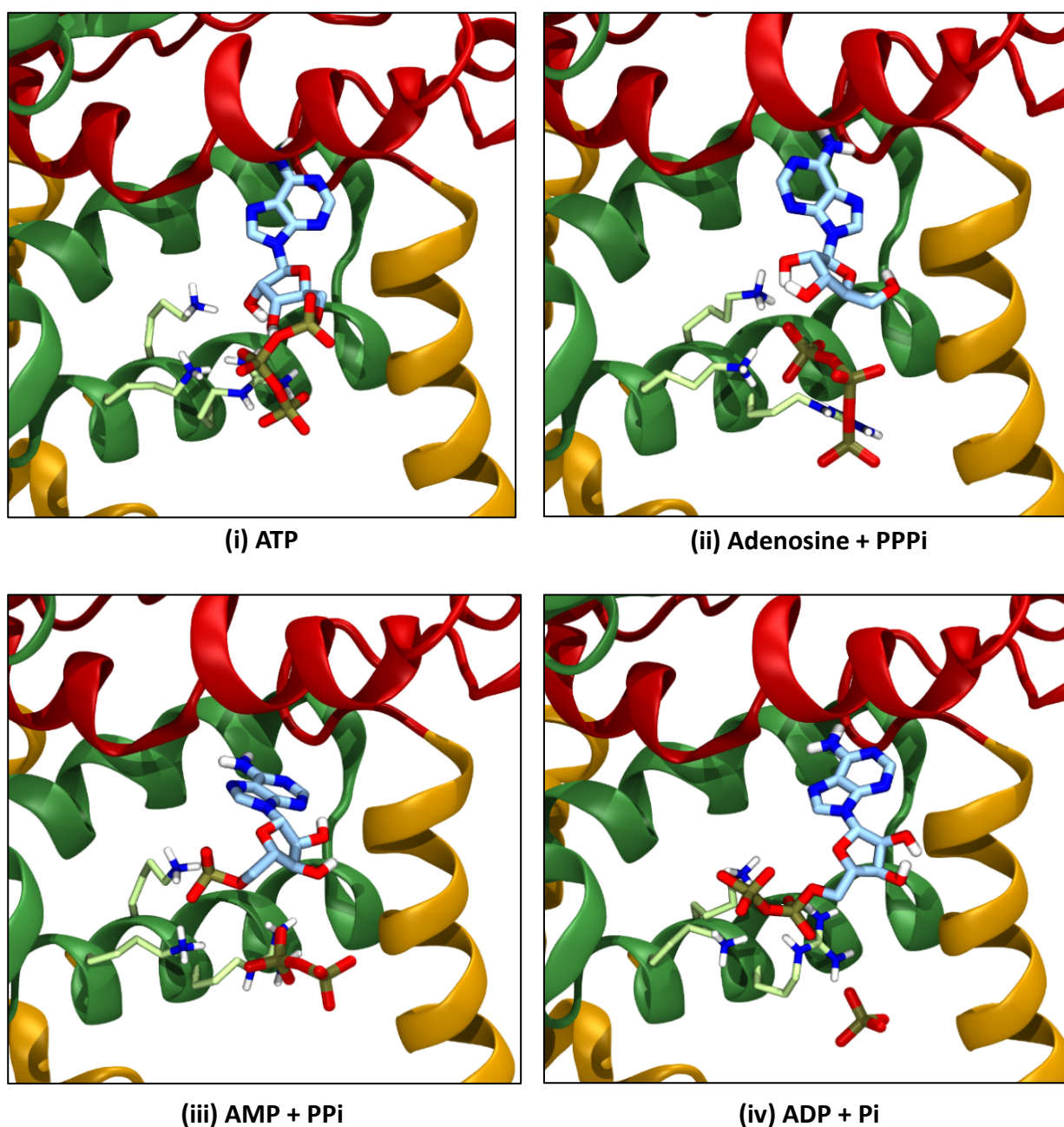


Figure 7.10 Docking of complementary fragments of ATP

The RyR2 ATP binding site with the most ATP-like docking results for **(i)** ATP, **(ii)** adenosine and PPi, **(iii)** AMP and PPi, and **(iv)** ADP and Pi. In each case, the nucleoside ligand was docked first, the most ATP-like pose was adopted as part of the protein, and the phosphate ligand subsequently docked into the same site.

We can also observe that the N atom adjacent to the amino group of the adenine base of ATP (**Figure 7.8(A) inset**) is positioned in a suitable orientation to support a hydrogen bonding interaction between this atom and the C4889 residue (bond angle 124.5° and bond length 2.5 Å). This interaction may therefore be responsible for directing the adenine nucleotide to bind in this pocket. In the case of RyR1, Yan *et al.* noted the presence of a zinc-finger motif in the C-terminal subdomain, in close proximity to the adenine core of the bound ATP molecule²⁵⁸. Importantly, this is a feature also present in RyR2. Given this close proximity, and the affinity of sulfur containing groups (such as cysteine) for Zn^{2+} ions⁴⁰³, I also investigated whether retaining the bound Zn^{2+} in the structure was important for the docking of the ATP molecule. It was found that removing the bound Zn^{2+} from the RyR2 protein structure, did not appear to affect the docking of the ATP molecule (**Table 7.4**). The binding energies with Zn^{2+} were slightly lower than without, but the poses generated for the ATP molecule appeared to be more similar to the RyR1 cryo-EM without Zn^{2+} , than with it bound. The Zn^{2+} was therefore removed for further docking calculations.

It is important to stress here that this experiment does not provide evidence as to whether the Zn^{2+} itself is important physiologically for ATP binding. Indeed, Zn^{2+} has been found to be constitutively bound to RyR2, even when the concentration of Zn^{2+} in the surrounding cytosol is 100 pM or less.⁴⁰⁴ All that is being assessed is whether the presence or absence of Zn^{2+} produces binding modes more similar to that observed in the cryo-EM. Additionally, in this docking model, the residues around the zinc-finger domain were not treated as flexible, and therefore their position will be unaltered by the presence or absence of Zn^{2+} in the structure. The experiment does, however, show whether the charge from the Zn^{2+} ion in any way affects the docking of the ATP.

Having established docking parameters which could satisfactorily reproduce the binding of ATP to RyR2, my next objective was to use this model to explain the binding of the other

adenine nucleotides used in this study. My first aim was investigate if these other nucleotides could bind in a similar manner to ATP, and if so, to focus on those poses which bind in the same manner as that seen by des George *et al.*¹⁷¹ Applying the same docking protocol as had been used for ATP, I found that for ADP, AMP and adenosine the same binding modes could be observed as I had seen for ATP. Namely, that the lowest energy modes that the docking model predicted shared the same orientation of the adenine core being bound in the hydrophobic pocket and the phosphates (if present) interacting with the positively charged lysine and arginine residues. For each ligand, I selected the pose which most closely resembled the experimentally determined RyR1-ATP bound structure (which I will refer to as 'ATP-like'). These I have reported in **Table 7.4** along with the predicted docking energy, and the number of times that pose was predicted by Autodock. The lowest energy 'ATP-like' poses for each nucleotide are shown in **Figure 7.10**.

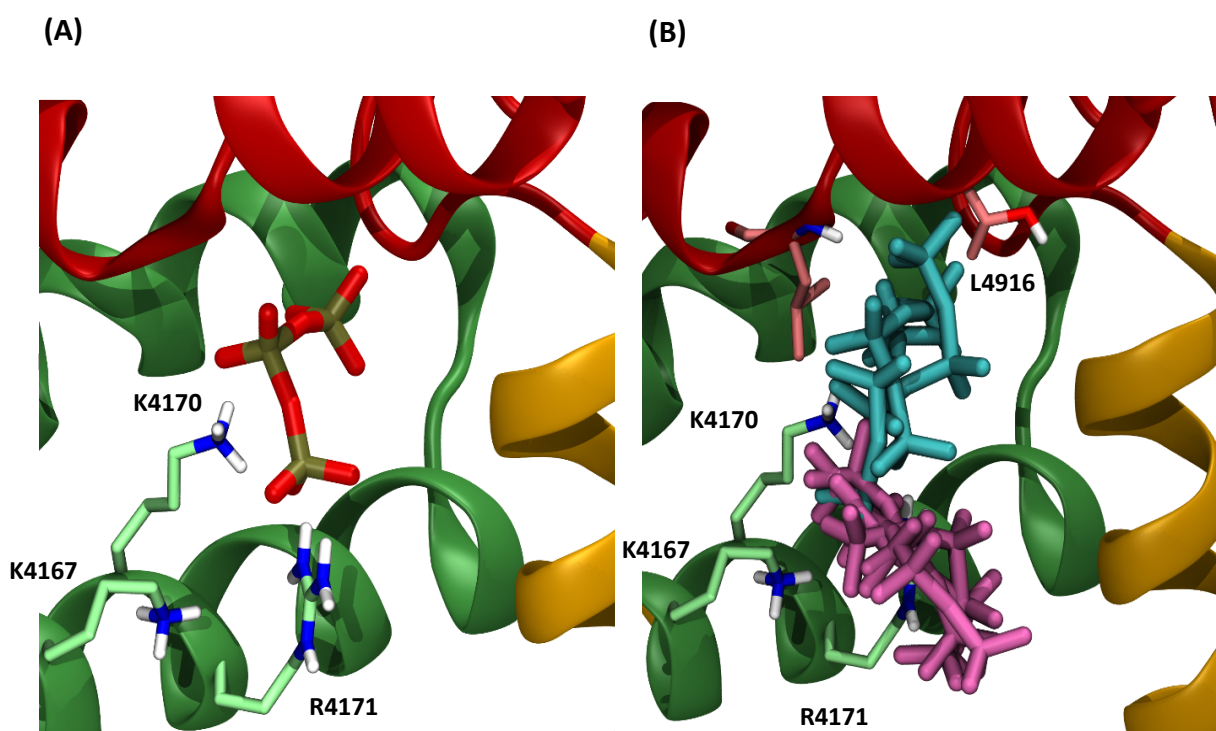


Figure 7.11 PPPi docking in the RyR2 ATP site

(A) A representative pose from the lowest energy cluster for docking of PPPi into the RyR2 ATP-site. The residues R4171, K4167 and K4170 are shown. **(B)** Poses from two clusters for docking PPPi into the RyR2 ATP-site. Four poses from each cluster are shown, the ATP-like cluster is shown in pink, while the cluster binding deeper in the pocket (with lower energy) is shown in cyan. Residues that appear to interact with these poses are also shown.

I also investigated how PPi itself could bind in the ATP site, using the same docking protocol. When PPi was docked alone, and therefore not connected to an adenine nucleotide, the binding poses were varied, and did not always replicate the position of the triphosphate groups of a bound ATP molecule. That is, they were not always positioned to interact with the R4171, K4167 and K4170 residues. The binding modes could be broadly grouped into two clusters, shown in **Figure 7.11**. In the lower energy binding mode of the two, PPi appeared to be bound in the hydrophobic pocket, within 2 Å of the L4976 and T4910 residues, in a suitable position for hydrogen bonding. This is a position similar to that typically occupied by the adenine core of ATP (**Figure 7.11(A)**). The other dominant binding mode contained poses where the PPi was clustered around the positively charged lysine and arginine residues lining the site. This mode is more similar to how the triphosphate groups of a full ATP molecule bind. In order to rationalise the [³H]ryanodine binding data obtained with multiple ligands together, my next aim was to use these docking results to indicate whether the complimentary phosphate ligand could also bind in the ATP site once the relevant nucleotide was already bound. To achieve this, I selected the lowest energy, most ‘ATP-like’ pose for each nucleotide as already shown, and this nucleotide was fixed as part of the RyR2 protein. This then allowed the docking simulation to be run a second time using a phosphate ligand, with the respective adenine nucleotide already bound in the site. For example Pi, was docked into a structure already containing ADP bound. This process was repeated for each nucleotide and phosphate combination.

The effect of adding these multiple ligands together is shown in **Figure 7.10**. **Figure 7.10** compares the lowest energy ‘ATP-like’ pose of the full ATP molecule with those already obtained by docking the other adenine nucleotides with subsequent docking of their complementary phosphate groups. We can observe that having ADP already bound in the site, appeared to obstruct Pi from being able to closely interact with the positively charged residues lining the pocket. This effect was also seen with AMP and PPi. However, when adenosine was

bound in the pocket, it appeared that PPPi was now able to closely associate with the positive residues, and that the combination of having adenosine and PPPi bound together appeared to be similar to that of a full ATP molecule.

7.2.4.3 *Effect of ATP fragments on RyR2 channel properties*

While the docking model has provided useful insight into how these molecules can bind to RyR2, we must turn to the single-channel data to understand how the ligands functionally effect the channels. **Figure 7.12** shows the maximum Po, and mean open time of RyR2 obtained with the ligands in this study. For comparison, this figure also includes data collected previously in the group, performed under the same experimental conditions^{156,374,376,392}. As previously demonstrated, ATP is the most efficacious ligand at inducing high RyR2 Po, This is because ATP is also the most effective ligand at increasing the duration of channel openings.^{156,374,405}

Finally, I assessed the relationship between the predicted binding energies generated for each ligand by Autodock, and these channel parameters. The correlation between each is shown in **Figure 7.13**. We can see that the predicted docking energies have a positive correlation with both maximum Po and mean open time ($R^2 = 0.6188$ and 0.6755 respectively).

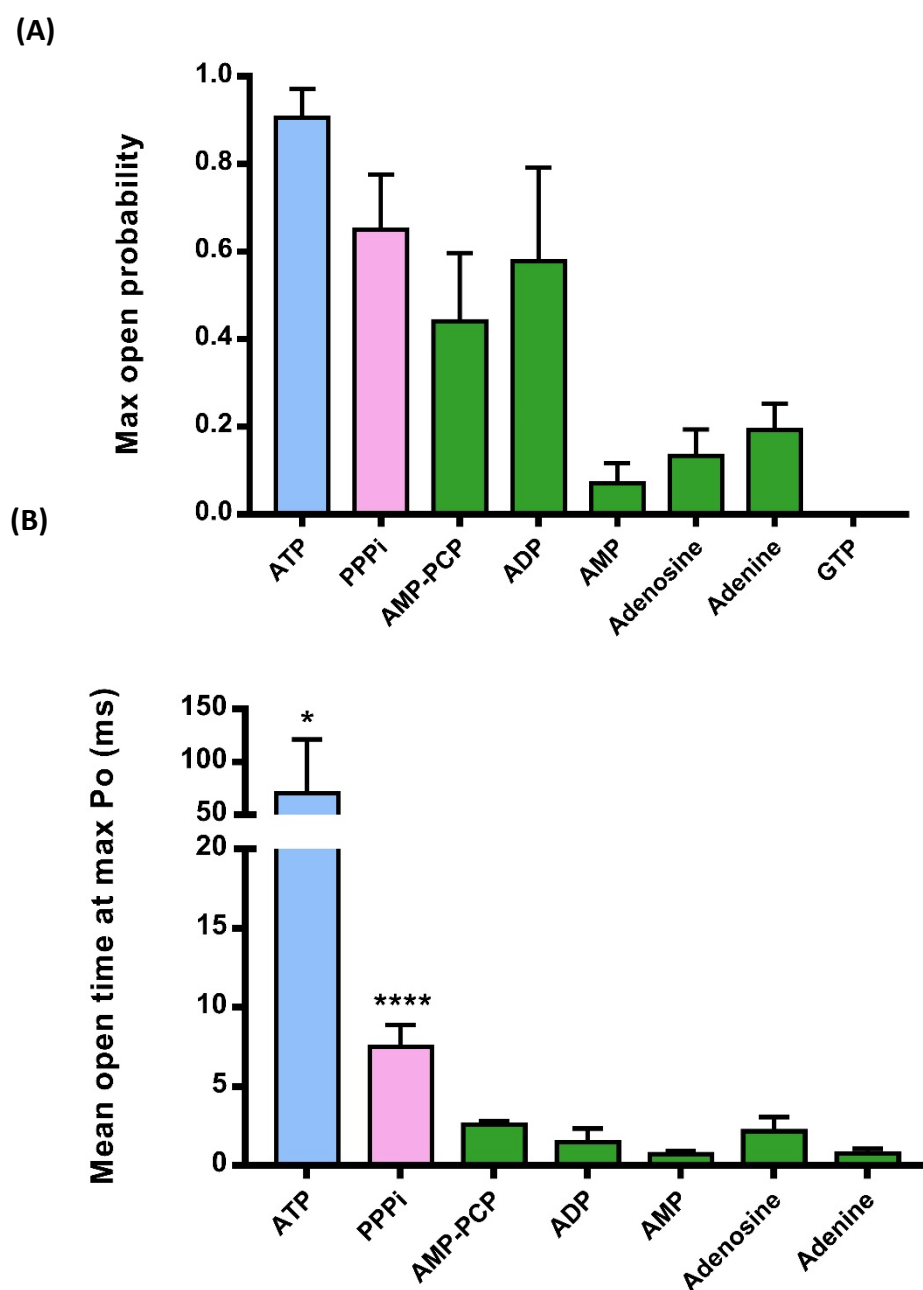
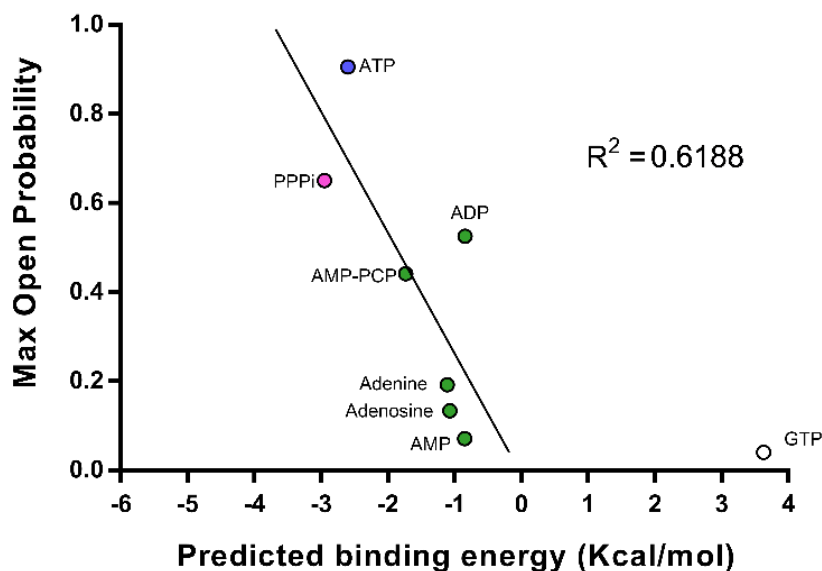


Figure 7.12 Effect of adenosine based nucleotides on RyR2 max open probability and mean open time.

(A) Comparison of the ability of ATP and other adenine or phosphate containing molecules to increase P_o . The maximum P_o obtained in single-channel experiments (with 10 μM free Ca^{2+}) by the compounds indicated in the bar chart is shown. (B) The mean open time at maximum P_o for ATP and various fragments or analogues of ATP is shown.; $n = 4$; Mean $\pm$ SEM is shown; $*P < 0.05$; $****P < 0.0001$; The data includes results from Dr Mano Sitsapesan and from previous work from the Sitsapesan group on adenine nucleotides performed under identical experimental conditions. The experiments not previously reported were performed by Drs Mano Sitsapesan & Wei Mun Chan, and the figures made by myself.

(A)



(B)

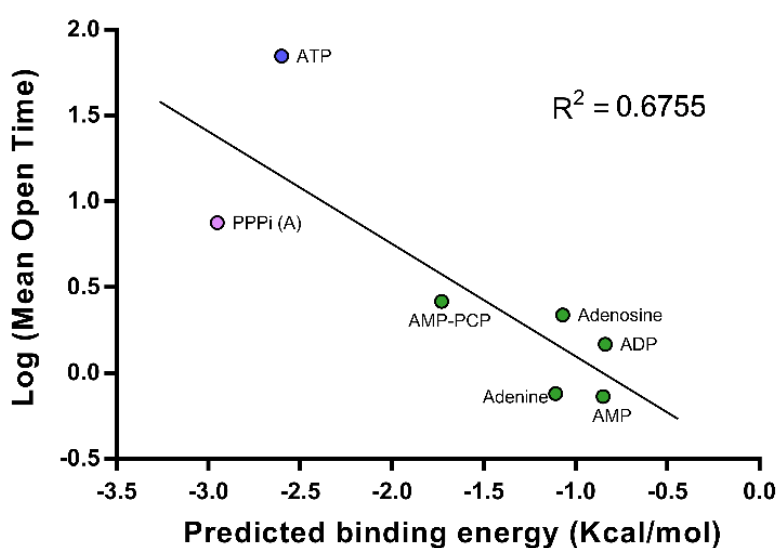


Figure 7.13 Relationship between max open probabilities, mean open time and predicted binding energy

Correlation between the maximum Po (A) or the mean open time (B) obtained for each ligand and Autodock predicted mean binding energies (kcal/mol) for the various ligands listed. As GTP did not bind in the ATP site, the point is shown, but the value has not been included in the statistical analysis. The Pearson correlation coefficients (R^2) were 0.6188 and 0.6755 respectively.

7.3 Discussion

ATP is crucial physiological regulator of RyR2, capable of driving the channel into high Po states.¹⁵⁶ While the binding of ATP is essential for proper regulation of the channel, the molecular mechanism of how ATP binds has been poorly understood. I have shown, consistent with and extending on previous studies, that this effect depends both on the adenine core of the nucleotide, as well as the triphosphate moiety. By constructing a model of the RyR2 ATP binding site, based on the cryo-EM structures of RyR1 with ATP bound, I have been able to rationalise how ATP fragments can work together to promote channel openings, and to explain their functional effects.

The single channel results obtained from the ligands in this study clearly indicate that triphosphate, in the form of PPPi is capable of having multiple effects on RyR2 channel gating; it can activate some channels to relatively high Po levels while irreversibly inactivating others. This effect is not immediately intuitive, since we know that GTP (itself containing three phosphate groups) is ineffective at activating RyR2^{374,391}, and the adenine core thought to be essential for achieving high RyR2 Po, as other nucleotides were similarly inactive³⁷⁶. Despite this, PPPi was able to increase RyR2 Po to approximately 0.6, a level not seen even with ligands such as ADP (lacking only one phosphate group). Thus after ATP, PPPi is the next most effective ligand at increasing RyR2 Po. It is also important to understand the mechanism by which Po is increased. **Figure 7.12** shows that ATP can lengthen the mean open time from approximately 1 ms (in control conditions of 10 μ M Ca^{2+}) to 70 ms and because of this property can almost fully open the channel. PPPi is the only other ligand that can increase mean open time above 5 ms and even this effect is rather minor in comparison to that of ATP. Additionally, as neither Pi or PPi appear to be effective, it seems that three phosphates linked together is the ideal molecular motif to increase RyR2 Po^{156,406}.

The dual effects of PPi were also surprising, as in approximately 50% of experiments this molecule appeared to activate RyR2, while in the other half it appeared to irreversibly inactivate the channel. The fact that irreversible inactivation was not observed with PPi concentrations below 10 mM does not necessarily mean it does not occur. It is likely that the rate of inactivation has a concentration and time dependence, and therefore at lower concentrations, may be too slow to be observed on the timescale of bilayer experiments (several minutes). Yet, in the presence of adenosine this inactivation was prevented, and only activation observed. The molecular modelling results suggest a possible explanation for this. We can see from the docking results of PPi alone (**Figure 7.11**) that when PPi is not connected to an adenine nucleotide, it preferentially binds in one of two locations within the ATP binding pocket. One of these favourable locations is surprisingly deep within the pocket, reducing its potential for interacting with the positively charged residues that line the pocket. Irreversible binding in this position may therefore result in channel closure, or the closed state becoming so stable that the energy barrier to channel opening is too high to overcome by the binding of other ligands. In the single channel experiments, pre-addition of adenosine protects against this irreversible inactivation. In addition, the observation that in the presence of adenosine, channel opening is promoted, suggests that adenosine directs the PPi molecule into a more favourable binding site for causing channel opening. I suggest that adenosine fills the site where the adenine portion of the ATP molecule would bind and blocks this site, preventing PPi from binding there. Therefore since one of PPi's two preferred binding locations is occupied, the other is then increasingly preferred. This means that the PPi is effectively directed to bind closer to the positively charged residues that the triphosphate would have interacted with, had it been part of an ATP molecule. This would explain why the addition of adenosine and PPi together can increase channel openings to a level approaching that of ATP. PPi is also more effective at stimulating [³H]ryanodine binding in the presence of adenosine and one explanation for this is that when both adenosine and PPi dock into the ATP binding site, PPi no longer irreversibly closes 50% of the channels and, instead, increase

Po more effectively than either compound alone. Again, the docking results support this hypothesis and suggest that the preferred binding pose for PPi, when adenosine is also bound, is more similar to that for the triphosphate groups of the ATP molecule. This therefore suggests that the adenine ring of ATP provides a directing effect, encouraging productive binding of PPi in a manner which leads to activation, and prevents binding in a mode which leads to inactivation.

The context in which the components of the ATP molecule bind to RyR2 is important. Combinations of fragments of the ATP molecule (ADP + Pi, AMP + PPi or adenosine + PPi) cannot exactly reproduce the effects of ATP. Even for adenosine and PPi, the combined effect of these two fragments causes a slightly lower maximum Po level and a lower stimulation of [³H]ryanodine binding than would be obtained with ATP alone. The docking experiments highlighted in **Figure 5** and **Table 2** provide a potential explanation. In the case of ADP, the adenine core of the molecule is predicted to bind in the hydrophobic pocket in a similar manner to ATP, with the diphosphate tail coordinating to both lysine residues (illustrated in **Figure 5B**), hence, the reduced interaction of ADP with the arginine residue may explain its reduced efficacy. When the remaining phosphate now is docked in addition, it is not possible for Pi to coordinate to any of these residues, as to do so would create unfavourable repulsion between the Pi and phosphates of ADP. This explains why the addition of Pi has no effect (**Figure 4C**). For AMP, the same is true. However, with only one phosphate, the AMP can only coordinate to one lysine, leaving the other available for interaction with PPi. We therefore see that the added PPi can interact with both a lysine and an arginine residue, and so a small increase in channel activity is observed. Finally, adenosine alone, with no phosphate groups, is no longer able to interact with the positive residues. These residues are now much more available for interaction with the added PPi ligand. As adenosine is not charged, the lack of repulsion allows PPi and adenosine together to bind in a similar manner to an ATP molecule (**Figure 5B**). We also observed (consistent with previous reports) that the mean RyR2 open

time appears to correlate with the number of phosphate groups on the nucleotide and decreases as they are removed.³⁷⁶

While the adenine moiety appears not to be an absolute requirement to increase RyR2 Po, it is essential for the tight binding in the ATP-binding pocket and to encourage optimal positioning to enable the phosphates to cause long open states and high Po together. This is consistent with my observation from the modelling data that the C4889 residues appears to be ideally positioned to interact with an adenine nucleotide. As can be seen in **Figure 7.8(A)(inset)**, the C4889 residue can form a hydrogen bond with the N1 atom of the adenine core adjacent to the amino group. This interaction is shown in simplified form in **Figure 7.14**. This would explain why it appears that adenine nucleotides are the only ones that are capable of activating RyR, while guanosine nucleotides for example are inactive¹⁵⁶.

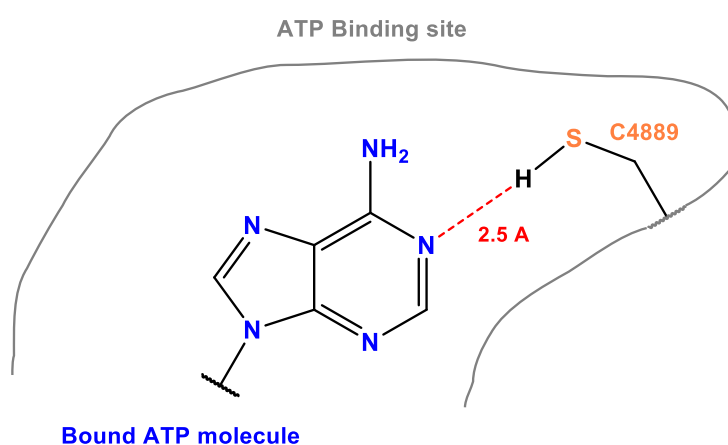


Figure 7.14 ATP molecule bound in position to hydrogen bond to C4889

A GTP molecule could not make this interaction with C4889 as the N1 atom is already bound to a hydrogen atom and hence is not available to act as a hydrogen bond acceptor (**Figure 7.14**). Indeed, one would expect that replacing the amino group of adenine with the carbonyl group of guanosine (as in GTP) would create repulsive effects due to the lone pairs on the carbonyl oxygen of GTP. Consistent with this, I found that when GTP was docked in the the

ATP-site under the same conditions as ATP, none of the poses resembled ATP and all poses has unfavourable (positive) predicted binding energies. The modelling results therefore suggest that perhaps this interaction with C4889 is enough to discriminate between ATP and GTP, and that an adenine nucleotide with three phosphate groups, is required to achieve full channel activation.

Des George *et al.* suggested that the binding of ATP promotes a rearrangement of the core of the channel (known as ‘priming’) which arms the channel for full opening²⁵⁴. In the presence of both Ca^{2+} and ATP, the channel can then be pushed to full open. **Figure 7.15** shows how this process happens. The transition between the closed and open channel structures involves dilation of the transmembrane pore, to create an aperture large enough to allow a Ca^{2+} to be conducted through the channel opening^{171,254,262}. This opening motion of the channel is caused by a bending and rotation of the S6 helix (**Figure 7.15**). From the cryo-EM studies reported by Des George *et al.* (2016), it appears that the ATP molecule is responsible for binding to residues on both the S6 helix and neighbouring S4-S5 helices. We can see therefore that ATP binds, and pulls these helices together, causing the conformational change that allows for channel opening.

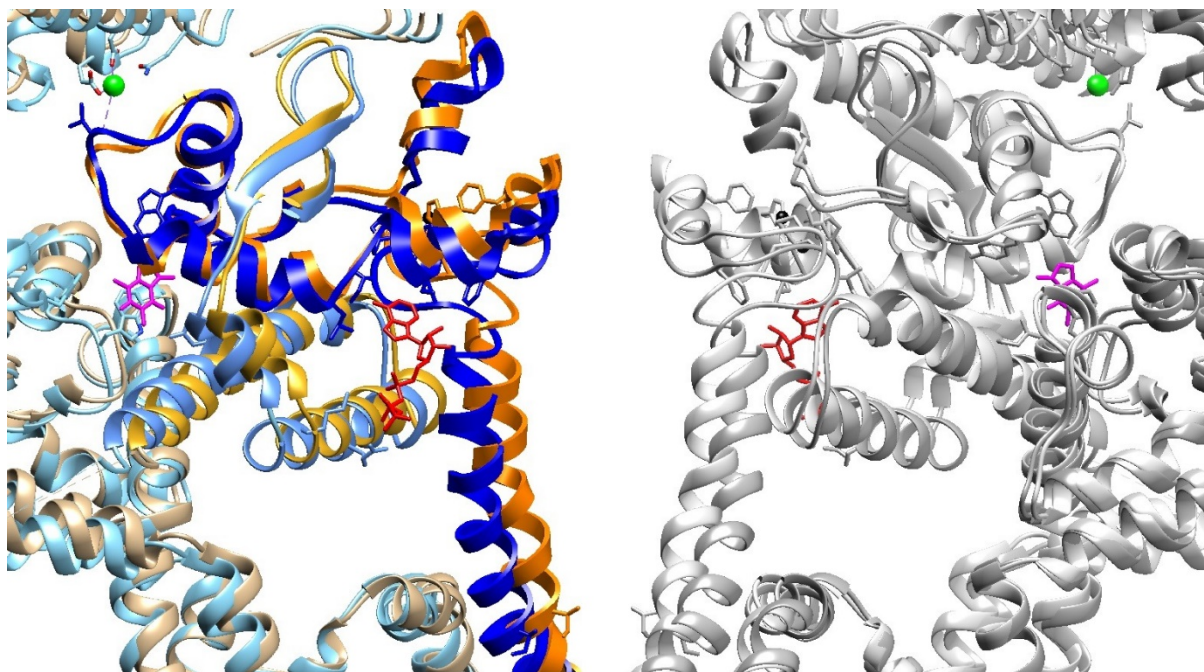


Figure 7.15 The effect of ATP binding to RyR on channel conformation adapted from Zalk et al. (2017)²⁶²

Upon ATP binding in the presence of Ca^{2+} , a conformational change is promoted to cause the channel to adopt the open state over the closed state. Movement of the S6 helix causes the pore aperture to become dilated allowing the passage of ions. In this figure, the closed state is shown in orange, and the open state in blue.

Given this finding, we can propose an explanation for why adenosine and PPi together are able to approximately replicate the increase in P_o with ATP, but not the increase in mean open time. Both adenosine and PPi can fit into the ATP binding site together (**Figure 7.10(ii)**), allowing both molecules to bind optimally. The adenosine can fit deep into the hydrophobic pocket as the ATP molecule would, and the PPi is able to interact optimally with the three positive residues that line the pocket of the site. This replicates how an ATP molecule would bind, with both of these functional groups joined together. We can therefore suggest that this binding interaction is enough to cause the conformational change required for channel opening, increasing the frequency of channel openings (and therefore P_o). However when an ATP molecule is bound, it is able to interact in both sites simultaneously and likely exerts a greater force on the helices involved. It therefore makes sense that the ATP molecule can

cause the same conformational change as adenosine + PPi, but that the change is more stable and that channel opening is maintained. This would result in the increase in mean open time seen with ATP, as the conformational change does not instantly revert back to the closed channel conformation.

It is also important to say a word about the limitations of this docking technique. While it is a powerful technique for understanding ligand binding, it is accepted that accuracy of predicted binding energies produced from docking calculations is generally quite low⁴⁰⁷ and so they should not be relied upon in isolation, nor should they be used as a direct prediction of experimental binding energies. Additionally it is also important to bear in mind that the resolution of the structures is also quite low at 4.2 Å.^{171,263} Rather, we can use these values to compare the ligands studied with each other and to assess the effect of adding or removing functional groups (such as removing phosphate groups). However, the fact that in this case Autodock is able to produce ATP-like binding modes for each of the adenine based ligands studied, but not for GTP etc is consistent with our observations from experiment. I have also shown that there is a broad relationship between the Autodock predicted mean binding energy, and the ability of a ligand to both increase Po and induce long open states (**Figure 7.13**). This gives me confidence that this model is a good one for understanding ligand binding to the RyR2 ATP-site.

The knowledge of how fragments of the ATP molecule can influence the function of RyR provides us with important physiological and pathological insight. In healthy cardiomyocytes the ATP concentration is reported to be between 4-12 mM³⁸⁸ whereas the levels of many other adenine nucleotides are very low or barely detectable^{161,162}. We would therefore expect that under normal conditions, ATP would occupy the adenine nucleotide binding sites providing a consistent activatory influence that is dependent on cytosolic free $[Ca^{2+}]$. This allows proper regulation of the channel, as the opening and closing can be modulated by the

rise and fall of $[Ca^{2+}]$. The adenosine portion of the ATP molecule prevents the triphosphate moiety from creating an irreversibly closed mode, which would otherwise cause major disruption of SR Ca^{2+} -release. Myocardial ischaemia is associated with rises in the levels of Pi, ADP, AMP and adenosine and eventually with decreases in ATP levels; the changes depending on the duration of the ischaemic insult^{161,162}. It is clear from the experiments in this chapter that multiple components of ATP would be unable to mimic the intact ATP molecule and that activation of RyR2 would be reduced, limiting the amount of Ca^{2+} released from the SR. The extent of disruption to EC-coupling would depend on the duration of ischaemia and the exact levels of ATP and its breakdown products.

From a pharmacological point of view, the ATP binding site on RyR channels is a druggable target. However, since the ATP-binding pocket is so similar for RyR1 and RyR2, any drug designed to bind to RyR2 will also bind to RyR1 and vice versa. Surprising though it may seem, it may still be possible to design an 'isoform-specific' ATP-analogue that can target a particular organ such as the heart. This is because of the synergistic nature of ligand regulation of RyR channels and the different mechanisms by which SR Ca^{2+} -release is triggered in different cell types. In the heart, SR Ca^{2+} release is ligand-triggered whereas in skeletal muscle, the voltage-sensing L-type Ca^{2+} -channels control SR Ca^{2+} release. Moreover, the effectiveness of ATP totally depends on the synergic effects of other important ligands such as cytosolic and luminal Ca^{2+} and Mg^{2+} , the actions of which are not identical across mammalian RyR channels. It is likely that the RyR-ATP site could also be responsible for the off-target effects of a range of other drugs as many different types of adenine-based compounds could potentially bind here. For example, there are a growing number of nucleoside analogue drugs such as vidarabine (antiviral) and immucillin-A (ebola) which are structurally related to ATP⁴⁰⁸. Further understanding of the structural features of ATP analogues that result in RyR2 activation or

inhibition will therefore facilitate drug discovery by early identification of undesirable chemical motifs in drug candidates.

In summary, we provide the first structure-based investigation into the binding of ligands in the RyR-ATP binding site, affording new insight into the likely mechanisms by which ATP and analogues regulate RyR2 gating. In future studies it is hoped that dynamical simulations can be performed. These will be challenging given the huge size of RyR and the complex synergistic effects of the various RyR regulators but have the potential for greater mechanistic understanding of ligand-driven RyR gating changes. Such knowledge is likely to have practical significance for improving treatments in cardiac disease where abnormalities in SR Ca^{2+} release contribute to impaired contractile function and the generation of life-threatening arrhythmias.

8

Conclusions

8.1 Concluding Remarks

Approximately 8 million people in the UK are prescribed statins for elevated cholesterol each year, and this number is increasing with recent changes to NICE recommendations resulting in many more patients becoming eligible for treatment.² However, muscular side-effects have been associated with their use and these continue to limit the ability of some patients to enjoy their full cardioprotective effects.⁴⁰⁹ Despite the extensive use of statins, a mechanistic explanation for their effects on the muscle, has remained elusive.

In this thesis, I have investigated whether reports that statins cause undesired Ca^{2+} -release in skeletal muscle can be rationalised via an interaction between statins and RyR1. Using electrophysiological techniques, I have investigated at the level of the single ion channel, whether statins induce activation of RyR1. I have found that all commonly prescribed statins, cause concentration-dependent activation of RyR1, increasing the release of Ca^{2+} from the SR. My data have shown that the mechanism of this activation is primarily the result of statins increasing the frequency of RyR opening through increasing sensitivity to cytosolic $[\text{Ca}^{2+}]$. I suggest that this mechanism may provide an explanation for the observation that statins cause Ca^{2+} -release in skeletal muscle.

As we have described, the primary function of RyR1 in skeletal muscle is to release Ca^{2+} from the SR, allowing for the proper cycling of $[\text{Ca}^{2+}]$ in the cell required for contraction. Therefore, a drug which disturbs this balance by influencing the Po of RyR channels *in vivo* is likely to have deleterious effects on the muscle. Indeed, it is well established that mutations to RyR1 which cause overactive RyR1 channels, and hence, inappropriate Ca^{2+} -release, result in damage to the skeletal muscle, rigidity and acidosis, such as in MH and CCD.¹⁷² Therefore, statin-induced Ca^{2+} release would be predicted to result in similar pathology. A schematic representation of this hypothesis is shown in **Figure 8.1**. At rest, there is a large Ca^{2+} gradient between the SR (internal $[\text{Ca}^{2+}] = 1 \text{ mM}$) and the surrounding cytosol ($[\text{Ca}^{2+}] = 100 \text{ nM}$).

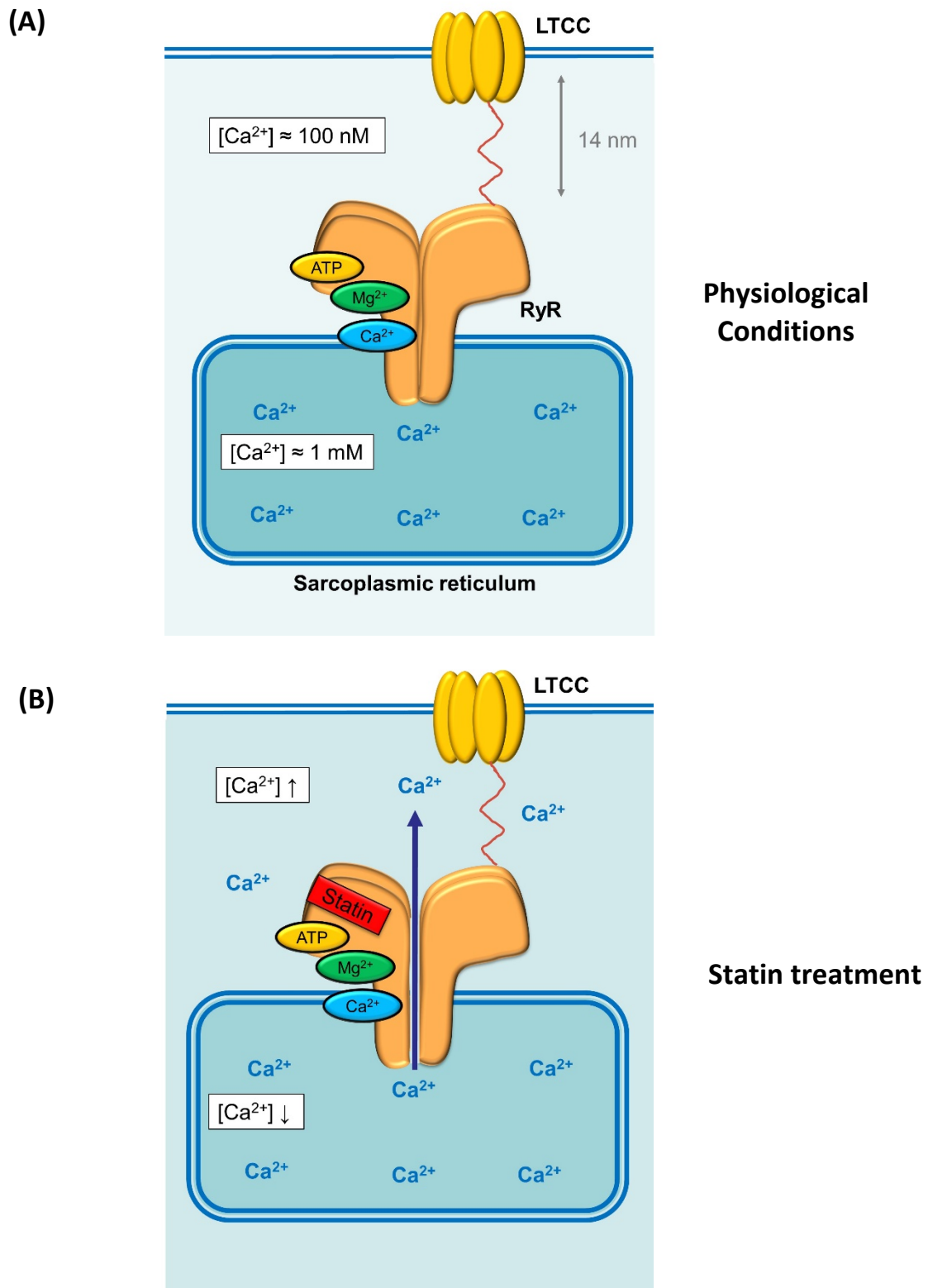


Figure 8.1 Possible mechanism by which statins cause undesired Ca^{2+} -release.

(A) At rest, under normal physiological conditions, RyR allows the SR to effectively contain Ca^{2+} in preparation for muscular contraction. **(B)** Following statin binding to RyR, the RyR becomes more sensitive to cytosolic Ca^{2+} which promotes undesired channel openings and Ca^{2+} release, increasing $[\text{Ca}^{2+}]$ in the cleft, and reducing SR Ca^{2+} content. Note that the distance between the LTCC and RyR is not shown to scale.

During contraction, as the LTCC induces the opening of the RyR, the $[Ca^{2+}]$ increases in the cleft region, to trigger the opening of neighbouring RyRs, and the cascade which finally results in muscle contraction. However, as I have shown, statins at a clinical concentration (10 -100 nM) increase the P_o of RyR1. Therefore as the RyR binds a statin molecule, P_o will be increased as the RyR becomes more sensitive to the surrounding cytosolic $[Ca^{2+}]$, inducing channel opening under rest conditions and resulting in a Ca^{2+} -leak. An increase in the cytosolic $[Ca^{2+}]$ in the region between the RyR and LTCC would be predicted to result, and hence the contractile apparatus of the cell could be inappropriately engaged. Whether the mechanism of statin side-effects can be attributed predominately to RyR1 activation, or whether this effect is just one among a number of parallel pathways such as mitochondrial dysfunction, as previously discussed, is not yet clear. However, that there is now a clear link between statins, RyR1 dysregulation and therefore muscle-related symptoms further study is strongly warranted.

To establish the extent to which statin myopathy may be attributed to RyR1 activation, a tool compound was envisaged which would retain potent HMG-CoA inhibition, but no longer influence RyR1 gating. To determine if these interactions were separable, the statin pharmacophore was synthesised alone, as a fragment. It was found that this moiety did not alone cause activation of RyR1. This indicates that the part of the statin molecule crucial for their effective inhibition of HMG-CoA reductase, is not also responsible for causing RyR1 activation. Therefore, these effects must be separable and an HMG-CoA reductase inhibitor with no RyR1 activity may be designed.

To achieve this goal, the location of the RyR1 statin binding site was investigated, to provide a knowledge of the key protein-ligand interactions involved. Competition experiments indicated that the RyR1 ATP-site is a plausible location for the binding of statins, as the addition of low concentrations of statins antagonised the activatory effect of ATP on single

RyR1 channels. High affinity binding of statins at the RyR1 ATP site might be expected to increase the sensitivity of the channel sufficiently to induce openings at resting cytosolic $[Ca^{2+}]$ *in vivo*, thus leading to undesired SR Ca^{2+} -release and a disturbance to the contractile function of skeletal muscle, with a similar phenotype to other RyR-linked diseases such as MH.

With the recent publication of full cryo-EM structures for RyR1 with ATP bound, a molecular modelling study utilising Autodock software was used to screen for analogues of atorvastatin with reduced affinity for RyR1. This approach revealed four compounds which had no effect on RyR1 activity at 100 nM, but were potent inhibitors of HMG-CoA. The discovery of these compounds will facilitate future work to establish the involvement of RyR1 in statin-induced myopathy and confirm that statin-induced Ca^{2+} -release in skeletal muscle, is unrelated to inhibition of HMG-CoA reductase.

Given the high level of homology between RyR1 and RyR2, this docking model of the ATP site also allowed results found with ATP ligands by previous group members to be rationalised. My results indicated that the phosphate groups and their interaction with positively charged residues which line the site are responsible for the increase in RyR2 mean open time observed with ATP treatment, however, the adenine core is required to direct these phosphate groups into the correct position. My results also provided a possible explanation for PPi-induced inactivation of RyR2 and the ability of adenosine to protect against this irreversible effect.

In contrast to RyR1, the effects of statins on the cardiac isoform RyR2 appeared to be more complex, with concentration-dependent activation *and* inhibition of RyR2 being observed for both simvastatin and atorvastatin. This finding is noteworthy given the reported antiarrhythmic nature of statins, including their ability to reduce instances of ventricular tachycardia and atrial fibrillation.⁷⁶ Given that statins are prescribed to patients who are at higher than average risk of cardiovascular events, their effects on the cardiac muscle and RyR2

are important to understand. There is no doubt that a drug causing RyR2 activation would be undesirable, in the light of the knowledge that increased diastolic Ca^{2+} -leak in cardiac muscle results in arrhythmic diseases such as in CPVT¹⁸⁴.

My experiments have helped shed light on the mechanism of statin side-effects and confirm the hypothesis that statins can induce Ca^{2+} -release at clinically relevant concentrations by activation of RyR1. With increasing numbers of patients becoming eligible for statin-treatment, it is more important than ever that the explanation for their side-effects is understood in order to facilitate the development of future classes of these drugs and allow more patients to benefit from their cardioprotective effects.

9

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9.1 References

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