Creatine transport and its regulation in skeletal and smooth muscle

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To Alfred, Margaret,
Nana and Daniel with love and thanks

ABSTRACT

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Despite the importance of creatine (Cr) in normal and pathological cellular metabolism, the control of intracellular Cr concentration, ([Cr]), is poorly understood. Cytosolic [Cr] is maintained at many times the passive electrochemical equilibrium value, and there is strong evidence for the existence of a Cr transporter in the cell membrane of several tissues. In this study a simple protocol for determining net creatine uptake or efflux was developed. Cr transport across the cell membrane of skeletal and smooth muscle, its modulation, and the relationship between transport and intracellular creatine were then investigated. Results from the cultured skeletal muscle cell line, G8, show that, as in other tissues, skeletal muscle Cr uptake is largely Na gradient - dependent, is electrogenic, and has a low K_m for [Cr]. In this cell line several modulators were shown to increase creatine uptake; the agonists included catecholamines, IGF-I and insulin, triiodothyronine and amylin. By the use of selective agonists and antagonists the receptor subtype mediating the stimulation by catecholamines and the likely signalling pathway was identified. Similarly a tyrosine phosphorylation pathway was identified as responsible for the signal transduction of insulin and IGF-I. Evidence is provided for a role of the sodium potassium ATPase as a common mediator of the uptake signal provided by each of the positive modulators. In addition we confirm the low net creatine efflux seen in other skeletal muscle preparations. Investigation of creatine transport in the isolated perfused porcine carotid artery also identifies a sodium dependent concentrative creatine uptake and a negligible net creatine efflux. The uptake was shown to be positively modulated by isoproterenol (isoprenaline). These results are discussed in terms of the biochemical and hence functional implications for the muscle affected.

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"Omnis cellula e cellula"

Rudolf Virchow, 1858

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ABBREVIATIONS

A	Ampere
APS	Ammonium persulphate
Asn	Asparagine
ADP	Adenosine diphospate
ATP	Adenosine triphosphate
BBCK	Brain isoenzyme of creatine kinase
ß-GPA	Beta guanidinoproprionic acid
Bq	Becquerel
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene related peptide
Ci	Curie
CK	Creatine kinase
Cr/cr	Creatine
Da	Dalton
dBcAMP	Dibutyryl cyclic adeonsine monophosphate
dBcGMP	Dibutyryl cyclic guanosine monophosphate
dH_2O	Distilled water
DMEM	Dulbecco's minimal essential medium
DNA	Deoxyribonucleic acid
ECACC	European collection of animal cell cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylene diaminotetracetic acid
FCS	Foetal calf serum
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
Glut	Glucose transporter
Gly	Glycine
Hepes	Hydroxyethylpiperazine ethanesulphonic acid
HRP	Horse radish peroxidase
HS	Horse serum
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFBP	Insulin-like growth factor binding protein
IGFR	Insulin-like growth factor receptor
IR	Insulin receptor
IRR	Insulin receptor related receptor
J	Joule
K	Partition coefficient, potassium

K_i	Dissociation constant for a protein-inhibitor complex
K_m	Michaelis constant
$K_{n\text{-octanol}}$	Octanol-water partition coefficient
Kp	Partition coefficient
M	Molar
MAP	Microtubule associated (or mitogen activated) protein kinase
mol	Mole
MSA	Multiplication stimulating activity
N_α	Alpha nitrogen
Na^+,K^+-ATPase	Sodium potassium pump (ATPase)
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCr	Phosphocreatine, creatine phosphate
Pi	Inorganic phosphate
PKA	Protein knase A
PKC	Protein kinase C
PLC	Phospholipase C
^{31}P-NMR	Phosphorus nuclear magnetic resonance
PSS	Physiological saline solution
PT	Phosphotyrosine
PTK	Phosphotyrosine kinase
PVDF	Polyvinylidene
R	Ideal gas constant
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SE	Standard error
SEM	Standard error of the mean
Ser	Serine
T	Absolute temperature
$T_{1/2}$, $t_{1/2}$	Halflife
T3	Triiodothyronine
T4	Tetraiodothyronine
TCr	Total creatine
TEMED	Tetramethylethylenediamine
Thr	Threonine
Tris	Trizma; Trishydroxymethylaminoethane
UK	United Kingdom
v/v	By volume
V	Volts
V_{max}	Maximal reaction velocity
VLDL	Very low density lipoprotein

Chapter 1

Introduction

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1.1 Creatine and phosphocreatine in skeletal muscle

The quantity of adenosine triphosphate (ATP) in skeletal muscle is sufficient to sustain contraction for less than a second. An alternative pool of high-potential phosphoryl groups exists in the form of phosphocreatine (PCr); at pH 7, the free energy of hydrolysis of phosphocreatine is 43 kJ/mol compared to 31 kJ/mol for ATP. Thus, energetically, it is possible for PCr to supply the energy required to cause the synthesis of ATP from ADP. In addition, the large quantity of phosphocreatine (≈ 25 mM) in the cell and the action of the enzyme creatine kinase (CK) allow ATP levels to be buffered during muscular exertion:

$$\text{Phosphocreatine} + \text{ADP} + H^+ \xleftarrow{\text{CK}} \text{Creatine} + \text{ATP} \quad [1]$$

Hence in active muscle the ATP level decreases little until most of the phosphocreatine is consumed [Wallimann, 1992]. The immediate precursor to phosphocreatine is the amino acid creatine [1] and these two are in equilibrium in the cell.

The creatine/CK system also has the following actions that facilitate the function of skeletal muscle:

1) During sudden energy demands the increased glycolytic activity increases the lactic acid concentration. The CK reaction, when synthesizing ATP, consumes H^+ and so contributes to a buffering action on the pH [Walker, 1979].

2) The ATP produced by the CK reaction is hydrolysed by the contractile proteins during muscular exertion and this hydrolysis contributes to the raised intracellular inorganic phosphate which stimulates glycogen phosphorolysis, glycolysis and oxidative phosphorylation [Walker, 1979].

3) The decrease in phosphocreatine concentration decreases the PCr inhibition of phosphofructokinase, glyceralde-3-phosphate dehydrogenase and pyruvate kinase, increasing the rate of glycolytic ATP production [Walker, 1979].

4) Creatine acts as an energy messenger between the mitochondria, sites of ATP synthesis and the cytosol, the major site of ATP utilzation during contractile activity [Walker, 1979].

5) Creatine is anabolic, causing the deposition of protein in skeletal muscle, particularly type II muscle fibres [Sipilä *et al.*, 1981; Bessman and Mohan, 1992; Ingwall *et al.*, 1974; Ingwall *et al.*, 1972].

1.2 Introduction to creatine transport

The creatine influx into, and efflux from, the cell are two of the key factors that determine cellular creatine concentrations. There is no synthesis of creatine in muscle cells [Walliman, 1992] and the only metabolism that occurs is the 1-2% conversion of total creatine (creatine plus phosphocreatine) stores to creatinine per day [Walker, 1979; Bloch, 1940]. The importance of the total creatine content (TCr) *in vivo* is demonstrated by the decreased creatine content in several pathological conditions, notably muscular dystrophy [Fitch, 1977] and myocardial infarction [Rix, 1993]. In contrast increasing cell creatine content by dietary supplementation in humans causes an increase in intracellular creatine levels that is correlated with an increase in maximal voluntary muscle contraction [Greenhaff *et al.*, 1993; Harris *et al.*, 1992].

Despite the importance of creatine fluxes, relatively little work has been done in characterising these processes; most characterisation has occurred in rat/mouse heart, skeletal muscle and brain. There has been no study of creatine uptake in smooth muscle. The

transporter that mediates influx has only recently been cloned [Guimbal and Kilimann, 1993; Schloss *et al.*, 1994]. The regulatory factors that control influx have begun to be addressed but the means of physiological regulation of these fluxes is unclear [Loike *et al.*, 1988]. The mechanism by which creatine leaves a cell is unknown.

1.3 Creatine Influx

Most work on creatine transport has focused on the influx of creatine and several tissues have been examined; particularly human muscle cells, rat (L6) and G8 mouse myoblasts, rat skeletal muscle *in vivo*, rat heart, rat and mouse brain, rat fibroblasts, human monocytes, human macrophages, human erythrocytes and mouse liver. As the kinetic constants for the uptake transporter are similar in different tissues, a simplifying assumption has been made that the transporter is essentially the same in the different tissues in an animal.

This saturable transport is highly specific for creatine, sodium dependent, concentrative and sensitive to metabolic inhibitors; it has become accepted as an active transport process [Möller and Hamprecht, 1989; Loike *et al.*, 1988; Bennett *et al.*, 1991; Fitch *et al.*, 1968; Fitch and Chevli, 1980; Ku and Passow, 1980]. The sensitivity of the transport to pronase has been interpreted as a protein mediated transport [Ku and Passow, 1980]. Kinetic data suggests that 2 sodium molecules are transported for every creatine molecule and that the K_m for sodium is 55 mM [Möller and Hamprecht, 1989]. The uptake of creatine appears to be little affected by pH over the range pH 6.9-7.9 [Syllm-Rapoport *et al.*, 1980] although it has recently been shown that decreasing the pH to 6.4 decreases creatine uptake in the mouse liver [Masson and Quistorff, 1994]. The electrogenic nature of this transport allows it to take advantage of the negative membrane potential as well as the inwardly directed sodium

gradient, to maximise the uptake of creatine.

The K_m of creatine uptake in L6 myoblasts is 40-60 μM [Loike *et al.*,1988], the K_m for uptake in the rat heart is 50 μM [Seppet *et al.*,1985] and in rat neuronal cells the K_m is 45 μM [Möller and Hamprecht, 1989]. These reports suggest that the transporter is the same in each of the rat tissues. In rat plasma the concentration of creatine is 140 μM [Syllm-Rapoport *et al.*, 1981] and in sera is 500 μM [Marescau *et al.*, 1986]; the transporters that are not in the central nervous system will always be saturated with substrate; however as the concentration of creatine around astroglial cells is unknown, the average occupancy and hence activity of the transporter are not known. Earlier research indicated a higher value for the apparent K_m in isolated rat extensor digitorum longus of 0.5 mM [Fitch *et al.*, 1966, 1968]. It is possible that there is a difference between the uptake in isolated tissue as opposed to whole cells; however *prima facie* this seems unlikely as more recent rat heart experiments also yield the lower K_m [Seppet *et al.*, 1985].

No figure has been published for the K_m of the transporter in human muscle. Some data does exist for human monocytes and macrophages [Loike *et al.*, 1986]; this shows the K_m in the normal cells to be approximately 30 μM. The K_m for saturable uptake in human erythrocytes is not dissimilar at 20 μM [Ku and Passow, 1980] or 87 ± 32 μM [Syllm-Rapoport *et al.*, 1980, 1981]. The physiological serum creatine concentration in man is in the range of 10-60 μM [Bennett *et al.*, 1991, Loike *et al.*, 1988]. So the transporter in these human cells will respond to physiological fluctuations in creatine by altering the creatine influx mediated by the transporter.

Several groups have reported that there is a second non-saturable component to the uptake [Loike *et al.*, 1986; Ku and Passow, 1980; Fitch and Shields, 1966] which is sodium independent [Loike *et al.*, 1986]. Under physiological conditions this component is considered

to represent at most 10% of the uptake and furthermore there have been few reports indicating saturation [Syllm-Rapoport *et al.*, 1981]. This latter observation and the fact that the second component does not change in transport rate with age of the cell, on addition of an active transport inhibitor or on the addition of metabolic poisons, indicate that this smaller component is a passive permeability exchange flux [Ku and Passow, 1980; Syllm-Rapoport *et al.*, 1980, 1981].

1.4 Inhibition of Creatine Uptake/Creatine Efflux

The inhibitors of active creatine uptake have been valuable experimental tools; they have demonstrated the specificity of the transporter [Fitch *et al.*, 1968, Fitch and Chevli 1980, Ku and Passow, 1980] and allowed studies of the effects of creatine depletion [Fitch and Chevli, 1980, Seppet *et al.*, 1985, Otten *et al.*, 1986, Shoubridge and Radda, 1984].

The uptake transporter is currently believed to require a guanidinium group and a nonpolar group (methylene or methyl) on N_α to recognise creatine or a structural analog [Möller and Hamprecht, 1989]. The most widely used inhibitor of creatine uptake is ß-guanidinoproprionate (ß-GPA) which has a K_i for competitive inhibition of approximately 0.2 mM [Fitch *et al.*, 1968; Möller and Hamprecht, 1989]; although several other inhibitors have been identified [Fitch and Chevli, 1980].

Inhibition of the creatine uptake transporter has shown that the rate of efflux is low; work done on rat skeletal muscle *in vivo* has shown that an average of 1.7 - 2.5% of total cell creatine is lost per day [Otten *et al.*, 1986; Meyer, 1989; Bloch *et al.*, 1940]. The similarity of this number to the rate of loss of creatine from the cell as creatinine (2% of the total cell creatine is lost per day) has led Bloch to propose that the efflux of creatine from the cell

occurs almost exclusively as creatinine [Bloch *et al.*, 1940]. If creatine is lost from the cell as creatine then its rate is so small as to make it difficult to measure [Fitch and Shields, 1966; Fitch and Sinton, 1964].

Erythrocytes have also proved a useful model for examining the creatine loss; here the **creatinine** flux is proportional to the creatine concentration up to a 5 mM creatine concentration and cannot be inhibited by ß-GPA or pronase. This implies that the creatinine flux is a non protein dependent, non-active transport process i.e. a simple passive efflux [Ku and Passow, 1980].

Net creatine loss has also been studied in cultured cells and is in the range of 4-8% per hour [Loike *et al.*, 1986, 1988]. Loike *et al.* obtained their efflux rates through net efflux and isotope exchange studies but the ability to cause near complete depletion of intracellular creatine after 24 hours in a low creatine medium, indicates unique behaviour *in vitro* that is not matched *in vivo*.

1.5 Moderation and Regulation of Creatine Transport

Some attempts have been made to investigate the effects of structurally similar molecules on creatine uptake. Phosphocreatine (PCr), creatinine, ornithine and glycine, glutamic acid, histidine, alanine, arginine, leucine, methionine or cysteine have little or no effect on creatine uptake rates [Loike *et al.*, 1988; Fitch *et al.*, 1968; Möller and Hamprecht, 1989].

The sodium dependent uptake of creatine in cultured L6 myoblasts, L6 myotubes, human myoblasts or human myotubes has been shown to be sensitive to extracellular creatine concentration [Loike *et al.*, 1988]. Myoblasts maintained for 24 hours in a medium containing 1 mM creatine exhibited 1/3 of the uptake activity of cells maintained for 24 hours in a

medium lacking creatine. The downregulation was slowed by inhibitors of protein synthesis. The preincubation of the myoblasts in a creatine supplemented medium containing phosphate buffered saline, results in an increased creatine uptake activity compared to myoblasts preincubated in choline buffer. It is concluded that extracellular creatine regulates the the expression of a protein that decreases the sodium dependent uptake activity only after creatine enters the cell.

Loike also investigated the effect of extracellular creatine on creatine efflux and found no alteration in the efflux rate. If creatine was leaving mainly by passive or facilitated diffusion, one might have expected an alteration in efflux rates; it is likely that the creatine is leaving mainly as creatinine with a small creatine component as they both move down their concentration gradients.

Adding triiodothyronine (T3) to mimic hyperthyroidism has been shown to increase the uptake and efflux of creatine in the rat heart [Seppet *et al.*, 1985] and this group call into question earlier publications that implied T3 induced inhibition of creatine uptake [Dinking *et al.*, 1959; Fitch *et al.*, 1960]. Seppet reports an increase in V_{max} of uptake. The simplest mechanistic explanation derives from an observation that T3 causes a 3 fold increase in transporter expression in rat myotubes [Brodie and Sampson, 1988]; assuming a similar effect in mouse myoblasts, the resulting tendency to increase the local or gross sodium gradient would then enhance the sodium dependent cotransport of creatine into the cell.

1.6 Creatine feeding studies

The potential therapeutic effects of creatine supplementation man have resulted in several small (n <30) studies that explore the link between creatine administration and muscle

function. All of the studies have been on skeletal muscle as this is the most functionally accessible muscle type. Most of the studies have used short duration (<10 days) and high dose (≈ 20 grams) creatine feeding protocols. The average age of the subjects was approximately 30 and almost all subjects were male. The fact that creatine is a natural substance present in large amounts in the diet may in part account for the few side effects these creatine feeding studies have produced. The creatine feeding studies have been performed with a control group given a placebo rather than performing a cross-over study due to the difficulty in obtaining washout within a reasonable time (*in vitro* work has shown that the $T_{½}$ of creatine efflux is approximately a month).

1.6.1 Raising intracellular creatine:

Harris *et al.* [Harris *et al.*, 1992] have shown that 1 gram of creatine a day is sufficient to raise plasma creatine concentrations in man. 5 g resulted in a mean peak creatine concentration of 795 mM in the plasma one hour after administration; this dose is equivalent to the creatine content of 1.1 kg fresh, uncooked steak. Using a protocol of 5 g of creatine 4-6 times per day an increase of total intracellular creatine of up to 50% was demonstrated. There also appeared to be a maximum intracellular creatine content of 160 mmol/kg dry weight of quadriceps femoris. The greatest increase occurred in those with lowest starting creatine content, for example vegetarians. There appeared to be no effect of age (20 - 62) or sex on total creatine content, however there were only 17 subjects in this study. A larger study by Forsberg *et al.* [Forsberg *et al.*, 1991] has demonstrated that women have higher total muscle creatine content than men.

1.6.2 Intracellular creatine and muscle function

Harris [Harris *et al.,* 1992] demonstrated that exercise enhanced creatine uptake in an exercised leg (one hour a day) in comparison with the contralateral leg. A similar result was obtained by Greenhaff [Greenhaff *et al.,* 1993]. No mechanism was offered for this effect. The increase of intracellular creatine has been shown to be correlated with an increased absolute torque and an ability to maintain maximal voluntary effort [Greenhaff *et al.*, 1993; Balsom *et al.,* 1993], the exercise protocol consisted of 5 x 30 unilateral knee extensions at a constant angular velocity of 180 degrees per second, with a 1 minute recovery period. Such a protocol was designed to cause a mixed fibre response (Type I and Type II) in the muscle tissue. The authors suggest that the improved muscle function depends on the increased initial PCr stores and the rate of PCr resynthesis after each bout of exercise. The latter was supported by Greenhaff *et al.* [Greenhaff *et al., 1993*] The accumulation of plasma ammonia, an accepted marker of muscle adenine nucleotide turnover was reduced during exercise following creatine feeding.

In contrast to these effects of enhanced muscle performance induced by creatine during predominantly anaerobic exercise, there appears to be no effect of creatine in improving predominantly aerobic exercise function [Balsom *et al.*, 1993]. There was a general increase in run time for a 6 km run that was attributed to the increase in weight (approximately 0.9 kg) and an increase in lactate acid production on the treadmill run that was attributed to the increase in type II muscle fibre mass, supporting more glycolytic activity.

The only long term creatine feeding study was performed to treat gyrate atrophy of the retina, a disease that causes progressive constriction of the visual fields and may lead to blindness in 30 - 40 years [Sipilä *et al.,* 1981], 1.5 grams were given per day. The diameter

of Type II muscle fibres increased from 34.1 +/- 7.1 to 49.9 +/- 7 (standard deviation) micrometres. There was no significant increase in the size of Type I muscle fibres. The visual tests showed that there was no further constriction of visual fields and the progression was slowed.

1.7 PCr studies

The PCr feeding studies are beyond the scope of this study, the method of delivery and biochemistry of PCr administration differ from creatine in:

a) Requiring intravenous administration.

b) Having a biophysical effect in membrane stabilisation throughout the entire organism because the amphipathic PCr binds to polar phospholipid headgroups. This decreases the efflux of small metabolites [Saks and Strumia, 1993].

c) Possessing more complex pharmacodynamics which indicate that the clearance of PCr from the blood has a component that is due to PCr hydrolysis and a component that is due to binding to cell membranes [Saks and Strumia, 1993].

1.8 Creatine and smooth muscle

There have been no studies to characterise creatine transport in smooth muscle, the bioenergetic functions of creatine in smooth muscle are believed to be similar to those in skeletal muscle.

1.9 Applications

Much of the research carried out into creatine transport has been performed with a view towards therapeutic interventions in dysfunctional muscle and nerves. In the absence of molecular knowledge of influx and efflux, current work frequently relies on perturbations that would indirectly cause an increase in intracellular creatine either by feeding creatine to the animal or by loading the extracellular medium with creatine. It has already been shown that exercise combined with creatine ingestion enhances the increase in cell creatine [Harris *et al.*, 1992]; it may be possible to increase the effect by combining similar protocols with appropriate stimulatory hormones/factors. As the pool of hormones or other factors that affect the transport of creatine become known, therapeutic interventions for particular disease states can be devised and, secondly, designing interventions specific for a patient by varying the ratios of the modulators becomes possible.

In using creatine as a supplement to enhance sporting performance, the circulating level of creatine is less likely to be a limit on the desired benefits than the activity of the transporter itself. It is accepted that under normal physiological conditions the maximum intracellular creatine concentration is of the order of 155 mmol/kg wet weight; this will probably only be exceeded if the maximum activity of the transporter is increased. It remains to be seen whether it will be fruitful to pursue an alternative approach that would depend on decreasing the efflux of creatine; however the little evidence that exists indicates a slow passive efflux - a process that is unlikely to be easy to moderate.

The aims of this study were to elucidate the mechanisms of creatine transport in skeletal and smooth muscle, to identify modulators of creatine transport and to describe the signalling pathways of the modulators.

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Chapter 2

Characterisation of creatine transport in the G8 cell

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2.1 The G8 cell line

The G8 cell line (European Collection of Animal Cell Cultures, ECACC No 89050906) is a myogenic cell line derived from the skeletal muscle of a Swiss Webster mouse [Christian *et al.*, 1977]. These myoblasts grow to confluence and can be passaged 18 times before they are no longer stable. In addition, the G8 cells can differentiate to the larger tapering multinucleate myotubes *in vitro*, these can then be triggered to contract under appropriate stimulus; hence they mimic the most important function of mature skeletal muscle. The other commonly used myoblast cell line L6 [Loike *et al.*, 1988], was not used in this study because of the difficulty of obtaining reproducible results [Polgreen, 1993]. G8 cells were used extensively in this work both because they possessed many properties in common with mature skeletal muscle and because of their ease of handling.

2.2 Cell culture methods

2.2.1 Maintaining cells in culture

G8 cells were grown in 750 ml flasks with 25 - 35 ml Dulbecco's minimal essential medium (DMEM) supplemented with 10% foetal calf serum (FCS), 10% horse serum (HS), and 2 mM glutamine. The glutamine was sterilised by filtration through 0.2 µm filters and added to the medium. The cells were incubated at 37°C under 5% CO_2. The medium was changed every 3-5 days. The cells were passaged on reaching confluence. The cells used were between passage numbers 5 and 16. Sterile technique was used in handling G8 cells throughout this work.

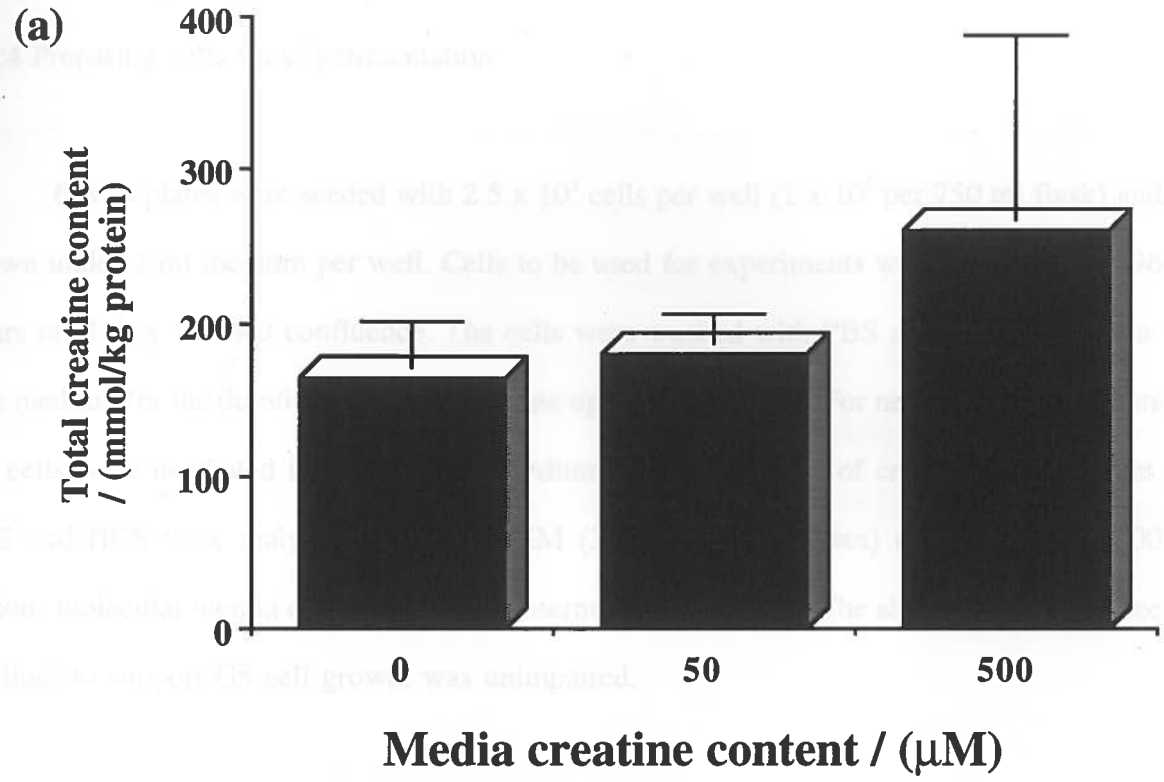
2.2.2 Passaging cells

Cells were washed with 10 ml of phosphate buffered saline (PBS); 10 ml of 0.5%/5.3 mM Trypsin-EDTA was added to the flasks for about 1 minute and the flasks agitated until the cells were dislodged from the surface of the flask. 10 ml of complete medium (containing sera) was added to halt the activity of the trypsin and the cell suspension was poured into a 50 ml tube and centrifuged for 2 minutes at 1200 rpm. The supernatant was poured off and the cells were resuspended in medium. An aliquot was taken for counting on a haemocytometer. The number of cells per ml was calculated and 5 x 10^5 cells were added to each 200 ml flask or 1 x 10^6 cells to each 750 ml flask.

2.2.3 Long term storage of cells

Cells were removed from a confluent flask with trypsin, (as for passaging), pelleted, resuspended and counted. The volume of cell suspension was adjusted with media to give a concentration of 10^6 cells per ml. Aliquots of 1 ml were dispensed into cryovials and cooled to -70°C overnight. The cells were then placed in liquid nitrogen for long term storage.

To defrost cells from storage, a vial was warmed to hand temperature and immediately on defrosting, the cell suspension was added to 9 ml of medium. The cells were spun for 2 minutes at 1200 rpm. The medium was poured off the pellet and resuspended in 2 ml of medium before being poured into a 200 ml flask with 10 ml of medium. The flask was incubated at 37 °C under 5% CO_2.



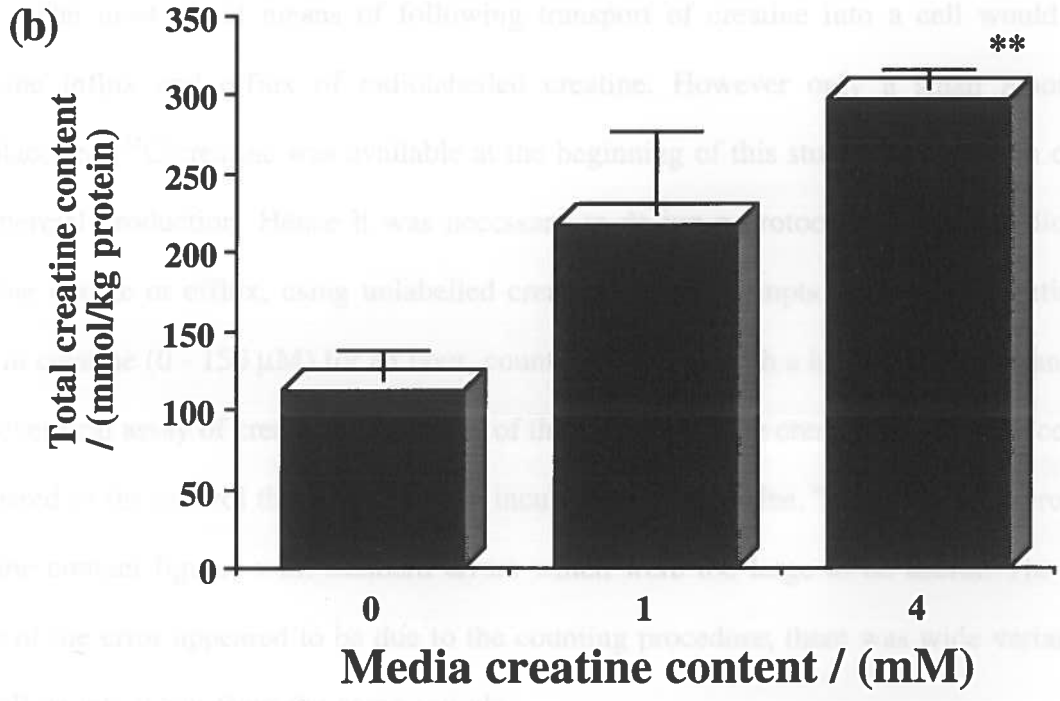


Figure 2.1 Modulation of intracellular creatine content by extracellular creatine
Myoblasts were incubated in the creatine concentrations indicated. Cells were of passage number 10, plating density 250,000 (n=6). (a) A 24 hour uptake experiment (b) A 48 hour uptake experiment.

2.2.4 Preparing cells for experimentation

6-well plates were seeded with 2.5 x 10^5 cells per well (1 x 10^6 per 750 ml flask) and grown under 2 ml medium per well. Cells to be used for experiments were grown for 48-96 hours until they reached confluence. The cells were washed with PBS and changed to sera-free medium for the duration of the net creatine uptake experiment. For net efflux experiments the cells were incubated in creatine free medium. For preparation of creatine-free medium, FCS and HCS were dialysed against DMEM (3 x 48 hour dialyses) using tubing of 500 daltons molecular weight cut off (Medicell International, London). The ability of creatine-free medium to support G8 cell growth was unimpaired.

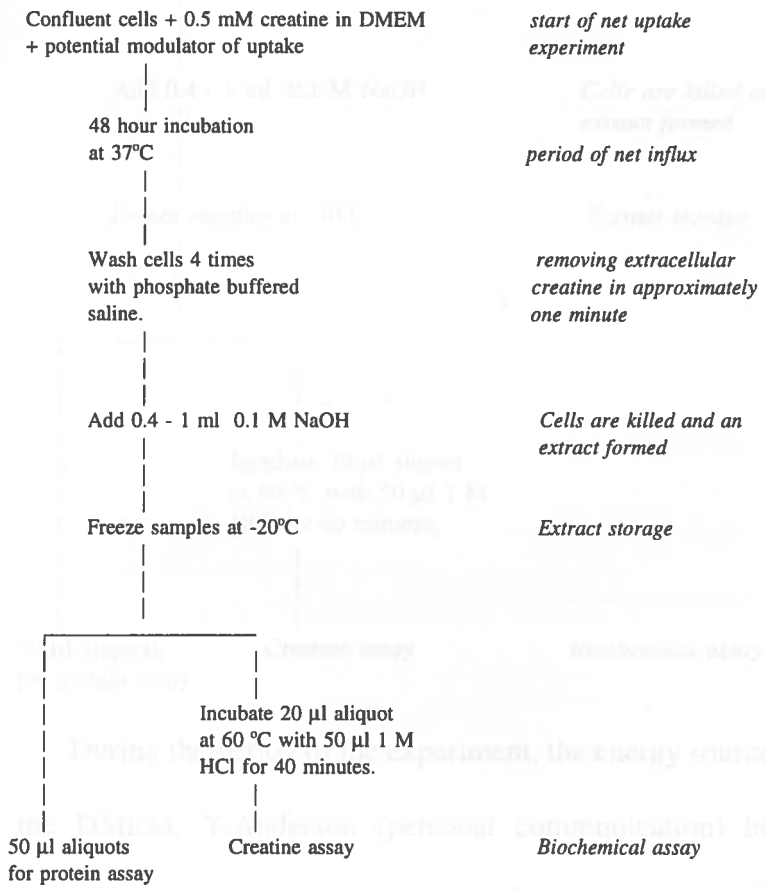
2.3 Transport protocols; developing a general methodology

The most direct means of following transport of creatine into a cell would be to examine influx and efflux of radiolabelled creatine. However only a small amount of radiolabelled [^{14}C]creatine was available at the beginning of this study; it was not in current commercial production. Hence it was necessary to design a protocol that could follow net creatine uptake or efflux, using unlabelled creatine. Early attempts involved incubating the cells in creatine (0 - 150 μM) for an hour, counting G8 cells with a haemocytometer and then a biochemical assay of creatine. At the end of the experiment the creatine content per cell was compared to the control that had not been incubated with creatine. This procedure produced creatine content figures with standard errors which were too large to be useful. The major cause of the error appeared to be due to the counting procedure; there was wide variation in the cell counts taken from the same sample.

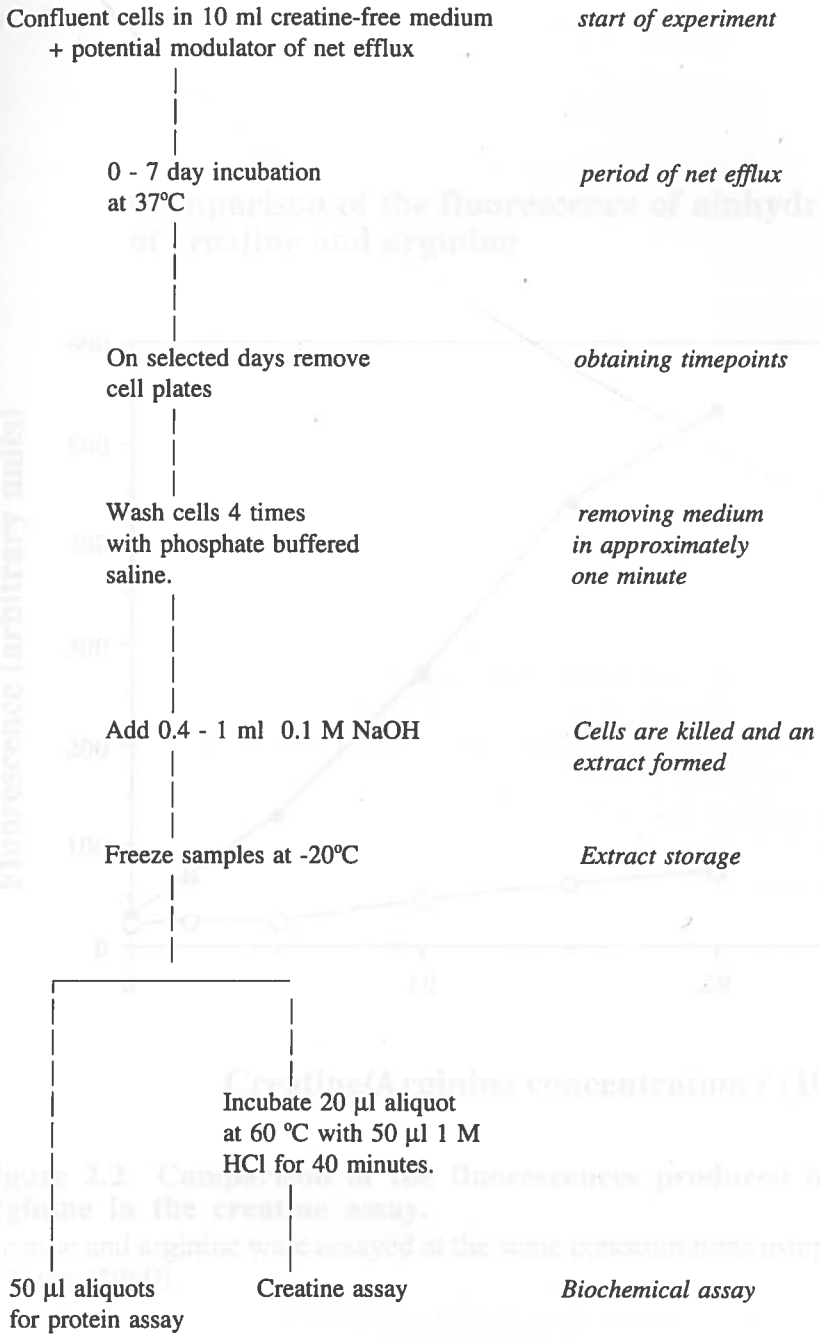
Creatine content was then measured in terms of unit mass of protein. The duration of the experiments was extended first to 24 hours (0 - 500 μM) and then to 48 hours (0 - 4 mM)

and the results of these two preliminary experiments are shown (Figure 2.1). Significant increases in creatine content compared to the control were obtained after the 48 hour incubation with creatine. In most of the G8 creatine uptake experiments in this work 48 hour incubations were used. This gave the greatest range over which creatine content could potentially be modulated compared with the control. The apparently high creatine content values shown in the earliest protocols probably arose out of protein hydrolysis at the time of cell extraction; subsequent work involved storing extracts on ice before freezing and this resulted in smaller creatine content values.

Summary of net uptake protocol



Summary of net efflux protocol



During the period of the experiment, the energy source is the 11.2 mM glucose present in the DMEM. Y.Anderson (personal communication) has demonstrated that G8 cells consumed less than 40% of the glucose. 5 mM pyruvate added to G8 cells in DMEM for 48 hours was not utilised. The inorganic phosphate concentration in DMEM + sera is 15 mM (Y.Anderson, personal communication).

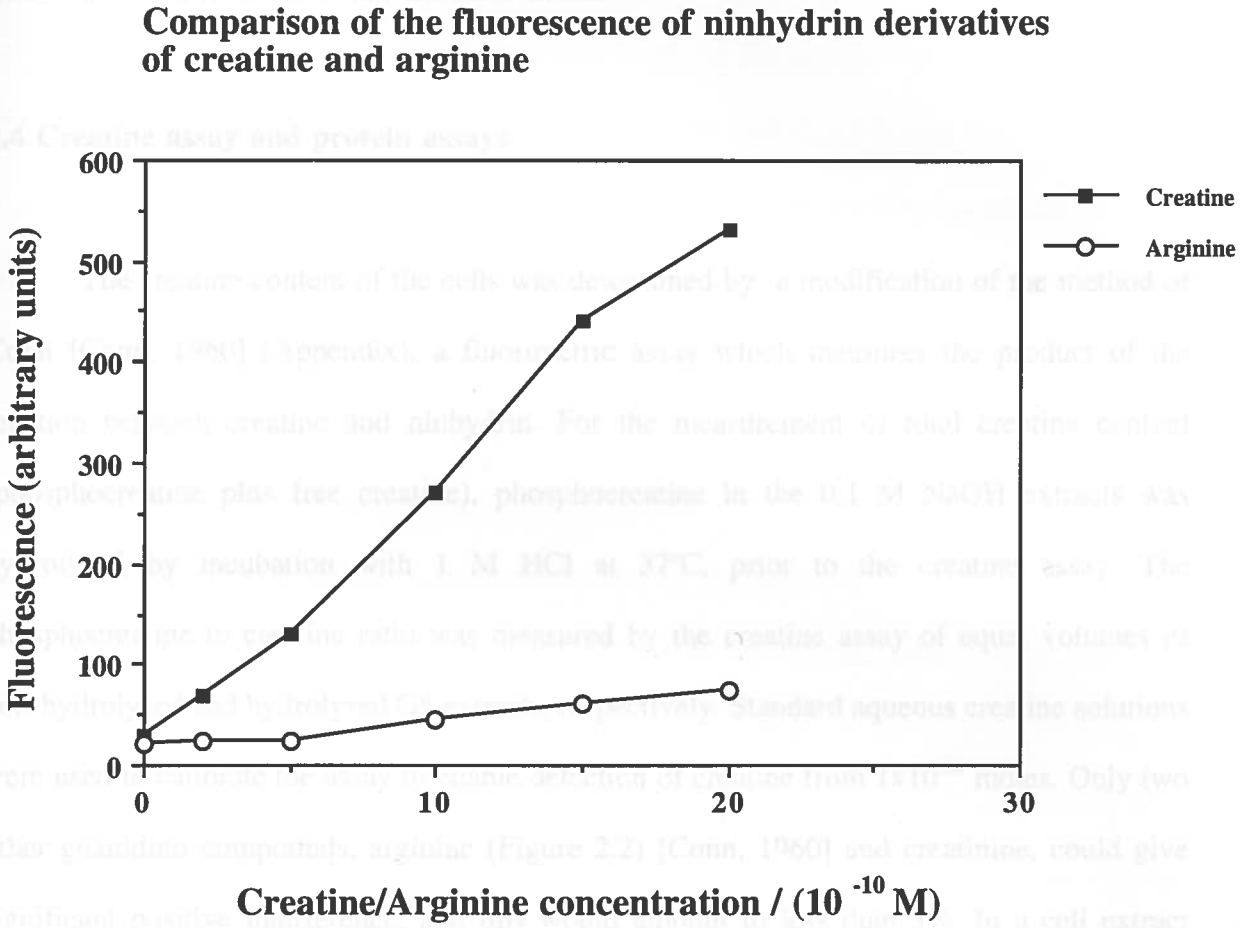


Figure 2.2 Comparison of the fluorescences produced by creatine or arginine in the creatine assay.
Creatine and arginine were assayed at the same concentrations using the method of Conn (1960).

The advantage of measuring creatine content rather than creatine fluxes arises out of the observation that the total muscular creatine content is an important determinant in muscle function [Greenhaff *et al.*, 1993]; the corresponding correlation between increased creatine fluxes and muscle function has not been made.

2.4 Creatine assay and protein assays

The creatine content of the cells was determined by a modification of the method of Conn [Conn, 1960] (Appendix), a fluorimetric assay which measures the product of the reaction between creatine and ninhydrin. For the measurement of total creatine content (phosphocreatine plus free creatine), phosphocreatine in the 0.1 M NaOH extracts was hydrolysed by incubation with 1 M HCl at 37°C, prior to the creatine assay. The phosphocreatine to creatine ratio was measured by the creatine assay of equal volumes of non-hydrolysed and hydrolysed G8 extracts, respectively. Standard aqueous creatine solutions were used to calibrate the assay to enable detection of creatine from $1x10^{-10}$ moles. Only two other guanidino compounds, arginine (Figure 2.2) [Conn, 1960] and creatinine, could give significant positive interference, and this would amount to less than 3%. In a cell extract recovery of added creatine was 100%.

Total protein content of the cells was determined by the method of Lowry [Lowry *et al.*, 1951]. G8 cells were extracted with 0.1 M sodium hydroxide and the calibration of the Lowry assay [Lowry *et al.*, 1951] was performed with bovine serum albumin, BSA, in 0.1 M sodium hydroxide solutions. All chemicals were of analytical reagent grade.

The protein content of each well was determined; this gave more accurate values of creatine content per unit mass of protein. In addition measuring the protein content of each well would reveal any anabolic or catabolic effects caused by the potential modulator of

uptake/efflux.

2.5 Determining K_m and V_{max}; radiolabel measurements

Cells were incubated in a Tris or Hepes buffered Ringer solution at pH 7.4 in air at 37°C. The Ringer solution contained 122 mM Na^+, 130 mM Cl^-, 5.6 mM K+, 2 mM Mg^{2+}, 2 mM Ca^{2+}, 5 mM glucose and 30 mM Tris or Hepes. For sodium-free incubations, sodium was replaced by equimolar choline. At the end of the experiment the cells were washed with PBS four times and 0.1 M sodium hydroxide extracts taken. Cell extracts and medium were counted by liquid scintillation using 0.1 ml in 4 ml of scintillant (ICN Biomedicals, Inc. Irvine, CA, U.S.A.) on a Beckman LS 1701 counter. The protein content per well was determined from 0.1 M sodium hydroxide extracts by the method of Lowry.

The sodium dependence and kinetic parameters of creatine uptake were measured using [^{14}C]creatine (Amersham International) at an activity range of 0.1 μCi/ml (3.7 kBq/ml) in the medium. Uptake was determined in both sodium-free and in sodium-containing Tris-Ringer. Uptake rates were calculated from the accumulation of [^{14}C]creatine in the G8 cell over a one hour incubation, during which labelling was linear.

2.6 Experiments performed

A) Measurement of PCr:creatine in G8 cells.

B) Creatine efflux: Cells were incubated in creatine-free medium and samples taken at 0, 3 and 6.5 days.

C) Intracellular and extracellular creatine: Cells were incubated in media containing creatine

concentrations of 0 - 2 mM.

D) K_m and V_{max}: Uptake of [^{14}C]creatine in sodium-free and sodium-containing media.

E) Effect of isoproterenol (isoprenaline) on creatine uptake: The effect of isoproterenol on net creatine uptake was tested at isoproterenol concentrations of 0 - 10 μM.

F) Effect of amylin on creatine uptake: The effect of amylin on net creatine uptake was tested at amylin concentrations of 0 - 90 nM.

G) Effect of Triiodothyronine, T3, on creatine uptake: The effect of T3 on creatine uptake was determined at T3 concentrations of 0 - 111 nM.

H) Effect of dexamethasone on cellular creatine content: The effect of dexamethasone on cellular creatine content was determined at dexamethasone concentrations of 0 - 60 nM.

2.7 Analysis and presentation of data

n-Octanol/water partition coefficients ($K_{n\text{-octanol}}$) were calculated *ab initio* by using the FRAGMENT method of Hansch and Leo [Hansch and Leo, 1979]. This procedure assigns constant values to a small number of fundamental functionalities and then attempts to incorporate factors resulting from particular structural features (e.g. unsaturation, interacting H-bonding groups) that affect partitioning in a predictable manner. This procedure yields accurate $K_{n\text{-octanol}}$ values for a wide range of organic molecules, including ions [Hansch and Leo, 1979].

All counts were corrected for background. The rate of [^{14}C]creatine uptake is expressed as mmol per kg per h. Creatine contents are expressed as mmol (total creatine) per kilogram of protein.

Results are expressed as mean ± standard error (SE). Statistical significance of the

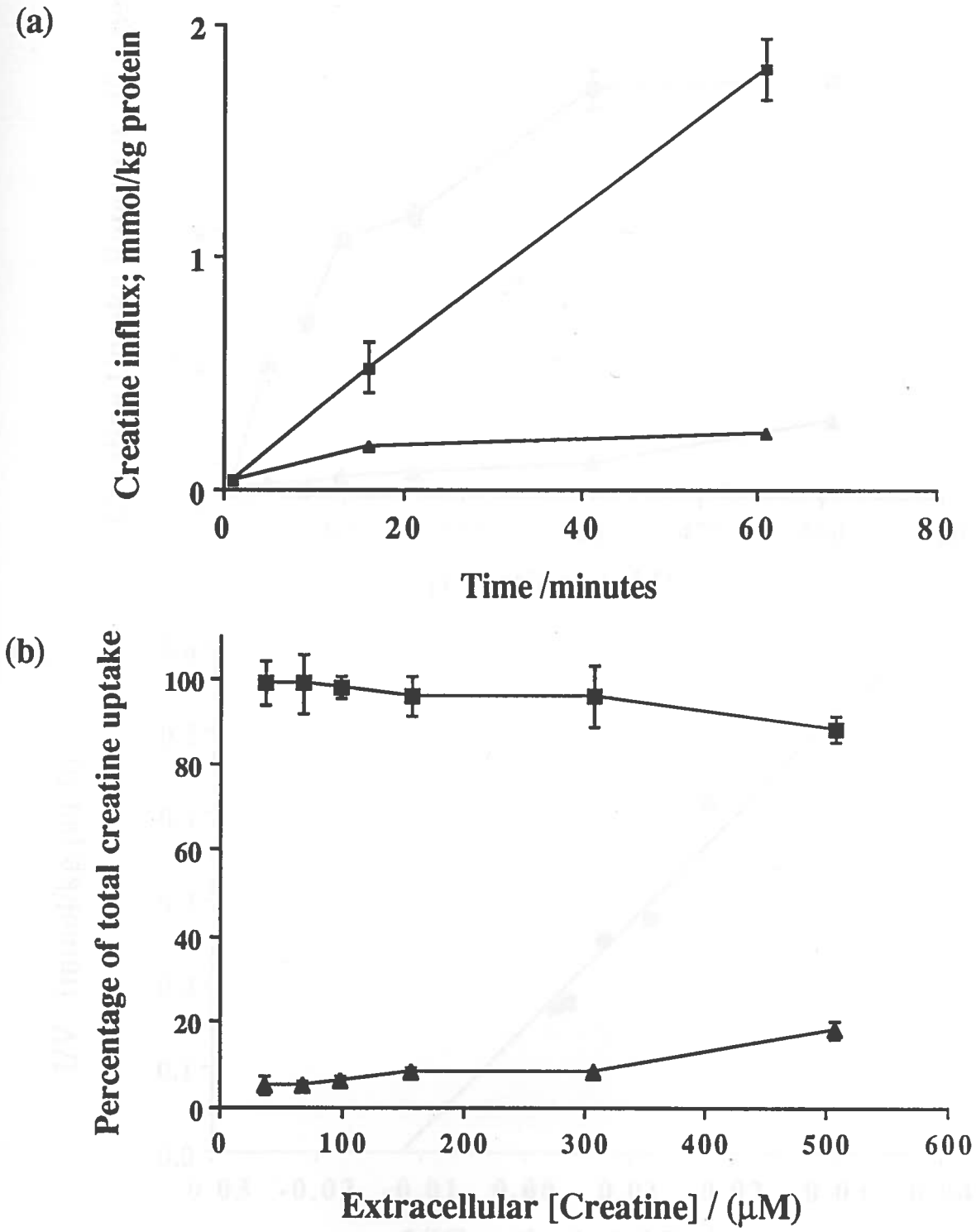


Figure 2.3 Sodium dependence of [^{14}C]creatine uptake into G8 myoblasts
(a) Time course. Myoblasts were incubated in 0.1 mM extracellular creatine, with sodium (squares) or without sodium (triangles). [^{14}C]creatine was added at time t=0. Cells were of passage number 10, plating density 2.5×10^5 (n=5). The error bars represent ±SEM.
(b) Concentration dependence of sodium dependent fraction. The graph shows the sodium dependent (squares) and sodium independent (triangles) percentages of total creatine uptake after 1 h incubation with [^{14}C]creatine, plotted as a function of extracellular [creatine]. Cells were of passage number 11, plating density 2.5×10^5 (n=6).

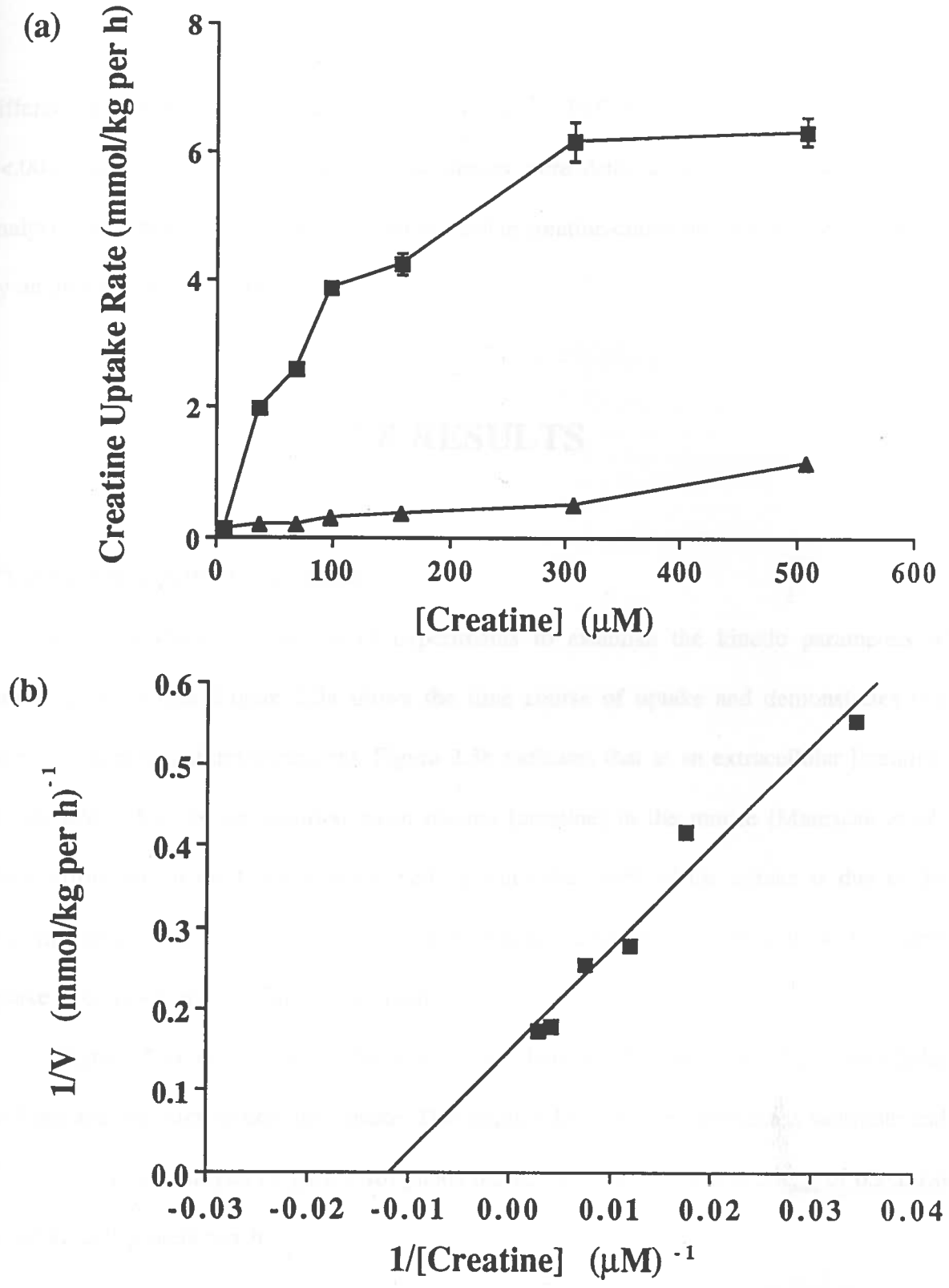


Figure 2.4 Kinetic analyses of [^{14}C]creatine uptake in G8 myoblasts

(a) Concentration dependence. The graph shows sodium dependent (squares) and sodium independent (triangles) creatine uptake rate measured from 0-1 h after the addition of [^{14}C]creatine, plotted as a function of [creatine]. Cells were of passage number 11, plating density 2.5×10^5 (n=6).

(b) A Lineweaver-Burk plot of the sodium dependent [^{14}C]creatine uptake data shown in Figure 2.4a.

differences was determined by the Student's t-test (* = P<0.05, ** = P<0.01, *** = P<.001 compared to control). Kinetic parameters were determined from Lineweaver-Burk analysis. Cell growth in creatine-free medium and in creatine-containing medium was assessed by an analysis of the variance.

2.8 RESULTS

[^{14}C]Creatine Uptake Experiments

Figure 2.3 shows the results of experiments to establish the kinetic parameters of unidirectional influx. Figure 2.3a shows the time course of uptake and demonstrates that uptake is largely sodium dependent. Figure 2.3b indicates that at an extracellular [creatine] of 200 μM, which is the reported mean plasma [creatine] in the mouse [Marescau *et al.*, 1986] (from which the G8 line is derived), greater than 90% of the uptake is due to the sodium dependent process, while at high extracellular [creatine] the non-sodium dependent uptake becomes somewhat more important.

Figure 2.4a demonstrates the relationship between [creatine] in the extracellular medium and the rates of creatine uptake. The sodium dependent component is saturable and Lineweaver-Burk analysis (Figure 2.4b) yields the K_m of 86 ± 15 μM and a V_{max} of 6.9 ± 1.0 mmol/kg cell protein per h.

Intracellular [Creatine] in G8 myoblasts

In extracts of cells the phosphocreatine to free creatine ratio was 2.0 (plating density 250,000/well, n=6, passage number 9). During creatine-free incubation, there is net creatine

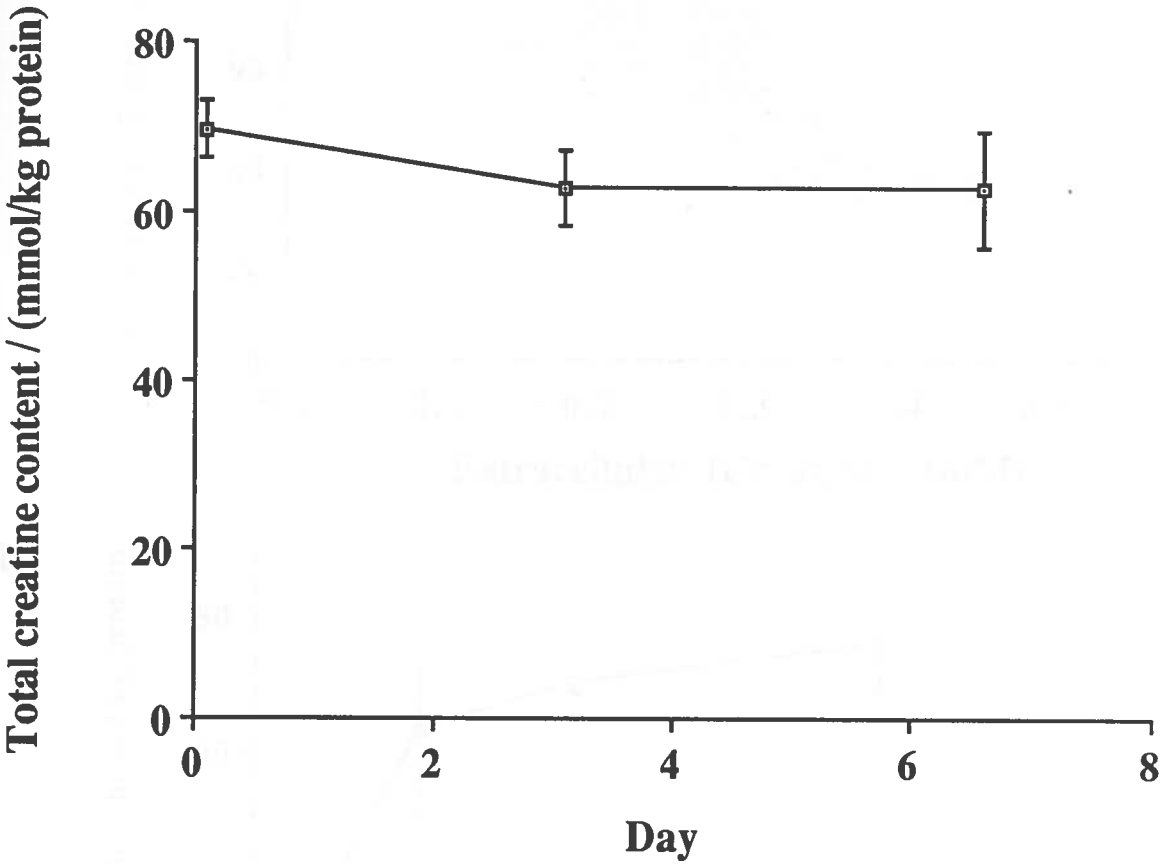


Figure 2.5 Creatine efflux from G8 myoblasts
A plot of total intracellular creatine against time for G8 myoblasts incubated in a creatine -free medium. The cells were of plating density 250,000 (n=6).

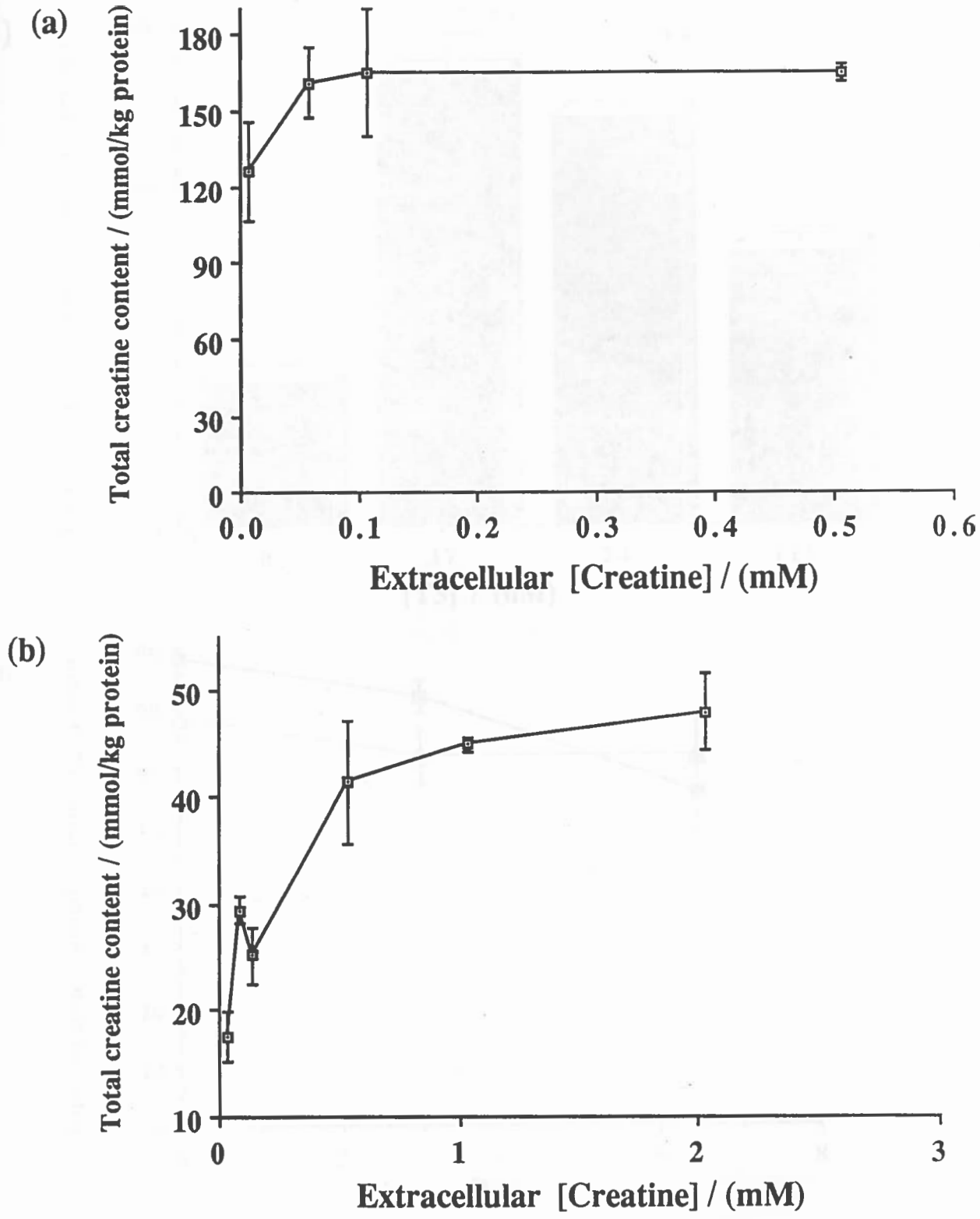


Figure 2.6 The relationship between intracellular and extracellular [creatine] in G8 myoblasts

A plot of total intracellular [creatine] against extracellular [creatine] for G8 myoblasts. Results are shown for experiments on two different batches of cells. (a) shows results from myoblasts that were of passage number 14, plating density 2.5×10^5 (n=6). (b) shows results from cells of passage number 12 and plating density 5×10^5 (n=6). Both experiments were conducted over 48 hours.

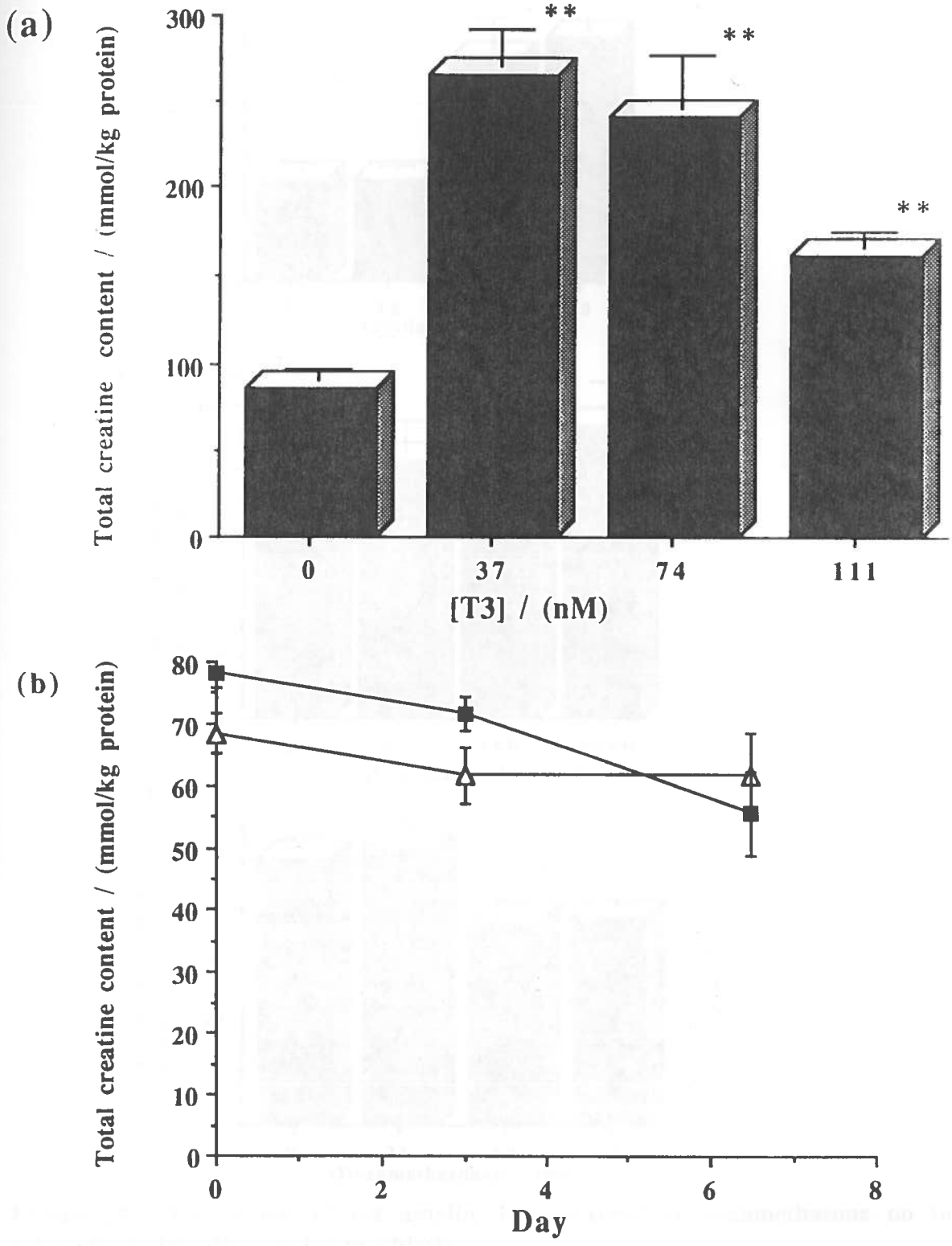


Figure 2.7 The effect of 3,3′,5-triiodothyronine (T3) on total intracellular [creatine] in G8 myoblasts.

(a) A graph of total intracellular [creatine] against applied [T3], with 0.5 mM creatine in the extracellular medium. The cells were of passage number 14 and plating density 2.5×10^5 (n=6). The experiment was conducted over 48 hours.

(b) A plot of total intracellular [creatine] against time for G8 myoblasts incubated in the presence (squares) or absence (triangles) of 77nM T3. The cells were of passage number 12 and plating density 2.5×10^5 (n=6).

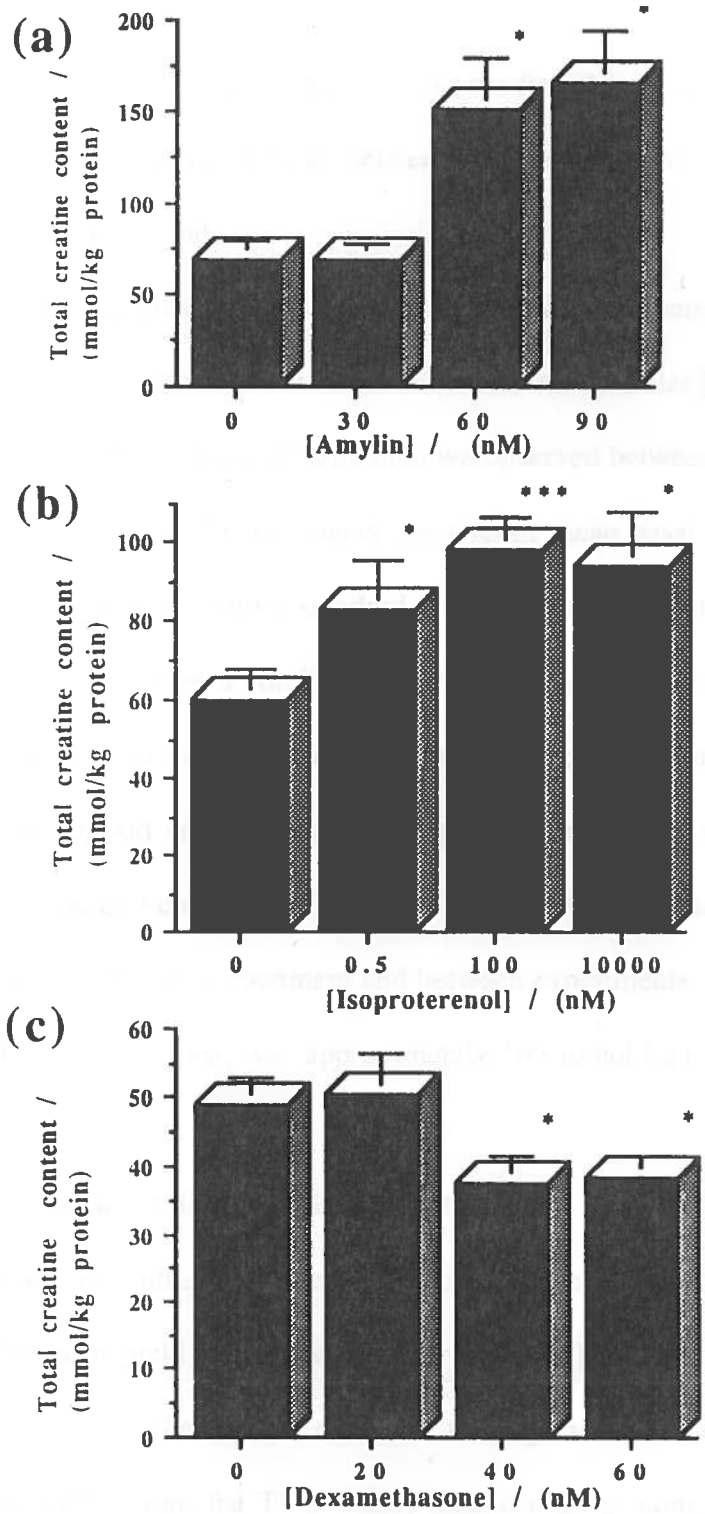


Figure 2.8 The effects of rat amylin, isoproterenol or dexamethasone on total intracellular [creatine] in G8 myoblasts.

(a) A graph of total intracellular [creatine] against applied [amylin], with 0.5 mM creatine in the extracellular medium. The cells were of passage number 10 and plating density 2.5×10^5 (n=6).

(b) A graph of total intracellular [creatine] against applied [isoproterenol], with 0.5 mM creatine also being in the extracellular medium. The cells were of passage number 16 and plating density 2.5×10^5 (n=6).

(c) A graph of total intracellular creatine against applied [dexamethasone], with 0.5 mM creatine in the extracellular medium. The cells were of passage number 10 and plating density 2.5×10^5 (n=6). All experiments were conducted over 48 hours.

efflux which amounts to 1.6% per day over the first three days (Figure 2.5). This experiment does not attempt to distinguish between the two possible forms of creatine efflux; as creatinine or as creatine.

Figure 2.6 makes two points. First, it shows that there is a fairly flat but apparently hyperbolic relationship between intracellular and extracellular [creatine]. Second, it illustrates the variation in absolute [creatine] which we observed between batches of cells. Considering thirteen different sets of experiments, the overall mean basal creatine content value was 85 ± 10 mmol/kg protein, with a standard deviation of 40 mmol/kg protein. The cause of this variation is likely to be a combination of different passage numbers, variation in hormone levels in sera, precise level of confluence and time taken to reach confluence. These calculations support an experimental design that includes its own control for basal creatine uptake (e.g. the first columns in figures 2.7a, 2.8a, 2.8b etc); such a control allows meaningful comparisons in any one experiment and between experiments. The highest [creatine] seen, in the absence of an agonist, was approximately 165 mmol/kg cell protein.

Factors Modulating Intracellular [Creatine]

A T3 concentration in the 10^{-8} M range causes an enhancement of creatine uptake by G8 myoblasts to yield a total intracellular [creatine] of about 260 mmol/kg of cell protein (Figure 2.7a). Over the 48 hour course of the experiment, there is no change in the net rate of creatine efflux from the T3 (77 nM) treated cells as compared with the control (Figure 2.7b).

Rat amylin causes significant enhancement of creatine uptake when present at 60-90 nM in the extracellular medium (Figure 2.8a). The enhancement in uptake caused by isoproterenol (isoprenaline) occurs at very low concentrations (Figure 2.8b); 0.5 nM appears

to cause a maximal increase in creatine uptake. The addition of dexamethasone causes a diminishment of net creatine uptake (Figure 2.8c).

2.9 DISCUSSION

Creatine Uptake

The total [creatine] in a G8 cell is the result of an active creatine uptake balanced by passive creatine efflux, a model that has been applied to phosphate metabolism in L6 myoblasts [Kemp *et al.*, 1992; Kemp and Bevington, 1993]. Under steady state conditions, this model predicts a hyperbolic relationship between intra- and extracellular [creatine], and this is broadly consistent with Figure 2.6. However presumably because of the slow efflux of creatine (Figure 2.5), it proved impossible to achieve zero intracellular [creatine] with zero extracellular [creatine] as the model predicts for a true steady state.

Figure 2.3 shows creatine uptake values similar to previously published data for L6 rat myoblasts [Loike *et al.*, 1988]. This transport appears to be saturable (Figure 2.4) with a K_m of 86 ± 15 μM, which is similar to K_m values reported for creatine transporters in several other tissues (Table 2.1).

Animal	K_m	Tissue/cell type	Reference
Rat	**40/60** μM	myoblasts	Loike *et al.*, 1988
	50 μM	heart	Seppet *et al.*, 1985
	45 μM	rat neuron	Möller & Hamprecht, 1988
Human	**30** μM	monocytes and macrophages	Loike *et al.*, 1986
	20 μM	erythrocytes	Ku & Passow, 1980
	87 ± 32 μM	erythrocytes	Syllm-Rapoport *et al.*, 1980, 1981
Mouse	**86** ± 15 μM	myoblasts	Present work.
Rabbit	**46** μM	expressed in HEK-293	Schloss *et al.* 1994
	35 μM	expressed in COS-7	Guimbal & Kilimann, 1993

Table 2.1: K_m values for the creatine uptake transporter.

It is not clear if this similarity suggests significant structural homology between the transporters. As extracellular [creatine] was 0.5 mM, uptake was clearly concentrative and hence active. This active transport can be attributed to the sodium creatine cotransporter which makes use of the free energy of the sodium concentration gradient. Furthermore, cotransport of sodium and creatine (creatine being a zwitterion that has no net charge at physiological pH) must be electrogenic in the positive-inwards sense (unless it is accompanied by some unidentified anion). Creatine uptake is therefore driven both by the sodium gradient and by the inside-negative membrane potential. Although work on rat and mouse neural cell cultures suggests an uptake of 2 Na^+ per creatine molecule [Möller and Hamprecht, 1989] it is not clear if this figure is generally applicable. If we say that n Na^+ are transported with one creatine, then by equating the free energies of the sodium and creatine concentration gradients [Kemp and Bevington, 1993] we can calculate the maximum possible accumulation of creatine (inside/outside) as

$$(\text{external [Na]}/\text{internal [Na]})^n .\exp(-\text{membrane potential}/RT)$$

where R and T are the gas constant and absolute temperature. In skeletal muscle, membrane

potential is -70 mV and internal [Na] = 10 mM [Dunn *et al.*, 1993], thus the maximum accumulation ratio would be 200 if n=1, rising to 3000 if n=2. In adult humans, where resting muscle free [creatine] ≈ 4 mM [Arnold *et al.*, 1984] and plasma [creatine] ≈ 50 μM [Marescau *et al.*, 1986], the actual accumulation ratio is only 80, and so the sodium creatine transporter is not near equilibrium and may then be a potential site for the control of intracellular [creatine].

This analysis strictly relates only to free [creatine]. The total [creatine] pool size (PCr+creatine) is determined by free [creatine] and by the PCr:creatine. The PCr:creatine is compatible with figures obtained from skeletal muscle *in vivo* [Meyer, 1989; Arnold *et al.*, 1984].

Figure 2.6 shows that the maximum intracellular [creatine] measured was 165 mmol/kg protein. Assuming that most of the dry cell mass is due to protein then this would be approximately equivalent to 165 mmol of creatine per kilogram dry weight of *in vivo* muscle; this total creatine content is similar to the limit of achievable intracellular [creatine] in human muscle of 160 mmol/kg dry weight recently reported during creatine feeding studies [Harris *et al.*, 1992]. The result suggests a fundamental similarity in [creatine] homeostasis in the G8 and human muscle cells. Cellular creatine concentration is determined by the influx rate, which may be influenced by the activity of the ubiquitous Na^+,K^+-ATPase - this controls the transmembrane sodium gradient as well as the membrane potential.

The existence of a maximum intracellular creatine content implies a regulation of creatine that is effective over a short term; considering the high intracellular creatine concentrations of this zwitterion, the possibility of the involvement of the cellular osmotic balance is not unreasonable.

Creatine Efflux

The steady state model does not distinguish between facilitated diffusion and simple passive diffusion of creatine out of the cell. Such a discrimination is important because the existence of a facilitated transport process might provide another pharmacological target for the control of intracellular [creatine].

Thus we can regard cellular [creatine] as the result of active influx and passive efflux. Of these, we have measured unidirectional active influx isotopically. We have not measured unidirectional efflux because of the difficulties of prelabelling/isotope efflux experiments, but we have studied net efflux. The rate constant of net creatine efflux is 0.03 per day (expressed in terms of the whole creatine pool). If efflux is approximately first-order this should be a reasonable estimate [Kemp and Bevington, 1993] of the rate constant of unidirectional efflux (carrier-mediated or diffusion). This rate constant is close, as one would predict, to the rate constant for the fall in muscle total creatine during replacement with the creatine analogue ß-guanidinoproprionic acid (ß-GPA) *in vivo*, which is 0.02 per day [Meyer, 1989]. In terms of the simple model [Kemp and Bevington, 1993], this rate constant should also equal the ratio of the unidirectional influx rate to the steady-state cell [creatine]. However, in the present case this ratio is approximately 2 per day, which is therefore 200 times larger than expected. **To put this another way, net creatine efflux, 0.1 mmol/kg per hour, is much slower than expected given the relatively rapid rate of unidirectional creatine influx, 6.9 mmol/kg per hour, measured**. There is a parallel with Pi transport, where rates of Pi exchange in myoblasts at least [Kemp *et al.,* 1993] are considerably greater than would be expected from the very low net efflux permeability of the sarcolemma *in vivo*, as evidenced by the very small Pi efflux during exercise, when extensive PCr hydrolysis raises cell [Pi] considerably [Kemp and Bevington, 1993]. This suggests that contrary to the assumptions of

the simple model [Kemp and Bevington, 1993] and to observations of net Pi fluxes in perfused rat heart [Polgreen *et al.,* 1993], creatine unidirectional influx and efflux may be tightly coupled to transport in the myoblast. Such coupling may depend on the downregulation of the transporter when the intracellular creatine is raised [Loike *et al.*, 1988].

Dexamethasone has substantial structural homology with androstan-3ß-ol (the latter being structurally equivalent to a cholesterol molecule from which the alkyl chain has been removed) and on the basis of the structure of dexamethasone and its calculated octanol/water partition coefficient (log $K_{n\text{-octanol}} \approx 0$), one would expect the molecule to partition appreciably into the hydrophobic membrane milieu and enhance simple passive diffusion of a small molecule by enhancing membrane fluidity [Houslay and Stanley, 1982]. The data shown in Figure 2.9 is compatible with this explanation but does not preclude an effect of dexamethasone on the creatine uptake transporter. Considering the slow efflux rates indicated in Figures 2.5b and 2.7b, even a small concentration of dexamethasone would be expected to be effective in causing an enhancement of a passive creatine efflux.

The net loss of creatine from the cell has been termed "creatine efflux"; however it is not yet clear whether the creatine is lost from the cell as creatine or creatinine. If the exit is a simple passive process, then on the basis of the calculated octanol/water partition coefficients of creatine (log $K_{n\text{-octanol}} \approx -10$) and creatinine (log $K_{n\text{-octanol}} \approx -6$) one would expect creatinine to be the major efflux state of cellular creatine. Early work on feeding rats isotopic creatine suggests that the major form of creatine excretion from the tissues is creatinine [Bloch *et al.*, 1941].

Modulators of creatine transport and accumulation

The importance of the Na^+,K^+-ATPase in the control of intracellular [creatine] is

further demonstrated by the factors that increase intracellular [creatine]. We have shown that 3,3′,5-triiodothyronine, isoproterenol (isoprenaline) and rat amylin raise intracellular [creatine]; each of these is known or believed to enhance the activity of the Na^+,K^+-ATPase. This is supported by the observation that ouabain inhibits creatine uptake in astroglial cells [Möller and Hamprecht, 1989] and human erythrocytes [Bennett *et al.*, 1994].

In rat myotubes T3 has a maximal effect on the Na^+,K^+-ATPase at concentrations of 5×10^{-8} M [Brodie and Sampson, 1988]; the peak activity correlates well with the dose dependence shown in Figure 2.8a. These effects, which were elicited over 48 hours and depended on protein synthesis, were sufficient to give a small but significant enhancement of the membrane potential (4-17 mV) [Brodie and Sampson, 1988]. This suggests the occurrence of ion gradient changes which may have been large enough to influence sodium dependent creatine transport. Increasing the activity of the pump allows the maximum intracellular [creatine] of the G8 cell to rise above its original maximum of 165 mmol/kg protein. Could these effects be large enough? If T3 can treble the rate of the Na^+,K^+-ATPase [Brodie and Sampson, 1988], then in the simplest case this would treble the transmembrane sodium gradient. In addition the small enhancement of the membrane potential would also increase the thermodynamic drive to influx. In terms of the discussion above, this could increase the steady-state [creatine] (a measure of the driving force for creatine uptake) by nearly 5 times (if the Na:creatine stoichiometry factor is 1) or 15 times (if it is 2). In rat heart an effect of hyperthyroidism is to increase the both active creatine influx and creatine efflux [Seppet *et al.*, 1985]; the net result being to lower intracellular [creatine] because of small circulatory creatine concentrations. It would be interesting to determine whether thyroxine, T4, or high concentrations of T3 have an effect in enhancing efflux in the G8 cell. Figure 2.7a is compatible with an effect of T3 on enhancing creatine efflux at high concentrations

[Seppet *et al.*, 1985]; the low concentration used to examine the effect of T3 on efflux, Figure 2.7b, shows no significant enhancement of efflux.

Isoproterenol (isoprenaline) is a non-specific agonist at $ß_1$ and $ß_2$ adrenergic receptors. $ß_2$ adrenoreceptors have been shown to mediate the effect of catecholamines in increasing the activity of Na^+,K^+-ATPase in the rat soleus muscle [Clausen and Flatman, 1980]. This effect is mediated by cAMP [Clausen and Flatman, 1977] and leads to a significant enhancement of the membrane potential of 5.8 mV, which is in accordance with our hypothesis. It is possible then that a catecholamine mediated increase in intracellular [creatine] is part of the normal longterm physiological adaptation to exercise. **As efflux is slow, pulses of catecholamine released during exercise would cause transient increases in creatine uptake (Figure 2.8b) but a cumulative increase in intracellular [creatine]; this would then improve muscle function both because of increased deposition of actin and myosin [Bessman, 1992; Ingwall *et al.*, 1972, 1974] and because of increased [PCr].** Interestingly, in the heart T3 causes an increase in expression of ß receptors [Ganong, 1989]; which would permit synergistic actions of T3 and catecholamines in the modulation of intracellular [creatine] at this physiologically important site.

Amylin has approximately 50% homology with calcitonin gene-related peptide (CGRP) and binds to the CGRP receptor in the rat skeletal muscle L6; they both act to increase intracellular cAMP concentration [Zhu *et al.*, 1991] by activation of adenylate cyclase [Poyner *et al.*, 1992]. The fact that amylin is reported to have its maximal effect in the L6 myocytes at a minimum of 100 nM is consistent with the data presented in Figure 2.8a. CGRP acting at the CGRP receptor has also recently been reported to increase the activity of the Na^+,K^+-ATPase in the rat soleus muscle [Andersen and Clausen, 1993], enhancing the membrane potential by 2.5-9.1 mV. This is consistent with our general hypothesis. The location of CGRP

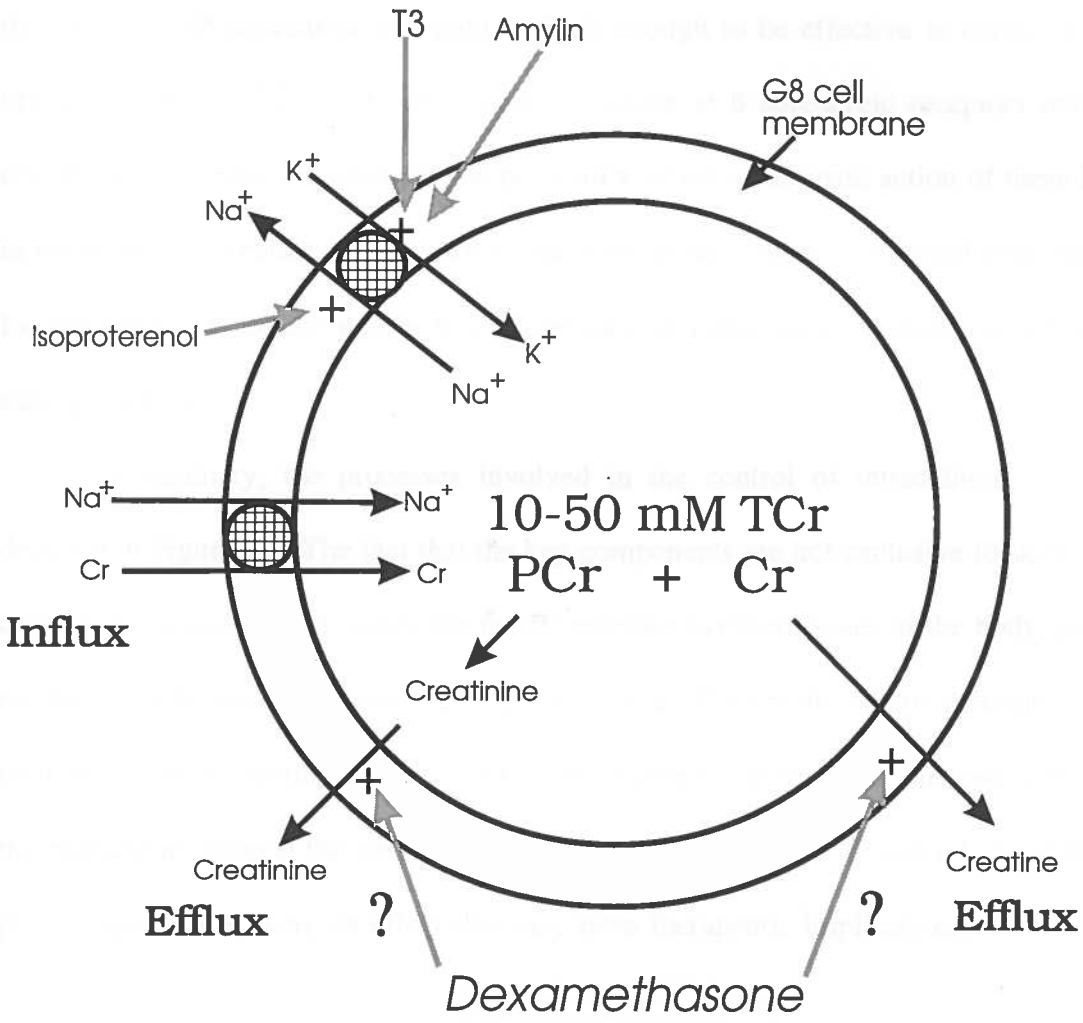


Figure 2.9 Summary of Creatine Transport Processes in the G8 Myoblast
The above is a hypothetical model of creatine transport in the G8 cell, summarising present and previous work (see text).

in motor neurons and the presence of the CGRP receptor on skeletal muscle and cardiac myocytes and its role as a neuropeptide [Leighton and Cooper, 1988; Zhu *et al.*, 1991] indicate a role in muscular adaptation to exercise by increasing creatine uptake. At the post-synaptic membrane and the remainder of the plasma membrane it is not inconceivable that the local CGRP concentration would be high enough to be effective in enhancing creatine uptake. As both CGRP and catecholamines acting at ß adrenergic receptors make use of cAMP as a secondary messenger, the possibility of the synergistic action of these hormones in the control of creatine uptake *in vivo* cannot be ignored; again this would have implications for the sensitivity of the uptake to the presence of either catecholamines or CGRP/amylin during exercise.

In summary, the processes involved in the control of intracellular [creatine] are depicted in Figure 2.9. The fact that the key components are not exclusive to skeletal muscle cells (nor to mouse cells) makes the model relevant to other tissues in the body, particularly cardiac muscle, nervous tissue and smooth muscle. The results obtained support a role for total intracellular creatine as part of an adaptive process to repeated exercise. The effects of the modulators suggest the possibility of raising the intracellular [creatine] above the normal physiological maximum, an effect that may have therapeutic implications.

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Chapter 3

Catecholamines and creatine uptake

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3.1 Introduction

An animal cell can be considered as a component of a multicellular organism whose action is directed by a set of extracellular signals it receives. The process by which the cell converts these signals into a cellular functional response is termed intracellular signal transduction. Because of the importance of creatine in the bioenergetics of muscle and nerve cells it seemed likely that there would be a signal transduction pathway to control its intracellular concentration. Hence an attempt was made to identify and elucidate one such pathway.

Bearing in mind that the G8 cell line is a muscle myoblast cell line it is possible that the extracellular signals *in vivo* are growth factors (e.g IGF-I), neurotransmitters (e.g. CGRP), metabolites (e.g. glucose or creatine) and/or hormones (e.g. adrenaline). However voluntary muscle function is under sympathetic control and this is enhanced by the release of the catecholamines, noradrenaline and adrenaline, that occurs during exercise [Rogers *et al.,* 1991; Bangsbo *et al.,* 1992; Chandler *et al.,* 1993]. Hence an effect of catecholamines on creatine uptake would serve to couple muscle action to metabolite supply. The work described in this chapter is concerned with investigating the effect of catecholamines on cellular creatine content and determining the mechanism of such an effect.

3.1.1 Characteristics of receptor mediated signal transduction

The following are characteristics of signal transduction pathways mediated by plasma membrane receptors [Barritt, 1992]:

1) The extracellular message is carried in the blood or other extracellular fluid; this is termed the first messenger (or primary messenger) e.g. noradrenaline in the plasma.

2) When the receptor binds to the primary messenger a conformational change occurs in the receptor that activates a functional domain. The receptor may be transmembrane and transmit the signal directly to an intracellular target or may activate an intramembrane messenger e.g. a G-protein.

3) All known plasma membrane receptors for extracellular signals are proteins. Receptors generally possess an extracellular domain that is hydrophilic, glycosylated and binds to the agonist. The transmembrane domain is usually composed of one or more short strands of polypeptide chain that has a hydrophobic character (20 amino acids in an alpha helical arrangement).

4) The diffusible molecule or ion that carries the signal from the receptor to the cytosolic target protein is the second messenger (or secondary messenger) e.g. cyclic AMP.

5) The binding of the secondary messenger to intracellular target protein (e.g. protein kinase A, PKA) alters the activity of this protein and initiates a series of intracellular events which result in the cellular response.

6) A cellular response that is caused by a small concentration of an extracellular signal requires amplification of the original signal. This can occur at several levels:

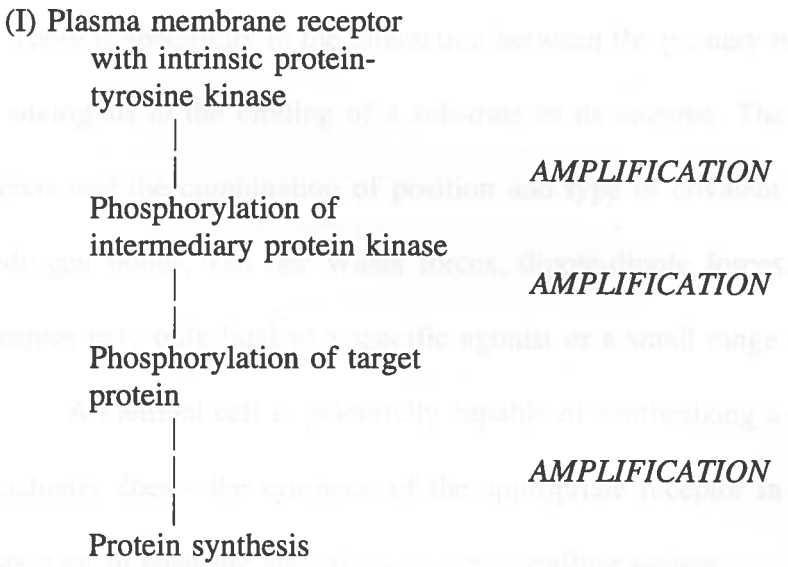


Figure 3.1 A schematic diagram of a generalized tyrosine kinase signalling pathway.

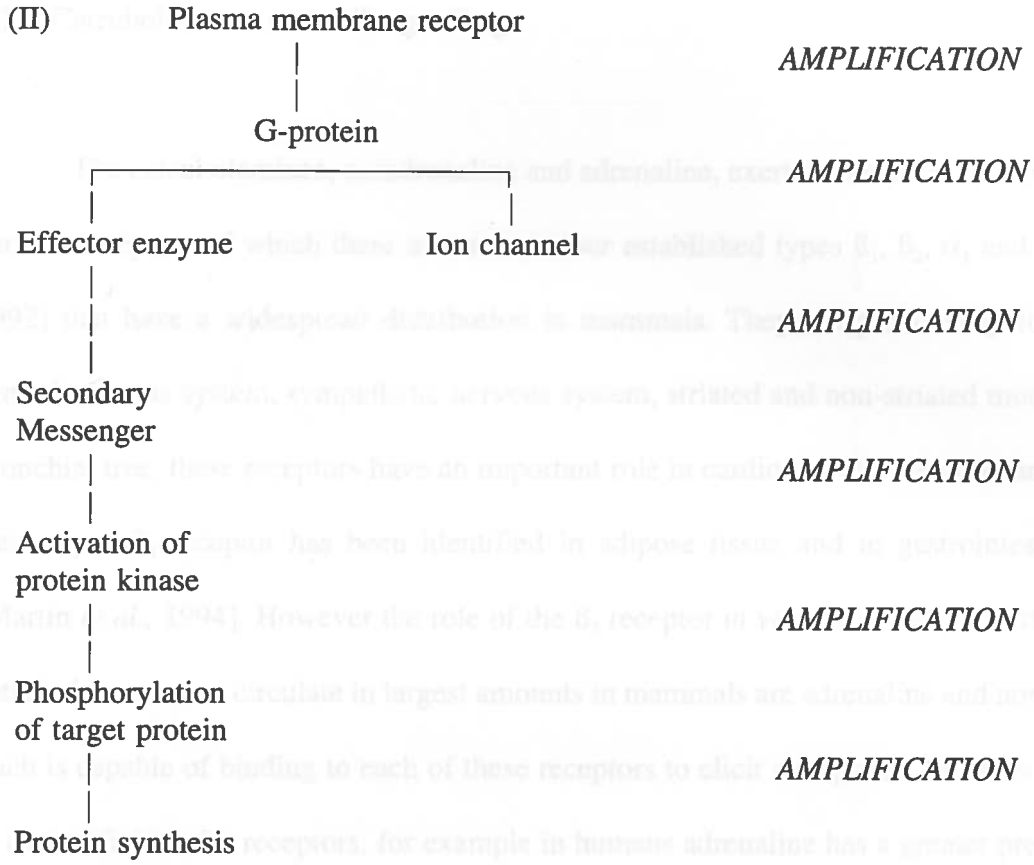


Figure 3.2 A schematic diagram of a generalized serine/threonine kinase signalling pathway.

6) There is specificity in the interaction between the primary messenger and its receptor, this is analogous to the binding of a substrate to its enzyme. The specficity is caused by steric effects and the combination of position and type of covalent and non-covalent forces (e.g. hydrogen bonds, van der Waals forces, dipole-dipole forces, ionic interactions). Hence a receptor may only bind to a specific agonist or a small range of agonists.

An animal cell is potentially capable of synthesizing a larger range of receptors than it actually does - the synthesis of the appropriate receptor in the type of cell concerned is important in ensuring specificity in the signalling system.

3.1.2 Catecholamines and cell signalling

The catecholamines, noradrenaline and adrenaline, exert their effects by binding to cell surface receptors of which there are at least four established types $ß_1$, $ß_2$, α_1 and α_2 [Barritt, 1992] that have a widespread distribution in mammals. They are particularly found in the central nervous system, sympathetic nervous system, striated and non-striated muscle and the bronchial tree; these receptors have an important role in cardiovascular function and exercise. Recently a $ß_3$ receptor has been identified in adipose tissue and in gastrointestinal tissue [Martin *et al.,* 1994]. However the role of the $ß_3$ receptor *in vivo* is not fully established. The catecholamines that circulate in largest amounts in mammals are adrenaline and noradrenaline. Each is capable of binding to each of these receptors to elicit a response, however they differ in their affinities for receptors, for example in humans adrenaline has a greater preference for β_2 receptor than noradrenaline [Weatherall *et al.*, 1987].

The adrenergic receptors differ in the signal transduction pathways that they trigger [Barritt, 1992]. The α_1 adrenergic receptor works through a G protein that activates phospholipase C, which then results in increased intracellular calcium mobilisation and phosphatidylinositol activation. The α_2 adrenergic receptor works through a G protein (G_i) that inhibits adenylate cyclase, to decrease intracellular cyclic AMP production. Both β_1 and β_2 adrenergic receptors act through the stimulatory G protein (G_s) and both β_1 and β_2 receptors stimulate adenylate cyclase to increase intracellular cAMP levels [Barritt, 1992].

The G-proteins are important in transducing the signal from the catecholamine across the cell membrane to an effector [Ross and Gilman, 1977; Taylor, 1990]. The structures of the oligomeric G_s and G_i are well characterised, but the G protein that stimulates phospholipase C has been recently identified as a member of the Gq/GII family [Ushikubi *et*

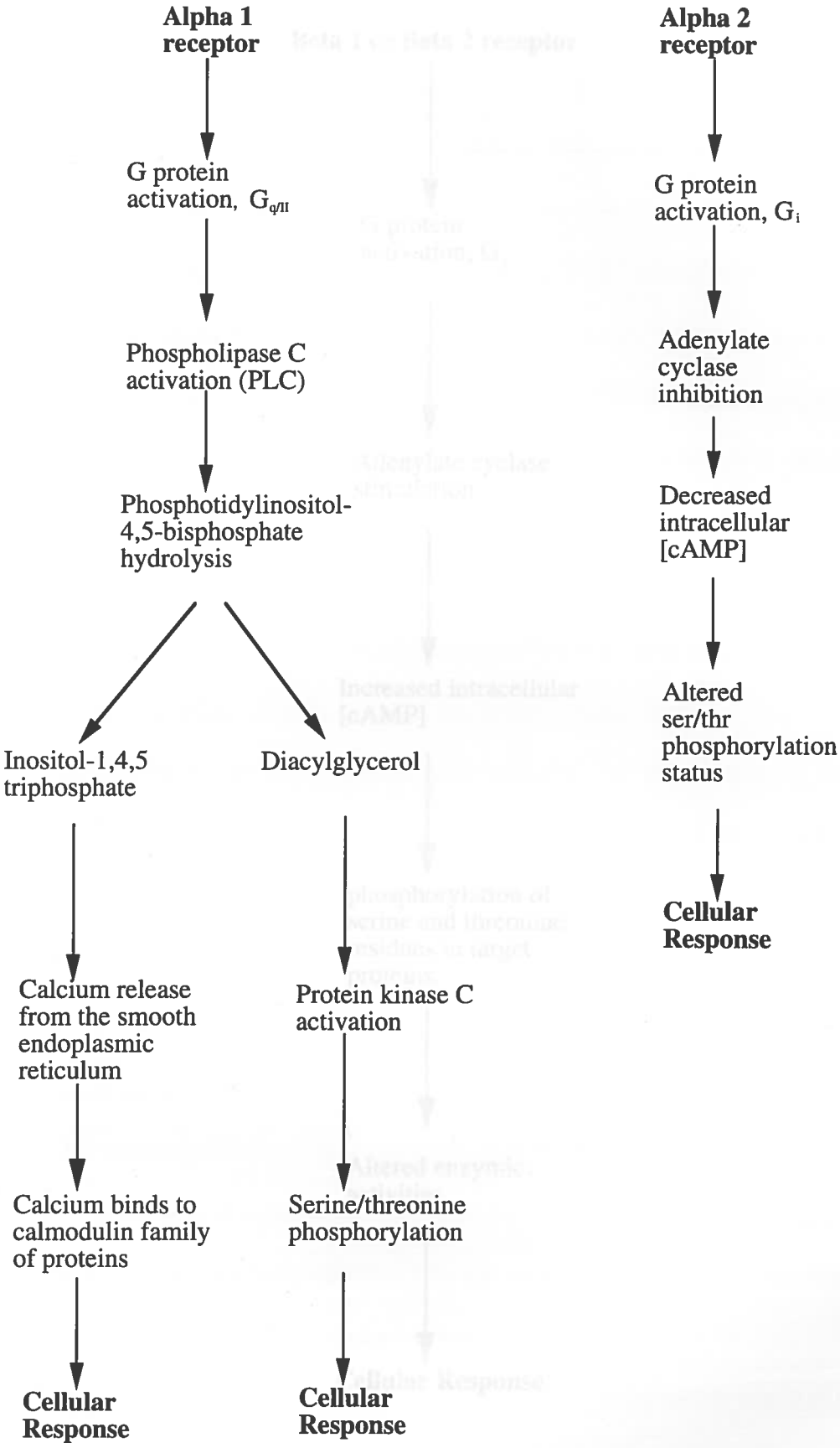


Figure 3.3 Generalised schematic diagram of the signal transduction pathways following alpha1 and alpha2 receptor activation.

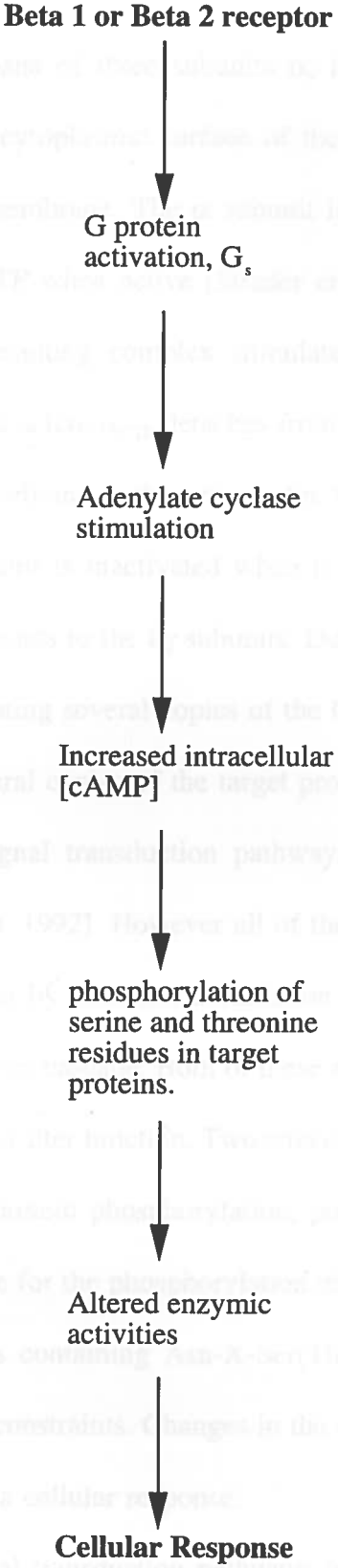


Figure 3.4 Generalised schematic diagram of the signal transduction pathway following beta receptor activation.

al., 1994]. The former consist of three subunits α, ß and γ [Barritt, 1992]. Each of the subunits is exposed to the cytoplasmic surface of the plasma membrane, but none of the subunits span the plasma membrane. The α subunit is bound to GDP when the former is inactive and is bound to GTP when active [Strader *et al.*, 1994]. When the catecholamine binds to its receptor the resulting complex stimulates the inactive α_{GDP} ßγ oligomer to exchange GDP for GTP. The active α_{GTP} detaches from the rest of the G-protein and diffuses in the plane of the plasma mebrane to the activate the target protein (e.g. adenylate cyclase) [Strader, 1992]. The α subunit is inactivated when it hydrolyses the bound GTP to GDP, subsequently the α subunit binds to the ßγ subunits. During this process the receptor-agonist complex is capable of activating several copies of the G protein and the activated α subunit is capable of activating several copies of the target protein [Barritt, 1992].

At this point the signal transduction pathways of the adrenergic receptors differ (figures 3.3 and 3.4) [Barritt, 1992]. However all of the pathways act to control the activity of intracellular proteins either by allosteric modulation (e.g. calcium binding to calmodulin) or by a protein phosphorylation cascade. Both of these approaches depend on conformational changes in protein structure to alter function. Two enzymes (with several isozymes) have been shown to be ubiquitous in protein phosphorylation, protein kinase A and protein kinase C (PKC). They have preference for the phosphorylation of serine/threonine residues in proteins that have primary structures containing Asn-X-Ser(Thr) residues [Hardie, 1993], exposed under the appropriate steric constraints. Changes in the activity of the target proteins of PKC and PKA ultimately lead to a cellular response.

Important in all signal transduction pathways is a means of terminating the signal. Apart from a decrease in extracellular hormone concentration, this can be achieved by two desensitisation processes [Barritt, 1992]. Homologous desensitisation can occur as

a result of the catecholamine decreasing the sensitivity of the cell to the same catecholamine, for example protein kinase A is stimulated by β receptor activation but can also phosphorylate and inactivate the β receptor. The catecholamines may also have their effectiveness on a cell diminished by internalisation and degradation of receptor-agonist complexes - this too is a method of homologous desensitisation. Heterologous desensitisation can occur as a result of a catecholamine decreasing the responsiveness of the cell not only to the catecholamine itself but also to other hormones that share parts of the same signalling process. For example the inactivation of the stimulatory G_s protein [Chambers *et al.*, 1994] by protein kinase C will lead not only to a diminished effect of the catecholamines but also of other hormones using G_s, for example glucagon and vasopressin.

3.2 Methods

The methods and analysis used are described in sections 2.3, 2.4 and 2.7. K-free media was custom made by GIBCO; this was DMEM in which the potassium salt was replaced by a sodium salt. All chemicals were obtained from Sigma unless stated otherwise.

3.3 Experiments performed

A) The effect of noradrenaline on creatine uptake: Noradrenaline hydrochloride (arterenol) was applied to cells at concentration range of 0 - 10 μM.

B) The effect of adrenergic antagonists on basal creatine content: Butoxamine hydrochloride ($ß_2$ antagonist), atenolol ($ß_1$ antagonist), propranolol hydrochloride (non-selective ß antagonist) and labetalol hydrochloride (non-selective α and ß antagonist) were tested at the highest

concentrations used in uptake experiments.

C) The effect of labetalol on noradrenaline mediated creatine uptake: Labetalol hydrochloride (non-selective α and ß receptor antagonist) was tested at a concentration of 1 μM.

D) The effect of methoxamine on creatine uptake: Methoxamine hydrochloride (α_1 agonist) was applied at a concentration range of 0 - 20 μM.

E) The effect of phentolamine on noradrenaline mediated creatine uptake: Noradrenaline hydrochloride was applied at a concentration of 0.1 μM and phentolamine hydrochloride (α antagonist) was applied at a concentration of 100 μM.

F) The effect of propranolol on noradrenaline mediated creatine uptake: Noradrenaline hydrochloride was applied at a concentration of 0.1 μM and propranolol hydrochloride (non-selective ß antagonist) was applied at a concentration of 10 μM.

G) A comparison of butoxamine and atenolol on noradrenaline mediated creatine uptake: Noradrenaline hydrochloride was applied at a concentration of 0.1 μM and butoxamine hydrochloride ($ß_2$ antagonist) and atenolol($ß_1$ antagonist) were applied in a concentration range of 0 - 10 μM.

H) The effect of clenbuterol on creatine uptake: Clenbuterol hydrochloride ($ß_2$ agonist) was applied at a concentration of 100 μM.

I) The effect of dibutyryl-cAMP on creatine uptake: Dibutyryl-cAMP (membrane permeant cAMP analogue) was applied at a concentration range of 0 - 200 μM.

J) The effect of ouabain on creatine uptake: 2 mM ouabain was applied to the cells, as a 60 minute preincubation.

K) The effect of potassium-free medium on creatine uptake: Potassium-free DMEM was applied to the cells.

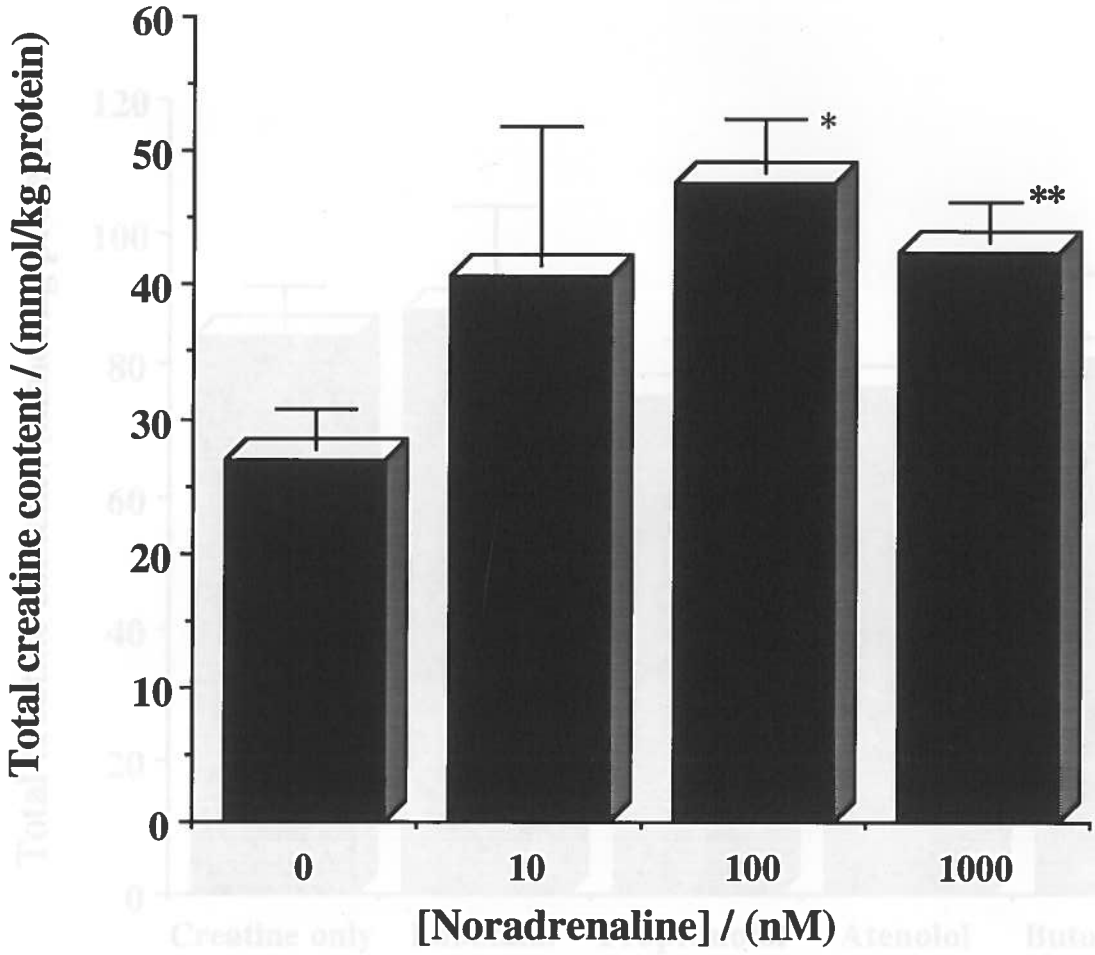


Figure 3.5 The effect of noradrenaline on creatine uptake
A graph of total intracellular [creatine] against applied [noradrenaline], with 0.5 mM creatine in the extracellular medium. The cells were of passage number 10 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

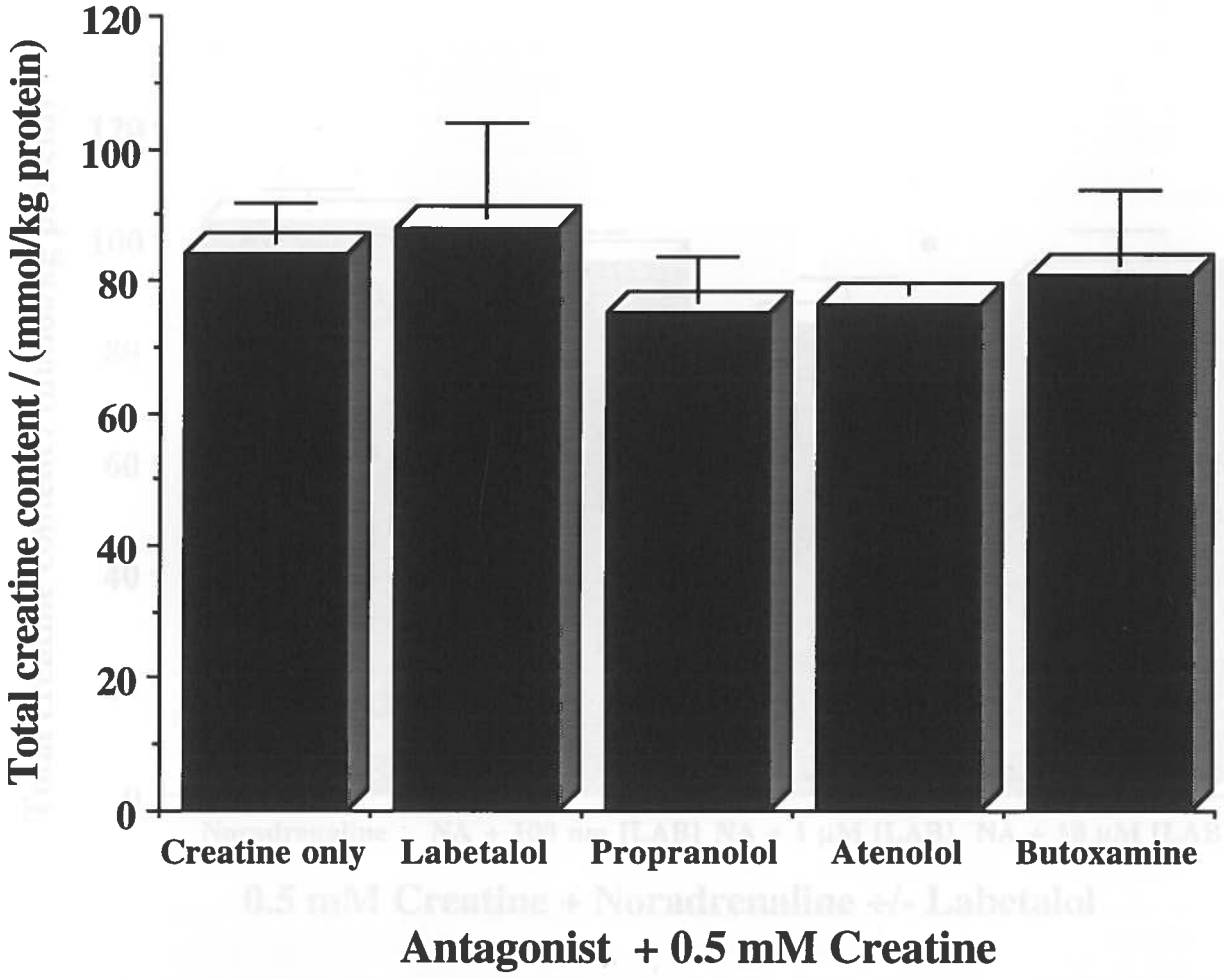


Figure 3.6 The effects of antagonists on basal creatine uptake
A graph of total intracellular [creatine] against 10 μM applied antagonist, with 0.5 mM creatine in the extracellular medium. The cells were of passage number 13 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

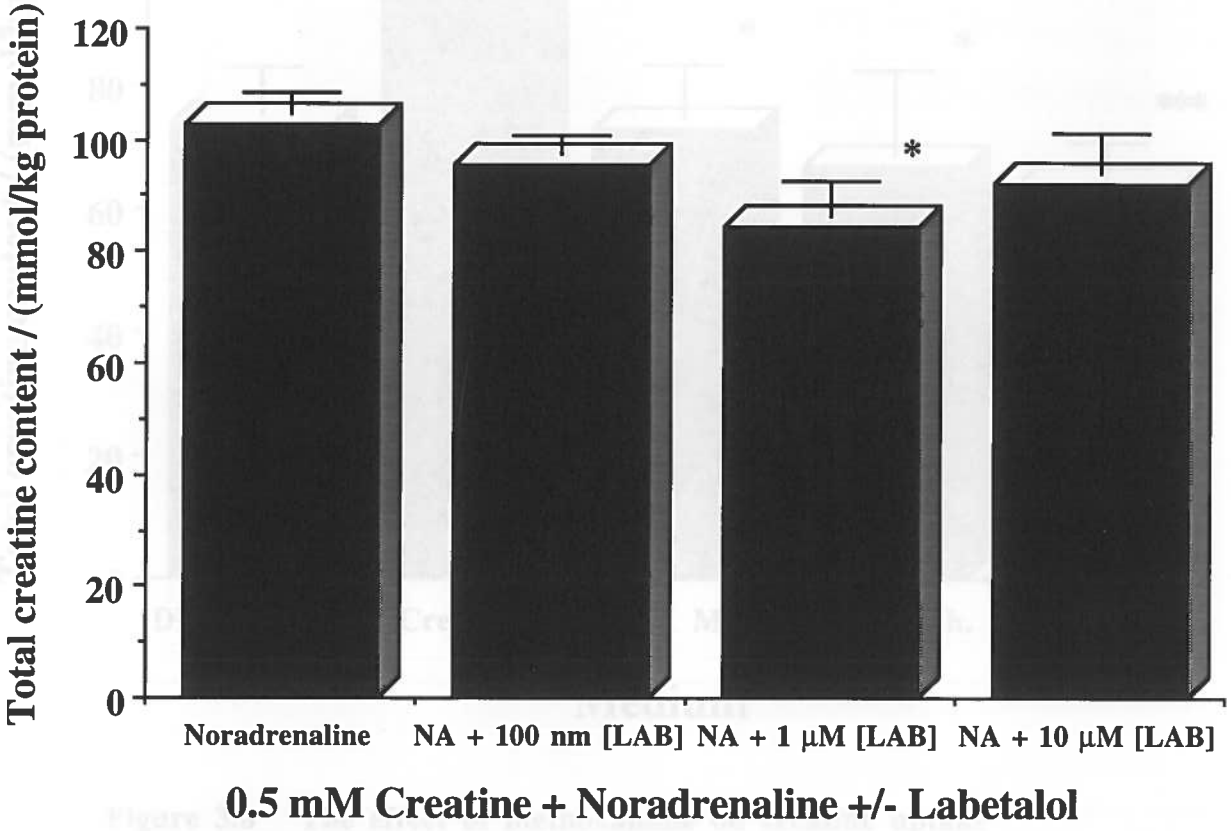


Figure 3.7 The effect of labetalol on noradrenaline mediated creatine uptake
A graph of total intracellular [creatine] against applied [labetalol] in the presence of noradrenaline 0.1 μM. The cells were of passage number 13 (n=6). The experiment was conducted over 48 hours. LAB = labetalol, NA = noradrenaline.

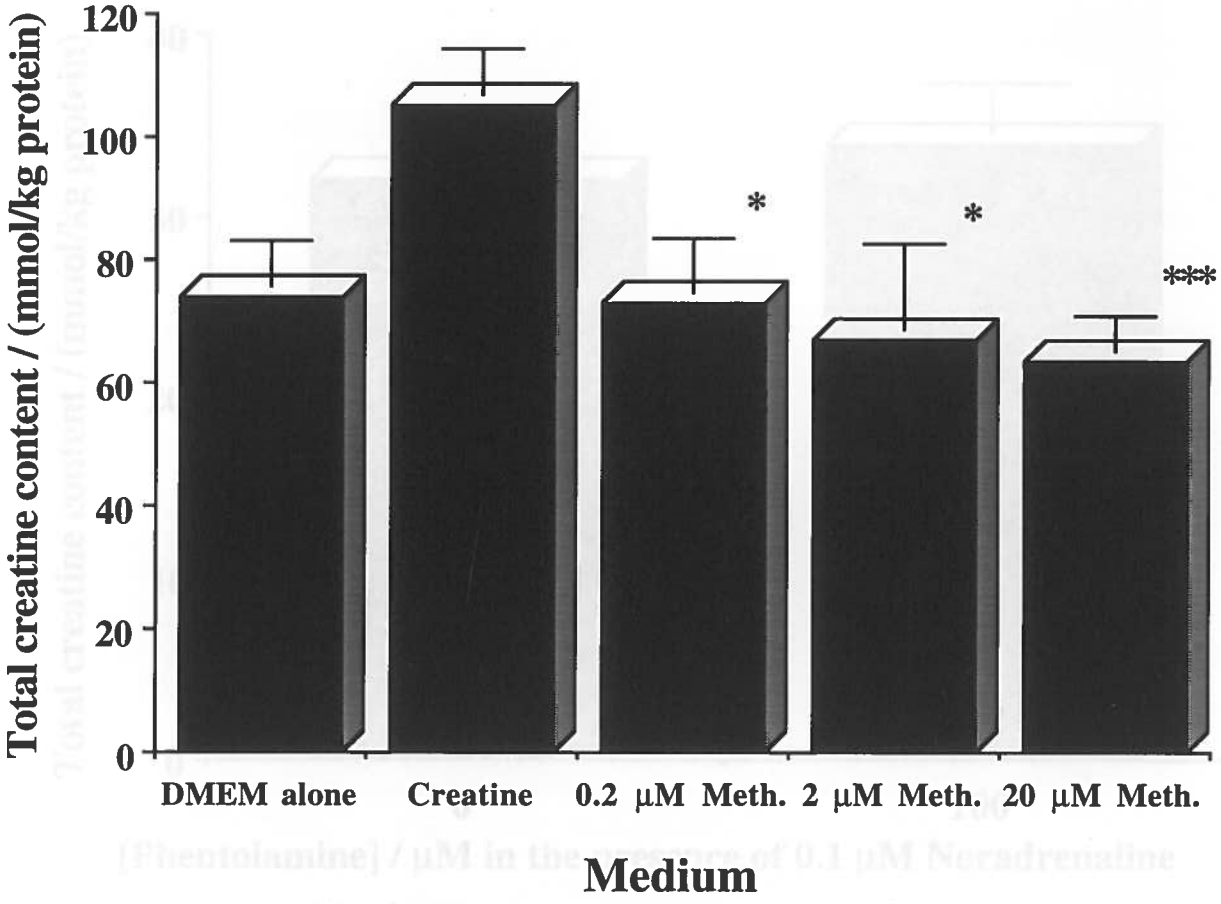


Figure 3.8 The effect of methoxamine on creatine uptake
A graph of total intracellular [creatine] against applied methoxamine (meth.), with 0.5 mM creatine. The cells were of passage number 7 and plating density 250,000 (n=6). The experiment was conducted over 48 hours. DMEM alone contained no creatine.

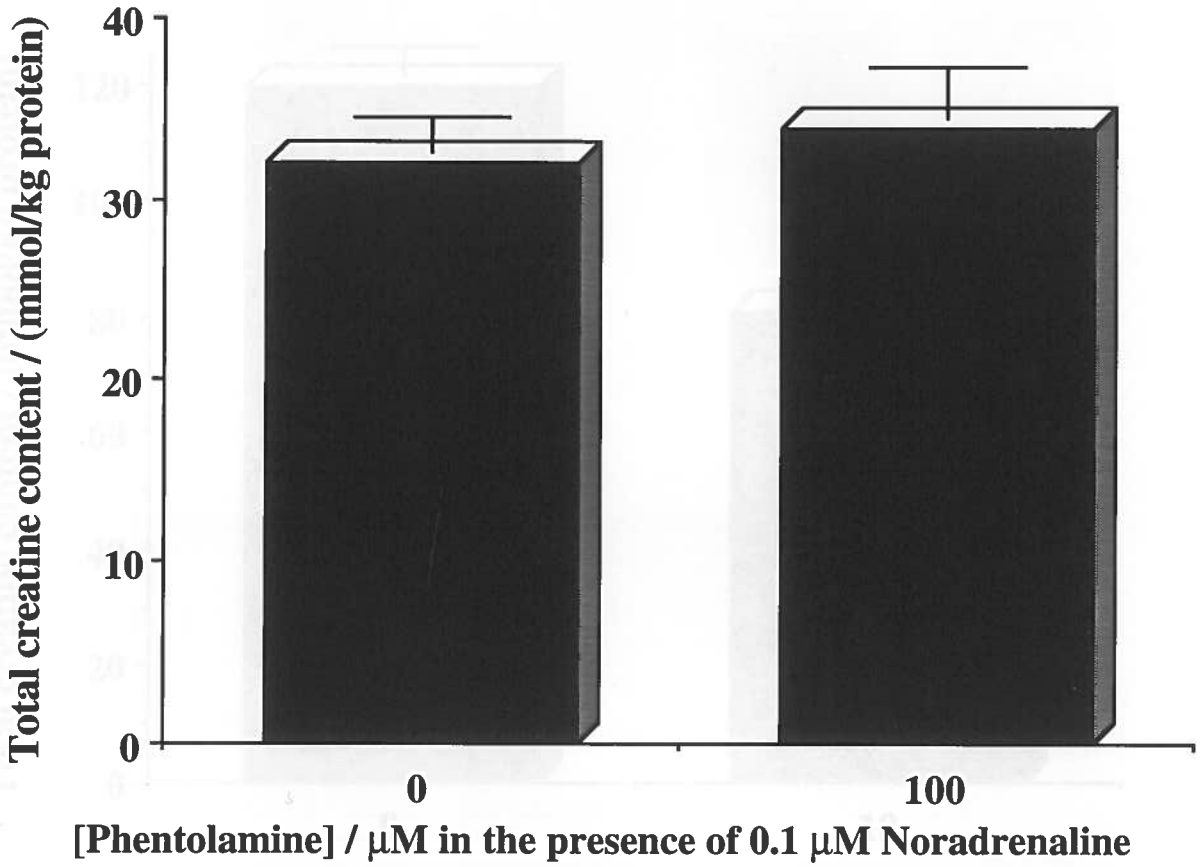


Figure 3.9 The effect of phentolamine on noradrenaline mediated creatine uptak
A graph of total intracellular [creatine] against applied phentolamine, with 0.5 mM creatine. The cells were of passage number 11 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

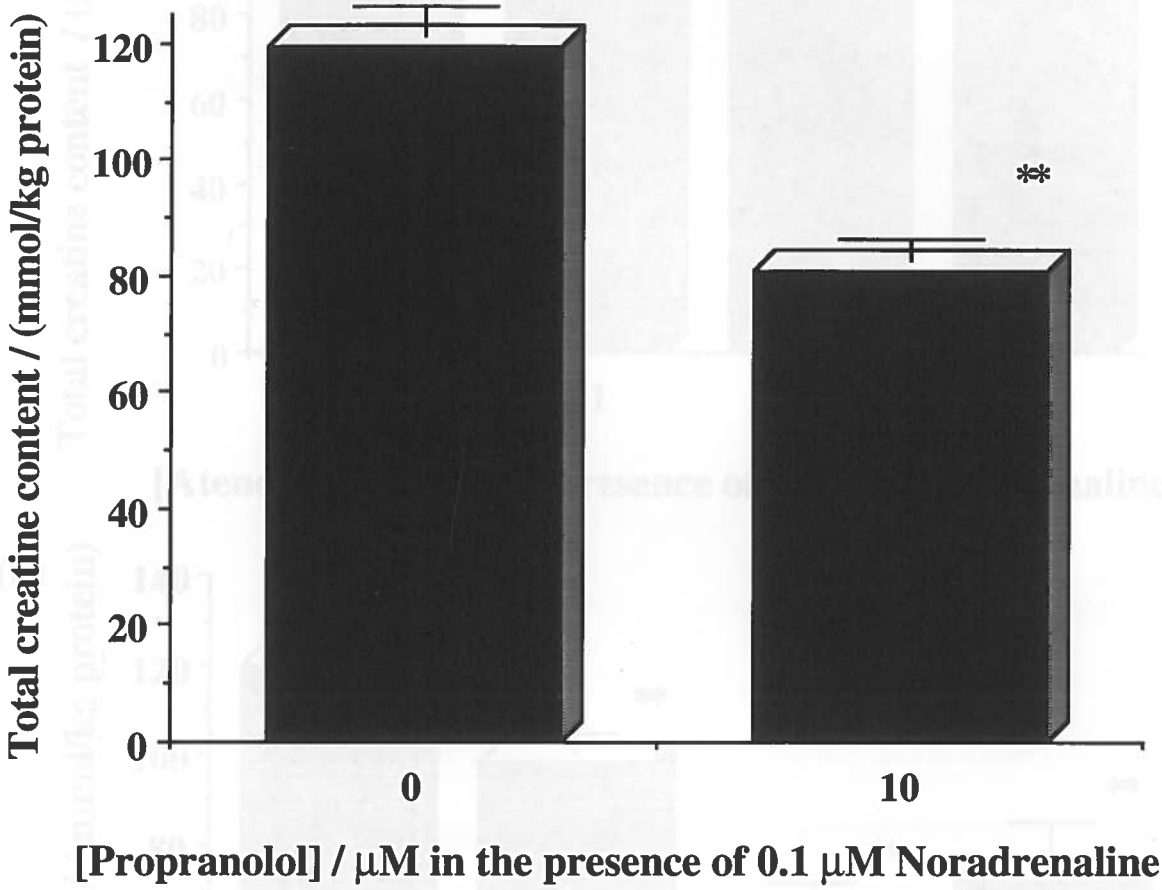


Figure 3.10 The effect of propranolol on noradrenaline mediated creatine uptake A graph of total intracellular [creatine] against applied propranolol, with 0.5 mM creatine. The cells were of passage number 8 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

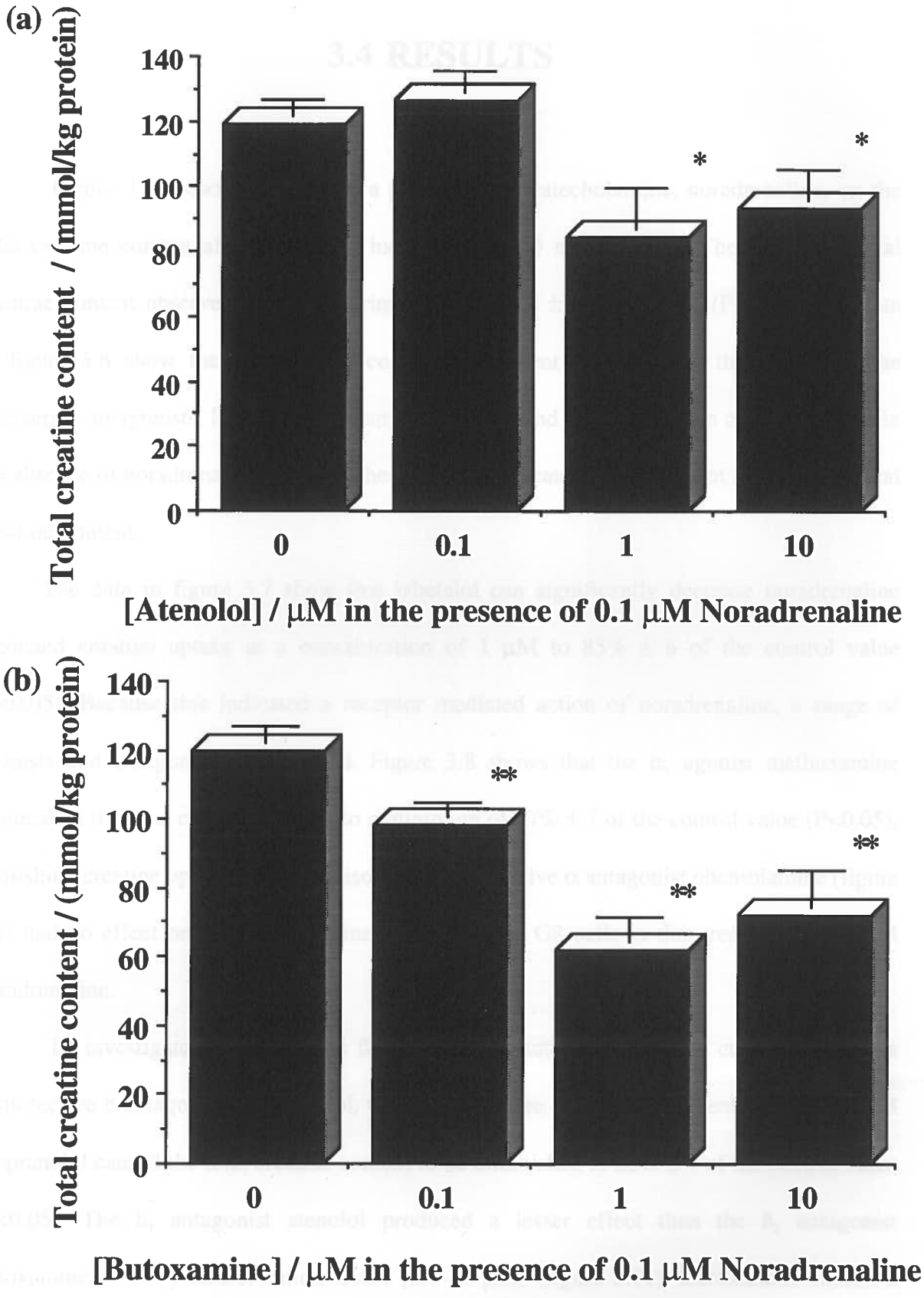


Figure 3.11 Effects of butoxamine and atenolol on noradrenaline mediated creatine uptake
Graphs of total intracellular [creatine] against atenolol (a) or butoxamine (b), with 0.5 mM creatine in the extracellular medium. The cells were of passage number 9 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

3.4 RESULTS

Figure 3.5 shows the effect of a physiological catecholamine, noradrenaline, on the total creatine content after a 48 hour incubation in 0.5 mM creatine. The maximum total creatine content observed in this experiment was 180% ± 30 of control ($P<0.05$). The data in figure 3.6 show the results of a control experiment to determine the effects of the adrenergic antagonists, labetalol, butoxamine, atenolol and propranolol, on creatine uptake in the absence of noradrenaline. None of these antagonists caused a significant alteration in total creatine content.

The data in figure 3.7 show that labetalol can significantly decrease noradrenaline mediated creatine uptake at a concentration of 1 μM to 85% ± 6 of the control value ($P<0.05$). Because this indicated a receptor mediated action of noradrenaline, a range of agonists and antagonists were tested. Figure 3.8 shows that the α_1 agonist methoxamine diminished the total creatine content to a minimum of 57% ± 7 of the control value ($P<0.05$); abolishing creatine uptake. In comparison the non-selective α antagonist phentolamine (figure 3.9) had no effect on the total creatine content of the G8 cells in the presence of 0.1 μM noradrenaline.

To investigate the role of the ß receptors in controlling the total creatine content a nonselective ß antagonist, propranolol, was tested (figure 3.10); at a concentration of 10 μM propranolol caused the total creatine content to be diminished to 68% ± 4 of the control value ($P<0.05$). The $ß_1$ antagonist atenolol produced a lesser effect than the $ß_2$ antagonist butoxamine at every concentration tested (0.1-10 μM) (figure 3.11). Butoxamine caused a maximum decrease in total creatine content to 52% ± 6 of control ($P<0.01$) whereas atenolol caused a maximum decrease to 70% ± 11 of the control value ($P<0.05$). The data in figure

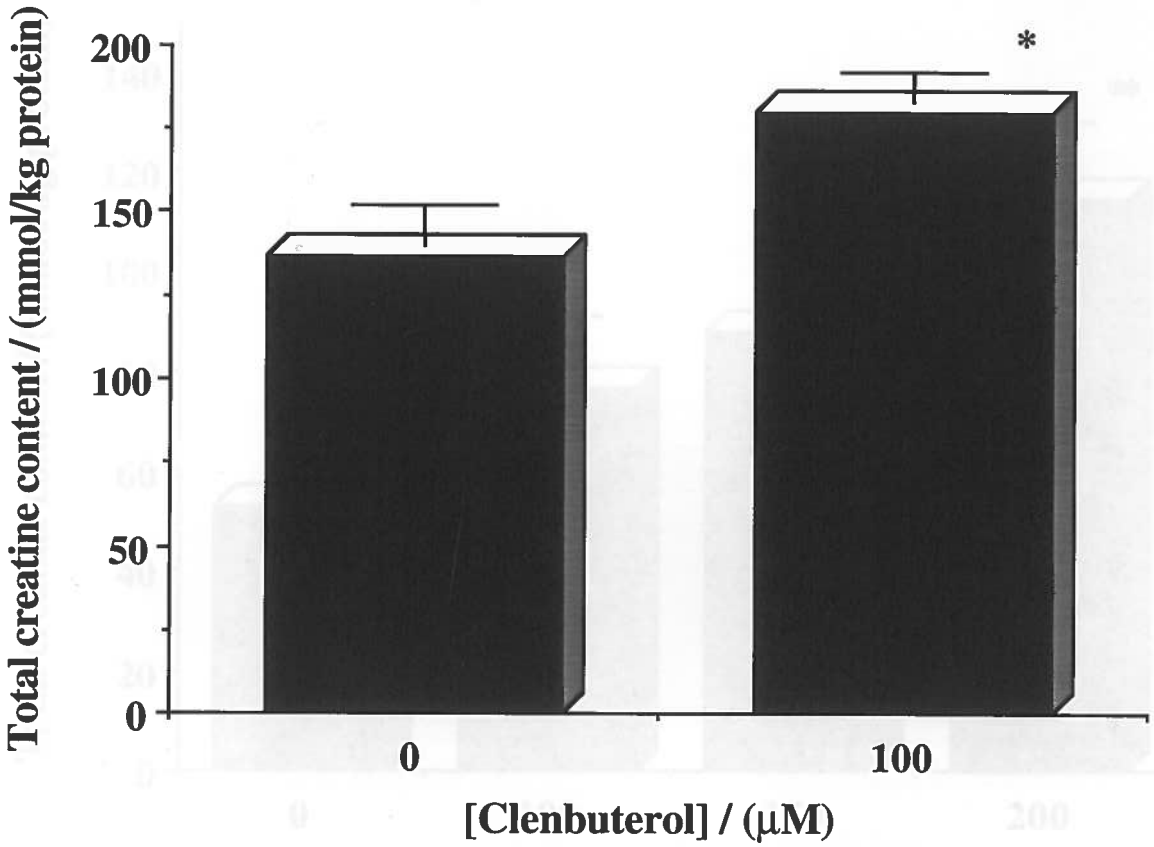


Figure 3.12 The effect of clenbuterol on creatine uptake
A graph of total intracellular [creatine] against applied [clenbuterol], with 0.5 mM creatine in the extracellular medium. The cells were of passage number 11 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

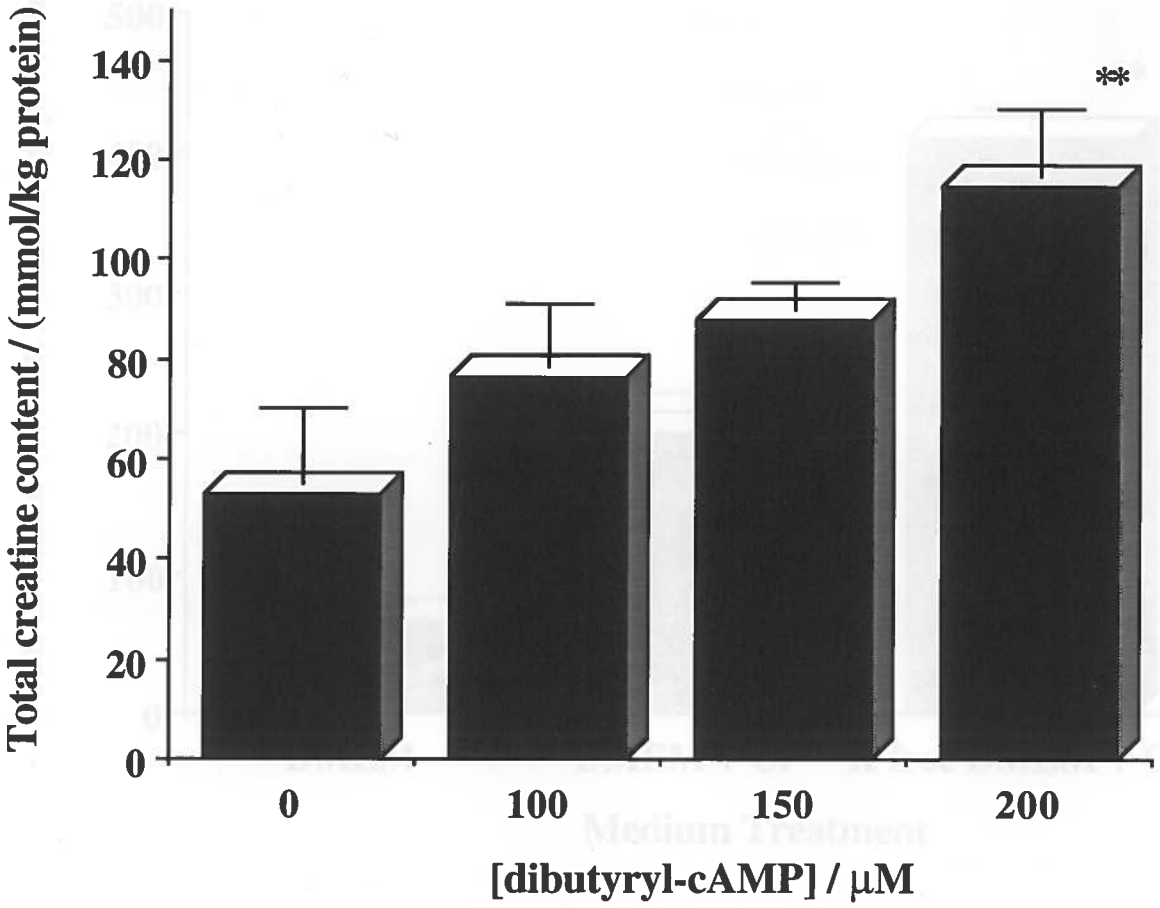


Figure 3.13 The effect of dibutyryl-cAMP on creatine uptake
A graph of total intracellular [creatine] against applied [dibutyryl-cAMP], with 0.5 mM creatine. The cells were of passage number 10 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

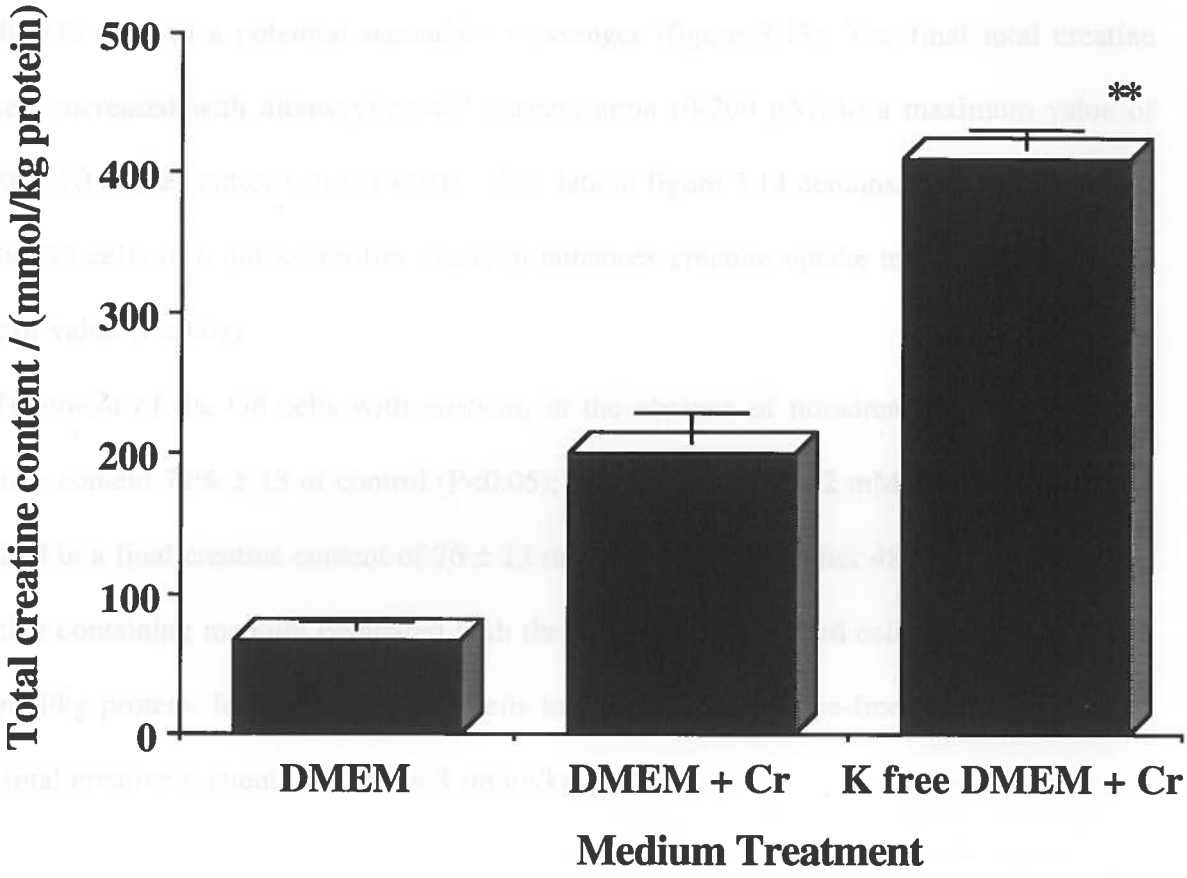


Figure 3.14 The effect of potassium-free medium on creatine uptake
A graph of total intracellular [creatine] against media treatments. DMEM contained no creatine. The cells were of passage number 12 and plating density 250,000 (n=6). Creatine was present at 0.5 mM. The experiment was conducted over 48 hours.

3.12 show that the $ß_2$ agonist increases the total creatine content of the G8 cells to 130% ± 13 of the control value ($P<0.05$).

The cAMP analogue dibutyryl-cAMP was then used to investigate the responsiveness of the G8 cells to a potential secondary messenger (figure 3.13). The final total creatine content increased with dibutyryl-cAMP concentration (0-200 μM) to a maximum value of 230% ± 70 of the control value ($P<0.01$). The data in figure 3.14 demonstrate that incubation of the G8 cells in a potassium-free medium enhances creatine uptake to 200% ± 20 of the control value ($P<0.01$).

Treatment of the G8 cells with ouabain, in the absence of noradrenaline, resulted in a creatine content 72% ± 13 of control ($P<0.05$); preincubation with 2 mM ouabain for 1 hour resulted in a final creatine content of 76 ± 12 mmol/kg of protein after 48 hours in a 0.5 mM creatine containing medium compared with the non-ouabain exposed cells' content of 106 ± 8 mmol/kg protein. In comparison, the cells incubated in a creatine-free medium (DMEM) had final creatine contents of 79 ± 4.3 mmol/kg protein.

3.5 DISCUSSION

Catecholamine controlled creatine uptake

The importance of the effect of nanomolar concentrations of isoproterenol (isoprenaline) in enhancing creatine uptake, is supported by the increased creatine uptake produced by the physiological agonist noradrenaline. Such an effect was demonstrated at concentrations that would be expected to mimic physiological catecholamine activity. The identification of a signal transduction pathway for this agonist required the systematic use of

adrenergic antagonists, each of which was shown to have no effect on basal creatine uptake in the absence of the catecholamine stimulus (figure 3.6); phentolamine was not tested as this α antagonist had no effect in decreasing total creatine in G8 cells exposed to noradrenaline and creatine simultaneously (figure 3.9). The effectiveness of micromolar quantities of labetalol, a non-specific α and ß receptor antagonist, in inhibiting the catecholamine stimulus (figure 3.7) demonstrates that the action of noradrenaline is receptor mediated. The α_1 adrenergic agonist methoxamine (figure 3.8) failed to elicit an enhancement of creatine uptake and the failure of the α antagonist phentolamine to inhibit the action of noradrenaline indicated that a ß receptor population was responsible for transducing the action of noradrenaline. The effect of the non-specific ß receptor antagonist propranolol (figure 3.10) in causing a significant reduction in the noradrenaline mediated creatine uptake confirmed this conclusion.

In an attempt to identify the subtype of ß receptors responsible for the stimulation of creatine uptake the $ß_1$ and $ß_2$ adrenergic receptor antagonists atenolol and butoxamine (figure 3.11) respectively were applied to G8 myoblasts in the presence of 0.5 mM extracellular creatine and 10^{-7} M noradrenaline. Although both atenolol and butoxamine inhibited the noradrenaline mediated creatine uptake, at each concentration butoxamine appeared to produce greater inhibition of the creatine uptake than atenolol; this difference was greatest and statistically significant ($P<0.05$) at 0.1 μM antagonist. This suggests that the $ß_2$ receptors play a dominant role in mediating the catecholamine signal.

In accordance with this the $ß_2$ agonist clenbuterol was shown to be active in enhancing creatine uptake (figure 3.12). As creatine has previously been shown to be anabolic [Bessman and Mohan, 1992] and clenbuterol has been shown to have a muscle-specific anabolic effect by enhancing protein deposition in rat skeletal and heart muscle through $ß_2$ receptors [Choo

et al., 1992; Sillence *et al.*, 1991] it is possible that the anabolic effects of clenbuterol are elicited in whole or part by creatine.

ß receptors in mammalian muscle are known to elicit their effects through the secondary messenger cAMP. We show that the membrane permeant cAMP analogue, dibutyryl cAMP, produces a concentration dependent increase in creatine uptake (figure 3.13). $ß_2$ adrenoreceptors have been shown to mediate the effect of catecholamines in increasing the activity of Na^+,K^+-ATPase in the rat soleus muscle [Clausen and Flatman, 1980]. Furthermore the importance of the Na^+,K^+-ATPase in creatine uptake, in L6 myoblasts and rat/mouse astroglial cells, has recently been demonstrated [Bennett *et al.*, 1994; Möller and Hamprecht, 1989]; both groups have shown that the uptake of radiolabelled creatine is decreased on ouabain treatment. This increased activity of the Na^+,K^+-ATPase leads to a significant enhancement of the membrane potential of 5.8 mV [Clausen and Flatman, 1977]. The potassium-free DMEM was used to indirectly test the hypothesis that the membrane potential would be a controlling factor in creatine uptake. A potassium-free medium would lead to a hyperpolarisation of the plasma membrane, as this is controlled by the potassium electrochemical gradient, removing extracellular potassium increases the potassium concentration gradient. Hence the potassium-free medium would be expected to enhance creatine uptake and total creatine content. Figure 3.14 depicts data that support this hypothesis.

Our experiments show that 2 mM ouabain pretreatment of G8 myoblasts completely abolishs net creatine uptake without affecting cell viability. In addition earlier work has shown that triiodothyronine, isoproterenol (isoprenaline) and amylin all increase creatine uptake in G8 myoblasts (sections 2.8 and 2.9); these agonists are known to or believed to enhance the activity of the Na^+,K^+-ATPase [Odoom *et al.*, 1993; Conway and Clark, 1995; Radda *et al.*,

Catecholamines and Creatine Uptake

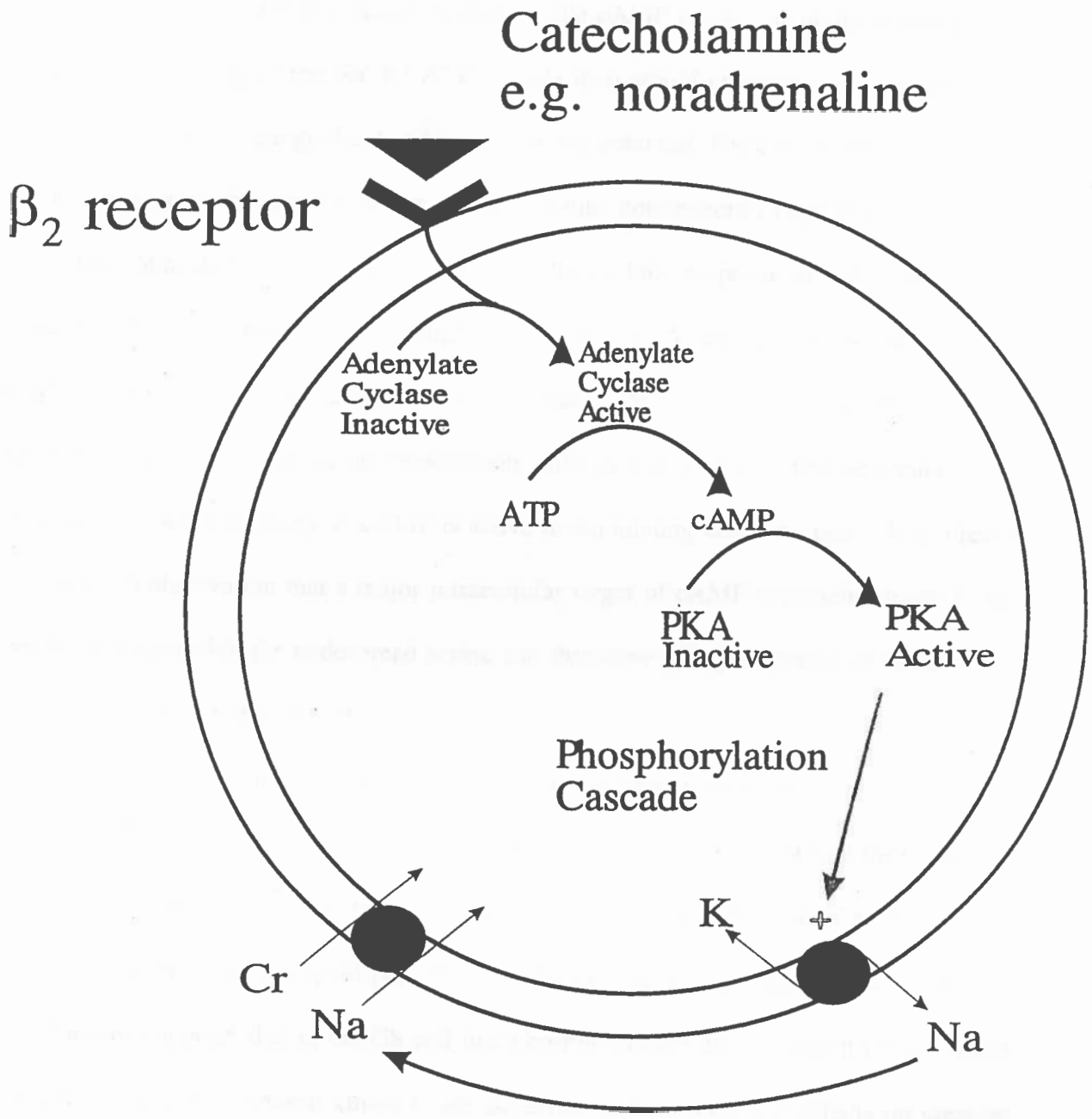


Figure 3.15 Summary of proposed regulatory pathway for creatine uptake
The diagram represents a postulated regulatory pathway for total creatine content in the G8 cell. The connection between the activity of PKA and the sodium potassium ATPase is indirect.

1995]. Together these observations suggest that creatine uptake is controlled predominantly by a $ß_2$ adrenergic receptor that increases intracellular cAMP levels eventually resulting in an increase in the activity of the Na^+,K^+-ATPase, this then would enhance locally or generally the transmembrane sodium gradient and the membrane potential. The electrochemical gradient would then increase the activity of the sodium creatine cotransporter (figure 3.15).

The connection between cyclic AMP and the cellular response of enhanced creatine uptake has been assumed to be through protein kinase A and protein serine/threonine phosphorylation. The following observations provide evidence for phosphorylation being an important component of the signal transduction pathway that controls creatine uptake:

1) We have shown that dibutyryl-cAMP is active in stimulating creatine uptake. It is already an established observation that a major intracellular target of cAMP is protein kinase A, the latter being responsible for widespread serine and threonine phosphorylation of intracellular protein [Barrit, 1992; Hardie, 1993].

2) Protein kinase A and protein kinase C often act in opposition when active along the same signal transduction pathway [Nishizuka, 1984]. The data in figure 3.8 show that the α_1 agonist methoxamine decreases creatine uptake. Furthermore α_1 receptor stimulation works through a G protein to stimulate phospholipase C and indirectly activate protein kinase C [Barritt, 1992]. Hence it appears that in the G8 cell line phosphorylation due to protein kinase A and phosphorylation due to protein kinase C are occurring to have opposite effects on creatine uptake.

3) The Na^+,K^+-ATPase is known to be phosphorylated and inhibited by protein kinase C *in vitro* [Lowndes *et al.*, 1990]. Phosphorylation of the Na^+,K^+-ATPase would be predicted from our model of the control of creatine uptake. However it is not known whether PKA has a direct stimulatory effect on the Na^+,K^+-ATPase, together with the reported indirect effect in

stimulating the Na^+,K^+-ATPase [Clausen and Flatman, 1977,1980].

This chapter presents evidence for a signalling pathway initiated by noradrenaline on receptor binding, causing an increase in intracellular cyclic AMP levels, resulting in increased activity of the sodium potassium ATPase and hence resulting in an increased activity of the sodium creatine cotransporter. However this need not be a complete description of the process, for example it possible that the phosphorylation cascade also has an effect directly on the sodium creatine cotransporter, as well as passing through the Na^+,K^+-ATPase. There is also the possibility of interaction between intracellular signalling pathways, for example the effects of H^+ or Ca^{2+} on the action of noradrenaline. However such interactions would be partly dependent on the existence of other signal transduction pathways to control creatine uptake; this is pursued in the following chapters.

Exercise and creatine regulation

The physiological importance of this signal transduction becomes clearer when allied to the fact that although net creatine uptake in these skeletal muscle cells can be enhanced rapidly by catecholamines, net efflux is much slower in G8 myoblasts ($t_{1/2}$ of approximately a month) [Odoom *et al.*, 1993, Conway and Clark, 1995] (sections 2.8 and 2.9). This slow efflux has been noted in several tissues [Otten *et al.*, 1986; Meyer, 1989; Bloch *et al.*, 1941]. Hence a bout of exercise would be expected to cause a lasting increase in total intracellular creatine content and a consequent improvement in muscle function. Such a model is consistent with human creatine feeding studies that show exercise enhancement of creatine content in the leg [Harris *et al.*, 1992]. The fact that creatine appears to have an effect on anaerobic muscle function rather than aerobic muscle function [Balsom *et al.*, 1993] is consistent with the increase in creatine phosphate concentration on creatine administration, and the increase

in body (muscle) weight caused by creatine.

Muscle contraction in the rat soleus muscle has been shown to prevent the desensitisation of ß receptors caused by prolonged exposure to catecholamines [Bergersen *et al.*, 1994]. This study *in vitro*, if valid *in vivo*, suggests that the ß receptors are more likely to be conserved at the cell surface if the individual continues to exercise, thus maintaining the sensitivity of the cell to a creatine uptake signal. This would be a valuable adaptation to repeated exercise.

The sensitivity of the muscle cell to a creatine uptake signal would be further enhanced if the activity of adenylate cyclase can be increased *in vivo*. Nieto [Nieto *et al.*, 1994] has shown that activity of the catalytic unit of adenylate cyclase is increased after a 10 week progressive running program in rats. If this result, obtained in the liver, is applicable in striated muscle then the increase in adenylate cyclase activity with prolonged exercise regimes would be part of an adaptation to exercise.

The role of the Na^+,K^+-ATPase suggested from the G8 work *in vitro* is supported by independent tissue studies [Smirnova *et al.*, 1994]. It has been shown that rats swimming for 15 or 90 minutes had an increased Na^+,K^+-ATPase activity in the myocardium. The number of ^{3}H-ouabain binding sites increased with the intensity of skeletal muscle exercise, provided exhaustion was not reached. Furthermore, prevention of muscular movement through denervation, tenotomy or plaster immobilisation decreased the concentration of ^{3}H-ouabain binding sites in skeletal muscle. The authors concluded that acute exercise-induces activation of Na^+,K^+-ATPase function in skeletal muscle. It is reasonable to assume that there will be a concomitant increase in the trans plasma membrane sodium gradient and enhanced creatine influx *in vivo*.

It is likely that adaptation to repeated exercise occurs at the level of the signal

transduction pathway for creatine uptake rather than in the levels of released and circulating catecholamines because of the small difference in catecholamine release observed in well trained and minimally trained healthy subjects during exercise [Rogers *et al.*, 1991].

Clenbuterol in animal and human physiology

Clenbuterol is frequently used as an anabolic agent in the livestock industry; its mechanism of action is known to be through β_2 receptors; although we have not investigated that here, work at lower clenbuterol concentrations (0.1 micromolar) in the L6 rat cell line, has shown that absolute protein deposition is significantly increased after 24 hours [McMillan *et al.*, 1992]. Both creatine and clenbuterol cause a shift in proportion from type I (slow) to type II (fast) muscle fibre composition in skeletal muscle bundles [Sipilä *et al.*, 1981; Duncan *et al.*, 1994] and both are believed to cause deposition of protein actin [Ingwall *et al.*, 1972, 1974; Carter *et al.*, 1991]. It would be interesting to see whether creatine could replace clenbuterol as an anabolic food supplement, so avoiding the side effects caused by β receptor activation throughout the animal, in favour of the localised effects of creatine on muscle.

Clenbuterol has been used to improve post-operative recovery in patients with orthopaedic knee problems [Maltin *et al.*, 1993]. The major intracellular metabolite of creatine, creatine phosphate has also been shown to improve post-operative recovery in similar patients [Agnese *et al.*, 1992]. (Although administered as creatine phosphate, this is hydrolysed in the plasma to creatine, which is then absorbed by the tissues.) It is reasonable to suggest that the effect of clenbuterol in enhancing creatine uptake by the striated muscle is responsible for at least part of the therapeutic effect.

Although the therapeutic effects of clenbuterol are promising it has also been noted that clenbuterol treated rats have a decreased maximum training intensity after 4 weeks

administration, this being believed to be due to collagen deposition in the heart. Perhaps the more specific anabolic effects of creatine might avoid such problems.

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Chapter 4

IGF-I, Insulin and creatine uptake

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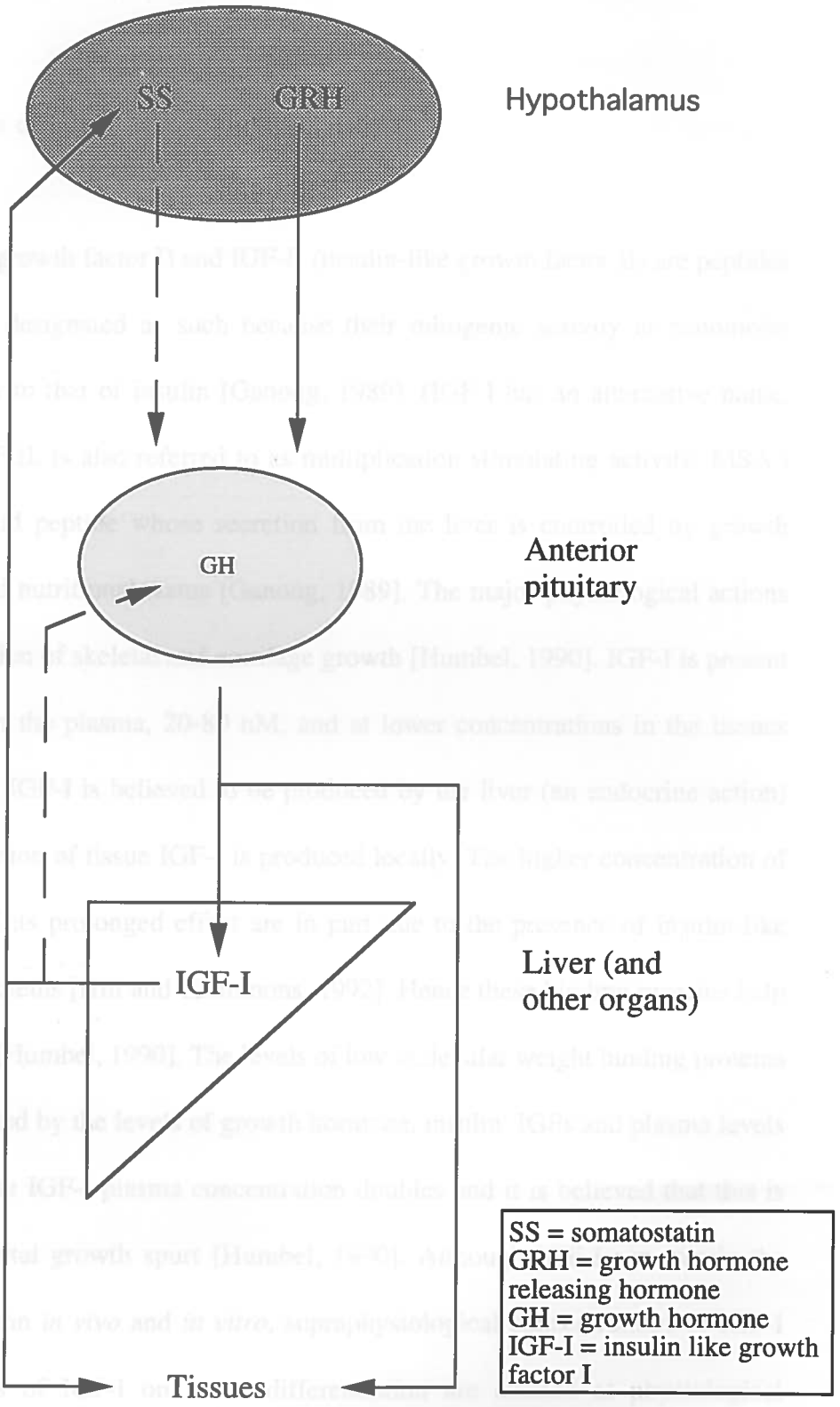


Figure 4.1 Feedback control of IGF-I secretion
The dashed arrows indicate inhibitory effects and the solid arrows indicate stimulatory effects. [From Ganong, 1989]

4.1 Introduction

4.1.1 Physiological roles of IGF-I, IGF-II and insulin

IGF-I (insulin-like growth factor I) and IGF-II (insulin-like growth factor II) are peptides present in human sera, designated as such because their mitogenic activity at nanomolar concentrations is similar to that of insulin [Ganong, 1989]. (IGF-I has an alternative name, somatomedin C and IGF-II, is also referred to as multiplication stimulating activity, MSA.) IGF-I is a 70 amino acid peptide whose secretion from the liver is controlled by growth hormone (figure 4.1) and nutritional status [Ganong, 1989]. The major physiological actions of IGF-I are the stimulation of skeletal and cartilage growth [Humbel, 1990]. IGF-I is present at high concentrations in the plasma, 20-80 nM, and at lower concentrations in the tissues [Humbel, 1990]. Plasma IGF-I is believed to be produced by the liver (an endocrine action) whereas a greater proportion of tissue IGF-I is produced locally. The higher concentration of IGF-I in the plasma and its prolonged effect are in part due to the presence of insulin-like growth factor binding proteins [Hill and Clemmons, 1992]. Hence these binding proteins help to avoid hypoglycaemia [Humbel, 1990]. The levels of low molecular weight binding proteins in plasma may be regulated by the levels of growth hormone, insulin, IGFs and plasma levels of glucose. At puberty the IGF-I plasma concentration doubles and it is believed that this is responsible for the pubertal growth spurt [Humbel, 1990]. Although IGF-I can mimic the metabolic effects of insulin *in vivo* and *in vitro*, supraphysiological concentrations of IGF-I are required; the effects of IGF-I on tissue differentiation are elicited at physiological concentrations.

IGF-II is a 67 amino acid peptide that is produced by diverse tissues [Ganong, 1989]

and, unlike IGF-I, its serum concentration is not controlled to as great an extent by growth hormone. Like IGF-I, IGF-II is present at higher concentrations in the plasma than in the tissues and also binds to circulating IGF binding proteins [Humbel, 1990]. There is no change in plasma IGF-II concentration at puberty and its major physiological role is unclear; some investigators believe that it is a foetal growth factor. The known effects of IGF-I and II on metabolic processes in cartilage are as follows [Ashcroft and Ashcroft, 1992]:

1) Increased transport of amino acids across the cell membrane.

2) Stimulation of protein synthesis.

3) Increased rates of DNA and RNA synthesis.

4) Stimulation of proteoglycan and collagen synthesis.

5) Decreased rate of protein degradation.

Human disorders characterised by an increased serum IGF are acromegaly and gigantism. Human disorders that can result in decreased serum IGF are cirrhosis of the liver, diabetes, primary hypothyroidism and undernutrition [Ganong, 1989; Humbel 1990].

Insulin is a peptide consisting of two chains, A and B, of total molecular weight 6000 daltons. It is secreted by the beta cells of the islets of Langerhans in the pancreas and these cells store insulin until they receive the stimulus to secrete. The major physiological stimulus is an increased serum glucose concentration [Ashcroft and Ashcroft, 1992]. This peptide hormone is secreted into the circulatory system and has the metabolic effects outlined in Table 4.1 [from Ashcroft and Ashcroft, 1992].

Table 4.1: The metabolic effects of Insulin

Effects on protein metabolism

1) Insulin increases the rate of transport of glucose across the cell membrane in adipose tissue and muscle.

2) Insulin increases the rate of glycolysis in muscle and adipose tissue.

3) Insulin stimulates the rate of glycogen synthesis in a number of tissues, including adipose tissue, muscle and liver. It also decreases the rate of glycogen breakdown in muscle and liver.

4) Insulin inhibits the rate of glycogenolysis and gluconeogenesis in the liver.

5) Insulin increases the rate of glucose oxidation by the pentose phosphate pathway in liver and adipose tissue.

Effects on lipid metabolism

1) Insulin inhibits the rate of lipolysis in adipose tissue.

2) Insulin stimulates fatty acid and triacylglycerol synthesis in adipose tissue and liver.

3) It increases the rate of very low density lipoprotein (VLDL) formation in the liver.

4) It increases lipoprotein lipase activity in adipose tissue and this increases the uptake of tryglyceride from the blood into adipose tissue.

5) It may decrease the rate of fatty acid oxidation in liver.

6) It increases the rate of cholesterolgenesis in the liver.

Effects on protein metabolism

1) Insulin increases the rate of transport of amino acids into muscle, adipose tissue, liver and other cells.

2) Insulin increases the rate of protein synthesis in muscle, adipose tissue, liver and other tissues.

3) Insulin decreases the rate of protein degradation in muscle.

Insulin deficiency can result in diabetes mellitus, this occurs as an insufficiency in insulin secretion and is characterized by an elevated fasting blood glucose concentration.

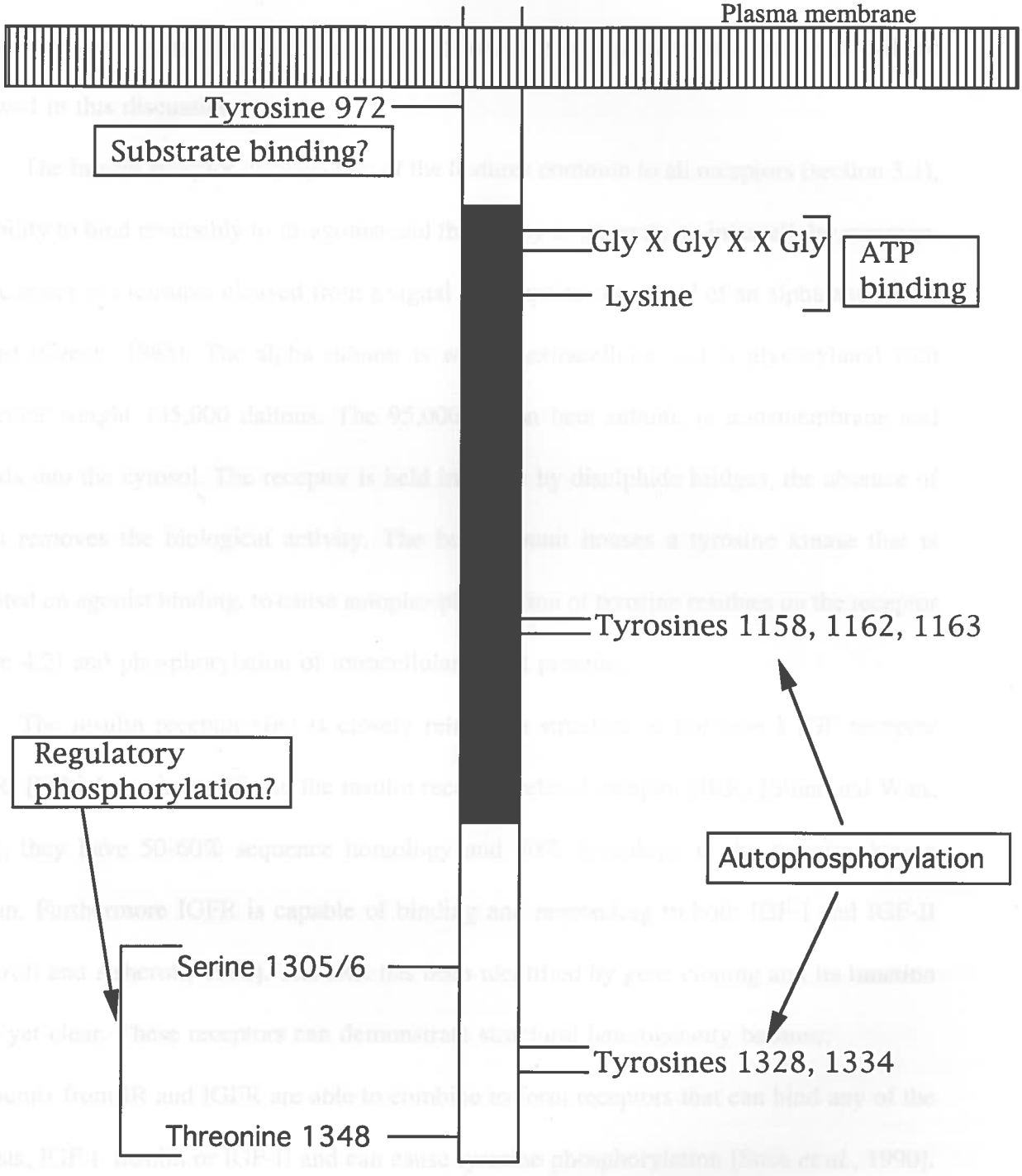


Figure 4.2 Intracellular portion of the insulin receptor
Residues of functional importance are shown. The conserved kinase domain is indicated by shading [From Ashcroft and Ashcroft, 1992].

4.1.2 IGF-I, IGF-II, insulin and cell signalling

The similarity in structure and function of the insulin and IGF receptors lead investigators to assume that their signalling pathways are similar; such a precedent will be followed in this discussion.

The insulin receptor displays two of the features common to all receptors (section 3.1), the ability to bind reversibly to its agonist and the ability to generate an intracellular message. The receptor is a tetramer cleaved from a signal polypeptide composed of an alpha and a beta subunit [Czech, 1985]. The alpha subunit is wholly extracellular and is glycosylated with molecular weight 135,000 daltons. The 95,000 dalton beta subunit is transmembrane and extends into the cytosol. The receptor is held in shape by disulphide bridges, the absence of which removes the biological activity. The beta subunit houses a tyrosine kinase that is activated on agonist binding, to cause autophosphorylation of tyrosine residues on the receptor (figure 4.2) and phosphorylation of intracellular target proteins.

The insulin receptor (IR) is closely related in structure to the type I IGF receptor (IGFR) [Ullrich *et al.*, 1986] and the insulin receptor related receptor (IRR) [Shier and Watt, 1989]; they have 50-60% sequence homology and 80% homology in the tyrosine kinase domain. Furthermore IGFR is capable of binding and responding to both IGF-I and IGF-II [Ashcroft and Ashcroft, 1992]. The IRR has been identified by gene cloning and its function is not yet clear. These receptors can demonstrate structural heterogeneity because:

i) Subunits from IR and IGFR are able to combine to form receptors that can bind any of the agonists, IGF-I, insulin or IGF-II and can cause tyrosine phosphorylation [Soos *et al.*, 1990].

ii) The glycosylation patterns of the receptors are variable [Ashcroft and Ashcroft, 1992].

iii) At the level of the genome variable splicing can lead to different peptides forming the

backbone of the receptor [Ashcroft and Ashcroft, 1992].

It is assumed that this heterogeneity has a physiological relevance but this has not yet been shown.

The binding of the agonist to the receptor, IR, IGFR or IRR, causes the conformational changes that activate the tyrosine kinase and prevent a second agonist molecule binding to the receptor [Gammeltoft, 1984]. Although the three-dimensional structure of the insulin receptor has not been determined, the ATP binding site necessary for the tyrosine kinase activity is indicated by the consensus sequence Gly-X-Gly-X-X-Gly (figure 4.2). Aside from autophosphorylation, the receptor can be phosphorylated at serine and threonine residues that results in decreased activity of the tyrosine kinase [Ashcroft and Ashcroft, 1992]. However the existence of this phosphorylation as a physiological desensitisation process is still controversial. Tyrosine specific protein phosphatases have been postulated as another means of deactivating the receptor but have not yet been shown to be operating physiologically to cause desensitisation. Subsequent to the activation of the insulin receptor several hypotheses have been advanced to explain the intracellular signalling:

1) The receptor kinase initiates a cascade of phosphorylation which begins as a tyrosine phosphorylation cascade and ends as a serine threonine cascade [Czech *et al.*, 1989; Czech *et al.*, 1988]. A possible agent in this cascade is MAP kinase (microtubule-associated protein kinase/mitogen activated protein kinase), this being activated downstream of the insulin receptor through multisite phosphorylation at tyrosine and threonine residues and is itself a serine protein kinase. Hence MAP kinase could respond to a tyrosine phosphorylation cascade and trigger a serine phosphorylation cascade. Another candidate for mediator in the phosphorylation cascade is Raf-1, this serine/threonine protein kinase can also be tyrosine phosphorylated and serine phosphorylated [Ashcroft and Ashcroft, 1992], although the latter

seems to be more important in its activation [Li *et al.*, 1991].

2) It is possible that the binding of insulin to its receptor triggers low molecular weight secondary messengers which allosterically regulate the activity of target enzymes. Possible candidates are phospho-oligosaccharides, for example inositol glycans, released by the hydrolysis of membrane lipids [Saltiel, 1990].

3) Lowering of cytosolic cAMP levels has also been implicated as a mediator of the actions of insulin. In the liver insulin induces tyrosine phosphorylation and activation of a cyclic AMP phosphodiesterase that hydrolyses cAMP [Pyne *et al.*, 1989]; however although there is no evidence for such an insulin triggered effect in skeletal muscle, there may be an IGF-I stimulated effect.

4) From work done in a myocyte cell line, there is limited evidence for G protein mediation in insulin action [Moises and Heidenreich, 1990].

5) As well as providing a potential means for homologous desensitisation, endocytosis of the insulin receptor may be involved in intracellular signalling by delivering the activated hormone receptor complex to internal sites [Khan *et al.*, 1989].

The fact that both the insulin like growth factors can increase the transport rate of amino acids into the cell and that insulin can increase the rate of transport of glucose, suggests that they might offer a paradigm for the regulation of creatine transport. Extensive studies have been carried out on the regulation of glucose transport in adipose tissue and skeletal muscle, and have revealed the following:

Insulin increases the transport of glucose into the skeletal muscle cell by enhancing a protein mediated facilitative diffusion process. This occurs by increasing the rate of translocation of the major transporter isoform, Glut-4, to the cell membrane [Klip *et al.*, 1987]. The process is rapid with a halflife of 2-3 minutes and this active process results in

a 10 fold increase in the number of transporters at the cell surface [Ashcroft and Ashcroft, 1992]. Interestingly, there is an increase in the maximum rate of uptake (V_{max}) without there being a change in K_m. Taken together with the observation that phorbol esters, by initiating protein phosphorylation, can cause the same amount of transporter translocation while achieving a lesser maximum uptake, this also suggests that insulin increases the inherent activity of the transporter once it is inserted into the membrane [Ashcroft and Ashcroft, 1992]. It has been assumed that this latter effect is a result of transporter phosphorylation because okadaic acid, a protein phosphatase inhibitor, can stimulate glucose uptake.

This chapter is concerned with the examination of the effects of insulin, IGF-I and IGF-II on creatine uptake, a biochemical interpretation of the effects on the creatine transporter, an investigation of intracellular signalling pathways and a consideration of the possible physiological significance. This will be discussed in the context of the independent work described above.

4.2 Methods

The methods for creatine uptake experiments in G8 cells and analyses used are described in sections 2.3, 2.4 and 2.7. The method for the gel electrophoresis is taken from Laemmli [Laemmli, 1970] and the Western blotting protocol is from Amersham, UK, and is described here. In order to establish whether insulin and IGF-I are coupled to protein tyrosine phosphorylation (PTK) in the G8 cells and to assess the effects of these stimuli on tyrosine phosphorylation, cells were stimulated and lysed before proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a polyvinylidene difluoride, PVDF, membrane by Western blotting. The blot was then exposed to the mouse

anti-phosphotyrosine (anti-PT) monoclonal antibody (Upstate Biotechnology Inc, New York) which binds specifically to phosphorylated tyrosine residues. This antibody was detected using the enhanced chemiluminescence detection system (ECL)(Amersham, UK). All chemicals were obtained from Sigma unless stated otherwise.

4.2.1 Western Blotting and Immunodetection with Anti-phosphotyrosine

To prepare samples for protein tyrosine phosphorylation analysis, cells (0.5 x 10^6 cells/500 µl/sample) were stimulated with 6 nM IGF-I or insulin, for varying times at 37°C, or left untreated as in the control samples. After stimulation samples were pelleted by microcentrifuge and washed twice in phosphate buffered saline, PBS. 25 µl of SDS (sodium dodecyl sulphate)-PAGE buffer was added to the clean pellet and samples were sonicated for 30 seconds to disrupt DNA and decrease the viscosity of the sample.

Gel electrophoresis separates molecules on the basis of their charge density. The process involves denaturation of proteins and exposure to an electric field whilst being supported in a polyacrylamide matrix. The polyacrylamide gel acts as a molecular sieve whose properties can be varied by changing the polyacrylamide concentration. Hence the larger proteins migrate through the gel more slowly. As proteins generally have similar charge densities after binding SDS, SDS-PAGE separates them on the basis of their molecular mass.

Polyacrylamide resolving gels, 10%, were made and left to set for 1.5 hours. A stacking gel, 4.5%, was then added to the top of the first gel with combs in place. Samples prepared as above were loaded onto the gel, such that an equal concentration of proteins was present. They were run along side rainbow molecular weight markers ranging from 220 to 14.5 kDa at 200 V in electrode buffer for 45 minutes.

After gel electrophoresis proteins were transferred to Hybond-PVDF membrane by supplying an electric current of 84 mA in blotting buffer. The membrane provides a suitably resilient matrix for immunological analysis of the samples. The blots were blocked using 1%, bovine serum albumin (BSA) in PBS at room temperature for 2 hours, so as to reduce non-specific binding of antibodies to the membrane. This was followed by an overnight incubation, at room temperature, with the primary antibody, murine monoclonal anti-phosphotyrosine antibody (1 μg/ml), in PBS, 0.1% Triton X-100 (PBS-T) and 0.01% sodium azide. The immunoblot was washed for 30 mins with 5 changes of PBS-T and incubated with the secondary antibody anti-mouse-HRP (1/5000 of stock concentration), in PBS with 5% milk for 1 hour. The immunoblots were washed as before and then developed using the ECL detection system.

The ECL system allows the detection of proteins, in small concentrations, by chemiluminescence. Chemiluminescence involves the excitation of a substance, effected by a chemical reaction, from a lower energy state to an excited state. In the excited state the substance will emit electromagnetic energy and return to its ground state. The primary antibody recognises phosphotyrosine on the target protein, a secondary antibody is conjugated to horse radish peroxidase as well as recognising the primary antibody. Under alkaline conditions the peroxidase on the secondary antibody will oxidise luminol and produce light. Luminol has a maximum light emission at 428 nm and with the help of the enhancer, this light can be detected by blue light sensitive autoradiography film.

Equal concentrations of protein in sample buffer were loaded on to the gel to allow relative intensities of bands to be determined. The experiment was pperformed in duplicate and the results are displayed in Plate 4.1.

Phosphate Buffered Saline

2.68 mM KCl
1.47 mM KH_2PO_4
137 mM NaCl
8.06 mM $Na_2HPO_4.7H_2O$

SDS-PAGE buffer

Tris HCl (pH6.8)	- 1.2 ml
Glycerol	- 1.0 ml
10% (w/v) SDS	- 2.0 ml
0.1% (w/v) Bromophenol blue	- 0.5 ml
2-mercaptoethanol	- 0.5 ml
dH_20	- 0.5 ml

Polyacrylamide Resolving Gel	**10%**
dH_2O	- 6.08 ml
1.5M Tris-HCl (pH 8.8)	- 3.75 ml
Bis-acrylamide	- 5.00 ml
10% SDS	- 0.15 ml
10% APS	- 0.0375 ml
TEMED	- 0.012 ml

4% Polyacrylamide Stacking Gel

dH_2O	- 6.10 ml
1.5M Tris-HCl (pH 8.8)	- 0.83 ml
Bis-acrylamide	- 1.3 ml
10% SDS	- 0.10 ml
10% APS	- 0.05 ml
TEMED	- 0.01 ml

Electrode Buffer

For 6 litres:Tris-HCl	- 18 g
Glycine	- 86.4 g
SDS	- 6.0 g
dH_2O	- 6.0 l

Blotting Buffer

For 6 litres:Tris-HCl	- 18 g
Glycine	- 85.5 g
Methanol	- 1.2 l
dH_2O	- 4.8 l

Table 4.2 : Solutions used in Western Blotting

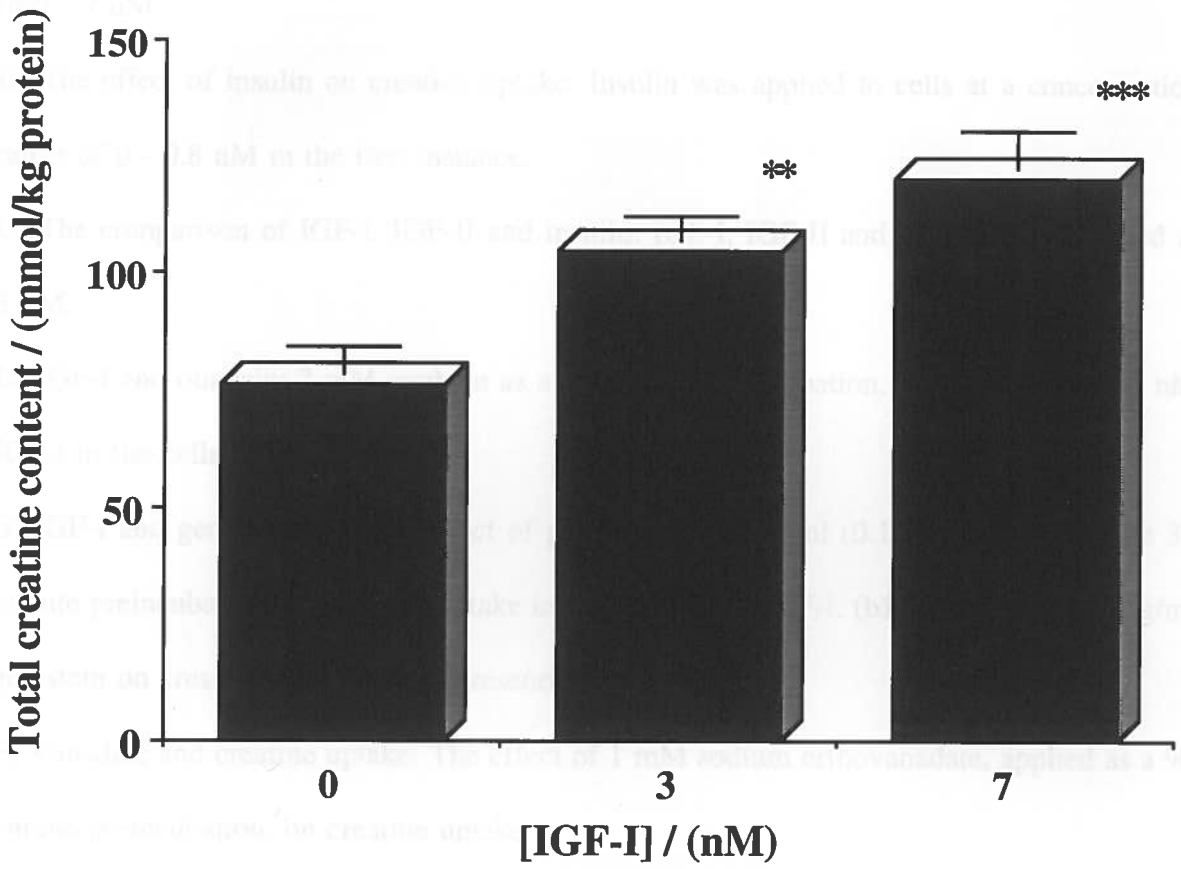


Fig 4.3 Effect of IGF-I on creatine uptake
A graph of total intracellular [creatine] against applied IGF-I. The cells were of passage number 12 (n=6). The experiment was conducted over 48 hours.

4.3 Experiments performed

A) The effect of IGF-I on creatine uptake: IGF-I was applied to cells at a concentration range of 0 - 7 nM.

B) The effect of insulin on creatine uptake: Insulin was applied to cells at a concentration range of 0 - 0.8 nM in the first instance.

C) The comparison of IGF-I, IGF-II and insulin: IGF-I, IGF-II and insulin were applied at 3 nM.

D) IGF-I and ouabain: 2 mM ouabain as a 30 minute preincubation, was applied with 3 nM IGF-I to the cells.

E) IGF-I and genistein: (a) The effect of genistein at 30 μg/ml (0.11 mM) applied as a 30 minute preincubation, on creatine uptake in the absence of IGF-I. (b) The effect of 30 μg/ml genistein on creatine uptake in the presence of IGF-I.

F) Vanadate and creatine uptake: The effect of 1 mM sodium orthovanadate, applied as a 90 minute preincubation, on creatine uptake.

G) IGF-I and cycloheximide: (a) The effect of 70 μM cycloheximide, applied as a 60 minute preincubation, on creatine uptake in the absence of IGF-I and (b) in the presence of IGF-I.

H) Agonist stimulated protein tyrosine phosphorylation: IGF-I and insulin were applied at 6 nM. Each agent was applied for 0, 1, 5 and 10 minutes before reactions were stopped as described in methods.

4.4 RESULTS

Figure 4.3 shows that the physiological agonist IGF-I is capable of stimulating creatine

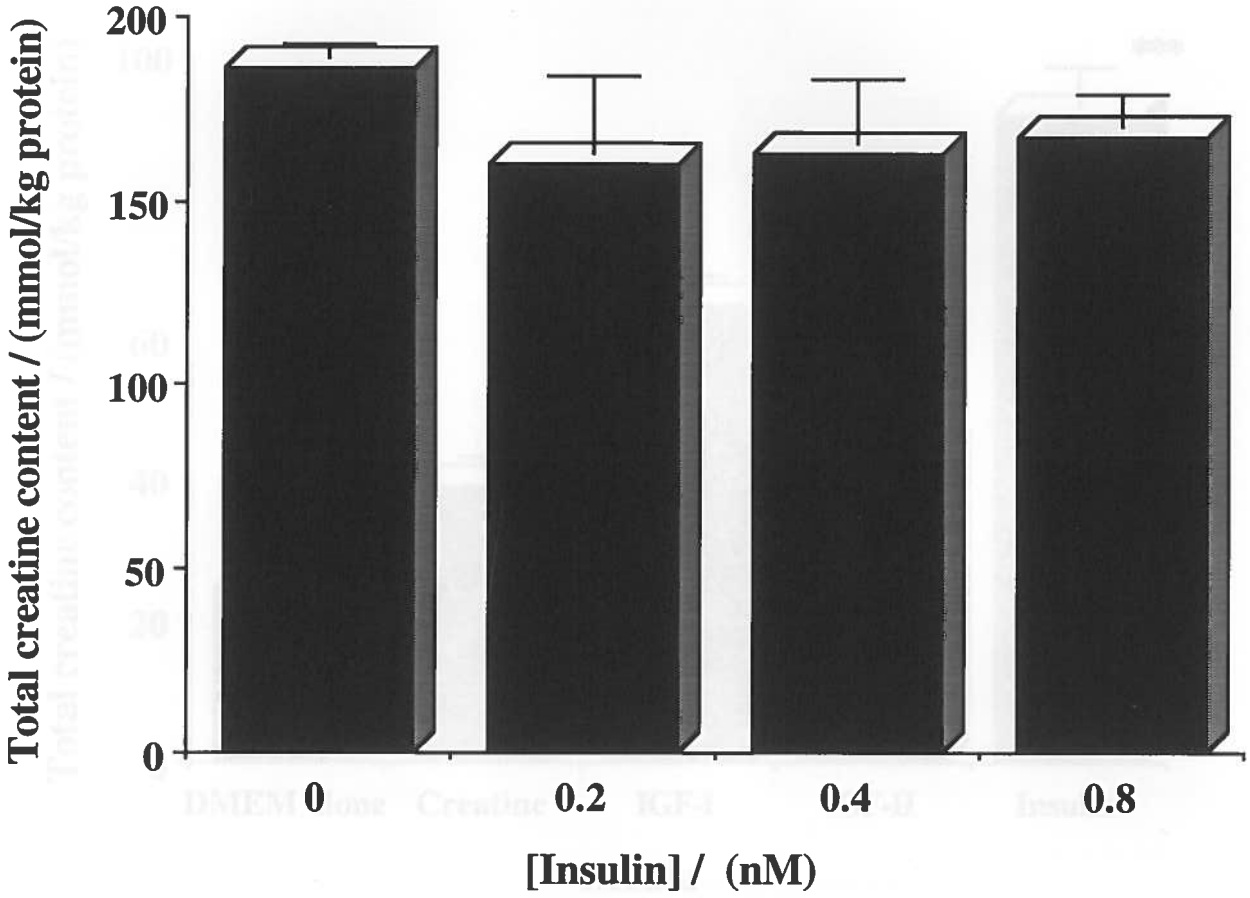


Figure 4.4 The effect of insulin on creatine uptake
A graph of total intracellular [creatine] against applied [insulin]. The cells were of passage number 8 (n=6). The experiment was conducted over 48 hours.

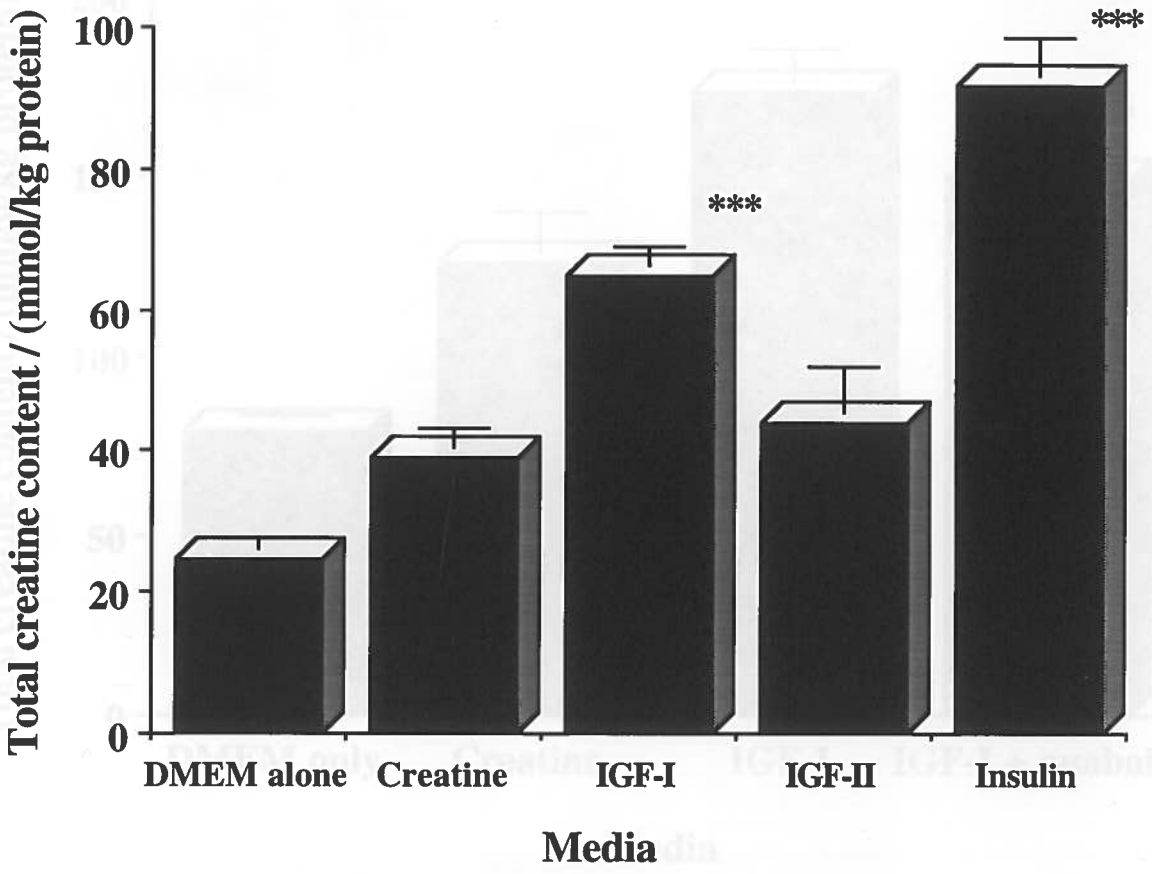


Figure 4.5 Comparison of the effects of IGF-I, IGF-II and insulin on creatine uptake
A graph of total intracellular [creatine] against applied agonist. The cells were of passage number 9 (n=12). The experiment was conducted over 48 hours. Statistical significance is calculated relative to creatine alone IGF-I, IGF-II and Insulin were applied at 3 nM.

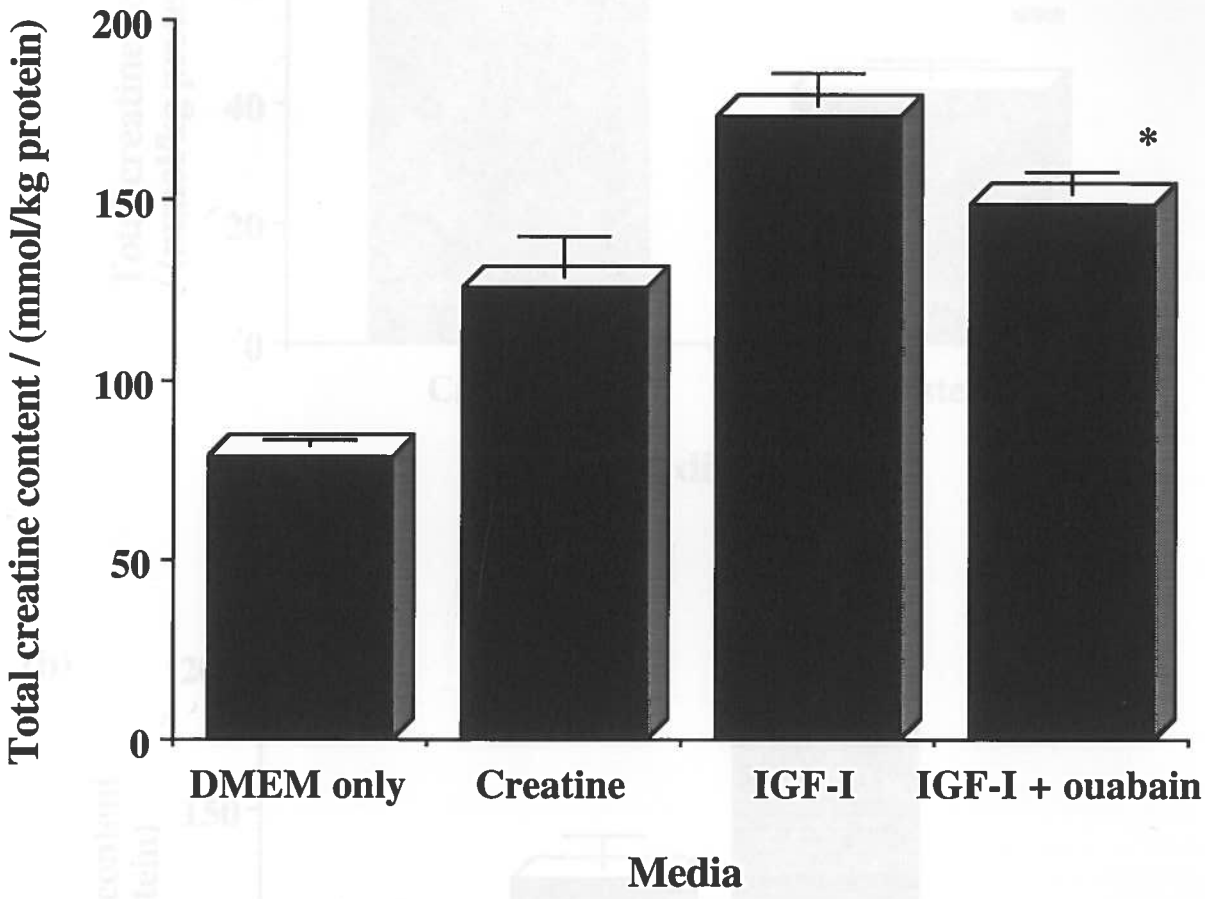


Figure 4.6 Ouabain inhibition of creatine uptake in the presence of IGF-I
A graph of total intracellular [creatine] against media treatments. The cells were of passage number 10 (n=12). Statistical significance is determined relative to IGF-I. The experiment was conducted over 48 hours. IGF-I was applied at 3 nM, ouabain at 2 mM.

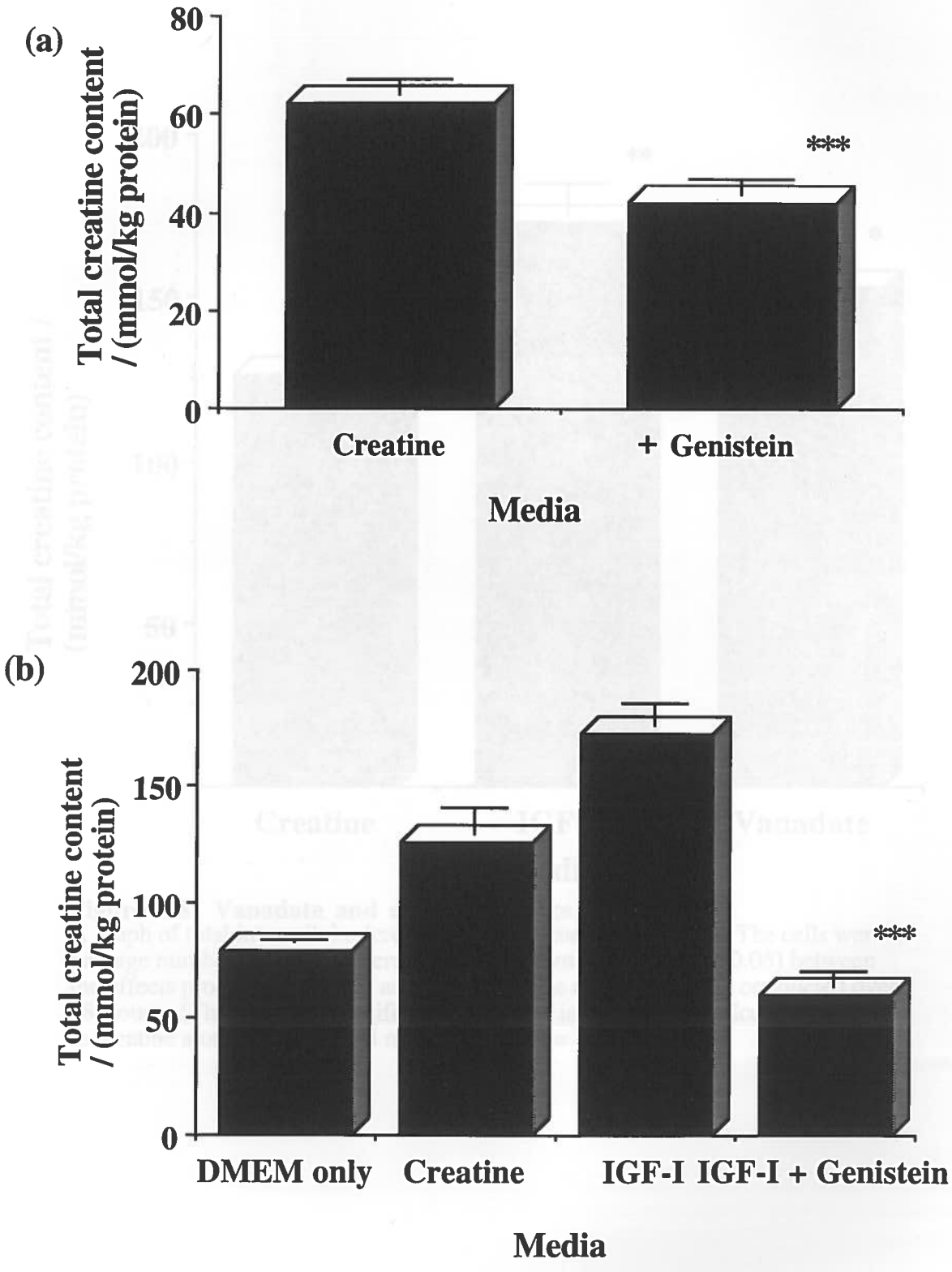


Figure 4.7 The effect of genistein on creatine uptake in the presence of IGF-I
Graphs of total intracellular [creatine] against media treatments. Cells were of passage number 11 (a) and 8 (b) (n=12). In (b) statistical significance is tested relative to IGF-I. The experiments were conducted over 48 hours. [Genistein] = 0.11 mM, [IGF-I] = 3 nM.

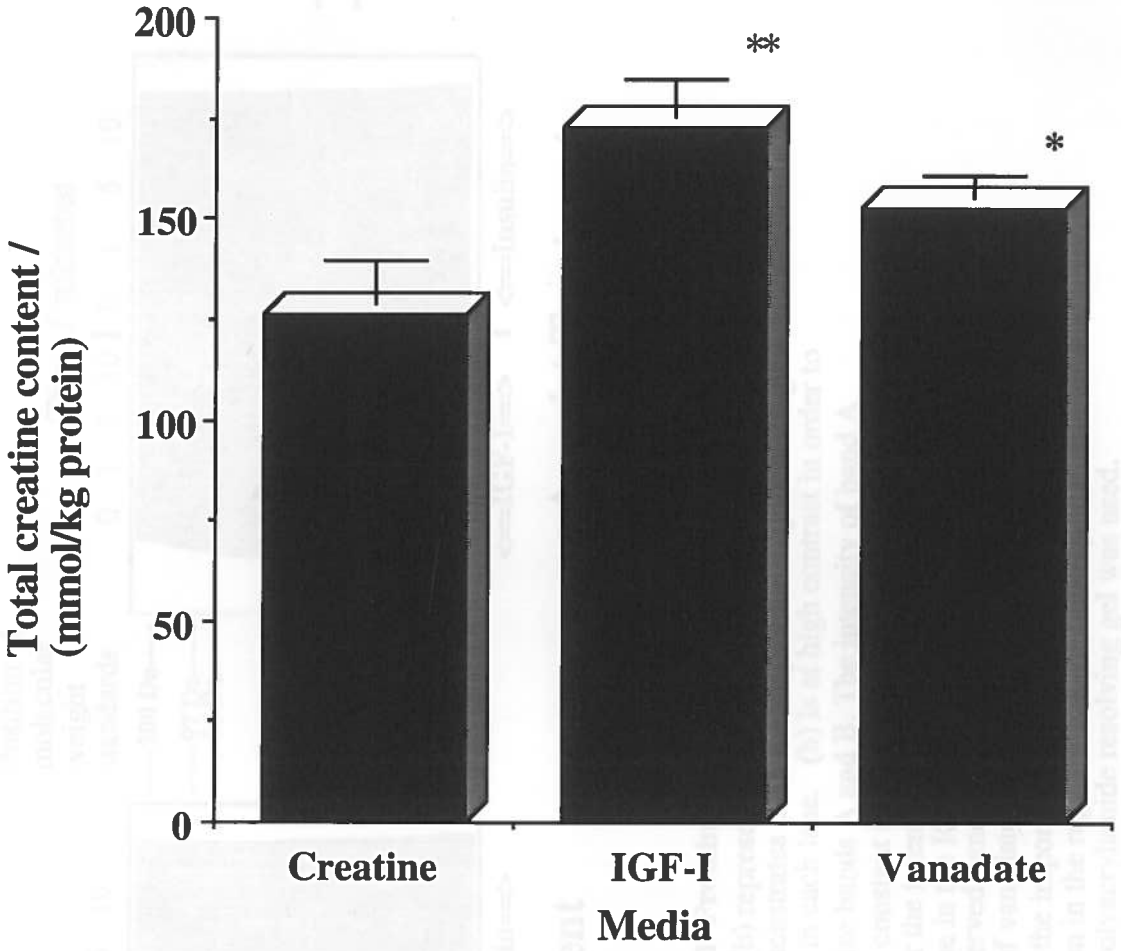


Figure 4.8 Vanadate and creatine uptake
A graph of total intracellular [creatine] against media treatments. The cells were of passage number 8 (n=12). There is no significant difference (P>0.05) between the effects produced by IGF-I and vanadate. The experiment was conducted over 48 hours. (The statistical significance indicated in the figure is calculated relative to creatine alone). [IGF-I] = 3 nM, [Vanadate] = 1 mM

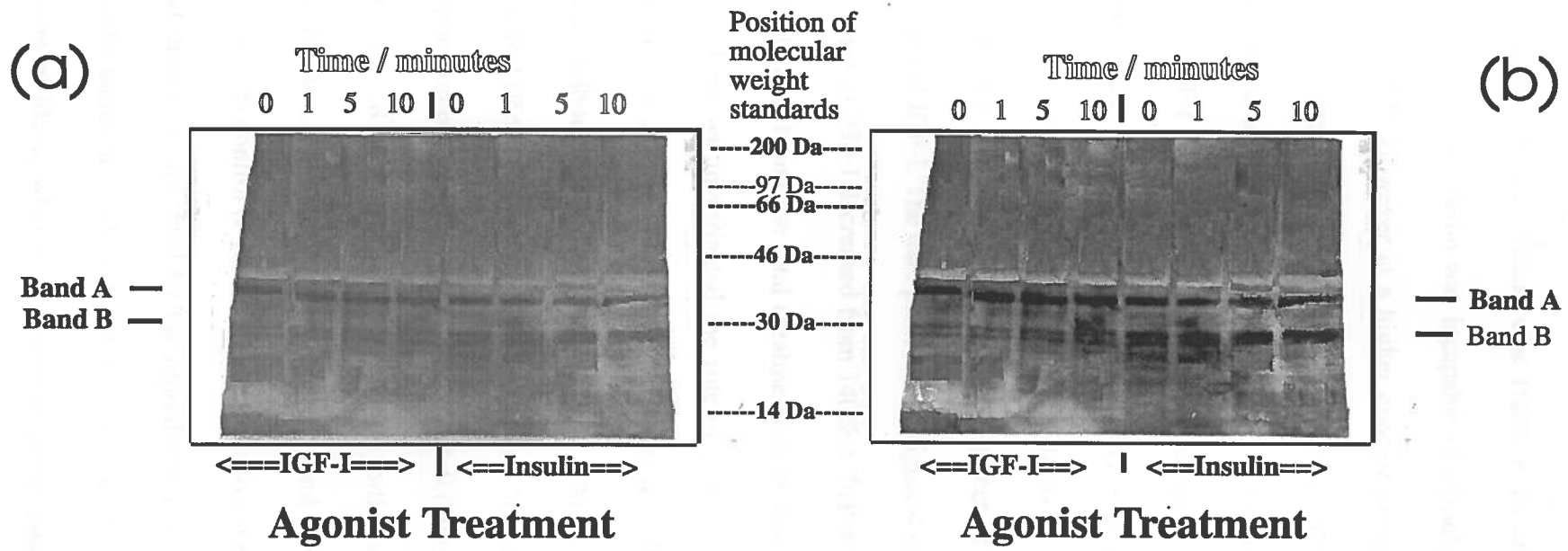


Plate 4.1 Protein phosphotyrosine formation
(a) and (b) represent the same gel. (a) is at low contrast and demonstrates the similarity of the major banding patterns in each lane. (b) is at high contrast in order to emphasize bands A and B. The intensity of band A remains constant in the IGF-I and insulin series of lanes however the intensity of band B (a doublet) increases with time in the IGF-I and the first three insulin lanes. The observed banding patterns, taken together with the effects of vanadate and genistein on creatine uptake, indicate the importance of protein phosphotyrosine formation in the regulation of creatine uptake.
A 10% polyacrylamide resolving gel was used.

uptake after a 48 hour incubation in 0.5 mM creatine. The maximum total creatine content observed in this experiment was 160% ± 16 of control ($P<0.05$). The data in figure 4.4 demonstrate that insulin was incapable of stimulating creatine uptake at low concentrations (0 - 0.8 nM). However at a higher concentration, 3 nM, insulin stimulated creatine uptake (figure 4.5) to 230% ± 6 of the control value ($P<0.05$). Figure 4.5 also compares the effect of the same concentration of IGF-I, IGF-II and insulin on the total creatine content after 48 hours. IGF-I increased the total creatine content to 140% ± 13 of the control value ($P<0.05$) whereas IGF-II had no significant effect ($P>0.05$).

Previous work had shown a sensitivity of creatine uptake to Na^+,K^+-ATPase activity (sections 2.9 and 3.5) so we tested the effect of 2 mM ouabain on creatine uptake in the presence of IGF-I. The data presented in figure 4.6 show that after preincubation with ouabain the effect of IGF-I decreased from 140% ± 7 ($P<0.05$) to 118% ± 11 of the control value (the control value being the total creatine content that occurs in the absence of IGF-I).

Next we investigated the role of protein tyrosine phosphorylation on total creatine content. The data in figure 4.7(a) show that in the absence of IGF-I, the protein tyrosine kinase inhibitor genistein (30 μg/ml, 0.11 mM) decreased total creatine content to 68% ± 5 of control ($P<0.001$) and the data in figure 4.7(b) demonstrate that genistein diminished total creatine content to 48% ± 6 of control ($P<0.001$), when applied prior to IGF-I. The results in figure 4.8 show that 1 mM sodium orthovanadate, an inhibitor of protein tyrosine phosphatases, significantly increased the total creatine content in the absence of IGF-I to 120% ± 10 of control ($P<0.05$). The stimulation of protein phosphotyrosine formation by IGF-I and insulin, is indicated by the reproduction of the Western blot shown in Plate 4.1. The multiple bands in each column demonstrate that several proteins are phosphorylated at tyrosine residues, when the agonist is either insulin or IGF-I. IGF-I and insulin produce

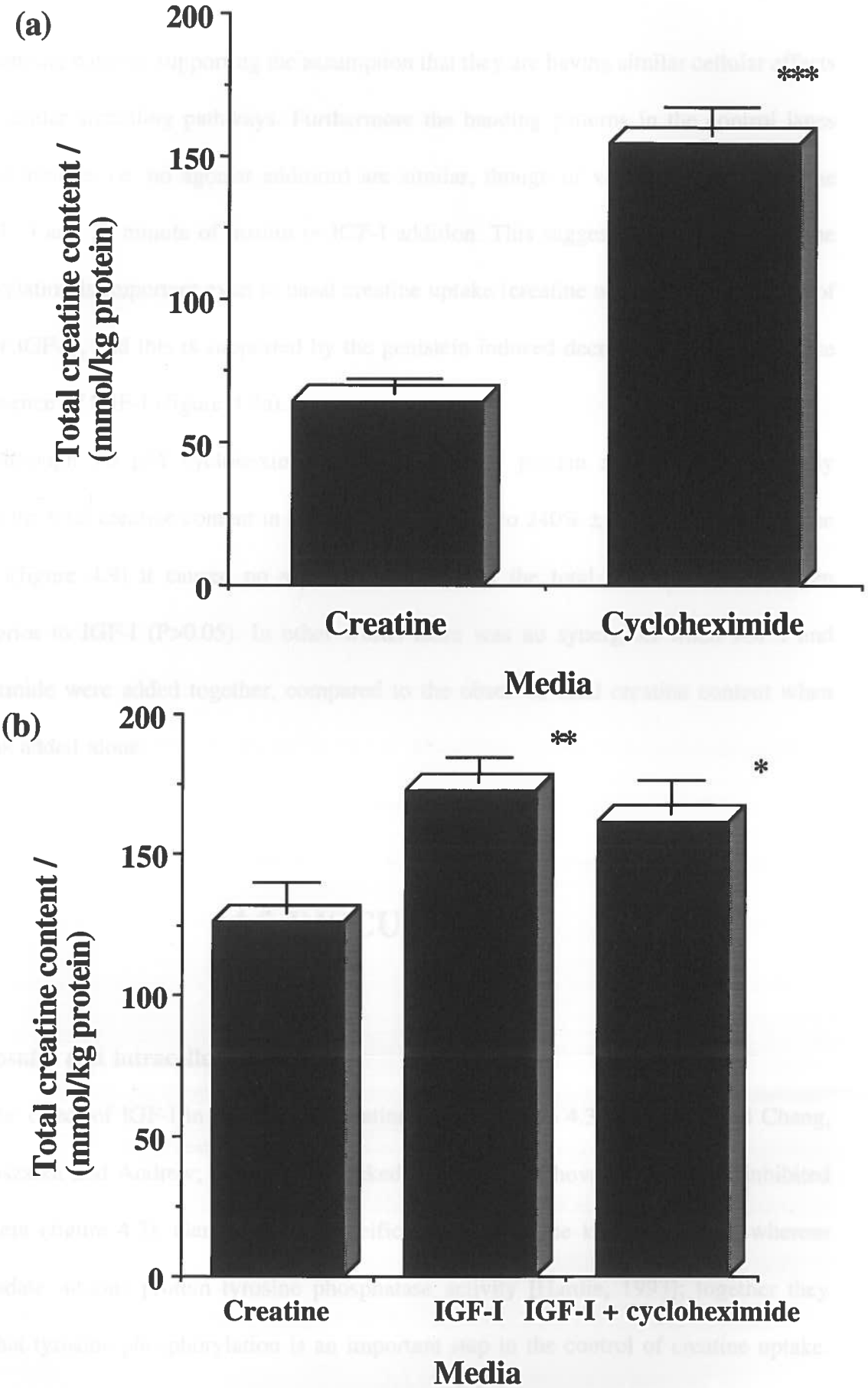


Figure 4.9 The effect of cycloheximide on creatine uptake in the presence and absence of IGF-I

Graphs of total intracellular [creatine] against media treatments. Cells were of passage number 9 (a) and 8 (b) (n=12). The experiments were conducted over 48 hours. [IGF-I] = 3 nM, [Cycloheximide] = 70 μM

similar banding patterns supporting the assumption that they are having similar cellular effects through similar signalling pathways. Furthermore the banding patterns in the control lanes (time = 0 minutes i.e. no agonist addition) are similar, though of varying intensity, to the lanes at 1, 5 and 10 minute of insulin or IGF-I addition. This suggests that protein tyrosine phosphorylation is important even in basal creatine uptake (creatine uptake in the absence of insulin or IGF-I), and this is supported by the genistein induced decrease in creatine uptake in the absence of IGF-I (figure 4.7a).

Although 70 μM cycloheximide, an inhibitor of protein synthesis, significantly increased the total creatine content in the absence of IGF-I to 240% ± 24 of the control value ($P<0.001$)(figure 4.9) it caused no significant change in the total creatine content when applied prior to IGF-I ($P>0.05$). In other words there was no synergism when IGF-I and cycloheximide were added together, compared to the observed total creatine content when either was added alone.

4.5 DISCUSSION

IGF-I, Insulin and intracellular signalling

The effect of IGF-I in stimulating creatine uptake (figure 4.3)(Haugland and Chang, 1975; Koszalka and Andrew; 1972) is mimicked by sodium orthovanadate and is inhibited by genistein (figure 4.7). Genistein is a specific protein tyrosine kinase inhibitor whereas orthovanadate inhibits protein tyrosine phosphatase activity [Hardie, 1993]; together they suggest that tyrosine phosphorylation is an important step in the control of creatine uptake. Protein tyrosine kinase activity in the mediation of the action of growth factors has been

reported previously [James and Bradshaw, 1984] where receptor autophosphorylation leads to activation and a cascade of tyrosine phosphorylation of intracellular proteins. This ultimately yields the cellular response [Ashcroft and Ashcroft, 1992]. The relevance of tyrosine phosphorylation in the action of insulin and IGF-I is indicated by the multiple bands representing phosphotyrosine residues formed when either insulin or IGF-I is applied (Plate 4.1). These effects occur within 10 minutes of agonist application which is far sooner than any increase in total creatine content as determined by the techniques used here (section 2.3). It is possible that phosphotyrosine formation is causative in stimulating creatine uptake. Future analytical use of the Western blotting technique to identify the relevant proteins would require further developments to enhance resolution, primarily by decreasing the background noise caused by residual antibody binding to the blocking protein; here the data presented is intended show the presence of tyrosine phosphorylation.

It is known that the activity of the of Na^+,K^+-ATPase is important in creatine uptake (sections 2.9 and 3.5) so we tested ouabain, a specific inhibitor of the Na^+,K^+-ATPase, for an effect on creatine uptake in the presence of IGF-I; we show that the effect of IGF-I is diminished by treatment with ouabain (figure 4.6). In addition it is known that insulin can increase the activity of the Na^+,K^+-ATPase in rat skeletal muscle [Marette *et al.*, 1993]. Hence an indirect signal through the Na^+,K^+-ATPase may at least in part be responsible for the effects of insulin on creatine uptake. As there is extensive cross-reactivity [Ashcroft and Ashcroft, 1992] of insulin and IGF-I at their receptors, it is possible that either receptor may be mediating the effect on creatine uptake. However as IGF-I seems to be effective at physiological concentrations (<20 nM) [Humbel, 1990] and insulin is effective only at pathological/supraphysiological concentrations (figures 4.4 and 4.5), it is likely that the IGF-I receptor (IGFR) is controlling creatine uptake. Our work indicates that a receptor specific for

HYPOTHESIS:

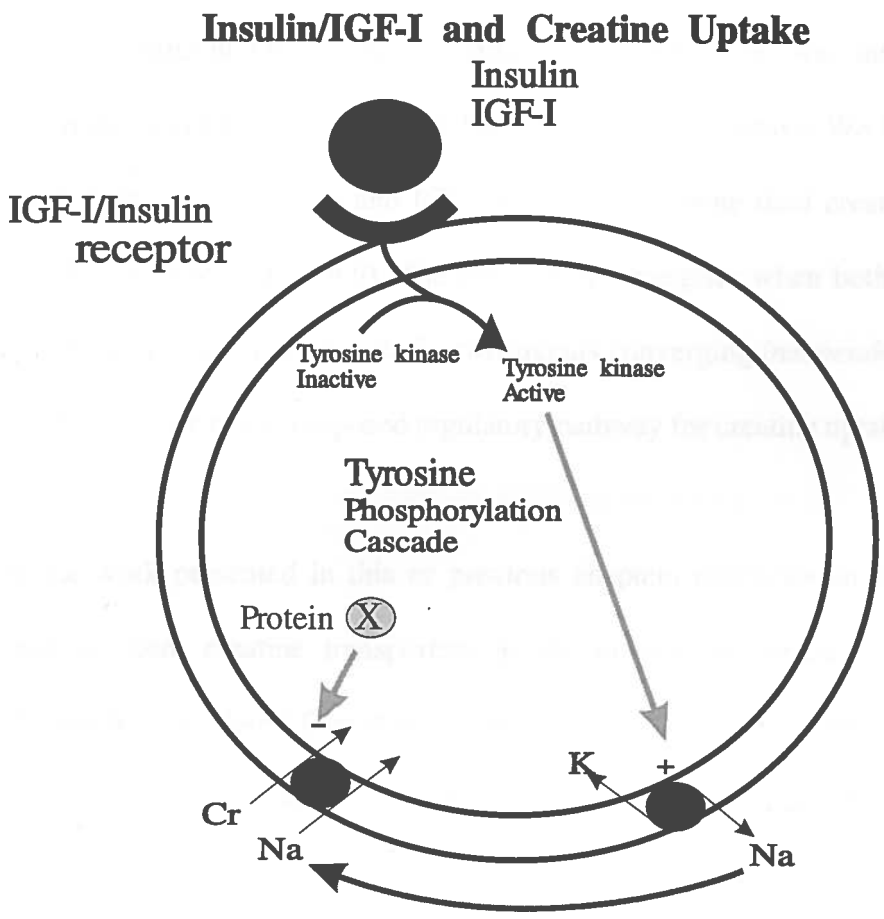


Figure 4.10 Proposed pathway for IGF-I/Insulin regulation of creatine uptake
The pathway represents a summary of the data presented in chapter 4. (- indicates inhibition and + indicates stimulation.)

IGF-II is probably not involved in the stimulation of creatine uptake at physiological concentrations (figure 4.5) and so the role of IGF-II *in vivo* remains unclear [Humbel, 1990].

The action of cycloheximide in stimulating creatine uptake (figure 4.9a) is similar to an earlier observation made in L6 myoblasts [Loike *et al.*, 1988]. This was interpreted as being due to a decrease in synthesis of a protein that inhibits creatine uptake. We have shown that treatment with both cycloheximide and IGF-I results in the same final creatine content as treatment with IGF-I alone (figure 4.9). The absence of synergism when both IGF-I and cycloheximide are present is consistent with the two signals converging *independently* on the transport protein. A summary of the proposed regulatory pathway for creatine uptake is shown in figure 4.10.

None of the work presented in this or previous chapters precludes an effect of an agonist in recruiting more creatine transporters to the plasma membrane in a manner analogous to the insulin stimulated Glut-4 transporter [Klip *et al.*, 1987]. However, at the moment, there has been no investigation to determine the existence of an intracellular creatine transporter pool.

The pathways that control the effects of insulin on intermediary metabolism often work by decreasing cytosolic cAMP levels [Barritt, 1992] and the pathways for the other subsets of insulin's actions are frequently controlled by increasing PKC activity [Barritt, 1992]. As both of these effects would tend to decrease creatine uptake (section 3.5) this may explain the lack of sensitivity of creatine uptake to applied insulin in the G8 cells, because of the balance of all of the effects of insulin on the cell. There is further scope for the interactions between signalling pathways because of the following observations:

1) Catecholamines increase the sensitivity to insulin [Ashcroft and Ashcroft, 1992]. Hence it is possible to envisage a situation in which prolonged exposure to catecholamines makes the

insulin induced creatine uptake a physiological event. Alternatively, if catecholamines increase the sensitivity to the functionally and biochemically similar IGF-I, then creatine uptake would be enhanced when appropriate levels of both IGF-I and adrenaline/noradrenaline are circulating in the plasma.

2) Insulin increases the number of IGF-II receptors at the cell membrane [Ashcroft and Ashcroft, 1992]. As both IGF-I and IGF-II appear to act through IGFR, this observation suggests that insulin may enhance the effect of IGF-I in stimulating creatine uptake.

Interactions between signalling pathways allow for flexibility and gradation in the ultimate cellular response because of the possibility of multiple complex interactions (e.g. antagonistic, sequential, redundant, hierachical and coordinated paradigms).

Physiological significance of the effect of IGF-I

It is currently believed that foetal and adult IGF-I is synthesised in the liver and in fibrous mesenchymal cell types (the latter being located around and within organs) [Hill and Clemmons, 1992]. It is subsequently transported to target sites by insulin-like growth factor binding proteins (IGFBP) [Humbel, 1990; Hill and Clemmons, 1992]. Because human IGFBP-I is associated with human fetal skeletal muscle it is believed to regulate bioavailability of IGF-I at its skeletal muscle receptor. As the expression of IGF-I is under the control of growth hormone [Humbel, 1990], it is then reasonable that a hormone responsible for transmitting a mitogenic signal, could also enhance the uptake of bioenergetic metabolites required for the growth and function of skeletal muscle. Hence the increase in creatine uptake produced by IGF-I suggests a possible physiological role for IGF-I in muscle growth and development. Furthermore creatine is itself an anabolic factor [Bessman and Mohan, 1992; Sipilä, 1981] and so may be responsible for mediating at least some of the growth promoting

effects of IGF-I.

There is a release of growth hormone during exercise [Ganong, 1989] and this then causes an increase in synthesis of IGF-I [Humbel, 1990] which we have shown in turn increases creatine uptake. In the same way that we have postulated that the action of catecholamines may be part of a positive physiological adaptation to exercise (sections 2.9 and 3.5) IGF-I may also have such a role. With each bout of exercise growth hormone is released, subsequently IGF-I is released with a resultant increase in creatine uptake at skeletal muscle. The increase in total cellular creatine content caused by each pulse of exercise would be sustained because of the slow efflux of creatine. In addition IGF-I may also be important in creatine metabolism because of the secondary effects on phosphate metabolism. IGF-I is known to stimulate inorganic phosphate uptake in L6 rat myoblasts, hence IGF-I can regulate both creatine and inorganic phosphate uptake into muscle cells [Polgreen, 1993]. This is of particular importance because creatine and inorganic phosphate are the two components of the bioenergetically active creatine derivative, creatine phosphate.

In preparation:

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Odoom, J.E., Kemp, J.K. & Radda, G.K. (1995) IGF-I modulation of creatine content in a skeletal muscle cell line. In preparation for Biochemistry Society Transactions.

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Chapter 5

Creatine transport and the carotid artery

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5.1 Introduction

The previous chapters of this thesis have been concerned with a cellular model of creatine transport. The material presented in this chapter considers a tissue model of creatine transport and so the results presented are more relevant to the complete animal. The porcine carotid artery was selected because:

a) It is a good model of the human carotid artery [Clark, 1994; Clark and Dillon, In Press].

b) It has been extensively studied and characterised by other workers [Clark, 1994; Clark and Dillon, In Press; Khromov *et al.*, 1994; Campbell and Paul, 1992].

c) It is a tissue whose stability *ex vivo* has been demonstrated; so uptake experiments on the isolated tissue for 48 hours are feasible [Clark, 1994; Clark and Dillon, In Press].

d) No creatine transport studies have been carried out on this non-striated muscle which also relies upon the bioenergetic role of creatine for function.

Previous studies of creatine transport and content have focused on skeletal and heart muscle and the implications of the regulation of creatine metabolism for clinical states [Greenhaff *et al.*, 1993; Dinking *et al.*, 1959; Fitch and Sinton, 1964; Seppet *et al.*, 1985]. However as the creatine content of vascular smooth muscle [Clark and Dillon, In Press], is an order of magnitude lower than either skeletal muscle or heart muscle [Walker, 1979], and as the circulatory system is bounded by vascular smooth muscle, any protocol that alters the creatine content in striated muscle by oral application [Harris *et al.*, 1992], is likely to have a major effect on the creatine content of vascular smooth muscle because of the increased plasma creatine concentration.

Once transported into the smooth muscle cell, Cr is rapidly phosphorylated by the equilibrium reaction catalysed by creatine kinase (CK) [Clark and Dillon, In Press]:

CK

Creatine + ATP <--------> Phosphocreatine + ADP + H^+.

23% of the total CK activity in smooth muscle is due to the "brain" isozyme, BBCK, bound to contractile proteins [Clark, 1994]. Bound BBCK alone could supply the contractile proteins with sufficient ATP to relax guinea pig carotid arteries from a state of high tension rigor [Clark, 1994], through the hydrolysis of PCr. Increased free ADP can inhibit smooth muscle relaxation [Fuglsang *et al.,* 1993; Nishye *et al.,* 1993] an effect that is diminished by PCr [Khromov *et al.,* 1994]. Hence creatine plays a central role in cellular bioenergetics and contractility through PCr/CK in smooth muscle. It is well known that in several cardiovascular pathologies the total creatine content of muscle is decreased [Das and Harris, 1993; Preedy *et al.*, 1994], additionally alcoholics have a greater tendency to cardiomyopathy and decreased tissue creatine content. As alcohol alters membrane fluidity we set out to test the hypothesis that alcohol was responsible for tissue creatine depletion, and so may contribute to cardiovascular pathology. The regulation of net creatine uptake into the porcine carotid artery is also investigated.

5.2 Methods

The approach used to study the total creatine content was modified from the protocols designed for G8 cells (sections 2.3 and 2.4). For comparison with the effect of alcohol on the total creatine content in the porcine carotid artery, an experiment with G8 cells was performed to test the effect of alcohol on the total cellular creatine content. For this latter experiment the methods were the same as those described in sections 2.2, 2.3 and 2.4.

Tissue preparation

Porcine carotid arteries were collected from healthy male or female pigs in an abattoir at the time of slaughter and immediately placed in cold (4°C) physiological saline. The lumen was rinsed and connective tissue removed. Carotid arteries were kept in cold physiological saline (for a maximum of 24 hours) until the experiments were commenced. Creatine uptake experiments were initiated by placing 1 cm long carotid rings (approximately 0.07 g) in the physiological saline solutions (PSS) with the agent of interest. PSS contained; 116 mM NaCl, 5.4 mM KCl, 25.3 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM glucose at pH 7.4. To this solution was added 0.5 mM creatine for the NMR experiments or 0.15 mM creatine for the non-NMR experiments. Sodium pyruvate (5 mM) was used as an alternative substrate. Solutions were maintained at 22°C with constant stirring and aeration with 95% O_2 / 5% CO_2 and changed every 12 hours. The uptake experiments were carried out over 48 hours while the efflux experiments were carried out over 72 hours (the efflux experiments were performed in PSS without creatine). The comparison of creatine uptake mediated by the selective ß adrenergic agonists was performed over 24 hours and in PSS containing 100 µM creatine. At the end of the experiments the carotid arteries were rinsed thoroughly, blotted, rinsed again, blotted and weighed. After weighing the carotid arteries were immediately placed in 5 ml of 0.1 M NaOH, homogenised with a polytron homogeniser and analysed on the same day.

Creatine assay

See section 2.4. Here results are calculated as µmol/g wet weight and expressed as percentages of the control value.

NMR methods

One carotid artery, 3 cm long, was placed in a 5 mm diameter NMR tube and superperfused at 22°C at a constant flow rate of 20 ml/min with PSS aerated with 95% O_2 / 5% CO_2. Perfusate was siphoned away from the carotid arteries and recirculated at a constant volume of 5 l and changed every 24 hours. The carotid arteries were equilibrated for a minimum of 20 minutes in the NMR tube before being placed in the 9.6 Tesla wide-bore (72 mm) magnet. The probe was home built and was tuned and matched to 161.96 Mhz. The magnetic homogeneity was adjusted by shimming on the proton resonance using the Bruker Biospec Spectrometer. Spectra were collected using 2048 data points with a 60° pulse and a 2.14 second relaxation delay. Each spectrum consists of 1500 transients with Lorentzian line broadening corresponding to 30 Hz. NMR spectra were analysed using NMR1 (NMRi, Syracuse, NY) to determine peak area and position. The pH was calculated using the intracellular Pi peak position as previously described [Kushmerick *et al.*, 1986].

Octanol/water partition coefficients

See section 2.7.

Materials

Isoprenaline (isoproterenol) and clenbuterol were obtained from Sigma (Sigma Chemical Company Limited, Dorset, U.K.), dobutamine was obtained from Eli Lilley (Eli Lilley Company Limited, Basingstoke, U.K.) and ZD2079 was donated by Zeneca (Zeneca Pharmaceuticals, Cheshire, U.K.). All other standard reagents were obtained from Sigma.

Data Analysis: Data were analysed by the Student's t test. Values are presented as mean ±

standard error, SE (* = P<0.05, ** = P<0.01, *** = P<.001 compared to control).

5.3 Experiments performed

A) Incubation in PSS: porcine carotid arteries were incubated in physiological saline solution for 72 hours.

B) The effect of primary alcohols on total creatine content: The primary alcohols, 0.1%-(v/v) ethanol, 0.1%-(v/v) propan-1-ol and 0.1%-(v/v) butan-1-ol, were tested for an effect on total creatine content over 24 hours.

C) The effect of alcohols in the presence of 5 mM creatine: The primary alcohols, 0.1%-(v/v) ethanol, 0.1%-(v/v) propan-1-ol and 0.1%-(v/v) butan-1-ol, were tested for an effect on total creatine content over 24 hours in the presence of 5 mM creatine.

D) The effect of alcohols in the presence of 0.1 mM creatine: The primary alcohols, 0.1%-(v/v) ethanol, 0.1%-(v/v) propan-1-ol and 0.1%-(v/v) butan-1-ol, were tested for an effect on total creatine content over 24 hours in the presence of 0.1 mM creatine.

E) The effect of ethanol on total creatine content in the G8 cell: The effect of 0 - 2% ethanol on the total creatine content of the G8 cell over a 48 hour incubation.

F) Modulators of total creatine content: membrane permeable dibutyryl cAMP (0.2 mM), membrane permeable dibutyryl cGMP (0.2 mM), isoprenaline (isoproterenol) (0.1 μM), sodium pyruvate (5 mM), Na^{+} (60 mM), dobutamine (40 μM) a $ß_1$ agonist, clenbuterol (40 μM) a $ß_2$ agonist or ZD2079 (40 μM) a $ß_3$ agonist, were each tested for an effect on total creatine content in the porcine carotid artery.

G) The effect of extracellular creatine on total tissue creatine. Carotid arteries were incubated in PSS containing creatine concentrations of 0 - 0.5 mM.

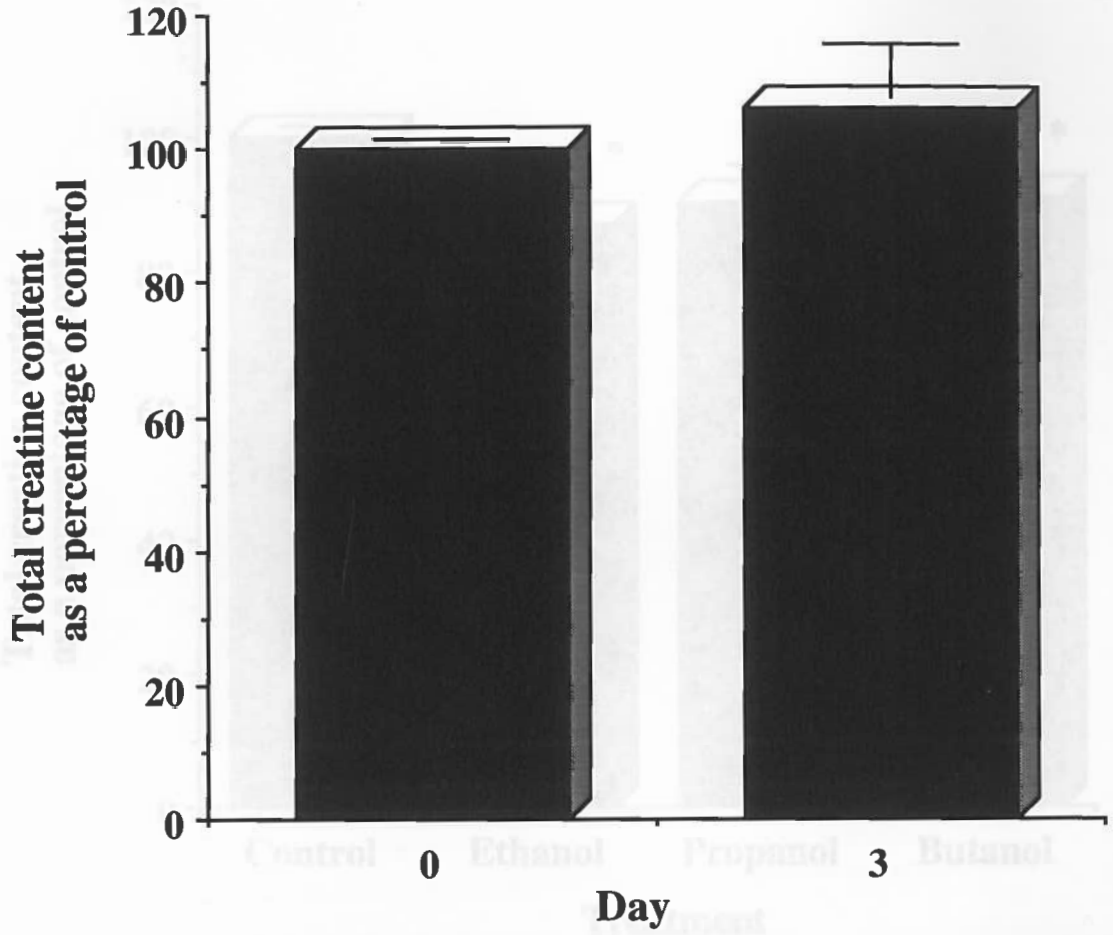


Figure 5.1 Creatine efflux from the porcine carotid artery over 72 hours A graph of total tissue creatine content against time. The arteries were incubated in PSS (n=7). There is no statistically significant difference (P>0.05) between the total creatine contents at day 0 and day 3. Control is day 0.

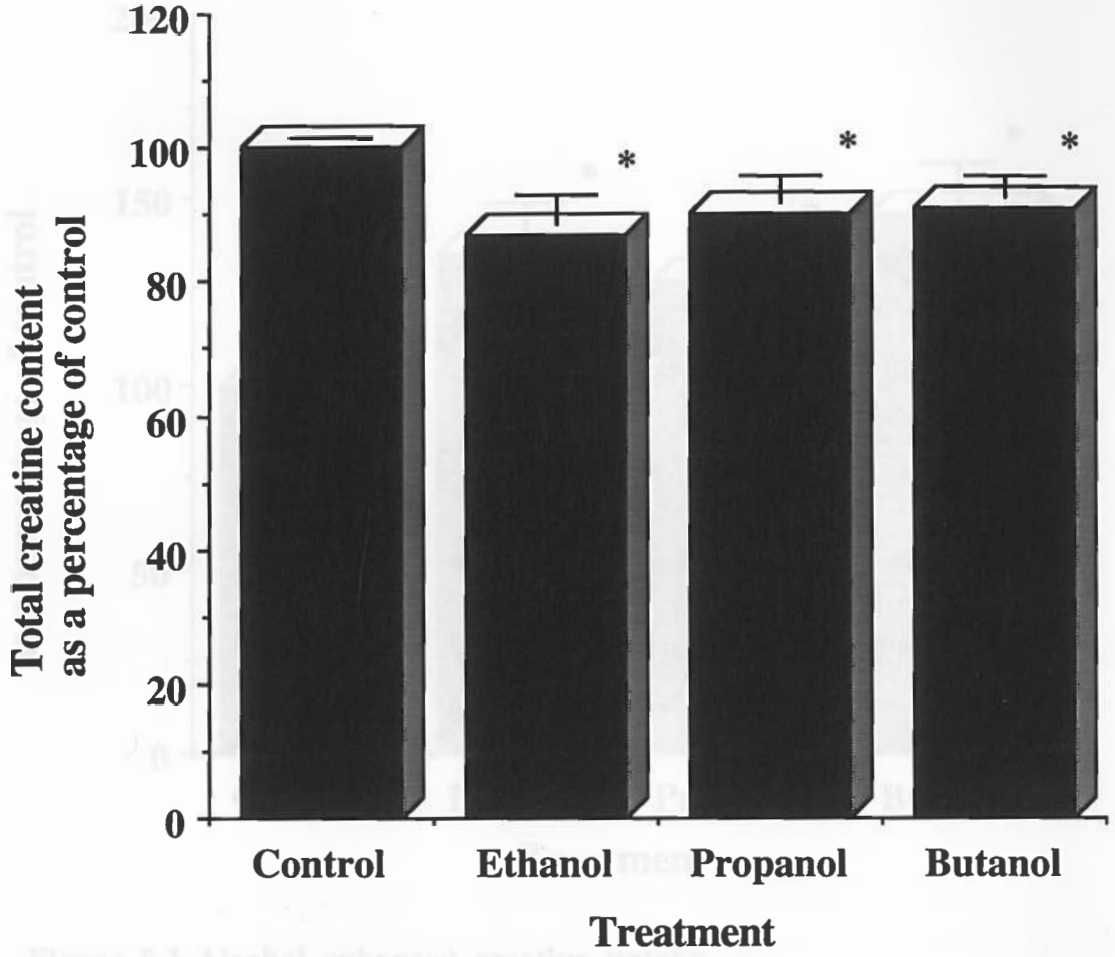


Figure 5.2 Alcohol induced total creatine depletion
A graph of total tissue creatine content against PSS treatment. The carotid arteries were incubated in PSS for 24 hours (n=15). The alcohols, at 0.1% (v/v), caused a statistically significant decrease in total creatine compared to the control ($P<0.05$).

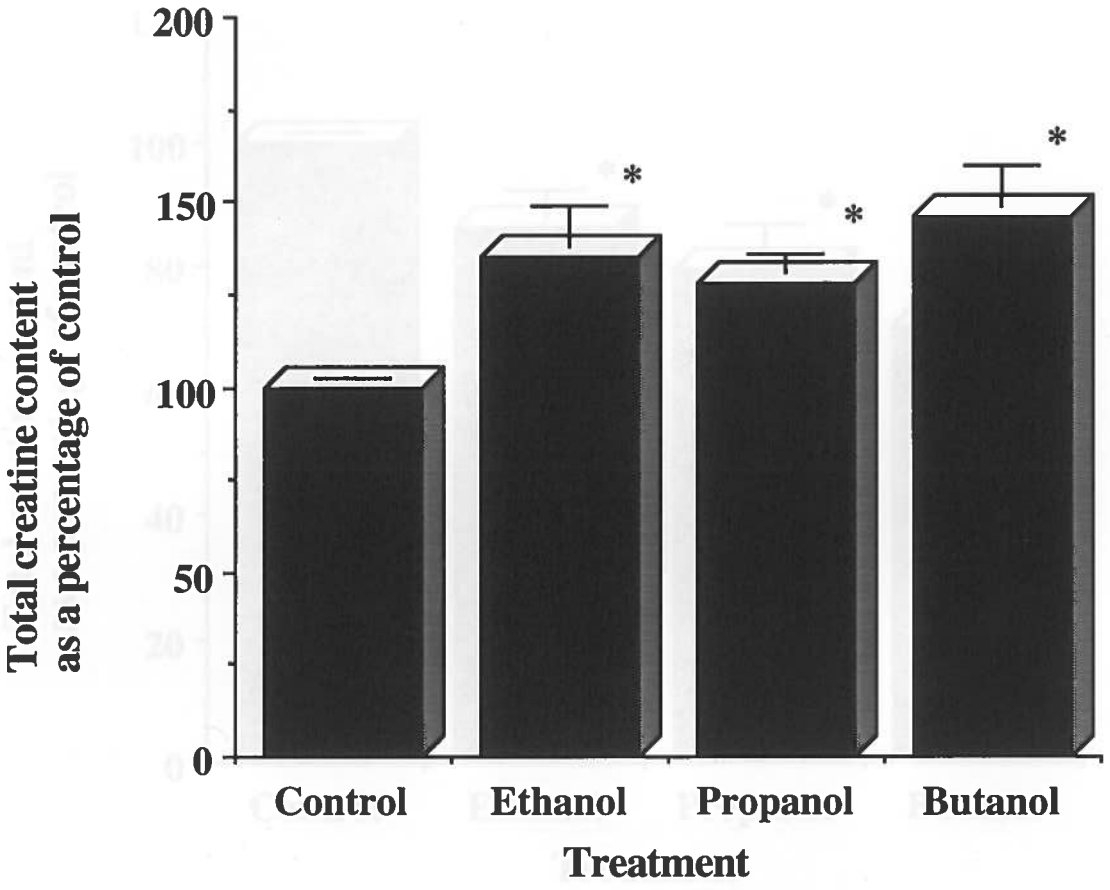


Figure 5.3 Alcohol enhanced creatine uptake
A graph of total tissue creatine content against PSS treatment. The carotid arteries were incubated in PSS containing 5 mM creatine for 24 hours (n=10). The alcohols, at 0.1% (v/v), caused a statistically significant increase in total creatine compared to the control ($P<0.05$).

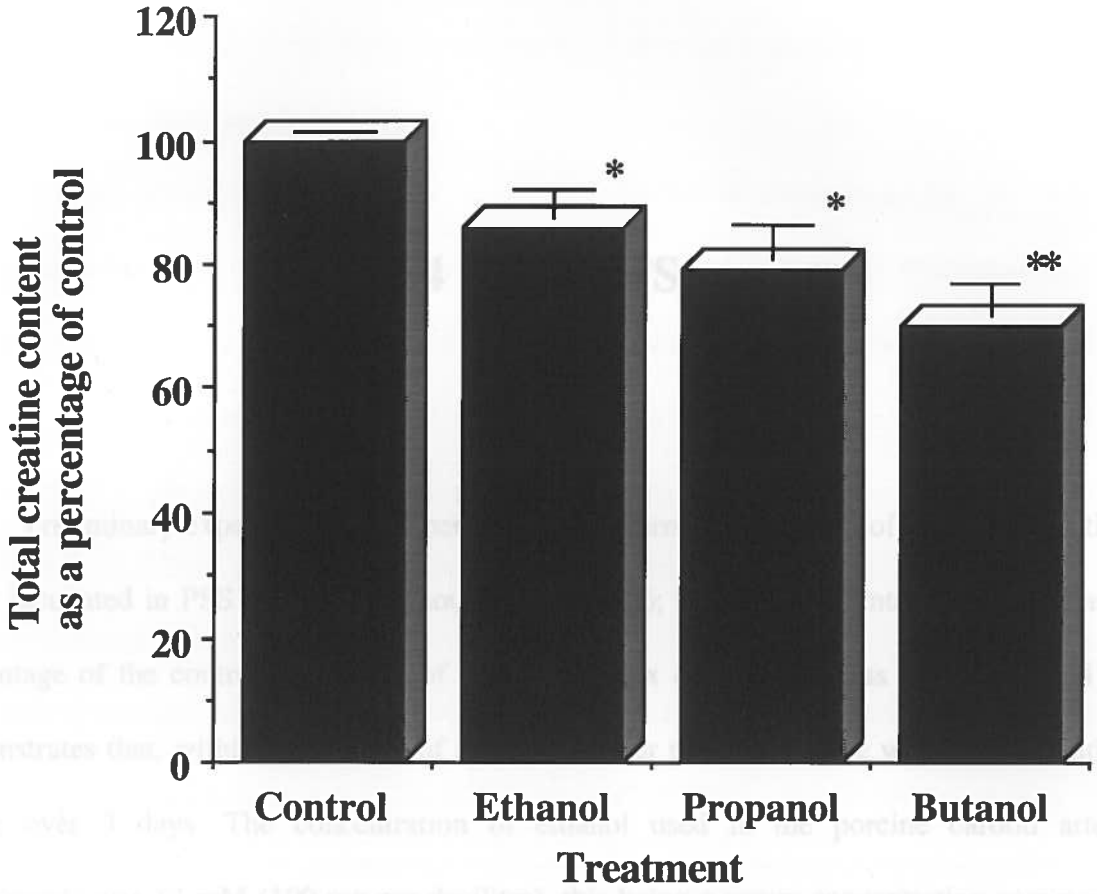


Figure 5.4 Alcohol induced creatine depletion in the presence of 0.1 mM creatine

A graph of total creatine content against PSS treatment.The carotid arteries were incubated in PSS containing 0.1 mM creatine for 24 hours (n=8). The alcohols, at 0.1% (v/v), caused a statistically significant increase in total creatine compared to the control ($P<0.05$).

H) NMR examination of perfused carotid arteries after 48 hours: The carotid arteries were incubated in PSS for 48 hours.

5.4 RESULTS

Preliminary experiments were performed to determine the extent of creatine depletion when incubated in PSS for up to 72 hours (Figure 5.1); the creatine content expressed as a percentage of the control at the end of the 72 h efflux experiment, was 106 ± 8% and so demonstrates that, within the bounds of accuracy of our protocols, there was no net creatine efflux over 3 days. The concentration of ethanol used in the porcine carotid artery experiments was 17 mM (100 mg per decilitre), this being a serum concentration commonly achieved in man. The data in Figure 5.2 (n=15) show that 0.1% (v/v)-ethanol, propan-1-ol and butan-1-ol caused the total tissue creatine content to decrease significantly to 87% ± 4, 90% ± 4 and 91% ± 3% of control respectively after a 24 hour incubation in a creatine-free solution, compared to the control ($P<0.05$). There is no significant difference in the extent of the depletion caused by ethanol, propan-1-ol or butan-1-ol ($P>0.05$).

On the basis of the observed alcohol induced depletion of total creatine content and to test the hypothesis that this was due to a membrane effect (e.g. enhanced fluidity leading to more/greater pore formation) we performed the experiments with the creatine concentration gradient favouring creatine entry, Fig 5.3 (n=10). The tissue was incubated in PSS containing

0.1% alcohol and 5 mM creatine for 24 hours. Ethanol, propan-1-ol and butan-1-ol each significantly increased the creatine content to 135% ± 11, 128% ± 5 and 146% ± 11 of the control value respectively ($P<0.05$). Here, the total creatine concentration in the carotid artery was enhanced by the alcohols when the extracellular creatine concentration was greater than the intracellular creatine concentration.

To test whether the tissue had an intrinsic ability to compensate for the alcohol-induced creatine loss over a 24 hour period and to determine the extent of creatine loss in conditions of a physiological plasma creatine concentration, we examined the effects of the alcohols on total creatine content in the presence 0.1 mM creatine; this concentration representing a physiologically achievable plasma creatine concentration [Rose, 1933; Marescau *et al.*, 1986]. The results of this experiment are depicted in Fig 5.4 (n=8). The total creatine contents after treatment with ethanol, propan-1-ol and butan-1-ol were 86% ± 4, 79% ± 6, 70% ± 5 of the control (all significantly less than the control, $P<0.05$) and there is no statistically significant difference between the effects of propan-1-ol and butan-1-ol ($P>0.05$).

The data in Table 5.1 allow comparison of the octan-1-ol:water partition coefficients calculated here (column A) using the Fragment method [Hansch and Leo, 1979] with reported partition coefficients obtained by measurement in octan-1-ol:water systems (column C) [Hansch and Leo, 1979], and with reported measurement in the membranes of the stratum-corneum from a human cadaver (column D) [Hansch and Leo, 1979]. Using the calculated log Kp of butan-1-ol, 1.15, as an example this is equivalent to 1 molecule of butan-1-ol partitioning into the aqueous phase for every 14 molecules partitioning into the hydrophobic phase (column B).

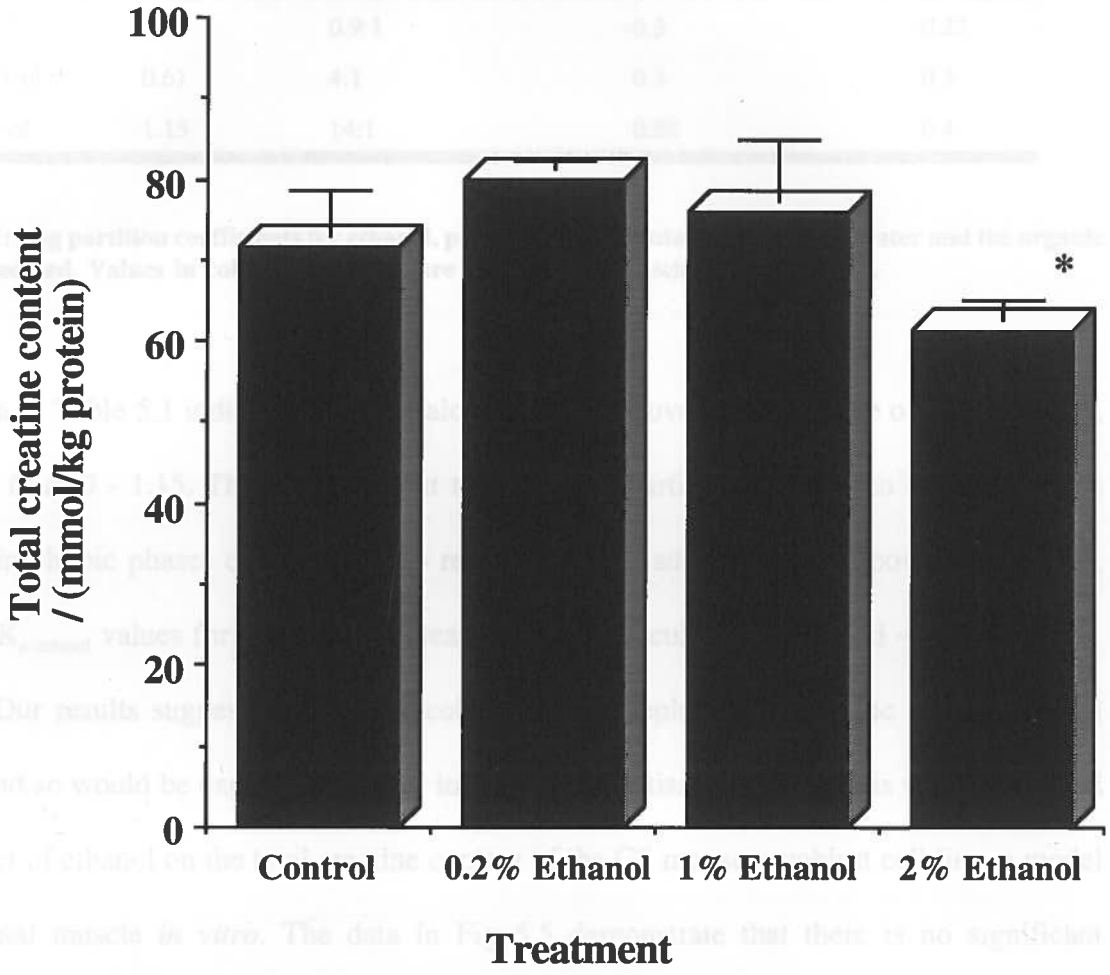


Figure 5.5 Effect of alcohol on total creatine in the G8 cell. A graph of total intracellular [creatine] against applied [ethanol] as % (v/v), with 0.5 mM creatine in the extracellular medium. The cells were of passage number 9 and plating density 250,000 (n=6). The experiment was conducted over 48 hours.

	A	B	C	D
Alcohol	Calculated logKp (Octan-1-ol:water)	Concentration Ratio (Octan-1-ol:water)	logKp (Octan-1-ol:water)	logKp (skin:water)
Ethanol	-0.05	0.9:1	-0.3	-0.22
Propan-1-ol	0.61	4:1	0.3	0.3
Butan-1-ol	1.15	14:1	0.88	0.4

Table 5.1: Log partition coefficients for ethanol, propan-1-ol and butan-1-ol between water and the organic phase specified. Values in columns C and D are taken from Hansch and Leo (1979).

The data in Table 5.1 indicate that these alcohols do not cover a large range of logKp values, varying from 0 - 1.15. This is equivalent to molecular partitioning between the hydrophilic and hydrophobic phases of 1:1 and 1:14 respectively. In addition to the above calculations, the log $K_{n\text{-octanol}}$ values for creatine and creatinine were calculated as -10 and -6 respectively.

Our results suggested that the alcohol induced depletion of creatine was a physical effect and so would be expected to occur in other cells or tissues. To test this we investigated the effect of ethanol on the total creatine content of the G8 mouse myoblast cell line, a model of skeletal muscle *in vitro*. The data in Fig 5.5 demonstrate that there is no significant depletion at 0.2% (v/v)-ethanol in the extracellular medium over a 48 hour incubation whereas 2% (v/v)-ethanol resulted in a depletion of total cellular creatine content to 86% ± 3 of the control value. The mean control value was 61.5 ± 2.3 mmol/kg protein. Cell viability was confirmed by trypan blue extrusion (>95% of the cells extruded trypan blue).

The Cr:PCr ratio for fresh carotid rings prior to incubation was 1.38 (± 0.18): 1 (n=7 from 4 pigs), this is similar to other reported values [Clark and Dillon, In Press]. The basal creatine content was 1.66 ± 0.29 mmol/kg wet weight (n=8).

Medium	Creatine content as a percentage of control
Control (without creatine)	71 ± 4
Control (with 0.15 mM creatine)	100## (± 6)
dBcAMP (0.2 mM)	126* ± 3
dBcGMP (0.2 mM)	117* ± 3
Sodium pyruvate (5 mM)	142** ± 7
Na^+ (60 mM)	64*# ± 13
Isoprenaline (0.1 µM)	146** ± 11

Table 5.2: Modulators of net creatine uptake (n=10).* = P<0.05 ** = P<0.01 compared to control with no addition.

There is no significant difference between this value and the control (without creatine).
This value is significantly greater than the control without creatine (P<0.01).

Table 5.2 is a summary of the results of independent experiments. Decreasing the sodium concentration to 60 mM led to a decrease of net uptake that resulted in a creatine content no greater than carotid rings incubated in the absence of creatine. Hence the net creatine uptake is sodium dependent. We have also found that net creatine uptake is stimulated by the oxidative substrate pyruvate (Table 5.2).

Table 5.3 shows a series of experiments conducted to examine the effects of ß adrenergic receptor agonists, dobutamine ($ß_1$), clenbuterol ($ß_2$) and ZD2079 ($ß_3$) on net creatine uptake (n=10).

Medium	Creatine content as a percentage of control
Control	100 ± 5
Dobutamine (40 µM)	94 ± 6
Clenbuterol (40 µM)	123*± 9
ZD2079 (40 µM)	84 ± 12

Table 5.3: Effect of ß adrenergic receptor agonists on net creatine uptake (n=10). * = P<0.05 compared to control (containing creatine and no agonist)

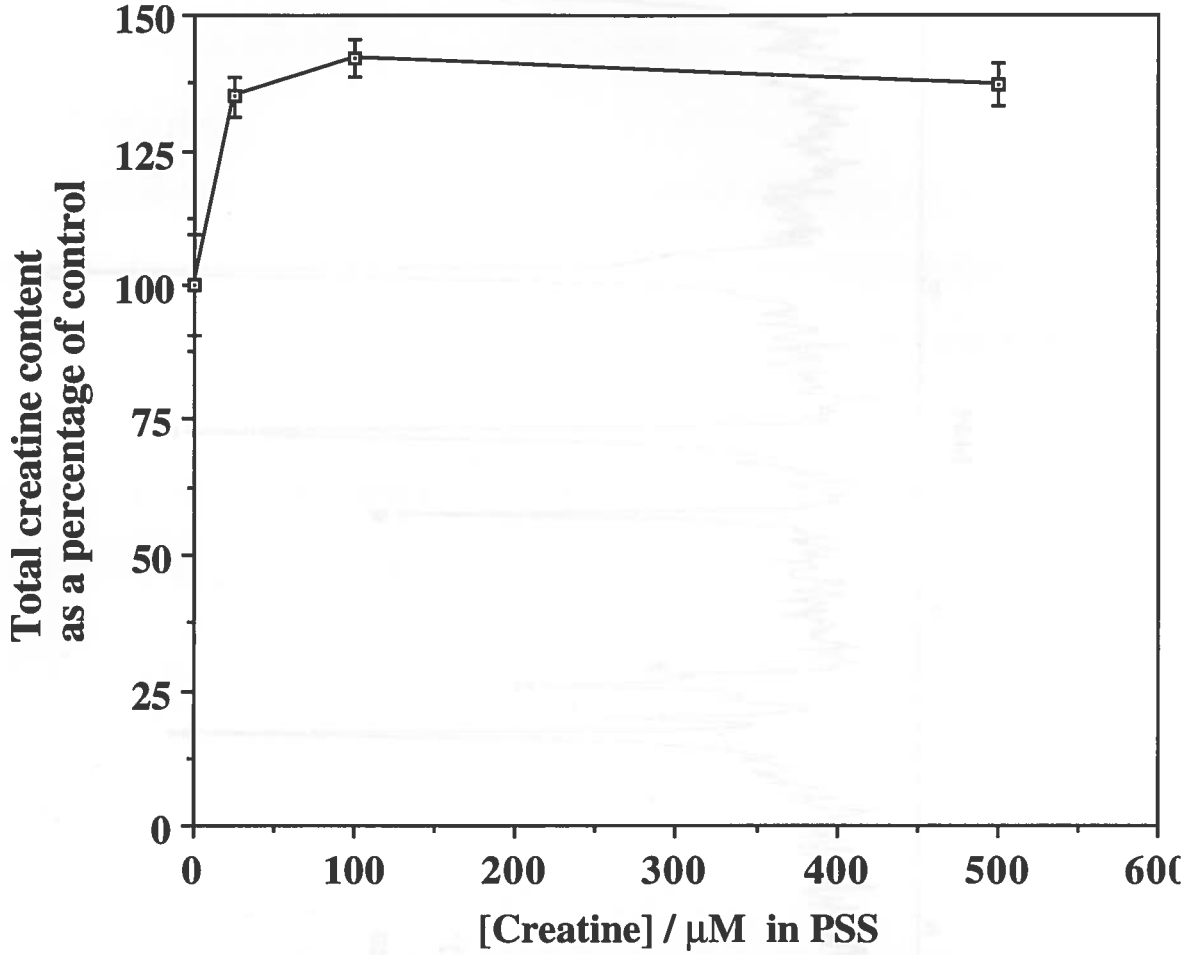
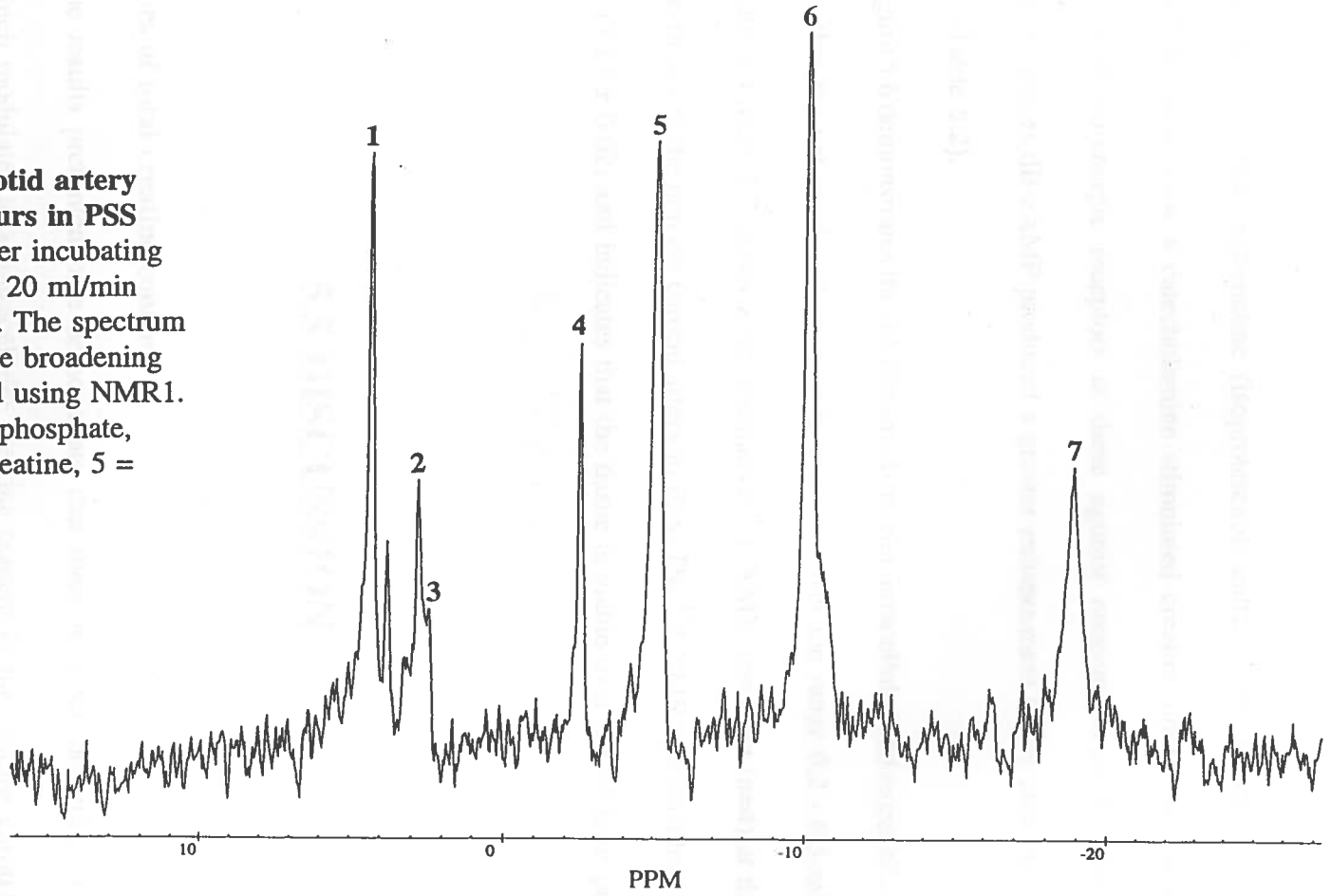


Figure 5.6 Dependence of total creatine content on extracellular creatine A graph of total tissue creatine content against applied creatine (0-0.5 mM). The carotid arteries were incubated in PSS containing creatine for 24 hours (n=10).

Figure 5.7 ^{31}P-NMR spectrum of the porcine carotid artery after 48 hours in PSS

This representative spectrum (n=4) was produced after incubating a carotid artery at 22°C at a constant flow rate of 20 ml/min with PSS aerated by 95% O_2 / 5% CO_2 for 48 hours. The spectrum consists of 3 x 1500 transients with Lorentzian line broadening corresponding to 30 Hz. NMR spectra were analysed using NMR1. 1 = Phosphomonoesters, 2 = Extracellular inorganic phosphate, 3 = Intracellular inorganic phosphate, 4 = Phosphocreatine, 5 = γ ATP, 6 = α ATP, 7 = β ATP.



Taken together with the isoprenaline (isoproterenol) enhancement of net creatine uptake (Table 5.2), the data show a catecholamine stimulated creatine uptake that appears to be mediated by $ß_2$ adrenergic receptors at these agonist concentrations. Of the secondary messenger analogues dB-cAMP produced a greater enhancement of net creatine uptake than dB-cGMP (Table 5.2).

Figure 5.6 demonstrates the relationship between intracellular and extracellular creatine and shows that the net uptake of creatine is saturable over the range 0.2 - 0.5 mM creatine in the medium. Figure 5.7 shows a representative ^{31}P-NMR spectrum (n=4) at the end of a 48 hour perfusion of the porcine carotid artery in PSS. The ^{31}P-NMR spectra allow calculation of the pH (7.17 ± 0.02) and indicates that the tissue is viable over the 48 hour period.

5.5 DISCUSSION

Modulators of total creatine content

The results presented here demonstrate that there is a Na^+-dependent net creatine uptake which modulates total intracellular creatine content in the porcine carotid artery. A sodium dependent creatine uptake has also been identified in nervous tissue, skeletal muscle and cardiac muscle [Möller and Hamprecht, 1989; Loike *et al.*, 1988; Seppet *et al.*, 1985]. In addition, we have provided evidence for a $ß_2$ adrenergic receptor pathway that may be important in stimulating net creatine uptake. We also demonstrated that there is a modulation of the creatine transporter linked to presence of the oxidative substrate, pyruvate.

Throughout this study, the measurement of changes in the total intracellular creatine

relative to tissue wet weight, was used as an index of creatine uptake. We have shown that there is negligible net efflux of creatine over 72 hours in this tissue and this is consistent with earlier work in both muscle cell lines and tissues (section 1.4) [Odoom, In Press]. Since there is no synthesis of creatine in muscle [Walker, 1979] a change in total cell creatine can only arise out of increased transport. This methodology could be adapted to study creatine transport in cardiac and skeletal muscle and the agonists identified would offer new pharmacological approaches to controlling intracellular creatine.

Viability of the porcine carotid artery at the end of the experiments is supported by the NMR spectrum (Figure 5.7) which yields an average pH of 7.17 ± 0.02. The total reported creatine concentration of the porcine carotid artery is 2 mM (Clark & Dillon, In Press) (similar to the value of 1.66 ± 0.29 mmol creatine/kg wet weight demonstrated by our studies) hence a net uptake of creatine in the presence of an extracellular creatine concentration of 0.15 mM (Table 5.2, Figure 5.6) demonstrates a concentrative and therefore active transport process. In addition, the plot of extracellular creatine against intracellular creatine content reaches a plateau, at low creatine concentrations, and so indicates a carrier mediated creatine transport into the tissue. Decreasing the Na^+ concentration in the medium to 60 mM decreased the net uptake to a level that was indistinguishable from that of carotid arteries incubated without creatine (Table 5.2), demonstrating a sodium dependence of net creatine uptake. Together these observations support our proposal for a secondary active transport of creatine in this tissue. Such a cotransport would harness the potential energy of the sodium gradient to drive creatine uptake. Although we have identified a creatine uptake process we were unable to measure any significant net creatine efflux when the carotid arteries were incubated in only PSS for up to 72 hours. The calculated log $K_{n\text{-octanol}} \approx -10$ (creatine) and log $K_{n\text{-octanol}} \approx -6$ (creatinine) suggest that the inability to measure a significant efflux is due to the low

affinity of these molecules for the hydrophobic membrane milieu.

We tested the non-selective catecholamine analogue isoprenaline (isoproterenol) for an effect on creatine uptake because of the importance of the sympathetic nervous system in controlling vascular smooth muscle. The results in Table 5.2 demonstrate the enhancement caused by isoprenaline. To identify the receptor subtype responsible for increasing net creatine uptake, the selective agonists dobutamine ($ß_1$), clenbuterol ($ß_2$) and ZD2079 ($ß_3$) were tested. We found no enhancement of creatine uptake by dobutamine or ZD2079, but clenbuterol produced a significant increase in creatine uptake (Table 5.3), a similar result is discussed in section 3.5.

We identified a possible secondary messenger involved by using the analogues, dibutyryl cAMP (an analogue of cAMP) and dibutyryl cGMP (an analogue of cGMP), to alter creatine uptake (Table 5.2). The greater increase in creatine uptake was produced by dibutyryl cAMP. Hence we propose that the $ß_2$ receptor is probably working through cyclic AMP in the porcine carotid artery to stimulate creatine uptake. This is similar to the isoprenaline (isoproterenol) mediated stimulation of creatine uptake discussed in section 3.5. It was proposed that this was part of the physiological adaptation to repeated exercise; the slow efflux together with the rapid uptake means that each bout of exercise could yield a sustained increase in intracellular creatine over time. This could then enhance muscle function due to the increased PCr levels [Harris *et al.*, 1992] and the anabolic effects of creatine [Bessman and Mohan, 1992]. As in skeletal muscle, the efflux of creatine from the porcine carotid artery is also negligible compared to uptake and is stimulated by catecholamines. One possible physiological significance of the catecholamine stimulated creatine uptake in vascular smooth muscle might be to aid vascular contractility or dilatation during exercise. Some supporting evidence comes from the demonstration of a dependence of vascular contractility on

phosphocreatine content [Clark, 1994].

The oxidative substrate, pyruvate, increased creatine uptake compared to glucose. This suggests a metabolic component to the pathway regulating creatine uptake. Mechanistically, there are at least two plausible explanations for this effect:

a) Pyruvate has a direct effect either on the creatine transporter or on one of the signalling molecules involved in its control. b) Pyruvate alters the energetic balance of the vascular smooth muscle to indirectly increase *active* creatine uptake.

The creatine/PCr/CK system is an important part of the energy metabolism of vascular smooth muscle. This is further reinforced by the control systems for creatine uptake that we have found in the isolated perfused carotid artery and implies that total cellular creatine may be an important regulator of smooth muscle energetics. In particular, the observation of catecholamine controlled creatine uptake may have important implications for vascular smooth muscle function with physical training. It is possible that future studies on the control of smooth muscle function by bioenergetic interventions may become important in pathological processes involving vascular contractility, for example hypertension.

Alcohol and creatine depletion

We have shown that 0.1% (v/v)-extracellular alcohol over 24 hours is sufficient to deplete the porcine carotid artery of approximately 10% of its total creatine stores (creatine + phosphocreatine), compared with the control value (Figure 5.2). This effect occurs with each of the short chain alcohols used and the biological material tested; ethanol, propan-1-ol and butan-1-ol each caused this depletion in the porcine carotid artery. 2% (v/v)-extracellular ethanol depleted the G8 myoblast cell line to 86% ± 3 of the total creatine content of the control after 48 hours (Fig 5.5). The difference in the ethanol concentration required to cause

significant depletion in the porcine carotid artery and the G8 cell may reflect a stabilizing effect of the cytoskeletal-plasma membrane association in the G8 myoblast cell line, provided by the greater actin and myosin content. Furthermore, it is also possible that active skeletal muscle tissue may be more susceptible to the effects of alcohols as it has recently been shown that muscle contraction potentiates the effects of alcohol induced solute loss [Amaladevi *et al.*, 1995].

In both the porcine carotid artery and the G8 cell line the intracellular creatine concentration is greater than the extracellular (or media) creatine concentration [Clark and Dillon, In Press; Odoom et al., 1993] hence the concentration gradient favours net creatine efflux. If such an enhanced passive efflux were responsible for our observations then one would predict that exposing the porcine carotid artery to a high extracellular creatine concentration, that is reversing the creatine concentration gradient, would enhance creatine uptake in the presence of an alcohol. Fig 5.3 shows that such an increased uptake occurred. The movement of creatine with the concentration gradient (Figures 5.2 and 5.3) indicates a passive efflux process.

Figure 5.4 demonstrates that an extracellular creatine concentration lower than the intracellular creatine concentration resulted in net creatine efflux in the presence of the alcohol. This latter result is also important because it suggests that in the presence of a physiological creatine concentration the porcine carotid is alone unable to compensate for this creatine depletion, over a 24 hour period. Butanon-1-ol in the presence of creatine appears to produce a larger effect than ethanol or propan-1-ol (Figures 5.3 and 5.4); it is not clear if this represents an additional interaction between creatine and butan-1-ol.

Alcohols are membrane fluidisers [Odoom *et al.*, 1990; Stibler *et al.*, 1991] that can be used to enhance passive non-protein mediated transport processes [Odoom *et al.*, 1990] by

intercalating into the phospholipid bilayer. It is established [Hansch and Leo, 1979] that the affinity of the solute for the hydrophobic membrane environment is proportional to the octan-1-ol:water partition coefficient and the magnitude of the log Kp is proportional to the alkyl chain length (Table 5.1), as each $-CH_2-$ group confers an increment of hydrophobicity [Hansch and Leo, 1979]. The data in Table 5.1 is consistent with the partition coefficients being of sufficiently large a value to be above a putative threshold for eliciting membrane perturbation and hence creatine depletion [Amaladevi *et al.*, 1995].

A 0.1% blood ethanol level is achieved by recreational drinkers and by chronic alcoholics [Jones and Sternebring, 1992] (this is also the legal blood alcohol limit for being responsible for a motorised vehicle in the UK). As the fluidising action of the alcohols is a physical effect that is not dependent on the short chain aliphatic alcohol used or the cell/tissue studied, a reported study of the effect of ethanol on human erythrocytes is pertinent. Stibler [Stibler *et al.*, 1991] demonstrated that erythrocytes from chronic alcoholics have lower membrane fluidity than normals and resist the fluidising effects of alcohols - hence there was a marked decrease in the partition of ethanol itself into the plasma membrane. Our observations and these results are consistent with an *in vivo* model that postulates a short term depletion of total creatine on exposure to alcohol, the duration of the effect being diminished by the adaptive processes of the plasma membrane and probably by the ingestion of creatine. Interestingly, work done on rat skeletal muscle (from rats chronically administered ethanol) and in erythrocytes from human alcoholics, shows that the activity of the Na^+,K^+-ATPase is increased (as is the leakage of the sodium solute) in response to ethanol [Johnson and Crider, 1989]. Such a role for the Na^+,K^+-ATPase would not only tend to restore the sodium gradient to the pre-ethanol treatment value, but would also normalise the solute entrance of any sodium dependent uptake process, in this case creatine uptake.

Chronic alcoholics are more likely to develop cardiac myopathy, coronary vascular disease and hypertrophy [Das and Harris, 1993; Preedy *et al.*, 1994]. As each of these is characterised by a decreased total tissue creatine it is possible that an alcohol induced creatine depletion plays at least a contributory role to the development of these pathologies. The following points support this hypothesis:

1) Creatine depletion achieved with a creatine analogue, ß-guanidinoproprionic acid, induced hypertrophy in rat hearts [Clark *et al.,* 1994].

2) Type-II muscle fibre atrophy and decreased muscle protein synthesis is common in alcoholics [Das and Harris, 1993]; creatine is known to preferentially stimulate Type-II muscle fibre synthesis and actin and myosin synthesis [Bessman and Mohan, 1992; Ingwall *et al.*, 1972].

3) The link between muscle function and membrane fluidity is indicated by the membrane stabilising effect of the cardioprotective drug phosphocreatine [Saks and Strumia, 1993].

However it is also possible that creatine is one of several factors that lead to cardiovascular pathology, and so the deficiency in tissue carnitine in ischaemia and heart failure may be important observations [Reibel *et al.,* 1987]. Both creatine and carnitine are similar sized molecules whose concentration gradients favour net efflux from cells *in vivo*; it is reasonable to suggest that the membrane damage that occurs results in an increased rate of solute loss.

Non-alcohol related creatine depletion occurs in several cardiovascular dysfunctions, most notably during heart failure [Jones and Sternebring, 1992]. Furthermore elevating creatine content in striated muscle by 10% appears to enhance skeletal muscle function [Greenhaff *et al.*, 1993]. The observed depletion of creatine content and the correlation with muscle function, make it reasonable to suggest that creatine levels are relevant in the

development of several cardiovascular pathologies.

PUBLICATIONS:

1) Odoom, J.E., Willott, C.A., Radda, G.K. Clark, J.F. (1995) Modulation of the total creatine pool in the porcine carotid artery. Submitted to the Journal of Physiology.

2) Odoom, J.E., Willott, C.A., Kemp, G.J., Radda, G.K. Clark, J.F. (1995) Depletion of the total creatine pool from the carotid artery and skeletal muscle cells by primary alcohols. Submitted to Cardiovascular Research.

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SUMMARY AND OVERVIEW

Summary

The following advances in the cellular study of creatine transport have been made from this work:

(a) A new cellular model of skeletal muscle has been characterised and used to study creatine uptake and efflux. The kinetic (e.g. K_m and V_{max} of uptake, net efflux measurements) and metabolic (e.g. PCr:Cr) characteristics are described and are similar to skeletal muscle *in vivo*.

(b) A simple fluorimetrically based protocol has been developed to test modulators of creatine uptake over a medium term (24 - 48 hours). This protocol has been extended to a tissue study, the porcine carotid artery.

(c) The first reports have been provided of a range of agonists that can increase creatine uptake. Important amongst these are noradrenaline and IGF-I.

(d) Plausible models for the physiological roles of IGF-I and noradrenaline are provided, as is evidence for their modes of signal transduction.

(e) The net rate of efflux of creatine is shown to be susceptible to modulation over a 24 hour period by alcohols. The alcohols have their effect through increasing passive efflux of creatine, probably through an effect on membrane permeability. This is a physical effect which may be important in alcoholic cardiovascular illness.

(f) Evidence is provided for a regulatory role of the Na^+,K^+-ATPase in creatine uptake and an action in the adaptation to creatine efflux is suggested.

Overview

The overview is addressed by considering areas in which answers to the following

questions would be particularly illuminating:

From chapter 1:

1) What are the long term effects of feeding creatine on each of the tissues in the body? To date only the Sipilä [Sipilä *et al.*, 1981] study has been more than three months long. Is it reasonable to predict an effect of creatine dosing on the synthesis of creatine by the liver? A homeostatic mechanism that responds to chronic elevated plasma creatine is a possibility. It seems unlikely that low dose creatine feeding (<2 g per day) would lead to liver damage, as this is within the dietary range for man. However most of the creatine feeding studies that have produced a beneficial effect on muscle function have been of the order of 20 g of creatine per day. Furthermore is a prolonged anabolic stimulus, produced by creatine dosing, likely to affect cellular growth to increase the risk of tumour formation?

Can chronic high dose creatine feeding lead to significant passage of creatine through the blood brain barrier and so affect central bioenergetics? Despite creatine's low permeability across biological membranes, a prolonged sufficiently high dose would be expected to alter central creatine concentrations. Would a change in creatine concentration lead to a positive or negative effect on neuronal function?

2) What type of sporting activities will creatine benefit? What type of normal daily activities in the non-athlete will creatine improve? One can imagine creatine being of benefit in posture and movement of the elderly as here it is type II muscle fibres that appear to atrophy disproportionately, whereas creatine may be of less benefit, for example, to active meat eating young women who are more likely to have high muscle creatine contents.

3) The loss of tissue creatine that occurs with chronic myocardial dysfunction appears to be due in part to an effect on membrane permeability. Would giving creatine to such individuals slow the progression of these pathologies or would only an action at the plasma membrane

be significantly therapeutic?

From chapter 2:

1) Although the G8 cell line may be a model of human skeletal muscle, is there a model of human cardiac muscle upon which biochemical manipulations can be performed? With the range of possible applications to cardiovascular illness such studies may ultimately lead to clinical applications.

2) What is the precise timecourse of creatine uptake in the G8 cell? This would more precisely identify the optimum duration for uptake experiments.

3) How do the effects on creatine uptake of amylin and CGRP compare? As these two agonists can stimulate the other's receptors, one might expect CGRP to stimulate creatine uptake.

4) Is T3 the only thyroid hormone that affects creatine transport? If the effect of T3 is receptor mediated then the effects of other thyroid hormones would be expected to be lesser because of likely structure-activity relationships at the receptor.

5) What changes do amylin, T3 and isoprenaline (isoproterenol) produce in membrane potential? The work presented in this study would predict an enhancement of membrane potential.

6) What effects do combinations of these agonists have upon creatine uptake in the G8 cell? How high can the cellular creatine content be driven by agonists at physiological concentrations? Would very high intracellular creatine levels be detrimental to muscle function?

From chapter 3:

1) What is the effect of G protein stimulators and inhibitors on creatine uptake? If the signalling pathways postulated are accurate then an effect of G proteins on creatine uptake

would be predicted.

2) Does the activity of adenylate cyclase change? The measurement of an increase in activity of adenylate cyclase on catecholamine administration would support the proposed pathway (figure 3.15).

3) How do the activities of PKA and PKC change on agonist addition? The summary presented in figure 3.15 would be further supported by measuring the predicted changes in activity of protein kinase A and C on agonist application.

From chapter 4:

1) What is the effect of the inhibition or stimulation of MAP kinase on creatine uptake? Direct determination of changes in activity of MAP kinase on agonist application could confirm the involvement of MAP kinase.

2) Can inhibitors of creatine uptake be studied? The isolation and characterisation of the creatine uptake inhibitory protein (Figure 4.10) may provide another site for pharmacological action.

3) Is the insulin or the IGF receptor important in insulin's action? It may be possible to use cross-linking agents to confirm which of the receptors are responsible for agonist induced creatine uptake.

4) Can the proteins with phosphotyrosine residues, important in regulating creatine uptake, be identified? A systematic use of agonists and other stimulators of protein tyrosine phosphorylation, should allow identification of bands (western blotting) correlated with stimulation of creatine uptake. Using analytical protein techniques these could then be identified.

5) Where are the creatine transporters? It may be possible to determine the cellular locations of the creatine transporter by a form of fluorescence microscopy. This would resolve the

existence of an intracellular store of transporters. Furthermore, modifying the creatine transport methodology (section 2.3) to determine the speed of responsiveness of the cells to IGF-I or Insulin, may also provide evidence for the presence of an intracellular store of creatine transporters.

From chapter 5:

1) Does the effect of alcohol on tissue creatine content extend to cardiac tissue? One would expect an effect here if the alcohols affect membrane permeability. In addition, extending the study to a human source of smooth muscle, e.g. human placenta, may also be pertinent.

2) Can the sensitivity of the NMR examination of creatine uptake be increased to obtain a timecourse of creatine uptake?

3) Which adrenergic receptors are important mediating the effects of isoproterenol? The use of adrenergic antagonists to confirm the existence of a $ß_2$ receptor mediated uptake process and investigating the effect of α adrenergic receptors would be important.

The above questions can be divided into those that require biochemical investigation and those that require clinical/physiological investigation.

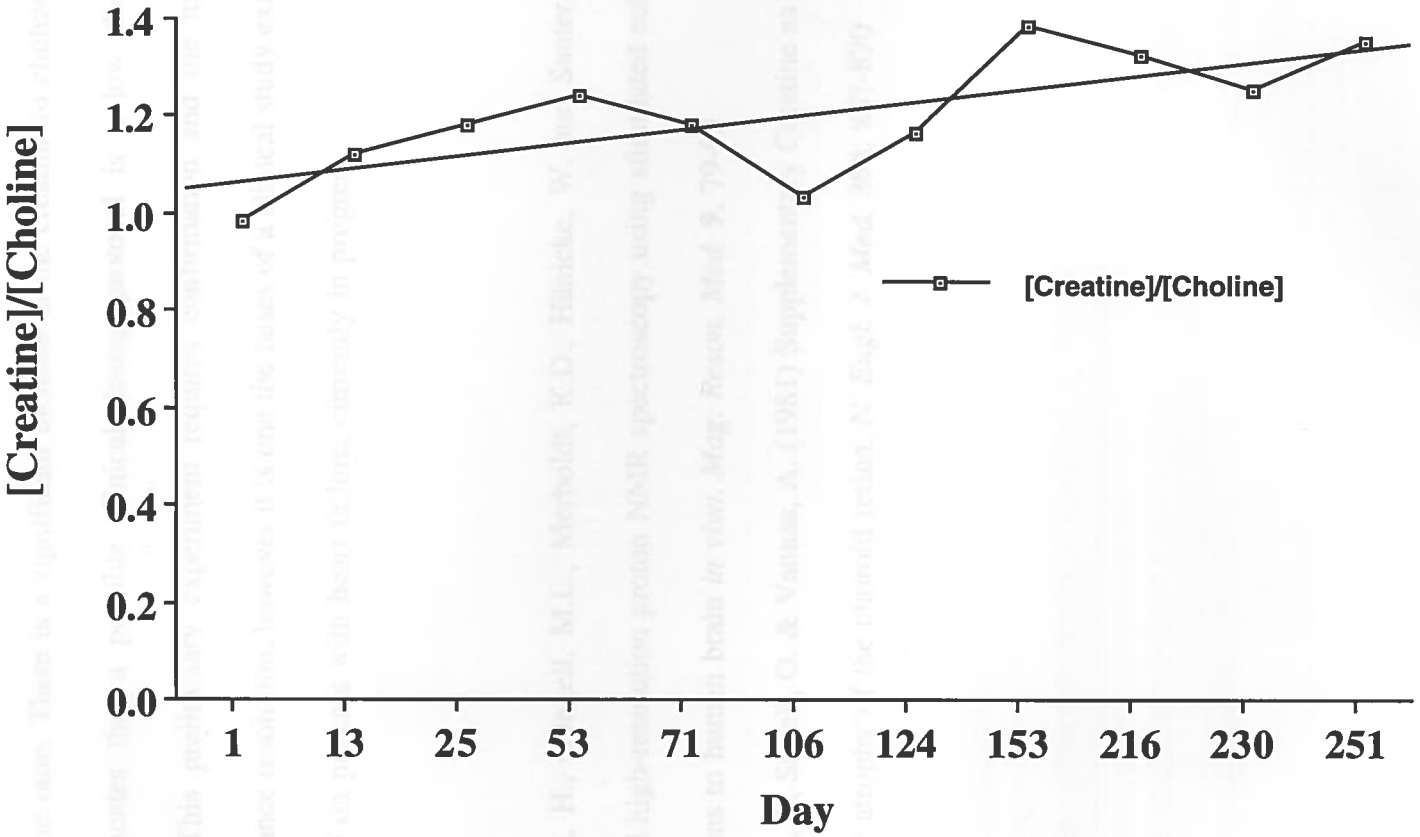
Those that require biochemical investigation are hindered by the lack of molecular knowledge concerning the transporter itself. The recent cloning of the transporter has shed light on the primary structure of the creatine transporter but isolation, purification and structural studies are necessary for further progress. By comparison with other membrane proteins with 7 transmembrane sequences, it should be possible to identify exposed Asn-X-Ser/Thr sequences that could represent regulatory phosphorylation sites. In addition the number of isoforms of the transporter should soon become clear. Major progress will be made in the understanding of transporter regulation when reconstitution studies are possible; the

installation of the transporter into a phospholipid vesicle would allow investigation of factors that directly affect the activity of the transporter. Allied to these approaches could be the development of nuclear magnetic resonance procedures to follow creatine fluxes in cells and tissues; this would relate the strands of study *in vitro* and *in vivo*. Finally, ubiquitous in the analyses of the results presented in this thesis is the action of the Na^+,K^+-ATPase; the most direct means of identifying changes in Na^+,K^+-ATPase activity would require the use of rubidium and sodium radiolabels and in principle such studies could be performed on the tissues examined here.

The investigation of clinical and physiological function of creatine uptake would require a non - invasive approach to an investigation of the whole animal. This could be achieved by applying a technique such as magnetic resonance. Accordingly, we have performed a preliminary experiment on a human volunteer. This involved giving 2g of creatine per day as a single dose while the volunteer was instructed to maintain a normal lifestyle (30 year old male, physically active). The creatine content on the mixed muscles of the calf (soleus and gastrocnemius) was followed over 251 days.

Method

Localized proton MR spectra were obtained on a whole body magnet operating at 2 tesla by means of the STEAM sequence (stimulated echo acquisition mode, [Frahm *et al.*, 1989]). Water suppression was achieved using 3 CHESS pulses. A $(3\ cm)^3$ volume in the calf muscle was selected from a spin-echo image (the volume was largely soleus, but included some gastrocnemius muscle). The spectra were acquired with an interpulse delay of 6 s, a mixing time (TM) of 30 ms, and an echo time (TE) of 68 ms. Eight or sixteen scans were accumulated. The spectra were processed with 2 Hz exponential line-broadening, and the peak



Creatine feeding in a human subject
An active male was given a 2g dose of creatine per day and the creatine/choline ratio determined as outlined in the text. The data is fitted to a line (linear regression) described by [Creatine]/[Choline] = 1.0 + 0.028(Day) and R square = 0.530

areas of choline (3.25 ppm) and creatine (3.04 ppm) were quantified with a computer-aided fitting routine (Glinfit, Bruker).

The result is depicted as timecourse on the adjacent page and is expressed as a creatine to choline ratio. There is a significant increase in the creatine to choline ratio with time which indicates that a possible clinical dosing protocol is a low dose creatine administration. This preliminary experiment requires confirmation and the methodology improved to enhance resolution, however it is one the bases of a clinical study examining the effect of creatine on patients with heart failure, currently in progress.

References

Frahm, J., Bruin, H., Gyngell, M.L., Merboldt, K.D., Hänicke, W. and Sauter, R. (1989) Localized high-resolution proton NMR spectroscopy using stimulated echoes: initial applications to human brain *in vivo*. *Mag. Reson. Med.* **9**, 79-93

Sipilä, I., Rapola, J., Simell, O. & Vannas, A. (1981) Supplementary Creatine as a treatment for gyrate atrophy of the choroid retina. *N. Engl. J. Med.* **304**, 867-870

Appendix I

Creatine Assay (Conn, 1960)

a) Hydrolysis of PCr in biological sample (in 0.1 M NaOH)

20 µl of extract + 480 µl dH_20 + 50 µl 1 M HCl and vortex
Heat for 20 minutes in a 60°C waterbath
Add 450 µl dH_20

b) Standards

10 mM creatine stock diluted 1/1000 = 10 nmol/ml
Make 1ml samples containing 0-5 nmol/ml

c) Fluorimeter

Turn on fluorimeter 20-30 minutes before use.
Excitation wavelength = 410 nm Excitation slit width = 10 nm
Emission wavelength = 525 nm Emission slit width = 20 nm

d) Procedure

Make fresh 1% ninhydrin in 70% EtOH and 10% KOH in 70% EtOH
Mix 1 ml standards, blanks and samples with 0.5 ml of ninhydrin and mix
Add 0.5 ml OH at time = 0

Read fluorescence at 8 minutes

Calculate unknown creatine concentrations by linear regression.

Appendix II

Variation in basal creatine contents in the G8 cell line

Throughout this work there was noticeable variation) in the basal creatine content exhibited by the control values for each of the experiments (section 2.8, page 28. Although this variation does not affect the interpretation of the data, it warrants further discussion. The factors in cell handling and culture medium components that may be causing such variation include:

a) The time taken for the cells to reach confluence prior to a transport experiment. If one batch of cells takes 3 days to reach confluence, compared with 5 days for another, then the latter will have 67% more time to take up creatine from the complete medium. (The complete medium contains sera which itself contains creatine).

b) The precise level of confluence may affect the extent to which the cells are stimulated to, or inhibited from, taking up creatine. (It should be noted that judging confluence by sight is a subjective process). Such a mechanism would depend on intercellular communication analogous to the contact inhibition of cell growth in culture. Dr K. Polgreen (Polgreen, 1993; Chapter 2 references) showed in her work that the uptake of phosphate by L6 myoblasts is dependent on the extent of confluence.

c) Passage number; there may be a contribution to the variation caused by the change in passage number. The G8 cells are recommended for use up to passage

18, however some aging of the cells must be expected.

d) The effects of stimulatory factors in the complete medium. The complete medium contains sera that is a natural biological quantity. Now although some components can be controlled by the supplier, the levels of hormones are not. Hence the levels of noradrenaline, adrenaline, insulin, IGF-I and amylin can be expected to vary in different sera preparations. There may also be an effect due to added agents (e.g. clenbuterol - currently used in the livestock industry) and agents that affect creatine uptake which we have not yet identified.

e) The state of the cells. The work by Loike (Loike et al., 1988; Chapter 2 references) suggests that cultured myoblast cells can express surprisingly large efflux rates (the data in his paper suggest that a 50% depletion of total creatine can occur with an overnight incubation in a creatine free medium). Perhaps the cells were disturbed enough to affect membrane permeability without significantly decreasing cell viability; could such an effect be occurring here?

Ideas similar to the above may apply to the variation in the total creatine content of the porcine carotid artery.

Appendix III

Creatine and Metabolism

A) The following extract is taken from Walker, J.B. (1979) Creatine: Biosynthesis, regulation, and function *Adv. Enzymol.* **50**, 177-242.

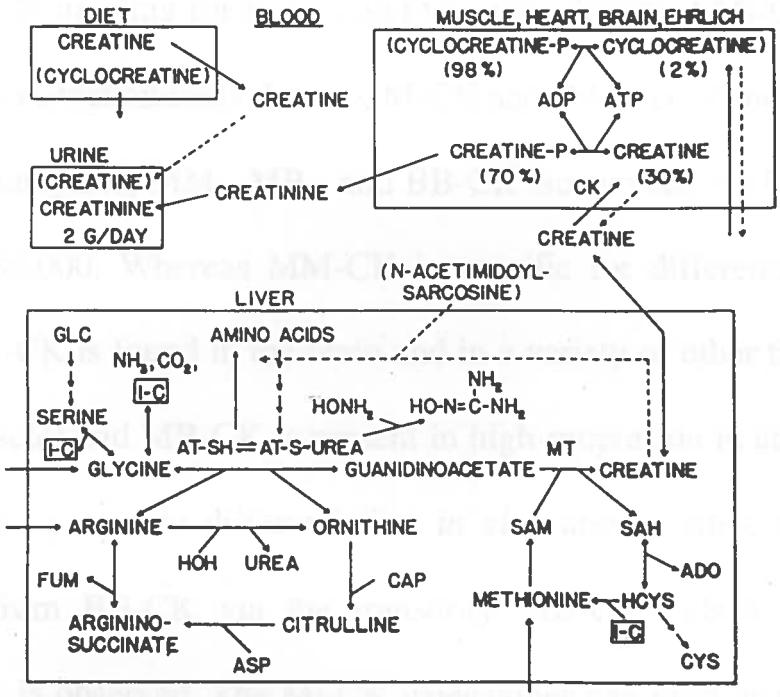


Fig. 1. Reactions involved in the biosynthesis and metabolism of creatine. *De novo* biosynthesis of creatine can occur in liver and pancreas of man and in liver of chicks. In contrast to human liver, human pancreas and chick liver have low arginase levels and cannot synthesize citrulline from ornithine. Controls modulate excessive biosynthesis of creatine during ingestion of creatine-containing diets and periods of inadequate food intake and thus spare amino acid precursors of creatine and the important one-carbon pools. Tissues containing creatine kinase take up and release creatine and its synthetic analog cyclocreatine by unknown mechanisms; percentages phosphorylated in resting muscle are given in parentheses. The creatine–creatine-P pool is continuously depleted throughout life by the formation and excretion of creatinine, about 2 g/day in man; this loss must be replaced from the diet or by biosynthesis. AT—SH, glycine amidinotransferase; MT, guanidinoacetate *N*-methyltransferase; CK, creatine kinase; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; CAP, carbamoyl phosphate; 1-C, one-carbon derivative.

B) **Creatine kinase**

i) **Isoenzymes**

More than 20 isoenzymes of creatine kinase have been sequenced, however there are only four commonly occurring subunits of creatine kinase in vertebrates. There are two cytosolic forms M-CK and B-CK (M standing for muscle and B standing for brain), and two mitochondrial Mi-CK isoforms (Mi standing for mitochondrial). *In vivo*, M-CK and B-CK combine to give the three dimeric isoenzymes; MM-, MB-, and BB-CK isoenzymes with approximate M_r of 80,000-86,000. Whereas MM-CK is specific for differentiated sarcomeric muscle, BB-CK is found in the brain and in a variety of other tissues (including smooth muscle) and MB-CK is present in high proportion in cardiac muscle. In addition during muscle differentiation *in vivo* and *in vitro*, a developmental transition from BB-CK via the transitory MB-CK hybrid to the MM-CK homodimer, is observed. The Mi-CK isoenzymes can exist as octamers as well as dimers *in vivo*.

ii) **Functions of creatine kinase**

Creatine kinase catalyses the following reaction;

Cr + ATP <=> PCr + ADP + H+

and so creatine kinase is an important component of the creatine-phosphocreatine-creatine kinase axis. This system has the following

functions:

a) It is a "temporal" energy buffer which replenishes ATP in the initial phase of exercise, so acting prior to full activation of glycolysis. Hence ATP levels are not severely depleted at the onset of exercise or during early exercise (subsequently the increased rate of oxidative phosphorylation protects ATP levels).

b) The system acts as an "energy carrier" to couple the sites of cytosolic and mitochondrial ATP synthesis to ATP utilisation (the latter in the cytoplasm at contractile proteins).

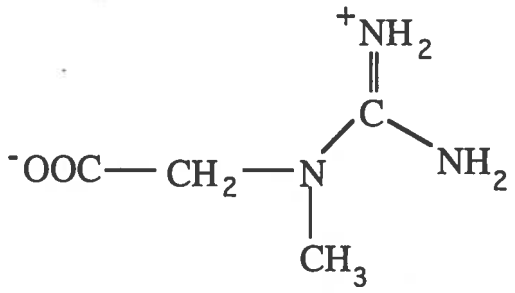
c) The creatine kinase/phosphocreatine system prevents the rise in intracellular ADP concentration which might otherwise inactivate cellular ATPases.

d) The hydrolysis of ATP that occurs with muscle action would release protons and cause acidification. The action of the PCr/creatine kinase system in allowing resynthesis of ATP prevents this effect. This proton buffering effect is important in the early phase of severe exercise.

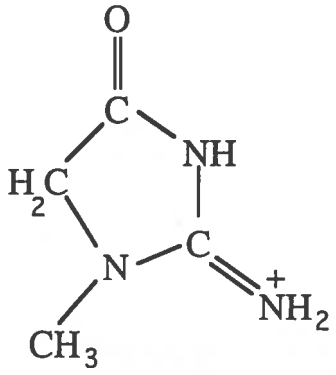
e) The PCr/Creatine kinase system also controls the cytosolic concentrations of inorganic phosphate through the hydrolysis of PCr. The increased phosphate levels are necessary to stimulate glycogenolysis.

Appendix IV

Creatine and Creatinine Structures:



Creatine



Creatinine