Physiological CO_2 exchange does not depend on membrane channels

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Diffusion in aqueous compartments, tortuosity due to macromolecules and permeation across lipid-based membranes are factors that determine CO_2 transport capacity (Fig 1A) (Hulikova & Swietach, 2014). Several studies over the past two decades have proposed that the lipid matrix of membranes is a major barrier to passive CO_2 transport, and may be circumvented by a parallel flux through gas channels (Cooper *et al.*, 2002; Boron, 2010; Endeward *et al.*, 2014). Other studies, however, challenge this hypothesis by arguing that the low intrinsic CO_2 permeability of cell membranes has been incorrectly attributed to the lipid matrix but is, instead, a property of unstirred layers (USLs) adjacent to the membrane (Verkman, 2002; Missner & Pohl, 2009). If the dominant resistance to CO_2 transport lies outside the lipid matrix, then permeation across membrane-spanning gas channels cannot meaningfully supplement CO_2 flux. This assertion can be explained mathematically by breaking-down total permeability (P_t) into its components (Fig 1B), namely permeability across the lipid matrix (P_{lm}), through gas channels (P_{ch}), and across the adjacent intra- and extracellular USLs (P_i, P_e):

$$\frac{1}{P_t} = \frac{1}{P_i} + \frac{1}{P_{lm} + P_{ch}} + \frac{1}{P_e} \quad \ldots (Eq\ 1)$$

Numerically, an increase in P_{ch} will always raise total permeability. But in order for this effect to be biologically meaningful P_{ch} must be >> P_{lm}, while P_{lm} must be << P_i and P_e. These criteria are met for the transport of small ions and water across membranes, because the lipophobicity of these molecules reduces P_{lm} profoundly. In contrast, CO_2 is a peculiar gas that is soluble in both water and lipid, and is therefore expected to have a higher P_{lm}. Although the scientific community remains polarised about the exact value of P_{lm} relative to P_i, P_e and P_{ch}, there is consensus that *(i)* artificial bilayers have high CO_2 permeability (P_{art}~10^4 μm/s) and *(ii)* P_{lm} must be at least two orders of magnitude lower than P_{art} in order for CO_2 channels to conduct a meaningful transmembrane CO_2 flux (Boron *et al.*, 2011; Endeward *et al.*, 2014). We postulate that, in most tissues, CO_2 transport will not benefit from membrane channels, although we identify exceptional circumstances where they may play a role.

Resolving the membrane CO_2 permeability constant. Considering a spherical cell of radius 10 μm, even a 1000-fold decrease in membrane permeability from P_{art}=10^4 μm/s predicts a rate constant of CO_2 exchange between intra- and extracellular compartments of merely a third of a second. Time-delays shorter than this cannot be determined reliably, therefore alternative ways are needed for reporting membrane permeation. Gros, Endeward and co-workers used the mathematical relationship between rapid trans-membrane fluxes and the slow rate of ^{18}O

exchange between CO_2/HCO_3^- and water to estimate membrane permeability. We have taken an alternative approach of measuring the diffusive spread of CO_2 in a spherical multi-cellular tissue growth ("spheroid"). As CO_2 diffuses into these spheroids, the gas passes through a sequence of intracellular space–membrane–extracellular space that is repeated multiple times in series. The large number of such repeats in a spheroid of radius 100-150 µm greatly increases time-delays associated with CO_2 spread, but retains the same relationship between membrane and aqueous compartment permeability as in a single cell (Hulikova & Swietach, 2014). Thus, if membrane CO_2 permeability were low, time-delays would become readily resolvable. However, we find that CO_2 diffuses across spheroids of human epithelial or fibroblast cells as fast as predicted for aqueous solutions containing protein at the concentration found in tissue, i.e. the spread of CO_2 is limited by macromolecular tortuosity but not membranes. The observation that the same results were obtained in fibroblasts with aquaporin-assisted water/NH_3 permeability and in colorectal epithelial cells without aquaporin activity argues against a role for CO_2 permeation across water channels. Moreover, treatment with mercurials did not affect CO_2 diffusivity although this reduced water and NH_3 permeability in aquaporin-positive cells.

Are P_i and P_e negligible compared to membrane permeability? In an attempt to minimise USLs, measurements of CO_2 permeability have been made on superfused cells or in stirred cell-suspensions. However, USLs are a natural, albeit poorly characterised, feature of cells in tissue. The size of USLs will vary greatly between cell types, but they are very likely to be larger than the width of membranes (10 nm). As the size of USLs increases, so does their share of total resistance to CO_2 flux, and this limits the scope for membrane channels to improve CO_2 venting. This can be observed by contrasting the proposed 10-fold facilitatory effect of gas channels on CO_2 transport in vigorously-stirred red cells (USL width ~0.6 µm) (Endeward & Gros, 2009) with the lack of channel-dependent facilitation in poorly-stirred red cells (USL width >10 µm) (Yang *et al.*, 2000). The USL in circulating red cells is 1-2 µm thick (Klitzman & Duling, 1979) and confers half of the resistance to CO_2 movement, but USLs in capillary-perfused tissue will be larger, not least because of the larger dimensions of cells and the finite degree of convection (stirring equivalent). When quantifying physiological CO_2 exchange between intra- and extracellular compartments, diffusion across aqueous compartments must be factored in.

Is P_{lm} several orders of magnitude lower than P_{art}? It is widely accepted that P_{lm} of biological membranes is lower than P_{art}, but the magnitude of this difference is debated. For CO_2 channels to have a physiologically-meaningful effect, P_{lm} would have to be at least 100-fold smaller than P_{art} (Boron *et al.*, 2011). Volume-exclusion by integral proteins reduces P_{lm}, but typically by no more than two-fold. Tortuosity due to macromolecules would restrict CO_2 access to/egress from the membrane, but this will also reduce diffusivity in abutting aqueous compartments, thereby having a neutral effect on the relative distribution of resistance between the USLs and the membrane. Cholesterol has been proposed to tighten membranes to CO_2 flux by orders of magnitude (Itel *et al.*, 2012), but other studies have proposed this effect to be much smaller (Missner *et al.*, 2008; Zocher *et al.*, 2012). On the other hand, molecular dynamics simulations suggest that cholesterol has little effect on the permeation of lipophilic gases, such as NO (Zocher *et al.*, 2013). Measurements of O_2 permeation at 37°C indicate that raising bilayer cholesterol content to 50% reduces membrane permeability by a factor of ~3 only (Subczynski *et al.*, 1989; Widomska *et al.*, 2007). It may be argued that poorly water-soluble O_2 and NO are not adequate models for predicting membrane permeability to CO_2, a more hydrophilic gas. However, 50% cholesterol reduces the permeability of

artificial lipid bilayers to polar molecules (water and NH_3) by less than an order of magnitude (Lande *et al.*, 1995; Mathai *et al.*, 2008). In the case of CO_2, to attain the required P_{art}/P_{lm} ratio of at least 100, an additional factor appears to be necessary. This factor may be specific to biological membranes, adding to the argument that artificial lipid bilayers are not an accurate model for understanding transport across cell membrane (Boron, 2010). However, any additional restriction to CO_2 transport across biological membranes would also be expected to reduce permeability to O_2, water and NH_3. But measurements of O_2, water or NH_3 permeability across cholesterol-containing lipid bilayers are not greatly different to those obtained with biological membranes apparently lacking channel-mediated pathways (Widomska *et al.*, 2007; Boron *et al.*, 2011; Hulikova & Swietach, 2014). On the balance of evidence, cholesterol and volume-exclusion by integral proteins will significantly tighten the lipid matrix to the flow of CO_2, but not by as much as 2-3 orders of magnitude necessary to meet the gas channel criteria discussed above. The lipid matrix of most biological membranes is therefore unlikely to re-direct a majority of CO_2 flux through proteins.

Can P_{ch} be adequately large? The capacity of gas channels to augment CO_2 transport is limited by the magnitude of P_{ch}. In red cells, P_{ch} is estimated to be 2×10^3 μm/s (Endeward & Gros, 2009). However, this value is most likely at the upper end of the physiological range as red cells have an unusually high abundance of integral proteins (50% of membrane surface). Considering a more typical cell within a tissue, having a total USL width of at least 2.5 μm, integral proteins covering 25% of the membrane surface (Boron, 2010) and P_{lm} equal to that in gas channel-null red cells (240 μm/s, i.e. 40-times lower than P_{art}; NB: scaled to account for a lower protein fraction in typical cells), gas channels would accelerate CO_2 flux by no more than 2-fold. If, however, P_{lm} were only 10-fold lower than P_{art} (see preceding paragraph), gas channels would accelerate CO_2 transport by less than 20%. Even this calculation may be an over-estimate for most cells, if red cells are believed to have an unusually high proportion of putative gas channels among their integral proteins (Cooper *et al.*, 2002).

Conclusion. We conclude that CO_2 channels are unlikely to have a universally substantive effect on CO_2 transport, in contrast to the obligatory role of channels in transmitting ions and water. Exceptional circumstances, such as very short intracellular diffusion distances (high surface area/volume ratio), collapsed USLs (e.g. red cells squeezing through capillaries), and membranes of very low lipid content may optimise conditions for a facilitatory role for CO_2 channels, but this would not represent a general biological phenomenon. Further measurements on a comprehensive range of cell types, using methods of superior resolving power and not reliant on complex multi-variable analytical algorithms are needed for obtaining direct and calibrated measurements of the permeability components listed in Eq 1. Without this information, it is not possible to assess unequivocally the universal role of gas channels in CO_2 transport. In the meantime, we recommend that caution be exercised when attributing a role for gas channels in cell types that have not been subject to rigorous permeability analyses. Our conclusion does not conflict with the evidence for restricted CO_2 transport across apical membranes of some epithelia, as this may be explained by larger unstirred layers and higher diffusive tortuosity on either side of the membrane, without implicating unusually low membrane permeability.

References

Boron WF. (2010). Sharpey-Schafer lecture: gas channels. *Exp Physiol* **95,** 1107-1130.

Boron WF, Endeward V, Gros G, Musa-Aziz R & Pohl P. (2011). Intrinsic CO_2 permeability of cell membranes and potential biological relevance of CO2 channels. *Chemphyschem* **12,** 1017-1019.

Cooper GJ, Zhou Y, Bouyer P, Grichtchenko, II & Boron WF. (2002). Transport of volatile solutes through AQP1. *J Physiol* **542,** 17-29.

Endeward V, Al-Samir S, Itel F & Gros G. (2014). How does carbon dioxide permeate cell membranes? A discussion of concepts, results and methods. *Front Physiol* **4,** 382.

Endeward V & Gros G. (2009). Extra- and intracellular unstirred layer effects in measurements of CO_2 diffusion across membranes--a novel approach applied to the mass spectrometric 18O technique for red blood cells. *J Physiol* **587,** 1153-1167.

Hulikova A & Swietach P. (2014). Rapid CO_2 permeation across biological membranes: implications for CO_2 venting from tissue. *FASEB J* **28,** 2762-2774.

Itel F, Al-Samir S, Oberg F, Chami M, Kumar M, Supuran CT, Deen PM, Meier W, Hedfalk K, Gros G & Endeward V. (2012). CO_2 permeability of cell membranes is regulated by membrane cholesterol and protein gas channels. *FASEB J*.

Klitzman B & Duling BR. (1979). Microvascular hematocrit and red cell flow in resting and contracting striated muscle. *Am J Physiol* **237,** H481-490.

Lande MB, Donovan JM & Zeidel ML. (1995). The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *J Gen Physiol* **106,** 67-84.

Mathai JC, Tristram-Nagle S, Nagle JF & Zeidel ML. (2008). Structural determinants of water permeability through the lipid membrane. *J Gen Physiol* **131,** 69-76.

Missner A, Kugler P, Saparov SM, Sommer K, Mathai JC, Zeidel ML & Pohl P. (2008). Carbon dioxide transport through membranes. *J Biol Chem* **283,** 25340-25347.

Missner A & Pohl P. (2009). 110 years of the Meyer-Overton rule: predicting membrane permeability of gases and other small compounds. *Chemphyschem* **10,** 1405-1414.

Subczynski WK, Hyde JS & Kusumi A. (1989). Oxygen permeability of phosphatidylcholine--cholesterol membranes. *Proc Natl Acad Sci U S A* **86,** 4474-4478.

Verkman AS. (2002). Does aquaporin-1 pass gas? An opposing view. *J Physiol* **542,** 31.

Widomska J, Raguz M & Subczynski WK. (2007). Oxygen permeability of the lipid bilayer membrane made of calf lens lipids. *Biochim Biophys Acta* **1768,** 2635-2645.

Yang B, Fukuda N, van Hoek A, Matthay MA, Ma T & Verkman AS. (2000). Carbon dioxide permeability of aquaporin-1 measured in erythrocytes and lung of aquaporin-1 null mice and in reconstituted proteoliposomes. *J Biol Chem* **275,** 2686-2692.

Zocher F, van der Spoel D, Pohl P & Hub JS. (2013). Local partition coefficients govern solute permeability of cholesterol-containing membranes. *Biophys J* **105,** 2760-2770.

Zocher F, Zeidel ML, Missner A, Sun TT, Zhou G, Liao Y, von Bodungen M, Hill WG, Meyers S, Pohl P & Mathai JC. (2012). Uroplakins do not restrict CO2 transport through urothelium. *J Biol Chem* **287,** 11011-11017.

Figure legend. *Electrical circuit representation of CO_2 transport.* (A). Factors affecting CO_2 transport. [1] intracellular and [2] extracellular macromolecules, [3] gas channels, [4] cholesterol, [5] phospholipid bilayer. USL: unstirred layer. (B). Electrical representation of CO_2 transport, featuring resistance in the intra- and extracellular unstirred layers (R_i, R_e), through the gas channel pathway (R_{ch}) and the lipid matrix (R_{lm}).