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Intrinsic CO_2 Permeability of Cell Membranes and Potential Biological Relevance of CO_2 Channels**

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The past dozen years has seen a series of papers—published from the laboratories of Boron[1–6] and of Endeward and Gros[7–9]—that come to the conclusion that CO_2 passes through certain aquaporins (AQPs) and Rhesus (Rh) proteins. The past three years has seen another series of papers—published from the laboratory of Pohl[10–14]—that come to the conclusion that protein channels could not make a meaningful contribution to overall CO_2 membrane permeability because of a combination of: 1) a high CO_2 permeability of membrane lipids and 2) large (when compared to the membrane thickness) unstirred layers (ULs), which would render the CO_2 resistance of the ULs much higher than that of a biological membrane.

In response to the most recent paper from the Pohl laboratory, which appeared herein,[11] Boron, Endeward, Musa-Aziz and Gros drafted a lengthy letter to the Editor of *ChemPhysChem*, detailing a litany of issues to which they took exception in the Pohl papers. Pohl's response included material that spurred this group to lengthen their letter even further. Toward the end of this exasperating process, Pohl invited Boron, Endeward and Gros to a meeting that he was organizing in Strobl, Austria, sponsored by the German Biophysical Society. At this meeting, we each heard and discussed the other's formal presentations. During a long walk in the foothills of the Alps, we focused our discussions on the major issues of difference. Boron suggested that it would be far more productive to replace the two lengthy letters to the Editor—which in the end would come to no clear conclusion—with a single, shorter, joint letter that would summarize the major areas in which we agreed or disagreed. That evening, drinking Austrian beer under the stars, we came to the following conclusions.

1) We all agree that results from previous stop-flow measurements of CO_2 transport in liposomes[15,16] are probably not reliable because limitations in the dead time of the instrument and the efficacy of mixing would make it impossible to achieve the necessary time resolution (<1 ms). Nevertheless, it remains unexplained how, in the paper by Prasad et al.,[15] reconstitution of AQP1 protein increased apparent CO_2 permeability in these experiments. It is also not clear how $HgCl_2$ blocked this effect, and β-mercaptoethanol reversed the blockade.[15]
2) We all agree that the non-gas-channel portion of the red blood cell (RBC) membrane would have to be ~200-fold tighter to CO_2 than are Pohl's artificial lipid bilayers in order to explain the observation that the CO_2 permeability (P_{CO2}) of AQP1-null red blood cells (RBCs) treated with 4-4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) (to block the CO_2 permeability of the Rh complex) is ~0.015 $cm\,s^{-1}$,[8] whereas the P_{CO2} in Pohl's experiments[14] is ~3 $cm\,s^{-1}$ (i.e. ~200-fold higher).
3) Pohl is concerned that the osmotic water permeability (P_f) of both AQP1-null RBCs[17] and of planar lipid bilayers made from either synthetic or natural lipid mixtures[18–20] is $\sim 2\times 10^{-3}$ $cm\,s^{-1}$. Thus, compared to artificial lipid bilayers, the non-gas-channel portion of the RBC membrane would have to be specifically tight to CO_2. Boron, Endeward and Gros point out that AQP1-null RBC still possess water channels (AQP3 and UT-B) and thus RBCs may have a considerably lower "intrinsic" P_f. Also in control oocytes, P_f ($\sim 2.8\times 10^{-3}$ $cm\,s^{-1}$, not corrected for surface membrane amplification, ref. [21]) is similar to that in lipid bilayers, whereas P_{CO2} (~0.05 $cm\,s^{-1}$, ref. [8]) is only ~1/100 of Pohl's lipid bilayer value of 3 $cm\,s^{-1}$. If surface membrane amplification is taken into account, oocyte P_f and P_{CO2} each fall by about an order of magnitude. Pohl points out that in this case a comparison should be made between oocytes and planar bilayers tightened by cholesterol and sphingomyelin. These bilayers have a P_f of only 3×10^{-4} $cm\,s^{-1}$.[22] Thus, oocyte P_f would be equal to the lipid-bilayer value, and oocyte P_{CO2} would be 1/1000 of the lipid bilayer value. Endeward and Gros state that this comparison of P_f values between oocytes and planar lipid bilayers is flawed by the presence of an intrinsic aquaporin in oocyte membranes,[23] which conducts water but may not conduct CO_2. Nevertheless, the

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[**] *Comment to A. Missner and P. Pohl, ChemPhysChem* **2009**, *10*, 1405–1414. *The authors are listed in alphabetical order. The three groups of Boron, Musa-Aziz, of Pohl and of Endeward, Gros have contributed equally to this letter.*

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oocyte membrane appears to be specifically tight to CO_2. Boron and Gros state that this specificity could be a consequence of the protein content of biological membranes.

4) Pohl remains sceptical of Endeward's and Gros's ^{18}O approach, which extracts conclusions about rapid processes using a model that deals with data from very slow processes. The ^{18}O approach exploits the slow equilibration (over a period of several tens of minutes) of ^{18}O-labeled CO_2/HCO_3^- with H_2O. Although in vivo CO_2 equilibration with RBCs is complete within ~300 ms, the ^{18}O exchange, whose kinetics is decisively influenced by the RBC CO_2 permeability, requires minutes to hours to reach completion and can therefore be conveniently measured by mass spectrometry. Endeward and Gros explain that mathematical description of the process in conjunction with a fitting procedures yields well defined values for P_{CO2}.[8,24]
5) Gros and Endeward point out—and Boron agrees—that they have solid experimental evidence from variations of the viscosity of the RBC suspension[25] that the thickness of the unstirred layer in their ^{18}O experiments with RBCs is ~0.5 μm and thus affects the experimental RBC P_{CO2} only in a minor way. Endeward and Gros believe therefore that their "basal" P_{CO2} of 0.015 $cm\,s^{-1}$ indicates that the RBC membrane in the absence of CO_2 channels indeed is rather tight for CO_2 and that their "normal" RBC P_{CO2} of 0.15 $cm\,s^{-1}$ indicates that AQP-1 and another DIDS-inhibitable protein (Rhesus protein) act as CO_2 channels. Pohl argues that CO_2 diffuses over a distance of 0.5 μm within 50 μs, whereas the time constant for the reaction $H_2CO_3 \rightleftharpoons H_2O + CO_2$ is equal to 10 s. Thus, the ^{18}O uptake experiments with RBCs are entirely reaction-limited. Accordingly, a two exponential fit to the time traces of ^{18}O uptake (recorded with a time resolution of 1 s) returns time constants in the order of tens of seconds or larger.[8] Any information that is extracted about processes that are five orders of magnitude faster (diffusion through the UL) or more than six orders of magnitude faster (CO_2 diffusion through the membrane) may be subject to large uncertainties. Moreover, in combination with an intracellular UL of comparable size, the extracellular UL of 0.5 μm would represent a barrier so large that any contribution of a gaseous channel would be physiologically irrelevant, provided that the plasma membrane CO_2 permeability is in the range of 3 $cm\,s^{-1}$ (compare Figure 6 in ref. [14]). Endeward and Gros point out that in their measurements of CO_2 permeability in red cells two very fast processes, the permeation of labelled CO_2 across the membrane and the intracellular CO_2 hydration-dehydration reaction with a half-time of 0.35 ms (37 °C) determine the decay of ^{18}O in CO_2 as observed in the extracellular fluid compartment during the mass spectrometric experiment. The important absence of carbonic anhydrase (CA) activity from the extracellular compartment (and accordingly slow hydration–dehydration reaction with a half-time of 7 s at 37 °C) serves to maintain a gradient of ^{18}O-labelled CO_2 between the extra- and intracellular compartments and constitutes a prerequisite for observing the extra- to intracellular exchanges of labelled CO_2 and HCO_3^-. In order to be able to determine very high P_{CO2} values of the membrane, it is in fact also important that the intracellular hydration–dehydration reaction of CO_2 is very fast, because otherwise the entire process would indeed be reaction-limited. Thus, a red cell with its very high intracellular CA activity and a low and inhibitable extracellular CA activity is an ideal cell to reliably determine high membrane CO_2 permeabilities with the mass spectrometric ^{18}O method.[24–27] Regarding the influences of unstirred layers in their measurements of normal red cells, Endeward and Gros[25] have recently concluded that the total measured RBC diffusion resistance towards CO_2 is constituted of 1) the cell membrane, being responsible for 60% of the total resistance, 2) the intracellular unstirred layer, responsible for 15%, and 3) the extracellular unstirred layer, responsible for 25%.
6) Regarding the oocyte data from Boron's laboratory, we all agree that, in order for AQPs and Rh proteins to increase CO_2 permeability—as measured by intracellular or surface-pH measurements—the non-gas-channel portion of the oocyte membrane has to be perhaps 1000-fold tighter to CO_2 than are Pohl's artificial bilayers, which the data of Endeward, Gros and Boron[8] suggest to be indeed the case.
7) Regarding points (2), (3), and (6), Pohl and Boron–Endeward–Gros disagree on how to define the "tightness" of the non-gas-channel portion of the membrane to CO_2. Pohl's definition of tightness includes only CO_2 partition (s_{CO2}) into the lipid phase and CO_2 diffusion constant (D_{CO2}) through the lipid (i.e. classical solubility–diffusion theory). The Boron–Endeward–Gros definition also includes the effects of proteins on the efficiency of CO_2 access from aqueous phase to the lipid and the efficiency of CO_2 egress from the lipid to the aqueous phase (access–solubility–diffusion–egress theory, ref. [28]). According to this latter view, proteins loosely attached to the membrane or ectodomains of integral membrane proteins interacting with the lipid portions of the membrane[29] would decrease the efficiency with which CO_2 moves from the aqueous phase to the lipid phase (access) or vice versa (egress). Pohl argues that if there is such an effect, the proteins are likely to affect access and egress of all small substances in the same way. Otherwise it would be impossible to explain why the permeability coefficient routinely measured for lipid-soaked filters (parallel artificial membrane permeation assay[30]) correlates so well with the permeability coefficient of plasma membranes, as shown for example in ref. [31]. Specifically, any additional protein barrier which slows down CO_2 entry into the membrane should hamper H_2O partition to the same degree. Consequently, any model membrane which properly reflects plasma membrane H_2O permeability may be expected to return proper values for CO_2 permeability as well. Endeward and Gros reiterate that the value of the "intrinsic" P_f is not clear, neither for red cells nor for oocytes, due to the presence of other aquaporins when AQP1 is not expressed.
8) We all agree that for many cells (e.g. RBCs), integral membrane proteins make up more than 50% of the surface area and that—because CO_2 generally cannot penetrate these

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proteins—the presence per se of integral membrane proteins could reduce P_{CO2} for some membranes by more than half. However, we also all agree that, by itself, a 50% or even a 70% reduction in effective surface area would not be enough to achieve a 200- to 1000-fold reduction in tightness of the non-gas-channel portion of the membrane. However, Boron, Endeward and Gros suggest that integral membrane proteins could not only serve as anchors for loosely associated proteins (see access–egress) but also could order the lipids in their immediate environment (thereby markedly reducing the effective s_{CO2} and D_{CO2} for these lipids) and/or cover considerable portions of the lipid phases of the membrane (thereby further reducing access and egress). Pohl agrees but raises doubts about the CO_2 specificity of that process. Since lipid ordering is accompanied by 1) a decrease of the surface area per lipid molecule and 2) an increase in membrane viscosity, partition and diffusion of all small molecules are similarly affected. Consequently, any model membrane which properly reflects plasma membrane H_2O permeability may also be expected to return proper values for CO_2 permeability.

In general, we all agree that, in order for gas channels to be able to make a significant contribution to the macroscopic permeability of CO_2 (e.g., the movement of CO_2 through an extracellular aqueous unstirred layer, through a biological membrane, and through some distance of intracellular fluid), the unstirred layers must offer a relatively low resistance to CO_2 diffusion, the non-gas-channel portion of the membrane must offer a relatively high resistance to CO_2 diffusion, and the gas channels must offer a relatively low-resistance pathway around the non-gas-channel portion of the membrane.

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