Comment to the Editor

Response to Zeuthen and Zeuthen's Comment to the Editor: Enough Local Hypertonicity Is Enough

ABSTRACT In a Comment to the Editor, Zeuthen and Zeuthen criticize our treatment of the water cotransport hypothesis. In this response, we argue that we calculated water cotransport as if there were no significant local osmotic gradient generated in the first minute of Na/glucose cotransport. It is surprising to receive this type of criticism from Zeuthen and Zeuthen, as the same treatment was used in at least six studies from his laboratory where it is systematically assumed that "intracellular unstirred layers effects" are negligible. Zeuthen and Zeuthen also state that "the cotransport hypothesis predicts the measurements better than the osmotic hypothesis". We present a quantitative comparison that challenges this contention. We would like to conclude by stating that our article was not about comparing different numerical models but about an experimental measurement of the local osmotic gradient generated after 20, 40, or 60 s of cotransport. Osmotic gradients were indeed detected, and were of appropriate amplitude to explain virtually all water transport observed.

INTRODUCTION

Our article (1) aimed at measuring the amplitudes of the transmembrane osmotic gradients generated by the activation of Na/glucose cotransport transport in *Xenopus laevis* oocytes for transport periods of 20, 40, and 60 s. By measuring oocyte swelling rates during the postcotransport period, we were able to experimentally determine that the osmotic gradients reached 4.6, 5.0, and 5.3 mOsm after 20, 40, and 60 s of cotransport, respectively. These osmotic gradients were developed during the cotransport periods and are of appropriate magnitudes to account for the water movements measured at the end of each period. We concluded that the water transport associated with the stimulation of Na/glucose cotransport could be quantitatively explained by the presence of this osmotic gradient and, consequently, that there was no need to propose the presence of a putative secondary active water transport mechanism. We stand by this conclusion.

ZEUTHEN AND ZEUTHEN'S COMMENT

In contrast to our article, which is firmly based on experimental observations, the Zeuthen and Zeuthen Comment to the Editor is essentially about modeling. The first point raised by Zeuthen and Zeuthen is regarding our treatment of the water cotransport hypothesis. They criticize the fact that in our Fig. 4 (1), we compared the time course of the oocyte volume during the cotransport and the postcotransport periods to the prediction of the water cotransport hypothesis as if there were no concomitant, osmotically driven water transport. Their second point is a comparison of two numerical models describing water transport across the oocyte membrane and diffusion of an ''idealized'' transported osmolyte across the oocyte cytosol. The ''osmotic model'' uses the assumption that all water transport across the oocyte membrane is passive and driven by a local osmotic gradient. The ''cotransport model'' includes a passive water transport and a secondary active water transport with a stoichiometry of 250 water molecules per glucose transported. They argue that ''. . .at the termination of sugar transport combined with an osmotic challenge, the cotransport hypothesis and the osmotic hypothesis predict roughly the same rate of cell swelling''. They also state that ''the cotransport hypothesis gives the best fit to the recorded volume changes''. Let's consider these two points in detail.

POINT NO. 1: TREATMENT OF THE WATER COTRANSPORT HYPOTHESIS

Since 1996, the Zeuthen laboratory's publications have repeatedly presented the water cotransport hypothesis as if it were the dominant transport mode during the first minute of Na/glucose cotransport. For example, in 1996, they calculated that the passive water flux due to osmolyte accumulation in the oocyte unstirred layer would account for only 3% of the observed value (2). In 1997 and in 2002, they reasoned that the transmembrane osmolarity gradient can be neglected since ''Unstirred layer effects are ruled out on the basis of experiments on native oocytes incubated with the ionophores gramicidin D or nystatin'' (3–5). Even in 2006, Zeuthen and Zeuthen published the claim that ''The combination of high resolution measurements and precise modeling showed that water transport across the membrane can be explained by cotransport of water in the membrane proteins and that intracellular unstirred layers' effects are minute'' (6). In fact, according to the same authors, it would take between 5 and 10 min of cotransport before a steady state is achieved where 1/3 of water transport is secondary active and 2/3 are passive (7).

In agreement with the model described by the Zeuthen laboratory in these publications, we chose to represent the

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Address reprint requests to Jean-Yves Lapointe, Groupe d'étude des protéines membranaires (GÉPROM), Université de Montréal, C.P. 6128, succ. centre-ville Montréal, Québec H3C 3J7, Canada. E-mail: jean-yves.lapointe@umontreal.ca.

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component corresponding to water cotransport as if the contribution of osmotic water flow was indeed ''minute''. As has been shown in the articles from Zeuthen's laboratory already mentioned, the observed cell swelling was compared with the water flow corresponding to a stoichiometry of 250 water molecules per glucose transported. It is to our surprise that Zeuthen and Zeuthen, in this Comment to the Editor, stated that our numerical model did not ''incorporate the correct equations to describe cotransport of water''. The numerical model they proposed differs from ours simply by taking into account the early, transport-dependent osmotic gradient. We fully agree that considering the generation of local osmotic gradient is necessary to have an adequate description of the oocyte cell swelling. We would go even further and say that local osmotic gradients can actually explain all water movement.

POINT NO. 2: '' ... THE COTRANSPORT HYPOTHESIS GIVES THE BEST FIT TO THE RECORDED VOLUME CHANGES''

This statement is somewhat misleading because the model used by Zeuthen and Zeuthen with an intracellular diffusion coefficient of 0.15×10^{-5} cm^2/s, does not suitably reproduce the observed cell volume time course. Experimentally, the cell swelling observed after 60 s of cotransport is consistent with an intracellular hypertonicity of 5.3 mOsm (1). As stated both in our article and in the Comment to the Editor, the two models used predict similar local osmotic gradients of 1.5 and 2.0 mOsm in the presence or absence of water cotransport, respectively. This discrepancy between data and model should be no surprise since the limitations of a simple diffusion model involving spherical symmetry and a single type of transported osmolyte were discussed in our article. For example (see Fig. 4 of Charron et al. (1)), an intracellular diffusion coefficient five times smaller than that used by Zeuthen and Zeuthen has to be used to mimic the cell volume of an oocyte submitted to a 20-s cotransport period. By the way, this diffusion coefficient for an osmolyte representing both Na and glucose in the diffusion model is 50 and 15 times smaller than the diffusion coefficients for Na and glucose in free aqueous solution, respectively. It seems inaccurate to mention in the discussion of the Comment to the Editor that the diffusion model requires intracellular diffusion coefficients that are ''three orders of magnitude'' smaller than in free aqueous solution.

In Fig. 1, we present an illustration of the predictions provided by the water cotransport hypothesis and the osmotic hypothesis using an intracellular diffusion coefficient of 0.15×10^{-5} cm^2/s and a water permeability of 6.5×10^{-4} cm/s, as suggested in the Comment to the Editor. The diffusion model used has been described in Duquette et al. (8) and, with the inclusion of water cotransport with a given stoichiometry, it is completely equivalent to the model used in Zeuthen and Zeuthen's comment. It should be noted that

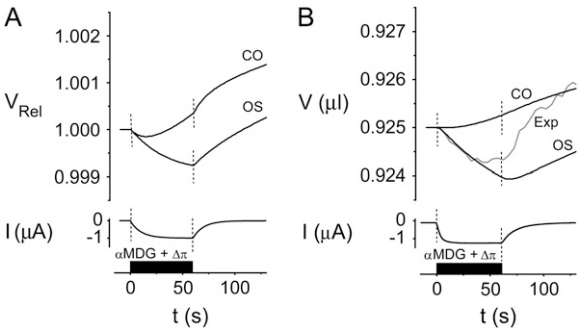

FIGURE 1 Comparison between experimental results and the predictions of the cotransport (CO) or the osmotic (OS) hypotheses. In panel *A*, we have simulated the time course of the relative oocyte volume (V_{Rel}) as presented in the Comment to the Editor assuming a water permeability of 6.5×10^{-4} cm/s, an intracellular diffusion coefficient of 0.15×10^{-5} cm^2/s, an accessible intracellular volume of 50%, and a net transport of three osmolytes per glucose. As done by Zeuthen and Zeuthen, the osmotic shock caused by the addition of 5 mM α-methyl-D-glucose was assumed to be instantaneous, and a water cotransport stoichiometry of 250 water molecules per glucose was used for the trace labeled ''CO''. In panel *B*, the model predictions are superimposed to the experimental result originally presented in Fig. 1 of Charron et al. (1). Parameters were identical to the parameters used in panel *A* with the exception of water permeability. Water permeability was adjusted to 7.8×10^{-4} cm/s to represent the experimentally measured value and for the net number of osmolytes transported per glucose molecule, which was adjusted to obtain the observed steady-state oocyte volume (0.928 μl at $t \geq 1000$ s). Note that the rapid volume changes observed upon adding or removing α-methyl-D-glucose have disappeared because the changes in external osmolarity were assumed to follow the time course of the cotransport current.

in their simulation, Zeuthen and Zeuthen have assumed that addition of glucose stimulated the cotransporter with a time constant of 10 s but, for reasons that we cannot comprehend, they assumed that the osmotic effect of glucose (+5 mOsm) was instantaneous. The smooth lines in Fig. 1 *A* were obtained by applying the same assumption of an instantaneous effect concerning the osmotic shock. This curve is quite similar to that seen in Fig. 1 *B* of the Comment to the Editor.

Now let's compare the prediction of the two models with a real oocyte volume measurement. We use the data shown in the first figure of our article (1), representing an oocyte successively submitted to hypertonic shocks of 5 mM mannitol and of 5 mM α-methyl-glucose (αMG) for 60-s periods. A water permeability of 7.8×10^{-4} cm/s was necessary to reproduce the mannitol hypertonic shock, and this value was subsequently used to predict the volume change associated with the 60-s cotransport period. If it is assumed that the osmotic shock is applied with a time course corresponding to the cotransport current, the smooth curve labeled ''CO'' in Fig. 1 *B* is obtained. This is far from a good fit to the experimental data, as the initial cell shrinkage is not reproduced and the fast swelling rate that follows the return of the isosmotic, glucose-free solution is considerably underestimated by the model. If the water cotransport stoichiometry is

reduced from 250 to 0 (see curve labeled OS in Fig. 1 *B*), then the initial cell shrinkage upon adding hypertonic glucose is clearly observed but the swelling rate of the postcotransport period is not accounted for any better than by the cotransport model. Consequently, the contention that ''. . .the cotransport hypothesis gives the best fit to the recorded volume changes'' is, at best, questionable.

In their comment, Zeuthen and Zeuthen argued that ''If the L_p is increased by coexpression of AQP1, the difference between the predictions of the cotransport hypothesis and the osmotic hypothesis became smaller, and not larger as argued in (1)''. Again, we did not aim to compare two numerical models but sought to measure the size of transport-dependent osmotic gradients once the cotransport period is over. We agree that the overall benefit of coexpressing AQP1 is debatable. Quantitatively, an oocyte expressing AQP1 is seven times more permeable to water; but the osmotic gradient is about half of what it would have been in the absence of AQP1 (1), and it dissipates more rapidly once the cotransport is terminated. The greatest advantage to using AQP1 is that, under these conditions, 90% of water movement is mediated by AQP1, and one can use phlorizin to block the cotransporter without having to worry about any significant change in the passive water permeability of the oocyte.

As discussed in our article, the model for intracellular diffusion in an oocyte may be too simple to correctly describe any accumulation of osmolytes in the vicinity of the oocyte membrane. For one thing, the diffusion of Na and glucose should be treated individually and not as one model osmolyte. It is also possible that the cotransport activity is not distributed equally all around the oocyte, i.e., between the animal and vegetal poles of the oocyte. The model also has to respect electroneutrality. As Na moves across the oocyte membrane, an equivalent amount of charge moves at the current injection electrode. Intracellular ion displacement will take place in the cytosol to compensate for the charge movement occurring at the plasma membrane and at the current electrode. An improved model should take into account salt accumulation or depletion at these two sites.

CONCLUSION

The presence of a local osmotic gradient was experimentally detected after cotransport periods as brief as 20 s. The amplitude of this osmotic gradient is appropriate to explain virtually all observable water transport, and consequently there is no need for a significant water cotransport mechanism. The model proposed by Zeuthen and Zeuthen, which includes intracellular diffusion of an idealized osmolyte and water cotransport across the membrane, is unable to account for the cell volume observed. Our conclusion is that a local osmotic gradient exists but that an adequate model that explains how an osmotic gradient of 5 mOsm can be generated in 60 s has yet to be proposed.

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Jean-Yves Lapointe

Groupe d'étude des protéines membranaires (GÉPROM) and Département de Physique Université de Montréal Montreal, Canada