J Membrane Biol (2013) 246:665–667
DOI 10.1007/s00232-013-9585-0

LETTER TO THE EDITOR

Response to "What Do Aquaporin Knockout Studies Tell Us about Fluid Transport in Epithelia?" Maclaren OJ, Sneyd J, Crampin EJ (2013) J Membr Biol 246:297–305

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Received: 31 July 2013 / Accepted: 4 August 2013 / Published online: 22 August 2013

Dear Sir,

Epithelial isotonic fluid transport was originally assumed to be based upon osmotic equilibration following active salt transport, and with the discovery of aquaporins (AQPs) present in epithelial membranes and dominating the osmotic permeability, it became apparent that genetic knockout (KO) of these would be a specific test of the osmotic theory. Such experiments were conducted for several AQPs in water transporting epithelia and have been reviewed in this journal (Hill 2008; Hill et al. 2004).

In their recent article on KO studies in isotonic transporting epithelia (Maclaren et al. 2013), the authors criticize the two previous articles on the grounds that the conclusions presented there based on mice AQP KOs were premature and mistaken. They argue that our conclusion that the KOs, far from underpinning the theory of epithelial fluid production by cell osmosis, have in fact undermined it is unwarranted: if an appropriate set of equations is used—which they claim to present—then the results can be seen to accord well with the osmotic theory. We show here that their analysis is incorrect and that our previous conclusions are fully justified.

Our main points are threefold: (1) that the authors have not used the osmotic model correctly in predicting what would happen when the osmotic permeability (P) is reduced by KO; (2) that the large reduction in overall salt flow in KO studies remains unexplained, and the authors' claim to predict this result from an osmotic model is untrue; and (3) that their claim that a basis for this effect can be found in a recent article of theirs in this journal (Maclaren et al. 2012) is unsupported.

The authors repeatedly comment on our failure to realize the "nonlinearity" of the problem, meaning that we assume a reduction in osmotic permeability should be reflected in a proportional reduction in fluid flow; we do not, but linearity had been uncritically assumed and we were right to stress this; we assume all isotonically transporting epithelia to be secretory, and nonlinearity is implicit in our articles and in the arguments and equations we present here.

We are in agreement that the initial treatment is correct; i.e., the transepithelial water flow (J_v) is given by

$$J_v = P(C_3 - C_1) \tag{1}$$

where we have used their symbols, C_1 and C_3 being the source bath and exudate osmotic concentrations and P, the overall (lumped) cell osmotic permeability. In the setup known as a "unilateral system" (which can often be realized in experiments) the exudate is created by the two flows of salt (J_s) and water (J_v), i.e., $C_3 = J_s/J_v$; but from this point the development of the osmotic model is unaccountably abandoned. If we substitute this expression for C_3 into Eq. 1 we obtain the quadratic in J_v

$$J_v^2 + PC_1J_v - PJ_s = 0 \tag{2}$$

the solution of which, using the positive root only, is

$$J_v = \frac{P}{2}\left(\sqrt{C_1^2 + 4J_s/P} - C_1\right) \tag{3}$$

and this can easily be solved for J_v and C_3 ($=J_s/J_v$) when P is altered by KO, given the values of P, C_1, and J_s (the rate of salt pumping based ultimately on the Na-pump rate).

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(1) The authors make no reference whatsoever to the epithelial systems studied in which AQP KOs had no effect (Hill et al. 2004); these cannot simply be dismissed as examples of extreme nonlinearity. They use as a central example the paper on KO of AQP5 in mouse salivary glands (Ma et al. 1999), which we discussed; and we concentrate on this too. The authors decided that the wild-type glands have a primary secretion that is 5–10 % hypertonic; this is not the value given by Ma et al. (1999), who measured a wild-type secretion tonicity not significantly different from plasma; in fact, the mean is *hypotonic*. As this is a crucial point, we draw attention to the fact that there is ductal reabsorption of salt in most salivary glands, which dilutes the primary fluid transported from the acini. It is commonly observed that when the fluid transport rate is high, the saliva tonicity approaches the isotonic value (Young and Schogel 1966; Young and Van Lennep 1979), which indicates that the primary secretion is virtually isotonic; but significant hypertonicity is not observed. We use the upper quasi-isotonic value of $1.01C_1$ as found by Ma et al. (1999) for wild-type mice (mean + SE).

The exact P of the glandular acini cannot be determined with any accuracy because of its complex internal geometry, and the only assumption we can make in choosing P is that the ratio of P to J_s is constant per unit membrane area. With $C_1 = 300$ mOs/l and using the experimental value of J_s equal to 3.2×10^{-8} osm/(cm^2 s), a P value of 35 cm^4/(osm s) is required to generate this 1 % hypertonicity in the wild type according to Eq. 3. In the AQP5 KOs the fall of P by 90 % (a reasonable upper limit for the fall in most animal membrane systems to the basal lipid value) can be seen in the accompanying figure, which shows solutions from Eq. 3 compared to the findings of Ma et al. (1999).

In KO 1 the relative saliva osmolarity (C_3) rises by 9.3 % and the fluid flow (J_v) falls by only 7.5 %. These are very small changes indeed and do not accord with the experimental KO results, which show changes that are about five to eight times greater, respectively. It is a consequence of the fact that, far from assuming proportionality, the approach to osmotic equilibration in a unilateral system is asymptotic in P. The authors' presentation of results that fit the findings of Ma et al. (1999) is solely a consequence of beginning with the unrealistic and experimentally unjustified assumption that the quasi-isotonic primary secretion of salivary glands is up to 10 % hypertonic.

(2) Their section "Model Features" presents the fall in salt transport observed by Ma et al. (1999) as a natural prediction of the osmotic model, but again this is not true and is only based on their Eq. 9.

$$\frac{J_s^k}{J_s^w} = \frac{r_v J_v^w \left(1 + \frac{r_v}{r_p}\frac{\Delta C^w}{C_1}\right) C_1}{J_v^w \left(1 + \frac{\Delta C^w}{C_1}\right) C_1} = \frac{r_v \left(1 + \frac{r_v}{r_p}\frac{\Delta C^w}{C_1}\right) C_1}{\left(1 + \frac{\Delta C^w}{C_1}\right) C_1} \tag{4}$$

where w and k represent wild type and KO and r_v and r_p are the fractions of J_v and P remaining in the KO system, respectively. If this unnecessarily complicated equation is simplified using these fractions and Eq. 1, it reduces to

$$\frac{J_s^k}{J_s^w} = \frac{J_v^k C_3^k}{J_v^w C_3^w} \tag{5}$$

which is used in our article to point out that the salt transport has fallen to about half (50–60 %) in the cases we consider (Hill et al. 2004 [Fig. 2]). This equation is an expression of the conservation of salt (the transepithelial flux of salt being equal to the flux of solution times its salt concentration) and will apply to any fluid transfer model whatsoever. It should be noted that J_s is now not that of Eq. 3, which represents salt pumping at a membrane which drives volume flow in the osmotic model but has become merely an operational definition, the value deduced from observed volume flow and concentration. We read, "Note that our analysis does not specify why or by what mechanism there is a reduction in salt transport but simply that self-consistency of the osmotic mechanism requires that this should be observed." But it is not the self-consistency of any specific mechanism, only the conservation of salt flux. What then is the point of this exercise? To use Eq. 5, which can only return a fall in J_s as observed in the KO experiments, or to calculate the expected saliva osmolarity and rate from the original wild-type osmotic model using Eq. 3? This is what we understand by testing a model against experiment.

If we insert the experimental value after KO for J_s of 1.89×10^{-8} osm/(cm^2 s) (accepting that it might be a "pleiotropic" effect of the AQP5 KO) directly into Eq. 3 and reduce P to 10 %, it now represents the osmotic model of a KO mouse. The results in Fig. 1 KO 2, show that the saliva osmolarity (C_3) is now only increased by 6 % (as opposed to the 43 % observed) and there is an *expected* major decrease in secretion rate (J_v) as predicted in our article (Hill et al. 2004). Decreasing J_s in the osmotic model (Eq. 3) has the effect of further lowering the hyperosmolarity, which is the major finding of Ma et al. (1999). This is why we call the large reduction in salt flow inexplicable.

(3) The model referred to (Maclaren et al. 2012) is a complex computer simulation involving ion fluxes, osmotic relations and Ca oscillations in salivary cells (Gin et al. 2007; Palk et al. 2010), which requires experimental verification. The purported decrease in salt transport (J_s, equated to Cl transport), when P is decreased to 10 % by

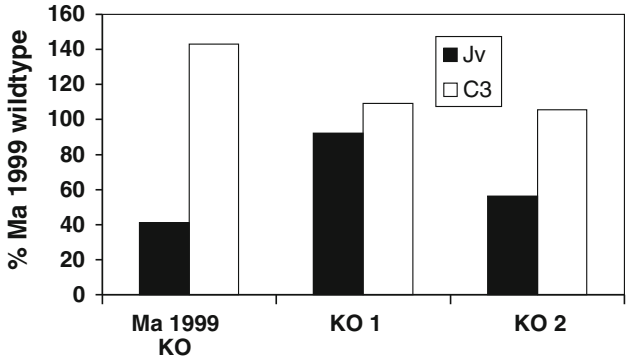

Fig. 1 Knockout (KO) results: volume flows J_v and saliva osmolarity C_3 as a percentage of experimental wild-type values from Ma et al. (1999). *Ma 1999 KO* experimental KO results of Ma et al. (1999); *KO 1* and *KO 2* were calculated with the osmotic model (Eq. 3) using a reduction of 90 % in P from the wild type and either the wild-type (*KO 1*), or the knockout (*KO 2*) value of J_s from Ma et al. (1999)

KO, first appears in Table 1; but we cannot find any explicit demonstration of this in the article. J_s is stated to fall by 27 % (the Ma et al. value is 41 %) and Maclaren et al. (2012) say, "Further decreases in chloride-transport rates not accounted for in the model could be due to changes in duct absorption rates in KOs (affecting J_s) or other changes in cell chloride secretion (affecting I_{Cl} and J_s) in KOs." That is, the experimental fall in J_s is not fully explicable even in their treatment. The problem here is that, apart from the obscurity of the explanation, there is no way to relate the osmotic permeabilities (P) used in the computer simulation to those of the salivary gland derived from the results of Ma et al.—the fall in J_s is apparently very dependent on the initial value of P.

If we assume that AQP KOs have other complex effects in epithelia, then we obviously undermine the whole rationale of using them as specific tools for testing the osmotic theory in the first place. The answer to the question of Maclaren et al.'s paper's title "What Do Aquaporin Knockout Studies Tell Us about Fluid Transport in Epithelia?" is, therefore, that simple osmotic equilibration cannot be the mechanism of epithelial fluid transport.

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