

## Review

## Sodium–proton exchange in crayfish

Leonard B. Kirschner\*

*School of Biological Sciences, Washington State University, Pullman, WA 99164-4236, USA*

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**Abstract**

Recently, three proton pump inhibitors were shown to have no effect on proton excretion and little on Na uptake in tapwater-adapted (TW) crayfish, while all three reduced Na–H exchange in salt-depleted (SD) animals. It appeared that the exchange was mediated by the Na channel–H pump in SD crayfish but not in TW animals. An alternative, a 2Na–1H exchanger, might function in the latter.

To test this, the effects of amiloride (AM) and ethylisopropyl AM (EIPA) on Na fluxes were observed. AM inhibits the Na channel but is a much weaker blocker of Na–H exchangers. In contrast, EIPA inhibits exchangers but acts weakly on the Na channel. If an exchanger functions in TW crayfish, we should expect EIPA to block Na influx in them with AM having a weaker action. The reverse should be true in SD animals.

Experimental data showed that EIPA was a potent inhibitor of Na influx in TW crayfish with half-maximal inhibition at about 0.2  $\mu\text{M}$ . However, AM proved to be equipotent. In SD crayfish, EIPA was as effective as in TW animals, and again AM was equally potent.

The data fail to show the expected differential action. Therefore, AM and its analogues cannot be used to distinguish between the two models of Na–H exchange in crayfish.

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**Keywords:** Sodium transport; Sodium–proton exchange; Amiloride; Ethylisopropyl amiloride; Proton pump; V-ATPase

**1. Introduction**

Modern research on epithelial sodium transport began more than a half century ago in the laboratory of Hans Ussing in Copenhagen [1–3]. This work made the frog skin an icon for epithelial ion movement comparable to the role played by the squid giant axon in neurophysiology. It culminated in a model of sodium–proton exchange in both frog skin and turtle bladder that developed largely in two laboratories [4–7]. The model, a sodium channel and an ATP-driven proton pump (V-ATPase) obligatorily coupled by the apical membrane potential of the epithelial cell, permitted the “exchange” of these ions between cell and medium. Sodium entering the cell is then extruded into the body fluid by a sodium–potassium pump on the basolateral membrane. The data supporting the model are compelling, and the model itself convincing.

Attempts to find this system in fully aquatic, freshwater (FW) animals, such as fish and invertebrates, have produced

a mixed and less than compelling picture. For example, the coupling between net  $\text{Na}^+$  and proton fluxes in tapwater-adapted (TW) crayfish was not 1:1 as required by the model, and amiloride (AM) affected the former more than the latter [8]. In addition, the transepithelial potential, generated largely by  $\text{Na}^+$  movement in frogs, is totally different in crayfish [9,10]. On the other hand, coupling of the two ion fluxes is 1:1 in salt-depleted (SD) crayfish as required by the model [8,11]. Recently, the V-ATPase has been shown to be present in gills of tapwater-adapted crayfish, and the enzyme concentration increased markedly when the animals were salt-depleted [12]. In what follows, I proposed to review some of these data and to extend the picture in crayfish.

**2. Experimental observations**

Fig. 1 shows the resting (control) fluxes of  $\text{Na}^+$  and net proton flux in two groups of the crayfish *Procambarus clarki*. One group was adapted to local tapwater (TW). The other was salt-depleted (SD) in deionized water for 10–14 days. Fluxes were measured in a bathing medium consisting

\* Fax: +1-509-335-3184.

E-mail address: kirschl@wsu.edu (L.B. Kirschner).

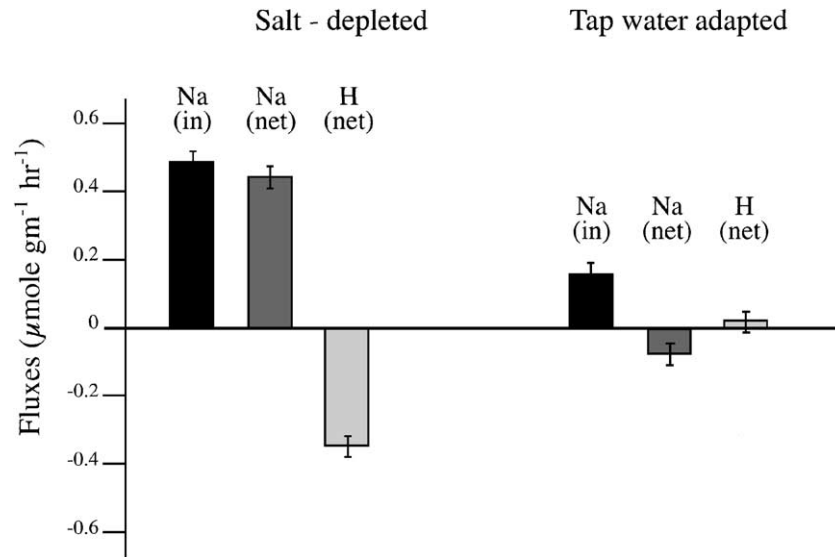


Fig. 1. Control fluxes in TW-adapted and salt-depleted crayfish. For each group, the first (black) bar is  $\text{Na}^+$  influx; middle (dark gray) bar is net  $\text{Na}^+$  flux and the third bar is net  $\text{H}^+$  flux. The latter is the sum of titratable acid and ammonia fluxes. The error bars show  $\pm 1$  S.E.  $N=24$  animals for both groups. Adapted from Ref. [16].

of  $\text{Na}_2\text{SO}_4$  ( $[\text{Na}]=0.5$  mM) buffered by tris-HEPES (HEPES 1 mM, pH 7.5). There was a substantial influx of  $\text{Na}^+$  in the TW animals, but essentially no net flux of  $\text{Na}^+$  or  $\text{H}^+$  (the sum of titratable acid and ammonia movements). These animals were in both sodium and acid–base balance. In the SD group, Na influx was about three times higher and there were substantial net fluxes of both  $\text{Na}^+$  and protons. The ratio of the latter pair was 1; that is, they were coupled 1:1 as required by the channel pump model. The data are consistent with those from earlier studies [8,11].

The model was tested by exposing both groups to known inhibitors of the V-ATPase. Dicyclohexylcarbodiimide (DCCD) has been shown to inhibit proton efflux in both turtle bladder and frog skin [13,14]. *N*-ethylmaleimide (NEM) inhibited  $\text{H}^+$  efflux in frog skin [14], and bafilomycin

inhibited both  $\text{Na}^+$  uptake and proton efflux in frog skin [15]. Fig. 2 shows the action of DCCD (0.1–0.2 mM) on fluxes in crayfish. There was a modest inhibition of  $\text{Na}^+$  influx (about 35%) in the TW animals but no significant effect on net sodium flux. This indicates that  $\text{Na}^+$  efflux was also reduced by the same amount. More important, the inhibitor had no effect on net proton movement. However, in SD animals, there was significant reduction ( $\sim 40\%$ ) in sodium influx and nearly equal reductions in both net  $\text{Na}^+$  and  $\text{H}^+$  movements. Inhibition was only partial, but an attempt to use a higher concentration (0.5 mM) resulted in death of the animals. The results with NEM and with concanamycin A, a relative of bafilomycin, were similar [16]. These data suggest that sodium–proton exchange in the SD animals is mediated by the model developed for frog

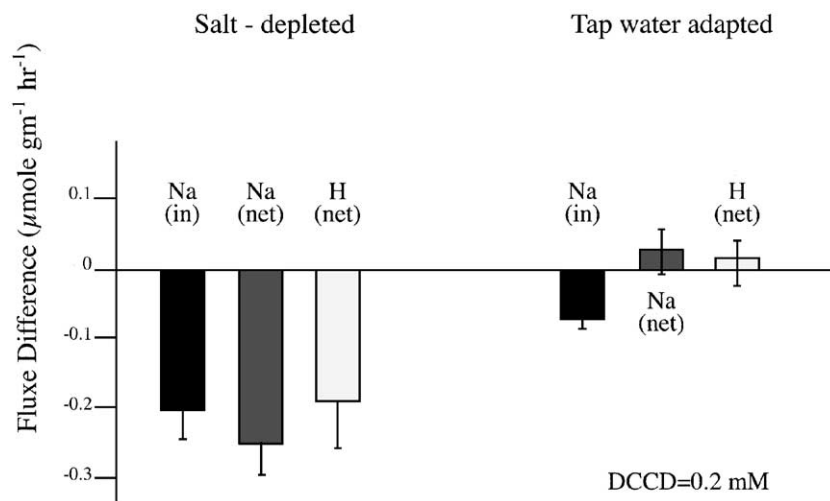


Fig. 2. The effect of DCCD on  $\text{Na}^+$  and  $\text{H}^+$  fluxes. Note that the bars represent changes in the fluxes. The inhibitor was added in 7  $\mu\text{l}$  DMSO.  $N=10$  (TW animals); 6 (SD animals). Error bars =  $\pm 1$  S.E. Adapted from Ref. [16].

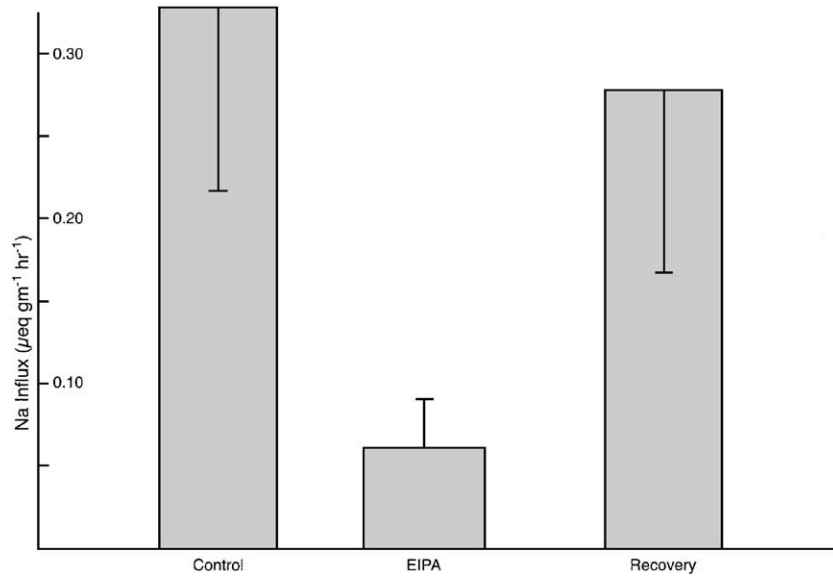


Fig. 3. The effect of EIPA (1 µM) on Na<sup>+</sup> influx. On removal of the inhibitor, recovery was essentially complete. Error bars show 1 S.E.  $N=3$  (TW adapted) animals.

skin, but they provide no evidence for operation of this system in TW-adapted animals.

It is clear that there is an “exchange” of Na<sup>+</sup> and H<sup>+</sup> in crayfish [16–18]. If this is not mediated by the Na<sup>+</sup> channel–H<sup>+</sup> pump system, what alternative is there? There has been described a 2Na<sup>+</sup>–1H<sup>+</sup> exchanger in several crustacean epithelia including the gills of a hyperregulating crab [19–21]. The question then becomes, “Can such an exchanger mediate the fluxes in FW?” The driving force for sodium entry (and proton efflux) across the apical mem-

brane of a FW animal gill was estimated recently [16] and can be expressed as

$$\Delta\mu = RT\ln\left(\frac{[\text{Na}^+]_c}{[\text{Na}^+]_m}\right)^2\left(\frac{[\text{H}^+]_m}{[\text{H}^+]_c}\right) + zF\Delta\Psi \quad (1)$$

where  $R=8.314 \text{ J deg}^{-1} \text{ mol}^{-1}$ ,  $T$  is  $\sim 293^\circ\text{C}$ ,  $z=+1$ ,  $F=96.5 \text{ kJ mol}^{-1}$  and  $\Delta\Psi$  is the apical membrane potential (volts, bathing medium reference). Subscripts c and m refer to values in cell and medium. If  $[\text{H}^+]_m = [\text{H}^+]_c$  (near pH 7), the relationship simplifies to

$$\Delta\mu = RT\ln([\text{Na}^+]_c/[\text{Na}^+]_m) + zF\Psi \quad (2)$$

Na<sup>+</sup> entry and proton extrusion will occur when  $\Delta\mu < 0$ . Using published values (from frog skin) for  $[\text{Na}^+]_c$  and the

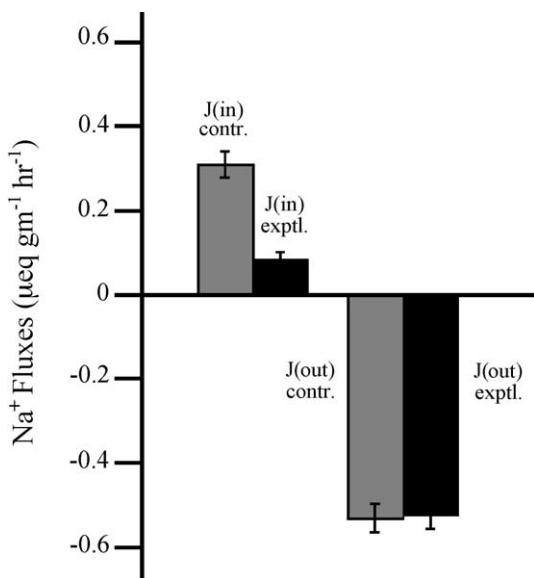


Fig. 4. The effect of EIPA(1 µM) on Na<sup>+</sup> fluxes. Efflux was unaffected by the inhibitor. Error bars show  $\pm 1$  S.E.  $N=9$  (TW adapted) animals.

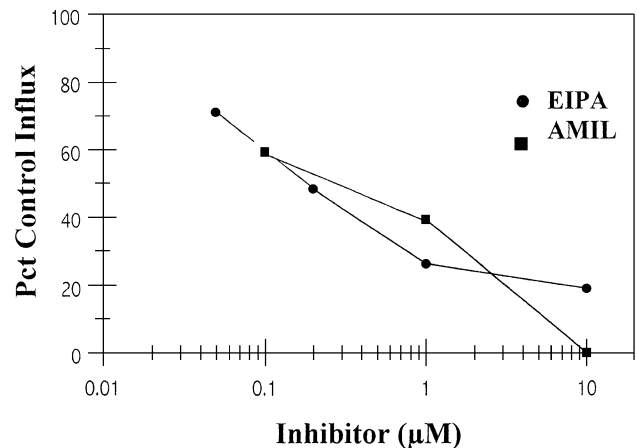


Fig. 5. Concentration dependence of influx inhibition by EIPA and amiloride in TW animals. The IC<sub>50</sub> was about 0.2 µM for both compounds. The number of measurements for the data points varied from 3 to 9.

apical membrane potential, it was possible to show that animals living in very dilute media (the salt-depleted group) could not use the exchanger to maintain a steady state. However, the TW-adapted animals would be able to regulate in external  $[\text{Na}^+]$  of 1–2 mM as long as the medium pH was the same as the cell, which is usually 7.0–7.3 in transport epithelia. If the environmental pH is higher, regulation could occur at even lower  $[\text{Na}^+]_m$  and the converse in more acid media. Operation of such an exchanger has a clear energetic advantage in eliminating the ATP expended by the proton pump which is about half of the total for the channel–pump system (for  $\text{H}^+/\text{ATP}$  values: Refs. [13,22]).

It has been reported that while AM is a powerful inhibitor of the epithelial  $\text{Na}^+$  channel, its action on  $\text{Na}^+ - \text{H}^+$  exchangers is much weaker. Additionally, there are a number of AM derivatives which act on the exchanger at very low concentrations but have a much weaker action on the channel [23]. Two of these are ethylisopropyl AM (EIPA) and hexamethylene AM (HMA). Action of the derivatives has been studied only on 1:1 exchangers, but it seemed possible that they might act similarly on a 2:1 exchanger, and hence provide a test for the presence of this system in crayfish.

The action of EIPA on sodium influx in TW animals is shown in Fig. 3. The final concentration was 1  $\mu\text{M}$  (added in dimethyl sulfoxide, DMSO, which had no effect on the fluxes), and influx was inhibited on average (three animals)  $81 \pm 8\%$ . Inhibition was reversible after removing the EIPA. Efflux was unaffected by the inhibitor (Fig. 4). The concentration dependence of inhibition by EIPA is shown in Fig. 5. The concentration for half-maximal inhibition ( $\text{IC}_{50}$ ) was about 0.2  $\mu\text{M}$ . HMA was effective at comparable concentrations (data not shown). The compounds clearly had a powerful effect on  $\text{Na}^+$  transport, and this seemed to indicate that an exchanger was implicated. At this juncture, it was expected that AM would have a much smaller effect, since previous work has shown that it is a relatively weak

inhibitor of exchangers. Instead, as shown in Fig. 5, it was equipotent with EIPA and acted in the same concentration range. One possible explanation is that AM is more active against a charged exchanger than one that is neutral.

Since there is now good evidence that the channel–pump model operates in SD animals, the action of AM and its derivatives on  $\text{Na}^+$  fluxes in this group was assessed. As shown in Fig. 6, both AM and EIPA were strong inhibitors of  $\text{Na}^+$  influx contrary to the expectation that the analogue would act more weakly. The  $\text{IC}_{50}$  for EIPA was again about 0.2  $\mu\text{M}$ . It appeared to be a little higher (about 0.7  $\mu\text{M}$ ) for AM for AM, but since only three measurements were made for each AM point, the apparent difference might merit closer scrutiny.

### 3. Discussion

The evidence is good that the  $\text{Na}^+$  channel–proton pump model operates in salt-depleted crayfish. The  $\text{Na}^+ - \text{H}^+$  exchange is 1:1 as required, and net fluxes of both ions are inhibited by AM [8] and by inhibitors of the proton pump [16].

As noted earlier, evidence for the channel–pump model is not nearly as strong in fully aquatic animals adapted to tapwater ( $[\text{Na}^+] \sim 1 \text{ mM}$ ), and the failure of three proton pump inhibitors to affect net  $\text{Na}^+$  or  $\text{H}^+$  fluxes in crayfish suggested that it might be useful to look for an alternative mechanism. The  $2\text{Na}^+ - 1\text{H}^+$  exchanger is such an alternative, since it could maintain the exchange in typical FW. It is worth noting that even if evidence for operation of this system is found, questions will remain (e.g. Since the exchange is asymmetric, how is electrical neutrality maintained?).

There appear to be at least three approaches to testing for the presence of this exchanger. One is to demonstrate its presence (or absence) directly in vesicles prepared from the gills as was done when it was first described. A second might be to prepare antibodies against the exchanger and attempt to immunolocalize it. The third is to take advantage of the reported difference in sensitivity of exchangers and the  $\text{Na}^+$  channel to AM and some of its analogues substituted in the 5  $\text{NH}_2$  position on the ring. The  $\text{IC}_{50}$  for AM acting on frog skin is about 0.2–0.5  $\mu\text{M}$  [24,25]. It was one to two orders of magnitude larger on a (1:1)  $\text{Na}^+ - \text{H}^+$  exchanger [26,27]. Conversely, an analogue like methylisopropyl AM (MIPA) has an  $\text{IC}_{50}$  of  $< 1 \mu\text{M}$  on an exchanger [27], while on the channel, its  $\text{IC}_{50}$  was 344  $\mu\text{M}$  [25]. There was no assurance that a charged exchanger would interact with the AM compounds as does the neutral exchanger, but the possibility seemed worth investigating.

The results, shown in Figs. 3–6, are equivocal. The powerful action of EIPA on  $\text{Na}^+$  influx in TW-adapted crayfish suggested that an exchanger was operating, but the equally potent action of AM was unexpected. There has been no previous work on the effect of the analogues on an

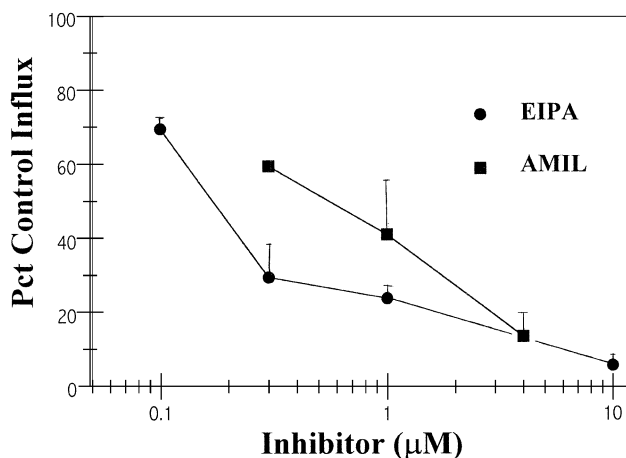


Fig. 6. Concentration dependence of influx inhibition for SD animals. The  $\text{IC}_{50}$  for EIPA is about the same as for TW animals. Error bars show 1 S.E.  $N=3$  for each point.

asymmetric exchanger, but an  $IC_{50}$  of 9  $\mu$ M was reported for the action of AM on a  $2Na^+ 1H^+$  exchanger from the hepatopancreas of the FW prawn *Machrobrachium rose-bergii* [19]. This is an order of magnitude higher than the value found here ( $<0.5 \mu$ M) and is consistent with some of the values reported for 1:1 exchangers. The actions of these compounds in SD animals are equally hard to understand. It is clear that the exchange in these animals is mediated by the channel–pump system, and the  $IC_{50}$  for AM, about 0.2  $\mu$ M, is consistent with its action on  $Na^+$  channels. However, the  $IC_{50}$  for EIPA is at least as low in these animals; its effect on the channel is usually much weaker than for AM.

The data show that AM and EIPA are equally potent in blocking  $Na^+$  influx in both TW-adapted and SD crayfish rather than displaying the differential actions reported on both the channel and exchanger. It has been shown that the  $IC_{50}$  for AM was decreased by an order of magnitude when frog skin (i.e. the channel) was bathed by 2.5 mM  $Na^+$  rather than by Ringer's solution which was usually used [28]. Similarly, inhibition of the exchanger by an analogue was markedly increased when  $Na^+$  in the medium was reduced from 60 to 3 mM [29]. Since the experiments reported here are run at  $[Na^+] = 0.5$  mM, it is possible that the very low concentration, in addition to increasing potency of the inhibitors, causes a loss in discrimination.

Whatever the basis for these observations, it is clear that the AM family does not show the differential actions in crayfish that have been reported in other preparations. Therefore, these compounds cannot provide a test for the presence of an exchanger.

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