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MgATP counteracts intracellular proton inhibition of the sodium–calcium exchanger in dialysed squid axons

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Intracellular Na^+ and H^+ inhibit Na^+–Ca^{2+} exchange. ATP regulates exchange activity by altering kinetic parameters for Ca_i^{2+}, Na_i^+ and Na_o^+. The role of the Ca_i^{2+} regulatory site on Na_i^+–H_i^+–ATP interactions was explored by measuring the Na_o^+-dependent $^{45}Ca^{2+}$ efflux (Na_o^+–Ca_i^{2+} exchange) and Ca_i^{2+}-dependent $^{22}Na^+$ efflux (Na_o^+–Na_i^+ exchange) in intracellular-dialysed squid axons. Our results show that: (1) without ATP, inhibition by Na_i^+ is strongly dependent on H_i^+. Lowering the pH_i by 0.4 units from its physiological value of 7.3 causes 80 % inhibition of Na_o^+–Ca_i^{2+} exchange; (2) in the presence of MgATP, H_i^+ and Na_i^+ inhibition is markedly diminished; and (3) experiments on Na_o^+–Na_i^+ exchange indicate that the drastic changes in the Na_i^+–H_i^+–ATP interactions take place at the Ca_i^{2+} regulatory site. The increase in Ca_i^{2+} affinity induced by ATP at acid pH (6.9) can be mimicked by a rise in pH_i from 6.9 to 7.3 in the absence of the nucleotide. We conclude that ATP modulation of the Na^+–Ca^{2+} exchange occurs by protection from intracellular proton and sodium inhibition. These findings are predicted by a model where: (i) the binding of Ca^{2+} to the regulatory site is essential for translocation but not for the binding of Na_i^+ or Ca_i^{2+} to the transporting site; (ii) H_i^+ competes with Ca_i^{2+} for the same form of the exchanger without an effect on the Ca_i^{2+} transporting site; (iii) protonation of the carrier increases the apparent affinity and changes the cooperativity for Na_i^+ binding; and (iv) ATP prevents both H_i^+ and Na_i^+ effects. The relief of H^+ and Na^+ inhibition induced by ATP could be important in cardiac ischaemia, in which a combination of acidosis and rise in $[Na^+]_i$ occurs.

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The electrogenic Na^+–Ca^{2+} exchanger is a membrane-bound protein that reversibly moves Ca^{2+} in exchange for Na^+ and is primarily responsible for Ca^{2+} extrusion in many cells. This mechanism is particularly important during the rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that occurs during cell signalling and secretion (for overview of recent work see Blaustein & Lederer, 1999). The two main types of regulation of this transporter, which are likely to take place in the large intracellular loop of the exchange protein are: (i) ionic regulation, which comprises Na_i^+-dependent inactivation, Ca_i^{2+} regulation and H_i^+ modulation; and (ii) metabolic regulation, which includes MgATP and phosphagen modulation (for references see Hilgemann, Philipson & Vassort, 1996). It is also known that these modes of regulation are related. For instance, intracellular Na^+ inhibition of the Na^+–Ca^{2+} exchanger seems to result not only from simple competition between Na_i^+ and Ca_i^{2+} for the transporting sites, but also from bringing the carrier into a Na^+-occluded state (Hilgeman *et al.* 1992). Also, in both squid axons and cardiac cells, cytoplasmic calcium and MgATP decrease the extent of Na_i^+ inhibition (Requena, 1978; Hilgemann & Matsuoka, 1992). Furthermore, it has been shown that Na_i^+ acts as a cofactor by increasing H_i^+ inhibition of the exchanger (Doering & Lederer, 1993).

Proton inhibition of the Na^+–Ca^{2+} exchanger was first shown in squid axons (Baker & McNaughton, 1977; DiPolo & Beauge, 1982) and later characterized in cardiac sarcolemmal vesicles (Wakabayashi & Goshima, 1981; Philipson *et al.* 1982). The high sensitivity of the exchanger to inhibition by intracellular protons and the strong synergism of the H_i^+–Na_i^+ interaction (Doering & Lederer, 1993, 1994) make this mode of regulation important not only under physiological conditions, but also under pathological conditions such as ischaemia and hypoxia where there is an increase in H_i^+ and intracellular Na^+. Although important information exists on H_i^+–Na_i^+ interaction and Na_i^+–ATP antagonism, little is known of the effects of metabolic modulation of the Na^+–Ca^{2+} exchanger by ATP on H_i^+ inhibition and H_i^+–Na_i^+ interaction.

Dialysed squid giant axons, which allow accurate control of intracellular pH_i, pCa_i, Na_i^+ and ATP, provide an excellent preparation in which to examine the mechanisms by which intracellular protons affect the Na^+–Ca^{2+} exchanger and, in particular, how H_i^+–Na_i^+–Ca_i^{2+} interactions are modulated by intracellular ATP. In the present paper, we show that the Ca_i^{2+} regulatory site is central to H_i^+ and ATP modulation of the exchanger. In addition, we develop a simple kinetic model of the exchanger that explains most of the features of the observed effects of ATP on the H_i^+–Na_i^+–Ca_i^{2+} interactions. Some of these findings were presented in abstract form (DiPolo & Beaugé , 1999).

METHODS

Squid giant axons were obtained (after decapitation) from two squid species: *Loligo pealei* (Marine Biological Laboratory, Woods Hole, MA, USA) and *Loligo plei* (Instituto Venezolano de Investigaciones Cientificas; Fundaciencia-IVIC, Venezuela) and dialysed with highly permeable capillaries of regenerated cellulose fibres (210 μm o.d.; 200 μm i.d.; molecular mass 18 kDa; Spectrapor Number 132226; Spectrum, Houston, TX, USA). The standard dialysis medium had the following composition (mM): Tris-Mops, 385; NaCl, 45; $MgCl_2$, 2; glycine, 285; and Tris-EGTA, 1; pH 7.3 and temperature between 17 and 18 °C. The standard external solution consisted of (mM): NaCl, 440; $CaCl_2$, 0.3; $MgCl_2$, 60; and Tris-Cl, 10; pH 7.6. The osmolarity of all solutions was adjusted to 940 mosmol l^{-1}. The estimation of $[Ca^{2+}]$ was made using the WinMaxc computer program (Version 2.00, 1999; Chris Patton, Hopkins Marine Station, CA, USA). To control the $[Ca^{2+}]_i$ in a pH range between 6.9 and 8.8 we employed two pH-independent calcium buffers: BAPTA and dibromoBAPTA. The former (1–3 mM) was used to buffer $[Ca^{2+}]_i$ from 0.3 to 0.7 μM, the latter (1–3 mM) to buffer $[Ca^{2+}]_i$ from 1.2 to 10 μM. Higher values of $[Ca^{2+}]_i$ were taken as equal to the $CaCl_2$ added in excess to that required to obtain 10 μM in the presence of dibromoBAPTA. Removal of external sodium was compensated with lithium. In order to stop any endogenous production of ATP, 1 mM NaCN was always present in the external media. Addition of ATP (3 mM) to the dialysis medium was done at a constant free $[Mg^{2+}]_i$ of 1 mM. The Ca^{2+} pump component of Ca^{2+} efflux and the operation of the Na^+–K^+ pump were eliminated by adding 100 μM vanadate to the dialysis media. Na^+ channels were blocked with 100 nM TTX in the external solutions. Before including $[^{45}Ca^{2+}]$ in the dialysis solution, axons were routinely dialysed for about 45 min with a standard medium containing 0.2 mM EGTA that was free of calcium and ATP. In all experiments each axon served as its own control, since steady-state $^{45}Ca^{2+}$ effluxes or ^{22}Na effluxes were always measured before and after a given experimental condition. BAPTA and dibromoBAPTA were purchased from Molecular Probes (Eugene, OR, USA). All other reagents were from Sigma (St Louis, MO, USA).

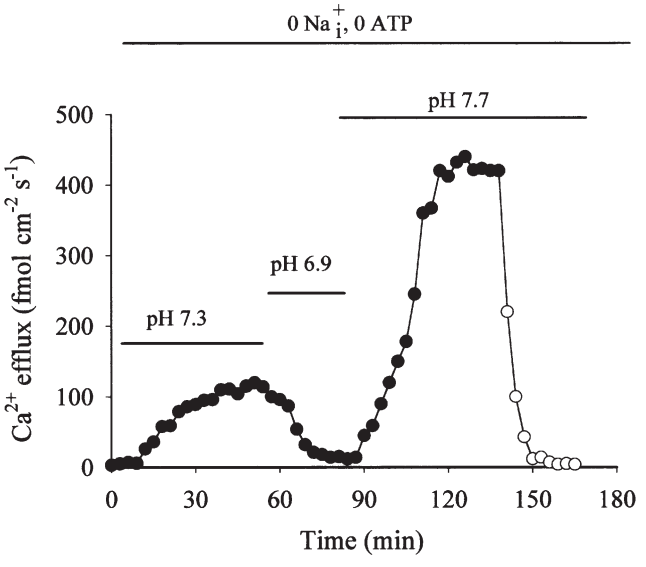

Figure 1. Effect of intracellular protons on the forward Na^+–Ca^{2+} exchange in the absence of Na_i^+ and ATP

Ca^{2+} efflux in the presence (●) and absence (○) of Na_o^+. Note the strong inhibition by protons of the forward Na^+–Ca^{2+} exchange. Axon diameter, 650 μM. Temperature, 17.5 °C.

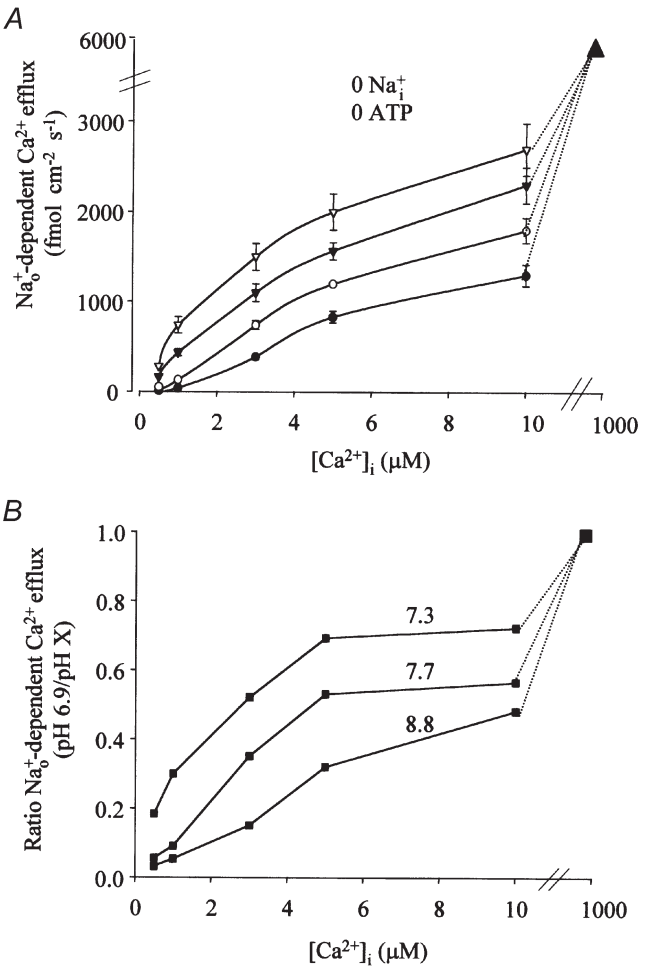

Figure 2. Ca_i^{2+}-dependent activation of forward Na^+–Ca^{2+} exchange flux at different values of pH_i in the absence of Na_i^+ and ATP

A, Na_o^+-dependent Ca^{2+} efflux at different values of $[Ca^{2+}]_i$ at pH 6.9 (●), 7.3 (○), 7.7 (▼) and 8.8 (▽). The error bars indicate S.E.M. The mean temperature was 17 °C. *B*, fractional inhibition by H_i^+ expressed as the ratio of the forward Na^+–Ca^{2+} exchange at pH 6.9 relative to that at pH 7.3, 7.7 and 8.8 as a function of $[Ca^{2+}]_i$. The graph was constructed using data from the experiments shown in Fig. 2*A*.

RESULTS

Inhibition of the Na^+_o–Ca^{2+}_i exchange by H^+_i and Na^+_i in ATP-depleted axons

Our initial experiments were designed to characterize the effect of intracellular protons on the steady-state levels of Na^+_o-dependent Ca^{2+} efflux without interactions with Na^+_i and ATP. For that purpose, Na^+ and ATP were completely removed from the cytosol before changes in pH_i. Figure 1 shows the effects, in a single axon, of changing the pH_i between 6.9 and 7.7 at a constant $[Ca^{2+}]_i$ of 1.2 μM. At a physiological pH of 7.3 the efflux of Ca^{2+} stabilizes at about 100 fmol cm^{-2} s^{-1}. Lowering pH_i by only 0.4 pH units to 6.9, causes a marked inhibition (about 80 %), while raising it to 7.7 increases the exchange rate to a level 20 times higher than that seen at pH 6.9.

In Fig. 2*A* the collected data from 11 axons illustrates the effect of different values of pH_i on the steady-state Na^+_o-dependent Ca^{2+} efflux with $[Ca^{2+}]_i$ ranging from 0.7 to 1000 μM. With $[Ca^{2+}]_i$ between 0.7 and 10 μM and in the absence of Na^+_i and ATP, protons always have an inhibitory effect on the forward Na^+–Ca^{2+} exchange (i.e. Ca^{2+} efflux, Na^+ influx). However, at 1 mM $[Ca^{2+}]_i$ protons have little effect on the exchanger. this result is predicted by the model depicted in Fig. 9. From the results shown in Fig. 2*A* it is possible to determine whether proton inhibition is affected by $[Ca^{2+}]_i$. Figure 2*B* shows a plot of the ratio of the forward Na^+–Ca^{2+} exchange at pH 6.9 relative to that at pH 7.3, 7.7 and 8.8. Clearly, the fractional proton inhibition decreases with increasing $[Ca^{2+}]_i$, becoming non-existant at 1 mM $[Ca^{2+}]_i$, indicating antagonism between the two ligands at the intracellular side of the exchanger.

In giant excised patches of guinea-pig cardiac sarcolemma, intracellular Na^+ acts as a cofactor in proton inhibition of the Na^+–Ca^{2+} exchanger (Doering & Lederer, 1994), presumably by acting on the large intracellular loop of the exchange protein (Philipson *et al.* 1982). We explored whether this Na^+_i–H^+_i synergism is present in dialysed squid axons. Figure 3*A*, *B* and *C* shows the results from three different axons in which the effect of $[Na^+]_i$ on the steady-state Na^+_o-dependent Ca^{2+} efflux was explored at physiological (7.3), acidic (6.9) and alkaline pH (8.8). In

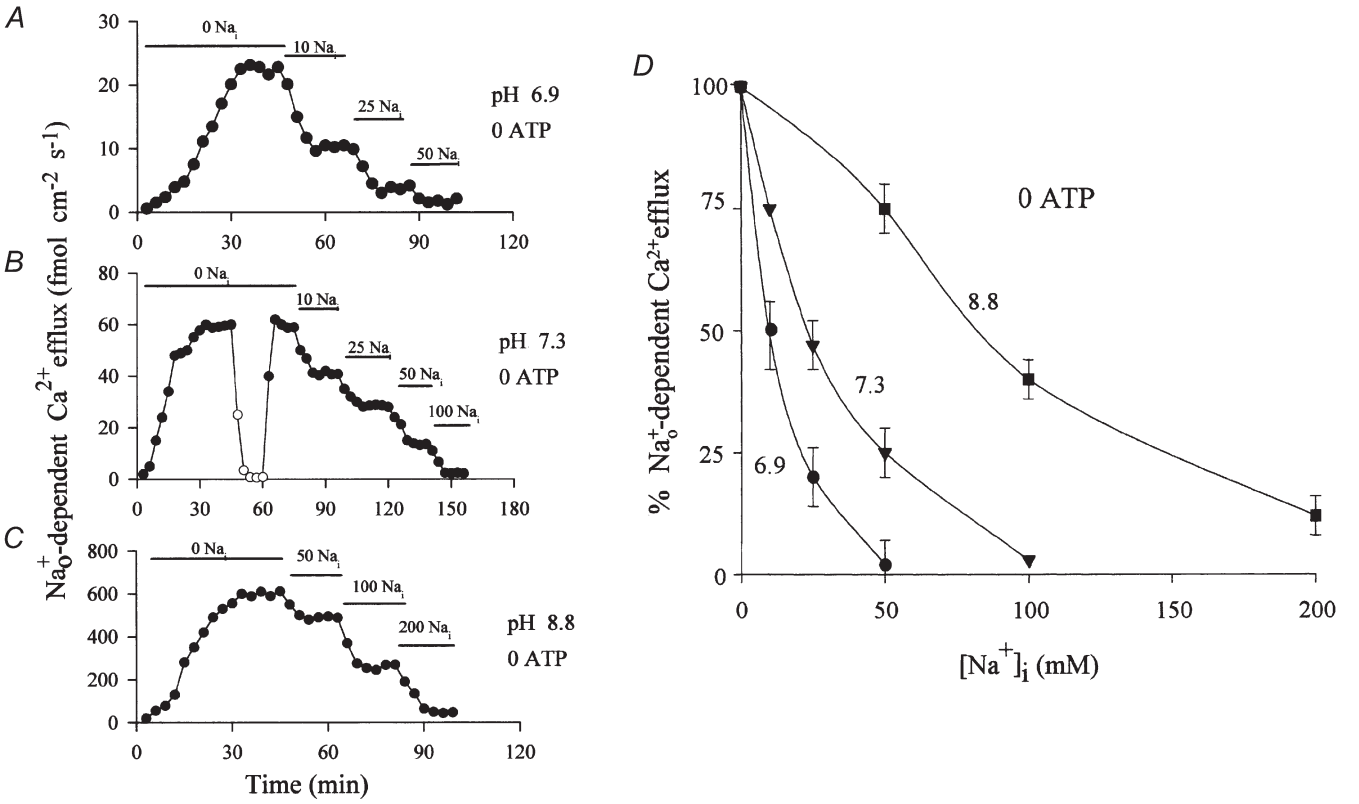

Figure 3. Effect of Na^+_i on the forward Na^+–Ca^{2+} exchange at different values of pH_i in the absence of ATP

Na^+_o-dependent Ca^{2+} efflux at pH 6.9 (*A*) 7.3 (*B*) and 8.8 (*C*) in the presence (●) and absence (○) of Na^+_o. All concentrations are millimolar. Notice the marked synergism between Na^+_i and H^+_i in inhibiting the exchanger at acidic pH_i. *D*, Na^+_i-dependent inhibition of forward Na^+–Ca^{2+} exchange at different values of pH_i in the absence of ATP. Ordinate, percentage Na^+_o-dependent Ca^{2+} efflux (Na^+_o–Ca^{2+}_i exchange). The error bars indicate S.E.M. The mean temperature was 17 °C. Notice the exquisite sensitivity of the exchange activity to Na^+_i at the acidic pH.

these experiments the protocol consisted of obtaining a steady-state baseline of Ca^{2+} efflux in the absence of Na_i^+ and ATP, and then following the changes in Ca^{2+} efflux as the concentration of Na_i^+ was increased. Two important results can be observed. First, in the absence of Na_i^+, the values of the Na_o^+-dependent Ca^{2+} efflux were 22, 61 and 600 fmol cm^{-2} s^{-1} at pH values of 6.9, 7.3 and 8.8 respectively, indicating that Na_i^+ is not essential for proton inhibition. Second, an increase in $[Na^+]_i$ caused progressive inhibition of the forward Na_o^+–Ca_i^{2+} exchange at every pH_i investigated. In addition, Na_i^+ became a more powerful inhibitor as pH was reduced, i.e. Na_i^+ inhibition was more noticeable at higher $[H^+]_i$. Figure 3*D* summarizes the results of several experiments in which the percentage Na_o^+-dependent Ca^{2+} efflux (Na_o^+–Ca_i^{2+} exchange) was explored in the range from 0 to 200 mM $[Na^+]_i$ at acidic (6.9), physiological (7.3) and alkaline (8.8) pH in axons completely depleted of ATP. It is clear that: (i) H_i^+ and Na_i^+ inhibit synergistically; (ii) the H_i^+–Na_i^+ cooperativity, noticeable at acid pH_i, is greatly diminished at pH_i 8.8 (the $K_{0.5}$ values for this effect of Na_i^+ were 10, 40 and 90 mM at pH 6.9, 7.3 and 8.8 respectively); and (iii) at pH 8.8 the curve for Na_i^+ inhibition suggests that more than one Na^+ ion is involved, whereas a single Na^+ ion kinetics seems to account for the results at pH_i values of 7.3 and 6.9.

The effect of ATP on H_i^+ and Na_i^+ interactions

In squid axons, stimulation of Na^+–Ca^{2+} exchange by ATP involves changes in several kinetic parameters, including a decreased capability of intracellular Na^+ to inhibit the forward Na_o^+–Ca_i^{2+} exchange (Requena, 1978; DiPolo & Beaugé, 1986). To determine whether ATP affects proton inhibition of the exchanger, and its synergism with intracellular Na^+ inactivation, we recorded the ATP stimulation of the Na_o^+-dependent Ca^{2+} efflux, in the absence and presence of Na_i^+, from pH_i 6.9 to 8.8. Figure 4*A* shows that in an axon dialysed without Na_i^+ and ATP at a physiological pH of 7.3, addition of ATP causes a

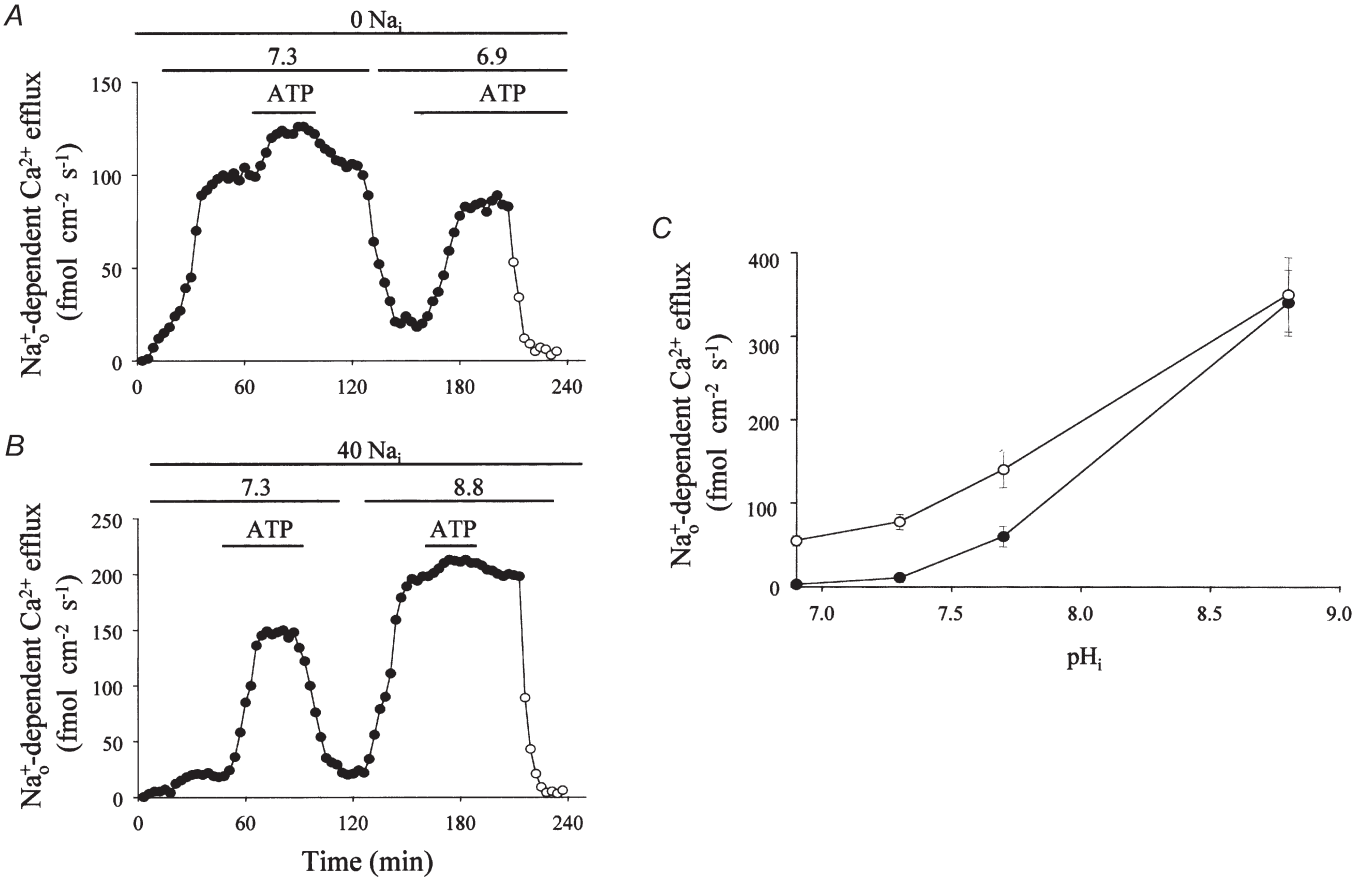

Figure 4. Effect of ATP on forward Na^+–Ca^{2+} exchange in the presence and absence of Na_i^+ at different values of intracellular pH_i

A, Na_o^+-dependent Ca^{2+} efflux in the absence of Na_i^+ in the presence (●) and absence (○) of Na_o^+. Notice the small effect of ATP at pH 7.3 compared to the large activation at pH 6.9. Axon diameter, 525 μm. *B*, Na_o^+-dependent Ca^{2+} efflux in the presence of a physiological $[Na^+]_i$ of 40 mM in the presence (●) and absence (○) of Na_o^+. Observe the large effect of ATP at pH 7.3 and its miniscule effect at pH 8.8. Axon diameter, 620 μm. *C*, H_i^+-dependent inhibition of forward Na^+–Ca^{2+} exchange at physiological Na_i^+ (40 mM) in the absence (●) and presence (○) of ATP (3 mM). The error bars indicate S.E.M. The mean temperature was 17 °C. Notice that the major fractional activation of the Na^+–Ca^{2+} exchange by ATP occurs between pH 6.9 and 7.3.

modest 20 % increment in the Na^+_o-dependent Ca^{2+} efflux, which slowly disappears upon removal of the nucleotide. Lowering the pH_i to 6.9 in the absence of both Na^+_i and ATP produces the already described marked inhibition of the Na^+–Ca^{2+} exchanger. Interestingly, at this acidic pH and in the absence of Na^+_i, ATP induces a pronounced (400 %) stimulation of the Na^+_o-dependent Ca^{2+} efflux. Figure 4*B* shows a representative axon from a different set of experiments in which the effect of ATP was tested in the presence of Na^+_i at both physiological (7.3) and alkaline pH_i (8.8). Contrasting with the results shown in Fig. 4*A*, at pH 7.3 ATP greatly increases (about 600 %) the rate of the Na^+_o-dependent Ca^{2+} efflux. After the Na_o-dependent Ca^{2+} efflux had reached a baseline value following the removal of ATP, pH_i was changed to 8.8, causing a tenfold increase in the forward Na^+–Ca^{2+} exchange. Remarkably, at alkaline pH_i readdition of ATP caused a quite small increment (less than 10 %) in the exchange activity. A summary of results of these experiments is given in Fig. 4*C*, which shows the degree of activation of the Na^+_o-dependent Ca^{2+} efflux by ATP in the pH_i range of 6.9–8.8, at a constant $[Na^+]_i$ (40 mM) and $[Ca^{2+}]_i$. It can be concluded that activation of the Na^+_o-dependent Ca^{2+} efflux by ATP is highly dependent on pH_i, being larger at acidic and almost non-existant at alkaline pH.

In order to obtain further insight into the ATP–H^+_i–Na^+_i interactions we measured the $[Na^+]_i$ inhibition of the Na^+_o-dependent Ca^{2+} efflux at pH_i 6.9 and 8.0 in the absence and presence of intracellular ATP. In the experiment shown in Fig. 5*A*, the axon was first dialysed without Na^+_i and ATP, causing the Ca^{2+} efflux to reach a level near 50 fmol cm^{-2} s^{-1}. Addition of 10 and 25 mM Na^+_i in the absence of ATP caused a 55 and 80 % inhibition of the exchange flux respectively. This inhibition completely reverted upon removal of Na^+_i. Figure 5*A* also shows that even in the absence of Na^+ and at pH 6.9, ATP caused a large activation of the Na^+_o-dependent Ca^{2+} efflux, but in this case much higher concentrations of Na^+_i were required to inhibit the exchange flux. This clearly shows that ATP at a physiological concentration (3 mM) causes a marked relief of the H^+_i–Na^+_i inhibition of the Na^+–Ca^{2+} exchanger. It should be pointed out that a similar protective effect of ATP was observed at [ATP] near its apparent affinity constant of 0.3 mM (experiments not shown).

Figure 5*B* and *C* summarizes the results of several experiments on the effect of ATP on Na^+_i inhibition of the forward Na^+–Ca^{2+} exchange at pH_i 6.9 and 8.8, at a constant buffer $[Ca^{2+}]_i$ of 1.2 μM. In Fig. 5*B* at pH 6.9, in the virtual absence of ATP, 10 mM Na^+_i brings a strong 50 % inhibition of the Na^+_o-dependent Ca^{2+} efflux, which is almost complete at 50 mM. However, in this acidic condition, ATP reduces Na^+_i inhibition, which is almost non-existant at 10 mM Na^+_i and only 50 % at 100 mM Na^+_i. In contrast, Fig. 5*C* shows that when the experiments were performed at pH 8.8, at the same constant buffer $[Ca^{2+}]_i$, inhibition by Na^+_i was markedly reduced and barely modified by the addition of ATP. In other words, Na^+_i inhibition of the Na^+–Ca^{2+} exchanger is antagonized by both ATP and alkalinization.

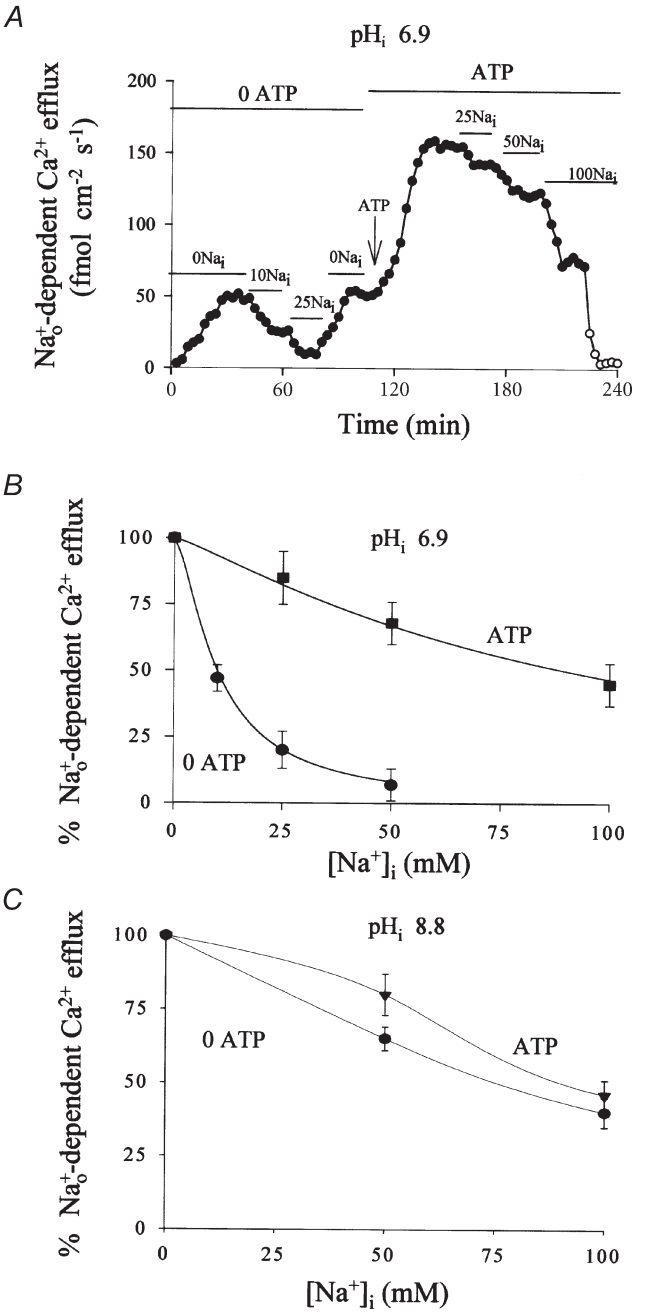

Figure 5. ATP relief of Na^+_i–H^+_i inhibition of forward Na^+–Ca^{2+} exchange

A, Na^+_i-induced inhibition of Na^+_o-dependent Ca^{2+} efflux at pH 6.9 in an axon dialysed first without ATP, then with 3 mM ATP. Notice first, the large activation in the exchange activity induced by ATP in the absence of Na^+_i and second, the relief of Na^+_i inhibition. *B*, Na^+_i-dependent inhibition of forward Na^+–Ca^{2+} exchange at pH 6.9 in the presence and absence of ATP. *C*, Na^+_i-dependent inhibition of forward Na^+–Ca^{2+} exchange at pH 8.8 with and without ATP. The error bars indicate S.E.M.

The effect of H_i^+ and ATP on the Ca_i^{2+} regulatory site

In squid axons ATP stimulation of the Na^+–Ca^{2+} exchanger occurs at limiting $[Ca^{2+}]_i$ via an increase in the affinity of the intracellular Ca_i^{2+} regulatory site (DiPolo & Beaugé, 1987). Therefore, another set of experiments was carried out to test whether the ATP–H_i^+ antagonism shown above might be associated with interactions of these ionic substrates at the Ca_i^{2+} regulatory site. In dialysed squid axons there are two ways to explore the apparent affinity of the Ca_i^{2+} regulatory site for Ca^{2+}. One approach uses the Ca_i^{2+} stimulation of the reverse partial reaction of the exchanger (Na_i^+-dependent Ca^{2+} influx), while the other uses the Ca_i^{2+} stimulation of the homologous Na_o^+–Na_i^+ exchange (DiPolo & Beaugé, 1987). For the sake of simplicity (to measure effluxes is technically less complicated than to estimate influxes), we decided to use the Na_o^+–Na_i^+ partial reaction of the exchanger. Figure 6*A* and 6*B* shows two experiments designed to measure the apparent affinity of the exchanger for Ca_i^{2+} at pH_i 6.9 and 8.8 in the absence of ATP. Initially, a baseline value of Na^+ efflux was obtained at a physiological Na_i^+, (40 mM) in the absence of ATP and Ca_i^{2+} (see Methods), followed by activation of Na_o^+-dependent Na^+ efflux through progressive increases in $[Ca^{2+}]_i$. At pH 6.9 (Fig. 6*A*), Ca_i^{2+} activates the Na_o^+–Na_i^+ exchanger with low affinity. In this particular axon, 50 % activation took place at a $[Ca^{2+}]_i$ greater than 10 μM. An important implication for the mechanisms of H_i^+ inhibition of the exchanger is seen in Fig. 6*B*, which shows a similar experiment carried out at pH 8.8. In this case the apparent affinity for Ca_i^{2+} activation of Na_o^+–Na_i^+ exchange increases to around 0.5 μM; i.e. about 20-fold higher than that observed at acid pH. It is worth noticing in Fig. 6*A* and *B* that the level of the Na_o^+-dependent Na^+ efflux attained at saturating $[Ca^{2+}]_i$ is almost the same at pH 6.9 and 8.8,

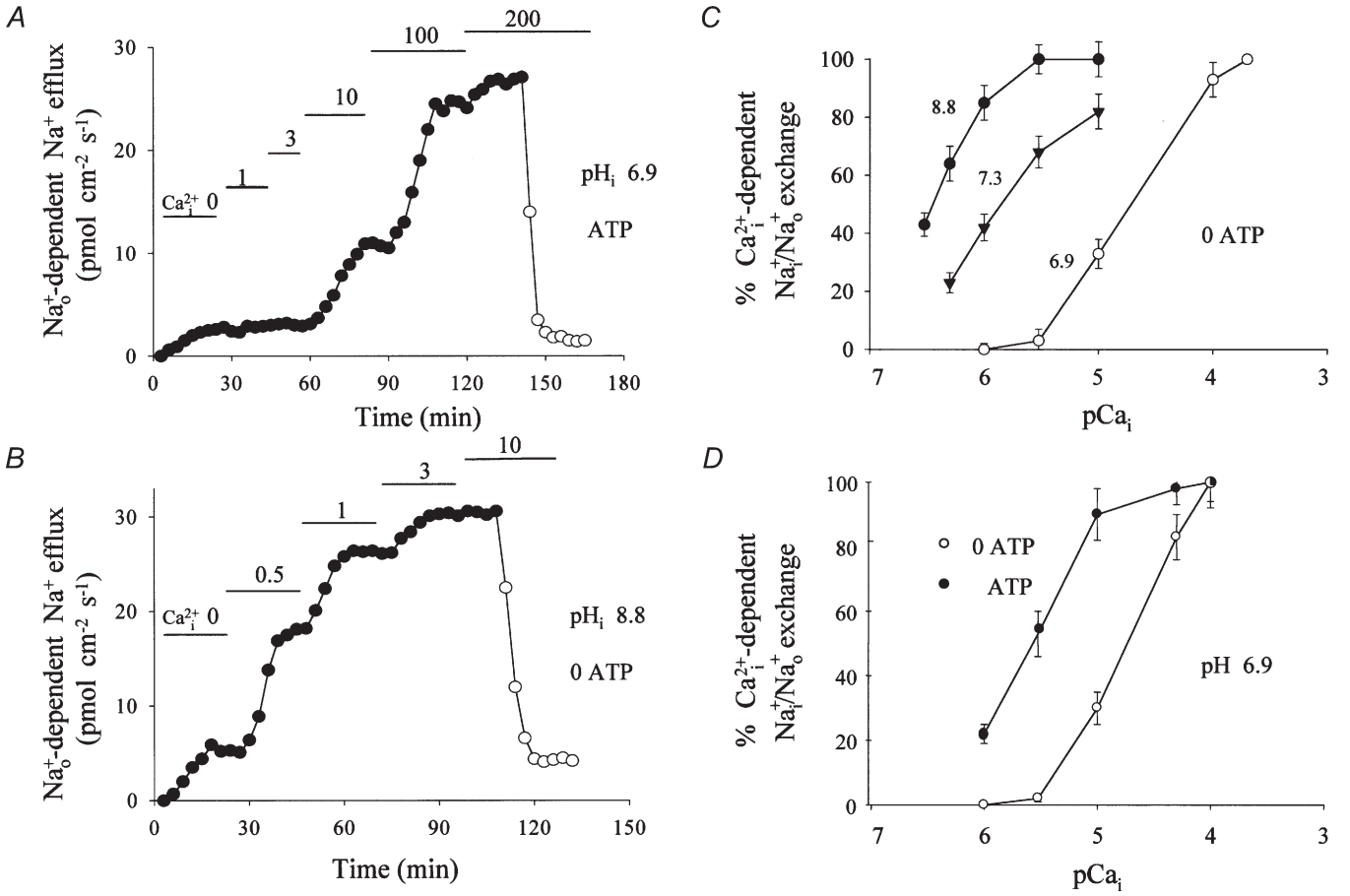

Figure 6. Effect of acid and alkaline pH_i on the $Ca^{2*}{}_i$-dependent Na_o^+–Na_i^+ exchange

Steady-state Na_o^+-dependent Na^+ efflux at pH 6.9 induced by increasing the $[Ca^{2+}]_I$ from 0 to 200 μM (*A*) and steady-state Na_o^+-dependent Na^+ efflux at pH 8.8 induced by increasing $[Ca^{2+}]_i$ from 0 to 10 μM (*B*) in the presence (●)and absence (○) of Na_o^+. The $[Ca^{2+}]_i$ was controlled with dibromoBAPTA (see Methods). Notice the large change in the apparent affinity of the Na_o^+–Na_i^+ exchange for Ca_i^{2+} between acid and alkaline pH. *C*, percentage Ca_i^{2+}-dependent Na_o^+–Na_i^+ exchange at pH 6.9 (○), 7.3 (▼) and 8.8 (●) in the absence of ATP at a physiological $[Na^+]_i$. The measurements at 0.3 μM Ca_i^{2+} were obtained with BAPTA as Ca^{2+} chelator. All other measurements were carried out with dibromoBAPTA. The error bars indicate S.E.M. *D*, percentage Ca_i^{2+}-dependent Na_o^+–Na_i^+ exchange at pH 6.9 in the presence (●) and absence (○) of 3 mM ATP. The error bars indicate S.E.M. The mean temperature was 17.5 °C.

suggesting that the effect of protons reflects changes in the affinity of the Ca^{2+}_i regulatory site rather than in the rate of Na^+ translocation.

Figure 6*C* collects the results of several experiments on the Ca^{2+}_i dependence of Na^+_o–Na^+_i exchange in the absence of ATP at a constant physiological $[Na^+]_i$ of 40 mM. Notice that the apparent affinity of the regulatory site for Ca^{2+}_i is strongly pH_i dependent, varying from 0.3–0.5 μM Ca^{2+}_i at pH_i 8.8 to 2 μM at pH_i 7.3 and 20 μM at pH_i 6.9. This illustrates that, in the absence of ATP, relatively minor variations in $[H^+]_i$ from its physiological value of 7.3 have pronounced effects in the exchange activity, which are mostly the consequence of changes in the binding affinity of the Ca^{2+}_i regulatory site. The role of ATP in this process is clearly seen in the series of experiments shown in Fig. 6*D*. At pH 6.9, addition of ATP reduces the $K_{0.5}$ for Ca^{2+}_i stimulation of Na^+_o–Na^+_i exchange about sevenfold, from 20 to about 3 μM. Interestingly, this increase in Ca^{2+}_i affinity induced by the nucleotide is close to that seen in its absence when the pH_i is raised from 6.9 to its physiological value of 7.3 (see Fig. 6*C*).

Additional evidence supporting the hypothesis that H^+_i and ATP antagonize each other by modifying the affinity of the Ca^{2+}_i regulatory site comes from the use of the sulfhydryl blocking reagent *p*-chloromercuryphenylsulphonic acid (*p*CMBS). This compound was shown to modify the reactivity of the Ca^{2+}_i regulatory site, preventing the increase in its affinity for Ca^{2+}_i induced by ATP, but without affecting the V_{max} of the Na^+–Ca^{2+} exchanger (DiPolo & Beaugé, 1993). The effect of increasing pH_i from 7.3 to 8.8 in the presence and absence of *p*CMBS on the two partial reactions of the exchanger is illustrated in Fig. 7 (Na^+_o–Na^+_i exchange in Fig. 7*A* and Na^+_o–Ca^{2+}_i exchange in Fig. 7*B*). Figure 7*A* shows that after obtaining a steady-state Na^+ efflux in the absence of Ca^{2+}_i and ATP at pH 7.3, addition of 1.0 μM Ca^{2+}_i activates the Na^+_o-dependent Na^+ efflux. The efflux of Na^+ is increased further when pH is raised to 8.8 and returns to its previous level at pH_i 7.3. Addition of 1 mM *p*CMBS causes a complete inhibition of the Ca^{2+}_i-activated Na^+_o-dependent Na^+ efflux and under these conditions alkalinization to pH_i 8.8 does not modify the level of Na^+_o–Na^+_i exchange. As reported previously (DiPolo & Beaugé, 1994), *p*CMBS does not cause a simple unspecific inhibition, since when $[Ca^{2+}]_i$ was raised to 100 μM, normal levels of exchange were obtained (cf. Fig. 6*B*). In the experiment shown in Fig. 7*B* the axon was submitted to a similar protocol but in this case the forward Na^+_o-dependent Ca^{2+} efflux was the measured partial reaction. Before *p*CMBS was applied, Na^+_o–Ca^{2+}_i exchange was stimulated by intracellular alkalinization from pH 7.3 to 8.8. After addition of *p*CMBS, similar changes in pH were completely ineffective. Also in this case, raising $[Ca^{2+}]_i$ to 100 μM increased the Na^+_o-dependent Ca^{2+} efflux to normal values. Therefore, in agreement with the results shown in Fig. 6*A* and *B*, the experiments with *p*CMBS strongly suggest that intracellular proton inhibition of the Na^+–Ca^{2+} exchanger occurs at the Ca^{2+}_i regulatory site without significantly affecting either the affinities of the Na^+_i or Ca^{2+}_i transporting sites or the translocation rates of these cations.

DISCUSSION

Primary inhibition by H^+_i and competition with Ca^{2+}_i

In dialysed squid axons, in the complete absence of Na^+_i and ATP, intracellular protons strongly inhibit the forward Na^+_o–Ca^{2+}_i exchange even at $[Ca^{2+}]_i$ as high as 10 μM (see Fig. 2*A*) while inhibition is absent at 1 mM $[Ca^{2+}]_i$. In contrast to the cardiac exchanger, in which there is no conclusive evidence that calcium ions displace protons (Doering & Lederer, 1993), our experiments show

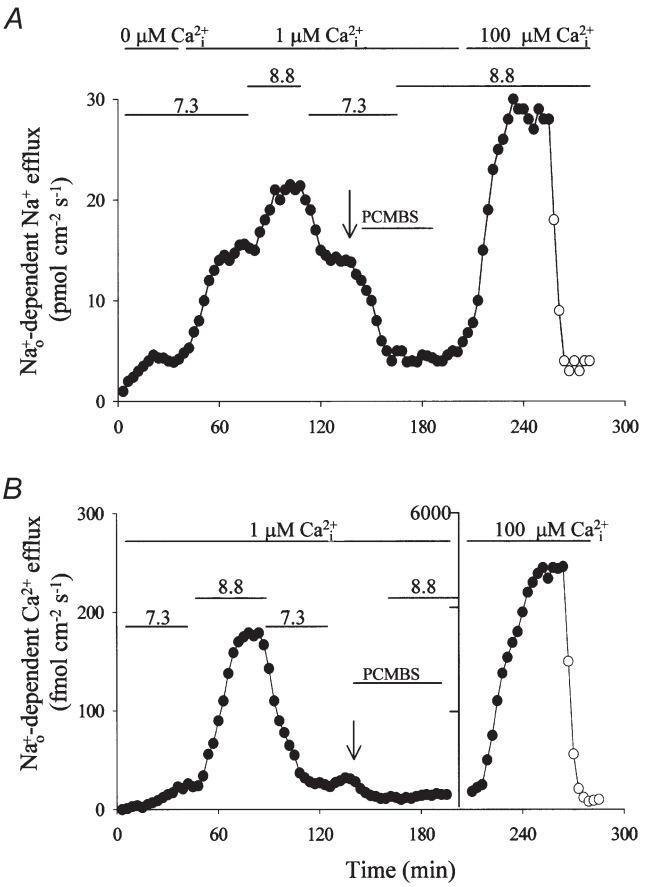

Figure 7. The effect of *p*CMBS on the alkalinization-induced increase in Na^+_o–Ca^{2+}_i and Na^+_o–Na^+_i exchange in axons dialysed without ATP

Na^+_o-dependent Na^+ efflux (*A*) and Na^+_o-dependent Ca^{2+} efflux (*B*) in the presence (●) and absence (○) of Na^+_o. The numbers above the lines represent the buffered intracellular pH. The arrows indicate the addition of 1 mM *p*CMBS. Notice that *p*CMBS completely blocks the alkalinization-induced increase in both Na^+_o–Ca^{2+}_i and Na^+_o–Na^+_i exchange. Notice also that at the end of both experiments increasing Ca^{2+}_i to saturating values reactivates the fluxes to normal levels. The mean temperature was 17.5 °C.

a significant decrease in the fractional proton inhibition when $[Ca^{2+}]_i$ is increased (see Fig. 2*B*) . In other words, in the squid, Ca_i^{2+} and H_i^+ are antagonists. The Na_o^+–Na_i^+ exchange experiments, which unequivocally provide a method for estimation of the affinity for Ca_i^{2+} of the regulatory Ca^{2+} site, indicate that modulation by protons involves interaction(s) with that modulatory site. Other experimental evidence favours this conclusion. First, the sulfhydryl agent *p*CMBS, which in squid axons markedly reduces the affinity of the regulatory site without affecting the V_{max} of the exchanger, completely blocks the stimulation of the Na^+–Ca^{2+} exchanger induced by alkaline pH (see Fig. 7*A* and *B*). Second, at $[Ca^{2+}]_i$ of 1 mM, protons have little effect on the rate of the forward exchange (see Fig. 2*A*). It could be argued that the same thing happens in the cardiac Na^+–Ca^{2+} exchanger because in that preparation alkalinization of the cytoplasmic side of inside-out patches shifts the secondary Ca_i^{2+} dependence of the outward exchange current towards lower $[Ca^{2+}]_i$ (Hilgemann *et al.* 1992). Nevertheless, these results are not conclusive because, as pH_i was increased, a significant outward current through the reverse Na^+–Ca^{2+} exchange occurred, even in the complete absence of cytosolic Ca^{2+} (Hilgemann *et al.* 1992). In our experiments, we found no evidence that alkaline pH_i may activate the Na^+–Ca^{2+} exchanger in the absence of Ca_i^{2+} (see Fig. 6*B*).

Na_i^+–H_i^+ interactions in metabolically depleted axons

An important finding in the cardiac Na^+–Ca^{2+} exchanger is that protons act as cofactors in Na_i^+ inactivation (Doering & Lederer, 1994). Similar interactions between sodium and protons are shown in the present experiments in ATP-depleted nerve fibres, where the H_i^+–Na_i^+ synergism is quite remarkable. As shown in Fig. 3*D*, at physiological pH (7.3) the activity of the Na^+–Ca^{2+} exchanger is inhibited by more than 60 % at a physiological $[Na^+]_i$ of 40 mM. In the range of pH and $[Na^+]_i$ explored in this work (pH 6.9–8.8 and $[Na^+]_i$ 0–200 mM), it is noticeable that 10 mM Na_i^+ at pH 6.9 causes 50 % inhibition of the forward Na^+–Ca^{2+} exchanger compared to about 100 mM Na_i^+ at pH 8.8.

ATP antagonizes intracellular sodium–proton synergism

In squid axons as well as in the cardiac Na^+–Ca^{2+} exchanger, ATP intrinsically regulates the exchange activity (Hilgeman & Matsuoka, 1992; DiPolo & Beaugé, 1999). In both preparations, when ATP levels are depleted (> 90 %) either by treatment with metabolic inhibitors (Haworth *et al.* 1987) or by intracellular dialysis (DiPolo, 1973), the exchange activity drops by more than 80 %. It is thought that the activation of the cardiac Na^+–Ca^{2+} exchanger (NCX1) by ATP is a result of the synthesis of PIP_2 (Hilgemann, 1997; Berberián *et al.* 1998), particularly of that strongly bound to the carrier protein (Asteggiano *et al.* 2001). In the squid, MgATP regulation of this exchanger seems to occur by a different mechanism, apparently unrelated to PIP_2 production (DiPolo *et al.* 2000) and probably associated with a phosphorylation–dephosphorylation process that involves a soluble cytosolic regulatory protein (DiPolo *et al.* 1997). The squid preparation has been extensively used to study the effects of ATP on the kinetics of interactions between transported and non-transported ligands with the Na^+–Ca^{2+} exchanger (DiPolo & Beaugé, 1999). Until now, it was known that regulation of the squid Na^+–Ca^{2+} exchanger by ATP involves: (i) an increase in the affinity of both intra- and extracellular sites of the transported cations (Na^+ and Ca^{2+}); (ii) an increase in the affinity of the Ca_i^{2+} regulatory site for calcium ions; and (iii) a decrease in the inhibition by Na_i^+.

The main contribution of the present study is to have found a link between the ionic (H_i^+–Na_i^+–Ca_i^{2+}) and the ATP-induced modulations of the squid Na^+–Ca^{2+} exchanger. A finding that has been difficult to explain in squid axons is that, in the absence of intracellular Na^+ and at a physiological pH of 7.3, the effect of ATP is rather small (DiPolo & Beaugé, 1984). A possible explanation for this effect can be seen in Fig. 4*A* in which, at pH 6.9 and in the absence of Na_i^+, ATP stimulation of the exchange activity is much greater than at pH 7.3. The relationship between ATP modulation and H_i^+ inhibition becomes even more clear in the experiment of Fig. 4*B* in which, at physiological Na_i^+, ATP has a large stimulatory effect on the exchange activity at pH 7.3, but that stimulation vanishes at alkaline pH. This dependence of the ATP effect on $[H^+]_i$ indicates that the larger fractional activation of the Na^+–Ca^{2+} exchanger induced by ATP at physiological $[Na^+]_i$ occurs mostly between pH_i 6.9 and 7.3. In these conditions the magnitude of the ATP effect is 12.5-, 4.0-, 2.3- and 1.05-fold at pH values of 6.9, 7.3, 7.7 and 8.8 respectively (see Fig. 4*C*).

It is known from previous studies that Na_i^+ inhibition of the Na^+–Ca^{2+} exchange activity is modulated by multiple factors including Ca_i^{2+}, ATP and H_i^+ (Miura & Kimura, 1989; Hilgemann & Matsuoka, 1992; DiPolo & Beaugé, 1999). As shown in Fig. 5*B*, the synergism between Na_i^+ and H_i^+ on the inhibition of Na^+–Ca^{2+} exchange activity is strongly attenuated or prevented by ATP. In fact, at pH 6.9 the exchange activity at a physiological $[Na^+]_i$ of 40 mM is almost 90 % inhibited in the absence of ATP, while inhibition amounts only to 10 % in the presence of ATP. As shown in Fig. 5*C*, at pH 8.8 the flux difference between the presence and absence of ATP is seen only at physiological $[Na^+]_i$, while both curves tend to superimpose at high $[Na^+]_i$ (> 100 mM). This observation, which may look bizarre, can be accounted for by the kinetic model proposed below (see Fig. 9 and Appendix). In Fig. 5*B* and *C* it can be seen that in the presence of 40 mM internal Na^+ the sizeable fraction of stimulation by ATP decreases as pH is raised from 6.9 and becomes nil at pH 8.8. This is

another indication that ATP modulation of the exchanger occurs by protection from intracellular proton inhibition. Our present hypothesis on the relationship between intracellular proton inhibition and modulation by ATP (see below) predicts that, at acidic pH, ATP substantially increases the affinity of the regulatory Ca^{2+}_i site and therefore, in the absence of ATP, increasing the pH_i from 6.9 to 8.0 should markedly increase the affinity of the

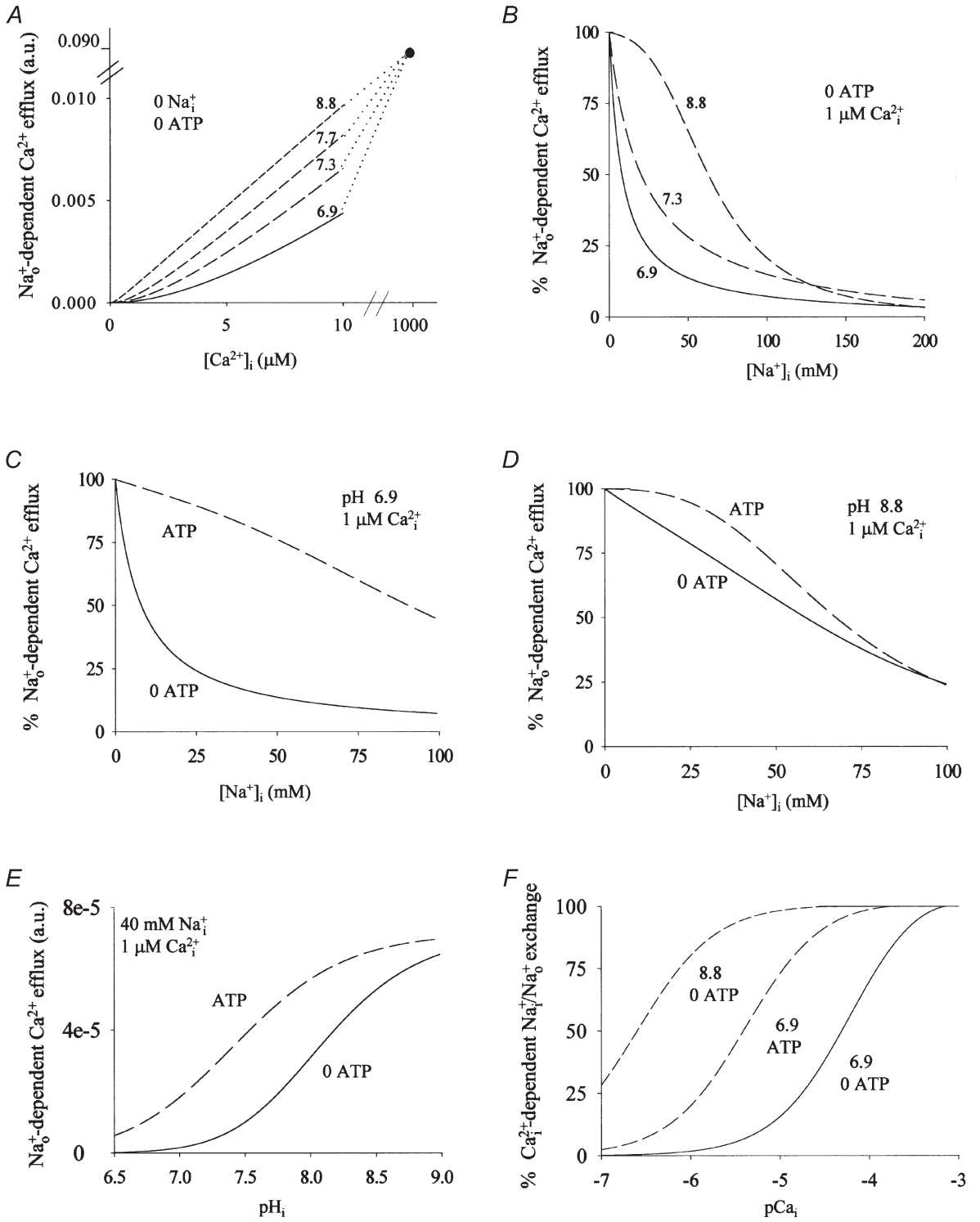

Figure 8. Kinetic model simulations for the Na^+_i, H^+_i, Ca^{2+}_i and ATP interactions in the regulation of the squid Na^+–Ca^{2+} exchanger

Notice that with the values of the constant used, at physiological pH, Ca^{2+}_i and Na^+_i, the fraction of carriers available for translocation is quite small. See text for details.

regulatory Ca_i^{2+} site for Ca^{2+} ions. These predictions are fulfilled in the experiments described in Fig. 6*C* and *D*.

The experiments reported here may explain some of the controversial aspects relating to the affinity of the regulatory Ca_i^{2+}-binding site reported in intact myocytes ($K_{½}$ of 20 nmol l^{-1}; Miura & Kimura, 1989) compared to giant patch preparations (100–400 nmol l^{-1}), excised myocyte blebs (Hilgemann *et al.* 1992), oocytes expressing the cloned Na^+–Ca^{2+} exchanger (Matsuoka *et al.* 1997) and large inside-out macropatches excised from intact myocytes (Fujioka *et al.* 2000). Taking into consideration the present results, the absence of ATP in the reported giant patch experiments may account for the underestimation of the affinity of the Ca_i^{2+} regulatory site.

Analysis of the proposed kinetic model

The steady-state regulation kinetics of the Na^+–Ca^{2+} exchanger were well simulated by the model shown in Fig. 9 in the Appendix. This model incorporates basic elements from other studies (Hilgemann *et al.* 1992; Doering & Lederer, 1994) and emphasizes the central role played by the intracellular regulatory site for Ca_i^{2+}, in particular the H_i^+– Na_i^+ cooperative inhibition of the exchanger and the protection exerted by MgATP against this inhibition. Figure 8 shows the results of several key simulations of the ATP–H_i^+–Na_i^+ interactions using the model illustrated in Fig. 9*B*. Figure 8*A* simulates the Na_o^+-dependent Ca^{2+} efflux as a function of $[Ca^{2+}]_i$. At any given pH_i, the Na_o^+-dependent Ca^{2+} efflux increases with increasing $[Ca^{2+}]_i$. The increase is greater as the pH_i is shifted from acidic to alkaline. Furthermore, the model predicts the results of Fig. 2*A* where, at 1 mM $[Ca^{2+}]_i$, pH_i has little effect on the level of the exchange flux. Qualitatively this simulation reproduces all the experimental findings of Fig. 2. However, quantitatively (and this is so far the only drawback of the model) it fails to show the initial saturation kinetics seen in Fig. 2. In Fig. 8*B*, the modelled effects of Na_i^+ on the forward Na^+–Ca^{2+} exchange at different values of pH_i and in the absence of ATP are almost an exact replica of the experiments of Fig. 3*D*, which show the synergism between Na_i^+ and protons in inhibiting the exchanger. In addition, the simulations of the effects of ATP in reverting Na_i^+–H_i^+ inhibition (Fig. 8*C* and *D*) reproduce quite well the experimental data of Fig. 5*B* and *C*. Figure 8*E* shows a simulated Na_o^+-dependent Ca^{2+} efflux and its pH_i dependence, corresponding to Fig. 4*C*. The simulated effects of intracellular pH in the range of 6.9–8.8 in the presence of 40 mM Na_i^+ with and without ATP are almost identical to the experiments shown in Fig. 4*C*, i.e. the model predicts, and the experiments corroborate, that the fractional activation of the Na^+–Ca^{2+} exchanger by ATP occurs at the acidic end of the pH range and vanishes at the alkaline end. A simulation of the increase in the affinity of the Ca_i^{2+} regulatory site with increasing pH_i is shown in Fig. 8*F*.

In summary, a model based on the hypothesis that proton interaction with the exchanger and its reversion by ATP occur at the intracellular Ca^{2+} regulatory site predicts all the experimental results described in this study.

Possible pathophysiological implications of the ATP relief of Na_i^+–H_i^+ cooperativity

Myocardial infarction is a complex syndrome with multiple variables, including a rise in intracellular Na^+, acidosis and ATP depletion. Early ischaemic episodes (ischaemic preconditioning) change the time course of myocardial damage in sustained final ischaemia (Murry *et al.* 1986; Li *et al.*1990). Reduced ATP consumption during ischaemic preconditioning, or repetitive acidosis, appears to be the most important factor in the preservation of both ATP and pH_i and has been implicated as playing a major role in protection of the myocardium during ischaemia (Murry *et al.* 1990; Kida *et al.* 1991; Lundmark *et al.* 1999). In addition, decreased ATP hydrolysis during the preconditioning phenomenon would make more ATP available at the time of reperfusion to maintain membrane homeostatic mechanisms such as the Na^+,K^+-ATPase (Grinwald, 1992), and possibly Na^+–Ca^{2+} exchange and the Ca^{2+} pump. Although the definitive mechanism(s) of preconditioning ischaemia has not been elucidated, it seems that glycogen depletion (Schaeffer *et al.* 1995), reduction in glycolysis (Murphy *et al.* 1991), limitation of acidosis and preservation of ATP play a critical role. In that regard, our experimental finding of a protective effect of ATP on the Na_i^+–H_i^+ synergistic inhibition of the Na^+–Ca^{2+} exchanger might be important for maintaining a functional Na^+–Ca^{2+} exchanger during sustained ischaemia, thus avoiding the rundown of a major Ca_i^{2+} extrusion mechanism in the heart.

APPENDIX

Figure 9*A* shows a simplified model summarizing the effect of H_i^+–Ca_i^{2+} and ATP on the Na^+–Ca^{2+} exchange activity, emphazising H_i^+–Ca_i^{2+} competition, H_i^+–Na_i^+ synergism and ATP protection of these interactions. Figure 9*B* shows a model of the Na^+–Ca^{2+} exchanger in which, for simplicity, only the intracellular ionic interactions have been taken into account. $\mathbf{E_1}$ is the state of the exchanger in which the ion binding site faces the intracellular medium. $Ca_r.\mathbf{E_1}$ is the cytoplasmic carrier loaded with Ca^{2+} at the regulatory site. $Ca_r.\mathbf{E_1}$.Ca and $Ca_r.\mathbf{E_1}$.3Na are the cytoplasmic-facing carriers loaded with $1Ca^{2+}$ or $3Na^+$ and ready to perform either Ca^{2+} or Na^+ efflux, respectively. $H.\mathbf{E_1}$, $H.\mathbf{E_1}$.Na and $H_2.\mathbf{E_1}$.Na are carriers binding H^+ and Na^+ at their inhibitory sites.

The elements we considered in designing the model, and which are shown in the Results, were the following. (a) Proton inhibition occurs even in the absence of Na_i^+ (first dead end, $H.\mathbf{E_1}$), but the presence of Na_i^+ strongly

potentiates that inhibition (second dead end, H_2.$\mathbf{E_1}$.Na). The way in which Na^+_i acts results in simulated curves which seem to require a single Na^+ ion at acid pH but become more complex as pH becomes more alkaline. In simulating the experimental data using the kinetic model we found that the best results were obtained by considering that two protons interact with the exchanger, one by competing with Ca^{2+}_i at the regulatory site and the other by taking the H.$\mathbf{E_1}$.Na state into a dead end, H_2.$\mathbf{E_1}$.Na. The model then assumes two types of Na^+_i inhibition, one favouring inhibition by H^+_i, while the other, independent of $[H^+]_i$, occurs by competition with Ca^{2+}_i at the transporting sites. (b) ATP antagonizes inhibition by protons in the absence but, more conspicuously, in the presence of Na^+_i. (c) Protons induce a striking reduction in the apparent affinity of the intracellular Ca^{2+} regulatory site. Conversely, Ca^{2+}_i antagonizes H^+_i inhibition. (d) Experiments where $[Ca^{2+}]_i$ was increased to as much as 1 mM show, in addition to counteracting H^+_i inhibition, the following: (i) Ca^{2+} efflux through the forward Ca^{2+}_i–Na^+_o exchange does not reach saturation at 1 mM; and (ii) no inhibition of the Na^+_i–Na^+_o exchange through the Na^+–Ca^{2+} exchanger is seen; this happens at all pH values and with or without ATP. According to these results, in the proposed model the minimal true dissociation constant for the Ca^{2+}_i transporting site becomes 10 mM.

Given the above, the basic features of this model are the following. (1) It takes into account only interactions between ligands at the cytoplasmic side. It makes no distinction whether the efflux of the cations is in exchange for external Ca^{2+} or Na^+. We consider this to be sufficient at this stage. A more elaborate model can be designed by adding the external part of the transport cycle. (2) The binding of all ions is instantaneous (rapid random equilibrium). In addition, the binding of $3Na^+_i$ are simultaneous. (3) The binding of Ca^{2+} to the regulatory site is essential for the binding of Na^+_i or Ca^{2+}_i to the transporting sites. (4) Intracellular protons compete with Ca^{2+}_i for the same form of the exchanger (not necessarily the same binding site), resulting in competitive inhibition by H^+_i at the regulatory Ca^{2+}_i site. (5) The binding of one proton to the carrier allows the binding of one intracellular Na^+ ion to form the H.$\mathbf{E_1}$.Na complex. The site(s) at which this Na^+ ion binds is not specified. (6) The formation of the H.$\mathbf{E_1}$.Na complex allows the binding of a second proton to form the dead end inhibitory H_2.$\mathbf{E_1}$.Na complex. (7) MgATP, through a process requiring phosphorylation, acts on the carrier by decreasing the apparent affinity for H^+_i and its Na^+_i counterpart binding to the carrier. However, the intimate mechanism for this ATP protection is not explicit.

For the rapid equilibrium solution of the present scheme we took the following values.

Dissociation constants:
K^r_{Cai}, true affinity for the Ca^{2+}_i regulatory site, 1×10^{-7} M;
K^t_{Cai}, true affinity for the Ca^{2+}_i transport site, 1×10^{-2} M;
K^t_{Nai}, true affinity for each Na^+_i transport site, 6×10^{-2} M;
K_{H10}, true affinity for the first proton site, 1×10^{-9} M;
K_{H20}, true affinity of the H.$\mathbf{E_1}$.Na_2 for the second proton, 1×10^{-8} M; and
K_{Nai0}, true affinity for Na^+_i of the H.$\mathbf{E_1}$ complex, 1×10^{-1} M.

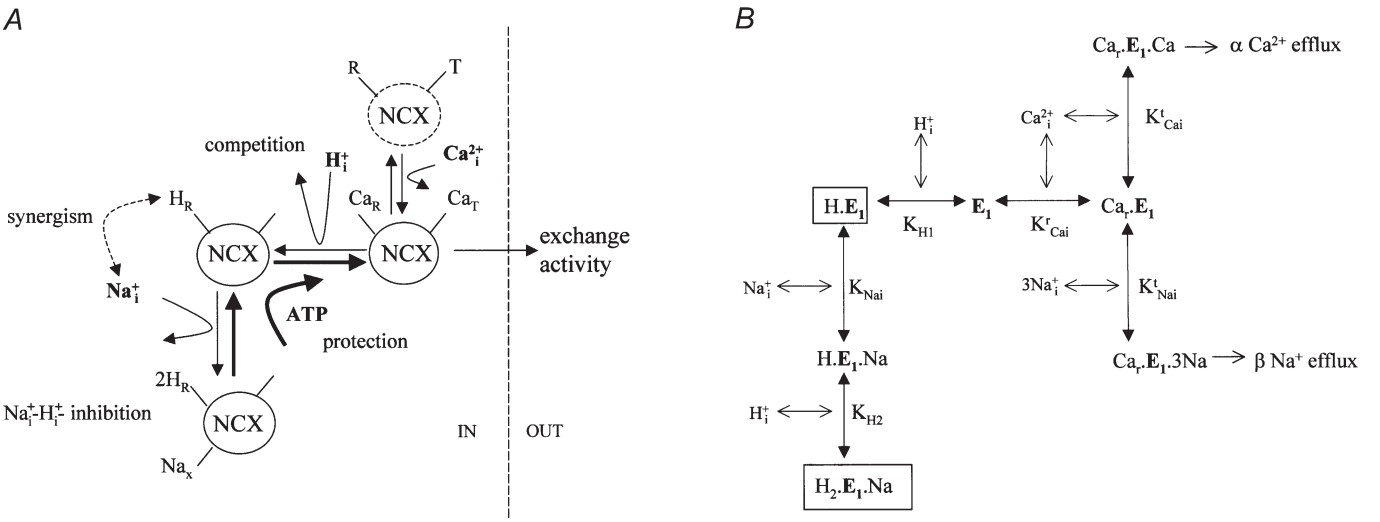

Figure 9

A, cartoon showing ligand interactions between Na^+_i–H^+_i (competition–synergism) and the protective effect of ATP on these interactions. NCX, Na^+–Ca^{2+} exchanger; R, regulatory site; and T, transport site. *B*, state diagram of the Na^+–Ca^{2+} exchanger. See text for details.

Other constants:
n = number of Na^+ ions binding to the H.$\mathbf{E_1}$ complex = 1;
K_{ATP}, K_m for the effects of MgATP, 200 μM;
factor_ATP_K_{H1}, decrease by ATP of the apparent affinity for H_i^+ binding to $\mathbf{E_1}$ = 2;
factor_ATP_K_{H2}, decrease by ATP of the apparent affinity for H_i^+ binding to H.$\mathbf{E_1}$.Na = 2;
factor_ATP_K_{Nai}, decrease by ATP of the apparent affinity for Na_i^+ binding to H.$\mathbf{E_1}$ = 10;

$$K_{H1} = K_{H10} \times (1 + (\text{factor\_ATP\_}K_{H1} \times [ATP]/(K_{ATP} + [ATP])));$$

$$K_{H2} = K_{H20} \times (1 + (\text{factor\_ATP\_}K_{H2} \times [ATP]/(K_{ATP} + [ATP])));$$

and

$$K_{Nai} = K_{Nai0} \times (1 + (\text{factor\_ATP\_}K_{Nai} \times [ATP]/(K_{ATP} + [ATP]))).$$

These last three equations express the fact that in the presence of ATP the affinity of the carrier for H_i^+ (K_{H1} and K_{H2}) and of the H.$\mathbf{E}_1$ complex for Na^+(K_{Nai}) is reduced.

The fluxes are proportional to the relative concentration of the 'active' carrier form: Ca_i^{2+} bound to the regulatory site plus either Ca_i^{2+} or Na_i^+ bound to the transporting sites. In this way, the resulting equations have a common denominator (D) and different numerators for Ca^{2+} (numCa^{2+}) and Na^+ (numNa$^+$) efflux. Thus,

$$\text{numCa}^{2+} = [Ca^{2+}]/K^t_{Cai} \times K^r_{Cai};$$

$$\text{numNa}^+ = [Ca^{2+}] \times [Na^+]^3/K^r_{Cai} \times K^{t}_{Nai}{}^3; \text{and}$$

$$D = 1 + [Ca^{2+}]/K^r_{Cai} + [Ca^{2+}]^2)/K^t_{Cai} \times K^r_{Cai} + [Na^+]^3/K^t_{Nai}{}^3 + [Ca^{2+}] \times [Na^+]^3/K_{cr} \times K_n{}^3 + [H^+]/K_{H1} + [H^+] \times [Na^+]^n/(K_{H1} \times K_{ni}{}^n) + [H^+]^2 \times [Na^+]^n/K_{H1} \times K_{H2} \times K_{Nai}{}^n).$$

Notice that with the values of the constants used, at physiological pH_i, Ca_i^{2+} and Na_i^+, the fraction of carriers available for translocation is quite small.

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