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THE EFFECT OF pH ON Ca^{2+} EXTRUSION MECHANISMS IN DIALYZED SQUID AXONS

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The effect of internal and external pH, on the components of the Ca^{2+} efflux have been investigated in internally dialyzed squid axons. (1) Internal pH: a fall in intracellular pH (below 7.3) inhibited both the ATP-dependent uncoupled (Ca^{2+} pump) (50% at pH_i 6.3) and the Na_o^+-dependent Ca^{2+} efflux (forward Na^+/Ca^{2+} exchange) (50% at pH_i 6.8). Internal alkalinization to pH 8.8 had no effect on the uncoupled component but markedly increased (4-fold) the Na_o^+-dependent Ca^{2+} efflux. (2) External pH: altering the external pH from 7.3 to 9.0 had no effect on the Na_o^+-dependent Ca^{2+} efflux mechanism. In the absence of Ca_o^{2+}, alkalinization to pH_o 8.8 caused a reduction in the magnitude of the uncoupled Ca^{2+} pump. This inhibition is markedly enhanced by the presence of Ca^{2+} in the external medium. As for the case of the sarcoplasmic reticulum Ca^{2+}-ATPase, this combined inhibitory effect of high pH_o and Ca_o^{2+} is most probably related to a reversal of the cycle of the ATP driven Ca^{2+} pump. The marked differences in the pH dependence of the components of the Ca^{2+} efflux support the model of two separate mechanisms of Ca^{2+} extrusion in squid axons: Ca^{2+} pump and Na^+/Ca^{2+} exchange.

Introduction

Recent interest has been focused on pH changes that occur during certain physiological processes. For instance, Meech and Thomas [1] have demonstrated a pH_i decrease in *Helix* neurones following an increase in $[Ca^{2+}]_i$, and Brown et al. [2], found acidification of photoreceptors after exposure to light. In intact cells, alteration in $[Ca^{2+}]_i$ as a consequence of changes in the $[H^+]$ gradient across the membrane, could be due primarily to the result of alterations in the intracellular Ca binding [3,4], and/or to modifications in the membrane Ca transport systems [5,6].

In experiments on intact squid axons, it has been reported that internal acidification either by injecting acid or by exposing the axons to CO_2, reduces the Ca^{2+}-dependent Na^+ efflux [6], the associated Na_i^+-dependent Ca^{2+} influx [7] and the efflux of $^{45}Ca^{2+}$ [7]. In contrast, intracellular alkalinization to about pH 8.0, increases the Ca_o^{2+}-dependent Na^+ efflux [6].

The present study was undertaken to explore the effects of external and internal pH on Ca^{2+} extrusion in squid axons under internal dialysis conditions. The precise control of the internal medium is essential, since it eliminates possible variation in ionic (Na_i^+, K_i^+, Cl_i^-, Ca_i^{2+}, Mg_i^{2+}) and metabolic (ATP, ADP, P_i) conditions induced by pH changes, thus allowing a more direct analysis of pH effects upon the Ca^{2+} extrusion mechanism present in this preparation (ATP driven Ca^{2+}

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pump and Na^+/Ca^{2+} exchange).

The results show that: (1) reducing the internal pH causes a fall of both the ATP dependent uncoupled and the Na_o^+-dependent Ca^{2+} efflux components. Increasing the internal pH, markedly enhances the Na_o^+-dependent component without changing the magnitude of the uncoupled Ca^{2+} efflux. (2) Increasing the external pH from 7.3 to 9.0 has no significant effect on the Na_o^+-dependent Ca^{2+} efflux, in contrast with an inhibition of the ATP dependent uncoupled component observed at alkaline pH_o. An interesting observation is that the inhibition of the ATP driven Ca^{2+} pump mechanism found at high pH_o is significantly potentiated by external calcium ions. This effect, originally found and studied in the sarcoplasmic reticulum Ca^{2+} pump [8,9] has been related to facilitation of the reversal cycle of the Ca^{2+} pump (ATP synthesis).

The present experiments further support the proposed model [10,11] of two separate mechanisms for Ca^{2+} extrusion in excitable cells: ATP driven 'uncoupled' Ca^{2+} pump and Na^+/Ca^{2+} exchange mechanism.

Methods

The experiments were carried out on two squid species: *Doryteuthis plei* at the Instituto Venezolano de Investigaciones Científicas in Caracas, Venezuela, and the squid species *Loligo pealei* at the Marine Biological Laboratory in Woods Hole, MA, U.S.A. The general dissecting procedure, dialysis technique, efflux and influx experiments are described in detail elsewhere [12,13] and the reader is referred to these papers for more information.

Solution. The artificial sea water had the following composition (mM): K^+, 10; Na^+, 440; Mg^{2+}, 50; Ca^{2+}, 10; $Tris^+$, 10; Cl^-, 580; EDTA, 0.1; CN^-, 1. The osmolarity was 1000 mosM and the normal pH (18–19°C) 7.6. The removal of Na^{2+}, Ca^{2+} or Mg^{2+} was compensated with equiosmolar amounts of $Tris^+$. Ca^{2+}-free sea water contained 60 mM Mg^{2+} and 0.5 mM EGTA. The standard dialysis solution had the following composition (mM): K^+, 310; Na^+, 30–60; Mg^{2+}, 4 in excess of the ATP concentration; $Tris^+$, 30; Cl^-, 98; aspartate, 310; EGTA, 1 or 2; glycine 310. Removal of Na^+ or K^+ was compensated with equiosmolar amounts of $Tris^+$. The osmolarity was adjusted to 980 mosM. All internal solutions contained 10 μg/ml oligomycin. The pH of both external and internal solutions was buffered from pH 6 to 8.8 with Tris-maleate (50 mM). The nominal ionized Ca^{2+} concentration values are based on a CaEGTA dissociation constant of 0.15 μM. Since ΔpH will change the CaEGTA dissociation constant, the $[Ca^{2+}]_i$ was corrected for this effect [14] and its concentration raised (micromolar range) to saturate both the ATP dependent 'uncoupled' and the Na_o^+-dependent Ca^{2+} efflux components [11,15]. ATP (vanadium free) was obtained from Sigma Co. as Tris salt, neutralized with Tris hydroxide and stored at −20°C as 250 mM solution.

All reagents used in the present work were of analytical grade. Radioactive solutions were made by adding solid $^{45}CaCl_2$ (15–30 mCi/mg, New England Nuclear) directly to the internal or external solution. Radioactive samples containing 4 ml artificial sea water mixed with 5 ml of scintillator solution and counted in a liquid scintillation counter for times long enough to give a standard error of about 1%.

Results

The effect of internal pH on the components of the Ca^{2+} efflux

Fig. 1 shows the effect of internal pH on the ATP dependent uncoupled Ca^{2+} efflux. In order to fully activate this component, the axon was dialyzed with a $[Ca^{2+}]_i$ of 150 μM. Also, both Na^+ and Ca^{2+} were removed from the external medium to avoid contributions from the Na_o^+- and Ca_o^{2+}-dependent components of the Ca^{2+} efflux. In the presence of ATP, and at a physiological pH_i of 7.3 [16], Ca^{2+} efflux reaches a steady value of about 240 fmol $\cdot$ cm^{-2} $\cdot$ s^{-1}. Decreasing the pH_i to 6.0, inhibits the uncoupled Ca^{2+} efflux by 80%. This reduction, is totally reversible upon returning the pH_i to its original value of 7.3. Fig. 1 also shows that alkalinization of the internal medium to pH_i 8.5, does not affect the steady level of the uncoupled efflux. When the internal pH was decreased to pH 6.5 from its value at 8.5, the Ca^{2+} efflux decreases by about 40%. In a few experiments (not shown) in which the internal pH was

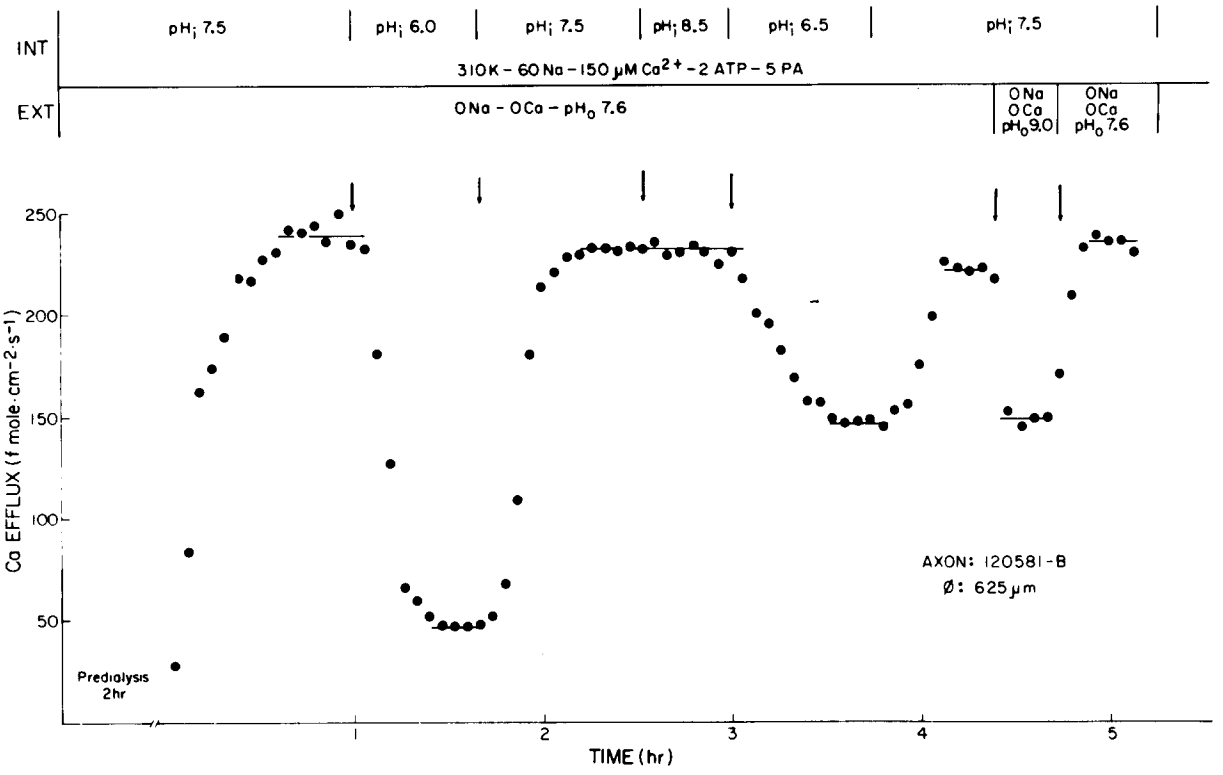


Fig. 1. The effect of internal and external pH on the ATP-dependent uncoupled Ca^{2+} efflux from a dialyzed squid axon. Ordinate: Ca^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscissa: time in hours. The axon was predialyzed for 2 h to obtain a good control of the intracellular medium prior to the addition of the radioactive dialysis solution. The arrows indicate changes in pH. The horizontal lines show the steady-state Ca^{2+} efflux levels. PA, phosphoarginine.

reduced below pH 6.0 the uncoupled efflux was almost completely abolished.

Fig. 2 shows the effect of internal pH on the Na_o-dependent Ca^{2+} efflux. As for the case of the experiment of Fig. 1, the axon was dialyzed with a micromolar $[Ca^{2+}]_i$ to fully activate this component. The possible contribution of the uncoupled Ca^{2+} efflux to the total efflux was eliminated by removing the ATP from the dialysis medium. In the absence of Na_o^+ and Ca_o^{2+}, Ca^{2+} efflux is very small and not different from the Ca^{2+} 'leak' value expected for this $[Ca^{2+}]_i$ [10]. Addition of Na_o^+ in the absence of Ca_o^{2+}, causes a marked increment in the Ca^{2+} efflux (Na_o^+-dependent component) to a steady level of about 1000 fmol·cm^{-2}·s^{-1}. A decrease in the pH_i from 7.5 to 6.0 decreases the efflux by 70%. As shown in Fig. 2, this effect is clearly reversible. When the intracellular medium was alkalinized to pH 8.5, the Na_o^+-dependent component increases 4-fold. The fact that virtually all of the increase in Ca^{2+} efflux observed at high pH_i is sensitive to external Na^+, indicates that internal alkalinization has no effect on the 'leak' of Ca^{2+}. Similarly, lowering the pH_i to about 6, had no effect on the Ca^{2+} 'leak'.

Fig. 3 summarizes the pH_i dependency of both the ATP dependent uncoupled, and the Na_o^+-dependent Ca^{2+} efflux components. The magnitude of the Ca^{2+} efflux at pH_i 7.3 has been plotted versus the pH of the internal medium. An increase in the internal pH from 7.3 to 8.5, caused a significant (400%) increase in the magnitude of the Na_o^+-dependent Ca^{2+} efflux, in marked contrast with the absence of effect on the uncoupled mechanism. In the pH range explored in this experiment, no sign of saturation was observed for the stimulating effect of high pH_i on the carrier mediated Na^+/Ca^{2+} exchange mechanism. Fig. 3 also

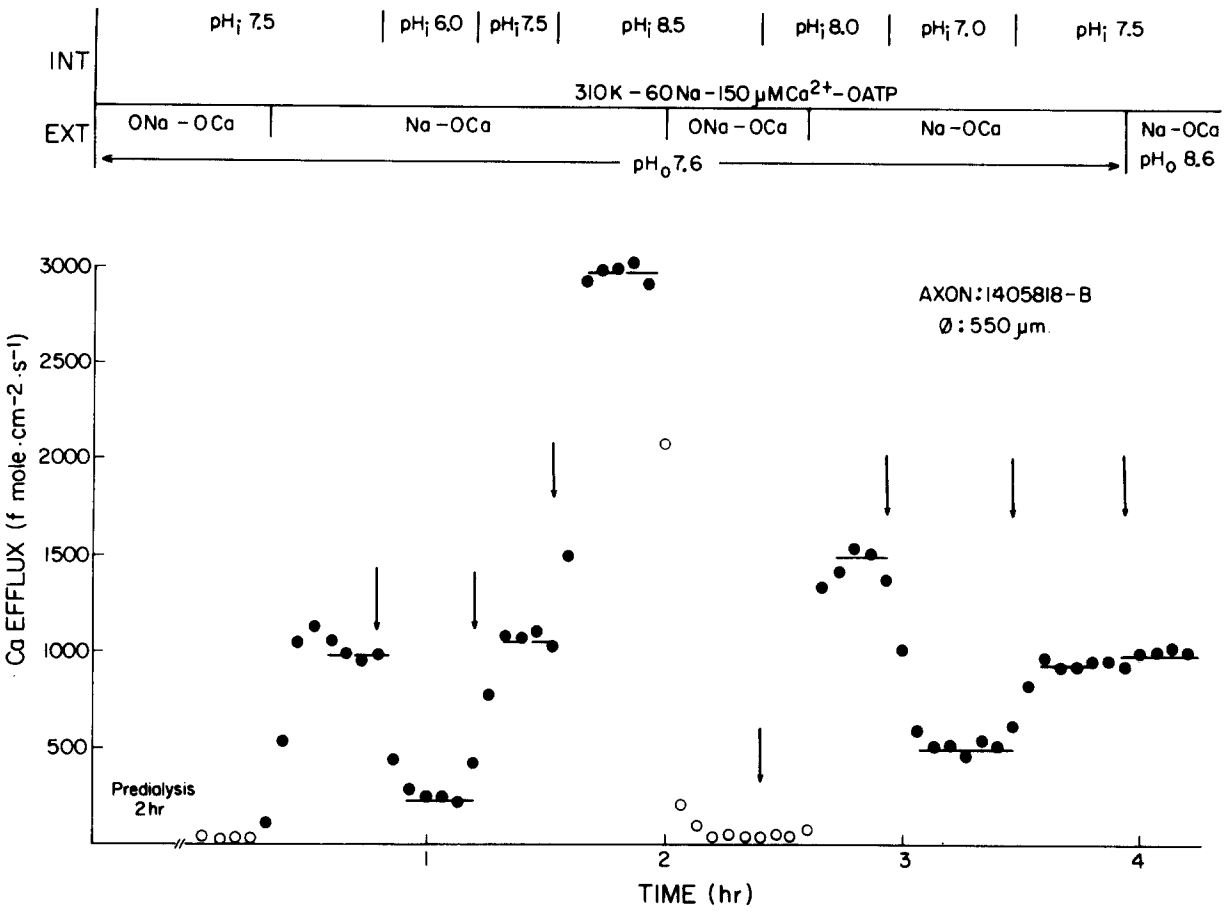
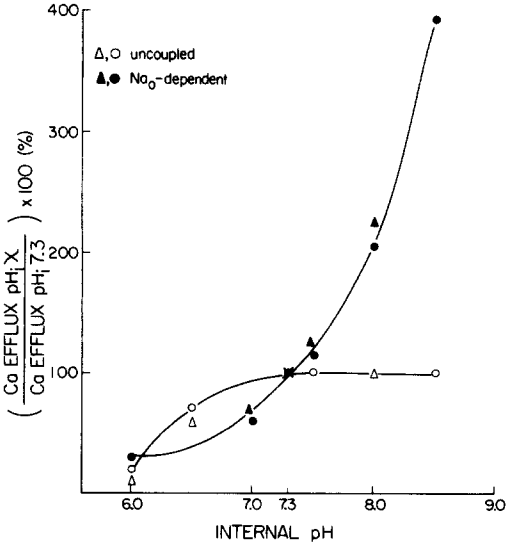


Fig. 2. The effect of internal and external pH on the Na_o^+-dependent Ca^{2+} efflux from a dialyzed squid axon. Ordinate: Ca^{2+} efflux in fmol · cm^{-2} · s^{-1}. Abscissa: time in hours. The axon was predialyzed for 2 h in order to remove completely the internal ATP. The arrows indicate changes in pH. Temperature 18°C.



shows that decreasing the pH_i inhibits both components of the Ca^{2+} efflux. For the case of the uncoupled mechanism, 50% inhibition is obtained at pH_i 6.3, as compared with a similar inhibition of the Na_o^+-dependent mechanism at pH_i 6.8.

The effect of external pH on the components of the Ca^{2+} efflux

In Fig. 2, it was shown (last part of the experiment), that in an axon in which virtually all of the

Fig. 3. Effect of internal pH on both ATP-dependent uncoupled (open symbols) and Na_o^+-dependent Ca^{2+} efflux (closed symbols) components. Ordinate: steady-state Ca^{2+} efflux at different pH_i values relative to that at pH 7.3. The symbols refer to different axons. Temperature 17–19°C.

Ca^{2+} efflux was Na_o^+-dependent (high $[Ca^{2+}]_i$, no ATP), increasing the external pH from 7.5 to 8.5, caused no significant effect on the level of the efflux. In similar experiments, the magnitude of the Na_o^+-dependent mechanism was not affected when the external pH was varied between 7.3 to 8.9. No attempt was made to study the effect of acidic pH_o on the Ca^{2+} efflux since in most of our experiments at pH_o below 7.2 the axons became progressively 'leaky' to calcium ions.

In the axon of Fig. 1 the effect of high external pH was explored on the magnitude of the ATP dependent uncoupled efflux. Alkalinization of the external medium from pH 7.6 to 9.0 in the absence of Na_o^+ and Ca_o^{2+} causes a 35% inhibition of this component. The reversibility of this effect is clearly seen after decreasing the pH_o to its initial value of 7.6. Fig. 4 summarizes the results of several experiments in which the magnitude of the uncoupled, and Na_o^+-dependent Ca^{2+} efflux components (at pH: 7.5), is plotted versus the extracellular pH. In the pH range 7.3–8.8, the magnitude of the Na_o^+-dependent mechanism remain clearly unaffected as compared with a significant decrease in the uncoupled efflux with increasing external pH. The fact that changing the external pH by more than one pH unit (7.3 to 8.8) causes no effect on the Na_o^+-dependent Ca^{2+} efflux, demonstrates a good control of the internal pH by the dialysis. This conclusion is based on the observation that internal alkalinization (fraction of pH unit) is effective in raising the Na_o^+-dependent Ca^{2+} efflux (see Fig. 3).

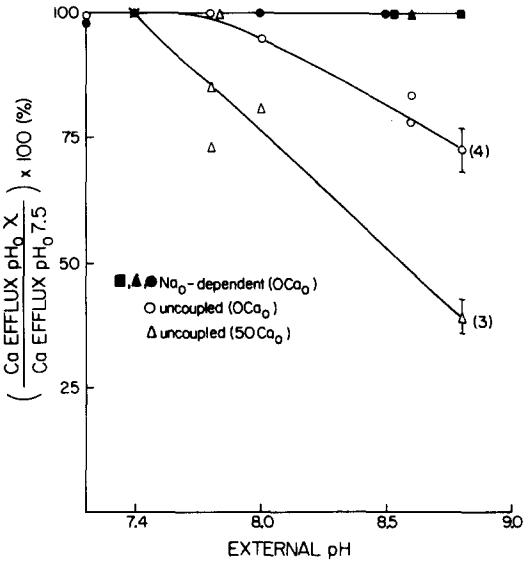


Fig. 4. Effect of external pH on both ATP-dependent uncoupled (open symbols) and Na_o^+-dependent Ca^{2+} efflux (closed symbols) components. Ordinate: Steady-state Ca^{2+} efflux at different pH_o values relative to that at pH_o 7.5. Note the absence of effect of pH_o on the Na_o^+-dependent Ca^{2+} efflux component (●,▲). ○, Uncoupled Ca^{2+} efflux at 0 Ca_o^{2+}. △, Uncoupled Ca^{2+} efflux in the presence of 50 mM Ca_o^{2+}. Temperature 17–19°C.

The combined effect of Ca_o and alkaline pH_o on the ATP-dependent uncoupled Ca^{2+} efflux

The dialysis technique, offers the possibility to manipulate independently both surfaces of the membrane, thus allowing to modify the environment of a 'site' when it faces the external or the internal medium. Experiments on sarcoplasmic reticulum vesicles, have shown that when the environment of the low-affinity Ca^{2+} site of the Ca^{2+} transport ATPase is changed properly (high Ca^{2+}, alkaline pH), it is possible to reverse the cycle of the Ca^{2+} pump (ATP synthesis) [8,9]. Since the ATP driven Ca^{2+} pump in squid axons must pick calcium ions from the inside (high-affinity site), translocate them across the membrane, and release to the outside (low-affinity site), in this section we have explored whether proper modifications of the external medium affects the uncoupled Ca^{2+} efflux in a manner to be expected for a 'potential' reversal of the Ca^{2+} pump.

Fig. 5 shows an experiment designed to explore the effect of external calcium ions on the ATP-dependent uncoupled efflux at normal (pH 7.8) and alkaline pH_o (8.8). At pH_o 7.8, and in the presence of 10 mM Ca_o^{2+}, the uncoupled efflux reaches a steady value of 27 fmol · $cm^{-2} \cdot s^{-1}$. Addition of 20 mM Ca_o^{2+} has no effect on the efflux level. However, a further increase in Ca_o^{2+} to 50 mM reduces the efflux value to about 20 fmol · $cm^{-2} \cdot s^{-1}$. As is also seen in Fig. 5, this small, but significant inhibitory effect of high Ca_o^{2+} on the uncoupled efflux observed at pH_o 7.8 is totally reversible, since removal of Ca_o^{2+} brings the efflux to 29 fmol · $cm^{-2} \cdot s^{-1}$. In the second part of the experiment of Fig. 5, the effect of Ca_o^{2+} on the uncoupled component was explored at a higher pH_o (8.8). Under this condition Ca_o^{2+} had a more

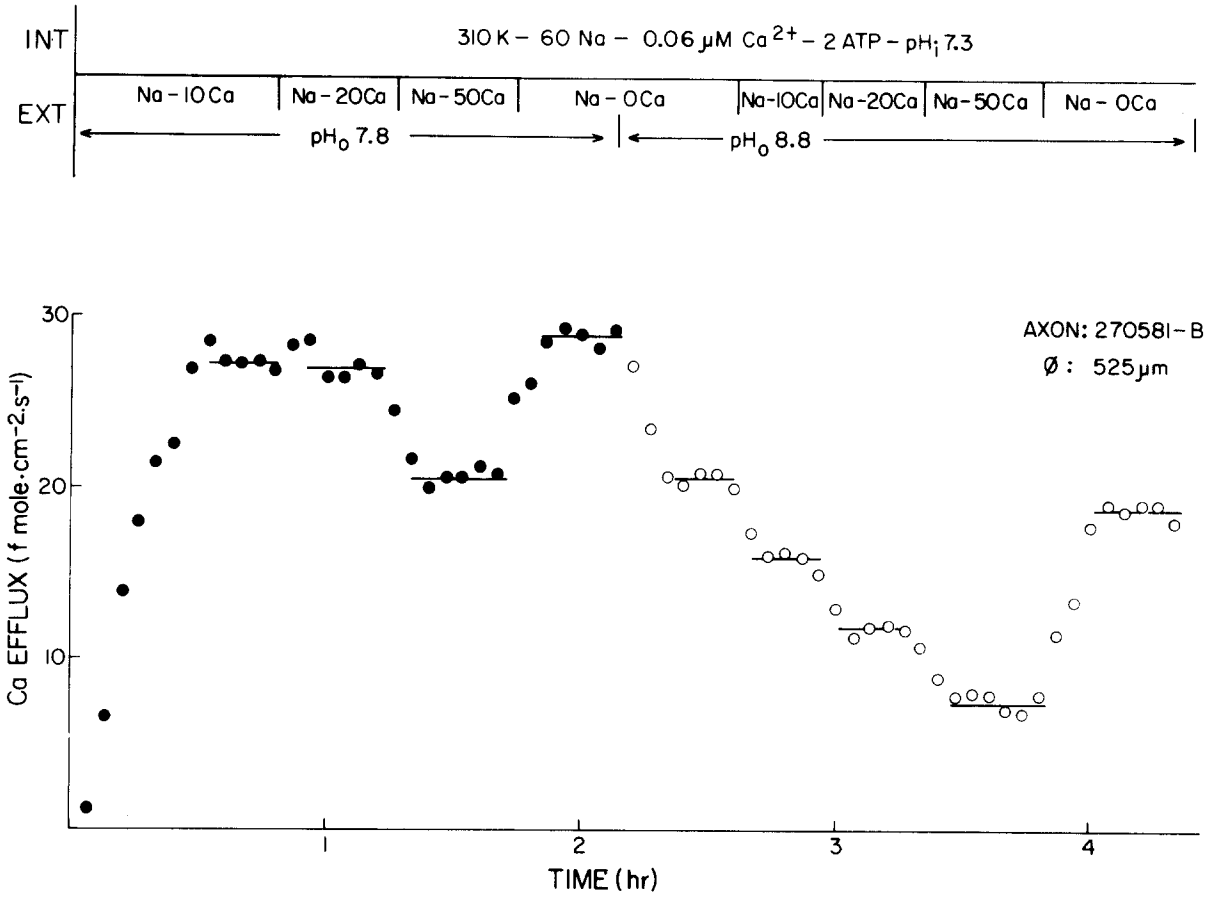
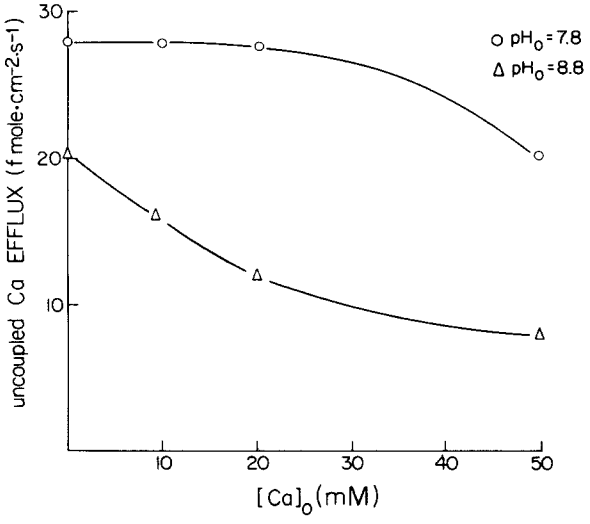


Fig. 5. The effect of external pH and Ca_o^{2+} on the magnitude of the uncoupled Ca^{2+} efflux component. Ordinate: Ca^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscissa: time in hours. Closed circles: Ca^{2+} efflux measurements at pH_o 7.8. Open circles: Ca^{2+} efflux at pH_o 8.8. Note the large inhibition of the uncoupled efflux induced by alkaline pH_o and Ca_o^{2+}.



pronounced inhibitory effect. At 50 mM Ca_o^{2+} and at alkaline pH_o, the uncoupled Ca^{2+} efflux is decreased by 65% from its value at 0 Ca_o^{2+}. The dependence of the uncoupled Ca^{2+} efflux on Ca_o^{2+} and pH_o is shown in Figs. 4 and 6. Two interesting observations can be deduced from them: (i) in the absence of Ca_o^{2+} the uncoupled component decreases with increasing pH_o. (ii) Increasing the Ca_o^{2+}, decreases the uncoupled efflux. This effect is markedly potentiated at alkaline pH_o.

Fig. 6. The effect of external pH on the inhibition of the uncoupled Ca^{2+} efflux by external Ca^{2+}. Data obtained from the same axon (Fig. 5). Ordinate: ATP-dependent uncoupled Ca^{2+} efflux in fmol·cm^{-2}·s^{-1}. Abscissa: external [Ca^{2+}] in mM. O, Efflux at pH_o 7.8. Δ, Efflux at pH_o 8.8.

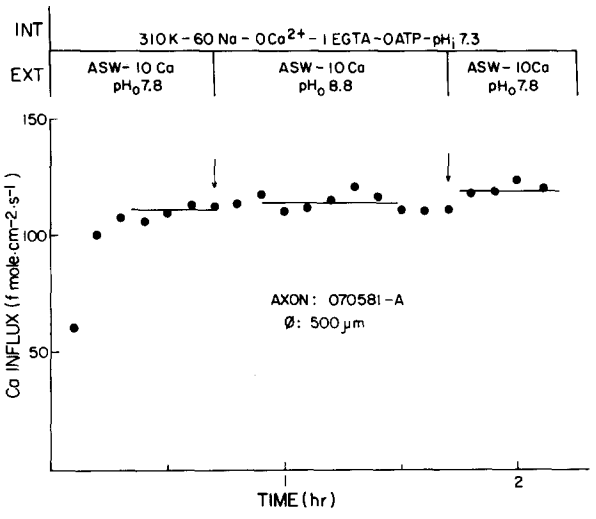


Fig. 7. Effect of external alkalinization on Ca^{2+} influx from an axon bathed in 10 mM Ca^{2+} artificial sea water (ASW). Ordinate: Ca^{2+} influx in fmol·cm^{-2}·s^{-1}. Abscissa: time in hours. Note the absence of changes in the Ca^{2+} permeability at high pH_o. Temperature 18°C.

The inhibition of the Ca^{2+} efflux by Ca_o^{2+} seen at alkaline pH_o can not be explained by a reduction in the Na_o^+-dependent component, first because at the $[Ca^{2+}]_i$ used in these experiments, the contribution of this component to the total Ca^{2+} efflux is rather small [11,17], and secondly, because as shown in Fig. 4, changes in the pH_o in the range from 7.3 to 8.8, causes no alterations of the forward Na^+/Ca^{2+} exchange. Since in red blood cells, raising the external pH increases the Ca^{2+} permeability [5], it could be argued that the observed effect of Ca_o^{2+} and pH_o is not directly on the Ca^{2+} pump mechanism (uncoupled Ca^{2+} efflux) but secondary to an increase in the Ca^{2+} influx as a result of external alkalinization (Ca^{2+} permeability increase). Such situation could lead to a decrease in Ca^{2+} specific activity near the inner membrane, thus resulting in a decrease in the Ca^{2+} efflux level. In order to test this hypothesis, Ca^{2+} influx experiments were carried out at two different pH_o values (7.8 and 8.8) and under similar external experimental conditions to those used to measure the ATP-dependent uncoupled component. The experiment in Fig. 7 clearly shows that external alkalinization has no effect on the level of the Ca^{2+} influx, thus making the above assumption very unlikely.

Discussion

In this work, we have studied the effect of external and internal $[H^+]$ changes on the plasma membrane Ca^{2+} extrusion mechanism present in squid axons (ATP driven Ca^{2+} pump and Na^+/Ca^{2+} exchange mechanism).

The observation that decreasing the internal pH below 7.3 causes an inhibition of the uncoupled Ca^{2+} efflux could have important physiological implications. In fact, since this mechanism is the main responsible for the maintenance of the resting $[Ca^{2+}]_i$ in nerve fibers [11,17], conditions that alter intracellular pH could result in changes in the level of $[Ca^{2+}]_i$.

The strong inhibition of the uncoupled efflux seen at acidic internal pH, could be associated with a decrease in the affinity of the inner 'site' towards Ca^{2+}. In favor of this are the pH studies on the SR Ca^{2+} pump in the sarcoplasmic reticulum vesicle which show that acidification decreases Ca^{2+} binding affinity (high- and low-affinity 'sites') of the Ca^{2+}-ATPase, several orders of magnitude [8]. The reduction of the ATP-dependent uncoupled efflux by low pH_i, agrees rather well with a similar inhibition of the Ca^{2+}-ATPase from purified membranes from squid optic nerve fibres at acid pH [18]. This squid axon membrane (Ca^{2+}, Mg^{2+})-ATPase, which is presumably the biochemical expression of the active Ca^{2+} transport mechanism (uncoupled Ca^{2+} efflux) [19] has an optimum in its activity at pH 7.3, decreasing 50% at about pH 6.5, and being fully inhibited at pH 5.0. Although the mechanism by which internal acidification affects the Ca^{2+} pump is unknown, it is interesting that most of the inhibitory effect occurs in the narrow range from pH_i 6 to 6.5. One explanation could be that some structure near or at the internal active Ca^{2+}-binding site (a negative charged proton-accepting group) contains residues which might modify its affinity for calcium ions depending on the pH_i value (possibly carboxyl or imidazole groups).

Acidification of the internal medium (below pH_i 7.3) causes also an inhibition of the Na_o^+-dependent Ca^{2+} efflux (forward Na^+/Ca^{2+} exchange) (see Fig. 2). However, the sensitivity of this component to a decrease in pH_i, is greater than that of the ATP dependent uncoupled mecha-

nism. In fact, at pH_i 7.0 the Na_o^+-dependent component is only 60% of its value at pH_i 7.3, compared to 90% for the uncoupled efflux. This inhibition of the Na_o^+-dependent Ca^{2+} efflux by internal acidic pH, can be correlated with the observation that in squid axons the Na_i^+-dependent Ca^{2+} influx (backward Na^+/Ca^{2+} exchange) is also inhibited at low pH_i [6].

The experiments on the effect of high internal pH upon the Na^+/Ca^{2+} exchange mechanism (Na_o^+-dependent Ca^{2+} efflux), are of special interest. Alkalinization to pH_i 8.5 causes a strong activation of this component (4-fold) as compared with the absence of effect on the ATP-dependent uncoupled system. This result further supports the model of two separate Ca^{2+}-transport systems initially proposed in squid axons [10,11], and recently postulated in other preparations (Ref. 20, for a review on this topic, see Ref. 21). An experimental result related to the present finding is that internal alkalinization increases the Ca_o^{2+}-dependent Na^+ efflux in intact squid axons [6]. Similarly, Na^+-dependent Ca^{2+} uptake (Na^+/Ca^{2+} exchange) by myocardial and neuroblastoma cells in culture showed an increased rate with increase in pH [22].

The effect of external pH on the ATP-dependent Ca^{2+} efflux should be compared with that of the passive carrier mediated process (Na^+/Ca^{2+} exchange): (i) raising the pH_o from 7.3 to 9.0, does not alter the magnitude of the Na_o^+-dependent Ca^{2+} efflux (forward Na^+/Ca^{2+} exchange). (ii) The ATP-dependent uncoupled component exhibits a complex dependence on pH_o. In the absence of Ca_o^{2+}, external alkalinization always induces a modest inhibition of the uncoupled efflux. In the presence of external Ca^{2+} external alkalinization greatly enhanced the inhibition of this component. The combined inhibitory effect of alkaline pH_o and external Ca^{2+} on the uncoupled Ca^{2+} efflux, could be of significant importance considering its possible relation with reaction steps in the cycle of the Ca^{2+} pump. Extensive studies on the Ca^{2+}-ATPase from sarcoplasmic reticulum vesicles [23,24] have shown that these vesicles can build up a $[Ca^{2+}]$ gradient at the expense of ATP hydrolysis (forward reaction). Under appropriate conditions, they can also catalyze a steady exchange between P_i and the phosphate of ATP (reverse reaction) [25,26]. In this preparation, simultaneous alkalinization of the low-affinity Ca^{2+} 'site' (interior of the vesicle) in the presence of saturation concentrations of Ca^{2+}, greatly enhanced the reversal of the Ca^{2+} pump [7,8]. The results presented in this paper, suggest that under the above conditions a similar mechanism might be operating for the case of the Ca^{2+} pump in squid axons. Hence, a possible explanation for the effect of pH_o and Ca_o^{2+} on the uncoupled Ca^{2+} pump, is that an increase in Ca^{2+} affinity of the low-affinity 'site' (site facing the external medium) induced by alkaline pH_o will favor a reversal reaction of the pump in the presence of high concentrations of Ca_o^{2+}. This in turn, would induce a decrease in the forward reaction of the Ca^{2+} pump (ATP dependent uncoupled Ca^{2+} efflux).

In summary, the marked differences in the pH dependency for the two modes of Ca^{2+} efflux, added to the inhibition of the uncoupled Ca^{2+} efflux by Ca_o^{2+} (reversal of the pump) gives further strong support for the existence of two separate mechanisms of Ca^{2+} extrusion in squid axons.

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