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# The effects of manganese and changes in internal calcium on Na-Ca exchange fluxes in the intact squid giant axon

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**The effects of manganese chloride were studied on Na-Ca exchange fluxes from intact squid axons. Ca uptakes and $Ca_o$-dependent sodium efflux were inhibited half-maximally by 3–7 mM $MnCl_2$. Mn inhibition appears less during $Na_o$-$Ca_i$ exchange (half-maximal inhibition; 30 mM) than that during $Ca_o$-$Na_i$ exchange, even when both fluxes were activated with 100 mM Na. The effects of changes in $[Ca_i^{2+}]$, effected by Ca-EGTA injection or inhibition of mitochondrial Ca uptake by ruthenium red, were examined on the reverse ($Ca_o$-$Na_i$) exchange mode. Ca-EGTA mixtures, designed to raise $[Ca_i^{2+}]$ above 2 $\mu$M, inhibited $Ca_o$-$Na_i$ exchange fluxes. Ruthenium red inhibited mitochondrial Ca buffering to effect increases in $Ca_i$ in the absence of Ca chelators; it activated $Na_o$-$Ca_i$ exchange fluxes but had little effect on $Ca_o$-$Na_i$ exchange despite similar reported $K_m$ for $Ca_i$. The results reflect the difficulty in demonstrating the stimulatory effect of $[Ca_i^{2+}]$ on $Ca_o$-$Na_i$ exchange fluxes in intact axons.**

## Introduction

Calcium asymmetry across plasma membranes is established and maintained by two major efflux mechanisms; an ATP-dependent pump and $Na_o$-$Ca_i$ exchange [9,26]. Both processes have been studied extensively in the squid axon where, depending on the levels of ATP and $Na_i$, $Na_o$-$Ca_i$ exchange has low affinity for $Ca_i$ ($K_m$ of the order of 1–10 $\mu$M) but large Ca transporting capacity, and the Ca pump high affinity ($K_m$ approx. 0.2–0.3 $\mu$M) but low transporting capacity [13,23]. Although no selective inhibitor of Na-Ca exchange has yet been determined [26,28], a few studies have been conducted using the alkaline earth metals (e.g., Mn, Ni and Co) to inhibit exchange activity in dialysed squid axons [13], sarcolemmal vesicles [37], smooth muscle [1] and exchange currents in ventricular myocytes [29]. However, few data are available on the effects of these cations on unidirectional Na-Ca exchange fluxes in intact cells (cf. bullfrog ventricle: Ref. 16).

Na-Ca exchange activity in intact, dialysed or perfused cells is asymetric with a lower Ca affinity at the outside membrane face (during $Ca_o$-$Na_i$ exchange) compared to the inside face (during $Na_o$-$Ca_i$ exchange) [8,13,22,30]. This feature is not readily observed when investigated in membrane vesicles, where a symmetrical nature with high Ca affinities on both membrane faces is normally found [32,33,35]. $Ca_o$-$Na_i$ exchange displays a greater difference in studies between vesicles and intact or dialysed cells; it is also stimulated by $Ca_i$ during unidirectional flux studies on dialysed and perfused cells [21,25,34] but not generally in vesicles [32]: one problem is the occurrence of Ca accumulation within the intravesicular space [36]. $Ca_o$-$Na_i$ exchange fluxes in the dialysed squid axon are half-maximally activated by $Ca_i$ of 2 $\mu$M [25], whilst in the perfused barnacle muscle cell the value is much lower at 0.3 $\mu$M [34]. Furthermore the exchange current in guinea-pig myocytes is half activated by a much lower $Ca_i$ level of 0.02 $\mu$M [30]. This catalytic or stimulatory effect of $Ca_i$ on $Ca_o$-$Na_i$ exchange has not yet been clearly described in intact cells, although evidence indicating this has been presented by injection of Ca chelators to lower $Ca_i$ [2,6,7]. The experiments in this study were designed to examine in the intact squid axon stimulation of $Ca_o$-$Na_i$ exchange fluxes by $Ca_i$.

## Materials and Methods

*Axon preparation.* Hindmost stellar giant axons were dissected from *Loligo forbesi*. The preparation of axons

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Mops, 3-(*N*-Morpholino)propanesulfonic acid.

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for influx and efflux studies and the measurement of resting potential and ionised calcium with aequorin was as described in detail in Allen and Baker [3] and Baker et al. [9].

*Flux measurements.* $^{45}Ca$ influx measurements were carried out at room temperature (21–23°C). Axons were preincubated for 5–10 mins in test media lacking $^{45}Ca$ and then transferred to the same media containing $^{45}Ca$ for 10–15 min. Uptake into axoplasm was determined by the methods of Baker et al. [8]. All influxes were done in the presence of 0.6 $\mu$M tetrodotoxin and $10^{-5}$ M ouabain to inhibit Ca entry through Na channels and Na-K exchange pump activity, respectively. Efflux determinations were performed from axons that had been carefully cleaned of small nerve fibres and adhering connective tissue, mounted in a perspex chamber and axially microinjected with isotope (3–5 mm column). Temperature was maintained at 17–18° C. Artificial sea water superfusing the axon was sampled at 1–2 min. intervals and counted in a gammacounter ($^{22}Na$) or by liquid scintillation counting ($^{45}Ca$).

*Solutions.* Axons were immersed in artificial sea waters (ASW) of composition (in mM); NaCl, 400; $MgCl_2$, 100; KCl, 10; $CaCl_2$, 10; $NaHCO_3$, 2.5; pH 7.8. Changes in cation concentrations are detailed in each figure legend. To minimize the effects of extracellular calcium binding on $^{45}Ca$ efflux, the calcium content of Ca-free solutions was adjusted to 50 $\mu$M [12].

*Materials.* $^{45}Ca$ and $^{22}Na$ were obtained as their chloride salts from Amersham International (U.K.). Chemicals were Analar grade or equivalent. LiCl was obtained from Fisher (U.S.A.). Mn, Ni and Co, as chloride salts, were used from freshly prepared solutions.

## Results

*Effects of manganese (Mn) on calcium influxes*

In squid axons Ca influx is increased when external Na is replaced by choline, Li or K and in each case the flux is strongly dependent on internal sodium [8]. This entry is partly due to removal of competition between external Na and Ca at the Ca binding site of $Ca_o$-$Na_i$ exchange, and partly through activation at a separate monovalent cation activating site which increases the affinity of the $Ca_o$-$Na_i$ exchanger for external Ca [3]. The contribution of Ca-Ca exchange to Ca influx, which is also activated by monovalent cations, is relatively small in axons containing ATP [14,21].

Table I summarizes experiments measuring Ca uptakes from artificial sea waters (ASW) lacking sodium but containing 10 mM Ca: the inclusion of $MnCl_2$ (10–25 mM) reduced Ca influxes by an average of 64% ± 4.5. These findings show that under conditions which activate $Ca_o$-$Na_i$ exchange fluxes to differing levels, Mn inhibition of Ca influx is quite similar.

TABLE I

*The effects of Mn on calcium uptakes from lithium and choline waters*

Sodium content of artificial sea water was replaced by the monovalent cations listed under Condition. Axons exposed to choline sea water were freshly dissected from living squid, whereas other axons had been sodium loaded by storage at 4°C for 3–6 h. All solutions contained 0.6 $\mu$M TTX. Mean calcium uptakes ± standard error are given.

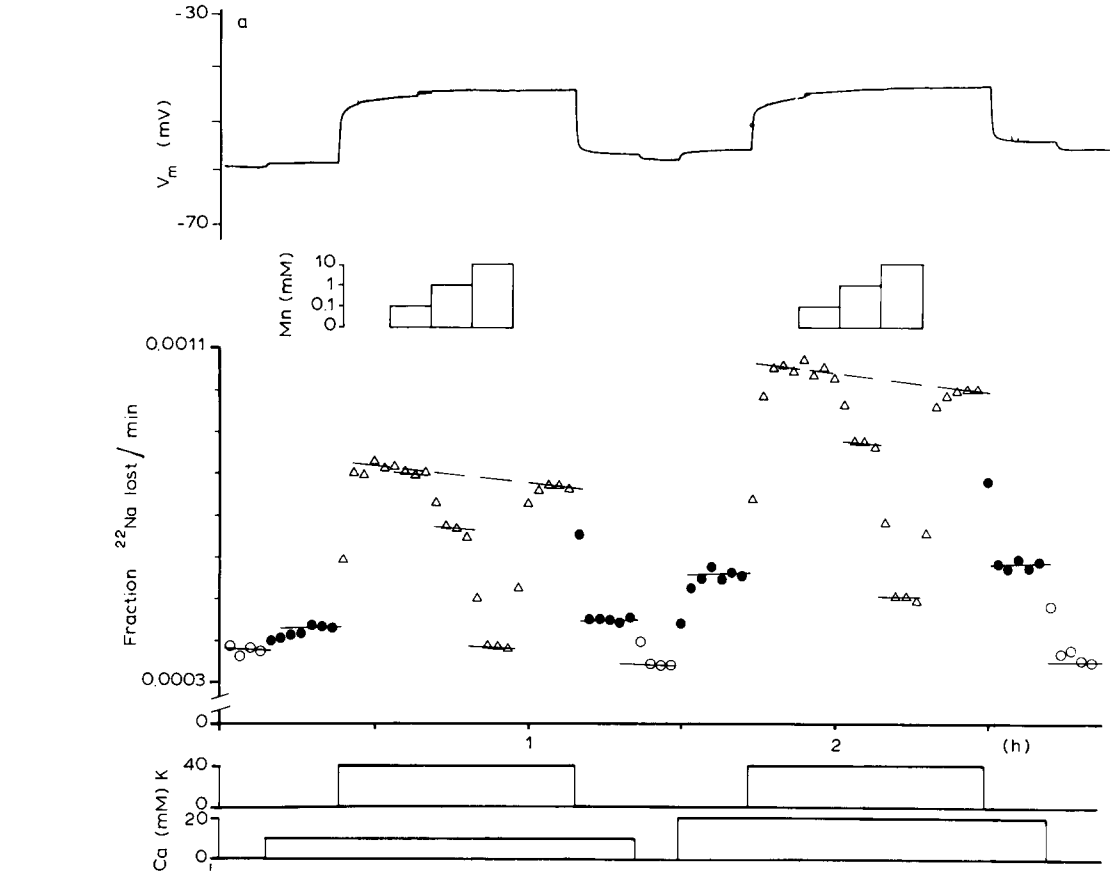
| Condition | Ca uptakes ($pmol \cdot cm^{-2} \cdot s^{-1}$) | | % reduction |
|---|---|---|---|
| | Control | Test | |
| Lithium sea water | | | |
| 25 mM $Mn^{2+}$ | 4.2 ± 0.8 ($n = 7$) | 1.4 ± 0.2 ($n = 5$) | 67 |
| Choline sea water | | | |
| 10 mM $Mn^{2+}$ | 0.51 ± 0.1 ($n = 3$) | 0.2 ± 0.1 ($n = 3$) | 61 |
| 50K-Choline sea water | | | |
| 10 mM $Mn^{2+}$ | 3.96 ± 1.2 ($n = 3$) | 1.30 ± 0.4 ($n = 3$) | 67 |

However, to determine the magnitude of Ca influx via $Ca_o$-$Na_i$ exchange, it would still be nescessary to examine further the effects of changes in $Na_i$ under these conditions.

*Effects of manganese on $Ca_o$-$Na_i$ exchange ($Ca_o$-dependent $^{22}Na$ efflux)*

Another way to study $Ca_o$-$Na_i$ exchange in squid axons is to monitor $Ca_o$-dependent $^{22}Na$ efflux from either intact axons injected with $^{22}NaCl$ [3,8] or from axons intracellularly dialysed with media containing $^{22}NaCl$ [25,25]. The experiment illustrated in Fig. 1a was performed on an intact axon superfused with choline sea water; addition of 10 mM calcium activated a small component of efflux due to the low Ca affinity of the exchange to $Ca_o$ in the nominal absence of small monovalent cations (half-maximal activation occurs with 50–60 mM $Ca_o$ [3,8]. 40 mM potassium increased sodium efflux primarily due to activation at a monovalent cation activating site to increase the affinity for $Ca_o$ (half maximal activation with 17 mM $Ca_o$; [3]). Mn reversibly inhibited the $Ca_o$-dependent $^{22}Na$ efflux into potassium-choline mixtures containing 10 mM and 20 mM calcium. This inhibitory effect of Mn was seen in each of seven studied axons. Half-maximal inhibition by Mn lies close to 2–3mM (Fig. 1b). By simply assuming that Ca and Mn compete for the same site and taking the value of 17 mM for the Ca concentration for half-maximal activation of the $Ca_o$-dependent $^{22}Na$ efflux [3], the apparent affinity of the exchanger for Mn is approx. 1.5 mM.

Fig. 2 shows an experiment examining Mn inhibition of $Ca_o$-$Na_i$ exchange in the nominal absence of small monovalent cations, where the exchanger has a lower affinity for $Ca_o$ (50–60 mM required for half-maximal



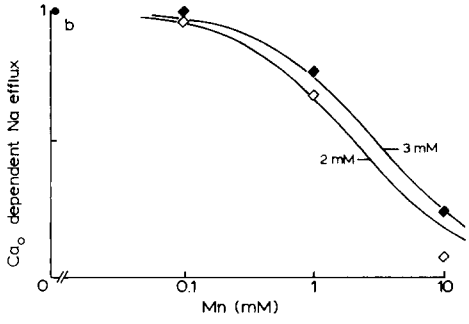




Fig. 1. The effects of Mn on the potassium stimulated calcium-dependent sodium efflux. (a) Raw data showing the inhibition of the $Ca_o$-dependent Na efflux by Mn and its reversibility at two levels of calcium. Raising calcium from 10 mM to 20 mM clearly increases the magnitude of the sodium efflux measured in the absence and presence of potassium. External medium: choline sea water containing $10^{-5}$ M ouabain to inhibit the sodium pump. (b) Relationship between Mn concentration (abcissa) and the normalized potassium-stimulated $Ca_o$-dependent Na efflux (ordinate). Open diamonds, 10 mM $Ca_i$; closed diamonds, 20 mM Ca. Curves drawn with calculated apparent $K_i$ for Mn of 2 mM and 3 mM.

activation). The axon was preinjected with the photoprotein aequorin to permit recordings of changes in free Ca brought about by exchange activity [3,19]. Addition of Mn (5–20 mM) brought about a parallel reduction in both $Ca_o$-dependent $^{22}$Na efflux and aequorin glow. This latter reduction in glow may in part reflect reduced entry of Ca via the exchange and also attenuation of the Ca-aequorin reaction due to entry of Mn. From this experiment, 50% inhibition of the $Ca_o$-dependent $^{22}$Na efflux required approx. 10 mM Mn. Raising Mn to higher levels affected the osmolarity of the bathing medium, but inhibition was not complete at 20 mM Mn. If we again simply assume competition between Ca and Mn for the same external site, the experiment indicates that the affinity of the exchanger for Mn is now approx. 3.5 mM.

Fig. 3 extends these observations to two other alkali earth metals; Co and Ni. Co and Ni inhibited $Ca_o$-dependent $^{22}$Na efflux in a manner similar to Mn; 1–2 mM produced half-maximal inhibition of the $Ca_o$-dependent $^{22}$Na efflux into choline sea waters containing 40 mM K. These inhibitory effects of Co and Ni were observed in each of three studied axons. In the nominal absence of Ca none of these cations at 10 mM was able to activate Na efflux but instead each brought about a further small reduction in efflux, suggesting that the exchange does not transport these cations or has very low affinity under these conditions.

*Effects of manganese on $Na_o$-$Ca_i$ exchange ($Na_o$-dependent $^{45}$Ca efflux)*

Fig. 4 illustrates the effects of Mn on the $Na_o$-depen-

dent ($Na_o$-$Ca_i$ exchange) and the uncoupled (ATP-dependent Ca pump) $^{45}Ca$ efflux from an unpoisoned axon. In nominally Ca-free sea waters, which prevents Ca-Ca exchange activity [11,16], Mn partially inhibits both components of Ca efflux ($n = 2$ axons); both fluxes are 50% inhibited by approx. 30 mM Mn (Fig. 4b). Similar experiments also carried out on cyanide-poisoned axons, where the uncoupled efflux is missing and the $Na_o$-dependent component of Ca efflux is greater, required even higher Mn concentrations to effect half-maximal inhibition in 400 mM $Na_o$ (approx. 50 mM Mn, $n = 2$ axons).

In the presence of ATP, half-maximal activation of the $Na_o$-dependent Ca efflux required close to 50 mM sodium, whilst in the nominal absence of ATP between 150 and 200 mM is required [10,11,14]. By simply assuming that Mn and Na compete for the same sites on the exchange, then the apparent affinity of the exchanger for Mn is approx. 3–4 mM in unpoisoned axons and approx. 15 mM in poisoned axons. However, to determine this value more precisely more data are required. In another set of experiments (two axons), conducted in choline sea water containing 100 mM sodium which permits activation of both forward and reverse exchange modes [4,8,11,15], the Mn concentration to effect half-maximal inhibition of the $Na_o$-dependent $^{45}Ca$ efflux was 30 mM (apparent $K_i$ of 10 mM taking $K_m$ for Na of 50 mM), and 7 mM for $Ca_o$-dependent $^{22}Na$ efflux (apparent $K_i$ of 1 mM taking $K_m$ for Ca of 2 mM).

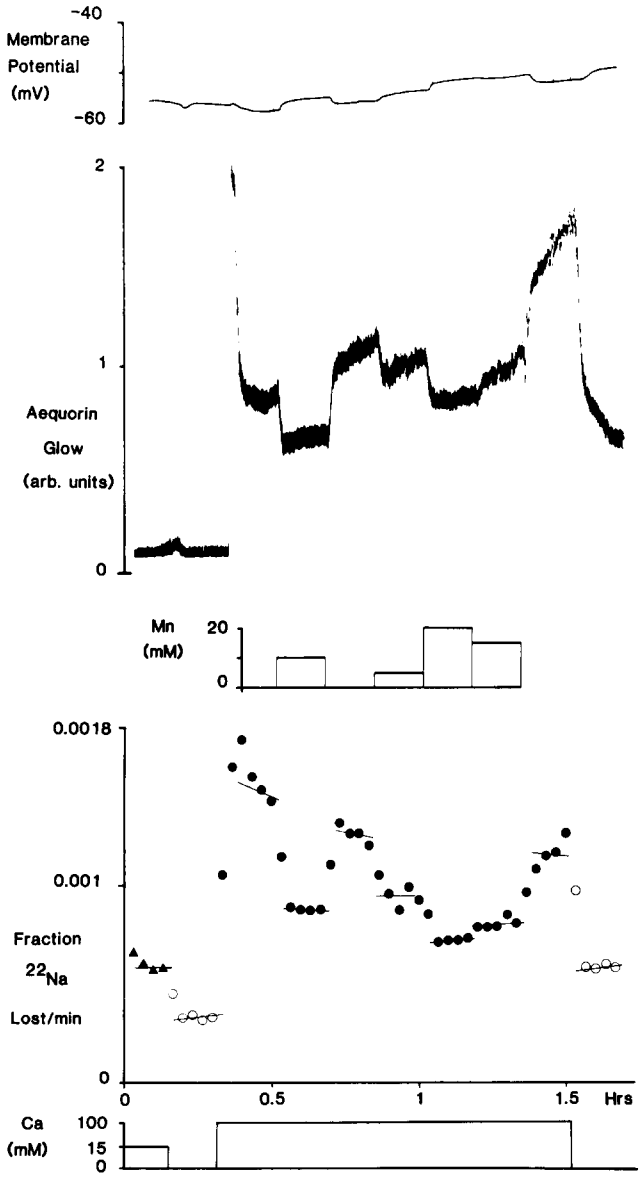



Fig. 2. The effects of Mn on $^{22}Na$ efflux and aequorin glow in choline sea water. The calcium-dependent sodium efflux into choline sea water containing 100 mM calcium and changes in intracellular free calcium monitored by aequorin glow. Magnesium-free solutions (except in 15 mM Ca) containing $10^{-5}$ M ouabain.

From these data Mn inhibition of both forward and reverse exchange modes appears dependent on not only the activating cations (Na or Ca), but also the levels of ATP. To fully explore these possibilities in detail more experiments are necessary, under more carefully defined kinetic conditions.

*Effects of manipulations in $Ca_i$ by Ca chelators on $Ca_o$-$Na_i$ exchange*

Injection of EGTA and EDTA into intact squid axons inhibits $Ca_o$-dependent $^{22}Na$ efflux and the associated calcium inflow [6,7]. A simple explanation for this effect has been provided in dialysed squid axons, perfused barnacle muscle fibres and dialysed guinea-pig myocytes; $Ca_i$ half-maximally activates $Ca_o$-$Na_i$ exchange activity between 2 $\mu$M [25], 0.3–0.7 $\mu$M [34] and 0.02 $\mu$M [30], respectively. This role for $Ca_i$ during $Ca_o$-$Na_i$ exchange has not been clearly demonstrated in intact cells.

An attempt to raise and stabilize internal free Ca close to 3 $\mu$M in an intact axon is illustrated in Fig. 5. After the monitoring of the K-dependent $^{22}Na$ efflux (Na pump activity) into sodium ASW and the $Ca_o$-dependent $^{22}Na$ efflux into lithium ASW ($Ca_o$-$Na_i$ exchange), the axon was injected along a 2.5 cm length by a Ca : EGTA mixture ratio of 0.9 : 1.0. This solution overlapped the column of $^{22}Na$ inside the axon and gave a final concentration of 2.49 mM Ca and 2.77 mM EGTA. Removal of K shows that the Na pump is largely unchanged but the subsequent substitution of Na for Li reveals that the $Ca_o$-dependent $^{22}Na$ efflux is almost completely inhibited, with little change in the baseline fluxes. This suggests that if the Ca-EGTA buffer evoked a rise in $Ca_i$ above prevailing levels then $Ca_o$-$Na_i$ exchange is not greatly stimulated. One possibility is that Ca-EGTA is unable to maintain high levels of free Ca inside the axon; the transient increase in sodium efflux during chelator injection in Fig. 5 does indeed suggest this. However, this transient was not a consistent observation and may be an injection artefact.

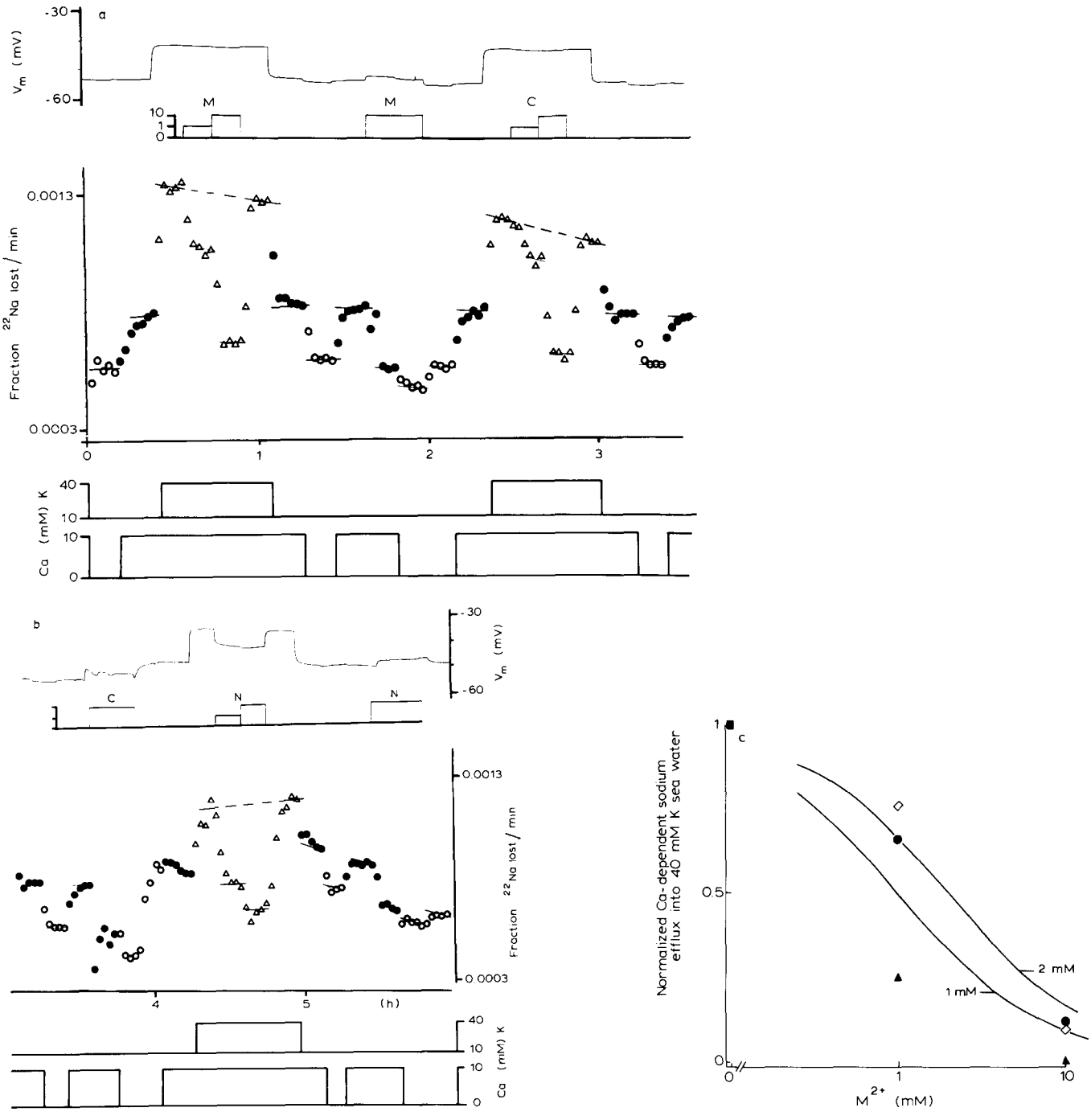



Fig. 3. Comparison of the effects of Mn, Co and Ni on the Ca-dependent $^{22}$Na efflux into choline and choline-potassium sea water mixtures. (a + b) Raw data to show the effects of 1 mM and 10 mM cation on the components of sodium efflux in the presence and nominal absence of calcium. Letters: (in mM) M = Mn, C = Co, N = Ni. Open circles, Ca-free media; closed circles, media containing 10 mM Ca; triangles, media containing 10 mM Ca and 40 mM K. Ouabain ($10^{-5}$ M) was present in all solutions to inhibit sodium pump activity. In this experiment sodium efflux in Ca-free sea water was apparently activated by Co, but this was not seen in other experiments. (c) Normalized data showing the concentration-dependent inhibition of potassium activated Ca-dependent $^{22}$Na efflux by divalent cations ($M^{2+}$). Circles, Mn; triangles, Ni; diamonds, Co. Curves drawn with apparent $K_i$ of 1 mM and 2 mM. Ni appears to be the most potent of the group in inhibiting $Ca_o$-$Na_i$ exchange fluxes, but note Ni also hyperpolarized the membrane potential in potassium (b).

Table II summarizes all observations on the effects intracellular EGTA and BAPTA buffers on the $Ca_o$-dependent $^{22}$Na efflux in seven axons, BAPTA being a less pH dependent Ca chelator. Inhibition by Ca : EGTA mixtures was still seen when injected at a final low concentration of 0.15 mM : 0.13 mM with 33 mM Mops

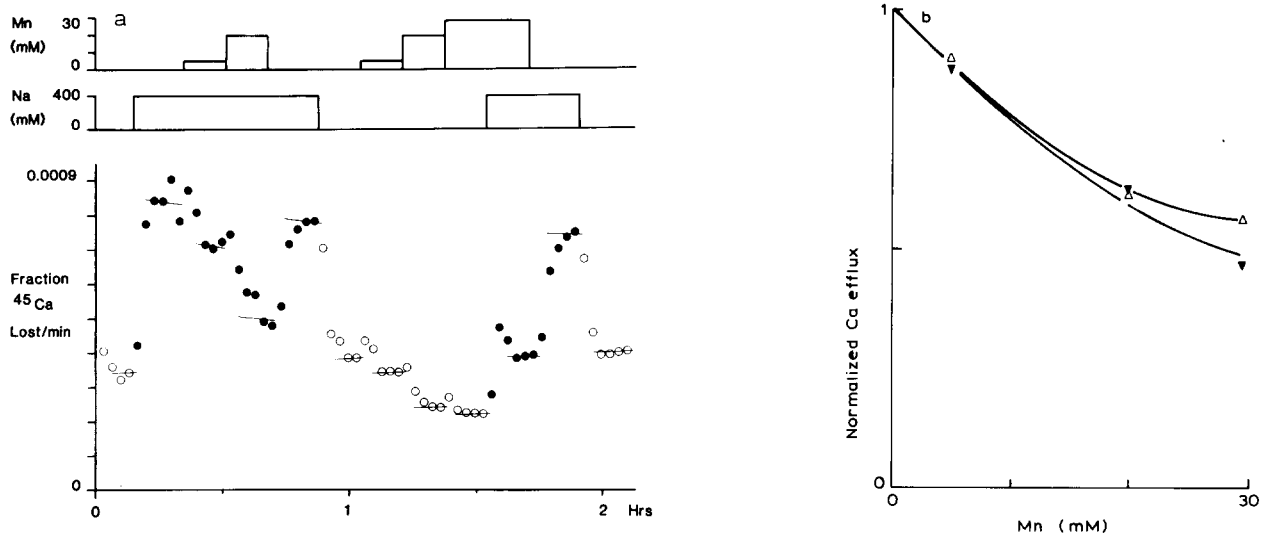



Fig. 4. Effects of Mn on the Na and Na-independent components of calcium efflux. (a) Raw data showing in the first half of the figure activation of $Na_o$-$Ca_i$ exchange by Na and inhibition by differing concentrations of Mn; in the second half of the figure Mn is added to the Na-independent (uncoupled 'Ca pump') calcium efflux. (b) Normalized calcium efflux from (a) after correcting for changes in baseline. Open triangles, uncoupled calcium efflux; closed triangles, Na-dependent calcium efflux. Curves drawn by eye. Media contained 50 $\mu$M calcium.

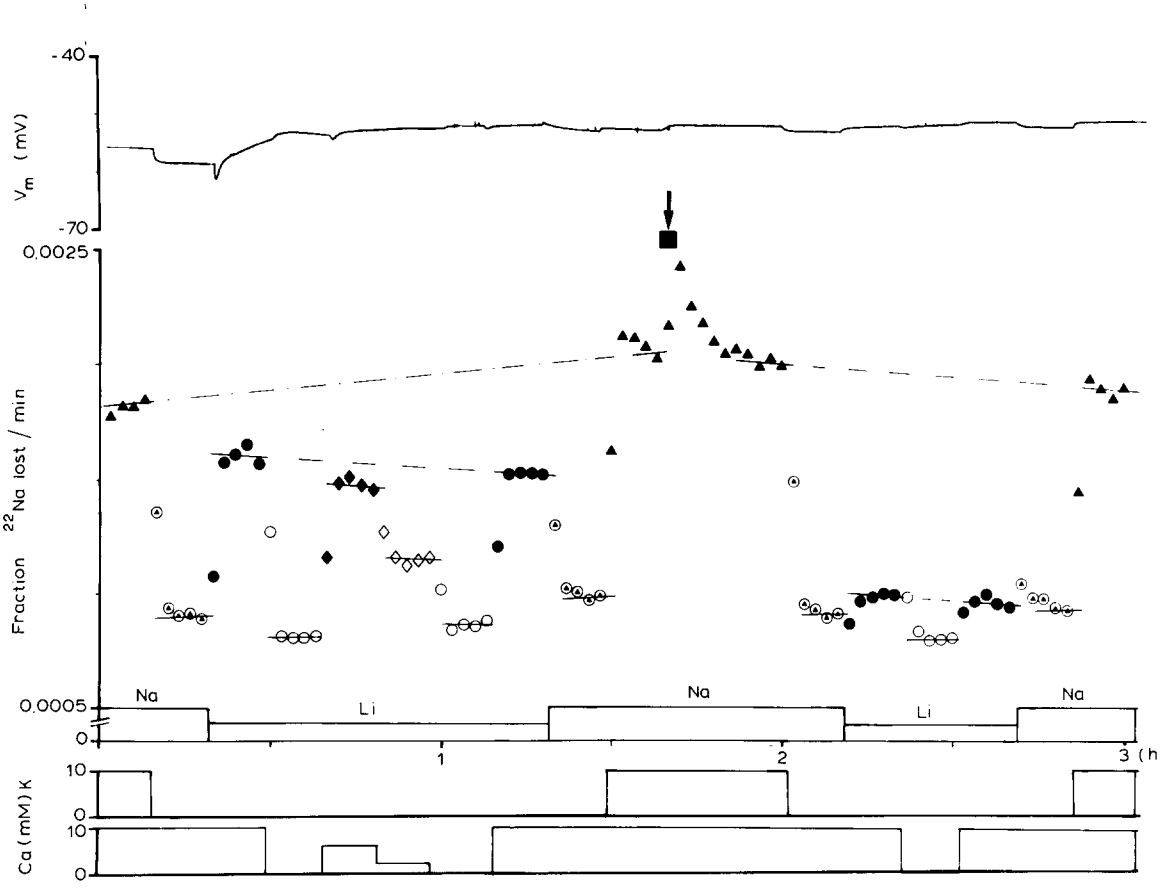



Fig. 5. Effects of intracellular Ca-EGTA on the sodium pump and the Ca-dependent sodium efflux. In sodium-sea water, **Na**, removal of K inhibits the sodium pump, whilst in lithium sea water, **Li**, changes in Ca affect $Ca_o$-$Na_i$ exchange. Arrow indicates period (approx. 1 min) during which a final concentration of 2.77 mM EGTA, 2.49 mM Ca and 2.77 mM Hepes was injected into axon interior. Estimated free calcium: 3 $\mu$M. Axon was preinjected with $^{22}$Na, then the injector loaded with Ca-EGTA was positioned inside the axon. Axon was initially sodium-loaded by stimulation at 100 Hz for 25 min in artificial sea water containing $10^{-5}$ M ouabain.

as pH buffer (pH 7.3), and when Ca was in excess of Ca chelator. All chelator mixtures included the pH buffer Mops to prevent possible axoplasmic acidification due to activation of mitochondrial Ca-H exchange [18]. Control experiments showed that Mops injected alone had no effect on the $Ca_o$-dependent $^{22}Na$ efflux (see also Ref. 3). The data show that calcium chelator mixtures designed to raise internal $Ca_i$ were unable to effect stimulation of $Ca_o$-dependent $^{22}Na$ efflux in intact axons.

*Effects of manipulations in $Ca_i$ by ruthenium red on $Ca_o$-$Na_i$ exchange*

As calcium chelator mixtures failed to stimulate $Ca_o$-dependent $^{22}Na$ efflux in intact axons, another means was sought to effect an increase in $Ca_i$ in intact axons. Ruthenium red is an established inhibitor of mitochondrial Ca uptake [19,31] and at $\mu$M levels effectively blocks Ca sequestration in isolated squid axoplasm [13]. Fig. 6 illustrates an experiment that uses intracellular injection of ruthenium red to inhibit mitochondrial Ca buffering during operation of $Ca_o$-$Na_i$ exchange. At a final concentration of 170 $\mu$M, ruthenium red evoked a rapid, large increase in free Ca when calcium is added externally, but was without large effect on the $Ca_o$-dependent $^{22}Na$ efflux monitored into lithium sea water. Prior to ruthenium red injection, changes in the $Ca_o$-dependent $^{22}Na$ efflux had little effect on free Ca at the low setting of the amplifier gain, but after its injection re-activation of $Ca_o$-$Na_i$ exchange is associated with a large rise in free Ca; reflecting calcium entry on the exchange which is usually removed from the cytosol. Despite the large changes in free calcium, the efflux into lithium sea water is little changed. This effect of ruthenium red was seen in each of three studied axons.

In the presence of ATP $Na_o$-$Ca_i$ exchange in squid axons is half-maximally stimulated by $Ca_i$ of approx. 2 $\mu$M [14,23], and the experiment illustrated in Fig. 7 examines the effects of ruthenium red on the components of calcium efflux from an intact axon. The figure

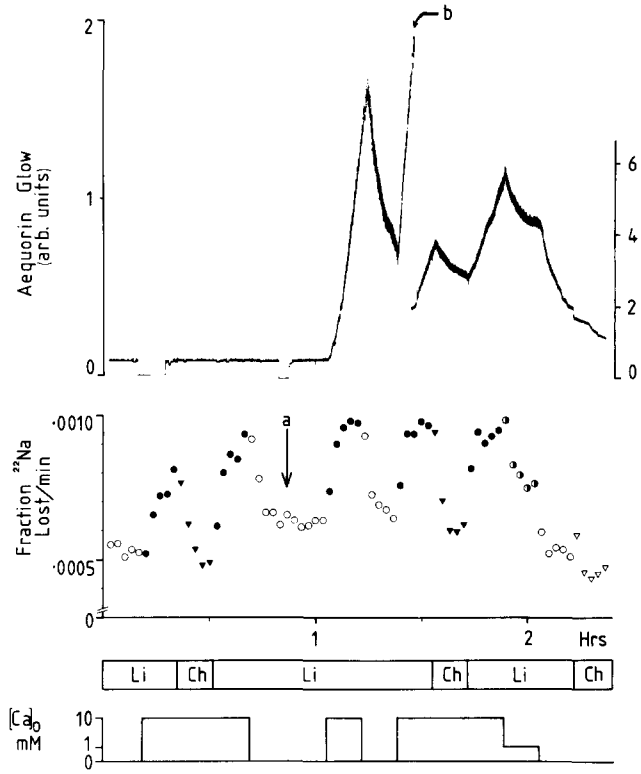



Fig. 6. The effects of intracellular ruthenium red on the calcium-dependent sodium efflux. Axon was preinjected with aequorin and then 25 min later with $^{22}Na$. At Arrow a, a final concentration of 170 $\mu$M ruthenium red with 3.4 mM Mops (pH 7.3) was injected intracellularly. Readdition of calcium activated the calcium-dependent sodium efflux and lead to a large increase in free calcium. Arrow b denotes point at which amplifier gain was reduced. Li, lithium sea water; Ch, choline sea water. The photomultiplier tube was switched off for two periods during the experiment.

shows that injection of ruthenium red (final concn. 210 $\mu$M) had no immediate effect on the $Na_o$-dependent $^{45}Ca$ efflux, but after a period of exposure to low Na sea water containing 10 mM Ca to impose a $Ca_i$ load, the Ca efflux is markedly increased following return to Na sea water. The uncoupled ($Na_o$ & $Ca_o$-independent) calcium efflux (ATP-dependent 'calcium pump') is little

TABLE II

*The effects of EGTA- and BAPTA-calcium mixtures on the $Ca_o$-dependent $^{22}Na$ efflux*

All chelator:Ca mixtures included pH buffer (Mops or Hepes) at a concentration equal to or in excess of chelator and buffered to pH 7.3. Percentage inhibition of the $Ca_o$-dependent $^{22}Na$ efflux was measured relative to the control flux before buffer injection.

| Axon diameter ($\mu$m) | Chelator:Ca ratio | Final concn. (mM) | % inhibition | Time measured after injection (min) |
|---|---|---|---|---|
| 743 | 1:1.2 | 4.2 EGTA:5 Ca | 80 | 15 |
| 657 | 1:1.1 | 5.4 EGTA:5.9 Ca | 24 | 40 |
| 686 | 1:1.1 | 4.9 EGTA:5.4 Ca | 15 | 10 |
| 857 | 1:1.2 | 0.13 EGTA:0.15 Ca | 50 | 60 |
| 886 | 1:0.5 | 0.3 BAPTA:0.15 Ca | 100 | 40–50 |
| 657 | 1:0.9 | 5.4 BAPTA:4.8 Ca | 100 | 40–50 |
| 771 | 1:0.9 | 5.4 BAPTA:4.8 Ca | 100 | 40–50 |

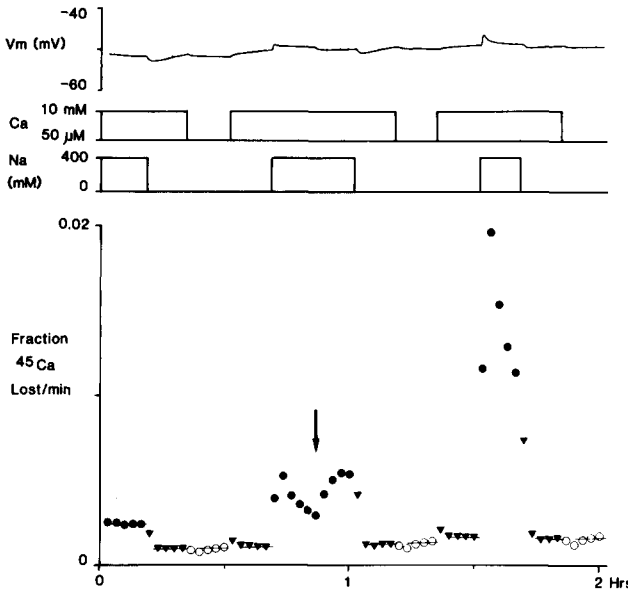



Fig. 7. The effects of intracellular ruthenium red on the sodium-dependent calcium efflux. Arrow on figure denotes period during which axon was injected with a final concentration of 210 μM ruthenium red and 4.2 mM Mops (pH 7.3). Open circles, Na-dependent calcium efflux; closed circles, uncoupled calcium efflux; triangles, calcium-dependent calcium efflux. Choline replaced sodium.

changed, reflecting the different $Ca_i$ affinities and $V_{max}$ between the two Ca efflux routes (see Introduction). It is noticeable that after re-introduction of external Na the $^{45}Ca$ efflux rises and then falls rapidly, suggesting that $Na_o$-$Ca_i$ exchange is very effective in lowering the increased levels of free $Ca_i$ with respect to the calcium pump. When applied externally, ruthenium red at 30 μM had no obvious effect on the $Na_o$-dependent $^{45}Ca$ efflux ($n = 2$ axons).

## Discussion

The results presented in this study examine some of the properties of Na-Ca exchange fluxes and their sensitivity to external Mn, intracellular Ca chelators and intracellular ruthenium red. Na-Ca exchange fluxes could be easily monitored by measuring the $Ca_o$-dependent $^{22}Na$ efflux ($Ca_o$-$Na_i$ exchange) and $Na_o$-dependent $^{45}Ca$ efflux ($Na_o$-$Ca_i$ exchange) from intact axons.

*Inhibition by manganese of Na-Ca exchange fluxes*

Manganese chloride between 10 and 25 mM inhibited Ca uptakes evoked by low sodium sea waters. This manipulation potentiates Ca influx occuring through $Ca_o$-$Na_i$ exchange in squid axons [9], the contribution of Ca-Ca exchange under these conditions is relatively small when measured in the presence of ATP [13,21]. Chapman and Ellis [20] provided evidence that Na-withdrawal contractures of frog atrial trabeculae were sensitive to Mn and could be antagonized by raising calcium, and studies on smooth muscle using Na-sensitive electrodes show that during the fall in $Na_i$ caused by a reduction of $Na_o$, Mn and Ca compete [1]. These studies are consistant with Mn action on $Ca_o$-$Na_i$ exchange. Indeed, between 1 and 7 mM Mn brought about half-maximal inhibition of the $Ca_o$-dependent $^{22}Na$ efflux from intact squid axons. It still remains unclear whether Mn inhibition is dependent on the extent of activation by monovalent cations, and a more extensive study is required for this.

It is of interest to note that $Na_o$-$Ca_i$ exchange appears less sensitive to Mn inhibition than $Ca_o$-$Na_i$ exchange. This apparent difference in sensitivity to Mn was maintained even when examined in similar media containing sufficient Na (100 mM) to monitor both modes of exchange: at 100 mM sodium activates $Ca_o$-$Na_i$ exchange in a manner similar to lithium and potassium [8]. Blaustein [13] has earlier reported in dialysed squid axons that 10 mM Mn leads to an approx. 27% reduction of the $Na_o$-dependent $^{45}Ca$ efflux, a value consistant with the observations in this report. Studies in bullfrog ventricle [16] suggest that Mn may activate $^{45}Ca$ efflux through the exchange process and also inhibit $^{45}Ca$ efflux through an ATP-dependent pathway. The results in this present study showed that Mn at 10 mM inhibited the uncoupled ATP-dependent $^{45}Ca$ efflux with low affinity, a finding similar to that of Brommundt and Kavaler [16], but provided no clear evidence for $Mn_o$-$Ca_i$ exchange.

In sarcolemmal vesicles Mn half-maximally inhibits $Na_i$-dependent Ca uptake between 100 μM to 200 μM when measured in the presence of 20 μM $Ca_o$ [37]. As the affinity during $Ca_o$-$Na_i$ exchange in vesicles is approx. 20–40 μM [35], it may be that $Ca_o$-$Na_i$ exchange in squid axon could be more susceptible to Mn inhibition than $Ca_o$-$Na_i$ exchange in vesicles. Unfortunately, as there was also evidence of Mn-$^{45}Ca$ exchange in these studies [37], studies on Mn effects at the Na binding site of the exchanger were prevented. Kimura et al. [29] have showed that 1 mM Mn effectively blocks the Na-Ca exchange current in single ventricular cells, but have not yet examined the relative effects of Mn on the two exchange modes. The squid axon would be a useful preparation to examine in detail Mn inhibition of Na-Ca exchange under activation by monovalent cations and ATP.

*The role of $Ca_i$ during $Ca_o$-$Na_i$ exchange*

The stimulatory or catalytic role of $Ca_i$ during $Ca_o$-$Na_i$ exchange requires approx. 0.2 μM for half-maximal activation in dialysed squid axons in Na containing sea waters [21] and 2 μM when measured in low Na (Tris-substituted) sea waters [25]. In perfused barnacle muscle cells half-activation occurs at 0.3 μM $Ca_i$ into lithium sea water and 0.7 μM into Tris sea water [34], and in

dialysed ventricular cells half-activation occurs at 0.02 $\mu$M $Ca_i$ into lithium sea water [30]. It remains to date uncertain whether this range of values (from 0.02 $\mu$M to 2 $\mu$M) is simply dependent on experimental conditions or species. Furthermore, results on sarcolemmal vesicles suggest that raising $Ca_i$ (between 100 $\mu$M and 500 $\mu$M) acts to decrease the $K_m$ for $Ca_o$, from 200 $\mu$M to 33 $\mu$M [36].

Attempts to examine a stimulatory or catalytic effect of $Ca_i$ in intact squid axons were unsuccessful in this study: injecting Ca-EGTA or Ca-BAPTA buffers to effect increases in $Ca_i$ inevitably lead to inhibition of the $Ca_o$-dependent $^{22}Na$ efflux. Isolated axoplasm injected with Ca-EGTA mixtures with free Ca above basal levels activates powerful Ca uptake mechanisms which can remove Ca from EGTA [13], and this could occur in intact axons. However, the $Ca_o$-$Na_i$ exchange flux in each case was inevitably inhibited compared to control, clearly suggesting $Ca_i$ is returned to below control levels by calcium sequestion or Ca chelators may have a direct effect on the exchange. If the former suggestion is true then studies disturbing Ca homeostasis by introduction of Ca chelators may underestimate the relative contribution of $Ca_o$-$Na_i$ exchange; if we take the latter, then Ca chelators may affect operation of $Ca_o$-$Na_i$ exchange. This latter possibility is more plausable since in sarcolemmal vesicles EGTA per se can activate exchange movements at constant free Ca [38].

In dialysed squid axons half-maximal activation of $Ca_o$-dependent $^{22}Na$ efflux into lithium and Tris sea waters occurs at approx. 3 mM $Ca_o$ [5]. However, in intact axons half-maximal activation is reached with 2–3 mM $Ca_o$ in lithium sea water and 50–60 mM $Ca_o$ in Tris or choline sea waters [3,8]. The possibility remains, therefore, that the introduction of intracellular EGTA may effect subtle changes in exchange kinetics (another less likely conclusion would be that exchange kinetics simply differ between the squid used: *Loligo forbesi* [3,8], *Loligo plei* and *Loligo pealei* [5,25]).

Ruthenium red, a mitochondrial Ca uptake blocker [18,31], proved useful to effect excursions in $Ca_i$ in the absence of Ca chelators to clearly activate $Na_o$-$Ca_i$ exchange, but not $Ca_o$-$Na_i$ exchange fluxes. This is despite similar reported half-maximal activations of close to 2 $\mu$M in the presence of ATP [14,23,25]. The observations suggest that in the absence of Ca chelators the activation curve for $Ca_i$ on $Ca_o$-$Na_i$ exchange fluxes may be shifted far to the left or to the right and deserves further investigation. To fully examine this possibility is difficult as Ca entry on the exchange will always contribute to $Ca_i$. It would be interesting, however, to measure the $Ca_o$-dependent $^{22}Na$ efflux in intact axons after introducing Ca-EGTA buffers with constant $Ca_i$ but differing EGTA concentrations, and after inhibition of endogenous Ca sequestration.

How $Ca_i$ modulates $Ca_o$-$Na_i$ exchange remains elusive. It clearly activates currents related to its operation [18,27,30], but values differ over a large range when unidirectional fluxes are included (from nM to $\mu$M [25,34]). Whether this reflects the importance of $Ca_o$-$Na_i$ exchange during electrical activity between the different preparations remains to be determined.

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