Biochimica et Biophysica Acta, 646 (1981) 450–456
Elsevier/North-Holland Biomedical Press

BBA 79374

INFLUENCE OF VANADATE ON CALCIUM FLUXES AND NET MOVEMENT OF CALCIUM IN INTACT SQUID AXONS

P.F. BAKER and R. SINGH

Department of Physiology, King's College London, Strand, London WC2R 2LS (U.K.)

(Received March 3rd, 1981)

Key words: Vanadate inhibition; Ca^{2+} transport; (Squid axon)

Intracellular vanadate at a concentration of 100 μM inhibits the uncoupled efflux of Ca^{2+} from intact axons but has little effect on the exchange fluxes and on the Ca^{2+}-dependent Na^+ efflux. External vanadate has no effect on the Ca^{2+} efflux. In addition and most importantly intracellular vanadate inhibits the Ca^{2+} efflux in the presence of external Na^+ and Ca^{2+} suggesting that the uncoupled efflux is operative under physiological conditions. Measurements of the net movements of Ca^{2+} under near physiological conditions have confirmed this conclusion.

Introduction

The regulation of intracellular ionised calcium is a complex process involving a variety of binding systems within the cytosol and transport processes in the plasma membrane (see Refs. 1, 2). These transport processes have been characterised most completely in the giant axons of the squid [2,3] where, in addition to the voltage-sensitive Ca^{2+} channels, it is possible to distinguish operationally at least three forms of calcium flux: (i) calcium-calcium exchange, (ii) calcium-sodium exchange and (iii) a residual, uncoupled, flux that persists in the nominal absence of both external calcium and sodium. Under appropriate conditions the exchange fluxes can carry calcium either into or out of the axon whereas the uncoupled process seems only to expel Ca^{2+} from the cell. The relative sizes of the fluxes can be changed experimentally. Thus metabolic poisons (which lower ATP and raise ionized Ca^{2+} in the cytosol) abolish the uncoupled flux, but greatly enhance the exchange fluxes; similarly extracellular lanthanides inhibit the uncoupled flux at concentrations which do not inhibit sodium-calcium exchange [4]. Although these observations on intact axons and parallel studies in dialysed axons [5,6] suggest that uncoupled Ca^{2+} efflux may reflect an ATP-dependent process essentially different from sodium-calcium exchange, the role of the uncoupled flux in calcium homeostasis under physiological conditions remains rather uncertain.

The recent report that intracellular vanadate inhibits the ATP-dependent component of Ca^{2+} efflux but not the Na^+-dependent component of efflux from dialysed squid axons [7] provides another potentially valuable tool with which to examine the relative importance of the fluxes under physiological conditions. An essential first step is to show that the results in dialysed axons can be repeated in intact axons. In this paper we show that internal vanadate inhibits the uncoupled Ca^{2+} efflux at concentrations which have little effect on the exchange fluxes. In addition and most importantly vanadate inhibits Ca^{2+} efflux and the net extrusion of Ca^{2+} from axons in normal sea-water implying that the uncoupled Ca^{2+} efflux is operating under physiological conditions.

Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

0005-2736/81/0000–0000/$02.50

Methods

Experiments were performed on the hindmost stellar giant axons of the squid *Loligo forbesi*. All axons were cleaned of adhering small nerve fibres.

Isotopes and other substances were introduced into the axon by microinjection and fluxes measured as described previously (see Ref. 4).

In those experiments in which extracellular Arsenazo III was used, the axons were first washed for about 5 min in a rapidly flowing stream of nominally Ca^{2+}-free sea water lacking Arsenazo III before exposure to sea water containing the dye. The Ca-Arsenazo III content of the superfusate downstream from the axon was monitored continuously as the difference in absorption at the wavelengths 650 and 700 nm using a dual wavelength, rotating wheel spectrophotometer (see Ref. 12).

In those experiments where vanadate was used, a stock solution of vanadate was made up by dissolving vanadium pentoxide in dilute NaOH to give a final vanadate concentration of 4 mM and adjusting the pH to 7.0.

Results and Discussion

Fig. 1 illustrates an experiment on an unpoisoned axon. In this preparation the Ca^{2+}-dependent, Na^+-dependent and uncoupled components of the Ca^{2+} efflux amount to 15, 25 and 60%, respectively, of the total flux. The effects of external and internal vanadate were examined under conditions where the uncoupled flux can be studied in the absence of exchange fluxes. Addition of 100 μM vanadate to the external medium had no effect on the residual Ca^{2+} efflux; but injection of vanadate to a final intracellular axoplasmic concentration of 100 μM rapidly reduced this component of the Ca^{2+} efflux. The vanadate-sensitive flux amounted to 45% of the total Ca^{2+} efflux. Subsequent reintroduction of extracellular Na^+ and Ca^{2+} revealed Na^+- and Ca^{2+}-dependent fluxes which were approx. 10% lower than those observed before treatment with vanadate. In other experiments of this type higher concentrations of external vanadate (1 mM) sometimes produced a small reduction in uncoupled flux but internal vanadate was always a much more effective inhibitor.

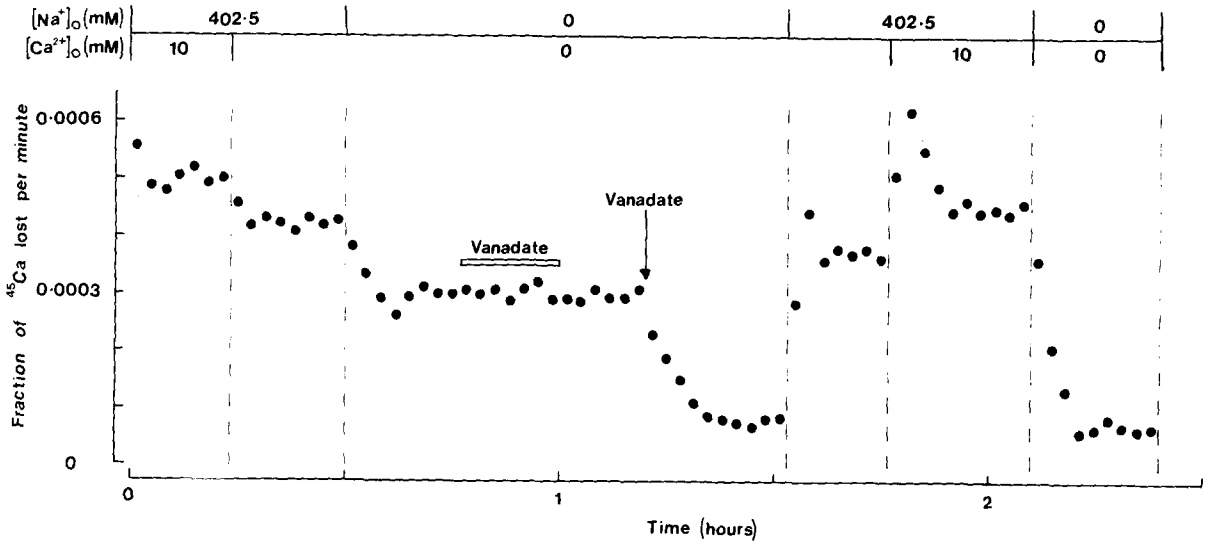

Fig. 1. Effect of vanadate on the Ca^{2+} efflux from an intact unpoisoned axon of *Loligo forbesi* (diameter 821 μm). The composition of the standard artificial sea-water was (mM): 402.5 Na^+; 100 Mg^{2+}; 10 Ca^{2+}; 10 K^+; 2.5 HCO_3^-; 630 Cl^-; (pH 7.8). Temperature 14°C. Na^+ was replaced isosmotically by lithium. Axon was loaded with $^{45}Ca^{2+}$ by microinjection (see Ref. 4). Application of external 100 μM vanadate is indicated by the open bar and injection by the vertical-arrow. The external solution was flowed at about 0.6 ml · min^{-1} and sampled at 2-min intervals.

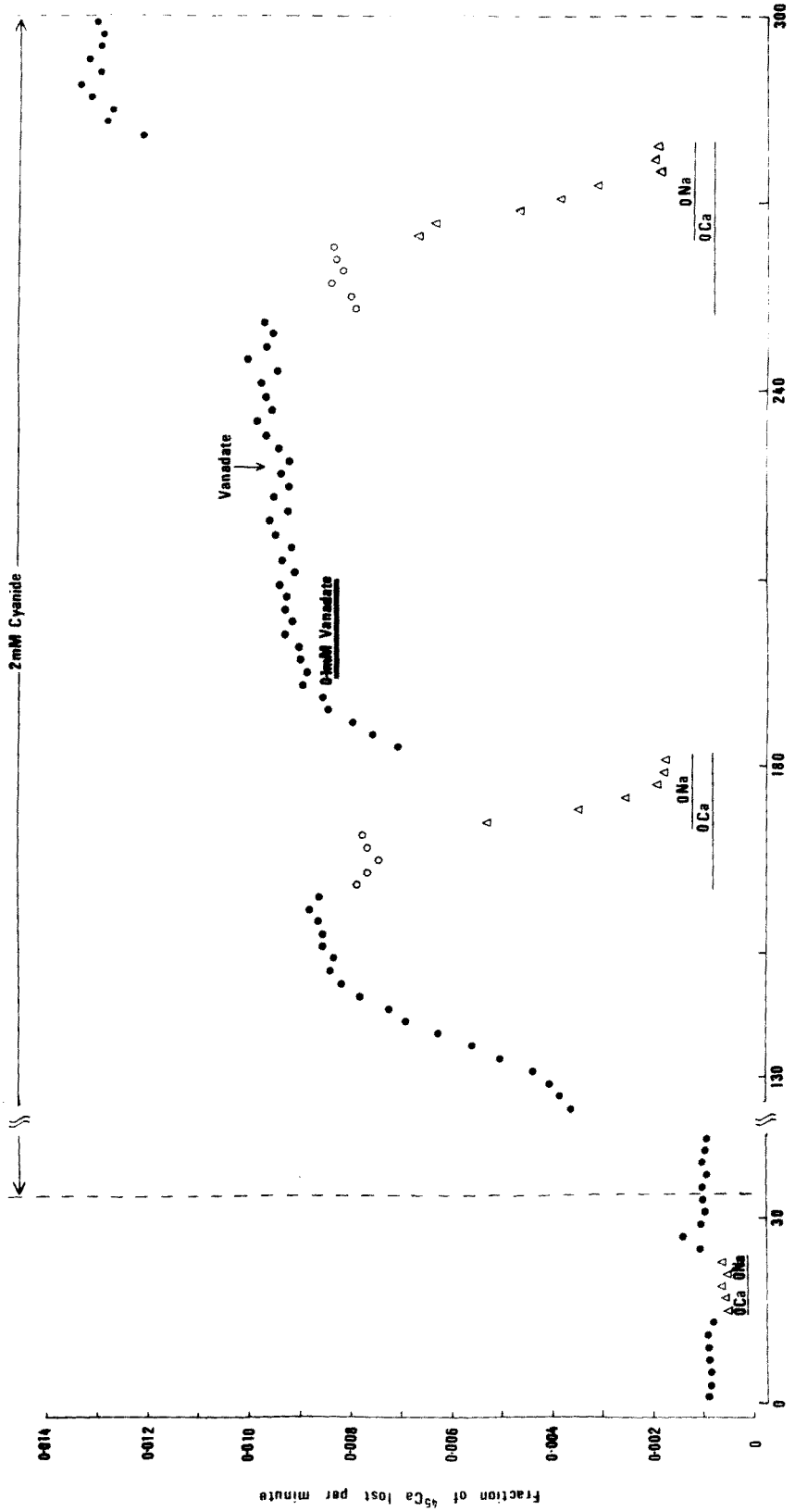

Fig. 2. Lack of effect of vanadate on the Ca^{2+} efflux from an intact axon of *Loligo forbesi* poisoned with 2 mM cyanide (diameter 761 μm) ○, Ca^{2+} efflux measured in nominally Ca^{2+}-free solution; △, Ca^{2+} efflux measured in nominally Ca^{2+}-free lithium sea-water. Application of external 100 μM vanadate is indicated by the solid bar and injection of vanadate (final axoplasmic concentration, 100 μM) by the vertical arrow. Cyanide was included in the external solution for the periods indicated by the horizontal arrow. Temperature 14°C.

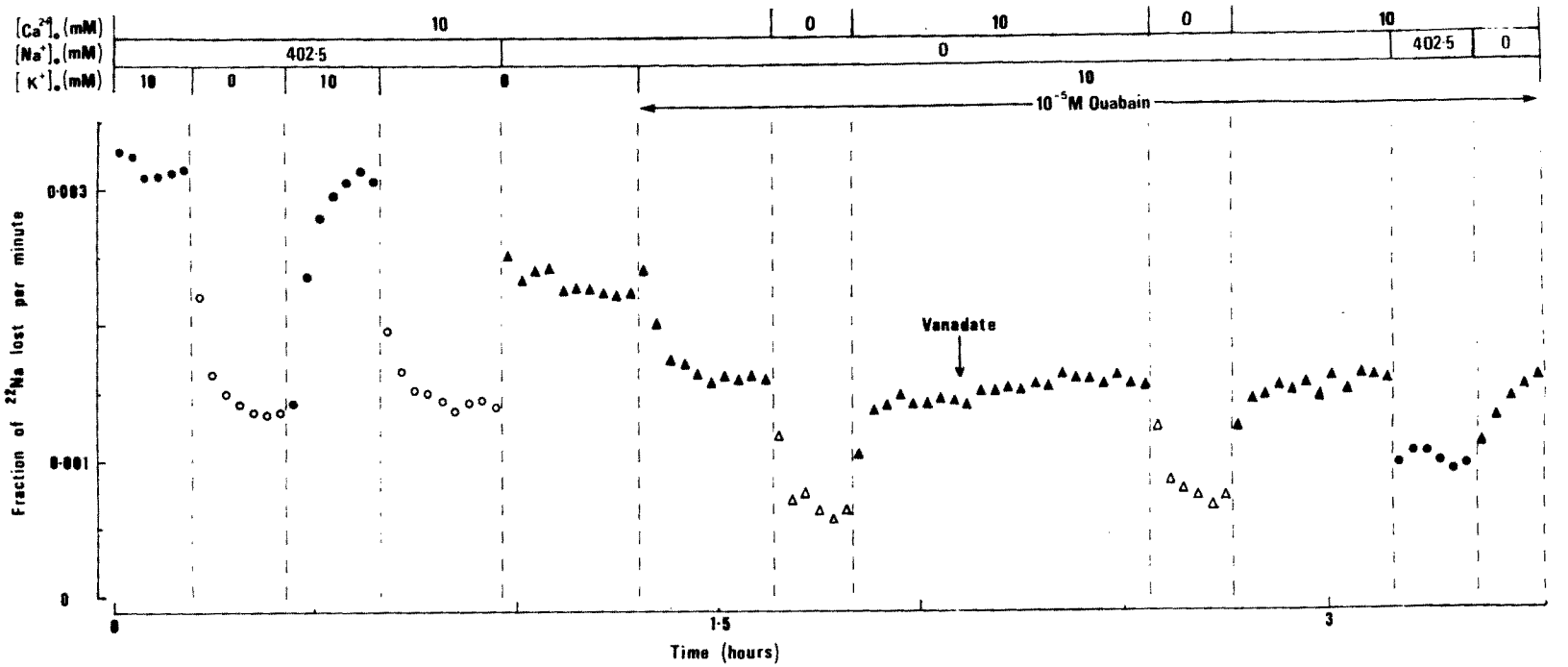

Fig. 3. Lack of effect of internal vanadate (final axoplasmic concentration 100 μM) on the Ca^{2+}-dependent component of the Na^+ efflux from an intact axon of *Loligo forbesi* (diameter 800 μm). Temperature 14°C. External Na^+ was replaced isosmotically by lithium (▲). The removal of external K^+ (○) and Ca^{2+} (△) was carried out without changing the other constituents. Exposure to 10^{-5} M ouabain is indicated by the horizontal bar.

Also, in some axons the size of the Ca^{2+}-dependent component of Ca^{2+} efflux increased somewhat following vanadate injection.

In view of the finding that vanadate affects only the uncoupled Ca^{2+} efflux, this inhibitor should have no effect on the Ca^{2+} efflux from fully-poisoned axons where there is no detectable uncoupled flux [4], nor should it affect the Ca^{2+}-sensitive component of Na^+ efflux which is presumed to reflect the Na^+-dependent Ca^{2+} efflux operating in reverse [8,9]. Figs. 2 and 3 confirm these predictions. In a separate experiment injection of vanadate to give a final axoplasmic concentration of 100 μM only produced 18% inhibition of the ouabain-sensitive Na^+ efflux which suggests that uncoupled Ca^{2+} efflux in the squid is more sensitive to vanadate than is the sodium pump.

Having shown that intracellular vanadate can inhibit the uncoupled Ca^{2+} efflux in the absence of external Na^+ and Ca^{2+}, and that this effect is specific for the uncoupled component of the Ca^{2+} efflux, the question of key importance is to determine whether a similar reduction in efflux can be demonstrated under fully physiological conditions (i.e. in sea-water containing both Na^+ and Ca^{2+}). Fig. 4A shows such an experiment. Injection of vandate to give a final axoplasmic concentration of 100 μM clearly reduces the Ca^{2+} efflux by 20%. Subsequent removal of external Na^+ and Ca^{2+} reveals that the inhibition is confined to the residual flux. This experiment provides direct evidence for the operation of the uncoupled Ca^{2+} efflux in axons of *Loligo forbesi* under physiological conditions. However, comparison with Fig. 1 suggests that the inhibition produced by intracellular vanadate is larger in the absence of external calcium and sodium than in their presence. Fig. 4B shows that this difference is related to the absence of calcium: inhibition in the presence of 10 mM Ca^{2+} averages 15 ± 2.2% (S.E. of the mean) of the initial flux in the presence of Na^+ and Ca^{2+} whereas in the absence of external Ca^{2+} it averages 62 ± 3.4% (S.E. of the mean). While in no way altering the conclusion that uncoupled Ca^{2+} extrusion occurs under physiological conditions, these observations suggest either that external calcium may modify the effectiveness of internal vanadate as an inhibitor of the uncoupled Ca^{2+} pump or that internal vanadate may promote Ca^{2+}-Ca^{2+} exchange in unpoisoned axons. DiPolo (DiPolo, R., personal communication) has recently reported essentially similar findings in dialysed axons.

It might be argued that although intracellular vanadate inhibits Ca^{2+} efflux under physiological conditions, it might also inhibit Ca^{2+} influx and as influx cannot be measured with the same accuracy as efflux, vanadate may not be altering the net movement of calcium. We have examined this possibility in two ways. The net loss of Ca^{2+} from Ca^{2+}-loaded axons was followed either by superfusing the axons with arsenazo III (and following the net loss of Ca^{2+} into the extracellular fluid by monitoring the absorbance of dye in the calcium form) or by direct analysis of the axoplasm. The use of arsenazo III is only possible at low levels of extracellular Ca^{2+}; but experiments can easily be carried out in the presence of 470 mM Na_o^+, 50 mM Mg_o^{2+} and 4.6 μM Ca^{2+}. Axons were loaded with Ca^{2+} by brief exposure to lithium sea-water containing 10 mM Ca^{2+} and subsequently superfused with Na^+ sea-water containing 4.6 μM Ca^{2+} and 300 μM arsenazo III. The net loss of Ca^{2+} is apparent as an increase in the absorbance of dye in the calcium form. This rises rapidly to a peak and subsequently falls over about 60 min. The size of this

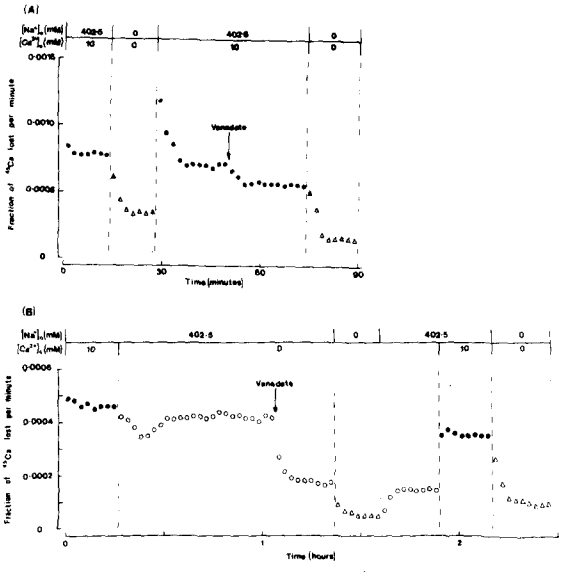

Fig. 4. Effect of internal vanadate (100 μM final axoplasmic concentration) on the Ca^{2+} efflux into (A) standard artificial sea-water containing 400 mM Na^+ and 10 mM Ca^{2+} (•), (axon diameter 802 μm) and (B) Ca^{2+}-free Na^+ sea-water (○) (axon diameter 670 μm). Tests of efflux into Ca^{2+}-free lithium sea-water are also shown (△). Temperature 14°C.

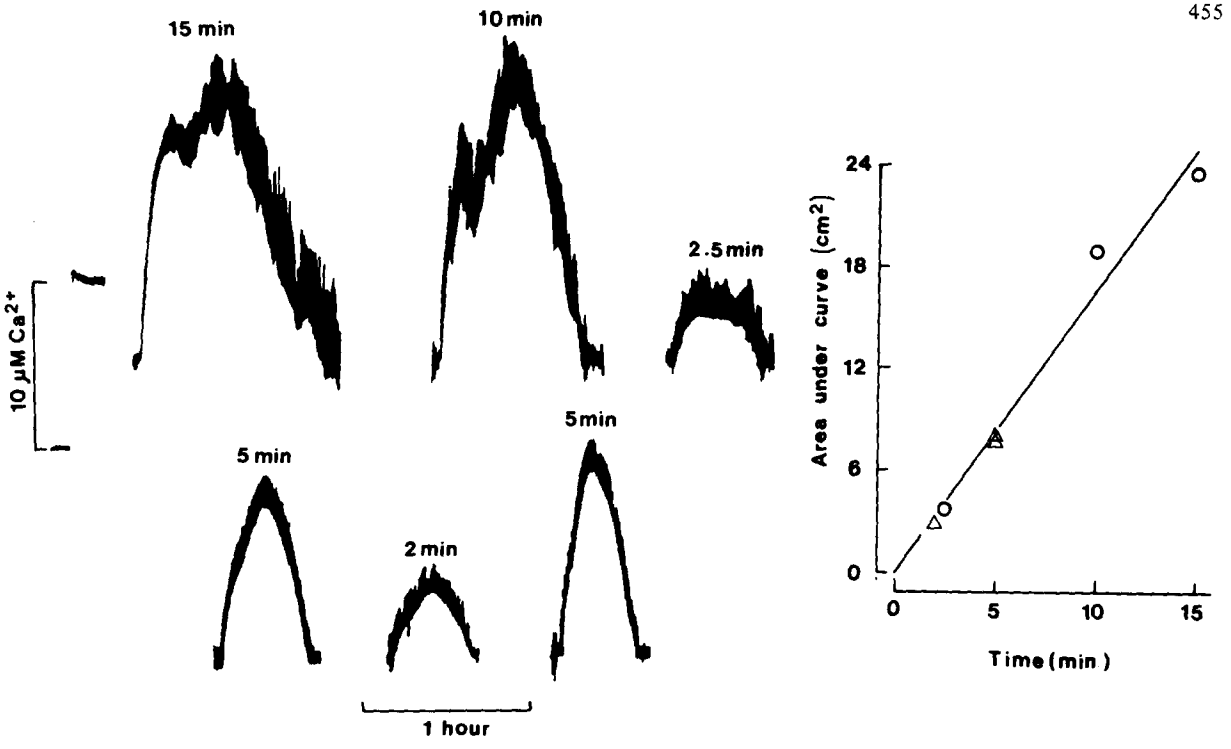

Fig. 5. Use of extracellular Arsenazo III to monitor net loss of calcium. The upper and lower sets of traces relate to measurements on two separate axons. After loading with Ca^{2+} by exposure to lithium sea-waters containing 10 mM Ca^{2+} for varying lengths of time (shown above the traces), the axons were washed in nominally Ca^{2+}-free Na^{+} sea-water for 5 min before exposure to the same solutions containing 300 μM Arsenazo III. Temperature 20°C. The graph on the right shows that the area under the Ca-Arsenazo III transient is linearly related to the duration of exposure to lithium sea-water i.e. the Ca^{2+} load.

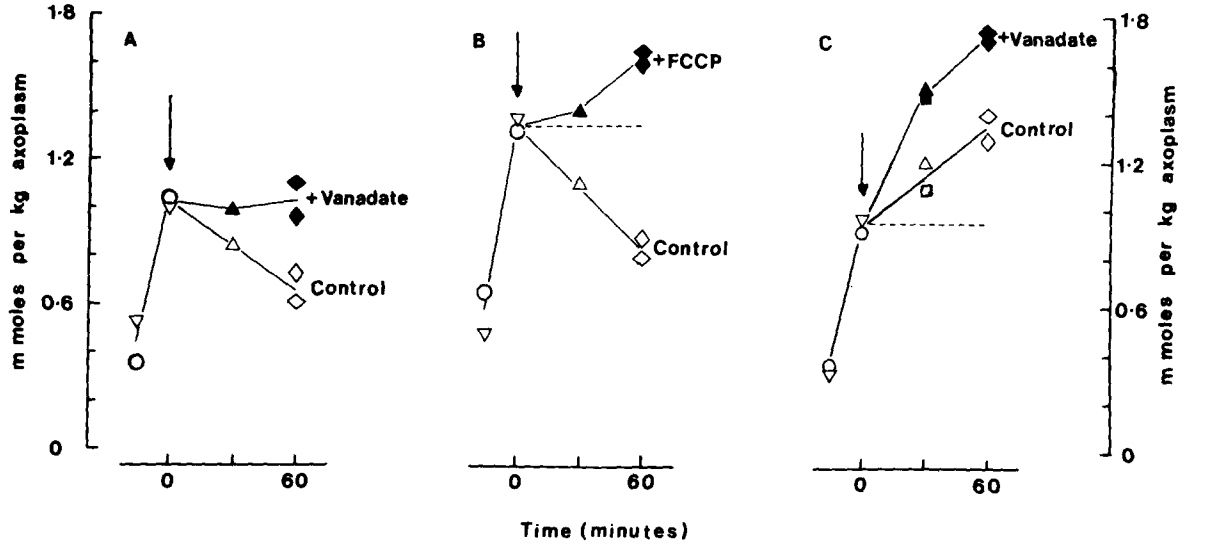

Fig. 6. Influence of vanadate, FCCP and Na^{+}-free (choline) sea-water on net movements of Ca^{2+} in axons of *Loligo forbesi* measured by atomic absorption spectrometry. Axons immersed in sea-water containing (mM): 402.5 Na^{+} (or choline C): 100 Mg^{2+}; 3 Ca^{2+}; 10 K^{+}; 2.5 HCO_3^{-}; 616 Cl^{-}; (pH 7.8). Comparisons were made between paired axons from the same animal, a different symbol is used for each pair. At zero time axons were injected (arrow) over their full length with a solution containing 24 mM Ca^{2+} and 400 mM EGTA (pH 7.2) and injected a second time with vanadate (4 mM) or FCCP (80 $\mu g \cdot ml^{-1}$) as indicated. At the time indicated axoplasm was extruded and a sample from the middle section of the fibre taken for analysis. All axons were excitable. Temperature 21°C.

transient is linearly related to the Ca load (Fig. 5). Lowering the temperature to 0°C or injection of vanadate (final concentration 100 μM) both greatly reduce the net loss of Ca^{2+} monitored in this way.

Although the Arsenazo method gives evidence for net extrusion of Ca^{2+} that is sensitive to internal vanadate, the extracellular Ca^{2+} against which the Ca^{2+} is pumped is far below physiological. This is avoided in the second method which permits measurements under fully physiological conditions. Axons were loaded with Ca^{2+} by microinjection of a suitable Ca^{2+}-EGTA buffer. This permitted a large Ca^{2+} load whilst enabling the ionized Ca^{2+} to be stabilised in the physiological range. Axons loaded with a buffer giving total axoplasmic calcium and EGTA concentrations of approx. 1 mM and 10 mM, respectively, and immersed in Na^+ sea water containing 3 mM Ca^{2+} lost 30–75% of their imposed Ca^{2+} load in 1 h. This rate of net loss is close to 1 pmol · cm^{-2} · s^{-1} which is higher than that expected from measurements of ^{45}Ca efflux. When vanadate (final intracellular concentration 100 μM) or the uncoupling agent FCCP (final intracellular concentration 2 μg · ml^{-1}) were injected along with the Ca^{2+} buffer, the axons failed to effect a net loss of the Ca^{2+} load (Fig 6A and B). In the absence of external sodium the axons gained Ca^{2+} and the rate of Ca^{2+} gain was increased following injection of vanadate (Fig. 6C).

These results show that under near physiological conditions (ionized Ca^{2+}, 40–70 nM) a process that is sensitive to internal vanadate contributes to the net extrusion of Ca^{2+} from squid axons. The experiments with FCCP suggest that this flux also requires ATP. The properties of the process effecting the net extrusion of Ca under near physiological conditions closely resemble those of the uncoupled Ca^{2+} pump which, in turn, has many of the features of the Ca^{2+}-ATPases that seem to be a characteristic feature of many plasma membrane preparations (see Refs. 13, 14). At first sight our results are difficult to reconcile with those of Requena et al. [10] who reported net extrusion of Ca^{2+} from fully poisoned squid axons. However, poisoning markedly raises the ionized Ca [11] and at elevated levels of internal Ca^{2+}, Na_o^+-dependent extrusion of Ca^{2+} undoubtedly plays an important role in the net extrusion of Ca^{2+}. Our experiments in which Ca_i^{2+} and Ca_o^{2+} are maintained constant and close to their physiological resting values suggest that the vanadate-insensitive, Na_o^+-dependent extrusion of Ca^{2+} is less important than the vanadate-sensitive, uncoupled Ca^{2+} pump under these conditions.

References

1 Baker, P.F. (1976) in Calcium in Biological Systems, S.E.B. Symposium, Vol. 30, pp. 67–88, Cambridge University Press, Cambridge
2 Baker, P.F. (1978) Ann. N.Y. Acad. Sci. 307, 250–268
3 Baker, P.F. (1972) Prog. Biophys. Mol. Biol. 24, 177–223
4 Baker, P.F. and McNaughton, P.A. (1978) J. Physiol. 276, 127–150
5 DiPolo, R. (1977) J. Gen. Physiol. 69, 795–813
6 DiPolo, R. (1978) Nature, 274, 391–392
7 DiPolo, R., Rojas, H.R. and Beaugé, L. (1979) Nature, 281, 228–229
8 Baker, P.F., Blaustein, M.P., Manil, J. and Steinhardt, R.A. (1967) J. Physiol. 191, 100–102P
9 Baker, P.F., Blaustein, M.P., Hodgkin, A.L. and Steinhardt, R.A. (1969) J. Physiol. 200, 431–458
10 Requena, J., Mullins, L.J. and Brinley, F.J. (1979) J. Gen. Physiol. 73, 327–342
11 Baker, P.F., Hodgkin, A.L. and Ridgway, E.B. (1971) J. Physiol. 218, 709–755
12 Scarpa, A. (1979) in Detection and Measurement of Free Ca^{2+} in Cells, (Ashley, C.C. and Campbell, A.K., eds.), pp. 85–115, Elsevier, Amsterdam
13 Schatzmann, H.J. (1981) in Membrane Calcium Transport (Carafoli, E., ed.), Academic Press, London
14 Sulakhe, P.V. and St. Louis, P.J. (1980) Prog. Biophys. Mol. Biol. 35, 135–195