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THE EFFECTS OF VANADATE ON CALCIUM TRANSPORT IN DIALYZED SQUID AXONS

SIDEDNESS OF VANADATE-CATION INTERACTIONS

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(1) Vanadate (VO_3^-) fully inhibits the ATP-dependent uncoupled Ca efflux (Ca pump) in dialyzed squid axons. (2) Vanadate inhibits with high affinity. The mean apparent affinity ($K_{1/2}$) obtained was 7 μM. (3) Inhibition by vanadate is dependent on Ca_o. External Ca lead to a release of the inhibitory effect. ($K_{1/2} \simeq 3$ mM). This antagonic effect can be reverted by increasing the vanadate concentration. Internal K^+ increases the affinity of the intracellular vanadate binding site. External K^+ has no effect on the inhibition. (4) Vanadate has no effect on the Na_o- dependent Ca efflux component (forward Na-Ca exchange) in the absence of ATP. In axons containing ATP vanadate modified this component.

Introduction

Squid axons are able to maintain a low intracellular $[Ca^{2+}]_i$ against a large Ca electrochemical gradient (for references see Ref. 1) Although Na-Ca exchange has long since been postulated as the responsible mechanism for the asymmetric Ca distribution in squid nerves [2–4], strong evidence has been recently obtained indicating that an ATP-dependent Ca pump is also involved in Ca regulation in these cells (for references see Ref. 5). The properties of this ATP-driven Ca pump and of the Ca dependent ATPase activity present in squid nerves axolema (Ref. 6, and Beaugé, L.A., Dipolo, R., Osses, L., Barnola, F. and Campos, M. (1981), Ref. 30) are similar to those of the Ca pump and Ca^{2+}-ATPase activities observed in other preparations [7,8]. Among the common denominator for all Ca pumps, and related ATPase activities, is that vanadate is a powerful inhibitor [9–13]. It is well known that inhibition by vanadate on active transport mechanisms has peculiar interactions with other ligands [9,12,14] and that these interactions have shown definite sidedness [15–18].

The vanadate-ligand interaction on the inhibition of the Ca^{2+}-ATPase activity has already been described for sarcoplasmic reticulum [10] and the human red blood cell [9,11,12]. However, there is still a lack of data on the sideness of these interactions. Together with red blood cell ghosts, dialyzed squid axons provide a unique preparation to study the sidedness of membrane related processes. So far, it has been demonstrated that vanadate inhibits the ATP-dependent Ca pump in squid axons acting on the intracellular surface [13,19]. The present work is an extension of the first observation and deals with the interactions between vanadate, calcium, sodium and potassium on the inhibition of the calcium pump in squid nerve fibers. The results show that vanadate

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Abbreviations: EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid; EDTA, ethylenediamine tetraacetic acid.

inhibition can be antagonized by extracellular Ca ions and reinforced by intracellular potassium. An additional interesting observation is that the Na-Ca exchange mechanism, which is unaffected by vanadate in the absence of ATP, can be modified by the inhibitor in ATP-containing axons.

Methods

The experiments were carried out on two squid species: *Dorytheutis plei* at the Instituto Venezolano de Investigaciones Científicas in Caracas, Venezuela, and *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 [20,21] 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 pH (18–19°C) 7.6. The removal of Na, Ca or Mg was compensated with equiosmolar amount of Tris. Ca-free sea water contained 60 mM Mg and 0.5 mM EGTA. The standard dialysis solution had the following composition (mM): K^+, 310; Na^+, 60 for the experiments with *Loligo pealei* and 40 with *Dorytheutis plei*; Mg^{2+}, 4 in excess of the ATP concentration; $Tris^+$, 30; Cl^-, 98; Aspartate, 310; EGTA, 1 or 2; glycine 330; pH (18–19°C) 7.3. 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 nominal ionized Ca concentration values are based on a CaEGTA dissociation constant of 0.15 μM [22]. ATP (vanadium free) was obtained from Sigma Co. as Tris salt, neutralized with TrisOH and stored at −20°C as a 250 mM solution. Na orthovanadate (from Fischer) was prepared as a 100 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 solutions. Radioactive samples containing 4 ml artificial sea water were mixed with 5 ml of scintillator solution and counted in a liquid scintillation counter for times long enough to give standard error of about 1%.

Results

The effect of vanadate on the uncoupled Ca efflux in the absence of Na_o and Ca_o

Fig. 1 shows the effect of internal vanadate on the ATP dependent uncoupled Ca efflux in an axon dialyzed with a physiological $[Ca^{2+}]_i$. In the absence of ATP, Ca efflux diminished progressively to a steady-state value of about 3.5 fmol · cm^{-2} · s^{-1}, no different from the expected 'leak' of CaEGTA [5]. Removal of Na_o and Ca_o has no effect on the efflux level, and addition of ATP raises the Ca efflux to about 43 fmol · cm^{-2} · s^{-1}. Internal vanadate at concentrations of 4.0, 8.0 and 50 μM causes increasing inhibitions on the Ca efflux. In this particular experiment vanadate at a concentration of 8 μM causes nearly 50% inhibition.

Fig. 2 shows the results of several experiments in which the percent inhibition of the ATP dependent uncoupled Ca efflux is plotted as a function of the internal vanadate concentration. The $K_{1/2}$ obtained from Fig. 2 is close to 7 μM, thus indicating that vanadate inhibits the uncoupled Ca efflux with high affinity. The fact that vanadate may bind to axoplas-

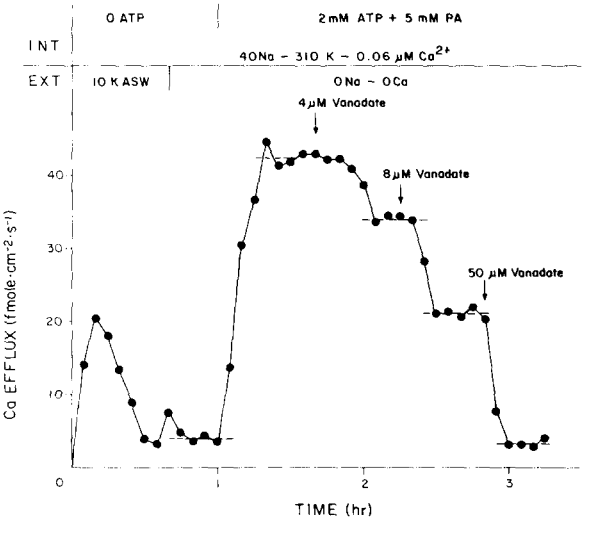

Fig. 1. The effect of different vanadate concentrations applied internally on the efflux of Ca in dialyzed squid axon. The transitory rise in the Ca efflux during the first 30 minutes of dialysis is due, in part, to the time course of ATP wash-out and in part to the time taken for isotope equilibration. Unless otherwise state, all solute concentrations are in mM. ASW, artificial sea water.

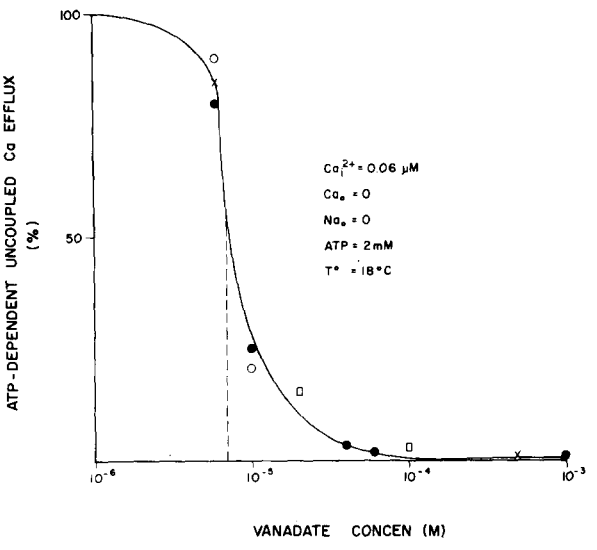

Fig. 2. Percentage of uncoupled Ca efflux remaining as a function of vanadate concentration in the dialysis fluid. The line through the points was drawn by eye. $K_{1/2}$ is close to 7 μM.

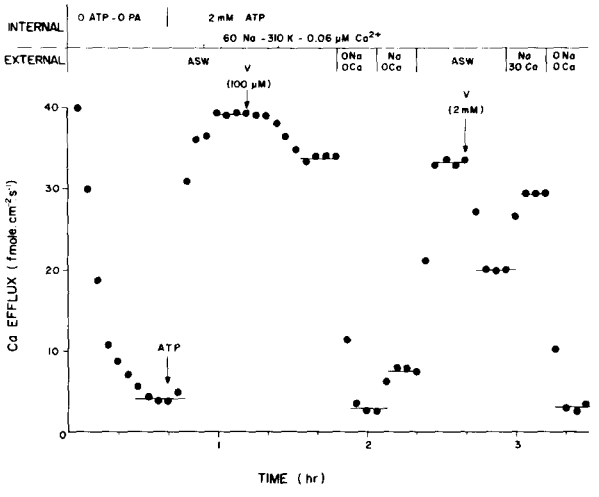

Fig. 3. The effects of Na_o and Ca_o on the inhibition of the uncoupled Ca efflux by vanadate. Note that in the presence of Na_o and Ca_o, 100 μM vanadate causes only a small inhibition in the efflux. The much larger inhibition caused by 2 000 μM vanadate can be partially reverted by 30 mM Ca_o. ASW, artificial sea water.

mic constituents during a dialysis experiment, could introduce a significant error in the actual vanadate concentration near the membrane. Since this could be particularly important at low vanadate concentrations, the measured $K_{1/2}$ may be much smaller than 7 μM. In favor of this idea, is the result of Fig. 1 which shows that the time taken to obtain a steady Ca efflux inhibition, after addition of vanadate, is longer with 4 μM vanadate than with 8 or 50 μM.

The effect of vanadate on the uncoupled Ca efflux in the presence of Na_o and Ca_o

Fig. 3 is an experiment designed to explore whether the inhibition of the uncoupled Ca efflux by vanadate is affected by the presence of external sodium or calcium. Similar to the experiment of Fig. 1, in the absence of ATP and at physiological $[Ca^{2+}]_i$, Ca efflux stabilizes close to the CaEGTA 'leak'. ATP, in the presence of external Na and Ca, increases the Ca efflux from about 3.5 to 40 fmol · cm^{-2} · s^{-1} (a value similar to that obtained for the uncoupled Ca efflux in the absence of Na_o and Ca_o (See Fig. 1)). Interestingly, addition of 100 μM vanadate to the internal dialysis fluid causes only a slight reduction in the efflux (17%), as compared to that seen in the absence of Na_o and Ca_o (see Fig. 1). Simultaneous removal of Na_o and Ca_o decreases the efflux to 'leak' values. In this experiment, the separate effects of Na_o and Ca_o on the Ca efflux shows, that the presence of full Na_o, causes a small increment in the efflux. However, addition of full Ca_o, reverts the inhibition by vanadate. Fig. 3 also shows that increasing the vanadate concentration to 2 000 μM, causes a partial inhibition of the Ca efflux, which can be again reverted by raising the external Ca to 30 mM. The fact that the magnitude of the Na_o-dependent component, under the present conditions (low Ca_i^{2+}, ATP present), is not modified by vanadate [5,6], indicates that Na_o has no effect on the vanadate inhibition of the uncoupled Ca efflux. This finding contrasts with that of Ca_o which clearly decreases the extent of the vanadate inhibition.

Another possible explanation for this phenomena is that vanadate induces a Ca-Ca exchange, rather than Ca_o reverts the binding of vanadate to an internal inhibitory site (transconcentration effect). If this were the case, one should be able to observe an increase in Ca influx (of about 30 to 40 fmol · cm^{-2} · s^{-1} induce by vanadate under similar experimental conditions of those of Fig. 3. An experiment,

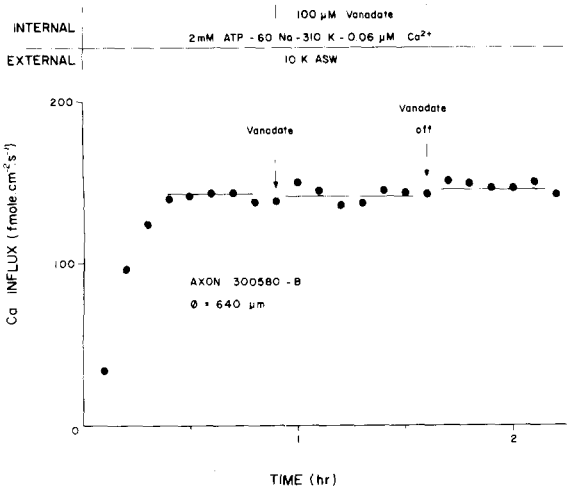

Fig. 4. The effect of internally applied vanadate on the Ca influx in a dialyzed squid axon. Unless otherwise stated, all solute concentrations are given in mM. The external Ca concentration was 10 mM. Temperature: 18°C. Note that 100 μM vanadate, under conditions similar to that of the experiment of Fig. 3, has no effect on the Ca influx level. ASW, artificial sea water.

designed to explore this possibility, is shown in Fig. 4. After a steady Ca influx of about 140 fmol · cm^{-2} · s^{-1} was obtained from an axon superfused with artificial sea water (10 mM Ca) and dialyzed with 0.06 μM Ca^{2+} and 2 mM ATP, 100 μM vanadate were added to the internal medium. Clearly, no significant change in the influx level was observed even after 30 min of dialysis. This, and other similar experiments, strongly argues against a Ca-Ca exchange as an explanation for the Ca_o-vanadate interaction.

The effect of different external Ca concentrations on the degree of vanadate inhibition, at constant vanadate concentration, is shown in Fig. 5. For this particular experiment 3 mM Ca_o is able to revert 50% of the vanadate inhibition of the uncoupled efflux. The mean $K_{1/2}$ for the Ca_o effect from three different experiments was 3.7 mM.

The effect of K^+ on the inhibition by vanadate

Potassium ions facilitate inhibition by vanadate of the Ca^{2+}-ATPase from human red blood cells [9,12]. In the experiment of Fig. 6, the degree of inhibition of the uncoupled Ca efflux by vanadate was explored in an axon in which both internal and external K^+

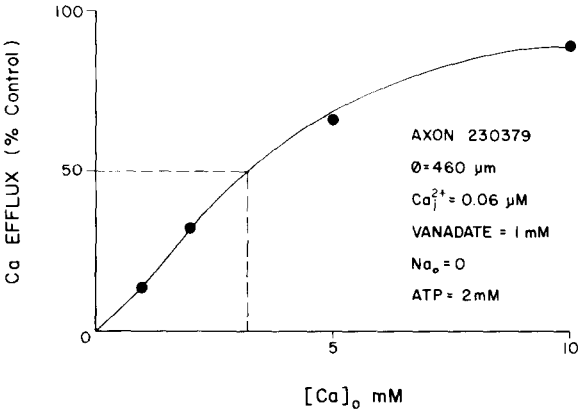

Fig. 5. Percentage of uncoupled Ca efflux remaining in the presence of 1 mM vanadate as a function of the concentration of Ca in the external medium. External Ca was substituted in equiosmolar quantities with Mg. The points were obtained from a single experiment. The $K_{1/2}$ obtained is close to 3 mM Ca_o.

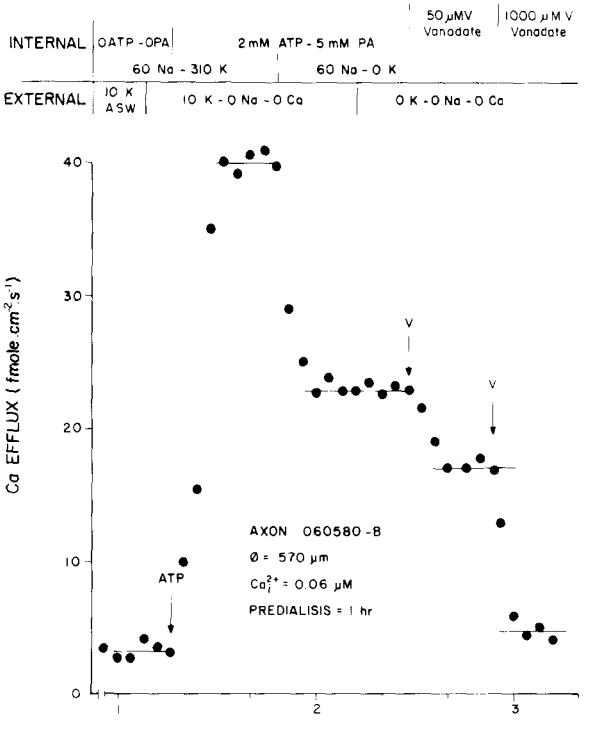

Fig. 6. The effect of K_i^+ on the inhibition of the uncoupled Ca efflux by vanadate. Internal K^+ was substituted with equiosmolar amounts of $Tris^+$. The discontinuity in the time scale at the beginning of the experiment correspond to the ATP wash-out time and isotope equilibration. ASW, artificial sea water.

were removed. In the initial part of the experiment, the activation of the uncoupled Ca efflux by ATP was carried out in the presence of 310 mM K_i^+. This treatment causes the efflux to increase from a value of about 4 to 40 fmol $\cdot$ cm^{-2} $\cdot$ s^{-1}. The removal of K_i^+ drops the Ca efflux by about half and subsequent withdrawal of K_o^+ does not affect the steady efflux level. Under this zero potassium condition, 50 μM vanadate causes only a partial inhibition of the remaining uncoupled Ca efflux, whereas 1 000 μM completely abolish it. This result is in contrast with the degree of vanadate inhibition in the presence of K_i^+ (see Fig. 1); thus indicating that as in other Ca transport systems [9,12], the vanadate interaction with the Ca pump in squid axons is dependent on K_i^+.

The partial requirement of the uncoupled Ca efflux for internal potassium has been described in detail in squid axons by DiPolo and Beaugé (submitted for publication). A similar role of K^+, as cofactor in the activation of a Ca pump, has been described for the sarcoplasmic reticulum [27] and red blood cells [28]. Few experiments were done to explore whether K_o may alter the degree of inhibition of the uncoupled Ca efflux by vanadate. The apparent affinity of 7 μM was not significantly modified by the removal of K_o alone.

Effect of vanadate on the Na_o-dependent Ca efflux in the absence of ATP

The absolute requirement of the uncoupled Ca efflux towards ATP [5,6], contrast with the behaviour of the Na_o-dependent component in which near maximum values can be obtained in the absence of ATP [26]. This property can be used as a tool for separating these two modes of Ca extrusion. We have therefore explored the effect of vanadate on the Na_o-dependent Ca efflux (forward Na-Ca exchange) in dialyzed axons depleted exhaustively of ATP. Since in squid axons the magnitude of the Na_o-dependent component of the Ca efflux is small at physiological $[Ca^{2+}]_i$ [5,6], in these series of experiments the $[Ca^{2+}]_i$ was artificially raised to about 0.7 μM.

In the axon of Fig. 7 intracellular ATP was completely removed by pretreating the fiber with 1 mM CN^- for 1 h followed by a predialysis (1 h) with a medium containing no ATP. Under this high internal Ca^{2+}, no ATP conditions, Ca efflux is totally dependent on Na_o and Ca_o since their removal drops the

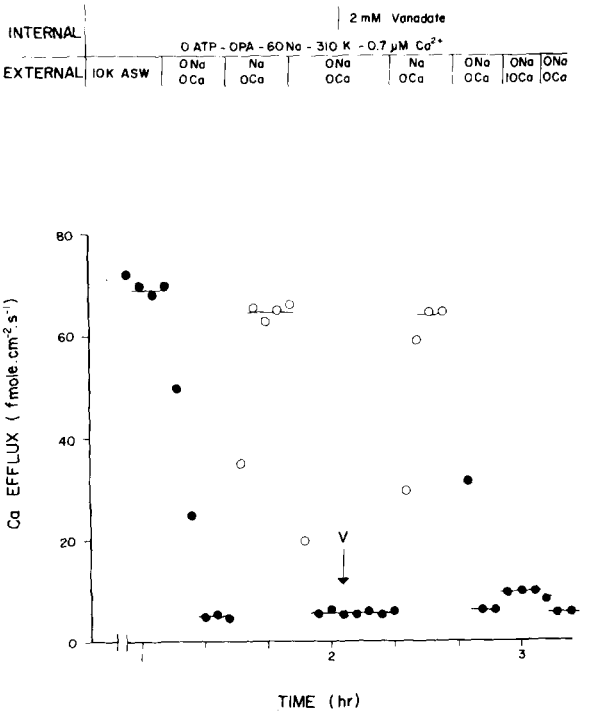

Fig. 7. Effect of vanadate on the Na_o and Ca_o dependent Ca efflux in an ATP depleted axon containing a high $[Ca^{2+}]_i$. The axon was preincubated for 1 h in artificial sea water (ASW), containing 1 mM KCN to lower its ATP content, and then dialyzed for 1 h with an ATP-free dialysis medium. The discontinuity in the time scale represents the predialysis period. Note that in the presence or in the absence of vanadate neither the Na_o- nor the Ca_o-dependent components change in magnitude.

efflux to 'leak' values. Readdition of Na_o increases the Ca efflux almost to the same initial base line, indicating that more than 90% of the total Ca efflux is Na_o-dependent, the rest is mostly Ca_o-dependent an a small fraction Ca leak. When the activating effect of Na_o on the Ca efflux was tested again but in the presence of 2 000 μM vanadate, no significant inhibition of this component was observed. It can also be seen at the end of the experiment, that the small Ca_o-dependent Ca efflux component is not affected by vanadate.

Fig. 8 shows an experiment in which the effect of vanadate was explored in an axon in which all the internal sodium was removed. This treatment is known to increase dramatically the Na_o-dependent Ca efflux (for references see Ref. 29). After a pre-

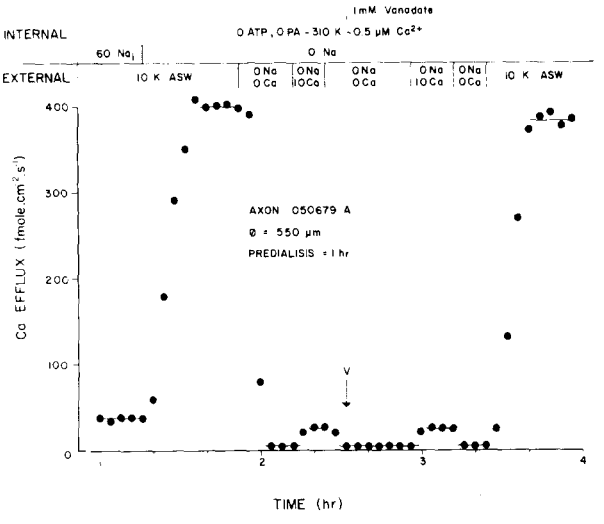

Fig. 8. The effect of vanadate on the Na_o-dependent Ca efflux in an axon dialyzed with 0.5 μM Ca^{2+}, and without Na_i and ATP. The axon was pretreated with CN^- and dialyzed for 1.5 h with ATP-free dialysis solution. Internal Na was substituted by Tris. Note that the large Na_o-dependent fraction of the Ca efflux is totally unaffected by vanadate. ASW, artificial sea water.

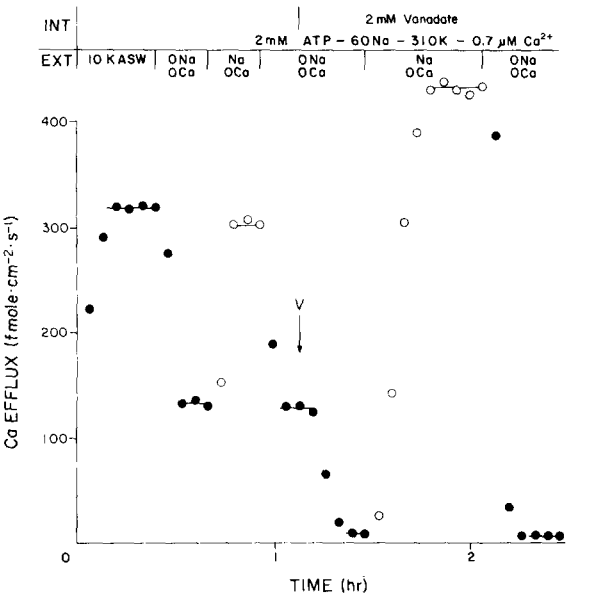

Fig. 9. The effect of vanadate on the Na_o-dependent Ca efflux in an axon dialyzed with 0.7 μM Ca^{2+} and 2 mM ATP. The open circles represent the value of the Ca efflux in the presence of full Na_o and no Ca_o. Closed circles represent either Ca efflux in the presence or in the absence of both Na_o and Ca_o. Unless otherwise stated all solute concentrations are given in mM. ASW, artificial sea water.

dialysis time of about 40 min with a solution containing 0.5 μM Ca_i^{2+}, 60 mM Na_i and no ATP, the Ca efflux reaches a steady state of about 35 fmol · cm^{-2} · s^{-1}. Removal of Na_i increases the efflux of Ca to 400 fmol · cm^{-2} · s^{-1}. As is shown in Fig. 8, most of this increment is Na_o dependent (90%), and only a small fraction Ca_o sensitive. The inclusion of 1 000 μM vanadate in the dialysis medium (more than 100 times the $K_{1/2}$ for the inhibition of the uncoupled component) causes no effect either on the Ca_o-dependent or on the large Na_o-dependent component.

The lack of effect of vanadate on the Ca_o-dependent Ca efflux observed in the present experiments, is another indication that the Ca_o-promoted Ca efflux obtained in the presence of vanadate in ATP fuelled axons perfuse with low $[Ca^{2+}]_i$ (see Fig. 3), is likely to be release of the vanadate inhibition by Ca_o and not to an induction of a Ca-Ca exchange by the inhibitor.

Effect of vanadate on the Na_o-dependent Ca efflux in the presence of ATP

The results presented above clearly show (Fig. 7 and 8) that vanadate, even at millimolar concentrations, has no effect on the Na_o-dependent Ca efflux in the absence of ATP. In squid axons, it is well known that ATP is able to activate with low affinity the Na_o-dependent Ca efflux component [24,25]. Therefore we decided to explore whether vanadate might affect the Na_o-dependent Ca efflux in the presence of ATP.

Fig. 9 shows an experiment in which an axon was dialyzed from the beginning with 0.7 μM Ca_i^{2+} and 2 mM ATP. After the Ca efflux has reached a steady state of about 320 fmol · cm^{-2} · s^{-1} the removal of both Na_o and Ca_o reduces the Ca efflux to 135 fmol · cm^{-2} · s^{-1}. Readdition of Na_o brings the efflux to 300 fmol · cm^{-2} · s^{-1}. In this particular experiment, and under the above experimental conditions, 53% of the total Ca efflux is Na_o-dependent, 39% uncoupled, 6% Ca_o-dependent and 2% 'leak'. The addition of 2 mM vanadate in the absence of external Na and Ca completely inhibits the uncoupled fraction. An interesting finding is that the addition of Na_o increases the Ca efflux to a value that not only surpasses the un-

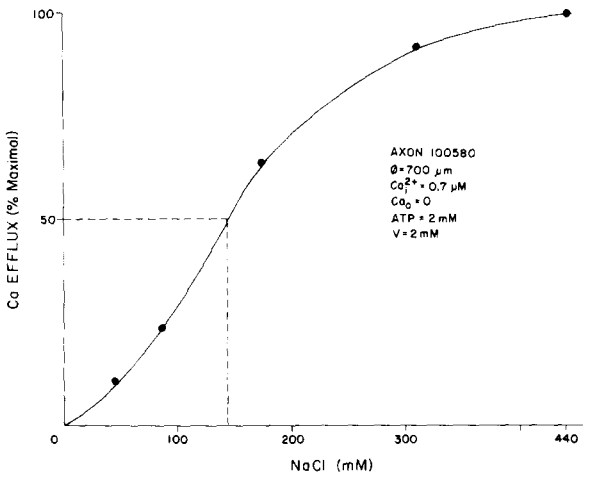

Fig. 10. The activation of the Ca efflux by Na_o in an axon containing ATP and vanadate. Ordenate: Percent of the maximal Na_o-dependent Ca efflux. Abscissa: external Na concentration. External Ca was replaced with equiosmolar amounts of Mg. The curve through the points was drawn by eye. The $K_{1/2}$ is close to 145 mM.

coupled component, but, also the Na_o-dependent one. This effect was found to be completely reversible upon removal of external Na.

Fig. 10 is an experiment designed to measured the activation of the Ca efflux by Na_o in an axon dialyzed with high $[Ca^{2+}]_i$, ATP and vanadate. For this particular experiment Na_o activates the Ca efflux along a sigmoidal curve with a $K_{1/2}$ of about 145 mM. The mean apparent affinity ($K_{1/2}$) from a total of three experiments was 120 mM.

Discussion

The results presented in this paper, confirm and extend previous observations reported by this laboratory [13], in dialyzed squid axons that vanadate fully inhibits with high affinity ($K_{1/2} \simeq 7$ μM) the ATP dependent uncoupled Ca efflux but does not affect the Na_o-dependent efflux of Ca in the absence of ATP. Recently, a similar finding has been obtained in unpoisoned intact axons of the squid by other laboratory [19].

The sites with wich vanadate interacts, are internally located, since no inhibition was found by externally applied vanadate. A similar situation occurs with the vanadate inhibition of the $(Na^+ + K^+)$-ATPase and the Na pump [16,17] and with the Ca^{2+}-ATPase and Ca pump [23] in other systems. Regarding the ATP dependent uncoupled Ca efflux, we have found that inhibition by vanadate is potentiated by potassium ions acting on the cytoplasmic side. The results are compatible with internal potassium increasing the affinity of the intracellular vanadate binding sites. Interestingly, external potassium ions had no effect on the degree of this inhibition. This concurs with recent observations on vanadate inhibition of the Ca pump in red blood cells [23]. On the other hand, it differs from the vanadate-K interactions on the $(Na^+ + K^+)$-ATPase where vanadate inhibition is potentiated by potassium ions acting on extracellular sites [14–16].

Our results also show that Ca ions acting on extracellular sites can markedly diminish vanadate inhibition of the uncoupled Ca efflux. This is in agreement with observations recently reported for the Ca pump in red cells [23]. In favor of a release of the vanadate inhibition caused by the external Ca ions, is the result of Fig. 4. In this experiment, no change in the influx of Ca was found in an axon dialyzed with vanadate under conditions of near maximum release of inhibition (vanadate = 100 μM, Ca_o = 10 mM; see Fig. 3). This indicates that the Ca_o effect cannot be accounted for by an induction of a Ca-Ca exchange due to vanadate. We have further shown, that the vanadate-calcium antagonism can be overcome by increasing the concentrations of either ligand. This suggests that the effect of Ca_o may also be on the affinity of the vanadate binding sites.

The small increment in Ca efflux induced by external Na in ATP-containing axons, dialyzed with physiological $[Ca^{2+}]_i$ concentrations (see Fig. 9), is similar, with or without vanadate. This argues against any significant effect of Na_o ions on vanadate inhibition under the above experimental conditions. On the other hand, an interesting finding that comes from the present experiments, is the observation that in axons containing ATP, dialyzed with high Ca_i^{2+} concentrations and poisoned with vanadate, there is an Na_o-dependent Ca efflux which is higher than that seen in axons with similar $[Ca^{2+}]_i$, and ATP, but without vanadate (see Fig. 9). As is clear from the experiments reported, in the absence of external sodium and calcium, the uncoupled Ca efflux is fully inhibited by vanadate, even in the presence of

high internal ionized Ca concentration. Since it is unlikely that internal vanadate, in the presence of external Na, actually activates the uncoupled Ca efflux, the possibility remains that these Na_o effects take place through the ATP promoted Na_o-dependent Ca efflux mechanism. This idea is supported by the fact that the $K_{1/2}$ for Na_o of the ATP-promoted Na_o-dependent Ca efflux (between 50 and 100 mM, see Refs. 24–26) is close to the Na_o-promoted Ca efflux in ATP-containing vanadate-poisoned axons. No data on vanadate effect on the Ca_i-dependent Na influx were collected, since for the ionized Ca_i concentrations used, the magnitude of the Na influx is too small to be detected when considering a 3–4 Na/Ca coupling ratio.

The absence of vanadate inhibition on the Na_o-dependent Ca efflux (in the presence or absence of ATP, at high or at low Ca_i) contrasts with the marked inhibition of the uncoupled Ca efflux. This favors the hypotehsis [5] of two independent parallel Ca transport systems in squid axons. Furthermore, the fact that vanadate not only fully inhibits the uncoupled Ca efflux, but also has a similar type of interaction with other ligands (including sidedness), as in the Ca pump in red blood cells, strongly favors the idea that the uncoupled Ca efflux in squid axons is a true ATP-driven Ca pump.

The use of vanadate as a tool for examining Ca fluxes in intact preparations (Na_o and Ca_o containing medium) should be viewed with caution, due to the sidedness effect of external Ca and Na on the vanadate effects. Furthermore, these effects are seen even at ATP concentrations in the micromolar range. This indicates that extreme caution should be taken when analysing the effect of vanadate on Ca fluxes in preparations were a thorough ATP wash-out is not certain.

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