Biochimica et Biophysica Acta 814 (1985) 13–22
Elsevier

BBA 72493

ATP-dependent calcium accumulation by non-mitochondrial organelles of axoplasm isolated from *Myxicola* giant axons

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(Received September 27th, 1984)

Key words: Ca^{2+} transport; Ca^{2+}-ATPase; Axoplasm; Non-mitochondrial uptake; Nerve; (*Myxicola* giant axon)

Axoplasm from freshly isolated *Myxicola* giant axons was mixed with small volumes of 'artificial axoplasm' containing ^{45}Ca and either CaEGTA/EGTA or CaDTPA/DTPA buffers giving various nominal values of $[Ca^{2+}]$. The axoplasm samples were centrifuged at $100\,000 \times g$ for 30 min to form a pellet and the percentage of ^{45}Ca bound to the pellet was determined. The fraction of bound calcium rose with increasing values of $[Ca^{2+}]$ along an S-shaped curve. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was used to reveal the presence of mitochondrial Ca uptake. At physiological values of $[Ca^{2+}]$, around 100 nM, Ca uptake was insensitive to FCCP. As $[Ca^{2+}]$ was elevated, increasing sensitivity to FCCP was noted above $[Ca^{2+}] = 0.5$ μM. At low values of $[Ca^{2+}]$, including the physiological range, Ca binding was significantly reduced by vanadate and quercetin, agents known to inhibit Ca uptake mediated by Ca^{2+}-activated ATPase reactions. Inhibition of Ca binding by these agents was approximately 50% at physiological values of $[Ca^{2+}]$. ATP depletion decreased the percentage of Ca binding by the pellet at physiological $[Ca^{2+}]$. The results suggest that about 50% of the Ca buffering by particulate matter in axoplasm is via organelles requiring intact Ca^{2+}-ATPase reaction at physiological values of $[Ca^{2+}]$.

Introduction

Both squid and *Myxicola* giant axons maintain very low values of the ionized calcium concentration, $[Ca^{2+}]_i$. Measurements of $[Ca^{2+}]_i$ in squid giant axons using optical methods [1] or Ca^{2+}-sensitive electrodes [2] gave an estimate between 20 and 100 nM. Application of the aequorin method to axoplasm from *Myxicola* giant axons gave values of $[Ca^{2+}]_i$ between 68 and 125 nM [3]. The total Ca content of freshly isolated squid axons is 60 μmol/kg axoplasm [4] and values in the range 100–500 μmol/kg axoplasm have been reported for both squid and *Myxicola* giant axons [3]. Clearly most of the calcium in axoplasm is buffered to give values of ionized calcium concentration about 1000-times lower than the total calcium concentration.

How this buffering is achieved is a matter of some physiological importance. Mitochondria are active in buffering an induced Ca load in intact squid giant axons, as deduced from the inhibitory effect on Ca buffering of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) [5,6]. In axoplasm isolated from squid [3] and *Myxicola* giant axons [3,7] an ATP-

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid.

dependent and an ATP-independent component of Ca binding has been described. The energy-dependent component has been identified as mitochondrial, based on its requirement for either ATP or succinate plus orthophosphate and its sensitivity to the mitochondrial inhibitors cyanide and oligomycin [3]. However, the threshold for Ca^{2+} uptake by mitochondria in intact squid axons lies in the range 200–400 nM, [5], and the mitochondrial component of Ca binding described by Baker and Schlaepfer, [3], shows a low affinity for Ca ($K_m = 50$ and 10 μM for squid and *Myxicola* axoplasm, respectively). As these values are several-fold higher than the resting physiological value of $[Ca^{2+}]_i$ in freshly dissected axons, it is not likely that mitochondrial Ca buffering is of much consequence in a resting nerve fiber.

The question of the Ca buffering entities in squid or *Myxicola* giant axons active at physiological values of $[Ca]_i$ remains largely unanswered. The energy-independent component of Ca binding shows high affinity for Ca ($K_m = 80$ and 160 nM for squid and *Myxicola* axoplasm, respectively) and a limited capacity [3]. This high-affinity component has not yet been identified.

In morphological studies it has been found that, besides mitochondria, the smooth endoplasmic reticulum of squid axons can also accumulate Ca [8]. It has also been demonstrated that essentially all of the non-mitochondrial component of Ca buffering in axoplasm of squid axons is located in organelles [9]. In rat nerve terminal (synaptosome) preparations, a non-mitochondrial ATP-dependent Ca sequestering process which resembles the Ca uptake mechanism of skeletal muscle sarcoplasmic reticulum has been described [10]. Based on the similar affinities for Ca and ATP and similar effects of poisons and other agents on both systems, Blaustein et al. [10] postulate that the non-mitochondrial ATP-dependent Ca uptake mechanism present in their synaptosome preparation is the smooth endoplasmic reticulum. Baker and Schlaepfer [3], on the other hand, found no evidence for an ATP-dependent but cyanide and oligomycin-insensitive component of Ca binding in either squid or *Myxicola* axoplasm.

The purpose of the present research was to investigate further the Ca-binding organelles in nerve fibers using centrifuged axoplasm from *Myxicola* giant axons. The aim was two-fold, to determine any important differences between the behavior of Ca-binding organelles in *Myxicola* axons and those in squid axons and to look for new properties of the non-mitochondrial Ca-buffering components. Due to the variability of axoplasm isolated from different batches of *Myxicola* and the relatively small yield of axoplasm, the present results are semi-quantitative and no kinetic analysis has been done.

Methods

The general method was a modification of the procedure described by Tiffert and Brinley [9]. Freshly dissected giant axons from the marine annelid *Myxicola* were given two consecutive rinses of 3 min each in Na-free 'artificial axoplasm' (320 mM potassium aspartate/354 mM glycine/3 mM $MgCl_2$/50 mM K_2Tes buffer (pH 7.3)). Axoplasm samples were taken from the blotted axons by cutting a longitudinal slit and removing the axoplasm with fine forceps. The weighed axoplasm samples from several axons were then pooled and diluted 1 : 4 with Na-free artificial axoplasm containing 2 mM Mg-ATP. The diluted axoplasm was then used in several protocols. The protocol procedures differed slightly depending upon the particular experiment. In experiments to measure Ca binding at different values of $[Ca^{2+}]$ and to determine the effects of FCCP and added calcium-ionophore (A23187) on calcium binding, ^{45}Ca/CaEGTA/EGTA mixtures that provided different final values of $[Ca^{2+}]$ were added to the diluted axoplasm samples and allowed to equilibrate for 15 min. Longer equilibration times (up to 30 min) produced no significant difference in the percentage of ^{45}Ca taken up by the pellet. The amount of CaEGTA/EGTA buffer added to axoplasm was such as to provide a final buffer concentration of around 500 μmol/kg axoplasm. For values of $[Ca^{2+}] = 3$ μM and above, the EGTA buffer was replaced with DTPA. The effective dissociation constants under the present conditions of ionic strength and pH were $0.15 \cdot 10^{-6}$ M and $3 \cdot 10^{-6}$ M for CaEGTA and CaDTPA, respectively [9].

In a typical protocol, the initial sample of diluted axoplasm was divided into two equal lots

prior to addition of ^{45}Ca. Each lot was then used for a different final value of $[Ca^{2+}]$. Control Ca binding was determined by drawing the axoplasm mixture into PE90 polyethylene tubing. Tubes containing 10 μl of sample were then cut and heat-sealed and centrifuged in a Beckman air-driven ultracentrifuge for 30 min at $100\,000 \times g$. FCCP was added to one lot of the remaining labelled axoplasm and FCCP + A23187 was added to the remaining lot. Tubed samples were again taken and the tubes centrifuged as before for 30 min at $100\,000 \times g$.

After centrifugation, the tubes were quickly frozen on solid CO_2 and sequentially sliced into 2 mm segments. The segments were placed into 6-ml scintillation counting vials with 1 ml of 1 mM $CaCl_2$. After overnight storage to elute the ^{45}Ca, 5 ml of scintillation counting fluid was added to each vial and radioactivity was counted in a Beckman LS-230 scintillation counter. The ^{45}Ca counts for a given polyethylene tube were then plotted versus segment number (sample 1 is at top of tube) and the percentage of counts in the pellet was taken as the measure of Ca binding. The pellet was clearly visualized in the bottom portion of the tube, occupying a length of about 4–6 mm. In practice, a residual ^{45}Ca count is evident in all of the upper tube segments. This count of ^{45}Ca per unit length is constant and must represent the ^{45}Ca not moved by centrifugation. Much of this ^{45}Ca must be in solution as $^{45}CaEGTA$, though some could be bound to molecules present in axoplasm that are not displaced by centrifugation. Most of the ^{45}Ca accumulation above the residual amount distributed in solution occurred in the bottom two segments. Whenever Ca accumulation occurred, the residual ^{45}Ca in solution was subtracted from the total ^{45}Ca counts in the segment showing accumulation. The amount of residual ^{45}Ca to subtract was found by multiplying the average residual ^{45}Ca counts per unit length times the length of the segment showing accumulation. No attempt was made to correct for the volume fraction of particulate matter in the bottom segments when determining residual ^{45}Ca as a volume marker was not employed. This means that a maximum residual ^{45}Ca is subtracted that is perhaps greater than the actual residual amount. The actual Ca binding to particles in the pellet could therefore be somewhat greater than the calculated values.

In other protocols involving inhibitors of calcium uptake, the inhibitor was added prior to addition of ^{45}Ca and allowed to act for 15 minutes before addition of ^{45}Ca and either EGTA or DTPA buffer mixtures. The procedure after addition of ^{45}Ca was similar to that already described. This procedure was followed for the inhibitors vanadate, quercetin, and apyrase.

In treating the numerical data, each datum point represents ^{45}Ca bound to the pellet from a single sample of pooled axoplasm. Each value with inhibitor present thus had, in the experimental plan, a corresponding control value on the same sample of axoplasm. Ideally, the numbers of experiments shown in the tables in parentheses should be the same for controls and samples treated with an inhibitor. A technical difficulty was encountered, however, that often upsets this correspondence. Some polyethylene tubes leak during centrifugation at the heat-sealed junction. Improved technique minimized the number of such leaks but a certain unavoidable number of leaks occurred randomly. As control tubes and tubes from inhibitor-treated samples can leak randomly and independently, the numbers of recorded observations often are different for control and inhibitor-treated samples, the number of control values sometimes being more than and sometimes less than the number of values with inhibitor. In calculations of % ^{45}Ca released or ^{45}Ca uptake inhibited using a certain modifying agent (see tables), only values paired from the same sample of axoplasm are used so that, again, numbers of experiments reported for those data columns do not always agree with numbers in the other columns. This procedure was followed because of the improved statistical significance obtained using data from the same sample of axoplasm. It should be noted, however, that conclusions based on average values rather than paired values would not be qualitatively different.

Results and Discussion

^{45}Ca accumulation by the pellet as a function of ambient $[Ca^{2+}]$. Effect of FCCP and Ca ionophore A23187

Tiffert and Brinley [9] demonstrated that

calcium uptake by organelles of isolated squid axoplasm is a function of the ambient free calcium concentration, $[Ca^{2+}]$, and part of the accumulated Ca is released by FCCP. That organelles of isolated axoplasm of *Myxicola* axons show a similar behavior is seen from Fig. 1, which describes the distribution of ^{45}Ca cpm along the tubes of centrifuged axoplasm at two different ambient $[Ca^{2+}]$ values. In these experiments samples of isolated axoplasm were mixed with ^{45}Ca as described in the legend for Fig. 1. The accumulation of ^{45}Ca by the pellet (bottom sections) above the general solution levels is evident at both (0.56 and 3.0 μM) free calcium concentrations. In the presence of 8 μM FCCP (final concentration in axoplasm suspension) there was a marked reduction in ^{45}Ca cpm corresponding to the pellet; this is shown in Fig. 1 for a nominal $[Ca^{2+}]$ of 3.0 μM. The agent FCCP is an uncoupler of oxidative phosphorylation that prevents Ca uptake and releases accumulated Ca in mitochondria by collapsing the proton gradient [14]. From Fig. 1 it is evident that at 3.0 μM free calcium concentration part of the ^{45}Ca accumulated by the pellet indicates mitochondrial Ca uptake. The ^{45}Ca accumulated by the pellet remaining after addition of FCCP could be due to ^{45}Ca stored in buffering organelles other than mitochondria.

The calcium ionophore A23187 increases membrane permeability to calcium and releases stored calcium in mitochondria [15], sarcoplasmic reticulum [17] and endoplasmic reticulum of nerve terminals [10]. Therefore, in the presence of A23187 all ^{45}Ca accumulation by membrane-bound vesicles should be abolished. Endoplasmic reticulum in squid axons is capable of Ca accumulation [8] and part of the FCCP-insensitive Ca accumulation by organelles of squid axoplasm is released upon addition of A23187 [9]. The experiments described in Fig. 2 were carried out to test the effect of the free calcium concentration on the percent of total ^{45}Ca

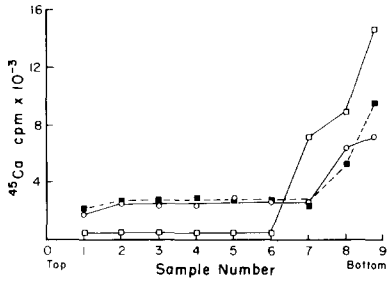

Fig. 1. Distribution of ^{45}Ca cpm along the column of centrifuged axoplasm at two different ambient $[Ca^{2+}]$ values. Each point represents the cpm contained in each 2 mm segment of axoplasm. Samples of isolated axoplasm were diluted 1:4 with Na-free 'artificial axoplasm' supplemented with 2 mM Mg-ATP as indicated under Methods. The reason for using a Na-free solution was to avoid any effect of Na on Ca release from mitochondria [11]. This solution also contained 3 mM Mg above the concentration of Mg-ATP in order to simulate the physiological concentration of Mg in axoplasm (2–3 mM) [12,13] as it is known that Mg^{2+} has a significant inhibitory effect on the rate of Ca uptake by mitochondria [14]. An appropriate amount of either ^{45}Ca-EGTA buffer having a nominal free calcium concentration ($[Ca^{2+}]$) of 0.56 μM (○———○) or ^{45}Ca-DTPA buffer with a nominal $[Ca^{2+}]$ of 3 μM (□———□; ■———■) was then added to the axoplasm suspension and the procedure described under Methods was followed. The points (■———■) refer to axoplasm poisoned with 8 μM FCCP at a nominal free calcium concentration of 3 μM. Ordinate: ^{45}Ca cpm in sample. Abscissa: sample number. Sample 1 is at top of tube. Fractions 7, 8 and 9 represent the pellet.

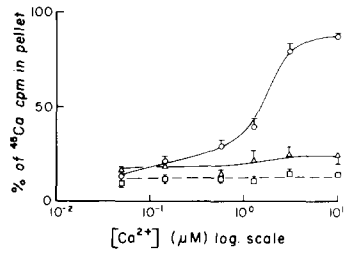

Fig. 2. Effect of FCCP and Ca-ionophore A23187 on ^{45}Ca accumulation by the pellet at several free calcium concentrations. Isolated axoplasm samples were processed as described in Fig. 1 and after 15 min of equilibration of the samples with ^{45}Ca-buffer, they were divided into three lots. One of them was used as control (○———○) to measure calcium accumulation by the pellet in the absence of poisons. To the second lot (△———△) was added 8 μM FCCP (final concentration in sample) and to the third lot (□———□), 8 μM FCCP plus 10 μM A23187 (final concentrations in samples). After allowing 15 min for equilibration, the samples were drawn into polyethylene PE90 tubing and processed as described under Methods. From curves similar to those in Fig. 1, the percentage of the total ^{45}Ca that was accumulated by the pellet was determined (see Methods) and plotted against ambient $[Ca^{2+}]$ provided by either Ca-EGTA/EGTA or Ca-DTPA/DTPA buffer ratios. Ordinate: percentage of ^{45}Ca accumulated. Abscissa: free calcium concentration, log scale. The free calcium concentrations are nominal (see Ref. 9).

accumulated by the pellet and also to determine the free calcium concentration at which the poisons FCCP and A23187 are effective inhibitors of Ca accumulation. On the basis of the above considerations on the effects of FCCP and A23187, a FCCP-sensitive ^{45}Ca accumulation is mitochondrial and a FCCP-insensitive A23187-sensitive ^{45}Ca accumulation is due to organelles other than mitochondria. Fig. 2 describes the percent of ^{45}Ca accumulated by the pellet as a function of the ambient free calcium concentration (μM) in axoplasm isolated from *Myxicola* axons. These experiments were carried out as described in the legend for Fig. 2. As seen in Fig. 2, the control curve (absence of poisons) has a marked sigmoidal shape, rising steeply above a nominally ambient $[Ca^{2+}]$ of 560 nM. The mitochondrial uncoupler FCCP had no effect on ^{45}Ca accumulated by the pellet at low values of $[Ca^{2+}]$, around 50 and 140 nM, close to the physiological values of $[Ca^{2+}]$ in axoplasm, but its effect increased steeply at concentrations above 560 nM (Fig. 2 and Table I). At $[Ca^{2+}] = 10$ μM about 75% of the control ^{45}Ca-accumulation is abolished by FCCP. The calcium-ionophore A23187 when added to FCCP-treated axoplasm had a noticeable effect at low values of $[Ca^{2+}]$ and caused a further reduction in ^{45}Ca accumulation at higher values of $[Ca^{2+}]$ (Fig. 2 and Table I). From Fig. 2 and Table I it thus seems clear that the pellet was able to accumulate calcium at physiological concentrations of the ion (50–100 nM) in a FCCP-insensitive A23187-releasable compartment. The results in Fig. 2 are presented numerically in Table I where the percent of total ^{45}Ca accumulated by the pellet and the percent of ^{45}Ca released by either FCCP or FCCP + A23187 treatment at several nominal $[Ca^{2+}]$ are given. These results are consistent with the generally accepted notion that the non-mitochondrial Ca-buffer is located in organelles and plays a significant role in Ca buffering in nerve tissue at physiological values of $[Ca^{2+}]$, whereas mitochondrial Ca buffering is of lower affinity and becomes effective at higher values of $[Ca^{2+}]$ [5,8–10].

The ^{45}Ca accumulated by the pellet and remaining in the presence of FCCP and A23187 could be due to Ca binding to low- and high-affinity sites on mitochondrial membranes and membrane-bound organelles [17,18] or to an effective con-

TABLE I

EFFECT OF FCCP AND FCCP+A23187 ON ^{45}Ca ACCUMULATION BY THE PELLET OF CENTRIFUGED *MYXICOLA* AXOPLASM

Values given in this table correspond to the experiments described in Fig. 2. For details see legend for Fig. 2. The percentages of ^{45}Ca released by FCCP are referred to the control values (absence of inhibitors). The percentages of ^{45}Ca released by A23187 addition are referred to the values of ^{45}Ca accumulated in the presence of FCCP. The percentages of total ^{45}Ca cpm accumulated by the pellet in the presence of inhibitors were always compared with control values obtained on lots from the same total sample of pooled axoplasm. Values are mean ± S.E. The numbers between parentheses indicate number of experiments. The given values for free calcium concentrations are nominal. A possible increase of free $[Mg^{2+}]$ due to hydrolysis of ATP in the presence of FCCP and A23187 cannot be disregarded. Such an increase in Mg^{2+}, however, would not seem to inhibit ^{45}Ca accumulation by mitochondria, as the FCCP-sensitive component was the same in the control (3.0 mM Mg; 68% release) as in the presence of 10 mM Mg (61%, mean of two experiments).

$[Ca^{2+}]$ (μM)	Percentage of total ^{45}Ca cpm in pellet			Percentage of ^{45}Ca cpm released [a]	
	Control	FCCP	FCCP + A23187	FCCP	A23187
0.047	13.5 ± 1.8 (12)	15.8 ± 2.0 (14)**	11.1 ± 1.5 (8)†	0	40.9 ± 13 (6)
0.14	20.4 ± 3.4 (7)	19.7 ± 1.5 (6)**	12.1 ± 1.3 (8)†	0	49.0 ± 6.5 (4)
0.56	29.2 ± 2.6 (9)	14.7 ± 1.7 (9)††	12.4 ± 1.0 (5)°	45.7 ± 7.2 (7)	30.9 ± 6.1 (5)
1.26	39.8 ± 4.3 (7)	21.9 ± 4.8 (4)†	10.7 ± 0.8 (6)†	35.6 ± 16 (4)	59.5 ± 3.6 (3)
3.0	79.0 ± 2.9 (5)	25.0 ± 4.0 (6)††	15.3 ± 1.9 (6)°°	64.8 ± 5.8 (5)	49.5 ± 2.8 (4)
10.0	86.9 ± 1.3 (6)	24.6 ± 5.7 (7)*	14.1 ± 0.8 (3)	68.2 ± 5.8 (4)	66.0 [b]

[a] Based on paired data from same sample of axoplasm (see Methods).

[b] Mean of two experiments. From *t*-values for paired data: $\dagger P < 0.05$; $\dagger\dagger P < 0.005$; $^{\circ} P < 0.025$; $^{\circ\circ} P < 0.01$; $^{*} P < 0.001$; **non-significant.

centration in axoplasm of these agents lower than that required for total inhibition, or both. The latter possibility seems unlikely as the ratio of inhibitor to membrane is high and, from the reasoning of Tiffert and Brinley [9], should have been appropriate. Also, in some additional non-reported experiments, application of higher concentrations of inhibitors produced no greater inhibition of calcium buffering. The incubation time to test the effect of the inhibitor was adequate, since longer incubation times (up to 30 min) produced no change in the percentage of inhibition.

Effect of ATP depletion on ^{45}Ca accumulation by the pellet of centrifuged axoplasm isolated from Myxicola axons

The absence of ATP in the axoplasm suspension should not inhibit mitochondrial Ca uptake in the presence of substrate as this organelle can use energy derived from electron transport or ATP hydrolysis to accumulate Ca. On the other hand, if a Ca-pump which uses energy derived from ATP hydrolysis is present in the membrane of non-mitochondrial organelles of *Myxicola* axoplasm, as is the case in endoplasmic reticulum of rat brain synaptosomes, red blood cell membranes, and the sarcoplasmic reticulum [10,19,20], ATP depletion should strongly inhibit Ca accumulation at low $[Ca^{2+}]$ values, i.e., at $[Ca^{2+}]$ below the threshold for mitochondrial Ca uptake. Some of the experiments described in Table II were designed to test the effect of ATP depletion on ^{45}Ca accumulation by pellet as a function of the ambient free calcium concentration. The enzyme apyrase was used to deplete the ATP of the axoplasm suspension as described in the legend for Table II. From Table II it is clear that ATP depletion produced a noticeable inhibition of ^{45}Ca at values of $[Ca^{2+}]$ within the physiological range (50–100 nM). At higher values of $[Ca^{2+}]$ the presence of ATP does not seem to be essential for Ca accumulation by the pellet. The effect of FCCP addition to ATP-depleted axoplasm was dependent on the free calcium concentration. At low $[Ca^{2+}]$ (47 nM) the percent of total ^{45}Ca cpm in pellet was 4.1 ± 0.6 (mean $\pm$ S.E., $n = 15$) in apyrase-treated axoplasm as compared with 6.8 ± 0.6 (mean $\pm$ S.E., $n = 14$) in axoplasm treated with apyrase plus FCCP. At 3.0 μM $[Ca^{2+}]$, however, the percent of total ^{45}Ca cpm in the pellet was 77.6 ± 1.0 ($n = 12$); 54.8 ± 2.3 ($n = 10$) and 8.9 ± 0.79 ($n = 17$), all mean $\pm$ S.E., for control, ATP-depleted and ATP-depleted plus FCCP-treated axoplasm, respectively. From the above values it is clear that the mitochondria could accumulate Ca in the absence of ATP provided the $[Ca^{2+}]$ was above a threshold value. The results presented in this section indicate that at physiological $[Ca^{2+}]$ an ATP-dependent Ca-accumulation process in cellular organelles other than mitochondria is active in *Myxicola* axoplasm. Our results do not agree with those of Baker and Schlaepfer [3] who found no energy-dependent Ca-sequestering system in squid and *Myxicola* axoplasm other than the mitochondrial one. However, the conditions under which the organelles were isolated and maintained in their experiments were completely different from ours. They dispersed axoplasm by exposing it to large volumes of 0.5 M KCl containing 1 mM calcium in order to liquefy the axoplasm. This high Ca load must surely have resulted in organelles massively loaded with calcium at the beginning of the experiment. Crude mitochondrial preparations were then made by passing the liquefied axoplasm through filter paper to retain organelles. This filtering step seems unlikely to permit a clean separation between mitochondria and endoplasmic reticulum organelles.

Effect of vanadate on ^{45}Ca accumulation by pellet

Vanadate inhibits the Ca^{2+}-ATPase and Ca-pump of the red blood cell membrane, sarcoplasmic reticulum, brain microsomal membrane and squid axolemma [22–26]. Mitochondrial ATPase is not inhibited by vanadate [27]. Since the results of the preceding section demonstrated the presence of an ATP-dependent ^{45}Ca accumulation by organelles other than the mitochondria in *Myxicola* axoplasm, vanadate was used in the present experiments to test for the presence in this preparation of a Ca-pump similar to the one found in the membranes mentioned above. The experimental protocols were identical to those used with apyrase, except that 100 μM vanadate was used instead. This vanadate concentration was chosen to assure inhibition of any Ca^{2+}-ATPase present in *Myxicola* axoplasm, as different sensitivities of Ca^{2+}-ATPases to vanadate have been reported

TABLE II

EFFECT OF ATP-DEPLETION AND ATPase INHIBITORS ON THE PERCENTAGE OF TOTAL ^{45}Ca cpm ACCUMULATED BY PELLET OF CENTRIFUGED *MYXICOLA* AXOPLASM

Axoplasm was isolated from *Myxicola* axons and resuspended in Na-free 'artificial axoplasm' as described under Methods. The pooled axoplasm samples were divided into three lots. One of them was used as control with addition of 2 mM Mg-ATP (final concentration in axoplasm suspension). The enzyme apyrase, a nonspecific ATPase which produces a dramatic reduction in ATP concentration of giant axons [20] was added to the second and third lots of axoplasm (5 units/ml, final concentration in sample) and was allowed to act for 15 min prior to ^{45}Ca addition. To the third lot of axoplasm suspension FCCP was added (8 μM final concentration) in addition to apyrase. ^{45}Ca buffer mixture to give a certain $[Ca^{2+}]$ was added to the three lots and after 15 min the procedure described under Methods was followed. The same procedure described for apyrase was followed for testing vanadate and quercetin effects, vanadate (100 μM final concentration), or quercetin (50 μM final concentration) replacing apyrase. The given values for free calcium concentrations are nominal. Values in this table express the percentage of total ^{45}Ca cpm in pellet and are mean ± S.E. The numbers between parentheses indicate number of experiments.

$[Ca^{2+}]$ (μM)	Control	Apyrase	% Inhibition [a]	Control	Vanadate	% Inhibition [a]	Control	Quercetin	% Inhibition [a]
0.047	25.3 ± 1.5 (13)	4.1 ± 0.6 (15)††	82.6 ± 2.5 (13)	19.6 ± 1.1 (15)	11.3 ± 1.1 (14)†††	48.8 ± 3.0 (12)	17.0 ± 1.9 (7)	9.6 ± 1.1 (6)*	38.4 ± 5.8 (6)
0.14	35.9 ± 4.0 (8)	14.1 ± 2.3 (9)††	63.7 ± 5.3 (7)	27.8 ± 2.7 (5)	13.5 ± 1.3 (5)*	48.8 ± 7.5 (5)	40.4 ± 1.5 (5)	23.8 ± 1.1 (4)††	40.8 ± 4.5 (4)
0.56	40.5 ± 1.2 (3)	25.2 ± 1.5 (4)†	36.3 ± 7.0 (3)	27.2 ± 1.4 (4)	22.9 ± 2.0 (4)†	16.3 ± 3.2 (4)	50.4 ± 1.4 (3)	28.9 ± 1.8 (4)††	42.4 ± 3.4 (3)
1.26	39.1 ± 1.8 (7)	35.0 ± 1.6 (9)†††	12.7 ± 1.5 (7)	32.0 ± 2.9 (3)	26.9 ± 3.0 (3)°°	12.8 ± 5.5 (3)	38.8 ± 5.3 (3)	38.4 ± 1.7 (4)°°	0.5 ± 4 (3)
3.0	77.6 ± 1.0 (12)	54.8 ± 2.3 (10)††	28.5 ± 3.3 (7)	76.3 ± 2.0 (12)	65.8 ± 3.2 (12)**	12.9 ± 3.9 (11)	77.8 ± 2.7 (8)	66.8 ± 2.8 (8)**	14.6 ± 7.0 (8)
10.0	76.4 ± 3.0 (3)	67.6 ± 1.0 (3)††	8.9 ± 2.9 (3)	82.1 ± 1.4 (3)	82.5 ± 1.8 (4)††	0	76.6 ± 2.4 (3)	75.2 ± 2.3 (4)°°	0.7 ± 0.4 (3)

[a] The percentages of inhibition of ^{45}Ca uptake are based on paired data from the same sample of pooled axoplasm (see Methods). $^{\dagger\dagger\dagger}P < 0.001$ (for t-values for paired data); $^{\dagger}P < 0.01$; $^{\dagger\dagger}P < 0.001$; $^{**}P < 0.025$; $^{*}P < 0.005$; °°non-significant.

[24,25]. Vanadate caused a marked inhibition of ^{45}Ca accumulation by the pellet at low values of $[Ca^{2+}]$. It also caused significant inhibition of ^{45}Ca accumulation at the higher $[Ca^{2+}]$ as well, though on a percentage basis the inhibition was considerably less. A more complete presentation of the data obtained in the presence of vanadate is found in Table II, where percentage of inhibition is stated for various values of $[Ca^{2+}]$.

Effect of quercetin on ^{45}Ca accumulation by the pellet

The flavonoid quercetin has been shown to inhibit the Ca^{2+}-ATPase and Ca-pump of red blood cells and sarcoplasmic reticulum [28–30]. If the vanadate-sensitive ATP-dependent Ca-buffering system just described shared the general properties of other well-known Ca pumps (erythrocyte membranes and sarcoplasmic reticulum), quercetin should inhibit ^{45}Ca accumulation at low $[Ca^{2+}]$. The experimental protocols were identical to the ones described for apyrase and vanadate, except that 50 μM quercetin replaced apyrase. As expected for ^{45}Ca accumulation via a Ca-pump, the presence of quercetin caused an inhibition (about 50%) of the ^{45}Ca uptake at low, physiological values of $[Ca^{2+}]$ (Table II). That quercetin has no effect on mitochondrial ^{45}Ca accumulation is shown by the fact that the percentage of ^{45}Ca-released by FCCP at 10 μM $[Ca^{2+}]$ is $68.2 \pm 5.8\%$ (mean $\pm$ S.E., $n = 4$) in the absence of quercetin and $68.7 \pm 1.0\%$ (mean $\pm$ S.E., $n = 4$) in its presence (not shown in the table).

It should be noted that ^{45}Ca accumulation values obtained in the presence of inhibitors were always compared with control values obtained on lots from the same total sample of pooled axoplasm. The effect of FCCP in the presence of inhibitors was also tested on lots from the same total sample of pooled axoplasm. Thus there will be minor differences between the values presented in the tables and those deduced from the figures. Within the range of experimental error, there does not appear to be any difference in the actions of apyrase (depletion of substrate), quercetin and vanadate on ^{45}Ca sequestration by organelles in the axoplasm of *Myxicola* giant axons. The three agents caused a strong reduction in the percentage of ^{45}Ca accumulated by the pellet only at low $[Ca^{2+}]$ within the physiological range. This observation, together with the fact that at low $[Ca^{2+}]$ the system was insensitive to FCCP, is consistent with the presence within *Myxicola* axoplasm of non-mitochondrial Ca-buffering organelles that sequester Ca^{2+} by a mechanism of high affinity for Ca^{2+} requiring ATP and functional ATPase.

Percentage of inhibition of ^{45}Ca accumulation as a function of $[Ca^{2+}]$

The action of FCCP and A23187 on Ca sequestration by axoplasm (percentage of bound ^{45}Ca released) is tabulated in Table I in a manner similar to the data presentation for the other inhibitors. The results with FCCP are seen to be the reciprocal of those obtained for all the other inhibitors employed. This can best be visualized by plotting the percent inhibition versus $[Ca^{2+}]$ for all of the inhibitors used. This is done in Fig. 3 for the inhibitors vanadate, quercetin, apyrase and FCCP. The best smooth curves are drawn by eye through the data points. It is clear that two reciprocal curves emerge from the data, one fitting the FCCP data and one giving a fair fit to the data for all of the other inhibitors, the ones which inhibit Ca-ATPase, or in the case of apyrase, unfuel the system. Within the ranges of experimental error, there is no evidence for significant differences among the class of Ca-ATPase inhibiting compounds. There is some suggestion that apyrase is the most effective inhibitor, but no claim for significance is made.

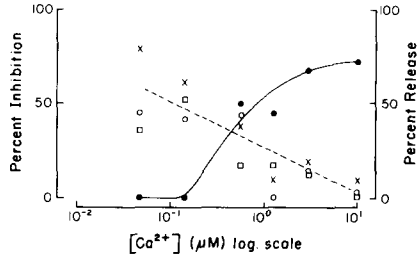

Fig. 3. Percentage of inhibition of ^{45}Ca accumulation as a function of $[Ca^{2+}]$. Values in this figure correspond to the experiments described in Tables I and II. Ordinate: percentage of inhibition. Abscissa: free calcium concentration, log scale. The free calcium concentrations are nominal. Left-hand scale: percent inhibition for apyrase (×———×), vanadate (□———□) and quercetin (○———○). Right-hand scale: percent of release for FCCP (●———●).

The present results strongly suggest the existence in *Myxicola* axoplasm of non-mitochondrial membrane-bound vesicles possessing a Ca-pump with high affinity for Ca^{2+}. That Ca^{2+} is sequestered into membranous organelles is suggested by the effect of the Ca-ionophore A23187 on ^{45}Ca release from the pellet in the presence of the uncoupler FCCP. The lack of effect of FCCP at low $[Ca^{2+}]$ seems to preclude any significant Ca-buffering by mitochondria at physiological $[Ca^{2+}]$. That Ca uptake at physiological concentrations is mainly an ATP-dependent process is shown by the strong inhibitory effect of apyrase, which reduces the ATP-concentration to near zero. The high affinity of the organelles for Ca is evident as saturation is approached at about 50 nM Ca^{2+}. The inhibitory effect of the Ca-pump ATPase inhibitors of other cells (vanadate and quercetin) suggests the presence in *Myxicola* axoplasm of a mechanism quite similar to the Ca-pump of erythrocytes and the sarcoplasmic reticulum. Finally, the comparison of the inhibitory effect of the inhibitors tested (Fig. 3) clearly demonstrates the presence of two entirely different Ca-buffering systems: one operative at low $[Ca^{2+}]$ (50–100 nM) and the other mitochondrial in origin (FCCP-sensitive) and becoming operative at $[Ca^{2+}]$ higher than 500 nM.

Electron microscopy of whole axoplasm was carried out in order to determine whether a non-mitochondrial membrane bound compartment could be visualized. Although the results were in general agreement with the observations of Gilbert [31], both mitochondria and reticulo-vesicular elements were more common (see Fig. 4). Indeed, it was difficult to find broad organelle-free fields

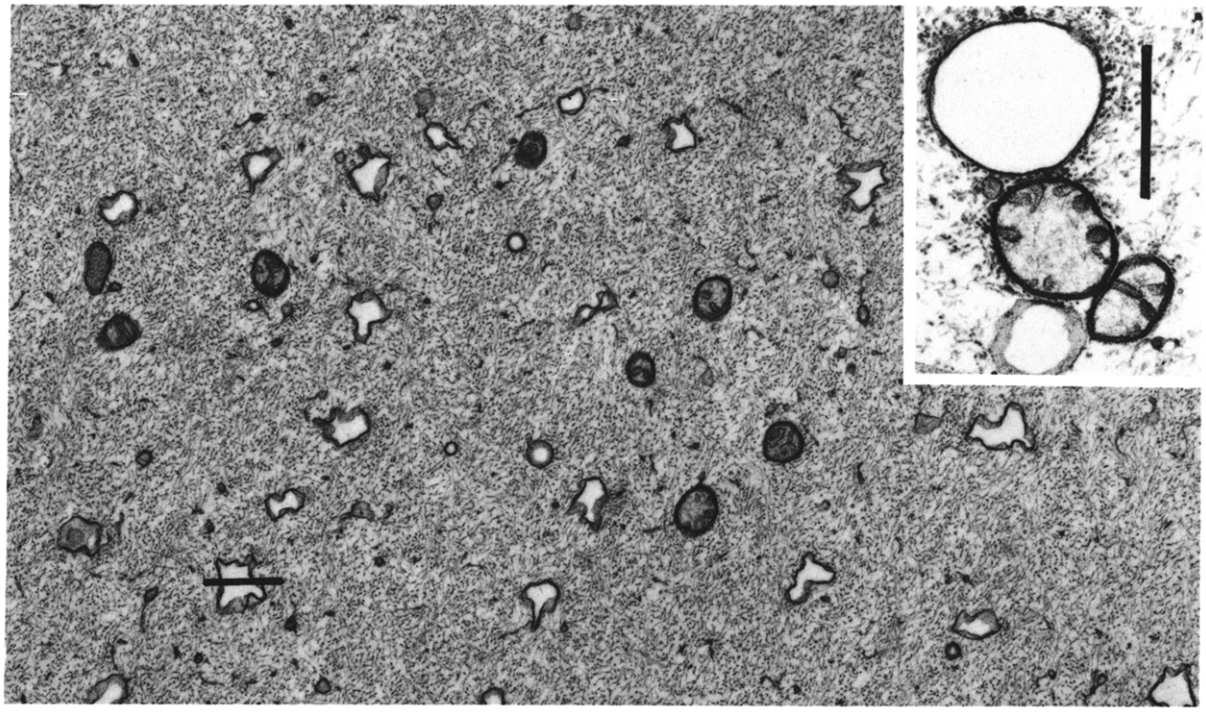

Fig. 4. Ultrastructural appearance of chemically fixed *Myxicola* axoplasm. Fixation for electron microscopy was carried out by immersing the dissected axoplasm in 0.5 M sodium cacodylate containing 5% glutaraldehyde with 0.9 ml 30% hydrogen peroxide per 100 ml solution [33]. The pH was adjusted to 7.4 just before use; fixation was for 1 h at room temperature. After washing in 0.5 M cacodylate, axons were post fixed in a 250 mM sucrose solution containing 1% osmium tetroxide and 1.5% potassium ferrocyanide [34]. They were then dehydrated and embedded for electron microscopy. The bars indicate 0.5 μM. Within the neurofibrils of the cytoskeleton, electron lucent tubular, vesicular and cisternal membrane bound compartments can be seen. The close association that can occur between these structures and mitochondria is illustrated in the inset. Magnification, 20000×; inset 40000×.

such as Plate 4 of Gilbert's paper. The present Fig. 4 illustrates the general appearance of our material after chemical fixation. McGraw et al. [32] have drawn attention to the frequent proximity of mitochondria and vesicular or cisternal elements of smooth endoplasmic reticulum in synaptosomes. The vesicles in *Myxicola* axoplasm are also frequently associated with mitochondria as shown in Fig. 4. These observations make clear that there is a substantial non-mitochondrial, membrane-bound compartment which could be responsible for the measured FCCP-insensitive Ca buffering and which may be very similar to the smooth endoplasmic reticular system which buffers Ca in synaptosomes. The *Myxicola* axoplasm specimens which were previously studied by Gilbert [31] using the electron microscope had sample weights of up to 70 mg. This is much larger than those used in the present study (maximal weight was 20 mg). Gilbert had noted that mitochondria and vesicles were more concentrated at the periphery of his samples; if smaller specimens show less differentiation of peripheral and core regions, the more uniform distribution of organelles found in the present work would be accounted for. The reason for the close association between the vesicle system and mitochondria remains enigmatic.

Acknowledgements

The authors wish to acknowledge the able technical assistance of Ms. Amy Law and Mr. Ernie Ritchey. They also thank Ms. Esther M. Scheffel for preparation of the manuscript. The work was supported by research grants to R.A. Sjodin from the National Science Foundation (PCM-8022820) and from the National Institute of Communicable Diseases and Stroke (NS 07626).

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