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EFFECT OF TEMPERATURE AND METABOLIC INHIBITORS ON ^{45}Ca OUTFLOW FROM SQUID GIANT AXONS

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SUMMARY

Labelled Ca^{2+} were incorporated into giant axons from the squid *Dosidicus gigas* by immersing them in sea water containing $^{45}Ca^{2+}$. The washout technique was used and two kinetics for $^{45}Ca^{2+}$ outflow were demonstrated (time constants of 0.140 and 0.005 min^{-1}, respectively). The second kinetics of $^{45}Ca^{2+}$ outflow was greatly affected by temperature and by some metabolic inhibitors. Lowering the temperature of the external sea water resulted in a reduction of the outflow and addition of uncouplers of oxidative phosphorylations or electron transfer inhibitors to the sea water induced an increase in the outflow. Ouabain, at a concentration which has been shown to effectively inhibit Na^+ efflux from axons of the same species, does not affect the second kinetics of $^{45}Ca^{2+}$ outflow. It is claimed here that this second kinetics is related to an intra-axonal compartment and that the rate of this Ca^{2+} outflow is determined by a steady state of calcium uptake and calcium release by some intra-axonal structures.

INTRODUCTION

Previous investigations have studied the exchange of Ca^{2+} between a variety of cells and their bathing solutions, using labelled Ca^{2+} which has been incorporated into the cells either externally or internally. For externally loaded cells two rates of outflow were described[1,2]. The first outflow was interpreted as the removal of Ca^{2+} from the extracellular space and the second outflow as the removal of Ca^{2+} from the intracellular space. For the internally loaded cells, the few experiments done were limited to giant axons which can be micro-injected with labelled Ca^{2+} (refs. 3–6). Here again, two rates of outflow were described[4–6]. A compartmental analysis of this last result[5,6] led to the following conclusions: (i) the permeability of the axon membrane to Ca^{2+} is much higher than has been previously thought and, (ii) as Ca^{2+} appeared to be bound largely by the axoplasm, an inward gradient and transport exists that can be reversed only by an active transport mechanism similar to that known for Na^+ and K^+. The slow outflow appeared to be related to the unbinding of Ca^{2+} from some intra-axonal structures. For this reason, some metabolic inhibitors would not have an effect upon this outflow as has been observed before[3].

The purpose of this communication is to present evidence indicating that Ca^{2+} is accumulated by some intra-axonal structure. It is claimed here that inhibitors of energy transfer and electron transfer reactions will prevent such accumulation, and as

a consequence of this, they will induce an increase in Ca^{2+} outflow due to an intra-axonal liberation of this Ca^{2+}.

METHODS

Giant axons from the squid, *Dosidicus gigas*, were extensively cleaned, and after being securely tied at both ends, they were externally loaded with $^{45}Ca^{2+}$. To externally load the axon fibers with $^{45}Ca^{2+}$ they were immersed in cold (4°) artificial sea water of the following composition: 430 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 5 mM Tris–HCl at pH 8, 10 mM $CaCl_2$, and $5 \cdot 10^3$ μC/mM of $^{45}Ca^{2+}$. To determine the time course of Ca^{2+} outflow, fibers were taken out of this artificial sea water and were immersed in 1 ml of sea water without $^{45}Ca^{2+}$ at room temperature of about 18°. Every 10 or 20 min the external sea water was changed; an aliquot of the removed sample was dried on a planchet and counted in a gas-flow counter (D-47 Nuclear Chicago automatic counter). At the end of each experiment, fibers were externally stimulated 100 times/sec while action potentials were either externally or internally recorded[7]. Those fibers that were unable to conduct 100 full-size propagated action potentials (from 90 to 105 mV) were discarded.

RESULTS AND DISCUSSION

Ca^{2+} outflow as a function of time was determined in 43 axon experiments. Fig. 1 shows the data obtained from one of these experiments. This is a semilogarithmic plot of counts/min collected during a 10-min period as a function of time. Using a conventional procedure[8], two straight lines were obtained from each experiment. The slopes of these lines were called λ_1 for the fast outflow and λ_2 for the slow outflow. These lines have been drawn in the figure. Table I gives the slopes computed from each experimental curve similar to that shown in Fig. 1. Clearly there are two different slopes: $\lambda_1 = (1.4 \pm 0.76) \cdot 10^{-1}$ min^{-1}, and $\lambda_2 = (5.4 \pm 1.8) \cdot 10^{-3}$ min^{-1}. In order to compare this data with previous data, one has to recall[5,6] that these λ's do not represent rate constants. The meaning of these slopes will depend on the kinetic model chosen. For internally loaded axons, for example, λ_2 will correspond to the following expression:

$$\lambda_2 = \frac{k_{21} \cdot k_{13}}{k_{12} + k_{13}}$$

where k_{21} is rate constant of the unbinding of Ca^{2+} from the axoplasm, k_{13}, the rate constant of Ca^{2+} outflow through the axon membrane, and k_{12}, the rate constant of the calcium binding[5,6]. In the case of externally loaded axons, it is difficult, if not impossible, to make a good model. Thus, the method used has several limitations: (i) the binding of Ca^{2+} to cellular membranes is so intense that even when chelating agents are added to the external sea water, all the Ca^{2+} fails to leave the axon surface; thus, the extrapolation of the slow exponential outflow to zero time, which is necessary to compute the different rate constants[5,6], contains an important error[9]; (ii) due to binding of Ca^{2+} to axoplasmic elements, the uptake and release of Ca^{2+} in axoplasm will not be uniform but will initially involve a greater change in a cortical layer; the kinetics will approach that of a thin cylindrical layer and not that of a solid cylinder[9];

TABLE I

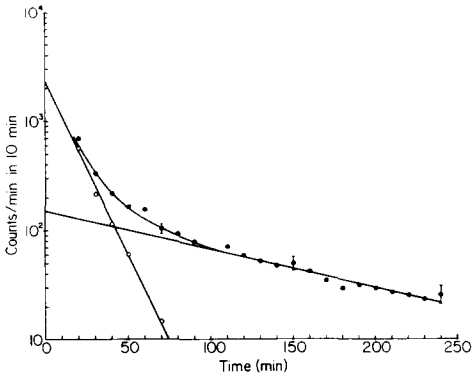
CONSTANTS OF $^{45}Ca^{2+}$ OUTFLOW FROM *Dosidicus gigas* AXON LOADED BY IMMERSION

Axon	*Diameter* (μ)	*Slopes*	
		λ_1 ($min^{-1} \times 10$)	λ_2 ($min^{-1} \times 10^3$)
1	760	1.38	3.6
2	770	1.00	—
3	—	0.69	3.4
4	743	0.86	6.9
5	—	1.00	4.3
7	750	1.00	—
8	790	1.72	—
9	700	1.38	—
10	745	3.45	—
11	714	1.38	6.3
12	600	1.38	4.0
13	657	1.00	5.3
14	700	1.47	5.7
15	829	1.38	6.3
16	740	1.38	6.3
17	864	1.14	5.8
18	900	1.36	4.2
19	672	1.40	4.9
20	720	3.45	—
21	720	2.30	—
22	745	0.70	8.0
25	864	—	3.8
26	912	—	5.0
27	912	—	3.8
28	1000	—	11.5
29	720	—	3.8
30	768	—	6.3
33	720	—	5.0
35	768	—	4.3
39	1008	—	4.3
40	672	—	9.9
43	770	1.38	6.3
Av. ± S.E.		1.4 ± 0.76	5.4 ± 1.8

and (iii) there are at least two extra-axonal compartments, connective tissue (basal membrane) and Schwann cells. It is not clear how to separate the kinetics of Ca^{2+} exchange between the Schwann cells and the external sea water, from the kinetics of Ca^{2+} exchange between the connective tissue and surrounding solution. Therefore, we will limit our considerations to descriptions of the Ca^{2+} outflow from externally loaded axons and the dependence of Ca^{2+} outflow described by λ_2 on various factors such as temperature and metabolic inhibitors.

It was found that temperature has a distinct effect upon Ca^{2+} outflow described by λ_2. In fact, when the temperature of the external sea water is lowered from 21° to 4°, the amount of Ca^{2+} collected in a 20-min period is reduced to at least 20% of its expected value, as shown in Fig. 2. This phenomenon, observed in five experiments, suggested that λ_2 was related to the Ca^{2+} outflow from an intracellular space, and that this outflow was associated with a metabolic process, as has been suggested before[6,10]. To determine whether or not this Ca^{2+} outflow was in fact dependent on metabolism,

several metabolic inhibitors which interact with the ATP synthesis at different levels were tested[15,17].



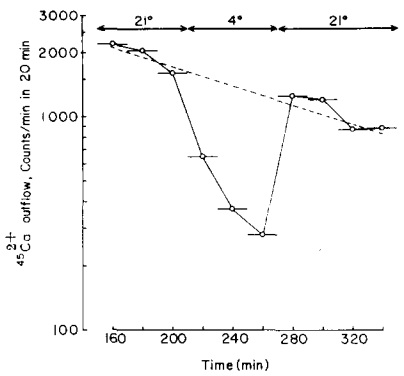


Fig. 1. Semi-logarithmic plot of Ca^{2+} outflow as a function of time. Fiber immersed in sea water with $^{45}Ca^{2+}$ during 270 min. Vertical axis represents counts/min collected in 10-min periods in a logarithmic scale. Horizontal axis represents experimental time in min. The vertical bars represent two times the standard error computed from the number of counts. The straight line drawn to fit the experimental points after 110 min is a regression line of the natural logarithm of counts/min on time in min. The other straight line, drawn to fit the circles, was also obtained as a regression line. In this case circles represent differences between experimental values and ordinates on the previous line. Temperature of the external sea water 20°. Axon diameter: 745 μ. Axon length: 70 mm.

Fig. 2. Effect of temperature upon $^{45}Ca^{2+}$ outflow. Fiber externally loaded for 600 min. Vertical axis represents counts/min collected in 20-min periods in a logarithmic scale. Abscissa represents experimental time in min (note that the experiment started 160 min after removal of the fiber from the sea water with $^{45}Ca^{2+}$). Horizontal lines represent the time during which each sample was collected. Dashed line was drawn with a slope of 0.005 min^{-1}. Axon diameter: 912 μ. Axon length: 80 mm.

Fig. 3a depicts the data obtained with a representative uncoupler, showing that 2 mM 2,4-dinitrophenol induces an increase in the amount of $^{45}Ca^{2+}$ collected in a 20-min period, and therefore, in the amount of Ca^{2+} released by the fiber. One also can see that once 2,4-dinitrophenol is removed from the external sea water, the amount of Ca^{2+} released by the fiber decreases toward a new and smaller value. A similar result was obtained with NaN_3, another uncoupler used (see Fig. 3b). In the case of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, however, the amount of Ca^{2+} released by the fiber did not decrease, as shown in Fig. 4. Table II summarizes the results obtained in 23 experiments.

A tentative interpretation of these effects can be given, considering that Ca^{2+} uptake and Ca^{2+} liberation from intracellular structures and, in particular, from mitochondria could modify the time course of Ca^{2+} outflow. It is known that some inhibitors of oxidative phosphorylation inhibit Ca^{2+} uptake by isolated mitochondria[11–13], and mitochondria have been shown to be present in giant axons[14]. The general results obtained with uncoupling agents, electron and energy transfer inhibitors, when externally applied to axon fibers, have shown that they enhance Ca^{2+} outflow, which can be interpreted as an indication of an intra-axonal release. It seems, therefore, that these inhibitors increase the amount of intra-axonal-free Ca^{2+}, perhaps by lowering the Ca^{2+} uptake capacity of the mitochondria.

Further evidence that these inhibitors induce an intra-axonal liberation of Ca^{2+}

was obtained by computing the time constant of the increase in Ca^{2+} liberation from the fiber. The time constant obtained is similar to the first rate constant measured by LUXORO and co-workers[5,6] in axons internally loaded with $^{45}Ca^{2+}$, *i.e.* $5 \cdot 10^{-2}$ min^{-1}, which is proportional to the permeability of the axon membrane to Ca^{2+}.

The inhibitors used in this work interact with the ATP synthesis at different levels[15,17]. Although amytal and cyanide, which interact with the electron carrier chain, did produce the effect observed with the uncouplers, they were not as effective. Since a prompt inhibition of the Na^+ efflux has been demonstrated by us in intact axons from *D. gigas* using 6 mM amytal and 3 mM cyanide[16], the permeability to them was

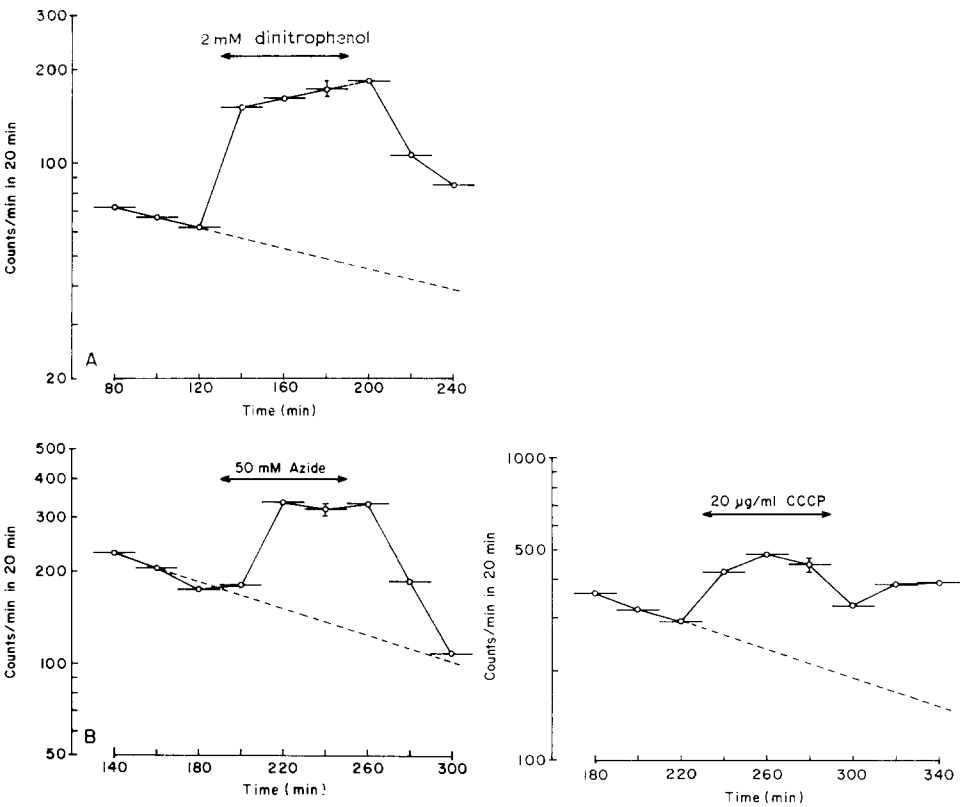


Fig. 3. Effect of 2,4-dinitrophenol and NaN_3 upon $^{45}Ca^{2+}$ outflow. A. Fiber externally loaded for 240 min. Dashed line was drawn with a slope of 0.0036 min^{-1}. The vertical bar on experimental point obtained at 170 min represents two times the standard error computed from the number of counts. The difference between this point and that one on the regression line is significant (t-test gave $P < 0.001$). Temperature of the external sea water about 20°. pH of the external sea water about 7.8. Axon diameter: 829 μ. Axon length: 60 mm. B. Fiber externally loaded during 1400 min. Vertical bar on experimental point at 240 min represents two times the standard error computed as before. The difference between this point and that one on the regression line (dashed line) is significant. Axon diameter: 720 μ. Axon length: 65 mm.

Fig. 4. Effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) upon $^{45}Ca^{2+}$ outflow. Fiber externally loaded for 720 min at 4°. Semi-logarithmic plot of Ca^{2+} outflow as a function of time. Temperature of the external sea water about 20°. Dashed line was drawn with slope of 0.005 min^{-1}. Vertical bar on experimental point at 280 min is two times the standard error. The difference between this point and that one on the regression line is significant. Axon diameter: 768 μ. Axon length: 64 mm.

TABLE II

EFFECT OF METABOLIC INHIBITORS UPON $^{45}Ca^{2+}$ OUTFLOW

The inhibitors were dissolved in the external sea water.

Inhibitor	*Total number of experiments*	*Concn.*	*Type of inhibitor**	*Effect upon $^{45}Ca^{2+}$ outflow*	
				Increase	*None*
2,4-Dinitrophenol	3	2 mM	Uncoupler	3	0
NaN_3	2	50 mM	Uncoupler	2	0
Carbonyl cyanide *m*-chlorophenylhydrazone	6	20 μg/ml	Uncoupler	5	1
Oligomycin	2	2 μg/ml	Uncoupler	0	2
KCN	4	3 mM	Electron transfer	2	2
Amytal	4	6 mM	Electron transfer	2	2
Antimycin	2	5 μg/ml	Electron transfer	0	2
Ouabain	4	0.01 mM	Transport	0	4

* Points of inhibition of uncoupling agents, electron and energy tranfer inhibitors can be found in refs. 15 and 17.

not rate limiting. Thus, amytal and cyanide may be acting on the mitochondria through an indirect effect on Ca^{2+} outflow. On the other hand, we do not know whether the permeability to antimycin was rate limiting. The same may be true in the case of oligomycin, which has been shown to inhibit the incorporation of ^{32}P to ATP in the mitochondria[17].

The lack of ouabain effect from the outside indicates that the Ca^{2+} movements under investigation are not coupled to Na^+ transport. The concentration of ouabain used has been shown to effectively inhibit the Na^+ efflux from axons of the same species[16].

At present it is not clear why the temperature induces a decrease in Ca^{2+} outflow. A simular result has been obtained with axons internally loaded with $^{45}Ca^{2+}$ (ref. 6). One possible interpretation is that there exists a Ca^{2+} carrier within the membrane whose mobility is reduced by a reduction in the temperature.

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