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MEASUREMENTS OF INTRACELLULAR IONIZED CALCIUM IN SQUID GIANT AXONS USING CALCIUM-SELECTIVE ELECTRODES

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Ca^{2+}-selective electrodes have been used to measure free intracellular Ca^{2+} concentrations in squid giant axons. Electrodes made of glass cannulas of about 20 μm in diameter, plugged with a poly(vinyl chloride) gelled sensor were used to impale the axons axially. They showed a Nernstian response to Ca^{2+} down to about 3 μM in solutions containing 0.3 M K^+ and 0.025 M Na^+. Sub-Nernstian but useful responses were obtained up to p*Ca* 8. The electrodes showed adequate selectivity to Ca^{2+} over Mg^{2+}, H^+, K^+ and Na^+. To calibrate them properly, a set of standard solutions were prepared using different Ca^{2+} buffers (EGTA, HEEDTA, nitrilotriacetic acid) after carefully characterizing their apparent Ca^{2+} association constants under conditions resembling the axoplasmic environment. In fresh axons incubated in artificial seawater containing 4 mM Ca^{2+}, the mean resting intracellular ionized calcium concentration was 0.106 μM ($n = 15$). The Ca^{2+}-electrodes were used to investigate effects of different experimental procedures on the $[Ca^{2+}]_i$. The main conclusions are: (i) intact axons can extrude calcium ions at low $[Ca^{2+}]_i$ levels by a process independent of external Na^+; (ii) poisoned axons can extrude calcium ions at high levels of $[Ca^{2+}]_i$ by an external Na^+-dependent process. The level of free intracellular Ca attained at these latter conditions is about an order to magnitude greater than the resting physiological value.

Introduction

One of the most extensively studied preparations from the point of view of calcium homeostasis has been squid giant axon [1–3]. The accurate knowledge of the resting intracellular ionized calcium concentration (resting $[Ca^{2+}]_i$), is of paramount importance: (a) to define the real electrochemical calcium gradient which must be accounted by any energetic model of calcium regulation; and (b) because the two postulated Ca^{2+} extrusion mechanisms (Ca^{2+} pump and Na^+/Ca^+ exchange) differ widely in their affinity for Ca^{2+} and therefore the relative importance of each of them will depend on the level of $[Ca^{2+}]_i$ [3,4].

Measurements of the level of $[Ca^{2+}]_i$ in squid giant axons have been made using either the Ca^{2+}-sensitive dye Arsenazo III [5] or the photoprotein aequorin [5,6]. A more recent method to measure free calcium ion concentration ($[Ca^{2+}]$) consists in the use of Ca^{2+}-sensitive electrodes which have become available for biological research with an improved selectivity (with respect to H^+ and Mg^{2+}) and low detection limit for Ca^{2+}

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; HEEDTA, *N*-(2-hydroxyethyl)-ethylenediamine-*N*,*N*′,*N*′-triacetic acid; Mops, morpholinopropanesulfonic acid. p*Ca*, negative log of the ionized calcium concentration.

[7,8]. One of the main advantages of Ca^{2+}-selective electrodes is that they provide direct measurements of the Ca^{2+} concentration, in contrast to optical calcium indicators in which the deconvolution of the optical measurements are subject to complicated calibration procedures (Refs. 5, 9, Palade and Vergara, unpublished results). Furthermore, Ca^{2+}-electrodes can be useful tools to verify results obtained with other Ca^{2+}-measuring techniques.

In this paper we report results of experiments in which the neutral carrier electrode [7] has been employed to determine the resting intracellular ionized calcium concentration in squid giant axons. In addition, we have investigated factors which are known to affect the levels of intracellular Ca^{2+}, such as variations in the external Ca^{2+} and Na^{+} concentrations, and metabolic poisoning. Our results, demonstrate that Ca^{2+}-electrodes are advantageous tools for this type of study, giving accurate measurements of the axoplasmic free $[Ca^{2+}]$. In fresh axons incubated in 4 mM Ca^{2+} artificial seawater at 19°C, the mean resting $[Ca^{2+}]_i$ was 0.106 μM. Changes in the passive Ca^{2+} entry (tetrodotoxin poisoning and changes in the external calcium) significantly affected the levels of intracellular Ca^{2+}. Poisoning of the axons interfered with their ability to recover their resting $[Ca^{2+}]_i$ level after an imposed Ca^{2+} load.

Methods

The experiments were performed on giant axons dissected from the tropical squid *Doryteuthis plei* between the months of February and May of 1982. The axons were dissected in artificial seawater and carefully cleaned under a dissecting microscope. A standard horizontal dialysis chamber [10] was used to mount the axon. The axon was cannulated at one end with a glass cannula of 250 μm prior to the insertion of the Ca^{2+} and voltage electrodes, independently introduced.

Electrodes

The reference electrode, which was used to connect the experimental chamber to earth, consisted of an Ag/AgCl pellet and a 1 mm polyethylene bridge filled with 1 M KCl in 2% agar. The internal potential pipettes were pulled from 1.5 mm tubes (Kimax 46485) into glass cannulae (50–75 μm O.D., 3 cm in length) and filled with 0.5 M KCl. An Ag/AgCl pellet was introduced into the glass tube for potential measurements. The Ca^{2+} pipettes were pulled from the same glass into cannulae of about 20 μm O.D. and 3 cm length. The glass tubes were precleaned in 55% HNO_3 (with small aliquots of ethanol) and rinsed in boiling distilled water. The pipettes were silanized using a procedure similar to that described by Tsien and Rink [8], using pure tri-*n*-butylchlorosilane (Pfaltz & Bauer, Stanford, CT, U.S.A.) vaporized at 200°C. The electrodes were backfilled with a calibrating solution of p*Ca* 7 (see Table I) and the tips were plugged with a neutral ligand cocktail (10% of the neutral ligand *N,N'*-di(11-ethoxycarbonyl)undecyl-*N,N'*,4,5-tetramethyl-3,6-dioxaoctane 1,8-dioamide and 1% sodium tetraphenylborate in (*o*-nitrophenyl)octyl ether, kindly supplied by Professor W. Simon), mixed with 10% (w/w) poly(vinyl chloride) dissolved in an excess of tetrahydrofuran. The amount of sensor introduced into the tip was controlled by negative pressure (with a 100 μl Hamilton syringe) and resulted, after evaporation of the tetrahydrofuran, in columns 50–100 μm in length. The electrode resistances, measured by 0.1 pA current pulses, were typically within the range of 0.5–1 GΩ.

Calibration procedure

The most critical aspect in the use of Ca^{2+}-selective electrodes is their accurate calibration with Ca^{2+} buffer solutions. Since the apparent association constant for any Ca^{2+} buffer is very sensitive to pH (especially close to pH 7), ionic strength, ionic composition and temperature, the calibration solutions must take into account these factors. In the present experiments we have adopted the method designed by Bers [11] to evaluate the apparent association constant of different Ca^{2+} buffers under our experimental conditions. We have selected three Ca^{2+} buffers with different Ca^{2+} affinities (EGTA, HEEDTA, nitrilotriacetic acid), to buffer the ionized Ca^{2+} effectively over a wide range of p*Ca* (p*Ca* 3–8).

Large Ca^{2+}-electrodes (Orion 90-02) and an electrometer amplifier (Orion Research Ionalyser 901) were used for the determinations of the apparent association constants. As an example, Fig. 1

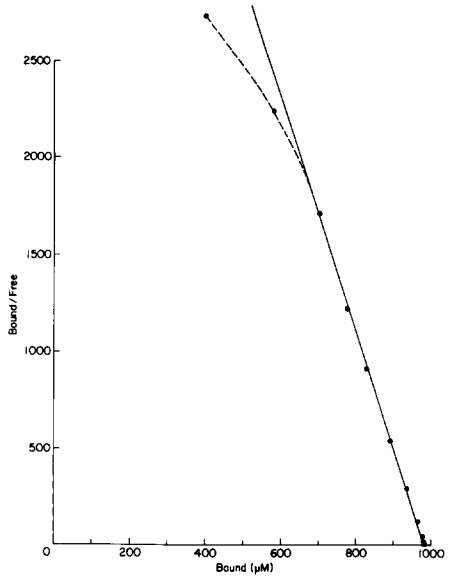

Fig. 1. Scatchard plot for the determination of the apparent association constant of EGTA. The solutions contained (mM): K^+, 300; Mops, 200; Cl^-, 100; EGTA, 1; pH (18.5 °C) 7.3. The solutions were prepared in highly purified distilled water of resistivity greater than 18 MΩ·cm. The Ca^{2+}-selective electrode used for these determinations (Orion 90-02) was verified to have a Nernstian behaviour from p*Ca* 2 to p*Ca* 5 in solutions without EGTA. Several solutions with 1 mM EGTA and different amounts of total Ca^{2+} added were prepared in order to span a wide range of $[Ca^{2+}]$ (calculated from an assumed K'_{Ca} of $6.6 \cdot 10^6$ M^{-1}). New (corrected) values of $[Ca^{2+}]$ were calculated from the electrode potentials in each of these solutions. The bound Ca^{2+} concentrations were calculated by subtracting the $[Ca^{2+}]$ from the total Ca^{2+}. The bound/free ratios and the bound values obtained in each of these solutions constitute the data for this figure. The linear regression fitted to the data points which did not obviously deviate from the straight line (all except the higher two), gave a value for K'_{Ca} of $5.96 \cdot 10^6$ M^{-1} and a total effective concentrations of ligand (n) of 0.983 mM. See text and Ref 11.

shows a typical Scatchard plot obtained with EGTA at the ionic conditions, pH and temperature closely resembling the ionic intracellular environment of the squid axon. The apparent association constant (K'_{Ca}) of EGTA, calculated from this plot, is $5.96 \cdot 10^6$ M^{-1} (dissociation constant of 0.17 μM). As pointed out by Bers [11], the total concentration of EGTA calculated from this plot (0.983 mM) is lower than that calculated from the manufacturers molecular weight (1 mM). Determinations similar to those shown in Fig. 1 yielded for HEEDTA and nitrilotriacetic acid the following affinity constants, respectively: $1.84 \cdot 10^5$ M^{-1} and $4.78 \cdot 10^3$ M^{-1}. For nominal concentrations of 1 mM of these buffers, the real concentrations were 1.02 mM for HEEDTA and 0.945 mM for nitrilotriacetic acid. These values were used to prepare calibrating solutions of different p*Ca* (p*Ca* 3–8). Table I shows the composition of these solutions.

The Ca^{2+}-selective intracellular electrodes were calibrated in the experimental chamber immediately before mounting the axon. The electrode potential was measured with an electrometer differential amplifier (WPI, FD-223) with respect on an earth bath potential and displayed via a pen chart recorder (Hewlett-Packard, 7132A) at a slow speed. Fig. 2 shows the response of one typical calcium electrode to solutions of different p*Ca*. The electrode gave a measurable response up to p*Ca* 8. It is shown in Fig. 2A that substitution of 25 mM K^+ for Na^+ (close to the physiological internal $[Na^+]$; Ref. 12) modifies the electrode potential by about 4 mV at p*Ca* 7, demonstrating an interference by Na^+ which should be considered in measuring the free Ca^{2+} concentrations. It is also evident from Fig. 2A that the electrode shows no hysteresis during the overall calibration process. In our determinations, the electrodes were practically insensitive to pH (in the range between 6.5 and 7.3) and to Mg^{2+} (between 0 to 5 mM). Fig. 2B shows the calibration plot of the electrode potential (arbitrarily set to 0 at p*Ca* 3) vs. the p*Ca*

TABLE I

COMPOSITION OF STANDARD CALIBRATION SOLUTIONS

Total ligand concentration: 10 mM. Ionic composition: 100 mM KCl + 200 mM potassium Mops, pH 7.3; temp., 18°C. NTA, nitrilotriacetic acid.

p*Ca*	Ligand	$CaCl_2$ (mM)
3	NTA	9.3
4	NTA	3.3
5	HEEDTA	6.5
6	HEEDTA	1.6
7	EGTA	3.9
8	EGTA	0.6
∞	EGTA	0.0

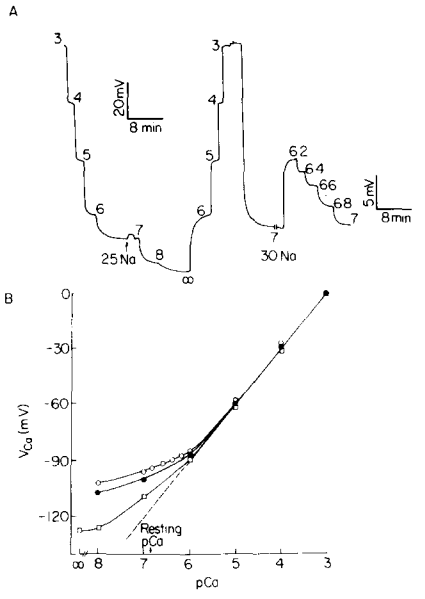

Fig. 2. Electrode response and calibration curve of a typical Ca^{2+}-selective electrode. A. Calibration recording of the electrode response to solutions of different p*Ca* (p*Ca* ∞ refers to a solution with no added Ca and with 1 mM EGTA). The flow rate of the solutions in the chamber during the calibration procedure was set to approx. 0.5 ml/s that corresponded to about 2 chamber volumes per s. As a result of this, the solutions were equilibrated within less than 3 s. The last calibrating run (p*Ca* 7–6.2) was taken at higher gain and in the presence of 30 mM Na^+. B. Calibration plot of the electrode potential vs. p*Ca* in calibrating solutions of different ionic composition (in mM). ○, data obtained in K^+, 290; Cl^-, 98; Na^+ 30; ●, data obtained in K^+, 320; Cl^-, 98; Na^+, 0; □, data obtained in K^+ 100; Cl^- 98; Na^+, zero; pH 7.3 (20°C). The electrode tip diameter was 20 μm, the length of the column 80 μm, and the resistance 0.7 GΩ (at the high ionic strength, p*Ca* 7 solution).

in different calibrating solutions. The Ca^{2+} electrode response at low ionic strength had a wider range of Nernstian behaviour (29.5 mV/p*Ca*) than at high ionic strenght. Under the latter condition, the electrode is Nernstian up to p*Ca* 6. Between p*Ca* 6 and 7, the electrode potential varied only 10–15 mV. This behaviour is expected from the greater K^+ interference in these calibration solutions.

The response time of the electrodes could be estimated from curves such as those shown in Fig. 2A. The equilibration of the electrode response was slow, even with respect to Ca^{2+}-microelectrodes, made by others and ourselves using the same sensor [8]. The long and thin pipettes contributed to large stray capacitances that, in combination with the large electrode resistances, probably resulted in these slow response times. This limitation does not affect the conclusions reached in this paper because they were used to study either steady-state or very slowly changing Ca^{2+}-related phenomena. Nevertheless, it may be desirable in the future to improve the frequency response of these large tip electrodes by shortening the glass shanks, using larger diameter pipettes, and including a floating Pt wire inside the pipettes. We are currently investigating these problems.

During the actual measurements in the axon, the membrane potential (V_m) was recorded by an independent voltage pipette and electronically subtracted from the Ca^{2+}-electrode potential (V_{Ca}) by the carefully matched differential amplifier (WPI, FD-223). The resulting differential potential ($V_{Ca} - V_m$) was compared to those obtained in the calibrating solutions in order to express it in p*Ca* units.

The excitability of the axon was checked in external artificial seawater by stimulation with brief (0.5 ms) current pulses and recording the action potentials with the internal voltage electrode. Care was taken in verifying that each axon was excitable throughout the experiments, preferentially after changes in the external solutions. The only exceptions to this procedure were those experiments in which tetrodotoxin was used.

External solutions

The artificial seawater had the following composition in mM: K^+, 10; Na^+, 440; Mg^{2+}, 50; Ca^{2+}, 4; $Tris^-$, 10; Cl^-, 580; EDTA, 0.1. The osmolarity was 1000 mosM and the pH (18.5–20°C) 7.8. The removal of Na^+ was compensated with equivalent amounts of Tris. Ca^{2+} was substituted by Mg^{2+}.

Results

The resting intracellular ionized calcium

The standard procedure to measure the resting $[Ca^{2+}]_i$ is illustrated in Fig. 3. Before impaling the axon with the Ca^{2+}- and voltage-electrodes, the former was placed in a solution of p*Ca* 3 and backed to zero voltage (initial basal level in Fig. 3).

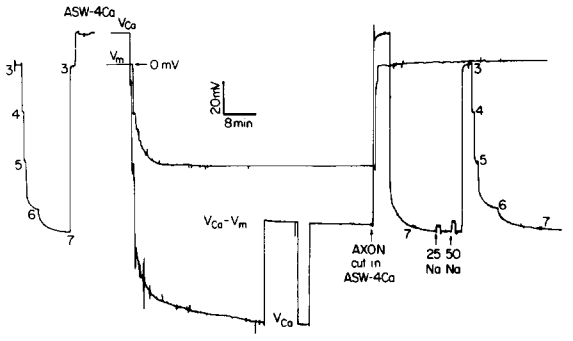

Fig. 3. Procedure for measuring resting free calcium activities in the squid giant axon. At left of the figure is the preimpalement calibration record of the Ca^{2+}-selective electrode. In this particular experiment the highest p*Ca* used was p*Ca* 7. At p*Ca* 3, the potentials of both electrodes (V_{Ca} and V_m) were set to zero. Subsequently the experimental chamber was flooded with artificial seawater (ASW) and the axon was mounted and impaled. The voltage- and Ca^{2+}-selective electrodes were axially introduced in the axon (with the aid of micromanipulators) until their tips reached the centre of the chamber. In some experiments the electrodes were moved to the periphery of the axon. After the electrodes were positoned there followed a long equilibration period, at the end of which the value of ($V_{Ca} - V_m$) was measured. At the end of the experiment, the axon was cut and a second set of calibration records was taken. Axon diameter, 500 μm; temperature, 19°C.

TABLE II

RESTING p*Ca* IN INTACT SQUID AXONS

Each row represents results from a particular experiment.

	$-V_m$ (mV)	$-(V_{Ca} - V_m)$ (mV)	p*Ca*
	60	92.0	6.6
	59	95.0	6.9
	59	96.0	7.1
	61	93.2	6.9
	62	93.5	6.8
	59	98.0	7.3
	59	94.6	6.9
	59	96.0	7.2
	60	95.0	7.0
	61	95.8	7.2
	58	94.6	6.9
	62	95.5	7.0
	64	98.0	7.3
	61	92.0	6.7
	62	93.2	6.8
Mean ±SD:	60.4 ± 1.64	94.8 ± 1.9	6.97 ± 0.21 (0.106 ± 0.065 μM)

This was followed by its calibration in solutions of differing p*Ca* (in this particular case only up to p*Ca* 7). In this and many other experiments, the time to reach a steady-state Ca^{2+}-electrode potential inside the axon was many minutes. Subsequently, the voltage-electrode potential (V_m) was subtracted from that of the Ca^{2+}-electrode (V_{Ca}) to obtain a reading of the resting free intracellular Ca^{2+} ($V_{Ca} - V_m$). Finally, the axon was cut in artificial seawater, and this was followed by a second calibration procedure. In this calibration is shown the effect on the Ca^{2+}-electrode potential of replacing 25 and 50 mM K^+ by Na^+. Several electrodes gave sub-Nernstian responses after withdrawal from the axon. This was particularly frequent in electrodes with tip diameters larger than 30 μm and might be due to partial dislodgement of the resin during the longitudinal impalement. Experiments in which this behaviour was observed were rejected.

Table II shows results from 15 different axons in which a procedure identical to that of Fig. 3 was followed. All of them fulfilled the following criteria: (i) resting potentials more negative than −58 mV; and (ii) similar calibration curves (between p*Ca* 3 and p*Ca* 8) before and after impalement. The mean p*Ca* value in Table II was obtained after correction for interference by 25 mM Na^+, measured for each electrode at p*Ca* 7.

The effect of external Ca^{2+}

Fig. 4 shows the results of an experiment in which the effects of changes in the external Ca^{2+} concentration and of tetrodotoxin were analysed. In artificial seawater, the resting $[Ca^{2+}]_i$ was within the range observed in fresh axons ($1 \cdot 10^{-7}$ M). Increasing the external $[Ca^{2+}]$ to 60 mM resulted in a steady increase in the free $[Ca^{2+}]_i$. Removal of both Na^+ and Ca^{2+} in the external solution not only stopped this increase but resulted in a slow return to its basal level. This recovery was found to be independent of the presence of external Na^+. In the second part of the experiment, the axon was exposed again to 60 mM Ca^{2+} outside, producing a similar steady increase in the $[Ca^{2+}]_i$

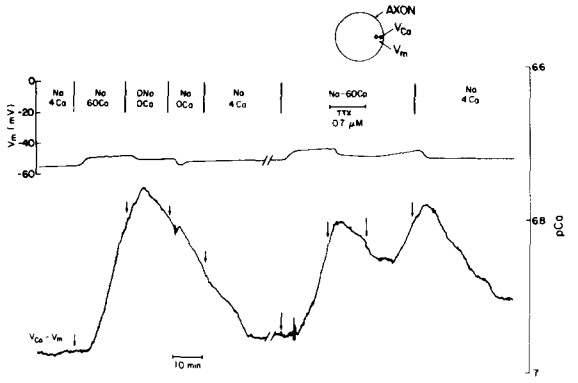

Fig. 4. The effects of changes in the external Ca^{2+} and Na^+ on the free intracellular Ca^{2+} recordings. The arrows indicate the changes in solutions. The interruption in both the membrane potential and Ca^{2+} traces represents a period of about 60 min during which the axon equilibrated in artificial seawater. The inset on top of the figure is a diagram of the approximate location of the electrodes in this experiment. The p*Ca* calibration scale at the right of the figure (from p*Ca* 7 to 6.6) was obtained by direct calibration of the electrode (before and after the impalement) in solutions of p*Ca* 7, 6.8, and 6.6. TTX, tetrodotoxin.

with about the same time-course. Addition of 700 nM tetrodotoxin, a well-known blocker of the sodium channel [13], stopped and reversed this trend in a way compatible with a reduction in the Ca^{2+} influx. This effect was reversible upon removal of the blocker. In other experiments (not shown), the removal of the external Ca^{2+} for brief periods of time, or the addition of tetrodotoxin, resulted in a reduction in the $[Ca^{2+}]_i$ to levels lower than the mean value shown in Table II. The effects reported in Fig. 4 were recorded from an axon in which the voltage and calcium electrodes were positioned as shown diagramatically in the figure inset. Other experiments, in which the electrodes were positioned closer to the axis of the axon, gave slower and smaller responses than those shown in Fig. 4, suggesting that changes in the $[Ca^{2+}]_i$ are not homogeneously detected inside the axon. Nevertheless, the free resting intracellular ionized calcium was homogeneously constant (in the radial direction from the centre to about 10 μm from the membrane) in several axons in which this was explored.

The effect of metabolic poisoning

The involvement of metabolic energy in the control of the resting free ionized calcium concentration in squid axons has been debated in recent years [4,14,15]. We have used Ca^{2+}-selective electrodes to analyse this problem for the following reasons: (a) they directly measure the free ionized calcium activity, which is the variable of critical importance; and (b) metabolic poisons are known to markedly affect the intracellular melieu by decreasing the internal pH and increasing the internal sodium and magnesium [12,16]. These changes would interfere with measurements of the $[Ca^{2+}]_i$ by Ca^{2+} indicators; but, as mentioned in Methods, Ca^{2+}-selective electrodes are very insensible to changes in pH and Mg^{2+} concentration.

Fig. 5 shows a typical experiment to test the effect of metabolic poisoning. After the electrode reading stabilized, the axon was incubated for 2 h in a solution without Na^+ and Ca^{2+} but with CN^- and iodoacetate. The external Na^+ and Ca^{2+} were removed to avoid an increase in the intracellular concentration of these ions during poisoning. Under these conditions, no change in the $[Ca^{2+}]_i$

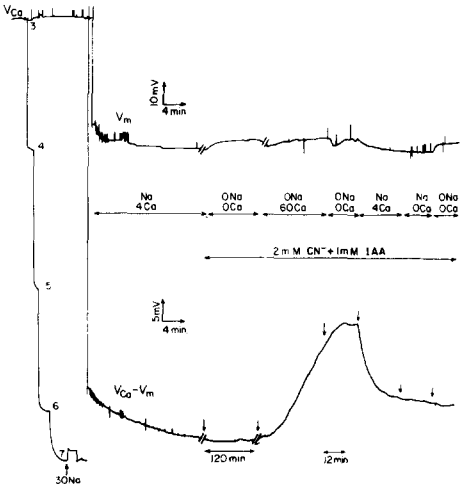

Fig. 5. The effect of metabolic inhibitors and changes in the external Ca^{2+} and Na^+ on the free intracellular Ca^{2+} activity. The time calibration bar of 12 min applies to most of the record except during the period between separating bars in which it was 120 min. The recording was continuous throughout the experiment, the bars indicating only a change in timescale. The vertical arrows indicate the solution changes. Sodium cyanide (CN^-) and sodium iodoacetate (IAA) were added to the external solutions where indicated. Axon diameter, 450 μm; temperature, 20°C.

was observed, thus confirming findings of DiPolo et al. [5]. When the external Ca^{2+} was changed to 60 mM, the $[Ca^{2+}]_i$ increased drastically into the micromolar range. The removal of external Ca^{2+} eliminated any further rise in internal free Ca^{2+}, this reaching a steady value at about 6 μM. Subsequently, superfusion with the standard artificial seawater caused a rapid fall in the $[Ca^{2+}]_i$ to a level close to 1.3 μM. Interestingly, this new steady value was virtually unaffected by the removal of the external Ca^{2+} or both Ca^{2+} and Na^+. In four similar experiments, the mean $[Ca^{2+}]_i$ changed from 0.13 μM before poisoning to 10 μM during poisoning and Ca^{2+} loading (zero Na^+, 60 mM Ca^{2+}). In the unloading solutions (440 mM Na^+, 4 mM Ca^{2+} and 440 mM Na^+, zero Ca^{2+}), the $[Ca^{2+}]_i$ partially recovered to a mean value of about 1.5 μM. It should be noted that during the course of the experiment, the membrane potential showed fluctuations of the order of only ± 5 mV due to external solution changes. At the end of the experiment the axon was slightly hyperpolarized.

Contrary to the behaviour observed in the experiment shown in Fig. 5, axons previously loaded with Ca^{2+} (zero Na, 60 mM Ca^{2+}, external solution), showed a large increase in the free intracellular Ca^{2+} during the exposure to the metabolic inhibitors. We also tried to test the mitochondrial inhibitor FCCP as an intracellular Ca^{2+}-releasing agent. Unfortunately, this compound was found to interfere with the Ca^{2+}-selective electrode (Alvarez-Leefmans, F.J., personal communication).

Discussion

The present study shows that Ca^{2+}-selective electrodes can be used to measure accurately free intracellular ionized calcium concentrations in squid giant axons. This was possible even in an internal environment of high ionic strength (300 mM K^+) and in the presence of tens of millimolar sodium ions (25–30 mM). Each electrode was tested and calibrated individually in solutions resembling the axoplasmic composition. The apparent association constants of three Ca^{2+} buffers were carefully measured under the above conditions before the calibrating solutions were prepared. This procedure allowed us to calibrate the Ca^{2+} selective electrodes confidently in the proper ionic environment.

The resting $[Ca^{2+}]_i$ in 15 axons that met our criteria of goodness was 0.106 ± 0.065 μM when the axons were bathed in 4 mM external Ca^{2+}. This value for the external Ca^{2+} was used because it is close to that measured in the squid hemolymph [17]. Our resting $[Ca^{2+}]_i$ is in the range reported for two other squid species: *Loligo pealei* (DiPolo et al. [5] and *Loligo forbesi* (Baker, [6]). Similar values (also obtained with Ca^{2+}-selective electrodes) have been reported in neurons of *Helix aspersa* [18], barnacle muscle fibres [19], cardiac Purkinje fibres [20], and frog skeletal muscle fibers (Ref. 8, and López, Alamo, Caputo, DiPolo and Vergara, unpublished data).

The possibility of manipulating the position of the tip of the electrodes inside the axon could be useful in studying the radial distribution of the intracellular Ca^{2+} buffer systems. This is reinforced by our finding that the Ca^{2+}-electrode in the axoplasm seems to measure highly localized Ca^{2+} activities.

The electrodes used in these experiments were able to measure accurately small changes in the ionized Ca^{2+} activity induced by external Ca^{2+} concentration changes, even in intact axons in which all the intracellular Ca^{2+} buffer systems were present. An interesting result is the effect of tetrodotoxin shown in Fig. 4. This Na^+-channel blocker reversed the rise in the $[Ca^{2+}]_i$ induced by 60 mM external Ca^{2+}. This effect is in agreement with the observations of DiPolo et al. [21] demonstrating that about 70% of the resting Ca^{2+} influx in intact and dialyzed axons is tetrodotoxin-sensitive. Two additional observations in Fig. 4 are worth discussing: (a) after a small Ca^{2+} load (60 mM Ca^{2+}, 440 mM Na^+), the axon is able to recover fully its original resting $[Ca^{2+}]_i$; and (b) the rate of recovery is independent of the external Na^+ concentration. The decrease in the $[Ca^{2+}]_i$ observed after the Ca^{2+} load could be due to the combined effect of Ca^{2+}-extrusion mechanisms, intracellular Ca^{2+} redistribution, and intracellular buffering. Experiments from another laboratory demonstrated that no Ca^{2+} uptake by energy-dependent intracellular structures occurs at $[Ca^{2+}]_i$ below 200 nM [22,23]. On the other hand, energetically independent intracellular Ca^{2+} buffers rapidly reach equilibrium after a Ca^{2+} load [22,24]. Therefore, the long recovery process observed in

Fig. 4 could be due only to membrane Ca^{2+}-extrusion mechanisms and/or passive intracellular Ca^{2+} redistribution. However, since the intracellular ionized calcium concentration returns to the original resting level after a Ca^{2+} load (see Fig. 4), a membrane extrusion mechanism must be involved. The insensitivity to external Na^+ of the recovery process shown in Fig. 4 agrees with the low turnover rate of the Na^+/Ca^{2+} exchange at low $[Ca^{2+}]_i$ [4] and favours the involvement of the Ca^{2+} pump (ATP-dependent, Ref. 25). This finding is also in agreement with recent experiments by Baker and Singh [15] showing that net Ca^{2+} extrusion at physiological $[Ca^{2+}]_i$ is mediated by an ATP dependent mechanism.

The experiments with metabolic inhibitors (Fig. 5) are of particular interest because: (a) no recovery from an imposed large Ca^{2+} load (60 mM Ca^{2+}, zero Na^+) was observed in the absence of external Na^+ and Ca^{2+}; and (b) the recovery observed in the presence of external Na^+ never reached the original resting $[Ca^{2+}]_i$ levels but instead stabilized at much higher concentrations (micromolar). These results are in apparent contradiction with those of Requena et al. [14] showing that poisoned axons can regulate their Ca^{2+} content by a mechanism independent of the ATP levels and totally dependent on external sodium (Na^+/Ca^{2+} exchange). However, these authors measured the total intracellular Ca^{2+} content, whereas in our experiments we monitored the free ionized Ca^{2+} activities. It is clear from these results that although the total Ca^{2+} might be effectively lowered by the Na^+/Ca^{2+} exchange, the $[Ca^{2+}]_i$ remains significantly high.

In summary, Ca^{2+}-selective electrodes are useful tools in studying Ca^{2+} transport in squid axons under physiological and other experimental conditions. Our results are consistent with the presence of two different mechanisms of Ca^{2+} extrusion in this preparation; one working at low $[Ca^{2+}]_i$, the uncoupled Ca^{2+} pump (high-affinity system), and the other working mostly at high $[Ca^{2+}]_i$, the Na^+/Ca^{2+} exchange (low affinity system).

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