Uptake and Release of ^{45}Ca by *Myxicola* Axoplasm

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ABSTRACT The binding and release of ^{45}Ca by axoplasm isolated from *Myxicola* giant axons were examined. Two distinct components of binding were observed, one requiring ATP and one not requiring ATP. The ATP-dependent binding was largely prevented by the addition of mitochondrial inhibitors, whereas the ATP-independent component was unaffected by these inhibitors. The ATP-independent binding accounted for roughly two-thirds of the total ^{45}Ca uptake in solutions containing an ionized $[Ca^{2+}] = 0.54$ μM and was the major focus of this investigation. This fraction of bound ^{45}Ca was released from the axoplasm at a rate that increased with increasing concentrations of Ca^{2+} in the incubation fluid. The ions Cd^{2+} and Mn^{2+} were also able to increase ^{45}Ca efflux from the sample, but Co^{2+}, Ni^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+} had no effect. The concentration-response curves relating the ^{45}Ca efflux rate coefficients to the concentration of Ca^{2+}, Cd^{2+}, or Mn^{2+} in the bathing solution were S-shaped. The maximum rate of efflux elicited by one of these divalent ions could not be exceeded by adding a saturating concentration of a second ion. Increasing EGTA concentration in the bath medium from 100 to 200 μM did not increase ^{45}Ca efflux; yet increasing the concentration of the EGTA buffer in the uptake medium from 100 to 200 μM and keeping ionized Ca^{2+} constant caused more ^{45}Ca to be bound by the axoplasm. These results suggest the existence of high-affinity, ATP-independent binding sites for ^{45}Ca in *Myxicola* axoplasm that compete favorably with 100 μM EGTA. The ^{45}Ca efflux results are interpreted in terms of endogenous sites that interact with Ca^{2+}, Cd^{2+}, or Mn^{2+}.

INTRODUCTION

Only a small portion of the total calcium in nerve appears as ionized Ca^{2+} in the cytosol (Hodgkin and Keynes, 1957; DiPolo et al., 1976; Baker and Schlaepfer, 1978); most intracellular calcium is sequestered within mitochondria and other organelles or is bound to cytoplasmic molecules. The relative contribution of these intracellular Ca-binding entities to Ca^{2+} buffering is, however, not well understood and is the subject of current investigations (Requena and Mullins, 1979). Because cytoplasmic free Ca^{2+} is normally too low to activate mitochondrial Ca uptake (Brinley et al., 1978; Baker and

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Schlaepfer, 1978; DiPolo et al., 1976; Requena et al., 1977), mitochondria may not be the major physiological Ca buffer in nerve. Ca^{2+} regulation by other intraneuronal organelles has recently been emphasized. A nonmitochondrial, ATP-dependent Ca uptake was observed in a disrupted synaptosome preparation (Blaustein et al., 1978*a* and 1978*b*), and Ca was shown to be taken up by the smooth endoplasmic reticulum of squid axon (Henkart et al., 1979). Baker and Schlaepfer (1978) found, in *Loligo* and *Myxicola* axoplasm, both mitochondrial and nonmitochondrial Ca binding; the latter component required no metabolic energy. The purpose of our study was to examine ^{45}Ca uptake by and efflux from isolated *Myxicola* axoplasm. Conclusions are drawn concerning localization of this uptake, energy-dependent and -independent components of calcium uptake, and the selectivity of axoplasmic sites that influence the rate of release of bound ^{45}Ca from the energy-independent fraction.

METHODS

Animals

Myxicola infundibulum (Marine Research Associates, Deer Island, New Brunswick, Canada) were stored up to 1 mo in a seawater aquarium maintained at 9°C. Animals used in this study were collected between July 1978 and September 1979.

Experimental Procedures

Axons were dissected in the usual manner (Binstock and Goldman, 1969), soaked for 10 min in a buffered isotonic solution containing $MgSO_4$ and $MgCl_2$, and blotted on filter paper. The axolemma was cut longitudinally with fine scissors and the axoplasm lifted away with forceps. Axoplasm from a single worm was divided into roughly equal portions and each portion was weighed on a piece of metal foil. The axoplasm formed a flat disk ~3 mm in diameter and 100–200 μm thick. The axoplasm samples were then delivered to a loading solution containing a specified ^{45}Ca/EGTA (ethyleneglycol-*bis*(β-aminoethyl ether)-N,N′-tetraacetic acid) ratio for a timed incubation: usually 90 min. At the end of the loading period, the axoplasm was transferred through a series of at least five 1-ml volume, isotope-free washout baths; each bath lasted 2 min. The activity remaining in the axoplasm and that of the washout solutions were determined by liquid scintillation counting.

Solutions

The composition of the normal soak solution was as follows: Na aspartate, 20 mM; K aspartate, 300 mM; glycine, 354 mM; K_2 TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 10 mM; $MgCl_2$, 10 mM; KH_2PO_4, 10 mM; and EGTA, 0.1 mM. Although stock solutions were passed through a Chelex column (Bio-Rad Laboratories, Richmond, Calif.), the normal soak solution contained 6 μM Ca contamination, which was included in the determination of the Ca/EGTA ratio. The pH of this solution was adjusted to 7.30 at 10°C. Small additions of Na cyanide (NaCN), ATP, or the divalent salts, $CaCl_2$, $MnCl_2$, $CdCl_2$, $NiCl_2$, $BaCl_2$, $SrCl_2$, or $MgCl_2$, were as a 1:100 dilution of neutral stock solutions. Oligomycin (5 μg/ml) was added as a 1:1,000 dilution of an ethanol stock solution. A23187 (from Dr. R. Hamill, Lilly Research Laboratories, Indianapolis, Ind.) was dissolved first in dimethyl sulfoxide (DMSO) (50 mg/ml), then the solution suspended as 2 parts DMSO solution/1,000 parts of normal uptake medium (to make an opalescent, colloidal

suspension). The suspension was then diluted to 5 μg A23187/ml of the uptake mixture.

In solutions containing EGTA, the concentration of the ionized divalent cation $[M^{2+}]$ is calculated from a knowledge of the total divalent cation concentration $[M_T]$, the total [EGTA], and the effective dissociation constant K_d, of the M-EGTA complex

$$[M^{2+}] = \frac{K_d}{2}\left[\sqrt{\left(1 + \frac{[EGTA] - [M_T]}{K_d}\right)^2 + 4\frac{[M_T]}{K_d}} - 1 - \frac{[EGTA] - [M_T]}{K_d}\right]. \quad (1)$$

Calculations of the ionized divalent cation concentrations in EGTA-buffered solutions containing more than one divalent ion were made by solving the relevant equations using an iterative method (cf. Schwarzenbach and Flaschka [1969]). Throughout the text the designation "Ca/EGTA" refers to the total Ca (i.e., radioactive ^{45}Ca and nonradioactive ^{45}Ca) and the total EGTA (i.e., complexed and free) present in the medium. The greatest uncertainty in the determination of $[M^{2+}]$ in our experiments is in the value of the ion-EGTA dissociation constant, K_d, under the ionic conditions prevailing in the uptake solutions. (Most literature values of K_d are for conditions of 0.1 M ionic strength or pH 7.0.) Our solutions are pH 7.3, 0.35 M ionic strength, and have 10 mM free Mg. The effect of pH on K_d can be obtained from standard references (Schwarzenbach and Flaschka, 1969). Increasing pH from 7.0 to 7.3 decreases the dissociation constant by a factor of 3.24. The effect of ionic strength on K_d is estimated using results from DiPolo et al. (1976). These workers found that increasing ionic strength from 0.1 to 0.35 M roughly doubled the effective dissociation constant of the Ca-EGTA complex. The same factor is assigned here for all divalent cation-EGTA dissociation constants (see Table II).

Although EGTA binds Mg poorly, a fraction of the total EGTA will chelate Mg in solutions containing 10 mM of this ion

$$\frac{[EGTA]_{free}}{[Mg\text{-}EGTA]} = \frac{K_d}{[Mg^{2+}]} = \frac{80\text{ mM}}{10\text{ mM}},$$

where K_d for Mg = 80 mM = 1,000 × antilog($-pK$ Mg) (from Table II). Therefore, the effect of 10 mM $[Mg^{2+}]$ is to reduce free EGTA by 11%. Because the values of K_d are somewhat approximate, we do not make corrections for the presence of Mg.

Method of Data Expression

The amount of Ca taken up by the axoplasm was determined, in terms of μmol/kg axoplasm, from a knowledge of the specific activity of ^{45}Ca in the loading solution, the activity remaining in the axoplasm at the end of the washout period, and the weight of the axoplasm. The rate coefficient of ^{45}Ca efflux was obtained by dividing the activity per min leaving the axoplasm by total activity in the axoplasm at the time of the efflux determination. Where the rate coefficient of ^{45}Ca efflux in the presence of a divalent cation was compared with the rate coefficient of ^{45}Ca efflux in the absence of the divalent cation (as in Fig. 6), the average of three points in divalent-containing solutions was compared with a line through the average of the three preceding and the three succeeding points in solutions not containing the divalent ion.

RESULTS

^{45}Ca Uptake by Isolated Axoplasm

In accordance with previous observations of ^{45}Ca uptake by isolated axoplasm of giant nerve fibers (Baker and Schlaepfer, 1978), our results with *Myxicola* showed both ATP-dependent and ATP-independent components of Ca binding. A typical experiment demonstrating the effect of ATP on ^{45}Ca uptake by *Myxicola* axoplasm is shown in Fig. 1. At a free $[Ca^{2+}]$ of 0.54 μM (80 μM Ca/100 μM EGTA, pH 7.3), ^{45}Ca adhered to the axoplasm at a rate of ~1 μmol/kg per min in the absence, and 1.5 μmol/kg per min in the presence, of 1 mM ATP. The *lowest curve* in Fig. 1 is an estimate of the fraction of this uptake that

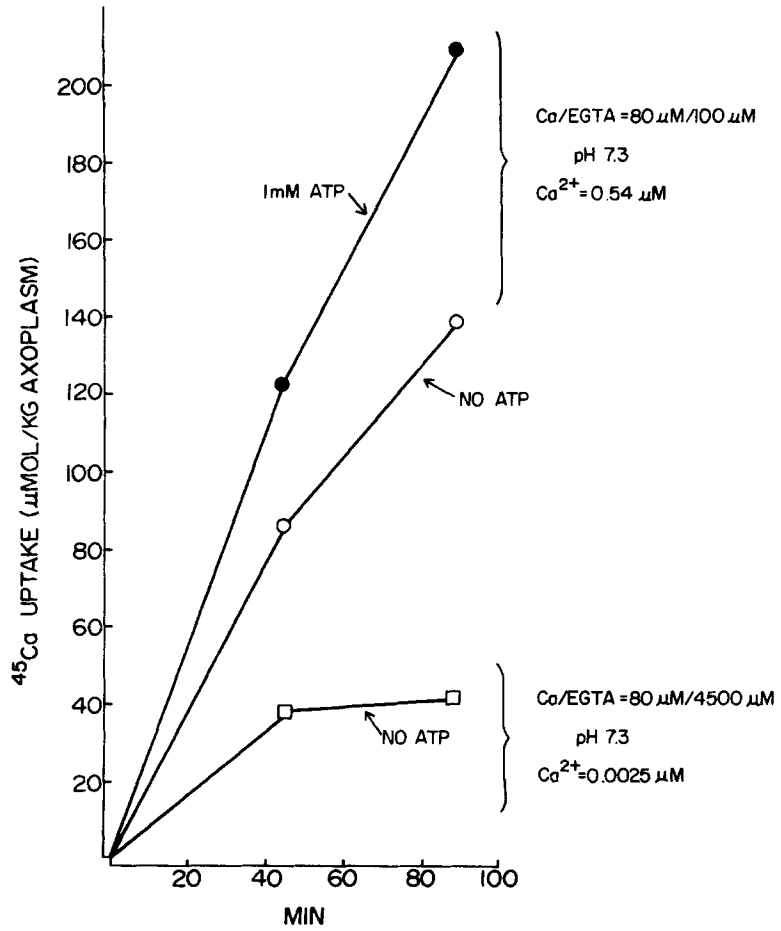


FIGURE 1. ATP-dependent and ATP-independent ^{45}Ca uptake at Ca_i^{2+} = 0.54 μM (Ca/EGTA = 80 μM/100 μM, pH 7.3); Temperature, 8–10°C. 1 mM ATP (●) and ATP-free (○) represent axoplasm taken from the same animal. □, corresponding to the ^{45}Ca uptake in excess EGTA, represent ^{45}Ca-EGTA marker space obtained with a separate preparation.

may be a result of nonspecific retention of ^{45}Ca into the diffusional space of the axoplasm. This estimate of nonspecific retention was obtained by adding excess EGTA to the incubation medium, a treatment that lowered $[Ca^{2+}]$ to the nanomolar range, thus preventing any specific ^{45}Ca attachment to axoplasmic sites (Baker and Schlaepfer, 1978). As discussed below, the excess EGTA procedure provides a valid estimate of the nonspecific retention of ^{45}Ca only if ^{45}Ca penetration of the axoplasm is independent of the concentration of EGTA in the incubation medium. Note that ^{45}Ca uptake measured in excess EGTA (*lowest curve*) is apparently saturated after only 45 min in the soak solution.

The component of ^{45}Ca uptake that required ATP was examined in the presence and in the absence of the mitochondrial poison cyanide plus oligomycin to determine the portion of this component that was a result of mitochondria. In solutions containing 0.54 μM Ca^{2+}, ATP increased ^{45}Ca uptake by 84 ± 8 μmol/kg (mean ± SEM) over a 90-min uptake period (three experiments). Cyanide (CN; 2 mM) and oligomycin (5 μg/ml) reduced this to 13 ± 4 μmol/kg (three experiments). Although there may be a small ATP-dependent uptake that persists in the presence of these poisons, most of the energy-dependent Ca binding at $[Ca^{2+}] = 0.54\ \mu M$ is blocked by the inhibitors. Therefore, most of the increase in ^{45}Ca uptake caused by the addition of ATP is apparently a result of mitochondrial action. Table I shows ^{45}Ca uptake into axoplasm samples incubated in the absence of ATP in the presence and absence of CN and oligomycin. The lack of effect of poisons in the absence of ATP suggest that mitochondria do not retain sufficient stores of energy to continue sequestering Ca. The Ca ionphore A23187 (5 μg/ml) also had no effect on ^{45}Ca uptake in the absence of ATP (data not shown).

This report is concerned primarily with ^{45}Ca binding in the absence of ATP. Fig. 2 shows the ATP-independent ^{45}Ca uptake as a function of free Ca^{2+}. The low range of ionized Ca^{2+} (Fig. 2 *a*) was set with 100 μM EGTA; no EGTA was used in the bath solutions containing high ionized Ca^{2+} (Fig. 2 *b*). The experimental procedure for these experiments involved soaking samples

TABLE I

EFFECT OF CN AND OLIGOMYCIN ON ^{45}Ca UPTAKE IN THE ABSENCE OF ATP

	^{45}Ca Uptake at 90 min*		
Preparation	Without CN and oligomycin	With CN and oligomycin‡	ΔCN and oligomycin
		μmol/kg axoplasm	
9/24/79A	78	78	0
9/24/79B	69	59	−10
9/24/79C	99	98	−1
Mean ± SEM	82 ± 8.9	78 ± 11.3	−4 ± 3.2

* $[Ca^{2+}] = 0.54\ \mu M$.

‡ [CN] = 2mM; [oligomycin] = 5 μg/ml.

for 90 min in solutions containing isotope and then rinsing the samples in isotope-free solutions for 10 min to remove superficial radioactivity before assaying them for ^{45}Ca. Because the ^{45}Ca content of the axoplasm was

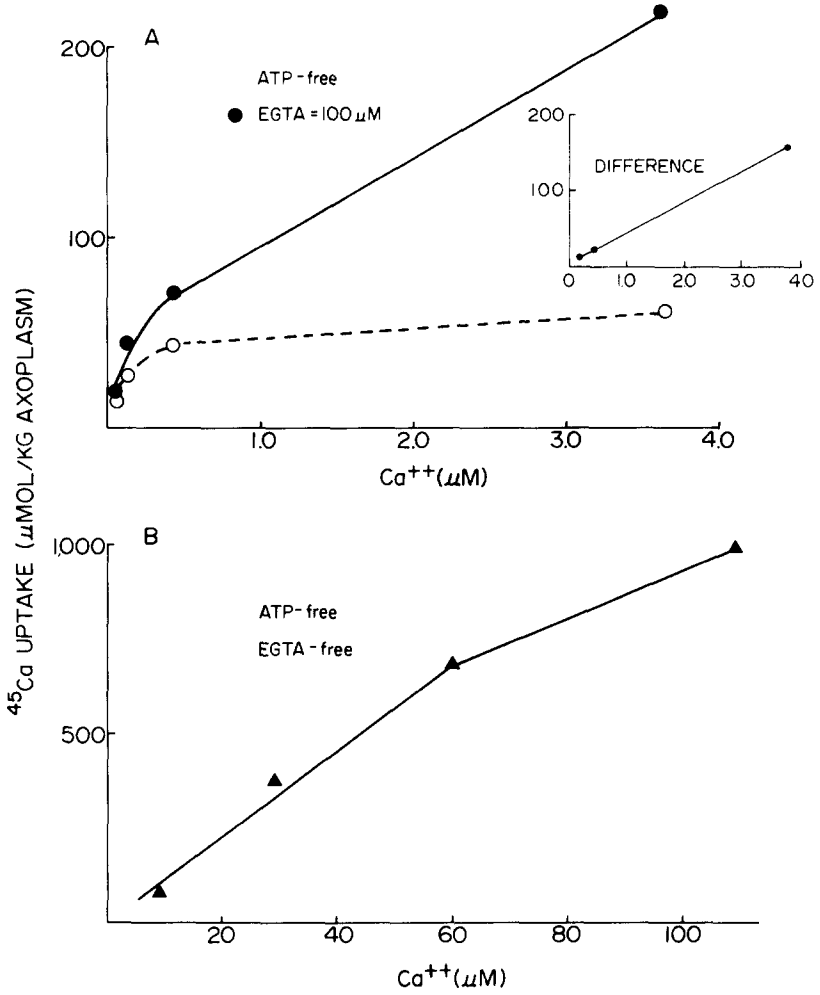


FIGURE 2. Effect of Ca^{2+} on 90-min Ca uptake in ATP-free solutions with and without EGTA. (*A*) ●, total ^{45}Ca uptake from a medium containing the specified ionized Ca^{2+} concentration set with 100 μM EGTA; ○, nonspecific retention, which was estimated by the procedure described in Fig. 1. (*B*) Uptake at higher concentrations of free Ca^{2+} was obtained in the absence of EGTA (▲).

determined after a 10-min washout period, any rapidly released component of bound ^{45}Ca would not be detected by this method. The dotted line in Fig. 2*a* provides an estimate of the ^{45}Ca retained as a result of simple penetration of the test solution into the axoplasmic nonbinding spaces. As in Fig. 1, this

estimate was made by adding excess EGTA to the uptake solutions and rests on the assumption that the concentration of EGTA does not affect ^{45}Ca penetration into the cytosol. This nonspecific ^{45}Ca uptake was linearly dependent on the total ^{45}Ca; i.e., the ^{45}Ca-EGTA concentration of the incubation bath.

Experiments were done to determine the effect of EGTA on ^{45}Ca uptake. Samples of axoplasm from a single worm were divided into two groups and incubated in separate, labeled uptake solutions. Both solutions contained identical amounts of ^{45}Ca and had ionized Ca^{2+} levels set with EGTA buffers to $[Ca^{2+}] = 0.54$ μM. However, in one solution Ca^{2+} was set by a Ca/EGTA concentration ratio of 80 μM/100 μM, whereas in the other solution, a buffer ratio of 160 μM/200 μM was used. The ^{45}Ca uptake and efflux then were measured while keeping the EGTA concentration for each group constant throughout the experiment. In two experiments done at 200 μM EGTA, uptake was 3.0 and 2.4 times that from the 100-μM EGTA solution; the rate coefficients of ^{45}Ca efflux were 25 and 20% lower from the 200-μM EGTA than they were from the 100-μM EGTA solution. These results indicate that additional EGTA buffer in the incubation medium enhanced the ^{45}Ca uptake by axoplasm. An effect of EGTA on ^{45}Ca uptake can also be seen by comparing uptake in Fig. 2*a* (measured with EGTA) to uptake, extrapolated toward low Ca^{2+}, in Fig. 2*b* (measured without EGTA).

Experiments were also done to determine if the EGTA complex itself was bound by axoplasm. That this was not the case was confirmed in several tests where a comparison was made between uptake of ^{22}Na, [^{14}C]EDTA, and ^{45}Ca with excess EGTA. All three methods of measuring the total axoplasmic nonbinding space gave the same value; thus, it seems unlikely that any of these substances are bound by axoplasm. In addition, the EGTA-promoted Ca uptake cannot be attributed to enhanced nonspecific ^{45}Ca retention because any nonspecific component should be proportional to the total amount of ^{45}Ca present. Yet, in the experiment described above in which the amount of ^{45}Ca label was the same in the two uptake media, more isotope was retained by the samples incubated in media containing the higher EGTA concentration. An interpretation of these results is given in the Discussion.

Properties of ^{45}Ca Release from Axoplasm

Fig. 3 (●) shows several features of ^{45}Ca release from isolated samples of *Myxicola* axoplasm. After a preliminary loading with ^{45}Ca (90-min incubation in 80 μM ^{45}Ca/100 μM EGTA; $[Ca^{2+}] = 0.54$ μM; see Methods), the axoplasm was transferred to the washout solutions containing 100 μM EGTA. Release of ^{45}Ca into the washout solutions occurred with an initial rate coefficient of ~0.05 min^{-1}. The efflux rate coefficient quickly decayed with time (●) and its magnitude was dependent on the concentration of free Ca^{2+} in the bath. The Ca-induced partial increment in the efflux rate coefficient was independent of the fraction of ^{45}Ca remaining in the sample as is revealed by plotting these data on a logarithmic scale. Thus, most of the loaded ^{45}Ca had the same Ca^{2+} sensitivity. If, to minimize ^{45}Ca binding to the axoplasm (Baker and Schlaep-

fer, 1978), the preliminary loading was done in solutions containing a large excess of EGTA over total Ca, the subsequent ^{45}Ca efflux had entirely different characteristics. Fig. 3 (○) shows that after incubating axoplasm in solutions containing 4,500 μM EGTA, ^{45}Ca efflux was more rapid, more constant, and did not respond to elevated Ca^{2+} in the efflux bath. Isotope remaining in the axoplasm after incubation with excess EGTA is likely to be located exclusively in the diffusional spaces of the axoplasm, and the ^{45}Ca efflux from samples

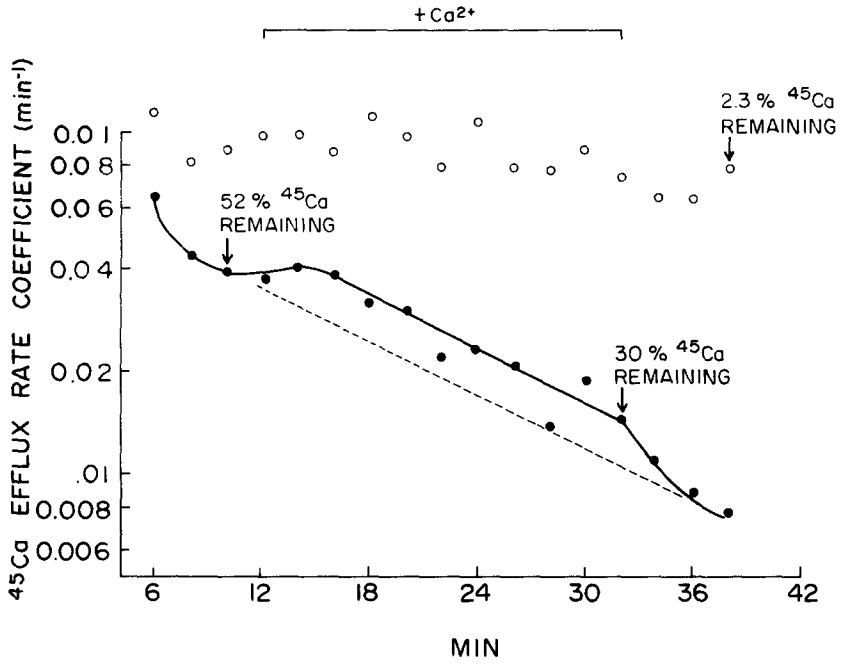


FIGURE 3. ^{45}Ca efflux from samples of axoplasm into solutions containing 100 μM EGTA. During the period designated by the *horizontal bracket* the efflux solutions contained 100 μM Ca + 100 μM EGTA, which, according to the Eq. 1 yields $[Ca^{2+}] = 3.8$ μM ($K_d = 0.14$ μM). The preliminary incubation conditions differed for the two groups of samples: ●, normal uptake medium (Ca/EGTA = 80 μM/100 μM, $Ca^{2+} = 0.54$ μM); ○, medium containing excess EGTA (Ca/EGTA = 80 μM/4,500 μM). Both solutions were ATP-free, ^{45}Ca-specific activity was the same in both uptake media, and all samples were taken from the same axon. The preliminary incubation was for 90 min.

loaded under such conditions will not reflect properties of the axoplasmic binding sites. The characteristic features of ^{45}Ca efflux shown in the lower trace of Fig. 3 were observed only when the preliminary incubation conditions were such as to favor specific binding of ^{45}Ca.

The effect of bath EGTA on the ^{45}Ca efflux rate coefficient was examined in an experiment shown in Fig. 4. All samples were incubated in test solutions of 80 μM Ca/100 μM EGTA, and washout was begun in a Ca-free solution

containing 100 μM EGTA. During the designated period, one-half the samples were switched to 200 μM EGTA and the other one-half continued to be washed in 100 μM EGTA. Fig. 4 shows that doubling the EGTA concentration did not enhance ^{45}Ca efflux, demonstrating that providing more carrier did not increase the rate at which ^{45}Ca was removed from the axoplasm. The efflux rate appears to respond only to the ambient free Ca^{2+}; lack of sufficient EGTA was not rate-limiting to the efflux process.

The experiment shown in Fig. 5 reveals the relationship between efflux rate coefficients and the duration of the uptake period. Axoplasm was soaked for

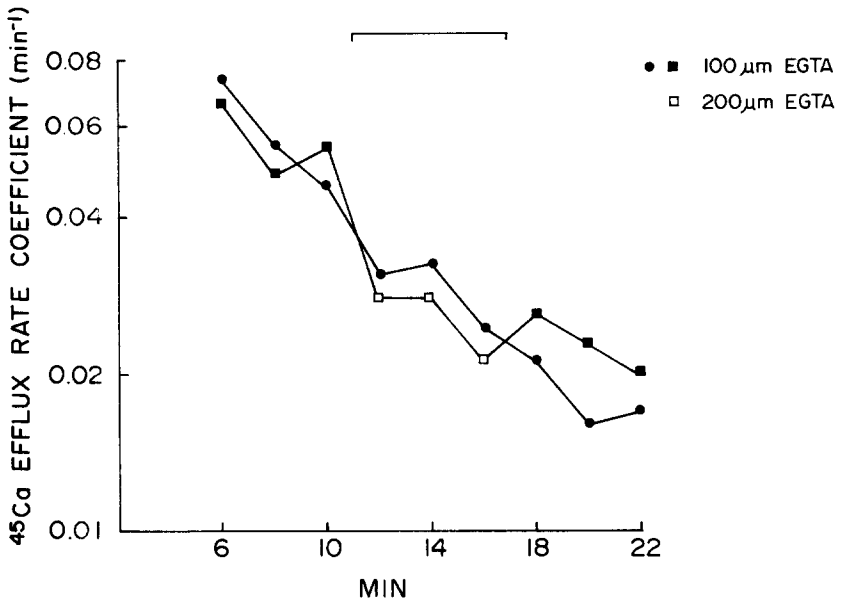


FIGURE 4. Effect of increasing EGTA concentration during the efflux period. All samples were loaded with ^{45}Ca by soaking in identical solutions containing Ca/EGTA = 80 μM/100 μM. One-half the samples were transferred to a 200-μM EGTA solution during the period marked by the *horizontal bracket.* The efflux baths were nominally Ca-free and contained no ATP.

increasing periods of time in solutions containing $[Ca^{2+}]$ = 0.54 μM (Ca/EGTA = 80 μM/100 μM, pH 7.3), and the corresponding washout rates in Ca-free and Ca-containing ($[Ca^{2+}]$ = 3.8 μM) solutions were measured. The initial rate coefficient of efflux appeared to be lower from the samples that contained more ^{45}Ca (i.e., the samples subjected to a longer incubation). The ^{45}Ca bound to the axoplasm at 15 min clearly leaves more rapidly than ^{45}Ca bound during longer periods of uptake. This result could be a result of diffusional effects whereby the 15-min retention represents binding to sites located nearer the surface that, because of their proximity to the bath solution, discharge their ^{45}Ca with less of a diffusional delay.

Divalent-Cation Substitution: Effects on ^{45}Ca Efflux

In addition to Ca^{2+} the seven divalent cations, Co^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+}, and Mg^{2+} were surveyed to test their ability to displace ^{45}Ca from samples of axoplasm; of these, only Cd^{2+} and Mn^{2+} were effective in the concentration range <10 μM. Two experiments representative of these studies are shown in Fig. 6. Mn^{2+} at a concentration of 0.84 μM increased the rate of ^{45}Ca efflux by roughly the same factor as did 3.8 μM Ca^{2+}. By contrast, 0.84 μM Co^{2+} had no effect. The increase in efflux rate coefficient brought about

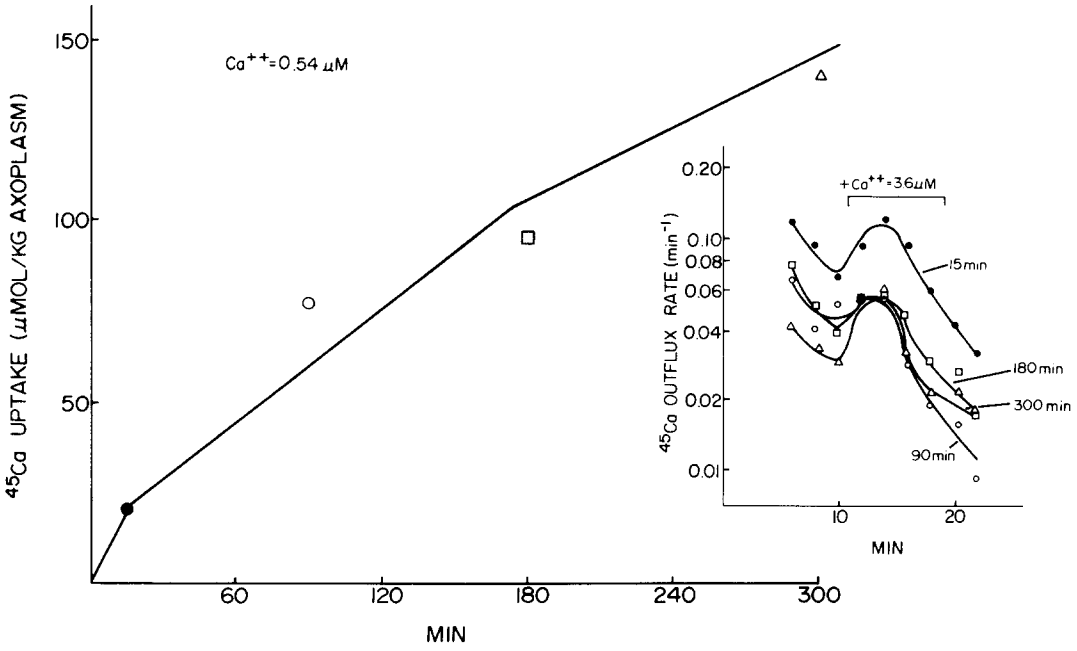


FIGURE 5. Ca uptake ($[Ca^{2+}]$ = 0.54 μM, pH 7.3, 8–10°C) as a function of time, followed by a washout into isotope-free solutions. After soaking the axoplasm samples in the uptake solutions for the designated period of time, each sample was transferred through an identical series of efflux solutions. The ^{45}Ca retention shown on the figure was that remaining in the axoplasm at the end of the 10-min washout. Solutions were ATP-free.

by Ca^{2+}, Cd^{2+} or Mn^{2+} did not require ATP during the ^{45}Ca-uptake or -washout periods, suggesting that the divalent ion-sensitive efflux was not from ATP-dependent structures. Moreover, when these experiments were done in the presence of 1 mM ATP (both in the uptake and efflux solutions), the rate coefficient of ^{45}Ca release was actually slightly lower than in those experiments done in the absence of ATP, which is consistent with the ATP-dependent retention of ^{45}Ca being less exchangeable than the ATP-independent component.

Fig. 7 *a–c* show the relationship between the relative increase in ^{45}Ca efflux and the concentration of ionized Ca^{2+}, Mn^{2+}, or Cd^{2+} in the bath solutions. In these experiments, total EGTA was held constant at 100 μM and the total

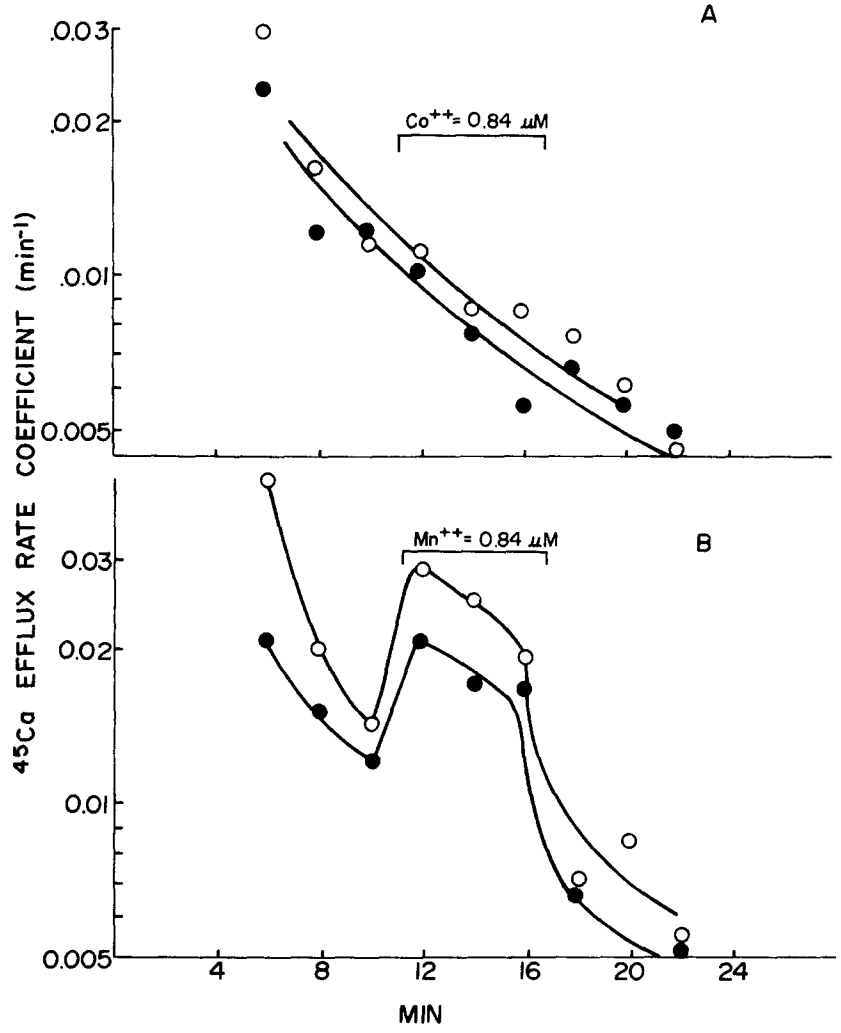
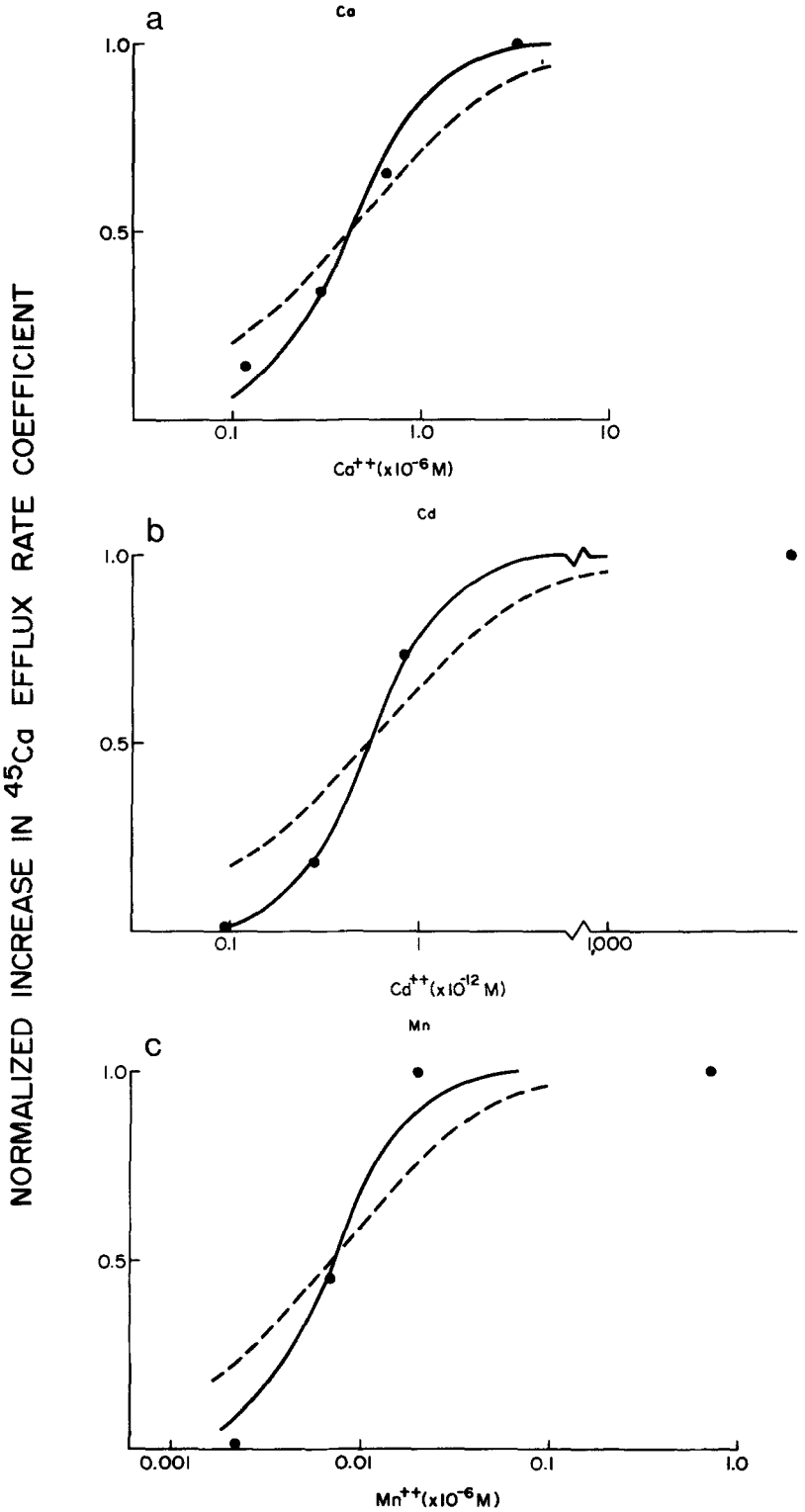


FIGURE 6. Effect of 0.84 μM Co^{2+} and Mn^{2+} (100 μM divalent cation with 100 μM EGTA) on ^{45}Ca efflux from preloaded samples of *Myxicola* axoplasm. 90-min-uptake periods were in the presence (●), or in the absence (○) of 1 mM ATP; [Ca^{2+}] = 0.54 μM, pH 7.3, 8–10°C, [EGTA] = 100 μM. The divalent cations were added during the period indicated by the *horizontal brackets*.

divalent ion concentration was increased. The free ion concentrations shown on the horizontal axis of the figure were calculated as described in Methods. Ca, Mn, and Cd began to increase ^{45}Ca efflux when their total concentrations


NORMALIZED INCREASE IN ^{45}Ca EFFLUX RATE COEFFICIENT
a
Ca
1.0
0.5
0.1
1.0
10
$Ca^{++}(x10^{-6}M)$
b
Cd
1.0
0.5
0.1
1
1,000
$Cd^{++}(x10^{-12}M)$
c
Mn
1.0
0.5
0.001
0.01
0.1
1.0
$Mn^{++}(x10^{-6}M)$

in the bath were approximately one-half the total concentration of bath EGTA. Thus, the cytoplasmic sites that interact with these ions to increase ^{45}Ca efflux apparently do so when the free-ion concentration is equal to the ion-EGTA dissociation constant. It should be emphasized that data like that shown in Fig. 7 do not represent an equilibrium saturation of Ca-binding sites in *Myxicola* axoplasm. These results merely indicate an effect of ambient bath divalent ions (or of the concentration of the ion-EGTA complex) on the rate of ^{45}Ca release. For comparison, curves shown in Fig. 7*a–c* (- - -) are plots of a unimolecular kinetic reaction whereas the solid curves assume a square-law relationship.

In Table II the divalent cations are arranged in order of their increasing crystal ionic radii (Pauling, 1960). This ordering reveals that the three effective cations for increasing the rate of ^{45}Ca release, Ca^{2+}, Mn^{2+}, and Cd^{2+}, are very close in size, suggesting a size requirement of the axoplasmic site. The fourth

TABLE II

EFFECT OF DIVALENT CATIONS ON RELEASE OF ^{45}Ca FROM *MYXICOLA* AXOPLASM

Divalent cation	Ligand structure	Crystal ionic radius	One-half maximum concentration that increases ^{45}Ca efflux rate	Affinity for EGTA*
		Å		pK_d^{EGTA}
Mg	Rutile (six ligands)	0.66	>10mM	1.1
Ni		0.72	>10μM	9.5
Co		0.74	>10μM	8.2
Mn		0.81	9.5nM	8.2
Cd	Fluorite (eight ligands)	0.98	0.35pM	12.6
Ca		1.0	0.38μM	6.9
Sr		1.14	>10μM	4.4
Ba		1.32	>10μM	4.2

* Calculated for pH 7.3; ionic strength, 0.35 M.

column shows the concentration of free divalent cations that increased the ^{45}Ca efflux by one-half the maximum amount. For those divalent ions that had no effect on ^{45}Ca efflux (Co^{2+}, Ni^{2+}, Ba^{2+}, Sr^{2+}, and Mg^{2+}) the highest concentration that was used is shown in column four. The fifth column lists the effective pK_d of divalent cations for EGTA (determined from Schwarzenbach and Flaschka [1969], as described in Methods). Note that although Co^{2+}

FIGURE 7. *Opposite.* Concentration-response curves representing the relative increase in ^{45}Ca efflux in the presence of Ca^{2+}, Mn^{2+}, and Cd^{2+}. In these experiments the EGTA concentration was held constant at 100 μM and the concentration of the divalent ion was varied. The response was a steep, saturating function of the free-ion concentration; the *solid curve* is plotted according to the equation $V = V_{max}/[1 + (K_d/M^{2+})^2]$; the *dotted curve* is a plot of $V = V_{max}/[1 + (K_d/M^{2+})]$. Solutions were ATP-free.

and Mn^{2+} have the same affinity for EGTA, only Mn^{2+} increased the ^{45}Ca efflux.

That Ca^{2+}, Cd^{2+}, and Mn^{2+} probably act at the same cytoplasmic site(s) to increase ^{45}Ca efflux is shown in Fig. 8. If each ion species had been acting at different sites, then adding saturating concentrations of two species together should have approximately doubled the ion-evoked ^{45}Ca efflux. Clearly, this was not the case.

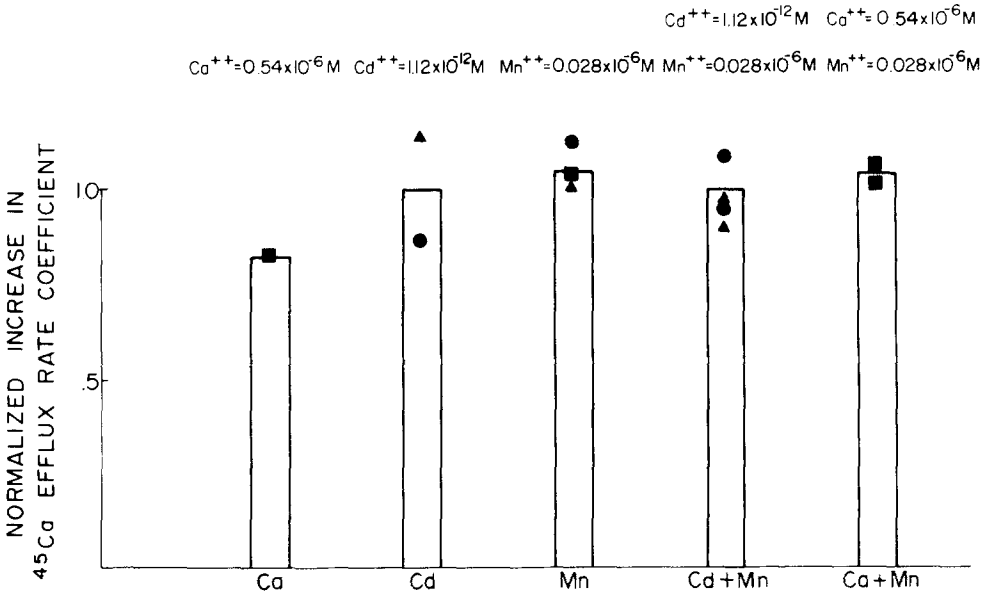


FIGURE 8. This figure shows that the maximum increase in efflux caused by the addition of one divalent ion (Ca, Cd, or Mn) is not exceeded when a second ion is added. The symbols represent axoplasm samples from three axons; the average affect observed in samples from a single axon was normalized to the value 1. Free EGTA was held constant at 20 μM. Bath divalent-ion concentrations: Ca^{2+}, 0.54 μM; Cd^{2+}, 1.12 pM; Mn^{2+}, 0.028 μM; Cd^{2+}, + Mn^{2+}, 1.12 pM + 0.028 μM; Ca + Mn, 0.54 μM + 0.028 μM. An example of the divalent-ion and EGTA concentrations used to obtain these values of the free-ion concentrations are, $[Ca^{2+}]$ = 0.54 μM: [Ca] = 80 μM, [EGTA] = 100 μM; $[Ca^{2+}]$ = 0.54 μM, $[Mn^{2+}]$ = 0.28 μM; [Ca] = 80 μM, [Mn] = 100 μM, [EGTA] = 200 μM. All solutions were ATP-free.

DISCUSSION

Myxicola axoplasm is capable of accumulating Ca by at least two distinguishable mechanisms. One mechanism can be identified as mitochondrial Ca sequestration by its requirement for ATP and its sensitivity to CN plus oligomycin. The other mechanism is tentatively ascribed to ionic binding. It is independent of ATP and is not affected by mitochondrial inhibitors or by

the Ca ionophore A23187. As little is known of this latter mechanism, it has been the primary subject of this study.

Bound ^{45}Ca was probably not uniformly distributed throughout the axoplasm in these experiments. Two types of considerations outlined below suggest instead that most of this ^{45}Ca was concentrated in the surface region of the axoplasm. Consider the following computation. Our measurements of unidirectional ^{45}Ca uptake were in the range of 50 μmol/kg per h in solutions containing $[Ca^{2+}] = 0.54$ μM, [EGTA] = 100 μM. The ^{45}Ca efflux rate coefficient in 0.54 μM Ca^{2+} = 100 μM EGTA, varied from ~0.10 min^{-1} at the beginning of the efflux experiment to ~0.01 min^{-1} after 20 min of washout. Total Ca concentration of *Myxicola* axoplasm from Gilbert (1975) and our own (unpublished) measurements is ~200 μmol/kg axoplasm. Assuming that the efflux rate coefficients apply to all the axoplasmic Ca, the total Ca efflux would be 0.10 min^{-1} × 200 μmol/kg × 60 min/h = 1,200 μmol/kg per h initially, and 0.01 min^{-1} × 200 μmol/kg × 60 min/h = 120 μmol/kg per h after 20 min of washout, compared with the unidirectional ^{45}Ca uptake of 50 μmol/kg per h. Thus, we must conclude either that the axoplasmic sample is losing total Ca at a very fast rate at ambient $[Ca^{2+}] = 0.54$ μM, which seems unlikely (Baker and Schlaepfer, 1978), or that the rate coefficients 0.10 min^{-1} and 0.01 min^{-1} do not apply to the total 200 μmol/kg axoplasmic Ca. This is consistent with the notion that the exiting ^{45}Ca comes mainly from the surface of the axoplasm.

The second result suggesting nonuniformity in the distribution of axoplasmic ^{45}Ca is the effect of EGTA on ^{45}Ca uptake. The local rate of ^{45}Ca uptake at all points within the sample should depend only on the local concentration of ionized Ca^{2+}. If Ca^{2+} had been controlled by the EGTA buffer to a uniform concentration throughout the axoplasm sample, the same Ca uptake would have been observed in a buffer of 160 μM Ca/200 μM EGTA as was observed in 80 μM Ca/100 μM EGTA, because $[Ca^{2+}] = 0.54$ μM in both solutions. That more Ca was bound by the axoplasm when more EGTA buffer was present suggests that Ca^{2+} in the cytosol was not uniform. Our interpretation of these results is that the volume space involved in Ca binding (i.e., the depth of penetration of ^{45}Ca into the axoplasm) is increased by increasing the bath concentration of EGTA. This is to be expected if the axoplasmic sites compete favorably with 100 μM EGTA for available ^{45}Ca. Thus only part of the axoplasm, the surface layer, fully participates in ^{45}Ca uptake. If increasing the concentration of EGTA increases ^{45}Ca penetration of the axoplasm (thus increasing the nonbinding volume) then nonspecific ^{45}Ca uptake will be overestimated by the procedure of Figs. 1 and 2.

Calcium-binding sites never became fully equilibrated with ^{45}Ca even after 300 min of loading in 0.56 μM Ca^{2+} solutions (Fig. 5). However, equilibration in solutions with excess EGTA occurred within 45 min (Fig. 1). Thus, diffusion of the Ca-EGTA complex must be considerably more rapid than that of free calcium. Increasing the concentration of the ^{45}Ca/EGTA buffer undoubtedly distributes ^{45}Ca more uniformly. However, as the concentration of bath ^{45}Ca and EGTA increases at a constant Ca/EGTA ratio, the amount of ^{45}Ca

accumulated by the axoplasm becomes a smaller fraction of the ^{45}Ca concentration in the bath and therefore more difficult to detect. We did not carry out these experiments using concentrations of EGTA >200 μM for this reason.

^{45}Ca Release Kinetics

In view of these results, it is evident that spatial nonuniformity in the distribution of ^{45}Ca will complicate analysis of both ^{45}Ca uptake and efflux data. However, a partial explanation of some of the features of the ^{45}Ca efflux experiments may be the following. We assume that the 90-min incubation period loads ^{45}Ca into a peripheral shell of axoplasm. Washout of ^{45}Ca with a Ca-free, EGTA solution is viewed as a reversal of this loading process. It may be reasonable to expect that as ^{45}Ca begins to diffuse toward the surface (as the Ca-EGTA complex), its progress will be slowed by unoccupied axoplasmic sites. According to this view, addition of the divalent ions Ca, Mn, or Cd would increase the rate coefficient of efflux by occupying vacant Ca-binding sites along the diffusional pathway, consequently reducing this impediment to diffusion.

This interpretation is consistent with most of our data, yet Fig. 4 shows an experiment that may be difficult to reconcile with this scheme. If the rate of ^{45}Ca efflux were limited by unoccupied natural sites competing with EGTA for available ^{45}Ca, then increasing the concentration of EGTA should increase the efflux rate coefficient. In fact, the rate coefficient was slightly reduced when bath EGTA was changed from 100 to 200 μM. A view more consistent with this result is that the efflux rate coefficient is determined not by restricted diffusion but by the rate of release of ^{45}Ca from primary attachment sites. In any case, if the effect of Ca^{2+}, Mn^{2+}, or Cd^{2+} results from an interaction of these ions with a single site, then this site must have definite size limitation for those divalent ions that it will recognize.

Although we did not measure ionized Ca directly in this study, our measurements are certainly relevant to Ca^{2+} regulation in nerve cells because they provide information about the properties of a Ca binding entity that seems to be responsible for the majority of Ca buffering in the *Myxicola* axon cytosol. In addition, knowledge of the ion selectivity of sites implicated in calcium buffering will be valuable in the evaluation of data from intracellular injection experiments. Because an injection of divalent cations may have many effects, it is important to know whether these effects can be attributed to the injected divalent cations themselves or to the redistribution of bound intracellular calcium.

At present, the presumed proteins within the *Myxicola* cytoplasm that have the observed high affinity for calcium cannot be identified. It is known that 3.6% of the wet weight of *Myxicola* axoplasm is protein, and neurofilaments comprise a major portion of this. Whether or not the protein structures of the neurofilament network will bind Ca in a manner sufficient to explain our results is not known.

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