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CALCIUM BUFFERING IN PRESYNAPTIC NERVE TERMINALS

FREE CALCIUM LEVELS MEASURED WITH ARSENAZO III

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Summary

The particulate fraction from osmotically shocked synaptosomes ('synaptosomal membranes') sequesters Ca when incubated with ATP-containing solutions. This net accumulation of Ca can reduce the free $[Ca^{2+}]$ of the bathing medium to sub-micromolar levels (measured with arsenazo III). Two distinct types of Ca sequestration site are responsible for the Ca^{2+} buffering. One site, presumed to be smooth endoplasmic reticulum, operates at low $[Ca^{2+}]$ (less than 1 μM), and has a relatively small capacity. Ca sequestration at this site is prevented by the Ca^{2+} ionophore, A-23187, but not by mitochondrial poisons. The second (mitochondrial) site, in contrast, is blocked by the mitochondrial uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and oligomycin. Since the intraterminal organelles can buffer $[Ca^{2+}]$ to about 0.3—0.5 μM, this may be an upper limit to the normal resting level of $[Ca^{2+}]_i$ in nerve terminals. In the steady state, total cell Ca and $[Ca^{2+}]_i$ will be governed principally by Ca transport mechanisms in the plasmalemma; the intracellular organelle transport systems then operate in equilibrium with this $[Ca^{2+}]$. During activity, however, Ca rapidly enters the terminals and $[Ca^{2+}]_i$ rises. The intracellular buffering mechanisms then come into play and help to return $[Ca^{2+}]_i$ toward the resting level; the non-mitochondrial Ca sequestration mechanism probably plays the major role in this Ca buffering.

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

Intracellular free Ca^{2+} regulates transmitter release from nerve terminals [1]. The processes that may influence the intracellular free Ca concentration ($[Ca^{2+}]_i$) include Ca influx and Ca extrusion across the plasma membrane, sequestration into and release from intracellular organelles, and binding to soluble cytoplasmic buffering sites [2]. Ca movement across the plasma membrane must clearly be involved in the long-term regulation of Ca levels in nerve terminals, in order to eliminate the Ca that enters the terminals upon stimulation [3]. However, since all the Ca that enters nerve terminals is not immediately extruded [4], intracellular mechanisms are likely to play a major role in short term $[Ca^{2+}]_i$ regulation.

Brinley et al. [5] have used arsenazo III, a Ca^{2+}-sensitive dye, to measure Ca^{2+} buffering in squid axons. By measuring the differential absorbance of free and Ca-complexed arsenazo III, it is possible to monitor the free Ca^{2+} concentration as different amounts of total Ca are added. In the squid axon, most of the added Ca is bound or sequestered, and does not appear as free Ca^{2+} in the axoplasm. A similar result is found with extruded axoplasm from squid or *Myxicola* [6]. Both intact and extruded axoplasm display two types of buffering process: passive and active (energy-requiring). When mitochondrial sequestration of Ca was blocked in extruded *Myxicola* axoplasm by the addition of oligomycin and cyanide in the presence of ATP, no active, non-mitochondrial sequestration was demonstrable [6]. However, Henkart et al. [7] have observed that Ca is sequestered in the smooth endoplasmic reticulum in the axoplasm of squid giant axons.

In mammalian nerve terminals there is an intracellular, ATP-dependent, non-mitochondrial Ca sequestering mechanism that appears to operate at physiological $[Ca^{2+}]_i$ [8,9]. This mechanism sequesters ^{45}Ca in a membrane-bounded compartment that is insensitive to mitochondrial poisons. Morphological [10] as well as biochemical [8] evidence indicates that this non-mitochondrial Ca sequestration system is located in the smooth endoplasmic reticulum. Moreover, this sequestering mechanism [9] is similar, in a number of ways, to muscle saroplasmic reticulum. In order to establish a role for this sequestration system in the regulation of $[Ca^{2+}]_i$, it is necessary to demonstrate that the non-mitochondrial compartment can accomplish a net accumulation of Ca, thereby lowering the $[Ca^{2+}]$ in the surrounding medium. The present article is a description of experiments designed to explore this possibility; a preliminary report of our findings has been published [11].

Methods

Synaptosomes. Synaptosomes were prepared from rat brains by a modification of the methods of Gray and Whittaker [12] and Hajos [13]. Full details of the procedures are given by Krueger et al. [14]. After differential centrifugation in 0.32 M sucrose, the membranes were placed in a discontinuous sucrose gradient, and synaptosomes were recovered from the 0.8 M sucrose band. Before use, the synaptosomes were equilibrated with physiological saline (at 0°C) containing 145 mM NaCl, 3 mM KCl, 1.4 mM $MgCl_2$, 2 mM KH_2PO_4, and

20 mM Hepes adjusted to pH 7.4 with Tris base.

Disrupted synaptosomes. Equilibrated synaptosomes were centrifuged at 13 000 × *g* for 7 min (5°C) and the pellets (approx. 30 mg protein) were resuspended in 100 ml of hypotonic lysis solution (30°C) containing only 20 mM Hepes-Tris (pH 7.4), 1.4 mM $MgCl_2$, and 2 mM KH_2PO_4. After 5 min at 30°C, the disrupted synaptosomes were pelleted by centrifugation at 13 000 × *g* for 7 min (5°C); the pelleted material will be referred to as 'synaptosomal membranes'.

Incubation of synaptosomal membranes with Ca and arsenazo III. The synaptosomal membranes were resuspended in a 150 mM KCl medium (30°C), similar in composition to the physiological saline described above, but with all the NaCl replaced by KCl; the membranes were dispersed with a glass-Teflon homogenizer. This suspension usually contained 1—2 mg protein/ml. To initiate the incubation, 0.5 ml of the membrane suspension was pipetted into a 1.5 ml microcentrifuge tube containing 0.5 ml of incubation medium (30°C). The incubation media consisted of the 150 mM KCl medium with the addition of 100 μM arsenazo III, varying amounts of Ca, and an energy source (either ATP or ATP and phospho*enol*pyruvate plus pyruvate kinase). In some cases, the solutions also contained the mitochondrial poisons, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and oligomycin, with or without the Ca^{2+} ionophore, A-23187. Final incubation concentrations are given in the figure legends.

The suspensions of disrupted synaptosomes were incubated for 5 min at 30°C, and then the membranes were pelleted by centrifuging the individual incubation tubes for 3 min in a Beckman (Fullerton, CA) microfuge B (approx. 9000 × *g*) at room temperature. The supernatants were removed for $[Ca^{2+}]$ determinations, and the pellets were analyzed for total Ca.

Analysis of ionized and total Ca. Free $[Ca^{2+}]$ was determined by measuring the absorbance of the supernatants (containing 50 μM arsenazo III) against a similar supernatant containing 50 μM EGTA (pH 7.4) and no added Ca, in addition to the normal substrates and the arsenazo III. Any non-specific absorbance changes (e.g., due to light scattering) were compensated for by subtracting the absorbance at 750 nm (where arsenazo III absorbance is very low and Ca^{2+}-independent) from the absorbance at 655 nm (the peak of Ca^{2+}-dependent absorbance change) [15]. These data were used to calculate $[Ca^{2+}]$ values according to the following relationships:

$$A_{655} = \epsilon'_{D(655)} \cdot (D_t - CaD) + \epsilon'_{CaD(655)} \cdot CaD + N \quad (1)$$

where A_{655} is the absorbance at 655 nm, $\epsilon'_{D(655)}$ and $\epsilon'_{CaD(655)}$ are the apparent molar absorption coefficients (at 655 nm) of free dye and Ca-dye complex, respectively, and D_t and CaD are the total concentration of dye and the concentration of Ca-dye complex, respectively. All absorbance values that are not due to the dye, such as that produced by light scattering, are combined in N.

Since the absorption coefficients of both free dye and Ca-dye are very small at 750 nm, N can be eliminated from Eqn. 1 by subtracting the absorbance at 750 nm (including any non-specific absorbance contributions) from the absorbance at 655 nm:

$$A_{655-750} = \epsilon'_{D(655)}(D_t - CaD) + \epsilon'_{CaD(655)} \cdot CaD \quad (2)$$

Rearranged, Eqn. 2 yields:

$$CaD = \frac{A_{655-750} - \epsilon'_{D(655)} \cdot D_t}{\Delta\epsilon'} \quad (3)$$

where $\Delta\epsilon'$ is the differential molar absorption coefficient ($\epsilon'_{CaD(655)} - \epsilon'_{D(655)}$). The necessity of using a calculated value for D_t, as in Eqn. 3, can be eliminated by using a differential absorbance, denoted by $\Delta A_{655-750}$. This can be done by measuring the sample against a similar solution in which none of the dye is in the Ca-dye form, e.g., when very little Ca and an excess of EGTA are present:

$$\Delta A_{655-750} = A_{655-750} - \epsilon'_{D(655)} \cdot D_t$$
$$= \epsilon'_{D(655)}(D_t - CaD) + \epsilon'_{CaD(655)} \cdot CaD - \epsilon'_{D(655)} \cdot D_t$$

which yields:

$$CaD = \frac{\Delta A_{655-750}}{\Delta\epsilon'} \quad (4)$$

Once CaD is determined, the $[Ca^{2+}]$ can be calculated as:

$$[Ca^{2+}] = \frac{K \cdot CaD}{D_t - CaD}$$

This relationship is based on the assumption that Ca and arsenazo III form 1 : 1 complexes with a single dissociation constant, K [15,16]. It has recently been suggested that, at very low Ca : dye ratios, a 1 : 2 complex can form [17]. Since the validity of this stoichiometry is in question, and since the use of a more cumbersome treatment (based on 1 : 2 complexation) would not substantially alter the interpretation of the results presented here, all calculations have been carried out using a 1 : 1 stoichiometry.

Total Ca determinations were performed by adding 1 ml of 0.2% Triton X-100 plus 1% La_2O_3 to each microcentrifuge tube containing the membrane pellet, and incubating the tubes overnight to extract all Ca. The resulting solutions were analyzed for total Ca with an Instrumentation Laboratories Model 251 atomic absorption flame spectrophotometer. Internal Ca standards were added to duplicate samples to correct for quenching.

Total Ca content of the synaptosomal membranes is expressed as nmol Ca/mg protein. Protein content of the membranes was determined by analysis of pellets suspended in H_2O [18], rather than detergent.

Results

Ca^{2+} buffering in the presence of ATP

The synaptosomal membrane pellets contain morphologically identifiable fragments of synaptosomes, plasma membranes, and intracellular organelles, including mitochondria, synaptic vesicles and elements of the smooth endoplasmic reticulum (Schweitzer, E., unpublished observations). When these membranes are incubated in the appropriate medium, they lower the free $[Ca^{2+}]$ in the medium by sequestering Ca. Preliminary experiments indicated that this net sequestration of Ca reaches a maximum in less than 5 min. Longer incubations,

at least at low Ca loads, did not result in any further change in the distribution of Ca. Fig. 1 shows the free Ca^{2+} levels that result from incubating membranes with 1 mM ATP and varying Ca loads. When membranes are omitted from the incubation medium (□), the only buffering that occurs is due to passive binding of Ca to phosphate and ATP [15]. In contrast, when disrupted synaptosomes are added (●), the $[Ca^{2+}]$ is maintained at less than $1 \cdot 10^{-6}$ M in the presence of Ca loads of up to approx. 40 nmol Ca/mg protein. The difference between these two curves represents the total buffering capacity of the synaptosomal membranes.

Two separate components contribute to this buffering. The mitochondrial contribution can be seen by comparing the unpoisoned condition (●) with that which includes mitochondrial poisons (▲). When FCCP and oligomycin are present, there is little difference at the low end of the buffer range, but at higher Ca loads, the Ca is less well buffered, and the $[Ca^{2+}]$ rises rapidly. The addition of A-23187, a Ca^{2+} ionophore that releases Ca from membrane-bound compartments [9], results in an additional increase in the $[Ca^{2+}]$ (■). This increase occurs over the entire range of Ca loads, indicating that even at the lowest loads this non-mitochondrial compartment contains substantial Ca. When all Ca sequestraton is eliminated by the addition of A-23187, the buffer curve is similar to that in the absence of membranes at low Ca loads, demonstrating that the membranes are unable to buffer Ca^{2+} under these conditions. A-23187, added to Ca-dye solutions in the absence of membranes, had no effect on $[Ca^{2+}]$. At high Ca loads, however, the membranes exhibit low-affinity Ca binding, as indicated by the difference between the filled and open squares. Analysis of intact synaptosomes (by flame spectrophotometry) gives a value of 7.7 ± 0.4 nmol Ca/mg protein (mean ± S.D., from three different experiments) for the total Ca content of nerve terminals. Since, in this buffering experiment, the concentration of synaptosomal protein was 1.1 mg/ml, such a Ca content corresponds to approximately 7 μmol Ca/l suspension. This information suggests that the lower end of the buffer curve in Fig. 1 corresponds to conditions inside normal, resting terminals.

Fig. 1 also indicates that at very large Ca loads (greater than 100 μmol Ca/l), Ca^{2+} is buffered less well than at lower Ca levels. This is probably due to the depletion of ATP at higher Ca levels. This suggestion is supported by total (net) Ca analysis of the various membrane components, which reveals how much Ca they sequester under each condition. In this experiment, the Ca content of the membranes, in the absence of poisons, was maximal when the Ca load was about 63 μmol Ca/l, and decreased with larger loads. In contrast, this decline, with large Ca loads, was not observed when ATP levels were maintained by an ATP-regenerating system (see Fig. 4).

To illustrate more clearly the Ca^{2+} buffering at low Ca loads, the data from Fig. 1 have been replotted in Fig. 2. In this double-logarithmic plot, the differences in $[Ca^{2+}]$ in the physiological range are more prominent. Note that the abscissa refers to total Ca/mg synaptosomal protein. In this form, it is clear that, given sufficient time, both the non-mitochondrial and mitochondrial systems are capable of lowering the $[Ca^{2+}]$ to sub-micromolar levels (▲ and ●). Moreover, there is a significant increase in the $[Ca^{2+}]$ with the addition of mitochondrial poisons and A-23187 (■), demonstrating that both sequestration

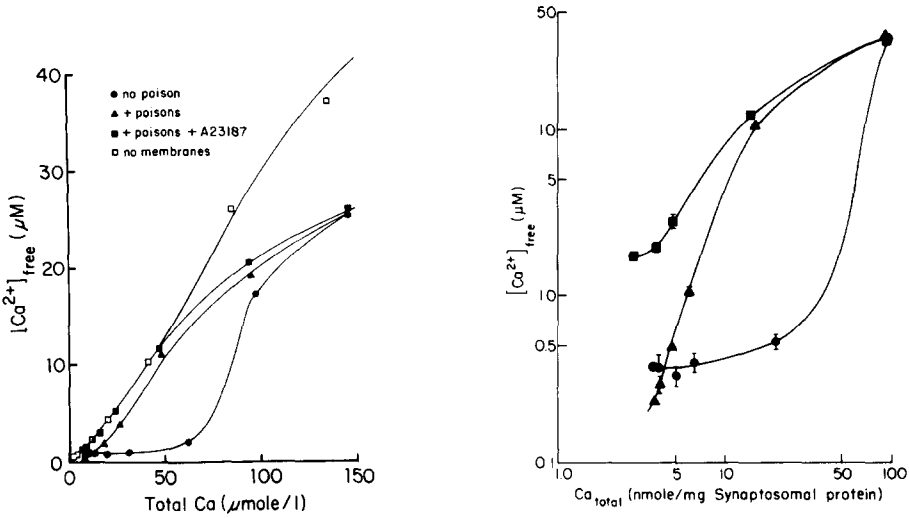

Fig. 1. Ca^{2+} buffering by synaptosome membranes. EGTA-washed synaptosomes were subjected to hypotonic lysis, and the membranes were separated from the soluble fraction by centrifugation at 14 000 × *g* at 5°C for 7 min. The synaptosomal membranes were then suspended in a high $[K^+]$ solution containing 100 mM arsenazo III. Aliquots (0.5 ml) of this suspension were added to an equal volume of substrate solution, and incubated for 5 min at 30°C. Final concentrations during the incubation were 145 mM KCl, 5 mM NaCl, 2 mM KH_2PO_4, 20 mM Hepes, buffered to pH 7.4 with Tris base, 2.4 mM $MgCl_2$, 1 mM ATP, and 50 μM arsenazo III. The concentration of synaptosomal membranes was 1.1 mg protein/ml (calculated on the basis of the amount of protein in the original, intact synaptosomes). Various amounts of Ca were added, as indicated. In addition to these substrates, some tubes contained mitochondrial poisons (10 μM FCCP plus 0.7 μg/ml oligomycin) or mitochondrial poisons plus A-23187 (10 μM), as indicated in the figure. After incubation, the tubes were centrifuged in a Beckman microcentrifuge at room temperature for 3 min to pellet the membranes. The supernatants were removed, and the absorbance of each was measured at 655 and 750 nm. $[Ca^{2+}]_{free}$ was calculated from these absorbance values using $\Delta\epsilon = 2.0 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ and a Ca-dye dissociation constant, $K_{CaD} = 24$ μM. The total Ca in each incubation tube was corrected by subtracting the amount of Ca bound to dye from the total Ca present; the latter was determined by atomic absorbance flame spectrophotometry. The data shown are means of duplicate determinations.

Fig. 2. Ca^{2+} buffering by synaptosome membranes (log-log plot). Synaptosomes were lysed in hypotonic solution and centrifuged at 13 000 × *g* for 7 min at 5°C to obtain synaptosome membranes and organelles (pellets). The pellets were suspended (2.6 mg synaptosomal protein/ml) in K^+-rich solutions (see Fig. 1 legend) containing 50 μM arsenazo III. These solutions contained no poison (●), mitochondrial poisons (10 μM FCCP plus 0.7 μg/ml oligomycin) (▲) or 10 μM A-23187 plus the mitochondrial poisons (■); varying amounts of $CaCl_2$ were also present (see abscissa). The suspensions were incubated for 5 min at 30°C, and then centrifuged for 3 min in a Beckman microcentrifuge. The free Ca^{2+} concentrations ($[Ca^{2+}]$free) in the supernatant solutions were determined from the differential absorbance ($A_{655nm} - A_{750nm}$) values for arsenazo III measured against parallel solutions containing 100 μM EGTA. To calculate $[Ca^{2+}]_{free}$, we used $\epsilon = 2.0 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ and $K_{CaD} = 24$ μM. Total Ca was determined by adding 10 μl of 20% sodium dodecyl sulfate (SDS) to parallel (1 ml) samples, adding La_2O_3 to 1%, and measuring total Ca by atomic absorbance flame spectrophotometry. The Ca bound to arsenazo III was subtracted from these values to give the corrected data for Ca_{total}. Note than the logarithmic plot emphasizes the lower end of the buffer range, as compared with Fig. 1. Error bars indicate range of duplicate determinations.

systems participate in Ca^{2+} buffering at Ca loads of less than 10 nmol Ca/mg protein.

Ca^{2+} buffering in the absence of ATP

Data from ^{45}Ca uptake experiments [8,9] indicate that both the mitochondrial and non-mitochondrial uptake systems require ATP. In the absence of

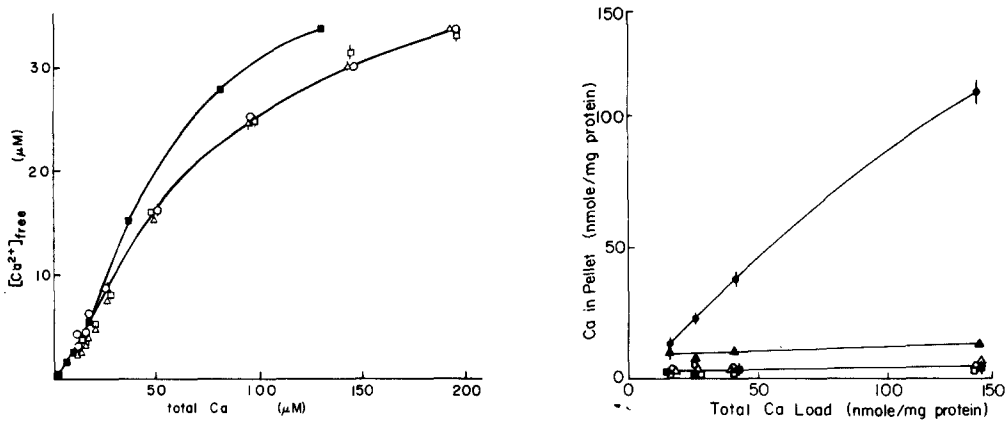

Fig. 3. Ca^{2+} buffering by synaptosomal membrane fractions in the absence of ATP. In this experiment, the experimental conditions were the same as in Fig. 1, except that ATP was omitted, and only 1.4 mM $MgCl_2$ was added. Membrane concentration 1.5 mg protein/ml. Error bars indicate ± S.D. ○, no poisons; △, + poisons (FCCP + oligomycin); □, + poison + A23187; ■, no membranes.

Fig. 4. Net Ca accumulation by synaptosome membrane fractions in the absence and presence of ATP. Synaptosomes were pelleted and resuspended in a hypotonic lysis solution. Buffered KCl was then added to bring the concentration of KCl to 150 mM. This suspension was incubated at 30°C for 5 min, and the membranes were pelleted by centrifugation at 12 000 × *g* for 7 min. The pellets were resuspended in 150 mM K^+ medium, and added to incubation media with or without ATP, poisons, and A-23187. The ATP media (filled symbols) contained 0.5 mM Mg ATP, 4 mM phospho*enol*pyruvate, and 10 units/ml pyruvate kinase; the remaining media (open symbols) contained none of these. In addition, no poison (●, ○), 10 μM FCCP and 0.7 μg/ml oligomycin (▲, △), or FCCP, oligomycin and 10 μM A23187 (■, □) were added. All suspension contained 0.39 mg synaptosomal protein/ml. Bars indicate ± S.D.

ATP, as illustrated in Fig. 3, there is no difference in the buffering abilities of the various membrane compartments in the absence or presence of mitochondrial poisons and A-23187. A comparison of Figs. 1 and 3 confirms that the Ca^{2+} buffering (i.e., net Ca sequestration), like the ^{45}Ca uptake system, requires ATP.

Fig. 4 shows Ca sequestration data from an experiment in which Ca accumulation was measured both in the presence and absence of ATP. As expected, Ca accumulation corresponds very well with the buffering capabilities of the membranes: in the absence of ATP, when no buffering is seen, there is no Ca accumulation. The inclusion of ATP promotes Ca accumulation in both the mitochondrial and non-mitochondrial compartments. With the smallest Ca loads, the mitochondrial Ca accumulation (difference between ▲ and ●) is relatively small compared to the non-mitochondrial accumulation (difference between ▲ and ■). With larger Ca loads, however, the mitochondria accumulate additional Ca, suggesting that they play a role when larger amounts of Ca enter the nerve terminals; this may occur in pathological conditions, such as injury. In this experiment, ATP levels are maintained, even at high Ca loads, by an ATP-regenerating system; therefore, the decrease in sequestration at high Ca loads, seen in Fig. 1, does not occur.

Even in the absence of ATP or the presence of mitochondrial poisons and A-23187, there is some residual Ca associated with the membranes. This residual amount is usually about 3 nmol Ca/mg protein. It is not clear where this Ca is located.

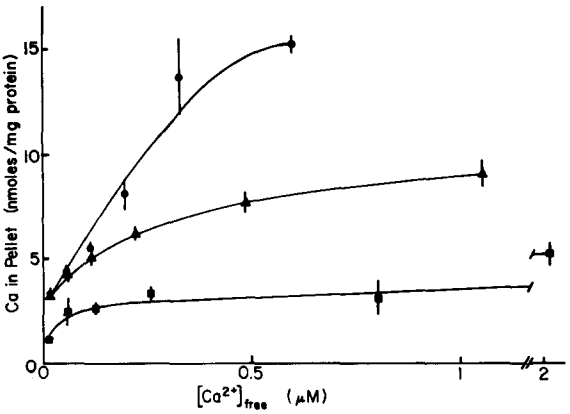

Fig. 5. Net Ca accumulation by synaptosome membrane fractions incubated in media with a low $[Ca^{2+}]$. This experiment shows the increased accumulation of Ca by both the mitochondria and the smooth endoplasmic reticulum when the $[Ca^{2+}]$ is raised. The synaptosomal membranes were incubated as described in Fig. 4 (+ATP condition), except that only 1 unit/ml pyruvate kinase was used, and 150 μM EGTA was added to all incubation media. The free $[Ca^{2+}]$ was determined from the differential absorbance of arsenazo III. Synaptosome concentration was 0.77 mg protein/ml. Error bars indicate ± S.D. •, no poison; ▲, + poisons (FCCP + oligomycin); ■, + poisons + A-23187.

Ca accumulation at low $[Ca^{2+}]$

One important question concerning the non-mitochondrial compartment is whether it can perform a net accumulation of Ca. In all the preceding experiments, the non-mitochondrial compartment was saturated at the lowest $[Ca^{2+}]$. The amounts of Ca introduced as contaminations, both in the preparation procedure and in the incubation solutions, are probably sufficient to fill this sequestration site. For this (non-mitochondrial) compartment to play a role in physiological Ca^{2+} buffering, there must be a range of $[Ca^{2+}]$ over which this compartment takes up Ca as $[Ca^{2+}]$ is increased. Fig. 5 shows the results of an experiment in which EGTA was added to bind some of the Ca in the suspensions, thereby reducing the $[Ca^{2+}]$ present during the incubation. Under these circumstances it is possible to examine the behavior of disrupted synaptosomes at a $[Ca^{2+}]$ below 1 μM, a range that probably corresponds to the normal levels of intracellular Ca^{2+}. As the $[Ca^{2+}]$ is increased from 0.01 to 1 μM, the amount of Ca in the non-mitochondrial compartment (difference between ▲ and ■ in Fig. 5) does, indeed, increase. Once again, the contribution of mitochondria to Ca buffering is small at low $[Ca^{2+}]$, but increases as $[Ca^{2+}]$ is raised. It seems, therefore, that both the non-mitochondrial and mitochondrial compartments can contribute to Ca^{2+} buffering in the nerve terminal at appropriate $[Ca^{2+}]$.

Discussion

The experiments described here provide evidence that there are two major membrane-bounded compartments in nerve terminals that are active in buffering internal Ca^{2+} levels. This control is accomplished by a net accumulation of Ca within the compartments. One of the buffering systems is associated with mitochondria (see also Ref. 2). Sequestration of Ca by this compartment can be blocked by the addition of FCCP and oligomycin. The present study indi-

cates that the mitochondria have a relatively large capacity for Ca, but that they accumulate relatively little Ca at $[Ca^{2+}]$ that may approximate that of normal cytoplasm (below $1 \cdot 10^{-6}$ M, Refs. 19 and 20). In the presence of ATP and large Ca loads, however, the mitochondria can accumulate considerable amounts of Ca.

The non-mitochondrial compartment, which has been identified as smooth endoplasmic reticulum [10], has a relatively smaller capacity for Ca accumulation, but a higher apparent affinity. Thus, at Ca loads that are most likely to be in the physiological range, much of the sequestered Ca is contained in the smooth endoplasmic reticulum compartment. These findings complement the relative affinities of the compartments as determined by the kinetics of ^{45}Ca uptake [9]. In addition, the data presented here, obtained with arsenazo III, demonstrate that synaptosomal membranes, including elements of the smooth endoplasmic reticulum and mitochondria, are capable of accumulating Ca from media containing sub-micromolar $[Ca^{2+}]$. Therefore, they should actively contribute to the control of intracellular Ca^{2+}.

The fact that the intra-terminal organelles can buffer small (physiological) Ca loads to a $[Ca^{2+}]$ of about 0.3—0.5 μM (cf. Figs. 1 and 2), may indicate that this is the upper limit for $[Ca^{2+}]_i$ in normal, resting terminals. Cell Ca and $[Ca^{2+}]_i$ in the steady state (i.e., at rest) are normally controlled by Ca transport mechanisms in the plasma membrane. Under these circumstances, the intra-terminal Ca-sequestering organelles are in equilibrium with $[Ca^{2+}]_i$, but do not actually control it. However, during periods of activity, when (net) Ca enters and $[Ca^{2+}]_i$ rises as a consequence of increased plasma membrane Ca conductance (cf. Ref. 21), the intra-terminal Ca-buffering mechanisms rapidly come into play: they tend to drive $[Ca^{2+}]_i$ back toward (but not quite to) the normal resting level. The Ca-sequestering organelles then maintain the $[Ca^{2+}]_i$ at a level slightly above the resting level until all of the Ca load has been extruded by the plasma membrane Ca transport system(s). Although all intra-terminal buffering mechanisms may be expected to contribute to this transient Ca buffering, data from our laboratory (Ref. 9 and the present report) suggest that the non-mitochondrial Ca sequestration mechanism plays the most important physiological role.

By combining the information on the distribution of Ca as a function of $[Ca^{2+}]$, as in Fig. 5, with measurements of the total Ca content of intact synaptosomes, it is possible to make some estimates regarding the disposition of Ca in intact nerve terminals. Approx. 20% of the total Ca is lost when synaptosomes are lysed. Assuming 7.7 nmol Ca/mg protein in intact synaptosomes, we would expect about 6.2 nmol/mg to be associated with the membrane-bounded compartments. This corresponds (see Fig. 5) to a $[Ca^{2+}]$ of about 0.1 μM. Moreover, Fig. 5 indicates that, at this level, 3.0 nmol of the total Ca are contained in the smooth endoplasmic reticulum, and 1.2 nmol are in the mitochondria. It thus appears that the smooth endoplasmic reticulum plays a substantial, and perhaps predominant role in buffering increases in intracellular $[Ca^{2+}]$ under normal physiological conditions.

These and earlier [8,9] results are consistent with the suggestion [9] that nerve terminal smooth endoplasmic reticulum has functional similarities to skeletal muscle sarcoplasmic reticulum, in terms of its ability to sequester Ca.

For example, Inesi and Scarpa [22] have employed the Ca-sensitivity dye, murexide, to show that the sarcoplasmic reticulum can lower $[Ca^{2+}]$ in vitro. In addition to the clear implications for the role of the smooth endoplasmic reticulum in terminating transmitter release, there remains the intriguing possibility that the nerve terminal smooth endoplasmic reticulum releases Ca upon stimulation (cf. Ref. 23), as does the sarcoplasmic reticulum in muscle [24].

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