

Calcium Buffering in Presynaptic Nerve Terminals

I. Evidence for Involvement of a Nonmitochondrial ATP-Dependent Sequestration Mechanism

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ABSTRACT A latent ATP-dependent Ca storage system is enriched in preparations of pinched-off presynaptic nerve terminals (synaptosomes), and is exposed when the terminals are disrupted by osmotic shock or saponin treatment. The data indicate that a fraction of the Ca uptake (measured with ^{45}Ca) is associated with the intraterminal mitochondria; it is blocked by ruthenium red, by FCCP, and by azide + dinitrophenol + oligomycin. There is, however, a residual ATP-dependent Ca uptake that is insensitive to the aforementioned poisons; this (nonmitochondrial) Ca uptake is blocked by tetracaine, mersalyl and A-23187. Moreover, A-23187 rapidly releases previously accumulated Ca from these (nonmitochondrial) storage sites, whereas the Ca chelator, EGTA, does not. The proteolytic enzyme, trypsin, spares the mitochondria but inactivates the nonmitochondrial Ca uptake mechanism. Chemical measurements of total Ca indicate that the ATP-dependent Ca uptake at the nonmitochondrial sites involves the net transfer of Ca from medium to tissue fragments. This system can sequester Ca when the ambient-ionized Ca^{2+} concentration (buffered with EGTA) is $<0.3 \mu\text{M}$; brain mitochondria take up little Ca when the ionized Ca^{2+} level is this low. Preliminary subfractionation studies indicate that the nonmitochondrial Ca storage system does not sediment with synaptic vesicles. We propose that this Ca storage system, which has many properties comparable to those of skeletal muscle sarcoplasmic reticulum, may be associated with intraterminal smooth endoplasmic reticulum. This Ca-sequestering organelle may help to buffer intracellular Ca.

INTRODUCTION

Calcium ions play a critical regulatory role in several fundamental physiological processes such as secretion and muscular contraction. In vertebrate skeletal

muscle, which has been particularly well studied, the concentration of ionized Ca^{2+} in the cytoplasm (sarcoplasm) of relaxed muscle is below $\sim 6-9 \times 10^{-7}$ M, the threshold for contraction (19); much of the muscle Ca is stored in the sarcoplasmic reticulum, and contraction is triggered when some of the stored Ca is released into the sarcoplasmic space after depolarization of the muscle plasma membrane (12, 19). Relaxation is then effected primarily by reaccumulation of the released Ca by the sarcoplasmic reticulum (25, 26). It is therefore clear that in this tissue the sarcoplasmic reticulum, a specialized form of smooth endoplasmic reticulum, plays a crucial role in intracellular Ca buffering.

Much less is known about the level of ionized Ca^{2+} in the cytoplasm of most other types of cells. The squid giant axon is an exception: several lines of evidence indicate that free Ca^{2+} is normally on the order of 10^{-7} M in the axoplasm (1, 2, 16). However, indirect evidence strongly suggests that the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in many other cells may also be on the order of 10^{-6} M or less. Perhaps most important is the observation that rat liver mitochondria utilize the energy from electron transport to accumulate Ca, rather than to phosphorylate ADP, when the ambient Ca concentration is in excess of about 10^{-6} M (17, 60). Furthermore, there is increasing evidence that the microvesicles ("microsomes") obtained from homogenates of a variety of cell types (11, 50, 51), including secretory cells (e.g., 32, 57, 61), can accumulate Ca at the expense of ATP hydrolysis. In general, these storage mechanisms, which may be associated with the smooth endoplasmic reticulum (e.g., 24), have a higher apparent affinity for Ca^{2+} (half-saturation constants on the order of 10^{-5} M Ca^{2+} or less) than do mitochondria (half-saturation constants on the order of $0.5-1 \times 10^{-4}$ M in media which may approximate the cytosol ionic composition; 33, 66). The implication is that these microsomal ATP-dependent Ca storage mechanisms play an important role in intracellular Ca^{2+} buffering and may help to maintain $[\text{Ca}^{2+}]_i$ in the range of 10^{-6} M or below, so that the mitochondria can phosphorylate ADP.

Microsomes from peripheral nerve (40) and from brain (e.g., 13, 54, 55, 63) have also been found to accumulate Ca by an ATP-dependent mechanism which resembles the Ca storage system of sarcoplasmic reticulum. Unfortunately, it is not possible to determine the origin (glial vs. neuronal) of microsomes prepared from whole brain homogenates. However, a preliminary report from our laboratory (35) has provided evidence that ATP promotes Ca sequestration in organelles other than mitochondria located within presynaptic nerve terminals; it was suggested that this storage system may help to buffer intraterminal Ca^{2+} . In view of the critical role which intracellular free Ca^{2+} plays in neurotransmitter release (e.g., 41, 48), a more detailed study of this Ca transport system was undertaken. The present report provides additional evidence that ATP promotes Ca sequestration in nonmitochondrial, vesicular intraterminal organelles; differential centrifugation studies indicate that these organelles are not identical with the transmitter-storing synaptic vesicles which are involved in transmitter release (15, 67). Some of the kinetic properties of the nonmitochondrial ATP-dependent transport system, and the possible role of this transport system in intraterminal $[\text{Ca}^{2+}]_i$ regulation, are discussed in the following article (7).

METHODS

Preparation of Brain Mitochondria and Presynaptic Nerve Terminals ("Synaptosomes")

Crude mitochondrial fractions ("P₂" of Gray and Whittaker [22]) were prepared from rat cerebral cortex homogenates by a slight modification (4) of the original Gray and Whittaker procedure. These tissue pellets ("P₂") were resuspended in isotonic salt solutions (cf. Table I) and constituted the preparations referred to below, as "brain mitochondria"; these preparations also contain many pinched-off, resealed ("intact") nerve terminals.

To obtain nerve terminal ("synaptosome") enriched preparations, the P₂ fractions were resuspended in 0.32 M sucrose and subjected to further fractionation on a one-step discontinuous sucrose gradient (23). (In one type of experiment, however, a two-step gradient [22] was used; see Table II for details.) The synaptosomes were recovered in the 0.8 M sucrose fraction of the gradient; they were returned to a more physiological, normotonic environment by adding small volumes of ice-cold Ca-free NaCl medium (Table I) to the sucrose suspensions over a 20–30-min period, to give a final volume three to four times the initial (0.8 M sucrose) volume. The synaptosomes in these suspensions will be referred to as "equilibrated" (with physiological saline); a large fraction of the terminals presumably have "intact" (resealed) surface membranes, as indicated by the retention of considerable functional integrity (5 and Results). All of the experiments reported below were made on freshly prepared tissue preparations because preliminary experiments indicated that a considerable fraction of the ATP-dependent Ca uptake activity was lost after overnight storage at 3 or –15° C.

⁴⁵Ca Uptake Studies

Small aliquots (containing 0.5–1.2 mg protein) of the equilibrated synaptosome suspensions were centrifuged at 15,000 *g* (max), for 6 min at 5° C. The supernatant solutions were decanted, and the synaptosome pellets were resuspended in a buffered solution; in most instances this was the hypotonic "lysis solution" (Table I) used to osmotically disrupt the terminals (but see Results). After a 1-min incubation at 30° C, additional aliquots of various solutions were added to the suspensions; these solutions contained Ca (labeled with ~50–100 μ Ci ⁴⁵Ca per micromole Ca; New England Nuclear, Boston, Mass.) and ATP and other solutes as dictated by the specific experiments (see Results for details). Incubation of the suspensions usually at 30° C was continued and, at various time intervals, small samples containing about 0.25 mg protein were pipetted onto 25-mm diameter, 0.3- μ m pore diameter Millipore filters (Millipore Corp., Bedford, Mass.), and filtered by suction. The filters were immediately rinsed with three 5-ml aliquots of wash solution (Table I).

Assay Methods

The ⁴⁵Ca content of the incubation solutions and the filters was determined by liquid scintillation spectroscopy, using a Triton X-100-toluene based scintillation fluid (52). Samples of incubation fluid without protein were also filtered, and the filters were washed and counted to correct for nonspecific binding of Ca. Generally, with fluids containing ATP, or CaEGTA buffers, these corrections accounted for <5% of the counts on the protein-containing filters.

In each experiment, several representative synaptosome pellets, obtained from the "equilibrated" suspensions, were assayed for protein content by the Lowry method (42);

bovine serum albumin was employed as a standard. The data could then be expressed in terms of "moles of Ca uptake per milligram protein per unit time."

Solutions and Special Reagents

The composition of representative solutions used in these experiments is given in Table I; further details will be presented in the Results section. Special reagents were added to some of the solutions in all experiments: ATP (Sigma Chemical Co., St. Louis, Mo.), Na azide (NaN_3), 2,4-dinitrophenol (DNP), oligomycin (Sigma), ruthenium red (Sigma), carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP; E. I. DuPont de Nemours, Wilmington, Del.), saponin (Sigma), A-23187 (the calcium ionophore; Eli Lilly, Indianapolis, Ind.), and trypsin (Sigma type I) and soybean trypsin inhibitor (Sigma type I-S). Details regarding the use of these agents will be given below.

EGTA (ethyleneglycol bis- β -aminoethyl ether *N,N'*-tetraacetic acid) was used to buffer the Ca in the medium in a number of experiments. Calculations of the nominal free Ca^{2+}

TABLE I
COMPOSITION OF REPRESENTATIVE SOLUTIONS

Solution	NaCl	KCl	CaCl ₂	MgCl ₂	KH ₂ PO ₄	HEPES†
	<i>mmol/liter</i>					
Lysis*	0	0	0	1.4	2.0	20
KCl medium*‡	5	145	Variable§	1.4	2.0	20
NaCl medium*‡	145	5	Variable§	1.4	2.0	20
Wash	5	145	1.2	1.4	2.0	20

* In many instances, other components such as mitochondrial poisons or ATP were added to these solutions; see Results for details.

‡ Indicates final concentration of incubation media; for example, 1 ml lysis solution was diluted with 1 ml of solution containing 10 mM NaCl + 290 mM KCl, to give a final concentration of 5 mM NaCl + 145 mM KCl.

§ See Results.

|| ATP was added as Mg ATP, so that the final Mg concentration was always 1.4 mM greater than the ATP concentration.

¶ All solutions were buffered to pH 7.5 at 22°C with Tris base.

concentrations, which are given in the text and figure legends, were based upon a CaEGTA apparent equilibrium constant of $7.6 \times 10^6 \text{ M}^{-1}$ (58).

Measurements of Net Ca Accumulation

In a few experiments, atomic absorption methods were used to determine the net amount of Ca accumulated by the suspended tissue particles. The incubation procedures were similar to those employed for the radioactive tracer studies, except that ^{45}Ca was omitted. At the end of the incubations, the suspensions were centrifuged at 15,000 *g* for 6 min at 5° C. The supernatant solutions were decanted and saved for Ca determination; the pellets were rinsed twice with 2-ml aliquots of ice-cold 0.32 M sucrose, and then resuspended in 0.2% Triton X-100 (Rohm & Haas Co., Philadelphia) in water, for Ca and protein analysis.

Subfractionation of the Synaptosome Lysates

Several experiments were made in an effort to separate the particles containing the ATP-dependent Ca uptake activity. Equilibrated synaptosomes from five rat brains (40–50 mg protein) were pelleted by centrifugation at 15,000 *g* (max) for 10 min at 5° C. The supernatant solution was decanted and discarded. The pellets were resuspended (~2 mg

protein/ml) in ice-cold lysis solution (Table I), and further dispersed by gentle homogenization in a loose-fitting glass (Tenbroeck) homogenizer (Kontes Co., Vineland, N.J.). These "whole lysates" were centrifuged at 15,000 *g* (max) for 20 min at 5° C (cf. 14); the supernatant solutions ("S₃") were decanted and saved for recentrifugation (see below). The pellets ("P₃") were resuspended in 0.32 M sucrose (~2-3 mg protein/ml), and the suspensions were carefully layered on top of a three-step sucrose gradient (modified from Whittaker et al. [67]), consisting of 7.5 ml each, of (top to bottom) 0.6, 1.0, and 1.2 M sucrose. The gradients were centrifuged at 110,000 *g* (max) for 105 min at 5° C, and the following fractions were recovered: (a) a "light band" at the 0.6-1.0 M sucrose interface; (b) a "heavy band" at the 1.0-1.2 M sucrose interface; (c) the pellet (which was resuspended in 1.0 M sucrose and diluted with H₂O to give 0.32 M sucrose); and (d) all the residual sucrose from the gradient (required in order to calculate recoveries: see Results). The material at the interfaces was removed by aspiration with a Pasteur pipette.

The S₃ supernatant solutions (from the 20-min spin) were centrifuged at 150,000 *g* (max) for 1 h at 5° C. The pellets ("P₄") obtained from this spin ("synaptic vesicles"; cf. 14, 15) were resuspended in 0.32 M sucrose.

The various fractions which were obtained from these centrifugations (summarized in the flow chart of Fig. 1) were assayed for ATP-dependent Ca uptake activity in the absence and presence of mitochondrial poisons, and for protein content, as described above.

RESULTS

Exposure of Latent ATP-Dependent Ca Uptake by Osmotic Shock

Previous studies have demonstrated that equilibrated, "intact" synaptosomes can take up Ca from physiological (~300 mosM) salt solutions (5), although the rate of uptake is, in part, a function of the Ca²⁺ concentration in the medium. In the experiment of Fig. 2, the nominal free Ca²⁺ concentration was only 0.55 μM, as compared to the more usual value for normal extracellular fluids, of ~1 mM; this accounts for the very low rate of Ca uptake by the "intact" terminals

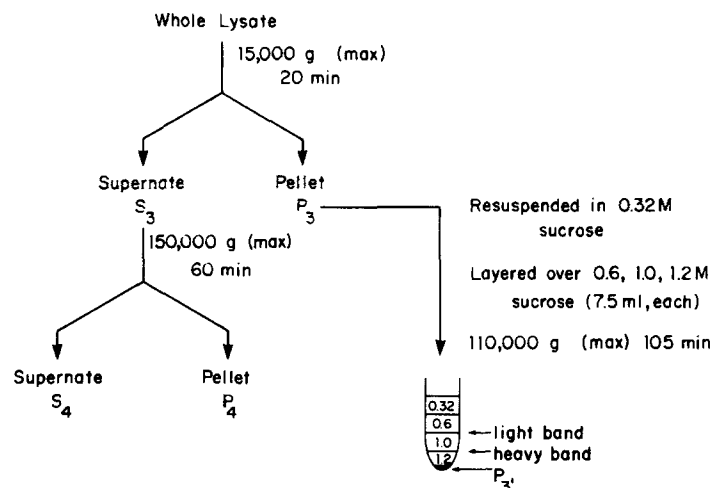


FIGURE 1. Flow chart for preparation of synaptosome lysate subfractions. See Methods for details.

(Δ , \blacktriangle). Moreover, the difference between the rate of uptake from Na-rich (Fig. 2 A) and from K-rich (Fig. 2 B) media is consistent with earlier observations (3) that the rate of Ca uptake is enhanced in "depolarizing" media (such as those with an elevated K concentration). The fact that ATP has relatively little effect on the rate of Ca uptake by these "intact" synaptosomes is not surprising: as noted in the Introduction, there is normally a large Ca electrochemical gradient

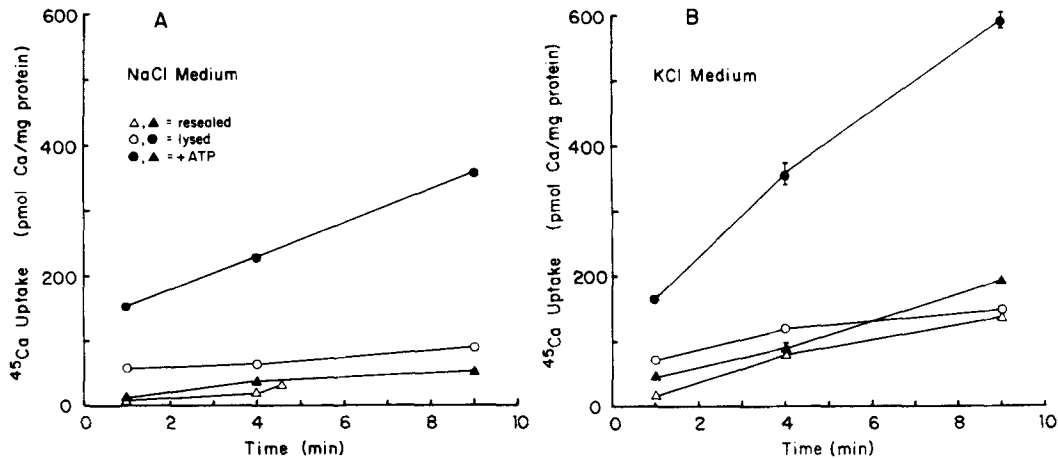


FIGURE 2. Time-course of ⁴⁵Ca uptake in disrupted and intact synaptosomes. Incubations were carried out in NaCl medium (A), or KCl medium (B). Pellets of equilibrated synaptosomes (see Methods) were suspended in Ca-free KCl medium (Table I) or in similar media where all the KCl was replaced by NaCl; after 1 min (time zero on graph) ⁴⁵Ca-labeled, EGTA-buffered Ca containing solutions (1.0 ml) were added to start the uptake. The data for these resealed ("intact") synaptosomes are indicated by triangles. Disrupted synaptosomes (circles) were prepared by suspending the equilibrated synaptosomes in 1.0 ml of a lysis solution (Table I). After 1 min (zero time on the graph) solutions containing EGTA-buffered Ca, labeled with ⁴⁵Ca, were added to start the uptake. These solutions also contained sufficient NaCl and KCl to provide final concentrations of 145 mM Na + 5 mM K (A) or 145 mM K + 5 mM Na (B); ATP (when present) was 1.0 mM. The free ionized Ca²⁺ concentration was buffered to 0.55 μ M using 70 μ M EGTA and 50 μ M Ca; the protein concentration was 0.66 mg/ml. All solutions contained 0.1 mM NaN₃, 0.1 mM DNP, and 0.7 μ g/ml oligomycin. (\bullet , \blacktriangle) Incubations in the presence of ATP; (\circ , Δ) incubations under ATP-free conditions. Each symbol represents the mean of three determinations; error bars are shown where the standard errors extend beyond the borders of the symbols.

which favors the inward movement of Ca across the plasmalemma, so that metabolically dependent transport processes are not needed to drive Ca into the terminals. Moreover, we would not expect ATP to act at the external surface of the plasmalemma.

Fig. 2 also shows that synaptosomes which are disrupted by osmotic shock (44, 67) take up Ca at a very low rate in the absence of added ATP. If, however, the media (whether Na-rich or K-rich) are supplemented with ATP, a marked stimulation of Ca uptake results. The most straightforward explanation is that

the Ca and (or) ATP in the bathing solutions do not have direct access to the Ca-accumulating organelles in the "intact" synaptosomes; disruption of the plasmalemma exposes these organelles, which then proceed to take up Ca by an ATP-dependent mechanism. Although the mitochondria are obvious candidates for this role, the fact that three mitochondrial poisons (NaN_3 , DNP, and oligomycin) were included in all the incubation media appears to eliminate this possibility (35, and below). We therefore conclude that this ATP-dependent Ca uptake, illustrated by the data in Fig. 2, is associated with other intracellular organelles.

The ATP-dependent Ca uptake by these disrupted terminals (Fig. 2B) amounted to ~ 450 pmol per mg protein after 9 min of incubation or about half the uptake observed with a 20-fold higher free Ca^{2+} concentration (Fig. 1 in Ref. 35). This may indicate that the half-saturation for this Ca uptake mechanism is on the order of $0.5 \mu\text{M}$ Ca (Table IV below, and Ref. 7). The fact that this uptake occurs from media with submicromolar concentrations of free Ca^{2+} , which may be appropriate for the intracellular environment (see Introduction), raises the possibility that this Ca uptake mechanism may play a role in intracellular Ca buffering. A detailed study of some of the properties of this Ca uptake system was therefore undertaken.

Origin of the Nonmitochondrial Ca-Storing Organelles

That this latent nonmitochondrial Ca uptake activity is manifest when synaptosome preparations are disrupted, may indicate that the responsible organelles are (presynaptic) intraterminal structures. However, an alternative possibility is that they may be derived from "resealed" glial cell fragments which, according to some workers (cf. 30), may contaminate synaptosome preparations. Although we are, as yet, unable to settle this point definitively, indirect evidence indicates that at least some, and perhaps most of the Ca accumulating organelles may be intraterminal structures. One line of evidence concerns a comparison between the distribution, on a two-step sucrose gradient, of nerve terminals and of latent ATP-dependent Ca uptake activity. Data from an experiment of this kind are shown in Table II; the results clearly indicate that the nerve terminals and the Ca uptake activity exposed by osmotic shock are comparably enriched in the "synaptosome" fraction (0.8–1.2 M sucrose interface) of the sucrose gradient. These data may be correlated with the observation that physiological activity (depolarization-induced Ca entry) specifically associated with presynaptic terminals is similarly enriched in the synaptosome fraction (3); the inference is that much of the latent Ca uptake activity is attributable to intraterminal structures, because it would be fortuitous if resealed vesicles derived from glial cells distributed in exactly the same way as nerve terminals, in the sucrose gradient. Unfortunately, there is no specific marker with which to assess glial contamination in synaptosome preparations.

In this context, it is also important to consider the origin of the nonmitochondrial ATP-dependent Ca uptake observed in "intact" synaptosome preparations (Fig. 2 and Tables II and III). This uptake, although somewhat variable from experiment to experiment, on the average accounted for ~ 20 – 30% of the total nonmitochondrial ATP-dependent uptake observed in "lysed" preparations.

One possibility is that the fraction of the uptake observed in "intact" preparations represents contamination by Ca-accumulating organelles from non-nerve terminal sources. However, some or all of this uptake, too, may be associated

TABLE II
DISTRIBUTION OF NONMITOCHONDRIAL ATP-DEPENDENT Ca UPTAKE
ACTIVITY IN SYNAPTOSOMAL AND OTHER FRACTIONS OF A TWO-STEP
SUCROSE GRADIENT

Tissue fraction*	Conditions†		Ca uptake‡			Relative distribution of nerve terminals¶
	"Intact" or "Lysed"	1 mM ATP	Total uptake	ATP-dependent uptake	Increment due to lysis	
			<i>pmol/mg protein × 3 min</i>			
P ₂ ("crude mitochondrial fraction")	Intact	0	137±12			
	Intact	+	276±13	139±18		1.0
	Lysed	0	536±43			
	Lysed	+	966±37	431±57**	292	1.0
0.32-0.8 M sucrose interface ("myelin")	Intact	0	37±2			
	Intact	+	62±1	25±2		0.18
	Lysed	0	85±2			
	Lysed	+	141±5	56±5**	31	0.11
0.8-1.2 M sucrose interface ("synaptosomes")	Intact	0	301±63			
	Intact	+	351±5	50±63		0.36
	Lysed	0	529±36			
	Lysed	+	1197±16	668±39**	618	1.55
Pellet below 1.2 M sucrose ("mitochondria")	Intact	0	23±1			
	Intact	+	175±7	152±7		1.09
	Lysed	0	27±2			
	Lysed	+	259±3	231±4**	79	0.54
					0.27	0.57

* A Gray-Whittaker (22) two-step sucrose gradient was used to subfractionate "P₂". Samples of P₂ and of each of the gradient layers were equilibrated with Ca-free NaCl medium. Subsequent steps were similar to those normally used for the "synaptosome" fraction.

† The incubation conditions were similar to those used for the experiment of Fig. 2 B, except that the solutions contained 10 μM CaCl₂, and no EGTA. All incubation solutions contained DNP, NaN₃, and oligomycin.

‡ The values shown are the means ± SE of three determinations.

§ The relative specific activity (RSA) of the Ca uptake in each fraction is the ATP-dependent or incremental uptake by the fraction, divided by the respective P₂ value.

¶ The relative distribution of nerve terminals was calculated from the data of Michaelson and Whittaker (1963. *Biochem. Pharmacol.* 12: 203); see Ref. 3 for details.

** Two-way analysis of variance indicates that the ATP-dependent Ca uptake in each fraction is significantly increased by osmotic shock ($P < 0.001$).

with intraterminal organelles, because morphological experiments with extracellular markers¹ indicate that ~20-30% of the nerve terminals in our synapto-

¹ Fried, R. C. and M. P. Blaustein. 1978. Retrieval and recycling of synaptic vesicle membrane in pinched-off nerve terminals (synaptosomes). *J. Cell Biol.* In press.

some preparations are damaged (i.e., the markers penetrate the cytoplasmic space in ~20–30% of the terminals). In view of the harsh procedures used to isolate the terminals, it does not seem surprising that a small, but significant, fraction of the terminals in the synaptosome preparations may be damaged. The finding that the relative specific activity of the total nonmitochondrial ATP-dependent Ca uptake of the lysed preparations most closely parallels the distribution of nerve terminals in the sucrose gradient (Table II) seems compatible with the hypothesis that most of this Ca uptake (including that fraction observed in “intact” preparations) may be associated with intraterminal organelles.

Exposure of Latent ATP-Dependent Ca Uptake by Saponin Treatment

It may, at first, seem somewhat disconcerting that a procedure as drastic as osmotic shock was used to disrupt the nerve terminals; the integrity of the intraterminal organelles which are thus exposed to the external media must, justifiably, be questioned. However, the procedures employed here are reminiscent of those used in a classical study of Ca transport in fragmented sarcoplasmic reticulum (18). In that case, functional integrity of the sarcoplasmic reticulum was maintained despite the fact that the tissue fractionation was carried out in markedly hypotonic media, whereas incubations with ^{45}Ca were performed in media containing 100 mM KCl. Furthermore, the fact that mitochondria appear to withstand the osmotic shock (see below), leads us to conclude that at least some intraterminal organelles survive this treatment.

Endo and his colleagues (20) have recently shown that saponin can be used to “chemically skin” muscle fibers: it makes the plasma membrane permeable to small ions and molecules, while leaving the sarcoplasmic reticulum functionally intact. Moreover, Martonosi (46) has reported that Ca uptake by isolated skeletal muscle sarcoplasmic reticulum vesicles is relatively unaffected by 5–6 h of exposure to solutions containing 1–2% saponin. This selective action of saponin presumably results from the fact that it interacts with cholesterol and may, therefore, be expected to affect primarily the properties of cholesterol-rich membranes, such as plasma membranes (36, 62); cholesterol-poor membranes, such as those of the endoplasmic reticulum and mitochondria (36) should be much less affected. Thus, as an alternative to the osmotic shock procedure, mentioned above, we also incubated “intact” synaptosomes in saponin-containing normotonic solutions to expose the intraterminal organelles directly to the bathing medium. Table III shows data from a representative experiment in which the effects of osmotic shock and saponin were compared. Saponin and osmotic shock both increased the poison-sensitive as well as the poison-insensitive ATP-dependent Ca uptake, relative to the values obtained when the terminals were maintained in normotonic solutions without saponin. Osmotic shock appeared to be somewhat more effective than saponin in exposing the poison-insensitive ATP-dependent uptake; these data are consistent with the observation that the saponin treatment released only about half as much occluded potassium from the synaptosomes,² as did osmotic shock. As might be

² Schweitzer, E. S. Unpublished observation.

TABLE III
EFFECT OF SAPONIN ON ATP-DEPENDENT ⁴⁵Ca UPTAKE BY "INTACT"
AND BY "DISRUPTED" SYNAPTOSOMES

Solutions (see Table I)	Preincubation*		⁴⁵ Ca Incubation‡		⁴⁵ Ca Uptake		
	Saponin μg/ml	Duration at 4°C min	Poisons present	ATP pres- ent	Total uptake§	Poison-insensitive ATP-dependent up- take	Mitochon- drial up- take¶
"Intact" synap- tosomes							
KCl Medium	0	1	0	0	244±57		
KCl Medium	0	1	0	+	5,297±28		4,967
KCl Medium	0	1	+	0	139±11		
KCl Medium	0	1	+	+	225±9	86±14	
KCl Medium	0	30	0	0	146±0		
KCl Medium	0	30	0	+	5,752±218		5,503
KCl Medium	0	30	+	0	106±10		
KCl Medium	0	30	+	+	209±10	103±14	
"Disrupted" synapto- somes							
Lysis Solution	0	30	0	0	192±6		
Lysis Solution	0	30	0	+	9,338±896		8,861
Lysis Solution	0	30	+	0	94±8		
Lysis Solution	0	30	+	+	379±6	285±10***	
KCl Medium	250	30	0	0	542±76		
KCl Medium	250	30	0	+	14,391±791		13,608
KCl Medium	250	30	+	0	67±2		
KCl Medium	250	30	+	+	268±19	201±19**	
Lysis Solution	250	30	0	0	210±40		
Lysis Solution	250	30	0	+	12,723±216		12,214
Lysis Solution	250	30	+	0	92±6		
Lysis Solution	250	30	+	+	391±37	299±37***	

* Equilibrated synaptosomes were resuspended in the Ca-free solutions containing the indicated concentrations of saponin.

‡ After the preincubation at 4°C for the period indicated in column 3, 0.1-ml aliquots of the suspensions were transferred to tubes containing 0.9 ml of media to give the final composition of KCl Medium (Table I), with 18 μM CaCl₂, labeled with ⁴⁵Ca. Some of these solutions also contained 0.1 mM NaN₃ + 0.1 mM DNP + 0.7 μg/ml oligomycin and (or) 1 mM ATP, as indicated by the "+" in columns 4 and 5, respectively. The incubations with ⁴⁵Ca were for 3 min at 30°C; the protein concentration in the incubated suspensions ranged from 0.23 to 0.29 mg/ml.

§ Each value is the mean of two determinations; the spread of the values is also indicated.

|| The difference values are shown with the standard errors. Two-way analysis of variance indicates that the poison-independent Ca uptake by the "disrupted" synaptosomes is significantly greater than that of the "intact" controls preincubated for 30 min ($P < 0.05 = **$; $P < 0.01 = ***$).

¶ ATP-dependent Ca uptake in the absence of poisons minus mitochondrial poison-insensitive ATP-dependent Ca uptake.

expected, the effects of saponin are very much dependent upon the saponin concentration and duration of incubation; the 250- $\mu\text{g}/\text{ml}$ concentration and 30-min incubation were found to be optimal. Note that when saponin was added after osmotic shock (Table III), it did not inhibit the poison-insensitive ATP-dependent Ca uptake. Digitonin, at a concentration of 500 $\mu\text{g}/\text{ml}$, had effects comparable to those of saponin (data not shown); this may be understandable in view of Martonosi's observation (46) that low concentrations of digitonin (≤ 1 mg/ml), like saponin, do not inhibit Ca uptake by sarcoplasmic reticulum. These results indicate that saponin (or digitonin) treatment and osmotic shock may be convenient alternative methods for exposing intraterminal organelles; the osmotic shock method was employed in all of the experiments described below.

Effect of Monovalent Cations on the ATP-Dependent Ca Uptake

Comparison of the data in Fig. 2 A and B indicates that the ATP-dependent Ca accumulation (i.e., uptake in the presence of ATP minus uptake from media without added ATP) by the disrupted terminals is considerably greater when K (Fig. 2 B), rather than Na (Fig. 2 A), is the predominant cation in the medium. Data from another experiment (Fig. 3) shows that Na and Li both reduced the rate of ATP-dependent Ca uptake, relative to the rate of uptake from the K-rich solution. Note that the ATP-dependent uptake from media in which hypotonicity was maintained during the incubation period with ^{45}Ca did not differ significantly from the uptake from normotonic, K-rich solution; this implies that Na and Li were somewhat inhibitory, an effect reminiscent of the inhibition, by Na, of Ca uptake in brain microsomes observed by Otsuka et al. (54, and see 13). The monovalent cation concentration dependence is illustrated in Fig. 4. The main point is that the ATP-dependent Ca uptake is reduced by $\sim 50\%$ when the Na concentration in the medium is increased from 5 to 40 mM; however, if the Na concentration is increased further (to 145 mM), there is little additional inhibition of the Ca uptake.

Two Types of Ca Storage Sites

As mentioned in the Introduction, it is well established that mitochondria (38), including those obtained from mammalian brain (37), can accumulate Ca at the expense of ATP. However, several lines of evidence indicate that the ATP-dependent Ca uptake described above (Figs. 2-4) is not associated with the mitochondria which contaminate our tissue preparations. For one, the three mitochondrial poisons (NaN_3 , DNP, and oligomycin), present in all the incubation solutions used for the experiments of Figs. 2-4, were sufficient to inhibit completely the ATP-promoted Ca uptake by "brain mitochondria" (Fig. 2 of Ref. 35). These poisons also inhibited the succinate-promoted Ca uptake by the mitochondria.³ Additional evidence is presented in Table IV; in this experiment, Ca uptake by brain mitochondria and by disrupted terminal preparations (which contain mitochondria) was compared at moderate (1.3-1.5 μM) and lower, more physiological (0.27-0.29 μM) free Ca^{2+} concentrations. At a nominal Ca^{2+} concentration of ~ 1.3 μM , 99% of the ATP-promoted Ca uptake by the

³ Blaustein, M. P., and R. W. Ratzlaff. Unpublished data.

mitochondrial fraction was blocked by the three poisons; the same poisons blocked only ~84% of the ATP-promoted uptake in the disrupted synaptosomes (when the free Ca^{2+} concentration was $\sim 1.5 \mu\text{M}$). The ATP-promoted Ca uptake by the brain mitochondria was reduced by ~96% when free $[\text{Ca}^{2+}]$ was lowered to $0.27 \mu\text{M}$; there was little residual ATP-dependent uptake in the presence of the three poisons, at either of the Ca^{2+} concentrations tested. The data imply that the apparent half-saturation (for Ca^{2+}), K_{Ca} , of the mitochon-

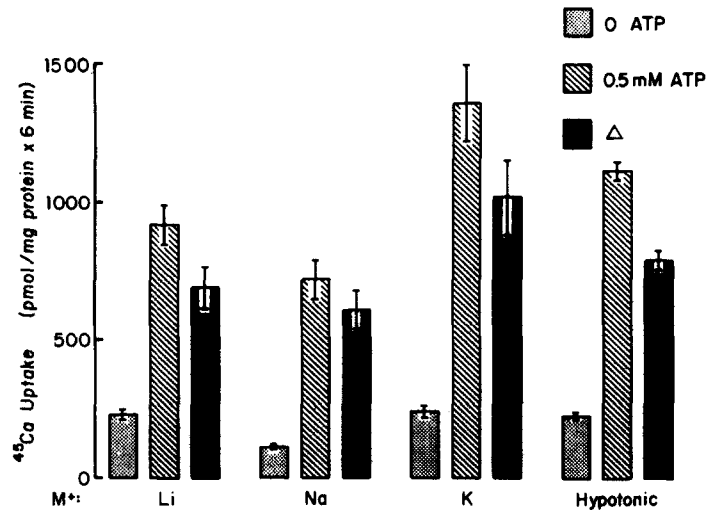


FIGURE 3. The effects of Na, K, and Li on ^{45}Ca uptake in disrupted synaptosomes. Pellets of equilibrated synaptosomes were disrupted by suspending them in 1.0 ml of lysis solution (Table I). After 1 min, ^{45}Ca -containing solutions (1.0 ml) were added to give a final Ca concentration of $10 \mu\text{M}$; some of the solutions also contained ATP, to give a final concentration of 0.5 mM . In some instances the ^{45}Ca solutions contained no alkali metal ions, ("hypotonic"); in other instances, the ^{45}Ca solutions contained sufficient KCl and NaCl or LiCl (see Table I) to give final concentrations of $145 \text{ mM NaCl} + 5 \text{ mM KCl}$ ("Na"), $145 \text{ mM LiCl} + 5 \text{ mM KCl}$ ("Li"), or $145 \text{ mM KCl} + 5 \text{ mM NaCl}$ ("Na"). All solutions contained 0.1 mM NaN_3 , 0.1 mM DNP , and $0.7 \mu\text{g/ml}$ oligomycin. Incubations were carried out at 30°C for 6 min; the protein concentration in the suspensions was 0.52 mg/ml . Stippled bars indicate Ca uptake in the absence of ATP, hatched bars indicate Ca uptake in the presence of ATP; the ATP-dependent Ca uptake (Δ) is represented by the solid bars. Each bar represents the mean of three determinations; the error bars indicate $\pm\text{SEM}$.

drial ATP-dependent Ca uptake system is considerably greater than $0.27 \mu\text{M}$ Ca^{2+} . By way of contrast, note that the poison-insensitive ATP-promoted Ca uptake by the disrupted synaptosomes was only reduced by ~55%, when the free Ca^{2+} was reduced from 1.5 to $0.29 \mu\text{M}$; moreover, at the lower Ca^{2+} concentration, the mitochondrial poisons had no significant effect on the magnitude of the ATP-dependent Ca uptake. These data are consistent with other observations (7) which indicate that the apparent half-saturation for the nonmitochon-

drial Ca uptake is $\sim 0.4 \mu\text{M Ca}^{2+}$; the data also imply that, at a Ca^{2+} concentration of $\sim 0.3 \mu\text{M}$, very little of the ATP-dependent Ca uptake can be attributed to the mitochondria.

Ruthenium red, an agent which is known to block Ca uptake by mitochondria (49, 65) but not by sarcoplasmic reticulum (21, 64), was also tested on our preparations. As shown in Fig. 5, ruthenium red inhibited only the fraction of the ATP-dependent uptake that was sensitive to the other mitochondrial poisons (NaN_3 , DNP, and oligomycin); concentrations of up to $50 \mu\text{M}$ had no effect on the residual (nonmitochondrial) ATP-dependent Ca uptake. The effect of the

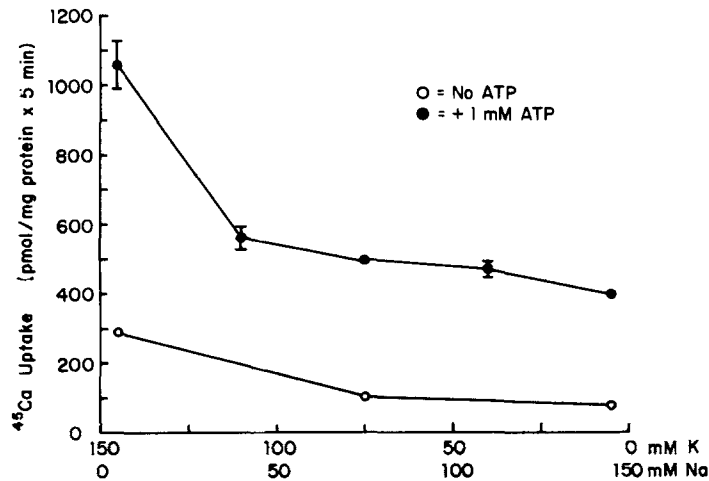


FIGURE 4. Effect of Na-K mixtures on ^{45}Ca uptake by disrupted synaptosomes. Equilibrated synaptosomes were disrupted with 1.0 ml of lysis solution; after 1 min, 1.0-ml aliquots of buffered solutions containing NaCl , KCl , and CaCl_2 labeled with ^{45}Ca were added. The final Ca concentration was $10 \mu\text{M}$; the final Na and K concentrations are indicated on the graph. All solutions contained 0.1 mM NaN_3 , 0.1 mM DNP , and $0.7 \mu\text{g/ml}$ oligomycin. Some of the ^{45}Ca -labeled solutions contained ATP, to give a final concentration of 1 mM ; (●) Ca uptake from the ATP-containing fluids. Incubations with ^{45}Ca were carried out for 5 min at 30°C . The protein concentration in the suspensions was 0.38 mg/ml . Each symbol indicates the mean of three determinations $\pm \text{SEM}$ (where the errors extend beyond the symbols).

mitochondrial uncoupler, FCCP (31), was also studied; this agent released previously stored Ca from the mitochondria but not from the $\text{NaN}_3 + \text{DNP} + \text{oligomycin}$ -insensitive sites (see Fig. 7 in Ref. 7).

Inhibitors of the Nonmitochondrial ATP-Dependent Ca Uptake

Two agents that are known to inhibit the Ca sequestration mechanism in sarcoplasmic reticulum, the local anesthetic, tetracaine (34), and the mercurial, mersalyl (27, 47), were tested on disrupted synaptosome preparations (data not shown). Tetracaine (10 mM) inhibited the nonmitochondrial ATP-dependent Ca uptake by $\sim 75\%$, whereas mersalyl ($50 \mu\text{M}$) blocked $\sim 96\%$ of this Ca uptake.

Mersalyl also inhibited 84% of the ATP-stimulated mitochondrial Ca uptake—an indication of its lack of specificity.

A rather striking result was obtained with the proteolytic enzyme, trypsin

TABLE IV
COMPARISON OF ^{45}Ca UPTAKE BY MITOCHONDRIA AND DISRUPTED SYNAPTOSOMES

[Ca ²⁺] μM	Conditions		^{45}Ca Uptake		Percent of ATP-dependent Ca uptake inhibited by poisons
	1 mM ATP	Mitochondrial poisons*	Total	ATP-dependent	
			<i>pmol/mg protein × 5 min</i>		
Mitochondria ("P₂")‡					
1.3	0	0	424 ± 71		
1.3	+	0	4,599 ± 175	4,175 ± 189	
1.3	0	+	366 ± 42		
1.3	+	+	407 ± 57	41 ± 71	99
0.27	0	0	144 ± 14		
0.27	+	0	308 ± 22	164 ± 26	
0.27	0	+	90 ± 3		
0.27	+	+	122 ± 3	32 ± 4	80
Disrupted synaptosomes§					
1.5	0	0	522 ± 36		
1.5	+	0	5,816 ± 81	5,264 ± 89	
1.5	0	+	242 ± 17		
1.5	+	+	1,067 ± 14	825 ± 22	84
0.29	0	0	66 ± 8		
0.29	+	0	441 ± 63	375 ± 64	
0.29	0	+	100 ± 11		
0.29	+	+	467 ± 39	367 ± 41	2

* 0.1 mM DNP + 0.1 mM NaN₃ + 0.7 $\mu\text{g/ml}$ in lysis and incubation fluids, where indicated ("+").

‡ Brain mitochondria from the P₂ fraction were suspended in normotonic Ca-free KCl medium (Table I) ± poisons (column 3). After 1 min, solutions containing EGTA-buffered Ca, labeled with ^{45}Ca , were added; some of these solutions also contained 1 mM ATP (column 2). The final concentrations of Ca and EGTA were: [Ca] (total) = 25 μM and [EGTA] (total) = 25 μM or 35 μM ; the calculated ionized Ca²⁺ concentrations are indicated in column 1 (see Methods). The protein concentration in the suspensions was 0.40 mg/ml.

§ Disrupted synaptosomes were prepared from pelleted-equilibrated synaptosomes by resuspension in lysis solution (Table I) ± poisons (column 3). After 1 min, solutions containing KCl and EGTA-buffered Ca, labeled with ^{45}Ca were added to provide a final composition identical to KCl medium (Table I); some of the solutions also contained ATP (column 3). The final concentrations of Ca and EGTA were: [Ca] (total) = 25 μM ; [EGTA] (total) = 25 μM or 35 μM ; the calculated ionized Ca²⁺ concentrations are indicated in column 1. The protein concentration in the suspensions was 0.38 mg/ml. All incubations were carried out for 5 min at 30°C. Each value in column 4 is the mean of three determinations ± SEM.

(Fig. 6), which is known to inhibit ATP-dependent Ca sequestration by sarcoplasmic reticulum (45). The data in Fig. 6 B show that incubation, with trypsin before lysis ("external trypsin"), or with simultaneously added soy bean trypsin inhibitor after lysis ("trypsin + inhibitor"), did not significantly affect the

nonmitochondrial ATP-dependent Ca uptake. However, when the disrupted terminals were incubated with trypsin before the addition of inhibitor ("internal trypsin"), this Ca uptake was completely abolished.

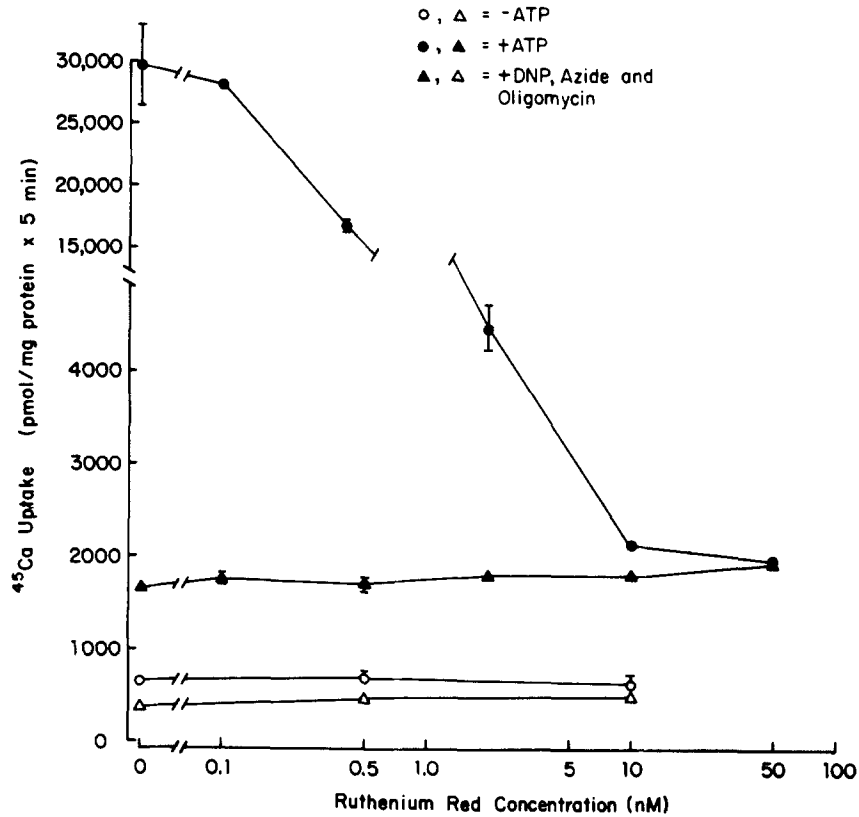


FIGURE 5. Effect of ruthenium red on ^{45}Ca uptake by disrupted synaptosomes. Equilibrated synaptosomes were disrupted by resuspension in lysis solution and were further dispersed by homogenization. To start the Ca uptake, 0.1-ml aliquots of the suspensions were transferred to tubes containing 0.7 ml of ^{45}Ca -labeled solution. After dilution the composition was identical to "KCl medium" (Table I) with $25\ \mu\text{M}$ CaCl_2 . Some of the solutions (triangles) contained $0.1\ \text{mM}$ NaN_3 + $0.1\ \text{mM}$ DNP + $0.7\ \mu\text{g/ml}$ oligomycin; some solutions (solid symbols) contained $1\ \text{mM}$ ATP. The ruthenium red concentration in the incubation fluids is indicated on the abscissa. The tissue samples were incubated with ^{45}Ca for 5 min at 30°C ; the protein concentration was $0.33\ \text{mg/ml}$. Each symbol represents the mean of three determinations; error bars (+ SEM) are shown where they extend beyond the symbols.

In contrast to the aforementioned observations, incubation with the enzyme had no apparent effect on the ATP-stimulated Ca uptake by mitochondria (Fig. 6 A). However, a high concentration of trypsin inhibitor, added either simultaneously with trypsin or after the trypsin incubation ("internal trypsin"), did partially inhibit the mitochondrial ATP-dependent Ca uptake; in the "external

trypsin" condition, the final concentrations of trypsin and trypsin inhibitor were much lower because the suspensions were diluted with 8 vol of lysis solution after the trypsin incubation, rather than before (as in the "internal trypsin" or

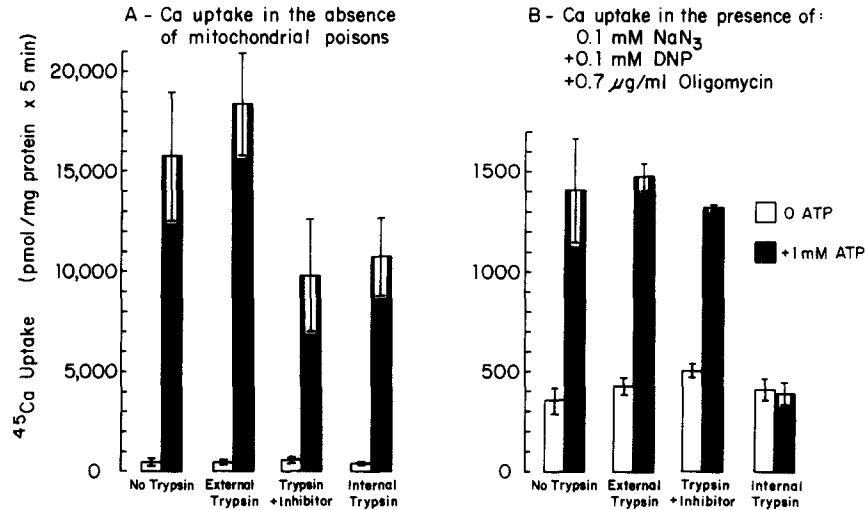


FIGURE 6. Effect of trypsin on Ca uptake by disrupted synaptosomes. Equilibrated synaptosomes were suspended in Ca-free NaCl medium at 30° and then lysed by adding a large volume of hypotonic lysis solution. After 1 min, the suspensions were adjusted to 145 mM K; Ca uptake was initiated by adding solutions containing ⁴⁵Ca and the transport substrates. Aliquots were assayed for ⁴⁵Ca uptake after a 5-min incubation at 30°C. The data shown are the mean values from three experiments ± SEM. In two experiments, trypsin digestion was carried out with 1 mg/ml trypsin for 5 min, and in one experiment, with 0.3 mg/ml for 10 min. No significant differences were apparent between these two digestion procedures. Trypsin digestion was terminated by adding 1.5 mg trypsin inhibitor/mg trypsin. Final concentrations during incubation were 145 mM K, 6.5 mM Na, 1.4 mM Mg, 2 mM PO₄, 7–15 μM Ca⁺⁺, ±1 mM ATP. The synaptosome protein concentrations in the suspensions were 0.11–0.37 mg/ml (protein concentration was constant in each experiment). (A) Ca uptake in the absence of mitochondrial poisons. In the first control, no trypsin was added. In the second control, trypsin and soybean trypsin inhibitor were added simultaneously after osmotic lysis of synaptosomes ("trypsin + inhibitor"). "External trypsin" indicates that the trypsin was present in the normotonic Ca-free NaCl medium; after 5 or 10 min at 30° C, trypsin inhibitor was added, and the terminals were then lysed and incubated with ⁴⁵Ca. "Internal trypsin" indicates that the terminals were lysed and trypsin was then added; after 5 or 10 min at 30° C, trypsin inhibitor was added, followed by the ⁴⁵Ca-containing fluids. (B) Ca uptake in the presence of mitochondrial poisons. Same conditions as in A, but with 0.1 mM NaN₃, 0.1 mM DNP, and 0.7 μg/ml oligomycin in the incubation media.

"trypsin + inhibitor" conditions). The fact that trypsin does not inhibit mitochondrial Ca uptake could be accounted for, if large molecules such as proteins are screened from the inner mitochondrial membranes, where the Ca transport system is located (39), by the outer mitochondrial membrane (56).

The Effect of the Ca Ionophore, A-23187

In our preliminary study (35, Fig. 3) we showed that the divalent cation ionophore, A-23187, abolished the ATP-dependent Ca uptake, and rapidly released previously bound Ca. Because, in that experiment, the concentrations of Ca and A-23187 were about equal (10 μ M each), the observations might be explained by a simple competition between the A-23187 and the tissue particles for Ca binding. To circumvent this possibility the experiment was repeated in the presence of a high concentration of Ca-EGTA buffer (50 μ M Ca and 70 μ M EGTA); the results obtained under these circumstances (data not shown) were identical to those observed in the earlier study: A-23187 abolished the ATP-dependent Ca uptake and released previously bound Ca. In this experiment the total Ca concentration (50 μ M) greatly exceeded the concentration of A-23187 (10 μ M); thus, the most straightforward explanation of the data is that the accumulated Ca is stored in membrane-bound vesicles which can be made permeable to Ca by the A-23187.

More conclusive evidence is given in Fig. 7. In this experiment we tested the effects of A-23187 and EGTA on Ca efflux from 45 Ca-loaded, disrupted synaptosomes. The main observation was that, in the presence of A-23187, >80% of the 45 Ca was lost in 30 s, and >90% was lost in 3 min; in contrast, only ~20% of the 45 Ca was lost after 5 min when the efflux solution contained 250 μ M EGTA (which should have reduced the free Ca^{2+} to below 10^{-7} M). The EGTA may be expected to compete effectively for superficially bound Ca, whereas A-23187 may simply make membranes permeable to Ca. Thus, it seems reasonable to conclude that most of the Ca is stored in membrane-bound organelles which survive the osmotic shock, and that the Ca is not simply bound to exposed surfaces (membranes).

Net Ca Sequestration

The experiments described in the preceding section indicate that ATP promotes the transport of 45 Ca into compartments which are not in direct communication with the bathing medium. One possibility is that this transport simply involves the exchange of 45 Ca for the endogenous 40 Ca in the tissue fragments (cf. 7); alternatively, of course, the ATP may promote net Ca sequestration. Measurements by atomic absorption spectrophotometry of net Ca concentrations in incubation fluids and tissue pellets provide strong support for the latter alternative. Fig. 8, which presents data from three experiments, shows that ATP promotes the net accumulation of Ca by the tissue pellets; although not illustrated here, there was a concomitant decrease in the Ca concentration of the ATP-containing incubation fluids, relative to the ATP-free controls (cf. 6).

As reported previously (6), the net transfer of Ca from medium to tissue, in the presence of ATP, was enhanced somewhat when oxalate was present in the medium; this is consistent with earlier observations (35) that oxalate enhances ATP-stimulated 45 Ca uptake by the tissue fragments. As in the case of sarcoplasmic reticulum (43), the results with oxalate can be readily explained if the organelles into which the Ca is transported are permeable to the oxalate so that, as the Ca accumulates inside the vesicles, the Ca-oxalate solubility product is

exceeded and Ca-oxalate precipitates; the intravesicular ionized Ca^{2+} concentration is thereby reduced, so that more Ca can be transported in. This may indicate that the Ca is transported against a concentration gradient. The effects observed with oxalate may be rather small (6) because phosphate ions can produce the same effect (cf. 63): phosphate ions are normally present in rather

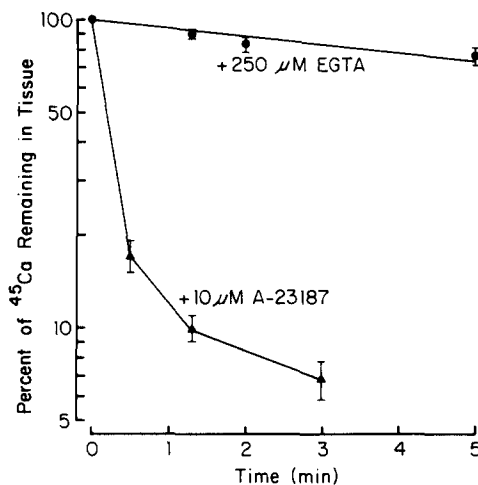


FIGURE 7. Effects of EGTA \pm ADP and ionophore A-23187 on Ca efflux from ^{45}Ca -loaded disrupted terminals. Previously lysed synaptosomes were loaded with ^{45}Ca by incubating them for 10 min at 30°C in KCl medium (Table I) containing $10\ \mu\text{M}$ CaCl_2 (labeled with tracer ^{45}Ca) and $1\ \text{mM}$ ATP. The suspensions were centrifuged at $15,000\ g$ (max) for 6 min at 4°C . The supernatant solutions were discarded, and the pellets were resuspended in Ca-free KCl medium (Table I). Aliquots were removed and filtered (zero-time samples), and additional Ca-free KCl medium was added to each sample; this solution also contained (final concentration after dilution) $250\ \mu\text{M}$ EGTA (●), or $10\ \mu\text{M}$ A-23187 (▲). The samples were incubated at 30°C ; portions of the suspensions were removed at the indicated times, and filtered through Millipore filters. The protein concentration in the efflux suspensions was $1.0\ \text{mg/ml}$; the fully loaded tissue fragments (i.e., at $t = 0$) contained approximately $900\ \text{pmol}$ Ca (as ^{45}Ca) per mg protein. Each point represents the mean of three determinations normalized to the fully loaded (100% Ca remaining at $t = 0$) condition; the bars indicate $\pm\text{SEM}$.

high concentration, even if no exogenous inorganic phosphate is added, because of ATP hydrolysis (7).

Distribution of Ca-Accumulating Particles in a Discontinuous Sucrose Density Gradient

In an effort to obtain information about the identity of the organelles responsible for the ATP-dependent Ca uptake, a series of subfractionation studies were undertaken with lysed synaptosomes (see Methods). The various centrifugation fractions (Fig. 1) were assayed for protein content and ATP-dependent Ca uptake activity; data from a representative experiment are shown in Table V.

One of the main observations was that only a tiny fraction (<1%) of the nonmitochondrial ATP-dependent uptake activity from the lysate was found in the synaptic vesicle pellet (P_4); moreover, the relative specific activity of the Ca uptake was rather low (~ 0.5). Nearly all of the activity sedimented in the more dense fraction (P_3). A similar result was obtained when P_3 was pelleted at 11,500

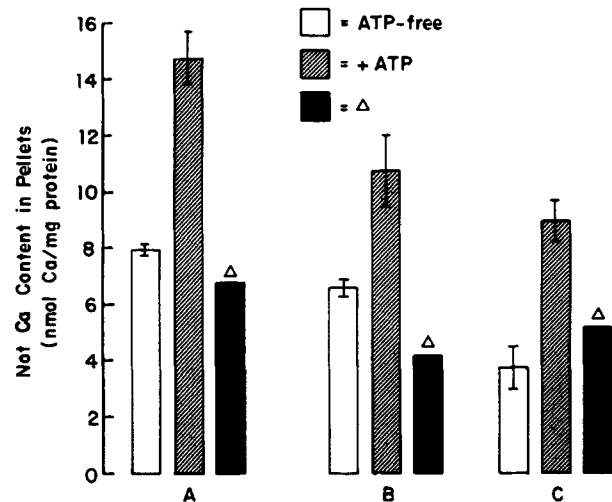


FIGURE 8. Effects of ATP on net Ca uptake by disrupted synaptosomes. Data from three experiments (A, B and C, respectively) are shown. Equilibrated synaptosome pellets were resuspended in 1.0 ml lysis solution; after 1 min, 1.0-ml aliquots of buffered solutions containing KCl, NaCl, and CaCl_2 were added to give a final Ca concentration of 6–8 μM , and a solution composition similar to “KCl medium” (Table I). All solutions contained 0.1 mM DNP, 0.1 mM NaN_3 , and 0.7 $\mu\text{g/ml}$ oligomycin; in some instances (hatched bars) the solutions contained ATP (final concentration = 1 mM). The protein concentration in the suspensions was 0.4–0.6 mg/ml. The suspensions were incubated at 30° C for 7 (A), 15 (B), or 21 min (C), and then centrifuged at 15,000 g (max) for 6 min at 4° C. Both supernatant solutions and pellets were assayed for Ca content (see Methods). The data shown represent the means of Ca determinations on three (B) or four (A and C) tissue samples; error bars indicate \pm SEM.

g for 20 min, even though, in this experiment, P_4 (the synaptic vesicle fraction) contained nearly 2% of the total lysate protein (data not shown). Upon sucrose gradient fractionation of P_3 , the Ca uptake was markedly enriched (specific activity = 4–6) at the 0.6–1.0 M sucrose interface (see Table V). Very little activity remained at or above the 0.32–0.6 M sucrose interface, and little activity was observed in the mitochondrial pellet (P'_3 ; see Refs. 14, 15, 67). About 80% or more of the nonmitochondrial Ca uptake activity was recovered routinely, after sucrose gradient fractionation.

In sum, although these data do not provide positive identification of the Ca sequestration sites, they do indicate that neither the “synaptic vesicles,” presumably, those associated with neurotransmitter storage (cf. 15, 67), nor the mitochondria are responsible for this activity.

DISCUSSION

Methodology: Use of Osmotically Disrupted Synaptosomes

Before evaluating the data obtained in this study, it is imperative to consider the validity of the osmotically disrupted synaptosome preparation that was employed in most of the experiments. As noted in Results, the use of osmotic shock seems to be justified by the evidence that organelles such as mitochondria and sarcoplasmic reticulum survive this type of treatment, and by the fact that comparable results are obtained when saponin is used to disrupt the terminals.

TABLE V
DISTRIBUTION OF ^{45}Ca UPTAKE ACTIVITY IN DISRUPTED
SYNAPTOSOMES SUBJECTED TO DIFFERENTIAL AND DISCONTINUOUS
SUCROSE GRADIENT CENTRIFUGATION

Fraction	ATP-dependent ^{45}Ca uptake <i>pmol/mg protein per 5 min*</i>	RSA†	Total activity <i>pmol/5 min</i>	Total protein <i>mg</i>
Whole lysate	652	(1.0)	33,056	50.7
P ₃	893	1.37	31,656	35.4
Light band (0.6–1.0 M inter- face)	3,873	5.94	7,281	1.88
Heavy band (1.0–1.2 M in- terface)	1,198	1.84	5,726	4.78
Pellet (P ₃)	265	0.41	3,678	13.9
Residual sucrose	1,076	(2.70)	<u>9,447</u>	<u>8.78</u>
Gradient totals			<u>26,132 (83%)§</u>	<u>29.3 (83%) </u>
S ₃	89	0.14	1,066	11.3
P ₄	341	0.52	136	0.4
S ₄	—	—	—	<u>10.5</u>
Overall totals			<u>27,334 (83%)§</u>	<u>51.5 (102%) </u>

* Incubated at 30°C in the presence of 0.1 mM NaN₃, 0.1 mM DNP, and 0.7 μg/ml oligomycin.

† Relative specific activity (=ATP-dependent uptake in fraction ÷ ATP-dependent Ca uptake in whole lysate).

§ Percent of activity recovered.

|| Percent of protein recovered.

In addition to osmotic shock and saponin treatment, we also attempted to expose intracellular organelles by employing very vigorous homogenization conditions. The latter procedure yielded preparations with relatively little, and poorly reproducible ATP-dependent Ca uptake activity, in contrast to the osmotic shock method. Several protocols for osmotic shock disruption were tested. When the hypotonic buffer ("lysis") solution contained 36 mM KCl, in addition to the constituents listed in Table I, the yield of ATP-dependent Ca uptake activity was reduced by ~20–30% (data not shown). On the other hand, marked variation in the duration of exposure to the standard lysis solution had but little effect: treatment for as short a period as 12 s or as long as 7 min (cf. Fig. 3) resulted in preparations with essentially identical ATP-dependent Ca uptake activity. Thus, the critical step appeared to be the initial exposure to the

very hypotonic solution. A 1-min exposure to the lysis solution followed by return to normotonic solution was arbitrarily chosen for use in most experiments.

Another important caveat, in terms of transmitter specificity, concerns the heterogeneity of the nerve terminal population in our synaptosome preparations. This will be a particularly crucial concern in evaluating the quantitative aspects of Ca storage (7); however, for present purposes it seems adequate to assume that most nerve terminals handle Ca in a uniform fashion, and that the Ca-storing systems under discussion are probably present in most types of terminals. Nevertheless, it should be clearly recognized that our data merely reflect the average of the activity in the heterogeneous population of terminals.

Finally, the question of whether or not the Ca-storing organelles are actually intraterminal structures must be reconsidered. Several observations are consistent with the hypothesis that they are derived from nerve terminals. In the first place, as will be illustrated in the subsequent article (7; see Figs. 9 and 10), nerve terminals apparently do have nonmitochondrial Ca sequestration sites with a high affinity for Ca. The present report shows that synaptosome preparations, in which a large fraction of the terminals are structurally and functionally intact (5), have a latent nonmitochondrial Ca uptake activity; this activity is manifest by procedures known to disrupt plasma membranes, thereby permitting the Ca sequestering organelles direct access to the ATP and (or) Ca in the media. Then, because the nonmitochondrial Ca uptake activity and the nerve terminals distribute in parallel fashion on preparative sucrose gradients (Table II and related text), we conclude that intraterminal organelles are probably responsible for most of this Ca uptake activity. However, we cannot eliminate the possibility that structures derived from other types of cells (e.g., glia) may also be involved.

The Sites of Ca Sequestration in Presynaptic Nerve Terminals

The evidence presented in this report clearly shows that there are at least two types of sites, presumably situated within presynaptic terminals, that can store Ca in the presence of ATP probably as a consequence of ATP hydrolysis (cf. 7). One type of Ca storage site has all the properties normally associated with mitochondrial Ca accumulation: Ca storage is promoted by ATP, and is blocked by a variety of mitochondrial poisons (DNP, oligomycin, NaN_3 , FCCP and ruthenium red). In preliminary studies (not shown), we also found that the mitochondrial poison-sensitive Ca uptake is promoted by a normal Krebs cycle intermediate, succinate. This Ca uptake is unaffected by saponin, presumably because the organelles involved have little cholesterol in their membranes; it is minimally affected by trypsin, which can be explained if the trypsin is prevented by the outer membrane (56) from reaching the active sites of Ca translocation at the inner membrane (39). Taken together, these observations indicate that one site of Ca sequestration is the intraterminal mitochondrion, as suggested by previous workers (59).

Our data also show that there is a second site of Ca sequestration, with somewhat different properties: the Ca uptake is supported by ATP, but not by succinate (data not shown); it is unaffected by mitochondrial poisons, but is

abolished by trypsin. This Ca storage system has a somewhat higher affinity for Ca than does the mitochondrial system (Table IV; and see 7).

The nonmitochondrial Ca storage system apparently involves membrane-bound organelles: the accumulated Ca is not removed by the Ca chelator, EGTA, but Ca is rapidly released and uptake is prevented by A-23187, an agent known to increase markedly the Ca permeability of natural membranes and lipid bilayers.

What is the Nature of the Second (Nonmitochondrial) Ca Storage Site?

A variety of organelles must be considered as possible candidates for sites of Ca sequestration: (a) plasma membrane vesicles; (b) synaptic (transmitter-storing) vesicles; (c) endoplasmic reticulum; and (d) mitochondria (which might, for example, have two different types of Ca storage mechanisms). To begin with, mitochondria may be ruled out by the fractionation studies (Tables II and V), because most of the poison-insensitive Ca uptake activity is found in the intermediate-density fractions, and relatively little activity appears in the mitochondrial pellets.

The possibility that the ATP-stimulated transport we observed may be a manifestation of Ca extrusion, i.e., Ca transport into everted (inside-out) plasma membrane vesicles formed at the time of lysis, must also be considered. However, two observations appear to eliminate this possibility. One is that the nonmitochondrial ATP-stimulated Ca uptake is not affected by low concentrations of saponin or digitonin. Saponin exposes both the mitochondrial and nonmitochondrial Ca uptake mechanisms (Table III), probably by disrupting the cholesterol-rich (9) plasma membrane. The second relevant observation is that the Ca sequestration proceeds in the virtual absence of Na, either inside or outside the vesicles (see Fig. 3). Net Ca extrusion from resealed presynaptic terminals requires external Na (4); this would be equivalent to Na inside the hypothetical everted plasma membrane vesicles, but the lysis solution contained no Na (Table I).

Recently, Blitz et al. (8) suggested that "coated vesicles" from brain may take up Ca by an ATP-dependent mechanism. Their conclusion rests primarily on the assumption that other organelles did not significantly contaminate their preparations. A variety of observations and considerations have led us to question the conclusion of Blitz et al., and to propose, instead, that the nonmitochondrial sites of Ca sequestration may be the intraterminal smooth endoplasmic reticulum. Synaptic vesicle membranes have a cholesterol content comparable to that of synaptic plasma membranes (10, 53); thus, the fact that the Ca uptake activity is resistant to saponin (Table III) may imply that synaptic vesicles are not involved in Ca sequestration. If the "coated vesicles" of Blitz et al. are, as they state, involved in synaptic vesicle membrane recycling, they presumably include components of the smooth ("uncoated") synaptic vesicle membranes.

A second critical observation is that synaptic vesicle fractions prepared by the method of DeRobertis et al. (14) contain little ATP-dependent Ca uptake

activity; most of the activity is found in more dense fractions (Table V).⁴ These data, too, are suggestive, but not conclusive.

Finally, as noted in the Introduction, there is considerable biochemical and morphological evidence that, in many types of cells including secretory cells, Ca is sequestered in smooth endoplasmic reticulum. One of the most convincing demonstrations is that of Henkart et al. (29). They found that the endoplasmic reticulum in the axoplasm of squid giant axons swelled when the axons gained Ca as a result of incubation in Ca-rich or Na-free sea waters (cf. 28); moreover, when oxalate was injected into the axoplasm, electron-dense precipitates, presumably, Ca oxalate, were formed in the endoplasmic reticulum of Ca-loaded axons (29).

In sum, these considerations are consistent with the view that endoplasmic reticulum, which is also seen in presynaptic terminals,⁵ and not synaptic vesicles, may be the site of nonmitochondrial ATP-dependent Ca sequestration in neuronal (including nerve terminal) cytoplasm.

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⁴ In most cell fractionation studies the smooth endoplasmic reticulum is recovered in a "microsome" fraction which does not generally sediment at 15,000 g. However, the homogenization conditions employed in these experiments are usually quite harsh, often involving, for example, tissue disruption in a Polytron homogenizer or Waring Blender. In contrast, when osmotic shock is used to disrupt cells, the endoplasmic reticulum may not fragment into microsomes; the endoplasmic reticulum may then sediment along with most other cell membranes, including the plasmalemma, as appears to be the case in our preparations.

⁵ McGraw, C. F., A. V. Somlyo, and M. P. Blaustein. 1978. Ultrastructural localization of calcium by electron probe analysis in presynaptic nerve terminals. *Soc. Neurosci. Symp.* In press.

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