Regulation of Cytosolic Free Calcium Concentration by Intrasyntaptic Mitochondria

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By the use of digitonin permeabilized presynaptic nerve terminals (synaptosomes), we have found that intrasyntaptic mitochondria, when studied “in situ,” i.e., surrounded by their cytosolic environment, are able to buffer calcium in a range of calcium concentrations close to those usually present in the cytosol of resting synaptosomes. Adenine nucleotides and polyamines, which are usually lost during isolation of mitochondria, greatly improve the calcium-sequestering activity of mitochondria in permeabilized synaptosomes. The hypothesis that the mitochondria contributes to calcium homeostasis at low resting cytosolic free calcium concentration ($[Ca^{2+}]_i$) in synaptosomes has been tested; it has been found that in fact this is the case. Intrasyntaptic mitochondria actively accumulates calcium at $[Ca^{2+}]_i$ around 10^{-7} M, and this activity is necessary for the regulation of $[Ca^{2+}]_i$. When compared with other membrane-limited calcium pools, it was found that depending on external concentration the calcium pool mobilized from mitochondria is similar or even greater than the IP_3 - or caffeine-sensitive calcium pools. In summary, the results presented argue in favor of a more prominent role of mitochondria in regulating $[Ca^{2+}]_i$ in presynaptic nerve terminals, a role that should be reconsidered for other cellular types in light of the present evidence.

INTRODUCTION

The characterization of the systems relevant to cellular calcium homeostasis in intracellular organelles with calcium sequestering activities, mainly mitochondria and endoplasmic reticulum (ER),¹ usually has been done with “in vitro” preparations of these organelles. The results obtained concerning the affinity for calcium and the capacity of both systems, as compared with the cytosolic free calcium concentration ($[Ca^{2+}]_i$) measured in intact cells with fluorescent dyes, have contributed to the elaboration of the widely held view that in neurons and other cell types the most important organelle gov-

erning calcium homeostasis at resting low $[Ca^{2+}]_i$ is the ER and not mitochondria. Thus, the ER is considered to have a high affinity for calcium in spite of a small total storing capacity, whereas mitochondria has a very high capacity for accumulating calcium but a very poor affinity for the cation (10^{-6} – 10^{-5} M) (for recent reviews, see Carafoli, 1987, 1988; McBurney and Neering, 1987; Blaustein, 1988; Meldolesi *et al.*, 1988; Tsien and Tsien, 1990). Two other points reinforce this way of thinking. One way of thinking is based on the rapidly exchangeable nature of the ER Ca^{2+} pool on the action of agonists such as inositol 1,4,5-tris-phosphate or cytosolic calcium itself, which mobilize calcium from this pool, generating an intracellular signal in terms of $[Ca^{2+}]_i$ (Schulz *et al.*, 1989; Rink and Merrit, 1990). The second way of thinking is based on the evidence obtained with electron probe x-ray microanalysis of crioprocessed samples that mitochondria does not accumulate calcium under resting conditions of low $[Ca^{2+}]_i$, whereas the cisterns of ER do (Somlyo *et al.*, 1985; LeFurgey *et al.*, 1986; Andrews *et al.*, 1987, 1988). However, the question has been raised as to whether the fast-freezing technique is insufficient

¹ Abbreviations used: BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic free calcium concentration; $[Ca^{2+}]_o$, external free calcium concentration; $\Delta\mu_{H^+}$, proton electrochemical gradient; CCCP, carbonyl cyanide m-chlorophenyl-hydrazine; EGTA, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-(tri-fluoromethoxy)-phenylhydrazine; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; $[^3H]$ -TPP, tetraphenylphosphonium; tbBHQ, tert-butyl-benzohydroquinone; Tris, tris(hydroxymethyl)amino methane.

to trap calcium and whether the use of anesthetics may have altered the real calcium content in mitochondria (Rottenberg and Marbach, 1990a).

It has been known for a number of years that brain mitochondria have a strikingly high capacity for calcium accumulation when incubated in the presence of adenine nucleotides and phosphate (Nicholls and Scott, 1980; Nicholls and Akerman, 1982; Vitórica and Satrústegui, 1985). These factors were thought to act on the calcium efflux pathway by way of allowing a stable accumulation of phosphate in mitochondria and by decreasing the matrix free calcium concentration through the formation of an inorganic phosphate/calcium complex. During the last years it has become apparent that these and other factors are also modulators of calcium uptake. Thus, Rottenberg and Marbach (1989, 1990b) have shown that very low concentrations of ADP increase the rate of calcium entry, especially at external calcium concentrations $> 2 \mu\text{M}$. It has also been reported that the polyamines spermine and spermidine enhance the affinity for calcium transport of the calcium uniporter, especially at low external calcium concentrations as may exist in the cytosol (Nicchitta and Williamson, 1984; Lenzen *et al.*, 1986; Jensen *et al.*, 1987; Jensen *et al.*, 1989a,b; Rottenberg and Marbach, 1990a). Moreover, physiological Mg^{2+} concentrations of below millimolar (Murphy *et al.*, 1989), which are known to inhibit the calcium uniporter of mitochondria from several tissues (Lenzen *et al.*, 1986), are not inhibitory in the brain (Rottenberg and Marbach, 1990b). These results raise the possibility that these and other yet unknown factors may act in combination to allow calcium uptake in brain mitochondria at the calcium concentration of resting neurons and synaptosomes.

The aim of the present work was to reexamine the function of brain mitochondria in synaptosomal calcium homeostasis in its cytosolic environment. To this end, we have studied the behavior of intrasynaptic mitochondria in digitonin-permeabilized synaptosomes and the effects of various regulators of calcium uptake and efflux on extramitochondrial calcium concentration. Our results indicate that brain mitochondria "in situ" may buffer cytosolic calcium at the calcium concentrations present in resting synaptosomes. Further, with the use of the calcium indicators quin2 and especially fluo3, which does not accumulate in intrasynaptic mitochondria (see below), and appropriate calcium mobilizing agents (respiratory inhibitors/uncouplers), we have assessed the presence of calcium within mitochondria at resting $[\text{Ca}^{2+}]_i$. In summary, it can be concluded that in addition to ER cisterns (Martínez-Serrano and Satrústegui, 1989; this paper, see below), intrasynaptic mitochondria accumulate calcium in an active manner even at resting low $[\text{Ca}^{2+}]_i$ and that this buffering capacity partially contributes to the whole synaptic regulation of $[\text{Ca}^{2+}]_i$.

METHODS

Materials

Sucrose, ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), EDTA, oligomycin, antimycin A, rotenone, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP), thapsigargin, succinic acid, ATP, ADP, pyruvic acid, malic acid, *N*-2-hydroxyethylpiperazine- N' -2-ethanesulfonic acid (HEPES), digitonin, cholesterol, ruthenium red, A23187, 4-Br-A23187, spermine, spermidine, and putresine were purchased from Sigma (St. Louis, MO); KPO_4H_2 , KCl, NaCl, glucose, tris(hydroxymethyl)aminomethane (Tris), and MgCl_2 were purchased from Merck (Darmstadt, West-Germany). 2,5-Di-*tert*-butyl-benzohydroquinone was purchased from Aldrich-Chemie (Steinheim, West-Germany). All other materials were of the highest quality available.

With exception of digitonin, all of the chemicals were used as supplied. Digitonin was repurified to remove digitonin-cholesterol complexes present in the commercial product according to the method of Jansky and Cornell (1980) and stored crystalline at 4°C . Freshly prepared stocks of 20 mM digitonin in water were used, sonicated ($4 \times 15''$), and heated to 60°C to avoid micelle formation just before use.

Synaptosome Purification

Male albino Wistar rats were used throughout this study. Synaptosomes from whole brain were prepared according to the method of Booth and Clark (1978) as modified in Vitórica and Satrústegui (1986). For calcium electrode determination of ambient free calcium, the synaptosomes were washed after the Ficoll gradient in the same buffers described earlier (Vitórica and Satrústegui, 1986) but were supplemented with 1 mM EGTA. The final wash and resuspension were done in 0.32 M sucrose, 25 μM EGTA, 10 mM Tris, pH 7.4, and synaptosomes were kept on ice for ≤ 90 min.

Measurement of Ambient Free Calcium With Ca^{2+} Selective Minielectrodes in Permeabilized Synaptosomes

Synaptosome permeabilization was performed by the addition of synaptosomes together with digitonin to the electrode chamber, containing the "incubation medium" (in mM): 110, KCl; 1, MgCl_2 ; 0.8, $\text{PO}_4\text{H}_2\text{K}$; 10, glucose; 20, HEPES, pH 7.4, containing 1–5 μM Ca^{2+} (the calcium concentration usually contaminating the medium) and appropriate substrates to follow calcium accumulation either in mitochondria or in ER.

Assays were performed with a noncommercial calcium electrode as described earlier (Vitórica and Satrústegui, 1985; Martínez-Serrano and Satrústegui, 1989). O_2 consumption (Clark-type electrode) and pH were measured simultaneously to ensure aerobic conditions and constant pH during the experiments (Figure 1A). All experiments were performed with magnetic stirring at 37°C . Calibration of the Ca^{2+} electrode signal (usually linear down to pCa 7) ($\text{pCa} = -\log_{10}[\text{Ca}^{2+}]$, M) was performed using calcium buffers as described by Tsien and Rink (1980).

Mitochondrial calcium accumulation was followed in the presence of 5 mM succinate (K^+ -salt) as energy source, 2 μM rotenone, and 10 μM oligomycin, the latter being present to avoid the synthesis of ATP, which could be used by ER to sequester calcium. Conversely, uptake of calcium by the ER (ATP-dependent calcium uptake) was measured in the presence of 10 μM CCCP (an uncoupler, to avoid regeneration of mitochondrial proton electrochemical gradient $[\Delta\mu_{\text{H}^+}]$ by ATP hydrolysis), 10 μM oligomycin, and 1 mM Mg/ATP and in the absence of mitochondrial respiratory substrates (to collapse any $\Delta\mu_{\text{H}^+}$ and hence calcium electrochemical gradient).

Loading of Fluorescent Dyes for $[\text{Ca}^{2+}]_i$ Measurements

For the determination of $[\text{Ca}^{2+}]_i$, the dyes used were quin2, fura2, and fluo3 (Tsien, 1980; Grynkiewicz *et al.*, 1985; Kao *et al.*, 1989;

Minta *et al.*, 1989). Dyes were loaded in synaptosomes from the hydrolysis of their cell-permeant acetoxymethylesters, quin2/AM, fura2/AM, and fluo3/AM (Molecular Probes, Eugene, OR). The loading procedures are identical to that described in Martínez *et al.* (1988) for quin2 and in Martínez-Serrano and Satrústegui (1989) for fura2. The characterization of fluo3 loading, hydrolysis, and compartmentation is described below.

After loading and washing, the synaptosomes were resuspended in physiological saline buffer (10 mg protein/ml) (in mM): 145, NaCl; 5, KCl; 1, MgCl₂; 0.4, KPO₄H₂; 10, glucose; 2.5, malate; 2.5, pyruvate; 10, Tris, pH 7.4. Synaptosomes were stored at room temperature in the dark for ≤2 h. CaCl₂ was added in each experiment, when required, from a 1-M stock solution.

Loading and Compartmentation of Fluo3

Our loading procedure for fura2 loading, from fura2/AM described elsewhere (Martínez-Serrano and Satrústegui, 1989), was modified by adding 1% bovine serum albumin (BSA) plus 0.005% Pluronic F127 (from Molecular Probes, dissolved in dimethyl sulfoxide to aid the solubilization of the highly hydrophobic dye (Minta *et al.*, 1989). After 30 min of incubation with the permeant form of the dye (5 μM fluo3/AM), the hydrolysis to the acid form was completed, as judged by the excitation and emission spectra of the dye extracted with digitonin, which are almost identical to those of the never esterified fluo3-acid (Minta *et al.*, 1989), with excitation and emission peaks at 506 and 526 nm, respectively (Figure 8A). Moreover, the fluorescence of the product obtained after hydrolysis increases around 40-fold after complexation with calcium, a criterion needed to confirm the quality of the fluo3 loaded into synaptosomes. Cytosolic fluo3 concentration achieved with this loading procedure can be estimated from the fluo3 extracted after permeabilization of synaptosomes with digitonin, making use of its molar extinction coefficient, $E_{506\text{ nm}}$ (Minta *et al.*, 1989), and the synaptosomal volume (2.7 μl/mg protein) (Martínez *et al.*, 1987). The data obtained with three different preparations of synaptosomes were 41 ± 5 (mean $\pm$ SD) μM, a value resembling those usually obtained for fura2 loading in similar conditions. K_d value used for fluo3/Ca²⁺ complex was 450 nM, as provided by the supplier, in spite of the contradictory results on temperature-dependence of this constant (Lattanzio, 1990; Merrit *et al.*, 1990).

Another crucial point in the characterization of the dye loading is its compartmentation. As shown in Figure 8B, the different amounts of fluo3 extracted with digitonin from fluo3-loaded synaptosomes parallel that of lactate dehydrogenase (a cytosolic marker), whereas no activity of citrate synthase (a mitochondrial activity) was detected. From these data, it can be concluded that synaptosomes are able to deesterify fluo3/AM to its acid form, fluo3, and that, unlike procedures developed for trapping the dye in mitochondria (Saavedra-Molina *et al.*, 1990), in our loading conditions there is no compartmentation of the dye inside mitochondria.

Measurement of Cytosolic pH in Synaptosomes Using 2', 7'-bis-(2-carboxyethyl)-5 (and-6)-carboxyfluorescein (BCECF)

Loading of synaptosomes with BCECF for cytosolic pH determination was performed in a similar way as fluo3 loading. Briefly, synaptosomes were incubated at 2 μg/ml with the cell-permeant form of the dye, BCECF/AM (Molecular Probes) in the same conditions as for fluo3, using 1% BSA as dispersing agent.

Hydrolysis of BCECF/AM in synaptosomes yields a product with the same spectral characteristics as free acid BCECF in terms of linearity of the response with pH (6.4–8) and excitation and emission spectra (Martínez-Serrano *et al.*, 1992). All measurements were performed at 506 nm and 526 nm, excitation and emission wavelengths respectively, with ± 3 nm bandwidth.

Measurement of ATP Content in Intact Synaptosomes

ATP concentration in synaptosomes was determined as described by Lemasters and Hackenbrock (1978) by using the ATP bioluminescence test constant light signal (CLS) (from Boehringer Mannheim, Mannheim, Germany) and following the specifications of the supplier. Luminescence was measured in a 1250-luminometer from LKB-Wallac (Turku, Finland).

Measurement of Synaptic Plasma Membrane Potential by the Distribution of Tetraphenylphosphonium (³H]-TPP)

Membrane potential determinations were based on the distribution at equilibrium of the lipophilic cation [³H]-TPP as described in Martínez *et al.* (1987).

Other Methods

Protein content of the synaptosomal suspension was determined by the biuret's method (Gornall *et al.*, 1949) using BSA (Sigma) as protein standard. Processing of fluorescence records to calcium values was performed off-line from digitized and stored fluorescence data as described (Martínez-Serrano and Satrústegui, 1989).

RESULTS AND DISCUSSION

Synaptosome Permeabilization

Synaptosomes were treated with digitonin to remove the permeability barrier for calcium provided by the plasma membrane, thus allowing a direct access to intrasynaptic organelles. Digitonin interacts with cholesterol at the membranes, leading to the formation of pores as observed by freeze-fracture electron microscopy and thus rendering the membrane freely permeable to small molecules and ions (Elias *et al.*, 1978). Because of the uneven distribution of cholesterol in the different cellular membranes (Korn, 1966; Elias *et al.*, 1978), theoretically it is possible to find a concentration that would disrupt selectively those membranes with the higher cholesterol content (the plasma membrane), leaving the membrane of calcium-accumulating organelles (mitochondria and ER) largely intact (see Fiskum, 1985, for a review on saponin permeabilization procedures).

Figure 1 shows the experiments that were carried out for the determination of these optimal permeabilization conditions. The different concentrations of digitonin were added together with the synaptosomal preparation, and calcium movements were recorded until a steady state was obtained, whereupon the calcium ionophore A23187 was added to release the entrapped calcium. The total amount of calcium taken up by the permeabilized synaptosome preparation, the ionophore-releasable calcium, and the respiratory rate observed were also computed.

The results shown in Figure 1B indicate that the optimal digitonin concentration to obtain the lowest set point (extra-organelle calcium concentration set by the organelle) values for mitochondria and ER was 100 μM.

Figure 1. Digitonin permeabilization of synaptosomal plasma membrane. (A) Simultaneous measurement of O_2 consumption (a), mitochondrial Ca^{2+} accumulation (b) (in the presence of 5 mM K^+ -succinate, 2 μM rotenone, 10 μM oligomycin, and 0.05 mM ADP), and ATP-dependent Ca^{2+} uptake in ER (c) (in the presence of 10 μM oligomycin, 2 μM rotenone plus 0.2 mM Mg/ATP). As observed, 1 μM A23187 induces the release of all of the calcium taken up in an energy-dependent manner. d, pH record. S, synaptosomes, 1 mg protein/ml. (B) Calcium accumulation and O_2 consumption at several doses of digitonin (for further details see text). (C) Integrity of the nonmitochondrial calcium compartment (the most labile to detergent action) in the continuous presence of 100 μM digitonin.

In addition, at this concentration, the calcium accumulated in ER and mitochondria and the ionophore-releasable calcium are close to maximal. This digitonin concentration agrees quite well with that found by Ronning *et al.* (1982) for the specific permeabilization of GH3 cell plasma membranes.

Permeabilization did not progress with time of exposure to digitonin. Thus, it was possible to observe uptake and release of calcium by ER at various times after digitonin addition, depending on the availability of Mg/ATP (Figure 1C). A similar situation could be observed for the mitochondrial Ca^{2+} pool, which remains in mitochondria for a time greatly exceeding the normal duration of the experiments reported in this paper.

Behavior of Intrasyntaptic Mitochondria In Situ

Calcium uptake in permeabilized synaptosomes depends on the existence of a $\Delta\mu_{H^+}$ in mitochondria or a functional Ca^{2+} -ATPase in ER. Thus, in the absence of exogenous respiratory substrates, ATP or an ATP-generating system, and provided that a proton ionophore

is present to collapse mitochondrial membrane potential, almost no calcium is taken up from the milieu on synaptosome addition (Figure 2A, trace a). Moreover, depending on the external free calcium concentration ($[Ca^{2+}]_o$), a net efflux of calcium may be observed. The requirement of an exogenous respiratory substrate is probably due to the loss of metabolites during permeabilization and may account for the fact that other authors have failed to observe any significant calcium uptake at $[Ca^{2+}]_o$ below 5 μM in mitochondria from permeabilized synaptosomes (Rasgado-Flores and Blaustein, 1987).

When synaptosomes are rendered permeable to external calcium with digitonin in the presence of respiratory substrates, calcium is taken up from the milieu mainly to a site that we have identified as mitochondria in the following grounds. 1) Calcium uptake is coupled closely with an increase in respiratory rate, and both Ca^{2+} uptake and respiratory rate increase with the amount of digitonin added until a plateau is reached (Figure 1B). 2) Calcium uptake is blocked in the presence of mitochondrial inhibitors (Martínez-Serrano and Sa-

Figure 2. Calcium movements in digitonin-permeabilized synaptosomes. Representative tracings of Ca^{2+} -specific electrode are shown. Synaptosomes (S, 1 mg protein/ml) were added to a cytosol-like medium containing 100 μM digitonin. (A) Calcium accumulation into synaptosomal organelles in the presence of different energy sources: a, no substrates, 10 μM CCCP; b, Ca^{2+} uptake in mitochondria and ER (5 mM K^{+} -succinate, 2 μM rotenone, 1 mM Mg/ATP, and 10 μM oligomycin); c, Ca^{2+} uptake in mitochondria (5 mM K^{+} -succinate, 2 μM rotenone, 10 μM oligomycin, 0.2 mM Na^{+} -orthovanadate). (B, C, and D) Calcium-buffering capacity of intrasynaptic mitochondria. The cytosol-like medium had the same composition as in A. (D) The effects of sodium on the calcium set point. In B, sodium concentration was 10 mM. Additions were Ca^{2+} , 5 nmol; EGTA, 5 nmol; FCCP and CCCP, 10 μM ; antimycin A, 0.2 μM ; A23187, 1 μM . (A, C, and D) 0.3 mM or (B) 50 μM ADP was present.

trústegui, 1989) (Figure 2, A, traces b and c, and C), but it is insensitive to the presence of vanadate and caffeine to inhibit Ca^{2+} -ATPase from ER (Martínez-Serrano and Satrústegui, 1989) (Figure 2A, traces b and c). 3) The calcium-storing organelle buffers the external calcium concentration around a "set-point" value, by either taking up calcium from the media or delivering calcium into it, as expected from a system with a kinetic balance between two components, influx and efflux (Figure 2, B and C). In brain mitochondria, the systems responsible for these calcium fluxes are an electrophoretic uniporter for calcium entry, driven by the proton electrochemical gradient, and a Na^{+} activated efflux system (for review, see Nicholls and Akerman, 1982).

Figure 2A shows the cooperation between the Ca^{2+} -sequestering activities of mitochondria and ER. As previously described (Martínez-Serrano and Satrústegui, 1989), in synaptosomes there is a vesicular, ATP-dependent, vanadate-sensitive calcium uptake, which leads to an extra calcium uptake in the presence of Mg/ATP as energy source for ER (in the presence of oligomycin to avoid ATP hydrolysis by mitochondrial H^{+} -ATPase) and lowers the combined set point to $\text{pCa} \approx 6.6$. Unlike other systems, the ER alone is unable to take up a substantial amount of calcium, and the pres-

ence of functional mitochondria is required to lower ambient calcium to submicromolar levels (Martínez-Serrano and Satrústegui, 1989) (Figures 2 and 3). This is probably due to the lower ER content of synaptosomes as compared with other systems, for example, neutrophils, Rinm5F cells, and bovine adrenal glomerulosa cells (Biden *et al.*, 1984; Prentki *et al.*, 1984; Rossier *et al.*, 1987). Thus, the cooperation between mitochondria and ER might vary among different tissues, and this fact could be a specific and differential characteristic of the presynaptic terminal.

Figure 3. Modulation of calcium buffering in permeabilized synaptosomes. Data were taken from experiments similar to those described in Figure 2. (A) Net calcium uptake (difference between the total calcium concentrations at the time of synaptosomes addition and once a steady state is reached) in mitochondria and ER. Incubation medium contained 5 mM K^{+} -succinate, 2 μM rotenone, 10 μM oligomycin (mitochondria) or 1 mM ATP, 10 μM oligomycin, and 2 μM rotenone (ER). Values in brackets represent the $[\text{Ca}^{2+}]_0$ at X axis intercept. (B) Replot of the data from A showing the calcium-buffering capacity of mitochondria and ER. Dotted line represents absence of calcium buffering; lines were tentatively drawn "by eye." NaCl and ADP concentrations (in mM) are indicated for each condition.

Factors That Regulate Mitochondrial Calcium Uptake in Permeabilized Synaptosomes

To study the behavior of intrasynaptic mitochondria with regards to calcium accumulation and calcium buffering in response to factors affecting calcium efflux and uptake, we have carried out the experiments shown in Figure 3. Figure 3A shows the net calcium uptake in mitochondria from digitonin-permeabilized synaptosomes as a function of the $[Ca^{2+}]_o$ at the start of the permeabilization, with varying sodium and ADP concentrations. As expected, the net calcium uptake is greater in the absence of sodium in the incubation medium, and the positive effect of ADP is potentiated in the most adverse conditions, i.e., presence of sodium. The intercept of the lines with the X axis could be taken as indicative of the lowest calcium concentration at which mitochondria could accumulate calcium from the milieu. This value decreases from ~ 400 to 300 nM in the absence of sodium. Interestingly, this is a calcium concentration quite similar to the resting synaptosomal $[Ca^{2+}]_i$ (Ashley *et al.*, 1984; Hansford and Castro, 1985; Adam-Vizi and Ashley, 1987; Martínez *et al.*, 1988; Verhage *et al.*, 1988, 1989, 1991; see below), and thus the possibility that mitochondria could participate in calcium homeostasis at resting $[Ca^{2+}]_i$ is opened.

For comparison, the results obtained for the ATP-dependent calcium uptake (in the presence of mitochondrial inhibitors) in ER are shown (Figure 3A, right). This calcium uptake is lower at any external calcium than that of mitochondria, but its apparent affinity for calcium is higher, the intercept with the X axis ~ 250 nM.

The calcium-buffering activity of mitochondria is illustrated in Figure 3B, which shows the final $[Ca^{2+}]_o$ where a steady state is achieved (the calcium set point) as a function of the initial calcium concentration. In the most adverse situation, i.e., high sodium and low ADP (Figure 2B), mitochondria were able to buffer external calcium at ~ 1 μ M and at ~ 0.3 μ M if 0.3 mM ADP was present (Figure 2, A, C, and D). However, in the absence of sodium and at any ADP concentration, mitochondria is able to buffer external calcium in a range ~ 200 – 300 nM. As observed (Figure 3B), the ER lacked any detectable calcium-buffering ability, probably due to its very low capacity for calcium storage.

There is a large body of results indicating that naturally occurring polyamines increase the affinity for calcium of isolated brain mitochondria (Lenzen *et al.*, 1986; Jensen *et al.*, 1987, 1989a,b; Rottenberg and Marbach, 1990a) and that of mitochondria present in saponin-treated hepatocytes (Nicchitta and Williamson, 1984; Fiskum, 1985) for net calcium uptake. Figure 4 shows typical calcium minielectrode (Figure 4A) or fluo3 (Figure 4C) tracings of mitochondrial calcium uptake in permeabilized synaptosomes incubated under adverse (10 mM NaCl, 50 μ M ADP) (Figure 4, A and B) or fa-

Figure 4. Polyamine stimulation of mitochondrial calcium uptake in permeabilized synaptosomes. (A) Synaptosomes (1 mg protein/ml) were added (S) to a cytosol-like medium containing 100 μ M digitonin, 5 mM K^+ -succinate, 2 μ M rotenone, 10 μ M oligomycin, 10 mM NaCl, 0.05 mM ADP in the absence (trace a) or presence of 1 mM spermine (trace b), 5 mM spermidine (trace c), or 5 mM putresine (trace d), and calcium movements were recorded with a calcium electrode. At the arrow, 1 mM spermine was added. (B) Calcium set points versus polyamine doses. $\bullet$, control (absence of polyamine, mean $\pm$ SEM); $\circ$, spermine; Δ , spermidine; $\square$, putresine. Sodium and ADP concentrations were 10 and 0.05 mM, respectively. Data correspond to one representative experiment. (C) Fluo3 tracings of mitochondrial Ca^{2+} accumulation and release in digitonin-permeabilized synaptosomes incubated in the presence of 0.3 mM ADP, in the absence of sodium, and at different initial external calcium concentrations. In this experiment, fluo3 hydrolyzed by synaptosomes from fluo3/AM was used as indicator of ambient free calcium. The following additions were made as indicated: SP, 1 mM spermine; F/O, 10 μ M FCCP plus 10 μ M oligomycin; Ca^{2+} , 1 mM $CaCl_2$; E/T, 10 mM EGTA plus 20 mM Tris. The dotted line shows the fluorescence recording of fluo3-loaded synaptosomes incubated in the presence of 1 mM spermine and at the same external calcium concentration as trace b, when permeabilized with 100 μ M digitonin (*). Following F/O addition, the record superimposes to trace b.

vorable (absence of NaCl, 0.3 mM ADP) (Figure 4C) conditions in the presence of different polyamines. Spermine and spermidine are able to decrease the mitochondrial set point from pCa 6.2 to 6.3 (625–500 nM), irrespective of the moment of the addition (Figure 4A). In the same way, putresine also stimulates net calcium uptake in mitochondria but to a lesser extent (Figure 4, A and B). When synaptosomes are permeabilized in the absence of sodium and presence of ADP (Figure 4C), set-point values in the presence of spermine are lower, attaining near 200 nM free calcium values. It is interesting to note that spermine and spermidine concentrations of 0.5–1 mM that are most active in stimulating

calcium uptake fall within the range of those found in vivo (Seiler and Lamberty, 1975; Laitinen *et al.*, 1982).

In summary, these results indicate that the regulatory effect of sodium, ADP, and polyamines on calcium efflux, uptake, and calcium set point seen with isolated brain mitochondria in vitro (Nicholls, 1978; Vitórica and Satrustegui, 1985; Rottenberg and Marbach, 1990a,b) are also present when the mitochondria is assayed in its cytosolic environment. Surprisingly, mitochondria appears to regulate extramitochondrial calcium at cation concentrations in the range of those present in the cytosol of resting intact synaptosomes.

Mobilization of Calcium From Intrasynaptic Mitochondria in Intact Synaptosomes and Its Detection With Fluorescent Dyes

As mentioned earlier, it is now believed by most investigators in the field that mitochondria is unable to participate significantly in the control of cytosolic calcium, unless at high $[Ca^{2+}]_i$, for example, after depolarization or in pathological situations. However, the experiments reported above suggest that mitochondria, in situ, have a calcium-buffering activity that could make them relevant to the regulation of the resting synaptosomal $[Ca^{2+}]_i$. If calcium is present in mitochondria under resting conditions, then it follows that calcium mobilization from intrasynaptic mitochondria should give rise to an increased $[Ca^{2+}]_i$ in intact synaptosomes. The aim of the following experiments was to study whether an increase in $[Ca^{2+}]_i$ could be observed after the addition of uncouplers or respiration inhibitors that abolish the calcium electrochemical potential in mitochondria and

whether this could be nonambiguously attributed to mitochondrial calcium mobilization. To this end we have used various combinations of cytosolic calcium indicators and mitochondrial respiration inhibitors chosen on the basis of their spectral characteristics.

Figure 5A (upper traces) shows the effects of dissipating intrasynaptic mitochondrial $\Delta\mu_{H^+}$ by the addition of KCN together with oligomycin, to prevent ATP run-down by mitochondrial ATPase, in quin2-loaded synaptosomes. Neither 10 μ M oligomycin nor 1 mM KCl (the same $[K^+]$ present in the addition of KCN) had any effect on resting $[Ca^{2+}]_i$ (upper panel); however, the addition of 1 mM KCN (neutralized to obtain the active form, cyanhydric acid) (Slater, 1967) induced an increase in cytosolic calcium.

It frequently has been reported that quin2 and fura2 become entrapped inside cytosolic organelles, and therefore, it could be argued that the $[Ca^{2+}]_i$ signals observed after KCN addition arise from intraorganelle quin2. To avoid this problem, we have used fluo3 as calcium indicator because we have assessed that this probe does not accumulate in intrasynaptic mitochondria (see METHODS). The spectral characteristics of fluo3 (excitation in the visible range, 506 nm) allowed the use of the powerful uncoupler FCCP. Further, fluo3 has another advantage over quin2, i.e., its relative insensitivity to pH changes around physiological values (Kao *et al.*, 1989; but see Lattanzio, 1990); moreover, the drop in cytosolic pH that has been observed after FCCP/oligomycin addition (Arslan *et al.*, 1985; Martínez-Serrano *et al.*, 1992), would lead to an increase in the K_d for the Ca/fluo3 complex (Lattanzio, 1990) and to a corresponding reduction rather than increase in

Figure 5. Calcium mobilization from mitochondria in intact synaptosomes. (A) Quin2-loaded synaptosomes were treated with 1 mM KCN plus 10 μ M oligomycin to collapse mitochondrial $\Delta\mu_{H^+}$. Representative traces of quin2 fluorescence are shown. A plot of the increase in $[Ca^{2+}]_i$ measured versus $[Ca^{2+}]_i$ at the moment of the addition of drugs is shown. X-axis intercept value was 94 nM. The different $[Ca^{2+}]_i$ before drug addition were obtained by KCl depolarization ranging from 5 to 65 mM KCl. (B) Fluo3-loaded, calcium-depleted synaptosomes were equilibrated during 5 min in the absence or presence of 0.5 or 1 mM $CaCl_2$ before drug addition (10 μ M FCCP plus 10 μ M oligomycin). Tracings are the mean (without filtering) of nine individual $[Ca^{2+}]_i$ records from three rats. A representation of the increase in $[Ca^{2+}]_i$ on drug addition as a function of $[Ca^{2+}]_i$ is also shown.

dye fluorescence. We have not corrected for pH-dependent changes in dye K_d and therefore, the increases in $[Ca^{2+}]_i$ shown may be underestimated (crude calculation yields a 50% underestimation in $[Ca^{2+}]_i$ increase at the peak of the signal after FCCP/oligomycin addition when using fluo3, due to a transitory fall in 0.1 pH units measured with BCECF).

Figure 5B shows that the addition of FCCP/oligomycin to fluo3-loaded synaptosomes induced a rapid rise in $[Ca^{2+}]_i$ that was followed by a slower but sustained increase in the presence of external calcium. This was not due to autofluorescence or other artifacts because in the absence of external calcium, $[Ca^{2+}]_i$ returns to its initial value within seconds (see Figure 5B, upper panel, lower trace, and Figure 6). The same results were obtained using antimycin/oligomycin to collapse mitochondrial $\Delta\mu_{H^+}$, even though the fluorescence increase was slightly slower, probably because the collapse of $\Delta\mu_{H^+}$ is mainly due to passive H^+ leak. The results obtained with antimycin rule out that the calcium signal seen after FCCP addition could arise from a nonmitochondrial compartment, such as ER subfraction and/or secretory vesicles where neurotransmitter and ion accumulation is driven by a $\Delta\mu_{H^+}$ (Cidon and Sihra, 1989; Schulz *et al.*, 1989; Yoo and Albanesi, 1990). Recently, similar findings about the effects of FCCP, antimycin, and oligomycin have been obtained in *Helisoma* neurons, although FCCP alone showed a delayed (after 10 min)

effect on nonmitochondrial calcium compartments (Jensen and Rehder, 1991).

Previous studies have shown a rise in $[Ca^{2+}]_i$ in various cell types after the addition of mitochondrial uncouplers or respiration inhibitors (Pozzan *et al.*, 1982; Tsien *et al.*, 1982; Arslan *et al.*, 1985; Dagani *et al.*, 1989; Dubinsky and Rothman, 1991; Jensen and Rehder, 1991). However, this rise was small and could be due to a number of reasons different from the release of mitochondrial calcium to the cytosol (changes in cell pH, plasma membrane depolarization; see below), including depletion of cell ATP. Indeed, the addition of a number of respiration inhibitors and uncouplers results in a fall in ATP levels both in synaptosomes (Scott and Nicholls, 1980) and different cell types (Masaki *et al.*, 1989; Nieminen *et al.*, 1990), and it has been proposed that ATP depletion is among the mechanisms responsible for the toxic consequences of mitochondria de-energization (Masaki *et al.*, 1989; Nieminen *et al.*, 1990).

In synaptosomes, aerobic glycolysis is stimulated markedly on mitochondrial de-energization induced by rotenone or an uncoupler (Scott and Nicholls, 1980; Kauppinen and Nicholls, 1986), and ATP synthesis generated in the Embden-Meyerhof pathway is sufficient for the continued function of ATP-dependent processes, including reversal of the mitochondrial ATPase and maintenance of the mitochondrial mem-

Figure 6. Comparative size of synaptosomal membrane-limited calcium pools. Synaptosomes were loaded with fluo3 or fura2 as described in METHODS. The use of both dyes was necessary because FCCP with fura2 or caffeine with fluo3 produced autofluorescence or quenching artifacts, respectively. After 10 min of preincubation in 1 mM $CaCl_2$ in the external medium, synaptosomes were centrifuged (30 s, $10\,000 \times g$) and resuspended (1 min before time zero) in calcium free media (A and B) or 20–30 μM EGTA supplemented media (C and D). The latter media were prepared so that external and cytosolic calcium concentrations were the same. The tracings are the average of at least three individual records. K_d values for the dye/ Ca^{2+} complex used were 224 and 450 nM, for fura2 and fluo3, respectively; the difference in absolute $[Ca^{2+}]_i$ measured with both dyes under the same conditions probably reflects the variations in K_d observed with pH and temperature by Lattanzio (1990) and Lattanzio and Bartschat (1991). Additions were 10 μM FCCP/oligomycin (F/O), 10 μM thapsigargin (Th), 10 μM 2,5-di-tert-butyl-benzohydroquinone (tbBHQ), 10 mM caffeine (Caf), and 200 nM 4-Br-A23187 (I).

brane potential lowered by only 20 mV (Scott and Nicholls, 1980). The extent to which ATP levels are preserved on mitochondrial de-energization is important to interpret the consequences of this process and depends on the supply of an adequate glycolytic source and on the degree of ATP utilization in mitochondria among other factors. Thus, on the one hand, omission of glucose synergizes with rotenone-induced mitochondrial de-energization to produce a large ATP drop, and this is accompanied by an increase in $[Ca^{2+}]_i$, much larger than that produced by rotenone alone (Dagani *et al.*, 1989), presumably due to the combined effects of the inhibition of the calcium pumps (plasma membrane and ER) and mitochondrial calcium mobilization on the collapse of $\Delta\mu_{H^+}$. In hepatocytes, fructose, a substrate for glycolytic ATP formation, provides a good protection against toxicity from cyanide (Nieminen *et al.*, 1990).

On the other hand, the addition of oligomycin to rotenone-inhibited synaptosomes to prevent reversal of mitochondrial ATPase leads to a complete collapse of the mitochondrial membrane potential (Scott and Nicholls, 1980) and, presumably, to an increased preservation of ATP levels. Indeed, it has been observed that hepatocytes are protected against the toxic effects of the uncoupler CCCP by fructose together with oligomycin, a finding that can only be explained by an increase in the availability of ATP (Nieminen *et al.*, 1990). In our experimental conditions, the addition of oligomycin together with FCCP leads also to a relative preservation of ATP levels; these were 0.911 ± 0.03 mM (before FCCP/oligomycin) and 0.385 ± 0.02 mM (4 min after FCCP/oligomycin addition). The latter concentration is still above that required to obtain maximum rate for the plasma membrane and ER calcium pumps of synaptosomes (Michaelis *et al.*, 1987; Martínez-Serrano and Sastrústegui, 1989), and consequently, it may be concluded that the increase in $[Ca^{2+}]_i$ observed is not due to a reduction in ATP-driven calcium efflux but reflects calcium release from mitochondria. Moreover, we have observed that the addition of FCCP/oligomycin to ^{45}Ca -loaded synaptosomes produced a net calcium efflux (see also Vitórica and Sastrústegui, 1986), indicating that the plasma membrane extrusion systems respond actively to the increased $[Ca^{2+}]_i$ obtained after calcium mobilization from mitochondria.

An objection that has been raised in experiments using uncouplers to mobilize mitochondrial calcium is that these agents depolarize the plasma membrane (Sihra *et al.*, 1984) by allowing proton influx driven by the plasma membrane potential and a corresponding decrease in cell pH (Arslan *et al.*, 1985). According to this idea, FCCP-dependent $[Ca^{2+}]_i$ signal could result from H^+ displacement of calcium from calcium binding sites or depolarization-dependent calcium influx. The first possibility may be ruled out because we have observed that antimycin A or KCN addition that also increases $[Ca^{2+}]_i$

does so without any drop in pH (see also Duchen *et al.*, 1990). The following results argue against the second. 1) The addition of FCCP/oligomycin leads to an increase in cytosolic calcium in the absence of external calcium (Figure 5B, upper panel, and Figure 6), even when the plasma membrane has been previously depolarized by an increase in K^+ concentration in the bathing medium (Figure 7B). 2) The addition of FCCP or FCCP/oligomycin did not depolarize the plasma membrane potential measured through $[^3H]$ -TPP distribution until ≥ 2 min after drug addition (see also Scott and Nicholls, 1980). 3) As mentioned above, net $^{45}Ca^{2+}$ efflux follows FCCP addition. Taken together, these results clearly indicate that the FCCP/oligomycin-dependent $[Ca^{2+}]_i$ increase must be due to calcium mobilization from mitochondria.

We have next studied the dependence of the calcium mobilization signal with respect to the $[Ca^{2+}]_i$ at the point of inhibitor/uncoupler addition (Figure 5, A and B, lower panels). Raising $[Ca^{2+}]_i$ by depolarization ($[KCl]$ ranging from 5 to 65 mM) (Figure 5A) or varying $[Ca^{2+}]_o$ (Figure 5B) resulted in an increase in the calcium mobilization signal on KCN or FCCP addition, suggesting

Figure 7. Handling of calcium loads in intact synaptosomes with functional or inhibited mitochondria. Both calcium-depleted (A) and calcium-equilibrated (B) fluo3-loaded-synaptosomes were exposed to a calcium load entering the plasma membrane. In A, 1 mM $CaCl_2$ was added (Ca) before or after FCCP/oligomycin (F/O) cocktail. In B, calcium load was induced by depolarization with 65 mM KCl in 0.5 (left) or 1 mM total $CaCl_2$ (right). The additions of FCCP/oligomycin, 65 mM KCl or 65 mM KCl plus 0.5 mM $CaCl_2$, are indicated. Twelve (A) or 8 (B) individual $[Ca^{2+}]_i$ records were averaged without filtering in each case. At least three different preparations of synaptosomes were used.

that organelle, cytoplasmic, and extracellular calcium exchange rapidly. The extrapolation of the $[Ca^{2+}]_i$ value at which no mobilization of calcium would occur lies around (Figure 5A) or below (Figure 5B) 100 nM. This surprising result suggests that, above 10^{-7} M $[Ca^{2+}]_i$, intrasynaptic mitochondria accumulates calcium actively against its calcium chemical gradient.

To further check this point and to quantitatively compare the size of the mitochondrial calcium pool with that of other calcium pools in the synaptosome, we have tested the effect of some well-known calcium mobilizing agents in the absence of external calcium. After a preincubation in media containing 1 mM $CaCl_2$, synaptosomes were resuspended in micromolar or nanomolar external calcium (Figure 6). In the presence of 1.2 μ M calcium (determined with fluo3 at the end of each run) in the external medium (nominally "calcium free"), the $[Ca^{2+}]_i$ rise obtained after FCCP/oligomycin addition was found to be larger than that obtained after the addition of the Ca^{2+} -ATPase inhibitor thapsigargin, which blocks Ca^{2+} accumulation in IP_3 -sensitive pools (Bian *et al.*, 1991) (Figure 6A); this and the caffeine-induced $[Ca^{2+}]_i$ rise were similar (Figure 6B). Interestingly, tert-butyl-benzohydroquinone (tbBHQ), an inhibitor of Ca^{2+} -ATPases in liver microsomes (Moore *et al.*, 1987), seems to have no effect in synaptosomes. This difference in thapsigargin and tbBHQ effects probably reflects the inhibitory action of tbBHQ in the cyclooxygenase pathway and the concomitant loss of the Ca^{2+} -mediated self-

amplifying system for emptying the IP_3 -responsive calcium pool as recently described in human platelets (Brüne and Ulrich, 1991a,b).

When the experiment was done under more adverse conditions (external free calcium equal to cytosolic free calcium), the size of mitochondrial, thapsigargin-, and caffeine-sensitive calcium pools was similar (Figure 6C). Under these conditions, an additional calcium pool (larger than those previously shown) releasable by 200 nM 4-Br/A23187 was found. The nature of this new calcium pool is yet unresolved because it is insensitive to prior addition of FCCP/oligomycin, thapsigargin, or caffeine and because its depletion does not affect the $[Ca^{2+}]_i$ signals arising from the other pools (Figure 6C).

In summary, at external $[Ca^{2+}]$ of micromolar or above, the mitochondrial calcium pool seems to be more important than those from the ER, and all pools seem to have the same size when $[Ca^{2+}]_o$ equals $[Ca^{2+}]_i$. It is important to point out here the different nature of mitochondrial and ER calcium pools. Although the former is a calcium-cycling organelle, whose calcium content steeply depends on available calcium (Thayer and Miller, 1990), the ER-located calcium pools require the presence of an agonist to release their calcium. This explains the different dependence of mitochondrial and ER calcium pools with respect to $[Ca^{2+}]_o$ and emphasizes the importance of the calcium-sequestering activity of mitochondria even under $[Ca^{2+}]_i$ conditions, where previously it has been considered irrelevant. In agreement

Figure 8. Characterization of Fluo3 loading and compartmentation in rat brain synaptosomes. (A) Excitation and emission spectra of fluo3 extracted with 160 μ M digitonin from synaptosomes loaded with 5 μ M fluo3/AM. (B) Calcium-dependent fluorescence extracted with digitonin from fluo3-loaded synaptosomes. (Lambda exc = 506 nm, lambda emm = 526 nm) and lactate dehydrogenase (LDH) or citrate synthase (CS) activities extracted from fluo3-loaded (closed) or control (open) synaptosomes. One hundred percent value refers to the signal or activities measured after 0.5% Triton X-100 extraction. As may be seen, permeabilization is independent of the presence of fluo3. One hundred percent values were LDH, 160 $nmol \cdot min^{-1} \cdot mg \text{ protein}^{-1}$; CS, 237 $nmol \cdot min^{-1} \cdot mg \text{ protein}^{-1}$. (C) Calibration of fluo3 fluorescence signals. As shown, even high doses of ionomycin (up to 20 μ M) resulted ineffective to obtain F_{max} , whereas 160 μ M digitonin (D) produced total saturation of the dye. As described by other authors (Kao *et al.*, 1989; Merrit *et al.*, 1990), $MnCl_2$ only partially quenched the fluorescence of the dye (*), so that Ca^{2+} chelation with 10 mM EGTA plus 20 mM Tris (E/T) at basic pH was preferred to obtain F_{min} .

with this conclusion, the results shown in Figure 7 show that mitochondria participates in buffering calcium loads entering across the plasma membrane in fluo3-loaded synaptosomes. As observed, calcium entry obtained by raising $[Ca^{2+}]_o$ in the absence or presence (Figure 7, A and B, respectively) of simultaneous K^+ -depolarization results in lower final $[Ca^{2+}]_i$ when mitochondria is functional with regard to calcium uptake than when it is not (i.e., in the presence of FCCP/oligomycin).

Conclusions

Calcium signals arising from interference with mitochondrial function have also been detected in other systems, including quin2- and fura2-loaded synaptosomes (Ashley *et al.*, 1984; Arslan *et al.*, 1985; Nachshen *et al.*, 1986; Duchen *et al.*, 1990; and see above). Moreover, Adams *et al.* (1985) and Hochner *et al.* (1989) have found evidence that in the squid giant synapse and in crayfish neuromuscular junction interference with mitochondrial function results in an increase in spontaneous neurotransmitter release, whereas evoked release is depressed; these effects are mediated by a rise in $[Ca^{2+}]_i$ and possibly by an increase in Ca^{2+} -dependent K^+ conductance, as shown in mouse dorsal rat ganglion neurons (Duchen, 1990). However, the relevance of these findings with respect to the contribution of mitochondria to calcium homeostasis has been questioned. In most cases it was unclear whether interference with ATP production, calcium entry from the external medium, or pH-dependent K_d changes of fluorescent dyes caused the calcium signals or their actions.

The results presented herein clearly show that brain mitochondria participates in calcium homeostasis in the synaptic terminal and disagree with the current belief that mitochondria can not take up substantial calcium *in vivo* under resting conditions. The behavior of intrasynaptic mitochondria, both in permeabilized and intact synaptosomes, indicates a more important role of mitochondria in the regulation of cytosolic calcium than that assumed until now. It is likely that spermine, spermidine, and adenine nucleotides together with yet unknown factors may be responsible for the high calcium affinity of mitochondria *in vivo*.

Buffering of calcium loads entering to the terminals is diminished when calcium uptake in intrasynaptic mitochondria has been abolished (Figure 7). It is interesting to note that under physiological electric stimulation of sympathetic neurons there is a rapid and inward $[Ca^{2+}]_i$ increase in the cell body, attaining near micromolar values in a short lapse of time, and that local nonlinearities of the signal can be clearly detected in the cytosol (Lipscombe *et al.*, 1988; Hernández-Cruz *et al.*, 1990), suggesting the existence of discrete buffering systems that perhaps arise from the presence of mitochondria. The increase in $[Ca^{2+}]_i$ is probably higher in presynaptic ter-

minals (Smith and Augustine, 1988; Adler *et al.*, 1991) due to the localization of calcium channels to these areas and the increase in the surface-to-volume ratio, and therefore, the calcium buffering capacity of intrasynaptic mitochondria may play an important role in presynaptic calcium homeostasis.

It would be interesting to know whether the role of mitochondria in calcium homeostasis is specific to the preparation we have used or may be more widely distributed. Recent work in rat dorsal root ganglia neurons (Duchen, 1990; Duchen *et al.*, 1990; Thayer and Miller, 1990) has revealed an important contribution of mitochondria to somatic calcium homeostasis and calcium-modulated phenomena. However, in other neuronal preparations, the participation of mitochondria in calcium homeostasis may be less prominent (compare the results in Thayer and Miller, 1990, with those shown in Thayer *et al.*, 1988) (Figure 6). Furthermore, the relative contribution of the calcium-regulating systems in different zones within the same neuron might vary, so that extrapolation of the experimental results obtained in one system should be done with caution. Present investigations in our laboratory indicate an important role of mitochondria in calcium homeostasis both in acutely dissociated neurons from rat hippocampus and primary cultures of rat cortical neurons (Villalba, Martínez-Serrano, Gomez-Puertas, Blanco, Bogónes, and Satrustegui, unpublished data), suggesting that this role is in fact not confined to the synaptic terminal.

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