Calcium Binding to the Cytosol and Calcium Extrusion Mechanisms in Intact Synaptosomes and Their Alterations with Aging*

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Alberto Martínez-Serrano‡, Pablo Blanco‡, and Jorgina Sartrústegui§

From the Departamento de Biología Molecular, Centro de Biología Molecular, Universidad Autónoma de Madrid, Consejo Superior de Investigaciones Científicas, Cantoblanco, 28049-Madrid, Spain.

A simple method to measure cytosolic calcium binding in intact presynaptic nerve terminals (synaptosomes) from rat brain, which is based on the simultaneous determination of \([Ca^{2+}]_i\) and total \([^{46}Ca^{2+}]\) in quin2-loaded synaptosomes undergoing a switch from high- to low-calcium containing medium, is presented. Binding to the cytosolic compartment alone was obtained following depletion of calcium storing organelles in the presence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone/oligomycin plus caffeine. Synaptosomes, as compared to various cells types, have a high calcium binding capacity to the cytosolic compartment; maximum binding, \(Ca^\cdot B_{max}\), was 4.76 mM and calculated \(s_{0.5}\) was 218 nM. Calcium binding to the cytosolic compartment as a function of aging was also determined; \(Ca^\cdot B_{max}\) was reduced to 1.84 nM and \(s_{0.5}\) increased to 492 nM in 30-month-old rats, indicating that the buffering of high calcium loads is impaired in old animals. The results obtained for binding of calcium to mitochondria and caffeine-sensitive calcium stores are consistent with an age-dependent reduction in calcium bound to mitochondria, whereas caffeine-sensitive calcium stores were unaffected. Finally, we have estimated the net rates of calcium extrusion in intact synaptosomes, and found that calcium efflux through the Na/Ca exchanger and \(Ca^{2+}\)-ATPase was markedly reduced in old rats.

Calcium is an important second messenger controlling many aspects of neuronal function and excitability, some of which associated with very rapid changes in cytosolic free calcium concentration. After the signal has ceased, it is necessary to normalize \([Ca^{2+}]_i\) to resting, prestimulation levels, in order to be able to respond to a new stimulus, and to avoid cytotoxic effects of a potential prolonged exposition to high calcium levels (1, 2).

The mechanisms for restoring \([Ca^{2+}]_i\) levels in neurons include both calcium extrusion at the plasma membrane (Na/Ca exchanger and \(Ca^{2+}\)-ATPase) and calcium sequestration in intracellular organelles (mitochondria and endoplasmic reticulum, ER) (see Refs. 3–5, for recent reviews), these mechanisms also being present in presynaptic nerve endings (3, 6–13).

The cytosolic compartment, i.e. that excluded from membrane-limited structures, also plays a crucial role in calcium homeostasis-related events, due to its dual ability to sense and transduce calcium-mediated signals and, on the other hand, to contribute to calcium sequestration by its intrinsic calcium buffering power. In the last years, a great effort has been made to identify and characterize various proteins that serve as calcium signal transducers. However, in order to understand the mechanisms governing calcium homeostasis as a whole, it is also important to know the calcium binding capacity of the cytosol, an important yet seldom studied factor, since calcium is largely complexed to ligands (inorganic and organic, including proteins and phospholipids) and thus, a very small fraction of cellular calcium is in the ionized free form (4).

In this work, we present a method to study calcium homeostasis in intact synaptosomes which is based on the parallel determination of synaptosomal \([Ca^{2+}]_i\) and \(^{46}Ca^{2+}\) bound to synaptosomes, that allowed us the dissection of different calcium binding compartments, and also from these data, the estimation of the true extrusion activities of plasma membrane systems for calcium efflux.

A second purpose of this work was to study calcium binding to the different synaptosomal compartments during aging. In the last few years, there has been increasing evidence of an altered calcium homeostasis in rat brain with advanced age, including reduced calcium transport through voltage-gated calcium channels (14) and decreased activity of plasma membrane \(Ca^{2+}\)-ATPase and Na/Ca exchanger (15), decreased calcium transport in mitochondria (16, 17) and an increase in cytosolic free calcium concentration in synaptosomes prepared from rat brain (18, 19). The relative importance of these modifications depends on the calcium binding capacity of the cytosolic compartment which may also be altered with aging. Therefore it was important to analyze any changes in calcium binding to the cytosol in old rats.

The main results obtained show that (i) the cytosol binds a considerable fraction of the total synaptosomal calcium in a physiological range of free cytosolic calcium ([\(Ca^{2+}]_i\)]; (ii) there is a great reduction in the calcium binding capacity of the cytosol in synaptosomes derived from old rat brain; (iii) in agreement with experiments with the isolated organelles.
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and lysed or permeabilized synaptosomes (3), the calcium binding capacity of the caffeine-sensitive subcompartment of ER is of minor importance when compared with that of the cytosol or mitochondria.

Once the calcium binding constants of synaptosomes (from 3- and 30-month-old rats) were known, it was possible to determine the true rate of calcium efflux from [Ca**(2+)**] progress curves, under conditions giving rise to net calcium loss. The results obtained indicate that the rate of calcium extrusion through the Na/Ca exchanger accounted for five to six times that through the Ca**(2+)-ATPase, under conditions where the maximum efflux was observed. On the other hand, aging resulted in a pronounced decrease in both Ca**(2+)-ATPase- and Na/Ca exchanger-mediated efflux, a result consistent with previous findings (15).

**EXPERIMENTAL PROCEDURES**

Adult (3 months) and old (30 months) healthy male Wistar rats were used in this study. Synaptosomes were isolated from total brain using discontinuous Ficoll gradients following the method of Booth and Clark (20) as modified in Ref. 16. The final pellet of synaptosomal protein was resuspended in (mM): NaCl, 145; KCl, 5; MgCl₂, 1; KPO₄/H₃PO₄, 0.4; glucose, 10; pyruvic acid, 2.5; malic acid, 2.5; Tris-HCl, 10, pH 7.4; and 50 μM quin2/AM, and 0.1% bovine serum albumin for quin2 loading as described before (18). After the loading period, synaptosomes were washed twice with the same medium devoid of quin2/AM and bovine serum albumin, and resuspended in 0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, at 20–30 mg of protein/ml, and stored on ice until used (never longer than 2 h). Internal quin2 concentrations ([QIT]) were the same in adult or old rat synaptosomal preparations, 1.1 mM, as reported earlier; the quality of the product obtained from hydrolysis did not vary with age (18).

Quin2-loaded synaptosomes were then used in parallel experiments to measure cytosolic free calcium concentration and total calcium ("Ca**(2+)**"). The experiments consist in a preincubation-depolarization-resperolization protocol as indicated in Fig. 1A, and the measurements were done during the repolarization period, where the calcium concentration was drastically reduced.

The composition of the preincubation medium was (mM): NaCl, 145; KCl, 5; MgCl₂, 1; KPO₄/H₃PO₄, 0.4; CaCl₂, 0.5; malic acid, 2.5; pyruvic acid, 2.5; Tris-HCl, 10, pH 7.4; variations from this during depolarization and repolarization are indicated in the figures. Calibration of quin2 fluorescence in terms of [Ca**(2+)]** was made at the end of each determination as described in (18). In all cases synaptosomes were preincubated in the presence of 20 μM TPEN to avoid differential quenching of quin2 and distinct heavy metal content of the synaptosomal preparations, from adult or old rats. The "Ca**(2+)**" content of synaptosomal suspensions was determined by a filtration technique that removes any external bound calcium (16). The total calcium content, [Ca**(2+)]**, may be defined as

\[
[Ca^{2+}]_{t} = [Ca^{2+}]_{r} + [Ca^{2+}]_{p}
\]

where [Ca**(2+)] and [Ca**(Q)] represent Ca**(2+)** bound to the synaptosomal calcium-binding sites and to quin2, respectively. This fraction of calcium bound to quin2 represents a considerable amount of total calcium-binding sites and to quin2, respectively. This fraction of calcium bound to quin2, calculated from the values of [Ca**(2+)**], [Kₐ], and quin2 (115 mM), and internal quin2 concentration ([QIT]), 1.1 mM (18), according to

\[
[Ca^{2+}] = \frac{[Ca^{2+}]_{r} + 1}{(1 + [Ca^{2+}]_{r}/K_{d})} \times [QIT]
\]

The synaptosomal volumes were 2.83 and 2.7  μl/mg of protein for adult and old rat brain synaptosomes, respectively (14).

The data obtained were fit by nonlinear regression to a simplified form of the Hill equation assuming a single class of identical calcium-binding sites,

\[
[Ca^{2+}] = \frac{([Ca^{2+}]_{r} + [Ca^{2+}]_{p})/(K'_{a} + [Ca^{2+}]_{p})}{[Ca^{2+}]_{t}}
\]

The overall goodness of each fit was evaluated in terms of its standard error, sum of squares, and correlation coefficient. The results for free parameters are given together with their standard errors; the confidence intervals shown in the figures are the result of the least favorable combination of the three standard errors of the parameters occurring in the fit. Clearly, these confidence intervals depend on the standard error of the estimation of the parameters, which is inversely proportional to the number of experimental data. For convenience, we have used only four time points, so that confidence intervals must be taken as relative, and always as an overestimation of the real situation. The values of the parameters n and K" obtained from the fits have no physiological relevance in terms of cooperativity.

For visual inspection of the calcium buffering capacity of intact synaptosomes and their cytosolic compartments, the derivative of Hill Equation 3 with respect to free cytosolic calcium,

\[
\frac{d[Ca^{2+}]_{t}}{d[Ca^{2+}]_{r}} = \frac{(n \times (K^{*} + [Ca^{2+}]_{r})^{n - 1})}{(K" + [Ca^{2+}]_{r})^{n}}
\]

was plotted versus free cytosolic calcium (Fig. 7, see "Discussion") using the parameter values obtained from the fits of bound calcium versus free cytosolic calcium.

Cytosolic pH measurements were performed using the fluorescent dye BCECF. Synaptosomes were loaded with the indicator by incubation with the cell permeant form, BCECF/AM (Molecular Probes) (20 μg/ml), exactly in the same way as described for fura2/AM (21). Excitation and emission wavelengths were 505 and 525 nm, respectively (both with ±3 nm bandwidth). To avoid interference from external dye during measurements, each sample was centrifuged (30 s, 12,000 × g) before assay. All experiments were carried out at 37 °C. To test the quality of the product obtained from the hydrolysis, synaptosomal BCECF was extracted with 100 μM digitonin and excitation and emission spectra were recorded at varying pH. As shown in Fig. 2A, fluorescence peaked at around 505 (excitation) and 525 (emission) nm, as expected from the spectral characteristics of the never esterified free acid form (22). Moreover, excitation maximum shifted from around 450 to 505 nm as pH increased. Signal linearity was assessed in a range of pH from 5 to 8; as may be seen (Fig. 2B), the dye behaved linearly with pH in the range 6.4–8. The quality of the de-esterified indicator did not vary between preparations of synaptosomes from different ages (3 or 30 months), according to the criteria mentioned before (see Fig. 2). Calibration of the signal was performed at the end of each run, by permeabilization of synaptosomal plasma membrane with 160 μM digitonin, which exposes the dye to the external pH (measured simultaneously with an Orion Research pH electrode). After stabilization of the signal in the presence of digitonin (around 30 s), different amounts of HCl were added so that the more acidic pH units versus nigericin + granicidin calibration procedure. However, we have followed the digitonin calibration because Nachshen and Drapeau (24) have reported that in synaptosomes from rat brain, this procedure allows a more accurate determination of pH, mainly at small loads of BICECF, as used in our experimental conditions.

**RESULTS**

**Determination of Calcium Binding to Intact Synaptosomes**—Fig. 1 summarizes the experimental approach used for the determination of the calcium binding constants. As shown in Fig. 1A, synaptosomes were preincubated in low K⁺ medium during 5 min so that appropriate ionic gradients could be generated (14) and then depolarized in an isosmotic medium in the presence of 2.3 mM CaCl₂ where the final [KCl] was 65 mM (10 min). After this depolarization period, a steady-state 4Ca**(2+)** accumulation was attained and a uniform labeling of total synaptosomal calcium pool may be assumed (not shown, but see Ref. 16).

After 10 min of depolarization, synaptosomes were repolarized by dilution in appropriate medium to obtain the desired
Fig. 1. Characterization of total calcium binding to intact rat brain synaptosomes. A, schematic diagram of the experimental procedure showing the successive changes in interesting ion concentrations during the preincubation-depolarization-repolarization protocol. For [Ca\(^{2+}\)]\(_i\), 0 means submicromolar calcium levels, as determined with quin2. B, fluorescence tracings from quin2-loaded synaptosomes from 3- and 30-month-old rats during the repolarization period; C and D, time courses of [Ca\(^{2+}\)]\(_i\), recovery and \(4\text{Ca}^{2+}\) efflux from quin2-loaded synaptosomes from 3-month-old rats after stimulation; E, bound calcium (calculated and corrected for Ca bound to quin2 as described under "Experimental Procedures") as a function of free cytosolic calcium in 3-month-old rats. The lines are the best fit to Equation 3. The parameters obtained were (parameter ± standard error): Ca-B\(_{\text{max}}\) = 7.35 ± 0.22 mM, n = 2.45 ± 0.26, K\(’\) = 0.032 ± 0.014 (correlation coefficient = 0.997); calculated \(a_0\) values was 246 nM. Confidence interval (shaded) was estimated as described. The results were obtained with six animals, each experiment was performed in triplicate. F, [Ca\(^{2+}\)]\(_i\), recovery (○) and \(4\text{Ca}^{2+}\) efflux (■) obtained with 30-month-old rats. Inset: bound Ca\(^{2+}\) versus [Ca\(^{2+}\)]\(_i\), (C). The parameters obtained were Ca-B\(_{\text{max}}\) = 2.32 ± 0.09 mM, n = 3.82 ± 1.05, K\(’\) = 0.004 ± 0.0007 (correlation coefficient = 0.992). Estimated \(a_0\) values was 294 nM. Confidence interval are also shown as a shaded area.

The final concentration of ionic species, 145 mM NaCl, and 5 mM KCl. In addition, this medium contained a high concentration of EGTA, 2 mM, to reduce final [Ca\(^{2+}\)]\(_i\), to submicromolar levels, while keeping the pH constant. As a result, a large calcium efflux from synaptosomes took place and the various calcium binding compartments became progressively desaturated. Under these conditions, net calcium efflux was principally due to passive efflux, since it was observed even in the absence of respiratory substrates and in the presence of metabolic poisons (1 mM KCN) (results not shown).

Fig. 1, B and C, show that [Ca\(^{2+}\)]\(_i\), returned to resting levels, of around 100–200 nM (18), after 7 min from the onset of repolarization, with a concomitant net loss in \(4\text{Ca}^{2+}\) content (Fig. 1D). Leakage of quin2 from synaptosomes during the 7-min duration of each experiment was estimated from the difference in external quin2 at the beginning and end of each run, and was found to be nondetectable both in 3- or 30-month-old rats. We have observed a decrease in internal pH of around 0.2 pH units (from 7.2 to 7.05–6.95) during these experiments (see "Experimental Procedures" and Fig. 2 for internal pH determinations). Fig. 1E shows the total Ca\(^{2+}\) associated with the synaptosome as a function of [Ca\(^{2+}\)]\(_i\), i.e. the calcium bound to the cytosol plus that located into organelles, mainly mitochondria and endoplasmic reticulum. Fig. 1F shows the result of a similar experiment carried out in 30-month-old rats, where a large decrease in Ca-B\(_{\text{max}}\) is observed.

In order to analyze the calcium binding capacity of the cytosolic compartment, calcium compartmentation in mitochondria and endoplasmic reticulum was avoided by carrying out similar experiments in the presence of FCCP and oligomycin (10 \(\mu\)M each mixture of FCCP/oligomycin), plus 10 mM caffeine. The use of FCCP together with oligomycin collapses any proton electrochemical gradient (\(\Delta\mu_{\text{H}^+}\)) across intrasynaptosomal compartements and prevents ATP run down by the mitochondriat ATPase, thus blocking any \(\Delta\mu_{\text{H}^+}\)-dependent calcium accumulation in intrasynaptosomal membrane limited structures. After blocking Ca\(^{2+}\) uptake in \(\Delta\mu_{\text{H}^+}\)-dependent calcium stores and in the caffeine-sensitive subcompartement of endoplasmic reticulum, the remaining Ca\(^{2+}\) is largely associated with cytosolic binding sites, since the presence of nonmitochondrial caffeine resistant calcium pools (i.e. 1,4,5-inositoltrisphosphate-sensitive one) in synaptosomes is controversial (Ref. 21, but see, Ref. 25).

The experiment was carried out in synaptosomes from 3- and 30-month-old rats and the results are illustrated in Fig. 3. As shown in panels A and B of Fig. 3, in the presence of FCCP, oligomycin, and caffeine, the individual [Ca\(^{2+}\)]\(_i\), values obtained are only relatively smaller than those of the control situation (Fig. 1C), while there is a great reduction in total \(4\text{Ca}^{2+}\) accumulation (compare Fig. 3A with 1C and Fig. 3B with 1D; note the difference in scales). Leakage of quin2 during the experiment was not affected by drug additions. The representation of calcium bound to the cytosol as a function of free internal calcium is shown in panel C. The parameters obtained from the fit of the data to Hill Equation 3, were: 3 months, Ca-B\(_{\text{max}}\) = 4.76 ± 0.04 mM, n = 3.88 ± 0.04, K\(’\) = 0.0027 ± 2.3–10\(^{-5}\); 30 months, Ca-B\(_{\text{max}}\) = 1.64 ± 0.25 mM, n = 3.09 ± 0.54, K\(’\) = 0.1119 ± 0.08. The calculated...
sO.6 values were 218 and 492 nM for adult and old animals, respectively. These data show that there is a great reduction in cytosolic Ca-Bmax and an increase in sO.6 in synaptosomes derived from 30-month-old rats, as compared to those of control (3-month-old) animals.

As it has been reported (26), the affinity for calcium of fluorescent Ca-sensitive dyes, such as quin2, depends, among other factors, on cytosolic pH, the affinity being increased as pH increases and vice versa. Under the experimental conditions used in this work, we have found no differences in cytosolic pH of synaptosomes from 3- or 30-month-old rats (6.89 ± 0.02, n = 14, and 6.90 ± 0.04, n = 6, respectively) and only a transitory effect of FCCP (Fig. 2) so that the measurement of cytosolic calcium using the protocol described in the text and Fig. 1, is not influenced by any change in pH.

Calcium Binding by Intrasympotic Organelles—While the study of calcium binding to the cytosol may be carried out in a direct way as shown above, the contribution of the calcium sequestering organelles can only be deduced after inhibiting their capacity to accumulate calcium. Thus, calcium binding to intrasympotic sellordiata may be inferred from the difference between calcium binding in synaptosomes incubated in the presence of caffeine alone (where mitochondria is operating) and FCCP/oligomycin plus caffeine (where neither mitochondria nor caffeine-sensitive calcium stores are operating, a condition representing the cytosolic compartment). On the other hand, binding to caffeine-sensitive calcium stores can be deduced from the data obtained in the presence of FCCP/oligomycin when compared with those of the cytosolic compartment. By employing conditions under which only one of the two organelles was operating at the time (i.e. mitochondria or caffeine-sensitive ER) the problem arising from calcium redistribution between the organelles can be avoided.

The experiments and subsequent calculations are shown in Fig. 4. It may be observed that calcium binding to mitochondria is clearly detected at [Ca2+]i below 0.2 μM, both in 3- and 30-month-old rats. This suggests that calcium accumulation in mitochondria occurs at calcium concentrations similar to those found under resting conditions (18), in agreement with other results from this laboratory. As shown in Fig. 4B, the maximum binding, Ca-Bmax, was only slightly smaller than that of the cytosol (Fig. 3C). Interestingly, synaptosomes from old rats showed a 4-fold reduction in Ca-Bmax (from 3 to 0.7 mM in 3- and 30-month-old rats, respectively, Fig. 4B).

The maximum binding to the caffeine-sensitive subcompartment of the ER in synaptosomes of 3-month-old rats was calculated to be in the range of 1 mM. This was not changed in old rats; its apparent (estimated by eye) sO.6 was 100–200 nM.

In Situ Estimation of Calcium Efflux Rates Through Na/Ca Exchanger and Ca2+ATPase Across the Synaptosomal Plasma Membrane—Once the calcium binding capacity of the intact synaptosome was known, it was possible to estimate the net rates of calcium efflux from the time course of [Ca2+]i, variation in quin2-loaded synaptosomes. Efflux rates were studied

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Determination of calcium binding constants of cytosolic Ca-binding sites in intact synaptosomes from adult (○) or old (●) rat brain. The drugs FCCP (10 μM), oligomycin (10 μM), and caffeine (10 mM) were present from the beginning of the depolarization period (see Fig. 1A). A and B, time courses of [Ca2+]i and 4Ca2+-content during depolarization. C, cytosolic bound calcium versus cytosolic free calcium concentration. The parameters obtained from the fit are shown in the text. The results were obtained with six or four animals, 3 or 30 months, respectively, and each experiment was performed in triplicate. *Shaded areas represent the confidence intervals for the fit, as explained under "Experimental Procedures." For comparison, plots of total calcium binding obtained from Fig. 1 are shown (dotted lines).
Fig. 4. Estimation of calcium binding to synaptosomal organelles. The experimental conditions are the same as described in the legend to Fig. 3, except that 10 mM caffeine or 10 μM FCCP/oligomycin (O) were added separately, as indicated. A, time courses of [Ca++]i decay for adult (O) and old (●) rat brain synaptosomes. B, estimation of calcium bound to mitochondria and to caffeine-sensitive subcompartment of ER. [Ca-B]caffeine and [Ca-B]FCCP/oligomycin plus caffeine and have been plotted against [Ca++]i, and the data were fit to Hill Equation 3 as described in the legend to Fig. 1. Then calcium bound to mitochondria at any [Ca++]i, expressed as [Ca-B]caffeine−[Ca-B]caffeine+FCCP/oligomycin plotted versus [Ca++]i. Similarly, calcium bound to caffeine-sensitive ER was expressed as [Ca-B]FCCP−[Ca-B]FCCP/oligomycin versus [Ca++]i. Due to the calculation procedure these four curves do not have equations by themselves. To illustrate the range of error in this estimation, individual S.E. of Ca-B data at discrete [Ca++]i measured are shown. The hinked curve obtained for calcium bound to mitochondria in young rats results from a slight variation in the slopes of the binding data in the presence of caffeine or FCCP/oligomycin plus caffeine and has no special meaning. The age in months is indicated for each curve.

after a preincubation-depolarization protocol similar to that described above, followed by a switch to a low calcium medium (total calcium and EGTA concentrations were 175 μM each, which consistently yielded micromolar (2–4 μM) external free calcium estimated with acid fluo 3) under conditions of repolarization or sustained depolarization in sodium- or choline-based media, where net calcium efflux takes place. The change in [Ca++]i, observed as a decrease in quin2 fluorescence, represents active calcium extrusion, since it was blocked by metabolic poisoning (absence of respiratory substrates and presence of 1 mM KCN, results not shown) (note the difference in the efflux experiments here and those used for the determination of intrinsic calcium binding constants). Cytosolic pH was 6.94 in choline- or 7.12 in sodium-based media; since this difference did not change the Kd value of quin2, the absolute [Ca++]i, values have not been modified.

Fig. 5 summarizes the results obtained with synaptosomes from 3- and 30-month-old rats. As shown in Fig. 5A, the return of [Ca++]i, to resting values was faster in sodium- than in choline-based media, due to the concerted operation of both Na/Ca exchanger and Ca++-ATPase. The electrogenic properties of the exchanger (27, 28) would reduce its function to extrude calcium under depolarization. However, due to the low calcium content of the milieu, the driving force for calcium of the exchanger is close to zero or favors calcium extrusion depending on [Na+]. Replacement of sodium by choline during the efflux period (final sodium concentration 7.57 mM) resulted in an additional reduction of calcium efflux (Fig. 5A). Moreover, depolarization had no effect on net rate of [Ca++]i, recovery in choline-based media (Fig. 5A), indicating that the Na/Ca exchanger does not operate to extrude calcium under these conditions, as reported by Sánchez-Armass and Blaustein (8). It may be observed that the cooperation of the two transport systems is necessary to achieve prestimulation [Ca++]i, levels.

Fig. 5B shows the time course of [Ca++]i, recovery in synaptosomes from adult and old rats, under repolarization conditions in sodium- or choline-based media. The rate and extent of [Ca++]i, recovery was much lower in choline-based media (Ca++-ATPase alone) than in sodium media (Ca++-ATPase and Na/Ca exchanger) in both age groups. Fig. 5B also shows that in sodium-based media the return to resting [Ca++]i, is significantly slower in 30-month-old animals than in adults, in particular at the beginning of the repolarization.

The total calcium content associated with synaptosomes in the course of these experiments were obtained from the [Ca++]i, values (Fig. 5) with the use of the function that describes calcium binding to synaptosomes in the absence of inhibitors (Fig. 1, E and F). The results are shown in Fig. 6A. The maximum rates of calcium efflux observed under these conditions (i.e. initial slopes of the calcium efflux plots) are again greater in sodium- than in choline-based media for both ages. The data obtained in the first condition, representing the Ca++-ATPase plus Na/Ca-mediated calcium efflux were (nanomole of Ca++/mg of protein·min): 10.62 (3 months) and 2.87 (30 months); for the second condition, where the Na/Ca exchanger was not operating, the corresponding values were
A, providing different efflux rates by substituting sodium for choline chloride in repolarization or sustained depolarization. This gave rates of calcium efflux through the Na/Ca exchanger was about 6 times higher than that through the pump in 3-month-old rats, but 1.6 times lower than the efflux through the calcium pump in 30-month-old rats. This is mainly due to a 90% loss of the pump activity in old animals.

These conclusions are illustrated in Fig. 6B, which shows the derivative of total calcium content with respect to time (obtained from the data in Fig. 6A), expressed as a function of cytosolic calcium at each time (data from Fig. 5B). As may be observed, the difference between dCa/dt in sodium- and choline-based media attained a greater value in adult than in old rats, at all [Ca2+]i values.

**DISCUSSION**

**Calcium Binding Capacity of Synaptosomal Compartments**—Our results show that the in situ determination of bound calcium as a function of free calcium may be used to estimate the calcium binding capacity of intact synaptosomes. In this paper we report a simple methodological approach to estimate calcium binding (Fig. 1), which is based on the computation of total and free calcium concentrations in parallel determinations, and assuming a spatial uniformity of [Ca2+]c signals and 45Ca labeling of the calcium pool. Quin2 was chosen for these experiments because this probe had already been used in studies of cytosolic calcium binding (see below) and because under our experimental conditions the change in relative fluorescence of the probe was larger than those of other calcium indicators (fura2, fluo3). Moreover, unlike fura2, quin2 does not accumulate in intracellular organelles.

It could be argued that the computation of total and free calcium concentrations in our experiments does not truly quantify the intrinsic properties of intracellular buffering but may reflect the specific conditions of our efflux experiments. On the other hand, an accurate binding quantification should be independent on the efflux conditions and efflux rates. To test the validity of the binding assay we have measured total and free calcium concentrations in efflux experiments identical to those described in Fig. 3 (i.e. in the presence of 2 mM EGTA, caffeine, FCCP, and oligomycin) under conditions providing different efflux rates by substituting sodium for choline chloride in repolarization or sustained depolarization conditions. This gave rates of calcium efflux (both 45Ca and [Ca2+]i) in the rank order sodium > choline (repolarization) > choline (depolarization). The curves of [Ca-B] versus [Ca2+]i, obtained in each case were the same as obtained previously (but with more data points in the upper range of the curve for [Ca2+]i) and the parameters were also the same: choline, repolarization — Ca-Bmax = 4.384 ± 0.138 mM, s0.5 = 171 nM; choline, depolarization — Ca-Bmax = 4.688 ± 0.248 mM s0.5 = 127 nM. Therefore, the results obtained are independent on the efflux conditions used and truly reflect the intrinsic binding capacity of the cytosol.

The role of the cytosolic compartment in the maintenance of calcium homeostasis is still poorly understood. The results obtained (Fig. 3) indicate that the concentration of cytosolic calcium-binding sites in synaptosomes, Ca-Bmax, is within the millimolar range (4.76 mM), and about 5–10 times larger than that reported for human platelets (32), neutrophils (31), and skeletal muscle (30). This high calcium binding capacity of synaptosomes may result from the characteristics of this preparation, where the surface-to-volume ratio is particularly high, and is thus enriched in membranes which are known to have a high calcium binding capacity (33). On the other hand, it should be pointed out that presynaptic terminals undergo high frequency stimulation and subsequent calcium entry so that a particularly high concentration of cytosolic calcium-binding sites could be a specific characteristic of these structures to protect them from the cytotoxic effects of calcium overloads.

We have found that the apparent affinity or s0.5 for calcium of these binding sites is around 200 nM, very similar to that found in other systems (140–550 nM) (31, 32). This calcium concentration is frequently attained in physiological conditions upon stimulation of the terminal and it corresponds to the free calcium concentration where the cytosol has its greater calcium buffering capacity. For a better understanding of the calcium binding capacity meaning and its importance, the derivative of bound calcium versus free internal calcium was calculated (Fig. 7). As observed, the larger variation in bound calcium as a function of the increase in free calcium takes place at [Ca2+]i, around 0.1 μM for the whole synaptosome (curves labeled as "total") and around 0.175 μM for the cytosolic compartment (other words, the existence of a calcium binding activity in the cytosol with such a s0.5 value and with a low saturation fraction at resting [Ca2+]i (around 5–10%), provides the basis for an effective buffering of calcium loads. Moreover, the relatively high concentration of these sites (Fig. 3) could make them of great importance in the buffering of calcium entering through the plasma membrane, specially at high frequency stimulation.

In this context it is important to note the large variation in

![FIG. 6. Estimation of total calcium efflux in synaptosomes. A, calculations were done from data on Figs. 1 and 4, as explained in the text. Open symbols, 3-month-old rats; closed symbols, 30-month-old rats. Circles, sodium-based media; squares, choline-based media. B, plot of −dCa/dt versus [Ca2+]i, from data on Fig. 5B and 6A. Conditions are expressed in the figure.](image-url)
calcium binding capacity found in synaptosomes derived from 30-month-old rats (Figs. 1, 3, and 7). Thus, Caₐ₋max decreases while s₀,ₐ increases by a factor of 2.3–2.5. The combined effects of decreased affinity and capacity for calcium binding implies a lower capacity for calcium binding as [Ca²⁺] increases (Fig. 7), suggesting that cytosolic calcium buffering at high calcium loads is severely impaired in old animals. Since effective buffering of intracellular free calcium during periods of neuronal excitation is crucial to cell survival (2), this defect probably increases neuronal vulnerability in old animals.

It has been recently reported that the expression of the brain cytosolic 28-kDa calcium-binding protein calbindin (34, 35) is reduced during rat aging (36) in specific brain areas, including cerebellum and corpus striatum. Our results may indicate that the decrease in calcium binding activity of the cytosolic compartment observed during rat aging could be associated with reduced calbindin expression.

The calcium binding capacity of the mitochondrial compartment is greater than that of the caffeine-sensitive ER, in agreement with results obtained in permeabilized synaptosomes (6, 37). The maximum calcium binding to mitochondria decreased with age so that the quotient between that and Caₐ₋max in the cytosol decreased from 0.65 to 0.38, in 3- and 30-month-old rats, respectively, a result that agrees with previous findings (16, 38). An impairment in the activity of the calcium uniporter of mitochondria appears to underlie this defect (16, 39). The maximum value of calcium bound to the caffeine-sensitive ER found here (around 1 mM, 2.7 mmol of Ca²⁺/mg of protein) was not varied during aging and equaled that of the ATP-dependent calcium pool found in permeabilized synaptosomes (6).

Tosomes (6, 8, 13, 35) are hampered by the loss of the cytosolic calcium pools (21), these results cannot reflect true binding constants since: (i) they were obtained (5 s), there is an instantaneous “back-flow” or calcium re-entry and conclusions about true efflux rates are not warranted.

The procedure used in this work is based on the knowledge of the characteristics of calcium binding to synaptosomes to calculate the rate of the extrusion systems from observed [Ca²⁺], decline (Fig. 5). The rates of calcium efflux observed under our experimental conditions, 9.14 and 1.48 nmol of Ca²⁺/mg of protein-min for the exchanger and Ca²⁺-ATPase, respectively, are within the range of those reported by other authors (7, 8, 11, 41), both in intact synaptosomes and synaptic vesicles.

With respect to the relative physiological importance of the two transport systems, our results support those of Sánchez-Armass and Blaustein (8), indicating that the concerted operation of Na/Ca exchanger and Ca²⁺-ATPase is required for an efficient recovery of pre-stimulation [Ca²⁺], levels, after a long-lasting depolarization, since this is markedly impaired in Na-free media (Fig. 5B).

Our results show that aged rats have lower rates of calcium extrusion both through the Na/Ca exchanger and the Ca²⁺-ATPase. Michaelis et al. (15) have found a decrease in the Vₐ₋max of the Ca²⁺-ATPase and an increase in the Kₐ of the Na/Ca exchanger in synaptic membranes from 23–25-month-old rats, that probably underlies these defects. It appears that the increased [Ca²⁺] found in old rats (Fig. 5) do not compensate for the change in Kₐ of the exchanger (15). However, Ca²⁺-ATPase-mediated calcium extrusion is still more affected by age even though the decrease in Vₐ₋max observed in vitro was relatively small (15); it would be interesting to know whether endogenous factors such as calmodulin and other Ca-binding proteins may be responsible for the increased defect observed in in situ versus in vitro experiments.

The defects in calcium extrusion added to those in cytosolic calcium binding may help to explain the alterations in calcium homeostasis during aging. Key questions relate to the role of specific Ca-binding proteins in the defect in cytosolic calcium binding and calcium extrusion through Na/Ca exchanger and Ca²⁺-ATPase. Further studies are in progress addressing these points.

REFERENCES
Calcium Binding to the Cytosol in Synaptosomes and Aging