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THE REGULATION OF CYTOSOLIC CALCIUM IN RAT BRAIN SYNAPTOSOMES BY SODIUM-DEPENDENT CALCIUM EFFLUX

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SUMMARY

1. When pinched-off presynaptic nerve endings (synaptosomes) isolated from rat brain are incubated in a low-Na (24–36 mM) medium, they take up ^{45}Ca in a time-dependent manner. In a medium containing 1 mM-Ca, this Na-dependent ^{45}Ca uptake amounts to ~10 nmol/mg protein at 1 min, and to ~40 nmol/mg protein at 20 min. The Na-dependent Ca uptake is not reduced when the synaptosomes are loaded with concentrations of quin 2 as high as 2 mM.

2. The increase in ^{45}Ca uptake is paralleled by an increase in the free cytosolic Ca concentration $[Ca]_i$, as monitored with the fluorescent Ca indicators quin 2 or fura 2. $[Ca]_i$ increases from the value of ~ 200 to ~ 500 nM within 3–5 min, and thereafter, remains at this elevated level.

3. When synaptosomes that have been loaded with ^{45}Ca (for 1 min, in a low-Na medium) are diluted into an Na-containing medium, there is a rapid efflux of the Ca load. After correcting for Ca that is taken up during the efflux period, calculations show that the total Ca in the synaptosomes returns to the control level within 1 min. Measurements of total chemical Ca parallel the measurements made with radiotracer Ca, and confirm that the Ca loaded into the nerve terminals during a 5 min incubation in a low-Na medium is extruded from the nerve terminals within 1 min in a normal-Na medium.

4. The efflux of Ca from the synaptosomes is paralleled by a drop of $[Ca]_i$ to its basal level, also within 1 min.

5. The mitochondrial uncoupler, carbonyl cyanide *p*-trifluoromethyloxy-phenylhydrazone (FCCP, 1 μM), has no effect on either Na-dependent Ca uptake or efflux in synaptosomes. FCCP causes a slight (100–200 nM) increase in $[Ca]_i$ in synaptosomes resuspended in either a Na or a low-Na medium. This indicates that little of the Ca that is taken up by the synaptosomes in a low-Na medium is sequestered by the mitochondria.

6. These results suggest that Na-dependent Ca efflux (probably Na–Ca exchange) plays an important role in allowing nerve terminals to recover rapidly from a Ca load.

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INTRODUCTION

Two mechanisms probably account for the long-term ability of nerve terminals to maintain free cytosolic Ca concentration ($[Ca]_i$) at low levels, both at rest, and following nerve-terminal activity. One mechanism is a Ca-ATPase or Ca 'pump' (Gill, Grollman & Kohn, 1981), and the other is a Na–Ca exchanger (Blaustein, 1984). In this study we examine the ability of Ca-loaded nerve terminals to extrude Ca and to lower $[Ca]_i$. Net movement of Ca is monitored by measuring ^{45}Ca uptake and efflux in independent, parallel experiments. In other corresponding experiments, $[Ca]_i$ is directly monitored using the intracellular Ca indicators, quin 2 and fura 2. We find that synaptosomes are able to extrude almost all of a Ca load in a Na-dependent manner, within 1 min. This is also the time required for $[Ca]_i$ to return to control level.

METHODS

Preparation of synaptosomes

Synaptosomes were prepared from rat forebrains by a modification (Krueger, Ratzlaff, Stricharz & Blaustein, 1979) of the method of Hajos (1975). In brief, the 0·8 M (nerve-terminal enriched) fraction of a sucrose gradient was equilibrated by the gradual addition of ice-cold Na solution that contained (mM): NaCl, 145; KCl, 5; $MgCl_2$, 3; glucose, 10; diethylenetriamine pentaacetic acid (a chelator of heavy metals), 0·001; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 10; pH adjusted to 7·4 with NaOH. The diluted suspension was centrifuged, and the pellet was resuspended in Na solution containing 1 mM-Ca. The diluted synaptosome suspension was warmed at 30 °C for 20 min before proceeding with the experiments.

Determination of $[Ca]_i$

The level of $[Ca]_i$ in synaptosomes was determined using the fluorescent Ca indicator, quin 2, as described previously (Nachshen, 1985*a*). In brief, 50 μM of the acetoxymethyl ester derivative of quin2 (quin2/AM; Lancaster Synthesis Ltd., Windham, NH, U.S.A.) was added to the resuspended synaptosomes. After 30 min at 30 °C, the suspension was diluted with 20 vol. of fresh Na solution, and centrifuged. The pellet was resuspended in 1·5 ml of Na solution, and maintained at 30 °C. For measurement of fluorescence, 50 μl of the stock synaptosome suspension was diluted with 1·5 ml of Na solution, and centrifuged for 45 s in a Beckman Microfuge. The pellet was resuspended in 100 μl Na solution and added to 2 ml of normal-Na or low-Na solution in a quartz cuvette. The composition of the low-Na solution was identical to that of the normal-Na solution, except that the NaCl was replaced with choline Cl or *N*-methyl glucamine Cl, and the pH was adjusted with Tris buffer instead of NaOH.

Fluorescence was measured with a Farrand System 3 spectrofluorometer. Excitation and emission wavelengths of 339 nm (2 nm slit) and 520 nm (10 nm slit) were employed. The excitation light was filtered with a 340 nm transmission filter, and the emission light was filtered with a cut-off filter (50% transmission at 506 nm). The temperature of the sample was maintained constant by a water jacket around the cuvette holder, and the sample was stirred with a magnetic stirrer.

The free-Ca concentration in the synaptosomes is related to fluorescence intensity (F) by the following equation (Tsien, Pozzan & Rink, 1982):

$$[Ca]_i = K_D \times (F - F_{min})/(F_{max} - F), \quad (1)$$

where F_{min} is the Ca-independent fluorescence, measured with [Ca] < 1 nM, F_{max} is the maximal quin 2 fluorescence measured with [Ca] $\geqslant$ 1 mM, and K_D is the apparent quin-2–Ca dissociation constant ($\sim$130 nM at 30 °C; Nachshen, 1985*a*).

After the fluorescence of a quin 2-loaded synaptosome sample was measured, 10 mM-EGTA was added to the cuvette, along with sufficient Tris to raise the external pH to 9·0 or more. This addition caused an immediate drop in the fluorescence signal due to the stripping of Ca from any quin 2 in

the suspension that was not inside the synaptosomes, and enabled us to estimate the quin 2 leak. In general, this leak amounted to no more than 4–8 % of the total quin 2 in the sample. Next, 100 μM of digitonin was added to permeabilize the synaptosome plasma membrane, thereby allowing F_{min} to be determined. Finally, 11 mM-Ca was added, to saturate the quin 2, and allow F_{max} to be estimated.

In all experiments, synaptosome autofluorescence was determined, with control, 'non-loaded' synaptosomes, that were prepared in parallel with the quin 2-loaded batch.

In some experiments the novel Ca indicator fura 2 was used. Loading with fura 2/AM (Molecular Probes, Junction City, OR, U.S.A.) proceeded as described for quin 2, except that a loading concentration of only 2·5 μM was employed. Fluorescence was measured with an excitation wavelength of 325 nm (5 nm slit) and an emission wavelength of 520 nm (10 nm slit). Eqn. (1) was used to calculate $[Ca]_i$ with K_D having a value of 240 nM (Grynkiewicz, Poenie & Tsien, 1985).

^{45}Ca loading and determination of ^{45}Ca efflux

All Ca radioflux measurements (unless stated otherwise) were made using quin 2-loaded synaptosomes (see above). Aliquots of the synaptosome suspension, typically 50 μl, were ejected into tubes containing 200–300 μl Na-free solution with 1 mM-Ca, and 0·5–1 μCi of ^{45}Ca, to induce Ca loading (see below). The tubes with radiosotope were vigorously vortexed during the addition of the synaptosomes to insure effective mixing. After 1 min, uptake of radiotracer was stopped by the addition of 4·5 ml ice-cold Na-free solution containing 1 mM–EGTA and no added Ca (quench solution), followed by filtration (Schleicher & Schuell No. 25 glass-fibre filters). The filters were immediately rinsed twice with additional aliquots of quench solution. Radioactivity retained on the filters was determined using standard liquid scintillation spectroscopy techniques, and this served as a measure of the Ca load taken up by the synaptosomes. Efflux was determined by loading the synaptosomes with ^{45}Ca as described above, and then adding 1·5 ml of either Na or Na-free solution (30 °C). Efflux of ^{45}Ca was allowed to proceed for timed intervals, ranging from 4 s to 5 min. At the end of the efflux intervals, the solutions were rapidly filtered and the filters rinsed twice with quench solution. The amount of ^{45}Ca retained by the synaptosomes as compared to the initial Ca load was taken as a measure of efflux.

Determination of ^{45}Ca uptake

In experiments designed to measure ^{45}Ca uptake during the efflux intervals, 20 μl aliquots of the synaptosome suspension were added to 80–100 μl of the Na-free solution as above, except that ^{45}Ca was omitted from the medium. The mixture was then diluted with 2 ml of Na or Na-free solution containing ^{45}Ca (1 μCi/μmol). At time intervals that corresponded to the time intervals used to determine efflux, the suspensions were filtered, and the filters were rinsed with four aliquots of quench solution, as described above. The ^{45}Ca retained by the filters was then determined.

In another series of experiments, in order to measure the time course of ^{45}Ca uptake in normal- or low-Na media, an aliquot of the synaptosome suspension was added to Na-free solution containing ^{45}Ca. After timed intervals ranging from 1 s to 20 min, uptake was stopped by the addition of quench solution, and filtration, as described above.

All samples were done in replicates of three to five. Values are given as means ± the standard errors of the means. Protein samples were taken from the suspension of synaptosomes in each experiment, and protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). In all experiments, tissue blanks were determined, by adding the synaptosome suspension to a medium with ^{45}Ca containing an aliquot of quench solution. The tissue blank values have been subtracted from the data presented in this manuscript.

Determination of total chemical Ca

Aliquots of synaptosome suspension (in normal-Na solution with 1 mM-Ca) were pipetted into microfuge tubes, and pelleted by centrifugation for 1 min (10000 *g*). The supernatant was removed, and the synaptosomes were resuspended in 1 ml of normal- or low-Na solution containing 1 mM-Ca, for various times. Transmembrane Ca movement was terminated by the addition of 0·5 ml ice-cold Na solution without Ca, containing 3 mM-$LaCl_3$. The samples were centrifuged, the supernatant was discarded, and the samples were resuspended in the Na solution with $LaCl_3$. After a final centrifugation, the pellets were resuspended in a solution consisting of 1 % La (as the chloride), 2 % HCl and 1 % Triton X-100, and left overnight. The Ca content of the samples was subsequently

determined with a Perkin-Elmer 457 Atomic Absorption Photometer. In some cases, prior to the addition of the La solution, the low-Na samples were centrifuged and resuspended in normal-Na solution. After either 1 or 5 min, Na solution with La was added, and the samples were processed as above.

Theory: Ca influx and efflux

Consider an experiment in which Ca is present both inside and outside the synaptosomes. We are interested in establishing the magnitude of the Ca fluxes by measuring intrasynaptosomal concentrations of ^{45}Ca.

The experiment is performed twice, first with the radiocalcium inside the synaptosome (A), and secondly with the radiocalcium outside the synaptosome (B), as starting conditions. It is clear that throughout the course of each experiment there will be mixing of 'hot' and 'cold' Ca, and both influx and efflux of each species. Nevertheless, it is also clear that the synaptosomal concentration of chemical Ca, as well as chemical unidirectional fluxes at each point in time, will be identical for both experiments A and B.

It is assumed that in the efflux (A) experiment there is some cold Ca within the synaptosomes, so that at $t = 0$:

$$c_A = rc_A^*, \tag{2}$$

where c_A and c_A^* are the cold and hot Ca concentrations, respectively, inside the synaptosomes in the A experiment, and r is a proportionality constant. Then, for all times (t):

$$c_A = rc_A^* + c_B^*, \tag{3}$$

where c_B^* is the concentration of hot Ca in the uptake (B) experiment. The total Ca concentration in the nerve terminals, $c_{A,T}$, will be equal to the sum of the hot and the cold Ca concentrations. Therefore:

$$c_{A,T} = c_A^* + c_A = (1+r)\, c_A^* + c_B^*. \tag{4}$$

The initial pool of cold Ca is depleted in parallel with the hot pool; efflux of total (chemical) Ca (J_T^e) may then be represented by:

$$J_T^e = J_A^e + J_A^{e*}, \tag{5}$$

where J_A^e and J_A^{e*} are the efflux of cold and hot Ca, respectively. Since

$$J_A^e = J_A^{e*} \times c_A / c_A^*, \tag{6}$$

eqn. (5) can be rewritten as:

$$J_T^e = J_A^{n*}[1 + r + c_B^*/c_A^*], \tag{7}$$

where J_A^{n*} is the measured net rate of ^{45}Ca efflux from the synaptosomes, and is equivalent to J_A^{e*} (since ^{45}Ca re-entry is negligible in this experiment). The influx of total Ca into the synaptosomes, J_T^i, is formally equivalent to the influx of hot Ca in the B experiment,

$$J_T^i = J_B^{i*} = J_B^{n*} - J_B^{e*}, \tag{8}$$

where J_B^{n*} is the measured rate of ^{45}Ca uptake in the B experiment, and J_B^{e*} is the efflux of ^{45}Ca in the same experiment. Since:

$$J_B^{e*} = J_A^{e*} \times c_B^*/c_A^*, \tag{9}$$

eqn. (8) may be rewritten as:

$$J_T^i = J_B^{n*} - J_A^{n*} \times c_B^*/c_A^*, \tag{10}$$

Combining eqns. (7) and (10), we obtain the net flux of total (chemical) Ca in the synaptosomes:

$$J_T^n = J_T^i + J_T^e = J_A^{n*}(1+r) + J_B^{n*}. \tag{11}$$

RESULTS

Na-dependent increase in Ca uptake and in $[Ca]_i$

When synaptosomes are incubated in a normal-Na medium (containing 1 mM-^{45}Ca) there is a time-dependent uptake of radioisotope (Fig. 1, filled circles). Uptake is greatly enhanced if most of the Na in the solution is replaced with either choline or *N*-methyl glucamine (Fig. 1, filled triangles). This extra uptake appears to be

mediated by a Na–Ca exchanger working in the reverse mode (Blaustein & Oborn, 1975), similar to that found in many different types of cells (Blaustein, 1984). Recently, it has been reported that cytosolic quin 2, at concentrations as low as 150 μM, selectively inhibits Na-dependent Ca uptake in squid axons (Allen & Baker, 1985). Since in our experiments we wished to compare Na-dependent Ca uptake with

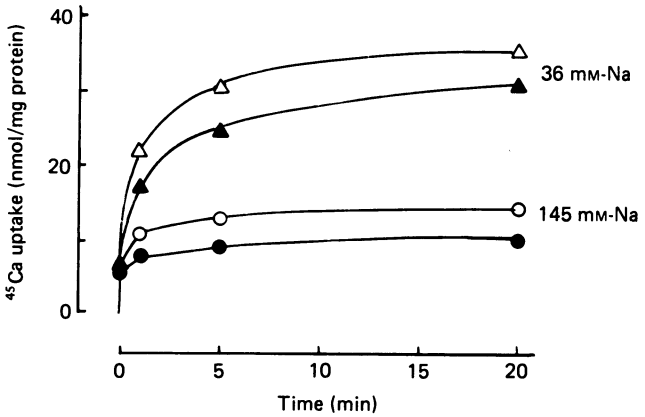

Fig. 1 The time course of Ca uptake in normal-Na (145 mM; ○, ●) or in low-Na (36 mM; △, ▲) solution. The experiment was performed using either normal synaptosomes (filled symbols), or synaptosomes that had been loaded with 50 μM-quin2/AM (open symbols), as described in the Methods section. The quin 2 content of the synaptosomes was spectrofluorometrically determined as 6 nmol/mg protein.

Na-dependent changes in $[Ca]_i$ monitored with quin 2, we examined whether quin 2 inhibited Ca uptake in synaptosomes. Fig. 1 shows that quin 2 has no inhibitory effect on Ca uptake in either normal- or low-Na media in synaptosomes. Instead, it causes a slight increase in the Ca uptake, that can be attributed to the extra Ca buffering capacity added to the nerve terminals by the intracellular quin 2. In this experiment, the amount of quin 2 taken up by the nerve terminals amounted to $\sim$ 6 nmol/mg protein, roughly twice the amount of the extra Ca uptake induced by the quin 2, as would be expected, if approximately half the quin 2 were associated with Ca ($[Ca]_i \simeq K_D$ for quin 2).

The Na-dependent Ca uptake is initially paralleled by an increase in cytosolic Ca, monitored with quin 2 (Fig. 2, filled triangles and circles). When synaptosomes are resuspended in a low-Na (24 mM) medium, there is a time-dependent increase in $[Ca]_i$, that reaches a plateau within 3 min. Thereafter, $[Ca]_i$ remains at this elevated level. This result is consistent with a role for Na–Ca exchange in regulating $[Ca]_i$ in mammalian presynaptic nerve terminals.

^{45}Ca efflux, total chemical Ca, and recovery of $[Ca]_i$ from a Ca load

The results of the previous section indicate that external Na is important in regulating the resting level of $[Ca]_i$ in synaptosomes. External Na is also important in allowing synaptosomes to recover from a Ca load (Blaustein & Ector, 1976). Synaptosomes were loaded with ^{45}Ca by incubating them in 29 mM-Na solution for

1 min. Subsequently, they were diluted into a large volume of a normal-Na or Na-free medium, in a Na-free medium (without Ca, to prevent ^{40}Ca–^{45}Ca exchange), the efflux of radiotracer with time is negligible (not shown). From the time course of efflux of ^{45}Ca in a medium with Na and Ca (shown in Fig. 3) it is evident that there is a

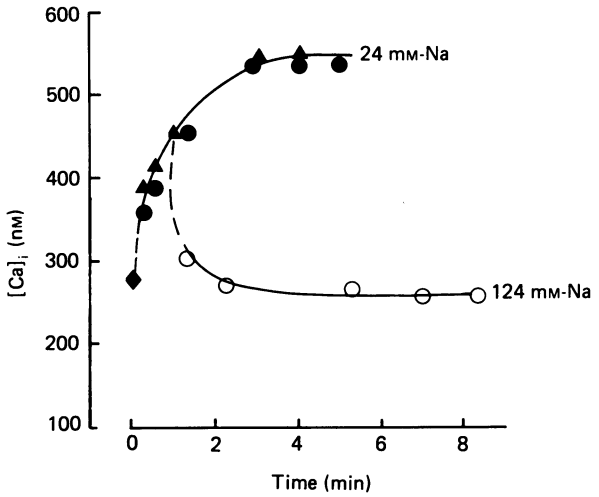

Fig. 2. The effect of Na on $[Ca]_i$ in quin 2-loaded synaptosomes. One batch of synaptosomes was initially resuspended in Na medium. $[Ca]_i$ remained at the level indicated by the diamond for 15–20 min (not shown). A second (●) and a third (▲) batch of synaptosomes were resuspended in a medium containing 24 mM-Na. A final batch of synaptosomes was resuspended in a small volume (100 μl) of low-Na solution for 1 min, and subsequently diluted into a normal-Na medium (○).

precipitous drop in the ^{45}Ca content of the nerve terminals when they are returned to a normal-Na medium. The actual change in chemical Ca during the efflux period differs from the change in ^{45}Ca content in two respects, however. First, there is some cold Ca in the nerve terminals at time zero in Fig. 3, in addition to the hot Ca that was loaded during the uptake period. This cold Ca will, it is assumed, efflux along with the ^{45}Ca. The cold exchangeable Ca content amounts to ~ 10 nmol/mg protein (Blaustein, 1975; also, see Fig. 1, 20 min time point). Since the uptake of hot Ca during the loading interval is approximately twice this value, r (the ratio of cold to hot Ca; see eqn. (2)) is ~ $\frac{1}{2}$. Secondly, there will be some uptake of cold Ca during the efflux period. This uptake was directly measured by loading the synaptosomes with cold Ca in 29 mM-Na solution for 1 min, and subsequently diluting them into a large volume of Na solution containing 1 mM-^{45}Ca. The time course of uptake of ^{45}Ca into the synaptosomes during the efflux intervals is also shown in Fig. 3 (squares), and is equivalent to c_B^* (eqn. (3)). The total Ca in the nerve terminals, calculated from eqn. (4), is shown in Fig. 3 (see Table 1). From these calculations it appears that slightly more than half of the total Ca in the nerve terminals after Ca loading is lost within 1 min, bringing the total Ca remaining close to the level expected in normal-Na solution. The rates of total Ca influx and efflux, as well as the net flux, calculated from eqns. (7), (10) and (11) (see Table 1), respectively, are shown in Fig. 4. These

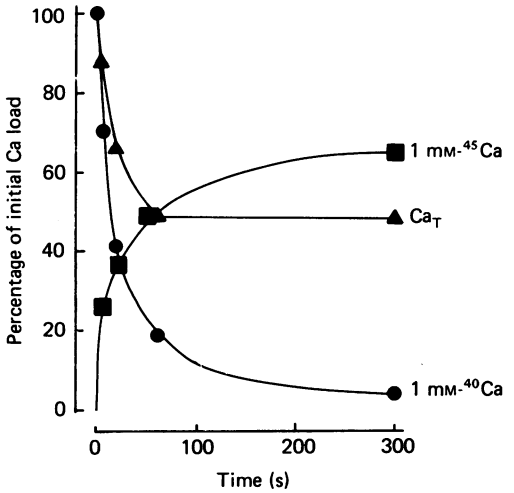

Fig. 3 The efflux of ^{45}Ca from ^{45}Ca-loaded synaptosomes (●), and the uptake of ^{45}Ca by ^{40}Ca-loaded synaptosomes (■). Synaptosomes were Ca loaded by 1 min pre-incubation in a low-Na (29 mM) medium, containing 1 mM of either ^{45}Ca or ^{40}Ca. The synaptosomes were subsequently diluted with a large volume of normal-Na (145 mM) solution, containing 1 mM of either ^{40}Ca (to measure efflux; ●), or ^{45}Ca (to measure uptake; ■). Results from six experiments have been averaged, and the data normalized to the Ca load after pre-incubation in a low-Ca medium (= 100%). The Figure also shows total chemical Ca remaining in the synaptosomes (Ca_T), calculated from uptake and efflux levels, according to eqn. (4), also normalized to 100% (▲).

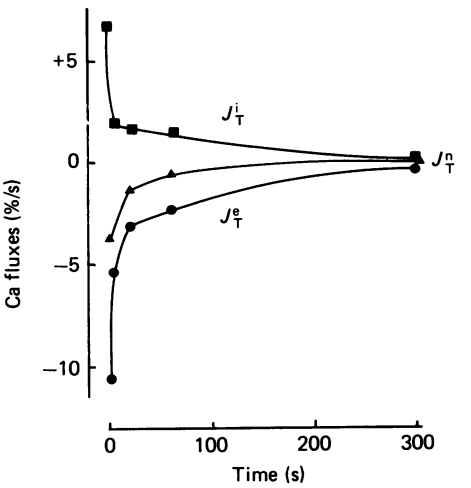

Fig. 4 The calculated rates of efflux (J_T^e, ●) and of influx (J_T^i, ■) of total chemical Ca, in Ca-loaded synaptosomes that have been diluted into a normal-Na medium. Rates of radiotracer fluxes were taken from Fig. 3, and total rates were then calculated according to eqns. (7) and (10). Also shown is the net flux rate (J_T^n▲), calculated according to eqn. (11).

calculations show that there is a very rapid net efflux of Ca from the nerve terminals, amounting to ~ 10 %/s, that declines to low values, ~ 1 %/s, by 1 min.

In order to confirm the results of the above calculations, and to show that the ^{45}Ca measurements faithfully reflect the movement of total chemical Ca, we measured chemical Ca in the synaptosomes (four experiments). A representative experiment is shown in Fig. 5. Synaptosomes that have been resuspended in a Na medium (Fig. 5, filled circles) have a total Ca content of ~10 nmol/mg protein. Ca increases to

TABLE 1.

$c_{A,T}$ (%)	J_A^{e*} (%/s)	J_B^{n*} (%/s)	J_T^e (%/s)	J_T^i (%/s)	J_T^n (%/s)
100	−7·0	6·67	−10·5	6·67	−3·83
88	−2·9	0·91	−5·4	1·99	−3·41
66	−1·25	0·51	−3·01	1·65	−1·36
49	−0·49	0·21	−2·14	1·62	−0·52
49	−0·008	0·009	−0·112	0·11	−0·002

$c_{A,T}$ is the total concentration in the nerve terminal, calculated from eqn. (4), and the c_A^* and c_B^* values shown in Fig. 3, with r, the initial ratio of cold to hot Ca taken as 1/2. $c_{A,T}$ has been normalized, to the $c_{A,T}$ value at $t = 0$. J_A^* and J_B^* are the rates of efflux and uptake, respectively, calculated from the slopes of curves in Fig. 3 (filled circles and squares) and J_T^e, J_T^i, and J_T^n are the total Ca efflux, influx, and net flux, respectively, calculated from eqns. (7), (10) and (11).

~20 nmol/mg protein after 1 min incubation in low-Na (24 mM) solution, and continues to increase, to ~30 nmol/mg protein after 20 min (Fig. 5, triangles). When synaptosomes that have been Ca loaded for 5 min in low-Na solution are resuspended in a normal-Na medium, the Ca content recovers to the base-line level within 1 min. These results parallel the results obtained with radiotracer (Fig. 3), and confirm that the synaptosomes can recover from a Ca load within 1 min in normal-Na solution.

Fig. 2 shows that the loss of Ca after Ca loading is paralleled by a rapid recovery of $[Ca]_i$. This was ascertained by loading quin-2-containing synaptosomes with Ca in 29 mM-Na solution for 1 min, and subsequently diluting them into a normal-Na solution in the cuvette of the spectrofluorometer. As can be seen in Fig. 2, there is a very rapid initial drop in $[Ca]_i$ that occurs too quickly to be adequately resolved with the current system. Within 1 min, $[Ca]_i$ has returned to its resting basal value. Thus, both the ^{45}Ca and the total chemical Ca measurements, and the measurements of $[Ca]_i$ show a rapid recovery from Ca loading that is substantially complete within 1 min.

The effect of FCCP on Na-dependent Ca uptake and on $[Ca]_i$

As may be seen in Fig. 1, there is a very large increase in Ca uptake when synaptosomes are incubated in a low-Na medium. In order to learn something about the deposition of this extra Ca uptake, we examined the effects of carbonyl cyanide *p*-trifluoromethyloxy-phenylhydrazone (FCCP) on ^{45}Ca uptake, efflux, and cytosolic Ca levels. FCCP is a mitochondrial uncoupler, that abolishes the mitochondrial membrane potential, thereby blocking mitochondrial Ca uptake and inducing a loss of sequestered Ca from the mitochondria (Scott, Akerman & Nicholls, 1980). We

found that FCCP (1 μM) has no effect on Ca uptake in either normal- or low-Na media. Neither does it have any effect on Na-dependent Ca efflux from synaptosomes that have been pre-loaded with ^{45}Ca in a low-Na medium (data not shown). Previous work from this laboratory (Nachshen, 1985*a*) has shown that FCCP induces a slight

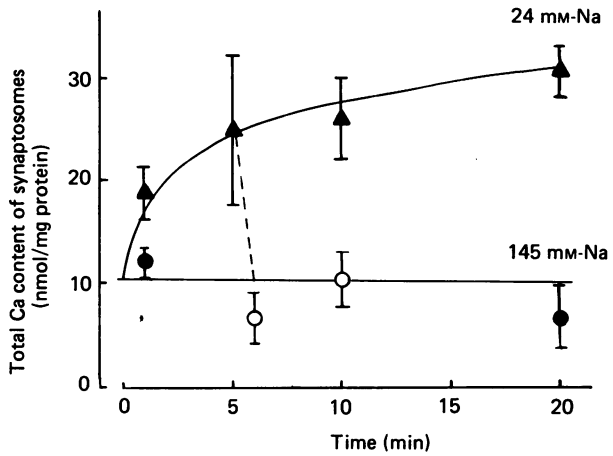

Fig. 5. The measured total chemical Ca content of synaptosomes in a normal-Na (145 mM) and low-Na (24 mM) solution. Synaptosomes were resuspended in a normal-Na (●) or low-Na (▲) solution containing 1 mM-Ca. After 5 min incubation in a low-Na medium, some of the samples were pelleted and resuspended in a normal-Na medium (○) for 1 or 5 min. Further details are supplied in the Methods section. Synaptosomes were not loaded with quin 2 in this experiment.

increase in $[Ca]_i$, but that this increase is probably not caused by the release of Ca from the mitochondria. Fig. 6 shows that the addition of FCCP to synaptosomes that have been resuspended in a low-Na medium causes an increase in $[Ca]_i$ that is of the same magnitude as the increase seen in a normal-Na medium. These results suggest that little, if any of the extra Ca taken up by the synaptosomes in a low-Na medium is sequestered by the mitochondria.

DISCUSSION

The mathematical analysis of the experiments in this study provides an accurate description of the Ca fluxes that are measured and their relation to the actual unidirectional fluxes. In the efflux (A) experiment, a pool of ^{45}Ca is pre-loaded and its efflux is inferred from the measured decline in ^{45}Ca content. This isotopic flux is an underestimate of the total chemical Ca efflux, however, due to (1) the presence of a pool of ^{40}Ca with the synaptosomes at the start of the experiment and (2) the further influx of ^{40}Ca from the bath during the experimental time period. With time, the difference between the isotopic flux and the total efflux increases. In the influx (B) experiment, the net accumulation of ^{45}Ca within the synaptosomes is measured, and an isotopic influx is inferred. This flux is again an underestimate of the total Ca

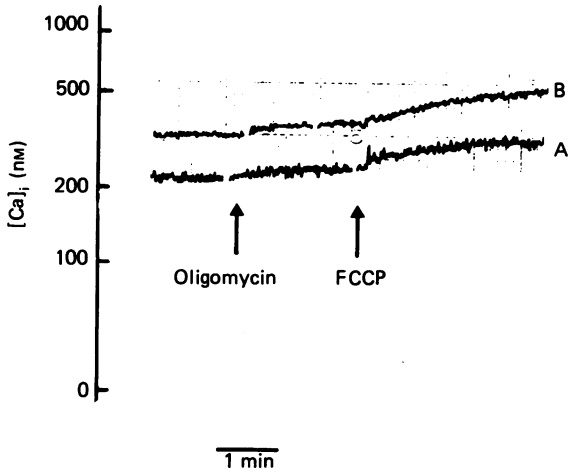

Fig. 6. The effect of FCCP (1 μM) on $[Ca]_i$ in fura-2-loaded synaptosomes, resuspended either in normal-Na (145 mM; A) or low-Na (29 mM; B) solution. At the first arrow, oligomycin (4 ug/ml; an inhibitor of mitochondrial ATPase) was added, to prevent the rundown of synaptosomal ATP in the presence of uncoupler. At the second arrow, FCCP (1 μM) was added.

influx, since a portion of this entering Ca will exit from the synaptosomes during the experimental time period. Furthermore, the difference between the estimated flux and the actual influx increases with time. The primary assumption of this analysis is that the chemical Ca fluxes of the two experiments are identical, so that each experiment provides information about the other. The basic result is that, beyond the dilutional effect of a synaptosomal cold Ca pool ($r > 0$ in the A experiment), the two isotopic fluxes underestimate the unidirectional fluxes by the same amount. Thus the difference of the two isotopic fluxes (from the A and B experiments) yields an accurate estimate of the net chemical Ca flux. This is the content of eqn. (11). Our approach eliminates the necessity of estimating unidirectional fluxes from measurements made at $t \simeq 0$, and provides a powerful tool for analysing Ca movements in synaptosomes.

Our calculations are based on the assumption that ^{40}Ca entering during the efflux period mixes freely with ^{45}Ca that is taken up during the pre-incubation ('loading') interval. If, however, the 'hot' Ca is sequestered, the subsequent efflux that is measured will underestimate the actual loss of total Ca. Thus, our analysis sets a lower limit of ~ 1 min for the clearance of Ca from the nerve terminals.

The general validity of this approach is confirmed by measurements of total chemical Ca. We find that the extra Ca accumulated by the nerve terminals during a 5 min incubation in a low-Na medium is extruded within 1 min after the synaptosomes are returned to a normal-Na medium (Fig. 5). This result is in excellent agreement with the predictions based on measurements of ^{45}Ca fluxes.

Allen & Baker (1985) have reported that quin 2, at concentrations as low as 150 μM, blocks Na-dependent Ca uptake in squid axons. We find no evidence for inhibition of this type, either in measurements of ^{45}Ca uptake, or in measurements of $[Ca]_i$, with

synaptosomal quin 2 concentrations of 1–2 mM. Allen & Baker (1985) have suggested that the inhibition of Na-dependent Ca uptake in squid axons by quin 2 is due to the stabilization of cytosolic Ca at low levels (less than micromolar). One implication of this finding is that Na-dependent Ca uptake would be inoperative in the squid axon at normal low ($\sim$0·1 μM) concentrations of cytosolic Ca. It is, therefore, possible that Na-dependent Ca uptake in synaptosomes is less sensitive to a low $[Ca]_i$ than that of the squid, and could operate at normal levels of $[Ca]_i$. Na-dependent Ca uptake is, of course, unlikely to be activated physiologically by a lowering of external Na. It could, however, be activated by depolarization of the nerve terminals, since the exchanger is electrogenic (Blaustein, 1984).

When synaptosomes are incubated in a low-Na medium, there is an increase in both ^{45}Ca uptake, and in $[Ca]_i$. Note, however, that the increase in $[Ca]_i$ ($\Delta[Ca]_i$) is modest, as compared with the increase in Ca uptake ($\Delta[Ca]_T$; also, see Fig. 5, triangles). If the synaptosome internal volume is roughly 4 μl/mg protein (Blaustein, 1975), then within 3 min, the synaptosomes take up $\sim$6 mM-Ca. The actual change in $[Ca]_i$ is, however, much smaller (only $\sim$250 nM). Thus, the buffering capacity of the synaptosomes (β) is given by:

$$\beta = \Delta[Ca]_T/\Delta[Ca]_i, \tag{12}$$

and is $\sim$25000 after a 3 min incubation in low-Na solution. Furthermore, whereas $[Ca]_i$ levels off after only 3–5 min (see Fig. 2), Ca uptake increase for up to 20 min, so that at 20 min, the value of β is roughly doubled.

It is unclear where the extra Ca taken up in a low-Na medium is sequestered by the nerve terminals. FCCP, an agent that uncouples the mitochondria and thereby abolishes the mitochondrial membrane potential releasing any Ca stored inside this organelle, had no effect on Ca uptake or efflux. The addition of FCCP to synaptosomes caused an increase in $[Ca]_i$ of the same magnitude in a normal-Na as in low-Na medium. Thus, the extra Ca is not stored in the mitochondria. The buffering capacity of the smooth endoplasmic reticulum, $\sim$2 nmol/mg protein (Schweitzer & Blaustein, 1980), could only account for a small fraction of the extra Ca uptake induced by incubation in a low-Na medium. It is possible that intrasynaptosomal proteins accommodate much of the extra Ca uptake. Further research is, however, needed to clarify the role of these proteins in buffering cytosolic Ca.

The main conclusion of this study is that Na-dependent Ca efflux in nerve terminals operates at a rate sufficient to clear a large Ca load ($\sim$20 nmol/mg protein) and to restore $[Ca]_i$ within 1 min. Recently, similar results have been found with moderate Ca loads (0·8 nmol/mg protein), induced by brief (10 s) depolarizations (S. Sanchez-Armass & M. P. Blaustein, unpublished observations). No information is, as yet, available about the efficacy of the exchanger with more physiologic Ca loads, such as might occur during the firing of a single action potential, on the order of 6 pmol/mg protein (Nachshen, 1985*b*). None the less, it is clear that Na-dependent Ca efflux, presumably Na–Ca exchange (Blaustein, 1984), plays an important role in maintaining $[Ca]_i$ at basal levels.

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