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Authors

Komulainen, H
Bondy, SC

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THE ESTIMATION OF FREE CALCIUM WITHIN SYNAPTOSOMES AND MITOCHONDRIA WITH FURA-2; COMPARISON TO QUIN-2

H. KOMULAINEN* and S. C. BONDY†

Laboratory of Behavioral and Neurological Toxicology, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709, U.S.A. and †Department of Community and Environmental Medicine, College of Medicine, University of California, Irvine, CA 92717, U.S.A.

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Abstract—The utility of the acetoxymethyl esters of two tetracarboxylic acids, fura-2 and quin-2, in the determination of ionic calcium levels within synaptosomes and mitochondria was compared. Synaptosomes and isolated mitochondria both accumulated the esters but mitochondria had a much more limited capacity to hydrolyze them. Dye-loaded synaptosomes maintain their external membrane potential of magnitude similar to values for unloaded controls and do not accumulate radioactive Ca^{2+} in excess with time. Both fluorescent compounds yielded similar values (about 300–400 nM) for free intrasynaptosomal calcium $[\text{Ca}^{2+}]_i$. Mitochondrial Ca^{2+} could be measured only with fura-2. Isolated mitochondria contained 0.9–1 μM free Ca^{2+} in a similar extrasynaptosomal medium. Fura-2 tended to overestimate $[\text{Ca}^{2+}]_i$ while quin-2 tended to underestimate it due to chelation of these dyes with intrasynaptosomal trace elements. Fura-2 requiring the use of two excitation wavelengths was significantly superior to the single wavelength method using quin-2. Advantages included reduced danger of erroneous readings due to (i) synaptosomal sedimentation, (ii) photobleaching of the dye, (iii) underestimation of intrasynaptosomal calcium during correction for dye leakage by manganese entry into synaptosomes. Fura-2 interfered less with synaptosomal Ca^{2+} transients than quin-2, probably due to lower intrasynaptosomal concentration of dye needed. Both unstimulated and K^+ -stimulated $^{45}\text{Ca}^{2+}$ uptake were increased in quin-2-loaded synaptosomes but only K^+ -stimulated uptake in fura-2 loaded ones. This series of advantages makes fura-2 of superior utility in the determination of free intrasynaptosomal calcium.

Levels of free calcium are very low within most eukaryotic cells while extracellular levels are generally over one thousand times higher ($>1\text{ mM}$). Several mechanisms exist that maintain this steep gradient. Calcium is extruded against a concentration gradient out of the cell, and in the cell sequestered into mitochondria and/or in the endoplasmic reticulum (Schatzmann, 1985). Such means are generally energy requiring and depend on the structural and metabolic integrity of the cell. The presence of relatively high concentrations of calcium in extracellular fluid and

the considerable amount of bound calcium within cells, has made the quantitative assay of free intracellular calcium difficult. Recently, great progress has been made in this area by the introduction of acetoxymethyl esters of tetracarboxylic acids which are accumulated by isolated cells and subsequently enzymically hydrolysed. The product of hydrolysis complexes with intracellular calcium to give a characteristic fluorescent signal (Rink and Pozzan, 1985). The intensity or wavelength shift of the emitted fluorescence allows estimation of free intracellular calcium ($[\text{Ca}^{2+}]_i$).

In view of the distinctive role of calcium in neurotransmission, the determination of $[\text{Ca}^{2+}]_i$ within the synapse is of special interest. Synaptosomes resemble intact cells in that they possess a relatively impermeable external plasma membrane enclosing a variety of organelles, and in an appropriate medium, they are capable of maintaining oxidative phosphorylation and an external membrane potential. However, they differ from living cells in that they are

*Address correspondence and reprint requests to: Dr H. Komulainen, National Public Health Institute, Department of Environmental Hygiene and Toxicology, P.O.B. 95, SF-70701 Kuopio, Finland (present address).

Abbreviations: $[\text{Ca}^{2+}]_i$, free intrasynaptosomal calcium; fura-2/AM, acetoxymethyl ester of fura-2; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; quin-2/AM, acetoxymethyl ester of quin-2; SDS, sodium dodecyl sulfate; TPEN, tetrakis (2-pyridylmethyl)-ethylenediamine; TPP⁺, tetraphenylphosphonium.

unable to sustain themselves for an extended period. Synaptosomes have a very limited anabolic capacity being virtually devoid of ribosomes and this constrains their adaptive abilities (Whittaker, 1984). The assay of synaptosomal $[Ca^{2+}]_i$ thus involves distinctive uncertainties and problems.

Our study was directed toward three major goals:

1. To assess utility of fura-2 in the determination of synaptosomal $[Ca^{2+}]_i$ and to compare it to quin-2, which has previously been used for this purpose (Ashley *et al.*, 1984; Richards *et al.*, 1984; Nachshen, 1985). The possibility of using these dyes in the assay of mitochondrial free Ca^{2+} was also studied.
2. Delineation of optimal conditions for, and special hazards connected with, the measurement of free calcium within synaptosomes.
3. To accurately determine intrasynaptosomal calcium concentrations. Changes in this concentration in response to alterations in the surrounding medium were also investigated. This work constitutes the first report wherein fura-2 is used in the determination of synaptosomal and mitochondrial $[Ca^{2+}]_i$. It is also the initial documentation of the effects of calcium-chelating dyes on synaptosomal membrane potential and on the movement of calcium through this membrane.

EXPERIMENTAL PROCEDURES

Preparation of synaptosomes and mitochondria

Adult male Fisher rats (F344/N), 3–4 months old weighing 290–340 g were used in this study. Brains were rapidly removed after decapitation and the anterior part of the cerebrum (including the striatum) was dissected out, weighed and homogenized in 10 vol of 0.32 M sucrose at 0°C as described previously (Komulainen and Tuomisto, 1981). The homogenate was centrifuged (1500 g 10') to give a post nuclear supernatant and was then centrifuged (17,800 g 30') to give a P_2 precipitate. The P_2 fraction was used in some studies but generally this was layered over 1.2 M sucrose to prepare synaptosomes and mitochondria by a modification of the procedure of Gray and Whittaker (1962) described by Dodd *et al.* (1981). Electron microscopic evaluation showed the appearance of the synaptosomal fraction to closely resemble that published by Dodd *et al.* (1981). The P_2 pellet or purified synaptosomes were resuspended in Hepes buffer (pH 7.4) at concentrations equivalent to 0.037 g-equiv/ml or 0.15 g-equiv/ml respectively. This results in a protein concentration of approximately 1.2 mg/ml for the P_2 fraction and 1.6 mg/ml for the synaptosomal fraction. Mitochondria were resuspended in Hepes buffer at a concentration of 0.3 g-equiv/ml (220–240 µg/ml protein). The composition of the Hepes buffer was (mM): NaCl, 125; KCl, 5; NaH_2PO_4 , 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 5; glucose, 6; $CaCl_2$, 1; Hepes, 25. The final pH was adjusted to 7.4 with NaOH.

Fluorescent dye loading

Synaptosomes or the P_2 fraction were loaded with quin-2 or fura-2 by a modification of the method of Tsien *et al.* (1982), described by Ashley *et al.* (1984). Briefly 1 ml of synaptosomal or P_2 suspension was incubated in 25 or 50 µM quin-2-acetoxymethyl ester (quin-2/AM) or in 5 µM fura-2-acetoxymethyl ester (fura-2/AM). Dimethylsulfoxide (DMSO) was used to dissolve these esters and was present in the final incubation mixture at a concentration of 0.5–1% (v/v). Control tubes also contained DMSO in the absence of esters.

After 20 min incubation in a shaking waterbath (37°C) samples were diluted in 9 ml Hepes buffer at 37°C and incubation continued for a further period (25 min for fura-2 or 40 min for quin-2 unless otherwise stated). Synaptosomes were then rapidly centrifuged (5 min, 12,300 g) and the pellet resuspended in 5 ml ice-cold Hepes buffer. The final g-equiv/ml was then 0.03 for synaptosomes and 0.008 for the P_2 fraction.

Mitochondria were loaded in a similar way except they were spun down at 17,800 g (10 min) and resuspended in 2 ml of Hepes buffer (0.15 g-equiv/ml).

Fluorescence determination

The emitted fluorescence of quin-2 and fura-2 was measured with an Aminco SPF-500 spectrofluorometer (American Instrument Co., Urbana, Ill). For quin-2, the excitation wavelength was 337 nm (bandwidth, 2–4 nm) and emission was recorded at 500 nm (bandwidth, 15–20 nm). Excitation of fura-2 was at 340 and 380 nm (bandwidth, 1 nm) and emission determinations were made at 510 (bandwidth, 8 nm). In order to attain adequate signal intensity, the instrument was operated in the ratio mode. The cuvette holder was thermostatically maintained at 37°C. A magnetic stirrer was used to mix the sample intermittently and after addition of chemicals. For each assay, 0.5 ml of loaded and unloaded tissue was rapidly centrifuged (30–60s, 13,000 g) in a microcentrifuge (235B Fisher Scientific, Pittsburgh, Pa). The resulting pellet was then resuspended in 1 ml Hepes buffer at 37°C. This buffer had a similar composition to that described above except that $NaHCO_3$ and NaH_2PO_4 were absent to prevent the precipitation of Ca^{2+} at elevated pH (required during the determination of minimal fluorescence, F_{min}). The tube was rinsed with another 1 ml Hepes buffer and the total 2 ml sample (140–160 µg synaptosomal protein) was then placed in a quartz cuvette at 37°C and left to equilibrate for 10 min.

The completeness of hydrolysis of the dye esters was verified by examination for the fluorescence emission spectra (quin-2) or excitation spectra (fura-2) of the samples. These spectra were similar to those published previously (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985). The fluorescence emission peak of unhydrolyzed quin-2/AM at 430 nm disappeared when its hydrolysis was complete to quin-2. The hydrolysis of fura-2/AM was assessed indirectly by the determination of R_{max} , ratio of the fluorescence (excitation 340 nm/380 nm) when fura-2 was saturated with Ca^{2+} . The rise of R_{max} corresponded to fura-2 formed.

The concentration of intrasynaptosomal quin-2 (Table 1) was estimated by comparison of the value of F_{max} to the fluorescence induced by known concentrations of quin-2 added to lysed, unloaded synaptosomal suspensions. Fura-2 content was similarly determined by measuring fluorescence of accumulated fura-2 at the excitation wavelength 340 nm.

Fig. 1. Determination of free intrasynaptosomal calcium $[Ca^{2+}]_i$ with quin-2. Synaptosomes (1.1–1.3 mg protein) were loaded in 50 μ M quin-2/AM (upper tracing). Autofluorescence was assayed with untreated synaptosomes (lower tracing). Fluorescence of samples (140–160 μ g protein in 2 ml Hepes buffer) was recorded as described in the Experimental section, in thermostatted cuvettes. Materials added to achieve the final concentrations shown were 500 μ M $MnCl_2$ (20 μ l); 0.5 M EGTA in 3 M Tris (20 μ l); 3 M Tris (30 μ l), 10% sodium dodecyl sulfate (SDS) 20 μ l; 0.5 M $CaCl_2$ (25 μ l). $[Ca^{2+}]_i$ was calculated from F , F_{max} and F_{min} as described.

The approximate intrasynaptosomal volume was taken as 3.3 μ l/mg synaptosomal protein (Marchbanks, 1975). No attempt was made to quantitate mitochondrial dye concentration because mitochondrial volume in these conditions is not known.

Calculation of free intrasynaptosomal calcium $[Ca^{2+}]_i$

Quin-2 fluorescence was calibrated essentially as described by Tsien *et al.* (1982) using the modification of Jacob *et al.* (1987). F (fluorescence of quin-2 in synaptosomes), F_{min} (fluorescence at very low Ca^{2+} < 10 nM) and F_{max} (fluorescence of quin-2 after its complete saturation with Ca^{2+}) were obtained as illustrated in Fig. 1. F was read within 20 s after addition of $MnCl_2$ to a final concentration of 5 μ M to the cuvette, in order to correct for any quin-2 outside the synaptosomes. Alkaline EGTA (5 mM) was used to chelate all Ca^{2+} after membrane lysis with 0.1% sodium dodecyl sulfate in order to obtain F_{min} . Excess Ca^{2+} (6 mM) subsequently allowed measurement of F_{max} . $[Ca^{2+}]_i$ was calculated using the formula:

$$[Ca^{2+}]_i = K_d \left(\frac{F - F_{min}}{F_{max} - F} \right)$$

where K_d is the dissociation constant of the quin-2 Ca^{2+}

complex. K_d was taken as 115 nM, assuming intracellular pH to be near 7.05 and $[Mg^{2+}]_i$ to be around 1 mM (Tsien *et al.*, 1982).

Extrasynaptosomal fura-2 was quenched by 40 μ M Mn^{2+} . A higher concentration was used than for quin-2 because fura-2 has 10-fold less affinity for Mn^{2+} (Grynkiewicz *et al.*, 1985). Calibration with fura-2 was carried out similarly as with quin-2 but the ratio R of the fluorescence excitation at 340 nm to that at 380 nm was the critical variable. R_{min} , the ratio in the presence of excess EGTA, was similar both in lysed, loaded synaptosomes and with fura-2 added directly to buffer but R_{max} (excess calcium present) was lower in lysed synaptosomes than with fura-2 in buffer. Therefore, an individual calibration was performed on each batch of synaptosomes in order to use correct R_{max} for calculations. $[Ca^{2+}]_i$ was calculated using the formula of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d \left(\frac{R - R_{min}}{R_{max} - R} \right) \left(\frac{S_{f2}}{S_{b2}} \right)$$

K_d for the fura-2- Ca^{2+} complex was taken to be 224 nM (Grynkiewicz *et al.*, 1985). S_{f2} and S_{b2} denote fluorescence of fura-2 at zero calcium and full calcium saturation respectively, at the excitation wavelength of 380 nm.

Mitochondrial free Ca^{2+} was calibrated and calculated as described for synaptosomes.

$^{45}Ca^{2+}$ uptake by synaptosomes

Unstimulated $^{45}Ca^{2+}$ uptake during loading of synaptosomes with dyes, and thereafter, was assayed using the experimental conditions described above. The 1 mM $CaCl_2$ used contained ^{45}Ca at a final sp. act. of 0.72 Ci/mol, throughout the study. After loading with fura-2 or quin-2, synaptosomes were resuspended in 5 ml ice-cold buffer, kept on ice and 50 μ l aliquots were removed in duplicate at various subsequent times. These were filtered on glass fibre filters (Type A/E, Gelman, Inc., Ann Arbor, Mich.) and washed twice with 5 ml cold buffer. Retained radioactivity was assayed in 10 ml Aquasol (New England Nuclear, Boston, Mass.) with a liquid scintillation counter (Beckman LS7500, Beckman Instruments, Irvine, Calif.).

Depolarization induced Ca^{2+} uptake was studied in dye-loaded synaptosomes by the addition of 0.1 ml of $^{45}Ca^{2+}$, containing 0.5 μ Ci in either 0.5 M KCl (depolarization) or 0.5 M NaCl (unstimulated uptake), to 0.9 ml of synaptosomal suspension in Hepes buffer, pH 7.4 (0.015 g equiv/ml) at 37°C. After 15 s, uptake was stopped by the addition of 5 ml cold Hepes buffer (nominally Ca^{2+} -free), followed by rapid filtration on glass fibre filters (Wu *et al.*, 1982). Depolarization induced Ca^{2+} uptake was defined as the difference between high K^+ and high Na^+ samples.

Determination of synaptosomal potential

The determination of the electrical potential across the synaptosomal membrane ($\Delta\psi$) was carried out by the procedure of Ramos *et al.* (1979). This method essentially consists of assay of the accumulation of a permeant lipophilic action (tetraphenylphosphonium, TPP^+) by 1 ml of the final suspension of loaded synaptosomes used for the fluorometric studies. The final concentration of $[^3H]TPP$ was 2 μ M and incubation was for 10 min at 37°C in Hepes buffer, pH 7.4. 5 μ M valinomycin was also present in order to depolarize any mitochondria that could contribute to TPP^+ accumulation (Scott and Nicholls, 1980). Incubation

was stopped by the addition of 2 ml cold 0.2 M NaCl and synaptosomes were filtered on glass fibre filters, washed with 2×5 ml 0.2 M NaCl and counted, as described for the $^{45}\text{Ca}^{2+}$ assay. Concentrations of TPP^+ inside and outside the synaptosomes were calculated and $\Delta\psi$ (mV) was derived as described by Lichtshtein *et al.* (1979):

$$\Delta\psi = -61 \log \frac{[\text{TPP}^+]_i}{[\text{TPP}^+]_o}$$

$[\text{TPP}^+]_i$ and $[\text{TPP}^+]_o$ are the concentrations of tetraphenylphosphonium within the synaptosomes and in the surrounding medium respectively.

Protein determination

Synaptosomal protein was assayed by the method of Lowry *et al.* (1956) using bovine serum albumin as a standard. Values were corrected for color produced by the Hepes buffer.

Materials

All chemicals were from Sigma Chemical Co. (St. Louis, Mo.) except fura-2, fura-2/AM and tetrakis (2-pyridylmethyl)ethylenediamine (Molecular Probes, Junction City, Ore.). $^{45}\text{CaCl}_2$ and [Phenyl- ^3H]tetraphenylphosphonium bromide (35.5 Ci/mmol) from New England Nuclear (Boston, Mass.).

Statistical analysis

Differences between groups were assessed using Fisher's Least Significant Difference Test after a one-way analysis of variance. The accepted level of significance in all cases was $P < 0.05$ using a two-tailed distribution. Linear regression analysis was used to study correlations when indicated.

RESULTS

Dye loading and hydrolysis in synaptosomes

During the loading incubation, between 20 and 30% of dye entered synaptosomes, was hydrolysed and thereby entrapped. This was true of the acetoxymethyl esters of both quin-2 and fura-2, and was independent on the concentration of esters used (5–100 μM). However, the hydrolytic capacity of synaptosomes was limited. At 25 μM quin-2/AM, complete hydrolysis was achieved in 30 min at 37°C but was incomplete at 60 min when 100 μM quin-2/AM was used. Hydrolysis of entrapped fura-2 was complete after a 45 min incubation, when the loading mixture contained 5 μM fura-2/AM, but there was enough cytoplasmic fura-2 after 20 min to allow a quantifiable Ca^{2+} -signal (Fig. 2). Fura-2/AM when added to fura-2-loaded synaptosomes did not interfere significantly with determination of ionic calcium levels either. This is because the additional fluorescence is cancelled out in the equation used (see Experimental Procedures).

The total amount of fura-2 in samples, declined by 10–12% between 30 and 60 min of loading incubation, probably because of disruption of some synaptosomes maintained at 37°C for a prolonged time. When suspensions were maintained at 0°C, dye contents within synaptosomes remained stable for hours.

Fig. 2. Rate of hydrolysis of fura-2/AM (5 μM) and derived Ca^{2+} levels within synaptosomes. R_{max} is ratio 340 nm/380 nm of fluorescence when synaptosomes are lysed and all fura-2 formed is exposed to 1 mM Ca^{2+} . Points represent the mean of two separate experiments with replicated values differing by less than 9%. The Ca^{2+} concentration was assayed and calculated as described in the Experimental section.

Ordinate on the left: Ratio of fluorescence measured at 340 and 380 nm.

Table 1. Values of free intrasynaptosomal calcium 30 min after dye loading

Dye	Purified synaptosomes		
	Concentration of ester prior to loading (μM)	Intrasynaptosomal dye concentration attained (mM)	$[\text{Ca}^{2+}]_i$ (nM)
Fura-2	5	0.24 ± 0.01 (10)	369 ± 6 (40)
Quin-2	25	1.15 ± 0.04 (17)	265 ± 24 (10)
Quin-2	50	2.74 ± 0.04 (23)	$378 \pm 27^*$ (8)

Values are means $\pm$ SEM determinations in parentheses.

* $P < 0.05$ that value differs from corresponding value for 25 μM quin-2 ester loading.

The concentrations of dyes within synaptosomes and the calculated values for $[\text{Ca}^{2+}]_i$ are shown in Table 1.

Dye loading and hydrolysis in isolated mitochondria

Isolated mitochondria also accumulated these dye esters. The concentrations in mitochondria increased with the concentration of the ester in the medium. However, mitochondria had a very limited capacity to hydrolyze the esters to free acids. 5 μM fura-2 was hydrolyzed completely in 60 min, but at 20 μM most of it remained unhydrolyzed. Accordingly, the higher quin-2/AM concentrations ($> 25 \mu\text{M}$) remained unhydrolyzed indicating low saturation threshold of the hydrolysis.

Corrections for extrasynaptosomal dye

The extrasynaptosomal dye was quenched by Mn^{2+} . 5 μM Mn^{2+} decreased quin-2 fluorescence of samples by 3–4% which resulted in corresponding corrections of $[\text{Ca}^{2+}]_i$ of $19.9 \pm 4.2\%$ ($n = 10$), $24.7 \pm 2.0\%$ ($n = 23$) for 25 and 50 μM quin-2/AM respectively. The decrease of calculated $[\text{Ca}^{2+}]_i$ was initially rapid after the introduction of Mn^{2+} , presumably representing quenching of extrasynaptosomal quin-2 (Fig. 3). Thereafter, the value of measured $[\text{Ca}^{2+}]_i$ decreased more slowly, perhaps due to Mn^{2+} entering synaptosomes. The Mn^{2+} correction (40 μM) for $[\text{Ca}^{2+}]_i$ was below 10% when fura-2 AM was loaded from 5 μM solution. Although Mn^{2+} also quenched some fluorescence of intrasynaptosomal fura-2, the ratio R of unquenched fura-2 gave the Ca^{2+} signal. Thus, the calculated value of $[\text{Ca}^{2+}]_i$ was also stable with time (Fig. 3).

In contrast, EGTA added at a raised pH, caused a rapid and large decline in apparent $[\text{Ca}^{2+}]_i$ (Fig. 3). After 30 s, this decrease was already 40–50% of the uncorrected $[\text{Ca}^{2+}]_i$. Similar results were obtained using quin-2 (data not shown).

The level of free intrasynaptosomal calcium

The apparent $[\text{Ca}^{2+}]_i$ obtained with different dyes are in Table 1. The values were essentially similar also

with 3 μM or 10 μM as with 5 μM fura-2/AM (results not shown). $[\text{Ca}^{2+}]_i$ as measured by 5 μM fura-2/AM did not increase significantly with time. It followed the regression formulae $[\text{Ca}^{2+}]_i$ (nM) = $0.123 \times (\text{time in min}) + 366$ ($n = 38$). Addition of 40 μM TPEN, a permeant trace- and heavy-metal chelator, decreased $[\text{Ca}^{2+}]_i$ by $< 10\%$ in 1 min and this value remained depressed (Table 2).

The apparent $[\text{Ca}^{2+}]_i$ obtained with quin-2 depended on the quin-2/AM concentration during loading. 25 μM quin-2/AM gave significantly lower values than 50 μM quin-2/AM or 5 μM fura-2/AM (Table 1). TPEN increased the values obtained by 25 μM quin-2/AM 20–30% but did not affect significantly those derived using 50 μM quin-2/AM (Table 2). This suggests that free intrasynaptosomal trace elements quenched quin-2 and led to underestimation of $[\text{Ca}^{2+}]_i$ at low quin-2 concentration.

Fig. 3. Time course of the effect of addition of Mn^{2+} and EGTA, on calculated $[\text{Ca}^{2+}]_i$ of synaptosomes. These materials were added to dye-loaded synaptosomes (fura-2 about 0.2 mM, quin-2 2.5 mM) in a cuvette after their 10 min preincubation at 37°C. $[\text{Ca}^{2+}]_i$ was calculated as fluorescence changed (see Experimental section). Values are derived as the mean of two separate experiments with replicates differing by less than 12%.

Table 2. Effect of intrasynaptosomal chelation of free trace elements with TPEN, on $[Ca^{2+}]_i$.

Dye ester	Addition	$[Ca^{2+}]_i$ nM		
		Before addition	1 min after addition	3 min after addition
Fura-2 5 μ M	DMSO 1%	345 $\pm$ 9	345 $\pm$ 11	354 $\pm$ 10
	TPEN 40 μ M	365 $\pm$ 5	326 $\pm$ 6*	329 $\pm$ 8*
Quin-2 25 μ M	DMSO 1%	248 $\pm$ 17	217 $\pm$ 17	198 $\pm$ 13*
	TPEN 40 μ M	277 $\pm$ 19	290 $\pm$ 21†	271 $\pm$ 19†
Quin-2 50 μ M	DMSO 1%	344 $\pm$ 30	307 $\pm$ 24	278 $\pm$ 22
	TPEN 40 μ M	361 $\pm$ 11	343 $\pm$ 11	325 $\pm$ 18

$[Ca^{2+}]_i$ was measured as described in Materials and Methods. After 10 min equilibration of synaptosomes in a cuvette, a control value was read and either TPEN or DMSO (control) added. At indicated subsequent times, $[Ca^{2+}]_i$ was determined. Values are mean $\pm$ SEM of 4 (fura-2) or 7 (quin-2) determinations.

* $P < 0.05$: value differs from the value before addition.

† $P < 0.05$: value differs from the corresponding DMSO control value.

Calculated $[Ca^{2+}]_i$ increased significantly with time after quin-2 loading (Fig. 4). The average increase followed the regression formulae $[Ca^{2+}]_i$ (nM) = $0.814 \times (\text{time in min}) + 229$ ($r = 0.46$, $P < 0.01$, $n = 35$) or $= 0.707 \times (\text{time in min}) + 349$ ($r = 0.36$, $P < 0.02$, $n = 44$) for loading concentrations of 25 and 50 μ M quin-2/AM respectively. Thus, the rates of increase of $[Ca^{2+}]_i$ were 49 and 42 nmol/h.

In synaptosomes loaded in 25 μ M quin-2/AM and resuspended in Hepes buffer to which no calcium had been added $[Ca^{2+}]_i$ was 140 nM (Fig. 5). The value is

lower than in the presence of 1 mM Ca^{2+} -containing buffer. However, restoration of 1 mM Ca^{2+} to the extracellular medium, elevated rapidly $[Ca^{2+}]_i$ to 490 nM (Fig. 5). This was above the average value found in 1 mM Ca^{2+} -containing buffer (265 nM). Further increase of calcium to 3 mM in the suspension, resulted in a lesser elevation of $[Ca^{2+}]_i$ to 650 nM (Fig. 5).

Depolarization of synaptosomal membranes by 50 mM potassium chloride elevated $[Ca^{2+}]_i$ to around 700 nM in 10 s (Fig. 6). This value decreased slowly but remained significantly elevated over resting val-

Fig. 4. Variations of $[Ca^{2+}]_i$ in quin-2-loaded synaptosomes (about 2.5 mM) maintained for various times at 0°C. Values shown are from 10 individual homogenates. The linear regression line for all data is shown.

Fig. 5. Responses of synaptosomal $[Ca^{2+}]_i$ to changes in external Ca^{2+} concentration. Quin-2-loaded synaptosomes (about 1 mM) were initially suspended in nominally Ca^{2+} -free Hepes buffer (Ca^{2+} below $10 \mu M$). At various times indicated by an arrow, extrasynaptosomal Ca^{2+} was elevated by the addition of $20 \mu l$ of a concentrated $CaCl_2$ solution. Data are derived from a continuous record of a representative experiment.

ues for several minutes. When $200 \mu M$ verapamil, a blocker of a class of voltage-regulated calcium channels, was added prior to $50 mM K^+$, the magnitude of the depolarization induced influx of $[Ca^{2+}]_i$ was reduced but not completely abolished (Fig. 6).

Characteristics of dye-loaded synaptosomes

$^{45}Ca^{2+}$ uptake was not significantly different in synaptosomes during loading with the dyes and sub-

Fig. 6. The effect of synaptosomal depolarization on $[Ca^{2+}]_i$ in the absence or presence of a Ca^{2+} -channel blocker. Verapamil, where indicated, was added 4 min before KCl . Data are mean $\pm$ SEM derived from 4 separate experiments with $25 \mu M$ quin-2/AM in Hepes buffer-containing $1 mM Ca^{2+}$. Control value was $275 \pm 45 nM$ ($\pm 100\%$). * $P < 0.05$ as compared to control.

Fig. 7. Entry of $^{45}Ca^{2+}$ into synaptosomes during loading with quin-2 (about $2.5 mM$) or fura-2 (about $200 \mu M$) and thereafter. Procedure is detailed in the Experimental section. Values are means $\pm$ SEM of 3 experiments.

sequent maintenance of $0^\circ C$ than in unloaded synaptosomes incubated in parallel (Fig. 7). Loading with $25 \mu M$ quin-2/AM produced a similar ^{45}Ca penetrance to that found with $50 \mu M$ dye (data not shown).

In contrast, uptake of $^{45}Ca^{2+}$ into synaptosomes during a short 15 s incubation was elevated in dye-loaded synaptosomes relative to controls and this was more pronounced under depolarizing conditions (Table 3). Such uptake was somewhat greater in the presence of quin-2 than of fura-2 but this difference was not statistically significant.

The resting potential of dye loaded synaptosomes was not significantly different from that of untreated controls. Values (mV) for controls were 74.9 ± 4.0 (mean $\pm$ SEM, $n = 6$), after fura-2 loading (about $0.2 mM$), 72.6 ± 4.0 and after quin-2 loading (about $2.5 mM$), 79.4 ± 4.6 . Valinomycin was used to prevent a mitochondrial contribution to TPP^+ uptake. In the absence of valinomycin, apparent synaptosomal charge was $147 \pm 11 mV$. Values obtained in the presence or absence of valinomycin compare well with those reported by Ramos *et al.* (1979).

The level of free mitochondrial Ca^{2+}

Free mitochondrial Ca^{2+} in isolated mitochondria in Hepes buffer was $985 \pm 83 nM$ (mean $\pm$ SEM, $n = 5$).

DISCUSSION

The concentration of free Ca^{2+} within polarized synaptosomes was around $370 nM$. This is slightly

Table 3. Entry of $^{45}\text{Ca}^{2+}$ into dye-loaded synaptosomes

Synaptosomes	Ca^{2+} uptake (pmol/mg protein/15 s)	
	Polarized (5 mM K^+)	Depolarized (55 mM K^+)
Unloaded control	24.4 ± 1.1	49.9 ± 2.7
fura-2 loaded	31.2 ± 4.2	$66.5^* \pm 5.8$
quin-2 loaded	$35.6^* \pm 3.9$	$74.7^* \pm 3.3$

Values are mean of 5 separate determinations $\pm$ SEM.

* $P < 0.05$ that value differs from corresponding value of unloaded controls.

higher than the range (85–300 nM) reported by others using quin-2 (Ashley *et al.*, 1984; Richards *et al.*, 1984; Nachshen, 1985). Our values by 25 μM quin-2/AM are in this same range (Table 1). However, a higher quin-2/AM loading concentration, as well as fura-2/AM gave consistently higher $[\text{Ca}^{2+}]_i$ values (Table 1). The use of TPEN, a permeant trace metal chelator (Arslan *et al.*, 1985), revealed that some free intrasynaptosomal cations quenched quin-2 and this quenching was significant at low quin-2 concentrations causing underestimation of $[\text{Ca}^{2+}]_i$. Such quenching capacity has been reported for Zn^{2+} , Fe^{2+} and Mn^{2+} , all of which have at least an order of magnitude greater affinity for quin-2 than has Ca^{2+} (Hesketh *et al.*, 1983; Grynkiewicz *et al.*, 1985). Fura-2 has considerably less affinity for divalent cations other than calcium than has quin-2 (Grynkiewicz *et al.*, 1985). Fura-2 fluorescence was decreased slightly by TPEN suggesting that some other cation replaced Ca^{2+} in forming a fluorescent complex with fura-2 rather than quenching it. The interfering cation might be Zn^{2+} because it quenches quin-2 but produces a fluorescent product with fura-2 (Grynkiewicz *et al.*, 1985). The overestimation of $[\text{Ca}^{2+}]_i$ by fura-2 was only about 10% and does not prevent the use of this dye.

Fura-2 offers several advantages over quin-2 in assay of $[\text{Ca}^{2+}]_i$ (Grynkiewicz *et al.*, 1985) which made the measurement of $[\text{Ca}^{2+}]_i$ more reliable. Its intrasynaptosomal concentration could be kept lower than that required for quin-2 decreasing the danger of cytotoxicity (Tsien *et al.*, 1982). Furthermore, fura-2-loaded synaptosomes could be illuminated continuously over 15 min without significant photobleaching. The utilization of the ratio of two separate excitation wavelengths for fura-2 instead of a single wavelength eliminated the problem of sedimentation of synaptosomes since the ratio remains constant in the face of diminishing total fluorescence. This sedimentation tended to lead to underestimation of $[\text{Ca}^{2+}]_i$ with time when quin-2 is used (Table 2). We have found that continuous stirring with a small magnetic bar is not possible during the assay since this causes some light reflection.

The ratio-method was also less sensitive to potential errors due to correction for extrasynaptosomal dye. While Mn^{2+} method works well for heart cells (Jacob *et al.*, 1987), synaptosomes appeared to rather rapidly take up Mn^{2+} .

This caused a quenching of intrasynaptosomal quin-2 and underestimation of $[\text{Ca}^{2+}]_i$ if readings were taken later than 20 s after MnCl_2 addition (Fig. 3). Quenching of intrasynaptosomal fura-2 by Mn^{2+} is not so deleterious because the remaining unquenched fura-2 gives the appropriate Ca^{2+} signal. Thus the time lapse between Mn^{2+} addition and assay is not so critical with fura-2.

EGTA has been used to correct for extracellular dye in several types of whole cells (Wollheim and Pozzan, 1984; Arslan *et al.*, 1985). However, in the case of synaptosomes, $[\text{Ca}^{2+}]_i$ is rapidly reduced by EGTA (Fig. 3), and thus EGTA cannot be used for correction of extrasynaptosomal dye. Since it is unlikely that EGTA can cross cell membranes (Arslan *et al.*, 1985), this reduction of $[\text{Ca}^{2+}]_i$ probably reflects a rapid and continuous efflux of calcium across the synaptic membrane.

Although quin-2 has enabled the quantitative measurement of $[\text{Ca}^{2+}]_i$, its drawbacks include the tendency to delay intracellular Ca^{2+} -transients (Tsien *et al.*, 1982; Knight and Kesteven, 1982; Jacob *et al.*, 1987; Harvey *et al.*, 1985). This occurs already at 0.2–0.3 mM quin-2 concentrations, and is most likely due to Ca^{2+} -buffering capacity of quin-2. An increase in the resting and K^+ -stimulated uptake of $^{45}\text{Ca}^{2+}$ in quin-2-loaded synaptosomes might result from such buffering. Ca^{2+} cycles across the plasma membrane (Snelling and Nicholls, 1985) and quin-2 might slow this cycling by binding temporarily $^{45}\text{Ca}^{2+}$ in the cytosol. With time $^{45}\text{Ca}^{2+}$ probably redistributed in nerve endings and no long-term increase in $^{45}\text{Ca}^{2+}$ uptake was observed (Fig. 7). Fura-2 had slightly less effect on initial $^{45}\text{Ca}^{2+}$ uptake. Its intrasynaptosomal concentration was lower and its K_d for Ca^{2+} is higher than that of quin-2. Because neither quin-2 nor fura-2 affected the plasma membrane potential, depolarization of synaptosomes did not cause the increase in $^{45}\text{Ca}^{2+}$ uptake. $[\text{Ca}^{2+}]_i$ increased slowly with time

after quin-2 loading but not after fura-2-loading. The source of this Ca^{2+} was probably predominantly intracellular, because $^{45}\text{Ca}^{2+}$ uptake was not increased.

In spite of the slight changes in Ca^{2+} -flux caused by these dyes, $[\text{Ca}^{2+}]_i$ appeared to respond to changes in extrasynaptosomal medium quite rapidly. K^{+} -induced depolarization of the plasma membrane increased $[\text{Ca}^{2+}]_i$ to submicromolar level in seconds. The level of free Ca^{2+} was lower in synaptosomes resuspended in nominally Ca^{2+} -free buffer but restoration of millimolar physiological Ca^{2+} concentration elevated it quickly from below normal to above control levels (Fig. 5).

Mitochondria have been reported to hydrolyze quin-2/AM poorly (Tsien *et al.*, 1982) and this observation was confirmed by us using isolated brain mitochondria. However, the limited hydrolytic capacity appears to be sufficient to hydrolyze low ester concentrations in isolated free mitochondria. Due to a lower intrinsic fluorescence, quin-2/AM must be used at such high concentrations that mitochondrial hydrolysis is not complete and quin-2 cannot be used to measure free mitochondrial Ca^{2+} . In contrast, fura-2/AM seems to be suitable for such a purpose. This is a completely new extension in the use of these dyes. We determined free mitochondrial Ca^{2+} to be around $1\text{ }\mu\text{M}$ in isolated mitochondria in a medium which was designed for extracellular, rather than intracellular studies.

Mitochondrial hydrolysis, however, scarcely contributes to synaptosomal $[\text{Ca}^{2+}]_i$ in purified synaptosomes. At the low loading concentrations fura-2/AM is more likely to be hydrolyzed in the cytosol before it is able to reach intrasynaptosomal mitochondria (Rink and Pozzan, 1985). Minor contamination of purified synaptosomes by mitochondria may not affect $[\text{Ca}^{2+}]_i$, perhaps because the higher hydrolysis and subsequent loading capacity of synaptosomes relative to mitochondria may allow a preferential accumulation of fura-2 by synaptosomes.

Taken together, these data suggest that fura-2 offers many advantages over quin-2 in the assay of free intrasynaptosomal Ca^{2+} . Moreover, fura-2 can also be used to study free mitochondrial Ca^{2+} in isolated mitochondria. This is a new application for this Ca^{2+} -indicator.

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