Measurement of intrasynaptosomal free calcium by using the fluorescent indicator quin-2

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The recently synthesized calcium indicator quin-2 was incorporated into synaptosomes from guinea-pig cerebral cortex following uptake and internal hydrolysis of quin-2 tetra-acetoxymethyl ester. Incubation in physiological media containing 1 mM- or 2 mM- CaCl_2 led to equilibrium cytosolic ionized calcium concentrations of $85 \pm 10 \text{ nM}$ and $205 \pm 5 \text{ nM}$ respectively (mean $\pm$ S.E.M. from eight and eighteen preparations respectively). Cytosolic Ca^{2+} was elevated following increases in external Ca^{2+} concentration, plasma membrane depolarization, mitochondrial inhibition, calcium ionophore addition or replacement of external sodium by lithium. Preliminary experiments were performed to assess changes in cytosolic Ca^{2+} accompanying the release of the neurotransmitter acetylcholine.

Cytoplasmic free calcium levels are recognized to be important in a number of cellular functions, including neuronal transmitter release (Katz, 1969; Baker, 1972, 1976). Direct injection of calcium into the squid giant synapse promoted transmitter release (Miledi, 1973) and transient $[\text{Ca}^{2+}]_i$ increases following stimulation of bullfrog sympathetic neurones have been demonstrated using arsenazoIII (Smith *et al.*, 1983). However, $[\text{Ca}^{2+}]_i$ has proved difficult to quantify in the regions of interest, even under resting conditions, because of the technical difficulties involved in introducing ion-sensitive microelectrodes or chemical indicators into presynaptic terminals and synaptosomes without causing them extensive damage.

Recently, Tsien (1980) has developed a series of fluorescent calcium indicators related to an analogue of EGTA, which following esterification can cross intact cell membranes (Tsien *et al.*, 1982a) and report cytoplasmic free calcium levels after intracellular hydrolysis (Tsien *et al.*, 1982b). Moreover, the results may be readily calibrated (as described in detail in the latter paper). So far the only compounds to become commercially available are quin-2 and its acetoxymethyl tetraester quin-2

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic ionized calcium; $[\text{Ca}^{2+}]_e$, external ionized calcium; $\Delta\psi_p$, plasma membrane potential; $\Delta\psi_m$, mitochondrial membrane potential; *R*, *T*, *F* and *Z* have their usual thermodynamic significance; CCCP, carbonyl cyanide *m*-chlorophenylhydrazonate.

A/M, both as used by Tsien *et al.* (1982b). The acid- Ca^{2+} complex has an apparent dissociation constant (assuming 1:1 binding) of 115 nM in cytoplasmic conditions and allows estimation of Ca^{2+} levels from 10 nM to some 2 μM . This range is likely to encompass resting levels in excitable tissues (e.g. Brinley, 1980, found $[\text{Ca}^{2+}]_i$ in squid axoplasm to be 30–50 nM), but may be insensitive to stimulated levels [e.g. indirectly estimated in mammalian synaptosomes at 1–10 μM (Akerman & Nicholls, 1981a)].

The present paper describes a method for incorporating quin-2 into synaptosomes from guinea-pig cerebral cortex. $[\text{Ca}^{2+}]_i$ was measured under a variety of conditions and preliminary attempts were made to investigate the changes accompanying neurotransmitter release.

Materials and methods

Materials

Quin-2 and quin-2 A/M were obtained as solids from Lancaster Synthesis (Morecambe, Lancs., U.K.). $[\text{G-}^3\text{H}]\text{Quin-2 A/M}$ (sp. radioactivity 10.6 mCi/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.) as a 2 mg/ml solution in dimethylsulphoxide. Before storage the unlabelled ester was also dissolved in dimethylsulphoxide, at a concentration of 5 mM, and aliquots were divided between brown glass ampoules which were then sealed. All quin stocks (the free acid still as a solid) were subsequently

stored at -20°C in a dessicator. [*methyl*- ^{14}C]-Choline chloride (sp. radioactivity 58 mCi/mmol) and [*methyl*- ^3H]-choline chloride (sp. radioactivity 15 mCi/mmol) were obtained from Amersham International. ^3H -labelled acetylcholine was prepared from the latter by acetylation with acetic anhydride. Veratridine, oligomycin and calcium ionophore A23187 were all purchased from Sigma and dispensed from ethanolic stock solutions. Verapamil was obtained as an aqueous solution of the hydrochloride (2.5 mg/ml in 150 mM-NaCl) from Abbott Laboratories (Queenborough, Kent, U.K.). Physostigmine (eserine) sulphate was supplied by BDH. The other chemicals and reagents used were all obtained from either Sigma or BDH and were of at least reagent grade.

Media

A modified Krebs-Ringer incubation medium was used which in its standard form contained 10 mM-glucose, with 132 mM-NaCl, 5 mM-KCl, 1.2 mM- NaH_2PO_4 , 1.2 mM- MgCl_2 , 2 mM- CaCl_2 and 20 μM -Tris/HCl, pH 7.4, made up in distilled water from concentrated stock solutions. At times the CaCl_2 content was varied between zero and 5 mM, the final addition being up to 0.5% 1 M- CaCl_2 added slowly with stirring near the final pH. Above 5 mM- CaCl_2 , calcium phosphate tended to precipitate. In some experiments the KCl concentration was increased but always with an accompanying reduction in NaCl to maintain iso-osmolality. In order to reduce small-particle scatter during fluorescence measurements the final solutions were filtered under pressure through 0.45 μm filters (Millipore, Bedford, MA, U.S.A.). Other changes to the medium are described in the Results section.

Preparation of synaptosomes

For each experiment synaptosomes were prepared from the forebrains of one or two guinea-pigs essentially as described by Gray & Whittaker (1962) with the following modifications. Firstly, after removal the cortices were quickly scraped free of much of their white matter tracts before being subjected to homogenization and centrifugation. Secondly, the final P_2B (synaptosomal) fraction was pelleted (20000 g_{av} , 15 min) only after slow dilution (over 5 min) with an equal volume of ice-cold incubation medium. Following preparation the synaptosomes were suspended in medium at a concentration equivalent to 1 g of cortex/ml (1 g-equiv./ml), i.e. about 10 mg of synaptosomal protein/ml, and kept on ice for at least 10–15 min before continuing as described below.

Quin-2 loading of synaptosomes

This was based on the method described for

lymphocytes by Tsien *et al.* (1982a). Control suspensions without quin-2 A/M were carried through every stage in each experiment except that dimethylsulphoxide (which was initially 1%, v/v) had no detectable effect on their subsequent behaviour and was generally omitted. For quin-2 loading synaptosomes were diluted to 0.15 g-equiv./ml and incubated at 37°C for 20 min after adding, with gentle agitation, quin-2 A/M to a final concentration of 50 μM . Following a further 10-fold dilution with warm medium, incubation was continued for 40 min to allow completion of intracellular hydrolysis to quin-2 while encouraging the acetic acid and formaldehyde also formed to diffuse out.

After separation from the loading medium by centrifugation (9000 g_{av} , 5 min), the synaptosomes were immediately resuspended in cold medium at 0.015–0.03 g-equiv./ml and kept on ice for up to 2 h as portions were removed for fluorescence measurements. Immediately before each measurement 0.5–1.5 ml aliquots of both control and quin-2-loaded suspensions were centrifuged in micro-Eppendorf tubes (Janetzki TH11 microcentrifuge, H. Janetzki, Leipzig, German Democratic Republic) for 20 s and the pellets were resuspended in 1 ml of fresh warm medium in matched 1 cm square quartz fluorescence cuvettes.

Fluorescence measurements

All readings were made at or near the maximum sensitivity of an Aminco-Bowman spectrophotofluorimeter fitted with an off-axis ellipsoidal condensing system. Full uncorrected emission spectra were usually recorded (using a PL3 X-Y recorder, JJ Instruments, Southampton, Hants., U.K.) to confirm the presence of a discrete emission peak. The standard cell chamber was replaced by a water-jacketed multiple cell changer maintained at 37°C and fitted with 1 cm-high aluminium spacers underneath the cuvettes so that 1 ml of the suspension was sufficient for readings. Narrow excitation and emission cell slits of 0.5 and 0.1 mm respectively were required for satisfactory resolution and all the other instrument slits were set to maximum.

The suspensions were stirred by repeated gentle passage through a wide-bore (2 mm) Pasteur pipette and emission spectra were recorded after 10 min equilibration at 37°C . Additions were made from microsyringes directly to the suspensions, which were then stirred. After each sample cuvettes were thoroughly cleaned using methanol followed by distilled water and then allowed to dry at room temperature. Freedom from fluorescent impurities required minimal or no contact with plastics.

Transmitter release

Synaptosomes at 1 g-equiv./ml were pre-incubated in medium for 15 min at 37°C following which [^{14}C]choline chloride was added to a final concentration of 2 μM (0.1 $\mu\text{Ci}/\text{ml}$) and incubation was continued for a further 45 min (i.e. total incubation time as for quin-2 loading above). Transmitter release was measured immediately after loading when acetylcholine and choline were to be separated or total release was to be assessed after varying $[\text{Ca}^{2+}]_e$. However in other experiments these measurements had to be delayed (by about 45 min) while measuring $[\text{Ca}^{2+}]_i$ in samples loaded with quin-2 in a parallel incubation.

To measure release, 0.2 g-equiv. of synaptosomes was placed in micro-Eppendorf tubes and centrifuged for 20 s as previously described. The supernatant was aspirated and the material washed twice (by re-suspension and centrifugation) in warm calcium-free incubation medium (containing 50 μM -eserine when separating choline and acetylcholine). The pellet was finally suspended in the appropriate release medium (with eserine if required) and after 10 min at 37°C the supernatant was collected following a final centrifugation step. The released radioactivity was measured by scintillation spectrometry either directly or after extraction and separation of choline and acetylcholine in the presence of [^3H]acetylcholine internal standards (Wonnacott & Marchbanks, 1976) when double-channel counting was employed.

Other measurements

Protein was measured by the method of Miller (1959) using bovine serum albumin for standards. [$\text{G}-^3\text{H}$]quin-2 A/M carries no label on the ester portions, so its specific radioactivity is unchanged on conversion to the acid. Labelled quin and total transmitter release were measured in a standard Triton X-100/toluene mixture containing 2,5-diphenyloxazole/1,4-bis(5-phenyloxazol-2-yl)-benzene scintillant. Separated [^{14}C]choline and [^{14}C]acetylcholine were finally obtained as cellulose t.l.c. scrapings and were counted in a similar mixture without Triton but containing 3.5% (w/v) fumed silica to aid suspension. All scintillation counts were converted to d.p.m. by reference to counting standards.

Results

Intrasynaptosomal quin-2 and $[\text{Ca}^{2+}]_i$

Quin-2 A/M uptake and conversion to the free acid were followed fluorimetrically by exciting at 339 nm and observing a shift in the emission spectrum from a peak at 430 nm for the ester to a peak at 492 nm for the acid (Tsien *et al.*, 1982b). Zero-time and 60 min traces from such an experi-

ment are shown in Fig. 1 (inset). The emission peak heights at these and intermediate times are plotted in the main part of the Figure (all for the same amount of synaptosomal protein, after subtracting the background signal from unlabelled synaptosomes). The rapid disappearance of the 430 nm peak is accompanied by the appearance of the 492 nm peak characteristic of the commercial free acid, although initially the ester emission spectrum overlaps and augments that of the acid. Intrasynaptosomal quin-2 concentrations reached under the standard loading conditions were esti-

Fig. 1. Synaptosomal incorporation of quin-2 A/M and conversion to quin-2

The inset shows the uncorrected emission spectra from synaptosomal suspensions incubated with quin-2 A/M as described in the Materials and methods section, immediately after ester addition (---) and after 60 min incubation (—). The signal from synaptosomes alone is also shown (····). Emission peak heights ($\lambda_{\text{emission}}$) at 430 nm (●) and 492 nm (○) corresponding to the ester and free acid respectively are plotted in the main graph against incubation time, and are corrected for the unlabelled signal and represent the same synaptosomal protein concentrations. Excitation was at 339 nm throughout.

mated using G-³H-labelled ester. Assuming an intrasynaptosomal volume of 3 μ l/mg of protein (Marchbanks, 1975, Scott & Nicholls, 1980) cytoplasmic quin-2 concentrations were estimated to be 2.3 ± 0.1 mM (mean $\pm$ S.E.M. from five experiments). It was noticed that during the 60 min incubation virtually all the added radioactivity became associated with the synaptosomes.

During most fluorescence measurements the preparation was exposed to exciting light for only about 10 s. The emission spectrum was recorded, once every 3–5 min, and the possibility of significant photobleaching is unlikely given the small area of the light path (1.5 mm²). However, samples were subjected to continuous illumination in several experiments (e.g. Fig. 2) to give an indication of the time course of induced $[Ca^{2+}]_i$ changes. Fig. 2 also serves to illustrate the method of calibration. As already mentioned observations were always repeated on control suspensions (see the lower trace) to which additions were also made. Adding 10 μ l of normal medium gives rise to a brief mixing artifact, but 10 μ l of 3 M-KCl (to give a final KCl concentration of 35 mM) leads to a more

sustained increase in the signal which falls over about 60 s to a new constant level (which serves to confirm the relative lack of photobleaching).

Calibration of this and other recordings was by the method of Tsien *et al.* (1982b) except that careful attention was paid to the effects of sample scatter and autofluorescence which are more marked in these synaptosomal preparations than in the lymphocytes and thymocytes used by those authors or in adrenal chromaffin cells (Knight & Kesteven, 1983). For 1:1 stoichiometry of quin-2 and Ca^{2+} and an apparent dissociation constant for the cytosolic complex of 115 nM:

$$[Ca^{2+}]_i = \frac{[Ca \text{ complex}]}{[Free \text{ acid}]} \times 115 \text{ nM} \quad (1)$$

The ratio $[Ca \text{ complex}]:[free \text{ acid}]$ is given by $(F - F_{min.}): (F_{max.} - F)$, where F is the observed fluorescence, $F_{max.}$ the maximum signal in the presence of excess Ca^{2+} and $F_{min.}$ the minimum signal in the presence of excess EGTA at a pH > 8.3. To find $F_{max.}$ the synaptosomes were lysed by adding 20 μ l of 10% (w/v) Triton X-100 or 10 μ l of 10% (w/v) sodium dodecyl sulphate. Digitonin, as used by Tsien *et al.* (1982b) and Knight & Kesteven (1983), increased scatter when added to synaptosomes (at up to 100 μ M) perhaps due to the formation of micelles and was also less effective than Triton X-100 at releasing occluded lactate dehydrogenase (measured as in Marchbanks, 1967). Lactate dehydrogenase activities could not be measured in the presence of sodium dodecyl sulphate, but as this substance led to $F_{max.}$ levels at least as high as those given by Triton X-100, and less was required, it was often used. Synaptosomal lysis resulted in the exposure of the entrapped probe (less than 2 nmol in each experiment) to a $[Ca^{2+}]_e$ of 1 mM or more, easily enough to saturate quin-2 binding and give $F_{max.}$ As discussed by Tsien *et al.* (1982b), addition of 10 μ M-diethylenetriaminepenta-acetic acid (a chelator for heavy metal impurities complexing with quin-2 and depressing $F_{max.}$) did occasionally increase readings (by up to 5%). Subsequent addition of excess EGTA (10–20 μ l of 0.5 M) and sufficient 1 M-Tris or -NaOH to raise the pH to 8.3 yielded $F_{min.}$ In practice all readings were the difference between signals from quin-2-loaded synaptosomes and controls because of the very significant scatter and autofluorescence contributions. In Fig. 2 calibration indicates a resting $[Ca^{2+}]_i$ of 215 nM increasing to 670 nM immediately after the addition of 30 mM-KCl and falling after 1 min to a steady level of 370 nM.

$[Ca^{2+}]_i$ was measured in medium containing 1 mM- or 2 mM- $CaCl_2$ after quin-2 loading under the same conditions. In 2 mM- $[Ca^{2+}]_e$ eighteen pre-

Fig. 2. Intrasyntosomal quin-2 fluorescence following K^+ -depolarization: time course and method of calibration. Synaptosomes were quin-2 loaded (upper trace) or included as controls (lower trace) and their fluorescence monitored in normal medium as described in the Materials and methods and Results sections. Additions were made at A and B of 1% (v/v) normal medium and 1% (v/v) 3 M-KCl respectively (final KCl concentration 35 mM). The abrupt change at C represents arc wander in the SPF lamp. The measured fluorescence signal F is the difference between the two traces. $F_{max.}$, the maximum signal with saturating Ca^{2+} , is obtained after synaptosomal lysis by adding 1% (v/v) of 10% (w/v) sodium dodecyl sulphate. $F_{min.}$, the minimum signal, follows chelation of all the Ca^{2+} present with 1% (v/v) 0.5 M EGTA (final pH > 8.3). $[Ca^{2+}]_i = (F - F_{min.}/F_{max.} - F) \times 115$ nM.

parations gave a value for $[Ca^{2+}]_i$ of 205 ± 5 nM (mean $\pm$ S.E.M.), and in 1 mM $[Ca^{2+}]_e$ eight preparations gave a value of 85 ± 10 nM (mean $\pm$ S.E.M.).

Equilibration of Ca^{2+} across the non-depolarized plasma membrane

The considerable amounts of Ca^{2+} which can be accumulated by neuronal tissues (Baker, 1972, 1976) are distributed between various compartments which effectively act to buffer free cytosolic Ca^{2+} . Eventually an equilibrium with $[Ca^{2+}]_e$ must become established across the plasma membrane and, as discussed by Baker (1972, 1976), if the membrane is permeable to Ca^{2+} some form of active outward transport must be involved. It has already been shown above that $[Ca^{2+}]_i$ is reduced when quin-2 loading is carried out in 1 mM $[Ca^{2+}]_e$ rather than 2 mM. This may partly be a reflection of differences in total calcium accumulation, so a more controlled study of the effect of altering $[Ca^{2+}]_e$ surrounding pre-loaded synaptosomes was made.

Loading was carried out in 1 mM- $CaCl_2$ (to keep the initial $[Ca^{2+}]_i$ low). Following their transfer to cuvettes after the final microcentrifugation step the samples (loaded and control) were equilibrated in normal medium at 37°C (still with 1 mM- $CaCl_2$) before small additions of 1 M- $CaCl_2$ were made with gentle mixing as previously described. After 5 min the fluorescence signal reached a new equilibrium level and was recorded. Finally (after three or four such additions) calibration was performed as described above (adding enough EGTA to chelate all the calcium added). In some experiments aliquots of EGTA were also added and the fluorescence signal decreased slowly (up to 1 min) rather than abruptly, as might be expected if the changes were due simply to synaptosomal lysis. The results are summarized in Fig. 3. Under these conditions increasing $[Ca^{2+}]_e$ leads to a proportional increase in $[Ca^{2+}]_i$ as the concentration gradient for the cation is altered.

Plasma membrane depolarization and $[Ca^{2+}]_i$

The effect of KCl-induced depolarization has already been noted in Fig. 2. An initial marked rise in $[Ca^{2+}]_i$ presumably follows entry through voltage-sensitive calcium channels (Kostyuk, 1981), at least some of which may subsequently be inactivated by raised $[Ca^{2+}]_i$ (Tsien, 1983). $[Ca^{2+}]_i$ is reduced over the course of about 1 min as the cation continues to equilibrate with intrasynaptosomal stores and a new equilibrium is reached across the plasma membrane. Fig. 4 shows the results of measuring equilibrium $[Ca^{2+}]_i$ after 10 min of KCl-induced plasma membrane depolarization. Synaptosomes were quin-2-loaded in normal medium containing 1 mM- $CaCl_2$ and trans-

Fig. 3. Equilibrium $[Ca^{2+}]_i$ and $[Ca^{2+}]_e$. Each point represents the mean $\pm$ S.E.M. from four quin-2-loaded synaptosomal preparations in which equilibrium $[Ca^{2+}]_i$ was measured as described in the Materials and methods and Results sections in response to $[Ca^{2+}]_e$ from 1 to 5 mM. The line was drawn following linear regression analysis (correlation coefficient $r = 0.99$).

ferred to media still containing 1 mM- $CaCl_2$ but various KCl concentrations (with an equimolar reduction in NaCl). If it is assumed that $[K^+]_i$ exceeds $[K^+]_e$ at least to 100 mM the plasma membrane potential ($\Delta\psi_p$, mV) can be calculated from the reduced Goldman equation:

$$\Delta\psi_p = \frac{RT}{ZF} \ln \frac{[K^+]_e + P_{Na}/P_K \times [Na^+]_e}{[K^+]_i} \quad (2)$$

inserting $P_{Na}/P_K = 0.053$ (Keen & White, 1971). These values are plotted versus the corresponding mean $[Ca^{2+}]_i$ values (inset in Fig. 4) where linear regression analysis gives:

$$[Ca^{2+}]_i = 0.71 - 0.012 \Delta\psi_p \quad (3)$$

with a correlation coefficient r of 0.99. This equation is used to produce the theoretical curve in the main part of Fig. 4. $[K^+]_i$ is in fact likely to be near 70 mM given the values usually obtained for the K^+ content of synaptosomes (e.g. see Vyas & Marchbanks, 1981). This latter value is also

Fig. 4. Equilibrium $[Ca^{2+}]_i$ following KCl-induced plasma membrane depolarization

Quin-2 loaded synaptosomes were K^+ -depolarized and equilibrium $[Ca^{2+}]_i$ was measured as described in the Materials and methods and Results sections. In the main Figure each point represents the mean $\pm$ S.E.M. from four preparations except the first point which represents eight preparations. In the inset graph measured $[Ca^{2+}]_i$ is plotted against $\Delta\psi_p$ calculated from the reduced Goldman equation and linear regression analysis shows a high correlation ($r = 0.99$). The regression equation was used as described in the text to plot the theoretical curve in the main Figure.

consistent with the values of 45–50 mV obtained for $\Delta\psi_p$ by measuring rubidium diffusion potentials (Scott & Nicholls, 1980) or by using potential-sensitive fluorescent probes (Blaustein & Goldring, 1975; R. H. Ashley, M. J. Brammer & R. M. Marchbanks, unpublished work). However, there will still be a potassium diffusion potential in Fig. 4 for log $[K^+]_e$ values up to at least 1.8 (i.e. $[K^+]_e = 63$ mM) and there does appear to have been a depolarization-related influx of Ca^{2+} .

Veratridine on the other hand depolarizes synaptosomes by increasing sodium entry (Li & White, 1977). Quin-2-loaded synaptosomes equilibrated in normal incubation medium were exposed to 150 μ M veratridine added from a 10 mM ethanolic solution, with corresponding additions to

control suspensions. In general, as shown in Table 1 unless $[Ca^{2+}]_i$ was previously elevated veratridine induced only a small (though still significant) increase in $[Ca^{2+}]_i$. It was found to be particularly important to observe the behaviour of control suspensions as uptake of Na^+ increased scatter considerably. In several experiments the calcium channel blocker verapamil (200 μ M) was added to the synaptosomes 10 min before the end of the loading incubation, and in these samples there was no change in $[Ca^{2+}]_i$ on adding veratridine.

Mitochondrial inhibition and other manipulations of $[Ca^{2+}]_i$

The proportion of synaptosomes containing mitochondria depends to some extent on the method of subcellular fractionation employed, but under the conditions described here it is likely that most of them will contain at least one such organelle (Jones, 1975). The intracellular compartmentation of calcium is overwhelmingly determined by the cation's distribution across the inner membrane of any mitochondrion (Carafoli, 1982) and this is particularly the case in synaptosomes (Scott *et al.*, 1980). Even at relatively low mitochondrial membrane potentials ($\Delta\psi_m$) of around say 120 mV, calcium accumulation could theoretically reach the levels predicted by the Nernst equation:

$$\text{matrix } [Ca^{2+}]/[Ca^{2+}]_i = \exp(ZF\Delta\psi_m/RT) \quad (4)$$

leading, with the values for cytoplasmic Ca^{2+} reported in the present paper, to intramitochondrial calcium concentrations of several millimolar (although the presence of a sodium-dependent calcium efflux pathway in brain mitochondria, discussed later, means that in practice this theoretical uptake is lowered).

It was thus of interest to modify $\Delta\psi_m$ and observed the effect on $[Ca^{2+}]_i$. The respiratory uncoupler CCCP could not be employed because of its fluorescent properties (likely to be shared by other lipophilic weak acid proton translocators) but respiration could be inhibited by exposing the synaptosomes to KCN with concomitant KCl reduction (*cf.* Baker, 1972). According to the chemiosmotic theory (Mitchell, 1961, 1966) any reduction in $\Delta\psi_m$ will be accompanied by reversal of mitochondrial ATP synthetase using glycolytic ATP. This ATPase activity can be prevented by oligomycin so that glycolytic ATP is conserved in the presence of a reduced $\Delta\psi_m$, as shown for synaptosomes by Scott *et al.* (1980). Free mitochondria cannot of course respire in the medium used due to lack of a suitable substrate. Should the organelles still accumulate significant amounts of calcium in the presence of a high concentration of

sodium (brain mitochondria having a very active sodium-stimulated calcium efflux pathway; Carafoli, 1982) this is likely to be lethal in the presence of relatively high levels of inorganic phosphate.

From Table 1 it can be seen that respiratory inhibition in quin-2-loaded synaptosomes did lead to a large increase in $[Ca^{2+}]_i$, which was less marked in the presence of oligomycin (4 μ g/ml). It is likely that the relative preservation of glycolytic ATP on adding oligomycin enabled a plasma-membrane calcium pump to lower $[Ca^{2+}]_i$ despite the extra Ca^{2+} released from more depolarized mitochondria. In either case 150 μ M-veratridine increased $[Ca^{2+}]_i$ beyond the limit of measurability ($>2 \mu$ M), indicating that mitochondrial buffering is likely to be of major importance in limiting depolarization-induced $[Ca^{2+}]_i$ rises (though not necessarily the only mechanism as its effectiveness must be reduced on increasing cytosolic Na^+ as discussed above).

The contribution to synaptosomal calcium homeostasis of Na^+/Ca^{2+} exchange across the plasma membrane (Blaustein & Oborn, 1975) is disputed (Akerman & Nicholls, 1981b) and some experiments were performed on synaptosomes which were quin-2-loaded and then exposed to media where LiCl replaced NaCl. From Table 1 it is seen that this resulted in higher resting levels of $[Ca^{2+}]_i$ which were increased to $>2 \mu$ M after respiratory inhibition even in the presence of oligomycin. These results may be partly explained by the inhibition of Na^+/Ca^{2+} exchange or even by reversal of the exchange, but the conditions are too extreme to draw more firm conclusions.

Ca^{2+} movements may be directly facilitated by incorporation of the calcium ionophore A23187 into membranes. However, this compound is

fluorescent (Pfeiffer *et al.*, 1974), ultraviolet excitation producing an emission peak which obscures quin-2 fluorescence at ionophore concentrations in excess of 100 nM. Also if it is added to solutions already containing significant amounts of Mg^{2+} and Ca^{2+} less is incorporated into membranes (Selinger *et al.*, 1974), possibly because it is complexed in the medium.

In order to circumvent these problems synaptosomes were suspended in low Mg^{2+} medium with no added Ca^{2+} for the final 10 min of their incubation (Wonnacott *et al.*, 1978) for exposure to 200 nM-A23187, which medium was removed during the normal subsequent washing procedures. As shown in Table 1 this led to a considerable elevation of $[Ca^{2+}]_i$, probably due to a combination of mitochondrial efflux and Ca^{2+} entry across the plasma membrane.

Transmitter release and $[Ca^{2+}]_i$

Very little is known concerning the timing of neurotransmitter release in relation to $[Ca^{2+}]_i$ changes. Recently, using quin-2 in isolated bovine adrenal medullary cells (a preparation which may recreate certain aspects of neurosecretion in catecholamine-containing neurones), Knight & Kesteven (1983) followed catecholamine secretion in response to an acetylcholine challenge and observed a broad correspondence to a transient increase in $[Ca^{2+}]_i$ (which peaked at 300 nM from a resting level near 100 nM in 1 mM- $[Ca^{2+}]_e$). The events occupied some 5 min altogether. The protocol described here provides a means of measuring equilibrium levels of $[Ca^{2+}]_i$ rather than dynamic changes (cf. the maximum and final values of $[Ca^{2+}]_i$ in Fig. 2) and synaptosomes from cerebral cortex include a number of different transmitter systems. Nevertheless, some investigations of transmitter release were undertaken after synaptosomes were loaded with $[^{14}C]$ choline as described in the Methods section. In experiments where $[Ca^{2+}]_i$ was compared with released radioactivity synaptosomes from the same preparation were subjected to conditions as similar as possible apart from the necessary concentration differences during loading with either quin-2 or $[^{14}C]$ choline.

Innumerable studies have demonstrated that in the presence of calcium K^+ -depolarization of synaptosomes pre-loaded with radiolabelled choline results in the release of radioactivity, mainly incorporated into acetylcholine although labelled choline is released as well. It has been seen that K^+ -depolarization also leads to readily-measurable changes in equilibrium $[Ca^{2+}]_i$ (Fig. 4). Fig. 5 shows the results from three experiments where $[Ca^{2+}]_i$ was altered by varying $[K^+]_e$ and compared with total release over 10 min in synaptosomes from the same preparation subjected to

Table 1. Cytoplasmic free Ca^{2+} under various conditions
All values are means $\pm$ S.E.M. or the range of the number of preparations in parentheses. Using paired *t*-tests, *P*(*a*) < 0.05 and *P*(*b*) < 0.01.

Additions to normal medium	$[Ca^{2+}]_i$ (nM)	
	No veratridine	150 μ M-veratridine
Nil	219 $\pm$ 15 (<i>a</i>) (3)	374 $\pm$ 24 (<i>a</i>) (3)
Verapamil (200 μ M)	185 $\pm$ 5 (3)	185 $\pm$ 5 (3)
KCN (2 mM)	989 $\pm$ 20 (<i>b</i>) (4)	All $> 2 \mu$ M (3)
KCN (2 mM) and oligomycin (4 μ g/ml)	612 $\pm$ 39 (<i>b</i>) (4)	All $> 2 \mu$ M (3)
LiCl replacing NaCl	508 $\pm$ 96 (2)	—
LiCl replacing NaCl with KCN + oligomycin	All $> 2 \mu$ M (2)	—
A23187 (200 nM)	939 $\pm$ 38 (2)	—

Fig. 5. *Transmitter release and $[Ca^{2+}]_i$*
All labelled release was measured in three preparations (○, ●, ▲) over the 10 min immediately following exposure of [^{14}C]choline-loaded synaptosomes to media containing 5–100 mM-KCl (maintaining iso-osmoticity by decreasing NaCl). $[Ca^{2+}]_i$ was measured in quin-2 loaded synaptosomes from a parallel incubation as described in the Materials and methods and Results sections. The theoretical curve relating the rate of change of 10 min release to $[Ca^{2+}]_i$ is derived as described in the Results section.

similar conditions. As might be expected there is considerable scatter (largely as a result of three separate radioactive loading and release experiments) but inspection of the data does suggest that release, measured over a 10 min period (R_{10}), is saturable at higher $[Ca^{2+}]_i$, with maximal 10 min release ($R_{10,max}$) when $[Ca^{2+}]_i = 395$ nM (linear regression analysis of a double-reciprocal plot, $r = 0.88$). The rate of change of measured release with $[Ca^{2+}]_i$ may be proportional to the amount of transmitter remaining in a releasable pool $R_{10,max} - R_{10}$, i.e.:

$$dR_{10}/d[Ca^{2+}]_i = K(R_{10,max} - R_{10}) \quad (5)$$

which on integration and insertion of the numerical value for $R_{10,max}$ gives:

$$R_{10} = 395 - A(e^{-K[Ca^{2+}]_i}) \quad (6)$$

The constants A and K were obtained from a plot of $\log(R_{10,max} - R_{10})$ versus $[Ca^{2+}]_i$ and the theoretical curve obtained by using eqn. (6) is drawn in Fig. 5. (A much poorer fit is obtained if the relationship between 10 min release and $[Ca^{2+}]_i$ is assumed to be a simple hyperbolic one.)

Equilibrium $[Ca^{2+}]_i$ was also increased by raising $[Ca^{2+}]_e$ (Fig. 3) and this might be expected to result in transmitter release. Synaptosomes were therefore labelled with [^{14}C]choline as described in the Methods section in medium containing 1 mM-

Fig. 6. *Transmitter release and $[Ca^{2+}]_e$*
In the main Figure each point represents the mean $\pm$ S.E.M. of all radiolabelled release from three [^{14}C]choline-loaded synaptosomal preparations in the 10 min following exposure to media containing 0–5 mM- $CaCl_2$. In the bar chart separated [^{14}C]acetylcholine release under the same conditions is expressed as a percentage of total release (mean $\pm$ S.E.M. from four preparations).

$CaCl_2$ and total release was measured after resuspension in normal media containing 0–5 mM- $CaCl_2$. The results from three determinations are shown in Fig. 6 (main graph) and although release over 10 min does at first increase (in parallel with $[Ca^{2+}]_i$, Fig. 3) it peaks around 3 mM- $[Ca^{2+}]_e$ and then declines to around the level of calcium-independent release. In further experiments the released radioactivity was separated into [^{14}C]acetylcholine and [^{14}C]choline. After achieving pellet loadings of 1223 ± 83 and 2330 ± 144 (mean d.p.m./mg of protein $\pm$ S.E.M. from four experiments) respectively, up to 616 ± 82 and 1734 ± 126 (mean d.p.m./mg of protein; $\pm$ S.E.M., $n = 4$) respectively were released during 10 min at 37°C. Acetylcholine release was calculated as a fraction of total release from three or four experiments and is shown in the inset to Fig. 6 (the error bars representing $\pm$ S.E.M.). Fractional acetylcholine release also peaks at 3 mM- $[Ca^{2+}]_e$, suggesting that there is a real effect on transmitter output, with [^{14}C]choline release more that of a constant back-

ground leakage. Similar results have been obtained in at least one physiological preparation (rat hemidiaphragm) by Gage (1967). In that case it was postulated that a calcium-binding molecule, probably located in the plasma membrane and carrying two anionic sites, bound either Na^+ or Ca^{2+} or both but promoted neurotransmitter release only when present as CaX and inhibited release when present as Ca_2X (or NaX).

Conclusions

The results obtained here indicate that quin-2 can be incorporated into synaptosomes where it does appear to report realistic values for $[\text{Ca}^{2+}]_i$ (although as yet there are no other direct measurements for comparison). The intrasynaptosomal concentrations obtained do not seem to impair cellular functions (Tsien *et al.*, 1982b) and provided samples are loaded in the presence of adequate amounts of external calcium it appears that more is simply taken up to buffer the entrapped quin-2.

Plasma membrane depolarization, mitochondrial inhibition and ionophore addition result in consistent changes in $[\text{Ca}^{2+}]_i$ which can be reconciled with biochemical and neurochemical theory. Possible causes of a sustained $[\text{Ca}^{2+}]_i$ elevation in the presence of a reduced $\Delta\psi_p$ (which should promote Ca^{2+} efflux) include a relatively minor increase in Ca^{2+} influx (down a steep concentration gradient) or a relatively minor impairment of any $\text{Na}^+/\text{Ca}^{2+}$ exchange. However, $[\text{Ca}^{2+}]_i$ changes associated with transmitter release are of particular interest, and although equilibrium rather than dynamic changes were measured it is obvious that this technique holds considerable promise. At the neuromuscular junction transmitter is released in both a quantal and non-quantal fashion. Quantal release is calcium-dependent (Katz, 1969) unlike non-quantal release, which is often regarded more as transmitter 'leakage' (Katz & Miledi, 1977). The situation is less well characterized for central nervous system synapses but it appears to be likely that at least some non-quantal release occurs, and this may be reflected in experiments (such as Fig. 6) where unstimulated cholinergic synaptosomes suffer a continuous loss of, particularly, choline, probably as simple molecular leakage across the plasma membrane. For this reason, synaptosomal release is usually defined in terms of calcium-dependency, and non-dependent release (e.g. Vyas & Marchbanks, 1981) is accredited to membrane damage.

Consequently, in experiments such as that in Fig. 5, where release is induced by plasma membrane depolarization in the presence of calcium, most information regarding cytoplasmic Ca^{2+} and

the release mechanism is likely to be gained from a closer study of short-term $[\text{Ca}^{2+}]_i$ changes associated with release, particularly if transmitter output could be followed at shorter time intervals or even continuously. Thus far the effective upper limit of $2\text{ }\mu\text{M}$ for the $[\text{Ca}^{2+}]_i$ sensitivity of quin-2 appears surprisingly adequate but the probe might be expected to buffer $[\text{Ca}^{2+}]_i$ transients. The 'release' which is independent of plasma membrane depolarization but varies in response to $[\text{Ca}^{2+}]_e$ (Fig. 6) peaks when $[\text{Ca}^{2+}]_e$ reaches 3 mM, despite a continued rise in $[\text{Ca}^{2+}]_i$ (Fig. 3). At least under these conditions it may be supposed that transmitter output is limited not by free cytoplasmic calcium but by a calcium complex, probably located in the plasma membrane. It is also possible that $R_{i0,\text{max}}$ as described for Fig. 5 represents not a certain neurotransmitter pool but the maximum activity of a calcium complex, or release assembly. It appears likely that further studies using this new class of internal Ca^{2+} indicators will provide important information about the molecular events involved in synaptic transmission.

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