Calcium-ion transport by intact synaptosomes

Intrasynaptosomal compartmentation and the role of the mitochondrial membrane potential

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The association of Ca^{2+} with isolated nerve endings (synaptosomes) is investigated and resolved into two components, that bound to the outer surface of the plasma membrane and that transported across the plasma membrane. When synaptosomes are added directly to a Ca^{2+} -containing medium, there is an initial rapid uptake of Ca^{2+} across the plasma membrane, followed by a slow uptake that proceeds for 20 min. The rapid phase is not observed if the synaptosomes are initially pre-incubated in a Ca^{2+} -free medium. Rapid disruption of synaptosomes reveals that less than 3 nmol of transported Ca^{2+} per mg of synaptosomal protein can be ascribed to non-mitochondrial components, whereas the remainder, up to 79% of the total, is further transported into the mitochondrial matrix. Abolition of oxidative phosphorylation while the mitochondrial membrane potential is retained leads to a time-dependent increase in transported Ca^{2+} , whereas abolition of the mitochondrial membrane potential decreases both plasma-membrane transport and accumulation of Ca^{2+} in the mitochondrial matrix. It is concluded that intrasynaptosomal mitochondria are major regulators of synaptosomal Ca^{2+} .

The study of Ca^{2+} transport across the plasma membrane of isolated nerve endings (synaptosomes) and the subsequent distribution of the cation among the various sub-synaptosomal compartments is clearly central to the investigation of the Ca^{2+} -dependency of neurotransmitter release (for review see Raiteri & Levi, 1978). There is, however, little consensus in the literature. Thus although the uptake of Ca^{2+} across the plasma membrane has been shown to be enhanced by depolarization (Blaustein, 1975), and although efflux of Ca^{2+} from synaptosomes has been reported to involve an exchange of Ca^{2+} for Na^{+} (Blaustein & Oborn, 1975; Blaustein & Ector, 1976), estimates of synaptosomal Ca^{2+} content after a short period of incubation in media containing millimolar concentrations of Ca^{2+} vary from less than 1 to greater than 30 nmol of Ca^{2+} per mg of synaptosomal protein depending on the methodology employed (Blaustein, 1975; Blaustein & Ector, 1976; Goddard & Robinson, 1976; Jansson *et al.*, 1977; Wonnacott *et al.*, 1978).

One purpose of the present paper is to attempt to resolve these anomalies by examining critically the

Abbreviations used: pCa^{2+} , $-\log$ (free Ca^{2+} concentration); $\Delta\psi_m$, membrane potential across the mitochondrial inner membrane.

methodology for the determination of total synaptosomal Ca^{2+} content both to distinguish between superficially bound and transported Ca^{2+} and to minimize artefactual efflux of Ca^{2+} during the separation procedure.

The second purpose of the present paper is to investigate the subcellular distribution of the transported Ca^{2+} . Two classes of isolated sub-synaptosomal organelle accumulate Ca^{2+} . First, a non-mitochondrial vesicular fraction exhibits ATP-dependent Ca^{2+} uptake (Rahamimoff & Abramovitz, 1978*a,b*), although low rates are obtained and the origin of the fraction is unclear as an $\text{Na}^{+}/\text{Ca}^{2+}$ exchange activity, indicative of plasma membranes, can also be detected in the same preparation (Rahamimoff & Spanier, 1979). A similar low ATP-dependent Ca^{2+} uptake can be detected in hypo-osmotic lysates of synaptosomes (Blaustein *et al.*, 1978*a,b*). Purified cholinergic synaptic vesicles also exhibit an ATP-dependent uptake of Ca^{2+} (Michaelson *et al.*, 1980).

The second putative Ca^{2+} -accumulating organelle is the mitochondrion. Brain mitochondria of synaptosomal and non-synaptosomal origin have similar Ca^{2+} transport properties (Nicholls, 1978*a*). As with other mitochondria (for reviews see Nicholls & Crompton, 1980; Saris & Åkerman, 1980), the

distribution of Ca^{2+} across the brain mitochondrial inner membrane is the result of a kinetic balance between independent uptake and efflux pathways (Nicholls, 1978a,b; Crompton *et al.*, 1978; Nicholls & Scott, 1980a).

In the present paper the significance of the intrasynaptosomal mitochondria is assessed by investigating the effect of specific abolition of the mitochondrial membrane potential both on net plasma-membrane transport and on the distribution of Ca^{2+} between mitochondrial and non-mitochondrial compartments, employing rapid fractionation techniques of the type developed for the study of compartmentation within hepatocytes (Zuuren-donk & Tager, 1974; Tischler *et al.*, 1977; Murphy *et al.*, 1980).

Although some attempts have been made to determine the sub-synaptosomal distribution of Ca^{2+} by fractionation and separation (Blaustein *et al.*, 1978a,b; Wonnacott *et al.*, 1978), both the methods of disruption (lysis in hypo-osmotic media for periods of 1–5 min) and the subsequent separation [centrifugation for 60 min (Wonnacott *et al.*, 1978) or filtration followed by washing with media containing 1.2 mM- Ca^{2+} (Blaustein *et al.*, 1978b)] do not preclude an extensive artefactual redistribution of the cation.

It is concluded that under conditions *in vitro* intrasynaptosomal mitochondria play a significant role in the regulation of cytosolic Ca^{2+} , and that this in turn influences the net transport of Ca^{2+} across the plasma membrane.

Part of this work has been published as a Conference report (Nicholls & Scott, 1980b).

Experimental

Materials

Radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ficoll was obtained from Pharmacia, Uppsala, Sweden, and was exhaustively dialysed against water before use. Ruthenium Red, digitonin, veratrine, ouabain, oligomycin and all enzymes were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Ruthenium Red was recrystallized before use. Silicone fluid (Dow Corning 550) and dinonyl phthalate were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. All other reagents were of analytical grade. Unless otherwise stated sodium salts were employed throughout.

Synaptosomes and 'free' brain mitochondria

Synaptosomes and 'free' (i.e. non-synaptosomal) mitochondria were prepared from the cerebral cortices of Duncan–Hartley-strain guinea pigs of either sex aged 4–8 weeks, exactly as previously

described (Nicholls, 1978a; Scott & Nicholls, 1980). Synaptosomes were stored at 0°C as concentrated pellets in 250 mM-sucrose/5 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethane-sulphonate), pH 7.2, for not more than 3 h, before use.

The total Ca^{2+} content of the sucrose-stored synaptosomes, determined by atomic absorption was 0.56 ± 0.10 nmol/mg of synaptosomal protein (mean of six determinations), which is sufficiently low, compared with the $^{45}\text{Ca}^{2+}$ contents reported in the present paper, to be ignored.

Determination of synaptosomal Ca^{2+} accumulation

Synaptosomal pellets containing 7.5 mg of protein were resuspended in 1 ml of an air-saturated medium at 30°C and pH 7.4 which contained 122 mM-NaCl, 3.1 mM-KCl, 1.2 mM- MgSO_4 , 1.3 mM- CaCl_2 , 0.4 mM- KH_2PO_4 , 5 mM- NaHCO_3 , 20 mM-Tes and 10 mM-D-glucose. The suspension was then immediately transferred to a further 4 ml of incubation medium that contained additionally $^{45}\text{Ca}^{2+}$ (0.51 $\mu\text{Ci}/\text{ml}$ final concentration) and 167 μM - ^{3}H -sucrose (3.3 $\mu\text{Ci}/\text{ml}$ final concentration), together with further additions as indicated. The suspensions ('non-preincubated synaptosomes') were then incubated in polycarbonate flasks at 30°C in a shaking water bath. Alternatively, the incubation was allowed to proceed for 15 min in the total absence of added Ca^{2+} before addition of the cation ('preincubated synaptosomes'). Portions (500 μl) were withdrawn at defined times and layered on to 400 μl of a mixture of 60% (v/v) Dow Corning 550 silicone fluid and 40% (v/v) dinonyl phthalate contained within a 1.5 ml-capacity Eppendorf centrifuge tube. Where indicated 4.5 μl of a mixture of 220 mM-EGTA and 550 μM -Ruthenium Red was present in the centrifuge tube sufficient to give a final concentration in the incubation of 2 mM and 5 μM respectively. Centrifugation was initiated within 5 s, and continued for 60 s on an Eppendorf model 5412 bench centrifuge. In one experiment (Fig. 2) the delay between EGTA addition and centrifugation was varied. A portion of the supernatant was taken for counting ^3H and $^{45}\text{Ca}^{2+}$ radioactivities, after which the remaining supernatant was removed with a Pasteur pipette. The side of the tube was washed with water, and the silicone fluid was removed. The synaptosomal pellet was suspended in 200 μl of 12% (v/v) HClO_4 , and centrifuged for 60 s. A portion of the supernatant from this centrifugation was removed, neutralized with 3 M- K_2CO_3 /1 M-Tris base and counted for radioactivity as before.

In one experiment the synaptosomes were filtered through 0.6 μm pore size cellulose acetate filters (Sartorius, Göttingen, Germany), and $^{45}\text{Ca}^{2+}$ association was determined as described previously for mitochondria (Heaton & Nicholls, 1976).

Determination of the distribution of Ca^{2+} within synaptosomes

Synaptosomes were suspended and incubated with or without a 15 min Ca^{2+} -free pre-incubation as described above. At defined times 500 μl portions were transferred to Eppendorf centrifuge tubes. The portion was then taken up into a 2 ml plastic syringe that contained 1 ml of non-radioactive incubation medium together with 7.5 μM -Ruthenium Red, 3 mM-EGTA and various concentrations of digitonin as specified in the text. The concentration of EGTA was sufficient to decrease the free Ca^{2+} concentration to 1 μM . This precaution was necessary to avoid exposing the mitochondria released by the action of digitonin to the millimolar free Ca^{2+} concentrations of the incubation medium. Ruthenium Red was present in order to inhibit the mitochondrial Ca^{2+} -uniporter (see Nicholls & Crompton, 1980).

It should be noted that the mitochondrial Na^{+} -dependent Ca^{2+} -efflux pathway (Nicholls, 1978a; Crompton *et al.*, 1978) will be operative during the separation. At 30°C and pH 7.0 the activity of this pathway is about 10 nmol of Ca^{2+} /min per mg of mitochondrial protein (Nicholls, 1978a). An average separation time of 15 s will thus allow about 2 nmol of Ca^{2+} /mg of mitochondrial protein to efflux from the matrix during the separation. Values for matrix Ca^{2+} will thus be minimum estimates.

Fig. 1. Cross-section of disruptor and axial-loading rotor for the digitonin fractionation of synaptosomes (a) Extruded aluminium rotor; (b) Teflon insert; (c) swing-out centrifuge tube buckets; (d) 23-gauge needle; (e) 2 ml disposable syringe; (f) Perspex guide; (g) 1.8 kg weight.

The syringe containing synaptosomes and digitonin was immediately positioned in a Perspex guide located on the lid of a modified Eppendorf type 5412 bench centrifuge (Fig. 1). The centrifuge rotor was constructed to allow two swing-out centrifuge tubes, each containing 400 μl of 60% silicone fluid/40% dinonyl phthalate, to be loaded whilst the rotor rotates at full speed, thus greatly decreasing the total time needed to separate the soluble and particulate fractions of the digitonin-treated synaptosomes (Murphy *et al.*, 1980). The contents of the syringe were forced (within 5 s of mixing with digitonin) through a 23-gauge needle with a pressure of 2.8 kg/cm², generated with a 1.8 kg lead weight (Fig. 1). The final disruption of the synaptosomes is due to a combination of digitonin action (Zuurendonk & Tager, 1974; Nicholls, 1978a; Booth & Clark, 1979) and shear during passage through the needle (Tischler *et al.*, 1977).

Centrifugation was continued for 60 s, although control experiments indicated that the bulk of the synaptosomes sedimented through the silicon fluid within 15 s. Sampling of supernatants and pellets was performed as for intact synaptosomes.

Assay of marker enzymes during digitonin disruption of synaptosomes

Synaptosomes were incubated and disrupted in the presence of various concentrations of digitonin exactly as described above for ⁴⁵Ca²⁺, except that the radioisotopes were omitted. Enzymic activity was determined in the supernatant fraction after disruption and the pellet activity was estimated by difference with respect to portions of synaptosomal incubation solubilized with 0.1% (v/v) Triton X-100. Enzyme assays were performed at 30°C in a Cecil CE272 recording spectrophotometer. Lactate dehydrogenase (EC 1.1.1.27) was assayed in 50 mM-P_i/1 μM -rotenone/0.17 mM-NADH/4 mM-pyruvate/0.1% (v/v) Triton X-100. NAD⁺-linked glutamate dehydrogenase (EC 1.4.1.3) activity was assayed in a medium containing 200 mM-ammonium acetate, 10 mM-Tes, 10 mM-2-oxoglutarate, 1 mM-EGTA, 1 μM -rotenone, 5 mM-ADP, 0.17 mM-NADH and 0.1% (v/v) Triton X-100. Glucose 6-phosphatase (EC 3.1.3.9) was assayed in 50 mM-maleate/10 mM-glucose 6-phosphate/0.1% (v/v) Triton X-100, pH 6.5; after 30 min incubation the reaction was stopped by the addition of trichloroacetic acid (0.38 M final concentration), and liberated P_i was assayed. Acetylcholinesterase activity (EC 3.1.1.7) was assayed by the method of Ellman *et al.* (1961), except that 0.1% (v/v) Triton X-100 was added to the assay medium. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Statistical errors are presented as S.E.M.

Results and discussion

Ca²⁺ transport across the plasma membrane

The total Ca²⁺ content of a synaptosomal pellet consists of four components: 1, that in the extra-synaptosomal medium; 2, that associated with non-synaptosomal particles (e.g. free mitochondria) contaminating the preparation; 3, that bound superficially to the synaptosomal plasma membrane; 4, that transported into the synaptosome. To calculate the transported Ca²⁺ the other factors must be allowed for while at the same time avoiding artefactual movements of Ca²⁺ across the plasma membrane during the separation procedure.

Ca²⁺ accumulation by contaminating 'free' brain mitochondria was estimated in a control experiment in which free brain mitochondria were incubated for 20 min in synaptosomal incubation medium before centrifugation through oil. An association of 17 nmol of Ca²⁺/mg of mitochondrial protein was found. The 5% contamination of mitochondria in the present synaptosomal fraction (Nicholls, 1978a) therefore accounts for less than 0.9 nmol of Ca²⁺/mg of synaptosomal protein.

Allowance for contamination by incubation medium may readily be made, without perturbing the incubation conditions, by the inclusion of an extracellular marker, such as [³H]sucrose (the present paper), or ³⁵SO₄²⁻ (White & Keen, 1970). The time course of association of ⁴⁵Ca²⁺ with 'non-pre-incubated' synaptosomes after making this correction is shown in Fig. 2. Ca²⁺ association, i.e. binding plus uptake, is strongly biphasic. Control experiments in which rapid filtration replaced centrifugation showed that the rapid phase was complete within 10 s. This implies a minimum rate of association of 100 nmol/mg of synaptosomal protein per min or 200 times faster than the subsequent slow phase. The obvious conclusion (see Borle, 1975) is that a proportion at least of the rapid phase represents superficial binding of Ca²⁺ to the outer face of the plasma membrane.

The possibility of using La³⁺ to displace surface Ca²⁺ was investigated by Jansson *et al.* (1977). However, these authors found that 15 min were required for optimal displacement. EGTA, as introduced by Blaustein (1975), was suggested to be effective in displacing surface Ca²⁺ (Jansson *et al.*, 1977), but to lead to an extensive efflux of internal Ca²⁺ if separation was delayed. A similar conclusion was reached by Wonnacott *et al.* (1978). The EGTA-induced efflux is confirmed in the present study (Fig. 2). EGTA added after either 30 s or 10 min of incubation resulted in a decrease in ⁴⁵Ca²⁺ association with a time course that is the converse of that of the initial ⁴⁵Ca²⁺ association; namely an initial rapid phase followed by a subsequent slow phase.

Fig. 2. Influence of Ruthenium Red plus EGTA on the time course of ⁴⁵Ca²⁺ association with synaptosomes. Synaptosomal pellets, stored in 250 mM-sucrose/5 mM-Tes, pH 7.2, were suspended and incubated in ⁴⁵Ca²⁺-containing incubation medium as described in the Experimental section. At defined times, oil centrifugation of portions was initiated (O). Alternatively, the portions were mixed with Ruthenium Red (final concentration 5 μ M) plus EGTA (final concentration 2 mM) at defined times, and centrifugation was initiated within 10 s (●). The two discontinuous lines represent the time course of ⁴⁵Ca²⁺ loss from the synaptosomes when Ruthenium Red and EGTA were added either 1 min or 11 min after commencing the incubation, but centrifugation was delayed until the times indicated. Where error bars are shown, results are means for at least four independent experiments.

Ruthenium Red was added together with the chelator in these experiments to facilitate comparison with experiments in which synaptosomes were disrupted, although Ruthenium Red neither displaced superficial Ca²⁺ by itself nor affected the extent of EGTA-induced removal.

Although separation of synaptosomes within 10 s of EGTA addition minimizes artefactual efflux, the use of conventional centrifugation, with the necessity of resuspension and re-centrifugation (e.g. Blaustein, 1975), will of necessity lead to a substantial loss of Ca²⁺ after EGTA addition, and the risk of pellet anoxia, with a resultant loss of Ca²⁺ from intrasynaptosomal mitochondria, is present.

It is significant that washing of membrane filters with ⁴⁰Ca²⁺-containing medium or Sephadex-gel

filtration of synaptosomes in columns equilibrated with sucrose (Wonnacott *et al.*, 1978) do not appear to remove surface Ca^{2+} , as the time course and extent of Ca^{2+} association under these conditions is very similar to that observed here in the absence of EGTA (Fig. 2).

The time course of Ca^{2+} transport (i.e. that resistant to 10s exposure to EGTA) is depicted in Fig. 2. It is apparent that when sucrose-stored synaptosomes are exposed to a Ca^{2+} -containing incubation medium without prior pre-incubation in a Ca^{2+} -free medium, a limited fast phase of Ca^{2+} association remains. This phase, resulting in the association of 3.7 ± 0.2 nmol of Ca^{2+} /mg of protein (means $\pm$ s.d.; $n = 18$) within 1 min probably represents Ca^{2+} uptake occurring before the plasma membrane potential has stabilized, since it is not observed when synaptosomes are pre-incubated in Ca^{2+} -free media before Ca^{2+} addition (Fig. 2). The superficial Ca^{2+} removed by a 10s exposure to EGTA amounts to 12.6 ± 0.4 nmol/mg of protein ($n = 12$) for non-preincubated synaptosomes and 10.7 ± 1.0 nmol/mg of protein ($n = 8$) for synaptosomes preincubated for 15 min before Ca^{2+} addition.

The role of the mitochondrial membrane potential

Intrasynaptosomal mitochondria can be predicted to influence synaptosomal Ca^{2+} transport in two ways: by supplying ATP for putative Ca^{2+} -translocating ATPases located in the plasma membrane, endoplasmic reticulum or synaptic vesicles, or by accumulating Ca^{2+} themselves. These modes can be distinguished by their energy requirements, as the former would be dependent on cytosolic ATP, whereas the latter utilizes the membrane potential across the mitochondrial inner membrane (see Saris & Åkerman, 1980; Nicholls & Crompton, 1980). To some extent $\Delta\psi_m$ and cytosolic ATP are interdependent, since abolition of the mitochondrial membrane potential of necessity inhibits oxidative phosphorylation. However, it has been shown (Scott & Nicholls, 1980) that glycolytic ATP synthesis is sufficient, under the present conditions, to maintain cytosolic ATP at a thermodynamic potential that, although lowered, is sufficient for the continued operation of ATP-dependent ion pumps and to maintain the plasma membrane potential for at least 5 min. It is thus possible to abolish $\Delta\psi_m$ by the simultaneous addition of rotenone and oligomycin (Scott & Nicholls, 1980), while allowing putative ATP-dependent Ca^{2+} sequestration to continue for a limited time. As a control, the effect of the abolition of mitochondrial ATP synthesis while $\Delta\psi_m$ is maintained at 150 mV (Scott & Nicholls, 1980) can be investigated by the addition of oligomycin alone.

When non-pre-incubated synaptosomes are added to an incubation medium containing rotenone and

Fig. 3. Effect of pre-incubation, oligomycin and rotenone on the time course of Ca^{2+} transport into synaptosomes. Synaptosomes were incubated as described in the legend to Fig. 2, and $^{45}\text{Ca}^{2+}$ association was determined after centrifugation initiated within 10s of the addition of Ruthenium Red and EGTA. $\circ$, Control; $\bullet$, $1.7 \mu\text{g}$ of oligomycin/ml added at $t = 0$; $\square$, $1.7 \mu\text{g}$ of oligomycin/ml plus $4 \mu\text{M}$ -rotenone added at $t = 0$. Alternatively, synaptosomes were pre-incubated for 15 min in the absence of Ca^{2+} before addition of $^{45}\text{Ca}^{2+}$ at $t = 0$ ($\blacksquare$). Results are means for at least four independent experiments.

oligomycin (Fig. 3) the initial rapid phase of Ca^{2+} transport is retained. However, a lag of 10 min ensues before net Ca^{2+} accumulation resumes. With oligomycin alone (Fig. 3), no difference from the control is noted until 10 min, when net Ca^{2+} accumulation accelerates relative to the control. The difference between the time courses in the presence of oligomycin and in the presence of oligomycin plus rotenone reflects the effect of Ca^{2+} accumulation by the intrasynaptosomal mitochondria. The discontinuity in both time courses at 10 min suggests an effect on the plasma membrane due to the lowered ATP concentrations induced by the inhibition of oxidative phosphorylation (Scott & Nicholls, 1980), as a consequence either of the inhibition of a directly coupled Ca^{2+} -translocating ATPase or of a decrease in the ATP-maintained Na^{+} -electrochemical

gradient. That oligomycin increases rather than decreases net Ca^{2+} accumulation suggests that ATP-dependent Ca^{2+} sequestration by intra-synaptosomal organelles is of lesser importance.

Compartmentation of synaptosomal Ca^{2+}

Experiments with intact synaptosomes can only provide indirect evidence for the sub-synaptosomal location of Ca^{2+} . Direct confirmation necessitates disruption and fractionation. The problems associated with a meaningful determination of Ca^{2+} compartmentation are considerable, as artefactual uptake into mitochondria can occur during fractionation if the external free Ca^{2+} concentration increases above that experienced in the cytosol, or if the kinetics of the uptake or efflux pathways are modified by alterations in pH, Mg^{2+} , P_i or Na^+ (see Nicholls & Crompton, 1980). On the other hand artefactual efflux from the matrix can occur if the supply of O_2 or mitochondrial substrate is interrupted for more than a few seconds. Published attempts to determine sub-synaptosomal Ca^{2+} distribution (Blaustein *et al.*, 1978*a,b*; Wonnacott *et al.*, 1978) have relied on hypo-osmotic lysis, a technique that is open to criticism, not only because of the long separation time and interference with the mitochondrial environment, but also because only 50% release of lactate dehydrogenase is observed (Wonnacott *et al.*, 1978), and the mitochondria that are released are exposed to a highly non-physiological environment.

The synaptosomal plasma membrane has been shown to be sensitive to digitonin (Nicholls, 1978*a*;

Booth & Clark, 1979), and using an axial-loading rotor (Murphy *et al.*, 1980) the mean time to separate solubilized and particulate fractions may be decreased below 30s from the initial admixture of digitonin (see the Experimental section). EGTA is included with the digitonin, the chelator performing the dual roles of lowering the free Ca^{2+} concentration instantaneously to about $1\mu\text{M}$ and removing Ca^{2+} bound superficially to the plasma membrane (see above). To further decrease the possibility of artefactual Ca^{2+} uptake by the mitochondria during disruption and separation, Ruthenium Red is included in the digitonin solution.

A low digitonin concentration would be predicted only to disrupt the plasma membrane, and hence release the cytosol. As little Ca^{2+} would be expected to be free in the cytosol (see Baker, 1976), the demonstration of a high proportion of Ca^{2+} in the resultant pellet would not be surprising, and in particular would not resolve the roles of the mitochondria on the one hand or synaptic vesicles and endoplasmic reticulum on the other. To distinguish the roles of these organelles two approaches were taken. First oligomycin and rotenone were employed as discussed above to distinguish ATP-dependent and $\Delta\psi_m$ -dependent Ca^{2+} accumulation, and secondly a sufficiently high digitonin concentration (0.7 mg/ml of incubation) was employed, which resulted in the solubilization not only of $94 \pm 1.3\%$ of the lactate dehydrogenase activity ($n=5$), but also of 70% (mean for two experiments) of glucose 6-phosphatase activity. If this enzyme is a valid marker of synaptosomal endo-

Table 1. The effect of mitochondrial membrane potential on the distribution of Ca^{2+} between mitochondrial and non-mitochondrial synaptosomal compartments

Synaptosomes were suspended and incubated for 15 min as described in the legend to Fig. 2 in the presence of $1.3\text{ mM } ^{45}\text{Ca}^{2+}$ with initial additions of inhibitors as indicated (Expt. A). Alternatively, synaptosomes were pre-incubated for 15 min in Ca^{2+} -free incubation medium after which $1.3\text{ mM } ^{45}\text{Ca}^{2+}$ was added together with inhibitors as indicated, and the synaptosomes were incubated for a further 15 min (Expt. B). Synaptosomes were then disrupted with digitonin (0.7 mg/ml of incubation) in the presence of Ruthenium Red and EGTA and rapidly centrifuged as described in the Experimental section. Pellet $^{45}\text{Ca}^{2+}$ was determined, and solubilized $^{45}\text{Ca}^{2+}$ was calculated by difference with reference to a parallel intact synaptosomal centrifugation. Membrane potentials were determined in parallel (Scott & Nicholls, 1980), except that synaptosomes were separated by filtration rather than by centrifugation. Values in parentheses refer to numbers of experiments.

Initial additions	Intact synaptosomes			Disrupted synaptosomes		
	Membrane potentials (mV)		Total Ca ²⁺ (nmol/mg)	Pellet Ca ²⁺ (nmol/mg)	Solubilized Ca ²⁺ (nmol/mg)	Pellet Ca ²⁺ (%)
	Plasma	Mitochondrial				
Expt. A						
Control	45	148	13.8 ± 0.6 (4)	9.9, 11.8 (2)	2.9	79
Rotenone (4 µg/ml) + oligomycin (4 µg/ml)	40	<59	7.9 ± 0.5 (4)	2.3, 2.5 (2)	5.5	30
Oligomycin (4 µg/ml)	41	153	16.5 ± 0.7 (4)	12.9, 14.7 (2)	2.7	83
Expt. B						
Control	—	—	5.2 ± 0.4 (4)	2.2 ± 0.3 (4)	3.0	42
Rotenone (4 µM) + oligomycin (1.7 µg/ml)	—	—	2.7 ± 0.2 (4)	0.36 ± 0.09 (4)	2.3	13

plasmic reticulum (Stephens & Sandborn, 1976; Rahamimoff & Abramovitz, 1978a) this would imply extensive fragmentation of the membrane and release of Ca^{2+} stores. In addition, the turbulent shear inherent in the disruption results in a disruption of endoplasmic reticulum to give microsomes (Murphy *et al.*, 1980), whereas digitonin treatment causes a solubilization of synaptic vesicles as judged by electron microscopy (Booth & Clark, 1979).

In contrast, the same digitonin concentration caused a solubilization of only $12 \pm 2\%$ of glutamate dehydrogenase activity ($n = 5$). Interestingly, no detectable solubilization of acetylcholinesterase was detected under these conditions, suggesting that fragments of plasma membrane are still sedimented.

In Table 1, the results of digitonin disruptions of non-pre-incubated and pre-incubated synaptosomes are listed. It is apparent that under control conditions, non-mitochondrial Ca^{2+} amounts to less than 3 nmol/mg of protein, and that the greater Ca^{2+} accumulation of non-pre-incubated synaptosomes is reflected in a greater mitochondrial Ca^{2+} content, such that 79% of the total Ca^{2+} is pelleted in the presence of digitonin. In agreement with the results obtained with intact synaptosomes (Fig. 3), abolition of oxidative phosphorylation by oligomycin increases the Ca^{2+} in the pellet, whereas abolition of $\Delta\psi_m$ by oligomycin plus rotenone greatly decreases the pellet Ca^{2+} . This confirms that mitochondria, rather than digitonin-resistant vesicles catalysing ATP-dependent Ca^{2+} accumulation, are primarily responsible for Ca^{2+} sequestration under these conditions.

Conclusion

Table 2 summarizes the extents of the different pools of Ca^{2+} in a synaptosomal pellet obtained by oil centrifugation of 'non-pre-incubated' synaptosomes after a 15 min incubation in the absence of

inhibitors. It is apparent that a multiplicity of Ca^{2+} pools can be resolved. In particular, even the non-mitochondrial transported Ca^{2+} (Table 2) is heterogeneous since it includes not only the physiologically important free cytosolic Ca^{2+} , but also chelated cytosolic Ca^{2+} and Ca^{2+} accumulated by ATP-dependent organelles.

It is evident from the present paper that the mitochondria accumulate substantial amounts of Ca^{2+} *in situ*. As the membrane potential of these mitochondrial is in the region of 150 mV (Scott & Nicholls, 1980) the two essential pre-conditions are satisfied for the mitochondria *in situ* to buffer the cytosolic free Ca^{2+} concentration, namely the presence of matrix Ca^{2+} and the existence of a sufficient $\Delta\psi_m$ to impose a unidirectional cycling of Ca^{2+} between independent uptake and efflux pathways (Nicholls & Scott, 1980a). At 30°C and pH 7.0 in the presence of 1 mM free Mg^{2+} and 45 mM- Na^+ isolated brain mitochondria maintain an extramitochondrial free Ca^{2+} concentration close to 1 μM (D. G. Nicholls, unpublished work), and it is therefore reasonable to assume that the mitochondria *in situ* attempt to maintain a cytosolic free Ca^{2+} concentration at or below this value.

As reviewed by Baker (1972, 1976) the long-term maintenance of intracellular Ca^{2+} must be effected by the plasma membrane, and studies such as those of Brinley *et al.* (1977, 1978) suggest that under resting conditions the plasma membrane is capable of depleting the mitochondrial matrix of Ca^{2+} . During periods of electrical activity, however, the cytosolic Ca^{2+} rises (Llinas *et al.*, 1972; Brinley *et al.*, 1977) and it is then that mitochondrial Ca^{2+} sequestration could become significant, enhancing the termination of transmission.

In agreement with results obtained with extruded squid axoplasm (Baker & Schlaepfer, 1978), ATP-dependent Ca^{2+} accumulation appears to be of lesser importance. A more precise characterization of these non-mitochondrial systems would appear desirable, particularly as they have yet to be shown

Table 2. The distribution of Ca^{2+} between different fractions in a pellet obtained by oil centrifugation of a synaptosomal incubation

Results are listed for 'preincubated' synaptosomes incubated for 15 min in Ca^{2+} medium in the absence of inhibitors, under conditions described in the Experimental section. Values in parentheses refer to numbers of experiments.

	Ca^{2+} (nmol/mg of synaptosomal protein)
Contamination by incubation medium	2.6 ± 0.1 (20)
Bound to outer surface of plasma membrane	10.7 ± 1.0 (8)
Transported into synaptosome [of which mitochondrial = 2.2 ± 0.3 (4) and non-mitochondrial = 3.0]	5.2 ± 0.4 (4)
Total $^{45}\text{Ca}^{2+}$ in pellet	18.5
(Accumulation by contaminating 'free' mitochondrial)	<0.9
(Endogenous $^{40}\text{Ca}^{2+}$)	0.56 ± 0.1 (6)

to be capable of a reversible precise regulation of the free Ca^{2+} concentration in their milieu.

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