The role of mitochondria in the regulation of calcium ion transport in synaptosomes

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Research during the last 4 years has revealed that isolated mitochondria possess a sophisticated mechanism for regulating the distribution of Ca$^{2+}$ across their inner membrane, based on a steady-state cycling of Ca$^{2+}$ between independent Ca$^{2+}$ uptake and efflux pathways (for reviews see Carafoli, 1979; Nicholls & Crompton, 1980; Saris & Akerman, 1980).

One consequence of Ca$^{2+}$ cycling is that the isolated mitochondria are able to buffer selectively the extra-mitochondrial free Ca$^{2+}$ concentration in the micromolar region (Nicholls & Scott, 1980). The present paper examines whether there is any evidence that the mitochondria continue to exercise such a regulatory role when within the cytosol of intact synaptosomes.

Previous work from this laboratory (Nicholls & Scott, 1979, 1980) has shown that the first criterion, the maintenance of a high mitochondrial membrane potential, is satisfied by intact synaptosomes. When incubated at 30°C and pH 7.4 in the medium described in the legend to Fig. 1, a membrane potential of 45 mV was determined across the plasma membrane, and a potential of 148 mV across the inner membrane of the mitochondria in situ (Scott & Nicholls, 1980). This latter potential is sufficient to prevent the uniporter from reversing (Nicholls & Scott, 1980).

Experiments with the isolated brain mitochondria have indicated that selective extra-mitochondrial Ca$^{2+}$ buffering is already apparent when the matrix contains as little as 10 nmol of Ca$^{2+}$/mg of mitochondrial protein (Nicholls & Scott, 1980), indicating that this amount of Ca$^{2+}$ is sufficient to saturate the efflux pathway. It follows therefore that, if the mitochondria in situ can be shown, not only to maintain a high membrane potential, but also to contain sufficient Ca$^{2+}$ within their matrices, then the likelihood that the mitochondria will play a significant role in the regulation of cytosolic free Ca$^{2+}$ in the isolated synaptosome will be enhanced.

The use of Ca$^{2+}$ chelators during the preparation of synaptosomes results in an almost complete depletion of their endogenous Ca$^{2+}$. Thus synaptosomes prepared in this laboratory from guinea-pig cerebral cortex contain less than 0.5 nmol of Ca$^{2+}$/mg of synaptosomal protein, as determined by atomic absorption spectroscopy. When incubated in a Ca$^{2+}$-containing physiological salt medium, there is a very rapid association of about 18 nmol of Ca$^{2+}$/mg of synaptosomal protein, which is essentially complete within 10 s, followed by a much slower

Fig. 1. The time course of association of Ca$^{2+}$ with Ca$^{2+}$-depleted synaptosomes

Synaptosomes from guinea-pig cerebral cortex were incubated at 30°C and pH 7.4 in a medium containing 122 mM-NaCl, 3.1 mM-KCl, 1.2 mM-MgSO$_4$, 0.4 mM-KH$_2$PO$_4$, 5 mM-NaHCO$_3$, 20 mM - 2-[-2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulphonate, 10 mM-D-glucose, 1.3 mM-$^{45}$CaCl$_2$ (0.51 µCi/ ml) and $^{3}$H-hydroxy-sucrose (3.5 µCi/ml). At defined time points were withdrawn and centrifuged through silicone oil. Samples of pellet and supernatant were counted for radioactivity and net Ca$^{2+}$ association was calculated. Symbols: ○, control; O, 5 µM-Ruthenium Red and 2 mM-EGTA added immediately before centrifugation.
increase in association, which continues at a rate of 0.5 nmol of Ca\(^{2+}\)/min per mg of synaptosomal protein for the duration of the experiment (Fig. 1). The initial rapid phase can be greatly decreased by the addition, just before centrifugation, of Ruthenium Red together with sufficient EGTA to decrease the free Ca\(^{2+}\) concentration in the incubation medium to 1 \(\mu\)M (Fig. 1). It is therefore likely that the initial association represents Ca\(^{2+}\) bound superficially to the synaptosome, whereas the subsequent slow phase, which in contrast is not reversed by Ruthenium Red and EGTA, represents transport across the plasma membrane.

Contamination of the synaptosomal preparation with free mitochondria, i.e. those not bounded by a plasma membrane, can increase the apparent Ca\(^{2+}\) accumulation disproportionately if a mitochondrial substrate is present. Although the absence of such a substrate in the experiments shown in Fig. 1 and Table 1 should prevent the 5% contamination (on a protein basis) of the present synaptosomal preparation (Nicholls, 1978a) from introducing a significant error, control experiments were performed with preparations of free brain mitochondria, which indicated that the contamination free mitochondria in the synaptosomal preparation could contribute less than 0.9 nmol of Ca\(^{2+}\)/mg of synaptosomal protein.

To establish the distribution of accumulated Ca\(^{2+}\) within the synaptosome, it is necessary to disrupt the plasma membrane and fracnionate the organelles. As has been recognised for some time in the study of metabolite compartmentation within isolated hepatocytes the primary problem is methodological, and concerns the devising of techniques to minimize artefactual redistribution of the cation in the inevitable interval between disruption and separation. In view of the high mobility of Ca\(^{2+}\) across the inner mitochondrial membrane, the accuracy of an estimate of intra-synaptosomal Ca\(^{2+}\) distribution must be assessed critically in relation to the techniques employed, and for this reason it is necessary to discuss methodology at some length.

The procedure adopted here was developed for hepatocyte disruption (Murphy et al., 1980) and combines selective disruption by digitonin of the plasma membrane (Zuurendendk & Tager, 1974), complemented by shear induced by turbulent flow through a small diameter needle (Tischler et al., 1977), with the improvement in time resolution made possible by a rotor whose centrifugation is running at full speed. Under these conditions the mean separation time may be decreased to less than 30s from the initial admixture of digitonin.

On lysis of the plasma membrane the intra-cellular organelles are instantaneously exposed to the Ca\(^{2+}\) of the incubation medium. For this reason sufficient EGTA is added together with the digitonin to decrease the free Ca\(^{2+}\) concentration from 1.3 mM to 1 \(\mu\)M. As with intact synaptosomes, this treatment removes the majority of the Ca\(^{2+}\) bound superficially to the plasma membrane. The chelation of Ca\(^{2+}\) also serves to decrease the medium pH from 7.4 to 7.0, which approximates to the cytosolic pH in situ. Thus the intra-synaptosomal organelles undergo a minimal disturbance to pH or free Ca\(^{2+}\) concentration when the plasma membrane is disrupted. To further decrease the possibility of artefactual Ca\(^{2+}\) uptake by the mitochondria after disruption, 5 \(\mu\)M-Ruthenium Red is present in the incubation medium. One inherent error is due to the Na\(^+\)-dependent mitochondrial Ca\(^{2+}\)-efflux pathway, which will continue to operate during the short interval between disruption and separation. At 30°C and pH 7.0 the efflux pathway of guinea-pig brain mitochondria operates at 10 nmol of Ca\(^{2+}\)/min per mg of mitochondrial protein (Nicholls, 1978a), and if the intrasynaptosomal mitochondria contribute 10% to the synaptosomal protein (Scott & Nicholls, 1980), a 30 s separation time could lead to an underestimate of 0.5 nmol/mg of synaptosomal protein in the mitochondrial Ca\(^{2+}\).

With these precautions in mind, the experiment reported in Table 1 was performed to establish the distribution of Ca\(^{2+}\) within synaptosomes after 16 min incubation in a medium containing 1.3 mM Ca\(^{2+}\). The digitonin concentration employed to disrupt the synaptosomes was chosen not only to cause maximal release of cytoplasm, as monitored by the release of lactate dehydrogenase, and minimal release of mitochondrial matrix, monitored by NAD\(^+\)-dependent glutamate dehydrogenase, but also to largely disrupt endoplasmic-recticular membranes, as judged by solubilization of glucose 6-phosphatase activity. Any Ca\(^{2+}\) that can be still be pelleted during the centrifugation must therefore be associated either with the mitochondrial matrix, or with the fragmented plasma membrane, which, judged by acetylcholinesterase activity, may still be sedimented at digitonin concentrations far higher than those required to release lactate dehydrogenase activity.

After 16 min incubation under control conditions 79% of the total Ca\(^{2+}\) accumulated by the synaptosomes can be pelleted after digitonin disruption. As only 7% of lactate dehydrogenase and 28% of glucose 6-phosphatase activity are pelleted under these conditions, it follows that only a minor fraction of the Ca\(^{2+}\) can be associated with endoplasmic reticulum or be soluble in the cytoplasm.

To establish whether the pelleted Ca\(^{2+}\) is associated with the mitochondrial matrix, or is merely bound to fragmented plasma membrane, the experiment was repeated under conditions that selectively depolarize the mitochondria in situ, namely in the

<table>
<thead>
<tr>
<th>Initial additions</th>
<th>Membrane potentials (mV)</th>
<th>Total Ca(^{2+}) (nmol/mg)</th>
<th>Solubilized activity (%)</th>
<th>Pellet Ca(^{2+}) (nmol/mg)</th>
<th>Solubilized Ca(^{2+}) (nmol/mg)</th>
<th>Pellet Ca(^{2+}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45 148</td>
<td>13.8</td>
<td>93 13</td>
<td>10.9</td>
<td>2.9</td>
<td>79</td>
</tr>
<tr>
<td>Rotenone + oligomycin</td>
<td>40 &lt;30</td>
<td>7.2</td>
<td>93 13</td>
<td>2.4</td>
<td>4.8</td>
<td>33</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>41 153</td>
<td>15.3</td>
<td>95 16</td>
<td>13.8</td>
<td>1.5</td>
<td>90</td>
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Table 1. The effect of mitochondrial membrane potential on the distribution of Ca\(^{2+}\) between mitochondrial and non-mitochondrial compartments within synaptosomes

Synaptosomes (1.5 mg of protein/ml of incubation) were incubated for 15 min in the presence of 4 Ca\(^{2+}\) and [\(^{3}H\)]glucose as described in the legend to Fig. 1. To one portion, 5 \(\mu\)M-Ruthenium Red and 2 \(\mu\)M-EGTA were added before centrifugation to determine total accumulated Ca\(^{2+}\). Another portion was disrupted with digitonin (0.7 mg/ml of incubation) in the presence of Ruthenium Red and EGTA and rapidly centrifuged as described in the text. Pellet 4 Ca\(^{2+}\) was determined, and solubilized 4 Ca\(^{2+}\) was calculated by difference. Lactate dehydrogenase (LDH) and glutamate dehydrogenase (GDH) activities were assayed in the supernatant after disruption and centrifugation. Membrane potentials were taken from Scott & Nicholls (1980). Where indicated, 4 \(\mu\)M-rotenone and 4 \(\mu\)g of oligomycin/ml were present during the 16 min incubation.
presence of rotenone and oligomycin (Scott & Nicholls, 1980). This combination of inhibitors should prevent any Ca\(^{2+}\) uptake into the matrices of the mitochondria. As is shown in Table 1, the pelleted Ca\(^{2+}\) was greatly diminished under these conditions, whereas the solubilized Ca\(^{2+}\) actually increases. This clearly indicates that the major proportion of the synaptosomal Ca\(^{2+}\) is located in a compartment that is responsive to the mitochondrial membrane potential, and can therefore be identified with the mitochondrial matrix.

Abolition of the mitochondrial membrane potential of necessity inhibits oxidative phosphorylation. Thus it could be argued that the diminished pellet Ca\(^{2+}\) resulted from the inhibition of the ATP supply to a Ca\(^{2+}\)-dependent ATPase accumulating Ca\(^{2+}\) within digitonin-resistant vesicles. However, glycolysis allows significant ATP concentrations to be maintained under these conditions (Scott & Nicholls, 1980), whereas the presence of oligomycin alone, which inhibits the mitochondrial ATP synthase without lowering the membrane potential of the internal mitochondria (Scott & Nicholls, 1980), does not diminish the Ca\(^{2+}\) in the pellet (Table 1).

The conclusion is therefore that the mitochondrial matrix represents the major site of Ca\(^{2+}\) accumulation within isolated synaptosomes amounting to some 85 nmol of Ca\(^{2+}\)/mg of mitochondrial protein under these conditions. This is well within the capacity of brain mitochondria to accumulate Ca\(^{2+}\), but is sufficient to saturate the efflux pathway (Nicholls & Scott, 1980). It is therefore feasible that the mitochondria regulate cytosolic Ca\(^{2+}\) concentrations under these conditions. There is, however, one proviso to make: the total Ca\(^{2+}\) content of the synaptosome is still increasing at 16 min (Fig. 1), implying that the internal mitochondria are capable of decreasing the cytosolic free Ca\(^{2+}\) concentration sufficiently to impose a net inward Ca\(^{2+}\) flux across the plasma membrane. It remains to be established therefore whether this represents an approach towards a steady-state, or whether a proportion of synaptosomes with a defective plasma membrane Ca\(^{2+}\)-efflux pathway contributes disproportionately to the observed Ca\(^{2+}\) uptake by the total synaptosomal population.

Previous studies (Kendrick et al., 1977; Blaustein et al., 1978; Rahaminoff & Abramovitz, 1978) emphasized the role of ATP-dependent Ca\(^{2+}\) accumulation by intra-synaptosomal reticular membranes. However, the present study demonstrates that the mitochondria, with their elaborate Ca\(^{2+}\)-regulatory mechanism, play the predominant role within the cytosol of the isolated synaptosome.

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The role of calcium in the regulation of mitochondrial metabolism

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Studies in this laboratory have identified three mammalian intramitochondrial dehydrogenases that, in mitochondrial extracts or after purification, are activated by Ca\(^{2+}\) with half-maximum effects (k\(_{50}\)) values of Ca\(^{2+}\) being observed at close to 1 \(\mu\)M. These enzymes are pyruvate dehydrogenase, isocitrate dehydrogenase (NAD\(^{+}\)) (EC 1.1.1.41) and the 2-oxoglutarate dehydrogenase complex. The effects of Ca\(^{2+}\) on pyruvate dehydrogenase activity are brought about by changes in the proportion of the complex in its active non-phosphorylated form. Pyruvate dehydrogenase phosphate phosphatase is activated by Ca\(^{2+}\) (Denton et al., 1972, 1975), whereas pyruvate dehydrogenase kinase, at least from heart muscle, may be inhibited by Ca\(^{2+}\) (Cooper et al., 1974). The effects of Ca\(^{2+}\) on isocitrate dehydrogenase (NAD\(^{+}\)) and oxoglutarate dehydrogenase are more direct. In both cases, the presence of Ca\(^{2+}\) results in a greatly decreased K\(_{m}\) for their substrates (three- \(\Delta\)isocitrate and oxoglutarate respectively) with little or no change in \(V_{\text{max}}\). (Denton et al., 1978; McCormack & Denton, 1979). The enzymes appear to have very similar properties in mitochondria from all mammalian tissues so far studied including those from pig heart and the following rat tissues: white and brown adipose tissue, heart and skeletal muscle, liver, kidney and brain. Moreover, in all cases the effects of Ca\(^{2+}\) are closely mimicked by Sr\(^{2+}\), but the k\(_{50}\) values are some ten times higher.

All three of the Ca\(^{2+}\)-sensitive dehydrogenases are located exclusively in cells within the inner mitochondrial membrane and are generally considered to be important sites of regulation of intramitochondrial oxidative metabolism. In particular, the activity of all three dehydrogenases may be increased by elevations in the intramitochondrial ADP/ATP and NAD\(^{+}\)/NADH concentration ratios (see McCormack & Denton, 1979). This can be viewed as the 'intrinsic' means of regulation whereby the rate of NADH production in mitochondria is always matched to the requirements of the respiratory chain and thus ATP synthesis. We proposed that the activation of these same key dehydrogenases by Ca\(^{2+}\) ions may be an important means whereby 'extrinsic' control of intramitochondrial oxidative metabolism by such factors as hormones and neurotransmitters could be superimposed on this 'intrinsic' control. However, we realized the need to be cautious when ascribing regulatory importance to enzyme properties that have only been observed with separated enzymes. We have therefore sought more direct evidence by using intact mitochondria.

Initially, we were keen to establish the sensitivity of the dehydrogenases to Ca\(^{2+}\) when the enzymes were located within intact mitochondria. We approached the problem by studying the effects of changes in the extramitochondrial Ca\(^{2+}\) concentration on the pyruvate dehydrogenase activity and on isocitrate and oxoglutarate oxidation in uncoupled mitochondria from white and brown adipose tissue. In uncoupled mitochondria incubated in KCl-based medium there will be little or no pH gradient or membrane potential and thus the concentration of Ca\(^{2+}\) within the matrix compartment is close to 1 \(\mu\)M.