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INTRASYNAPTOSOMAL COMPARTMENTATION OF CALCIUM DURING DEPOLARIZATION-INDUCED CALCIUM UPTAKE ACROSS THE PLASMA MEMBRANE

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The distribution of Ca^{2+} between mitochondrial and non-mitochondrial compartments within intact synaptosomes is investigated during the net Ca^{2+} uptake induced by plasma membrane depolarization. The steady-state synaptosomal Ca^{2+} content (5.8 ± 0.3 nmol/mg protein) is increased by 77% by plasma membrane depolarization induced by veratridine plus ouabain (9.7 ± 0.6 nmol/mg protein) and by 100% by high K^+ (50 mM) (11.0 ± 0.9 nmol/mg protein). Prior abolition of the mitochondrial membrane potential, and hence inhibition of intrasynaptosomal mitochondrial Ca^{2+} accumulation, decreased the steady-state Ca^{2+} accumulation by 40% in both the control and the veratridine-ouabain depolarization, and by almost 60% in the case of high K^+ depolarization. Similar values were obtained for the release of Ca^{2+} from synaptosomes when the mitochondrial membrane was depolarized after a steady state had been attained. Control experiments demonstrated that contaminating free mitochondria were not responsible for the altered Ca^{2+} accumulation. That the decrease in the Ca^{2+} accumulation on mitochondrial depolarization corresponds to the extent of the mitochondrial pool was confirmed by rapid synaptosomal disruption with digitonin which gave values of 2.5 ± 0.5 nmol/mg protein, 4.4 ± 0.9 nmol/mg protein and 6.9 nmol/mg protein for control or veratridine/ouabain- and high-[K^+]-depolarized synaptosomes, respectively. The lesser contribution of intrasynaptosomal mitochrondria during veratridine/ouabain-induced depolarization is proposed to be a consequence of raised cytosolic Na^+ concentrations activating the mitochondrial Ca^{2+} efflux pathway. The results demonstrate that intrasynaptosomal mitochondria represent a metabolically responsive Ca^{2+} pool in situ.

Introduction

The ability of isolated mitochondria to buffer extramitochondrial free Ca^{2+} is well established [1–6]. Depending on the tissue from which the mitochondria are isolated and the incubation conditions the extramitochondrial [Ca^{2+}] is maintained at from $3 \cdot 10^{-7}$ M to $5 \cdot 10^{-6}$ M over a wide range of matrix Ca^{2+} loads [1,2,5,7] as long as a high mitochondrial membrane potential is maintained. This buffering of extramitochondrial [Ca^{2+}] is the result of the simultaneous operation of a membrane-potential-driven uptake and an efflux in which Ca^{2+} is exchanged for protons or Na^+ [5,6]. In brain as well as in some other types of mitochondria the efflux pathway is Na^+ dependent [8–10].

Mitochondrial buffering may be demonstrated in situ in the squid giant axon where cytosolic [Ca^{2+}] is about 10^{-7} M under resting conditions [11,12]. Intraaxonal mitochondria buffer the cytoplasm during

Abbreviations: $\Delta\psi_p$, membrane potential across the plasma membrane; $\Delta\psi_m$, membrane potential across the inner membrane of mitochondria in situ; Tes, 2-((2-hydroxy-1,1-bis-(hydroxymethyl)ethyl)amino)ethane sulphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

excitation-induced Ca^{2+} uptake across the axonal membrane, sequestering the excess Ca^{2+} and preventing the free cytosolic Ca^{2+} concentration from rising much above 10^{-6} M [13].

We have previously shown [14] that intrasynaptosomal mitochondria contain a large proportion of the total synaptosomal Ca^{2+}, and that this mitochondrial pool is dependent on the maintenance of a high mitochondrial membrane potential. The purpose of this paper is to investigate whether intrasynaptosomal mitochondria respond to an increased Ca^{2+} uptake across the plasma membrane upon depolarization [15] in a way which is similar to the mitochondria within the squid axon [13]. At the same time the role of the cytoplasmic Ca^{2+} pool in regulating net Ca^{2+} transport across the synaptosomal plasma membrane is investigated.

Materials and Methods

Radiosotopes were obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Ficoll was obtained from Pharmacia, Uppsala, Sweden and was dialysed against water before use. Veratridine and oligomycin were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Veratrine is a mixture of alkaloids and the veratridine content was taken as 25%.

Synaptosomes. Synaptosomes were prepared from cerebral cortices (including corpus striatum) of Duncan-Hartley-strain guinea pigs of either sex aged 4–8 weeks, by a modification of the method of Cotman and Matthews [16] employing a discontinuous Ficoll gradient, and stored as pellets in sucrose at 0°C for not more than 3 h as described previously [10,14,17].

Conditions of incubation and synaptosomal separation during $^{45}Ca^{2+}$ uptake experiments. The synaptosomal pellets were suspended into a modified Elliott's medium (122 mM NaCl, 3.1 mM KCl, 1.2 mM $MgSO_4$, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM Tes, 10 mM D-glucose, pH 7.4) at 30°C. After 15 min preincubation 1.3 mM $CaCl_2$ was added together with $^{45}Ca^{2+}$. Aliquots were taken into EGTA/Ruthenium Red and immediately spun through a 50% (v/v) mixture of silicone oil (Corning 550) and dinonylphthalate in an Eppendorf bench centrifuge (model 3412) for 2 min. The supernatant was aspirated off and 0.5 ml distilled water was added onto the top of the oil. The water and oil phases were aspirated and 50 µl of 1.2 M perchloric acid was added to the pellet. The pellet was suspended into the acid and extracted for at least 2 h. The suspension was neutralized by the addition of 1.8 M Tris base and 50 mM EGTA, transferred into scintillation vials and counted as described earlier [14,17]. [^{3}H]Sucrose was used as a marker of external water [14,17].

Disruption with digitonin. Digitonin disrupts cholesterol containing membranes. Treatment with digitonin was performed as described earlier [14]. Briefly, samples of the synaptosomal suspension (0.5 ml) were mixed in a syringe (2 ml) with Ca^{2+} free Elliott's medium containing digitonin, EGTA (2 mM final concentration) and ruthenium red (5 µm final concentration) whereafter the suspension was quickly injected through a 23-gauge needle into the disrupter as described earlier [14]. Marker enzymes, lactate dehydrogenase, glutamate dehydrogenase, glucose-6-phosphatase and acetylcholinesterase were measured from the resulting supernatant as described earlier. Fig. 1 shows enzyme profiles as a function of the digitonin concentration. It is seen that the concentration used in the present study (0.7 mg/ml) releases most of the lactate dehydrogenase (cytosol) and glucose-6-phosphatase (endoplasmic reticulum) but less than 20% of glutamate dehydrogenase (mitochondria).

Other Methods. Protein was determined by the biuret method [18].

Results

The effect of the mitochondrial membrane potential on depolarization induced Ca^{2+} uptake by synaptosomes

The combination of veratridine (to activate Na^+ channels in the synaptosomal plasma membrane) and ouabain (to inhibit the $(Na^+ + K^+)$-dependent ATPase) results in a rapid depolarization of the plasma membrane without affecting $\Delta\psi_m$ [19]. As shown in Fig. 2B addition of veratridine plus ouabain together with Ca^{2+} results in an increased rate and magnitude of Ca^{2+} accumulation.

Depolarization induced by high external $[K^+]$ produces a similar increase in synaptosomal Ca^{2+} accumulation (Fig. 2C) although the initial rate of

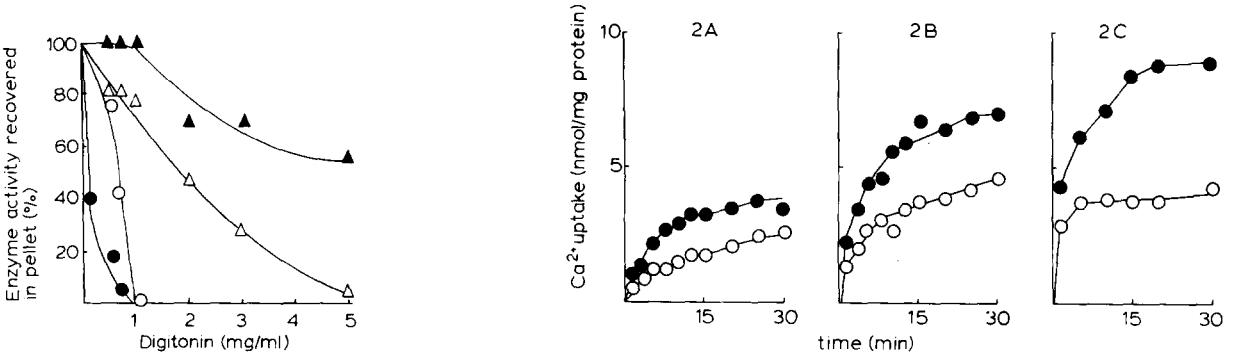

Fig. 1. The effect of digitonin concentration on the solubilization of synaptosomal enzyme activities. Synaptosomes (1.5 mg of protein/ml of incubation) were incubated and disrupted in the presence of varying digitonin concentrations as described in Materials and Methods. Enzyme activity was assayed in the supernatant fraction following 60 s centrifugation, and the pelleted activity calculated by difference with respect to the total incubation. Symbols: •———•, lactate dehydrogenase; ○———○, glucose-6-phosphatase; △———△, glutamate dehydrogenase; ▲———▲, acetylcholinesterase.

Fig. 2. The effect of rotenone plus oligomycin on Ca^{2+} uptake by synaptosomes. Synaptosomes (1.6 mg protein/ml) were preincubated for 15 min in the basal experimental Ca^{2+}-free medium. At $t = 0$ 1.3 mM $CaCl_2$ was added together with 2 μCi/ml [^{3}H]-sucrose and 0.8 μCi/ml $^{45}Ca^{2+}$. Further additions at $t = 0$ were (A) none; (B) 100 μM veratridine and 0.3 mM ouabain; (C) the external K^+ concentration increased to 50 mM with a K^+-based medium otherwise identical to the basal experimental medium (see Materials and Methods). •———•, controls; ○———○, 6 μM rotenone and 2 μg/ml oligomycin added 2 min before $t = 0$.

Ca^{2+} uptake is considerably higher than for veratridine plus ouabain. The time to achieve a steady-state Ca^{2+} accumulation is about the same for depolarized as for control synaptosomes.

The extent to which the depolarization-induced increase in net synaptosomal Ca^{2+} accumulation is dependent on Ca^{2+} accumulation within the intrasynaptosomal nitochondria can be investigated by eliminating the latter by specifically abolishing $\Delta\psi_m$. This may be accomplished by the combination of rotenone, to inhibit the respiratory chain, and oligomycin to prevent H^+ extrusion by the mitochondrial ATPase utilising glycolytic ATP [19]. Under these conditions glycolysis is able to maintain synaptosomal ATP at 50% of control values for at least 10 min. Depolarization of $\Delta\psi_m$ by these inhibitors has been shown to decrease Ca^{2+} uptake across the synaptosomal membrane by inhibiting uptake into intrasynaptosomal mitochondria.

In Fig. 2 the effect of rotenone plus oligomycin on the time course of Ca^{2+} accumulation is shown for control conditions and following depolarization of $\Delta\psi_p$ induced by veratrine plus ouabain or high [K^+]. In each case Ca^{2+} accumulation is inhibited relative to the control in which the mitochondria are functional. However, even in the presence of the mitochondrial inhibitors Ca^{2+} uptake is still enhanced by plasma membrane depolarisation.

All preparations of synaptosomes are contaminated to some extent with free mitochondria, and the possibility that some of the decreases Ca^{2+} uptake observed in the presence of rotenone plus oligomycin reflects an altered accumulation by these contaminating particles must be considered carefully. In a previous paper [14] the upper limit for the contribution of free mitochondria to Ca^{2+} accumulation by the present preparation of synaptosomes was estimated to be 0.9 nmol/mg protein. A more precise technique for estimating the free mitochondrial contribution is to follow Ca^{2+} uptake in the continuous presence of Ruthenium Red, which would inhibit the Ca^{2+}- uniporter of the free mitochondria without affecting intrasynaptosomal mitochondria. 10 μM Ruthenium Red (sufficient to inhibit totally the mitochondrial Ca^{2+} uniporter [20–22]) is without detectable effect on the time course of Ca^{2+} accumulation (not shown).

In contrast to Ruthenium Red, high concentrations of La^{3+}, an inhibitor of various Ca^{2+} transport systems [11,23,24] inhibit completely Ca^{2+} transport

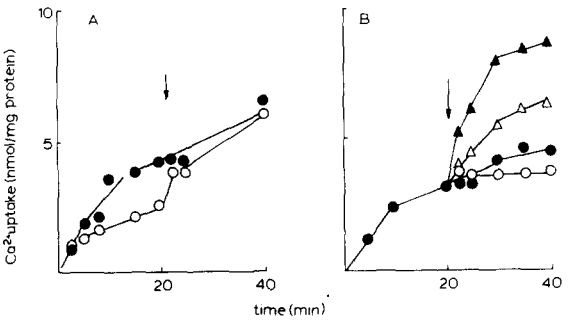

Fig. 3. The effect of rotenone plus oligomycin on veratridine- and ouabain-stimulated Ca^{2+} uptake by synaptosomes. Synaptosomes (1.4 mg protein/ml) were incubated as in Fig. 2 (o——o). In (A) 6 μM rotenone and 2 μg/l oligomycin were added at t = 100 μM veratridine and 0.3 mM ouabain added at t = 20 min (o——o). In (B) no addition (•——•), addition of 100 μM veratridine and 0.3 mM ouabain (▲——▲) 6 μM rotenone and 2 μg/l oligomycin (o——o), or veratridine/ouabain and rotenone/oligomycin (△——△) at t = 20 min.

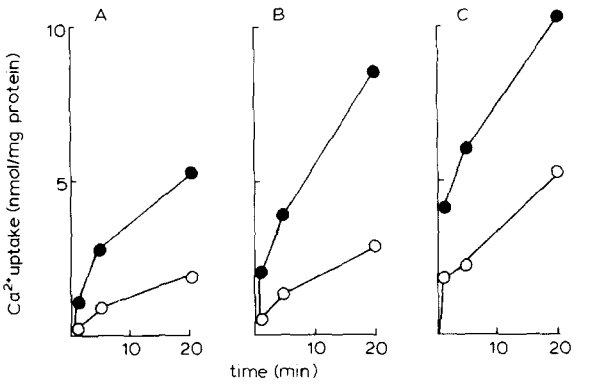

Fig. 4. Mitochondrial Ca^{2+} contents during depolarization-induced Ca^{2+} uptake by synaptosomes. Synaptosomes (1.8 mg protein/ml) were incubated as described in Fig. 2. (A) Control experiment. (B) Addition of 100 μM veratridine together with 0.3 mM ouabain at t = 0. (C) External K^+ concentration raised to 54 mM at t = 0. •——•, intact synaptosomes; o——o, samples treated with digitonin as described in Materials and Methods. The amount of lactate dehydrogenase released by digitonin was 86 ± 4% of the total and glutamate dehydrogenase released was 11 ± 4% of the total.

across the plasma membrane (not shown). A phosphate-free medium was employed to prevent $LaPO_4$ precipitation. La^{3+} has no significant effect on $\Delta\psi_p$ (not shown) suggesting that the synaptosomes remained functional in the presence of this inhibitor.

In Fig. 3B the effects of specific depolarization of either $\Delta\psi_m$ or $\Delta\psi_p$ are shown on synaptosomes which have been allowed to accumulate Ca^{2+} for 20 min in order to approach a steady-state Ca^{2+} distribution. Veratridine plus ouabain induce a rapid additional uptake of Ca^{2+} which is only partially inhibited if rotenone plus oligomycin are added together with veratridine plus ouabain. Rotenone plus oligomycin added alone at 20 min inhibit further Ca^{2+} uptake without inducing Ca^{2+} efflux from the synaptosomes.

A veratridine/ouabain-induced increase in Ca^{2+}-uptake can also be seen when $\Delta\psi_p$ is depolarized 20 min after the addition of Ca^{2+} to synaptosomes in the presence of rotenone plus oligomycin (Fig. 3A).

Intrasynaptosomal Ca^{2+}-distribution during plasma membrane depolarization

If non-mitochondrial Ca^{2+} pools are unaffected by mitochondrial depolarisation, it follows that the decrease in synaptosomal Ca^{2+} uptake corresponds to the extent of the mitochondrial Ca^{2+} pool when the mitochondria were functional.

To test this hypothesis directly, rapid separation of intrasynaptosomal mitochondria from the cytosol [14] was performed by disrupting the plasma membrane with low concentrations of digitonin in the presence of Ruthenium Red and EGTA to prevent redistribution of Ca^{2+}. Fig. 4 shows experiments using this technique. In the control experiment (Fig. 4A) about 40% of the intrasynaptosomal Ca^{2+} appears to reside in the mitochondria (about 2 nmol/mg protein at 20 min) in agreement with our previous results [14]. Upon depolarization with veratridine plus ouabain (Fig. 4B) there is a slight increase in mitochondrial Ca^{2+} but a very pronounced increase in the non-mitochondrial pool.

With high $[K^+]$ depolarization mitochondrial Ca^{2+} is more pronounced and there is a significant increase in the mitochondrial Ca^{2+} content even at 1 min of incubation. At 20 min of incubation with high K^+ about 60% of the total synaptosomal Ca^{2+} is present in the mitochondria (about 7 nmol/mg protein).

Net efflux of Ca^{2+} from synaptosomes induced by proton-translocator-induced depolarisation of $\Delta\psi_m$

If net plasma membrane Ca^{2+} transport for any

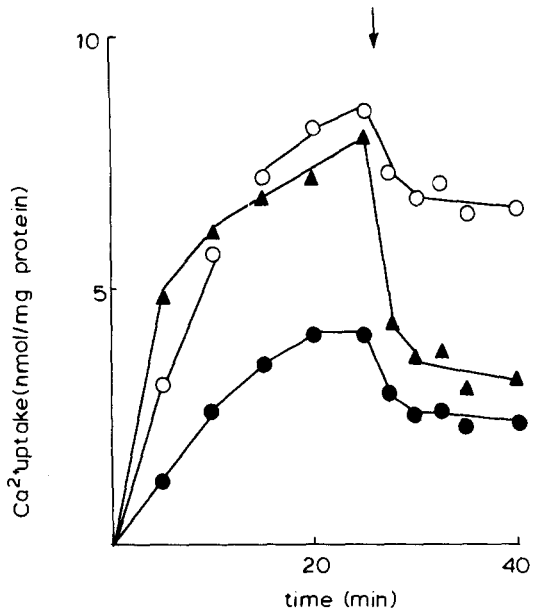

Fig. 5. Ca^{2+} release from synaptosomes induced by FCCP in combination with oligomycin. Synaptosomes (1.85 mg protein/ml were incubated as described in Fig. 2 (•———•). At t = 0 100 μM veratridine and 0.3 mM ouabain (○———○) or 48 mM K^+ (△———△) were added. At t = 25 min 5 μM FCCP and 1 μg/ml oligomycin were added.

given state of the plasma membrane potential is regulated by the non-mitochondrial Ca^{2+} pool, it follows that a rapid Ca^{2+} efflux from intrasynaptosomal mitochondria induced by addition of a proton translocator will increase the cytosolic [Ca^{2+}] and induce a net efflux of Ca^{2+} across the plasma membrane until the initial non-mitochondrial pool size is regained.

In Fig. 5 the effect of FCCP (plus oligomycin to prevent cytosolic ATP depletion) are plotted. In each case a transient efflux of Ca^{2+} is observed. The final steady state (and hence the non-mitochondrial pool) is lowest for control mitochondria, slightly higher for K^+-depolarized synaptosomes, and considerably higher for veratridine ouabain-depolarized synaptosomes. Thus the net Ca^{2+} release is low for the control and veratridine ouabain- and large for high-[K^+]-treated synaptosomes. Rotenone/oligomycin (Fig. 3B) added at 20 min inhibits further Ca^{2+} uptake by synaptosomes but, in contrast to FCCP, does not induce net Ca^{2+} release probably because of the slow mitochondrial Ca^{2+} efflux limited by the low proton permeability of the mitochondrial membrane i.e. because of lack of charge compensating ions. The final extent of the difference in total synaptosomal

TABLE I

MITOCHONDRIAL AND NON-MITOCHONDRIAL Ca^{2+} CONTENTS AS MEASURED AT 25 MIN WITH THE DIFFERENT METHODS

Conditions as in Figs. 2, 4 and 5. Results calculated as ±S.E. T, total; M, mitochondrial; NM, non-mitochondrial Ca^{2+} content.

Method		Condition					
		Control		Veratridine Ouabain		High [K^+]	
		nmol/mg protein	% of total	nmol/mg protein	% of total	nmol/mg protein	% of total
Disruption with digitonin	T	5.8 ± 0.3	100	9.7 ± 0.6	100	11.0 ± 0.9	100
	M	2.5 ± 0.5	42	4.4 ± 0.9	45	6.9 ± 1.1	62
	NM	3.3	58	5.3	55	5.0	38
	n	4		5		5	
Inhibition by rotenone oligomycin	T	3.5	100	6.5	100	9.9	100
	M	1.4	40	2.6	40	5.2	58
	NM	2.2	60	3.9	60	3.7	42
Release by FCCP/ oligomycin	T	4.1	100	8.5	100	7.9	100
	M	1.6	39	1.7	20	4.5	57
	NM	2.5	61	6.8	80	3.4	43

Ca^{2+} content is similar whether rotenone/oligomycin or FCCP/oligomycin are added at time zero or $t = 20$ min.

Comparison of pool sizes obtained with the different methods

In Table I the three independent means of estimating the relative pool sizes of mitochondrial and non-mitochondrial Ca^{2+} are compared. It is apparent that in the case of veratridine plus ouabain the largest increase in pool-size occurs in the non-mitochondrial compartment, whereas with K^+-depolarization a large proportion of the increased Ca^{2+} is found in the mitochondrial matrix.

Discussion

The results of the present study indicate that mitochondria within synaptosomes continue to respond to changes in their extramitochondrial $[Ca^{2+}]$. The negligible effect of Ruthenium Red, an effective non-competitive inhibitor of mitochondrial Ca^{2+} uptake (K_i about 30 nM, Ref. 22), on synaptosomal Ca^{2+} uptake suggests that Ca^{2+} uptake by contaminating free brain mitochondria does not contribute significantly to Ca^{2+} uptake by synaptosomes. This finding also indicates that Ruthenium Red does not penetrate the plasma membrane in contrast to suggestions made by Alnaes and Rahamimoff [25] based upon the effect of this inhibitor on neuromuscular transmission.

Effect of plasma membrane depolarization upon Ca^{2+} uptake by synaptosomes

It has earlier been shown [15] that depolarization of the synaptosomal plasma membrane increases the rate of Ca^{2+} uptake. As shown in the present study not only the rate but also the steady state level of Ca^{2+} uptake is significantly enhanced by plasma membrane depolarization. With high external $[K^+]$ the Ca^{2+} uptake consists of a fast phase, which is completed within 1 min, whereas in the presence of veratridine plus ouabain the rate of Ca^{2+} uptake increases during the whole time course of uptake to about 20 min. In both cases the magnitude of Ca^{2+} uptake at steady state is increased 2- to 3-fold as compared to the control.

Effect of the mitochondrial membrane potential on synaptosomal Ca^{2+} uptake

The three different experimental approaches used in the present study to assess the possible contribution of intrasynaptosomal mitochondria to the regulation of cytosolic Ca^{2+} activity produce closely comparable results (Table I). A consistent finding with all three methods is that the mitochondrial contribution to the enhanced synaptosomal Ca^{2+} uptake during depolarization is less pronounced with ouabain plus veratrine than with high $[K^+]$. Veratridine is known to increase the Na^+ content of synaptosomes [26] whereas high $[K^+]$ is expected to deplete the intrasynaptosomal Na^+ due to a stimulation of the (Na^+/K^+)-ATPase. In fact we have observed a decrease in the synaptosomal Na^+ content upon high K^+ depolarization (Åkerman, K.E.P. and Nicholls, D.G. (1981) Eur. J. Biochem. [38]). In mitochondria isolated from brain [9,10] as well as some other tissues [8,9] Na^+ activation of the efflux pathway means that the steady-state distribution of Ca^{2+} undergoes a stable change when extramitochondrial $[Na^+]$ is increased [27]. Thus the difference between the mitochondrial Ca^{2+} content of high$[K^+]$- and veratridine/ouabain-treated synaptosomes might be due to the activity of the mitochondrial Na^+/Ca^{2+} exchanger. Veratridine-induced release of Ca^{2+} from intracellular stores has also been observed in the endocrine pancreas [28].

Interrelation between mitochondrial and non-mitochondrial Ca^{2+}

When Ca^{2+} uptake by intrasynaptosomal mitochondria is inhibited by rotenone plus oligomycin there is a decrease in Ca^{2+} uptake by the synaptosomes, and if the mitochondrial membrane is depolarized with FCCP plus oligomycin under near steady-state conditions Ca^{2+} is extruded across the plasma membrane. This indicates that Ca^{2+} transport across the plasma membrane is affected by changes in Ca^{2+} buffering by intrasynaptosomal mitochondria. The determining factor in this case is probably the cytosolic Ca^{2+} concentration, indicating that both mitochondrial and plasma membrane net Ca^{2+} transport may be regulated by the cytosolic free Ca^{2+} concentration.

The non-mitochondrial Ca^{2+} varies significantly between the different experimental conditions. In the control experiment the size of this pool is about 2–3 nmol/mg synaptosomal protein and it increases to about 4–5 nmol/mg protein with high external $[K^+]$ or veratridine plus ouabain. Since the cytosolic free

Ca^{2+} in nervous tissue is thought to be of the order of 10^{-7} M [11,12] at rest and between 10^{-6} and 10^{-5} M during activation [11,13,17] a 2-fold increase in the total non-mitochondrial Ca^{2+} pool can only be reconciled with an order-of-magnitude change in free cytosolic [Ca^{2+}] if the majority of the Ca^{2+} under control conditions is bound to high-affinity, low-capacity binding sites. Additional nonmitochondrial Ca^{2+} during depolarization would saturate these sites and lead to a rapid subsequent rise in free Ca^{2+} concentration.

Ca^{2+} uptake by non-mitochondrial buffers has been suggested to play a role in the regulation of the cytosolic Ca^{2+} activity [29,30]. Microsomal fractions from brain preparations have indeed been shown to transport Ca^{2+} as well as possess ($Ca^{2+} + Mg^{2+}$)-stimulated ATPase activity [31–35]. However the location of these activities in the intact synaptosomes and their regulation remains to be elucidated. On the other hand Baker and Schleapfer [36] have reported that squid axoplasm contains significant amounts of a protein, which binds Ca^{2+} with high affinity. Thus the bulk of non-mitochondrial Ca^{2+} also in synaptosomes might be in the cytoplasm.

Role of mitochondria in the regulation of the cytosolic Ca^{2+} activity within synaptosomes

Since the cytosolic [Ca^{2+}] activity in nervous tissue at rest is thought to be of the order of 10^{-7} M [11,12] and mitochondria incubated in conditions approximating those of the cytosol strive to keep their external [Ca^{2+}] near 10^{-6} M [14,17], little contribution of mitochondria to the regulation of the cytosolic Ca^{2+} activity in resting conditions is expected. However as shown in the present study there is a significant increase in Ca^{2+} uptake by intrasynaptosomal mitochondria when synaptosomal Ca^{2+} uptake increases during depolarization with high [K^+]. In agreement with this it has been histochemically demonstrated that the mitochondrial Ca^{2+} increases upon stimulation of nerve terminals [37] and that FCCP causes a considerable increase in the cytosolic [Ca^{2+}] of stimulated squid axons [13] with a lesser effect upon non-stimulated ones.

Thus it appears that mitochondrial Ca^{2+} transport is of importance during activation. The mitochondria probably buffer the cytosolic Ca^{2+} near 10^{-6} M thus preventing the cytosolic [Ca^{2+}] from rising to very high values as well as aiding restoration of cytosolic [Ca^{2+}] during termination of transmission.

Acknowledgements

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