Biochimica et Biophysica Acta, 1016 (1990) 77–86
Elsevier

BBABIO 43178

Regulation of Ca^{2+} transport in brain mitochondria. I. The mechanism of spermine enhancement of Ca^{2+} uptake and retention

Hagai Rottenberg and Miriam Marbach

Pathology Department, Hahnemann University, Philadelphia, PA (U.S.A.)

(Received 10 May 1989)
(Revised manuscript received 6 October 1989)

Key words: Calcium ion transport; Brain mitochondrion; Spermine; Mitochondrion

Spermine enhances electrogenic Ca^{2+} uptake and inhibits Na^+-independent Ca^{2+} efflux in rat brain mitochondria. As a result, Ca^{2+} retention by brain mitochondria increases greatly and the external free Ca^{2+} level at steady-state can be lowered to physiologically relevant concentrations. The stimulation of Ca^{2+} uptake by spermine is more pronounced at low concentrations of Ca^{2+}, effectively lowering the apparent K_m for Ca^{2+} uptake from 3 μM to 1.5 μM. However, the apparent V_{max} is also increased. At low Ca^{2+} concentrations, Ca^{2+} uptake is diffusion-limited. Spermine strongly inhibits Ca^{2+} binding to anionic phospholipids and it is suggested that this increases the rate of surface diffusion which reduces the apparent K_m for uptake. The same effect could inhibit the Na^+-independent efflux if the rate of efflux is limited by Ca^{2+} dissociation from the efflux carrier. In brain mitochondria (but not in liver) the spermine effect depends on the presence of ADP. In a medium that contains physiological concentrations of P_i, Mg^+, K^+, ADP and spermine, brain mitochondria sequester Ca^{2+} down to 0.1 μM and below, depending on the matrix Ca^{2+} load. Moreover, brain mitochondria under the same conditions buffer the external medium at 0.4 μM, a concentration at which the set point becomes independent of the matrix Ca^{2+} content. Thus, mitochondria appear to be capable of modulating calcium oscillations in brain cells.

Introduction

Ca^{2+} transport in mitochondria has been studied extensively ever since mitochondria were first isolated four decades ago (for recent reviews see Refs. 1–3). Most of our knowledge of the Ca^{2+} transport system in mitochondria comes from studies with liver and heart mitochondria, whereas Ca^{2+} transport in mitochondria from other tissues has not been studied in as great detail.

Three major Ca^{2+} transport systems have been identified in liver, heart and brain mitochondria. The most active system is the electrogenic Ca^{2+} carrier, which, in vitro, under normal conditions (i.e., high membrane potential), catalyzes the uptake of Ca^{2+} against Ca^{2+} concentration gradients [4]. At electrochemical equilibrium calcium concentration gradients could be very large; however, under most physiological conditions the free calcium concentration in the mitochondrial matrix is well below the electrochemical equilibrium concentration [1–3]. This is the result of the activities of several systems that promote Ca^{2+} efflux against the calcium electrochemical potential gradient. In heart mitochondria, the most active efflux system is the Ca^{2+}–Na^+ exchange system, which is somewhat similar to the plasma membrane Ca^{2+}–Na^+ exchange carrier [5]. This system is also very active in brain mitochondria, but is much less active in liver mitochondria. Another efflux system, which is Na^+-independent, exists in mitochondria from all tissues, but is very poorly understood [1]. In addition, under some conditions, high concentrations of Ca^{2+} in the mitochondrial matrix induce a state of high membrane permeability which leads to discharge of matrix content, including Ca^{2+} [6]. The balance of the activities of the various Ca^{2+} transport systems determines the external Ca^{2+} concentration at which net transport vanishes [2]. When the matrix Ca^{2+} concentration increases above the saturation level of the efflux system, mitochondria will buffer the external Ca^{2+} level ("set point").

For many years it was believed that mitochondria must play a central role in cellular Ca^{2+} regulation [2].

Correspondence: H. Rottenberg, Pathology Department, Hahnemann University Broad and Vine, Philadelphia, PA 19102, U.S.A.

However, based on the results of recent investigations it is now generally believed that the mitochondria play no role in the regulation of cellular Ca^{2+} levels [1,3,7]. Rather, the function of the mitochondrial Ca^{2+} transport systems in all tissues is now considered to be directed only at the regulation of the free calcium concentration in the matrix. Matrix Ca^{2+} regulates the activities of several important dehydrogenases and thus controls the rate of electron transport [3].

Three lines of evidence have led to the demise of the hypothesis that mitochondria play a role in cellular Ca^{2+} regulation in the brain as well as any other tissue. Firstly, measurements of cellular free Ca^{2+} on one hand and the determination of the ability of mitochondria, in vitro, and in permeabilized cells and synaptosomes, to sequester Ca^{2+}, on the other hand, indicated an apparently unbridgeable gap between the range of cellular free Ca^{2+} in resting cells in vivo (0.1–0.2 μM) and the mitochondrial set point in vitro and in permeabilized cells and synaptosomes (0.5–10 μM) [1,7]. Secondly, measurement of mitochondrial Ca^{2+} content, in situ, with electron probe X-ray microanalysis [8] and also estimates based on the activity of Ca^{2+} activated dehydrogenases [3] indicated extremely low mitochondrial Ca^{2+} content, which is considered to be incompatible with the hypothesis that mitochondria play a role in cellular Ca^{2+} regulation. Lastly, there is no evidence that any of the mitochondrial Ca^{2+} release systems are activated by extracellular signals which lead to elevation of cellular Ca^{2+}. Since both the Ca^{2+} transport systems of the endoplasmic reticulum and the plasma membrane respond to these signals, it is believed that these systems are solely responsible for cellular calcium regulation [1,7,9,10].

Little attention, however, has been paid, until very recently, to the role of various cellular components in regulating Ca^{2+} transport in mitochondria. It has been shown recently that polyamines greatly enhance the ability of liver mitochondria to sequester Ca^{2+} in the physiological range [11]. These findings were recently extended to mitochondria from heart and brain [12–14]. Also, there are several observations on the ability of adenine nucleotides to improve Ca^{2+} retention in liver, heart, and particularly brain mitochondria [1,15]. However, the role of these agents in regulating Ca^{2+} transport by mitochondria, under physiological conditions, is still unclear and the mechanisms of their effects are completely unknown.

In a preliminary short report we showed that ADP greatly enhances the rate of electrogenic Ca^{2+} transport in brain mitochondria and suggested that its effect is due to locking the adenine-nucleotide carrier in the M-state. We also showed that the enhancement of Ca^{2+} uptake by spermine in brain mitochondria depends on the presence of ADP [16]. In this and the following paper we examine Ca^{2+} transport in brain mitochondria in greater detail, with particular emphasis on the mechanism of the effects of polyamines and adenine nucleotides. This paper deals with the effects of polyamines on Ca^{2+} transport in brain mitochondria and its mechanism.

Materials and Methods

Rat brain ('free') mitochondria were prepared from the forebrains of male Sprague-Dawley rats, in batches of two or three brains, by the Ficol gradient method essentially as described by Lai and Clark [17]. Isolated mitochondria contained approx. 15 nmol Ca^{2+}/mg protein as determined by atomic absorption. Rat liver mitochondria were prepared as described previously [18]. The rate of Ca^{2+} transport was measured routinely with the Ca^{2+} indicator Arsenazo III [19,20]. Absorbance difference of the suspension was followed at 685–675 nm using an Aminco DW-2A spectrometer. Rates and extents of Ca^{2+} transport were calculated on the basis of internal calibration obtained from the addition of known amounts of Ca^{2+} to the suspension. Free Ca^{2+} concentrations were estimated from calibration curves constructed from the titration of Arsenazo III with Ca^{2+} [20]. For each amount of total Ca^{2+}, the concentration of calcium-bound Arsenazo III was calculated from the value of the absorbance. From these values, free Ca^{2+} is calculated and the free Ca^{2+} is plotted against the absorbance to yield a calibration curve. Since the titration curves do not fit a simple, single dissociation constant, we used these curves directly for estimation of free Ca^{2+}. Since the Arsenazo III-Ca^{2+} complex is very sensitive to various cations and pH, we used separate calibration curves for each medium employed in this study (excluding Ca^{2+} buffers). In each experiment EGTA was added at the end of the experiment and the difference in absorbance between the experimental point and the EGTA reading was used to estimate free Ca^{2+} from a calibration curve in the appropriate medium. The calibration was also checked against Ca^{2+} electrode measurements. Ca^{2+} electrode (Orion 93-20) was also used in several experiments, in particular, when the accurate determination of low free Ca^{2+} levels was required. The electrode was calibrated by the iterative procedure described by Bers [21]. The electrode signal is linear with the Ca^{2+} concentration down to 0.2 μM Ca^{2+}. In a few experiments, Ca^{2+} transport was measured from the fluorescence of fura-2, using the excitation ratio method (340/380 nm) on a Spex Flurolog Spectrofluorometer (Series 2), as previously described [22].

Kinetic analysis (e.g., estimates of K_m and V_{max}) was preformed by linear regression analysis of Lineweaver and Burk plots. All the indicated values were obtained from plots in which the correlation coefficient was higher than 0.95.

Mitochondrial protein was determined by a modified Lowry method using the commercial reagent BCA (Pierce). All fine chemicals were from Sigma. All other reagents were of the highest analytical grade.

Results

Fig. 1 shows a typical experiment indicating the effects of spermine on Ca^{2+} transport and demonstrating the protocols we employed in most of the experiments described in this study. Mitochondria (0.5 mg protein/3 ml) are incubated in a medium which contains P_i (5 mM), Mg^{2+} (1 mM) and ADP (20 μM) together with rotenone and oligomycin. After 8 min incubation at room temperature, during which some of the endogenous Ca^{2+} is released, succinate is added. The formation of membrane potential by succinate oxidation induces Ca^{2+} uptake, reducing external free Ca^{2+} until a steady state is established. Addition of $CaCl_2$ (30 nmol) increases the external concentration which induces further uptake of Ca^{2+} until a new steady state is established. Addition of Ruthenium red (RR), which specifically inhibits the electrogenic Ca^{2+} carrier, results in Ca^{2+}-efflux due to the activity of the Na^+-independent efflux system. Addition of Na^+ increases the efflux as it activates Na^+–Ca^{2+} exchange.

In Fig. 1A we follow external Ca^{2+} from the absorption difference of Arsenazo III. Trace a shows the experiment in the absence of spermine, while trace b shows the same experiment in the presence of spermine. To evaluate the free Ca^{2+} concentration we add EGTA (300 nmol) at the end of the experiment. Because spermine itself interacts with Arsenazo III (Rottenberg, unpublished data), both the magnitude of the spectral changes and the Ca^{2+} affinity are affected and thus somewhat obscure the spermine effect. However, a calibration curve in a medium which includes spermine allows the calculation of the free calcium concentration in the presence of spermine.

It was observed that spermine enhances the rate of uptake (approx. 2-fold), lowers the steady-state to a lower external Ca^{2+} and inhibits the Ca^{2+} efflux rate. Fig. 1B shows the same experiment as followed by a Ca^{2+} electrode. Since spermine is without effect on the Ca^{2+} electrode signal, the figure shows the spermine effect more clearly. Finally, Fig. 1C shows the same effects of spermine as followed with the fluorescent indicator fura-2 (which is not affected by spermine). Both the rates and the free calcium concentrations calculated from each of the three measurements are comparable and allow the use of these three methods interchangeably.

Fig. 2 shows the effects on the rates of calcium transport which were obtained by increasing concentrations of spermine. For the uptake experiments we used a medium which simulates the cation composition of

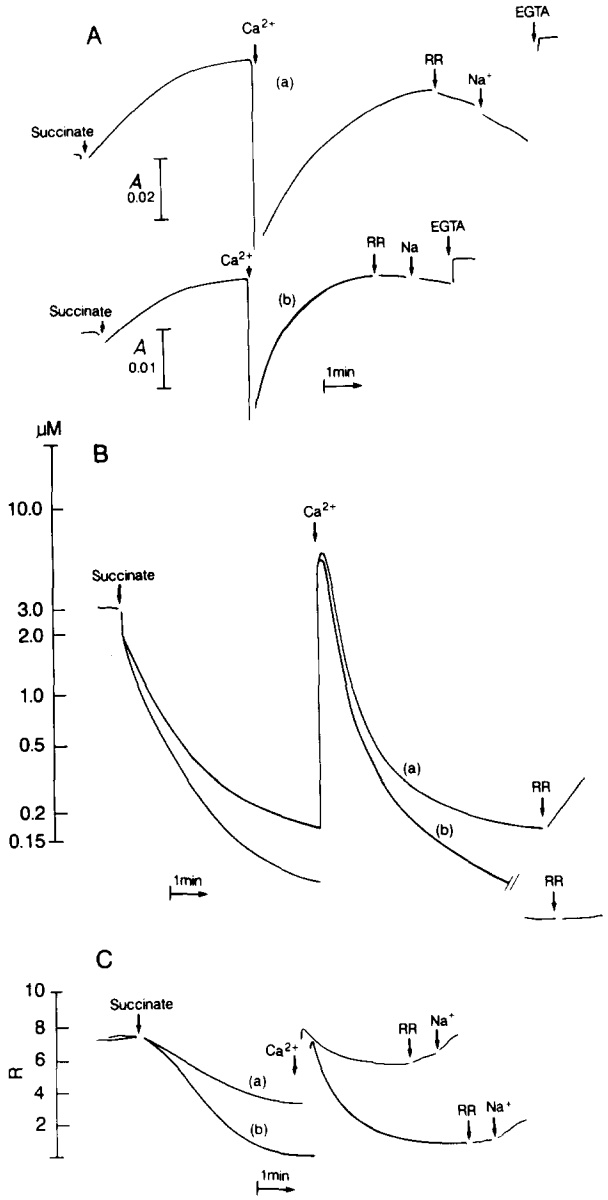


Fig. 1. The effect of spermine on Ca^{2+} transport. The basic medium contained 0.2 M sucrose, 0.08 M mannitol, 10 mM Tris-HCl, 5 mM Tris-P_i, 1 mM $MgCl_2$ (pH 7.4). The experiment was initiated by addition of 0.5 mg mitochondrial protein to 3 ml medium. Rotenone (1 μM) oligomycin (2 μg/mg protein) and ADP (20 μM) were then added and the suspension was incubated for 8 min at room temperature to release endogenous Ca^{2+}. Ca^{2+} uptake was started by the addition of 5 mM Tris-succinate. After the establishment of steady state, additional Ca^{2+} (30 nmol $CaCl_2$) was added. When steady state was established again, Ruthenium red (RR) was added (30 pmol), and later 10 mM NaCl was added. Finally, 300 nmol EGTA were added to bind all external Ca^{2+}. Curve a shows the experiment in the absence of spermine and curve b shows the experiment with 0.5 mM spermine in the incubation medium. In (A), 50 μM Arsenazo III was included in the incubation medium and Ca^{2+} transport was followed from the absorption difference (685–675 nm), as described in Materials and Methods. In (B) the free Ca^{2+} concentration in the medium was followed by Ca^{2+} electrode. In (C) 5 μM fura-2 was added and Ca^{2+} transport was followed by the fluorescence ratio method (340/380 nM), as described in Material and Methods.

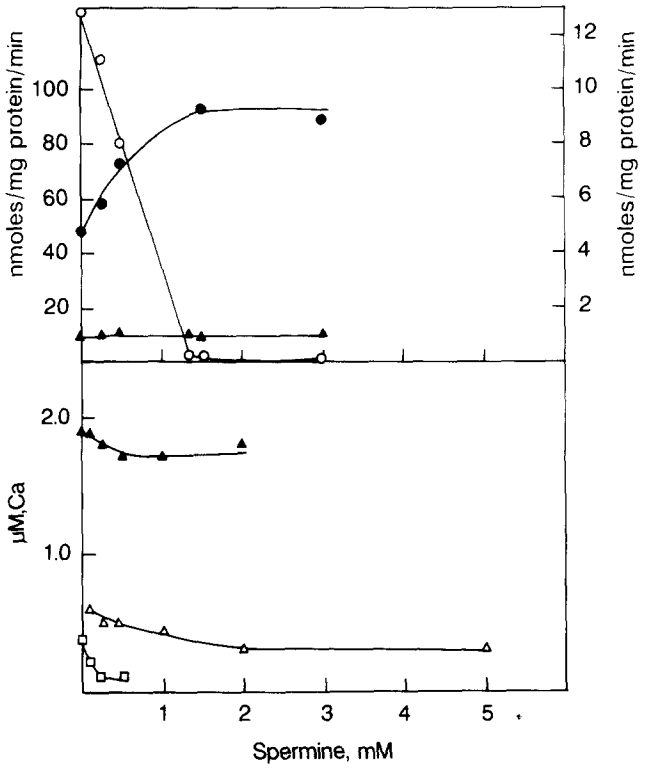


Fig. 2. The effect of spermine concentration on Ca^{2+} transport. (A) shows the effect of spermine on the rates of Ca^{2+} uptake and efflux. Medium and method was the same as in Fig. 1A, except for the addition of 100 mM KCl and 10 mM NaCl, and MgADP when present (100 μM). The rate of Ca^{2+} uptake after addition of 30 nmol $CaCl_2$ (as in Fig. 1A) in the absence of MgADP (▲) and the presence of ADP (●) is shown. The rate of Ca^{2+} efflux after addition of RR (30 pmol) in the system that contains Mg ADP (○) is also shown. (B) shows the final free Ca^{2+} concentration in the suspension after the uptake of added Ca^{2+} as measured by Ca^{2+} electrode in a medium identical to that of (A) except for the omission of Arsenazo III. The steady-state level in the absence of MgADP (▲) and the presence of MgADP (△) are shown. In addition, the steady-state level in a medium identical to that of Fig. 1B is also shown (□).

the cytoplasm (i.e., 1 mM Mg^{2+}, 100 mM K^+ and 10 mM Na^+) in the presence and absence of 0.1 mM MgADP. The top panel shows the dependence on spermine concentration of the stimulation of Ca^{2+} uptake. The protocol of the experiments were as in Fig. 1A and the results are those obtained after addition of 30 nmol Ca^{2+}. In this high salt medium, maximal effects were obtained at 1.5 mM spermine, with 0.5 mM exhibiting 50% stimulation of uptake (or inhibition of efflux). The figure also shows that in the absence of ADP, the Ca^{2+} uptake rates were very low and were not stimulated by spermine.

The bottom panel of Fig. 2 shows the effect of spermine concentration on the Ca^{2+} steady-state levels obtained after the addition of 30 nmol of Ca^{2+} as measured with Ca^{2+} electrode. In the presence of ADP the steady-state level was reduced from 0.9 μM without spermine to 0.3 μM at 2 mM spermine. The total dependence of the spermine effect on ADP was demonstrated again by a similar experiment in the same medium without ADP. Spermine had only a slight effect on the steady-state level in the absence of ADP, reducing it from 1.9 μM to 1.7 μM. As observed previously, the absence of ADP raised the steady-state level from 0.9 to 1.9 (Ref. 16, and see the following paper). The figure also shows the spermine effect on the steady-state level which was obtained in a low salt medium. Under these conditions the spermine effect was observed at much lower concentration (maximal effect is obtained at 250 μM) and because of the absence of Na^+, the steady-state level was reduced to below 0.1 μM. This large reduction of the steady-state level in the absence of Na^+ appears to be due to inhibition of the Na^+-independent efflux. The inhibition of Na^+-independent efflux as function of spermine concentration is shown in the top panel of Fig. 2.

Fig. 3 shows the dependence of Ca^{2+} uptake and retention on external Free Ca^{2+} which we observed in the presence and absence of spermine. Panel A shows the effect of spermine on the initial rate of uptake as a function of the free concentration of added Ca^{2+} (experimental protocol was as in Fig. 1A, except for the amount of added Ca^{2+} (10–250 nmoles/mg protein)). Spermine decreased the apparent K_m from 3 μM to 1.5 μM. The apparent maximal rate was also increased from 68 to 108 nmol/mg protein/min. Panel B shows the effect of spermine on the amount of Ca^{2+} which was taken up by mitochondria after the addition of Ca^{2+} as function of the initial external free Ca^{2+}. In the presence of ADP, large amounts of Ca^{2+} were accumulated with and without spermine. However, spermine greatly enhanced the ability of the mitochondria to accumulate Ca^{2+}. Fig. 4A shows the relationships between the steady-state level and the total accumulated Ca^{2+} (i.e., the sum of Ca^{2+} which was taken after succinate addition and after Ca^{2+} addition). In the absence of spermine, but in the presence of ADP, the steady-state level was increased gradually from 0.4 μM at low load of Ca^{2+} (75 nmol/mg protein) to 2.9 μM at high load (250 nmol/mg protein). There appear to be a narrow range, around 0.9 μM in which the mitochondria has a limited buffering power (set point). Spermine reduced the set point dramatically, allowing the mitochondria to accumulate very large amounts of Ca^{2+} without raising the steady-state levels above 0.4 μM. For comparison, the figure also outlines the results of similar experiments, but in the absence of ADP (see the following paper for the results). In this case, the steady-state levels increased asymptotically, effectively preventing the mitochondria from accumulating Ca^{2+}, regardless of external Ca^{2+} concentration.

The enhancement by spermine of Ca^{2+} accumulation paralleled its inhibition of Na^+-independent Ca^{2+} efflux, as shown in Fig. 4B. Without spermine the Na^+-independent efflux increased quickly with increasing

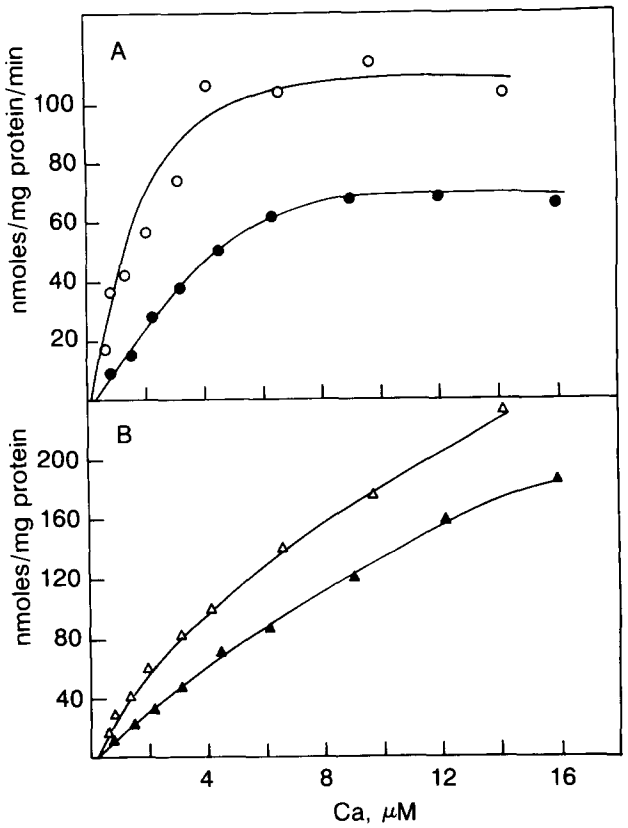


Fig. 3. The dependence of the spermine effects on the external free Ca^{2+} concentration. Medium was the same as in Fig. 1A, except for the addition of 100 mM KCl and 0.3 mM MgADP. Spermine, when added, was 1 mM. The protocol is the same as in Fig. 1A, except for the addition of various amounts of $CaCl_2$ (10–250 nmol/mg protein). The external free Ca^{2+} concentrations shown are those obtained after the addition of $CaCl_2$, as estimated from the calibration of Arsenazo III, and the rates are the initial uptake rates after Ca^{2+} addition. (A) shows the rate of Ca^{2+} uptake with (○) and without (●) spermine. (B) shows the amount of Ca^{2+} taken up by the mitochondria in the same experiments shown in (A). Net uptake with (△) and without (▲) spermine is shown.

Ca^{2+} load, while in the presence of spermine Na^+-independent efflux was very slow (< 1 nmol/mg protein per min) up to very high load. Fig. 4B also shows that Na^+-dependent efflux was only slightly affected by spermine and was largely independent of the Ca^{2+} load. Thus, this experiment demonstrates that in the presence of spermine and ADP, brain mitochondria will buffer external Ca^{2+} at about 0.4 μM.

To evaluate the relationship between Ca^{2+} load and the steady-state level at a lower Ca^{2+} load, we conducted the following experiments. Using the Ca^{2+} electrode reading as a guide, we titrated the external Ca^{2+} to low levels with the Ca^{2+} buffer nitrilotriacetate prior to the addition of succinate. Addition of succinate at low external Ca^{2+} induced a limited extent of uptake with low Ca^{2+} loading, and thus further reduced the steady-state level. The results of these experiments are shown in Fig. 5A. It is observed that below 90 nmol/mg protein, the steady-state level decreased with decreased Ca^{2+} load and reached a value well below 100 nM.

Another way to test the dependence of the Ca^{2+} steady-state level on the matrix Ca^{2+} load is to increase the mitochondrial concentration in the suspension. The results of these experiments are shown in Fig. 5B. For the same amount of Ca^{2+} in the medium, higher mitochondrial concentrations resulted in lower load and decreased steady-state level. Thus, both experiments demonstrate that in the presence of ADP and polyamines, and provided that the mitochondria are not overloaded with Ca^{2+}, brain mitochondria are capable of sequestering Ca^{2+} at the physiological range down to levels of cellular Ca^{2+} at rest.

The dependence of the spermine effect on the presence of ADP (Fig. 2) was not observed in liver mitochondria. This is shown in Fig. 6. Neither the lowering of the steady-state level nor the stimulation of the rate

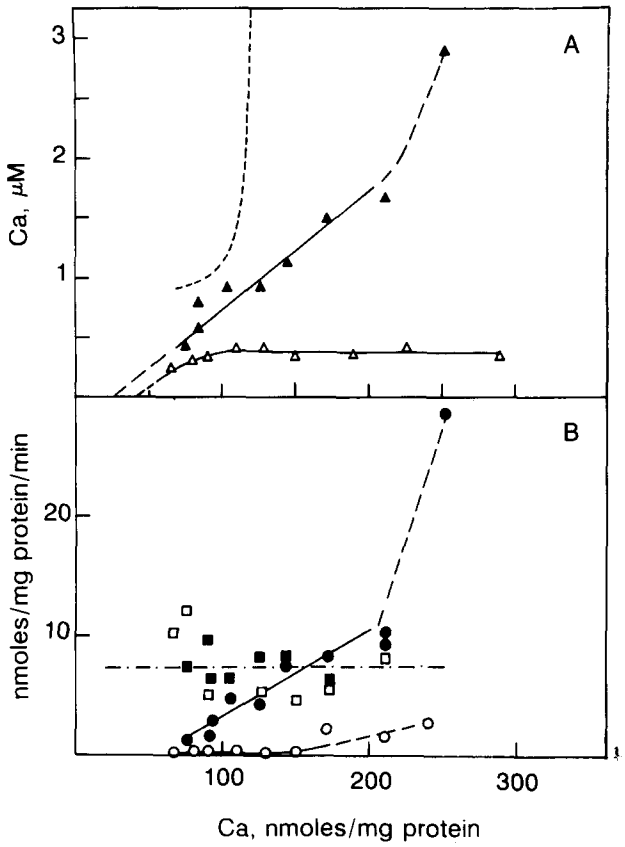


Fig. 4. The effect of spermine on the Ca^{2+} steady-state level and Ca^{2+} efflux. Data are from the same experiments shown in Fig. 3. (A) shows the final free Ca^{2+} concentration in the medium) after the accumulation of the added Ca^{2+} (Fig. 4B) plotted as function of the total content of the mitochondrial Ca^{2+} which included the Ca^{2+} accumulated after the succinate addition and after the $CaCl_2$ addition. The steady-state levels with (△) and without (▲) spermine are shown. The steady-state level in the absence of ADP is also indicated (— — —) for comparison (see following paper). (B) shows the rate of efflux induced by RR with (○) and without (●) spermine, and the rate of efflux induced by NaCl (■) with and without (□) spermine as a function of the total Ca^{2+} content of the mitochondria.

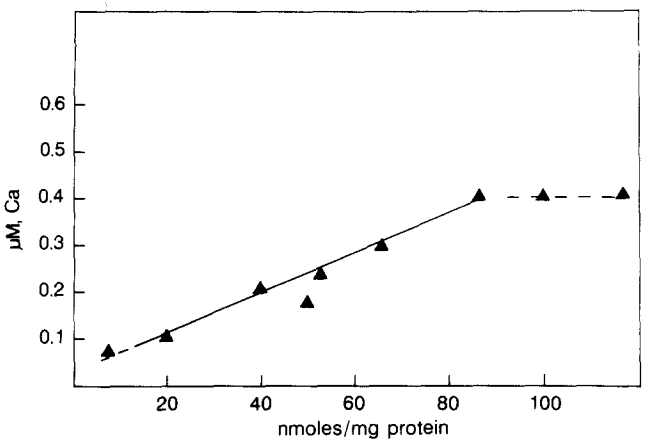
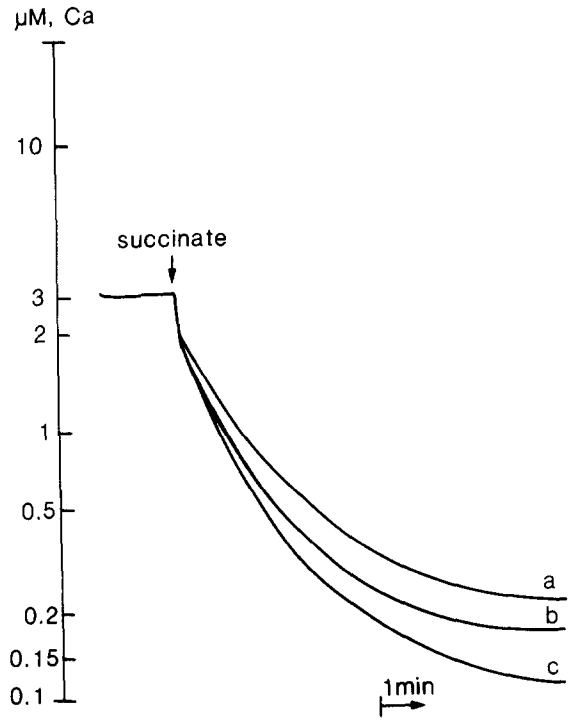


Fig. 5. The effect of Ca^{2+} load on the Ca^{2+} steady-state level. Medium was the same as in Fig. 3, except for the concentration of spermine (0.5 mM) and the omission of Arsenazo. In (A) increasing concentrations of NTA were added to obtain lower initial Ca^{2+} concentration and thus lower Ca^{2+} load. The figure shows the final steady-state level. In (B) increasing initial mitochondrial concentrations were used to lower Ca^{2+} load. The figure shows the kinetics of the change in external free Ca^{2+}. Trace a, 0.25 mg protein/ml; trace b, 0.5 mg protein/ml; trace c, 1.0 mg protein/ml.



of uptake is significantly affected by the presence of ADP.

Although spermine and other polyamines affect many cellular processes, the molecular mechanisms of these effects are completely unknown. We have observed that spermine can compete with Ca^{2+} and Mg^{2+} effectively and can release Ca^{2+} and Mg^{2+} from certain complexes (Rottenberg, unpublished data). Since binding of Ca^{2+} to the mitochondrial membrane surface can impede the rate of Ca^{2+} uptake, particularly at low external Ca^{2+} (see Discussion), spermine may act by weakening Ca^{2+} binding to anionic phospholipids on the membrane surface.

We have observed that under some conditions spermine can release small amounts of bound Ca^{2+} and protons from mitochondria (not shown). However, in mitochondria, a clear distinction between transport and binding, and between lipid surface binding and protein binding is difficult. We have, therefore, investigated the effect of spermine on Ca^{2+} binding to anionic phospholipids in liposomes prepared from a mixture of 80% neutral lipids (phosphatidylcholine) and 20% anionic phospholipids (phosphatidic acid). This mixture (PC/PA) corresponds roughly to the charged/neutral ratio in mitochondrial lipids. When small amounts of Ca^{2+} are added to suspension of these liposomes, the Ca^{2+} bind to the liposome surface. Addition of spermine released this bound Ca^{2+}, a process that was completed at 0.3 mM spermine. Adding spermine to the medium without the liposome was without effect, indicating that the release was from the liposomes surfaces and not from Ca^{2+}-complexes in the medium. Similar experiments with neutral liposomes (PC) showed no effect on Ca^{2+} binding, while in asolectin liposomes, which contain negative lipids, similarly strong effects on Ca^{2+} binding, were observed. Fig. 7 shows an experiment in which the release of Ca^{2+} from PC/PA liposomes containing 25 nmoles Ca^{2+}/mg lipid was followed with Ca^{2+} electrodes. In the absence of spermine, the liposomes bind more Ca^{2+}, lowering the medium free Ca^{2+} concentration. In contrast, when spermine was included in the medium, the liposomes released Ca^{2+}. The amount of released Ca^{2+} depends on the

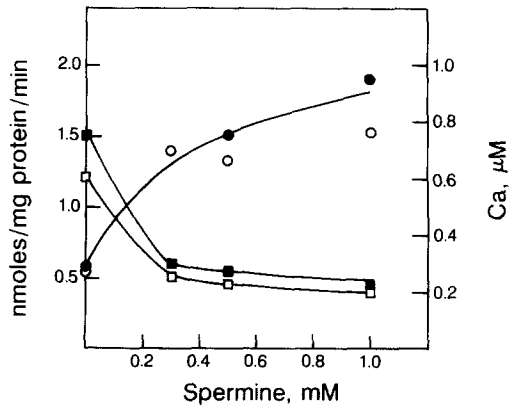


Fig. 6. The absence of significant ADP effect on the spermine effect on liver mitochondria. Medium and protocol was the same as in Fig. 1B, except for the addition of 100 mM KCl and the omission of Arsenazo III. Mg ADP when present was 0.3 mM. Ca^{2+} uptake rates and set point were measured by calcium electrode. Initial free Ca^{2+} concentration, after addition of Ca^{2+} was 3 μM. The initial uptake rate with (●) and without (○) ADP, and the steady-state level with (□) and without (■) ADP are shown.

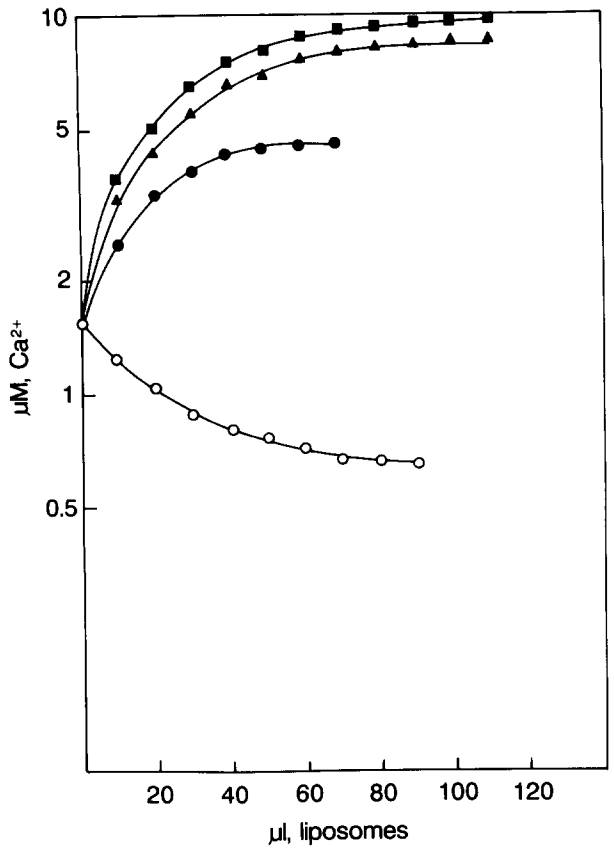


Fig. 7. The effects of negatively charged liposomes on free Ca^{2+} in the presence and absence of spermine. Liposomes were prepared from mixture of 80 mg phosphatidylcholine and 20 mg phosphatidic acid in Tris-HCl medium (pH 7.5, final concentration 116 mM). Before the experiment, the liposome suspension was sonicated until it cleared to increase the surface area and 25 nmol Ca^{2+}/mg lipids were added. Medium was a buffer solution (20 mM Tris-HCl (pH 7.5)) with free calcium concentration of 1.5 μM. Stock suspension of liposomes was added to 4 ml of medium and Ca^{2+} concentration was followed by Ca^{2+} electrode. Spermine concentration in the buffer was: 0 (○); 50 μM (●); 150 μM (▲); 250 μM (■).

total calcium, on the liposome concentration and on the spermine concentrations as shown in Fig. 7. For instance, with 250 μM spermine, at 5 μM free Ca^{2+} concentration, 22 nmol of Ca^{2+}/mg lipid were released. Thus, these experiments clearly demonstrate that spermine effectively compete with the binding of Ca^{2+} to anionic phospholipids. This action could explain its effect on the rate of Ca^{2+} uptake and efflux (see Discussion).

Discussion

Spermine effect on Ca^{2+} transport in brain mitochondria

Our findings that spermine increases the rate of Ca^{2+} uptake at low external Ca^{2+} and reduces the Ca^{2+} steady-state level in rat brain and liver mitochondria suspensions are in general agreement with previous observations [11–14]. However, there are several novel findings in our study.

(i) Brain mitochondria, in the presence of ADP and spermine, lower the set-point and buffers the external Ca^{2+} at 0.4 μM. Moreover, in the presence of spermine, mitochondria show a significant capacity to accumulate Ca^{2+} at extremely low Ca^{2+} levels.

(ii) The spermine effect in brain mitochondria completely depends on the presence of ADP, whereas, there is no such dependence in rat liver mitochondria. As we show in the following paper, there is also a very strong effect of adenine nucleotides on the rate of Ca^{2+} uptake in the absence of spermine, which is not observed in liver mitochondria. Our examination of the ADP effect strongly suggests that the ADP effect is exerted on the matrix surface of the inner membrane, possibly by stimulating Ca^{2+} dissociation from the electrogenic carrier (Ref. 16 and the following paper). If the rate of the electrogenic transport is limited by internal dissociation in the absence of ADP, then the lack of enhancement of uptake by spermine suggests that spermine affects an earlier step, most likely the access of Ca^{2+} to the carrier on the cytosolic surface. This conclusion is compatible with the fact that spermine appears to decrease the apparent K_m for external Ca^{2+} and that the effect of added spermine is immediate (not shown). Although it was recently demonstrated that spermine is taken up by mitochondria [23,24], this uptake is slow compared to the effect on Ca^{2+} uptake.

(iii) In our study, spermine inhibited the Na^+-independent efflux, while in previous studies it appeared to stimulates the efflux [11,13]. This difference correlates with the phosphate concentration in the medium. In the experiment shown here, the medium contain 5 mM P_i, whereas Nichitta and Williamson [11] included only 0.5 mM P_i and Jensen et al. [13] included 1.0 mM P_i. It has been shown previously that polyamines enhance phosphate accumulation by mitochondria, while preventing swelling [25]. Phosphate, in the presence of ADP, inhibits the efflux (see the following paper).

(iv) In our experiments, spermine stimulated Ca^{2+} uptake at all tested external calcium concentrations (up to 16 μM) where as in some previous studies, spermine inhibited the uptake above 4.5 μM Ca^{2+} [11,13]. This difference is probably related to the difference in the effect on the efflux as discussed above. Indeed, Lenzen et al., who also used high P_i observed no inhibition of Ca^{2+} uptake by spermine at any external Ca^{2+} [12]. Since the measured net uptake represents the difference between uptake and efflux, increased efflux at high Ca^{2+} (where the effect on uptake is already saturated) would result in inhibition of net uptake.

Mechanism of the spermine effect on Ca^{2+} transport

The range of specific effects of polyamines on biological processes is bewildering [26]. Despite the grow-

ing number of reported specific effects on increasingly diverse systems, hardly anything is known about their mechanism of action. In the course of our studies we observed that spermine competes effectively with Ca^{2+} and Mg^{2+} in various specific complexes (Rottenberg, unpublished data). Because the main effect of spermine on mitochondrial Ca^{2+} transport is to decrease the apparent K_m for external Ca^{2+} of the electrogenic carrier, it appears that spermine enhances the interaction between Ca^{2+} and the electrogenic carrier. This effect could not be due to simple electrostatic interaction with the membrane since the positively charged spermine should reduce the negative surface charge density (and hence the surface potential) and thus increase the apparent K_m for Ca^{2+} rather than decrease it [27]. Therefore, the effect must be more specific. The effect could not be due to competitive binding of spermine to the Ca^{2+} carrier, since this should inhibit the transport rather than stimulate it.

It is well known that the rate of binding of ligands (or ions) with membrane receptors at very low concentration depends on their surface diffusion rate [28]. If the receptor (carrier) concentration on the membrane surface is low, the probability of direct binding from the medium is negligible compared to the probability of surface diffusion of the ligand to the receptor. The rate of surface diffusion, would be inhibited if binding groups with high affinity for the ion exist on the membrane surface, because the rate of dissociation from these groups would limit the surface diffusion rate [29]. Since Ca^{2+} binds strongly to anionic phospholipids, the presence of these groups should impede surface diffusion of Ca^{2+} at low Ca^{2+} concentrations. Our demonstration that polyamine compete with Ca^{2+} binding to anionic phospholipids, and previous observations of spermine binding to anionic phospholipids [33] and mitochondria [24] provides the basis for an hypothesis that explains the polyamines effect as follows: at low external Ca^{2+}, the rate of Ca^{2+} uptake is limited by the rate of the formation of the Ca^{2+}-carrier complex. This rate, in turn, is limited by the rate of surface diffusion of Ca^{2+} which is slowed down by Ca^{2+} binding to anionic phospholipid. Polyamines interact with anionic phospholipids, competing with Ca^{2+} and thus enhance Ca^{2+} surface diffusion, which stimulates the rate of Ca^{2+} uptake.

In our model we assume that the rate of electrogenic transport is diffusion-limited. To evaluate whether external surface diffusion could be a rate limiting step in the Ca^{2+} transport process, we follow the approach of Berg and Purcell [28]. We express the rate of electrogenic Ca^{2+} transport by a carrier of low surface density, and under conditions where the rate is limited by diffusion as follows:

$$J = J_{max} Ns/(Ns + \pi a) \quad (1)$$

Where J_{max} is the rate that would be obtained if the mitochondrion were freely permeable to Ca^{2+}, N is the number of carriers per mitochondrion, s is the radius of the Ca^{2+} binding site and a is the radius of the mitochondrion. J_{max} is given by:

$$J_{max} = 4\pi a D C_\infty \quad (2)$$

Where D is the Ca^{2+} diffusion coefficient and C_∞ is the concentration (number of ions/cm^2) at the bulk phase [28]. For a mitochondrion with a radius of 1 μm, at bulk Ca^{2+} concentration of 1 μM and taking $D_{Ca} = 2 \cdot 10^{-5}$ cm^2/s we get from Eqn. 2: $J_{max} = 15 \cdot 10^6$ ions/s. Based on the determination of the electrogenic carrier of 1 pmol/mg protein [30], and that each mitochondrion contains about $0.5 \cdot 10^{-10}$ mg protein [31], we arrive at an estimate of 30 electrogenic carriers for each mitochondrion. Assuming that the radius of the Ca^{2+} binding site is twice that of the Ca^{2+} ion, i.e., 2 Å, we obtain from Eqn. 1 a transport rate of $2.86 \cdot 10^4$ ions/s per mitochondrion, which translates into approx. 58 nmol/mg protein per min. This calculated rate is of the same magnitude we observed under optimal conditions in the presence of spermine at 1 μM Ca^{2+}. Since binding of Ca^{2+} to the anionic phospholipids at the surface would effectively reduce the apparent diffusion coefficient at the vicinity of the surface [29], leading to lower transport rates, the assumption of diffusion-limited transport is quite reasonable. Note that this treatment does not consider at all the kinetics of the carrier itself as it is assumed not to be rate limiting. Rather, it is the trapping of the Ca^{2+} by the carrier which is assumed to determine the rate of transport. If the rates estimated by such calculations were orders of magnitude higher than the measured rates, we could conclude that access to the carrier could not be rate-limited. Close agreement of the measured and estimated maximal rates suggest that it is reasonable to assume that the diffusion of Ca^{2+} to the carrier is rate limiting for the transport, at low external Ca^{2+} concentrations.

The hypothesis that spermine increases the apparent Km for Ca^{2+} uptake by increasing the rate of surface diffusion could not explain the inhibition of efflux. If the rate of efflux is also limited by the diffusion of Ca^{2+} on the membrane surface, spermine should enhance the efflux. Indeed, it has been reported that in liver mitochondria spermine enhances the efflux, when Ca^{2+} transport is assayed at low phosphate concentrations [11]. Thus, as discussed above, the spermine effect on the efflux could be related to its effect on phosphate uptake [25]. However, under conditions where the efflux is limited by the rate of dissociation of Ca^{2+} from the carrier at the cytosolic surface, exactly the same mechanism, i.e., spermine inhibition of Ca^{2+} binding to negatively charged phospholipids, would lead to inhibition of Ca^{2+} efflux since binding of Ca^{2+} to adjacent nega-

tively charged phospholipids should enhance the efflux. Nevertheless, a full explanation of the spermine effect on efflux awaits a better understanding of the mechanism of Na^+-independent Ca^{2+} efflux. We cannot explain in full the effect of spermine on the apparent V_{max}. The inhibition of the Na^+-independent efflux can explain only a small part of the apparent increase in V_{max}. Since the V_{max} appears to be determined, in part, by the energetics of the system [2], it is difficult, at present, to interpret this effect. To evaluate the effects of spermine on influx it is necessary to measure initial rates of Ca^{2+} uptake into Ca^{2+}-free mitochondria which has proven quite difficult. In preliminary experiments, using fura-2 loaded mitochondria, the increase in internal Ca^{2+} on addition of substrate was faster than the response time of our apparatus [38]. Therefore, measuring the rate of transport externally into fura-2 loaded mitochondria by conventional techniques could not provide true initial kinetics. Fast kinetics methods may help to resolve this issue.

Specific effects of polyamines, which may be related to interactions with anionic phospholipids, have been observed previously. For instance, polyamines decreased lateral mobility of membrane proteins [32] and inhibited peroxidation of anionic phospholipids [33]. Polyamine effects on membrane enzymes which are phospholipid dependent, such as protein kinases [34], phospholipase C [35], and inositol phospholipid synthesis [36] may also depend on specific interaction with anionic phospholipids in competition with Ca^{2+} and/or Mg^{2+}.

It is interesting to note that the spermine effect on the Ca^{2+} transport in liver and heart mitochondria is modulated by Mg^{2+} [12,14]. Since in these systems Mg^{2+} inhibits the transport system in the absence of spermine, it appears that spermine competes with Mg^{2+} for a Mg^{2+} binding site. Since spermine enhances Ca^{2+} transport, this site could not be the transport site but a regulatory site, which could be a specific anionic phospholipid. In brain mitochondria, Mg^+ does not inhibit Ca^{2+} transport at the physiological range (see following paper) and hence, Mg^{2+} at low concentration does not modulate the spermine effect [12].

The spermine effect on mitochondria and regulation of cellular Ca^{2+}

As discussed in the Introduction, it is now believed by most investigators in the field that mitochondria do not play a significant role in cellular Ca^{2+} regulation. For example, in experiments with saponin treated synaptosomes, Rosgado-Flores and Blaustein [37] have shown that the mitochondria do not take up Ca^{2+} at submicromolar concentration of Ca^{2+}. However, in their experiments the permeabilized synaptosomes were incubated for a long period of time without polyamines and adenine nucleotides. The uptake was initiated by addition of ^{45}Ca and ATP and terminated after 1 s, a period which is not sufficient to generate sufficient concentration of ADP (see following manuscript). Thus, effectively, this amounts to a study of Ca^{2+} uptake by mitochondria in the absence of polyamines and ADP, which, as we showed here, are required for Ca^{2+} transport by brain mitochondria.

It is well documented that neurotransmitter-induced elevation of cytosolic Ca^{2+} depends on calcium release from 'calcium stores' which are associated with the endoplasmic reticulum and on opening of Ca^{2+} channels in the plasma membrane, which are also activated directly by depolarization [1,9,10]. There is no evidence that mitochondria play any role in this process. However, the calcium release is followed quickly by Ca^{2+} sequestration. The system participating in this process have not been fully accounted for [39]. It is, therefore, quite plausible, based on our results and previous results, to assume that mitochondria contribute to Ca^{2+} sequestration from the cytoplasm. Once loaded, mitochondria would release Ca^{2+} when cytosolic levels of Ca^{2+} are lowered below the set point. Therefore, mitochondria could modulate Ca^{2+} oscillations by enhancing Ca^{2+} sequestration during excitation and slowing the relaxation on approach to resting level below their set point. This model predicts oscillations of mitochondria Ca^{2+} which are out of phase with the oscillation of cytosolic Ca^{2+} [39].

Finally, there is increasing evidence that Ca^{2+} transport by mitochondria may be regulated by temporal and spatial oscillations of spermine and other cytoplasmatic factors. It has been shown that in synaptosomes, membrane depolarization activates ornithine decarboxylase and increases the level of polyamines and this is followed by enhanced ^{45}Ca cycling [40]. Polyamines were also reported to inhibit phospholipase C [35] and enhance inositol phospholipids synthesis [36], thus modulating also the activity of the inositol-lipids second messenger system. Combining the latter effects with the effects of polyamines on Ca^{2+} sequestration by mitochondria suggests that polyamines attenuate the rise in cytosolic Ca^{2+} and enhance the rate of relaxation of Ca^{2+} to resting levels. In addition, there is a sharp dependence of the mitochondrial Na^+–Ca^{2+} exchange system on Na^+ concentration, which may oscillate locally in excitation, and may also regulate calcium release by the mitochondria. Excitation increases ATP hydrolysis and may also lead to spatial and temporal changes in P_i, ADP and ATP, which may greatly affect Ca^{2+} transport by mitochondria (see following paper). The fact that mitochondria are highly concentrated at the most actively excitable dendrites [41] suggest that, at these locations, at least, mitochondria play an important role in modulating Ca^{2+} level.

The strongest evidence that appears to be incompatible with the role of mitochondria in modulating cellular

Ca^{2+} cycles, as discussed above, is the finding of very low Ca^{2+} content in brain mitochondria in situ by electron probe microanalysis [8]. However, these studies were conducted on brains of anesthetized rats, which tend to lower both cytosolic and matrix free Ca^{2+} and certainly do not reflect Ca^{2+} content during excitation. Moreover, it is quite possible that the fast freezing techniques used in these measurements was insufficient to trap the Ca^{2+} in the mitochondria. The extremely high turnover of the electrogenic carrier, together with its very low temperature coefficient [42] could result in a delay between the collapse of membrane potential and the total freezing of the system, which may be sufficient to allow all the matrix Ca^{2+} to leak out.

It has been argued that the results of studies on Ca^{2+} regulation of the mitochondrial dehydrogenases are incompatible with a role for mitochondria in the regulation of cellular Ca^{2+} [43]. However, what these studies suggest is that in cells, at rest, the mitochondrial free Ca^{2+} concentration, and presumably the total Ca^{2+} content is very low and thus do not indicate a role for mitochondria in maintaining the level of cytosolic free Ca^{2+}, at rest. But, there is nothing in these studies to suggest that during excitation of nerve cells, mitochondria do not take up substantial amounts of Ca^{2+} and thus modulate, locally, and transiently the cytosolic free Ca^{2+} concentration. In fact, it is the main conclusion of these studies that mitochondria take up Ca^{2+} during excitation. The question is only, how fast and how much. Our study suggests that brain mitochondria, at least, are capable of taking up substantial amounts of Ca^{2+} during this transition. The recent findings of large temporal and spatial oscillation of cytosolic Ca^{2+} [39] strongly indicate that estimates of Ca^{2+} levels in the cytosol and the mitochondria during excitation from measurement with cell suspension, or synaptosomes are underestimated, since the signal is integrated over space and time.

In conclusion, considering the combined effects of spermine and ADP on calcium transport in brain mitochondria, it is very likely that mitochondria participate in regulation of free Ca^{2+} oscillations in brain cells. Studies of the effects of mitochondrial inhibitors on Ca^{2+} oscillations should provide a better understanding of the role of mitochondria in these processes.

Acknowledgements

We thank Dr. William Thayer for critical reading of this manuscript. Supported by PHS grants GM-28173 and AAO-7238.

References

1 Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433.
2 Nicholls, D. and Akerman, K. (1982) Biochim. Biophys. Acta 683, 57–88.
3 Denton, R.M. and McCormack, J.G. (1985) Am. J. Physiol. 249, E543–554.
4 Rottenberg, H. and Scarpa, A. (1974) Biochemistry 13, 4811–4817.
5 Crompton, M. (1985) Curr. Top. Membr. Transp. 25, 231–276.
6 Crompton, M. (1985) in The enzymes of Biological membranes (Martonosi, A.N., ed.), Vol. 3, pp. 249–286, Plenum, New York.
7 Blaustein, M.P. (1988) Trends Neurolog. Sci. 11, 438–443.
8 Somlyo, A.P., Urbanics, R., Vadasz, G., Kovach, A.G.B. and Somlyo, A.V. (1985) Biochem. Biophys. Res. Commun. 132, 1071–1078.
9 Miller, J.R. (1988) Trends Neurolog. Sci. 11, 415–419.
10 Meldolesi, J., Volpe, P. and Pozzan, T. (1988) Trends Neurolog. Sci. 11, 449–452.
11 Nicchitta, C.V. and Williamson, J.R. (1984) J. Biol. Chem. 259, 12978–12983.
12 Lenzen, S., Hicketheir, R. and Panten, U. (1986) J. Biol. Chem. 261, 16478–16483.
13 Jensen, J.R., Lynch, G. and Baudry, M. (1987) J. Neurochem. 48, 765–772.
14 Kroner, H. (1988) Arch. Biochem. Biophys. (1988) 267, 205–210.
15 Nicholls, D.G. and Scott, I.D. (1980) Biochem. J. 186, 833–836.
16 Rottenberg, H. and Marbach, M. (1989) FEBS Lett. 247, 483–489.
17 Lai, J.C.K. and Clark, J.B. (1979) Methods Enzymol. LV, 51–60.
18 Hashimoto, K. and Rottenberg, H. (1986) Biochemistry 25, 1747–1755.
19 Scarpa, A., Brinley, F.J., Tiffert, T. and Dubyak, G.G. (1978) Ann. N.Y. Acad. Sci. 307, 86–112.
20 Ohnishi, S.T. (1978) Ann. N.Y. Acad. Sci. 307, 213–216.
21 Bers, D.M. (1982) Am. J. Physiol. 242, C404–C408.
22 Grynkiewicz, GF., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
23 Toninello, A., Dilisa, F., Siliprandi, D. and Siliprandi, N. (1985) Biochim. Biophys. Acta 815, 399–404.
24 Toninello, A., Miotto, G., Siliprandi, D., Siliprandi, N. and Garlid, K. (1988) J. Biol. Chem. 263, 19407–19411.
25 Toninello, A., Dilisa, F., Siliprandi, D. and Siliprandi, N. (1986) Arch. Biochem. Biophys. 245, 363–368.
26 Tabor, C.W. and Tabor, H. (1984) Annu. Rev. Biochem. (1984) 53, 749–790.
27 Rottenberg, H. (1989) Methods Enzymol. 171, 364–375.
28 Berg, H.C. and Purcell, E.M. (1977) Biophys. J. 20, 193–219.
29 Junge, W. and McLaughlin, S. (1987) Biochim. Biophys. Acta 890, 1–5.
30 Reed, K.C. and Bygrave, F.L. (1974) Biochem. J. 140, 143–155.
31 Lehninger, A.L. (1964) The Mitochondrion, W.A. Benjamin, New York.
32 Schindler, M., Koppel, D.E. and Sheetz, M.P. (1980) Proc. Natl. Acad. Sci. USA 77, 1457–1461.
33 Tadolini, B. (1988) Biochem. J. 249, 33–36.
34 Moruzzi, M.S., Barbiroli, B., Monti, M.G., Tadolini, B., Hakin, G. and Mezzetti, G. (1987) Biochem. J. 247, 175–180.
35 Wojcikiewicz, R.J.H. and Fain, J.N. (1988) Biochem. J. 255, 1015–1021.
36 Smith, C.D. and Synderman, R. (1988) Biochem. J. 256, 125–130.
37 Rasgado-Flores, H. and Blaustein, M.P. (1987) Am. J. Physiol. 252, C588–C598.
38 McCormack, J.G., Browne, H.M. and Dawes, N.J. (1989) Biochim. Biophys. Acta 973, 420–427.
39 Lipscombe, D., Maddison, D.V., Poenie, M., Reuter, H., Tsien, R.W. and Tsien, R.Y. (1988) Neuron 1, 355–365.
40 Iqbal, Z. and Koening, H. (1985) Biochem. Biophys. Res. Commun. 133, 563–573.
41 Wong-Riley, M.T.T. (1989) Trends Neurolog. Sci. 12, 94–101.
42 Akerman, K.E.O. (1977) J. Bioenerg. Biomembranes 9, 141–149.
43 Hansford, R.G. (1985) Rev. Physiol. Biochem. Pharmacol. 102, 1–72.