Biochimica et Biophysica Acta, 1016 (1990) 87–98
Elsevier

BBABIO 43179

Regulation of Ca^{2+} transport in brain mitochondria. II. The mechanism of the adenine nucleotides 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; Adenine nucleotide; Mitochondrion

ADP greatly enhances the rate of Ca^{2+} uptake and retention in Ca^{2+} loaded mitochondria. Atractyloside, a specific inhibitor of the ADP/ATP translocator, completely inhibits the ADP effect, while bongkrekate, another specific inhibitor of the translocator enhances the effect of ADP. These results indicate that locking the ADP/ATP translocator in the M-state is sufficient to produce the ADP effect. Cyclosporin A, a specific inhibitor of the Ca^{2+}-induced membrane permeabilization does not substitute for ADP, indicating that ADP directly affect the rate of electrogenic Ca^{2+} uptake. The effect of the translocator conformation on the rate of electrogenic Ca^{2+} uptake is independent of the concentration of P_i and is not caused by changes in membrane potential. However, locking the carrier in the M-state appears to increase the negative surface charge on the matrix face of the inner membrane. This may lead to an enhanced rate of Ca^{2+} dissociation from the electrogenic carrier at the matrix surface. The rate of Na^+-independent Ca^{2+} efflux is only slightly inhibited by locking the carrier in the M-state, presumably due to the same mechanism. In the presence of ADP, P_i inhibits the Na^+-independent efflux. In the presence of physiological concentrations of spermine, P_i and Mg^{2+}, the rate of Ca^{2+} uptake, Ca^{2+} retention and Ca^{2+} set points depend sharply on ADP concentration at the physiological range of ADP. Thus, changes of cytosolic ADP concentration may lead to change in the rate of Ca^{2+} uptake by mitochondria and thus modulate the excitation–relaxation cycles of cytoplasmic free calcium.

Introduction

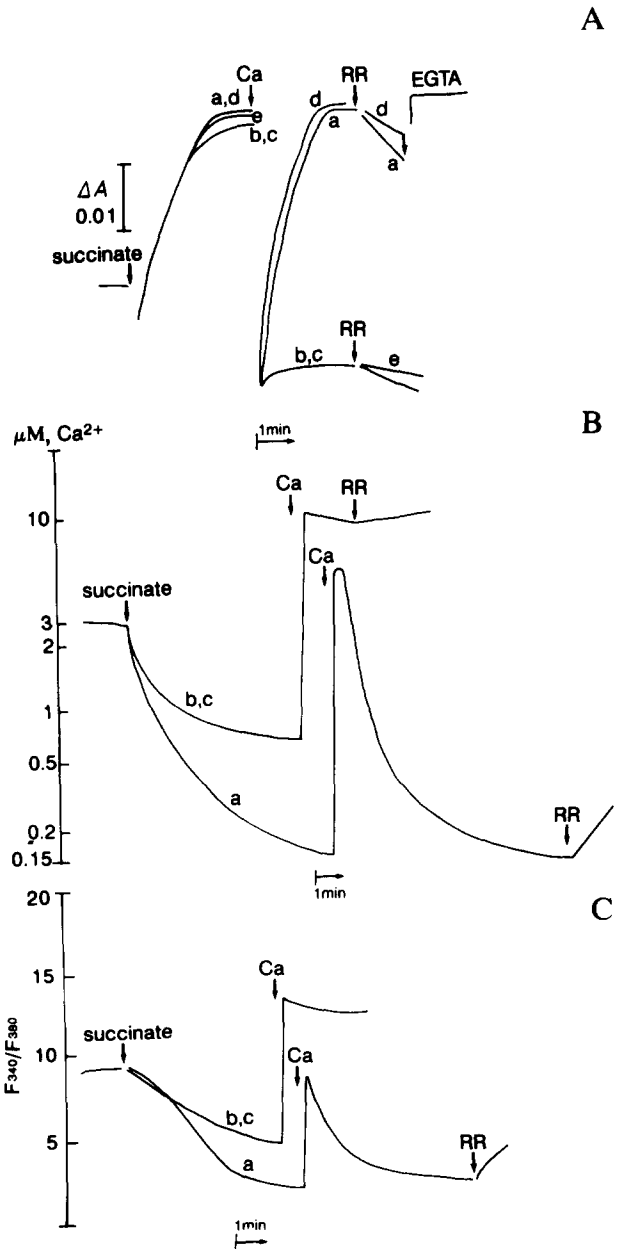
It has been known for a very long time that adenine nucleotides determine the ability of mitochondria to accumulate and retain large amounts of Ca^{2+} (cf. Refs. 1–4). However, the mechanism by which adenine nucleotides exert their effect on Ca^{2+} accumulation and retention is still unknown. It has been suggested by many authors that adenine nucleotides protect the mitochondria from P_i-induced swelling which damages the mitochondrial inner membrane permeability barrier (cf. Refs. 3, 5, 6). Since P_i is usually included in the incubation medium to enhance Ca^{2+} uptake, the requirement for adenine nucleotide was thought to be related to the P_i effect. This model has been expanded in great detail by the group of Crompton (cf. Ref. 7), who suggest that P_i and Ca^{2+} activate a pore which allows ions and metabolites to diffuse across the membrane, thus collapsing the membrane potential and releasing accumulated Ca^{2+}. The activation of the pore is assumed to be suppressed by ADP. It has been demonstrated by Nicholls and Scott [8] that brain mitochondria cannot accumulate large amounts of Ca^{2+} and retain a 'set-point' which is independent of the matrix Ca^{2+} unless ATP and oligomycin are included in the incubation medium. Nicholls and Scott [8] attributed the inability of brain mitochondria to accumulate Ca^{2+} without added oligomycin and ATP to Ca^{2+}-induced loss of adenine nucleotides and to the collapse of the membrane potential [8]. Other investigators attribute the ADP effect to inhibition of P_i efflux, which helps retain the insoluble Ca-P_i complexes [9].

Particularly intriguing is the effect of atractyloside, a specific inhibitor of the mitochondrial ADP/ATP translocator [10]. It has been shown that atractyloside potentiates Ca^{2+} loss from heart mitochondria [11] and brain mitochondria [8]. On the other hand, bongkrekate, another specific inhibitor of the ADP/ATP transloca-

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

tor, was shown to inhibit Ca efflux [12], although others found both stimulation and inhibition of efflux and also inhibition of the rate of uptake [13]. While these facts suggest that binding of ADP by the ADP/ATP translocator is involved in the ADP effect, the mechanism is still unknown.

In the preceding paper [14], we have demonstrated that spermine greatly enhances the ability of brain mitochondria to accumulate large amounts of Ca^{2+} and to maintain low set-points. This effect was totally dependent on the presence of adenine nucleotides. In a preliminary report we have demonstrated that in brain mitochondria, ADP specifically stimulates electrogenic Ca^{2+} uptake. Bongkrekate stimulates, while atractyloside inhibits the effect of ADP on the electrogenic transport. Therefore, we have suggested that locking the ADP/ATP carrier in the M-state conformation leads to the stimulation of electrogenic Ca^{2+} transport [15]. In this paper we elucidate in greater detail the mechanism of the adenine-nucleotide effect on Ca^{2+} transport in brain mitochondria and its possible physiological relevance.



Methods and Materials

The methods for the preparation of mitochondria, the determination of the rate and extent of Ca^{2+} transport, and free Ca^{2+} concentrations, and the determination of protein concentrations are described in the preceding paper [14]. Respiration rates were determined by a polarographic oxygen electrode. The distribution of [^{3}H]TPP$^+$, ^{86}Rb, and the determination of matrix volumes, and membrane potentials are as described previously [16]. All fine chemicals were from Sigma, except for cyclosporin A (Sandoz). All other reagents were of highest analytical grade. Bongkrekate was a generous gift from Dr. A. Halestrap, University of Bristol, and also from Dr. J.A. Duine, Delft University of Technology.

Results

We used three alternative methods for the measurements of Ca^{2+} uptake and efflux. The Ca^{2+} indicator,

Fig. 1. The effect of ADP on Ca^{2+} transport in brain mitochondria. The basic medium was composed of 0.2 M mannitol, 0.08 M sucrose, 10 mM Tris-HCl, 5 mM Tris-P_i, 1 mM $MgCl_2$ (pH 7.4). 0.5 mg mitochondrial protein was added to 3 ml medium and then 2 μM rotenone and 2 μg/mg protein of oligomycin were added as well. When added (traces a, c) 10 μM ADP was added before the addition of 5 mM Tris-succinate. The latter addition induced the uptake of Ca^{2+} which was present in the incubation medium. When steady state was reached, 30 nmol of $CaCl_2$ were added. After the added Ca^{2+} was taken up by the mitochondria, Ruthenium red (60 pmol/mg protein) was added to induce Ca^{2+} efflux. Trace a shows the experiment in the presence of 10 μM ADP. Trace b is without ADP and trace c is with 10 μM ADP and 8 nmol/mg protein of atractyloside. In trace d, 10 μM ADP and 1 nmol/mg protein cyclosporin A was added. In Trace e, 1 nmol/mg protein cyclosporin A was added. In (A), Ca^{2+} transport was followed by the absorbance difference of Arsenazo III. 50 μM Arsenazo III was added to the medium and the absorbance difference (685–675 nm) was recorded. The measured rates were calibrated by the addition of Ca^{2+} and the steady-state levels of free Ca^{2+} were obtained from the difference in the absorption between the steady state and the system with excess EGTA, using a calibration procedure described in the preceding paper [14]. In (B), Ca^{2+} was followed with a Ca^{2+} electrode. Free Ca^{2+} concentration was reduced to 3 μM by the addition of small amounts of nitrilotriacetate prior to the addition of succinate. In (C) Ca^{2+} uptake was followed by the fluorescent Ca^{2+} indicator fura-2, using the fluorescence ratio method as described in Material and Methods. Small amounts of nitrilotriacetate were added to lower the Ca^{2+} concentration to 3 μM before the addition of succinate.

Arsenazo III, was used routinely to measure rates and extent of transport and occasionally steady-state free Ca^{2+} concentrations. Ca^{2+} electrode was mostly used to measure steady state concentrations below 0.5 μM and Fura-2 was used occasionally for the same measurements. Fig. 1 shows the effect of ADP on Ca^{2+} transport in brain mitochondria and its reversal by atractyloside, a specific inhibitor of ADP/ATP translocator. In most of the experiments described below, we follow the protocol demonstrated in Fig. 1. First, mitochondria were incubated at room temperature in the presence of rotenone and oligomycin (with or without ADP) for 6 min. Then succinate was added and the suspension Ca^{2+} was accumulated. After steady state was established, a known amount of $CaCl_2$ was added and allowed to accumulate. When steady state was established again, Ruthenium Red (RR), a specific inhibitor of electrogenic Ca^{2+} uptake, was added to initiate efflux. Finally, excess EGTA was added to bind all free Ca^{2+} and allow determination of free Ca^{2+} concentration. As Fig. 1 shows, in the presence of ADP (trace a) succinate addition caused rapid accumulation of Ca^{2+}, and lowered the external Ca^{2+} below 0.2 μM; additional Ca^{2+} was taken up quickly and a new steady state was established at approximately the same external Ca^{2+}. When ADP was omitted (trace b), or when atractyloside was added in addition to ADP (trace c), the initial uptake was slower, the steady-state was shifted to higher external Ca^{2+} concentrations and the additional Ca^{2+} was not accumulated, bringing the steady-state to a very high value. This pattern was evident in experiments with Arsenazo III (Fig. 1A), Ca^{2+} electrode (Fig. 1B) and fura-2 (Fig. 1C). The rates and free calcium concentrations obtained by these methods are comparable and therefore we used these three methods interchangeably.

It was recently discovered [17,18] that cyclosporin A is a potent and specific inhibitor of the Ca^{2+}-induced permeabilization ('pore') of the inner membrane. To find whether the ADP effect can be attributed to inhibition of the latter process, we measured Ca^{2+} transport in the presence of cyclosporin A, with ADP (trace d) and without ADP (trace e). It is observed that cyclosporin alone did not significantly affect the rate or extent of Ca^{2+} uptake, but inhibited the efflux. When added with ADP, cyclosporin A slightly enhanced the ADP effect. These results clearly indicate that ADP enhances Ca^{2+} transport directly by stimulating the electrogenic uptake.

As Fig. 1 shows, atractyloside, a specific inhibitor of the ADP/ATP translocator, completely inhibits the ADP effect on Ca^{2+} transport. To find out whether this inhibition is due to its specific effect on the ADP/ATP translocator, we studied the concentration dependence of the atractyloside effect on Ca^{2+} transport in comparison to its inhibition of ADP-ATP exchange. The

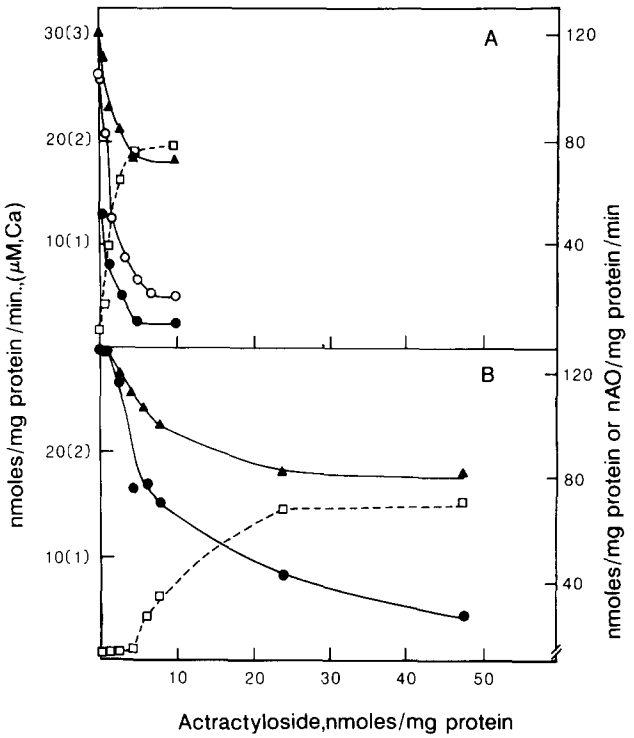


Fig. 2. The effect of atractyloside on Ca^{2+} transport and Respiration. Conditions for Ca^{2+} transport experiments are the same as in Fig. 1A. Panel A shows the effect of atractyloside in the presence of 10 μM ADP. The figure shows the rate of Ca^{2+} transport after the addition of 30 nmol Ca^{2+} (left-hand scale ●). The total extent of Ca^{2+} uptake (right-hand scale, ▲), the steady-state Ca^{2+} external concentration (left-hand scale in brackets, □), and the rate of State 3 respiration (○) in a medium composed of 0.2 M sucrose, 100 mM KCl, 5 mM Hepes, 5 mM Na_2HPO_4, 5 mM $MgCl_2$, 5 mM succinate and 10 μM ADP. Panel B shows the effect of atractyloside in the presence of 100 μM ADP. All other conditions are the same as in panel A.

later process was assayed by the inhibition of the stimulation by ADP of he rate of respiration (State 3). As Fig. 2A shows, 3 nmol/mg protein of atractyloside caused 50% inhibition of stimulation of the respiration by 10 μM ADP, while 10 nmol/mg protein of atractyloside completely inhibited the stimulation of respiration by ADP, indicating complete inhibition of ADP-ATP exchange. At the same concentration range, atractyloside inhibited the rate of Ca^{2+} uptake (after addition of 30 nmol Ca^{2+}, as in Fig. 1A) from 13 to 3 nmol/mg protein per min; inhibited the extent of uptake from 120 to 75 nmol/mg protein per min and raised the steady-state Ca^{2+} from 0.2 to 2.0 μM.

Since it is well known that atractyloside is a competitive inhibitor which competes with ADP for the cytosolic binding site of the translocator [10], it is expected that a higher concentration of atractyloside would be required to inhibit the ADP effect at higher concentration of ADP. Fig. 2B shows that this is indeed the case. Raising the ADP concentration to 100 μM shifted the inhibition titration curves with atractyloside to a higher

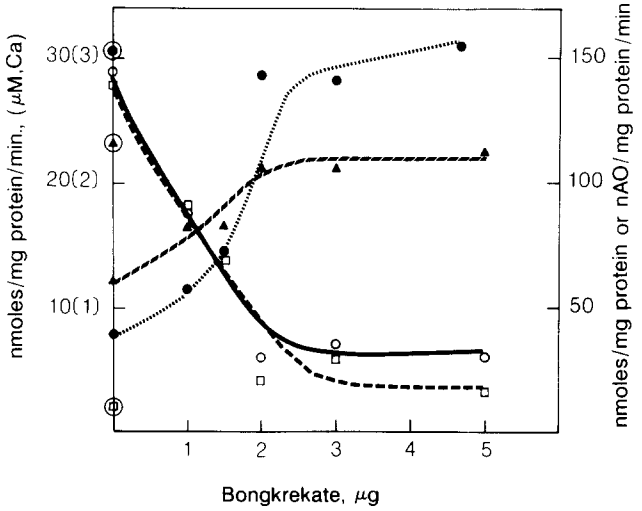


Fig. 3. The effect of bongkrekate on Ca^{2+} transport and respiration. Conditions for Ca^{2+} transport experiments are as in Fig. 1A, without added ADP. The figure shows the rate of Ca^{2+} uptake (left, ●) after the addition of 30 nmol of Ca^{2+}, the total amount of accumulated Ca^{2+} (right scale, ▲), the steady-state concentration of external Ca^{2+} (left-hand scale in brackets, □) and the rate of State 3 respiration (right scale, ○). Medium for respiration experiments is the same as in Fig. 2. For comparison, values, obtained with ADP (10 μM) without bongkrekate, are shown in brackets on the ordinate.

concentration range. Thus, the results presented in Fig. 2 clearly demonstrate that the effect of ADP on Ca^{2+} transport requires binding of ADP to the translocator. However, it is not clear whether the binding itself stimulated Ca^{2+} uptake or whether the exchange of ADP for ATP is the important factor, since atractyloside inhibits both.

To distinguish between these two possibilities we employed another specific inhibitor of the translocator, bongkrekate. Bongkrekate binds to a site on the matrix face of the translocator but also inhibits the exchange [10]. If the stimulation of Ca^{2+} transport by ADP is due to the ADP–ATP exchange, we expected bongkrekate to inhibit the ADP effect in parallel to its inhibition of the exchange. The addition of bongkrekate to a system supplemented with ADP inhibited the ADP–ATP exchange, as measured by the ADP effect on respiration, but, in contrast to atractyloside, it enhanced the stimulatory effect of ADP on Ca^{2+} transport. Moreover, in the absence of added ADP, bongkrekate stimulates Ca^{2+} transport in a manner completely analogous to ADP. This is shown in Fig. 3. Bongkrekate caused 50% inhibition of the stimulation of respiration by ADP at 1 μg and completely inhibited the stimulation of respiration at 2 μg. At the same concentration range, bongkrekate stimulated the rate of Ca^{2+} uptake, increased the extent of Ca^{2+} uptake and decreased the Ca^{2+} steady-state levels to the values which are the same as those obtained by the addition of ADP (circled points on the ordinate).

These surprising results suggest that the stimulation of Ca^{2+} transport is due to locking the ADP/ATP translocator in a particular conformation state – the M-state. It is known that the ADP/ATP translocator exists in either one of two conformation states, the M-state, in which the nucleotide binding site is accessible from the matrix, and the C-state, in which the site is accessible from the cytoplasm. Atractyloside locks the carrier in the C-state, while ADP or ADP and bongkrekate lock the carrier in the M-state [10]. Thus, the results clearly indicate that simply locking the carrier in the M-state, regardless of how this is accomplished, is sufficient for the stimulating effect on Ca^{2+} transport.

To further establish the mechanism of the effect, we tested a large number of nucleotides for their effect on Ca^{2+} transport (e.g., AMP, ATP, cAMP, GTP, GDP, GDP, GMP, cGMP, ITP, IDP, adenosine). Except for ATP, none of these were active. When preincubated with ATP, ATP was almost as effective as ADP. However, since the mitochondrial preparation has considerable adenylate kinase and ATPase activities, it is not clear whether the effect is due to ATP or the generation of ADP during incubation. To test this we examined the direct effect of added ADP and ATP on Ca^{2+} transport. This is shown in Fig. 4, in which mitochondria were preincubated without ADP, allowed to reach steady state after addition of Ca^{2+}, and then supplemented with either ADP or ATP. As observed, the effect of ADP was immediate, while the effect of ATP was delayed. This experiment suggests that the ATP effect is due largely to the production of ADP in the suspension. This agrees with the fact that ADP is more effective in locking the carrier in its M-state. Indeed, when mem-

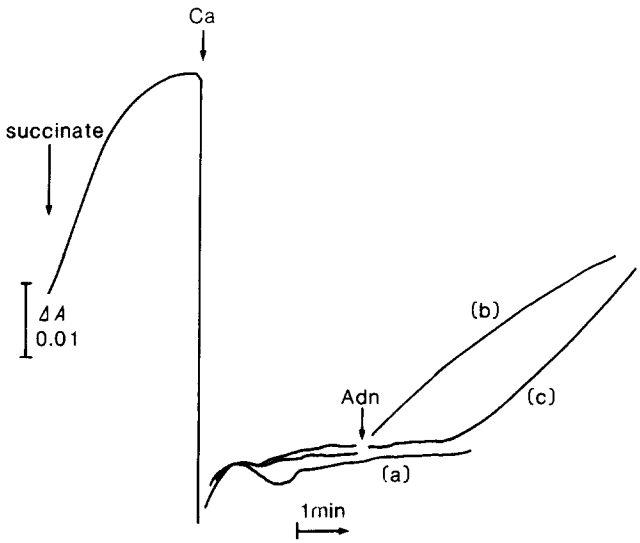


Fig. 4. The effect of added ADP and ATP on Ca^{2+} uptake. Conditions are the same as in Fig. 1A, trace b, except that 20 μM of nucleotides were added where indicated. Trace a – none; trace b – ADP; trace c – ATP.

brane potential is high there is a preferential affinity for ADP by the ADP/ATP translocator [10].

Fig. 5 shows the dependence of the rate of Ca^{2+} transport on ADP concentration. In low salt medium the effect is saturated at very low concentration, which is compatible with the high binding affinity of ADP to the translocator. Addition of 100 mM KCl (or other salts, not shown), appears to reduce the affinity for ADP. Since the mitochondrial surface potential is negative [19], screening of the surface potential by high salt should actually enhance the binding of the negatively charged ADP. However, the binding site itself is most probably positively charged and the affinity must depend strongly on electrostatic interaction [20]. Hence, it is the screening of the localized positive charges at the binding site which appears to reduce the affinity of ADP.

Under physiological conditions, mitochondria are exposed to relatively high concentration of free Mg^{2+}. This fact is of major importance in evaluating the competence of mitochondria to sequester Ca^{2+} in vivo. Fig. 6 shows the effect of Mg^{2+} on Ca^{2+} transport in brain mitochondria. Surprisingly, unlike liver and heart mitochondria [21], Mg^{2+} did not inhibit Ca^{2+} transport in brain mitochondria at the physiological range (up to 1 mM). In fact, in the absence of ADP, Mg^{2+} actually stimulated Ca^{2+} uptake at low concentration. This is partially due to inhibition of Ca^{2+} efflux as shown in the figure. In the presence of ADP, Mg^{2+} did not inhibit Ca^{2+} transport up to a concentration of 1 mM. Higher concentrations of Mg^{2+} did inhibit Ca^{2+} transport. This effect is probably due to a combination of factors: (a) screening of the negative surface potential which decreases the apparent K_m for Ca^{2+} [19]; (b) decreasing the free concentration of ADP; (c) decreasing the affinity of ADP (Fig. 5). However, this effect is of little physiological significance since cellular free Mg^{2+} is well below the range of this effect and physiological ADP concentrations are higher than those of Fig. 6 (see below).

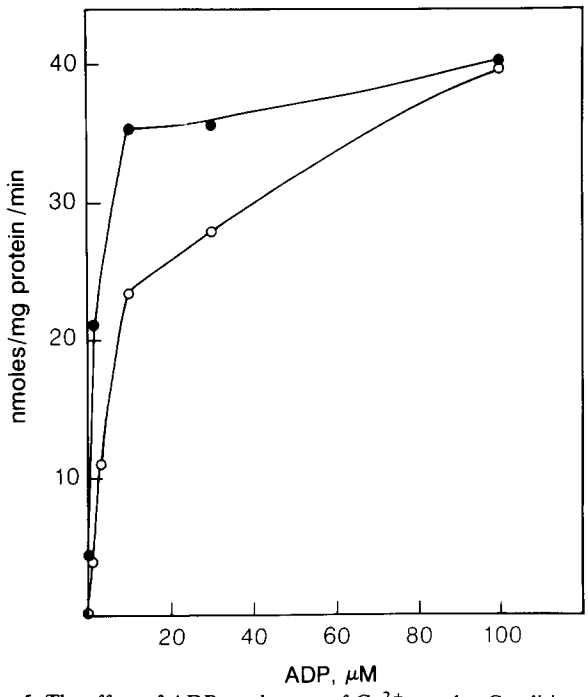


Fig. 5. The effect of ADP on the rate of Ca^{2+} uptake. Conditions are as in Fig. 1A, except for the absence of $MgCl_2$, the indicated ADP concentrations and the presence of 100 mM KCl in the experiments of curve b (○). The rate shown is that obtained after the addition of 30 nmol of Ca^{2+}.

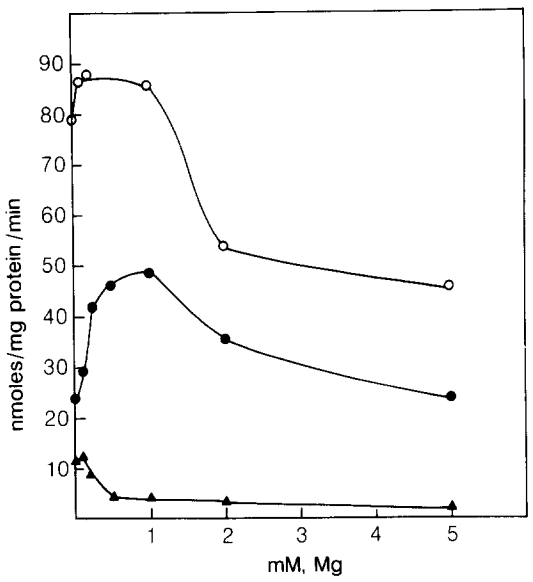


Fig. 6. The effect of $MgCl_2$ on Ca^{2+} transport. Conditions were the same as in Fig. 1A. The rates of Ca^{2+} transport shown are those obtained after the addition of 30 nmol of Ca^{2+}. The rates shown are of uptake in the presence of 100 μM ADP (○) and in the absence of ADP (●), and of Ruthenium red (60 pmol/mg protein) induced efflux in the presence of ADP (△).

Fig. 7 shows the dependence of Ca^{2+} transport on external free Ca^{2+} in the presence and absence of ADP. Panel A shows the effect of ADP on net uptake. In the absence of ADP, net uptake of added Ca^{2+} was limited to about 30 nmol/mg protein and did not increase, regardless of the external Ca^{2+} concentration (total net accumulation was limited to about 100 nmol/mg protein, see below). In the presence of ADP, net uptake increased linearly with the amount of added Ca^{2+} (and initial Ca^{2+} concentration) and the mitochondria could accumulate very large amounts of Ca^{2+}.

Panel B shows the effect of ADP on the rate of uptake of added Ca^{2+}. Without ADP the rate was saturated at about 2 μM Ca^{2+}, and reached a maximum of less than 20 nmol/mg protein per min. However, in the presence of ADP the rate continued to increase with increasing Ca^{2+} concentration, with apparent K_m of about 3 μM. As seen clearly from both Figs. 7A and Fig. 7B, the effect of ADP increased with the Ca^{2+} load

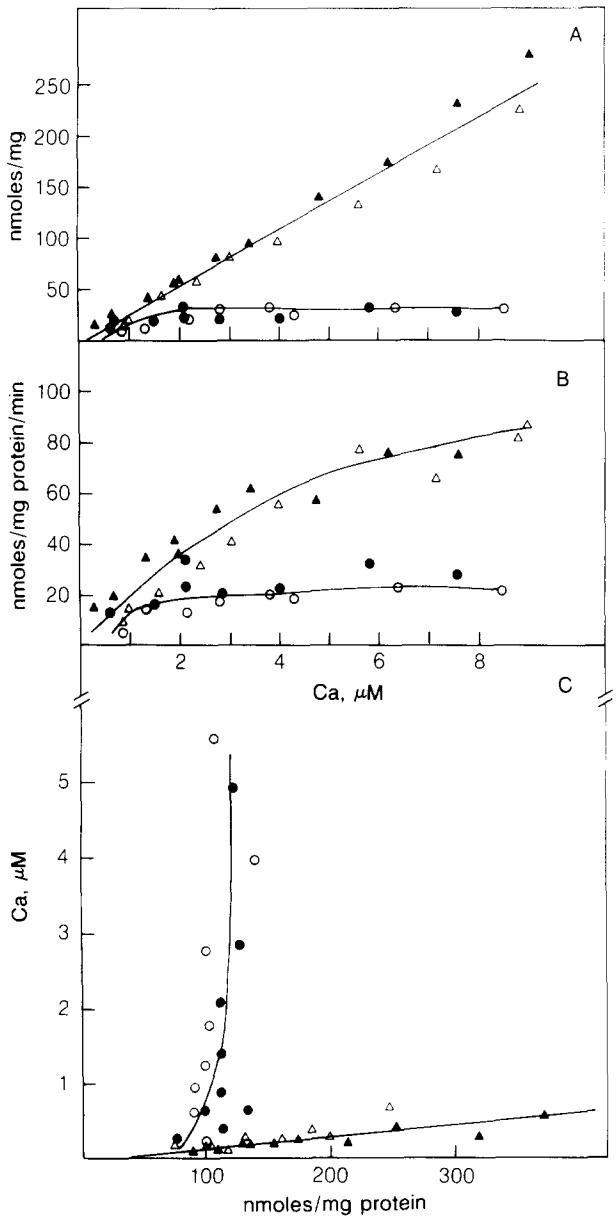


Fig. 7. The effect of external free Ca^{2+} concentration on Ca^{2+} transport. Conditions are the same as in Fig. 1A, except for the amount of added Ca^{2+} which was varied (5 nmol to 150 nmol). Panel A (top) show the effect of external Ca^{2+} on the extent of uptake in the absence of ADP (○, ●) and in the presence of 20 μM ADP (△, ▲). Panel B (middle) show the effect of external Ca^{2+} on the rate of Ca^{2+} transport in the absence of ADP (○, ●) and in the presence of 20 μM ADP (△, ▲). Panel C (bottom) shows the relationship between total Ca^{2+} load and steady concentration of free Ca^{2+} in absence of ADP (○, ●) and in the presence of 20 μM ADP (▲, △). The results shown are from two different mitochondrial batch preparations (empty and full symbols).

of the mitochondria. This is also clear from Fig. 1, since the effect of ADP on the initial Ca^{2+} uptake (after succinate) was not as strong as on the uptake of additional Ca^{2+}. Hence, the ADP effect appears to depend on matrix Ca^{2+} and must therefore be exerted on the matrix side of the inner membrane.

We have previously shown that the ADP effect, under these conditions, is mostly due to enhancement of electrogenic uptake and not inhibition of efflux [15]. These conclusions are supported further by the fact that in the presence of cyclosporin A, which inhibit the Ca^{2+}-induced permeabilization, ADP greatly enhance the rate of uptake (Fig. 1A). The rate-limiting step for electrogenic Ca^{2+} uptake on the matrix surface, which involves matrix Ca^{2+}, is most probably the dissociation of Ca^{2+} from the loaded carrier. Hence, it is possible that locking the ADP/ATP translocator in the M-state enhances Ca^{2+} dissociation from the electrogenic Ca^{2+} carrier on the matrix surface (see Discussion).

Fig. 7C shows the effect of ADP on the relationships between Ca^{2+} matrix loading and external Ca^{2+} at steady state. In the absence of ADP mitochondria did not accumulate more than 100 nmol Ca^{2+} per mg protein. Any further addition of Ca^{2+} quickly raised the external concentration and the steady-state level. Therefore, only unloaded mitochondria lowered external Ca^{2+} concentration, in the absence of ADP, and once loaded, mitochondria were completely ineffective in lowering external Ca^{2+}. ADP was found to greatly enhance the capacity of the mitochondria to accumulate Ca^{2+} while keeping a low set-point. Even though the set point is not totally independent of load and increased gradually with Ca^{2+} loading, the set-point was still kept within the physiological range up to very high loading. As we have shown in the preceding paper (Ref. 14, Fig. 4), spermine further enhances the ability of mitochondria to accumulate Ca^{2+} and maintain a truly constant set-point, essentially buffering external Ca^{2+} at 0.4 μM.

Another important metabolite which greatly affects the Ca^{2+} transport systems of the mitochondria is P_i. Fig. 8 shows the dependence of Ca^{2+} transport on the concentration of P_i with and without ADP. In the absence of ADP low concentrations of P_i greatly enhanced the rate and extent of Ca^{2+} uptake and reduced the Ca^{2+} steady-state Ca^{2+} level. However, increasing the concentration of P_i above 0.2 mM inhibited the rate and extent of Ca^{2+} transport and increased the steady-state Ca^{2+} level. The stimulatory effect of low P_i concentration is well known and results from the formation, in the mitochondrial matrix, of Ca-P_i complexes (hydroxyapatite) which lower the matrix Ca^{2+} concentrations [22]. The inhibitory effect of high phosphate, although well known, is not really understood and is usually attributed to stimulation of P_i and Ca^{2+} efflux [9] or a loss of the permeability barrier [7]. However, as Fig. 8 shows, there is no increased rate of efflux as a function of P_i concentration, at least under the conditions of these experiments. High concentrations of P_i are known to lower the matrix pH and this should inhibit the Ca^{2+}–H^+ exchange. On the other hand, P_i stimulate the Ca^{2+}-induced permeabilization which contribute to the efflux (Fig. 1A). The combina-

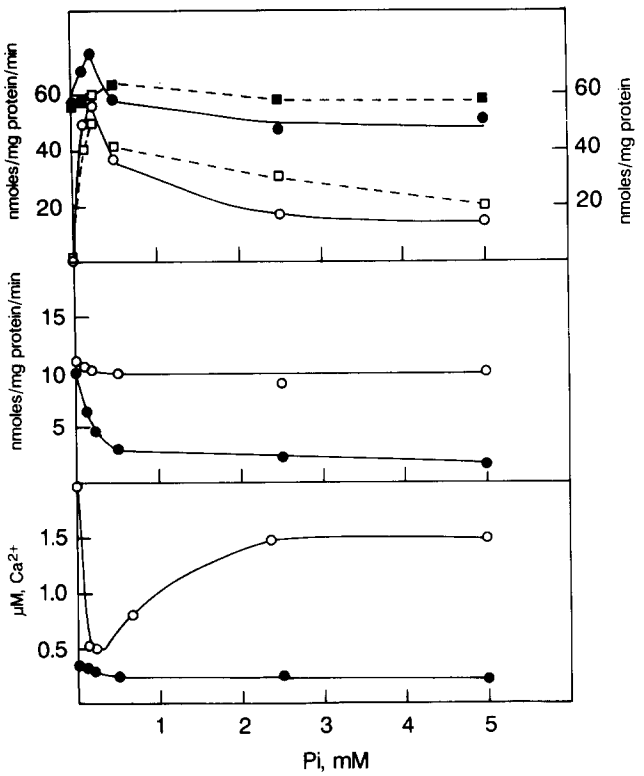


Fig. 8. The effect of P_i on Ca^{2+} transport. Medium and experimental conditions are as in Fig. 1A, except for the concentration of Tris-P_i that was varied as indicated, and ADP concentration which was 20 μM when present. Top panel shows the rate (left scale) and extent (right scale) of uptake after addition of 30 nmol Ca^{2+} (as in Fig. 1A). Rate in the absence of ADP (○); rate in the presence of ADP (●). Extent in the absence of ADP (□), extent in the presence of ADP (■). Middle panel shows the rate of the Na^+-independent efflux (after addition of RR) in the absence (○) and presence (●) of ADP. Bottom panel shows steady-state Ca^{2+} level (set points) in the absence (○) and presence (●) of ADP.

tions of these effects could produce the observed effects of high concentrations of P_i (see Discussion).

Fig. 8 shows that in the presence of ADP, the rates, extents and steady-state levels of Ca^{2+} transport become independent of P_i concentration. In the presence of 0.2 mM P_i, there is relatively small stimulation by ADP. ADP stimulate the uptake, either in the absence of P_i or in the presence of high P_i. The inhibition of the electrogenic uptake by high P_i concentrations is highly correlated with the lowering of the matrix pH. From the distribution of [^{14}C]DMO we estimated the matrix pH at 0.2 mM P_i under the conditions of Fig. 8 as 8.25. Increasing P_i progressively decreases the matrix pH to a value as low as 7.45 at 5 mM P_i (cf. Ref. 23). We have previously shown that the negative surface charge of the liver mitochondrial inner membrane decrease sharply with pH between pH 8 and 7 [24]. Preliminary measurements with brain mitochondria indicate similar patterns (results not shown). Thus it is possible that negative surface charge enhance the rate of dissociation of Ca^{2+} from the electrogenic carrier. Hence, the stimulatory effect of the ADP/ATP translocator conformation on Ca^{2+} uptake could be due to increased (negative) surface charge.

Table I shows a summary of experiments in which we tested the effect of the conformation of the adenine nucleotides carrier on the trans-membrane potential and the surface charge of the matrix face of the inner mitochondrial membrane. Membrane potentials were estimated from the distribution of ^{86}Rb in the presence of valinomycin [16]. We also included [^{3}H]TPP$^+$ in the incubation medium and measured its distribution simultaneously with the Rb^+ distribution. As we have shown previously, the excess binding of TPP^+, when compared to Rb^+, is a measure of the binding of TPP^+ to the matrix face of the inner membrane [25]. The apparent partition coefficient, K_i, obtained from such measurements, is a function of the surface potential, i.e.,

$$K_i = K_i^\circ e^{-F\psi_s/RT} \quad (1)$$

where K_i° is the true partition coefficient, ψ_s the surface potential, F the Faraday constant, R the gas constant and T the absolute temperature. When there is a change in the surface charge the surface potential would change as well, and we can obtain the difference in surface potentials form the difference in the apparent partition coefficients as follows:

$$K_{ia}/K_{ib} = (K_i^\circ e^{-F\psi_s/RT})/(K_i^\circ e^{-F\psi_s/RT}) \quad (2)$$

$$K_{ia}/K_{ib} = e^{-F\Delta\psi_s/RT} \quad (3)$$

$$\Delta\psi_s = (RT/F)\ln(K_{ia}/K_{ib}) = 59\log(K_{ia}/K_{ib}) \quad (4)$$

Table IA shows the results of such measurements with liver mitochondria. The first column shows membrane potential measurements (with ^{86}Rb) in a system (a) without ADP, (b) with ADP (20 μM) and (c) with ADP and atractyloside. There were no significant differences in the measured membrane potential under these conditions. The second column shows R_c, the mitochondria/medium distribution ratio of [^{3}H]TPP$^+$ measured simultaneously with the ^{86}Rb distribution. Assuming that the excess accumulation of [^{3}H]TPP$^+$ is due to binding to the internal inner membrane surface, we can calculate the apparent partition coefficient, K_i, under each of these conditions [25]. It is apparent that there are highly significant differences in the K_i values which are obtained for each of the three systems. ADP increases K_i compared to control, whereas the addition of ADP and atractyloside decreases the K_i. These differences allow us to estimate the difference in surface potential, using Eqn. 4. The results are shown in the last column. ADP increases the negative surface charge by 1.8 mV (compared to control), while atractyloside decreases the surface charge by 3.8 mV (compared to

TABLE I

Effect of adenine nucleotide carrier conformation on the transmembrane potentials and surface potentials

In A, medium was: 0.2 M sucrose, 5 mM Tris-HCl, 5 mM Tris-P_i, 5 mM $MgCl_2$ (pH 7.5). 2.5 mg mitochondrial protein/ml were preincubated with rotenone (2 μM), oligomycin (2 mg/mg protein), valinomycin 0.1 μM, ^{86}Rb (0.1 μCi/ml) and [^{3}H]TPP$^+$ (0.2 μCi/ml), and the indicated addition. Reaction started by addition of 5 mM succinate. Measurement of ion distribution and calculation are as in Ref. 25. In B, medium was: 0.22 mannitol, 0.08 M sucrose, 10 mM Tris-HCl, 1 mM Tris-P_i (pH 7.4). Other additions and procedures as in A, and as indicated in the table. The results are averages and standard deviations of four separate experiments. Statistical significance estimated by the two-tailed Student's *t*-test.

System	Rb^+ ($\Delta\psi$, mV)	TPP^+ (R_c)	K_i (μl/mg)	$\Delta\psi_s$ (mV)
A: Liver mitochondria				
(a) Control	170 ± 1	4.87 ± 0.18	5.4 ± 0.1	
				−1.8 (b − a)
(b) + ADP (20 μM)	168 ± 3	4.79 ± 0.33	5.8 ± 0.2 *	
				+3.8 (c − b)
(c) + ADP (20 μM) + atractyloside (25 nmol/mg)	167 ± 2	4.06 ± 0.14	5.0 ± 0.2 **	
B: Brain mitochondria				
(a) Control	134 ± 4	2.33 ± 0.16	11.5 ± 0.8	
(b)a + ADP (20 μM) + atractyloside (25 nmol/mg) + $CaCl_2$ (10 μM)	115 ± 3	1.07 ± 0.13	11.0 ± 1.0	
				−7.1 (c − b)
(c) + ADP (20 μM) + bongkrekate (4 μg/mg) + $CaCl_2$ (10 μM)	122 ± 6	1.81 ± 0.36	14.5 ± 0.4 ***	
				−5.9 (c − a)

* Significantly different from control ($P < 0.02$).
** Significantly different from (b) ($P < 0.01$).
*** Significantly different from (b) and (a) ($P < 0.01$).

ADP). While these numbers are small, the statistical analysis shows that they are highly significant. Considering the very high salt concentration in the matrix, most of the surface charge is screened and ψs is relatively small, probably not more than 2–3 mV [19]. Thus, changes in surface potentials of a few millivolts, under these conditions, indicate very large changes in surface charge density.

Table IB shows similar experiments with brain mitochondria. Here we use the same medium and incubation conditions as in our Ca^{2+} transport experiments, including the addition of 10 μM $CaCl_2$, but limiting the phosphate concentration to 1 mM. We compare here a control system without added Ca^{2+} (a) with a system containing Ca^{2+}, ADP and atractyloside (b) and one that contains Ca^{2+} and bongkrekate (c). There is a significant difference in $\Delta\psi$ between the three systems. This is most probably due to the fact that Ca^{2+} efflux rate is high in the presence of atractyloside and Ca^{2+} (Fig. 2), lower in the presence of bongkrekate (Fig. 3) and negligible in the absence of added Ca^{2+}. Thus, at steady state, Ca^{2+} cycling rate appears to determine the magnitude of $\Delta\psi$. The second column shows the value of R_c calculated from the distribution of TPP^+. The apparent partition coefficient of TPP^+ between the matrix and the inner membrane surface is calculated from the values of $\Delta\psi$ and R_c and shown in the third column. The difference between the control and the system with atractyloside does not appear to be significant. However, the difference between bongkrekate and atractyloside and also control and bongkrekate is very large and appear to be highly significant. The calculated difference in surface potential by Eqn. 4 between bongkrekate and atractyloside systems is −7.1 mV, and between bongkrekate and control is −5.9 mV.

The results of these experiments indicate that (a) there is no direct effect of the carrier conformation on the membrane potential. In the presence of Ca^{2+} there is a small difference in $\Delta\psi$, which is most probably the result of stimulation of Ca^{2+} cycling when the ADP/ATP translocator is in the C-state. (b) There is a very significant effect of the carrier conformation on the apparent surface potential of the matrix face of the inner membrane. Locking the carrier in the M-state appears to greatly increase the negative surface charge density (see Discussion).

To ascertain the physiological significance of our findings, we examined in detail the effect of ADP on

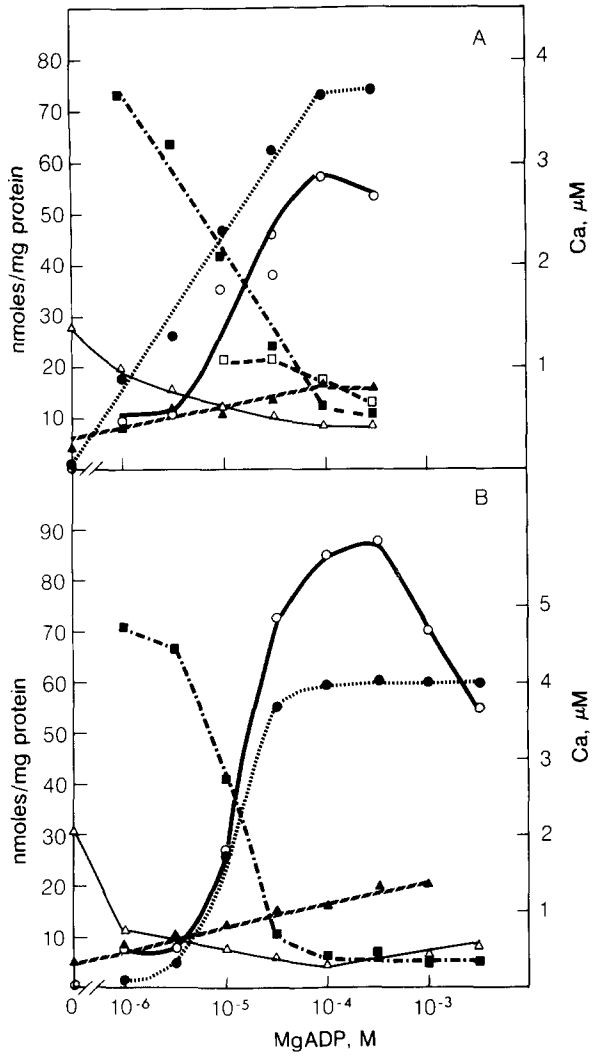


Fig. 9. The effect of MgADP on Ca^{2+} transport in a medium containing physiological concentration of cations. Medium was 0.22 M mannitol, 0.09 sucrose, 10 mM Tris-HCl, 0.5 mM $MgCl_2$, 10 mM NaCl, 100 mM KCl, 5 mM Tris-phosphate (pH 7.4). The protocol is the same as in Fig. 1A; 50 μM Arsenazo is added to the medium, then 0.5 mg mitochondrial protein added to 3 ml medium, rotenone (2 μM), oligomycin (2 mg/mg protein) and the indicated amount of MgADP are added. The reaction was started by the addition of 5 mM Tris-succinate. The Ca^{2+} present in the medium was accumulated to the extent indicated (▲), while the free external Ca^{2+} concentration reached the indicated level (△). Then 30 nmol Ca^{2+} were added and were taken up by the mitochondria at the indicated rate (○) until a new steady state was reached with the indicated net accumulation (●) and free external Ca (■). Finally, Ruthenium red was added (60 pmoles/mg p) to induce Ca^{2+} efflux (□). Panel A (top) is without spermine and panel B (bottom) is with 0.5 mM spermine.

various aspects of Ca^{2+} transport in a medium which stimulates the cation composition of the cytoplasm. Fig. 9 shows the dependence of Ca^{2+} transport parameters on the MgADP concentration in such a medium. To show more clearly the effect of spermine, we show the result without (Fig. 9A) and with (Fig. 9B) spermine. Fig. 9A shows that increasing MgADP over the range 10^{-6} to 10^{-4} M caused a gradual increase in the extent of Ca^{2+} uptake, a small increase in the rate of Ca^{2+} uptake at low Ca^{2+} load and a very large increase in the rate of uptake at high Ca^{2+} load. There was also a modest lowering of the Ca^{2+} steady-state level at low Ca^{2+} load and very dramatic lowering of Ca^{2+} steady-state levels at high Ca^{2+} load. The figure also shows that at high ADP concentration there was inhibition of Ca^{2+} efflux. The effect of ADP in the presence of spermine is shown in Fig. 9B. First, we note again that without ADP spermine had little effect on Ca^{2+} transport parameters in brain mitochondria (see preceding paper [14]). However, increasing the ADP concentration caused a synergistic enhancement of Ca^{2+} uptake rate and synergistic lowering of the steady-state level of free Ca^{2+}. Not only did spermine enhanced the ADP effect, it sharpened the concentration dependence of the MgADP. The effect was seen to center around 10^{-5} M ADP with a very steep slope, such that a relatively small change in ADP concentration had a very large effect on Ca^{2+} transport parameters. Since this is the physiological range of ADP concentration in the cytoplasm [26], it suggests that small modulation of the ADP concentration can cause a large modulation of Ca^{2+} transport parameters.

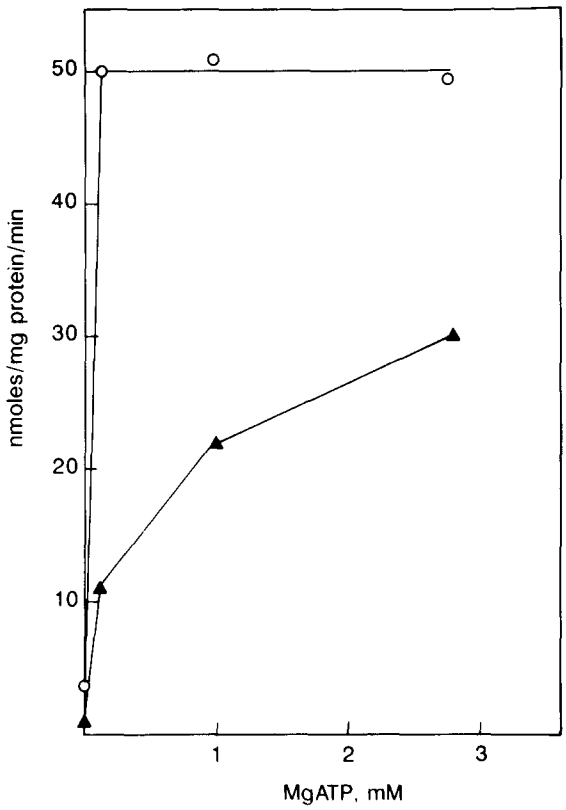


Fig. 10. The effect of MgATP and creatine kinase on the rate of Ca^{2+} uptake. Medium: 0.22 M mannitol, 0.08 M sucrose, 10 mM Tris-HCl, 5 mM Tris-P_i, 0.5 mM MgCl, 100 mM KCl, 10 mM NaCl, 10 mM creatine phosphate, and 5 mM succinate (pH 7.0). Mitochondria content was 0.17 mg protein/ml and Arsenazo was 50 μM. Ca^{2+} uptake rate was measured after the addition of 60 nmol of Ca^{2+} per mg protein. ○, Without creatine kinase (▲) with 10 μg creatine kinase.

However, in the cytoplasm the concentration of MgATP is in the mM range. Although we showed that in the micromolar range, ADP, but not ATP, stimulates Ca^{2+} uptake (see Fig. 4), it is possible that when MgATP is in the mM range it too can stimulate Ca^{2+} uptake. To test this possibility, we studied the effect of mM concentration of MgATP on Ca^{2+} transport in a medium that will maintain a micromolar concentration of ADP in the presence of millimolar concentration of ATP, namely, in the presence of creatine-kinase and creatine-phosphate.

This is shown in Fig. 10. In a medium similar to that of Fig. 9, in the presence of 10 mM creatine phosphate but without creatine kinase, MgATP was almost as effective as MgADP in stimulating Ca^{2+} uptake and the maximal rate of Ca^{2+} uptake was obtained at 100 μM MgATP. However, this could be due largely to conversion of ATP to ADP. When excess creatine kinase was added, creatine phosphate phosphorylated the liberated ADP, maintaining a very low concentration of ADP, and Ca^{2+} transport was greatly inhibited. The inhibition was gradually relieved by increasing concentrations of MgATP which increases the free ADP concentration. Since the MgATP concentration is not significantly different in the presence and absence of creatine kinase, this experiment clearly indicates that even in the presence of millimolar concentrations of MgATP and micromolar concentrations of ADP, it is the concentration of ADP that controls the rate of Ca^{2+} uptake. From the equilibrium constant of the creatine kinase reaction it can be inferred that the addition of creatine kinase did not change significantly the initial ATP concentration, while the ADP concentration varied in the range of 1 to 20 μM.

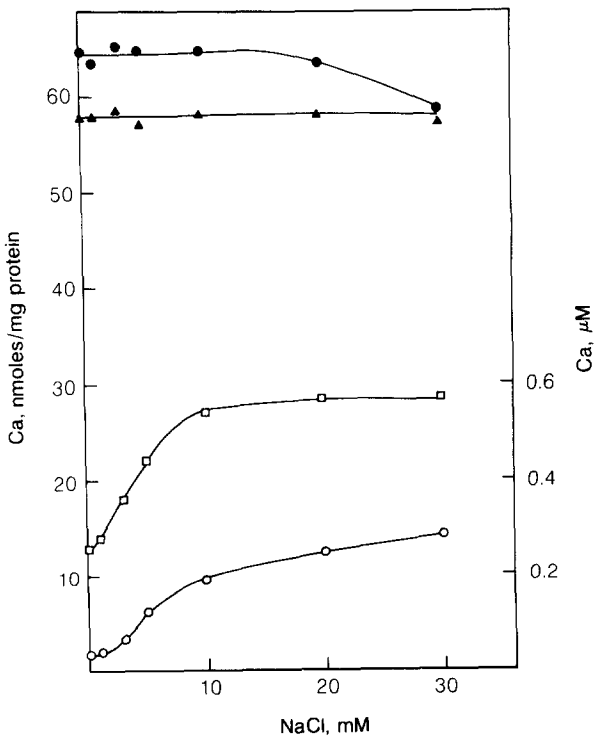


Fig. 11. The effect of NaCl on Ca^{2+} transport. Medium was the same as in Fig. 9, except for the absence of NaCl, which was varied as indicated. Mg ADP concentration was 0.3 mM. The figure shows the effect on the rate of uptake (●), the extent of uptake (▲), the rate of efflux (○) and the steady-state Ca^{2+} concentration (□).

A major contribution to the Ca^{2+} efflux in brain mitochondria is due to the activity of the Na^+–Ca^{2+} exchange system. This is shown in Fig. 11. It is observed that although Na^+ had little effect on the rate or extent of Ca^{2+} uptake (except at high concentration) it has a pronounced effect on the rate of efflux and on the steady-state level. The effect appears to be saturated at 10 mM and have sharp dependence on Na^+ concentration, as was previously observed in heart mitochondria [28]. This fact may also be of physiological significance.

Discussion

The mechanism of the adenine-nucleotide effect

Although it has been known for a very long time that adenine nucleotides determine the ability of mitochondria to accumulate and retain large amounts of Ca^{2+}, the various contrasting explanations which were offered previously were never fully substantiated [21]. Brain mitochondria are particularly sensitive to the effect of adenine nucleotides [8] and it is, therefore, an appropriate system to investigate the mechanism of this effect. Previous investigators attributed the effect of ADP to either prevention of phosphate-induced, Ca^{2+}-dependent, permeabilization and efflux or direct prevention of P_i efflux (cf. Ref. 9). Either way, the effect is attributed to inhibition of Ca^{2+} efflux [7]. Our results clearly demonstrate that in brain mitochondria this is a minor effect of ADP. (a) The effects of Ruthenium red and atractyloside on the rate of efflux are not additive [15], thus demonstrating that both inhibit the rate of uptake. The inhibition of uptake, as in Fig. 1, could not be accounted for by the small effects on efflux. (b) The ADP effect on the rate and extent of uptake is observed even in the absence of added phosphate (Fig. 8). Phosphate does not stimulate the Ruthenium red induced efflux. Moreover, in the presence of ADP, P_i actually inhibits the efflux. The rate of electrogenic uptake in brain mitochondria is stimulated by ADP to a larger extent in the absence of P_i than in the presence of low concentrations of P_i. (c) Cyclosporin A, a specific inhibitor of Ca^{2+}-induced membrane permeabilization [17,18], does not substitute for the ADP effect on the rate of uptake and steady-state level.

The explanation of the effect of adenine nucleotides, as due to the prevention of a loss of adenine nucleotides [8], is also contradicted by our results. Since the loss of adenine nucleotides require long incubations [8], how can atractyloside, when added to mitochondria which

were preincubated with ADP, induce instantaneous Ca^{2+} loss [15]? We also found that neither the atractyloside nor the bongkrekate effect on the rate of Ca^{2+} uptake can be attributed to effects on membrane potential [8]. In the absence of Ca^{2+} there is no effect on $\Delta\psi$. The small effect of these agents on membrane potential in the presence of Ca^{2+} are due to Ca^{2+} cycling and are related to their effect on the efflux. Since the rate of electrogenic uptake at high membrane potential is not limited by the magnitude of the membrane potential, these differences cannot account for the large difference in the rate of uptake.

Our results strongly suggest that it is the conformation of the adenine nucleotides translocator which determines the ability of the mitochondria to accumulate and retain large amounts of Ca^{2+} [15]. Very recently, the same conclusion was arrived, independently, regarding the effect of ADP on the Ca^{2+}-induced loss of permeability in liver mitochondria [29]. It was suggested previously that the conformation of the carrier also determine the permeability of monovalent cations [30]. The adenine-nucleotide carrier is the most abundant protein in the inner membrane, comprising 10% of the total inner membrane proteins. It is known that the translocator carries a large number of positive charges [20]. The transformation from the C-state to the M-state appears to cause a large conformation change of a charged hydrophilic segment on the matrix surface of the inner membrane. This change is associated with increased accessibility of lysines and arginines [20], and it is possible that this is due to removal of positively charged peptide segment away from the phospholipid surface. Since anionic phospholipids, in particular cardiolipin, are associated with the translocator [26], it is likely that the movement of positively peptide segment away from the surface will be associated with increased availability of these phospholipids. Although this model is highly speculative, it would explain the apparent increase of negative surface charge on the transition to the M-state. Increased surface charge could accelerate the dissociation of Ca^{2+} from the electrogenic translocator and thus enhance the rate of transport.

This model also explains why in brain mitochondria the spermine effect depends on ADP. In the absence of ADP, the rate-limiting step for electrogenic transport would be the final step, the dissociation of Ca^{2+} from the carrier in the matrix, whereas in the presence of ADP the rate-limiting step is the diffusion of Ca^{2+} on the cytosolic surface, a step which is enhanced by spermine [14]. The small inhibition by ADP of the efflux is largely due to its inhibition of the Ca^{2+} induced permeabilization [29]. Since both processes appear to depend on the conformation of the ADP/ATP translocator, it is likely that the mechanism of both processes depends on increased surface charge density. The fact that brain mitochondria are more strongly affected by ADP than liver mitochondria could be due either to higher concentrations of the adenine-nucleotide translocator or to different molecular species. It is known that there are at least two isoenzymes in mammals which differ in net charge and are not expressed equally in all tissues [32]. Differences in phospholipid composition of the inner mitochondrial membrane may also contribute to these tissue specific differences.

Adenine nucleotides as regulators of mitochondrial Ca^{2+} transport in brain cells

As Fig. 9B shows, in a medium which simulates the cation composition of brain cells (e.g., K^+, Na^+, Mg^+, spermine), there is a very sharp dependence of mitochondrial Ca^{2+} transport on ADP concentrations in the physiological range of ADP [22]. Raising ADP from 5 to 20 μM reduced the Ca^{2+} steady-state level from 4.5 to 0.4 μM and enhanced the rate of uptake from 5 to 75 nmol/mg protein per min. Hence, a very small variation in ADP concentration can result in a very large effect on Ca^{2+} transport. In brain cells, as in muscle, creatine kinase buffers ADP concentration at very narrow range. Moreover, ATP concentrations are in the millimolar range, a concentration range which normally would saturate the adenine nucleotide binding site. However, the fact that oxidative phosphorylation can proceed at very high rates, even when the ATP/ADP ratio is high, and that there is a range where the rate of ADP translocation control the rate of phosphorylation suggest that there is a preferential inward translocation of the ADP translocator which depend on ADP concentration. This is probably due to the potential-induced ADP selectivity of the carrier [10], together with the fact that the difference in the Mg^{2+} affinity and pK values of ATP and ADP species result in a considerable lower ratio of ATP^{-4}/ADP^{-3} than the total ATP/ADP ratio. Thus it is not unlikely that even when the ratio of total ATP/ADP is high, ADP concentration would determine the rate of the exchange and thus the fraction of translocator in the M-state. The experiments with the creatine-kinase system demonstrate that, in vitro, at least, it is possible to regulate the Ca^{2+} transport by micromolar concentrations of ADP even in the presence of millimolar concentrations of ATP.

At present, there is not much information on spatial and temporal variations in ADP concentrations in brain cells. However, since mitochondria are present at high concentration at active synapses [28], it is not inconceivable that excitation, which leads to enhanced ATP hydrolysis, could result in temporal and/or spatial oscillations in ADP concentration. Such oscillations, if existing, could modulate the cellular Ca^{2+} oscillation as discussed in the preceding paper.

While it is apparent that in most tissues respiratory rates are controlled by Ca^{2+} and not by ADP [34], it is

possible that in the brain, at least, ADP, through its effect on the rate of Ca^{2+} transport, enhance respiration during excitation. If this speculation could be substantiated, it would add a new, surprising dimension, to the classical concept of respiratory control [35].

Acknowledgements

We thank Dr. William S. Thayer for critical reading of this manuscript and Ms. J. Lavin for expert technical assistance. Supported by PHS Grants GM-28173 and AAO-7238.

References

1 Rossi, C.S. and Lehninger, A.L. (1964) J. Biol. Chem. 239, 3971–3980.
2 Carafoli, E., Rossi, C.S. and Lehninger, A.L. (1965) J. Biol. Chem. 240, 2254–2261.
3 Lehninger, A.L., Carafoli, E. and Rossi, C.S. (1967) Adv. Enzymol. 29, 259–320.
4 Meisner, H. and Klingenberg, M. (1968) J. Biol. Chem. 243, 3631–3634.
5 Carafoli, E. and Crompton, M. (1978) Curr. Top. Membr. Transp. 10, 151–216.
6 Bygrave, F.L. (1978) Biol. Rev. Cambridge, Philos. Soc. 53, 43–79.
7 Crompton, M. and Costi, A. (1988) Eur. J. Biochem. 178, 489–501.
8 Nicholls, D.G. and Scott, I.D. (1980) Biochem. J. 186, 833–839.
9 Vitorica, J. and Satrustegui, J. (1985) Biochem. J. 225, 41–49.
10 Klingenberg, M. (1985) in The Enzymes of Biological Membranes Vol. 4 (Martonesi, A.N., ed.), pp. 511–533, Plenum, New York.
11 Asimakis, K.E.O. and Sordah, A. (1977) Arch. Biochem. Biophys. 179, 200–210.
12 Peng, C.F., Stroub, K.D., Kane, J.J., Murphy, M.L., Wadkins, C.L. (1977) Biochim. Biophys. Acta 462, 403–413.
13 Harris, E.J. (1979) Biochem. J. 178, 673–680.
14 Rottenberg, H. and Marbach, M. (1990) Biochim. Biophys. Acta 1016, 77–86.
15 Rottenberg, H. and Marbach, M. (1989) FEBS Lett. 247, 483–486.
16 Rottenberg, H. (1989) Methods Enzymol. 172, 63–84.
17 Crompton, M., Ellinger, H. and Costi, A. (1988) Biochem. J. 255, 357–360.
18 Brockemeir, K.M., Dempsey, M.E. and Pfeiffer, D.R. (1989) J. Biol. Chem. 264, 7826–7830.
19 Rottenberg, H. (1989) Methods Enzymol. 171, 364–375.
20 Klingenberg, M. (1989) Arch. Biochem. Biophys. 270, 1–14.
21 Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433.
22 Zoccarato, F. and Nicholls, D. (1982) Eur. J. Biochem. 127, 333–338.
23 Klingenberg, M. and Rottenberg, H. (1977) Eur. J. Biochem. 73, 125–130.
24 Hashimoto, K., Angiolillo, P. and Rottenberg, H. (1984) Biochim. Biophys. Acta 764, 55–62.
25 Rottenberg, H. (1984) J. Membrane Biol. 81, 127–138.
26 Erecinska, M. and Silver, A.I. (1989) J. Cereb. Blood Flow Metab. 9, 2–19.
27 Gyulai, L.Z., Roth, J.S., Leigh, J.S., Jr. and Chance, B. (1985) J. Biol. Chem. 269, 3947–3954.
28 Crompton, M., Caparo, M. and Carafoli, E. (1976) Eur. J. Biochem. 69, 453–462.
29 LeQuoc, K. and LeQuoc, D. (1988) Arch. Biochem. Biophys. 265, 249–257.
30 Panov, A., Filippova, S. and Lyakhovich, V. (1980) Arch. Biochem. 199, 420–426.
31 Dress, M. and Beyer, K. (1988) Biochemistry 27, 8584–8591.
32 Powell, S.J., Medd, S.M., Runswick, M.J. and Walker, J.E. (1989) Biochemistry 28, 866–873.
33 Wong-Riley, M.T.T. (1989) Trends Neurolog. Sci. 12, 94–101.
34 Denton, R.M. and McCormack, J.G. (1986) Cell Calcium 7, 377–386.
35 Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 409–427.