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Control of the Mitochondrial Permeability Transition Pore by High-Affinity ADP Binding at the ADP/ATP Translocase in Permeabilized Mitochondria[1]

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Low levels of ADP binding at the ADP/ATP translocase caused inhibition of the Ca^{2+}-induced permeability transition of the mitochondrial inner membrane, when measured using the shrinkage assay on mitochondria, which have already undergone a transition. Inhibition was prevented by carboxyatractyloside, but potentiated by bongkrekic acid, which increased the affinity for inhibition by ADP. This suggests that inhibition was related to the conformation of the translocase. Ca^{2+} addition was calculated to remove most of the free ADP. Ca^{2+} added after ADP induced a slow decay of the inhibition, which probably reflected the dissociation of ADP from the translocator. We conclude that the probability of forming a permeability transition pore (PTP) is much greater when the translocase is in the CAT conformation than in the BKA conformation, and, in the absence of CAT and BKA, the translocator is shifted between the BKA and CAT conformations by ADP binding and removal, even in deenergized mitochondria with no nucleotide gradients.

KEY WORDS: Permeability transition; ADP/ATP translocase; kinetics; adenosine diphosphate; carboxyatractyloside; bongkekic acid; mitochondria.

INTRODUCTION

Mitochondria exposed to low levels of Ca^{2+} accumulate it rapidly without initial ill effect: the mitochondria retain good respiratory control and are in the "aggregated" configuration. Then, one by one, the mitochondria undergo a sudden change in permeability, causing a change to the "orthodox" configuration and an uncoupling of oxidative phosphorylation (Hunter *et al.*, 1976). This phenomenon we termed the Ca^{2+}-induced transition. A similar effect occurs upon incubation of mitochondria in the presence of phosphate, arsenate, or fatty acids; in each case, it requires the presence of endogenous Ca^{2+} (Hunter *et al.*, 1976).

During our studies on intact mitochondria, it became apparent that the slow rate at which they undergo the transition is, in part, a consequence of inhibitory agents contained in the matrix space (Hunter and Haworth, 1979). When a transition occurs, these agents leave the matrix space and the mitochondria thereafter undergo Ca^{2+} dependent transitions at an uninhibited rate (Haworth and Hunter, 1979). It was inferred from the effect of various agents on intact mitochondria, that internal NADH and ADP were two of these agents active in modulating the action of Ca^{2+}. ADP was shown to inhibit at two sites: one internal and a further effect of ADP binding at the ADP/ATP translocase (Hunter and Haworth, 1979).

The study of the mode of action of Ca^{2+} and its inhibition by nucleotides has been facilitated by

[1] Key to abbreviations. PEG, polyethyleneglycol; MOPS, morpholinopropane sulfonate; BKA, bongkrekic acid; CAT, carboxyatractyloside; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether) *N*,*N*′-tetraacetic acid; RR, ruthenium red, PTP, permeability transition pore.

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adopting two tactics: (1) using mitochondria that have undergone a transition and are permeable to added solutes in the presence of Ca^{2+} and (2) by using shrinkage induced by polyethyleneglycol (MW 1500) (PEG) as a rapid and quantitative assay of membrane permeability (Haworth and Hunter, 1979). This system has provided evidence that the Ca^{2+}-induced permeability is controlled by a nonspecific channel with a molecular weight cutoff around 1000 Da. It can open when two atoms of Ca^{2+} bind with high affinity (Haworth and Hunter, 1979). ADP (binding in the presence of carboxyatractyloside) and NADH exert a mixed-type inhibition of the Ca^{2+} activation with binding of two molecules of either nucleotide, and act synergistically when present at the same time (Haworth and Hunter, 1980a). The present report concerns the effect of ADP binding at the ADP/ATP translocase on Ca^{2+}-induced permeability using this simple system.

This work was conducted in 1979, but was not published at that time because of resistance to the concept of a mitochondrial permeability transition pore. The results are, however, discussed in light of subsequent work by others.

METHODS

Preparation of Treated Mitochondria

The preparation of hypotonically swollen beef heart mitochondria, which had undergone a Ca^{2+}-induced transition (treated mitochondria), was as previously described (Haworth and Hunter, 1979). Briefly, 10 ml mitochondrial suspension (50 mg/ml in 250 mM sucrose, 20 mM Tris-HCl, pH 7.6) was hypotonically swollen by addition to 200 ml 5 mM potassium MOPS, pH 7.2 at 0°C. The transition was then induced by addition of 1 mM arsenate and incubation for 30 min at 30°C. Then, 40 nmol EGTA/mg was added to chelate released endogenous Ca^{2+}. The suspension was centrifuged for 10 min at 27,000 $\times$ *g*, and the pellets resuspended to 20 mg/ml in the MOPS buffer at 0°C. Hypotonic treatment was employed so that the Ca^{2+}-dependent permeability could be measured conveniently, using the light-scattering assay of PEG-induced mitochondrial shrinkage (Haworth and Hunter, 1979) (see below).

Permeability Measurements

Light scattering was measured on a Perkin Elmer MPF 3 fluorescence spectrophotometer with excitation and emission wavelengths set at 520 n*M*. The instrument was fitted with a mechanical stirrer, which gave complete mixing of added solutions within approximately 2s. The cell holder was maintained at 30° in all experiments by a flow-through water jacket. The rate of shrinkage (*V*) was measured from the slope of the 90° light-scattering curve after the addition of 0.3 ml 35% PEG (MW 1500) to 2.7 ml mitochondrial suspension (see Fig. 1 of Haworth and Hunter, 1979). In brief, the principle of this assay is as follows. When a solute, which cannot permeate the inner membrane, is added to a mitochondrial suspension, a rapid efflux of water from the matrix space occurs, which is completed within a few seconds. At the new matrix volume, the increased osmotic pressure of internal solutes balances the increased osmotic pressure of external solutes. The case of treated mitochondria, where the membrane has a significant (Ca^{2+}-dependent) degree of permeability to solutes of low molecular weight as well as to water is now considered. When an impermeable solute such as PEG (MW 1500) is added to a suspension of treated mitochondria, the subsequent shrinkage continues beyond that achieved when the membrane is impermeable to internal solutes, which are now also leaving the matrix space; shrinkage will continue until the osmotic pressure of the PEG is balanced by the osmotic pressure of the matrix proteins and associated Donnan ions. Under the conditions used here, this final matrix volume is almost immeasurably small. It is clear that the more permeable the membrane is to internal solute, the faster the mitochondria will shrink during this extra shrinkage phase. This rate of volume flow is measured by light scattering. It has been shown empirically to be a good quantitative measure of membrane permeability: the Ca^{2+} dependence of permeability to salts measured by this method correlates well with the Ca^{2+} dependence of permeability of the inner membrane to $NADP^+$, measured at zero volume flow by a completely different method (Haworth and Hunter, 1979).

Calculation of Free ADP Concentration

Free ADP concentration was calculated from added ADP concentration using the MAXCHELATOR program (Bers *et al.*, 1994), available at www.stanford.edu/~cpatton

RESULTS

It was shown previously that low levels of ADP inhibited the Ca^{2+}-induced transition; the inhibition was potentiated by bongkrekic acid and abolished by atractyloside (Hunter and Haworth, 1979). It is seen that, under the conditions used, the effect of ADP at this site appeared to decrease the maximum Ca^{2+} induced permeability and also to increase the apparent K_m for Ca^{2+} (Fig. 1). It should be noted that this and other experiments reported here used a buffer at pH 6.8. This is because at this pH the apparent K_m for Ca^{2+} is sufficiently high to eliminate the need for Ca^{2+} buffers. The apparent K_m for Ca^{2+} is very sensitive to pH: while the K_m for Ca^{2+} at pH 6.8 is 37 μM, at pH 7.2 the apparent K_m for Ca^{2+} (in the absence of ADP or NADH) is <10 μM and is still decreasing with increasing pH (Haworth and Hunter, 1979). Endogenous levels of mitochondrial Ca^{2+} may, therefore, be expected to be active in the mechanisms described here. Figure 2 shows that, using a similar protocol, the apparent *K*; may be more usual for ADP was of the

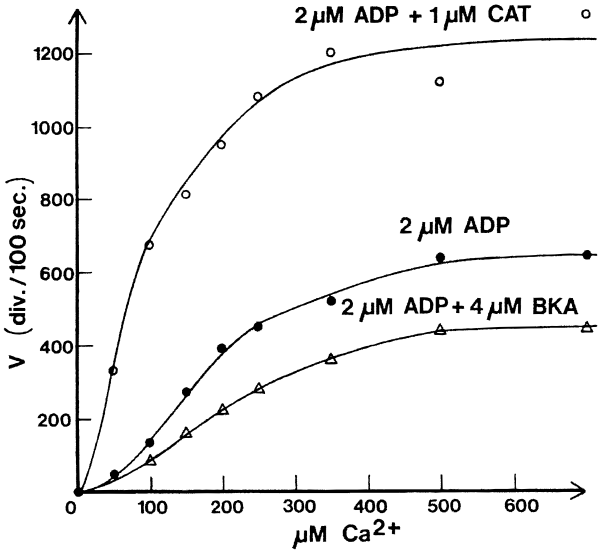

Fig. 1. Ca^{2+} dependence of permeability in the presence of ADP. To 2.5 ml 5 mM K^+MOPS, 25 mM K^+ cacodylate and 50 mM KCl pH 6.8 was added to 0.15 ml (3 mg protein) treated mitochondria (see Materials and Methods), ADP, and inhibitor shown. The suspension was mixed. After 15 s, $CaCl_2$ and 2.5 μg A23187 was added and the suspension was remixed. Shrinkage was initiated 1 min later by addition of 0.3 ml 35% PEG 1500. The rate of shrinkage (*V*) was measured from the slope of the 90° light-scattering curve at 50% light scattering increase after the addition of PEG (see Fig. 1 of Haworth and Hunter, 1979, for illustration). This rate would occur at different times after the addition of PEG, depending on the inner membrane permeability.

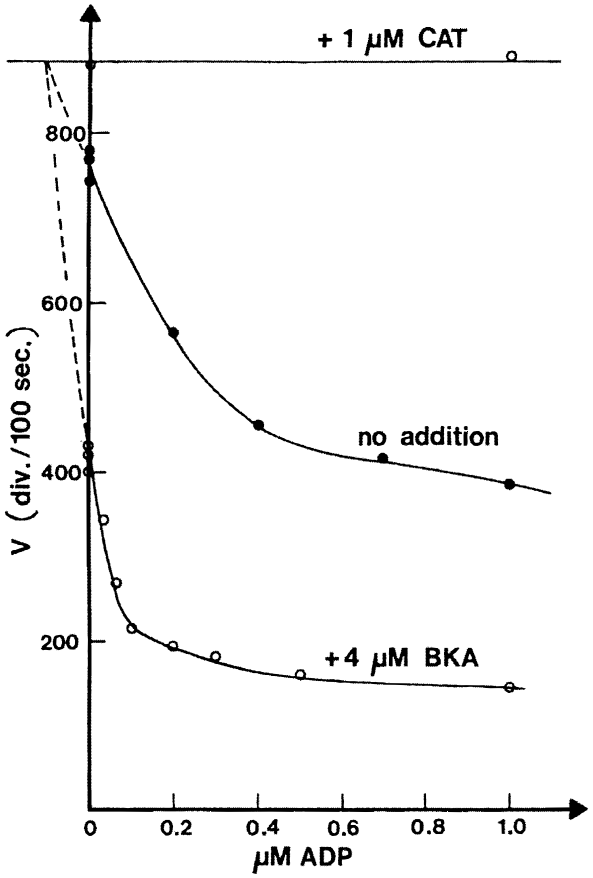

Fig. 2. ADP dependence of permeability in the presence of Ca^{2+}. Procedure as in Fig. 1, except that PEG was added just 15 s after the $CaCl_2$. The level of $CaCl_2$ was 500 μM.

order of 0.2 μ*M*, and this figure appeared to be further reduced in the presence of bongkrekic acid. Bongkrekic acid alone caused a significant degree of inhibition.

Further experiments showed, however, that the inhibition produced by high-affinity ADP binding (as shown in Figs. 1 and 2) was relieved with increasing time of incubation with Ca^{2+}. When the time between the addition of Ca^{2+} and the addition of PEG was varied, it was seen that the major effect of adding 2 μ*M* ADP was to delay the onset of permeability induced by subsequent Ca^{2+} addition (Fig. 3, compare open squares with open circles). In the absence of added ADP, the effect of added $CaCl_2$ is seen very rapidly (Fig. 3, open circles). We know from previous work (Hunter and Haworth, 1979) that the site of action of Ca^{2+}, when it induces the permeability change, is on the matrix side of the inner membrane. Could the ADP somehow be inhibiting the movement of Ca^{2+} into the mitochondria? Ruthenium red, which blocks the Ca^{2+}

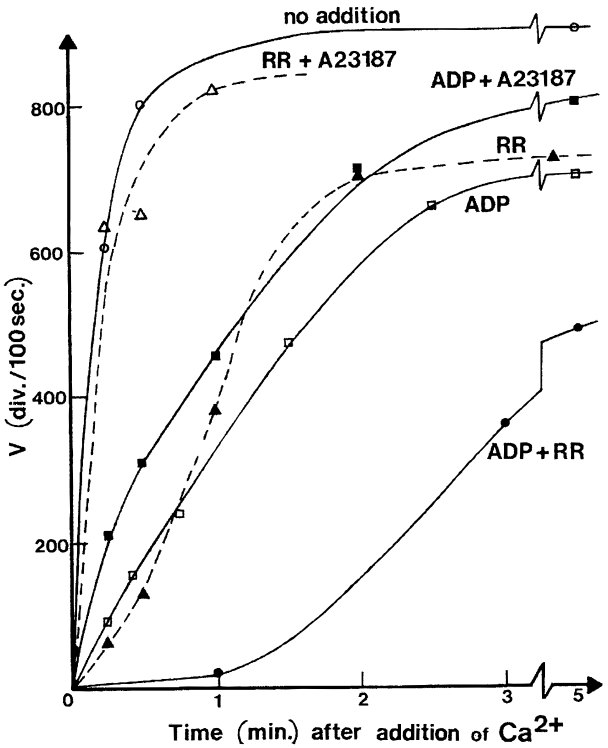

Fig. 3. Time dependence of the inhibition by ADP of the Ca^{2+}-induced permeability. Procedure as in Fig. 1, except that the time interval between addition of $CaCl_2$ and addition of PEG was varied as shown. A23187 (2.5 μg) or ruthenium red (RR) (5 nmol) were added along with 2 μ*M* ADP, where shown. The level of $CaCl_2$ was 500 μM.

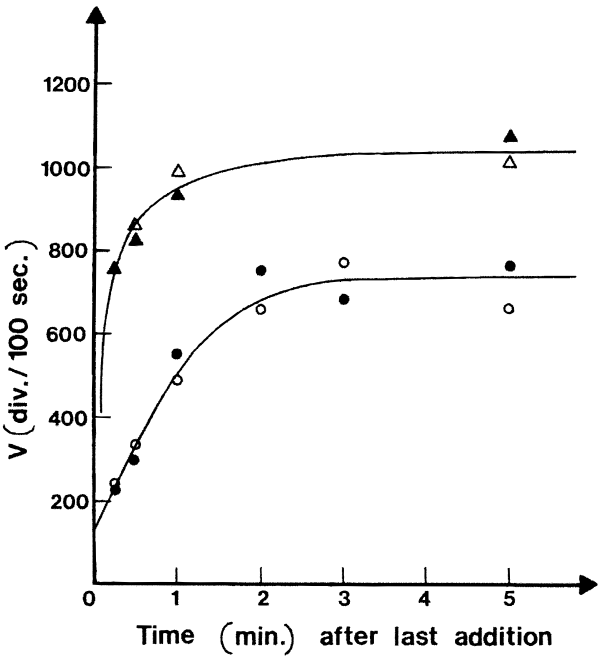

Fig. 4. Induction of ADP-dependent inhibition by removal of Ca^{2+}. Conditions were as in Fig. 3. A23187 (2.5 μg) was present in all samples. The orders of addition were as follows: ▲—▲ = no ADP, 30 s 500 μM $CaCl_2$; Δ—Δ = 500 μM $CaCl_2$, 30 s, 2 μM ADP; ❍–❍ = 2 μM ADP, 30 s, 500 μM $CaCl_2$; ●—● = 500 μM $CaCl_2$, 30 s, 2 μM ADP, 30 s, 700 μM EGTA, 1 min, 700 μM $CaCl_2$.

uniporter, was indeed found to delay the onset of the effect of added Ca^{2+} on permeability (Fig. 3, filled triangles). This inhibition by ruthenium red could be overcome by adding the Ca^{2+} ionophore A23187 (Fig. 3, open triangles). By contrast, the effect of ADP to delay onset of the effect of Ca^{2+} was only slightly relieved by A23187 (Fig. 3, filled squares). Additional A23187 provided no further relief (data not shown). Furthermore, the effect of ruthenium red and ADP was additive (Fig. 3, filled circles). This, therefore, suggests that the ADP effect was not to delay Ca^{2+} entry into the mitochondria. Neither doubling the level of ADP nor the addition of bongkrekic acid served to prolong the ADP effect.

The order of addition of ADP and Ca^{2+} was found to be critical. ADP addition before Ca^{2+} again delayed the effect of Ca^{2+} to increase permeability (as in Fig. 3) (Fig. 4, open circles). However, when ADP was added after Ca^{2+} (Fig. 4, open triangles), it exerted no effect. Furthermore, even when Ca^{2+} was added before ADP, ADP could be induced to act by the addition of EGTA. When Ca^{2+} was subsequently added, its action was slowed to the same extent as when ADP was added before Ca^{2+} (Fig. 4, filled circles; compare with open circles). Note that A23187 was present in all samples. Thus, the absence of Ca^{2+} was the critical factor for the action of ADP.

Since Ca^{2+} addition was the critical factor, which relieved the inhibition by ADP, the impact of Ca^{2+} addition on the level of free ADP was calculated for the conditions of Fig. 4, using the MAXCHELATOR program, which gave a K_d for Ca^{2+} binding to ADP of 1.12×10^{-5} M (Bers *et al.*, 1994). Thus, we calculate that 97.8% of the 2 μ*M* free ADP was bound by Ca^{2+} addition.

DISCUSSION

Our earlier observation that the Ca^{2+}-induced transition could be inhibited by the binding of ADP at the ADP/ATP translocase (Hunter and Haworth, 1979) has been extended here using the shrinkage assay to show that such binding also inhibits the Ca^{2+}-depen-

dent permeability of mitochondria, which have undergone a transition and lost their endogenous nucleotides. The maximum expression of inhibition does, however, appear to require the binding of ADP before the binding of Ca^{2+} and the inhibition is strongly attenuated with time. This attenuation is not caused by degradation of ADP, since inhibition can be restored by removal of Ca^{2+} (Fig. 4), nor does it reflect the rate of access of added Ca^{2+} to the mitochondrial matrix space (Fig. 3). Since Ca^{2+} addition results in the removal of free ADP, the most likely explanation for the time dependence of the loss of the ADP effect is that it either reflects the rate at which ADP is unbound from the translocase or else it reflects the rate at which the newly unbound translocase reverts essentially to the CAT configuration.

If the above interpretation is correct, then the best measure of the permeability characteristic of 2 μM free ADP in the presence of Ca^{2+} is that measured after Ca^{2+} has had time to enter, but before ADP has had time to dissociate much (as in Fig 2). The opposite effect of CAT and BKA seen here, as in our original observation with intact mitochondria (Hunter and Haworth, 1979), supports the notion that the PTP is affected by the conformational state of the ADP/ATP translocase rather than its operational state. It is a long-standing observation that BKA appears to raise the affinity of ADP binding to the carrier while atractyloside, of which CAT is a higher affinity derivative, displaces ADP (Weidemann *et al.*, 1970). The effect of BKA, ADP, and CAT on Ca^{2+}-induced permeability (Fig. 2) could, therefore, be rationalized in terms of an inhibition of this permeability by ADP binding to the translocase or possibly also by bongkrekate binding. Bongkrekic acid alone did show considerable inhibition (Fig. 2); however, there could also have been residual ADP in the preparation of treated mitochondria, the action of which could have simply been potentiated by BKA (Fig. 2, dashed lines). Binding of CAT and BKA are thought to stabilize the translocase into two opposite conformational states: the c-state (cytosol-facing) (Buchanan *et al.*, 1976) or CAT conformation (Fiore *et al.*, 1998) when CAT is bound, and the m-state (matrix-facing) (Buchanan *et al.*, 1976) or BKA conformation (Fiore *et al.*, 1998) when BKA is bound. These two states are distinguished by different antibody reactivity (Buchanan *et al.*, 1976) and by different reactivity to sulfhydryl reagents such as NEM. ADP binding made the translocase more susceptible to inhibition by NEM, while atractyloside abolished the inhibition (LeBlanc and Clauser, 1972).

The importance of the configuration of the ADP/ATP translocase in controlling Ca^{2+} release has also been demonstrated in liver mitochondria (Le Quoc and Le Quoc, 1988, 1989). CAT-sensitive inhibition of the transition pore has also been observed in liver mitochondria by Halestrap *et al.* (1997) using a shrinkage assay and mitochondria put through a transition by exposure to Ca^{2+}. These authors were unable to see effects of bongkrekic acid on the inhibition of the PTP by ADP. The reason for this is unclear, but could be related to different conditions of preparation of swollen mitochondria and/or different shrinkage assay conditions used by these authors. Novgorodov *et al.* (1992, 1994) have also used a shrinkage assay, but to study the low-affinity inhibition of the transition pore by ADP, as measured in the presence of CAT, using both liver and heart mitochondria. They observed a synergistic action of cyclosporin A, which inhibits cyclophilin binding, and ADP, and concluded that cyclosporin A increased the affinity of ADP binding. By contrast, Halestrap *et al.* (1997) saw no evidence for a direct interaction between the action of cyclosporin A and the effect of ADP, at either the CAT-sensitive or at the low-affinity site. They concluded, therefore, that the mechanisms of inhibition by ADP binding and by cyclophilin dissociation were independent (Halestrap *et al.*, 1997). These results also show how results gained using mitochondria in the shrinkage assay may be different, depending, perhaps, on the conditions of assay.

Is the effect of the translocase configuration on the PTP a reflection of the identity of the two? The results presented here could, in principle, merely reflect an interaction between the ADP/ATP translocase and the PTP, as we originally suggested (Haworth and Hunter, 1980b). This could, for example, reflect the impact of translocase configuration on surface potential (Novgorodov *et al.*, 1994), since the transition pore is influenced by membrane potential (Petronelli *et al.*, 1993). Increasing evidence suggests, however, that the translocator may itself incorporate at least a part of the PTP. Vesicles containing reconstituted purified translocase showed unselective channels, which open in a Ca^{2+}-dependent manner, when measured either by electrophysiology of excised patches (Brustovetsky and Klingenberg, 1996) or by solute flux measurements (Ruck *et al.*, 1998). A less purified complex containing the translocator, porin, hexokinase, and cyclophilin also retained sensitivity to the derivative of cyclosporin A, which specifically inhibits the transition (Beutner *et al.*, 1996). Recently,

a direct interaction between the translocator and cyclophilin has been demonstrated: when solubilized inner membrane was passed through an affinity column containing immobilized cyclophilin, cyclosporin-sensitive retention of the translocator protein was observed (Woodfield *et al.*, 1998; Crompton *et al.*, 1998). Porin was also retained from Chaps-solubilized whole heart mitochondrial membranes (Crompton *et al.*, 1998), but not from Triton X-100 solubilized liver mitochondrial inner membranes (Woodfield *et al.*, 1998). Liposomes incorporating the purified Chaps-solubilized complex showed cyclosporin-sensitive permeabilization by Ca plus phosphate (Crompton *et al.*, 1998). A limitation of all of the above studies is that no mention is made of any specificity for Ca^{2+} over Sr^{2+}, which is the hallmark of the Ca^{2+}-induced transition, either in intact mitochondria (Hunter and Haworth, 1979) or as measured by the shrinkage assay (Haworth and Hunter, 1979). Demonstration of any such specificity in these reconstituted systems would be further convincing evidence for their identity with the transition pore. A structural relationship between the translocase and the transition pore has been questioned on the basis of the following argument (Novgorodov *et al.*, 1994): there is evidence that the translocase configuration is determined by the nucleotide gradient, but there is no such gradient in mitochondria such as those used in the shrinkage assay where the transition pore is open; therefore, it is unlikely that effects of ADP binding to the translocase are mediated by changes in its configuration. However, the specific effects of CAT and BKA seen here suggest that the translocase configuration can be manipulated in these mitochondria in the absence of nucleotide gradients. The time dependence of the high-affinity ADP effect (Fig. 3) cannot be explained in terms of a time-dependent equilibration of the ADP added: when ADP is added after Ca^{2+}, thus with the pore open, it is ineffective (Fig. 4). However, its effect can be reestablished by the subsequent removal of Ca^{2+} (Fig. 4), which presumably reflects the restoration of 2 μM free ADP. It, therefore, appears that the probability of the translocator forming (or causing to be formed) a PTP via Ca^{2+} binding is much greater when the translocase is in the CAT conformation than in the BKA conformation and, in the absence of CAT and BKA, the translocator conformation is shifted between the BKA and CAT conformations by ADP binding and removal.

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