Mitochondrial ADP/ATP Carrier Can Be Reversibly Converted into a Large Channel by Ca^{2+} †

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ABSTRACT: Single-channel current measurements of excised patches with reconstituted purified mitochondrial ADP/ATP carrier (AAC) indicates the presence of a large low cation selective ($P_{K^+}/P_{Cl^-} = 4.3 \pm 0.6$) channel. The channel conductance has multiple sublevels and varies from 300 to 600 pS. It has low probability of current fluctuations at V_{hold} up to 80−100 mV of both signs and is reversibly gated at $V_{hold} > 150$ mV. The opening of the channel is Ca^{2+}-dependent (1 mM Ca^{2+}) and can be reversibly closed on removal of Ca^{2+}. It is strongly pH dependent and closes completely at pH_{ex} 5.2. The AAC-specific inhibitor bongkrekate inhibits the channel partially and completely in combination with ADP, whereas carboxyatractylate did not affect the conductance. The effects of these AAC-specific ligands prove that the channel activity belongs to AAC. The AAC-linked conductance can clearly be differentiated from the porin channel, rarely detected in our preparations. The properties of the AAC-linked channel coincide with the mitochondrial permeability transition pore (MTP), which is also affected by the AAC ligands [Hunter, D. R., & Haworth, R. A. (1979) *Arch. Biochem. Biophys. 195*, 453−459] and resembles the mitochondrial "multiconductance channel" [Kinnally, K. W., Campo, M. L., & Tedeschi, H. T. (1989) *J. Bioenerg. Biomembr. 21*, 497−506] or "megachannel" [Petronilli, V., Szabo, I., & Zoratti, M. (1989) *FEBS Lett. 259*, 137−143]. Therefore we conclude that the AAC, when converted into a large unselective channel, is a key component in the MTP and thus is involved in the ischemia-reperfusion damage and cytosolic Ca^{2+} oscillations. The channel opening in AAC is proposed to be caused by binding of Ca^{2+} to the cardiolipin, tightly bound to AAC, thus releasing positive charges within the AAC which open the gate.

The ADP/ATP carrier (AAC) is the most abundant membrane protein in mitochondria. Although it is a small protein with about 300 residues, it must provide a wide translocation path for the transport of very bulky solutes such as ATP and ADP. The structural model of the homodimeric AAC proposes a pore-like domain which is gated at either side (Klingenberg, 1981). Any condition in which the gates operating during the ADP/ATP exchange are not closed might result in a comparatively wide pore. It remains an open question whether this channel is at the two-fold axis of the homodimer AAC or within each of the two subunits. The first indication of this parafunction of the AAC as a pore came from studying the adenine nucleotide efflux from mitochondria which was induced by atractylate and inhibited by bongkrekate (Meisner & Klingenberg, 1968; Klingenberg et al., 1971b) and from carboxyatractylate-induced efflux of K^+ (Panov et al., 1980). Also mercurials were reported to induce unidirectional pore-like transport by the AAC in reconstituted vesicles (Dierks et al., 1990).

In the inner membrane of mitochondria, under certain conditions a large nonspecific pore, also called mitochondrial permeability transition pore (MTP), can appear [for review, see Bernardi et al. (1994) and Zorrati and Szabo (1995)]. The MTP could be induced by Ca^{2+} and inhibited by cyclosporin A (Hunter & Haworth, 1979; Fournier et al., 1987; Crompton et al., 1988; Broekemeier & Pfeiffer, 1989; Halestrap & Davidson, 1990). It was suggested that the MTP plays an important role in cell physiology and pathophysiology, i.e., in intracellular signal transduction as reflected in cytosolic Ca^{2+} oscillations induced by Ins(1,4,5)P_3 (Ichas et al., 1994; Evtodienko et al., 1994), and most strikingly in the ischemia reperfusion damage as well as in lethal cell injury by hydroperoxides (Griffiths & Halestrap, 1995; Nieminen et al., 1995). The nature of this pore is still under discussion. The involvement of the mitochondrial AAC has been proposed, based on the interaction of specific AAC inhibitors with the MTP (Hunter & Haworth, 1979; Panov et al., 1980; LeQuoc & LeQuoc, 1988; Halestrap & Davidson, 1990), whereas others suggest that the outer membrane porin might be also responsible for the MTP (Szabo et al., 1993; Szabo & Zoratti, 1993).

When patch clamp was applied to the inner mitochondrial membrane (Sorgato et al., 1987; Kinnally et al., 1989; Petronilli et al., 1989), a variety of channels were reported [for review, see Sorgato and Moran (1993), Tedeschi and Kinnally (1994), and Zoratti and Szabo (1995)]. Only the voltage-dependent anion channel (VDAC) was clearly identified with porin of the outer membrane (Benz, 1994; Colombini, 1994). In the inner mitochondrial membrane the "multiconductance channel" (Kinnally et al., 1989) or "megachannel" (Petronilli et al., 1989) was found. This channel is activated by Ca^{2+} and inhibited by cyclosporin A (Zorov et al., 1992; Szabo & Zoratti, 1992), supporting the identity with the cyclosporin A-sensitive MTP. However, the complexity of the mitochondrial membranes did not

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permit a clear assignment of these permeabilities. Recent claims that AAC incorporated into black lipid membrane can form a pore were not substantiated by the actual experiments presented (Tikhonova et al., 1994). The single-channel current recordings were very unstable, and there was no sensitivity to any AAC ligands.

Here we show using patch clamp with isolated and reconstituted AAC that this most abundant carrier can be converted reversibly into an unusually large slightly cation-selective channel. Its opening requires Ca^{2+} and is sensitive to AAC specific inhibitors and to the AAC substrate ADP. It is closed at low pH. The channel has well-defined multiple substates and is voltage gated. These findings open important new perspectives for studying the mechanism of the AAC-catalyzed nucleotide transport and the mechanism of the MTP, since we conclude to have identified the AAC as the major component responsible for the Ca^{2+}-induced MTP in mitochondria.

EXPERIMENTAL PROCEDURES

AAC Purification and Reconstitution. Giant vesicles for patch clamp were prepared by a dehydration−rehydration procedure (Criado & Keller, 1987; Schwarz et al., 1994) with modifications. For this purpose, first the AAC was incorporated into the small unilamellar vesicles as described (Brustovetsky & Klingenberg, 1994) with some modifications. Instead of mitochondria, mitoplasts were used as starting material in oder to eliminate porin as much as possible. The mitoplasts were prepared from bovine heart mitochondria by a 30 min osmotic shock in 5 mM Hepes, pH 7.2, at 0 °C. In the AAC purification procedure the ratio hydroxyapatite/ protein was increased to ≈400. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed as described by Laemmli (1970). The small AAC vesicles were diluted (1:10) with azolectin vesicles (50 mg/mL) prepared from the acetone-washed azolectin (type S-II, Sigma) by sonification in 5 mM MES/Tris, pH 7.2. The mixture was diluted (1:10) with 100 mM KCl/5 mM Hepes, pH 7.2. Five microliters of the diluted suspension was partially dehydrated under vacuum on a glass slide in an exsiccator with anhydrous $CaCl_2$ for 5 min at room temperature. The dehydrated vesicles were rehydrated with 10 μL of 50 mM KCl/2.5 mM Hepes, pH 7.2, during 2 h at 4 °C. The resulting giant vesicles with incorporated AAC had diameters of 20−80 μm and were suitable for patch-clamp experiments.

Patch Clamping. The pipettes for the microelectrodes were pulled from borosilicate glass tubings and then slightly heat polished. The pipettes were filled with standard bath solution containing 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM potassium gluconate, 5 mM MES, and 5 mM Tris at pH 7.2. The microelectrodes filled with standard solution had a resistance of 15−20 MΩ. The AAC-containing giant vesicles (5 μL) were added to the experimental chamber of 2.0 mL volume, which then was filled with standard bath solution. A giant vesicle, attached on the bottom, was touched by the microelectrode, and the seal was formed spontaneously by lifting the microelectrode or with very slight sucking. Recordings were performed with an Axopatch 200 amplifier (Axon Instruments) using pClamp 5.5 software. The current signals were low pass filtered at 1 kHz and digitized at a sampling rate 3.3 kHz. In all cases the channels have been examined starting from a low holding potential (±20 mV). The sign of the voltage is referred to the pipette electrode. The data were collected, stored, and analyzed in a personal computer, using Axon's pClamp software.

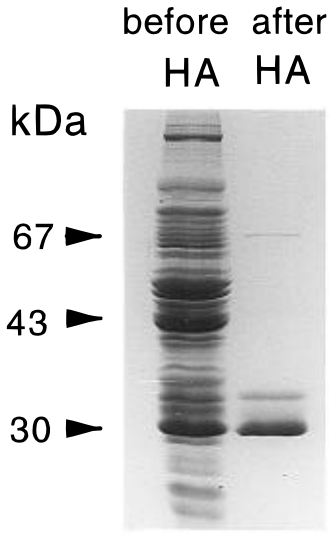

FIGURE 1: SDS−polyacrylamide gel of the solubilized bovine heart mitoplasts before and after hydroxyapatite (HA) purification used for reconstitution into giant vesicles.

RESULTS

To prove the possible conversion of the AAC into a large channel, we have applied a patch-clamp technique on isolated and reconstituted AAC. For this purpose AAC isolated from bovine heart mitochondria was incorporated into giant reconstituted vesicles obtained by a dehydration−rehydration procedure (Criado & Keller, 1987). We took precautions to prevent copurification of porin (VDAC) with the AAC by (i) using mitoplasts as a start material for the AAC isolation and (ii) by employing the detergent $C_{12}E_8$ instead of the more conventional Triton X-100.

Figure 1 shows SDS−PAGE of the solubilized bovine heart mitoplasts before and after purification with hydroxyapatite (HA). After purification two bands were seen: the main band (≈30 kDa) attributed to ACC and a faint band (≈35 kDa) probably corresponding to residual porin. Figure 2a shows the typical single-channel current recordings with a patch from the AAC containing giant vesicles. The channel was usually in the open state almost without current fluctuations up to the holding voltage of 80−100 mV of both signs. The mean conductance in the open state has varied in the different preparations from 300 to about 600 pS in symmetrical 100 mM KCl, with multiple sublevels of conductance. The conductance of neighboring patches without channel activity was in the range of 10−15 pS. At high voltage of both signs (150−180 mV), the channel revealed a drastic but reversible decrease of the conductance. Figure 2b gives the voltage ramp protocol (from 0 to +185 mV) illustrating the large decrease of the channel conductance at the gating voltage (V_{gate}). The transitions were accompanied by current fluctuations with fast kinetics which then were followed by the stable occupation of a lower conductance substate (Figure 2a). In most cases the whole transition occurred within 10−15 s after applying high holding voltage. Figure 3 shows current−voltage ($I-V$)

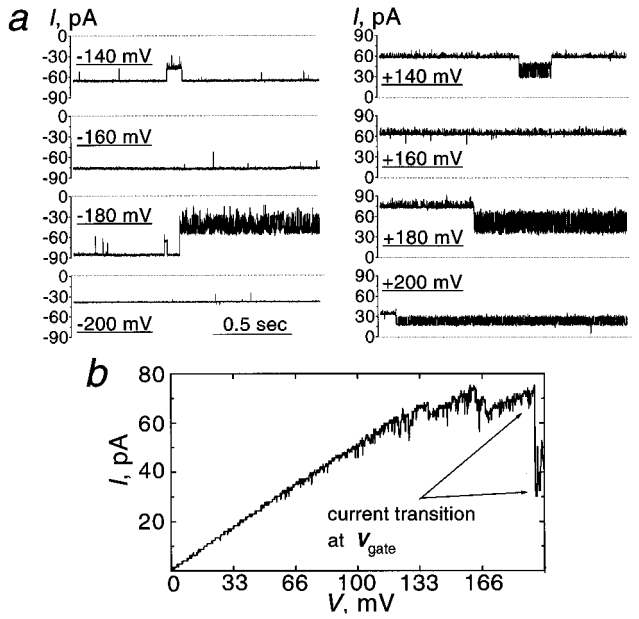

FIGURE 2: Single-channel current recordings of excised patch from giant vesicle containing reconstituted bovine heart ADP/ATP carrier. (a) The holding potential was changed manually. The data were digitized with a sampling rate of 3.3 kHz. (b) The voltage ramp protocol, demonstrating partial closure of the channel at the gating voltage (V_{gate}). The holding potential was automatically changed in the form of a ramp wave at a linear rate of 1 mV/s from 0 to +185 mV. The data were digitized with a sampling rate of 10 Hz.

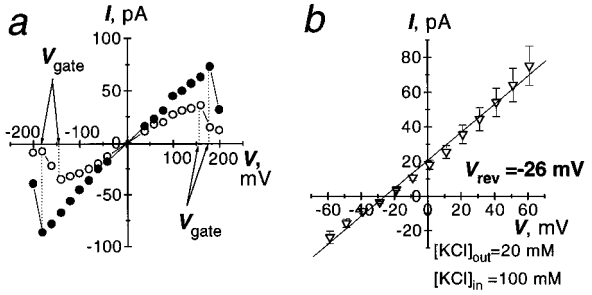

FIGURE 3: Current−voltage ($I-V$) relationships of the single AAC channels. (a) The typical current−voltage relationships for two channels with different mean conductances in symmetrical 100 mM KCl. The mean conductances of the presented channels determined by linear regression fitting were 430 pS (closed circles) and 300 pS (open circles), respectively. The arrows show the gating voltage (V_{gate}). The holding potential was changed manually; before recordings, the patches were exposed to the applied voltage for a few seconds. (b) The current−voltage relationship, obtained in asymmetrical (20 mM KCl_{out}/100 mM KCl_{in}) solutions for determination of selectivity for K^+ versus Cl^-.

relationships in symmetrical (a) and asymmetrical (b) conditions. As a rule, channels with higher mean conductance had higher gating voltage than channels with lower conductance (Figure 3a). To determine the selectivity of the channel, we have measured a reversal potential (E_{rev}) in the asymmetrical KCl solutions (Figure 3b). The measurements gave $E_{rev} = -26 \pm 2$ mV ($n = 6$) in 20 mM KCl_{out}/100 mM KCl_{in} indicating a low cation selectivity of the channel with a permeability ratio of $P_{K^+}/P_{Cl^-} = 4.3$.

The channel has a pronounced dependence on Ca^{2+}. On lowering the Ca^{2+} concentration to 0.1 μM in the pipette and bath solutions the channel was not detected as well as with Ca^{2+}-free solutions. A removal of Ca^{2+} from the bath medium by slow perfusion with Ca^{2+}-free solution decreased the activity of the channel (Figure 4a). The reperfusion with 1 mM Ca^{2+} solution caused reactivation of the channel. The pH dependence is another feature of the channel. While at pH 7.2 the channel has a high conductance, the decrease of pH of the bath medium to pH 5.2 resulted in a reversible inactivation of the channel (Figure 4b). The channel activity could again be restored by increasing the pH to the initial value.

To further identify the AAC as being responsible for the channel activity, we have applied the AAC specific inhibitors carboxyatractylate (CAT) and bongkrekate (BKA). CAT (20 μM) added to the bath medium did not affect the channel activity. However, BKA (10 μM) caused distinct and stable transitions to substates of lower conductance (Figure 5). Whereas BKA alone decreases the conductance only by about 30−40%, ADP (0.5 mM) added after BKA caused full closure of the channel (Figure 5). In contrast, ADP (0.5 mM) alone had a weak inhibitory effect, and further addition of BKA closed the channel almost completely (not shown). The effects of BKA and ADP could be reversed by washing out. CAT added after BKA at least in ≈25% of the successful experiments (5 of 19 experiments) was capable to reverse the effect of BKA resulting in a restoration of the channel conductance (not shown). Since CAT interacts with AAC only from the cytosolic side of the protein (Klingenberg et al., 1971a), we can assume that in our experiments ≈75% of patches had matrix side-out orientation of AAC. BKA interacts with AAC from the matrix side (Klingenberg & Buchholz, 1973), and in most trials it required only a short time (1−3 min) to inhibit the channel activity. This agrees with the conclusion that in the excised patches most of the AAC are in the matrix side-out orientation. As a consequence in most patches the positive holding voltage, referred to the pipette electrode, corresponds to the physiological polarity of the electrical potential on the inner mitochondrial membrane.

Because of the specificity of BKA and CAT for AAC, these results identify AAC as the component responsible for the channel activity. Cyclosporin A (5 μM), which is an inhibitor of the MTP (Hunter & Haworth, 1979; Fournier et al., 1987; Crompton et al., 1988; Broekemeier & Pfeiffer, 1989; Halestrap & Davidson, 1990) and of channels observed in mitochondria (Zorov et al., 1992; Szabo & Zoratti, 1992), does not inhibit the channel activity observed here. This is to be expected as the reconstituted system used here should not contain cyclophilin, the target for cyclosporin A.

We can clearly differentiate the here identified AAC-linked channel activity from the well characterized VDAC as shown in comparative recordings (Figure 6). Due to spurious contamination of porin, it was possible to occasionally detect a channel which has the characteristics of VDAC (Figure 6a). The closures of the channel already at low voltage (±40 mV) are typical for VDAC (Wunder & Colombini, 1991) and contrast to the behavior of the AAC-linked channel recorded for comparison under the same holding potentials (Figure 6b). Also, the VDAC-resembling channel is not influenced by BKA and ADP and has no dependence on the presence of Ca^{2+} in the bath and pipette solutions.

DISCUSSION

The occurrence of a large channel in the ADP/ATP carrier of mitochondria, as shown here, has multifocal significance.

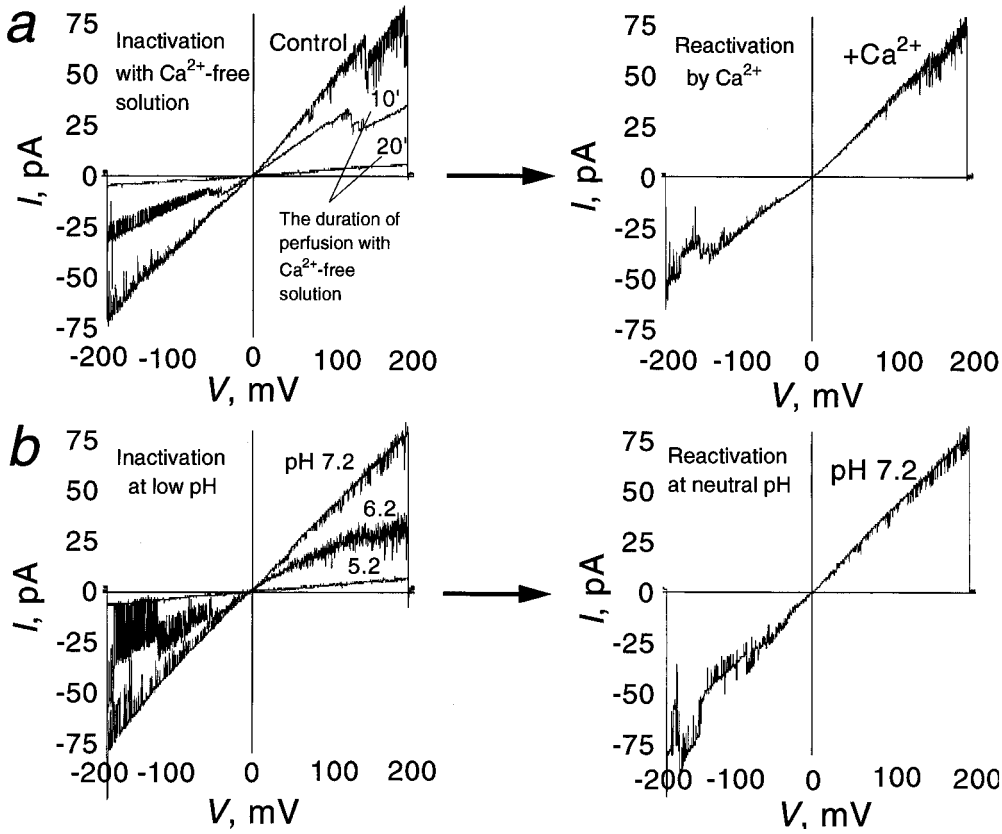

FIGURE 4: (a) Reversible inactivation of the AAC channel by removal of Ca^{2+}. Typical voltage ramp protocols, obtained in symmetrical 100 mM KCl with and without Ca^{2+}. Initially, the recordings were performed in the standard bath solution (with 1 mM Ca^{2+}), and then the bath medium was slowly replaced by perfusion with Ca^{2+}-free solution and recordings were repeated at 10 and 20 min of perfusion. The channel was reactivated by reperfusion of the experimental chamber with standard Ca^{2+}-containing solution for 20 min. (b) Reversible inactivation of the channel at low pH and reactivation at neutral pH. The representative voltage ramp protocols, obtained at pH 7.2, 6.2, and 5.2 in symmetrical 100 mM KCl. The measurements were started at pH 7.2, and then pH was decreased by slow perfusion. To reactivate the channel, the experimental chamber was reperfused with standard initial solution (pH 7.2) for 20 min. In both cases (*a*, *b*) the holding potential was automatically changed in the form of ramp wave at a linear rate of 4 mV/s from −195 to +195 mV, and the data were digitized with a sampling rate of 20 Hz.

First, it raises new exciting perspectives concerning the carrier mechanism and the structure of the AAC, particularly in view of the abundant data concerning the carrier mechanism of this paradigm of mitochondrial carriers. Ca^{2+} induces the AAC to form a high conductance channel, whereas the normal transport mode of AAC does not require any cation. Since in this reconstituted system other mitochondrial proteins are largely eliminated, Ca^{2+} must affect the AAC directly. As a tempting speculation, we propose that the effect of Ca^{2+} is linked to cardiolipin molecules which we have shown to be bound in unusually high amounts to the AAC (Beyer & Klingenberg, 1985). Six cardiolipin molecules tightly bound and probably more loosely bound molecules (Drees & Beyer, 1988) are required for the normal AAC translocation activity (Hoffman et al., 1994). The negative charges of the cardiolipin headgroups are visualized to bind to the positive charges, in particular to lysines located in the AAC primarily at the matrix side (Bogner et al., 1986). At high concentrations Ca^{2+} binds to the two phosphate headgroups of cardiolipin and thus releases positive charges at the AAC interface (Figure 7). It is well documented in the literature that Ca^{2+} binds to cardiolipin and its affinity is higher than that of Mg^{2+} (Nielsen, 1971; Rand & Sengupta, 1972). Ca^{2+} accumulated in the mitochondria therefore will complex with cardiolipin of the matrix side and compete with the lysine groups of the AAC more efficiently than the normally present Mg^{2+}. The positive charge excess might trigger an intramolecular repulsion which opens the gates of the preformed translocation paths located either between the two subunits or within each subunit. The large conductance indicates that the channel is relatively wide which could be easier accommodated between the subunits.

This also concerns the question whether the channel uses the pathway built into the AAC for the ADP/ATP exchange. Due to the large size of ADP and ATP, this pathway must be comparatively wide which might be translated into the high conductance channel. In the open channel state the transport pathway should have lost the coordinated gating at both sides of the channel. Most interesting are the opposite effects of the specific inhibitors of AAC, CAT and BKA, on the open channel. The inhibition of the conductance by BKA in cooperation with ADP occurs in the same manner also for the ADP/ATP exchange (Erdelt et al., 1972; Klingenberg & Buchholz, 1973). ADP transforms the AAC and then BKA fixes the AAC in the *m*-state. CAT inhibits the transport by fixing the carrier in the opposite *c*-state. However, CAT does not inhibit the conductance of the channel. It even stabilizes against the closure by BKA, which shows that it is bound to the AAC. This resembles the opening by atractyloside of the AAC in mitochondria for the leakage of adenine nucleotides and cations (Meisner & Klingenberg, 1968; Panov et al., 1980). The effect of CAT seems to argue against the identity of the translocation path and the open channel. Further work is necessary to

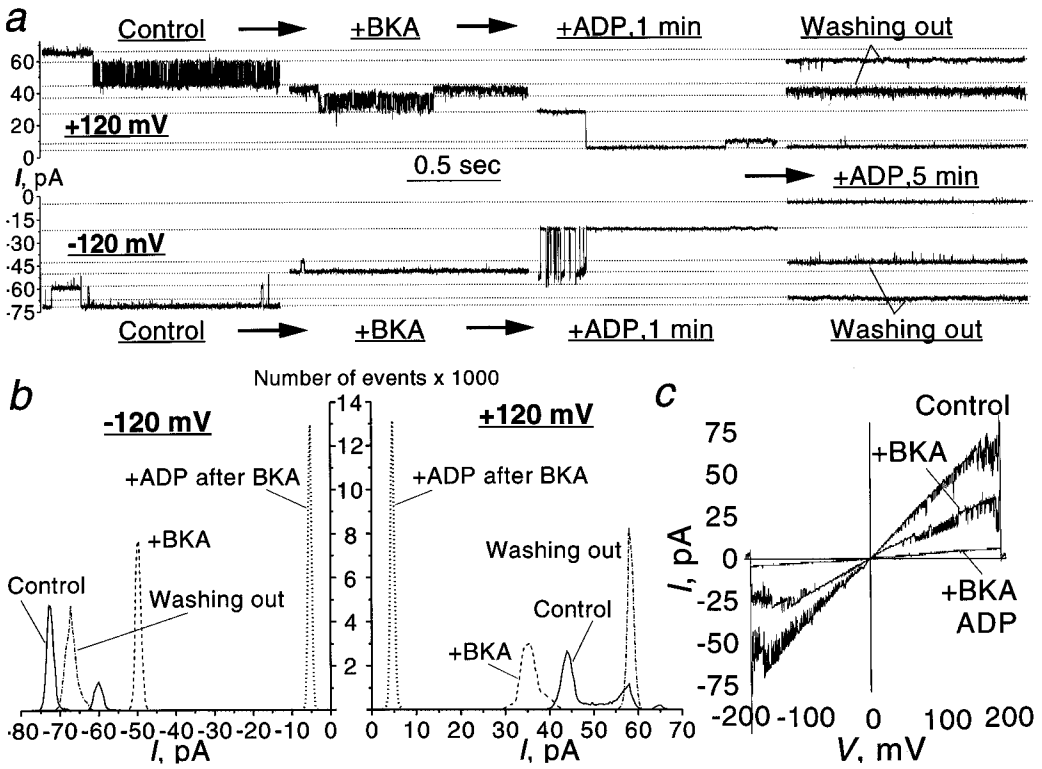

FIGURE 5: Inhibition of the channel conductance by the AAC specific inhibitor bongkrekic acid (BKA) and ADP. (a) The single-channel current recordings were obtained in two separate experiments both in the absence and in the presence of BKA (10 μM) and ADP (0.5 mM). BKA and ADP were added in the bath solution under very gentle stirring. The holding voltage was ±120 mV. The dotted lines indicate substates of the channel conductance. (*b*) The current amplitude distributions for 10 s of the recordings in the absence and in the presence of BKA (10 μM) and ADP (0.5 mM). (c) The voltage ramp protocols, obtained with and without BKA (10 μM) and ADP (0.5 mM); the holding potential was automatically changed in the form of ramp wave at a linear rate of 4 mV/s from −195 to +195 mV, and the data were digitized with a sampling rate 20 Hz.

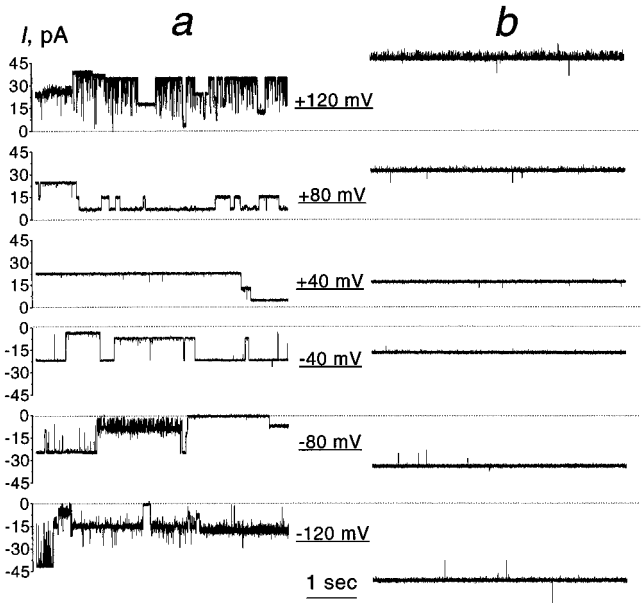

FIGURE 6: Representative single-channel current recordings with two different channels detected in excised patches of the AAC reconstituted giant vesicles. (a) Current recordings of a VDAC-resembling channel assigned to porin. (b) Current recordings due to the AAC-linked channel under the same holding voltages as in panel a.

interpret these striking results in terms of the structure/mechanism relationships in the AAC.

The second area for which these results have important consequences concerns the megachannel conductances in mitochondria. The present demonstration of the Ca^{2+}-dependent conversion of the AAC into a large lowly selective channel provides strong evidence that AAC is the conducting part of MTP. The main properties of the AAC channel reported here coincide with those of MTP, namely, Ca^{2+} dependence (Hunter & Haworth, 1979; Crompton et al., 1988; Broekemeier & Pfeiffer, 1989; Halestrap & Davidson, 1990), voltage gating (Bernardi, 1992), inhibition at low matrix pH (Petronilli et al., 1993), inhibition by BKA and ADP, and reactivation by CAT (Hunter & Haworth, 1979; Panov et al., 1980; LeQuoc & LeQuoc, 1988; Halestrap & Davidson, 1990). Most of these properties also resemble the "multiconductance channel" (Kinnally et al., 1989) or "megachannel" (Petronilli et al., 1989) found in patch clamp studies of mitochondria. Very recently a "multiconductance channel" was reported from AAC-deficient yeast mutant in giant liposomes reconstituted with mitochondrial inner membrane (Lohret et al., 1996). At variance with the conclusions derived from this work our results indicate that

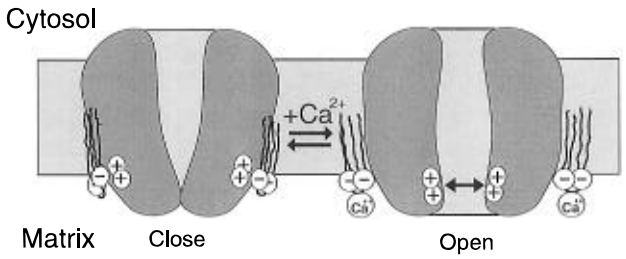

FIGURE 7: Tentative model for the Ca^{2+}-dependent conversion of the ADP/ATP carrier into a large unselective channel. The negatively charged headgroups of cardiolipin are shown with "−" and positively charged lysines of the AAC are shown with "+". For further explanations, see text.

the multiconductance channel activity in mammalian mitochondria can at least be partially due to the AAC.

In our reconstituted system the AAC channel should have been separated from the putative regulatory components, which might form the complex of MTP in mitochondria. For example, the cyclosporin A-binding protein cyclophilin (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991) was not present in our experiments, and thus the AAC channel had no sensitivity to cyclosporin A. So far we have ignored that besides Ca^{2+} also oxidants can induce MTP, probably in cooperation with Ca^{2+} (Chernyak et al., 1995). In this context, cysteinyl groups have been proposed to be involved in the voltage sensor of MTP (Petronilli et al., 1994a,b). With the channel in the isolated AAC at hand, it is now of keen interest to find out whether also the oxidative inducers of MTP opening are targeted to the AAC. At any rate, we believe to have proven that the AAC can be a key component in this physiologically and pathophysiologically important mitochondrial permeability phenomenon.

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