A Large Ca^{2+}-Dependent Channel Formed by Recombinant ADP/ATP Carrier from *Neurospora crassa* Resembles the Mitochondrial Permeability Transition Pore†

Nickolay Brustovetsky,‡ Maximilian Tropschug,§ Simone Heimpel, Doerthe Heidkämper, and Martin Klingenberg*

Institute for Physiological Biochemistry, University of Munich, Schillerstrasse 44, 80336 Munich, Federal Republic of Germany

Received January 8, 2002; Revised Manuscript Received July 8, 2002

ABSTRACT: Strong support for the central role of the ADP/ATP carrier (AAC) in the mitochondrial permeability transition (mPT) is provided by the single-channel current measurements in patch-clamp experiments with the isolated reconstituted AAC. In previous work [Brustovetsky, N., and Klingenberg, M. (1996) *Biochemistry 35*, 8483−8488], this technique was applied to the AAC isolated from bovine heart mitochondria. Here we used recombinant AAC (rAAC) from *Neurospora crassa* expressed in *E. coli*, since AAC from mammalian sources cannot be expresssed in *E. coli*. The rAAC is free from residual mitochondrial components which might associate with the AAC in preparation from bovine heart. Ca^{2+}-dependent channels with up to 600 pS are obtained, which are gated at >150 mV. The channel corresponds to a preferential matrix-outside orientation of rAAC in the patch membrane as shown with carboxyatractylate and a polar gating asymmetry. The channel is inhibited by ADP and bongkrekate, not by carboxyatractylate. Cyclophilin, isolated from *Neurospora crassa*, suppresses the gating, thus increasing conductivity at high positive voltage. Cyclosporin A abolishes the cyclophilin effect. ADP does not eliminate the cyclophilin effect but produces fast large-amplitude flickering of the channel without a stable decrease of the channel conductance. Also the pro-oxidant *tert*-butyl hydroperoxide reversibly suppresses voltage gating of the channel. The results show that the AAC can be a conducting component of the mPT pore, exhibiting similar characteristics as the mPT pore (response to Ca^{2+}, BKA, ADP), with a cyclophilin and pro-oxidant-sensitive gating at high voltage.

Upon Ca^{2+} overload and/or oxidative stress, the inner mitochondrial membrane (IMM)[1] can undergo a permeability transition (mPT) (*1, 2*). In this state, the membrane becomes permeable for solutes with a molecular mass up to 1.5 kDa. The mPT has been implicated in several types of cell injuries, such as the ischemia−reperfusion damage, in lethal cell injury by hydroperoxides (*3*−*5*), in programmed cell death, apoptosis (*6, 7*), and in glutamate-induced excitotoxicity (*9*−*12*).

Although it was originally thought that the pore involved in the mPT is formed by protein(s), its molecular identity was obscure [for a review, see (*1*)]. However, already in earlier experiments with isolated mitochondria, ligands of the mitochondrial ADP/ATP carrier (AAC) were shown to significantly modulate the mPT (*13*−*16*). Therefore, it was proposed that the AAC could be a critical constituent of the mPT (*15*−*17*).

Major support for the AAC as a central player in the mPT came from the patch-clamp experiments demonstrating that Ca^{2+} can reversibly activate a large channel of ≥500 pS in the membrane of vesicles containing the AAC extracted from the bovine heart mitochondria (*18*). The channel was inhibited by bongkrekate (BKA) and ADP, resembling the mPT pore, but was insensitive to carboxyatractylate (CAT). These results directly corroborated the central role of the AAC in the mPT.

In further developments, Ruck et al. (*19*) confirmed our results by using reconstituted AAC to demonstrate Ca^{2+}-induced release of several anions such as malate, ATP, and AMP loaded into the vesicles. ADP inhibited while atractyloside (ATR) and $HgCl_2$ induced permeability. In further experiments, possible involvement of hexokinase, creatine kinase, mitochondrial cyclophilin D (CyD), and porin was demonstrated (*20, 21*). This complex was also used to show an inhibitory effect of BCl-2 on the mPT (*22*) whereas BAX induced an ATR-sensitive channel (*23*). A cyclosporin-sensitive binding of CyD to the AAC was demonstrated with CyD, immobilized to Sepharose, and solubilized mitochondria (*24, 25*). Thus, the mPT pore may comprise several different proteins cooperating in the mPT pore formation and/or its regulation.

Whereas different proteins may participate in the regulation of the mPT pore, primarily the AAC is considered as a main

† This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

* To whom correspondence should be addressed. Phone: 49 89 5996473, fax: 49 89 5996473, e-mail: klingenberg@pbm.med.uni-muenchen.de.

‡ Present address: Department of Neuroscience, University of Minnesota, 6-145 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455. Phone: 612 626-2114, fax: 612 626-5009, e-mail: brous003@tc.umn.edu.

§ Present address: Institute for Biochemistry and Molecular Biology, University of Freiburg, Hermann Herder Strasse 7, D-79104 Freiburg, Germany. Phone: 49 761 2035244, fax: 49 761 2035253, e-mail: tropschug@uni-freiburg.de.

[1] Abbreviations: AAC, ADP/ATP carrier; rAAC, recombinant AAC expressed in *E. coli*; mPT, mitochondrial permeability transition; CyD, mitochondrial cyclophilin D from mammalian sources; CyN, cyclophilin from *N. crassa*; CsA, cyclosporin A; *t*-BOOH, *tert*-butyl hydroperoxide; CAT, carboxyatractyloside; BKA, bongkrekate; DTE, dithioerythritol; IMM, inner mitochondrial membrane; CL, cardiolipin.

10.1021/bi0200110 CCC: $22.00
Published on Web 09/04/2002

candidate for a conducting pathway of the mPT pore in the IMM. The AAC-linked channel found in our previous work (*18*) resembled the mPT-related "megachannel" detected earlier in electrophysiological experiments with mitoplasts (*26*, *27*). However, we failed to demonstrate one of the main features of the mPT pore—an inhibition by cyclosporin A (CsA)—which we attributed to a lack of CyD in the reconstituted system. Recent flux measurements in AAC-reconstituted proteoliposomes revealed that addition of CyD was necessary for simulation of the CsA-sensitive permeability transition in proteoliposomes (*25*, *28*). To investigate whether cyclophilin influences the AAC-linked channel leading to its sensitivity to CsA, here we used recombinant AAC (rAAC) of *Neurospora crassa* (*N. crassa*) expressed in inclusion bodies of *E. coli*. This approach allows us to exclude the possible influence of hypothetical mitochondrial proteinaceous cofactors which might accompany in small amounts the AAC isolated from the bovine heart mitochondria in our previous work (*18*).

While liver or heart mitochondria readily undergo the mPT following excessive Ca^{2+} uptake and/or oxidative stress, nothing is known about either the mPT in *N. crassa* mitochondria or the AAC involvement into this process. However, the present study was not aimed to examine the mPT in *N. crassa* mtiochondria or the role of the AAC in this phenomenon. A potential mPT in *N. crassa* mitochondria might depend not only on the AAC as a constituent of the inner membrane that provides a translocating pathway but also on the differences in the regulatory mechanisms and activity of the Ca^{2+} uptake system. On the other hand, the AAC from bovine heart mitochondria and from *N. crassa* are highly homologous proteins. Therefore, we believe that the AAC from *N. crassa* could be used as a model to study the AAC-linked channel regardless of whether mitochondria from *N. crassa* could undergo the classical mPT.

The results of the patch-clamp experiments presented in this paper extend those obtained with bovine heart AAC, producing channels with characteristics (size, voltage grating, responses to Ca^{2+}, ADP, BKA, CAT, *t*-BOOH, cyclophilin) similar to those associated with the mPT pore in mammalian mitochondria.

EXPERIMENTAL PROCEDURES

Materials. *N. crassa* AAC expressed in transformed *E. coli* cells was kindly given to us by Simone Heimpel and Gabriele Basset. Expression of the AAC from *N. crassa* has been chosen because expression of human and bovine AAC as well as AAC from *S. cerevisiae* have failed due to a codon usage which is unfavorable in *E. coli* (*29*). Transformation of *E. coli* cells with cDNA of *N. crassa* AAC, growth of these cells, isolation of AAC carrying inclusion bodies, solubilization, and purification by FPLC of the AAC were performed as described (*29*). Fractions containing primarily the rAAC were used for reconstitution into the giant liposomes suitable for patch-clamp experiments. Cyclophilin of *N. crassa* (CyN) was prepared from isolated mitochondria as previously described (*30*, *31*).

rAAC Reconstitution. Giant vesicles for patch-clamp were prepared by the dehydration−rehydration procedure (*18*, *32*) with some modifications. The purified rAAC was incorporated into the small unilamellar vesicles by a freeze−thawing procedure using liposomes prepared by sonication from the methanol−acetone-washed azolectin (type S-II, Sigma) (*33*). All solutions used for the rAAC reconstitution and in the dehydration−rehydration procedure were filtered through 0.2 μm Millipore filter to avoid bacterial contamination. The small rAAC-liposomes were diluted (1:10) with pure azolectin vesicles (50 mg/mL) prepared by sonication in 5 mM Tris buffer, pH 7.2. The mixture was diluted (1:10) with 100 mM KCl, 5 mM Hepes, pH 7.2. Then 7 μL of the diluted suspension was partially dehydrated under vacuum on a glass slide in a desiccator containing anhydrous $CaCl_2$ during 5 min at room temperature. The dehydrated liposomes were rehydrated with 10 μL of 50 mM KCl, 2.5 mM Hepes, pH 7.2, overnight at 4 °C. The resulting giant liposomes with incorporated rAAC (phospholipid/protein $\sim$ 1300) had diameters of 20−80 μm and were suitable for patch-clamp experiments.

Patch-Clamping. The patch-clamp experiments were performed as described earlier (*18*). The pipets for the microelectrodes were pulled from borosilicate glass tubings and then slightly heat-polished. The pipets were filled with standard bath solution containing 100 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM K-gluconate, 5 mM MES, 5 mM Tris at pH 7.4. The microelectrodes filled with standard solution had a resistance of 15−20 MΩ. To prevent interference of some compounds with the reference electrode, we used an agar bridge. The rAAC-containing giant vesicles (5 μL) were added to the experimental chamber of 3.0 mL volume, which then was filled with standard bath solution. In the experiments with CyN, we used a 0.2 mL chamber. To form a patch, a giant vesicle, attached to 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 EPC-7 amplifier (List Medical Instruments) using pClamp 6.0 software. The current signals were low-pass-filtered at 3.3 kHz and digitized at a sampling rate of 5 kHz. In all cases, the channels have been examined starting from a low holding potential (±20 mV). The sign of the holding voltage is referred to the pipet electrode. The data were analyzed using Axon's pClamp software.

RESULTS

Electrophysiological Channel Characteristics. Single-channel current measurements with patches derived from the rAAC-reconstituted proteoliposomes revealed a large channel resembling the AAC-linked channel found in our previous work (*18*). The main characteristics of the channel were analyzed to compare this channel and the AAC-linked channel obtained with the AAC from bovine heart mitochondria.

Figure 1 shows representative single-channel current recordings with a patch from the rAAC-reconstituted giant liposomes. The channel was in the open state without current fluctuations up to the holding voltage of 80−100 mV of both polarities, similar to that observed with bovine heart AAC (*18*). The mean conductance of the open state was 500−700 pS in symmetrical 100 mM KCl with several substates of conductance. At higher voltage, the channel revealed distinctly asymmetric behavior. At high positive voltage (160−200 mV), the channel conductance was dramatically

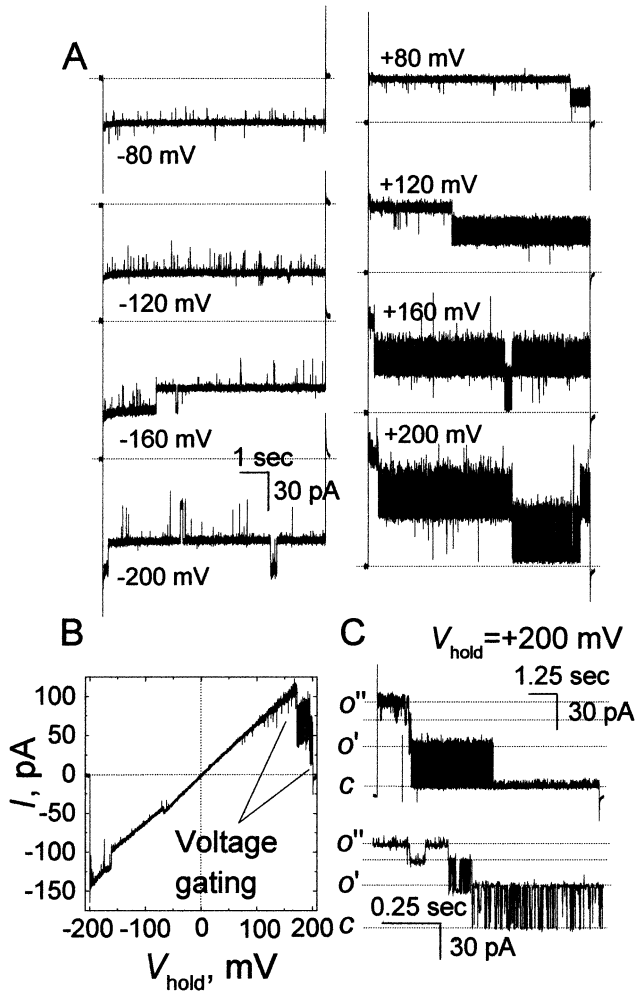

FIGURE 1: Representative single-channel current recordings of the excised patch from giant vesicles containing reconstituted rAAC (*Neurospora crassa*). (A) The holding voltage was applied as a step of 10 s duration. (B) Voltage ramp protocol, demonstrating pronounced voltage gating at high positive holding potential. The holding potential was automatically changed as a ramp wave at a linear rate of 20 mV/s from −200 to +200 mV. (C) Subconductance states of the rAAC-linked channel. The holding voltage (+200 mV) was applied as a 10 s step. The lower trace shows a part of the whole recording at higher time resolution.

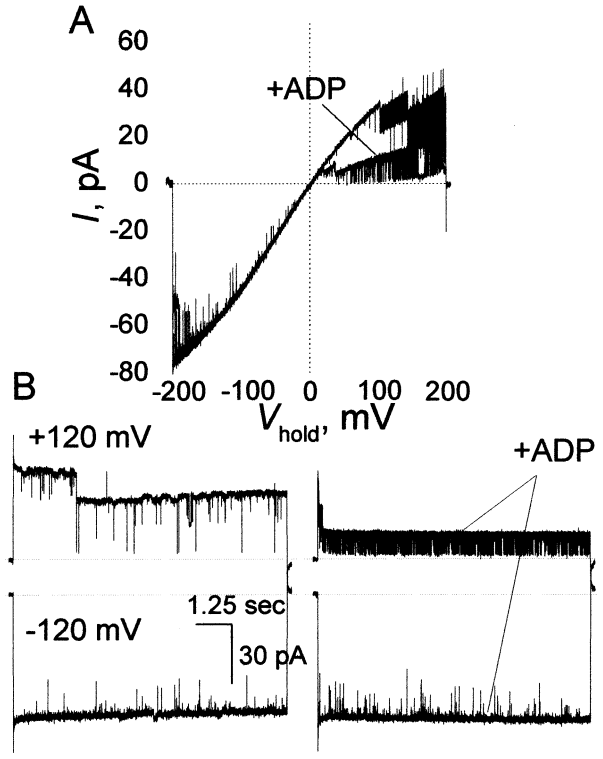

FIGURE 2: Inhibition of the rAAC-linked channel activity by ADP. (A) Two superimposed representative voltage ramp protocols obtained with and without ADP. Here and in panel B, 0.5 mM ADP was added into the bath solution 30 min prior to the recordings where it is indicated. In the voltage ramp protocol the holding potential was automatically changed in the form of a ramp wave at a linear rate of 20 mV/s from −200 to +200 mV. (B) Representative voltage step protocols obtained with and without ADP. In both cases, the inhibitory effect of ADP was observed only under positive holding voltage. Here and further, in the voltage step protocols the holding voltage (+200 mV) was applied as a 10 s step.

decreased (Figure 1). The transitions to the lower conductance substates were accompanied by current fluctuations with fast kinetics. At high negative holding potential, the conductance remained unchanged or decreased abruptly as a single-time event.

The distinct asymmetry of voltage gating is clearly illustrated in a voltage ramp protocol (Figure 1B). The induction of voltage gating is further analyzed in a voltage step protocol (Figure 1C) where a high holding potential (+200 mV) was applied as a rectangular pulse of 10 s duration. Pronounced voltage gating with intermediary fast transitions between half-open and closed states for about 4 s until the stable closed state was observed. The analysis of the recordings allowed us to distinguish the half-open state of the channel (*o′*), which was about 50% of the fully open state (*o″*). This could be due to the dimeric structure of the channel-forming protein since the native AAC has been shown to have a dimeric structure (*34*). Only in a few experiments (4 out of 45) have we observed fast current transitions occurring at high negative potential. A preferential, one-side-oriented incorporation of the rAAC into the membrane could explain this behavior, as discussed below.

To determine the cation/anion selectivity, voltage ramp protocols (from −100 to +100 mV) in symmetrical (100 mM KCl) and asymmetrical (20 mM KCl_{out}/100 mM KCl_{in}) solutions were obtained (not shown). From this we estimated a reversal potential (E_{rev}) of −12 mV corresponding to a low cation selectivity with a permeability ratio $P_{K^+}/P_{Cl^-} = 1.9$.

ADP−BKA. In previous work, we have demonstrated an inhibitory effect of ADP and bongkrekate (BKA) on the AAC-linked channel (*18*). This allowed us to definitely attribute the channel activity to the AAC. The channel formed by the rAAC from *N. crassa* also responded to ADP and BKA. ADP (0.5 mM) decreased the channel conductance under positive voltage whereas under negative holding potential ADP had no effect (Figure 2). Further, ADP shifted the gating voltage toward lower values and induced fast current flickering. Electrophoretic movements of ADP into the channel under positive voltage and its repulsion under negative voltage could be accountable for the observed asymmetry in the inhibitory effect of ADP.

The specific AAC ligand BKA (10 μM) also inhibited the channel activity but, different from ADP, symmetrically under positive and negative holding voltage (Figure 3). The difference between ADP and BKA effects could be explained by stronger binding of BKA to the AAC. Therefore, BKA

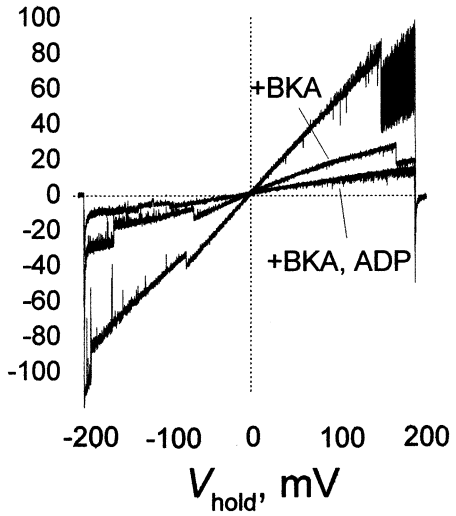

FIGURE 3: Inhibition of channel conductance by the AAC-specific inhibitor bongkrekate (BKA) and enhancement of the inhibition by ADP. BKA (10 μM) as well as ADP (0.5 mM) were added into the bath solution 30 min prior to recordings where it is indicated. The representative voltage ramp protocols obtained with and without inhibitors were superimposed.

upon binding to the AAC could presumably remain in this position regardless of the applied holding voltage. As in our previous work, the combined addition of ADP strengthened the effect of BKA, causing almost complete blockage of the channel. The cooperation between ADP of BKA was in line with the well-established role of ADP in facilitating the transition into the BKA binding "*m*"-state (*35*, *36*).

Previously we reported a reactivation of the channel activity, inhibited by BKA, on addition of carboxyatractylate (CAT) (*18*). In contrast to BKA, CAT is impermeant and can interact with the AAC only in cytosolic side-outside orientation. This feature of CAT, which reactivated 25% of the measured channels of the AAC from mitochondria, has been used to estimate the orientation of the AAC in the patch membrane (*18*). In the present work with rAAC, we failed to detect any reactivating effect of CAT, indicating that in this case we have mostly matrix side-out orientation of the AAC in the patch membrane.

Dependence on Ca^{2+}. Ca^{2+} is a main inducer of the mPT [for review, see (*1*)], and the rAAC-linked channel is activated by Ca^{2+}, similar to the channel with mitochondrial AAC. Addition of EGTA to bind Ca^{2+} in the bath solution (Figure 4A,B) or perfusion with Ca^{2+}-free bath solution (Figure 4C,D) drastically decreased the channel activity. The simultaneous decrease of pH in the bath solution following addition of EGTA could further enhance the inhibition of the channel in accordance with the pH influence on the mPT in de-energized mitochondria (*37*, *38*). The channel was fully reactivated by increasing both free [Ca^{2+}] and pH in the bath solution (Figure 4A). However, high [Ca^{2+}] alone was also sufficient to reactivate the channel after EGTA treatment (not shown). The channel closed after perfusion with Ca^{2+}-free bath solution could be reactivated by re-perfusion with Ca^{2+} (Figure 4C). Thus, the rAAC-linked channel appears to be Ca^{2+}-dependent.

The probability of channel detection significantly diminished with a decrease of Ca^{2+} concentration in both bath and pipet solutions. At 1 mM Ca^{2+} in 94 tested patches, 31 channels were detected; at 100 μM Ca^{2+}, 12 channels were

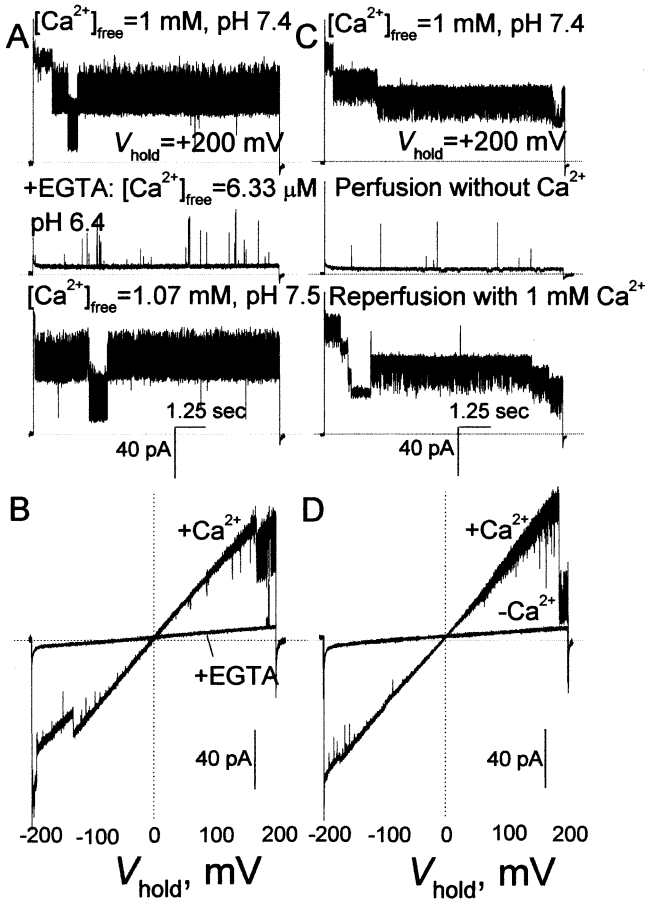

FIGURE 4: Reversible inactivation of the rAAC-linked channel due to removal of Ca^{2+} by EGTA (A,B) or by perfusion with Ca^{2+}-free bath solution (B,D). The representative voltage step and voltage ramp protocols (10 s each) were obtained with two different patches (panels A and B versus panels C and D). Where indicated, addition of 2 mM EGTA decreased $[Ca^{2+}]_{free}$ to 6.33 μM and shifted the pH to 6.4 in the bath solution. $[Ca^{2+}]_{free}$ has been calculated as described earlier (*58*). The pH in the bath solution was controlled by a miniature pH-electrode connected to a pH-meter. Restoration of high $[Ca^{2+}]_{free}$ and pH following addition of $CaCl_2$ and KOH into the bath solution reactivated the channel. In panels C and D, the channel was reversibly inactivated following 10 min perfusion with Ca^{2+}-free solution. Reperfusion with standard bath solution containing 1 mM Ca^{2+} caused reactivation of the channel within 5−10 min.

detected in 71 patches; and at 10 μM Ca^{2+}, only 2 channels were detected in 68 patches. Without Ca^{2+} in the bath solution, not 1 of the 57 examined patches demonstrated the channel activity. The channels, detected at various Ca^{2+} concentrations, revealed similar behavior. Thus, variations in the Ca^{2+} concentration influenced the probability of the channel detection but not the channel properties.

Oxidative Stress. Pro-oxidants promote mPT formation. Here we show that the pro-oxidant *tert*-butyl hydroperoxide (*t*-BOOH) reversibly influences the channel behavior of the reconstituted rAAC. The representative current traces obtained in voltage ramp as well as voltage step protocols with and without *t*-BOOH are shown in Figure 5. Treatment with *t*-BOOH (0.5 mM) locked the channel in the open state without fast flickering and transitions to the lower conductance substate under high positive holding potential. This effect could be reversed by dithioerythritol (DTE); 2 mM DTE added after *t*-BOOH restored voltage gating and fast current transitions between conductance substates (Figure 5).

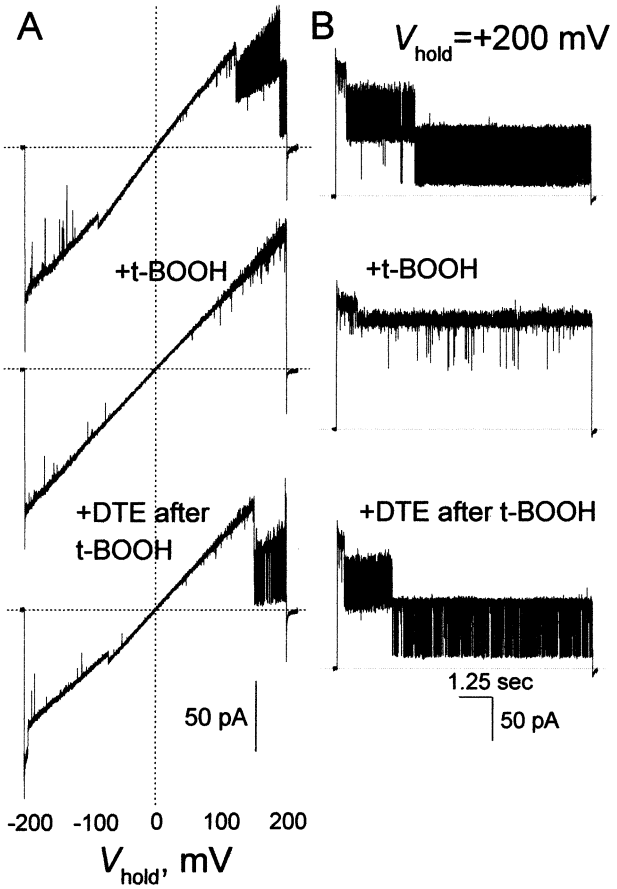

FIGURE 5: Prevention of voltage gating at high positive holding potential by pretreatment of the patch with *tert*-butyl hydroperoxide (*t*-BOOH). The representative voltage ramp and voltage step protocols (10 s) were obtained with the same patch. Where indicated, 0.5 mM *t*-BOOH and then 2 mM dithioerythritol (DTE) were added into the bath solution 30 min prior to recordings.

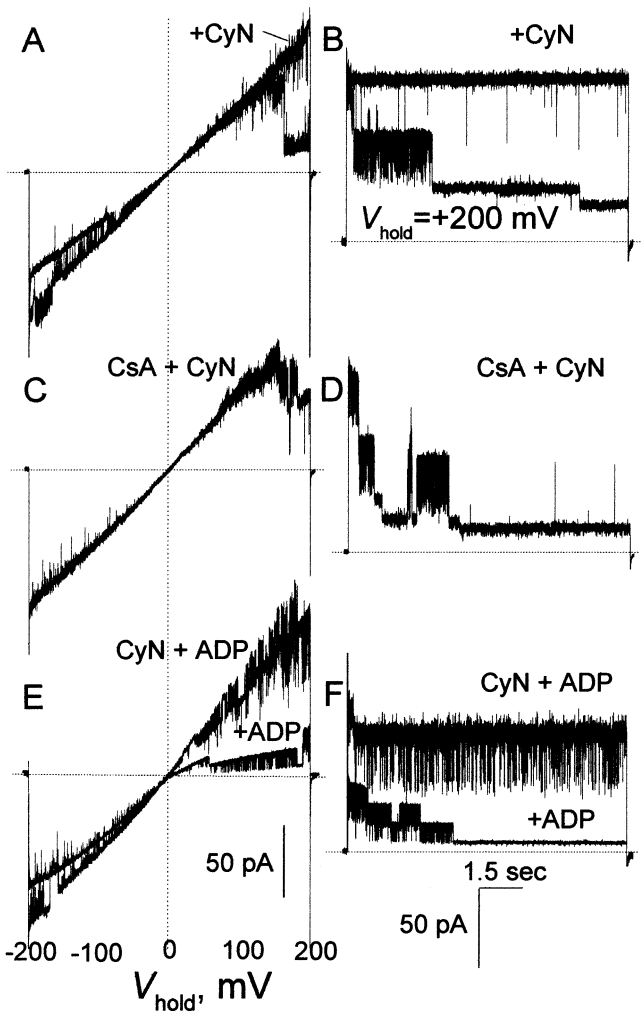

FIGURE 6: Suppression of voltage gating at high positive holding potential by cyclophilin (CyN) from *Neurospora crassa* (A,B). Cyclosporin A (CsA) abolishes the CyN effect (C,D). CyN prevents inhibition of the rAAC-linked channel induced by ADP (E,F). (A,B) Representative voltage ramp (20 s duration, 20 mV/s linear rate of voltage change, −200 − +200 mV range) and voltage step protocols obtained with and without CyN. CyN (3 μg/mL) was added 10 min prior to recordings. (C,D) Representative voltage ramp and voltage step protocols obtained in the presence of both CyN (3 μg/mL) and CsA (1 μM). CyN and CsA were added into the bath solution 10 min prior to recordings. (E,F) Representative voltage ramp and voltage step protocols obtained with CyN and ADP. ADP (0.5 mM) alone or in a combination with CyN (3 μg/mL) was added 10 min prior to recordings.

DTE, added without *t*-BOOH, did not influence the channel activity (not shown).

Cyclophilin. Although our previous patch-clamp experiments suggested that the AAC-linked channel might be a key component in the mPT pore (*18*), the channel did not respond to CsA, a potent inhibitor of the mPT. This is to be expected since CyD, the target for CsA, should have been lost during purification and reconstitution of the bovine heart AAC. On the other hand, binding of CyD to the AAC in mitochondria had been considered necessary for the opening of the channel. Here we used purified CyN isolated from *N. crassa* to test whether it can influence the channel behavior and induce the sensitivity of the channel to CsA. In *N. crassa*, only one form of CyN is present both in cytosol and in mitchondria (*31*). Figure 6 demonstrates representative voltage ramp (A) and voltage step (B) protocols with and without CyN and the inhibitory effect of CsA (C,D). CyN (3 μg/mL) added into the bath solution increased the probabaility of the channel detection. In the presence of CyN at 1 mM Ca^{2+}, in 31 tested patches 17 channels were detected (54.8% of patches compared to 33.0% in the absence of CyN). At the same time, CyD decreased the probability of voltage gating under high positive holding potential (Figure 6A,B). In the voltage step protocol, where high voltage was applied for a longer period of time, the effect of CyN was more pronounced (Figure 6B). However, the effect of CyP usually could be detected only in the first 20−25 min after addition of CyN. The cause for that is unclear. As expected, CsA added alone had no effect on the channel activity (not shown). But importantly, CsA added together with CyN prevented suppression of voltage gating (Figure 6C,D). Whether CsA could reverse the CyN effect remains unclear. Since the effect of CyN in our experiments was transient, we could not distinguish between CsA-induced reversal of the CyN effect and its spontaneous disappearance.

Normally, mitochondria contain millimolar concentrations of ADP and ATP. A combination of ADP and CsA has been reported to be required for the inhibition of the mPT pore (*39*). Therefore, we examined the effect of ADP on CyN-induced suppression of voltage gating. (Figure 6E,F). ADP alone (0.5 mM) inhibited the rAAC-linked channel, causing rapid flickering already under a moderate positive voltage (Figures 2 and 6E,F). CyN (3 μg/mL), added with ADP,

largely absorbed the inhibition by switching the channel into the higher level of conductance. However, the amplitude of flickering is significantly increased, whereas CyN alone locked the channel in the open state almost without flickering (Figure 6A,B). Thus, in the presence of CyN, the inhibitory tendency of ADP produces only fast, large-amplitude channel flickering without realizing the stable transition to the lower substate of conductance, as in the absence of CyN.

DISCUSSION

The ability to reconstitute the rAAC expressed in inclusion bodies of *E. coli* opens new possibilities to gain further support for the central role of the AAC in the mPT. Previous patch-clamp studies with reconstituted AAC from bovine heart mitochondria showed for the first time that AAC alone is competent for a large channel activity with typical characteristics of the mPT pore as studied on mitochondria (*18*). However, traces of the mitochondrial components that might be co-purified with the AAC derived from heart mitochondria could be sufficient to produce positive "hits" in single-channel patch-clamp experiments. In view of the ongoing discussion whether the mPT pore requires a complex of other components associated with AAC, we made use of the guaranteed absence of other mitochondrial components in the preparations of the rAAC.

Due to unfavorable codon usage by the AAC, the expression of bovine AAC was not possible in *E. coli* (*29*) and thus we had to resort to AAC from *Neurospora crassa*. However, *N. crassa* mitochondria do not display the mPT (unpublished experiments), probably solely because of the absence of a Ca^{2+} uptake system (*40*). Since the present study is aimed at elucidating the components for pore formation rather than the whole complex of the mPT phenomenon, the recombinant AAC (rAAC) from *N. crassa* might provide a valuable model for the pore opening in the mPT. This is confirmed by the finding that the currents obtained with rAAC fulfilled the basic criteria of AAC specificity as established previously, i.e., activation by Ca^{2+} and inhibition by ADP and BKA. Several other features are also similar, i.e., the high conductivity of up to 700 pS and the tendency for gating at a voltage >160 mV between full open and half-open states. These characteristics also resemble those of the "megachannel" with a high conductance found by patch-clamp of mitochondria (*26, 27*) and a decreased probability of the mPT pore opening at high potential (*37, 38*).

The existence of a complex comprising the AAC, CyD, VDAC (porin), BAX, and hexo- and creatine kinases was thought to be instrumental for the mPT (*20, 21*). However, the composition of the putative pore complex has not been well-defined. Subsequently, the same authors showed that isolated reconstituted AAC can form a pore for the diffusion of malate etc. in the presence of high Ca^{2+} (*19*). More stringently than with the AAC derived from bovine heart mitochondria, the present results demonstrate that challenged by high Ca^{2+}, AAC alone, without adherent components, can form a "megachannel" typical for the mPT and indicate that Ca^{2+}-modified AAC can be a conducting part of the mPT pore in the IMM. Also, interaction with CyD required only purified AAC and not a complex, as claimed by other groups (*24, 41, 42*). Although a regulatory role of additional components on the mPT pore seems quite possible, existing data do not support their direct involvement in the formation

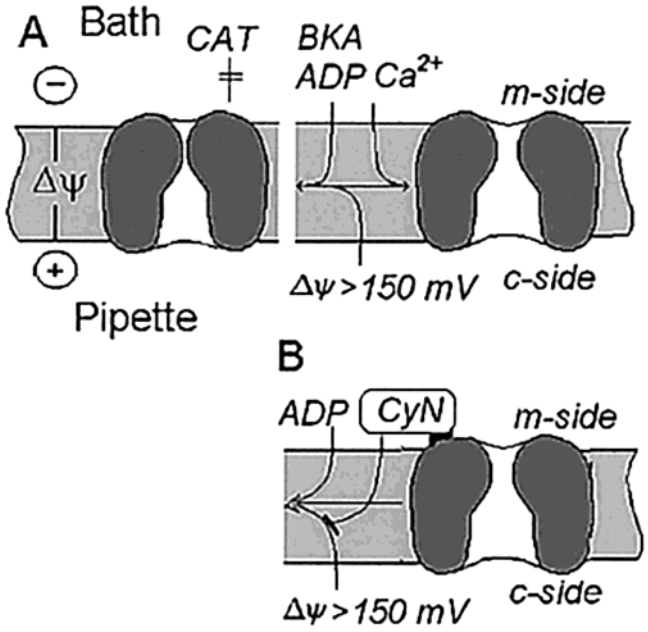

FIGURE 7: Scheme illustrating the orientation of the AAC in the patch membrane and the influence of some effectors on the channel opening. The AAC is oriented with the matrix (m) side to the bath solution. In this orientation, CAT cannot interact with the c-side of the AAC from the bath, and Ca^{2+} required on the m-side activates from the bath but not from the pipet volume. The observed polarity asymmetry of the high voltage gating ($\Delta\psi \geq 150$ mV) corresponds to the physiological polarity of positive voltage on the c-side (pipet volume). Part B illustrates the interaction of cyclophilin (CyN) with the m-side of the AAC. By interacting with UCP, CyN inhibits the high voltage gating ($\Delta\psi \geq 150$ mV), i.e., the transition into the closed state. CyN exerts this effect with and without the amplification of voltage gating by ADP.

of the conducting pathway in the IMM. BKA proved here and in other studies to be the most stringent and specific inhibitor of the AAC-linked channel (*6, 18*). Only in combination with ADP, it fully inhibits channel activity, in line with the cooperative effects of BKA and ADP or ATP on the ADP/ATP exchange, on the transformation of the AAC from the *c* into the *m* state, and on the binding of BKA to the AAC (*35, 36*). Notably, BKA appears to be an efficient blocker of the mPT-associated apoptosis (*7, 12*). Overexpression of the human AAC-1 (but not AAC-2) led to all features of apoptosis which could be prevented by BKA (*43*). These observations demonstrate a crucial role of the AAC in mPT-associated apoptosis.

Asymmetric Gating and Orientation of rAAC. Whereas with mitochondrial AAC the gating of the single channels was observed at both negative and positive voltage (*18*), with rAAC it occurred strongly preferred only at positive voltage. Since CAT is membrane-impermeable and reacts with the AAC only from the cytosolic side, the lack of activation by CAT indicates that the rAAC is inserted mostly in the matrix side-out orientation. In this orientation of the rAAC, the positive holding voltage inside of the electrode corresponds to the physiological situation in mitochondria with a membrane potential positive on the cytosolic side (see scheme in Figure 7).

The pronounced voltage gating of the rAAC-linked channel upon "physiologically oriented" positive holding potential correlates with a decreased probability of opening of the mPT pore in mitochondria upon high potential. This feature of

the AAC may translate into physiologically important behavior of the AAC in the cell. At low Ca^{2+} level, a high mitochondrial potential ensures the closed state of the intrinsic "megachannel" of the AAC, significantly diminishing a probability of its opening. Thus, the observed voltage gating of the AAC channel seems to have important physiological implications for the regulation of the mPT by maintaining the impermeable state of the inner memebrane.

Activation by Ca^{2+}. Ca^{2+} is the principle activator of the mPT pore. It also opens the megachannel of the reconstituted rAAC. The channel activity can be inhibited by EGTA or on removing Ca^{2+} by perfusion with Ca^{2+}-free solution as in the previous work (*18*). Mitochondria, treated with ruthenium red, proved to be resistant even to high Ca^{2+} level in the incubation medium; e.g., interaction of high Ca^{2+} with the AAC from the cytosolic side did not induce the mPT (*1*). Assuming preferential matrix side-out orientation of rAAC in the patch membrane, this can explain why high Ca^{2+} in the pipet solution could not preclude channel closure after removal of Ca^{2+} from the bath solution (see Figure 7). Once the rAAC-linked channel was sealed following Ca^{2+} sequestration by EGTA or perfusion with Ca^{2+}-free bath solution, high Ca^{2+} in the pipet solution could not re-open the AAC-linked channel.

High Ca^{2+} used to detect the rAAC-linked channel seems to be incompatible with physiological conditions. However, channels with similar characteristic could also be detected at lower Ca^{2+}, although with less probability of detection: at 1 M Ca^{2+}, in 33% of the patches; at 0.1 mM Ca^{2+} , in 8.5%; and at 0.01 mM Ca^{2+}, in 3%. Higher Ca^{2+} concentration increased the probability of channel detection without altering the channel properties. The different Ca^{2+} concentration dependence of the mPT is expected on the basis of observing single-molecule events in patch-clamp versus an estimated 10^{22} channels acting in a mPT of 1 mg of mitochondrial protein. Further, in mitochondria additional factors such as ROS, lipid peroxidation, and free fatty acids as well as possible interaction of the AAC with other proteins might decrease the Ca^{2+} threshold for channel activation. Thus, in mitochondria the additional factors could dramatically decrease the required Ca^{2+} concentration, whereas in the guaranteed absence of those factors with reconstituted rAAC, Ca^{2+} concentration primarily influenced the probability of channel occurrence.

Activation by Oxidative Stress. Pro-oxidants are well-established activators of the mPT [see review (*2*)]. Here we show that *t*-BOOH is able to prevent voltage gating, keeping the rAAC in the wide-open "megachannel" state even at high holding potential. These results give additional support to the role of the AAC in the mPT, for which pro-oxidants are major inducers. The reversibility by DTE indicates that SH groups are involved. The role of SH groups in pore opening of the mitochondrial AAC and also of other mitochondrial anion carriers had been observed previously by Dierks et al. using $HgCl_2$ (*44, 45*). Halestrap et al. (*4*), by showing that the pro-oxidants diamide, phenylarsine oxide, and *t*-BOOH counteract the inhibition of the mPT by ADP, suggest that the affinity of ADP for the AAC is lowered by pro-oxidants. Recently, NO, peroxynitrite, and 4-hydroxynonenal as well as the photosensitizer verteporfin were reported to permeabilize reconstituted AAC (*47, 48*). Oxidation of critical thiols of the AAC favors the mPT and apoptosis (*46*−*48*). Strikingly, photoinactivation of the AAC coincided with the prevention of the mPT pore opening by Ca^{2+} and P_i (*49*).

The outstanding susceptibility of the AAC to oxidation by *t*-BOOH was demonstrated by Giron-Calle and Schmid (*50*). On exposure of mitochondria to *t*-BOOH, the AAC was found to be the only protein with a marked increase of the molecular mass in SDS gels. According to amino acid analysis, a large portion of lysines seemed to have formed adducts with oxidized phospholipids. These findings were related to the exceptionally high binding of cardiolipin (CL) to the AAC (*51*). As CL contains a high amount of polyunsaturated fatty acid and the AAC is rich in lysines, the generation of reactive lipids directly at the surface of the AAC may lead to the massive formation of lysine adducts. In the present case, on short time exposure to *t*-BOOH, only SH groups seem to be involved, indicating that the oxidative reaction of the AAC progresses in stages until the irreversible alteration due to the formation of the adducts.

The interaction of the AAC with an unusual high number of CL molecules is visualized to facilitate the large conformational changes and gating during the ADP/ATP translocation (*52*). The disturbance of the CL−AAC interaction by the specific Ca^{2+} binding (*53*) to the CL headgroups has been previously proposed (*18*) to cause the wide opening to the ADP/ATP translocation channel, by abolishing ionic bonds between CL and lysines necessary for normal AAC gating. The repulsion of the free lysines causes the AAC translocation path to open and abolish the gating. A similar sequence of events involving CL is proposed for the activation by *t*-BOOH. According to this scenario, the impact on the AAC both of Ca^{2+} and of pro-oxidant is based on the particular CL−AAC relationship as the cause for the mPT pore formation.

Cyclophilin. Inhibition by CsA has been widely regarded as a hallmark of the mPT. It had been shown that the mPT formation correlates to the binding of CyD to the inner membrane and that CsA causes unbinding of CyD (*54*). More specifically, Woodfield and Crompton (*24, 25*) provided evidence that CyD binds to the AAC contained in the mPT pore complex. Pro-oxidants appear to increase binding of CyD, and this is regarded to be the reason for the increased sensitivity to Ca^{2+}. The inability of CsA in our previous experiments to inhibit the AAC-linked channel was attributed to the absence of CyD and indicated that CyD might be not obligatory for channel opening (*18*). In the meantime, it was stressed that high matrix [Ca^{2+}] can induce a CsA-insensitive mPT, and as a result, a more accessory role has been attributed to CyD in the mPT (*55*−*57*).

As shown here, the rAAC alone, containing no trace of CyN, was capable of forming a wide pore. With the present preparation of rAAC originating from *N. crassa*, we were able to apply a cyclophilin (CyN) from the same origin to study its influence on the channel. CyN increased the probability of channel detection. Thus, CyN could facilitate the Ca^{2+}-induced conversion of the rAAC into a large channel. CyN applied to the matrix side clearly stabilized the opening of the channel against gating at voltage >150 mV with positive polarity and thus increased the conductivity nearly 2-fold. Interestingly, the effect of CyN on increasing the conductivity was more dramatic in the presence of ADP, which would correspond to the physiological situation (see

Figure 7B). In this case, the presence of ADP causes fast, large-amplitude flickering of the channel opened by CyN. Consistent with the unbinding of CyN on formation of the CsA−CyN complex (*54*, *55*), CsA did not inhibit the basic channel activity, but abolished only the stimulatory effect of CyN, obviously by sequestering CyN. Under our conditions, high concentration of Ca^{2+} may further contribute to the partial independence from CyN, in accordance with reports that the mPT pore becomes insensitive to CsA at higher Ca^{2+} concentration (*39*, *55*−*57*). It seems that in this environment CyN binds only loosely to the AAC.

Thus, our results show for the first time that CyN can enhance directly the AAC-linked channel activity in the absence of other potential mPT components, and that CsA reverses this CyN effect. The results also show that CyN targets the potentially physiological voltage gating mechanism, significantly increasing conductance of the rAAC-linked channel under high positive voltage. This underscores a mechanism of regulation of the mPT pore in which cyclophilin has a modulating but no participating role in the conductivity of the AAC channel and thus in the mPT pore formation.

REFERENCES

1. Zoratti, M., and Szabo, I. (1995) *Biochim. Biophys. Acta 1241*, 139−176.
2. Bernardi, P., Colonna, R., Costantini, P., Eriksson, O., Fontaine, E., Ichas, F., Massari, S., Nicolli, A., Petronilli, V., and Scorrano, L. (1998) *Biofactors 8*, 273−281.
3. Griffiths, E. J., and Halestrap, A. P. (1995) *Biochem. J. 307*, 93−98.
4. Halestrap, A. P., Woodfield, K. Y., and Connern, C. P. (1997) *J. Biol. Chem. 272*, 3346−3354.
5. Nieminen, A. L., Saylor, A. K., Tesfai, S. A., Herman, B., and Lemasters, J. J. (1995) *Biochem. J. 307*, 99−106.
6. Zamzami, N., Susin, S. A., Marchetti, P., Hirsch, T., Gomez-Monterrey, I., Castedo, M., and Kroemer, G. (1996) *J. Exp. Med. 183*, 1533−1544.
7. Zamzami, N., Marchetti, P., Castedo, M., Hirsch, T., Susin, S. A., Masse, B., and Kroemer, G. (1996b) *FEBS Lett. 384*, 53−57.
8. Kroemer, G., Dallaporta, B., and Resche-Rigon, M. (1998) *Annu. Rev. Physiol. 60*, 619−642.
9. Ankarcrona, M., Dypbukt, J. M., Orrenius, S., and Nicotera, P. (1996) *FEBS Lett. 394*, 321−324.
10. Schinder, A. F., Olson, E. C., Spitzer, N. C., and Montal, M. (1996) *J. Neurosci. 16*, 6125−6133.
11. White, R. J., and Reynolds, I. J. (1996) *J. Neurosci. 16*, 5688−5697.
12. Budd, S. L., Tenneti, L., Lishnak, T., and Lipton, S. A. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 6161−6166.
13. Hunter, D. R., and Haworth, R. A. (1979) *Arch. Biochem. Biophys. 195*, 453−459.
14. Panov, A., Filippova, S., and Lyakhovich, V. (1980) *Arch. Biochem. Biophys. 199*, 420−426.
15. LeQuoc, K., and LeQuoc, D. (1988) *Arch. Biochem. Biophys. 265*, 249−257.
16. Halestrap, A. P., and Davidson, A. M. (1990) *Biochem. J. 268*, 153−160.
17. Haworth, R. A., and Hunter, D. R. (2000) *J. Bioenerg. Biomembr. 32*, 91−96.
18. Brustovetsky, N., and Klingenberg, M. (1996) *Biochemistry 35*, 8483−8488.
19. Ruck, A., Dolder, M., Walliman, T., and Brdiczka, D. (1998) *FEBS Lett. 426*, 97−101.
20. Beutner, G., Ruck, A., Riede, B., Welte, W., and Brdiczka, D. (1996) *FEBS Lett. 396*, 189−195.
21. Beutner, G., Ruck, A., Riede, B., and Brdiczka, D. (1998) *Biochim. Biophys. Acta 1368*, 7−18.
22. Marzo, I., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Vieira, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. (1998) *Science 281*, 2027−2031.
23. Marzo, I., Brenner, C., Zamzami, N., Susin, S. A., Beutner, G., Brdiczka, D., Remy, R., Xie, Z. H., Reed, J. C., and Kroemer, G. (1998) *J. Exp. Med. 87*, 1261−1271.
24. Woodfield, K., Ruck, A., Brdiczka, D., and Halestrap, A. P. (1998) *Biochem. J. 336*, 287−290.
25. Crompton, M., Virji, S., and Ward, J. M. (1998) *Eur. J. Biochem. 258*, 729−735.
26. Zorov, D. B., Kinnaly, K. W., and Tedeschi, H. (1992) *J. Bioenerg. Biomembr. 24*, 119−124.
27. Szabo, I., and Zoratti, M. (1991) *J. Biol. Chem. 266*, 3376−3379.
28. Halestrap, A. P., Doran, E., Gillespie, J. P., and O'Toole, A. (2000) *Biochem. Soc. Trans. 28*, 170−177.
29. Heimpel, S., Basset, G., Odoy, S., and Klingenberg, M. (2001) *J. Biol. Chem. 276*, 11499−11506.
30. Tropschug, M., Wachter, E., Mayer, S., Schonbrunner, E. R., and Schmid, F. X. (1990) *Nature 346*, 674−677.
31. Tropschug, M., Nicholson, D. W., Hartl, F.-U., Koehler, H., Pfanner, N., Wachter, E., and Neupert, W. (1988) *J. Biol. Chem. 263*, 14433−14440.
32. Criado, M., and Keller, B. U. (1987) *FEBS Lett. 224*, 172−176.
33. Labarca, P., Coronado, R., and Miller, C. (1980) *J. Gen. Physiol. 76*, 397−404.
34. Klingenberg, M. (1985) in *Bioenergetics of Electron and Proton Transport* (Martonosi, A. N., Ed.) pp 511−551, Plenum Press, New York and London.
35. Erdelt, H., Weidemann, M. J., Buchholz, M., and Klingenberg, M. (1972) *Eur. J. Biochem. 30*, 107−122.
36. Klingenberg, M., and Buchholz, M. (1973) *Eur. J. Biochem. 38*, 346−358.
37. Nicolli, A., Petronilli, V., and Bernardi, P. (1993) *Biochemistry 32*, 4461−4465.
38. Petronilli, V., Cola, C., and Bernardi, P. (1993) *J. Biol. Chem. 268*, 1011−1016.
39. Novgorodov, S. A., Gudz, T. I., Milgrom, Y. M., and Brierly, G. P. (1992) *J. Biol. Chem. 267*, 16274−16282.
40. Carafoli, E., and Lehninger, A. L. (1971) *Biochem. J. 122*, 681−690.
41. Bernardi, P. (1992) *J. Biol. Chem. 267*, 8834−8839.
42. Crompton, M., and Andreeva, L. (1994) *Biochem. J. 302*, 181−185.
43. Bauer, M. K. A., Schubert, A., Rocks, O., and Grimm, S. (1999) *J. Cell Biol. 147*, 1493−1502.
44. Dierks, T., Salentin, A., Heberger, C., and Kramer, R. (1990a) *Biochim. Biophys. Acta 1028*, 268−280.
45. Dierks, T., Salentin, A., and Kramer, R. (1990b) *Biochim. Biophys. Acta 1028*, 281−288.
46. Vieira, H. L., Belzacq, A. S., Haouzi, D., Bernassola, F., Cohen, I., Jacotot, E., Ferri, K. F., El Hamel, C., Bartle, L. M., Melino, G., Brenner, C., Goldmacher, V., and Kroemer, G. (2001) *Oncogene 20*, 4305−4316.
47. Belzacq, A. S., Jacotot, E., Vieira, H. L., Mistro, D., Granville, D. J., Xie, Z., Reed, J. C., Kroemer, G., and Brenner, C. (2001) *Cancer Res. 61*, 1260−1264.
48. Costantini, P., Belzacq, A. S., Vieira, H. L., Larochette, N., de Pablo, M. A., Zamzami, N., Susin, S. A., Brenner, C., and Kroemer, G. (2000) *Oncogene 19*, 307−314.
49. Salet, C., Moreno, G., Ricchelli, F., and Bernardi, P. (1997) *J. Biol. Chem. 272*, 21938−21943.
50. Giron-Calle, J., and Schmid, H. H. (1996) *Biochemistry 35*, 15440−15446.
51. Beyer, K., and Klingenberg, M. (1985) *Biochemistry 24*, 3821−3826.
52. Hoffmann, B., Stöckl, A., Schlame, M., Beyer, K., and Klingenberg, M. (1994) *J. Biol. Chem. 269*, 1940−1944.
53. Maddaiah, V. T., and Kumbar, U. (1993) *J. Bioenerg. Biomembr. 25*, 419−427.
54. Nicolli, A., Basso, E., Petronilli, V., Wenger, R. M., and Bernardi, P. (1996) *J. Biol. Chem. 271*, 2185−2192.
55. Halestrap, A. P. (1999) *Biochem. Soc. Symp. 66*, 181−203.
56. Crompton, M., Virji, S., Doyle, V., Johnson, N., and Ward, J. M. (1999) *Biochem. Soc. Symp. 66*, 167−179.
57. Connern, C. P., and Halestrap, A. P. (1996) *Biochemistry 35*, 8172−8180.
58. Alekseev, A. E., Jovanovic, A., Lopez, J. R., and Terzic, A. (1996) *J. Mol. Cell. Cardiol. 28*, 1193−1202.

BI0200110