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Reconstitution of the Native Mitochondrial Outer Membrane in Planar Bilayers. Comparison with the Outer Membrane in a Patch Pipette and Effect of Aluminum Compounds

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Summary. Detergent-free rat brain outer mitochondrial membranes were incorporated in planar lipid bilayers in the presence of an osmotic gradient, and studied at high (1 M KCl) and low (150 mM KCl) ionic strength solutions. By comparison, the main outer mitochondrial membrane protein, VDAC, extracted from rat liver with Triton X-100, was also studied in 150 mM KCl. In 1 M KCl, brain outer membranes gave rise to electrical patterns which resembled very closely those widely described for detergent-extracted VDAC, with transitions to several subconducting states upon increase of the potential difference, and sensitivity to polyanion. The potential dependence of the conductance of the outer membrane, however, was steeper and the extent of closure higher than that observed previously for rat brain VDAC. In 150 mM KCl, bilayers containing only one channel had a conductance of 700 ± 23 pS for rat brain outer membranes, and 890 ± 29 pS for rat liver VDAC. Use of a fast time resolution setup allowed demonstration of open-close transitions in the millisecond range, which were independent of the salt concentration and of the protein origin. We also found that a potential difference higher than approx. ± 60 mV induced an almost irreversible decrease of the single channel conductance to few percentages of the full open state and a change in the ionic selectivity. These results show that the behavior of the outer mitochondrial membrane in planar bilayers is close to that detected with the patch clamp (Moran et al., 1992, *Eur. Biophys. J.* **20**:311–319).

The neurotoxicological action of aluminum was studied in single outer membrane channels from rat brain mitochondria. We found that μM concentrations of Al Cl_3 and aluminum lactate decreased the conductance by about 50%, when the applied potential difference was positive relative to the side of the metal addition.

Key Words outer mitochondrial membrane · channel · VDAC · planar bilayer · patch clamp · aluminum

Introduction

While the discovery of highly conducting ion channels in the inner membrane of mitochondria is fairly recent (Sorgato, Keller & Stühmer, 1987; *for recent reviews see* Moran & Sorgato, 1992; Sorgato & Moran, 1993), that of VDAC (voltage-dependent anion channel) in the outer membrane of mitochondria (OMM) goes back to the midseventies (Schein, Colombini & Finkelstein, 1976). In general, the electrophysiological characterization of this channel has been accomplished at high salt, after insertion in planar bilayers of a detergent-extracted fraction of the outer membrane (OM) (*for reviews see* Colombini, 1989; Benz, 1990). Such studies have shown that, regardless of the origin, VDAC properties are similar. At large these features are: a high conductance (4.0–4.5 nS in 1 M KCl) displayed at low values of the membrane potential difference (up to approx. ± 20 mV), the ability to close to several subconducting states upon increase of the potential difference (with a concomitant change of the ionic selectivity from anionic to cationic), and a slow kinetics. However, single channel recordings of the OMM with the patch-clamp technique, either *in situ* or in proteoliposomes, have provided another picture of the OM electrical properties (Moran et al., 1992). Channels of smaller conductance values (from less than 50 to around 300 pS in 150 mM KCl), and with faster kinetics of the open-close transitions, were found.

That VDAC represents the major permeability pathway for the OMM seems well established (Mannella, Forte & Colombini, 1992). Therefore, to exclude that methodological causes were at the basis of the discordant results, the detergent-untreated OMM (from rat brain) and the low ionic strength solution (150 mM KCl) of the patch-clamp experi-

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ments have been used with planar bilayers. This paper reports the results of such investigation, as well as data of the behavior of the native OM at high ionic strength (1 M KCl), and that of the detergent-extracted rat liver VDAC in the 150 mM KCl-containing medium. The data obtained show that, when in artificial bilayers, the native (detergent-untreated) OM can indeed display several of the electrical features typical of the detergent-extracted VDAC, but also those first recognized with the patch-clamp technique (Moran et al., 1992).

Various metal trihydroxides have been shown to interact with *Neurospora crassa* VDAC so as to affect strongly its voltage sensitivity (Dill, Holden & Colombini, 1987; Zhang & Colombini, 1989, 1990; Colombini, 1991). Due to the possible neurotoxicological action of aluminum (Al) (Van der Voet et al., 1991), we studied the effect of two Al (III) compounds (added asymmetrically with respect to the bilayer) on a single OMM channel from rat brain. Our results show that the channel had the tendency to remain in the open state at negative potential differences (relative to the side of aluminum addition), but only before imposition of positive voltages as low as +20 mV, +30 mV. Under the latter conditions, the channel conductance was reduced to approximately 50%, and this state did not change either at subsequent higher voltage steps or after the washing out of the aluminum-containing solution.

Materials and Methods

Chemicals

Hydroxyapatite (Bio-gel HTP) was purchased from Biorad, celite 535 from Roth, Triton X-100 from Serva and soybean asolectin (L-α-phosphatidylcholine, type II-S) from Sigma. Aluminum lactate was obtained from Fluka, aluminum chloride from Prolabo.

Isolation of the Outer Mitochondrial Membrane and of VDAC

The OM from nonsynaptosomal rat brain mitochondria was isolated and biochemically characterized as detailed in Moran et al. (1990, 1992). Small aliquots were stored at −80 °C in 250 mM mannitol, 50 mM HEPES (pH 7.2) until use. Electron microscopy of the preparation is shown elsewhere (Moran & Sorgato, 1992).

VDAC was purified from isolated rat liver mitochondria essentially as in De Pinto et al. (1987), and stored in small samples at −80°C, at a protein concentration of approx. 10–50 μg/ml. When necessary, samples were thawed at room temperature, and never refrozen.

Preparation of Proteoliposomes

The isolated OM was reconstituted in asolectin liposomes either by freezing and thawing or by dehydration-rehydration. Asolectin was purified in two steps; first, according to Labarca, Coronado and Miller (1980), then according to Kagawa and Racker (1971). For the former preparation of proteoliposomes, 180 μl asolectin liposomes (prepared according to Criado and Keller, 1987, with a starting concentration of 100 mg/ml) were mixed with 5–20 μl of the OM, containing 50–200 μg of protein. The mixture was then centrifuged in an Airfuge at maximal speed for 50 min, at 4°C, the pellet resuspended in 50 μl of 50 mM MOPS KOH (pH 7.2) and divided in small aliquots. These were subjected to two cycles of freezing (in liquid nitrogen) and thawing (at room temperature), then stored at −80°C. Aliquots were seldom stored directly at −80°C without the two freeze-thaw steps. When needed, samples were thawed, and never refrozen. Proteoliposomes, rendered giant by dehydration-rehydration steps, were prepared according to Criado and Keller (1987), and used within the day of formation.

Planar Bilayer Setup and Electrical Recording

The planar bilayer setup used was specifically designed to allow perfusion of one chamber in 3–5 sec (Zilberstein, 1989). Briefly, the *cis* chamber (of total volume 50 μl) was holed longitudinally into a parallel pipedPlexiglass block, to which the *trans* chamber was inserted perpendicularly through a hole of 2 mm. The *trans* chamber was made of a 1-cm long Teflon tubing (with an inner diameter of 500 μm and an outer diameter of 2 mm), connected to the measuring electrode through a 2-cm long silicon tubing (with an inner diameter of 2 mm). The resulting *trans* chamber total volume was approximately 50 μl. The ground electrode was inserted to one end of the *cis* chamber. Through a second hole, perpendicular also to the *trans* chamber, the perfusing medium could be dropped into the *cis* chamber, the perfusate dropping out at the other end. Obviously, this construction allowed perfusion of (hence additions to) only the *cis* chamber.

Agar-agar AgCl-Ag electrodes were used. Potential difference values are defined as *trans* chamber minus *cis* chamber potentials. Any voltage step was imposed starting from 0 mV. Between two successive steps, the bilayer was usually kept at 0 mV for at least 10 sec. Currents were recorded using an Axopatch-200 amplifier (Axon Instruments), visualized on a digital storage Kikusui 5020A oscilloscope, and stored simultaneously on a chart recorder and on magnetic tapes. For the latter, a digital data recorder was used (VR-10A, Instrutech).

Incorporation of Proteoliposomes and VDAC in Planar Bilayers

Planar bilayers were formed at 0 mV, by spreading purified asolectin (15 mg/ml of *n*-heptane) across the 500 μm diameter *trans* chamber hole. The particular construction of the setup did not allow bilayers to form from solvent-free lipids by fusing monolayers present at each side of the hole (Montal & Mueller, 1972). Chamber solutions contained either 20 mM HEPES-KOH, pH 7.2, 1 mM $CaCl_2$, 1 M KCl (high ionic strength medium) or (mM) 10 HEPES-KOH, pH 7.2, 0.5 $CaCl_2$, 150 KCl (low ionic strength medium). After membrane blacked, 50 mV was usually applied for a few minutes, before returning to 0 mV. Fusion with planar bilayers of proteoliposomes at any salt concentration, or of detergent-extracted VDAC in the low ionic strength medium, was highly facilitated by the presence of an osmotic gradient. Thus, if the *trans* chamber contained the 1 M KCl solution, the *cis* solution was usually diluted threefold. Vice versa, if the *trans* chamber

contained the 150 mM KCl solution, the *cis* solution was either two times more concentrated or three times more diluted. After a few minutes, the chamber was perfused again with the same medium containing OM proteoliposomes (at a final protein concentration of 0.02–0.15 mg/ml), or VDAC (at a final protein concentration of 2–50 ng/ml). Immediately after the first step increase of membrane current, and regardless of its amplitude, the *cis* chamber was reperfused with the same solution present in the *trans* chamber to establish isosmolar conditions and eliminate unfused vesicles. The time required for protein insertion ranged from a few minutes to 30–40 min. If no current jump was detected within this time, the setup was prepared for a new assay.

At variance from previous reports, the conductance of a single channel was not calculated from the stepwise increase of current due to the insertion of channels in the planar bilayers. On the contrary, after one channel had been inserted and the *cis* chamber perfused, the current increase in response to at least three voltage steps of different magnitude was measured.

Chemicals were added to the same medium of the *cis* chamber, which was then perfused, at 0 mV, with 60-fold its volume. Perfusion took a few seconds and did not alter the baseline current. Electrode potentials, checked at the end of experiments with broken bilayers, ranged from 0.2 to 1 mV. All experiments were carried out at 22–25°C, the range of temperature most suitable for successful fusion events.

Data Analysis

Data stored on magnetic tapes were filtered with an 8-pole low-pass Bessel filter (Frequency Devices 902LPF2), at a cutoff frequency of 0.1–1 KHz, and then transferred to an Atari (1040ST) microcomputer using a 12-bit analog to digital converter (M2 LAB Instrutech). Sampling intervals were between 250–750 μsec. An Atari version (Instrutech) of TAC program (Sigworth, 1983), and programs kindly provided by Dr O. Moran (Genova, Italy), were used to display and analyze data.

Protein Determination

Protein concentration was determined according to Lowry et al. (1951). For details *see* Moran et al. (1990).

Results

Native OMM Properties

Proteoliposomes used in this work were made with the detergent-free OM (Sandri, Siagri & Panfili, 1988), isolated from rat brain mitochondria and enriched in asolectin phospholipids by freezing and thawing. We also used, although less frequently, large proteoliposomes prepared by dehydration-rehydration (Criado & Keller, 1987), since this was one of the preparations used in the patch-clamp experiments (Moran et al., 1992). We saw no difference in the results.

Regardless of the preparation, insertion of the native OM-containing liposomes in planar bilayers was possible only in the presence of an osmotic gradient and was easier, the higher the KCl concentration in the *trans* chamber (*see* Materials and Methods). Insertion is exemplified in Fig. 1, with the *trans* chamber (with the measuring electrode) containing 1 M KCl and the *cis* chamber (with the proteoliposomes) 330 mM KCl. As soon as current was evoked by proteoliposome insertion, perfusion of the *cis* chamber with the 1 M KCl-containing medium was carried out to eliminate the osmotic gradient and unfused proteoliposomes (last arrow).

Figure 2 shows the behavior, in symmetrical 1 M KCl, of a bilayer containing five channels (upper trace), or one channel (lower trace), from rat brain OMM, at 40 and −40 mV, respectively. Under these conditions, the mean value of the single channel full open state conductance was 3.7 ± 0.18 nS (inset to Fig. 2). This is lower than, but of the same magnitude order of, the value reported for VDAC extracted with detergents from mammalian tissues (Colombini, 1989; Benz, 1990). (For simplicity, the detergent-extracted VDAC will be referred to as VDAC.)

Figure 2 shows also that the detergent-free OMM has another feature typical of any VDAC preparation, i.e., the capability to close to subconducting states at membrane potentials higher than approximately ± 20 mV (Colombini, 1989; Benz, 1990). This is even more evident in Fig. 3 (filled circles), where the conductance variation upon voltage of a multichannel-containing bilayer is shown to retain the bell-shaped behavior characteristic of VDAC. The slope increase of the voltage sensitivity (Fig. 3, triangles) due to the negatively charged copolymer of metacrylate, maleate and styrene (König et al., 1977) is also in accordance with other reports when the applied potential is negative relative to the side of addition of the reagent (Fig. 3, right, triangles) (Colombini et al., 1987; Benz, Kottke & Brdiczka, 1990). (As explained in Materials and Methods, the polyanion could be added only to the *cis* chamber, whose potential is opposite to that shown in the figure.) However, at variance from Benz et al. (1990) and Benz and Brdiczka (1992), the effect of the copolymer does not change substantially at voltages of opposite sign (Fig. 3, left, triangles). Possibly, either the 10-fold higher concentration of the reagent used in our experiments, or a different susceptibility of the OM [isolated and handled otherwise than in Benz et al. (1990) and Benz and Brdiczka (1992)] is the reason for the discrepancy.

Other dissimilarities, however, can be noted between the macroscopic current of the native OM and VDAC, extracted with detergents. One is the conductance dependence upon voltage (Fig. 3) which is steeper than that of VDAC from rat brain

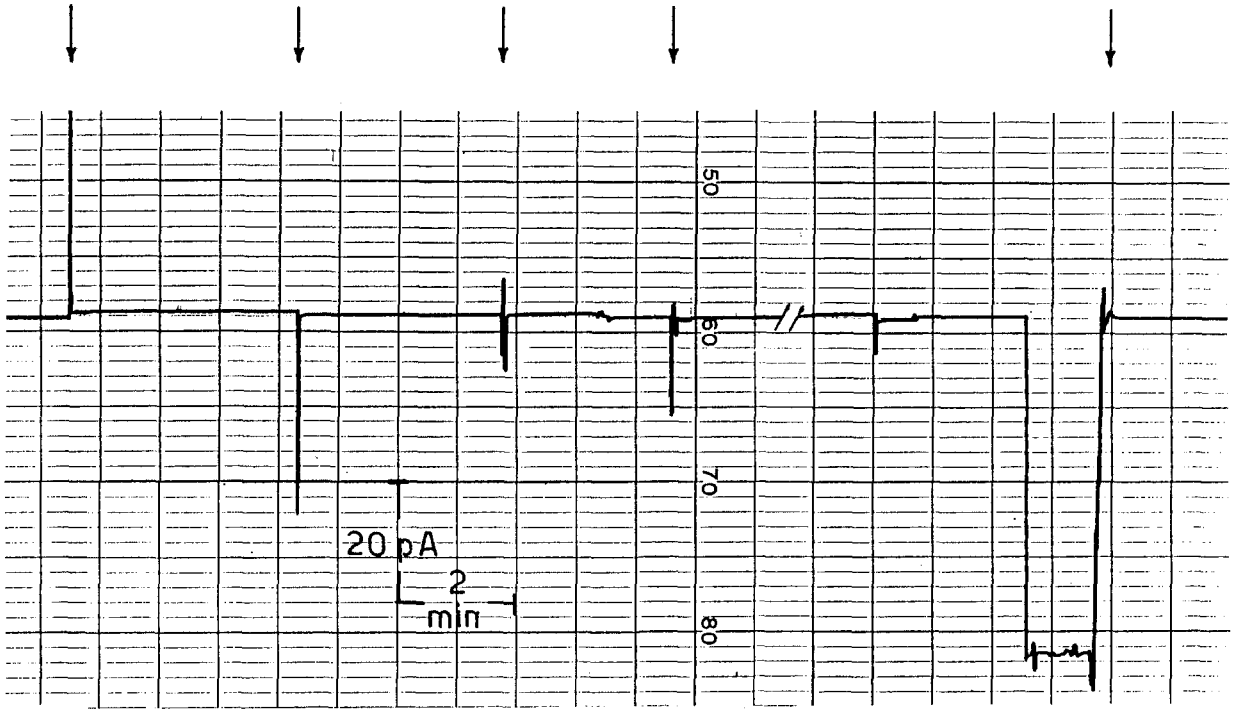

Fig. 1. Incorporation of OM-containing liposomes in planar bilayers in the presence of an osmotic gradient. The temporal progress of the experiment goes from the left to the right of the figure. Initially, both chambers contained the 1 M KCl solution. After formation of the planar bilayer (at 0 m V) and application of + 50 mV for 4 min (first arrow), the potential was set again to 0 mV (second arrow). The *cis* chamber solution was then substituted with the 330 mM KCl medium (third arrow) and subsequently with the same medium but containing proteoliposomes (fourth arrow) (*see* Materials and Methods). After the first increase of current, indicating the fusion of proteoliposomes with the planar bilayer, the *cis* chamber was immediately perfused with the 1 M KCl solution to eliminate the osmotic gradient and unfused vesicles (last arrow). Interruption in the trace indicates a time span of 8 min.

(Ludwig et al., 1986). Another difference refers to the relative conductance at membrane potentials higher than approx. ±40 mV, which is smaller than generally reported (*compare* Fig. 3 with Doring & Colombini, 1985; Ludwig et al., 1986; Benz & Brdiczka, 1992).

Finally, an interesting observation was the conductance level reached by single channels even at low potential differences (lower trace of Fig. 2, and control traces of Fig. 9) being much lower than expected from the multichannel behavior.

The conductance of VDAC from brain and liver mitochondria has been frequently shown to vary almost linearly with salt concentrations (Roos, Benz & Brdiczka, 1982; Ludwig et al., 1986). We therefore checked if our preparations also adhered to this pattern, by measuring currents at 150 mM KCl. Despite the lower rate of incorporation with low salt gradients (with 150 mM KCl in the *trans* chamber and 50 or 300 mM KCl in the *cis* chamber), we were able to follow repeatedly the behavior of a single channel of either rat liver VDAC (Fig. 4*A*) or rat brain OM (Fig. 4*B*) under symmetrical 150 mM KCl. [Note that upon the voltage pulse, VDAC either maintains the maximal conductance state (Fig. 4*A*, upper trace) or expresses it transiently (middle trace). Seldom was the full conductance also observed after a few seconds (5.5) from voltage imposition (Fig. 4*A*, lower trace).] Data collected from similar experiments, indicated that the single channel full open conductance was 890 ± 29 pS for rat liver VDAC, and 700 ± 23 pS for rat brain OM (insets to Fig. 4); these are approximately 30 and 15% higher than values previously reported for VDAC of the same tissues (Roos et al., 1982; Ludwig et al., 1986).

The plot of Fig. 3 was derived from experiments carried out with multichannel-containing bilayers, where, rarely, potential differences higher than ± 60 mV could be imposed. A different situation was encountered with bilayers containing only one channel. Here, regardless of the salt concentration, higher potentials were imposed without collapsing the bilayer, especially with the detergent-free OM. This type of protocol produced an interesting result, reported in Fig. 5, where an OM single channel (in symmetrical 1 M KCl) was subjected to increasing membrane potentials. However, from the percentage of the bilayer remaining conductance (shown at

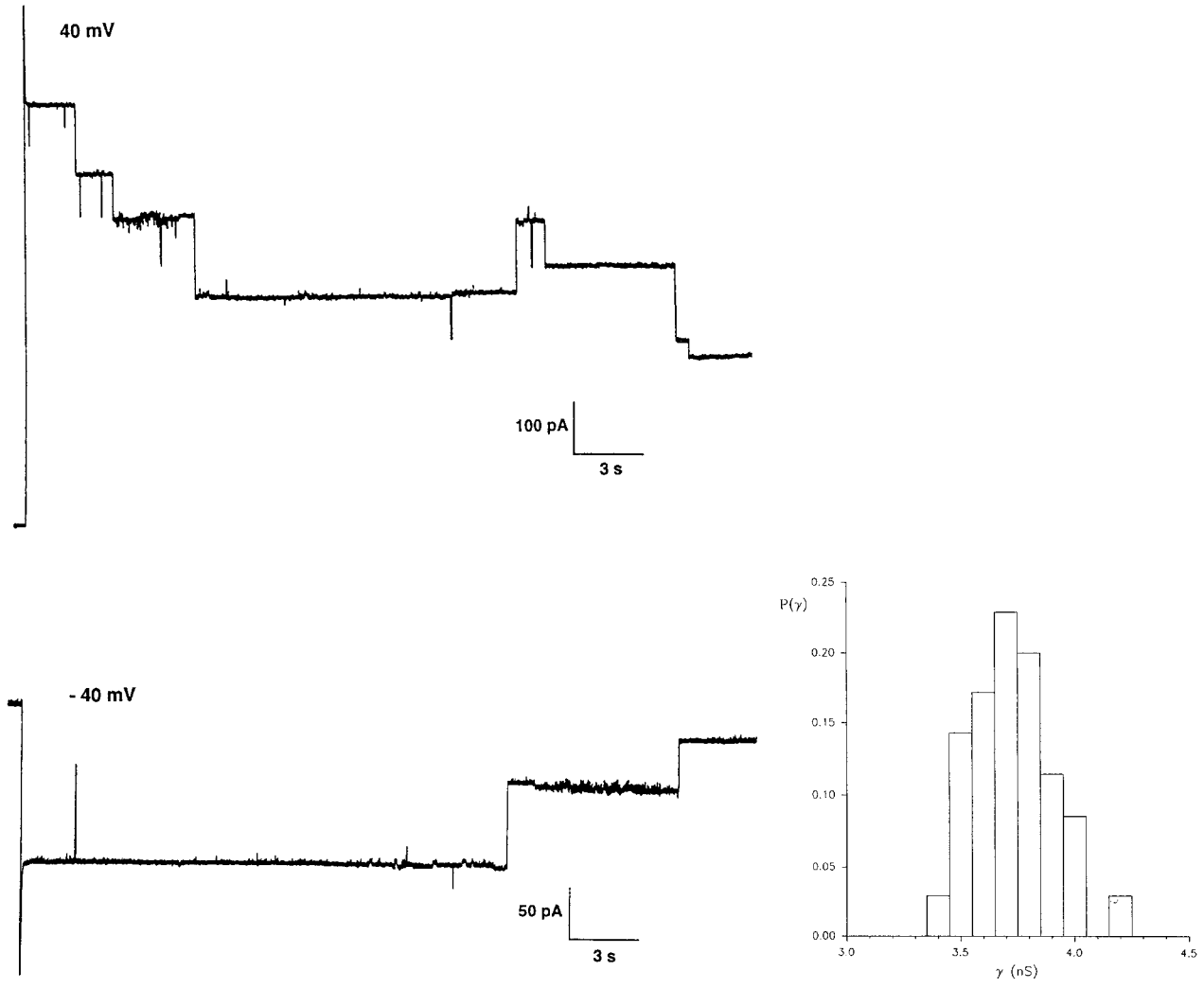

Fig. 2. Behavior, in symmetrical 1 M KCl, of a five channel- (upper trace) and one channel-containing bilayer (lower trace), after incorporation of rat brain OMM-containing liposomes. Potential differences (shown in the figure) were applied from 0 mV (far left traces). The inset shows the histogram of full open state conductances of single channels, measured after incorporation in response to voltage steps of different magnitude (*see* Materials and Methods). The number of experiments considered was 6 for a total of 35 determinations. The conductance mean value was 3.7 ± 0.18 nS (mean ± SD). Filter was 100 Hz. Incorporation occurred as described in the legend to Fig. 1.

the end of each trace), measured approximately 1 min (first three traces) or 30 sec (last trace) from the voltage step, it is evident that at potentials higher than −60 mV, the single channel goes to much more closed states than expected from the analyses with multichannel-containing bilayers (*compare* Fig. 5 with Fig. 3). Furthermore, this state, which we define as deactivated, was also stable, and unable to reverse completely even after letting the bilayer at 0 mV for several minutes before returning to the high potential difference. In other words, when back at high potentials, the channel would not open as much as before. The same type of results was found in low salt.

Importantly, this phenomenon must not be confused with what was shown in the lower trace of Fig. 2, nor with the control traces of Fig. 9. [*see also* Fig. 2 in Colombini (1986), and Fig. 5 in Holden and Colombini (1988).] Here, the quickly reached low conductance was reversible, in the sense that after it, higher conductance substates could follow. Also, it did not prevent the full opening of the channel at a subsequent voltage step.

Figure 5 also shows that the higher the potential difference, the quicker a very closed state is reached, pointing to a catalyzing effect by voltage. The time needed to reach this state was of the order of a few seconds at voltages around ±100

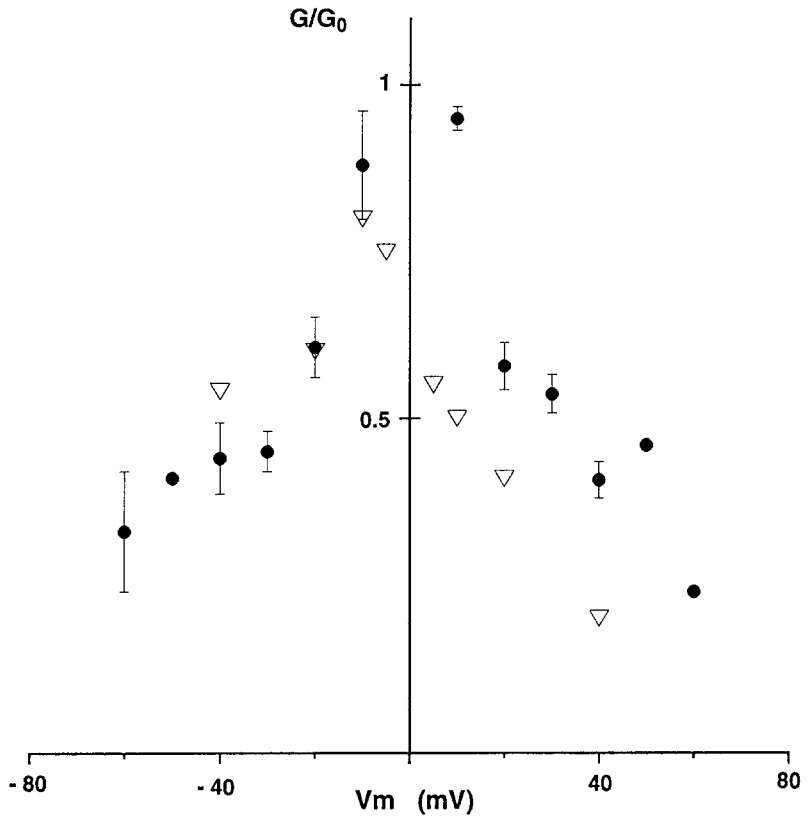

Fig. 3. Variation of conductance with voltage of multichannel-containing bilayers, after incorporation of liposomes with rat brain OMM, in the absence (filled circles) and presence (triangles) of polyanion (symmetrical 1 M KCl). Care was taken to analyze only those experiments (5) with bilayers containing a minimum of five channels and where the deactivation phenomenon did not occur (*see text*). The maximal opening conductance (G_o) could thus be detected, even if transiently, at any of the shown potentials. In the majority of cases, the conductance values (G), found after 30 sec from potential imposition, remained unaltered up to 2 min. If not, the (lowest) value detected at 2 min was that normalized to G_o. Bars indicate SD values calculated with between two and five experimental values. Polyanion, added only to the *cis* side (*see* Materials and Methods), had a final concentration of 4.8 μg/ml. Conductance values in the presence of polyanion were normalized relative to the initial maximal conductance of the control. *Vm*, the applied membrane potential difference, was imposed starting from 0 mV (*see* Materials and Methods). Incorporation occurred in the presence of an osmotic gradient (*see legend to* Fig. 1).

mV or higher; vice versa, at lower values, it was variable, from a few seconds to a couple of minutes. Thus, if in the need to control whether a particular substate was really deactivated (in line with previous reports—Colombini, 1989; Benz, 1990; Ermishkin & Mirzabekov, 1990—we observed as many as 10 substates), the bilayer was usually kept at 0 mV for some time before rechecking the opening conductance at high, or low (*see below*) voltages.

After the results of Fig. 5, it was natural to check if deactivation remained at potentials $< \pm 20$ mV, where VDAC should be always in its full open state. The experiments reported in Fig. 6 prove that this was the case, as the control current at +10 mV is drastically reduced after the application of 80 mV for 2.5 min. The same phenomenon occurs at +40 mV (Fig. 6). Actually, the second trace at +40 mV is symptomatic of an event frequently accompanying deactivation, i.e., the change of the kinetic behavior of the channel, with transitions between different levels of conductance in the msec range. Interestingly, another feature changed upon deactivation, i.e., the ionic selectivity. From slightly anionic for the untreated bilayer (the Cl^- over K^+ permeability ratio being 1.6 for VDAC, and around 2.0 for the OM), the selectivity turned to slightly cationic after deactivation (the VDAC K^+ over Cl^- permeability ratio being 1.5) (*data not shown*).

To our knowledge, the fast kinetics shown in Fig. 6 was never reported before for the OM in planar bilayers. On the contrary, we found it happening repeatedly with either preparation and at both salt concentrations, and more frequently at voltages higher than $\pm$ 30 mV. Figure 7 reports such behavior for brain OM (in symmetrical 1 M KCl), and Fig. 8 for a single VDAC channel (in symmetrical 150 mM KCl). From the records in an expanded scale (Figs. 7 and 8, lower panels), it is evident that the transitions can take less than 5 msec [the mean open time ranges from 2.5 to 12 msec (*data not shown*)]. Figures 7 and 8 also report the current amplitude histograms of sections of the shown flickering events, with the corresponding mean conductance values.

Effect of Aluminum

Panel *B* of Fig. 9 reports the effect of 10 μM Al lactate (Al $lact_3$) on an OMM single channel, in symmetrical 1 M KCl. The compound was added only to the *cis* chamber (*see* Materials and Methods). Compared to the control (panel *A*), no effect was evident at $\pm$20 mV. At +30 mV, the channel seemed

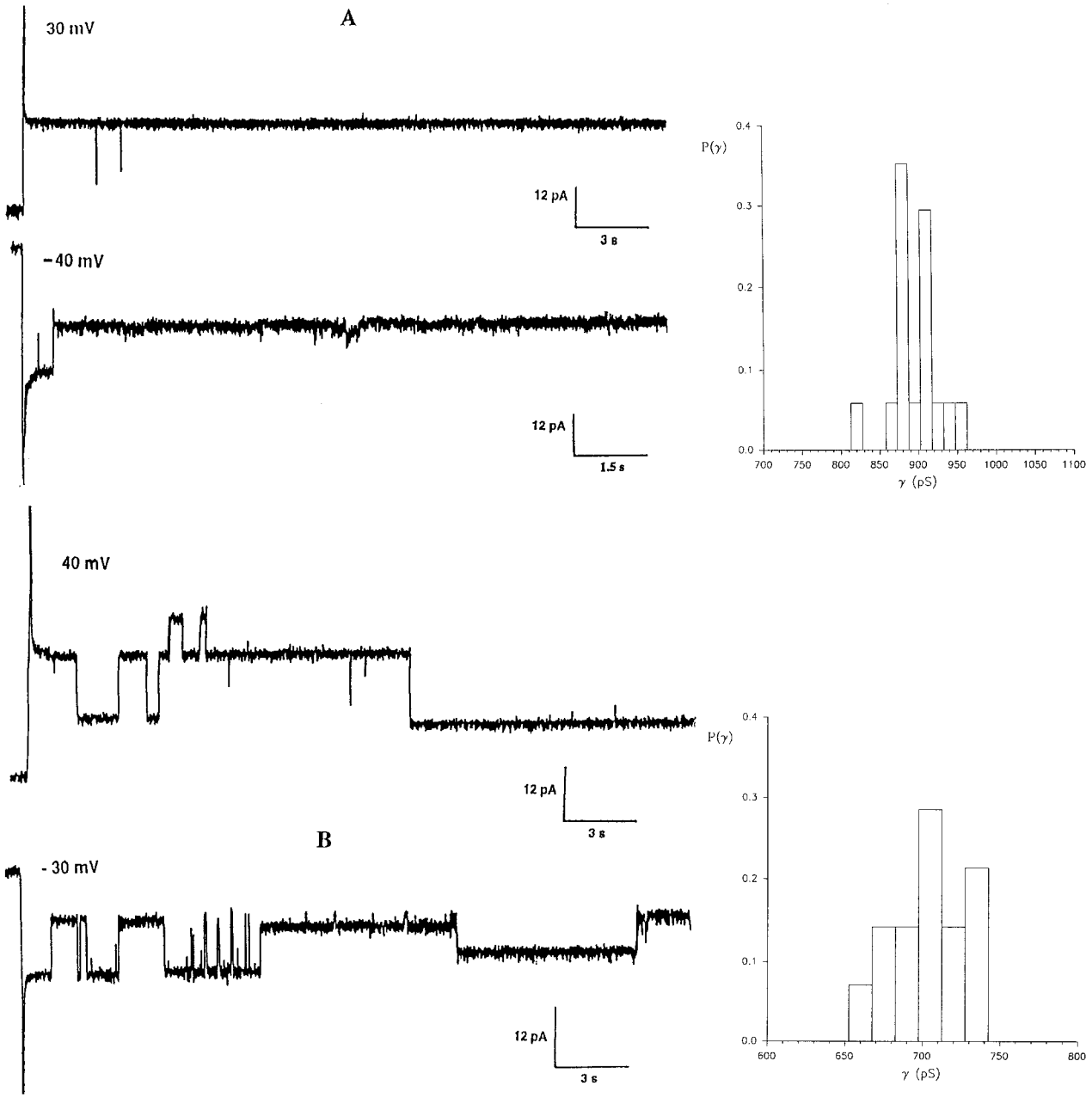

Fig. 4. Single channel recordings, in symmetrical 150 mM KCl, from bilayers containing VDAC extracted from rat liver with Triton X-100 (*A*) or rat brain OMM (*B*). Note in *A* that the maximal opening of VDAC is either long-lasting (upper trace), transiently expressed immediately (middle trace where the time scale is expanded) or occurs 5.5 sec after voltage imposition (lower trace). Potential differences (shown in the figure) were applied starting from 0 mV. Filter was 100 Hz. Insets show histograms of the full open state conductances of single channels, measured in response to voltage steps of different magnitude after protein incorporation (*see* Materials and Methods). For rat liver VDAC, the number of experiments considered was 5 for a total of 17 determinations. The single channel conductance had a mean value of 890 ± 29 pS. For rat brain OM, the number of experiments considered was 4 for a total of 14 determinations. The single channel conductance had a mean value of 700 ± 23 pS. Fusion details are given in Materials and Methods.

to remain more in the open state, but at −30 mV it rapidly reached a lower (around 55%) stable conductance level, maintained at subsequent voltage steps. (In all these calculations we refer to the most probable conductance state, from which additional, rapid close-open transitions may take place.) Panel *C* of Fig. 9 shows that Al action appears irreversible, as reperfusion of the metal-containing chamber was unable to restore the original behavior.

The same capability to close the channel at approximately half of its maximal conductance was also observed with 100 μM Al Cl_3 (Fig. 10).

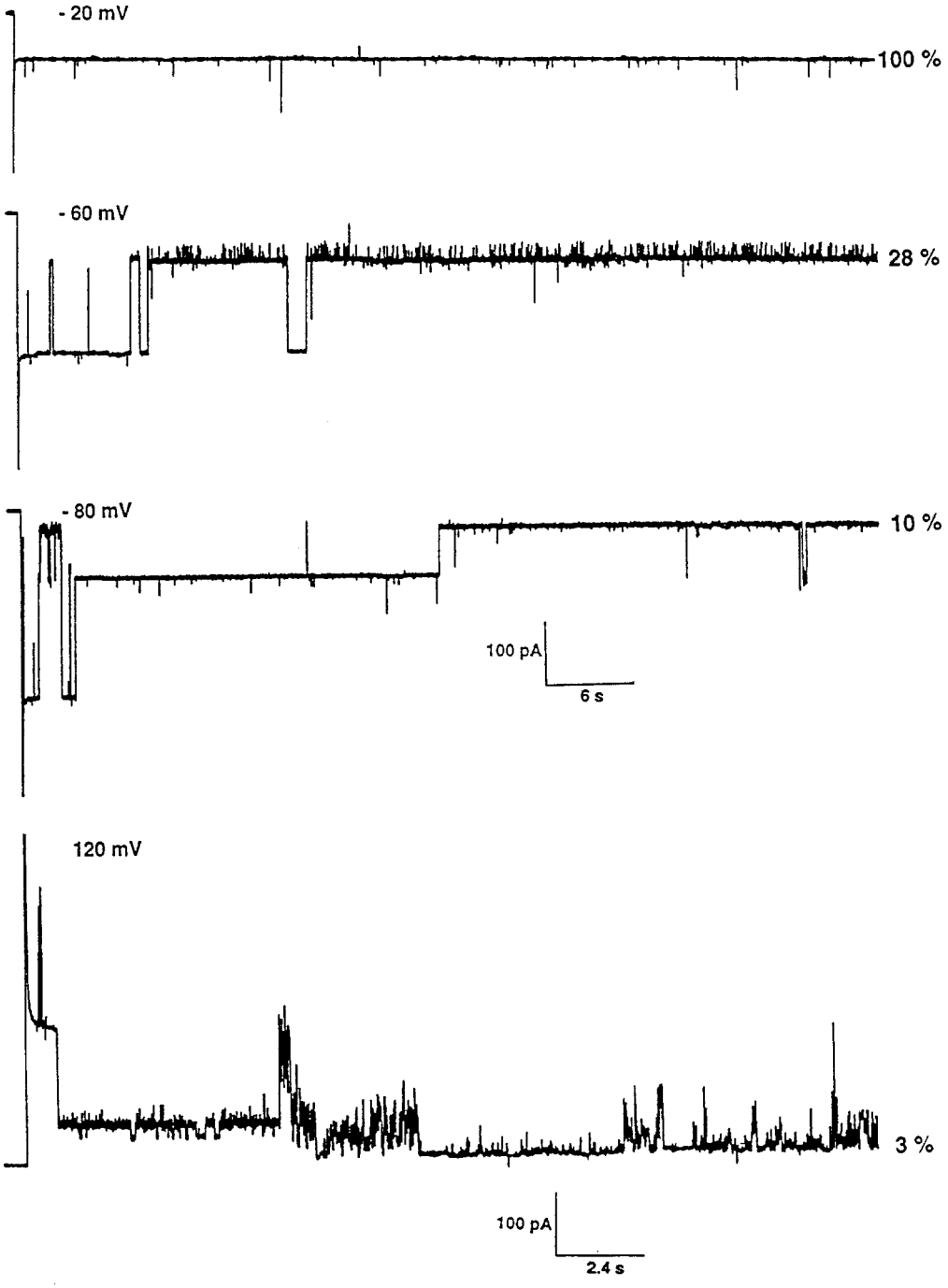

Fig. 5. Induction of very low conducting states in a single channel, after application of high potentials. Records were taken from a bilayer containing rat brain OMM, in symmetrical 1 M KCl. Each voltage step (shown in the figure) was applied starting from 0 mV. Note that the maximal opening current is possible to be detected at any potential. At the end of each trace, we give the percentage of left current, averaged over the last 6 sec (first three traces) and 3 sec (last trace) of the shown records. Proteoliposomes were incorporated as explained in the legend to Fig. 1. Filter was 100 Hz.

Discussion

The aim of the present work was to see if, by transposing to planar bilayers the experimental conditions used by us in patch clamping the OMM (Moran et al., 1992), it was possible to set the fracture with the data from other laboratories. On one side was the data obtained with detergent-extracted VDAC in planar bilayers, generally at high salt (Colombini, 1986, 1989; Benz, 1990), or by patch clamp-

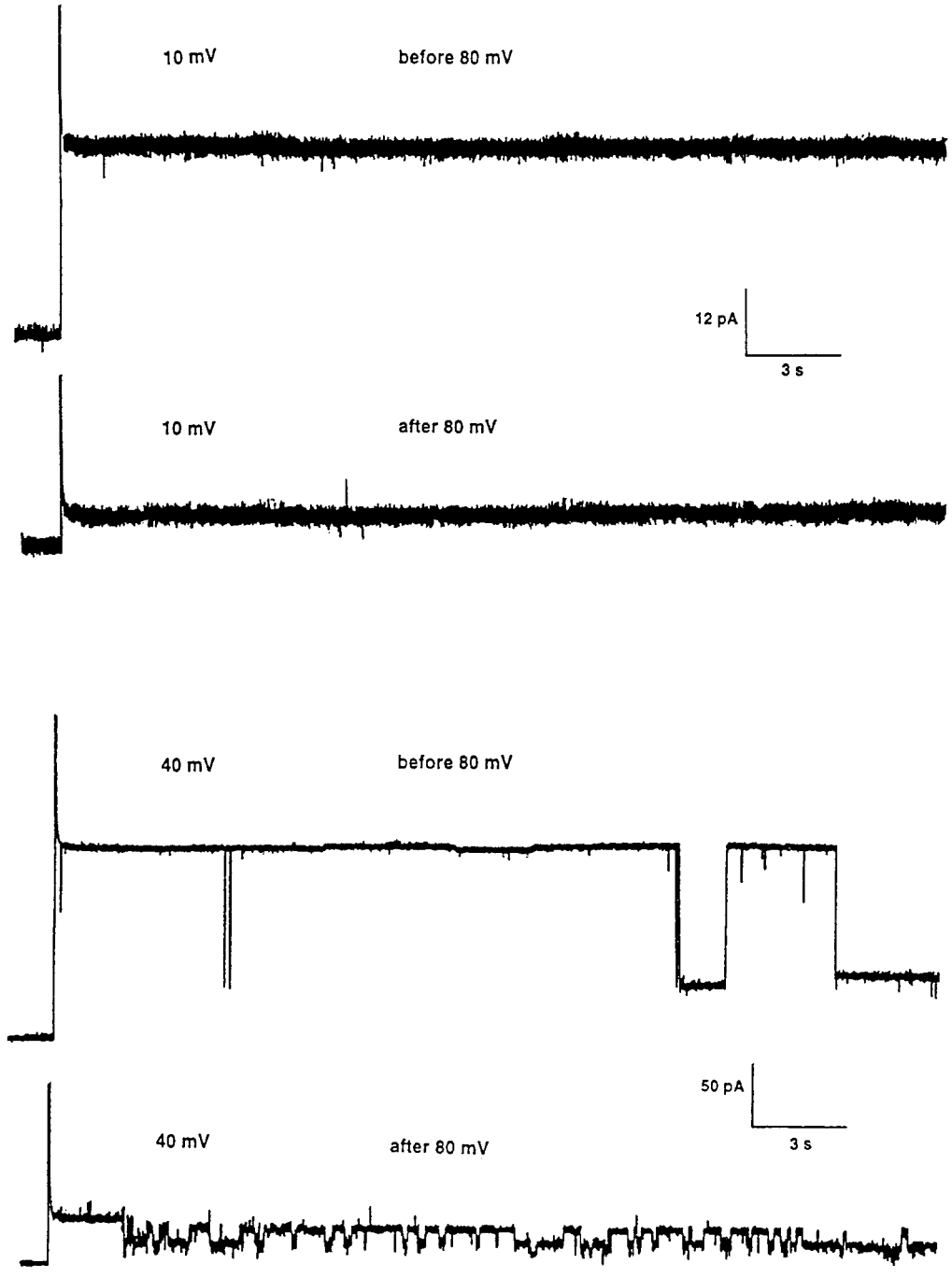

Fig. 6. Change in single channel behavior detected at low potentials after application of high potentials. Records of a bilayer, containing rat brain OMM in symmetrical 1 M KCl, were first taken at +10 mV and +40 mV (first and third trace) and then, at the same voltages, after application of +80 mV for 2.5 min (second and fourth trace). After each voltage step, the bilayer was kept at 0 mV for 15–30 sec. For other details, *see legend to* Fig. 5.

ing in low salt, proteoliposomes containing both mitochondrial membranes from *Neurospora crassa* (Wunder & Colombini, 1991) or integral rat liver mitochondria (Kinnally, Tedeschi & Mannella, 1987; Tedeschi, Mannella & Bowman, 1987; Kinnally et al., 1989; Tedeschi, Kinnally & Mannella, 1989). On the other side, were the single channel recordings obtained by patch clamping in low salt, either integral rat liver mitochondria or detergent-free rat brain OMM-containing liposomes (Moran et al., 1992).

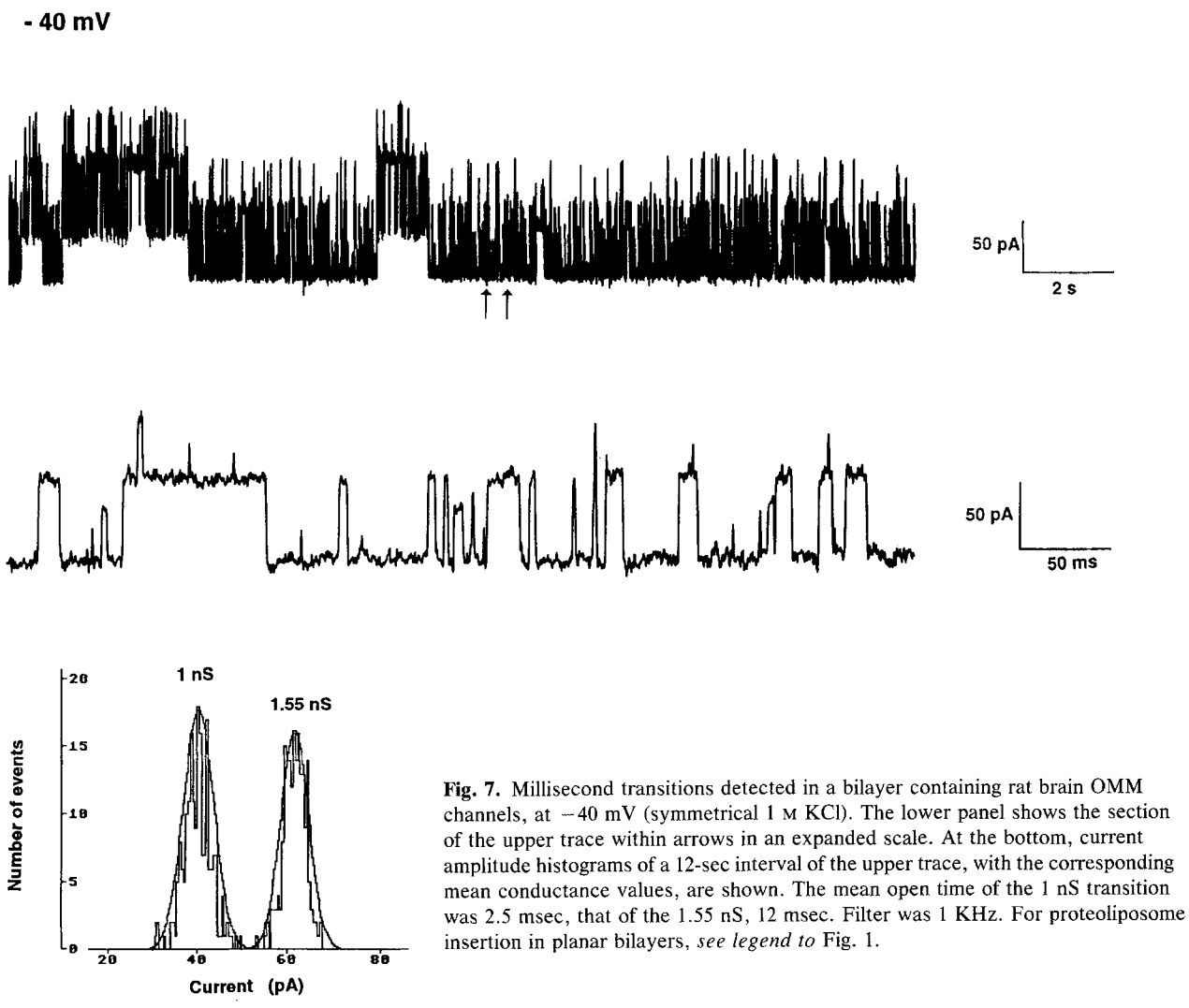

Fig. 7. Millisecond transitions detected in a bilayer containing rat brain OMM channels, at −40 mV (symmetrical 1 M KCl). The lower panel shows the section of the upper trace within arrows in an expanded scale. At the bottom, current amplitude histograms of a 12-sec interval of the upper trace, with the corresponding mean conductance values, are shown. The mean open time of the 1 nS transition was 2.5 msec, that of the 1.55 nS, 12 msec. Filter was 1 KHz. For proteoliposome insertion in planar bilayers, *see legend to* Fig. 1.

Thus we studied, in planar bilayers at low salt, the behavior of the same proteoliposomes used in our patch-clamp experiments. Concomitantly, we investigated the OMM in 1 M KCl and VDAC, extracted from rat liver with Triton X-100, in 150 mM KCl.

The results obtained in both media (Figs. 2–4) clearly show that, qualitatively, the detergent-free OM possesses several of the widely described features of detergent-extracted VDAC in artificial membranes (and confirmed with the patch clamp by Wunder and Colombini, 1991); i.e., the high conductance of the full open state of single channels and the presence of substates; the bell-shaped potential dependence, sensitive to polyanion, typical of multichannel-containing bilayers. This was not entirely expected after what had emerged by patch clamping, in 150 mM KCl, the same proteoliposomes used in this work, or integral mitochondria (Moran et al., 1992). In this latter work, channels were found with faster kinetics, a smaller conductance value (99, 152, 220, 307 and 530 pS), with different (or none, as for the 530 pS event) voltage dependence, and with opposite (cationic) selectivity.

One may conclude then that the conditions generally used with planar bilayers, i.e., presence of detergents and high ionic strength media, are not the main cause for the discrepant results. Actually, the high (although different values of liver VDAC and of brain OM) single channel conductance, found in planar bilayers at 150 mM KCl (Fig. 4), make the lower value (albeit identical for liver and brain OM) detected with the patch clamp (Moran et al., 1992) even more divergent.

At first instance, this conclusion seems to make it impossible to solve the problem at the basis of

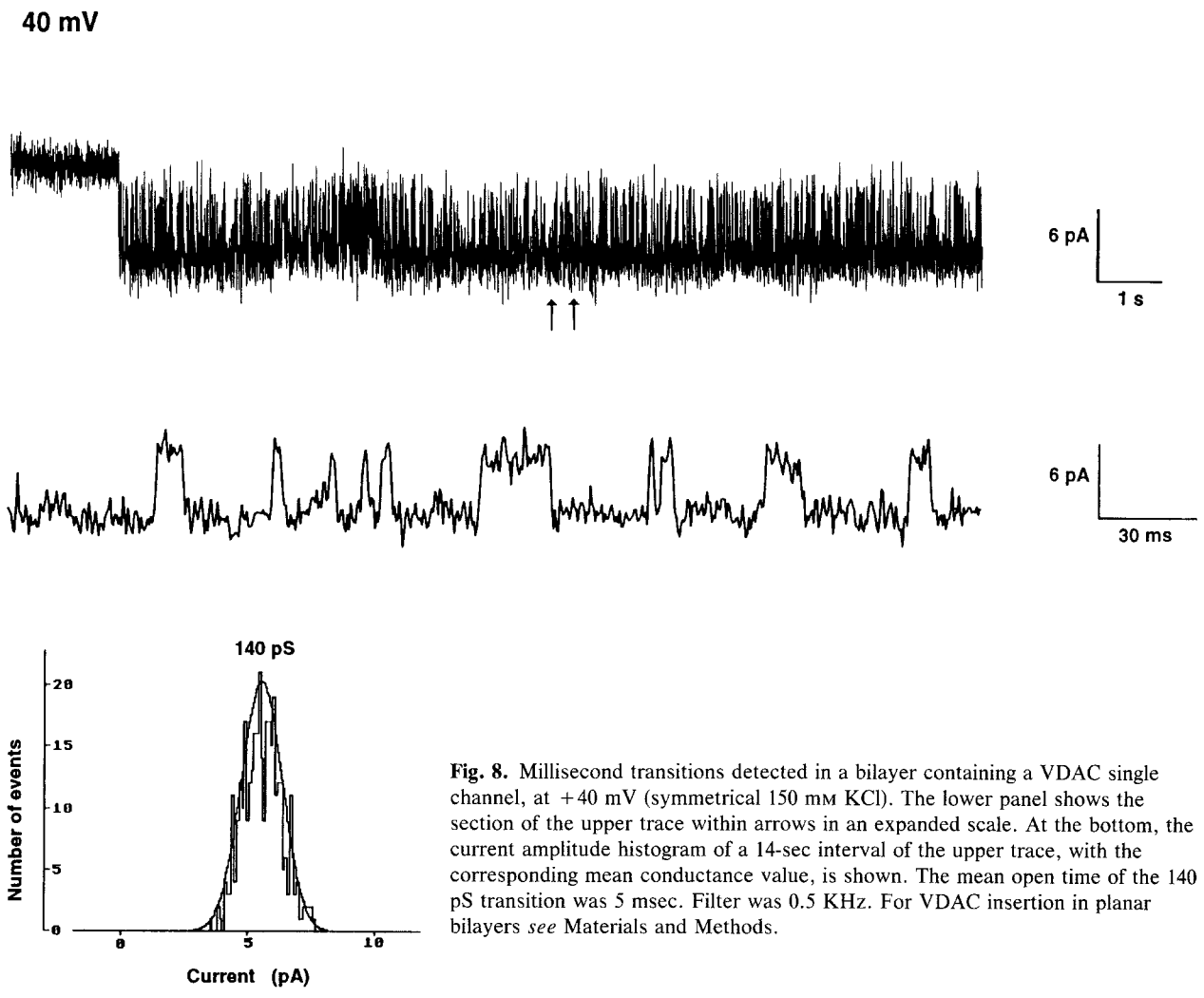

Fig. 8. Millisecond transitions detected in a bilayer containing a VDAC single channel, at +40 mV (symmetrical 150 mM KCl). The lower panel shows the section of the upper trace within arrows in an expanded scale. At the bottom, the current amplitude histogram of a 14-sec interval of the upper trace, with the corresponding mean conductance value, is shown. The mean open time of the 140 pS transition was 5 msec. Filter was 0.5 KHz. For VDAC insertion in planar bilayers *see* Materials and Methods.

this work. However, the instrumentation used was essential to reveal other, as yet unreported, features of the OM (or VDAC) in the planar bilayer itself. In many aspects, these novel features are very close to those detected with the patch clamp.

One support came from the high frequency digitalization of data stored in video tapes, which offered by far a better resolution of the events in terms of conductance and kinetics, allowing measurements of transient, full open states also at high membrane potentials (Figs. 4 and 5) and of small and rapid transitions (Figs. 6–8).

Another aid came from the use of a particularly small *cis*- (system-containing) chamber. Its rapid perfusion could stop incorporation almost instanteously, frequently after insertion of one channel. This brought the advantage of estimating single channel conductance in low salt (Fig. 4) [found higher than reported previously (Ludwig et al., 1986; Roos et al., 1982)], and imposition of high membrane potentials without breaking the bilayer. The outcome of this latter experimental protocol was the finding that the OM permeability was strongly restricted, the quicker and higher the potential (Fig. 5). Nor were these states (called deactivated) expressed only at high potentials. An altered (decreased) conductance, sometimes with a different kinetics, remained evident also at those low values where the channel should return to the fully open state (Fig. 6). The induction by voltage of these long-lived closed states resembles that observed in the presence of the so-called natural modulator, a soluble protein capable of increasing the extent and rate of VDAC closure (Holden & Colombini, 1988; Liu & Colombini, 1992).

Interestingly, the conductance values of single channel deactivated states are of the same order of magnitude of those reported for the patch clamping of the OM. For example, the residual conductances found at +10 mV and +40 mV after deactivation

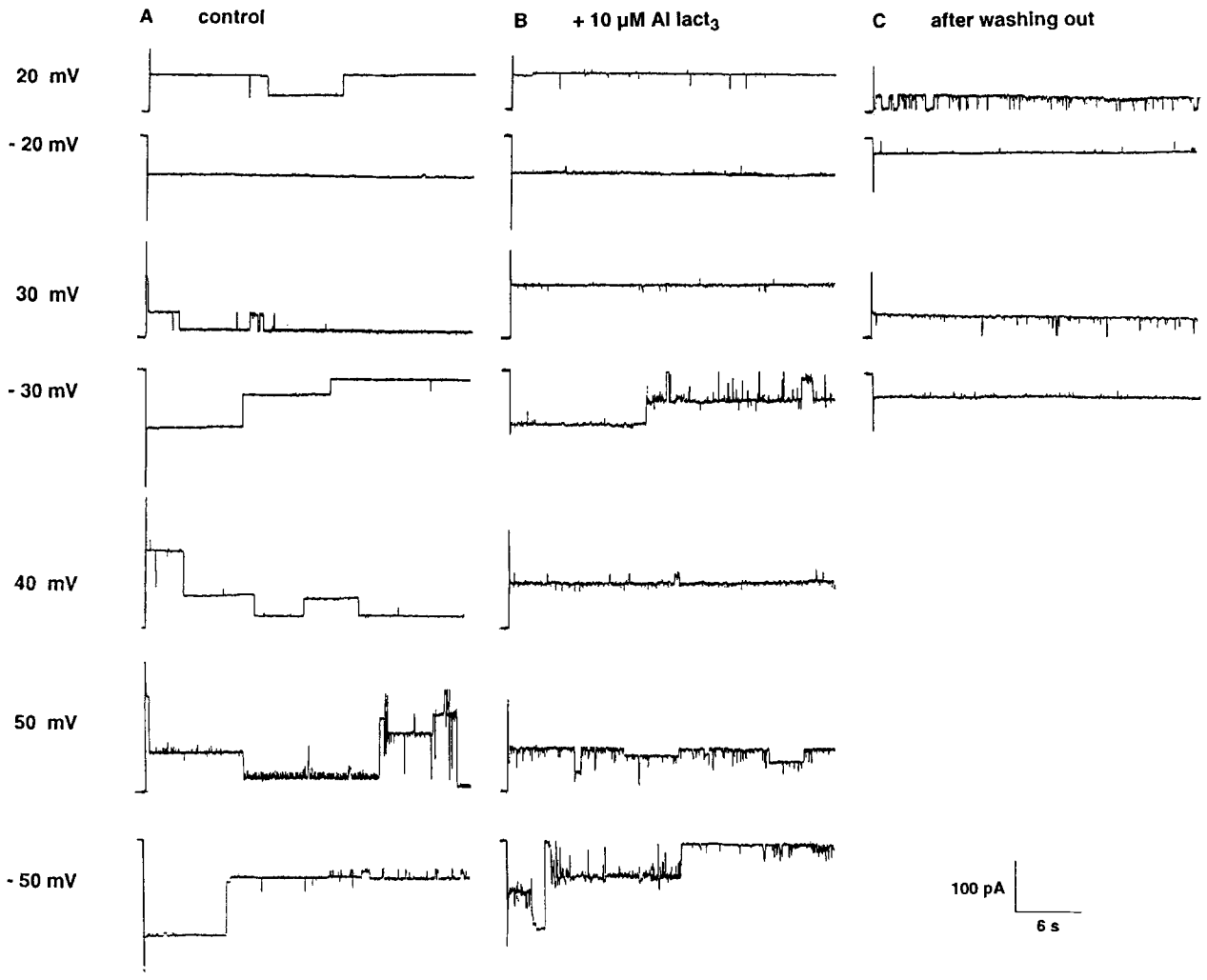

Fig. 9. Effect of Al $lact_3$ on a single channel of rat brain OMM (symmetrical 1 M KCl). Data from the same experiment are reported. (*A*) Records at different potentials (shown in the figure) in the absence of Al $lact_3$. (*B*) Records at the same potentials in the presence of 10 μM Al $lact_3$. (*C*) Records taken after Al $lact_3$ washing out. Al $lact_3$ was added after completion of the control experiments, shown in *A*, by perfusing the *cis* chamber (at 0 mV) with the 1 M KCl solution containing Al $lact_3$ at the indicated concentration. Al $lact_3$ was diluted from a stock solution (kept at room temperature) immediately before use. A period of about 2 min, at 0 mV, was allowed before checking the effect of the compound. When necessary, Al $lact_3$ was removed by perfusing the *cis* chamber (at 0 mV) with 60-fold the chamber's volume of the 1 M KCl medium. The experiment was run by imposing the different voltage steps in sequence, just as shown in the figure, with intervals of 10–15 sec at 0 mV between each step. Each record lasted between 1 and 1.5 min. The effect of Al $lact_3$ removal was checked after completion of the perfusion. In the figure, the last shown state is indicative of the state maintained by the channel until the end of the record. No change in the medium pH due to Al $lact_3$ was found. Filter was 100 Hz. Note that the sign of the potential relative to the side where Al was added is opposite to that shown (*see* Materials and Methods). For other experimental conditions, *see* Materials and Methods.

(Fig. 6) (corresponding to 95 and 152 pS in 150 mM KCl, if the right calculation for the different salt concentration is made), compare well with the 99 and 152 pS transitions routinely found with the patch clamp (Moran et al., 1992). The same is true for the amplitudes of the rapid open-close events (in the msec range) of the OM (Fig. 7) and of VDAC (Fig. 8). Here also, the 140 pS VDAC transition, in 150 mM KCl (Fig. 8), and the flickering events of the OM in 1 M KCl (Fig. 7) (which would correspond, in 150 mM KCl, to 185 and 286 pS), are values again close to those routinely detected with the patch clamp (Moran et al., 1992).

Finally, it is worth recalling that the deactivated states in planar bilayers and the OM in the patch pipette share the same cationic selectivity (this paper and Moran et al., 1992).

Thieffry et al. (1988) and Chich et al. (1991) have

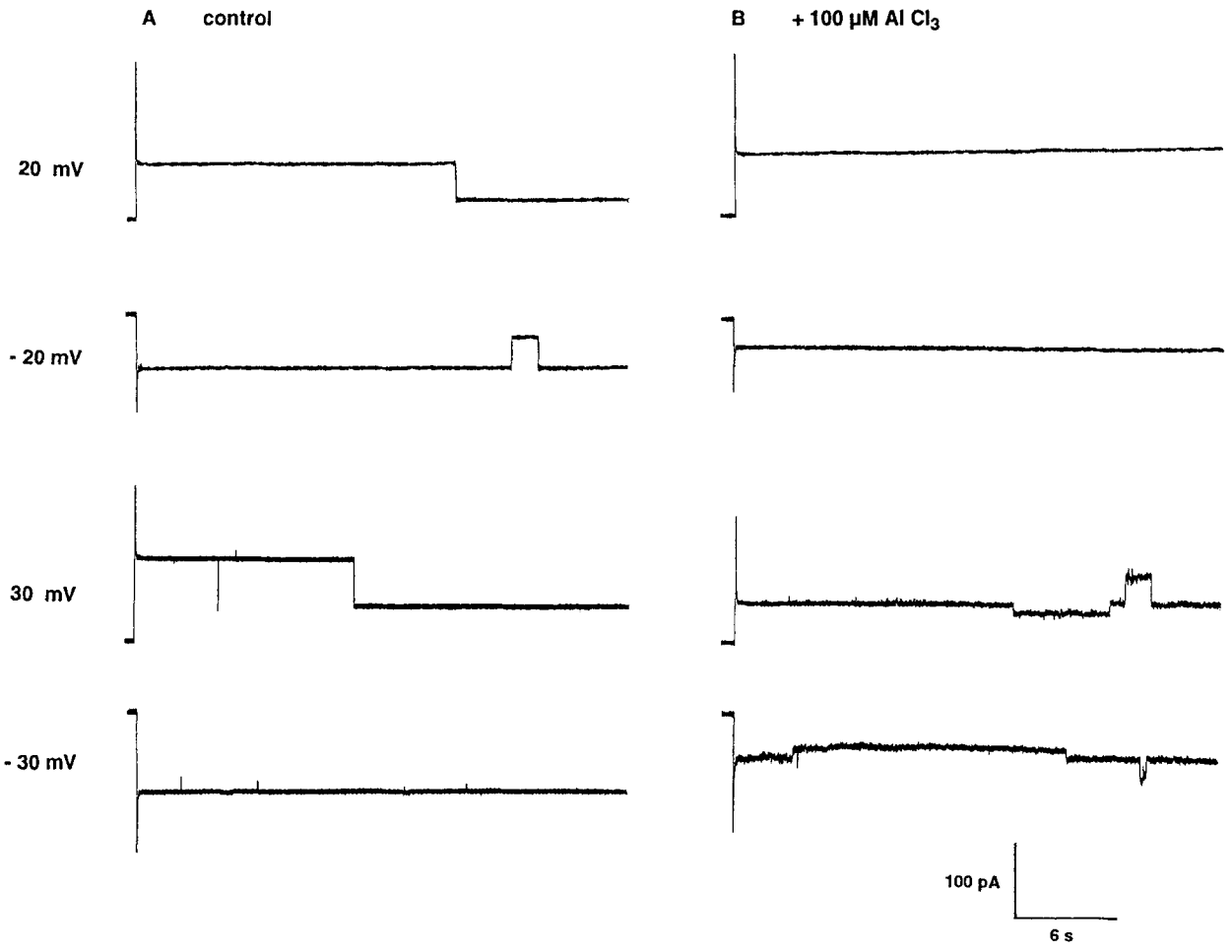

Fig. 10. Effect of 100 μM Al Cl_3 on a single channel of rat brain OMM (symmetrical 1 M KCl). (*A*) Records at different potentials (shown in the figure) in the absence of Al Cl_3. (*B*) Records at the same potentials in the presence of 100 μM Al Cl_3. Experimental conditions were identical to those detailed in the legend to Fig. 9.

observed in the OMM of a variety of mammalian cells conductances with a kinetic comparable to that described here. However, they have also proved that these conductances did not belong to VDAC (Fèvre et al., 1990). Thus, given the immunological proof of VDAC presence in our preparation (Moran et al., 1992) and the same behavior attributed to this channel in the control experiments, we have to conclude that what was studied in this work is VDAC.

With this in mind, the following possibilities should be considered. That in a patch, the low-conductance conformation of VDAC reflects the natural state of the channel. Or that, under the experimental conditions used by Moran et al. (1992), the channel is forced to assume the deactivated conformation for causes other than voltage. This is the same as hypothesizing that in planar membranes the channel assumes primarily the nondeactivated conformation.

To conclude this part of the Discussion, having established that the so far unorthodox (for VDAC) features of the OM detected in a pipette patch belong also to VDAC in planar bilayers, we still have to answer satisfactorily which one is the behavior expressed physiologically by the OMM: with a large electrical conductance and a slow, steady-state kinetics (Colombini, 1989; Benz, 1990; Wunder & Colombini, 1991), or with a lower conductance and fast kinetics (this paper and Moran et al., 1992), or both.

Aluminum is known to interact with several molecules and proteins essential for cell viability. Oortgiesen, van Kleef and Vijverberg (1990) found that addition of $Al(NO_3)_3$ to neuroblastoma cells induces the opening of particular plasma membrane channels. With respect to VDAC (from *Neurospora crassa*), Dill et al. (1987) and Zhang and Colombini (1989) have observed a potent effect by Al Cl_3 on the channel voltage sensor. Closure of the channels at a high potential difference was markedly slowed

down when $AlCl_3$ was symmetrically added (in both chambers). On the other hand, addition of indium (In) (a trivalent ion belonging to the same group of Al) to only one side of the bilayer showed that In inhibited VDAC closures when the potential was negative relative to the metal-containing side, but increased both the rate and the extent of closure at voltages of opposite sign (Zhang & Colombini, 1990).

We carried out similar experiments on a single OMM channel from rat brain, using either Al $lact_3$ or Al Cl_3, compounds most frequently used in the study of aluminum toxicology. The effect induced by micromolar concentrations of these compounds (Figs. 9 and 10, panels *B*) is quite clear. Already at low voltages, but of positive sign relative to the side of Al addition (*see* legends to the figures), aluminum seemed to lock the channel in a conformation of approximately 50–40% decreased conductance. Apparently, aluminum was firmly stabilizing this state, as application of potential differences of higher value, or of opposite sign, were unable to unlock the channel (Figs. 9 and 10, panels *B*). In line with this conclusion are the records taken after perfusion of the Al-containing chamber (panel *C* of Fig. 9), where the opening conductances remain inhibited (*cf.* records at ± 20 and +30 mV of panel *C* with those of panels *A* and *B*).

The response to voltages of opposite sign, due to the asymmetrical presence of Al (in our case) and In (in the case of Zhang & Colombini, 1990), is certainly comparable in many ways. Our experimental protocol, however, did not allow repeated observation of the metal effect at those potentials which, according to Zhang and Colombini (1990), locked the channel in the open state. The tendency of the channel to remain open was nonetheless detected (*see* Fig. 9, the record at +30 mV in the presence of Al $lact_3$), but was nullified at the subsequent (negative) voltage application.

Zhang and Colombini (1990) have proposed a model to explain the dependence of Al effect on the voltage sign. This model was based on the assumption of a stoichiometry of two for the functional channel, and on that the combination Al-voltage sign was either locking the channel in the fully open state or in the fully closed state. A recent investigation (Peng et al., 1992), carried out with VDAC extracted from yeast cells expressing both mutated and wild type VDACs, however, has indicated that only one polypeptide composes the channel, in accordance with the mass per unit area of VDAC estimated by scanning transmission electron microscopy (Thomas et al., 1991). In light of this, and of the use in our work of single channel-containing bilayers which allow a more direct definition of the metal action, the simplest explanation of our results envisages two sites of interaction with aluminum. One site probably affects the positively charged voltage sensor and causes insensitivity to voltage when the potential is negative relative to metal addition. The other becomes exposed upon application of potentials of opposite sign, and its occupancy by Al modifies the structure of the channel to approximately half of its conductance. Apparently, such conformation overrules the effect by negative potentials.

In discussing the action of aluminum on VDAC, however, one should also take into account the reactivity of Al with phospholipids (Corain, Nicolini & Zatta, 1992). By using planar bilayers without proteins, we found that μM concentrations of both Al Cl_3 and Al $lact_3$ increased the voltage value responsible for the membrane breakdown (*data not shown*).

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References

Benz, R.. 1990. Biophysical properties of porin pores from mitochondrial outer membrane of eukariotic cells. *Experientia* **46:**131–137

Benz, R., Brdiczka, D. 1992. The cation-selective substate of the mitochondrial outer membrane pore: single-channel conductance and influence on intermembrane and peripheral kinases. *J. Bioenerg. Biomembr.* **24:**33–39

Benz, R., Kottke, M., Brdiczka, D. 1990. The cationically selective state of the mitochondrial outer membrane pore: a study with intact mitochondria and reconstituted mitochondrial porin. *Biochim. Biophys. Acta* **1022:**311–318

Chich, J.-F., Goldschmidt, D., Thieffry, M., Henry, J.-P. 1991. A peptide-sensitive channel of large conductance is localized on mitochondrial outer membrane. *Eur. J. Biochem.* **196:**29–35

Colombini, M. 1986. Voltage gating in VDAC. *In:* Ion Channel Reconstitution. C. Miller, editor. pp. 533–551. Plenum, New York

Colombini, M. 1989. Voltage gating in the mitochondrial channel, VDAC. *J. Membrane Biol.* **11:**103–111

Colombini, M. 1991. Aluminium and membrane channels. *In:* Aluminium in Chemistry Biology and Medicine. M. Nicolini, P.F. Zatta, B. Corain, editors. pp. 33–43. Raven, New York

Colombini, M., Yeung, C.L., Tung, J., Kønig, T. 1987. The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion. *Biochim. Biophys. Acta* **905:**279–286

Corain, B., Nicolini, M., Zatta, P. 1992. Aspects of the bioinorganic chemistry of aluminium (III) relevant to the metal toxicity. *Coord. Chem. Rev.* **112:**33–45

Criado, M., Keller, B. U. 1987. A membrane fusion strategy for single-channel recordings of membranes usually non-accessible to patch-clamp pipette electrodes. *FEBS Lett.* **224:**172–176

De Pinto, V., Ludwig, O., Krause, J., Benz, R., Palmieri, F. 1987. Porin pores of mitochondrial outer membranes from high and low eukaryotic cells: biochemical and biophysical characterization. *Biochim. Biophys. Acta* **894:**109–119

Dill, E.T., Holden, M.J., Colombini, M. 1987. Voltage gating in VDAC is markedly inhibited by micromolar quantities of aluminum. *J. Membrane Biol.* **99:**187–196

Doring, C., Colombini, M. 1985. Voltage dependence and ion selectivity of the mitochondrial channel, VDAC, are modified by succinic anhydride. *J. Membrane Biol.* **83:**81–86

Ermishkin, L.N., Mirzabekov, T.A. 1990. Redistribution of the electric field within the pore contributes to the voltage-dependence of mitochondrial porin channel. *Biochim. Biophys. Acta* **1021:**161–168

Fèvre, F., Chich, J.-F., Lauquin, G.J.M., Henry, J.-P., Thieffry, M. 1990. Comparison of mitochondrial cationic channels in wild-type and porin-deficient mutant yeast. *FEBS Lett.* **262:**201–204

Holden, M.J., Colombini, M. 1988. The mitochondrial outer membrane channel, VDAC, is modulated by a soluble protein. *FEBS Lett.* **241:**105–109

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J. Biol. Chem.* **246:**5477–5487

Kinnally, K.W., Tedeschi, H., Mannella, C.A. 1987. Evidence for a novel voltage-activated channel in the outer mitochondrial membrane. *FEBS Lett.* **226:**83–87

Kinnally, K.W., Tedeschi, H., Mannella, C.A., Frisch, H.L. 1989. Kinetics of voltage-induced conductance increases in the outer mitochondrial membrane. *Biophys. J.* **55:**1205–1213

König, T., Kocsis, B., Meszaros, L., Nahm, K., Zoltan, S., Horvath, I. 1977. Interaction of a synthetic polyanion with rat liver mitochondria. *Biochim. Biophys. Acta* **462:**380–389

Labarca, P., Coronado, R., Miller, B.C. 1980. Thermodynamic and kinetic studies of the gating behaviour of a K^+-selective channel from sarcoplasmic reticulum membrane. *J. Gen. Physiol.* **76:**397–424

Liu, M.Y., Colombini, M. 1992. Regulation of mitochondrial respiration by controlling the permeability of the outer membrane through the mitochondrial channel, VDAC. *Biochim. Biophys. Acta* **1098:**255–260

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurements with the folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Ludwig, O., De Pinto, V., Palmieri, F., Benz, R. 1986. Pore formation by the mitochondrial porin of rat brain in lipid bilayer membranes. *Biochim. Biophys. Acta* **860:**268–276

Mannella, C.A., Forte, M., Colombini, M. 1992. Toward the molecular structure of the mitochondrial channel, VDAC. *J. Bioenerg. Biomembr.* **24:**7–19

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69:**3561–3566

Moran, O., Sorgato M.C. 1992. High-conductance pathways in mitochondrial membranes. *J. Bioenerg. Biomembr.* **24:**91–98

Moran, O., Sandri, G., Panfili, E., Stühmer, W., Sorgato, M.C. 1990. Electrophysiological characterization of contact sites in brain mitochondria. *J. Biol. Chem.* **265:**908–913

Moran O., Sciancalepore, M., Sandri, G., Panfili, E., Bassi, R., Ballarin, C., Sorgato, M.C. 1992. Ionic permeability of the mitochondrial outer membrane. *Eur. Biophys. J.* **20:**311–319

Oortgiesen, M., van Kleef, R.G.D.M., Vijverberg, H.P.M. 1990. Novel type of ion channel activated by Pb^{2+}, Cd^{2+}, and Al^{3+} in cultured mouse neuroblastoma cells. *J. Membrane Biol.* **113:**261–268

Peng, S., Blachly-Dyson E., Colombini, M., Forte, M. 1992. Determination of the number of polypeptide subunits in a functional VDAC channel from *Saccaromyces cerevisiae*. *J. Bioenerg. Biomembr.* **24:**27–31

Roos, N., Benz, R., Brdiczka, D. 1982. Identification and characterization of a pore-forming protein in the outer membrane of rat liver mitochondria *Biochim. Biophys. Acta* **686:**204–214

Sandri, G., Siagri, M., Panfili, E. 1988. Influence of Ca^{2+} on the isolation from rat brain mitochondria of a fraction enriched of boundary membrane contact sites. *Cell Calcium* **9:**908–913

Schein, S.J., Colombini, M., Finkelstein, A. 1976. Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from *Paramecium* mitochondria. *J. Membrane Biol.* **30:**99–120

Sigworth, F.J. 1983. An example of analysis. *In:* Single Channel Recording. B. Sakmann, E. Neher, editors. pp. 301–321. Plenum, New York

Sorgato, M.C., Keller, B.U., Stühmer, W. 1987. Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel. *Nature* **330:**498–500

Sorgato, M.C., Moran O. 1993. Channels in mitochondrial membranes: knowns, unknowns, and prospects for the future. *CRC Rev. Biochem. Mol. Biol.* **28:**127–171

Tedeschi, H., Mannella, C.A., Bowman, C.L. 1987. Patch clamping of the outer mitochondrial membrane. *J. Membrane Biol.* **97:**21–29

Tedeschi, H., Kinnally, K.W., Mannella, C.A. 1989. Properties of channels in the mitochondrial outer membrane. *J. Bioenerg. Biomembr.* **21:**451–459

Thieffry, M., Chich, J.-F., Goldshmidt, D., Henry, J.-P. 1988. Incorporation in lipid bilayers of a large conductance cationic channel from mitochondrial membranes. *EMBO J.* **7:**1449–1454

Thomas, L., Kocsis, E., Colombini, M., Erbe, E., Trus, B.L., Steven, A.C. 1991. Surface topography and molecular stoichiometry of the mitochondrial channel, VDAC, in crystalline arrays. *J. Struct. Biol.* **106:**161–171

Van der Voet, G.B., Marani, E., Tio, S., De Wolff, F.A. 1991. Aluminium neurotoxicity. *In:* Histo and Cytochemistry as a Tool in Environmental Toxicology. W. Graumann, J. Drukker, editors. pp. 235–242. G. Fisher Verlag, Stuttgart, New York

Wunder, U.R., Colombini, M. 1991. Patch clamping VDAC in liposomes containing whole mitochondrial membranes. *J. Membrane Biol.* **123:**83–91

Zhang, D.-W., Colombini, M. 1989. Inhibition by aluminium hydroxide of the voltage-dependent closure of the mitochondrial channel, VDAC. *Biochim. Biophys. Acta* **991:**68–78

Zhang, D.-W., Colombini, M. 1990. Group IIIA-metal hydroxides indirectly neutralize the voltage sensor of the voltage-dependent mitochondrial channel, VDAC, by interacting with a dynamic binding site. *Biochim. Biophys. Acta* **1025:**127–134

Zilberstein A. Ya. 1989. Nonelectrolytes inhibit amphotericin B ionic channel. *Biol. Membr.* **6:**1317–1329

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