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Inactivation of the Peptide-Sensitive Channel from the Yeast Mitochondrial Outer Membrane: Properties, Sensitivity to Trypsin and Modulation by a Basic Peptide

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Abstract. The yeast Peptide Sensitive Channel (PSC), a cationic channel of the mitochondrial outer membrane closes with slow kinetics at potentials of either polarity. The properties of this inactivation closely resemble those of the Voltage-Dependent Anion Channel (VDAC) slow kinetics closures. Addition of trypsin to one compartment suppresses the inactivation observed when this compartment is made positive, but does not affect the inactivation observed at potentials of reverse polarity. Both sides of the channel are sensitive. The reduced form of the Mast Cell Degranulating peptide (rMCD) increases the rate of inactivation, but only when the polarity of the compartment to which it is added is positive. The effect is not reversed by washing the peptide out, but is suppressed by trypsin. The peptide can bind to both sides of the membrane. The effect of rMCD on PSC closely resembles that of the ''modulator'' on VDAC. The similarities between PSC and VDAC suggest that the former might be a cationic porin of the mitochondrial outer membrane possessing a structure closely related to that of VDAC.

Key words: Mitochondrial channel — Inactivation — Bilayer — Yeast mitochondria — Porin

Introduction

In mitochondria, as in bacteria, the outer membrane is highly permeable and this permeability is attributed to large ionic channels, the porins. However, several structurally related bacterial porins have been described (Nikaido, 1994) whereas the high permeability of mitochondrial outer membrane has long been considered to originate in only one channel, the VDAC (Voltage Dependent Anion Channel) (Schein, Colombini & Finkelstein, 1976; Benz, 1994). We have described another channel of large conductance in the outer membrane of mammalian and yeast mitochondria, the PSC (Peptide Sensitive Channel) (Thieffry et al., 1988; Fèvre et al., 1990; Chich et al., 1991; Thieffry et al., 1992). This channel, which is slightly cationic, is blocked by various basic peptides (Henry et al., 1989; Fèvre, Henry & Thieffry, 1994; Juin et al., 1995). Among these peptides are mitochondrial presequence peptides, present at the N-terminal extremity of mitochondrial protein precursors. The effects of the 13 residue peptide pCytox IV(1–12)Y which is derived from the presequence of cytochrome c oxidase subunit IV precursor has been studied in detail (Henry et al., 1989; Fèvre et al., 1994). The characteristics of the blockade were consistent with the translocation of the peptide through the channel. This contention is supported by the observation that basic peptides active on the PSC were imported into intact mitochondria of a yeast strain in which the VDAC gene had been disrupted (Vallette et al., 1994).

These observations are interesting since they suggest that the PSC might be a component of the protein import machinery involved in mitochondrial biogenesis. Most of the mitochondrial proteins are synthesized in the cytoplasm as precursors with an N-terminal extension, the presequence, and they are then imported in an unfolded conformation through one or two mitochondrial membranes, depending upon their final destination (Kiebler et al., 1993). Dissection of the components involved in the translocation at the level of the outer membrane has progressed rapidly. However, the translocation pore is still unidentified and, because of its properties, the PSC is a good candidate.

The electrical properties of the PSC are different from those of the VDAC. The latter is characterized by slow kinetics with a symmetrical voltage dependence.

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For yeast or bovine adrenal medulla or adrenal cortex PSC, there exist rapid kinetics occurring only at one polarity of the transmembrane voltage (Fèvre et al., 1990; Thieffry et al., 1988; Thieffry et al., 1992). They have thus an asymmetrical voltage dependence. However, the study of yeast PSC using long durations of analysis showed that the channel closed after application of voltages of either polarity and reopened when voltage was returned to 0 mV or reversed (Thieffry et al., 1992). This slow kinetics of voltage dependence evokes that reported for VDAC and it might indicate some structural analogy between the two channels, as noted for the bacterial porins which have a common organization (Pauptit et al., 1992; Conan et al., 1992). In the present work, we describe the analysis of this activity, using a methodology close to that which has been developed for the study of VDAC.

Material and Methods

Preparation of Biological Membranes

Mitochondria were isolated from the porin-deficient mutant B_5 from *Saccharomyces cerevisiae* yeast strain DBY 747 which was kindly provided by Dr. G. Lauquin (Université Bordeaux II, France). They were prepared as described by Daum, Bohm and Schatz (1982).

Preparation of Proteoliposomes

Liposomes were prepared by sonicating to clarity a mixture (7:3) of bovine brain phosphatidylethanolamine and phosphatidylserine (Avanti Polar, Alabaster, USA) in 20 mM Hepes buffer (pH 7.5) at a final lipid concentration of 10 mg/ml. Biological membranes were centrifuged at 35,000 × *g* for 20 min and resuspended at 0.08–1 mg protein/ml in 0.15 M NaCl/20 mM Hepes buffer pH 7.5. A 75 μl aliquot of the phospholipid solution was added to 25 μl of the membrane solution. The mixture was frozen in liquid nitrogen and kept at −80°C. Before use, the mixture was thawed at room temperature and submitted to two additional cycles of freezing and thawing.

Electrical Recording

Planar lipid bilayers were formed by the Mueller-Rudin method (Mueller et al., 1963) using asolectin prepared from lecithin type II-S (Sigma Chemical, St. Louis, MO) according to Kagawa and Racker (1971). Control experiments were also carried out using bilayers formed from either bovine brain phosphatidylethanolamine and phosphatidylserine in the ratio 7:3 or diphytanoyl phosphatidylcholine (Avanti Polar, Alabaster, USA). The membranes were painted from a 20 mg/ml solution in *n*-decane over a 0.3 mm hole. Solutions were 10 mM Hepes-NaOH pH 7.4 (*trans*) and 150 mM NaCl, 10 mM Hepes-NaOH pH 7.4 (*cis*). 3–5 μl of the proteoliposome suspension described above were added to the *cis* compartment right against the lipid membrane and the transbilayer current was monitored under voltage clamp using a BLM-120 amplifier (Biologic, Claix, France). When a channel insertion was detected, the ionic strengths were made symmetrical by adding NaCl from a 3 M solution to the *trans* compartment. Data were filtered at 10 kHz and stored on videotape. Voltages are given by reference to that of the *trans* compartment defined as zero voltage.

Preparation of the Reduced form of the Mast Cell Degranulating Peptide (rMCD)

rMCD was prepared as previously described (Juin et al., 1995) from Mast Cell Degranulating Peptide (MCD) (NKGCIKRCIHPKIVHRKCNCKI) kindly provided by Dr. M. Lazdunski (Institut de Pharmacologie Moléculaire, Sophia-Antipolis, France).

Results

Inactivation of the Yeast PSC

Channels from the porin-deficient yeast strain B_5 were integrated in planar bilayers by fusion of proteoliposomes in the presence of an osmotic gradient. Insertion of a PSC was detected at 0 mV by an increase in the bilayer conductance exhibiting a cationic selectivity. In spite of strictly similar conditions, there was some variability in the reversal potentials which ranged from −40 to −57 mV. The properties of the channel were studied in symmetrical conditions after addition of 3M NaCl to the *trans* compartment.

In the open state, the PSC has a conductance γ_3 of 850 pS. It exhibits a complex voltage dependence. Following a voltage jump from 0 mV, the channel fluctuates with slow kinetics between several subconductance levels, the main ones γ_2 and γ_1 being respectively 330 and 660 pS below the open state (Fig. 1). For voltages of low amplitude (20 mV), the fluctuations between these three levels may remain stationary for periods of minutes. However, for voltages of higher amplitude, the channel inactivates. Following the voltage jump, and after a delay which depends on the voltage magnitude, the PSC no longer exhibits reopening from γ_2 to γ_3. After an additional delay, transitions from γ_1 to γ_2 no longer occur (Fig. 1).

Inactivation is observed for voltages of both polarities (Fig. 1). It is relieved by switching the voltage to 0 mV (Fig. 1*A*) or to a voltage of reverse polarity (*not shown*). For a given channel, the rate of inactivation increases with the voltage magnitude (Fig. 2). However, defining precisely the inactivated state appeared difficult. When inactivating voltages were applied for periods of one to several minutes, the channel frequently did not reopen following a switch to 0 mV or to a voltage of reverse polarity. It was then necessary to maintain the voltage close to 0 mV for several (up to 10) min before the channel reopens. Sometimes, reopening could also be obtained more rapidly by applying brief pulses of high magnitude (120–200 mV). In other cases, reopening could not be achieved. Thus, the inactivated state is not

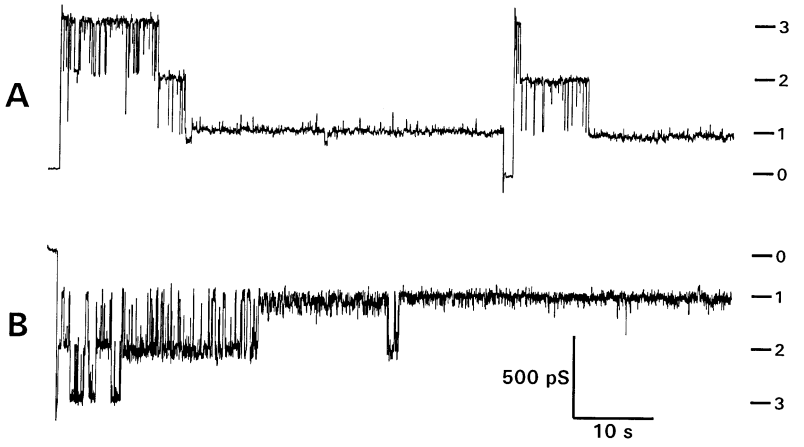

Fig. 1. Current recorded from a yeast PSC following a voltage jump from 0 to +40 mV (*A*) or −32 mV (*B*). The channel was incorporated by fusion to a diphytanoylphosphatidylcholine planar bilayer. The figures 0, 1, 2, 3 at the right of the traces indicate respectively the baseline and the three main conductances levels γ_1, γ_2 and γ_3 (fully open state). Data filtered at 50 Hz and sampled at 100 Hz.

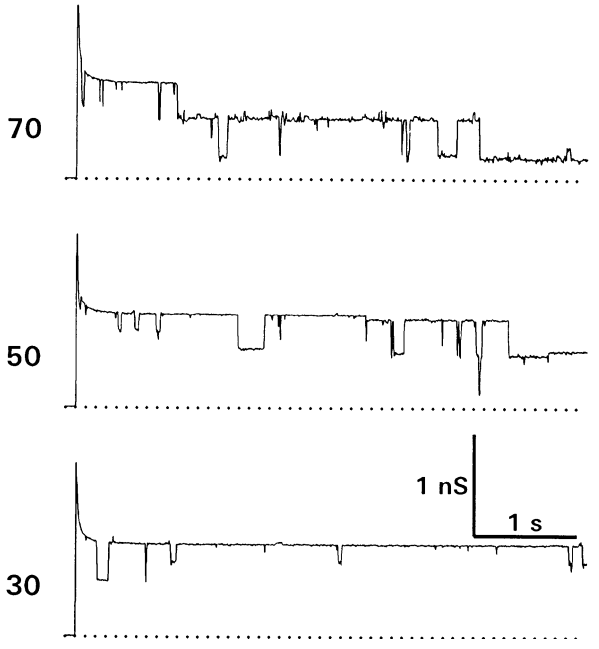

Fig. 2. Voltage dependence of a yeast PSC inactivation rate. Current recorded following voltage jumps from 0 mV to the value (in mV) indicated at the left of each trace. Data filtered at 100 Hz and sampled at 200 Hz.

a unique well-defined state and this prevented the quantitation of the rates of inactivation and recovery.

Since these properties were reminiscent of those of VDAC, we studied the response of PSC to a 5 mHz voltage sawtooth, a protocol often used to characterize the VDAC voltage dependence. Such a record is shown in Fig. 3*A*. In addition to the properties of inactivation exposed above, it shows that PSC has in fact more than three conductance levels. Below γ_1, there exist several subconductance levels which may appear as ill-defined noisy trace (*a*), transient closures to a stable level 60 pS below γ_1 (*b*) or fast flickering of about 100 pS amplitude (*c*). Current jumps of 400 pS may also occur from the γ_3 level. It may also be noted that closing and opening did not occur at the same transmembrane potential value, which appeared as hysteresis in *i/V* curves (Fig. 3*B*). Finally, like the VDAC, the PSC never completely closes.

All yeast PSCs examined in planar bilayers or tip-dip records inactivated. However, there was a large variability from channel to channel in the voltage sensitivity. This is illustrated in Fig. 4 which shows the mean conductance of two PSCs, computed over several sawtooth cycles, as a function of voltage. For both channels, the curves are roughly symmetrical, but the steepness of their voltage dependence differs. The same variability was observed in the responses to voltage jumps. The most sensitive channels inactivated within a few seconds following a 30 mV amplitude voltage jump from 0 mV whereas the least sensitive ones inactivated only after tens of seconds following a voltage jump of 80 mV amplitude. It was not possible to classify the channels in subsets having the same inactivation parameters, the latter appearing rather to vary within a continuum.

Effect of a Trypsin Treatment on PSC Inactivation

Inactivation is relieved by trypsin (Fig. 5). Both sides are sensitive to the protease. Addition of trypsin (10 μg/ml) to only one compartment suppressed the inactivation occurring when the compartment to which trypsin had been added was positively polarized with respect to the other one, but did not affect the inactivation occurring at

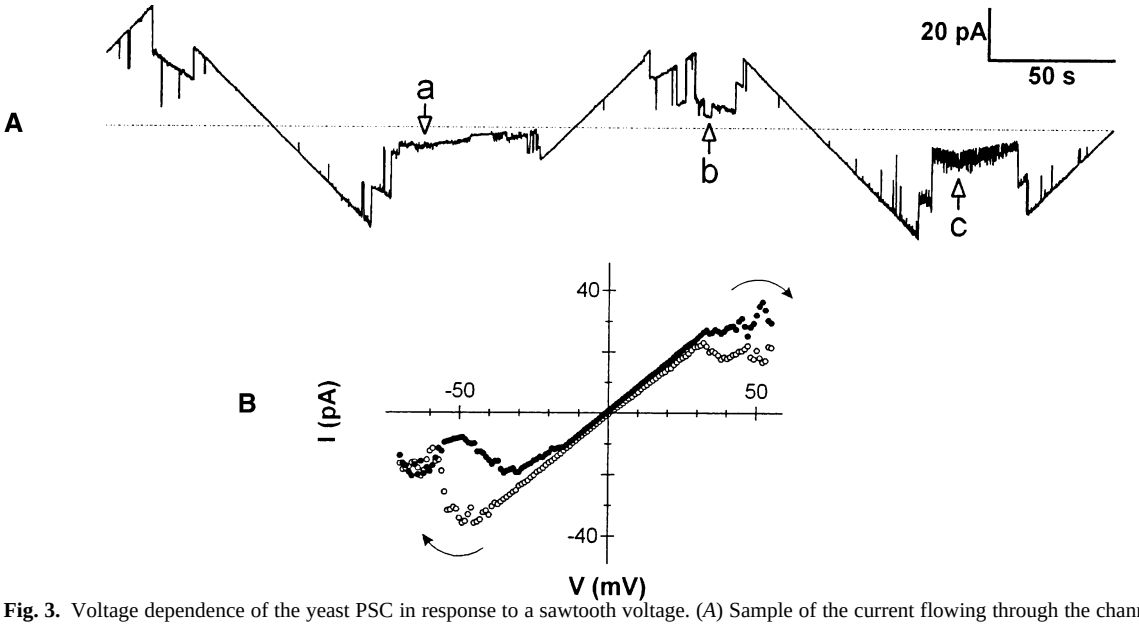

Fig. 3. Voltage dependence of the yeast PSC in response to a sawtooth voltage. (*A*) Sample of the current flowing through the channel in response to a 5 mHz sawtooth voltage. The voltage was varied between +55 and −70 mV. The dotted line indicates the 0 pA level. The arrows labeled *a*, *b* and *c* point to different forms of closures below the γ_1 level (*see text*). Data filtered at 10 Hz and sampled at 25 Hz. B: hysteresis of the *i/V* curve. The current was averaged over 5 cycles in 1 mV contiguous intervals during the ascending (●) or descending (○) parts of the voltage signal.

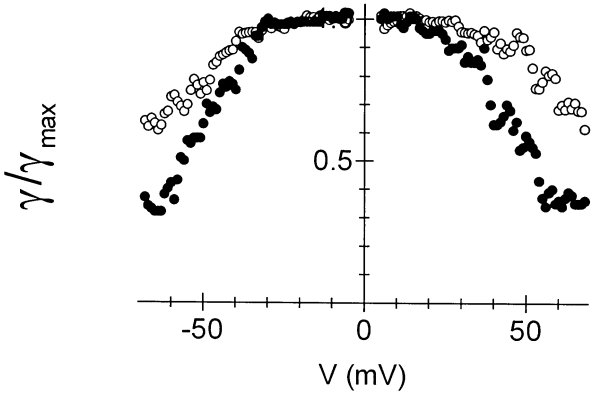

Fig. 4. Voltage dependence of the mean PSC conductance of two channels recorded in the same experimental conditions. The current flowing in response to a 5 mHz sawtooth voltage varying between −65 and +65 mV was averaged over several cycles in 1 mV contiguous intervals irrespective of the slope of the voltage signal, then divided by the voltage. The results are expressed as normalized conductance (mean conductance/maximum conductance). The charge q and the mid conductance voltage V_0 of the Boltzmann equation fitting the data were computed as described by Colombini (1989). Their values are respectively $q = 1.2$ and $V_0 = 48$ mV for channel 1 (●) and $q = 0.6$ and $V_0 = 71$ mV for channel 2 (○).

voltages of reverse polarity (Fig. 5*B*). The latter was in turn abolished by adding trypsin to the other compartment (Fig. 5*C*). Such a treatment neither modified the conductance levels nor suppressed the ability of the channel to close. As shown in Fig. 5, transitions to the lower conductance levels still occurred after proteolysis, but the channel fully reopened even at high potentials. The effects of trypsin are well summarized in plots of the mean current as a function of voltage (Fig. 5*D*). As expected, the trypsin effect was not reversed by washing the protease out.

Effect of Reduced Mast Cell Degranulating Peptide on PSC Inactivation

The Mast Cell Degranulating peptide is a 22 residue peptide from bee venom. It is extremely basic (9 positive charges and no negative charge) and two disulfide bridges lock it in a compact conformation. In this conformation, it had no effect on PSC inactivation (*data not shown*). This peptide can be irreversibly reduced by alkylation with iodoacetamide, without any change of its charge. The reduction results in a linearization of the structure and the reduced peptide (rMCD) had marked effects on the inactivation.

When added at low concentration (0.2 μM) to only one compartment (Fig. 6*B*), rMCD increased the rate of inactivation occurring when the compartment to which the peptide had been added was positively polarized (Fig. 6*B*, part of the curve below the dotted line), but did not affect the inactivation occurring at voltages of reverse polarity (Fig. 6*B*, part of the curve above the dotted line). The peptide affected both the closing and the opening of the channel, which in the presence of rMCD occurred at lower voltage magnitudes. As above, the effect of rMCD is well summarized in plots of the mean current as

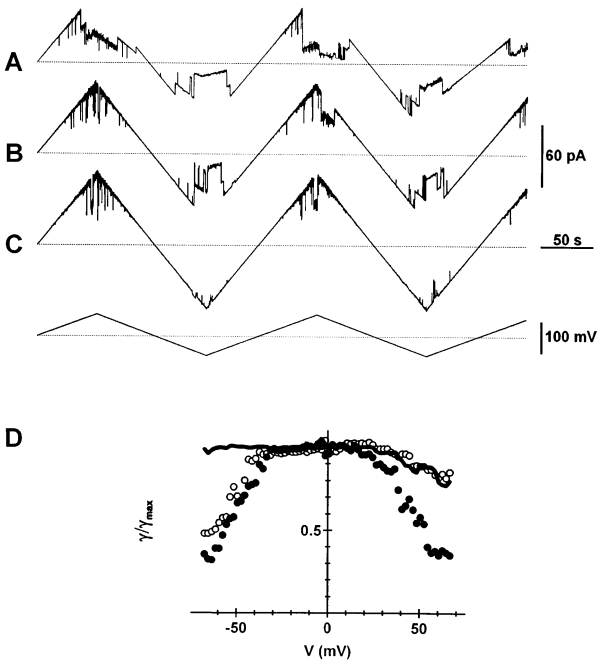

Fig. 5. Removal of PSC inactivation by trypsin. A voltage sawtooth varying between ±68 mV was continuously applied to the bilayer. (*A*) current flowing through the PSC prior to trypsin addition. (*B*) current recorded 5 min after addition of trypsin to the *cis* compartment at a final concentration of 10 μg/ml. (*C*) current recorded 5 min after trypsin addition to the *trans* compartment at the same final concentration. The protease was then present on both sides of the bilayer. (*D*) normalized mean conductance of the channel as a function of potential. The conductance was averaged over 2-sec contiguous intervals irrespective of the slope of the voltage signal before addition of trypsin (●), after action of trypsin *cis* (○) and after bilateral proteolysis (continuous line). Current filtered at 12.5 Hz and sampled at 25 Hz.

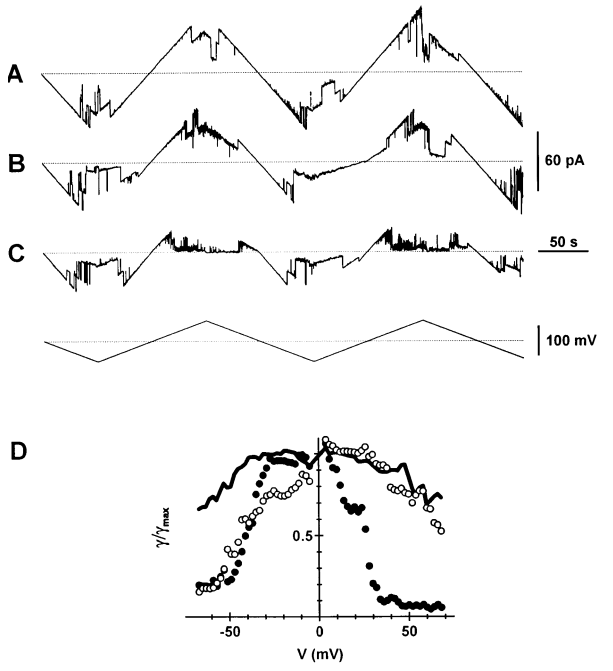

Fig. 6. Modification of PSC inactivation by rMCD. A voltage sawtooth varying between ±68 mV was continuously applied to the bilayer. (*A*) Current flowing through the PSC prior to rMCD addition. (*B*) Current recorded 5 min after addition of rMCD to the *trans* compartment at a final concentration of 0.2 μM. (*C*) Current recorded 5 min after rMCD addition to the *cis* compartment at the same final concentration. The peptide was then present on both sides of the bilayer. (*D*) Normalized mean conductance of the channel as a function of potential. The conductance was averaged over 2-sec contiguous intervals irrespective of the slope of the voltage signal before addition of rMCD (continuous line), after addition of rMCD *trans* (○) and in the presence of rMCD in both compartments (●). Current filtered at 12.5 Hz and sampled at 25 Hz.

a function of voltage (Fig. 6*D*). After addition of the peptide to the *trans* compartment (○), the mean conductance decreased at voltages of lower magnitude than in the control (solid line), but only at negative potentials.

The peptide was active on both sides of the channel. Consequently, when it was added to both compartments, it affected inactivation at both voltage polarities (Fig. 6*C* and Fig. 6*D*, ●). However, its effect was not fully symmetrical. In the experiment shown in Fig. 6, closures occurred at lower voltages at positive potentials and, at this polarity, voltages closer to 0 mV were required for the inactivated channel to reopen. It should also be noted that at this polarity, the PSC was then able to completely close (Fig. 6*C*, curve above the dotted line). Similar observations were done when voltage jumps from 0 mV were used in place of sawtooth voltages (*data not shown*).

The effect induced by rMCD was not reversed by washing the peptide out (Fig. 7). rMCD clearly decreased the time required for the channel to inactivate and this effect was unchanged 10 min after perfusion. However, the effect was abolished by addition of trypsin (10 μg/ml) to the compartment which either contained the peptide or had been perfused with a peptide-free buffer some minutes after peptide addition (Fig. 7*D*). The protease suppressed both the effect of rMCD and the inactivation (compare Fig. 7*A* and *D*). Channels modified by peptide added to one side were not affected by trypsin addition to the other side (*data not shown*). If the peptide had been added to both compartments, trypsin had to be added to both sides to suppress the rMCD effect.

Channels in which inactivation had been suppressed by trypsin were still sensitive to rMCD. After washing the protease out and exposure to rMCD, they inactivated again at a high rate following application of a positive voltage to the compartment where the peptide had been added (*data not shown*).

Finally, these experiments were repeated with bilayers having various compositions. No difference either in the inactivation itself or in the effect of rMCD was ob-

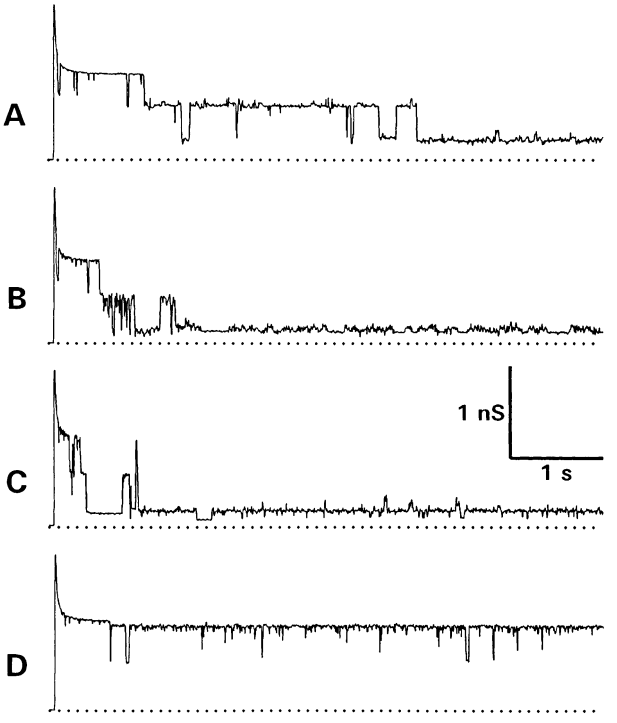

Fig. 7. Irreversibility of the rMCD effect. Current traces recorded following a voltage jump from 0 mV to +70 mV. (*A*) Control. (*B*) After exposure to 0.2 μM rMCD added to the *cis* compartment. (*C*) 5 min after washing the *cis* compartment with eight times its volume of rMCD-free solution. (*D*) After addition of trypsin to the *cis* compartment at a final concentration of 10 μg/ml. Data filtered at 100 Hz and sampled at 200 Hz.

served using asolectin, diphytanoylphosphatidylcholine or phosphatidylethanolamine-phosphatidylserine (7:3) bilayers.

Discussion

The data reported above show that yeast PSC inactivates following application of transmembrane voltages of both polarities. The properties of this inactivation evoke those of VDAC slow kinetics closures: (i) the channel closes following application of voltages of either polarity; (ii) the inactivated channel opens with fast kinetics following return to 0 mV or to a voltage of reverse polarity; (iii) there exists a family of ''closed'' states not completely non conductive; (iv) the rate of reopening depends on the time for which the voltage inducing inactivation has been applied (Colombini, 1986; Colombini, 1989; Colombini, 1994). However, PSC is clearly different from the VDAC. A possible involvement of porin, the molecule carrying the VDAC activity, in the PSC activity has to be discarded since all the experiments reported above were carried out using a yeast mutant strain in which the VDAC gene had been disrupted and in which there was no expression of an RNA specific for porin (Michedja, Guo & Lauquin, 1989). Independently, the selectivity of the PSC open state as well as that of the closed states observed at 0 mV in the presence of an ionic gradient are clearly cationic whereas VDAC is slightly anionic in its open conformation (Colombini, 1980). We have previously reported that, when the wild type is used in place of the porin-deficient mutant in the same experimental conditions as those used in the present study, VDAC is observed and exhibits indeed an anionic selectivity (Thieffry et al., 1994). We never found this activity in the porin-deficient mutant. Finally, PSC is blocked by basic peptides such as pCytox IV(1–12)Y which has no effect on VDAC (Thieffry et al., 1994).

Another similarity between PSC and VDAC is the way their voltage dependence is modified by charged molecules. The effect of rMCD, a highly charged basic peptide of linear structure, on the PSC is similar to that of the ''modulator protein,'' an acidic protein of the intermembrane space (Holden & Colombini, 1993), on the VDAC (Liu, Torgrimson & Colombini, 1994). Both molecules increase the steepness of the voltage-dependence by enhancing the closing rate and reducing the opening rate. They also facilitate transitions to low conductance levels rarely observed in the untreated channels (Fig. 6*C*). When added to only one compartment, they increase the steepness of the voltage dependence for transmembrane potentials of only one polarity. However, closures of the channel occur at positive potentials for rMCD and at negative potentials for the modulator, a property which might be expected if molecules carrying opposite charges act by plugging the channel. Finally, they bind tightly to both sides of the membrane and proteases (trypsin or pronase) are required to suppress the peptide-induced modifications.

The experiments carried out with trypsin show that the PSC inactivation is controlled by two structures acting independently and respectively exposed to the *cis* and *trans* compartments. Removal of these structures does not severely impair the PSC structure since the channel remains able to close transiently at the same conductance levels after proteolysis. Moreover, rMCD still binds tightly to both sides of the bilayer after pretreatment of the channel by trypsin and restores a voltage dependence resembling the original inactivation. This implies that removal of the rMCD effect by trypsin is due to proteolysis of the peptide itself. According to this view, PSC inactivation would not be an intrinsic property of the channel, but would originate in interactions with undetermined basic components. The binding sites for the rMCD-like components should be occupied on both sides of the channel to account for the symmetry of the inactivation. This hypothesis is supported by several observations: (i) as shown above, individual variations in

the voltage dependence of inactivation were large and might result from the diversity of the molecules bound; (ii) we have previously noted (Thieffry et al., 1992) that yeast PSC channels analyzed by patch clamp after insertion in giant liposomes did not inactivate, an observation which might be interpreted by the loss or the inactivation of the interacting component during the reconstitution procedure; (iii) PSC from bovine adrenal cortex did not show a clear inactivation, similar to that described for yeast. However, after exposure to rMCD, these channels inactivated like rMCD treated yeast channels (*unpublished results*).

We have previously shown that basic peptides, such as pCytox IV(1–22), a 22 residue peptide bearing 4 positive charges forming the N-terminal extremity of cytochrome c oxidase subunit IV precursor, irreversibly bind to either sides of the yeast channel (Fèvre et al., 1994). Once bound to one side, they confer to the PSC a characteristic asymmetrical voltage dependence, increasing the steady-state probabilities of the lowest conductance levels when the voltage gradient between the side exposed to the peptide and the unexposed side increases. For both pCytox IV(1–22) and rMCD, the effects do not depend on the composition of the planar bilayer. This argues against an electrostatic interaction of the charged peptides with the bilayer which would affect the channel environment. An alternate possibility is the existence of peptide binding sites on the channel or on a surrounding protein. The affinity for rMCD would have to be high since this peptide is active for inactivation at submicromolar concentrations. Moreover, it should be specific for the unfolded conformation since the native form of MCD, which is locked in a compact conformation by two internal disulfide bridges, has no effect on inactivation. We have previously proposed a ball and chain mechanism (Armstrong & Bezanilla, 1977) to explain the voltage dependence observed after binding of pCytox IV(1–22) (Fèvre et al., 1994). The same mechanism might account for the effect of rMCD on PSC and that of the modulator on VDAC.

It is somehow surprising to find peptide binding sites on both sides of the channel. It is tempting to associate the existence of such bilateral binding sites with the hypothesis that the PSC might be a component of the protein import apparatus which translocates unfolded proteins through mitochondrial membranes. It is now well established that presequence binding domains are present on both sides of the outer membrane translocation complex (Mayer, Neupert & Lill, 1995; Bolliger et al., 1995).

The similarities between PSC and VDAC reported above suggest that the two channels might have related structures. Though this hypothesis is speculative, it is worth noting that Mannella, Neuwald and Lawrence (1996) recently reported the existence of common structural features in porin, the molecule carrying the VDAC activity and TOM 40 (formerly ISP 42 in *Saccharomyces* and MOM 38 in *Neurospora*), the outer membrane import pore. Thus similarities between VDAC and PSC would not be surprising if PSC is the import pore, an hypothesis which is consistent with different sets of experimental results (Henry et al., 1996), but remains to be demonstrated.

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