Reversible and Irreversible Effects of Basic Peptides on the Mitochondrial Cationic Channel

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ABSTRACT We have previously shown that a 13-residue basic peptide, derived from the presequence of a mitochondrial precursor, blocked the cationic channel of the outer mitochondrial membrane. The properties of the blockade suggested that the peptide could go through the pore in the presence of a sufficient driving force. In an attempt to evaluate more precisely the relevance of such an interpretation, we have examined the effect on the same channel of basic peptides from 16 to 34 residues, most of which are parts of or derive from mitochondrial presequences. Two peptides were found to induce a reversible voltage-dependent blockade, the properties of which were the same as those of the blockade induced by the 13-residue peptide. The others had a similar effect, but triggered in addition a modification of the voltage gating that persisted after washing the peptide out. The modification was in turn abolished by trypsin added to the side of the channel previously exposed to the peptide. The protease acted on the bound peptide and not on the channel itself. The irreversible modification of the voltage gating, the mechanism of which remains obscure, was not specific for mitochondrial-addressing sequences.

INTRODUCTION

A cationic channel of large conductance is reliably observed in bilayers enriched in mammalian and yeast mitochondrial membranes. First characterized in membranes formed at the tip of microelectrodes by the tip-dip method (Thieffry et al., 1988), it was further identified both in patches of proteoliposomes and in planar bilayers enriched with the same material (Thieffry et al., 1992). Fractionation experiments, in which the frequency of observation of the channel was compared to the activity of enzymatic markers, indicated a localization in the outer membrane (Chich et al., 1991). Any possible relationship between this channel and the voltage-dependent anion channel (VDAC) the mitochondrial porin, was ruled out in yeast by using a porin-deficient mutant (Fèvre et al., 1990).

Yeast and mammalian cationic channels differ essentially by their voltage dependence. The mammalian channel is open for voltages of one polarity (corresponding to negative voltages in the cytoplasmic compartment) and closes with fast kinetics fluctuations for voltages of reverse polarity (Thieffry et al., 1988). By contrast, the yeast channel, which may flicker by bursts at sufficient voltages of one polarity (Fèvre et al., 1990), closes with slow kinetics after application of steady-state voltages of either polarity and reopens immediately when voltage is returned to 0 or reversed (Thieffry et al., 1992), a property that resembles the voltage dependence of VDAC (Colombini, 1989).

Although not identical, yeast and mammalian channels share several common properties. Both have a slight cationic selectivity ($P_{Na^+}/P_{Cl^-} \approx 4$), three main conductance levels and never completely close (Thieffry et al., 1992). Thus, they are likely to have the same physiological role. This idea is further supported by the fact that channels of either source are blocked by pL4(1-12)Y (referred to in previous papers as peptide M), a 13-residue peptide derived from a mitochondrial-addressing presequence. Moreover, the properties of the blockade are consistent with a translocation of the peptide through the channel (Henry et al., 1989; Thieffry et al., 1992). This observation was of great potential interest because it suggested that the channel could be involved in the mitochondrial biogenesis. Most of the mitochondrial proteins are synthesized in the cytoplasm as precursors, then imported into the organelle. The process is generally controlled by an N-terminal presequence that is rich in basic residues and possesses amphiphilic properties. It is now widely accepted that the precursors, once unfolded, are imported through an aqueous pore, the signal sequence being first translocated (Hartl et al., 1989). The cationic channel from mammalian or yeast origin, named peptide-sensitive channel (PSC) in view of its sensitivity to pL4(1-12)Y, is permeant to large molecules and thus is a good candidate as the translocation pore. It was interesting, therefore, to collect additional information about possible interactions between the channel and precursors or peptides that had already been tested as targeting sequences.

The use of whole precursors, although of considerable interest, is potentially difficult. In physiological conditions, these molecules have to be processed by a complex machinery before the translocation step (Hannavy et al., 1993), and the probability for this machinery to remain associated in a functional form with a channel incorporated in a lipid bilayer

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Abbreviations used: pAT III, RNASVLKSSKNAKRYLRCNLKA; pL4(1-12)Y, MLSLRQSIRFFKY; pL4(1-16), MLSLRQSIRFFKPATR; pL4(1-22), MLSLRQSIRFFKPATRTLCSSR; pL4(1-23)A19, MLSLRQSIRFFKPATRTLASSRY; pOAT(1-34), MLSKLASLQTVAALRRGLRTSVASATSVAT KKTE; PSC, peptide-sensitive channel; VDAC, voltage-dependent anion channel.

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is a priori extremely weak. We thus restricted our investigations to six peptides. Five are parts of or derive from natural presequences. The sixth one is unrelated to mitochondrial presequences but is, like the latter, amphiphilic and highly basic. It was formerly shown not to compete with precursors in import experiments (Glaser and Cumsky, 1990). We report below that these peptides induced reversible blocks similar to that caused by pL*4*(1-12)Y. In addition, some of them were found to modify irreversibly the gating properties of the channel by a mechanism that remains obscure and deserves further examination.

MATERIALS AND METHODS

Preparation of biological membranes

Bovine adrenal cortex mitochondria

Bovine adrenal cortex mitochondria were prepared by differential centrifugation as described earlier (Henry et al., 1989).

Yeast mitochondria

Mitochondria were isolated from the porin-deficient derivative B_5 from yeast strain DBY 747, which was kindly provided by Dr. G. Lauquin (Université Bordeaux II, France). They were prepared as described by Daum et al. (1982).

Preparation of proteoliposomes

Liposomes were prepared by sonicating to clarity a mixture (7:3) of bovine brain phosphatidylethanolamine and phosphatidylserine (Avanti Polar, Alabaster, AL) 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

Tip-dip experiments

Microelectrodes were pulled from Vitrex borosilicate hematocrit tubes (Modulohm I/S, Herlev, Denmark) using a P 87 puller (Sutter Instrument Co., San Rafael, CA). In 150 mM NaCl, their resistance was 3–10 MΩ. Their tips were fire-polished. Surface monolayers were formed by adding 5–20 μl of the proteoliposome suspension to 200 μl of a solution (150 mM NaCl, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.3). Microelectrodes were introduced into the bath under positive pressure. Bilayers were formed by the tip-dip method (Coronado and Latorre, 1983; Suarez-Isla et al., 1983; Wilmsen et al., 1983). Current measurements were made using a patch clamp amplifier EPC 7 (List, Darmstadt/Eberstadt, Germany). Data were filtered at 10 kHz and stored on video tape. The bath potential was taken as the reference voltage. Solution exchange was done by transfer of the pipette tip to a bath containing the new solution.

Planar bilayers

Planar lipid bilayers were formed by the Mueller-Rudin method (Mueller et al., 1963). Phospholipids (Avanti Polar) were either bovine brain phosphatidylethanolamine and phosphatidylserine in the ratio 7:3 or diphytanoyl phosphatidylcholine. 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*). Three to five μl of the proteoliposome suspension described above was 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, solutions were made symmetrical by adding NaCl from a 3 M solution to the *trans* compartment. Data were filtered at 10 kHz and stored on video tape. Voltages are given by reference to that of the *trans* compartment defined as zero voltage.

Peptides

Four peptides are parts of or derive from pL*4*(1-23), the 23 N-terminal residues of cytochrome c oxidase subunit IV precursor from yeast *Saccharomyces cerevisiae* (MLSLRQSIRFFKPATRTLCSSRY). pL*4*(1-12)Y (referred to as peptide M in previous papers) contains the first 12 residues, the minimal mitochondrial-addressing sequence required to translocate precursors synthesized in the cytosol (Hurt et al., 1985) and a C-terminal tyrosine. pL*4*(1-16) and pL*4*(1-22) correspond to the 16 and 22 N-terminal residues of pL4(1-23), respectively. pL*4*(1-23)A19 is identical to pL*4*(1-23) except in position 19, where an alanine is substituted for a cysteine. These peptides may be referred to in other works as pCyt OX IV in place of pL*4*.

pOAT(1-34) (MLSKLASLQTVAALRRGLRTSVASATSVATKKTE) contains the first 34 residues of the presequence of rat liver ornithine aminotransferase precursor.

pAT III (RNASVLKSSKNAKRYLRCNLKA) is a fragment of antithrombin III.

pL4(1-12)Y and pL*4*(1-23)A19 were synthesized by Neosystems (Strasbourg, France). pL*4*(1-16), pL*4*(1-22), and pAT III were generous gifts from Dr. M. G. Cumsky (University of California, Irvine, CA). pOAT(1-34) was kindly provided by Dr. H. Ono (Yamagata, Japan).

RESULTS

Effect of peptide pL4(1-16) on yeast channels

The effect of peptide pL*4*(1-16) was investigated in channels from yeast mitochondria. These channels exhibit closures with slow kinetics. After application of a transmembrane potential in the range of ±50 mV, they generally remain in their fully open state γ_3 (for durations of several seconds to minutes depending on the voltage magnitude) before closing to conductance levels γ_2 and γ_1, 330 and 660 pS below the maximum conductance level (850 pS in 150 mM NaCl), respectively. In the fully open state, the current records exhibit only rare and very brief closures (Fig. 1, *left*). When the pipette was transferred to a bath containing pL*4*(1-16) at concentrations of a few micromoles/l, a voltage-dependent blockade developed immediately (Fig. 1, *right*). Transient closures to levels γ_2 and γ_1 appeared at potentials below 50 mV. Their number increased with decreasing potentials while their duration first increased then decreased. Increasing the peptide concentration resulted in higher closure frequencies. The voltage dependence of the block magnitude appeared strikingly similar at different peptide concentrations (Fig. 2). Its magnitude increased for voltages decreasing from 50 to −10 mV, then slightly decreased. The blockade was reversible by transferring the pipette to a peptide-free bath (data not shown).

The characteristics of the effect described above are similar to those we have previously reported for the blockade

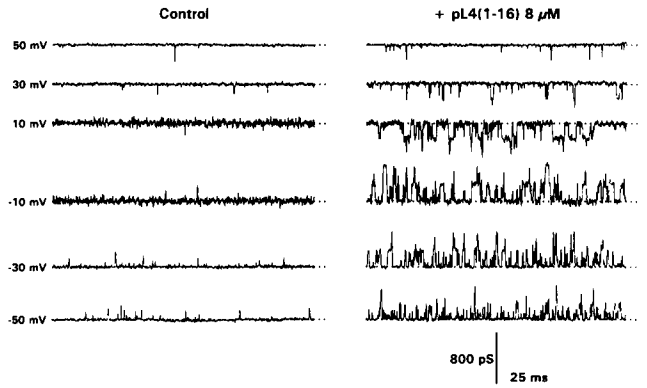

FIGURE 1 Blockade by pL4(1-16) of a porin-deficient yeast PSC incorporated in a bilayer formed at the tip of a microelectrode. Left: samples of current recorded at different potentials (indicated at the *left* of each trace) before exposure to the peptide. Right: activity recorded at the same potentials after transfer of the tip to a bath containing pL4(1-16) at a concentration of 8 μM. Data were filtered at 2.5 kHz and sampled at 10 kHz. In this figure and the following ones, the dotted line indicates the channel maximum conductance.

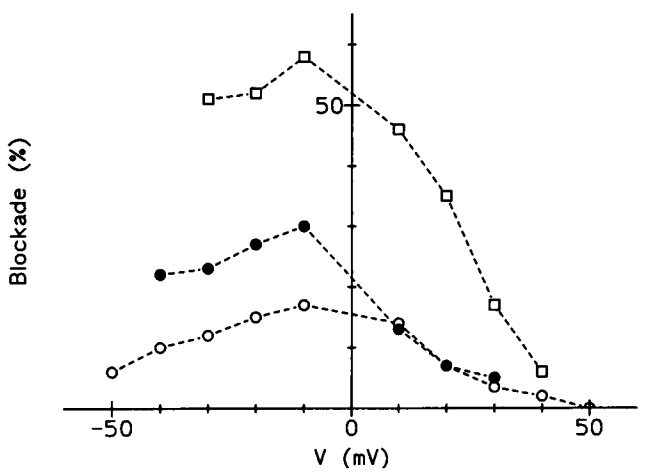

FIGURE 2 Voltage dependence of the magnitude of the blockade induced by pL4(1-16). The peptide concentrations were, respectively, 8 μM (○), 20 μM (●), and 50 μM (□). The magnitude is defined as the percentage of the mean current flowing through the channel which is suppressed by the peptide. The mean current was determined at each potential over periods of at least 10 s. Data are from three different experiments carried out by the tip-dip method using yeast mitochondria.

induced by pL4(1-12)Y (Henry et al., 1989). To compare more precisely the effects of the two peptides, the duration of the induced closures was analyzed as a function of voltage. This analysis is complicated because the channel has at least two closed states, γ_2 and γ_1. However, at low peptide concentrations ($\leq$10 μM), most of the peptide-induced closures occur according to the pattern γ_3-γ_2-γ_3. The closures to level γ_1 correspond mainly to jumps from level γ_2, and direct transitions from γ_3 to γ_1 are rare. We thus considered the channel as composed of two independent pores and restricted the analysis to the closures starting from the maximum conductance γ_3, discarding the events during which the channel conductance fell below γ_2. The results are shown in Fig. 3.

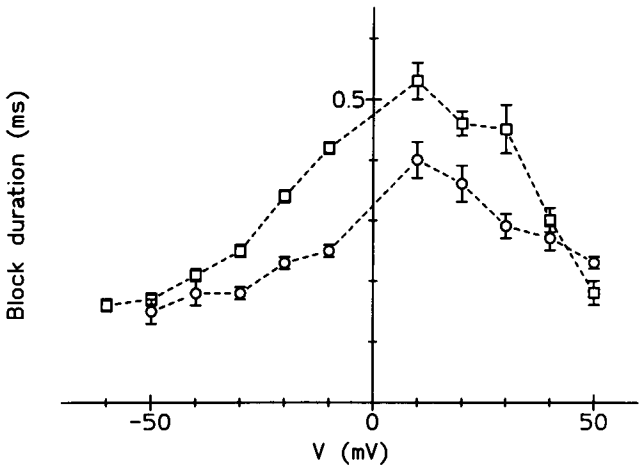

FIGURE 3 Voltage dependence of the duration of closures induced by pL4(1-12)Y (○) and pL4(1-16) (□) in tip-dip bilayers containing a yeast PSC. SE are indicated by bars. Data used for computation were filtered at 5 kHz and sampled at 20 kHz.

For both pL4(1-12)Y and pL4(1-16), the block duration first increased then decreased when the potential was decreased from 50 mV down to negative values, i.e., when the driving force for the peptide was increased. It was maximum around 0 mV and, at the same potential, pL4(1-16) induced longer closures than pL4(1-12)Y.

Effect of peptide pL4(1-22) on yeast channels

When PSCs from yeast mitochondria inserted in tip-dip bilayers were exposed to micromolar concentrations of pL4(1-22), a more complex effect developed. Brief closures (<1 ms), similar to those that occurred in the presence of pL4(1-16), appeared at potentials below 50 mV (Fig. 4). Again, their frequency increased with the driving force. In addition, longer (several tens of milliseconds) closures at levels γ_2 and γ_1 were observed simultaneously at low negative voltages (Fig. 4, *middle*, trace at −30 mV). When the peptide was washed out, the fast component was eliminated but the slower was not. Washing even increased the probability of the closed states (Fig. 4, *right*). The maximum conductance was not affected.

This irreversible effect was still observed 1 h after washing the peptide out. When the tip was transferred to a bath containing trypsin, the effect was eliminated within a few minutes (Fig. 5). The proteolysis did not preclude further action of pL4(1-22). If the channel was exposed to the same peptide concentration after trypsin washing, both fast reversible and slower irreversible closures were again induced, and washing the peptide out similarly increased the probability of the lowest conductance levels (Fig. 5, *bottom* traces).

Both the setting in of the irreversible effect and its elimination by trypsin developed progressively. It was not possible to detect precise times at which the peptide induced sudden changes in the gating behavior, changes which would be expected if the peptide bound to a specific receptor. In

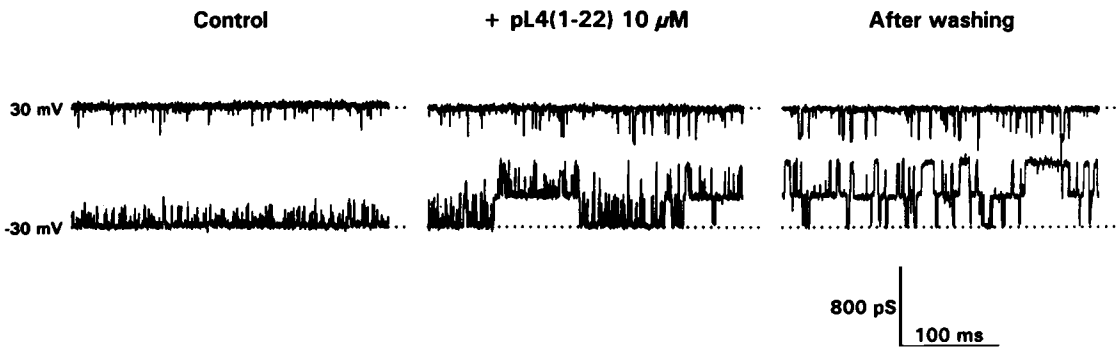

FIGURE 4 Effect of pL4(1-22) on a yeast PSC. The channel activity was recorded at ±30 mV (*left*), and the tip was transferred to a bath containing the peptide at a concentration of 10 μM. After 2 min, the activity was recorded at the same potentials (*middle*), and the tip was transferred to a peptide-free bath. The traces became immediately cleaner, but no longer changed during the following minutes. Right: samples of current traces recorded 10 min after removal of external peptide. Data were filtered at 2.5 kHz and sampled at 10 kHz.

mammalian PSC, we reliably observe that trypsin suppresses in two steps the spontaneous fluctuations (Chich et al., 1991; Thieffry et al., 1988); however, we could not detect similar clear-cut discrete changes during proteolysis of channels that had been exposed to pL4(1-22).

Although yeast PSC exhibits slow kinetics closures after application of voltages of either polarity, its electrical properties are not symmetrical. The presence of a fast flicker often occurring by bursts at potentials of one polarity and a steeper voltage dependence of the slow kinetics closures for voltages of the opposite polarity (Fèvre et al., 1990) make it possible to identify the orientation of the channel in the bilayer. In tip-dip bilayers, the yeast PSC is, for an unknown reason, almost always found with the same orientation. Because changing the pipette solution is difficult, we would rather use yeast PSCs fused to planar bilayers for comparing the effects of peptide addition to either compartment. Moreover, we took advantage of the possibility to use different phospholipids to rule out a possible dependence of the effect on the bilayer composition. PSCs were fused to diphytanoylphosphatidylcholine bilayers. Their orientation was determined, and the *trans* compartment was defined as that which corresponded to the bath in tip-dip records. When pL4(1-22) was added to this compartment, the complex effect described above was again observed, and the activity remaining after peptide removal was comparable to that recorded in tip-dip experiments (data not shown). The peptide was also active when added to the *cis* compartment (Fig. 6). After washing, the channel appeared strongly voltage-dependent. However, the voltage dependence was now opposite to that described above, the probability of the closed states increasing with increasing potentials. Channels exposed to the peptide on the *cis* side were not affected by trypsin addition to the *trans* side (Fig. 6, *bottom*). They progressively recovered their original properties within a few minutes when the protease was added to the *cis* compartment.

Effect of pL4(1-22) on adrenal cortex channels

The effect of pL4(1-22) on channels from adrenal cortex mitochondria was investigated in tip-dip records. It was quite similar to that described above for yeast channels. The peptide induced both fast reversible and slower irreversible closures, irrespective of the channel orientation in the bilayer (Fig. 7). After treatment, channels having the orientation shown in Fig. 7 *B* closed for potentials of either polarity. The induced gating resembled the natural gating except for its kinetics, which were slower.

pL4(1-22) also acted in the same way on channels whose voltage dependence had been abolished by trypsin before exposure to the peptide. The effect was relieved by trypsin and could be restored by a second exposure to the peptide (data not shown).

Voltage dependence of channels treated by pL4(1-22)

The voltage dependence of trypsinized adrenal cortex and yeast channels recorded after exposure to pL4(1-22) and the return to a peptide-free bath is illustrated in Fig. 8. The probabilities of the closed states, separated from the highest conductance level by two jumps of equal amplitude (250 pS for the cortical channel and 300 pS for the yeast channel), increase with decreasing voltages. The current patterns recorded at different potentials are reminiscent of the spontaneous fluctuations typical of the native mammalian PSC (Fig. 7). In the latter, the three main conductance levels are separated by smaller jumps (220 pS), and the kinetics are faster. The voltage dependence of peptide-modified channels (Fig. 8, *bottom*) is, like that of the native PSC, well described supposing a dimeric model in which the probability $p(V)$ of closure of each of two independent identical subunits follows the Boltzmann equation $p(V) = (1 + \exp[-zF(V - V_0)/RT])^{-1}$.

Effect of other peptides

Two other peptides were found to have the same properties as pL4(1-22). The first one, pOAT(1-34), is a natural presequence (Ono and Tuboi, 1990). The second one, pAT III, a fragment of antithrombin III, is a priori irrelevant to mitochondrial targeting. Both peptides induced fast reversible

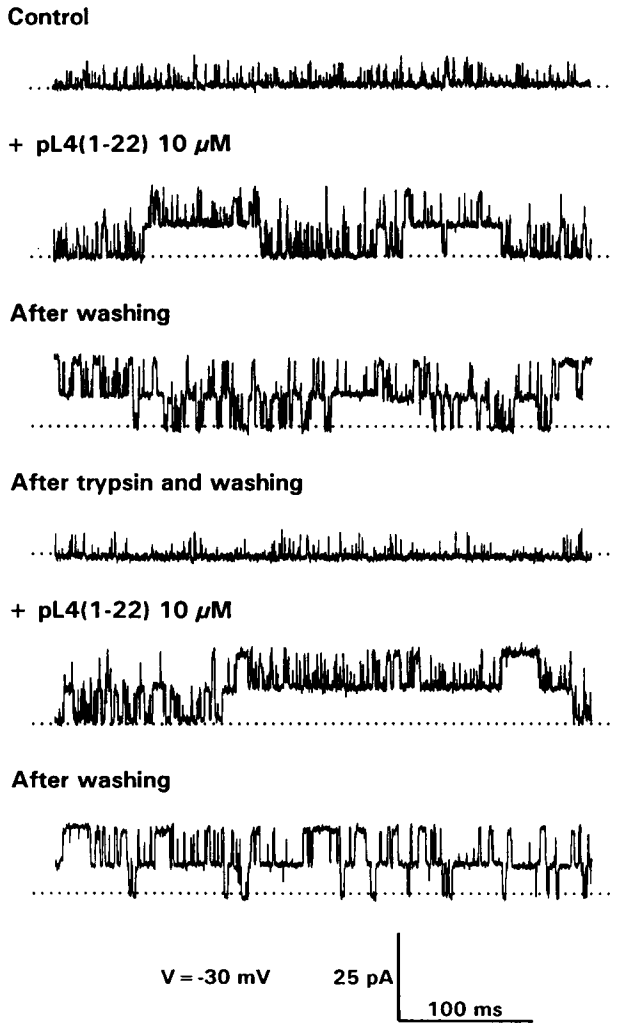

FIGURE 5 Suppression of the irreversible effect of pL4(1-22) by trypsin. From top to bottom: samples of current traces successively recorded at −30 mV from a tip-dip bilayer containing a yeast PSC at different steps of the following protocol. The activity was recorded in control conditions, then the tip was transferred to a bath containing pL4(1-22) at the concentration of 10 μM. After 2-min exposure, the peptide was eliminated and the tip was washed for a few minutes. The tip was transferred to a solution containing trypsin at the concentration of 10 μg/ml. Within a few minutes, the peptide effect was eliminated and the tip was washed. The bilayer was again exposed to the peptide for 2 min and finally transferred to a peptide-free bath. Data were filtered at 2.5 kHz and sampled at 10 kHz.

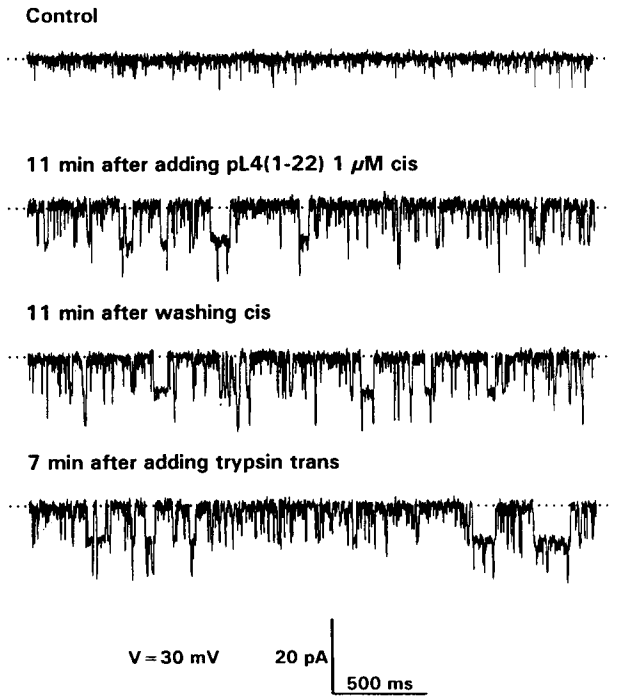

FIGURE 6 Effect of pL4(1-22) on a yeast PSC incorporated in a diphytanoylphosphatidylcholine planar bilayer. From top to bottom: samples of current traces successively recorded at +30 mV from a tip-dip bilayer containing a yeast PSC at different steps of the following protocol. The activity was recorded in control conditions, then pL4(1-22) was added at a final concentration of 1 μM to the *cis* compartment (equivalent to the pipette solution in the experiment shown in Fig. 4). After 11-min exposure, the *cis* compartment was perfused with a peptide-free solution. Finally, trypsin was added 11 min later to the *trans* compartment. The activity recorded at −30 mV (not shown) remained unchanged throughout the experiment. Data were filtered at 1.5 kHz and sampled at 10 kHz.

and slower irreversible closures and, as for pL4(1-22), washing the peptide out not only failed to restore the initial activity but still increased the probability of the lowest conductance levels. Binding of pAT III did not prevent further blockade of the modified channel by pL4(1-12)Y (data not shown).

We tested also pL4(1-23)A19, a peptide composed of the 23 first amino acids of the presequence of cytochrome c oxidase subunit IV precursor, except in position 19 where an alanine had been substituted for a cysteine. This peptide thus differed from pL4(1-22) by residue 19 and by the presence of an additional tyrosine at the C-terminal. Surprisingly, this compound was found to have the same effect as pL4(1-12)Y and pL4(1-16): it blocked the channel reversibly. The voltage dependence of block magnitude and block duration were similar to those of pL4(1-16) (data not shown).

DISCUSSION

The peptides tested in the present study separated into two groups. The first one includes pL4(1-12)Y, pL4(1-16), and pL4(1-23)A19. The peptides of this group induced a reversible voltage-dependent blockade. The peptides of the second group (pL4(1-22), pOAT(1-34), and pAT III) had the same effect but induced, in addition, modifications of the channel properties that were not reversed by washing the peptide out. We shall successively focus the discussion on three topics, the involvement of the PSC in mitochondrial biogenesis, the gating properties of mammalian and yeast PSC, and the nature of the irreversible action of peptides of the second group.

The present results do not provide clear-cut information concerning the possible involvement of the PSC in the process of precursor import. The properties of the blockade induced by peptides of the first group were similar to those previously reported for the reversible blockade induced by pL4(1-12)Y (Henry et al., 1989; Thieffry et al., 1992). They acted in the same way on both sides of the channel. The

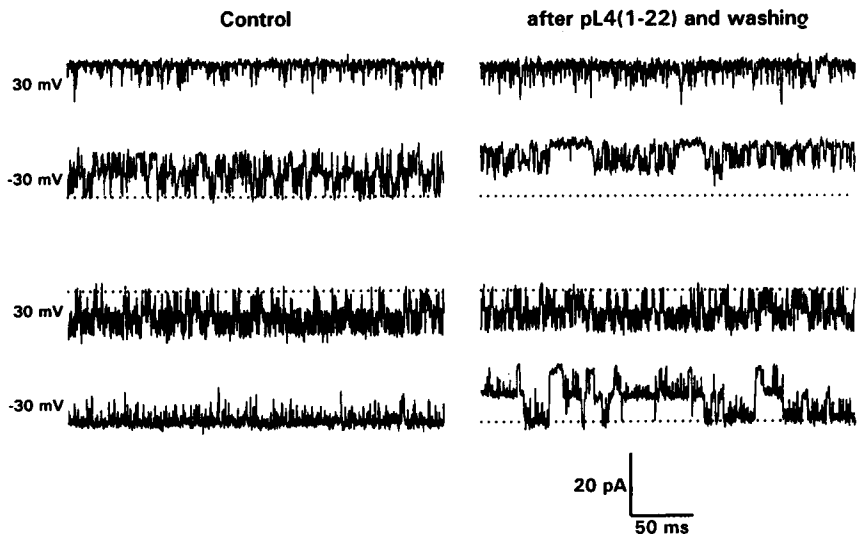

FIGURE 7 Effect of pL*A*(1-22) on adrenal cortex PSC incorporated in tip-dip bilayers. The activity of two channels incorporated in the bilayer with opposite orientation (*A* and *B*) was recorded before (*left*) and after exposure to pL*A*(1-22) at the concentration of 5 μM and return to a peptide-free bath (*right*). Samples of current traces were recorded at ±30 mV. Data were filtered at 2.5 kHz and sampled at 10 kHz.

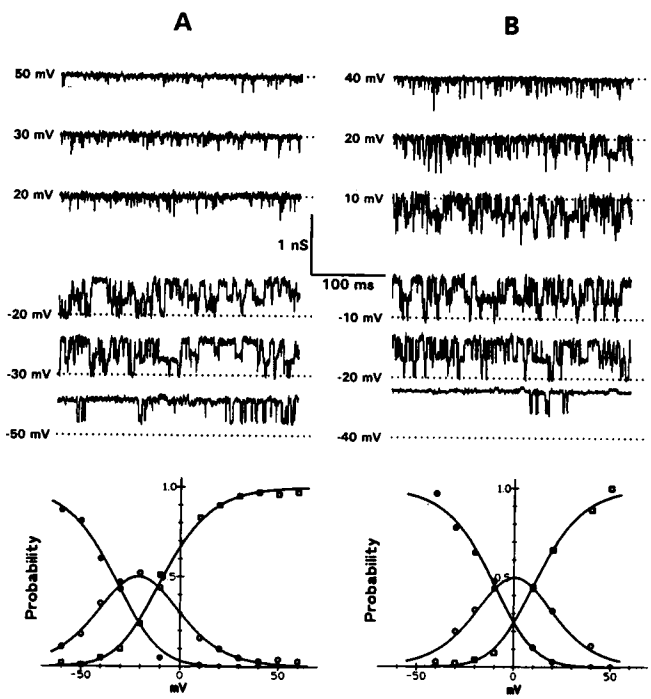

FIGURE 8 Voltage dependence of an adrenal cortex PSC pretreated by trypsin (*left*) and a yeast PSC (*right*) whose gating had been irreversibly modified by 5 μM pL4(1-22). For both channels, records were done after return to a peptide-free bath. Top: samples of current recorded at different potentials. Data were filtered at 2.5 kHz and sampled at 10 kHz. Bottom: voltage dependence of the probability of the three main conductance levels γ_3 (□), γ_2 (○), and γ_1 (●). The solid lines represent the probabilities of these levels for a channel formed of two independent two-state subunits whose open state probability follows the Boltzmann equation $p(V) = (1 + \exp[-zF(V - V_0)/RT])^{-1}$. The values of z and V_0 are, respectively, 1.8 and -21.5 mV for the adrenal cortex channel and 1.77 and 0 mV for the yeast channel.

voltage dependence of the blockade, which is related to the compartment to which the peptide is added and not to the orientation of the channel in the bilayer, suggests that the effect results from a plugging of the channel. However, the results are not compatible with a Woodhull type of block because block magnitude and block duration do not increase monotonically with the driving force applied to the peptide. As shown in Figs. 2 and 3, both parameters first increase (voltage decreasing from 50 to −10 mV) then decrease when the voltage becomes lower than −10 mV. We thus propose, as we previously did for pL4(1-12)Y (Henry et al., 1989; Thieffry et al., 1992), that peptides of the first group block the channel by plugging the pore but can go through when their electrochemical potential becomes sufficient. Potentials above 10 mV would be too small to pull the peptide through the pore, and block is relieved only when the peptide leaves the channel mouth, in agreement with the Woodhull model. Decreasing the potential below 10 mV increases the driving force on the peptide, and it can go through the pore. The block duration would then reflect the time necessary for the peptide to be translocated. The latter is expected to decrease when the driving force increases, which is in good agreement with the data of Fig. 3.

Peptides of the second group also induced reversible brief closures. However, the latter could not be analyzed in the same way because they were superimposed on the slower closures that persisted after washing the peptide out. We thus lack arguments favoring the hypothesis of their translocation through the channel. The only hint that they could go through the pore is the puzzling observation that the probability of the closed states increases when the peptide is washed out (Fig. 4). Due to the resemblance of their reversible effect, peptides of both groups are likely to act by the same mechanism. Thus, if closure results from a plugging of the pore, free peptide would compete with bound peptide at the site where the latter blocks the channel. As a consequence, a stronger block would result in the presence of peptide in the bath unless the free peptide could go through the pore.

The typical effect of peptides of the second group appears unspecific for mitochondrial presequences because it was also obtained using pAT III. Different from mitochondrial presequences, the latter does not inhibit import of mitochondrial precursors and, thus, is most likely devoid of

mitochondrial-addressing capability (Glaser and Cumsky, 1990). Moreover, inhibition of precursor import by pL4(1-22) was reported to be reversible (Glaser and Cumsky, 1990), which is in contradiction with the effect of the peptide on the channel. Thus, the present results do not correlate with the properties of the precursor import mechanism. By contrast, they are in good agreement with data recently obtained by F. Vallette and co-workers (Vallette et al., 1994). These authors have shown that pL4(1-12)Y and dynorphin B(1-13), a neuropeptide unrelated to mitochondrial import that blocks reversibly the PSC (Henry et al., 1989), were imported in the intermembrane space through an alternative pathway, different from that followed by matrix-targeted precursors. Interestingly, the import of pL4(1-12)Y and dynorphin B(1-13) was inhibited by pAT III as well as by pL4(1-22), which suggests that PSC is involved in this pathway. The possible relationship between this pathway and another alternative pathway, that by which cytochrome c heme lyase is directly translocated in the intermembrane space (Lill et al., 1992), is still unknown. The latter uses components of the main import machinery such as the general import protein (Lill et al., 1992) and one cannot exclude presently that the pathway described by Vallette and co-workers also uses such components.

The mammalian PSC differs from the yeast PSC by the presence of typical sustained and voltage-dependent rapid fluctuations that are eliminated by exposing the cytoplasmic side of the channel to trypsin (Chich et al., 1991; Thieffry et al., 1988). Formerly, we have interpreted this proteolysis as an action of the enzyme on the channel. The present results suggest another possible interpretation. On one hand, both PSC from adrenal cortex treated by trypsin and yeast PSC exhibit, after exposure to peptides of the second group, current patterns that resemble those of the adrenal cortex PSC before it is exposed to the protease (Fig. 8). On the other hand, trypsin abolishes the peptide action by proteolysis of the attached peptide. In physiological conditions, mammalian PSC, thus, might be in interaction with a cytosolic factor having properties similar to those of peptides of the second group. This interaction would be maintained throughout the protocol used for preparing the mitochondria and would be suppressed when trypsin cleaves the interacting factor. In yeast, such an interaction either might not exist or might occur with factors that have different properties. Alternatively, it could be lost during the steps preceding the incorporation of the channels in the bilayer. This model, however, is hardly consistent with the fact that peptides are active on the native adrenal cortex channels (Fig. 7), whereas occupancy of the sites by the bound factor would then be expected to prevent further binding of peptide.

The nature of the strong interaction that is responsible for the irreversible effect remains obscure. The formation of disulfide bonds between the peptide and the channel would require the presence of cysteine residues in all peptides of the second group, which is not the case for pOAT(1-34). The fact that trypsin interrupts the interaction is in no way indicative of a protein target. Channels treated by the protease before or after exposure to the peptide remained equally sensitive to peptide application. Thus, trypsin interrupts the interaction by acting on the peptide and not on the channel itself.

Two lines of evidence show that the peptides bind to components located on the side of the channel exposed to the peptide. First, the voltage dependence of the modified channel depends on the compartment to which the peptide is added and not on the orientation of the channel in the bilayer. Second, trypsin acts only when added to the side of the channel that has been exposed to the peptide (Fig. 6). Both facts exclude the possibility that the peptides added in *cis* or *trans* compartments could have a common target that they would reach either directly or after translocation through the membrane. Binding sites thus exist on both sides of the bilayer. Moreover, the progressive setting in of the effect suggests that they are present in multiple copies.

One possibility could be that the peptides bind to phospholipids. Like pL4(1-12)Y (Henry et al., 1989) and other addressing mitochondrial sequences (Lemire et al., 1989; Roise et al., 1988), the peptides used in the present study have amphiphilic properties and might interact with phospholipids. The most extensive data available about this type of interaction concern pL4(1-25), a longer part of the presequence containing pL4(1-22), and a labeled form of the same peptide. Both were shown to insert in artificial membranes (Roise, 1992; Tamm, 1986). The binding to the membrane surface occurs by partitioning of the peptide into the bilayer, and its characteristics suggest that the insertion would be rapidly reversible (Roise, 1992). From the latter data, it seems thus unlikely that the peptide-phospholipid interaction could be the basis of the irreversible effect of pL4(1-22), pOAT(1-34), and pAT III. However, direct evidence should be obtained using one of the latter peptides in binding experiments before a definite conclusion can be drawn. In fact, minor changes may alter dramatically the properties of such molecules (Glaser and Cumsky, 1990). This point is illustrated in the present work by the different effects of pL4(1-22) and pL4(1-23)A19, which have presumably very close amphiphilic properties.

Alternatively, the peptides could bind to the channel itself or to associated proteins. Interestingly, Mannella and coworkers have shown that pL4(3–22), a peptide differing from pL4(1-22) by the absence of the two amino terminal residues, induced structural changes in two-dimensional crystals prepared from outer mitochondrial membranes (Mannella et al., 1992). Their data indicated that the peptide bound to proteins rather than to lipid domains. According to the authors, the binding site would belong to the VDAC. In the present work, the experiments involving yeast channels were carried out using a porin-deficient mutant that excludes the VDAC as the peptide target. However, it remains possible that the pore arrays of membrane crystals analyzed by these authors contain PSC together with VDAC and that the sites identified as the peptide targets in the pictures belong, in fact, to PSC. On one hand, VDAC is not affected by pL4(1-12)Y (Thieffry et al., 1994). On the other hand, VDAC and yeast PSC appear to have common properties. Both have slow kinetics closures

and fast openings, do not completely close, and can translocate large molecules (Colombini, 1989; Thieffry et al., 1992). This suggests that both activities could be carried by related molecules that might be as difficult to discriminate in two-dimensional crystals as in electrical recordings (Thieffry et al., 1994). The idea is further supported by the fact that negatively charged molecules such as dextran sulfate (Mangan and Colombini, 1987), König's polyanion (Colombini et al., 1987) or the "modulator", an acidic protein of the intermembrane space (Holden and Colombini, 1988; Liu and Colombini, 1992), modify the gating of VDAC from both sides of the membrane in a way that resembles the effect of basic peptides on the PSC. Moreover, the effect of the modulator, like that of peptides of the second group, is not reversed by washing (M. Colombini, personal communication). Alternatively, the binding sites could be proteins associated to the channel. Whatever the nature of these sites, the data are consistent for both channels with a plugging of the pore by a charged molecule (negatively charged for VDAC, positively charged for PSC) attached to or near the channel, a mechanism similar to the ball and chain model accounting for the voltage-gating of Na^+ and K^+ channels originally proposed by Armstrong and Benzanilla (1977).

The unexpected effect of peptides of the second group obviously deserves further investigation because the determination of its mechanism appears to be a prerequisite for deciding whether interactions similar to those we observed are likely to occur in physiological conditions.

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