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Blockade of a Mitochondrial Cationic Channel by an Addressing Peptide: An Electrophysiological Study

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Summary. A voltage-dependent cationic channel of large conductance is observed in phospholipid bilayers formed at the tip of microelectrodes from proteoliposomes derived from mitochondrial membranes. This channel was blocked by a 13-residue peptide with the sequence of the amino terminal extremity of the nuclear-coded subunit IV of cytochrome *c* oxidase. The blockade was reversible, voltage- and dose-dependent. The peptide did not affect the activity of a *Torpedo* chloride channel observed under the same conditions. From experiments with phospholipid monolayers, it is unlikely that the peptide inserts into bilayers under the experimental conditions used. The blockade was observed from both sides of the membrane, being characterized by more frequent transitions to the lower conductance states, and a maximum effect was observed around 0 mV. Channels, the gating mechanism of which had been eliminated by exposure to trypsin, were also blocked by the peptide. For trypsinized channels, the duration of the closure decreased and the blockade saturated at potentials below −30 mV. These observations are consistent with a translocation of the peptide through the channel. Dynorphin B, which has the same length and charge as the peptide, had some blocking activity. Introduction of negative charges in the peptide by succinylation suppressed the activity.

Key Words ionic channels · mitochondria · addressing peptide · phospholipid bilayer · protein import

Introduction

Most of the mitochondrial proteins are synthesized in the cytoplasm as precursors, which are then translocated into the organelle (Hay, Böhni & Gasser, 1984; Hurt & Van Loon, 1986; Schatz, 1987; Hartl et al., 1989). The transport of these precursors is generally controlled by an N-terminal signal sequence, which, though not unique, is characterized by a high content of basic amino acids and the absence of acidic amino acids (Allison & Schatz, 1986; Von Heijne, 1986; Baker & Schatz, 1987). Though many characteristics of the import process are now well established, the precise mechanism of precursor translocation remains unknown. An early step in this process is the voltage-dependent translocation of the presequence (Gasser, Daum & Schatz, 1982; Schleyer, Schmidt & Neupert, 1982; Pfanner & Neupert, 1985). In view of the amphipathic nature of these presequences, a mechanism has been proposed according to which the translocation might involve the spontaneous insertion of the presequence into the cytoplasmic phospholipid monolayer, followed by electrophoresis of the positive charges through the membrane bilayer (Roise et al., 1986; Von Heijne, 1988), though the latter effect has been disputed (Skerjanc, Shore & Silvius, 1987). An alternative hypothesis proposes that the peptide is translocated through a membrane channel, the whole import process occurring in an aqueous environment (Hurt & Van Loon, 1986; Singer, Maher & Yaffe, 1987). However, at the present time no direct experimental evidence supports the latter hypothesis.

We have recently described a new channel of large conductance in mitochondria (Thieffry et al., 1988). This channel is rather cationic, whereas the other mitochondrial channels are slightly anionic (Colombini, 1986; Sorgato, Keller & Stühmer, 1987). Because mitochondrial signal sequences are positively charged, it was tempting to test whether this channel might be a candidate for protein translocation. As a first step, we describe in this communication the effect on the channel activity of a peptide with a mitochondrial addressing sequence. The tested peptide, peptide M (Table), has the first twelve residues of the N-terminal part of the cytochrome *c* oxidase subunit IV precursor from *Saccharomyces cerevisiae* (Schatz, 1987). When fused to cytosolic proteins, this sequence is known to direct the chimeric protein into the mitochondrial matrix (Hurt et al., 1985).

Materials and Methods

Materials

Phosphatidylethanolamine and phosphatidylserine (bovine brain) were purchased from Avanti Polar (Birmingham, AL). Peptide M was synthesized by Appligene (Strasbourg, France); stock solutions (10 mM) were prepared in dimethylsulfoxide.

Preparation of Mitochondria from Bovine Adrenal Cortex

Rapidly dissected bovine adrenal cortices (50 g) were cut into small pieces and homogenized in 250 ml of ice-cold 0.3 M sucrose/10 mM HEPES (pH 7.0) with a glass-Teflon homogenizer operating at 2000 rpm. The homogenate was centrifuged at 750 × *g* for 10 min, the pellet was discarded, and the supernatant was again centrifuged under the same conditions. The resulting supernatant was centrifuged at 8000 × *g* for 10 min. The 8000 × *g* pellet was resuspended in the same volume of homogenization buffer and washed twice by centrifugation under the same conditions. The final pellet was resuspended in 10 ml of buffer at a protein concentration of about 20 mg/ml. In some preparations, the mitochondrial pellet (10 mg of protein/ml) was further washed by incubation for 20 min at 4°C in sucrose buffer containing 0.1 mg of digitonin/mg of protein, followed by 20-fold dilution with sucrose buffer and centrifugation at 8000 × *g* for 10 min.

Preparation of Proteoliposomes from Mitochondria

Proteoliposomes were prepared as described (Thieffry et al., 1988) following the procedure of Ewald, Williams and Levitan (1985); the protein concentration of the proteoliposomes was generally decreased to 0.5 mg/ml.

Electrical Recording

Bilayers were formed at the tip of a microelectrode by the "tip-dip" method (Coronado & Latorre, 1983; Suarez-Isla et al., 1983; Wilmsen et al., 1983), as described (Thieffry et al., 1988). The bath and the microelectrode had the same composition: 150 mM NaCl, 1 mM $MgCl_2$, 20 mM HEPES (pH 7.3). Magnesium ions facilitated the formation of the seal, but the channel activity was affected neither by magnesium nor by calcium ions. The bath potential was taken as the reference voltage. Conductance histograms were computed from histograms of the current flowing through the whole patch including the leak current. Peptide M and its succinylated derivatives were solubilized in dimethylsulfoxide at 10 mM concentration and the solution was added to the bath at a maximum final solvent concentration of 1%. Dimethylsulfoxide at this concentration had no effect on current fluctuations. Other peptides were solubilized by the same procedure or directly in the aqueous buffer.

Succinylation of Peptide M

Succinic anhydride (25 μmol) was added to peptide M (2.5 μmol) solubilized in 0.25 ml of dimethylsulfoxide, and the mixture was incubated for 3 hr at room temperature. The mixture was resolved by HPLC, using a Nova-Pak C_{18} Column (Waters, Milford, MS) and H_2O: acetonitrile mixtures as the solvent. Four peaks were obtained which were assumed to be the unreacted peptide, the peptides succinylated on the terminal amino group and on the ε-amino group of Lys 12, and the twice succinylated peptide.

Phospholipid Monolayer Experiments

Surface pressure was measured by the Wilhelmy method using a 2,200 surface barostat from KSV Chemicals (Helsinki, Finland) and a 5-ml one-compartment rectangular through (30 × 40 mm) as described (Pattus et al., 1983).

Results

Effect of Peptide M on the Mitochondrial Cationic Channel

A voltage-dependent cationic channel of large conductance is observed in phospholipid bilayers formed at the tip of microelectrodes from liposomes enriched in mitochondrial membranes (Thieffry et al., 1988). This channel has three main levels of increasing conductance (1, 2, 3) separated by jumps of 220 pS in 150 mM NaCl. In the most often encountered conformation (Fig. 1*A*) the conductance fluctuates rapidly between these states at negative potentials, whereas at positive potentials the channel is mainly in the fully open state. The effect of adding 100 μM peptide M to the bath is shown in Fig. 1*B*. It mainly consisted of a decrease of the channel total conductance, due to more frequent brief transitions to the lower conductance states. The blockade was voltage dependent and was maximal at potentials around 0 mV where the channel mean conductance was reduced to about half the control value (Fig. 1, upper right).

The effect was observed immediately after transfer and could be reversed by transfer back to a peptide-free bath (Fig. 2*A*). However, if bilayers were exposed to 100 μM peptide M for more than 1 min, they were often disrupted following transfer back to the peptide-free bath. This fragility, which introduced a limit to the concentration of peptide used, suggested some detergent effect of the peptide (Roise et al., 1986). The blockade was dose dependent in the 10–100 μM peptide concentration range (Fig. 2*B*).

As reported previously (Thieffry et al., 1988), the channel may have two forms which differ slightly by their potential-dependence: type I and type II. Though data illustrated in the present paper concern only type I channels, identical results were obtained with type II channels.

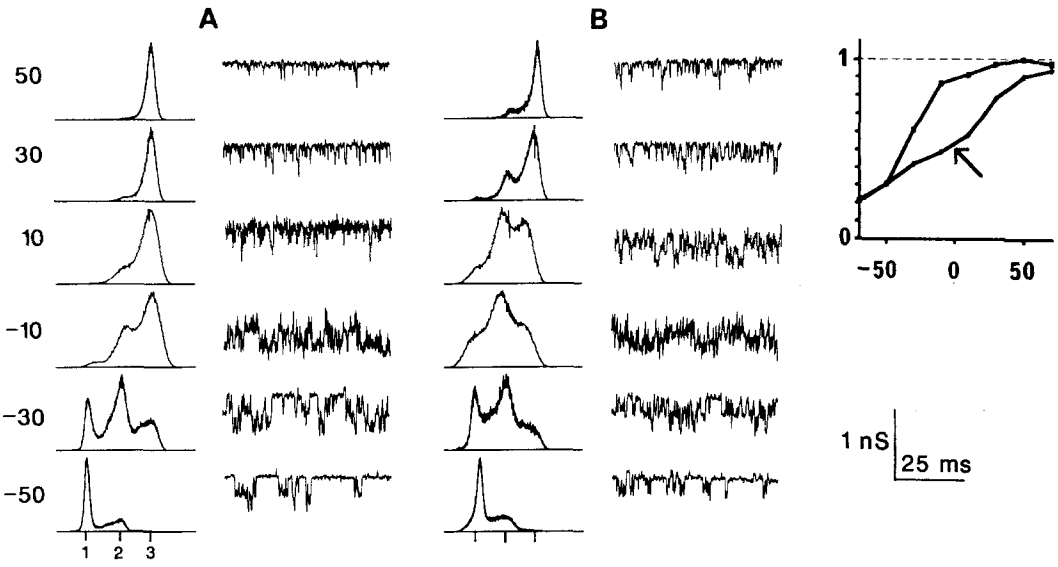

Fig. 1. Effect of peptide M on the current flowing through a mitochondrial cationic channel incorporated in a lipid bilayer at the tip of a microelectrode. Conductance histograms (left) and samples of current fluctuations (right) are shown at the pipette potentials indicated. (*A*) control; (*B*) after transfer of the tip to a bath containing 100 μM peptide M. Numbers *1*, *2* and *3* in histograms refer to the three main levels of increasing conductance. Conductance histograms were computed over periods of at least 5 sec at each potential. Filter: 3 kHz; sampling: 20 kHz. Upper right: Voltage-dependence of the channel mean conductance in control conditions and in the presence of 100 μM peptide (indicated by the arrow). The mean conductance is normalized between 1 (maximum conductance) and 0 (minimum conductance)

Peptide M Does not Block the Channel through the Lipidic Phase

In view of the detergent effect mentioned above, it was important to test if the blockade might result from a direct nonspecific interaction of the peptide with the phospholipid bilayer. This hypothesis is important to consider since mitochondrial presequences were previously shown to form amphiphilic helices and to perturb natural and artificial phospholipid bilayers (Ito et al., 1985; Epand et al., 1986; Roise et al., 1986; Von Heijne, 1986; Myers et al., 1987). In a first set of experiments, we tested the effect of the peptide on bilayers containing a well characterized chloride channel from *Torpedo* electric organ (Hanke & Miller, 1983), reincorporated by the same technique and under the same experimental conditions (Thieffry et al., 1987). Exposure of such channels to 100 μM peptide M had no effect either on the channel conductance or on its voltage dependence. Furthermore, no additional electrical events were detected in the -180, $+180$ mV range (*data not shown*).

Evidence for the insertion of presequences into phospholipid bilayers arises mainly from experiments in which the incorporation of the peptide into phospholipid bilayers is measured by the increase in surface pressure induced by adding the peptide to the sub-phase at different initial pressures (Roise et al., 1986, Tamm, 1986). Similar measurements performed under our experimental conditions (phospholipid and aqueous sub-phase compositions) indicated an incorporation of peptide M into the monolayer up to a limiting pressure of about 35 mN/m (Fig. 3). Under the same conditions, the surface pressure corresponding to the monolayer-bilayer equilibrium (i.e., the pressure of the monolayers used to form the tip bilayers) was about 50 mN/m. Therefore, the peptide is unlikely to insert into the bilayers we used.

Peptide M Does not Act on the Channel-Gating Mechanism

The current fluctuations observed in the presence of peptide M might be accounted for by a plugging of the peptide molecule in the channel. Alternatively, they might result from an action of peptide molecules on the gating mechanism, either through binding to an allosteric receptor or by direct electrostatic interactions. In this type of hypothesis, application of peptide M to the opposite side of the membrane would be expected to have no or a different effect. We took advantage of the asymmetry of the channel molecule, as evidenced by the asymme-

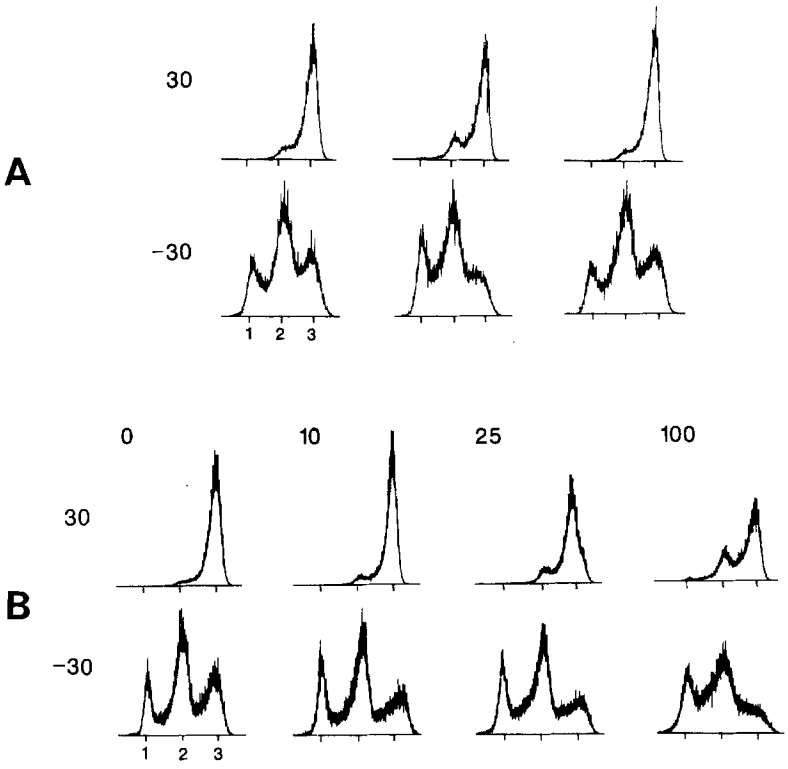

Fig. 2. Characteristics of the peptide-induced blockade. (*A*) Reversibility. An active patch (left) was transferred to a bath containing 100 μM peptide M (middle) and then to a peptide-free bath (right). (*B*) Dose-dependence. The effect of the peptide concentration was analyzed by transferring successively the same active membrane patch to baths containing peptide concentrations of 10, 25 and 100 μM. Data are shown as conductance histograms obtained at +30 and −30 mV. The numbers (*1*, *2*, *3*) on the histograms refer to the main levels of conductance as defined in Fig. 1. Filter: 2.5 kHz; sampling: 5 kHz

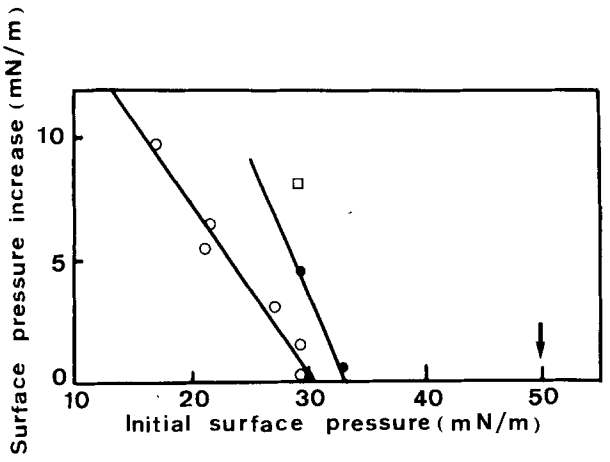

Fig. 3. Interaction of peptide M with a lipid monolayer. The increase of surface pressure resulting from the addition of peptide M to the bath was measured as a function of the initial pressure. Peptide M (○, 10 μM; ●, 100 μM) was injected in the sub-phase buffer (20 mM HEPES, pH 7.4/150 mM NaCl/10 mM $MgSO_4$) beneath the phospholipid monolayer (PE/PS, 70/30). One experiment (□) was performed in the absence of added magnesium ions, which indicated an inhibitory effect of these ions on the interaction. The equilibrium surface pressure of the monolayer is indicated by the arrow

try of its voltage dependence (Fig. 1*A*), to rule out the latter hypotheses. As reported earlier (Thieffry et al., 1988), the channel may insert in the patch bilayer in the opposite direction (Fig. 4*A*); the effect of 100 μM bath-applied peptide was tested on channels with such an orientation. A voltage-dependent blockade characterized by more frequent transitions to lower conductance levels was again observed (Fig. 4*B*). It increased with decreasing potentials down to −30 mV (Fig. 4, upper right). Below −30 mV, the blockade no longer increased, the channel mean conductance remaining at about half its control value.

Another line of evidence against an effect at the level of the gating mechanism was obtained using channels in which this mechanism had been virtually eliminated by exposure to trypsin (Thieffry et al., 1988). Current fluctuations were recorded as previously (Fig. 5, *Control*); the bilayer was then transferred to a bath containing 10 μg/ml trypsin, leaving the channel in the highest conductance state, with only some rare and brief closures at all potentials (Fig. 5, *Trypsin*). Transfer of the trypsinized channel to a peptide-containing bath restored rapid fluctuations between three levels separated by conductance steps of about 220 pS (Fig. 5, *Peptide M*). Again the channel blockade was characterized by brief transitions to the lower conductance levels. Blockade increased with decreasing potentials down to 10 mV. Below this value, the channel mean conductance remained constant at about half its control value (Fig. 5, upper right).

In native channels, closures induced by lower peptide concentrations were difficult to discriminate from the spontaneous activity. In trypsinized preparations, they were readily observed at a 10-μM

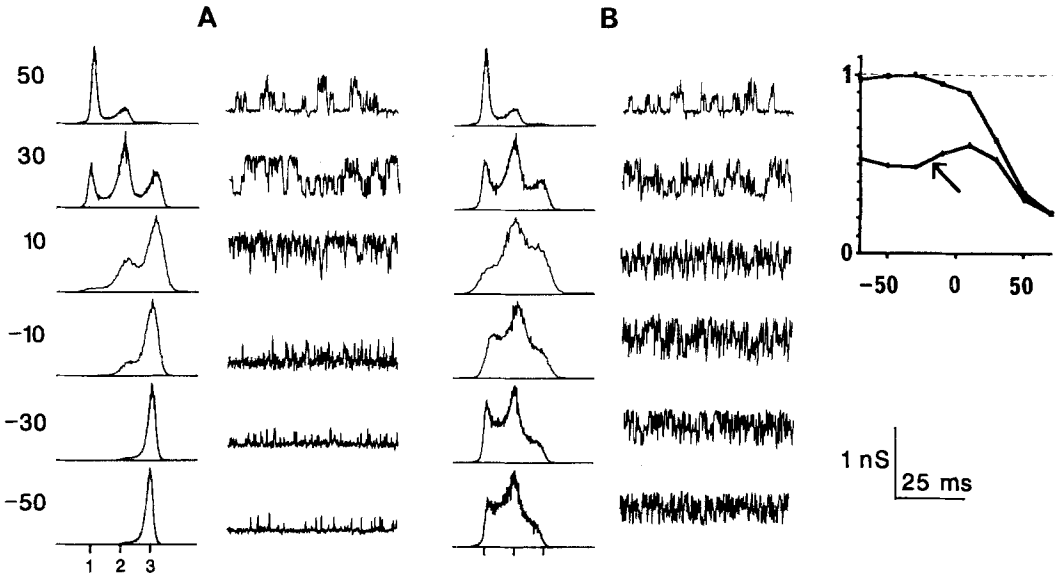

Fig. 4. Effect of peptide M on a channel inserted in the bilayer with the inverted orientation. (*A*) Control. (*B*) Effect of 100 μM bath-applied peptide. M. Upper right: voltage-dependence of the channel mean conductance in control conditions and in the presence of 100 μM peptide (indicated by the arrow). Conditions are the same as in Fig. 1

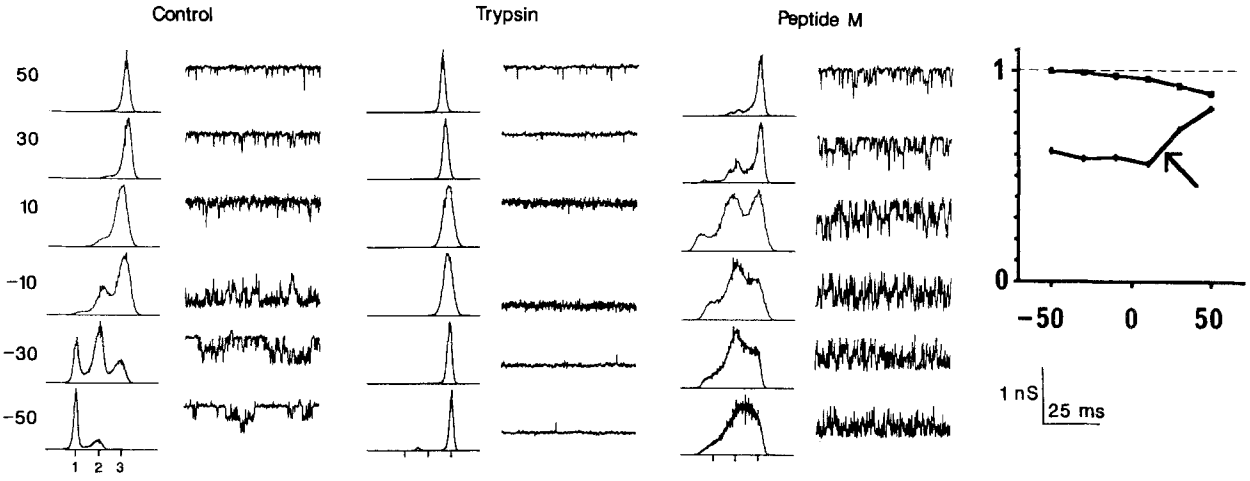

Fig. 5. Effect of peptide M on a mitochondrial channel treated by trypsin. *Control:* As in Fig. 1*A*. *Trypsin:* The bilayer was transferred to a bath containing 10 μg/ml trypsin. Within 40 sec, the voltage dependence of the channel was abolished, leaving the channel in the fully open state except for rare and brief closures. Currents were recorded as in control conditions after transfer to a trypsin-free bath. *Peptide M:* Transfer to a bath containing 100 μM peptide M induced a blockade of the trypsinized channel, restoring the conductance levels observed before proteolysis. Upper right: Voltage-dependence of the trypsinized channel mean conductance in the absence and the presence (indicated by the arrow) of 100 μM peptide

peptide concentration (Fig. 6). The frequency of peptide-induced closures increased with decreasing potentials while their duration decreased. The longest closed times, of the order of 2 msec, were observed at potentials between +20 and −20 mV. Below this value, the duration of the closures rapidly fell below the resolution time of the technique, leading to a decrease of the size of the current jumps at the lowest potentials.

The potential dependence of the blocking effect in the different cases described above is summarized in Fig. 7. It may be noted that the curves corresponding to channels inserted in the two orientations and to trypsinized channels incubated in the presence of 100 μM peptide superimposed in the 0–70 mV potential range. At negative potentials channels with the direct orientation are mostly in the closed state and the inhibition decreases. By contrast, channels with the reverse orientation and trypsinized channels are fully open; for both, a similar saturation of the blockade is observed. In the same figure, the effect of 10 μM peptide concentra-

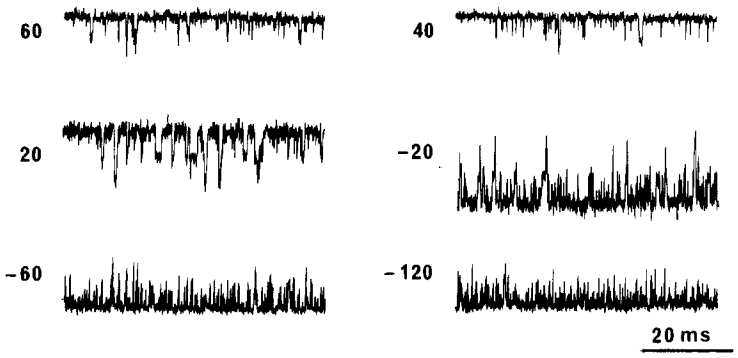

Fig. 6. Samples of current fluctuations recorded at different potentials from a trypsinized channel exposed to 10 μM peptide M. The experiment was performed as described in Fig. 5, except for the concentration of peptide M, which was decreased to 10 μM. Filter: 10 kHz; sampling: 20 kHz

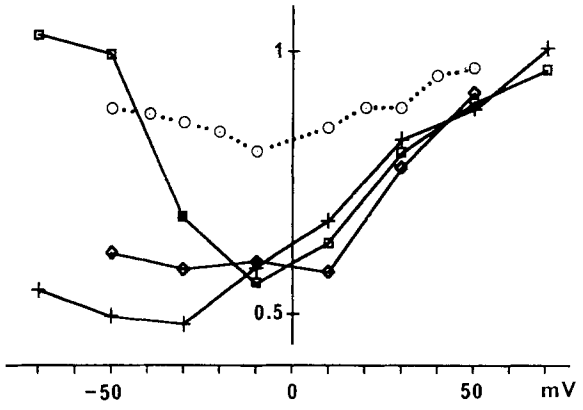

Fig. 7. Voltage dependence of the blockade. The blockade, defined as the ratio of the conductance in the presence of the peptide to control conductance, is shown on the same plot as a function of the voltage for four experimental conditions: channel inserted in the bilayer with the most often found orientation (experiment shown in Fig. 1), (□); channel inserted upside down in the bilayer (experiment shown in Fig. 4), (+); channels treated by trypsin and exposed to 10 μM (○--○) or to 100 μM (◇-◇) peptide M concentrations

tion on a trypsinized channel is also indicated (dotted line); the potential dependence of the blockade at 10 and 100 μM are similar.

Specificity of the Effect of Peptide M

The specificity of the effect observed with peptide M was investigated by testing various peptides (Table). Among the last five peptides of the Table, dynorphin B was the only one to have a small but significant blocking effect analogous to that of peptide M (Fig. 8). It may be noted that dynorphin B has the same length and the same charge as peptide M. The effect of the charge was further investigated by succinylation of peptide M. Such a chemical modification substitutes a negative charge for a positive one. Succinylation of peptide M gave three derivatives, modified on the terminal amino group, on the lysine residue in position 12, or in both positions. The three derivatives were isolated and tested. The twice succinylated peptide, which has a negative net charge, was completely inactive; the activity of the two partially succinylated molecules was greatly decreased.

Discussion

A 13-residue peptide was found to block the newly described cationic mitochondrial channel. Two main types of mechanism can be proposed to explain the experimental data: either an indirect action through the lipidic phase or a direct effect on the channel itself. Because mitochondrial addressing sequences strongly interact with natural and artificial bilayers (Von Heijne, 1988), the first hypothesis was thoroughly examined. It has even been proposed that the aggregation of the amphiphilic helices of the signal peptide might form ionic channels (Singer et al., 1987), as in the cases of alamethicin (Lattore & Alvarez, 1981) and mellitin (Hanke et al., 1983). We found no experimental evidence supporting this hypothesis: no additional conductance was found in bilayers containing *Torpedo* Cl^- channels recorded at different potentials from −180 to +180 mV and exposed to 100 μM peptide M.

It is conceivable that the peptide inserts in the bilayers and that the resulting surface pressure increase modifies the channel activity. We have observed an incorporation of peptide M in PE/PS (70 : 30) monolayers and the measured limiting pressure for the incorporation is similar to that previously reported (Roise et al., 1986; Tamm, 1986). However, in our case, the use of a different phospholipid composition and the presence of Mg^{2+} ions results in a much higher equilibrium surface pressure, which presumably prevents incorporation of peptide M into the bilayer. Furthermore, the addition of 100 μM peptide M had no effect on a *Torpedo* electric organ chloride channel, thus indicating the

Table Sequences of the tested peptides

Peptide	Sequence	Positive charges	Negative charges	Net charge
N-Terminal sequence of yeast cytochrome *c* oxidase subunit IV	M L S L R Q S I R F F K P 1 5 10	4	1	3
Peptide M	M L S L R Q S I R F F K Y 1 5 10	4	1	3
Succinylated derivatives of peptide M[a]	(Suc M) L S L R Q S I R F F K Y	3	2	1
	M L S L R Q S I R F F (Suc K) Y	3	2	1
	(Suc M) L S L R Q S I R F F (Suc K) Y	2	3	−1
Dynorphin B	Y G G F L R R G F K V V T	4	1	3
Dynorphin 1–17	Y G G F L R R I R P K L K W D N Q	6	1	5
Aplysia Rho_{44-54}	A D I E V D G K Q V E	2	5	−3
Hemoglobin βT_3	V N V D E V G G D A L G R	2	4	−2
Hemoglobin βT_4	L L V V Y P W T Q R	2	1	1

[a] Succinylated residues are indicated in parentheses.

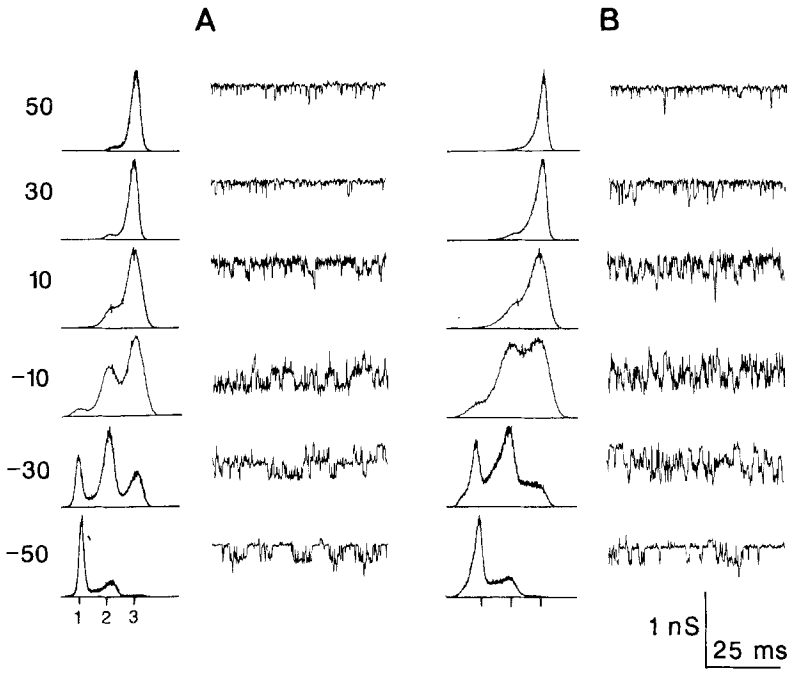

Fig. 8. Effect of 100 μM dynorphin B on the activity of the mitochondrial channel. Experimental conditions are the same as in Fig. 1. (*A*) Control (*B*) After transfer of the tip to a bath containing 100 μM dynorphin B

absence of a nonspecific effect. It is also noteworthy that dynorphin B had some blocking effect (Fig. 8) though the interaction of dynorphins with lipidic membranes is different from that of addressing peptides (Schwyzer, 1986).

The alternative hypothesis is that the peptide interacts with the channel in the aqueous phase, either by acting on the gating mechanism or by plugging the channel itself. The strongest evidence against the former possibility is the effect of peptide M on trypsinized channel (Figs. 5 and 6). Channels inserted inversely in the bilayer (Fig. 4*A*) are not sensitive to trypsin added to the bath (*unpublished data*). However, they are blocked by peptide M in the same way as channels with the normal orientation in which trypsin has eliminated the voltage dependence (Fig. 7). The site of action of the peptide is thus different from the trypsin-sensitive part of the molecule sensing the transmembrane potential. It is therefore most likely that the cationic peptide,

which acts similarly from both sides of the bilayer, is trapped in either of the channel mouths or in the pore itself. That the peptide could block the channel at conductance level 2 as well as at level 1 is not incompatible with such a mechanism. As previously reported (Thieffry et al., 1988), the channel properties are consistent with that of a dimeric complex on which trypsin acts in two steps. Blockade of one of the protochannels would result in a decrease of the conductance to level 2, whereas simultaneous blockade of both protochannels would correspond to level 1.

Accordingly, the voltage dependence of the blockade might indicate either that the cationic peptide is simply trapped in the channel or that it is translocated down its electrochemical gradient. In the former hypothesis, the mean time of channel occupancy by the peptide would be expected to increase as the voltage is decreased, thus resulting in an increase of the blockade efficiency. On the contrary, if the peptide is translocated, decreasing the voltage would be expected to accelerate the translocation since the driving force is increased. In this hypothesis, the mean time of channel occupancy by the peptide would decrease with decreasing voltage, leading to only partial blockade. The data of Figs. 6 and 7 are consistent with the hypothesis of a translocation of the peptide, since they indicate a decrease of the closure duration at potentials below −30 mV and a saturation of the blockade.

The translocation of polypeptidic chains through channels has been proposed by Singer et al. (1987). However, to our knowledge this hypothesis is not supported by direct experimental evidence. In the case of *tetanus*, *botulinum*, and *diphtheria* toxins, it has just been proved that the subunit responsible for the translocation of the active subunit through cell plasma membranes could form ionic channels in artificial bilayers (Hoch et al., 1985). The possibility for a channel to translocate unfolded polypeptidic chains implies that it is permeant to amino acids; in our case, large cations such as tetraethylammonium are permeant through the channel (Thieffry et al., 1988; *unpublished data*). This channel is thus a useful model to study the possibility of polypeptide translocation through channels.

A mitochondrial origin has been proposed for the studied channel (Thieffry et al., 1988). Current investigations suggest a localization on the outer membrane (J.F. Chich, D. Goldschmidt, M. Thieffry and J.P. Henry, *unpublished data*). The channel would thus be accessible from the cytosolic compartment and this raises the question of its possible involvement in the process of precursor translocation. The channel might be part of the system which allows the voltage-sensitive entry of the presequence into the mitochondrial matrix. Mitochondrial signal peptides competitively inhibit the precursor import process in the 1–20 μM concentration range (Gillespie et al., 1985; Ito et al., 1985; Ono & Tuboi, 1988), and the peptide effect was clearly seen at 10 μM concentration (Fig. 6). Inhibition of import and channel blockade are thus observed in about the same range of peptide concentration. In comparing the effect of peptides on the two processes, it should be noted that in the case of protein translocation the peptide inhibits the passage of a macromolecule whereas in our experiments the peptide interferes with a flux of small ions; the occupancy of the channel might thus be more efficient in the former type of experiments than in the latter. Also consistent with a possible involvement of the channel in precursor translocation is the fact that the succinylation of peptide M, which introduces negative charges in the sequence, decreases the channel blockade.

However, more direct experimental evidence will be required to substantiate this contention. The most direct evidence would be a block of the channel by full-size precursors. Yet the precursor structure might be under the control of the several steps of the import mechanism which precede the voltage-dependent one (Pfaller et al., 1988). Thus, it might be difficult to find experimental conditions leading to an unfolded structure of the precursor and to accessibility of the signal sequence. A less direct evidence would be the existence of a correlation between the blocking potency of different peptides and their ability to either translocate chimeric proteins (Allison & Schatz, 1986) or inhibit precursor entry into mitochondria (Ito et al., 1985; Gillespie et al., 1985; Ono & Tuboi, 1988). Finally, it would be of interest to identify the protein bearing the channel activity and to compare it to the various components of the import machinery (Gillespie, 1987; Ohba & Schatz, 1987).

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