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INVOLVEMENT OF THE PEPTIDE SENSITIVE CHANNEL IN THE TRANSLOCATION OF BASIC PEPTIDES INTO MITOCHONDRIA

Philippe Juin[a,b], Marc Pelleschi[a], Corinne Sagné[a], Jean-Pierre Henry[a], Michel Thieffry[b] and François-Marie Vallette[a*]

[a]Service de Neurobiologie Physico-Chimique, CNRS URA 1112, Institut de Biologie Physico-Chimique.13 rue P. et M. Curie. 75005 Paris (France)

[b]Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, Avenue de la Terrasse 91198 Gif/Yvette Cedex (France)

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The Peptide Sensitive Channel (PSC), a cationic channel of the mitochondrial outer membrane, is blocked by several highly basic peptides. Among these peptides, the most active are pCOX IV (1-12)Y, a mitochondrial addressing peptide and dynorphin B (1-13), a peptide unrelated to mitochondrial physiology. The voltage-dependent characteristics of the block duration of the PSC induced by these peptides and the fact that these peptides are imported into mitochondria in an *in vitro* assay suggest the involvement of the PSC in peptide translocation into mitochondria. We have analyzed the interaction of Mast Cell Degranulating peptide (MCD), a disulfide rich basic peptide, with yeast and mammalian mitochondria. Electrophysiological experiments with native and reduced forms of this peptide (nMCD and rMCD) showed an interaction of both forms with the yeast PSC. On the other hand, only rMCD blocked the electrical activity of the bovine adrenal cortex PSC. Similarly, although both forms inhibited the import of dynorphin B (1-13) into yeast mitochondria, only rMCD inhibited this import in bovine mitochondria. The correlation between electrophysiological and biochemical data strongly suggest that dynorphin B is translocated across the outer membrane at the level of the PSC.

In addition to the Voltage Dependent Anion Channel (VDAC), the mitochondrial outer membrane contains a cationic channel of large conductance, the Peptide Sensitive Channel (PSC) [1, 2]. This channel, although exhibiting some resemblance to the VDAC, appears to be a distinct entity as its activity is not impaired in a yeast mutant strain lacking the VDAC gene [3, 4]. The PSC is blocked by numerous basic peptides with a net charge superior to 2, including several addressing peptides such as pCyt OX IV (1-12) Y (the first 12 residues of cytochrome c oxidase subunit IV precursor followed by a tyrosine) [5, 6]. The electrophysiological characteristics of the blockade are consistent with a passage of these peptides through the

*Corresponding author. Fax: 33-1-40468331. e-mail: vallette@ibpc.fr.

Abbreviations: MCD: Mast Cell Degranulating peptide, native (nMCD) and reduced (rMCD) forms; Dynorphin B: a synthetic peptide corresponding to the first 13 amino acids of the dynorphin; PSC: Peptide Sensitive Channel; VDAC: Voltage Dependent Anionic Channel.

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channel [6]. However, other basic peptides unrelated to mitochondrial physiology, such as dynorphin B, also block the PSC in a similar manner [5-7]. We showed in a recent study that basic peptides could be imported into mitochondria by two distinct pathways. The first one, used by presequences at 30°C, shares some characteristics with the import pathway of matrix proteins [7]. The second one, used by all peptides active on the PSC (including dynorphin B (1-13)), operates even at low temperatures and could represent an alternate pathway to the intermembrane space [7]. The import at low temperatures in a VDAC-deficient mutant favours the hypothesis of a translocation through the PSC rather than a direct passage of the peptides through the lipid bilayer. However, a direct comparison between electrophysiological and biochemical data remained to be established to show that this peptide was translocated at the level of the PSC.

We have taken advantage of the electrophysiological properties on the PSC of the Mast Cell Degranulating peptide (MCD), a blocker of voltage-gated potassium channels [8]. This peptide carries 7 positive charges and is locked in a tight conformation by two disulfide bonds. As its native structure might prevent its translocation through a channel, we have compared the effect of native MCD (nMCD) and of its reduced forms (rMCD) on both PSC electrical activity and import of dynorphin B (1-13).

MATERIALS AND METHODS

Materials. Dynorphin B fragment 1-13 (YGGFLRRQFKVVT) was obtained from Sigma (St Louis, USA). Mast cell degranulating peptide (MCD) (IKCNCKRHVIKPHICRKICGKN) was a gift from Dr M. Lazdunski (UPR 411 CNRS). Peptide pRIHB (fragment of the retinoic acid induced heparin binding protein) (TRTGAECKQTMLTQR) was a gift from Dr D. Raulais (INSERM U 118).

Preparation of mitochondria. The yeast cells were grown and the mitochondria were prepared as described [7]. Bovine adrenal mitochondria were prepared by differential centrifugation [2].

Radiolabelling of dynorphin B. Dynorphin B at a concentration of 1 mM in 150 mM phosphate buffer (pH 7.4) was iodinated with 0.5 mCi Na ^{125}I (Amersham, France) using the Iodogen method according to the manufacturer's instructions (Pierce, USA).

Irreversible reduction of disulfide bonds of the mast cell degranulating peptide. The double-excess charge method [9] with dithiotreitol (DTT) and iodoacetamide was used to generate a reduced peptide (rMCD) with a net charge identical to that of the native peptide (nMCD). Briefly, 150 µl of 1M DTT were added to 1 mg of nMCD in 25 µl of 50 mM Tris-HCl (pH 7.5). The solution was stirred and left at room temperature in the dark for 30 min, then 300 µl of 2 mM iodoacetamide solution were added with constant stirring for 30 min at room temperature. The solution was injected into an analytical C18 HPLC column (Waters) and the peptide eluted in 20 min at a flow rate of 1.0 ml/min using a linear gradient of 10-50% acetonitrile in 0.05 % trifluoroacetic acid. The modified peptide was eluted as a single major peak separated from a minor component. The major peak corresponded mainly to rMCD as shown by comparison with the elution profile of nMCD.

Translocation assay. The assay was performed as described [7]; briefly, ^{125}I-dynorphin B (1 µM final concentration; 5 µCi) was incubated at 30°C with yeast or bovine adrenal cortex mitochondria (1 mg of protein/ml) in 100 µl import competent buffer (250 mM sucrose, 80 mM KCl, 10 mM $MgCl_2$, 10 mM malic acid, 8 mM succinic acid, 1 mM ATP-Mg^{2+}, 20 mM MOPS; pH 7.5) in the presence or the absence of o-phenanthroline (100 µM). At the end of the incubation, half of each sample was treated at 0°C with 20 µg/ml of proteinase K in order to hydrolyze unprotected peptides [7]. In competition experiments, 50 µM unlabelled peptides (r or nMCD, dynorphin B, pRIHB) were added to mitochondria prior to incubation with radiolabelled dynorphin B. Mitochondria were centrifuged and resuspended in the reducing loading buffer before analysis by SDS-PAGE (15% acrylamide/0.8% bis-acrylamide)

and autoradiography. The amount of radiolabelled peptide was quantified using a Phosphorimager (Molecular Dynamics).

Electrophysiological recordings. Tip dip experiments were performed as described [10]. Exposure of the channels to the peptides was achieved by transferring the tip of the pipette into a bath containing peptides at different concentrations.

RESULTS

Import of dynorphin B into mitochondria. Radiolabelled dynorphin B (1 μM) was incubated in the import competent buffer with yeast or bovine mitochondria and the amount of peptide associated to the organelle was estimated as a function of time. Within 2 min, most of the radiolabelled peptide was bound to the organelle, but was sensitive to mild proteolysis by proteinase K at 0°C (data not shown), suggesting that these peptides avidly bind to the cytosolic side of mitochondrial outer membrane. Similar results have been described by others for a synthetic mitochondrial presequence [11]. When 1 μM ^{125}I-dynorphin B was used, the amount of membrane bound peptide decreased gradually after 5 min in the assay medium and completely disappeared after 40 min at 30°C. This decrease did not correspond to a release of the peptide into the cytosolic phase as no radiolabelled peptide was found in the supernatant of the assay medium after centrifugation (data not shown). We have shown that the disappearance of this peptide required intact mitochondria and could be inhibited by incubation with 100 μM o-phenanthroline, a metalloprotease inhibitor [7]. We compared the time course of the intramitochondrial accumulation of dynorphin B in the presence of 100 μM o-phenanthroline (assayed by its proteinase K sensitivity) to that of its degradation in the absence of the inhibitor. The degradation of dynorphin B occurred at a rate identical to that of its accumulation within the mitochondria (fig. 1A). The import of dynorphin B is thus a two step mechanism in which the

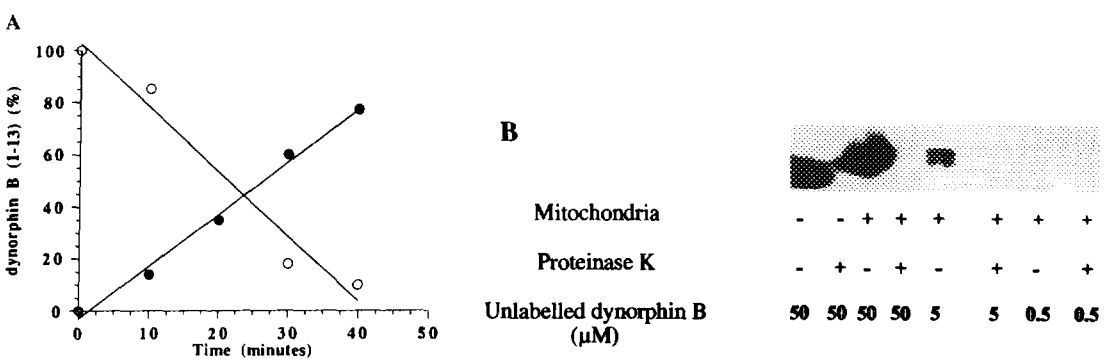

Figure 1. Characteristics of dynorphin B import. A) Accumulation and degradation of dynorphin B as a function of time. ^{125}I-labelled dynorphin B (1 μM) was incubated with yeast mitochondria (1 mg protein/ml) in the absence (○) or in the presence (●) of 100 μM o-phenanthroline. The incubation with o-phenanthroline was followed by incubation with proteinase K (20 μg/ml) for 10 min at 0°C. The amount of peptide associated with mitochondria was estimated after SDS-PAGE by quantification using a Phosphorimager.
B) Saturation of dynorphin B import into yeast mitochondria. Increasing concentrations of unlabelled dynorphin B (0.5, 5, 50 μM,respectively) were incubated with yeast mitochondria (1 mg of protein/ml) in the presence of 1 μM ^{125}I-labelled dynorphin B. Where indicated, import was followed by a proteinase K treatment. The amount of peptide associated with mitochondria was estimated by autoradiography after SDS-PAGE and compared to 1 μM ^{125}I-labelled dynorphin B incubated in the same conditions but without mitochondria.

translocation of the peptide into the organelle is immediately followed by a rapid proteolysis by a metalloprotease. Therefore, throughout this study, the degradation rate of dynorphin B was used as an index of its import into the mitochondria. In addition, the dynorphin B import process involved a saturable step at the level of the outer membrane as the amount of bound peptide remained constant over a period of 20 min when incubated at concentrations equal or superior to 50 μM and remained associated with the external side of the outer membrane as determined by the proteinase K sensitivity test (fig. 1B).

Effect of rMCD and nMCD on the import of dynorphin B into yeast or mammalian mitochondria. Due to the existence of a saturable step, basic peptides competing for binding and/or import were expected to increase the amount of radiolabelled dynorphin B associated with the cytosolic side of the mitochondria and thus sensitive to proteinase K digestion. We added to the import medium containing 1 μM [^{125}I]-dynorphin B, a 50-fold molar excess of unlabelled peptides (nMCD or rMCD) as putative competitors. The mixture was then incubated at 30°C for 30 min in the presence of either yeast or bovine adrenal cortex mitochondria (100 μg of protein). The effect of the addition of unlabelled peptides on dynorphin B import was compared to that of the addition of 50 μM unlabelled dynorphin B or 50 μM pRIHB, a control peptide inactive on import [7]. In both types of mitochondria, dynorphin B import was affected by the addition of an excess of unlabelled dynorphin B, but unaffected by pRIHB (fig. 2). The addition of nMCD and rMCD to the assay medium containing yeast mitochondria resulted in the accumulation of undegraded (i.e. not imported) dynorphin B (fig. 2A). A different situation was observed with bovine adrenal cortex mitochondria: dynorphin B import was inhibited by an excess of unlabelled rMCD whereas nMCD was without any effect (fig. 2B).

Effect of nMCD and rMCD on yeast and mammalian PSC. The effect of both forms of MCD on yeast and bovine adrenal cortex PSC was examined using channels incorporated in bilayers formed at the tip of microelectrodes. PSC is localized on the outer membrane [2], which

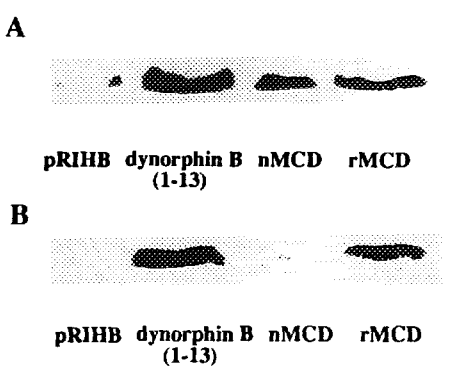

Figure 2. Inhibitory effects of nMCD and rMCD on the import of dynorphin B into yeast (A) or bovine adrenal cortex (B) mitochondria. ^{125}I-labelled dynorphin B (1 μM, 5 μCi) was incubated at 30°C for 30 min in the presence of mitochondria (1 mg of protein/ml) and 50 μM unlabelled peptides.^{125}I-labelled dynorphin B associated with the mitochondrial pellet was analyzed by SDS-PAGE and scanned with a Phosphorimager.

is most likely devoid of electrical polarization. We thus focused our attention to recordings carried out at potentials close to 0 mV (i.e between -10 and + 10 mV). When yeast PSC was exposed to 50 μM nMCD (the concentration used in import experiments), a voltage-dependent blockade developed immediately (fig. 3, bottom). The blockade appeared as a fast flicker between ill-defined conductance levels. Its magnitude increased when the voltage was shifted to negative values of higher magnitude (data not shown). When the mammalian channel was exposed to the same peptide concentration, the current traces were not clearly modified and statistical analysis of the current amplitude revealed only a very weak increase in the probability of the first closed level (fig. 3, top).

Conversely, rMCD acted on both mammalian and yeast PSC (fig. 4). Its effect on yeast PSC was similar to that observed with nMCD. As illustrated in figure 4, this effect was obtained at lower peptide concentrations indicating a stronger interaction between rMCD and the PSC than that observed with nMCD. With the mammalian channel, this peptide did not cause fast flickering, but induced longer closures at well-defined conductance levels (fig. 4).

It should be noted that, in addition to the effects described above, nMCD (on yeast PSC) and rMCD (on mammals and yeast PSCs) induced an inactivation of the channel at negative voltages. This inactivation, whose rate increased with the voltage magnitude, was sometimes observed some tens of seconds after switching the voltage from 0 mV to values as low as -10 mV. It could then be overcome with some delay by reversing the voltage polarity. This phenomenon will be described in more details in a forthcoming publication (Pelleschi, Henry and Thieffry, in preparation).

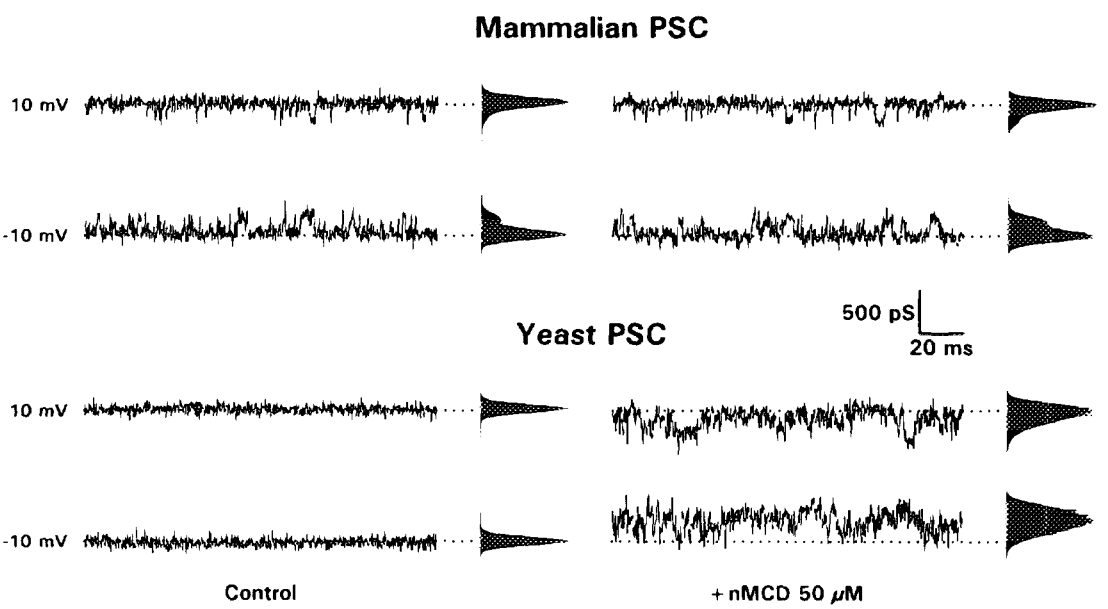

Figure 3. Effect of nMCD on mammalian and yeast PSC. Samples of current recorded at potentials close to 0 mV before (left) and after (right) transfer of the tip to a bath containing nMCD at the concentration of 50 μM. For this figure and the following one, the pipette potentials are indicated at the left of the control trace and the dotted line indicates the open state. Data filtered at 2.5 kHz and sampled at 5 kHz. The current distribution, computed over durations of at least 10 s, is shown at the right of each trace.

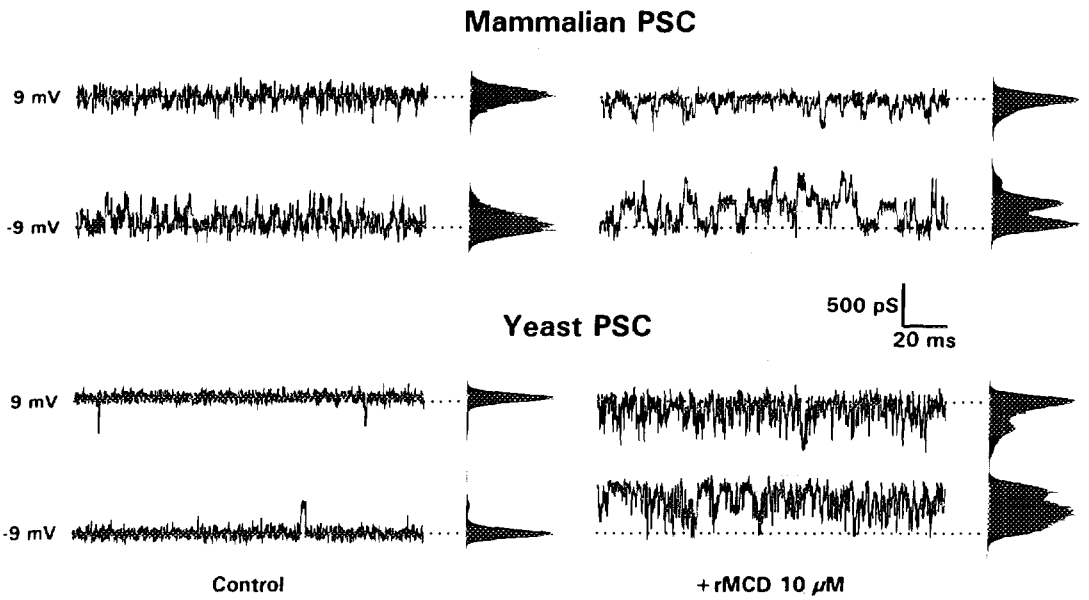

Figure 4. Effect of rMCD on mammalian and yeast PSC. Samples of current traces recorded before (left) or after (right) transfer of the tip to a bath containing rMCD at the concentration of 10 μM. Data filtered at 2.5 kHz and sampled respectively at 5 kHz for the mammalian channel and 10 kHz for the yeast channel. The current distributions are computed over durations of at least 8 s.

DISCUSSION

We have recently shown that the neuropeptide dynorphin B is imported into the intermembrane space of the mitochondria by a pathway different from that used *in vitro* at 30°C by matrix targeted preproteins and synthetic presequences [7]. The dynorphin B (1-13) import was inhibited by basic peptides active on the PSC, suggesting either an involvement of this channel in this process or an indirect electrostatic effect on peptides binding sites of the outer membrane negatively charged phospholipids. In addition, a direct import through lipids postulated by several authors (11-16) could have also been used by the dynorphin B (1-13).

We have therefore tried to corelate biochemical and electrophysiological data, although the interaction between peptides import *in vitro* and their elctrophysiological properties are not always straightforward (17).

We have showed in this paper that nMCD inhibited the import and blocked the PSC in yeast mitochondria but had only a very weak effect in mammalian mitochondria. On the contrary, rMCD was active on dynorphin import and PSC in mitochondria from both origins. The present data show that under physiological conditions (i.e. membrane potential around 0 mV), we observed only a partial blockade of the channel by the peptide (fig. 3 and 4). This might account for the partial inhibition of dynorphin B import by MCD peptides observed in our biochemical experiments (fig.2). However, a quantitative correlation between biochemical and electrophysiological experiments is difficult to establish. In the former, we evaluate the effect of a peptide (r or nMCD) on the translocation of an other peptide (dynorphin B). In the

latter, we record a flow of small ions which may be less affected than larger molecules by the tested peptide.

nMCD is locked in a compact conformation by two disulfide bridges. This difference in conformation is likely to explain the difference of effects observed on the PSC originating from different species. As shown by proteolysis experiments [1, 3], mammalian PSC has a cytosolic component responsible for fast fluctuations which is not found in yeast PSC. The channels of either origin have thus different cytosolic domains. A difference in the accessibility of the pore could be responsible for the lack of effect of nMCD on mammalian PSC. rMCD, because of its linear structure, would therefore be able to interact with both channels. It should be noted that a similar conformational constraint has been reported for protein import into mitochondria which requires also a linearization of the preprotein prior translocation [13].

Import of dynorphin B (1-13) is sensitive to nMCD in yeast mitochondria but import in the mammalian organelle is not sensitive to this form of MCD. Since the phospholipid composition of the outer membrane is likely to be similar in bovine and yeast mitochondria, a simple translocation of the peptide through the lipid phase [14-16] would imply similar sensitivities in both types of mitochondria. Such a mechanism can therefore be ruled out. On the contrary, sensitivity of dynorphin B (1-13) import in mammalian mitochondria parallels that of PSC activity. Indeed, nMCD is inactive on peptide import as well as on the electrical activity of PSC. Reduction of this peptide is necessary to reveal both an interaction with the mammalian channel and an inhibitory effect on dynorphin import. As electrostatic properties are unlikely to be different between nMCD and rMCD, the hypothesis of translocation of dynorphinB (1-13) through the PSC is strengthened by the correlation between import inhibition and channel blockade induced by nMCD and its reduced form.

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