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Inhibition of Protein Import into Mitochondria by Amphiphilic Cations: Potential Targets and Mechanism of Action

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In this paper we describe for the first time the inhibitory effect of three amphiphilic cations, trifluoperazine, propranolol and dibucaine on mitochondrial protein import. The amphiphilic cations did not affect binding of mitochondrial precursor proteins to mitochondria. Import into mitoplasts was affected in a similar manner to intact mitochondria, indicating that the protein import machinery of the inner membrane of mitochondria was responsible for the observed effect. At concentrations which completely inhibited protein import, the amphiphilic cations did not affect the membrane potential ($\Delta\Psi$) across the inner membrane. The inhibitory potency of amphiphilic cations reflects their lipid/water partition coefficient and relatively high concentrations of the drugs were required for complete inhibition, hence we propose that the mechanism of protein import inhibition by amphiphilic cations is due to membrane perturbing effects. We discuss the implications of our findings in view of the possible connection between various inner mitochondrial membrane channels and the protein import pore.

Key Words: **mitochondria; protein import; amphiphilic cations; inner membrane channels.**

The vast majority of mitochondrial proteins are nuclear encoded, translated on cytosolic ribosomes and imported into mitochondria. Two large distinct protein complexes, translocase of the outer (TOM) and the inner membrane (TIM) participate in the movement of preproteins across mitochondrial membranes. Translocation of precursor proteins targeted to the mitochondrial matrix requires a membrane potential ($\Delta\Psi$) and ATP hydrolysis (1). Translocation of the polypeptide chain through the inner mitochondrial membrane and simultaneous maintenance of the transmembrane proton gradient across the inner membrane requires tight regulation of the protein import channel. It is conceivable that the opening of the protein import channel may be regulated by the incoming precursor. Recently, it was shown that TIM23, one of the core components of the TIM machinery, exists in the import complex as a dimer and its dimerisation is abolished by a drop in the $\Delta\Psi$ or in the presence of an incoming precursor (2). Imported proteins arrested in transit across the mitochondrial membranes can be extracted by alkaline pH or urea indicating that upon translocation, precursors are not embedded into the lipid bilayer, but are imported through a hydrophilic environment (3). An unfolded polypeptide chain with the side residues can be roughly estimated to be about 1 nM in cross diameter. Moreover, mitochondria can import preproteins with a modified or branched chain (4). Therefore, large conductivities at the opened state for the TIM and TOM machineries can be expected. Electrophysiological experiments suggest that the inner mitochondrial membrane contains channels with a large conductivity and one of them, a multiple conductance channel (MCC) of yeast mitochondria, was recently identified as the TIM complex (5).

The MCC is a large conductance channel found in the inner mitochondrial membrane of mammals and yeast. In mouse kidney and yeast mitochondria, the addition of several mitochondrial presequence peptides resulted in transient blocking of MCC activity but not of other channel activities (6). In rat liver mitochondria, the addition of mitochondrial presequence pep-

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Abbreviations used: AOX, alternative oxidase; Cox, cytochrome oxidase; Dibucaine, (2-butoxy-N-[2-(diethylamino)-ethyl]-4-quinoline carboxamide hydrochloride); MCC, multiple conductance channel; mHsp70, mitochondrial heat shock protein of 70 kDa; MPP, mitochondrial processing peptidase; PK, proteinase K; Propranolol, [(±)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride]; PTP, permeability transition pore; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFP, trifluoperazine, [10-[3-(4-methyl-1-piperazinyl)propyl]-2- trifluoromethyl-phenothiazine dihydrochloride]; TOM, translocase of outer membrane; TIM, translocase of inner membrane.

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tides (pCoxIV and pCoxVI from *Neurospora crassa*) induced large amplitude swelling of mitochondria with the properties similar to the cyclosporin A-sencitive mitochondrial permeability transition pore (7). Comparison of electrophysiological data concerning the MCC or "megachannel" of mammalian mitochondria with properties of PTP suggest that they are identical (8). On the other hand, the MCC of yeast mitochondria, as mentioned above (5), was found to be identical to the TIM complex. Therefore, possibility of connection between TIM machinery and the other channels of large conductivity like the PTP of mammalian mitochondria should be investigated especially in view of the involvement of mitochondrial channels in various pathological and physiological events.

Inhibitory studies provide a good tool for a comparative analysis of the functional properties of protein complexes. Different transporters and channels of the inner membrane of mitochondria from various sources including the Na^+-Ca^{2+} carrier, the ATP-induced unspecific channel of the yeast mitochondria, the inner membrane anion channel of plant and mammalian mitochondria and the PTP of mammalian mitochondria were shown to be sensitive to the addition of amphiphilic cations (9–13). Amphiphilic cations represent a broad group of organic molecules with quite different pharmacological properties. The most common feature of these molecules is their potency of interaction with biological membranes, especially those enriched with anionic phospholipids. The amphiphilic cations have been shown to interact with either peripheral (14) or the lipid-embedded moiety (15) of membrane-associated proteins or to indirectly affect their functions through a general perturbation of the phospholipid bilayer (16). They are potent modulators of mitochondrial functions. Amphiphilic cations have been shown to have an inhibitory effect on energy-linked reactions. The inhibition of cytochrome c oxidase, F_0F_1-ATP synthase, mitochondrial respiration and $\Delta\Psi$ by amphiphilic cations has been (14, 17–19). On the other hand they have a protective effect on the mitochondrial damage induced by Ca^{2+} and prooxidants (20). The inhibitory effect of amphiphilic cations on the mitochondrial PTP was implicated in their protective effect against ischemia-reperfusion injury (21) and their anti-apoptotic effect (22).

In this paper we have studied the effect of three amphiphilic cations, the calmodulin inhibitor trifluoperazine, the β-adrenergic blocker propranolol and the local anaesthetic dibucaine on protein import into isolated potato tuber mitochondria used as a model system as well as into yeast and rat liver mitochondria. We conclude that amphiphilic cations have an inhibitory effect on protein import into mitochondria from different sources and that the inhibition occurs at the level of the inner membrane import machinery.

MATERIALS AND METHODS

Preparation of mitochondria and mitoplasts. Potato tuber mitochondria and mitoplasts were prepared as described previously (23). Yeast mitochondria were prepared according to (24). Rat liver mitochondria were prepared as described previously (25).

In vitro transcription/translation of the precursor proteins. A pTZ18U plasmid containing cDNA of the $F_1\beta$ precursor of the ATPase from *N. plumbaginifolia* and a plasmid containing cDNA clone of soybean alternative oxidase precursor (gift from Dr. J. Whelan) were used for *in vitro* expression of the precursors. *In vitro* transcription and translation were performed in a coupled reticulocyte lysate TNT system (Promega) in the presence of [^{35}S]-labeled methionine (Amersham) according to the manufactures instructions.

Mitochondrial in vitro protein import. Protein import experiments using potato tuber mitochondria and mitoplasts were carried out as described previously (23). To study the effect of amphiphilic cations on import, mitochondria were preincubated with respective amounts of each inhibitor for 5 min at 0°C prior to the addition radiolabeled precursor protein. Protein import experiments into yeast and rat liver mitochondria were carried out according to (24) and (25), respectively. Samples were analyzed on SDS–PAGE using 12% polyacrylamide gels in the presence of 4 M urea (26).

In vitro processing. Processing with the membrane fraction of potato tuber mitochondria was carried out as described previously (27).

Measurement of membrane potential and oxygen consumption. Measurements of membrane potential were performed using Rhodamine 123 according to (28). Freshly prepared potato tuber mitochondria were used for measurements. The final concentration of mitochondrial protein in the cuvette was 1 mg/ml. Inhibitors were added to the cuvette during measurements. Oxygen consumption was measured using a Clark oxygen electrode (Hansatech) as described in (23). Inhibitors were added into the incubation chamber during measurements.

Protein determination. Protein content was estimated according to the method of Bradford (29).

Statistics. $\Delta\Psi$ measurements and experiments for the calculation of half maximal inhibitory drug concentrations were performed at least three times.

RESULTS

Protein Import into Potato Tuber Mitochondria Is Sensitive to TFP, Propranolol and Dibucaine

Figure 1 shows import of the *in vitro* transcribed and translated precursors of the $F_1\beta$ subunit of the ATP synthase from *Nicotiana plumbaginifolia* ($pF_1\beta$) and soybean alternative oxidase (pAOX) into potato tuber mitochondria in the presence of different concentrations of amphiphilic cations, TFP, propranolol and dibucaine. Incubation of $pF_1\beta$ with plant mitochondria resulted in the appearance of an extra band above the band corresponding to the precursor form of the protein in addition to the mature form of precursor (Fig.1A). This band of higher molecular mass (p*), represents a modified form of the $F_1\beta$ precursor (von Stedingk *et al.*, submitted). Efficiency of import was calculated as the ratio of the mature, PK protected form, in the presence of inhibitors, to the PK protected mature form in the control sample in the absence of drugs. TFP, propranolol and dibucaine at submillimolar con-

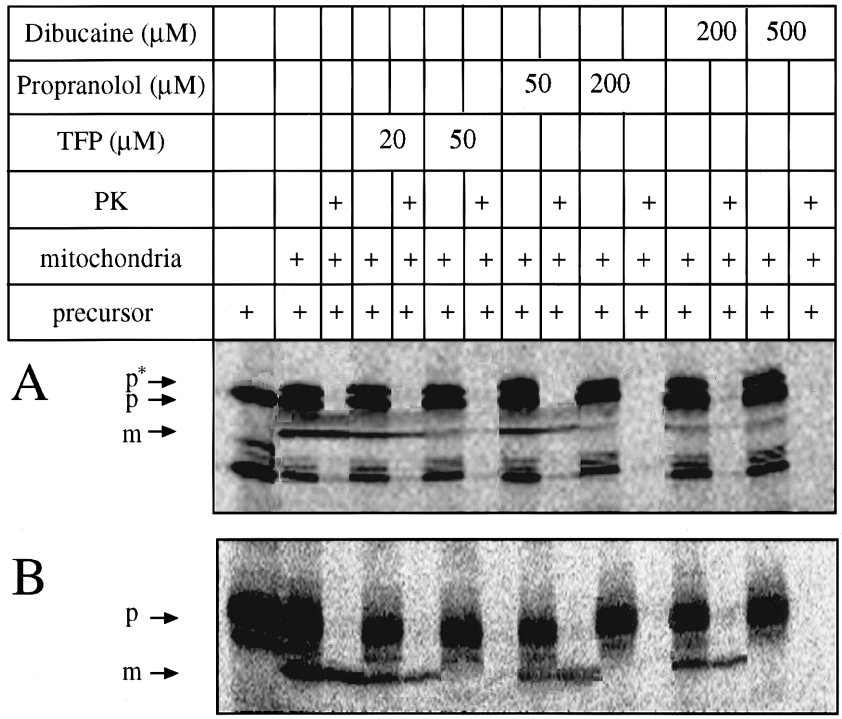

FIG. 1. Amphiphilic cations prevent import of precursor proteins into isolated potato tuber mitochondria. *In vitro* transcribed/translated radiolabeled $pF_1\beta$ and AOX were imported into mitochondria as described in Materials and Methods. Gels after SDS–PAGE were fixed, dried and scanned using a Fujix BAS 1000 MacBAS Bio-imaging Analyzer system. (A) The import of the $pF_1\beta$ into potato tuber mitochondria. The figure represents phosphoimage of the SDS–PAGE in the absence and in the presence of th the indicated amounts of TFP, propranolol and dibucaine. p, precursor protein; p*, modified precursor protein; m, mature protein. (B) As in A except that the pAOX was used in the import reaction.

centrations each had an inhibitory effect on import of the $pF_1\beta$ and pAOX into potato tuber mitochondria. Preincubation of mitochondria with amphiphilic cations also resulted in the inhibition of the formation of the PK sensitive mature forms of the precursors (see Fig. 1, PK untreated lanes). Cleavage of the presequence by mitochondrial processing peptidase (MPP), which in plant mitochondria is integrated into the cytochrome bc_1 complex of the respiratory chain, occurs at the matrix face of the inner mitochondrial membrane. As *in vitro* processing experiments using membrane bound mitochondrial processing peptidase and radiolabeled precursors did not reveal inhibitory effect of TFP, propranolol or dibucaine on processing (not shown), our results suggest that the precursor proteins did not reach the mitochondrial matrix and that the inhibition of protein import occurs before or at the step of translocation across membranes. Complete inhibition was achieved with TFP at 75 μM, propranolol at 300 μM and dibucaine at 350 μM (Fig. 2A). Table 1 summarize half maximal inhibitory concentrations of amphiphilic cations required for inhibition of protein import into isolated potato tuber, yeast and rat liver mitochondria. The inhibitory effect of the amphiphilic cations at these concentrations on the mitochondrial import was reversible since reisolation and washing of the mitochondria before import resulted in no inhibition (not shown).

Effect of Amphiphilic Cations on the Binding of Precursor Proteins to Mitochondria

Binding of precursor proteins to the mitochondrial surface is mediated by the protein import receptors of the outer membrane since proteolytic digestion of the mitochondrial surface proteins greatly diminishes binding of precursors to mitochondria (23). In our experiments, outer membrane receptor-mediated binding of precursors to mitochondria was calculated as the ratio of bound, PK sensitive precursor form in samples treated with amphiphilic cations at concentrations which completely inhibited protein import, to the amount of bound precursor in the control mitochondria. Results presented in Fig. 2B show that binding of neither $pF_1\beta$ nor pAOX was affected upon treatment with the amphiphilic cations.

Protein Import into Mitoplasts Is Sensitive to the Amphiphilic Cations

To identify the step in the protein import pathway which was sensitive to the drugs, we investigated pro-

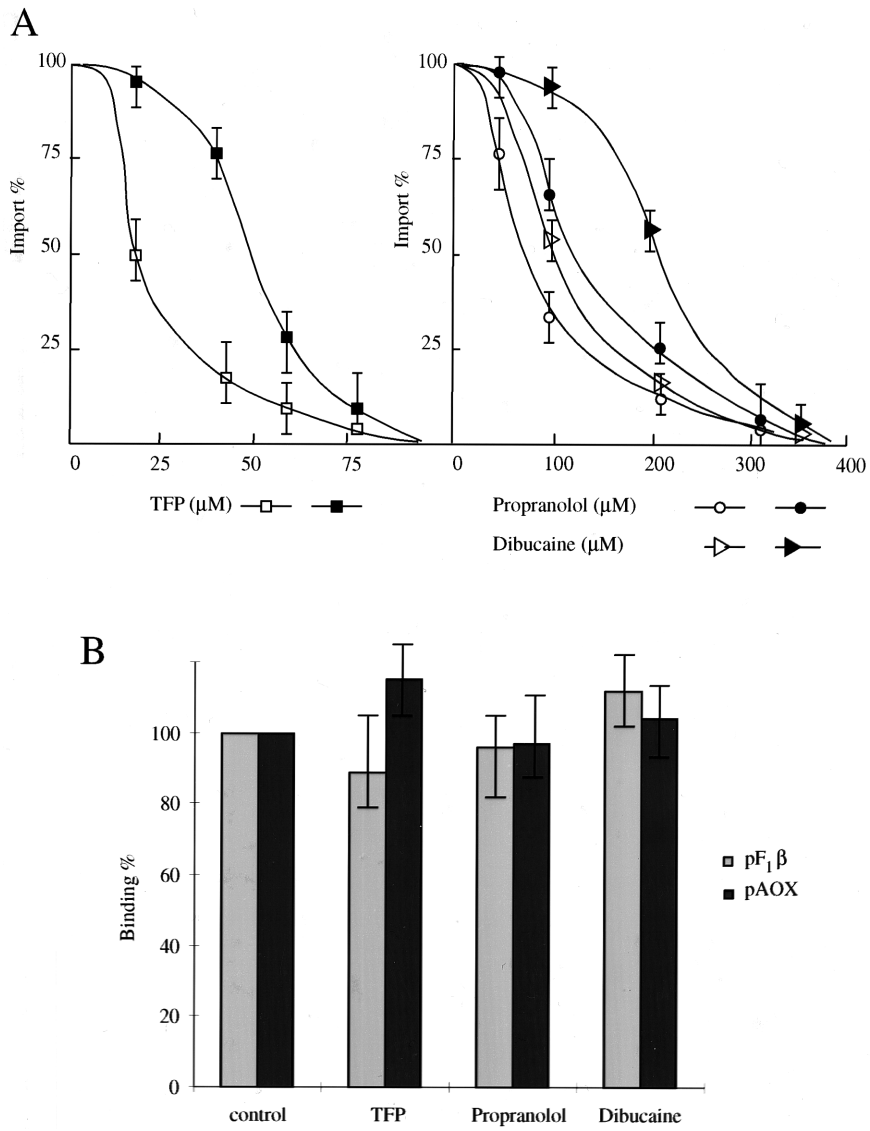

FIG. 2. Effect of the amphiphilic cations on the import and binding of precursors to the mitochondria. (A) Inhibition profile of the import of the *in vitro* transcribed/translated *N. plumbaginifolia* $pF_1\beta$ and *Pisum sativum* pAOX into potato tuber mitochondria upon addition of increasing amounts of the TFP, propranolol or dibucaine to the mitochondria. Import efficiency was estimated as percent of mature PK protected form in lanes where amphiphilic cations were present, compared to the mature form in the control sample without addition of drugs. Open squares, circles and triangles represent the inhibitory profiles for the import of the $pF_1\beta$ with TFP, propranolol and dibucaine. Filled symbols, as opened but with pAOX was an import substrate. (B) Efficiency of binding of the $pF_1\beta$ and pAOX to the mitochondria upon addition of the amphihpilic cations. Binding was calculated as a percent of the precursor form of the preproteins in presence of the amphiphilic cations to the amount of the precursor form bound to a control untreated mitochondria. Lane 1, binding efficiency of the $pF_1\beta$ and pAOX without the inhibitors taken as 100%. Lane 2, binding efficiency of the $pF_1\beta$ and pAOX in the presence of 100 μM TFP. Lane 3, as in lane 2, but with 500 μM propranolol. Lane 4, as in lane 2, but with 500 μM dibucaine.

tein import into mitoplasts prepared by osmotic rupturing of the outer mitochondrial membrane (23). Osmotic rupturing of the outer mitochondrial membrane resulted in 1.5- to 2-fold stimulation of the import of the $pF_1\beta$ and pAOX compared to protein import into intact mitochondria. This stimulation varied from preparation to preparation due to differences in the quality of potato tubers which were bought at the local market. The increased efficiency of protein import into mitoplasts might be explained by the more direct access of the precursor proteins to the TIM machinery, bypassing the step of translocation across the outer membrane. Results presented in Fig. 3 show that import of the $pF_1\beta$ and pAOX into mitoplasts was inhibited by the same concentrations of amphiphilic cations that inhibited import into intact mitochondria. Binding of the precursors to the mitoplasts surface was not affected by drug treatment.

TABLE 1

Comparison of Half-Maximal Inhibitory Concentrations of the Amphiphilic Cations Required for Inhibition of Import of the $pF_1\beta$ into Potato Tuber, Yeast and Rat Liver Mitochondria

IC_{50} (μM): Mitochondria	TFP	Propranolol	Dibucaine
Potato	20	80	110
Yeast	35	215	270
Rat	30	105	205

Effect of TFP, Propranolol, and Dibucaine on Energy-Linked Processes in Potato Tuber Mitochondria

We tested the effect of amphiphilic cations on membrane potential across the inner mitochondrial membrane. This was measured as a decrease of Rhodamine 123 fluorescence upon incubation of the mitochondria with succinate. Figure 4 shows time drive curves obtained upon incubation of the mitochondria with increasing amounts of the amphiphilic cations. Our results show no effect of amphiphilic cations on $\Delta\Psi$ at concentrations that completely inhibit protein import. The addition of TFP at a concentration of 200 μM caused in a decrease of the $\Delta\Psi$ by 40% compared to the complete dissipation of $\Delta\Psi$ upon addition of valinomycin to the mitochondria. Propranolol and dibucaine added at concentrations below 1 mM did not cause a significant decrease of $\Delta\Psi$. On the other hand, the incubation of mitochondria with higher amphiphilic cations concentrations (1 mM TFP and 5 mM propranolol and dibucaine) resulted in complete dissipation of $\Delta\Psi$ (not shown). Moreover, upon incubation with 1 mM TFP, the potato tuber mitochondria irreversibly aggregated indicating a damaging effect of high drug concentrations on the mitochondrial membrane. The effect of the drugs on mitochondrial oxygen consumption was also measured, but no effect was observed at concentrations that completely inhibited protein import (data not shown). TFP at concentrations >0.75 mM totally abolished the mitochondrial respiration; propranolol and dibucaine at concentrations > 1 mM did not have any inhibitory effect. In summary, amphiphilic cations at concentrations completely inhibiting protein import did not affect mitochondrial membrane potential or respiration, but high concentrations of the drugs affect mitochondrial functions.

Protein Import into Yeast and Rat Liver Mitochondria Is Completely Inhibited by Amphiphilic Cations

We isolated yeast and rat liver mitochondria in order to test the effect of TFP, propranolol and dibucaine on the import of the $F_1\beta$ precursor. Comparative data of the effect of amphiphilic cations on protein import into

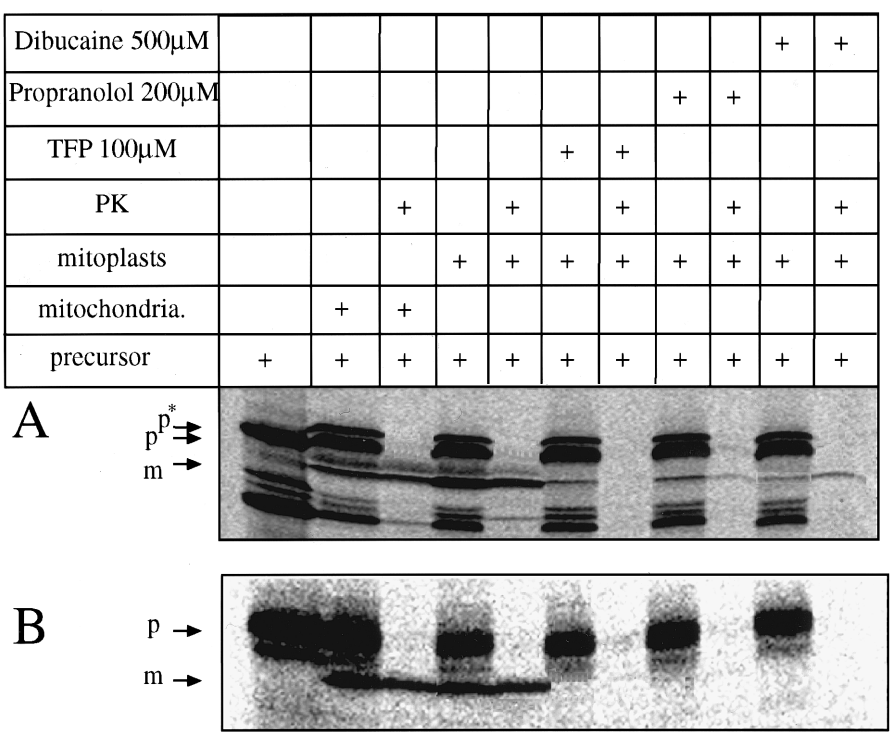

FIG. 3. Effect of the amphiphilic cations on the protein import into potato mitoplasts. Import was carried out as described in Materials and Methods. Labeling as in Fig. 1.

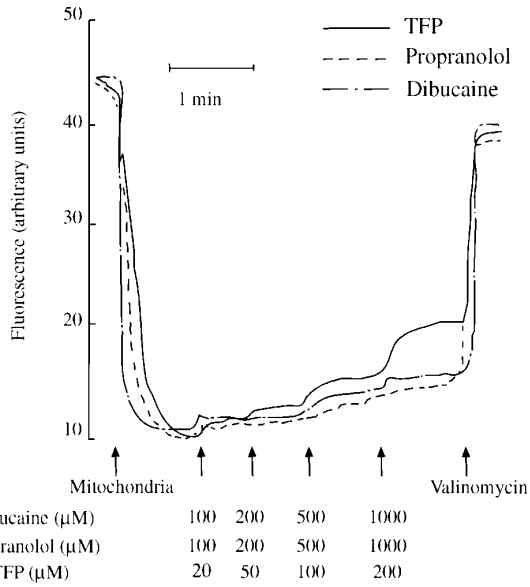

FIG. 4. Effect of the amphiphilic cations on the membrane potential of the isolated potato tuber mitochondria. Measurements were carried out as described in Materials and Methods. Concentration of valinomycin was 2 μM. Measurement curves represent time drive fluorescence quenching of Rhodamine 123 of mitochondria respiring with succinate as a substrate upon addition of the amphiphilic cations directly to the cuvette.

the mitochondria from different sources are summarized in Table 1. Our results show that there is a common inhibitory effect of the amphiphilic cations on protein import, however there are differences in the inhibitory potency between potato, yeast and rat mitochondria. These differences could reflect variations in membrane lipid composition of mitochondria from different sources, affinity of the drugs for their targets or variations in the mechanism of protein translocation across the inner membrane.

DISCUSSION

In this paper we have demonstrated for the first time that protein import into mitochondria from different species can be inhibited by the amphiphilic cations TFP, propranolol and dibucaine at micromolar concentrations. We showed that in the presence of the amphiphilic cations: (i) Binding of the $pF_1\beta$ and pAOX to the import receptors was not affected. (ii) The presequence did not reach the mitochondrial matrix. (iii) *In vitro* processing of the $pF_1\beta$ and pAOX with the membrane fraction of the mitochondria was not inhibited. (iv) Import into the mitoplasts was affected at the same drug concentration of the drugs that inhibited import into intact mitochondria. (v) Membrane potential and respiratory activity were not affected to a significant degree. The effect of the cations was equivalent in mitochondria from different organisms. On the basis of the above findings we conclude that translocation of the precursor across the inner mitochondrial membrane costitutes the critical step that is affected by the amphiphilic cations.

The mitochondrial protein import process and the components of the import machinery have a tendency to be conserved through the evolution. On their route to the matrix, preproteins have to be recognized by the surface receptors, translocated through the proteinaceous pores in the outer and the inner membranes and actively pulled into the matrix by the molecular chaperone mHsp70 and its cochaperones. Many of the components of the translocation import apparatus are not only conserved through evolution but are also absolutely essential for the mitochondrial biogenesis and organism viability. For example, the mHsp70 shows more then 80% sequence similarity to its bacterial counterpart, the TIM17 shows more than 70% similarity through the species from yeast to human (30); 50% similarity was reported for the TIM44 from yeast and mouse (31). The channel properties of the import complexes also have common features. The channels have a large conductance in the opened state and can be transiently blocked by the peptides derived from mitochondrial presequences. In yeast mitochondria a peptide-sensitive channel of the outer membrane was shown to be identical to the TOM complex (32) and the MCC of the inner membrane was found to be identical to the TIM complex (5). Recently, it was reported that the addition of the peptide representing the N-terminal part of the presequence of *N. crassa* COX IV induced massive swelling of isolated rat liver mitochondria (7). This pore was permeable to solutes $<$1500 Da and the range of permeabilities was similar to the classical Ca^{2+}-induced PTP. Despite the significant differences in structure of the compounds able to modulate channel activity, some of the inhibitors like TFP, propranolol and dibucaine were equally effective. Therefore, we decided to investigate the effect of these amphiphilic cations on different aspects of the protein import process. We investigated the effect of the amphiphilic cations on binding of precursor proteins to the mitochondrial surface, protein import into mitochondria and mitoplasts and on *in vitro* protein processing.

The traditional inhibitors of protein import are uncouplers and membrane potential dissipating compounds. Martin *et al.* (33) calculated that the value of $\Delta\Psi$ across the inner membrane required for the translocation of matrix targeted preproteins across the inner membrane has to be at least 40–60 mV. Assuming that the range of $\Delta\Psi$ in normally respiring mitochondria lies between 150 and 220 mV (34), depending on respiratory substrate and source of the mitochondria, it indicates that the precursors will reach the matrix even if $\Delta\Psi$ is only one third of the normal value. Previously, it was shown in our laboratory that import of the $F_1\beta$ precursor into potato tuber mitochondria was not inhibited upon addition of a non-

amidated form of antibacterial peptide, cecropin A (35) which was found to be a potent uncoupler of mitochondrial respiration. This indicates that import of the $pF_1\beta$ does not require a high membrane potential. Complete inhibition of protein import of the $pF_1\beta$ and pAOX into potato mitochondria was obtained at concentrations of amphiphilic cations which did not affect mitochondrial respiration and caused a decrease of $\Delta\Psi$ by 10%. Our results are consistent with the effect of TFP, propranolol and dibucaine on rat liver mitochondria, in which amphiphilic cations applied at concentrations completely inhibiting a presequence peptide-induced mitochondrial swelling, did not uncouple mitochondria but, moreover, protected the mitochondria against uncoupling action of the peptide (7).

What might be the mechanism providing the action of amphiphilic cations on the TIM machinery? There are several explanations for the action of these drugs. It was shown that membranes and membrane proteins are the main target of amphiphilic cations in biological systems (36). Such interactions will change the net membrane surface charges and might affect either the interaction of the positively charged presequence with the inner mitochondrial membrane or, indirectly, the properties of the TIM complex. Direct interaction with the components of the TIM machinery might be an alternative explanation for the inhibitory effect. Sirrenberg *et al.* (37) calculated amounts of TIM machinery in yeast mitochondria to be approximately 17 pM per milligramm of mitochondrial protein. It means that if amphiphilic cations would interact directly with the TIM complex, they would have a very low affinity for the potential binding site on the TIM machinery. This observation together with the fact that the tested compounds had different chemical structures but had a similar effect on protein import make the latter proposal less probable. TFP was found to be the most potent inhibitor of the protein import into all types of mitochondria tested. Among drugs tested TFP has highest lipid/water partition coefficient which might explain its greater damaging effect on mitochondrial function. On the basis of the above arguments, we propose that amphiphilic cations interact with the lipid bilayer and affect the inner membrane import machinery via local changes in membrane surface charge. This mode of action would explain the ability of the drugs to inhibit import into mitochondria from various organisms even though the nature of their protein import apparatus may be different.

From our experiments, it is clear that the amphiphilic cations used in this study are a useful tool in studying mitochondrial protein import. In contrast to the traditional protein import inhibitors such as valinomycin, these drugs seem to have a more specific inhibitory effect.

Valinomycin inhibits translocation to the matrix import through the complete collapse of the membrane potential, which also affects other mitochondrial functions such as electron transport and ATP synthesis. The amphiphilic cations tested achieved complete inhibition of protein import without significantly perturbing the membrane potential, indicating that their effect is more specific towards the import machinery.

The amphiphilic cations have been shown to have a protective effect on mitochondrial damage caused by ischemia-reperfusion injury and anti-apoptotic effects in several cell culture strains (21, 22). This is due to the inhibition of the Ca^{2+}-induced PTP (38), presumably via indirect effects mediated through the inhibition of phospholipase A2 (9). Since the formation of lysophospholipids is not currently considered to be the major cause of the PTP our findings in view of the channel-like nature of the protein import machinery may provide an alternative explanation. Amphiphilic cations may be used to better understand key physiological and pathological events in the cell.

In conclusion, we found a new mitochondrial target for the action of amphiphilic cations, the translocase of the inner membrane protein import machinery. The amphiphilic cations were previously shown to affect several inner mitochondrial channels and transporters.

Our results suggest that many as yet unidentified unspecific mitochondrial membrane channels might coincide with the inner membrane protein import machinery.

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