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Isolation of mitochondrial porin of the fly *Protophormia*: porin modification by the pesticide CGA 140′408 studied in lipid bilayer membranes

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Abstract

Mitochondrial porin from the fly *Protophormia* was solubilized with detergent from whole mitochondria and purified by chromatography across a hydroxyapatite (HPT) column. The purified protein had an apparent molecular mass of about 30 kDa on SDS-PAGE. Partial sequencing of the protein confirmed that it is porin. When reconstituted in planar lipid bilayer membranes, porin formed ion-permeable channels with single-channel conductances of 2.4 and 4.5 nS in 1 M KCl. At low voltage, *Protophormia* porin displayed the properties of a general diffusion pore and had a small selectivity for anions over cations. At transmembrane potentials starting with about 20–30 mV, the channel switched in closed state, which is still ion-permeable. Our results suggest that *Protophormia* porin possesses functional properties similar to those of other mitochondrial porins. Porin was also isolated and purified from mitochondria, which were treated with the carbodiimide CGA 140′408 It represents the active derivative of diafenthiuron a new acaricide and insecticide. This carbodiimide labels both a F_0-component of the inner membrane ATPase and outer membrane porin in a similar way as *N,N′*-dicyclohexylcarbodiimide (DCCD). Reconstitution experiments with the CGA 140′408-modified porin showed no significant effect of the modification on the single-channel conductance, suggesting that CGA 140′408 binds outside the channel. The voltage-dependence of the CGA 140′408-modified porin was changed with respect to the unmodified form. The closed configuration of the pesticide-modified channel was reached at smaller transmembrane potentials, suggesting a shift of the open to the closed state of *Protophormia* porin by pesticide binding. A possible contribution of this effect to the pesticide action is discussed.

Keywords: Voltage-dependent anion-selective channel; Mitochondrial porin; Pesticide; Diafenthiuron; DCCD; Lipid bilayer; (*Protophormia*)

1. Introduction

Mitochondria are surrounded by two membranes that separate the organelle from the cytosol and are involved in the exchange of metabolites between these compartments and in the import of nuclear-encoded proteins. The mitochondrial outer membrane shows a permeability for hydrophilic molecules up to a molecular weight of 4–5 kDa due to the presence of general diffusion pores called voltage-dependent anion-selective channels (VDAC) or mitochondrial porins in analogy to the pore-forming proteins in the outer membrane of Gram-negative bacteria [1,2]; for a recent review see Ref. [3]. Isolation and reconstitution of the mitochondrial porins from *Paramecium* [4], *Dictyostelium* [5], yeast [6], *Neurospora crassa* [7] and mammals [8,9] led to a detailed analysis of their biochemical and biophysical properties. At low membrane potentials all mitochondrial porins are weakly anion-selective in the 'open' state. At voltages higher than 20 mV the pore switches to the cation-selective 'closed' state.

The primary sequence of many mitochondrial porins is known at present [10–13]. The secondary structure of mitochondrial porins as channel-forming proteins is a matter of debate because crystallographic data are not available yet. One model assumes that the channel is formed by

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; BSA, bovine serum albumin; DCCD, *N,N′*-dicyclohexylcarbodiimide; DiphPC, diphytanoylphosphatidylcholine; EDTA, ethylene diamine tetraacetic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethansulfonic acid; HTP, hydroxy apatite; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; VDAC, voltage-dependent anion-selective channel.

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12 β-strands together with the N-terminal amphiphilic α-helical structure [14]. Another model [3,15] assumes that the mitochondrial outer membrane channel is formed by 16 amphipathic antiparallel β-strands similar to the arrangement of bacterial porins [16]. Mitochondrial porins play an important role in mitochondrial metabolism. Hexokinase and glycerokinase bind to porin on the cytosolic surface of mitochondria [17,18]. The binding of hexokinase to the mitochondrial surface leads to an oligomerization of the enzyme and to a considerable increase of its activity [19,20]. DCCD binds at low concentration to porin and the F_0-component of the mitochondrial ATPase [21–23]. The labeling of mammalian mitochondrial porin with DCCD occurs at glutamic acid 72, which is probably localized within a transmembrane β-strand [24].

Porin has recently been shown, for the first time, to be a target of a new acaricide and insecticide, diafenthiuron (see Fig. 1). This thiourea is not active per se but represents a prodrug that is converted to the related carbodiimide, CGA 140'408, acting as an inhibitor of mitochondrial respiration [25,26]. In vivo and in vitro studies provided evidence that the carbodiimide product irreversibly binds to two protein targets in mitochondria, the proteolipid of the F_0 moiety of ATPase and porin [27,28]. Since binding of ^{14}C-labeled CGA 140'408 to both proteins was competed for by DCCD, the binding sites may be the same for the two carbodiimides [21,22]. Intoxication of insects, treated with CGA 140'408, was correlated with reduced levels of both ATPase activity and ATP in vivo. Hence, binding to the F_0 component may, indeed, represent a major target mechanism of this pesticide [27,28]. Porin, however, by linking mitochondrial and cytosolic processes, may also contribute to the biological action when modified by CGA 140'408. This possibility is of interest under the aspect that this carbodiimide binds to porins from insects but not from rat [28,29]. Whether this contributes to the comparatively low mammalian toxicity of the parent thiourea [30], diafenthiuron, is unknown to date.

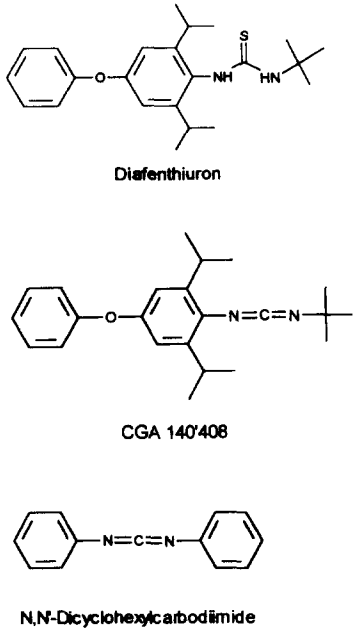

Fig. 1. Structural formulae of diafenthiuron, CGA 140'408 and DCCD.

The present work describes results from a first approach to examine the pesticidal contribution of binding of CGA 140'408 to mitochondrial porin. Porin was isolated from native and CGA 140'408-treated mitochondria of the fly *Protophormia* and the properties of the channels were compared in an artificial membrane system. Our results suggest a slight modification of the voltage-dependence of the insect porin after binding of this pesticidal carbodiimide. Whether this is relevant in the cellular environment is a question to be addressed in further studies.

2. Materials and methods

2.1. Insects

The fly *Protophormia terraenovae* has been bred in-house for several years. Larvae were fed commercial dog food which also served as substrate for egg laying. Adults were offered a mixture of sugar, milk and egg powders. In previous work on the binding of CGA 140'408 to mitochondrial porin, the closely related species *Calliphora erythrocephala* was used [27,28]. No difference was found between the two flies with regard to porin labelling by CGA 140'408 and DCCD (data not shown).

2.2. Isolation of mitochondria

About two-week-old adult flies were immobilized on ice and their flight muscles were squeezed out into cold isolation medium consisting of 160 mM potassium aspartate, 5 mM potassium phosphate, 2 mM EDTA and 1% BSA, adjusted to pH 7.4 with potassium hydroxide. Typically, 6 ml of medium were used per 40 flies. The muscle suspension was stirred with a small magnetic bar at 200 rpm for 4 min, filtrated through silanized glass wool and layered onto 10 ml of isolation medium supplemented with 40 mM sucrose. After centrifugation at $3000 \times g$ for 7 min, the mitochondrial sediment was resuspended in reaction medium containing 5 mM potassium phosphate, 1 mM EDTA and 320 mM sucrose (pH 7.4). Mitochondrial protein was determined according to Ref. [31] using the dye reagent from BioRad (Glattbrugg, Switzerland).

2.3. Modification of mitochondrial porin with CGA 140'408

The mitochondria were treated with CGA 140'408 basically as described in previous publications [27,28]. In brief, a solution of CGA 140'408 in acetone was added to the mitochondrial suspension (3.5%, v/v, final acetone concentration). The reaction was performed at a ratio of 42

nmol of CGA 140'408 per mg of mitochondrial protein in reaction medium for 5 h at 0°C. This high ratio, compared to about 2 nmol per mg used in previous work [27], was chosen to achieve an as high as possible degree of derivatization. The reaction was terminated by the addition of 9 volumes of cold acetone. After standing overnight at −18°C, precipitated protein was collected by centrifugation at 4000 × *g* for 10 min and the pellet was dried in a stream of nitrogen.

In a separate experiment, the extent of porin modification by CGA 140'408 was estimated from the extent of labeling by a successive treatment of the mitochondria with the more reactive DCCD. To this end, [^{14}C]DCCD (carbodiimide-labeled, spec. act. 2.18 GBq/mmol; Amersham, Zürich) was added to the assay after various times and the incubation continued for a fixed period of time. After precipitation and washing with cold acetone, the proteins were separated by SDS-PAGE and subjected to fluorography as described [28]. As estimated from a visual inspection of the fluorographs, more than 90% of reacting porin sites were blocked by CGA 140'408 after 5 h of incubation.

2.4. Isolation and purification of native and modified Protophormia porin

The porin of the unmodified and modified mitochondria was isolated essentially as has been described previously [9]. The mitochondrial pellet (24 mg/ml for the normal and 14 mg/ml for the pesticide-treated mitochondria) was suspended in solubilization buffer containing 10 mM Hepes (pH 7.0), 1 mM EDTA and 2% Genapol X-80 (Fluka, Neu-Ulm; Germany). After incubation on ice for 30 min, the suspension was centrifuged for 30 min at 100 000 × *g* in an ultracentrifuge (Beckman, Omega XL-90). The supernatant was applied to a ceramic hydroxyapatite (HTP) (Bio-Rad) column preequilibrated and eluted with the solubilization buffer. Fractions of 1 ml were collected. The porin containing fractions were stored at −25°C. They showed channel-forming activity in this form for at least half a year.

Purity and relative molecular mass of the *Protophormia* porin was estimated by 12% SDS-PAGE [32]. To remove most of the detergent, the protein was subjected to two-phase separation [33]. The resulting protein pellet was dissolved in 20 μl of sample buffer (containing 0.1 M Tris-HCl, 4% SDS, 10% mercaptoethanol, 20% (v/v) glycerol, 0.01% bromphenol blue) and incubated for 10 min at 100°C. The gels were stained either with Coomassie blue or with silver [34]. For sequencing, porin (50 μg) was precipitated using the two-phase method to remove the detergent [33]. Then the protein was digested with 2.5 μg trypsin at 37°C for 8 h. After addition of another 2.5 μg trypsin the digestion was continued for 12 h. The peptides generated by the proteolysis were separated by reverse-phase high-performance liquid chromatography (HPLC) using a 250 × 4 mm Vydac 2181P column equilibrated with 0.12% trifluoroacetic acid (TFA). The peptides were eluted over 90 min by a linear gradient of 0–35% acetonitrile containing 0.1% TFA (flow rate 0.7 ml/min). The effluent was monitored at 215 nm. The amino acid sequence of two peptides of interest was determined by the Edman degradation method using a gas phase sequenator (470A, Applied Biosystems) with on-line detection of the amino acids.

2.5. Lipid bilayer experiments

The method used for the reconstitution of porin into 'black' lipid bilayer membranes has been described previously in detail [35]. In brief, the apparatus consisted of a Teflon chamber with a thin wall separating two aqueous compartments. The Teflon wall had a small circular hole with an area of about 1 mm^2. Membranes were formed across the hole by painting onto the hole a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in *n*-decane. Porin was added to one or both sides of the membrane after the membranes had turned optically black in reflected light. The aqueous salt solutions (analytical grade; Merck, Darmstadt, Germany) were used unbuffered and had a pH of approx. 6.0. The temperature was kept at 25°C throughout. For single-channel and current–voltage curves the current through the membranes was measured with two Ag/AgCl electrodes (with salt bridges) switched in series with a voltage source and a current amplifier (model 427, Keithley, Cleveland, OH). The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder. In the case of zero-current membrane potential measurements the current amplifier was replaced by an electrometer (Keithley 602). These measurements were performed by establishing a salt gradient across membranes containing 100 to 1000 channels as has been described earlier [36].

3. Results

3.1. Isolation and purification of native and CGA 140'408-modified Protophormia porin

Porin of native and CGA 140'408-modified mitochondria of *Protophormia* was isolated and purified applying the same procedure. Treatment of mitochondria with the non-ionic detergent Genapol X-80 leads to solubilization of porin and other mitochondrial proteins. For purification of porin, the detergent extract was applied to a ceramic HTP-column. Like most other mitochondrial porins [9], native and modified *Protophormia* porin did not bind to the column and eluted with high purity just after the void volume. SDS-PAGE (silver staining) of the native *Protophormia* porin showed a single band with an apparent molecular mass of 29 kDa (Fig. 2). The purified CGA

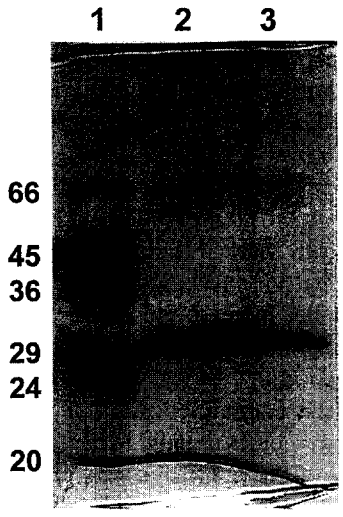

Fig. 2. 12% SDS-PAGE of *Protophormia* porin. Trace 1: molecular mass markers (66, 45, 36, 29 24 and 20 kDa); Trace 2: fraction 3 of the ceramic HTP-column of native porin purification. Trace 3: fraction 3 of the ceramic HTP-column of CGA 140'408-modified porin purification; silver staining.

140'408-modified porin displayed only a thin band in SDS-PAGE when it was stained with Coomassie blue. Nevertheless, silver-staining of the SDS-gel revealed a single protein band (Fig. 2), which was essentially free of contaminants.

3.2. Partial sequencing of the Protophormia porin

Partial sequencing of the *Protophormia* porin (PP) after tryptic digestion revealed two partial sequences which exhibited the highest homology to the human porins porin 31HL [12] and HVDAC2 [37]:

PP	PPSFSDLGKQARDI	
porin 31HL	AVPPTYADLGKSARDV	
HVDAC2	CIPPSYADLGKVARDI	
	1	16

and:

PP	FGGGGKYQ LDQDAAL	
porin 31HL	FGGSI YQKVNKKLE	
HVDAC2	FGGSI YQKVCEDLD	
	190	205

The first partial sequence represents part of the sequence near the N-terminus of the *Protophormia* porin, which is much better preserved than the other partial sequence showing similarity within the second half of the protein.

3.3. Single-channel experiments

The addition of native *Protophormia* porin to a final concentration of about 10 ng/ml to the aqueous solution on both sides of a lipid bilayer membrane resulted in a stepwise increase of the membrane conductance (Fig. 3). These conductance steps were caused by porin since they were not observed when only the detergent Genapol X-80 was added to the aqueous phase. Each step corresponded

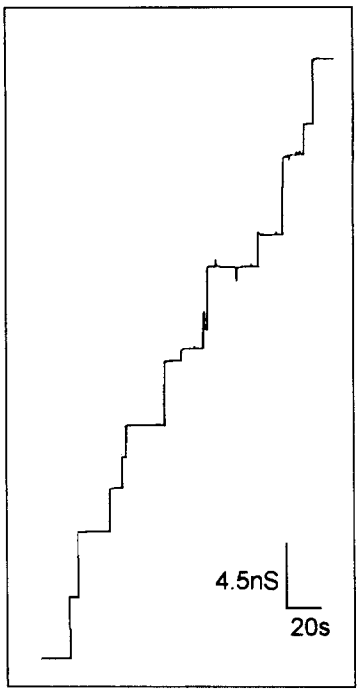

Fig. 3. Stepwise increase of membrane current after the addition of *Protophormia* porin to a black lipid bilayer membrane given as a function of time. The aqueous phase contained 10 ng/ml porin and 1 M KCl. The membrane was formed from 1% diphytanoyl phosphatidylcholine/n-decane. The applied voltage was 10 mV; $T = 25°C$.

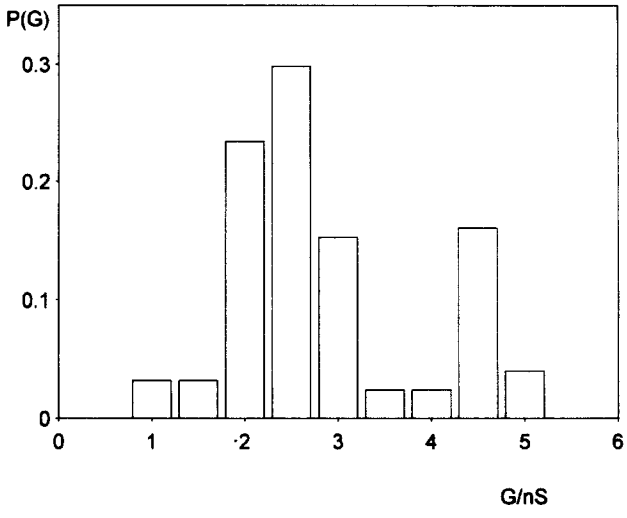

Fig. 4. Histogram of conductance fluctuations observed with membranes of diphytanoyl phosphatidylcholine/n-decane in the presence of purified native *Protophormia* porin. P(G) is the probability for the occurrence of a conductance step with a certain single-channel conductance (given in nS). The aqueous phase contained 1 M KCl. The applied voltage was 10 mV. The mean value of all upward-directed steps was 2.4 nS for the left-side maximum and 4.5 nS for the right-side maximum (in total 124 single events); $T = 25°C$.

Fig. 5. Single-channel recording of a diphytanoyl phosphatidylcholine/n-decane membrane after addition of 10 μl of CGA 140'408-modified *Protophormia* porin to the aqueous phases. Porin and transient channels were inserted into the membrane. The aqueous phases contained 1 M KCl (pH 6). The applied voltage was 10 mV; $T = 25°C$.

to the incorporation of one channel-forming unit into the membrane. Under the low voltage conditions of Fig. 3, most of the conductance steps were directed upwards. Only a few downward steps were observed. At higher transmembrane potentials the closing events became more frequent (see below). The on-steps at 20 mV had a single-channel conductance of 2.4 and 4.5 nS in 1 M KCl (mean value of the left-side and right-side maximum of the histogram in Fig. 4). It should be noted that similar single-channel distributions have also been found for most mitochondrial porins [3] including that from the fruit fly *Drosophila melanogaster* [38].

Lipid bilayer experiments were also performed with fractions of the HTP-column containing the CGA 140'408-modified *Protophormia* porin. Surprisingly, after the addition of small amounts of the fractions (10 μl containing about 5 μg/ml protein) to the aqueous phases bathing a lipid bilayer membrane, not only porin channels but also transient channels were observed (see Fig. 5), while the activity of mitochondrial porin was drastically reduced. The lifetime of the transient channels was about 2 s and their single-channel conductance was 2 nS in 1 M KCl. It is noteworthy that a similar channel has been observed in the mitochondrial outer membrane of a porin-deficient yeast mutant [39] and in pea mitochondria [40]. Preincubation of the porin containing fractions with 1% cholesterol (dissolved in 2% Genapol, 10 mM Hepes, 1 mM EDTA, pH 7) in a similar manner as has been described previously [41] resulted in a much higher channel-forming activity of the modified porin. Under these conditions the transient channels were no longer observed, probably because the 'normal' porin had an approx. 10-fold higher activity upon preincubation with cholesterol (data not shown), which masked the transient 2 nS channels. It is noteworthy that the incubation of the native porin with cholesterol did not increase the channel-forming activity. The average single-channel conductances of the modified *Protophormia* porin in 1 M KCl did not differ significantly from those of the unmodified porin. The left-hand maximum had in most cases a single-channel conductance of 2.1 nS and that of the right-hand maximum one of 4.5 nS, which compares to 2.4 nS and 4.5 nS for the native mitochondrial porin.

Single-channel conductances of the native and modified *Protophormia* porin were also measured in various salt solutions. The experiments with the modified *Protophormia* porin were always performed after preincubation of the porin containing fraction with 1% cholesterol as mentioned. It should be noted that cholesterol had no influence whatsoever on the single-channel conductance of native and modified porin. Its only effect was the increase of the channel-forming activity of the modified porin. The native and modified porin were permeable for different ions (Table 1). The histograms of conductance fluctuations for measurements in various salt solutions always showed a left-side and a right-side maximum similar to Fig. 4. There did not exist any significant difference between the single-channel conductances of the native and the modified porin in different salt solutions, so that the following considerations could be applied for both porins. Although the channel conductance was influenced considerably by the different salts and concentrations, the ratio between single-channel conductance G and specific conductance σ of the aqueous phase varied only little, i.e., the ions seemed to move within the pores as in the aqueous envi-

Table 1
Average single-channel conductance of native and modified *Protophormia* porin in different salt solutions

Salt	c(M)	Native porin			Modified porin		
		Right-side maximum	Left-side maximum		Right-side maximum	Left-side maximum	
		G(nS)	G(nS)	$G/\sigma(10^{-8}cm)$	G(nS)	G(nS)	$G/\sigma(10^{-8}cm)$
KCl	0.1	0.27	0.65	4.6	0.24	0.59	4.2
	0.3	0.76	1.60	4.1	0.80	1.42	3.7
	1	2.4	4.5	4.0	2.1	4.5	4.0
	3	6.1	13	3.8	5.9	12	3.5
LiCl	1	1.6	3.3	4.7	1.6	3.3	4.7
KAc	1	1.0	2.2	3.1	1.1	2.1	3.0

The aqueous salt solutions contained either about 10 ng/ml native porin or about 5 ng/ml modified *Protophormia* porin and less than 0.001% Genapol X-80; the pH was between 6.0 and 7.0. The membranes were made from diphytanoyl phosphatidylcholine/n-decane; $T = 25°C$; $V_m = 10$ mV. G represents the average single-channel conductance of the left-side and right-side maxima of the histograms (at least 100 single-channel recordings) from the native and the CGA 140'408-modified *Protophormia* porin, respectively. c is the concentration of the salt solution and σ is its aqueous specific conductivity.

Table 2
Ratio of the permeability for cations and anions of the native and modified *Protophormia* porin

Initial KCl concentration	V_m (mV)	P_{cation}/P_{anion} (mV)
Native porin		
10 mM KCl	7.1	1.4
50 mM KCl	5.5	1.3
CGA 140'408-modified porin		
10 mM KCl	24	3.3
50 mM KCl	3.8	1.2

V_m is defined as the difference between the potential at the dilute side (10 or 50 mM) and the potential at the concentrated side (100 or 500 mM. The pH of the aqueous salt solutions was 6 unless otherwise indicated; $T = 20°C$. The permeability ratio P_{cation}/P_{anion} was calculated with the Goldman-Hodgkin-Katz equation [37] on the basis of at least 3 individual experiments.

ronment. This finding suggests that both porins form wide water-filled channels. Nevertheless, the data of Table 1 show a certain extent of ion selectivity. The difference of the single-channel conductances in KAc and LiCl suggests that the porin is anion-selective; the exchange of K^+ by the less mobile Li^+ had a smaller effect on the left-side and right-side maximum of single-channel conductance than replacement of Cl^- by the less mobile Ac^-. In this respect it has to be noted that K^+ has the same aqueous mobility as Cl^- (both about 70 mS/ (cm · M)) and that Li^+ has the same one as Ac^- (both about 40 mS/cm · M) [42].

3.4. Ion selectivity of the native and the CGA140'408-modified porin

Zero-current membrane potential measurements were performed with the native and modified *Protophormia* porin to study their ion selectivity. Membranes were formed either in 10 mM KCl or in 50 mM KCl. After incorporation of 100–1000 porin channels into a membrane, salt gradients were established by the addition of small amounts of concentrated KCl solution (3 M) to one side of the membrane (on the other side the same volume of the more diluted KCl solution was added). From the measured zero-current membrane potential V_m due to the externally applied concentration gradient, c''/c', across the membrane, the ratio P_{cation}/P_{anion} of the permeability for cations and anions could be calculated using the Goldman-Hodgkin-Katz equation [36].

Table 2 shows the permeability ratios for the native and the modified *Protophormia* porin. The modified porin was preincubated with 1% cholesterol (in 2% Genapol, 10 mM Hepes, 1 mM EDTA, pH 7). The data indicate that both porins were slightly cation-selective under the conditions of the selectivity measurements. These results were in contrast to those obtained from the single-channel conductance measurements, which did indeed indicate anion-selectivity for both porins. The discrepancy could be explained by the influence of the ionic strength on the configuration (open/closed state) of the porin channel as has been shown previously for *Dictyostelium* porin [5]. At low salt concentrations (10 mM and 50 mM KCl) the channels could preferentially be in the closed state, which is cation-selective [6,43,44], whereas they are in the anion-selective open state at higher ionic strength [5].

3.5. Voltage dependence of the native and the CGA140'408-modified porin

At 10 mV transmembrane potential, the closing events in single-channel recordings of the native and modified *Protophormia* porin represented only a minor fraction of the current fluctuations. At higher voltages, beginning with about 20 mV, the number of closing events became more frequent, i.e., the porins are voltage-regulated. The voltage dependence of the native and the modified *Protophormia* porin was studied as follows. The voltage across a membrane bathing in 1 M KCl and containing a large number of pores was switched from zero to a given voltage, V_m (the range of the applied voltage was from 10 mV to 90–100 mV). The current measured immediately after application of the voltage, was a linear function of the membrane potential. Subsequently, the membrane current decayed exponentially to smaller values for voltages larger than 20 mV (not shown). The ratio of the steady-state conductance G (conductance after the decay was complete) divided by the initial conductance, G_0 (conductance at 10 mV transmembrane potential) showed a bell-shaped curve

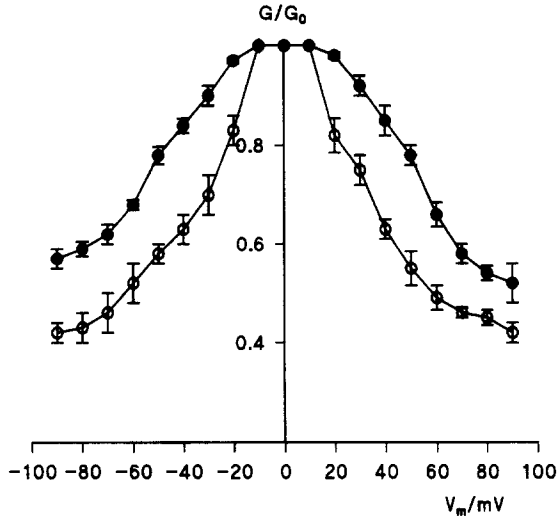

Fig. 6. Ratio of the conductance G at a given membrane potential (V_m) divided by the conductance G_0 at 10 mV as a function of the membrane potential V_m. The closed circles indicate the measurements with the native *Protophormia* porin, while the open circles show measurements with the CGA 140'408-modified one (preincubated with 1% cholesterol in 2% Genapol, 10 mM Hepes, 1 mM EDTA, pH 7.0). The aqueous phase contained 1 M KCl and either 10 ng/ml native porin or about 5 ng/ml modified porin. The membranes were formed from diphytanoyl phosphatiylcholine/n-decane. $T = 25°C$.

as a function of voltage for the native and the modified porin (Fig. 6). These results indicated that the CGA 140'408-modified porin exhibited a higher voltage-dependence than the native form. It is noteworthy that the incubation of the native porin with cholesterol did not influence its voltage-dependence.

4. Discussion

4.1. Properties of the Protophormia porin

In this study we have demonstrated that we were able to isolate and purify mitochondrial porin from the fly *Protophormia*. For the purification we followed an established procedure, the chromatography across a HTP-column [8]. *Protophormia* porin eluted just after the void volume of the ceramic HTP-column, indicating that the porin did not bind to the column material. It seems to be hidden in a detergent micelle similar to the situation in mitochondrial outer membranes of other organism, in which porin is also deeply buried [9]. *Protophormia* porin has an apparent molecular mass of about 30 kDa, which is also in good agreement with other mitochondrial porins [3]. Partial sequencing following tryptic digestion and Edman degradation confirmed that we are dealing here with a mitochondrial porin because the fragments showed a high homology to human mitochondrial porins porin 31HL and HVDAC2.

The porin function was verified by reconstitution of the purified *Protophormia* porin in lipid bilayer membranes. Channel reconstitution was not a rare event since up to 10000 channels were incorporated in a membrane of 1 mm^2 surface area. Control experiments demonstrated that the formation of channels is not simply an artifact caused by the interaction of detergents with the artificial membranes. The single-channel conductance of *Protophormia* porin was about 2.4 or 4.5 nS in 1 M KCl (left- and right-hand maxima of the channel distribution). Both maxima probably represent stable states of the same protein since they have been observed in mitochondrial porin preparations from different organisms [3]. Both states are characterized by a linear dependence of the single-channel conductance on the conductance of the bulk aqueous phase, which is typical for general diffusion channels.

Besides the mitochondrial porin channel, we also noticed another channel, which had a single-channel conductance of 2 nS in 1 M KCl and a short lifetime. This channel was frequently present in the sample of CGA 140'408-modified porin in particular. It is noteworthy that this channel has also been observed in porin-deficient yeast mitochondria [39] and in pea mitochondria [40]. Its role in mitochondrial metabolism is an open question. It has been argued that the 2 nS channel could have to do with the general import pathway for proteins into mitochondria [45].

The channel conductance of the 30 kDa protein was

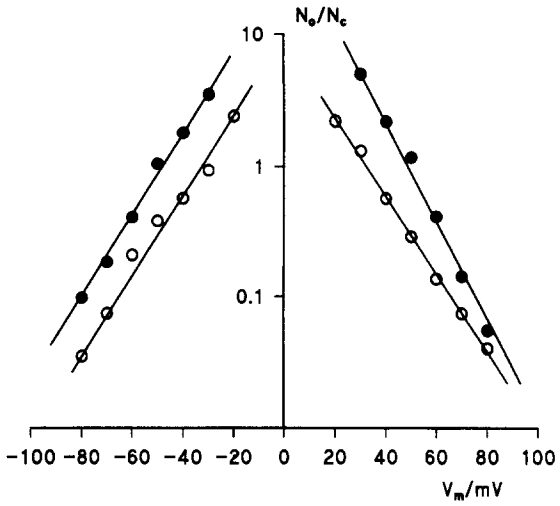

Fig. 7. Semilogarithmic plots of the ratio N_o/N_c of the native and the CGA 140'408-modified *Protophormia* porins as a function of the transmembrane potential V_m. The data were taken from Fig. 6. The midpoint potential of the N_o/N_c distribution (i.e., $N_o = N_c$) was −48 mV for negative potentials and 50 mV for positive potentials (native porin) and −32 mV for negative potentials and 33 mV for positive potentials (CGA 140'408-modified porin). The slope of the straight lines correspond to 32 mV (left side) and 26 mV (right side) for an e-fold change of N_o/N_c (native porin) and to 32 mV (left side) and 33 mV (right side) for an e-fold change of N_o/N_c (CGA 140'408-modified porin), which means that the number of gating charges was close to one in both cases.

voltage-regulated; transmembrane potentials higher than 20 mV resulted in a reduced conductance. The steady-state conductance, G, divided by G_o, the conductance in the absence of a transmembrane potential, displayed a typical bell-shaped curve (Fig. 6). To study the voltage-dependence in more detail, the data of Fig. 6 were analyzed using the equation [4]:

$$N_o/N_c = \exp(nF(V_m - V_o)/RT) \quad (1)$$

where F, R and T are standard symbols, n is the number of charges moving through the entire transmembrane potential gradient for channel gating and V_o is the potential at which 50% of the total number of channels are in the closed configuration. The open-to-closed ratio of the channels, N_o/N_c, may be calculated from the data in Fig. 6 according to

$$N_o/N_c = (G - G_{min})/(G_o - G) \quad (2)$$

G is in this equation the conductance at a given membrane potential V_m, G_o and G_{min} are the conductances at 10 mV (conductance of the open state) and very high potentials, respectively. The semilogarithmic plot of the ratio N_o/N_c as a function of the transmembrane V_m could be fitted to straight lines (see Fig. 7 for the native and for the modified *Protophormia* porin). The lines can be used for the calculation of the number of gating charges n (number of charges involved in the gating process) and the midpoint potential V_o (potential at which the number of open and closed channels is identical). The midpoint potential of the

native porin was about 50 mV for positive and negative potentials and decreased to 32 mV for CGA 140'408-modified porin. The gating charge was in both cases close to one, which was smaller than for other mitochondrial porins [2,8]. It is noteworthy that *Protophormia* porin was less voltage-dependent than most other mitochondrial porins but it was similar to that which has been observed previously for porin of *Drosophila melanogaster* [38].

4.2. Effect of CGA 140'408 on channel function

In the present study, we have also modified porin by treating mitochondria with the carbodiimide CGA 140'408 and studied the properties of porin channels in lipid bilayer membranes. This DCCD-analog binds to the F_0-component of the mitochondrial ATPase and to porin [27,28]. CGA 140'408 had no effect on the single-channel conductance of *Protophormia* porin. This result is in agreement with a previous study of DCCD-modified pig heart mitochondrial porin [21]. DCCD has no effect on the single-channel conductance but inhibits hexokinase binding to porin [46]. Glu-72 is the DCCD-binding site in mammalian porins [24]. This amino acid is presumably localized in a transmembrane β-strand because DCCD interacts only with negatively charged amino acid that are localized within a hydrophobic environment [22,23]. So far it is an open question whether CGA 140'408 also binds to a negatively charged amino acid localized in the hydrophobic environment of the *Protophormia* porin channel. In any case, it is clear from the result presented here that the binding site is outside the channel lumen, otherwise we should have observed a major effect on the single-channel conductance of *Protophormia* porin because of the bulky CGA 140'408 molecule. This means that the acidic residue faces probably into the lipid bilayer and could be shielded by cholesterol. It is noteworthy that the binding of DCCD to Glu-72 in mammalian porins leads to a similar situation. Also in this case the single-channel conductance is not influenced by DCCD-binding [21], although Glu-72 is clearly localized in a transmembrane β-strand [24].

The modification of *Protophormia* porin with the pesticidal carbodiimide CGA 140408 lowered the channel-forming activity of *Protophormia* porin (i.e., the number of channels observed at a given protein concentration). The addition of cholesterol led to an increase of its activity to values similar to that of native porin, whereas cholesterol had no influence on the channel-forming activity and the voltage-dependence of native porin. We have demonstrated recently that sterols are essential for the formation of channels from water-soluble mitochondrial porin from different organisms [41]. This could mean that the CGA 140'408-modified *Protophormia* porin lost part of the sterol presumably because of the chemical modification within the hydrophobic part of the channel-forming unit. This modification changed also the voltage-dependence. Native *Protophormia* porin was less voltage-dependent than CGA 140'408-modified porin. This result could mean that one of the external loops between two β-strands, which may be responsible for channel gating, has a smaller stability within the channel-forming unit in the modified form as compared with the native one. This means that we observed an increased potential dependence of channel function in the carbodiimide-bound form. However, the present results do not allow speculation as to whether that this modification may have an impact on porin channel function in the cellular environment. Furthermore, other roles of mitochondrial porin, such as representing docking sites for several enzymes such as hexokinase and glycerokinase [17,18,47] involved in the control of key metabolites [48], may be affected by binding of CGA 140'408 as it is known from DCCD [46]. Further studies addressing this question are under way.

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