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The Role of Sterols in the Functional Reconstitution of Water-Soluble Mitochondrial Porins from Plants

Francesco Carbonara,[1] Birgit Popp,[2] Angela Schmid,[2] Vito Iacobazzi,[1] Giuseppe Genchi,[1] Ferdinando Palmieri,[1] and Roland Benz[2]

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Water-soluble porins were prepared from native mitochondrial porins isolated from different plants (pea and corn). In the water-soluble form the porins have lost their channel-forming properties. The water-soluble porins were investigated for the influence of different sterols on their membrane activity and their channel-forming properties in lipid bilayer membranes. Our experiments demonstrated that the water-soluble porins regained channel forming activity when the protein was preincubated with different sterols in the presence of a detergent. The channels formed in lipid bilayer membranes after this procedure regain in many but not all cases the original properties of the native mitochondrial porins. Preincubation with other sterols led to a change in the single-channel conductance or to a complete loss of the voltage dependence. The sterols had also a strong influence on the channel-forming activity of the porins. Preincubation of water-soluble pea porin with the plant sterol β-sitosterol resulted in a considerable higher channel-forming activity than with all the other sterols used for preincubation. The role of the sterols in the channel-forming complex is discussed.

KEY WORDS: Mitochondrial porin; VDAC; *Zea mays, Pisum sativum*; sterol; reconstitution; voltage dependence; lipid bilayer membrane.

INTRODUCTION

The mitochondrial outer membrane acts as a molecular filter for hydrophilic solutes (O'Brien and Brierly, 1965; Pfaff *et al.*, 1968). Responsible for the molecular sieving properties of this membrane is the presence of an intrinsic outer membrane protein, the mitochondrial porin (Zalman *et al.*, 1980; Roos *et al.*, 1982) or VDAC[3] (voltage-dependent anion-selective channel) (Schein *et al.*, 1976; Colombini, 1979). Mitochondrial porins form water-filled channels that are slightly anion-selective in the open state and cation-selective in the voltage-induced ion-permeable closed states (Benz, 1994). Mitochondrial porins were isolated from a variety of eukaryotic cells and their pore-forming properties were studied in reconstitution experiments with planar lipid bilayers and liposomes (Colombini, 1980; Freitag *et al.*, 1982b; Lindén *et al.*, 1982). According to these investigations the mitochondrial pore has a diameter of about 2–3 nm in the open state, which agrees well with the electron microscopic analysis of mitochondrial outer membranes (Mannella *et al.*, 1986, 1989).

The biosynthesis of mitochondrial porins takes place on free cytoplasmic ribosomes (Mihara *et al.*, 1982; Freitag *et al.*, 1982a; Gasser and Schatz, 1983). The precursor is post-translationally imported into the mitochondria without cleavage of any signal sequence (Mihara *et al.*, 1982; Freitag *et al.*, 1982b; Gasser and

[1] Department of Pharmaco-Biology, University of Bari, Via Orabona 4, I-70125 Bari, Italy.

[2] Lehrstuhl für Biotechnologie, Theodor-Boveri-Institut (Biozentrum) der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany.

[3] Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; DiphPC, diphytanoyl phosphatidylcholine; EDTA, ethylenediamine tetraacetic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethansulfonic acid; SDS-PAGE, sodiumdodecylsulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; VDAC, voltage-dependent anion-selective channel.

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Schatz, 1983) and without requirement for a membrane potential. However, metabolic energy in the form of ATP (Pfanner *et al.*, 1988) or a detergent such as Triton X-100, probably acting as a chaperone, is needed for the insertion process (Pfaller *et al.*, 1985). According to secondary structure predictions the polypeptide chain of mitochondrial porins is arranged in a cylinder containing either 16 antiparallel, amphiphilic β-barrels similar to certain bacterial porins (De Pinto *et al.*, 1991; Benz, 1994) or 12 β-barrels and the amphiphilic N-terminal α-helix (Blachly-Dyson *et al.*, 1990). By precipitation of the isolated mitochondrial porin of *Neurospora crassa* with trichloracetic acid followed by treatment at high pH a water-soluble porin with qualities very similar to the precursor form is obtained (Pfaller *et al.*, 1985). This water-soluble porin requires cholesterol for successful reconstitution into a planar lipid bilayer as a channel (Pfaller *et al.*, 1985). Recent experiments showed that sterols are not necessary as a kind of insertion target in the membrane, but seem to be important for the formation of the channel unit in the aqueous phase before its insertion into the membrane (Popp *et al.*, 1995).

For a long time there had been little knowledge about mitochondrial porins in plants. Only the presence of a general diffusion channel in extracts of mung bean mitochondrial outer membranes (Zalman *et al.*, 1980) and in crude fractions of corn mitochondria (Smack and Colombini, 1985) has suggested the presence of mitochondrial porins. More recently porins have been isolated and characterized from a variety of plant sources: pea mitochondria (Schmid *et al.*, 1992), wheat mitochondria (Blumenthal *et al.*, 1993), and corn mitochondria (Aljamal *et al.*, 1993). More recently plant porins from the following sources have been sequenced: pea amyloplasts (Fischer *et al.*, 1994) and potato mitochondria (Meins *et al.*, 1994).

Sterols are major membrane components in eukaryotic cells (Asworth and Green, 1966; Glover and Green, 1957; Lasser and Clayton, 1966; Smith *et al.*, 1967). The sterols found in plants are typified by stigmasterol and β-sitosterol; the predominant sterol in fungi and yeast is ergosterol. So far it was not clear whether sterols play a similar role for the function of plant sterols than for mitochondrial porins from *Dictyostelium discoideum*, *Paramecium tetraurelia*, rat liver, and others (De Pinto *et al.*, 1989; Popp *et al.*, 1995). Furthermore, the successful reconstitution of unfolded membrane proteins and the control of their structure represents a major problem in the study of transport processes. In the present study we investigated the influence of different sterols on the reconstitution of unfolded mitochondrial porin from plants: corn (*Zea mays*) and pea (*Pisum sativum*). In particular, we studied the influence of the sterols on the function of the mitochondrial porins as a voltage-gated channel and on the single-channel conductance. The results of this study demonstrate that sterols play an important role for the formation of the porin channel and influence also the channel characteristics.

MATERIALS AND METHODS

Materials

Cholesterol (cholest-5-en-3β-ol) was obtained from Serva, Heidelberg, F.R.G.. 7-Dehydrocholesterol (cholest-5,7-dien-3β-ol), desmosterol (cholest-5,24-dien-3β-ol), ergosterol (cholest-5,7,22-trien-24-methyl-3β-ol), β-sitosterol (cholest-5-en-24-ethyl-3β-ol) and stigmasterol (cholest-5,22-dien-24-ethyl-3β-ol) were all bought from Sigma, epicholesterol (cholest-5-en-3α-ol) was obtained from Ferak, Berlin, F.R.G.. (For structures of the different sterols see Fig. 1.) Genapol X-80 was obtained from Fluka (Buchs,

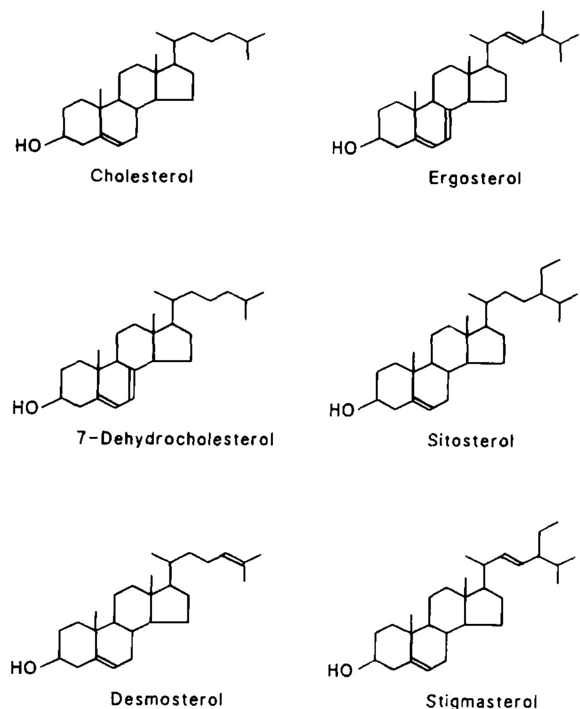

Fig. 1. Structures of sterols from mammalian, plant, or fungal origin. Epicholesterol has the same structure as cholesterol with the exception that the hydroxy group is in the β- and not in the α-position.

Switzerland). All salts and buffers were of analytical grade and obtained from Merck (Darmstadt, F.R.G.).

Purification of Porin

The mitochondrial porin from pea (*Pisum sativum*) was isolated from whole mitochondria by using a hydroxyapatite column as described previously (Schmid *et al.*, 1992). The mitochondrial porin from corn (*Zea mays*) was isolated and purified from whole mitochondria by using a hydroxyapatite:celite column (Aljamal *et al.*, 1993). Both purification procedures yielded pure mitochondrial porin as controlled by SDS-PAGE.

Preparation of Water-Soluble Porin

The native mitochondrial porins were precipitated according to the method of Wessel and Flügge (1984) to remove detergents, lipid, and sterol associated with the protein. To ensure that the associated sterol was completely removed, the resulting pellet was mixed a second time with 100 μl chloroform and 300 μl methanol was added. The probe was vortexed again and the protein was pelleted by centrifugation at 9000 × *g* for 5 min. The pellet was dried at room temperature. It was resolubilized by mixing with 1 volume of 0.1 M NaOH for 60 s and immediately neutralized by an equal volume of 0.2 M NaH_2PO_4 as described by Pfaller *et al.*, (1985). This procedure yielded water-soluble porin in 100 mM NaP_i, pH 6.8.

Preincubation with Sterols

Suspensions of sterols were made by taking small volumes from solutions of the sterol in chloroform, evaporating the chloroform, and then suspending the sterol in a concentration of 1% (w/v) in 1% Genapol, 0.5 mM EDTA, 5 mM Hepes, pH 7. For preincubation one volume of porin (10–20 μl), either water-soluble porin in 100 mM NaP_i-buffer or isolated porin in 2% Genapol, 10 mM Hepes, 1 mM EDTA as obtained from the last isolation step, was mixed with one volume of a 1% (w/v) sterol suspension and vortexed for 10 s. The obtained samples were stored on ice until use.

Lipid Bilayer Experiments

The methods used for the "black" lipid bilayer experiments have been described previously (Benz *et al.*, 1978). If not mentioned otherwise, membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (DiphPC, Avanti Polar Lipids, Alabaster, Alabama) in *n*-decane across circular holes (surface area about 0.1 mm^2) in the thin wall of a Teflon cell separating the two aqueous compartments. The temperature was kept at 25°C throughout. The aqueous solutions were either unbuffered and had a pH around 6 or buffered with 10 mM Hepes at a pH of 7. Porin was added from the stock solutions (either from the detergent solution as obtained from the isolation and purification procedure or in the water-soluble form) to the aqueous phase of the *cis* compartment (the compartment to which the voltage was applied) after the membranes had turned optically black in reflected light. The current through the membranes was measured with two calomel electrodes switched in series with a voltage source and a model 427 current amplifier (Keithley, Cleveland, Ohio). The amplified signal was monitored with a storage oscilloscope and recorded on a strip chart recorder.

RESULTS

The Channel-Forming Activity of Water-Soluble Porins from Plants Can Be Increased by Preincubation with Sterol Suspensions

Mitochondrial porins from plants lost almost 100% of their channel-forming ability after precipitation with chloroform and methanol. After the treatment with NaOH and resolubilization in NaH_2PO_4 as described above we observed a further decrease of the channel-forming activity. Water-soluble porin from corn mitochondria had very low channel-forming activity (less than 0.1% of the initial one) while with water-soluble pea porin formed only an insignificant number of channels at very high protein concentrations. With most of the sterols used in our reconstitution experiments the channel-forming activity showed a substantial increase of many orders of magnitude when the water-soluble porin was preincubated with the detergent–sterol suspension. This was found for porins from both pea and corn.

Differences in the Degree of Activation by Different Sterols

The preincubation of the plant porins led to a considerable increase of the channel-forming activity. It is noteworthy that the sterol used for the reconstitution of the porin had a strong influence on the reconstitution yield. In particular, the reconstitution of pea porin was strongly dependent on the sterol used for preincubation. Water-soluble porin from corn showed in contrast to this a good channel-forming activity upon preincubation with all sterols of Fig. 1 except for epicholesterol. Epicholesterol, which has in contrast to all naturally occurring sterols the hydroxyl group in the α- instead of β-position, only led to small channel-forming activity. Similarly, water-soluble pea porin was also hardly activated by epicholesterol and only weakly by stigmasterol. The highest activation of this porin occurred upon preincubation with the plant sterol β-sitosterol. This sterol activated water-soluble pea porin by a factor of 10 higher than all other sterols.

Influence of the Sterol on the Single-Channel Conductance

The single-channel conductance distribution of the reconstituted water-soluble forms was in general somewhat broader than for the native porins. However, the single-channel conductances of the reconstituted water-soluble forms of corn and pea mitochondrial porin did not differ significantly in most cases from that of the native forms of these porins (see Figs. 2A and 3A). We observed also some exceptions: After preincubation of corn porin with epicholesterol, most of the channels had conductances of around 0.9 nS in 1 M KCl (see Fig. 2B) instead of 2 or 4 nS that were observed for the native porin. Similarly, water-soluble pea porin preincubated with epicholesterol had an average single-channel conductance of 1.5 nS and no channels with the conductance of native pea porin of around 3.5 nS could be detected. Another exception was observed for the water-soluble porin from pea preincubated with stigmasterol. The channel-forming activity was not only quite low, but most of the channels had single-channel conductances of about 1 nS (Fig. 3C) whereas after preincubation with β-sitosterol most channels had conductances of approximately 3.5 nS (Fig. 3B). Tables I and II show a summary of all single-channel conductances obtained for the two native plant porins and the reconstituted systems with different sterols.

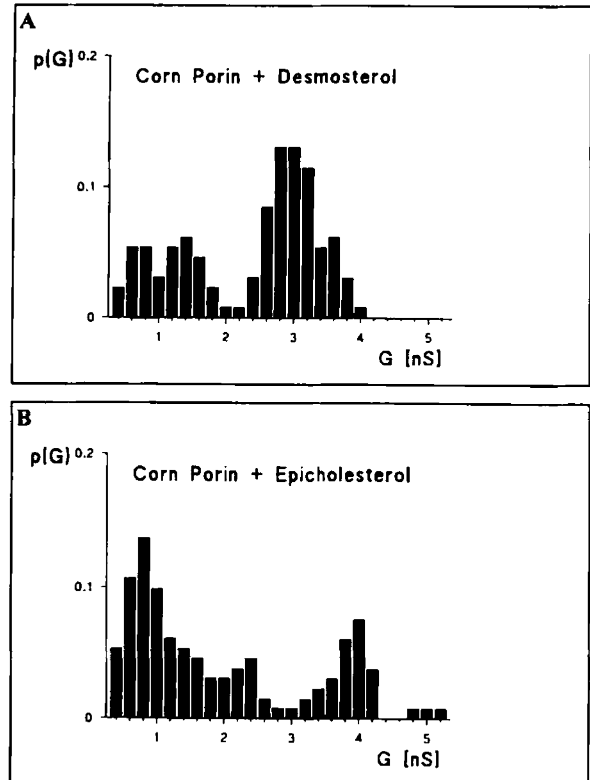

Fig. 2. Histogram of the probability p(G) for the occurrence of a given conductivity unit observed with membranes formed of DiphPC/*n*-decane in the presence of water-soluble corn porin, preincubated in a 1% desmosterol, 1% Genapol suspension (A) or a 1% epicholesterol, 1% Genapol suspension (B). p(G) is the probability that a given conductance increment G is observed in the single-channel experiments. It was calculated by dividing the number of fluctuations with a given conductance increment by the total number of conductance fluctuations. The aqueous phase contained 1 M KCl. The applied membrane potential was 10 mV; T = 20°C. The single-channel conductance averaged over all conductance increments was 2.4 nS for 130 single-channel events (A) and 2.0 nS for 131 single-channel events (B).

Voltage Dependence of the Plant Porins Can Be Abolished by Preincubation with Specific Sterols

With most sterols the voltage dependence of the water-soluble forms of both plant porins did not differ significantly from the one of the native form. It is noteworthy that upon preincubation with desmosterol both water-soluble porins completely lost their voltage-dependence (Figs. 4 and 5). The same loss of the

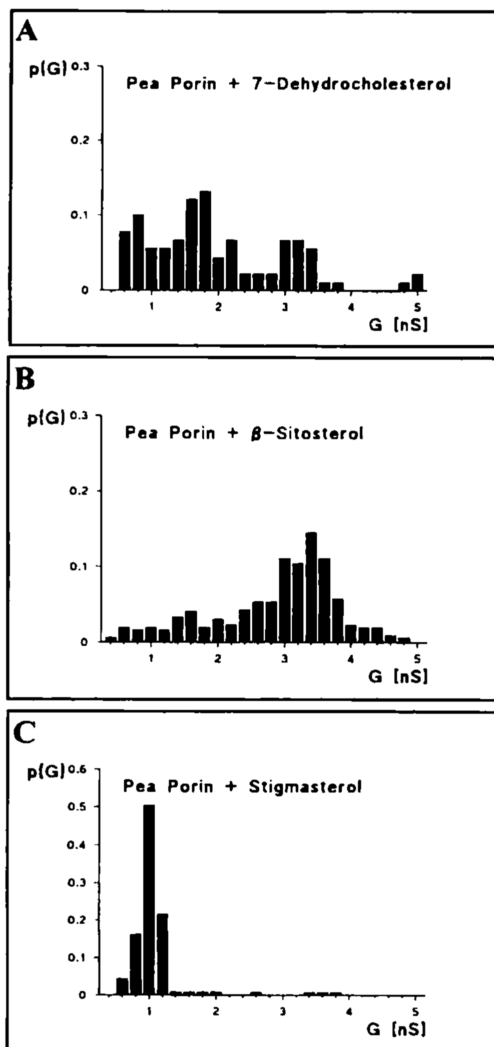

Fig. 3. (A) Histogram of the probability p(G) for the occurrence of a given conductivity unit observed with membranes formed of DiphPC/n-decane in the presence of water-soluble porin from pea preincubated in 7-dehydrocholesterol (A), β-sitosterol (B), or stigmasterol (C) respectively. p(G) is determined as in Fig. 2. The aqueous phase contained 1 M KCl. The applied membrane potential was 10 mV; T = 20°C. The single-channel conductance averaged over all conductance increments was 1.9 nS for 101 single-channel events (A), 2.9 nS for 294 single-channel events (B), and 1.1 nS for 111 single-channel events (C).

voltage dependence was observed when we preincubated the water-soluble pea porin with β-sitosterol. This means that the preincubation with sitosterol restored a high degree of channel-forming activity, but destroyed the voltage dependence. After preincubation of both plant porins with epicholesterol the channels were somewhat voltage-dependent, but a considerably higher voltage was needed for that (Figs. 4 and 5).

DISCUSSION

Sterols Play an Important Role in the Functional Reconstitution of Water-Soluble Mitochondrial Porin from Plants

The functional reconstitution of mitochondrial porins from *Dictyostelium discoideum, Paramecium tetraurelia,* and rat liver has been shown to be dependent on the presence of sterols (Popp *et al.,* 1995). Similarly we demonstrated in this study that water-soluble porins from plants (corn and pea) needed the addition of sterols for the formation of single-channel units that are active in the lipid bilayer assay. Water-soluble porin was obtained by the method developed previously for mitochondrial porin of *Neurospora crassa* (Pfaller *et al.,* 1985). Here we did not use the trichlorethanol precipitation that has been employed by Pfaller *et al.,* (1985) but removed lipids and detergents associated with the native plant porins with an extraction method using methanol/chloroform/water (Wessel and Flügge, 1984). After precipitation the porin had already very little channel-forming activity. It became completely unfolded at high pH. The water-soluble porin of *Neurospora crassa* acts similarly as the porin precursor (Pfaller *et al.,* 1985) and can be used as a model system for the investigation of the porin import process in the mitochondrial outer membrane.

The water-soluble plant porin had a very low channel-forming activity in lipid bilayer membranes. This activity could be strongly increased by preincubation of the porins with a sterol/detergent suspension. As already observed for water-soluble mitochondrial porin from *Dictyostelium discoideum, Paramecium tetraurelia,* and rat liver (Popp *et al.,* 1995), activation by preincubation with the sterol was much stronger than when the membrane contained sterol. We concluded from these results that the sterol molecules did not act as a promoter for the insertion into the membrane, but formed part of the channel unit capable for insertion. This means that our idea is that one or several sterol molecules bind to the outside of the channel-forming cylinder, thus shielding relatively polar parts of the protein and stabilizing the overall channel structure. In that way the sterol could facilitate preformation of the channel from the relatively polar protein and insertion into the membrane. In this respect it is noteworthy that mitochondrial porins isolated from *Neurospora crassa* and bovine heart are associated with sterols (Freitag *et al.,* 1982b; De Pinto *et al.,* 1989).

Table I. Single-Channel Conductances, Voltage Dependence, and Channel-Forming Activity of Native Mitochondrial Porin of *Zea mays* and Its Water-Soluble Form Preincubated in Different Sterols and Detergent[a]

Zea mays	Single-channel conductance [nS]	Voltage dependence [$G_{50\ mV}/G_0$]	Activity
Native porin	1.9; 4.2	0.57	n.d.
Water-soluble porin, preincubated in cholesterol	4	0.51	+++
Water-soluble porin, preincubated in epicholesterol	0.9; 4	0.83	++
Water-soluble porin, preincubated in ergosterol	3.9	0.67	+++
Water-soluble porin, preincubated in 7- dehydrocholesterol	3.8	0,62	+++
Water-soluble porin, preincubated in desmosterol	3.5	1	+++
Water-soluble porin, preincubated in sitosterol	3.8	0.67	+++
Water-soluble porin, preincubated in stigmasterol	3.5–4	0.72	+++

[a] The single-channel conductances represent the peaks of the single channel histograms. The channel-forming activity is characterized by +, ++, +++ from + meaning weak to +++ meaning very strong. The voltage dependence was derived from experiments similar to those shown in Figs. 4 and 5. The voltage dependence is characterized by the ratio $G_{50\ mV}/G_0$. $G_{50\ mV}$ is the stationary membrane conductance at a voltage of 50 mV. G_0 is the stationary conductance at 10 mV. n.d. means not determined. The aqueous phase contained 1 M KCl (pH 6.0) for all measurements. The applied membrane potential for the determination of the single-channel conductances was +10 mV; T = 20°C.

Table II. Single-Channel Conductances, Voltage Dependence, and Channel-Forming Activity of Native Mitochondrial Porin from *Pisum sativum* and Its Water-Soluble Form Preincubated in Different Sterols and Detergent

Pisum sativum	Single-channel conductance [nS]	Voltage dependence [$G_{50\ mV}/G_0$]	Activity
Native porin	1.5; 3.5	0.60	n.d.
Water-soluble porin, preincubated in cholesterol	3.5	0.55	+++
Water-soluble porin, preincubated in epicholesterol	1.5	0.92	+
Water-soluble porin, preincubated in egosterol	1.2; 2; 3.5	0.62	+++
Water-soluble porin, preincubated in 7- dehydrocholesterol	0.6–3.5	0.5	++
Water-soluble porin, preincubated in desmosterol	2; 3.6	1	+++
Water-soluble porin, preincubated in sitosterol	3.5	1	++++++
Water-soluble porin, preincubated in stigmasterol	1.1	0.6	+

[a] For explanation, refer to the legend of Table I.

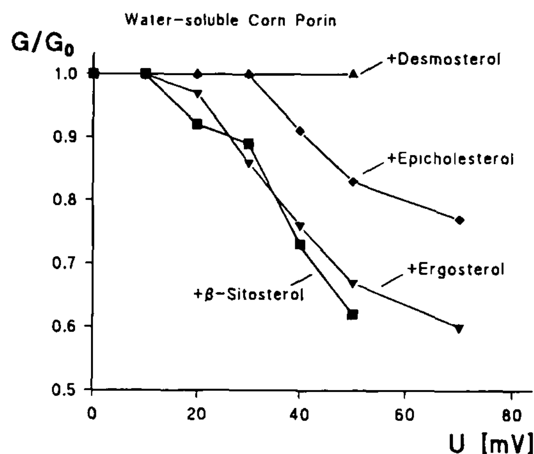

Fig. 4. Voltage dependence of reconstituted water-soluble corn porin preincubated in different sterols. The ratio of the conductance G at a given voltage V_m divided by the conductance G_0 at 10 mV is shown as a function of positive voltages. The membranes were formed of DiphPC/n-decane. The aqueous phase contained 1 M KCl (pH 6.0); T = 20°C.

Channel Characteristics Are Influenced by the Respective Sterol

The kind of sterol used for preincubation of the water-soluble porins could influence the channel properties such as single-channel conductance and voltage dependence. However, in many cases the channels obtained from the preincubated water-soluble porins were not significantly different from the native ones, e.g., water-soluble corn porin preincubated in cholesterol, ergosterol, 7-dehydrocholesterol, sitosterol, and stigmasterol or water-soluble pea porin preincubated in cholesterol, ergosterol, and 7-dehydrocholesterol. For some other sterols the water-soluble porin channels differed from the native ones. Preincubation with desmosterol resulted in both cases in channels without any voltage dependence. Water-soluble pea porin was also not voltage-dependent after preincubation with the plant sterol sitosterol, although the channel-forming activity was much higher than after preincubation with all other sterols. Loss of voltage dependence after preincubation with sitosterol and partially with desmosterol had also been observed for water-soluble porins from rat liver and *Paramecium* (Popp *et al.*, 1995). Water-soluble pea porin preincubated with stigmasterol was relatively inactive and showed only small single-channel conductances in the lipid bilayer assay.

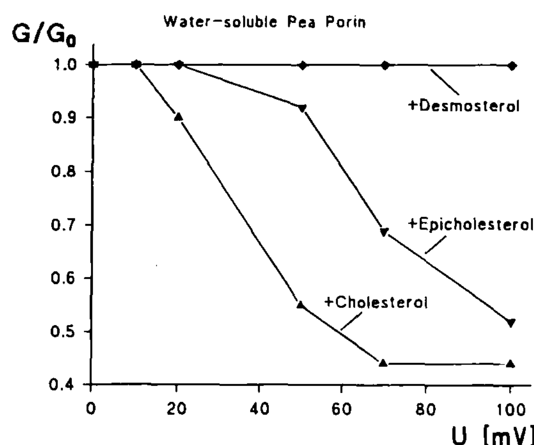

Fig. 5. Voltage dependence of reconstituted water-soluble pea porin preincubated in desmosterol, epicholesterol, or cholesterol suspension in detergent. The ratio of the conductance G at a given voltage V_m divided by the conductance G_0, at 10 mV is shown as a function of positive voltage. The membranes were formed of DiphPC/n-decane. The aqueous phase contained 1 M KCl (pH 6.0); T = 20°C.

The different single-channel conductances are probably caused by different conformations of the porin channel. Likewise for native porin from different species two maxima of the single-channel conductances are observed in most cases: one in the range 1.5–2 nS and the other between 3.5–4.5 nS (Benz, 1994). These two conductance levels are not caused by different isoforms of the channel protein because they are equally observed for porin expressed in *Escherichia coli* and purified from inclusion bodies (Fischer *et al.*, 1994; Popp, Fischer, Flügge and Benz, unpublished results). Moreover the behavior of the channel after application of higher voltage argues for several conformations that the porin can adopt and that cause different single channel conductances. At high membrane potential the channels do not close completely but go into several substates, that may be caused by a conformational change of the whole or parts (e.g., loops) of the channel. One could imagine that in the aqueous phase and in the membrane a kind of equilibrium between the different channel conformations exists. This equilibrium could be influenced by the membrane potential or by association with sterol or lipid molecules, e.g., the association of the unfolded pea porin with stigmasterol could drive the equilibrium to a conformation with a smaller pore diameter.

Loss of voltage dependence could be caused by steric hindrance through the side chains of some of the sterols. These side chains could prevent the conformational change after application of high voltages. It is noteworthy that preincubation of both porin species with epicholesterol in contrast to other investigations

(Popp *et al.*, 1995) led to only small single channels with small voltage dependence and low channel-forming activity. Epicholesterol has the hydroxyl-group in the α- instead of β-position, which suggests that the position of the hydroxyl group plays an important role in the structure of plant porin. This isomerization of the hydroxyl group has a strong influence on the overall structure of cholesterol and, for example, prevents interaction between the sterol and phospholipids in the membrane (Demel *et al.*, 1972), or with sterol-binding toxins (Alouf and Geoffroy, 1979).

Channel Model under Inclusion of Associated Sterol or Lipid

According to structural predictions mitochondrial porins are formed by a cylinder of amphiphilic antiparallel β-strands (Kleene *et al.*, 1987; Forte *et al.*, 1987). One model assumes 16 β-strands (De Pinto *et al.*, 1991; Benz, 1994) similar to those found by X-ray crystallography of bacterial porins (Weiss *et al.*, 1991; Cowan *et al.*, 1992). The other model, which we consider as less likely, assumes that the channel walls are formed by 12 β-strands and the N-terminal α-helix (Blachly-Dyson *et al.*, 1990). The overall porin proteins have a high polarity of about 0.5 (Forte *et al.*, 1987; Kayser *et al.*, 1989). Its amphiphilic β-strands are presumably folded in such a way that most of the polar residues point to the water lumen inside the channel, while the nonpolar residues are preferentially localized on the outer sphere. Some polar residues could contact the lipophilic outer environment of the channel. These could be shielded by sterol molecules. Association with the sterols and folding of the polypeptide chain could already take place in the water phase before insertion into the membrane. It is known that sterol molecules are present in low concentrations in the water phase (Backer and Dawidowicz, 1981). This whole channel complex can probably adopt several conformations with different single channel conductances and different selectivity properties (Benz and Brdiczka, 1992). The equilibrium between these different conformations could be influenced by voltage, by the lipid/sterol environment, by metabolites such as NADH (Zizi *et al.*, 1994), and by other molecules that bind to the channel. Examples for proteins binding to the mitochondrial porin are hexokinase and glycerokinase (Fiek *et al.*, 1992; Östlund *et al.*, 1983). Also, a protein called "VDAC modulator" (Holden and Colombini, 1988) and presumably located in the inter membrane space (Holden and Colombini, 1993) was found to increase the voltage sensitivity of the mitochondrial porin.

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