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Proteins of Cytosol and Amniotic Fluid Increase the Voltage Dependence of Human Type-1 Porin[1]

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Heat-stable proteins from human and porcine cytosol and human amniotic fluid were found to increase the voltage dependence of human type-1 porin reconstituted in planar phospholipid bilayers. Purification processes revealed that these regulatory molecules were characterized by anionic charge and apparent molecular weights of between 23 and 64 kDa. The human cytosol proteins exerted inhibitory activity only when added to the compartment with applied negative potential. The observed increase in voltage dependence of porin was due to the presence of specific proteins in cytosol and amniotic fluid, since human cerebral spinal fluid in comparable amounts had no significant effect on the channel properties. Furthermore, other anionic proteins and polypeptides investigated demonstrated no inhibitory activity, indicating that anionic charge alone could not mimic the molecular properties of the regulatory proteins. With respect to the well-documented expression of porin in the plasma membrane of various cells and species, the presented data give first clues for a biochemical regulation of the channel in this compartment.

KEY WORDS: Porin; VDAC; anion channel; chloride channel; voltage dependence; channel regulation; plasma membrane; regulatory protein.

INTRODUCTION

Eukaryotic porin (VDAC) is well known as a constituent of the mitochondrial outer membrane (Schein *et al.*, 1976). In this compartment it serves as the major route for the exchange of metabolites, particularly adenine nucleotides, between the cytoplasm and mitochondrion (Benz *et al.*, 1988; Huizing *et al.*, 1994; McCabe, 1994). Electrophysiological studies on the isolated and reconstituted porins of various species revealed channel activity with large conductances around 4 nS coupled with a slight anion selectivity at the presumed physiological outer membrane potential (reviewed in Sorgato and Moran, 1993; Benz, 1994). The main characteristic of all porins studied so far is their voltage dependence. At higher voltages ($\geq$ 30 mV) the channel switches to substates of lowered conductance by reducing the pore diameter with concomitant reversal of the ion selectivity. However, complete closure of the channel by voltage alone has not been achieved (Benz, 1994).

The sensitivity of porin to changes in the membrane potential, detected by delocalized net positive charges in the molecule (Bowen *et al.*, 1985; Thomas *et al.*, 1993), led to the assumption that porin channels do not simply act as a "coarse sieve" but can adopt well-defined substates depending on cellular energy demands. This notion was supported by the finding that synthetic anions like dextran sulfate (Mangan and Colombini, 1987) and König's reagent (Colombini *et al.*, 1987; Benz *et al.*, 1988 and 1990) increased the voltage dependence of eukaryotic porin. Subsequently, Colombini and coworkers provided the first data on the interaction of porin and physiological molecules with an impact on channel electrophysiology, showing that proteins of the mitochondrial intermembrane space cause porin to switch to a substate of reduced channel

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size (Holden and Colombini, 1988; Liu and Colombini, 1991 and 1992a; Holden and Colombini, 1993; Liu *et al.*, 1994). Furthermore, mitochondrial porin is known to function as binding site for kinases (Fiek *et al.*, 1982; Linden *et al.*, 1982; Östlund *et al.*, 1983) and was shown to be part of the peripheral benzodiazepine receptor (McEnery, 1992; McEnery *et al.*, 1992, 1993). Whether these associations have a regulatory influence on the channel properties is still unclear.

The interaction of porin with regulatory molecules has become particularly important since evidence for the multitopological expression of eukaryotic porin has accumulated (reviewed in Reymann *et al.*, 1995). As has been shown for the human cell line H2LCL, the *in vitro* electrophysiological properties are identical for porin isolated from the mitochondrial outer membrane and the plasma membrane (Benz *et al.*, 1992). According to recent data obtained by patch clamp studies, *in vivo* plasmalemmal porin appears to be part of the group of large conductance chloride channels (Janisch *et al.*, 1993; Dermietzel *et al.*, 1994). In order to maintain the plasma membrane's function as the physically and electrically impermeable divider between the intra- and extracellular matrix, strict regulatory control of porin to render the channel closed under nonstimulated conditions has to be postulated.

Because of the localization of porin in the plasma membrane, we investigated the influence of cytosol and amniotic fluid on the channel properties of human type-1 porin reconstituted in planar phospholipid bilayers (Heiden *et al.*, 1993). Here we report that proteins from both sources induce a significant increase in the voltage sensitivity of the human channel. These are the first data presented on possible biochemical regulation of the plasmalemmal porin.

MATERIALS AND METHODS

Purification of Porin 31HL and 31HM

Channel-active Porin 31HL and 31HM were prepared as described in detail elsewhere (Thinnes *et al.*, 1989; Jürgens *et al.*, 1991). In short, membrane proteins were solubilized with 2% Nonidet P40 and separated by ion exchange chromatography at CM-52- and DEAE-cellulose. Porin obtained after DEAE chromatography was used in the planar phospholipid bilayer experiments.

Preparation of Cytosol, Amniotic Fluid, and Cerebral Spinal Fluid

Human cytosol was obtained from the EBV-transformed, lymphoblastoid cell line H2LCL. Cells with a wet weight of 150 g were titrated to pH 7.5 and diluted fourfold with 10 mM Tris, 140 mM NaCl, pH 7.5 after thawing. To prevent proteolytic damage, the following inhibitors were added in the given final concentrations: phenylmethane sulfonylfluoride 85 μg/ml, Leupeptin 0.25 μg/ml, Pepstatin 0.25 μg/ml, Phosphoramidon 1 μg/ml. Cells were disrupted using a Teflon homogenizer. The homogenate was cleared of nuclei, mitochondria, and nondisrupted cells by centrifugation at 1500 × *g* for 10 min. The pooled supernatants were then ultracentrifuged (160,000 × *g*/h), which was repeated until the resulting supernatant (= cytosol) was optically clear. Prior to use, the cytosol was passed through 0.22-μm filters.

Porcine kidney cortex cytosol was prepared as described elsewhere (Krick *et al.*, 1991). Amniotic fluid was obtained from amniocenteses conducted during the 14th–23rd week past conception and was passed through 0.22-μm filters prior to use. Human Cerebral Spinal Fluid was obtained by puncture of the spine within the lumbar vertebra, canuling the dural sack. Insoluble constituents were removed by centrifugation (10 min/10,000 × *g*).

Fractionation of Cytosol and Amniotic Fluid

Removal of heat-labile proteins was achieved by boiling for 20 min and subsequent centrifugation for 20 min at 2000 × *g*. Extraction with chloroform was performed by vigorous shaking of cytosol and the organic phase in a ratio of 1:1 (v/v). The organic phase was discarded.

Protease VIII immobilized to agarose (Sigma, Munich; Germany) was used to digest the proteins of the biological probes. The enzyme was prepared as described by the manufacturer and was then added to aliquots of the probes in the amounts denoted. Digestion was stopped by short centrifugation at 10,000 × *g*, and the supernatant monitored for activity. In experiments with planar lipid bilayers, the free enzyme was added in the amounts indicated to both compartments of the chamber.

Fractionation by preparative isoelectric focusing was achieved using the Rotofor IEF system (Biorad, Munich, Germany). Probes were dialyzed against bidi-

stilled water to remove salt and buffering substances. After addition of 1.5% of Bio-Lyte ampholines (pH range 3–10) focusing was conducted for 6 h at a constant power of 12 W.

Gel filtration and anion exchange chromatography were carried out by FPLC (Pharmacia, Freiburg, Germany) using prepacked columns. Gel filtration used a Superose 12 column with a flow rate of 0.5 ml/min. Molecular weight determination of the active fractions was achieved by calibration with reference proteins. Anion exchange chromatography was performed on a Mono Q column. Buffer and flow rate conditions are described for the experiments in the respective figures.

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) and Laemmli and Favre (1973). Gels were stained with colloidal Coomassie brilliant blue as described elsewhere (Neuhoff *et al.*, 1988). Isolation of BSA after preparative SDS-PAGE by gel elution was conducted according to Barnikol-Watanabe *et al.* (1994).

Planar Phospholipid Bilayers Experiments

Set-up and generation of membranes are described elsewhere (Benz *et al.*, 1978). In short, a Teflon chamber containing two compartments, each filled with 8 ml of electrolyte solution (1 M KCl, 10 mM HEPES, pH 6), was used. Membranes made of 1% diphytanoyl phosphatidylcholine (DPPC) in *n*-decane were generated across the hole (diameter 1 mm) in the dividing septum. Porin 31HL or 31HM preincubated with cholesterol were added to both compartments in the denoted amounts. Insertion processes were monitored at a holding potential of 10 mV, applied over a pair of Ag/AgCl electrodes. Voltage dependence was determined after insertion of at least 100 porin molecules by following the time course of the amplified transmembrane current resulting from successive applied increasing voltages. Subsequently, aliquots of the probes and fractions to be tested were added to both compartments in the same experiment. After the destruction and following reformation of the membrane the voltage dependence was determined again under the conditions described above. The relative conductance (G/G_0) was calculated from the voltage-insensitive current at 10 mV (G_0) and the resulting current 9 sec after the application of a given voltage (G). Using this protocol an interaction between porin and regulating molecules can readily take place prior to membrane insertion.

RESULTS

Human type-1 porin was pretreated with cholesterol and inserted into membranes made of diphytanoyl phosphatidylcholine (DPPC). This had the effect of enhancing the channel activity and additionally diminished the voltage sensitivity of porin. Therefore, this experimental design was well suited to monitor regulatory influences of the added biological probes.

Influence of Cytosol on the Voltage Dependence of Human Type-1 Porin

Addition of cytosol of H2LCL cells to human type-1 porin led to a significant, dose-dependent increase of the voltage dependence of the pore. As shown in Fig. 1, the relative conductance (G/G_0) of porin at 80 mV dropped by 70% compared to the control in the presence of 1.25 μg/ml cytosol protein (final concentration). Significant effects (26% reduction) were observed with as little as 12.5 ng/ml cytosol protein. The effect of increased voltage sensitivity appeared at voltages above 20 mV, agreeing with the

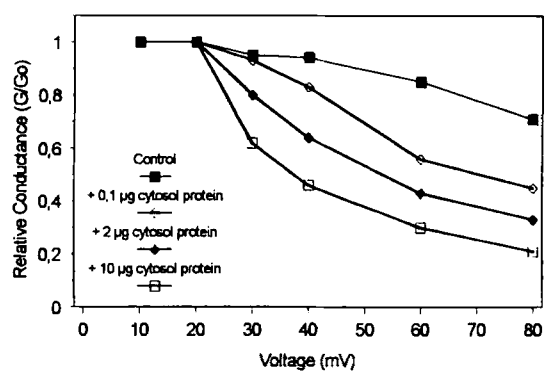

Fig. 1. Dose-dependent decrease of the relative conductance of human type-1 porin in the presence of human H2LCL cytosol. In each experiment 5 ng Porin 31HL and the given amounts of cytosol protein were added per compartment containing 8 ml of 1 M KCl, 10 mM HEPES, pH 6. The membranes were made of 1% diphytanoyl phosphatidylcholine (DPPC) in *n*-decane. Values for G/G_0 were calculated as described in Materials and Methods.

finding that the single-channel conductance was not affected at 10 mV (data not shown).

The inhibitory potency of cytosol proved to be quite resistant to various physical and chemical treatments. As displayed in Fig. 2, boiling and subsequent extraction with chloroform led only to a minor decrease of the regulatory activity of cytosol. Compared to the untreated cytosol, a loss of 18% inhibition at 80 mV was observed when equal volumes of the different probes were tested. It should also be noted that the above-mentioned treatment reduced the protein content of the cytosol by a factor of 4. The additional chloroform extraction did not alter the activity of previously boiled cytosol at all, so lipophilic molecules could be excluded as candidates for inhibitors. Preincubation of cytosol with 8 M urea exerted absolutely no effect on the inhibitory activity (data not shown). Since the sample is diluted 68:1 after addition to the Teflon chamber, refolding and reactivation of the inhibitory factors might be responsible for this finding. In contrast, the reducing agent mercaptoethanol (ME) diminished the activity of cytosol (Fig. 3). Preincubation for 30 min with 5 and 10% (v/v) ME reduced the inhibition at 80 mV compared to untreated cytosol about 25 and 44%, respectively. Complete inactivation of cytosol could not be achieved by increasing the amount of ME nor by prolonging incubation times. The voltage dependence of porin alone was not affected by similar concentrations of ME.

That protein component of the cytosol was responsible for the inhibitory activity was shown by digestion with bacterial protease VIII. As depicted in Fig. 4, addition of the protease to the chamber containing porin and cytosol led to a time-dependent con-

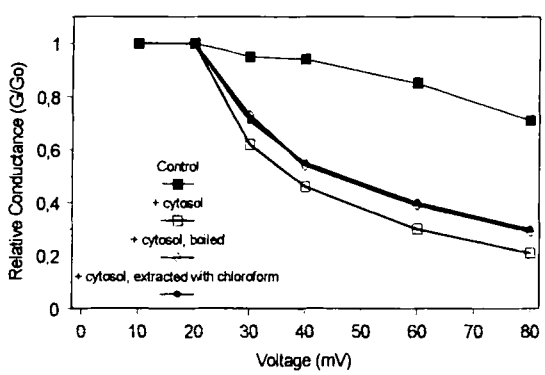

Fig. 2. Heat and chloroform stability of the modulating activity of H2LCL cytosol. Experimental conditions were as in Fig. 1. Cytosol was boiled for 20 min and subsequently extracted with one volume of chloroform. Native and pretreated cytosol were added in identical volumes.

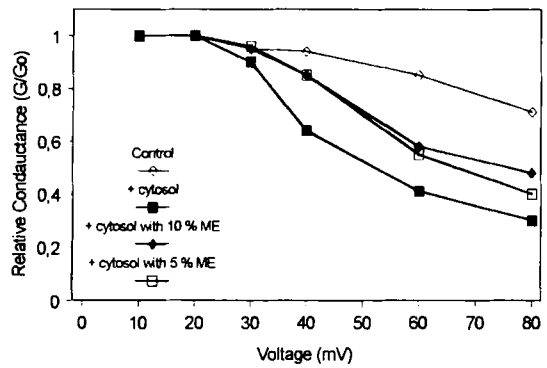

Fig. 3. Decrease of the modulating activity of H2LCL cytosol after treatment with mercaptoethanol (ME). Experimental conditions were as in Fig. 1. Cytosol obtained after boiling and chloroform extraction was incubated with the given amounts of ME for 30 min. The added cytosolic volumes of the samples were identical with native cytosol. Per compartment 8 ng Porin 31HL were added.

vergence of the relative conductances of the control and protease-treated samples. Complete abolition of the inhibitory activity was brought about by preincubation of cytosol with immobilized protease VIII for 4 h.

In order to determine whether the effect of cytosol was dependent on the charge of the applied potential, a different experimental approach was used. Cytosol and porin were added only to one compartment of the Teflon chamber and the cytosol was only allowed to interact with previously inserted porin molecules. Figure 5 shows that inhibitory effects were only observed when the relative negative potential was applied to the cytosol-containing compartment. Cor-

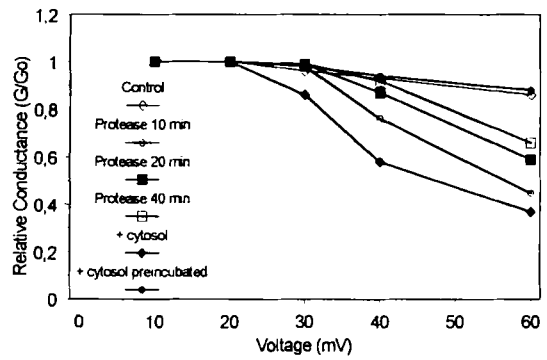

Fig. 4. Abolition of the modulating activity of H2LCL cytosol after treatment with bacterial protease VIII. Experimental conditions were as in Fig. 1. For monitoring the effect of the protease on cytosolic proteins already interacting with porin, 2.84 U of the enzyme were added to each compartment. Predigestion of cytosol was achieved in 4 h by 22 mU of the protease immobilized to agarose. The amount of Porin 31HL present per compartment was 10 ng.

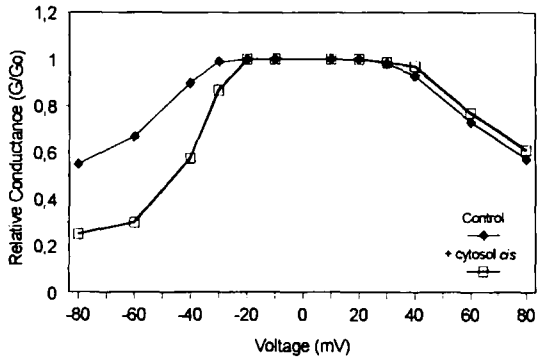

Fig. 5. Influence of the charge of the transmembrane potential on the action of H2LCL cytosol. 20 μg of cytosol protein and 30 ng of Porin 31HL were added to the same (*cis*) compartment and the opposite (*trans*) compartment was held at virtual ground. Membrane lipid and electrolyte solution were as described in Fig. 1.

responding results were obtained with cytosol added *trans*, i.e., in the chamber opposite porin addition (data not shown).

The voltage dependence of human type-1 porin was not modulated only by cytosol of the same species. As depicted in Fig. 6, the analogously prepared fraction of porcine kidney cortex exerted similar effects on the human channel, indicating the conservation of the molecular properties of the regulatory proteins in mammals. Again, the inhibitory activity was almost completely retained after boiling. To achieve similar levels of inhibition, four times the amount of protein compared to human cytosol had to be added. However, it should be taken into consideration that the assay system used is rather restricted in its ability to quantitate the changes in voltage dependence. The differences in the amount of protein required for similar levels of inhibition should therefore be regarded qualitatively.

Influence of Amniotic Fluid on the Voltage Dependence of Human Type-1 Porin

As a second source for molecules acting on human type-1 porin, amniotic fluid was chosen. It should be noted that this fluid, with importance for the functional lung development in the fetus, would interact with porin *in vivo* on the side of the plasma membrane opposite the cytosol. As shown in Fig. 7, the addition of amniotic fluid caused a decrease in relative conductance of porin, similar to the biological probes described above. In accord with the results achieved for cytosol, the inhibitory potency of amniotic fluid was nearly unaffected by boiling but could be removed completely by pretreatment with immobilized protease VIII. This indicates that proteins were also the acting factors in amniotic fluid. Similar levels of inhibition required fivefold excess of protein compared to human H2LCL cytosol.

Fractionation of Cytosol and Amniotic Fluid

After identifying the heat-stable part of the protein component as being the common effector of increasing the voltage dependence of human type-1 porin, attempts were made to enrich and isolate the acting

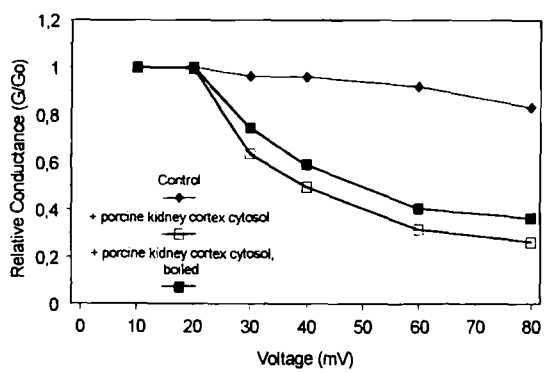

Fig. 6. Decrease of the relative conductance of human type-1 porin in the presence of porcine kidney cortex cytosol. Experimental conditions were as in Fig. 1. Native cytosol was added at 42 μg protein per compartment; added cytosol obtained after boiling for 20 min was of identical volume. The amount of Porin 31HM present per compartment was 10 ng.

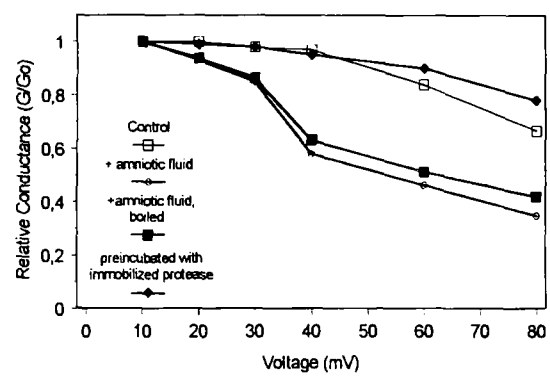

Fig. 7. Influence of amniotic fluid on the relative conductance of human type-1 porin. Experimental conditions were as in Fig. 1. Native amniotic fluid was added at 55 μg protein per compartment; added amniotic fluid obtained after boiling for 20 min was of identical volume. Predigestion of amniotic fluid was achieved in 4 h by 22 mU of bacterial protease VIII immobilized to agarose. The amount of Porin 31HL present per compartment was 9 ng.

molecules. Methods utilized were ion exchange chromatography, gel filtration, and isoelectric focusing.

Fractionation of cytosol from H2LCL cells and porcine kidney cortex by gel filtration revealed inhibitory activity by proteins with an apparent molecular mass of 23–64 kDa and 28–59 kDa, respectively. Pretreatment of cytosol by boiling, in order to enrich the acting factors, was not feasible because this resulted in their aggregation and elution in the void volume of the column. Another approach was the separation of H2LCL cytosol by preparative isoelectric focusing. Inhibitory activity was determined in the pH range from 4.3 to 5.6, showing the anionic nature of the responsible proteins. Nevertheless, the achieved separation was low, so that this method was not suitable for the first purification step. The best resolution of the inhibitory proteins was obtained by rechromatography of the active fractions after gel filtration on the anion exchanger Mono Q. As depicted in Fig. 8, inhibitory activity was narrowed down to a discrete fraction separate from the vast majority of the eluted probe components. However, analysis of this fraction by SDS-PAGE revealed that it still contained several different proteins. Assignment of the inhibitory activity to one of the visible bands is not yet possible.

Amniotic fluid contains a less complex mixture of proteins compared to cytosol and therefore seemed to be a promising starting point for the isolation of the inhibitory proteins. Gel filtration revealed an apparent molecular mass of 23–64 kDa, identical with the data obtained for H2LCL cytosol. On the other hand, the pI range of the acting proteins with pH 3.3–4.2 was shifted to significantly more acidic values, as determined by isoelectric focusing. The best resolution was obtained using anion exchange chromatography as the first separation step, leading to a single active fraction showing only a few protein bands, as depicted in Fig. 9. Subsequent gel filtration of corresponding fractions revealed, however, that the visible proteins were obviously not the ones responsible for the inhibitory effect on human type-1 porin. Therefore the acting proteins are very likely to be expressed only at very low levels, at least in the case of amniotic fluid, and thus were not yet detectable by SDS-PAGE.

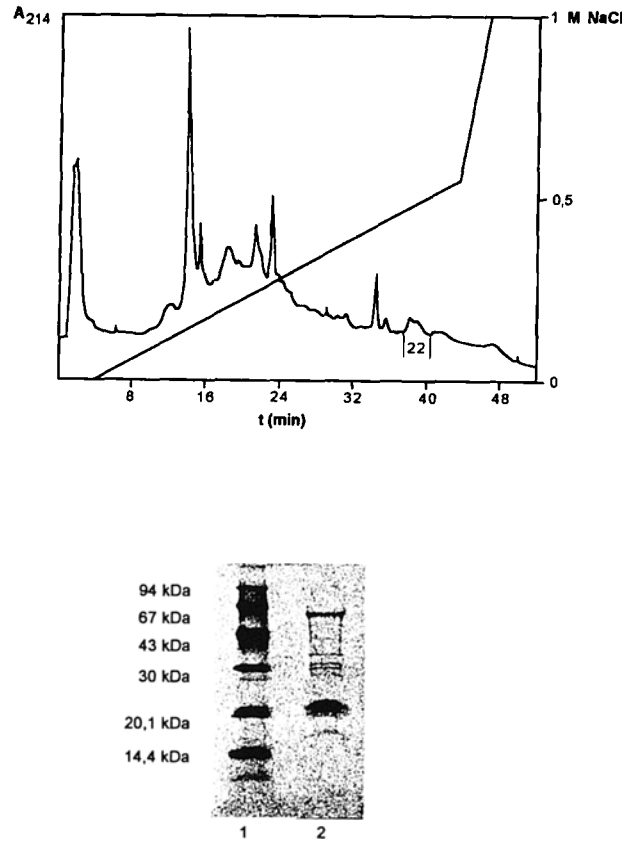

Fig. 8. Chromatography of the inhibitory fractions obtained after gel filtration on the anion exchanger Mono Q. The sample was loaded on the column at a flow rate of 1 ml/min in 10 mM Tris, pH 8. Elution of the proteins was performed by a salt gradient (0–1 M NaCl). The inhibitory fraction 22 was analyzed by SDS-PAGE (12.5%) and subsequent staining with colloidal Coomassie brilliant blue. Lane 1: Low molecular weight markers, lane 2: fraction 22.

Effects of Human Cerebral Spinal Fluid and Anionic Proteins on Human Type-1 Porin

Of the body fluids tested only human Cerebral Spinal Fluid was without significant effect on the electrophysiological properties of human type-1 porin. As shown in Fig. 10, addition of the sevenfold amount of protein compared with H2LCL cytosol nearly left the voltage dependence of porin unaffected. This finding indicates that the inhibitory effects of cytosol and amniotic fluid are due to the presence of specific proteins in these probes.

In a series of control experiments it was investigated whether anionic charge, the common property of the acting proteins of cytosol and amniotic fluid, was the sole basic necessity for inhibitory protein activity. However, neither glucose oxidase with pI 4.2 (final concentration 2.38 μg/ml) nor polyglutamic acid (M_{av} 17,5 kDa; final concentration 1.88 μg/ml) exerted significant effects on human porin (data not shown). Obviously, additional molecular parameters seem to play

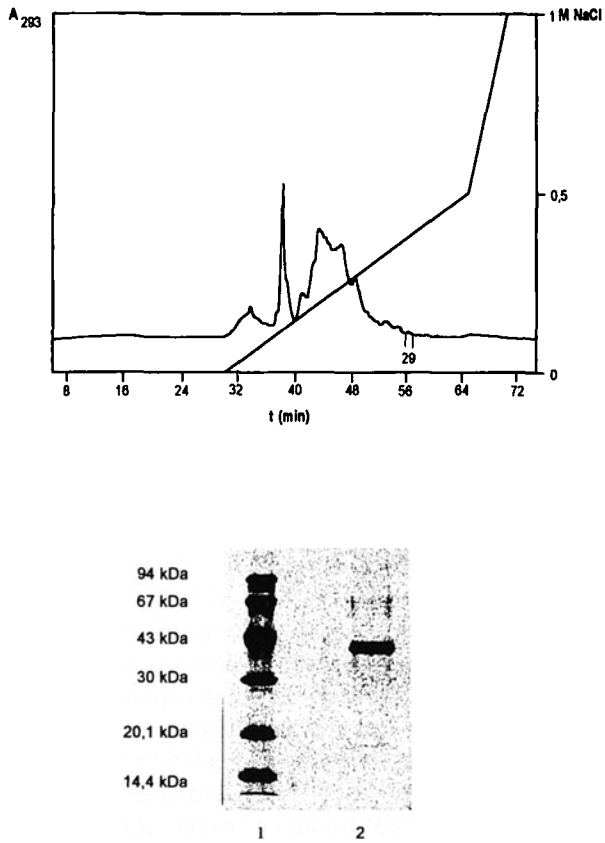

Fig. 9. Fractionation of amniotic fluid by anion exchange chromatography on Mono Q. The sample was loaded at a flow rate of 1 ml/min in 20 mM Tris, pH 7.5. Elution of the proteins and analysis of fraction 29 were as described in Fig. 8. Lane 1: Low molecular weight markers, lane 2: fraction 29.

the key role in the case of the inhibitory proteins in the examined biological probes.

An interesting result was the finding that inhibitory activity could be induced in an ineffective protein. Figure 11 shows that BSA alone did not affect the voltage dependence of human porin. After treatment with SDS the protein clearly caused a decrease in relative conductance. The level of inhibitory activity was dependent on the procedure by which SDS was transferred. Simple boiling of BSA in the presence of 2% SDS was far less efficient than submitting BSA to preparative SDS-PAGE and subsequent gel elution. Therefore, it would seem reasonable to assume that the absence of specific molecular properties in BSA, necessary for inhibitory activity, can be overcome by transferring negative charge in nonphysiological high density.

DISCUSSION

The increase in voltage dependence of porin reconstituted in planar phospholipid bilayers effected by cytosol and amniotic fluid shares a variety of common properties with data obtained with dextran sulfate, König's polyanion, and the mitochondrial VDAC modulator. The prerequisite met by the known regulators and the proteins described in this paper is the anionic charge (Mangan and Colombini, 1987; Colombini *et al.*, 1987; Liu *et al.*, 1994). As revealed by control experiments using different polypeptides and proteins, this molecular property in itself is not

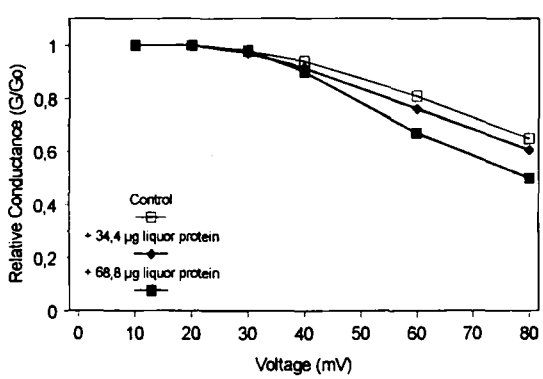

Fig. 10. Influence of human cerebral spinal fluid on the relative conductance of human type-1 porin. Experimental conditions were as in Fig. 1. The indicated amounts of liquor protein were added to each compartment. Porin 31HL present per compartment was 9 ng.

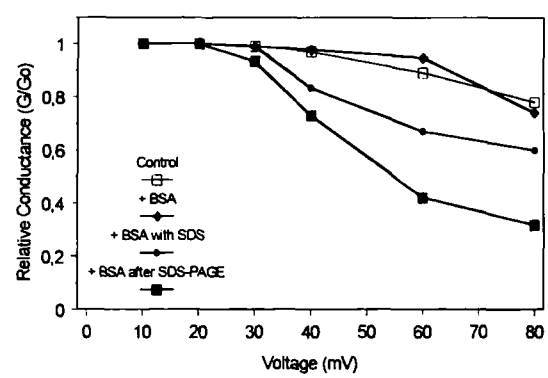

Fig. 11. Influence of BSA on the relative conductance of human type-1 porin. Experimental conditions were as in Fig. 1. BSA was added in 200 µg amounts per compartment in its untreated form or after boiling for 5 min in the presence of 2% SDS. The added amount of BSA obtained after preparative SDS-PAGE and subsequent gel elution was 80 µg. In each compartment 8 ng Porin 31HL were present.

sufficient for physiologically occurring factors to exert an inhibitory effect on eukaryotic porin when given in comparable amounts. Therefore additional specific features have to be present in the regulatory proteins described here. If the lack of a deactivating effect of urea holds true, it can be assumed that sequence rather than conformational information are the determinants of specific regulation. Moreover, the efficiency of both human and porcine cytosol points towards a conservation of these specific molecular determinants at least in mammals. Similar results were presented for the mitochondrial VDAC modulator, expanding conservation to plants and fungi (Liu and Colombini, 1991). The last common property of all previously described regulators and the proteins of cytosol and amniotic fluid is the finding that they exerted an inhibitory effect only when added to the side of the membrane with applied negative potential, irrespective of addition in *cis* and *trans* to porin (Mangan and Colombini, 1987; Colombini *et al.*, 1987; Holden and Colombini, 1988). Obviously, portions of porin necessary for interaction with these regulators are accessible from both sides of the membrane in planar phospholipid bilayers. The delocalized net positive charges serving as the voltage sensors in the porin molecule (Thomas *et al.*, 1993) can be considered as candidates for these interactive regions (Liu and Colombini, 1992a).

Besides the similarities discussed above, substantial differences between the VDAC modulator on the one hand and the regulatory proteins of cytosol and amniotic fluid on the other hand have to be stated. The most striking difference is the observed stability of the inhibitory activity of cytosol and amniotic fluid against various physical and chemical treatments. In contrast, the VDAC modulator demonstrated significant deactivation throughout the isolation procedure (Liu *et al.*, 1994). This discrepancy might be due to differences in the experimental design used by Colombini and coworkers compared to those used in this study. The influence of the VDAC modulator was monitored mainly by single-channel analysis and by interaction of the protein fraction with porin molecules which had already inserted into the bilayer (Holden and Colombini, 1988; Liu and Colombini, 1991). In contrast, we focused on multi-channel experiments and allowed interaction of porin and regulatory molecules prior to membrane insertion. It can be assumed that the overall property of the proteins to increase the voltage dependence of porin is rather stable under the experimental conditions described in this paper. However, the ability to interact with porin inserted into the membrane seems to be very sensitive and difficult to maintain over prolonged times of investigation. Supporting evidence for this assumption was acquired in our laboratory, as boiled or otherwise pretreated cytosol often failed to influence the voltage dependence of porin already integrated into the membrane. After destroying and reforming of the membrane in the same experiment, the inhibitory effect was observed again. Reducing agents caused opposite effects on the different regulatory proteins: the activity of cytosol was diminished by pretreatment with mercaptoethanol (ME), the VDAC modulator was stabilized by addition of 1 mM DTT (Liu *et al.*, 1994). Reasons for this finding are not clear at the moment, but dosage effects can be envisaged since ME was added in significantly higher amounts than DTT.

The data obtained by the isolation procedures described in this paper are not sufficient to answer the question whether the active proteins are identical in the mitochondrial intermembrane space, the cytosol, and amniotic fluid. The determined range of the apparent molecular weight using gel filtration shows strong similarities for cytosol and amniotic fluid, but is in contrast to the value of 100 kDa provided for the VDAC modulator (Liu *et al.*, 1994). However, the results of isoelectric focusing are in favor of a stronger relationship between cytosol and the VDAC modulator, as the active proteins of amniotic fluid showed significantly more acidic pI values (Liu *et al.*, 1994). The existence of special proteins or isoforms in each compartment would be in agreement with a separate and distinct regulation of porin residing in the different intracellular membranes and is the most probable explanation for the observed differences between the VDAC modulator and the data presented here at the moment. In addition, it should be taken into consideration that even in one compartment there may be different regulatory proteins present. Data pointing toward such a situation were presented by Liu *et al.* (1994), who showed that two separate fractions obtained after isoelectric focusing increased the voltage dependence of porin to a different extent. Ammonium sulfate fractionation of cytosol did not precipitate the inhibitory proteins in a narrow concentration range of this salt (data not shown), also indicating the probability of the existence of different regulatory molecules in each cellular compartment. However, it cannot be ruled out that the different inhibitory fractions

obtained may be due to proteolytic damage of a single regulatory protein or, as discussed for the VDAC modulator by Liu *et al.* (1994), varying extents of secondary protein modification like phosphorylation. A final answer to the question as to whether there are different regulators present awaits the isolation and identification of the acting proteins in cytosol, amniotic fluid, and the mitochondrial intermembrane space.

The data presented in this paper were obtained with isolated and reconstituted human type-1 porin using the *in vitro* system of planar phospholipid bilayers. Whether the observed increase in voltage dependence is of importance under physiological conditions remains to be elucidated. Given the similarities in charge and, presumably, the regulatory mechanism between the VDAC modulator and the inhibitory proteins described here, a finding of Liu and Colombini (1992b) strongly votes in favor of a relevance of this regulatory effect *in vivo*. As shown by these authors, the activity of ADP-stimulated respiration and of the adenylate kinase is reduced after addition of the modulator to intact mitochondria. The achieved reduction of pore size induced by the VDAC modulator seems sufficient in the case of porin residing in the mitochondrial outer membrane, since basic requirements for the regulation of adenine nucleotide transport are met under these conditions. In regard to porin expression in the plasma membrane, verified by various authors (e.g., Thinnes *et al.*, 1989; Cole *et al.*, 1992; Puchelle *et al.*, 1993; Lisanti *et al.*, 1994), the situation is obviously far more complex. In contrast to the outer mitochondrial membrane, potential differences necessary to induce the increase of voltage dependence exerted by the regulatory proteins are present across the plasma membrane. However, the data presented in this paper clearly show that a complete closure of porin was not achieved with regulatory proteins and physiological membrane potentials. A nonconductive state of the plasmalemmal porin under resting conditions is a prerequisite for maintaining nondispensable life functions like membrane energization. Therefore additional, to date unknown molecules might play a key role in the complete down regulation of the channel diameter. On the other hand, the observed *in vitro* effects of the proteins might have a different outcome under experimental conditions closer to the *in vivo* situation. Evidence in support of this hypothesis was recently provided by Dermietzel *et al.* (1994) by patch clamp studies. They showed that a large-conductance chloride channel (LCCC) could be completely blocked by addition of the well-characterized monoclonal anti-Porin 31HL antibodies (Babel *et al.*, 1991; Winkelbach *et al.*, 1994), indicating that porin is at least part of this widely distributed chloride channel. A common feature of the LCCCs is the so-called tonic inhibition: the channels are rarely observed in cell-attached patches, but occur after a short delay when the patches are excised or the membrane is permeabilized (Sadoshima *et al.*, 1989; Light *et al.*, 1990; Hurnak and Zachar, 1993). It is assumed that the LCCC is kept closed by a regulatory cytosolic factor under nonstimulated conditions, which is lost due to the treatments described above, thus allowing the fully open state of the channel (Bajnath *et al.*, 1993; Sun *et al.*, 1993; Hardy and Valverde, 1994). Data about the nature of this cytosolic factor have not yet been presented, but it can be hypothesized that the inhibitory proteins of porcine and human cytosol are potential candidates for it. Furthermore, there are several arguments that a plasmalemma-integrated channel complex, comprising VDAC and its modulator(s), in patch clamp experiments may figure as a maxi-, midi-, and even mini-chloride channel which we recently summarized (Reymann *et al.*, 1995).

In conclusion, the data presented in this paper clearly show that proteins interacting in a regulatory manner with eukaryotic porin are present in different compartments of the cell and body fluids. They share various homologies in their molecular properties and their way of action. Regarding the special situation of porin expressed in the plasma membrane, the presented data give first clues for the biochemical regulation of this channel. Further studies are necessary to isolate these regulatory proteins and to investigate their influence on porin under *in vivo* conditions.

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