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Halide permeation through three types of epithelial anion channels after reconstitution into giant liposomes

M. Duszyk, D. Liu, A. S. French, S. F. P. Man

Departments of Medicine and Physiology, University of Alberta, Edmonton, Alberta T6G 2C2, Canada

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Abstract. Anion-selective channels from apical membranes of cultured CFPAC-1 cells were isolated and incorporated into giant liposomes for patch clamp recording. Liposomes were formed from L-α-lecithin by a dehydration-hydration method. Ion channels were characterized using the excised inside-out patch clamp configuration. The most commonly observed anion channels were similar to those observed in native epithelial tissues. The linear 20 pS Cl^- channel had the halide permeability sequence $Cl^- > I^- \geq Br^- > F^-$, and showed anomalous mole-fraction behavior in solutions containing different proportions of Cl^- and F^- ions. The autwardly rectifying Cl^- channel had the halide permeability sequence $I^- > Br^- > Cl^- > F^-$, and also showed anomalous mole-fraction behavior, indicating that both these channels probably contain multi-ion pores. The third, voltage-dependent anion channel showed at least five different substrates, had a conductance of 390 pS in the main state, and showed two types of kinetics, fast (openings and closings <1 ms), and slow (openings and closings >1 s). The channel was seen more frequently after reconstitution into giant liposomes than in intact cells. It was not selective amongst the halides, and there was no deviation from a linear dependence of relative current on molar fractions, indicating relatively simple permeation through the pore. Differences in halide permeabilities suggest that different anion channels may be related to different membrane proteins. Comparison with the chloride channel proteins isolated biochemically from epithelial cell membranes is discussed.

Key words: Chloride channel – Epithelium – Ion permeation – Mole fraction – Liposomes

Introduction

Movement of an ion through a channel involves the replacement of ion-medium interactions with ion-channel interactions. As ions permeate a channel pore, they sense the electrostatic forces generated by amino acids that line the pore. These interactions determine the selectivity of the ion channel (Hille 1992), and are often used as a criterion to distinguish between different groups of ion channels. Anion channels are usually characterized by determining their relative permeability to different halides (Halm and Frizzell 1992; Wilk-Blaszczak et al. 1992). Another important feature of an ion channel is the number of ions which can be accommodated in the pore while they enter, cross, and leave the channel. Some insight into this phenomenon may be obtained by studies of mole-fraction dependence from concentration-dependent permeability ratios. In a single-ion channel, conductance or reversal potential should vary linearly with the ratio of mixed ionic concentrations. In a multi-ion channel, conductance often reaches a maximum or minimum as a function of ionic ratio.

This paper presents a biophysical characterization of ion channels from epithelial cells, incorporated into giant liposomes. Using the patch-clamp technique we show that the biochemical procedure for isolation of ion channel proteins preserves ion channel activity. We report the presence of at least four groups of anion channels reconstituted into giant liposomes, some of them seen previously in native tissues. For three groups of these channels, complete halide sequence permeabilities are given, as well as descriptions of their mixed halide mole-fraction behaviors.

Correspondence to: M Duszyk

Materials and methods

CFPAC-1, a pancreatic carcinoma cell line from a patient with cystic fibrosis (Schoumacher et al. 1990), was obtained from the American Type Culture Collection. Cells were plated on plastic tissue culture flasks (150 cm^2 growing area) at 1×10^4 cell/cm^2, in M199 medium (Sigma), supplemented with 5% FBS and antibiotics (gentamycin 50 μg/ml, streptomycin 100 μg/ml, and penicillin-G 60 μg/ml). Cells were cultured at 37% with 5% CO_2 in air, and the medium was changed twice a week. After

reaching confluence (~10 days), cells were washed with ice-cold homogenization medium (HM) consisting of 60 mM mannitol and 10 mM TRIS-HEPES pH = 7.4, and then scraped off the flasks with a cell scraper (Costar) into HM supplemented with protease inhibitors (25 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 175 μg/ml PMSF). The scraped material was homogenized on ice, and the unbroken cells were repelleted (300 g, 8 min), rehomogenized, and combined with the supernatant from the first homogenization. In order to separate apical from basolateral membranes, $MgCl_2$ (10 mM, 1:100 dilution of stock) was added and the suspension was stirred gently for 1 h at 4 °C (Langridge-Smith et al. 1983). Aggregated membranes (basolateral) were then spun down (6,500 g for 12 min) and the membranes remaining in the supernatant were pelleted with a high speed spin (100,000 g, 60 min) to produce a crude apical membrane preparation (fraction A0). A modified method of Neubig et al. (1979) was used to remove peripheral proteins from the apical membrane preparation. Fraction A0 was titrated to pH = 10.8 with 0.1 M NaOH and incubated on ice for 30 min. Following neutralization with HEPES, the fraction was spun at 150,000 g, for 40 min and the pellet produced an apical membrane fraction labelled A1. The enrichment of apical membrane enzyme markers in fraction A1, was assessed by measuring the activity of alkaline phosphatase as described by Murer et al. (1976). Protein concentration was determined using a BCA assay (Bollag and Edelstein 1991).

Fraction A1 was resuspended in ice-cold solubilization buffer (SB) consisting of: 150 mM NaCl, 175 μg/ml PMSF, 1 mM EDTA and 10 mM CHAPS (pH = 7.4), and shaken at 4 °C for 60 min. This material was then spun for 60 min at 43,000 g. The supernatant containing the detergent-solubilized proteins was supplemented with 2 mM egg phosphatidylcholine and stored at 4 °C at a protein concentration of about 1 mg/ml.

Giant liposomes were prepared by a modification of the method of Keller et al. (1988) and Riquelme et al. (1990). Phosphatidylcholine from soybeam (type 2-S, Sigma) was suspended at a concentration 10 mM in dialysing buffer (DB) consisting of (mM): 100 NaCl, 10 HEPES, pH = 7.4. CHAPS, 35 mM in DB, was mixed with the lipids and the purified fraction of proteins in the ratio of 2:5:3, and the mixture was incubated for 1 h on ice, following dialysis against 1000–2000 volumes of DB for 24 h using Spectrapor 3 dialysis tubing (Spectra). After dialysis, the mixture was ultracentrifuged for 60 min at 100,000 g. The pellet was suspended at 4 °C in 75 μl of DB supplemented with 5% (v/v) ethylene glycol. The suspension was then deposited as small drops (~10 μl) on a culture dish, and partially dehydrated for 4–5 h at 4 °C in a desiccator containing anhydrous $CaCl_2$. The samples were rehydrated by adding 10 μl of 50 mM NaCl on top of each dehydrated drop, at 4 °C overnight in Petri dishes (containing wet towel paper on the bottom). The resulting giant liposomes were pipetted off the rehydrated drops, spun at 300 g for 8 min, and the pellet was mixed with 200 ml of a DEAE-Sephadex A-50 (3 mg/ml, in DB), and incubated for 20 min at room temperature. This treatment anchored the liposomes to the gel beads and to the bottom of the dish, making it easier to get a patch clamp seal (Riquelme et al. 1990).

Single channel recordings were obtained using the patch clamp technique as described by Hamill et al. (1981). Patch pipettes were made from borosilicate microfilament glass using a two stage puller. The pipette tips were coated with Sylgard (Dow Corning) to reduce tip capacitance, and fire-polished. Pipette resistance was about 12 MΩ. Currents were detected with a List EPC-7 patch clamp amplifier and recorded on video tape. Single-channel analysis was based on the procedures described by Colquhoun and Sigworth (1985) to produce open and closed time distributions, channel amplitude and total open probability. Channel conductance and ionic selectivity was determined by fitting the current-voltage relationships with the Goldman-Hodgkin-Katz equation (Hille 1992). Reversal potentials were determined from current-voltage relationships for different anions, and the relative ion permeabilities were calculated from the following equation:

$$V_r = \frac{RT}{F} \times \ln \frac{[Cl^-]_o + (P_A/P_{Cl})[A^-]_o}{[Cl^-]_i + (P_A/P_{Cl})[A^-]_i}$$

where subsripts 'o' and 'i' denote the outside and inside solutions, respectively, P_A, is the permeability of an anion A^-, and R, T, and F are the gas constant, temperature and Faraday constant respectively.

Pipette offset potentials were measured and corrected before forming a seal. Changes in junction potential at the reference electrode due to changing halide solutions in the bath were calculated from the Henderson equation (McInnes 1939). The potential displayed by the patch-clamp amplifier was corrected for the liquid junction potential to obtain the potential across the patch (Neher 1992). Exchange solutions were provided by a multiple pathway flow system with very small dead space to minimize exchange times. All potentials are reported relative to zero in the extracellular (pipette) solution, and positive currents are outwards throughout.

Results

Cells used in this study were from six consecutive passages, starting at passage 24. Usually, cell membrane proteins were prepared from 50 flasks, giving about 260 mg of total proteins, or 0.035 mg/cm^2. Assays for the apical membrane marker alkaline phosphatase were undertaken to characterize the efficacy of the apical membrane preparation. On average, an 8 fold increase in specific activity of alkaline phosphatase was measured in the apical fraction, in comparison with the activity in the basolateral fraction. Further increase in the relative amount of integral proteins in the preparation was achieved by washing the apical fraction with the alkaline buffer. This treatment was previously shown to remove peripheral proteins (Neubig et al. 1979). After this high pH buffer treatment, alkaline phosphatase activity was below detection level, with 20% extraction of the total proteins. These proteins were subsequently used to prepare giant liposomes for patch clamp experiments.

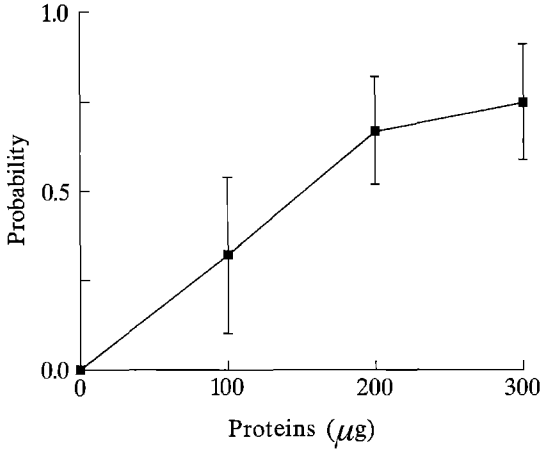

Fig. 1. The probability of finding an ion channel in a patch as a function of the amount of protein used to prepare the giant liposomes. The data present mean ± standard deviations from: 0 μg, 4 preparations ($n=24$); 100 μg, 7 preparations ($n=119$); 200 μg, 5 preparations ($n=42$); and 300 μg, 4 preparations ($n=28$)

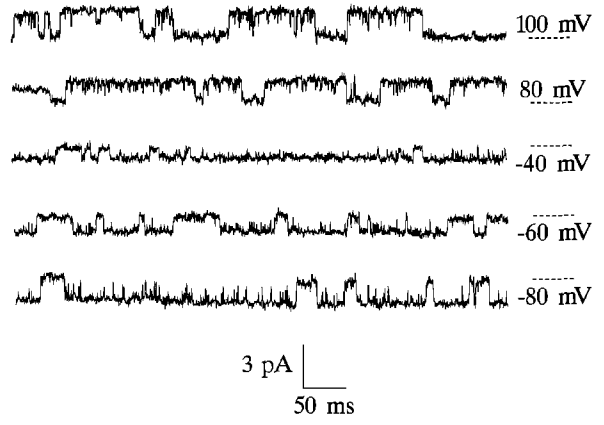

Fig. 2. Recordings of an anion channel in symmetric 140 mM Cl^- solutions. The channel conductance was 18.4 pS. The data were filtered at 800 Hz, and the closed condition is indicated by a dashed line

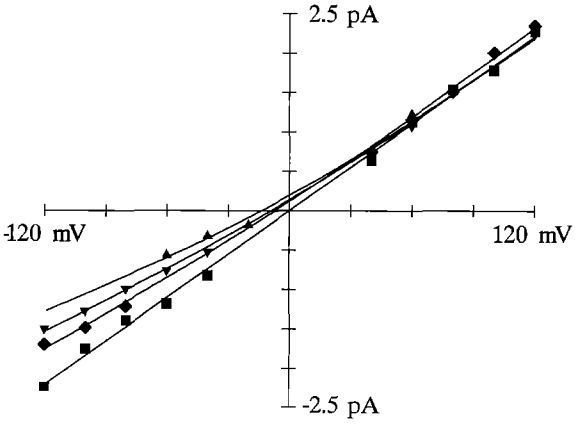

Fig. 3. Current-voltage relationships for a 20 pS anion channel with different halides in the bathing solution. Halide concentrations were (mM): 140 Cl^- (*squares*), 140 I^- (*diamonds*), 140 F^- (*upright triangles*), 140 Br^- (*inverted triangles*). The pipette solution contained 140 mM Cl^-. All potentials were measured in the pipette, relative to zero in the bath

Liposomes selected for recordings had diameters in the range 20–30 μm. Gigaohm seals between liposomes and the patch clamp electrodes were formed in about 90% of all trials, with little or no suction applied. Control experiments were performed with liposomes made from asolectin vesicles, but in the absence of apical membranes. Using a voltage range of ±120 mV no channel activity was detected in either lipsome-attached or excised, inside-out patches. The probability of finding active ion channels increased with the amount of protein used to prepare liposomes, and is shown in Fig. 1. Often, especially at higher protein concentrations, patches contained more than one active ion channel.

Biophysical properties were measured for three types of anion channels, the linear 20 pS chloride channel, the outwardly rectifying chloride channel, and the large (~390 pS) ion channel. Recordings of a chloride channel reconstituted into a giant liposome are shown in Fig. 2. The channel in this figure had a conductance of 18.4 pS, showed no rectification in symmetrical Cl^- solutions, and was similar to the 20 pS Cl^- channel described previously in different native epithelial tissues (Shoemaker et al. 1986; Duszyk et al. 1990). The current-voltage relationships obtained with different anions in the bathing solution are shown in Fig. 3. Membrane potential in this and other figures is reported relative to zero in the pipette. This convention has been adopted to present data in a similar manner to data from excised patches from native cells. Additionally, studies of protein orientations after reconstitution into liposomes indicated that from 75% to 95% of all proteins preserved their original orientations in cell membranes (Gennis 1989). The solid lines in Fig. 3 represent the best fits of the GHK equation to the experimental data. The resulting permeability ratios calculated from the reversal potentials were: Cl^- 1.0 ($n=9$), I^- 0.95±0.08 ($n=3$), Br^- 0.71±0.05 ($n=4$), F^- 0.59±0.05 ($n=6$). Single-tailed Student's *t*-tests applied to this data indicated that each pair of mean values in the sequence were significantly different ($P<0.05$) except for the difference $I^- > Br^-$.

Recordings of the outwardly rectifying chloride channel are shown in Fig. 4. This channel had a cord conductance at negative voltages of 48.2 pS and at positive voltages of 78.5 pS. The halide permeability of this channel was different to that of 20 pS anion channel. Figure 5 shows current-voltage relationships for this channel with different halide solutions in the bath. Interpolated values of the reversal potentials, with correction for liquid junction potentials, were used to calculate the following permeability ratios: Cl^- 1.0 ($n=7$), I^- 1.34±0.06 ($n=4$), Br^- 1.18±0.05 ($n=5$), F^- 0.52±0.05 ($n=4$). *T*-tests for these data indicated that the permeability sequence is $I^- \geq Br^- > Cl^- > F^-$ ($P<0.05$).

Recordings of the large, 390 pS ion channel are shown in Figs. 6, 7. The channel was mostly active at the voltages inside ±30 mV, being rarely open at higher voltages. This channel had at least 5 different substates and displayed complicated kinetics with both slow and fast channel openings. Transitions between different substates were often linked to changes in channel activity, e.g. from a slow mode with openings of the order of seconds, to a fast

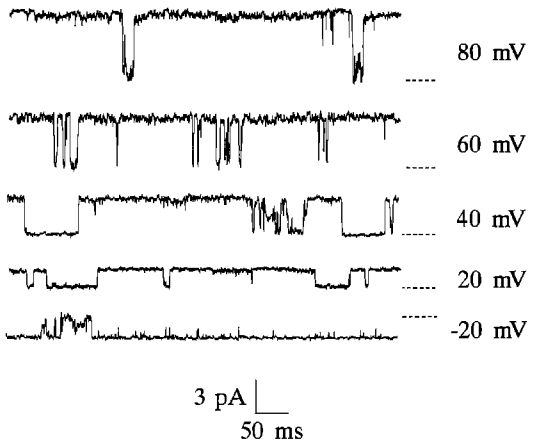

Fig. 4. Recordings of an outwardly rectifying anion channel. The pipette contained 140 mM Cl^- and the bath contained 140 mM Br^-. The data were filtered at 800 Hz, and the closed conditions is indicated by a dashed line

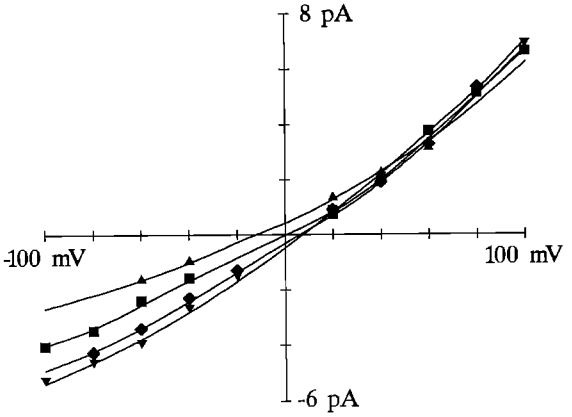

Fig. 5. Current-voltage relationships for an outwardly rectifying anion channel with different halides in the bathing solution. Halide concentrations were (mM): 140 Cl^- (*squares*), 140 Br^- (*diamonds*), 140 F^- (*upright triangles*), 140 I^- (*inverted triangles*). The pipette solution contained 140 mM Cl^-

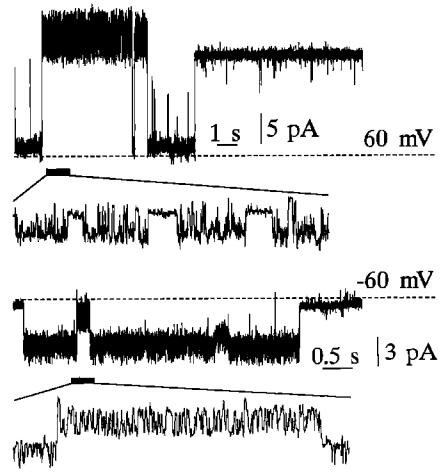

Fig. 6. Recordings of a large (392 pS) anion channel in symmetrical 140 mM Cl^- solutions. The channel revealed many substates and showed complicated kinetics with both slow (of the order of seconds) and fast (~1 ms) modes. The second and the fourth traces show recordings of the first and the third traces respectively (corresponding to the horizontal bars) on an expanded time scale. The data were filtered at 800 Hz, and the closed condition is indicated by a dashed line

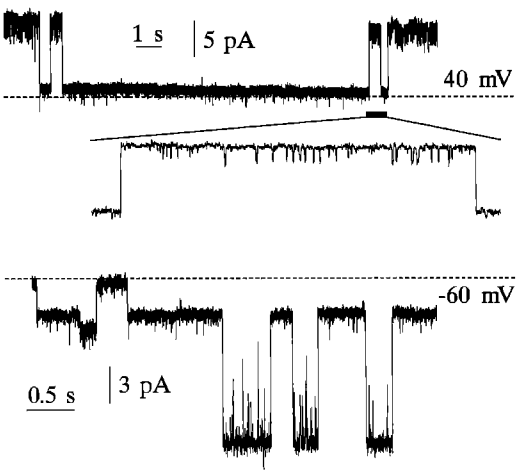

Fig. 7. Recordings of a large (392 pS) anion channel when the bath contained 140 mM Br^-, and the pipette contained 140 mM Cl^- solution. The middle trace shows a 1 s recording of the upper trace (corresponding to the horizontal bar) on an expanded time scale. The substate shown in the expanded scale had a conductance of 52 pS. Note the fast kinetics in this substate. The data were filtered at 800 Hz, and the closed condition is indicated by a dashed line

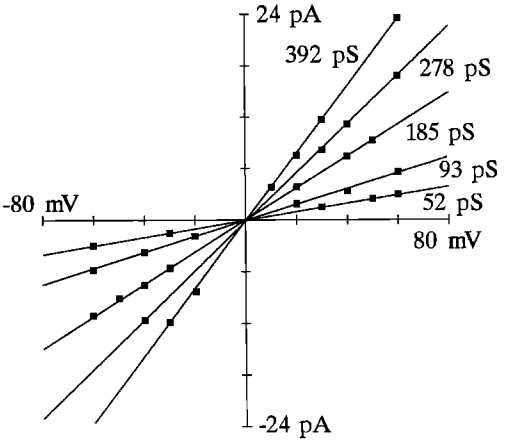

Fig. 8. Current-voltage relationships for the 392 pS ion channel and its major substates. Replacement of Cl^- in the bath with other halides had negligible effect on the channel or its substates conductances, indicating nonselectivity for anions

mode when the mean open time was about 100 μs. Differences in channel conductance when Cl^- in the bath was replaced by a different halide were within the experimental error for Cl^-, indicating that the channel is nonselective for Cl^- over other halides. The current-voltage relationships for this channel and its main substates are shown if Fig. 8. The channel was rarely seen fully open, being most frequently in a substate with conductance of 93 pS.

Variable mole-fraction behaviors were studied using different proportions of chloride and fluoride ions in a total of 140 mM halide. These two ions were used because they had the largest permeability differences for the 20 pS and rectifying channels. Figure 9 shows typical recordings of a frequently seen substate (93 pS) of a large channel in solutions having different concentrations of fluoride. The potential in the pipet was 20 mV in both cases. There was no significant difference in ion channel conduc-

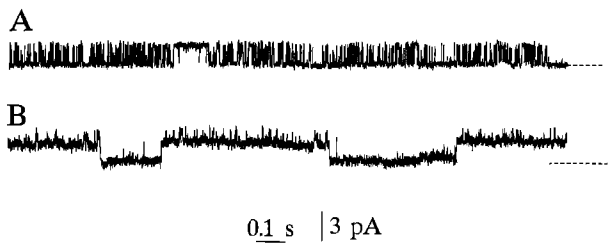

Fig. 9. Recordings of the most frequently seen substate (93 pS) of a large anion channel when the bath solution contained: (A) 70 mM F^- and 70 mM Cl^-, and (B) 140 mM F^-. The pipette contained 140 mM Cl^-. The voltage in the pipette was 20 mV in both cases. The data were filtered at 800 Hz, and the closed condition is indicated by a dashed line

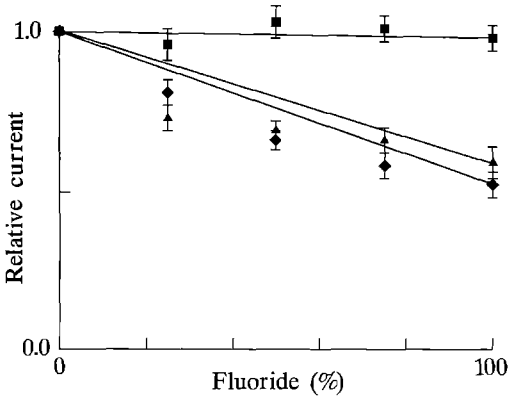

Fig. 10. Ion channel current at a voltage of 20 mV in the pipette, as a function of the mole fraction of fluoride. Squares denote relative current through the major substate of the 392 pS channel, triangles denote the relative current of the 20 pS chloride channels, and diamonds denote the relative current through the outwardly rectifying chloride channel

tance as a function of fluoride concentration. Summaries of the mole-fraction experiments for the 20 pS chloride channel, outwardly rectifying chloride channel, and the 93 pS substate of the large channel are shown in Fig. 10. The solid lines show the predicted linear relationships if the movements of the two ions were completely independent of each other. For the large channel there was no evidence of any deviation from a linear dependence of relative current on molar fractions. However, data for the 20 pS chloride channel and the outwardly rectifying chloride channel show clear deviations from linearity.

Discussion

We have described three different groups of anions channels: the 20 pS non-rectifying anion channel, the outwardly rectifying anion channel, and the large, ~390 pS nonselective voltage-dependent anion channel. The existence of these channels in native epithelia has been well documented (for reviews see Vaughan and French 1989; Anderson et al. 1992). The 20 pS anion channel has been shown to have a weak binding site inside the channel (about 3 kT), and its kinetic behavior could be described by a model having three open and three closed states (Duszyk et al. 1990). Recently, the halide permeability sequence for this channel was described (Wilk-Blaszczak et al. 1992), and anomalous mole-fraction behavior indicated that this channel probably contains a multi-ion pore. The results obtained here are in good agreement with the data from native tissues. After biochemical isolation and incorporation into giant liposomes, the channel preserved its selectivity sequence and showed similar behavior in anomalous mole-fraction experiments.

The outwardly rectifying chloride channel has been characterized recently by Halm and Frizzell (1992). These authors reported the permeability sequence $I^- > Br^- > Cl^- > F^-$, and described the channel as a multi-ion pore in which other permeant anions could affect chloride movement across the membrane. After reconstitution into liposomes, both, the permeability sequence and the variable mole-fraction data were in agreement with the results obtained in native tissues. This channel is different from the Ca^{++} dependent, rectifying anion channel described by Alton et al. (1991). The relative permeabilities of that channel under bi-ionic conditions gave the sequence $I^- > Cl^- = Br^- \gg F^-$, which is different from that reported here.

The presence of a voltage dependent, ~390 pS chloride channel in the apical membranes of epithelial cells has been documented before (Nelson et al. 1984; Hanrahan et al. 1985; Kolb et al. 1985; Schneider et al. 1985). However, the voltage dependence of activation and inactivation is not so well understood (Vaughan and French 1989). One explanation assumes that the channels are inactivated at significantly negative membrane potentials, including the resting membrane potential, activated with depolarization around zero or above, and then inactivated with a time constant of up to several seconds. More experiments will be needed to verify this hypothesis (Vaughan and French 1989). There are different reports about the 390 pS channel anion selectivity. Schneider et al. (1985) using pulmonary alveolar type II cells found its anion permeability sequence to be $I^- > Br^- \geq Cl^-$. Hanrahan et al. (1985) using rabbit urinary bladder epithelial cells found a selectivity sequence of $Cl^- \approx Br^- \approx I^- > F^-$. The permeability sequence described here is different from those given previously. These data suggest that although these channels have similar conductances and voltage dependence, they are not identical in different epithelia, as can be judged from their halide permeability sequences.

The 390 pS chloride channel in native epithelia is rarely open, making a detailed analysis of its properties difficult. For example, in human airway epithelial cells (Duszyk et al. 1989), this channel was found in 8 patches out of 495 studied (1.6%). The situation was different after reconstitution of the ion channel protein into liposomes. In the range of ± 30 mV, the channel was open for more than 50% of the time studied, and showed at least 5 different conductance substates. Additionally, the channel showed two obvious types of kinetics, fast (opening and closing events within less than 1 ms), and slow with the openings in the order of few seconds. Often, fast kinetic events appeared superimposed on the slow kinetics (Figs. 6, 7). The channel was not significantly selective for any of the halides, and did not show any anomalous

mole-fraction behaviour, indicating relatively simple permeation through the pore. The physiological role of this channel as well as its activation mechanisms are unknown. However, owing to its large conductance, one might expect that activation of this channel would have a dramatic effect on intracellular ion fluxes. The kinetic properties of this channel look quite similar to the OmpF and OmpC porin channels found in the membranes of *Escherichia coli* (Berrier et al. 1992). In both cases the channels show two different kinetic modes of openings and closings, and the conductance of the porin channel (200 pS in 0.1 M KCl) is not very different from the conductance of the channels reported here. However, porins form trimers, and essentially three steps, each corresponding to about one third of the trimer conductance have been observed (Berrier et al. 1992). This is clearly different from the data reported here, where at least 5 different conductance levels could be observed.

The reason for increased activation of the 392 pS channel in giant liposomes is unknown. One hypothesis to explain this behavior would be that the apical cell membrane contains inhibitor(s) of this channel which prevent its activation in cell-attached or excised inside-out modes. Another possibility is that biochemical isolation of the ion channel proteins, and especially the detergent treatment of cell membranes, could affect the ion channel protein function by chemical modification. This seems less probable, since CHAPS is considered to be one of the gentler and less denaturating detergents (Bollag and Edelstein 1991) and also because other ion channels were not obviously affected.

The channels described in this paper were not the only ones that were seen after protein reconstitution into liposomes. In some patches, channels with conductances below 10 pS were clearly present. However, these channels were not characterized in detail, because they were often in the same patch with larger channels, and it was difficult to distinguish between a small channel, and a subconductance of the larger one. The small channels will be characterized in separate experiments, in which the liposomes will be prepared with smaller amounts of apical proteins (50 μg or less). In this case it is more difficult to find ion channels, but there are much better chances that the patch will contain a single channel.

Relatively few attempts have been made to purify and to identify chloride channels from epithelial cell membranes. Ran and Benos (1991) identified a 38 kDa protein which was able to transport ^{125}I. Finn et al. (1989) reported inhibition of Cl^- conductance by monoclonal antibodies raised against 219 and 69 kDa proteins isolated from Necturus gallbladder. Breuer (1990) using a radioactive anion transport blocker DIDS, identified proteins of 65 and 31 kDa in renal thick ascending loop of Henle cells. Tsai et al. (1991) showed that the 219 kDa protein when reconstituted into lipid bilayers functioned as a Cl^- channel with a unit conductance of 62.4 pS.

More studies will be necessary to relate particular membrane proteins to observed chloride channels, but the methods of ion channel protein isolation and incorporation into giant liposomes, decribed in this paper, seems to be an appropriate starting point for these studies.

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