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Properties of Single- and Double-Barreled Cl Channels of Shark Rectal Gland in Planar Bilayers

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Summary. Chloride channels from the apical plasma membrane fraction of rectal gland of *Squalus acanthias* were characterized by incorporation into planar bilayers in the presence of cAMP-PK/ATP. In a total of 80 bilayer preparations, 21 Cl-selective channels were observed as single channels and 13 as pairs. This was a significantly greater number of double Cl channels than expected from a binomial distribution. The double Cl channels were divided into two groups based on kinetic and voltage-dependent behavior. One group had properties identical to the single channels (g_{b1}) while the other was consistent with a double-barreled channel (g_{b2}) with coordinated activity between protochannels. The single-channel slope conductances of g_{b1} and g_{b2} from −60 to +20 mV with a 250/70 mM KCl gradient were 41 and 75 pS, respectively. With symmetrical 250 mM KCl, the $I-V$ relation of g_{b1} showed outward rectification with 47.8 ± 6.6 pS at *cis* negative potentials and 68.9 ± 6.1 pS at *cis* positive potentials. g_{b1} was open from 70 to 95% at all electrochemical potentials from −80 to +40 mV. g_{b2} was steeply voltage dependent between −80 and −20 mV. Both g_{b1} and g_{b2} were insensitive to Ca (from 100 nM to 1 μM), blocked by 0.1 mM DIDS and highy selective for chloride. These data suggest that double-barreled Cl channels are related to the family of small, outwardly rectifying Cl channels of epithelial membranes.

Key Words shark rectal gland · apical membrane · planar lipid bilayers · Cl channels · anion selectivity

Introduction

It is now evident that there are several classes of Cl channels from a variety of tissues, including epithelial and excitable cells. While some Cl channels are involved in transcellular salt and fluid transport (Schlatter & Greger, 1985; Landry et al., 1987; Fong et al., 1988; Winters, Reeves & Andreoli, 1990; Dawson, 1991), others have been implicated in bicarbonate secretion (Gray, Greenwell & Argent, 1988), cell volume regulation (Montrose-Rafizadeh & Guggino, 1990; Welling & O'Neil, 1990; Strange, 1991) or maintenance of membrane potential and conductivity (Lukács & Moczydlowski, 1990). Because the latter two functions may be universal for all cell types, it is not unexpected that channels from primitive cell types would have properties in common with epithelial cells of complex organisms.

A double-barreled Cl channel of the noninervated face of the *Torpedo* electric organ was one of the first channels described in bilayer preparations (Miller & White, 1980). There has been recent interest in this channel because the primary structure was recently determined by expression cloning (Jentsch, Steinmeyer & Schwarz, 1990) and a similar channel has been described in the mammalian cortical collecting duct (Sansom, La & Carosi, 1990). The association between the *Torpedo* channel and other Cl channels will be important for understanding the structure function relation of voltage-gated anion channels.

In a recent study from this laboratory, we reported two anion channels from the apical plasma membrane of shark rectal gland in planar bilayers (La et al., 1991). Shark rectal gland (SRG), a model fluid-secreting epithelium, secretes Cl in a manner identical to tracheal cells (Silva et al., 1977; Greger et al., 1984; Moran & Valentich, 1991; Valentich & Forrest, 1991). It was recently shown that SRG contains a protein with 80% homology to CFTR, the defective gene product of cystic fibrosis (CF) (Grzelczak et al., 1990). At least three types of Cl channels have been described in SRG. The smallest channel, approximately 10 to 15 pS, was found in both the native (Gogelein, Schlatter & Greger, 1987) and cultured (La et al., 1991) SRG cell. This channel is the least characterized but does not appear activated by phosphorylating enzymes (Gogelein et al., 1987; La et al., 1991). The second, and most studied channel, g_{b1}, has a single-channel conductance of 40 to 50 pS (Greger, Schlatter & Gogelein, 1987; La et al., 1991). g_{b1}, which is activated by cAMP-PK, was found in planar bilayers (La et al., 1991) and in patch-

clamp experiments of both native (Greger et al., 1987) and cultured SRG cells (La et al., 1991). The third channel, g_{b2}, may be a double-barreled Cl channel. g_{b2} is not regulated by cAMP-PK but has voltage-dependent properties similar to double-barreled Cl channels of the electric organ from *Torpedo californica* (Miller & White, 1980) and the cortical collecting duct (Sansom et al., 1990).

The intent of this study is to fully characterize the properties of g_{bl} in planar bilayers and to determine its relation to the double-barreled Cl channel. Evidence suggests that the double-barreled channel has some distinct properties and may be related to g_{bl} and other outwardly rectifying Cl channels.

Materials and Methods

Planar Bilayer Methods and Solutions

Purified apical membranes from shark rectal glands (1.0–3.0 g) were prepared as described previously (Dubinsky & Monti, 1986; La et al., 1991). SRG were obtained from either Marinus Laboratories (Long Beach, CA) or Marine Biological Laboratories (Woods Hole, MA).

The planar bilayer techniques, described briefly here, in general will follow those of Miller (Miller, 1986). Lipid bilayers were made from 50% mixtures of phosphatydylserine and phosphatidylethanolamine in 20 mg/ml decane. The lipid mixture was spread across a 300 μM diameter hole connecting two chambers (volume = 3.5 ml each), designated *cis* and *trans*. Approximately 20 μl of a 7.5-mg/ml vesicle solution was added to the *cis* side in a gradient of 450 or 250 mM KCl (*cis*) to 70 mM KCl *trans*. Both chambers contained 10 mM 10-N-2 hydroxyethylpiperazine-N′-ethanesulfonic acis (HEPES) buffer and 1 μM $CaCl_2$ (pH = 7.2). Catalytic subunit of cAMP-PK (5 U/ml), 1 mM ATP and 2 mM Mg^{2+} were added to the vesicle preparation for 20 min prior to addition to the bilayer preparation. *Cis* and *trans* solutions were connected via KCl agarose bridges and Ag/AgCl wires to the headstage of a patch amplifier (List, Great Neck, NY) and ground, respectively. Because a previous study showed that Cl channels were inactivated by alkaline phosphatase from only the *cis* chamber (La et al., 1991), the orientation of the channels of this study will be designated as the intracellular side in the *cis* chamber.

Analysis of Data

All channel currents were recorded unfiltered on videotape, filtered at 400 to 800 Hz during playback, and digitized at 2000 to 4000 Hz for analysis using an IBM AT computer and pClamp program set (Axon Instruments, Foster City, CA).

Twenty to thirty seconds of data were analyzed at each command potential. The unitary current (i) was determined as the mean of the best-fit Gaussian distribution of the amplitude histograms. Channels were considered in an open state (S) when the current was $> (n - 1)i$ and $< (n + 1)i$. The probability of a channel existing in an open state (P_o) is defined as the time spent in S divided by the total time of the record. If two single channels or a double-barreled channel existed in a bilayer, P_o was calculated using the following equation, adapted from Light et al. (1988):

$$P_o = (fS_1 + fS_2)/2$$

where fS_1 and fS_2 are the frequency of observing the first and second current levels, respectively.

Differences between groups were tested using the unpaired t test or chi square test as appropriate.

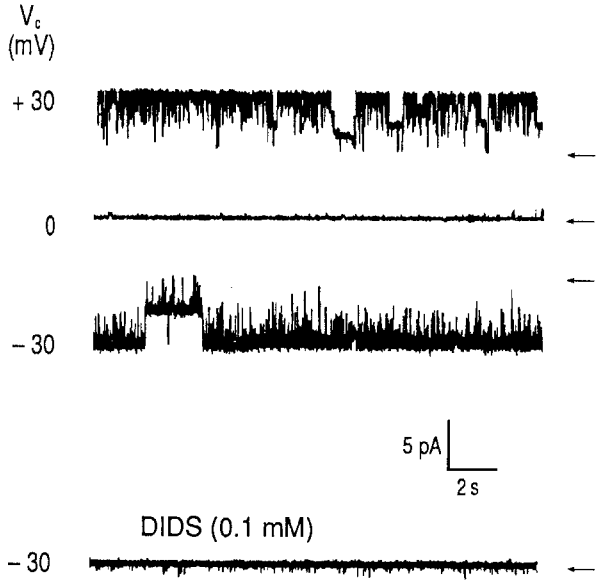

Fig. 1. Tracings of two channels, designated g_{bl}, in a bilayer with symmetric 250 mM KCl solutions. The intracellular side of the channel is oriented in the *cis* chamber. V_c is the command (cell) potential relative to *trans* ground. Outward currents are positive. Two g_{bl} channels were distinguished from double-barreled channels (*see* Fig. 5) by long dwell times (from 0.5 to 5 sec) at the first amplitude level. Both channels closed completely 1 min after addition of 0.1 mM 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS). Arrows indicate where both channels are closed.

Results

Voltage Dependence of g_{bl}

Figure 1 shows tracings of typical g_{bl} channels in symmetrical 250/250 mM KCl solutions. At all potentials g_{bl} was characterized by long closures interrupted by bursts of activity. The average duration of a closed state during a burst was 5 msec. Records long enough to analyze the long closures could not be obtained.

A summary of the I-V relations of g_{bl} in symmetrical 250/250 KCl solutions is shown Fig. 2. In five g_{bl} channels, the single-channel slope conductance was 47.8 ± 6.6 pS at potentials from −20 to −60 mV and 68.9 ± 6.1 pS at potentials from 0 to +40 mV.

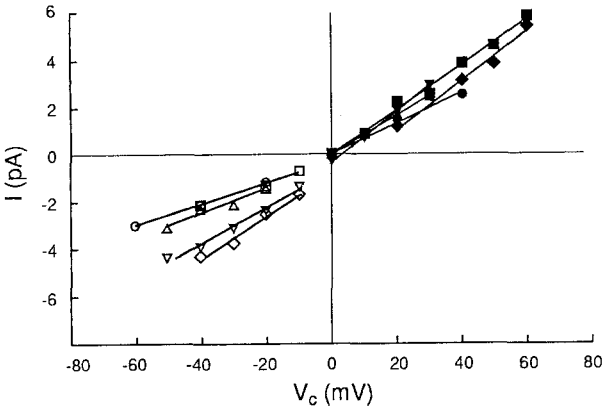

Fig. 2. *I-V* relations of five g_{b1} channels in symmetric 250 mM KCl solutions. Same open and filled symbols designate corresponding current values for a channel in which V_c is <0 and ≥0, respectively. The mean single-channel slope conductance from 0 to +60 mV (68.9 ± 6.1 pS) was significantly higher than the value from −10 to −60 mV (47.8 ± 6.6) using a paired *t* test.

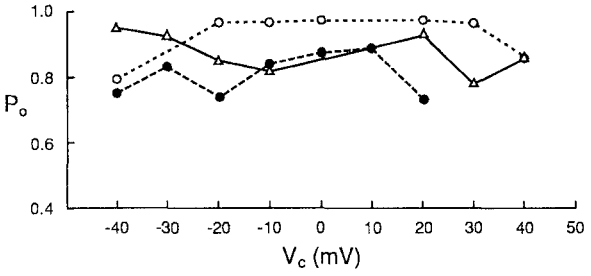

Fig. 3. Plot of open probability *vs.* cell potential for channel of Fig. 1. Filled and open circles are 250/70 and 250/250 mM KCl gradients, respectively and 1 μM Ca. Triangles are with 250/250 mM KCl and 100 nM Ca. The open probability for all electrochemical gradients from −80 to +40 mV was greater than 70%.

Figure 3 shows the voltage-gating relation of g_{b1}. The open probability (P_o) was not significantly different at all potentials between −60 and +30 mV with either 250/70 or 250/250 mM KCl solutions. There was no change in the relation of open probability *vs.* command potential when Ca activity was lowered in the bathing solution from 1 μM to 100 nM. This range was found to have a pronounced affect on Ca-activated K channels of the basolateral membrane vesicles of SRG (Collins, Carosi & Sansom, 1990) and other epithelial cell membranes (Hunter et al., 1986).

Selectivity of g_{b1}

Figure 4*A-D* shows the *I-V* relations for g_{b1} with a 450/70 mM (*cis/trans*) KCl gradient, before and after adding KCl, KNO_3, KBr and KI to the *trans* chamber in order to bring the final K and anionic concentrations equal in both chambers. Each graph represents at least three experiments. The reversal potential did not change when KSCN was added to the *trans* chamber (n = 3, *not shown*). The calculated relative ion permeabilities, using the Goldman-Hodgkin-Katz equation, the extrapolated reversal potentials and the activity coefficients from Robinson and Stokes (1959) were Cl (1.0) > NO_3 (0.75) > Br (0.45) > I (0.08) = SCN = K.

Double-Barreled Cl Channel

From a total of 80 bilayer preparations 21 Cl-selective channels were observed as single channels and 13 were noted as pairs. A chi square test for a normal binomial distribution (Goldstein, 1971) showed that this was a significantly high number of paired channels in a bilayer. Six of these channels, named g_{b2}, had properties distinct from the other seven, which had characteristics consistent with g_{b1}. It was shown in a previous study that g_{b2} was unaffected by phosphorylating or dephosphorylating enzymes. The biophysical properties of g_{b2} will be detailed here.

Figure 5 shows a typical g_{b2} in a bilayer. In contrast to g_{b1}, in which long closures occurred randomly between channels (at S_0 and S_1 levels, *see* Fig. 1), the long closures of g_{b2} typically exist at the S_0 level with both channels closing simultaneously.

Figure 6*A* shows tracings of g_{b2} in symmetrical 250/250 mM KCl solutions. The *I-V* relations for symmetric 250 mM KCl and 250/70 mM KCl are shown in Fig. 6*B* The single-channel conductance was 99 and 77 pS in symmetric and gradient solutions, respectively. Unlike g_{b1}, which showed outward rectification, the *I-V* relation was linear in symmetrical solutions. However, outward rectification may have been undetected because the range of positive potentials was not beyond 30 mV.

The reversal potential shifted 20 mV when NO_3 was added to the *trans* chamber to make anion concentrations equal in both chambers. The calculated relative permeabilities were Cl (1.0) > NO_3 (0.8) > K (0.1).

As shown in Fig. 7, there was a steep relation between the imposed electrochemical gradient and the open probability of each barrel of g_{b2}. When KCl was added to the *trans* chamber to change the KCl gradient from 250/70, the voltage-open probability relation shifted by approximately −20 mV, a value close to the theoretical Nernst Equilibrium potential for a 250/70 mM KCl gradient. These results contrast with those of g_{b1} (*see* Fig. 3) which did not show voltage dependency between −40 and +40 mV.

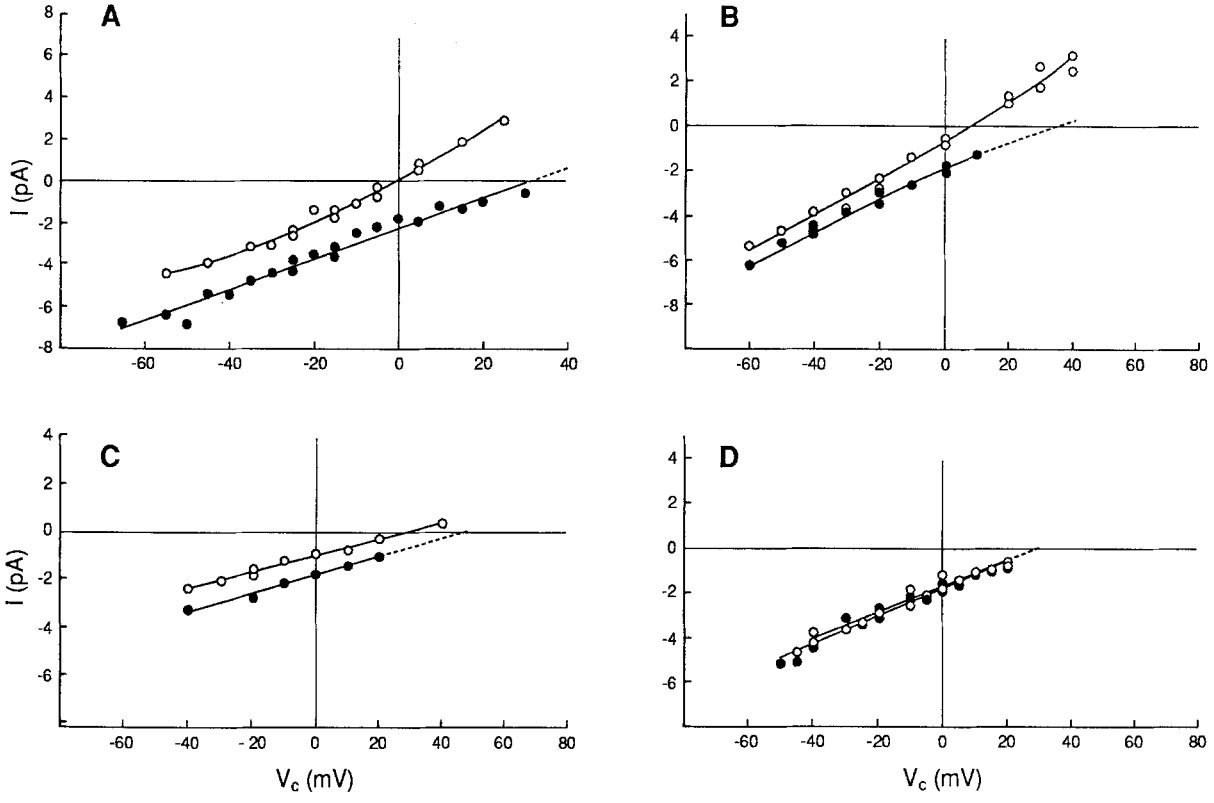

Fig. 4. *I-V* relations of 450/70 (*cis/trans*) mM KCl gradients (filled circles) and after adding (*A*) Cl, (*B*) NO_3, (*C*) Br and (*D*) I to bring the final anionic concentrations equal in both chambers. Each figure includes data from three paired experiments. Each plot was fitted with the best-fit second-degree equation. The anionic permeability was proportional to the shift in reversal potential.

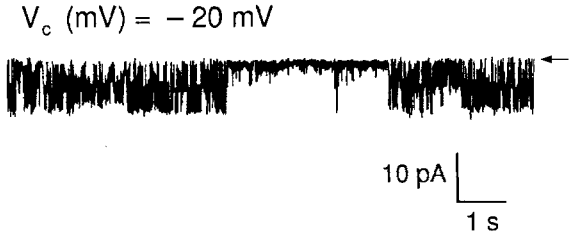

Fig. 5. Tracing of a double-barreled Cl channel, g_{b2}, with a 270/70 mM KCl gradient, showing coordinated closure of both subchannels to a long closed state. In contrast to the single-barreled channels (*see* Fig. 1) g_{b2} randomly fluctuates between three substates: S_1 and S_2 are one and two open subchannels, respectively, and both subchannels closed (S_0, at arrow).

The open probability for g_{b2} was unaffected when Ca was lowered in both chambers from 1 μM to 100 nM by addition of K_2EGTA.

The Table compares the open probabilities of g_{b1} and g_{b2} at various electrochemical gradients. The open probability of g_{b1} was not different at all potential ranges tested. The open probability of g_{b2} was similar to g_{b1} with gradients larger than −20 mV, but was significantly less at potentials less than −20 mV.

Discussion

A recent study from this laboratory showed that two types of Cl channels, g_{b1} and g_{b2}, were present when purified apical membrane of SRG were incorporated in planar bilayers (La et al., 1991). g_{b1} was observed only in the presence of cAMP-PK/ATP and was inactivated by dephosphorylation. g_{b2} was present regardless of the presence of kinase and was unaffected by alkaline phosphatase. The purpose of the present study was to define biophysical properties of g_{b1} and g_{b2} in order to determine the relation between these channels. It was found that, although the voltage dependence and kinetic properties differed, both channels were marked by similar ion selectivities, channel size, and an insensitivity to Ca.

Outward-rectifying Cl channels, first noted in epithelial cells, (Welsh, 1986) were also found in lobster neuron (Lukács & Moczydlowski, 1990).

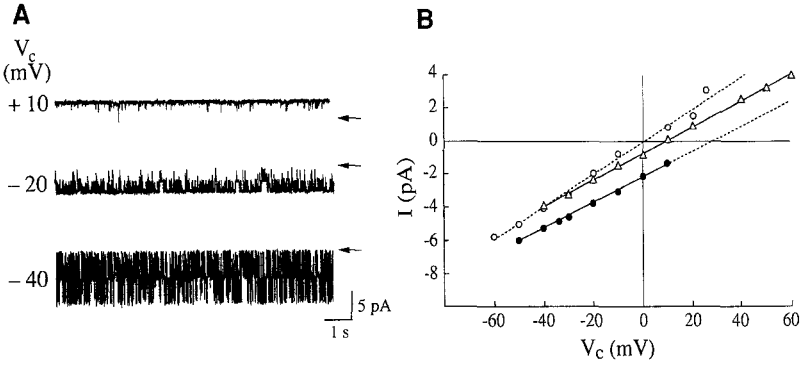

Fig. 6. (*A*) Tracings of g_{b2} in symmetric 250 mM KCl solutions at different command potentials. Arrows indicate where both channels are closed. (*B*) The *I-V* relation in 250/70 mM KCl (filled circles) and 250/250 mM KCl solutions (open circles), and 250/70 mM KCl + 180 mM KNO_3 (triangle). The shifts in reversal potential (32) were consistent with a selectivity of Cl (1.0)/NO_3 (0.8)K (0.1).

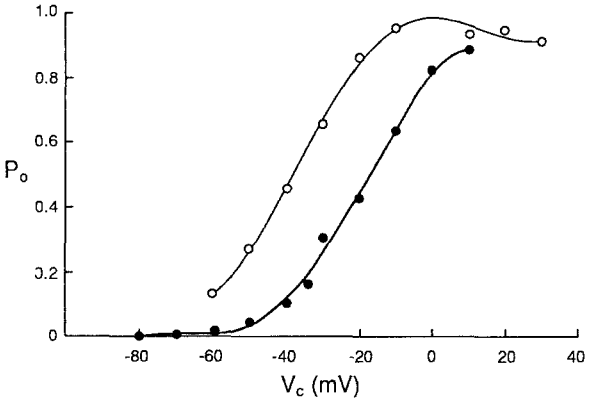

Fig. 7. Plot of open probability *vs.* command potential of the channel shown in Fig. 6*A*. Filled and open circles are with a 250/70 and 250/250 mM KCl gradient, respectively.

The common features of these channels are: (i) a relatively small conductance with higher single-channel conductance at positive cell potentials, (ii) at least two closed states, (iii) high sensitivity to stilbene anion channel blockers (Bridges et al., 1989; Lukács & Moczydlowski, 1990) and (iv) low selectivity for anions (Halm et al., 1988; Bridges et al., 1989; Hanrahan & Tabcharani, 1989). g_{b1} markedly differs from other outward rectifiers in its anionic selectivity sequence. All anions tested in this study were less permeable than Cl, with only nitrate having marked permeability. These results, however, are consistent with the findings of Greger who found that nitrate but not iodide or bromide were permeable to apical Cl channels of native shark rectal gland (Greger et al., 1987).

It is interesting that patch-clamp experiments of either the native or cultured SRG reveal a linear *I-V* relation (Greger et al., 1987; La et al., 1991). Furthermore, Sullivan, Swamy and Field (1991) expressed nonrectifying Cl current when mRNA from SRG was expressed in *Xenopus* oocytes. These results contrast with the present study that showed slight outward rectification of g_{b1} in the planar bilayer. The reasons for this discrepancy are not clear. It may be that either the rectification was too small to detect in the patch- and whole-cell configuration or g_{b1} becomes nonrectifying in the native membrane. It is interesting that a Cl channel of colonic enterocytes had a nonlinear *I-V* relation in planar bilayers (Reinhardt et al., 1987) and a linear relation in the natural membrane (Diener et al., 1989).

Double-Barreled Cl Channels

This study provides the following evidence that g_{b2} is a double-barreled channel and not two separate outward-rectifying Cl channels in the bilayer. First, according to a chi square test of a normal binomial distribution, if 23 single channels are observed, without triplets, then it is predicted that two outward rectifiers would appear in the same bilayer preparation on only seven occasions. There is less than a 5% chance that as many as 13 double-barreled channels would appear in the bilayer. Indeed, in seven bilayers, there were channels consistent with two g_{b1}. Unlike g_{b2}, long closures were noted for each amplitude level. Second, there is slight, if any, voltage-dependent gating of g_{b1}, as compared to the strong voltage dependence of g_{b2}.

Double-barreled Cl channels have been described in both renal epithelial (Sansom et al., 1990) and nonepithelial (*Torpedo* electric organ) cells (Miller & White, 1980). The common properties of these channels are the following: (i) two rapidly and independently gating subchannels, (ii) low permeability to other anions, (iii) an apparent coordinating master gate that controls both channels, (iv) increase in open probability of subchannels with depolarization, (v) a high anionic selectivity, and (vi) a sensitivity to stilbene anion channel blockers.

g_{b2} has all the properties described above for the *Torpedo* channel. However, g_{b2} also has characteristics in common with g_{b1}, including a similar single-

Table.

Channel	Open probability		
	$\Delta V \leq -40$ mV	-40 mV $< \Delta V \leq -20$ mV	$\Delta V > -20$ mV
g_{b1}	0.83 ± 0.02	0.86 ± 0.02	0.88 ± 0.02
(n)	(17)	(21)	(13)
g_{b2}	0.15 ± 0.02*	0.57 ± 0.05*	0.85 ± 0.03
(n)	(14)	(12)	(12)

ΔV is the imposed electrochemical gradient (*trans* ground). *$P < 0.001$ compared with values for g_{b1} using a paired t test.

channel conductance, a high anionic selectivity, a sensitivity to DIDS and insensitivity to Ca. Thus, although g_{b2} has different voltage-dependent properties from g_{b1}, these two channels may be closely related.

It is possible that g_{b2} is another form of g_{b1}. It was shown that lobster Cl channels had two "modes" of activity, a rapid flickering mode that is not voltage dependent, and a slower but voltage-dependent gating mode (Lukács & Moczydlowski, 1990). A tight association of two g_{b1} may result in g_{b2} with differing kinetics and voltage dependence. The transformation from g_{b1} to g_{b2} would have to result in a loss of regulation by kinase.

Postulated Roles of g_{b1} and g_{b2}

Because outward rectifiers have been shown to exist in a variety of cell types it is unlikely that they have a fluid-transporting function. Many studies have shown that cAMP- stimulated Cl current is DPC but not stilbene sensitive (Schoppa et al., 1989; Lencer et al., 1990). In addition, evidence now shows that outward rectifiers are not the channels responsible for the Cl secretion that is defective in cystic fibrosis. Tabcharani et al. (1990) described low conductance, nonrectifying Cl channels in T84 cells that are regulated by cAMP. Other studies (Drumm et al., 1990; Anderson et al., 1991; Kartner et al., 1991) showed that the gene product of CFTR could be expressed as a nonrectifying Cl current.

The most probable function of an outward rectifier would be to stabilize the membrane potential upon depolarization, a role suggested for the *Torpedo* channel (Miller & White, 1980). Strange (1991) has suggested that a volume regulatory role for Cl channels is possible in transporting epithelia, where Na entry must be matched exactly by Na-pump extrusion. A combination of either surplus Na entry or inhibited Na extrusion would result in depolarization and cell swelling. Activated Cl channels would release Cl thereby maintaining volume. A third possible role is to serve as a parallel conductance pathway for anion exchangers (Gray et al., 1988). Finally, it is possible that outward-rectifying Cl channels are part of endocytic vesicles that shuttle in and out of apical plasma membranes. It was recently shown that protein kinase A activates a stilbene-sensitive Cl conductance in endocytic vesicles from proximal tubules (Bae & Verkman, 1990).

The recent cloning of the *Torpedo* Cl channel (Jentsch et al., 1990) may enable studies of the oligomeric state channels. With the advent of novel cloning techniques it is now feasible to determine the molecular relation of single- and double-barreled outward-rectifiying Cl channels.

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