J. Membrane Biol. 118, 269–278 (1990)

The Journal of
Membrane Biology

Cl^- Channels in Basolateral Renal Medullary Membranes: III. Determinants of Single-Channel Activity

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Summary. We evaluated the effects of varying aqueous Cl^- concentrations, and of the arginyl- and lysyl-specific reagent phenylglyoxal (PGO), on the properties of Cl^- channels fused from basolaterally enriched renal medullary vesicles into planar lipid bilayers. The major channel properties studied were the anion selectivity sequence, anionic requirements for channel activity, and the effects of varying Cl^- concentrations and/or PGO on the relation between holding voltage (V_H, mV) and open-time probability (P_o).

Reducing *cis* Cl^- concentrations, in the range 50–320 mM, produced a linear reduction in fractional open time (P_o) with a half-maximal reduction in P_o at *cis* $Cl^- \approx 170$ mM. Channel activity was sustained by equimolar replacement of *cis* Cl^- with F^-, but not with impermeant isethionate. For *trans* solutions, the relation between Cl^- concentration and P_o was negatively cooperative, with 50% reduction in p_o at 10 mM Cl^-. Reducing *cis* Cl^- had no effect on the gating charge (Z) for channel opening, but altered significantly the voltage-independent energy (ΔG) for channel opening.

Phenylglyoxal (PGO) reduced Z and altered ΔG for Cl^- channel activity when added to *cis*, but not *trans* solutions. Furthermore, in the presence of *cis* PGO, reducing the *cis* Cl^- concentration had no effect on Z but altered ΔG. Thus we propose that *cis* PGO and *cis* Cl^- concentrations affect separate sites determining channel activity at the extracellular faces of these Cl^- channels.

Key Words Cl^- channels · bilayers · PGO inhibition · Cl^- dependence · open-time probability

Introduction

The experiments reported in this paper were designed to evaluate certain of the determinants of channel activity in Cl^- channels fused from basolaterally enriched renal medullary vesicles into planar lipid bilayers. Such Cl^- channels, when incorporated into planar lipid bilayers, exhibit linear current-voltage (I/V) relations when *cis* and *trans* solutions are symmetrical: in 320 mM KCl, the unit channel conductance (g_{Cl}, pS) is approximately 90 pS, and the gating charge (Z) required for channel opening is 1.11 [13].

These Cl^- channels exhibit several interesting, and in certain instances unique, features when the aqueous phases are asymmetrical, that is, when the *cis* and *trans* solutions are 320 and 50 mM KCl, respectively [13]. First, the I/V relations are consistent with classical Goldman-Hodgkin-Katz (GHK) rectification. The open-time probability (P_o) is also voltage dependent, so that $\bar{g}_{Cl}$, the time-averaged Cl^- conductance (that is, $P_o\ g_{Cl}$) is more strikingly voltage dependent than predicted from simple GHK relations. Second, P_o is exquisitely sensitive to reductions in *trans* but not *cis* ionized Ca^{2+} concentrations below ≈50 nM; thus, it is likely that the intracellular faces of the Cl^- channels are oriented to the *trans* solutions. Third, and of particular pertinence to the present studies, the Cl^- channels are completely inactivated when Cl^- concentrations in the *cis*, or extracellular, solutions are reduced to 50 mM; in contrast, reducing the *trans*, or intracellular, Cl^- concentrations to 50 mM has little effect on P_o.

The present studies evaluated the effects of two variables, varying aqueous Cl^- concentrations and the arginyl- and lysyl-specific reagent phenylglyoxal (PGO) [8, 9, 16], either individually or in concert, on Cl^- channel activity (P_o) and on the determinants of channel activity. The results show that reducing *cis* Cl^- concentrations, in the range 50–320 mM, produced a linear reduction in P_o. Alternatively, for *trans* solutions, the relation between Cl^- concentration and P_o was negatively cooperative. Finally, reducing *cis* Cl^- had no effect on the gating charge for channel opening, but altered significantly the voltage-independent energy component for channel-opening.

Second, we found that PGO, which inactivates amiloride-sensitive Na^+ channels [5], reduced the gating charge and altered the voltage-independent

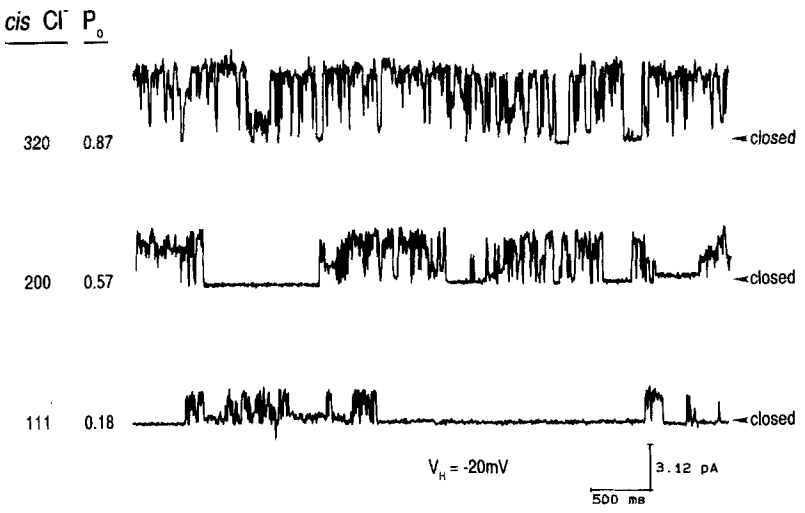

Fig. 1. A representative experiment showing the effect of varying *cis* Cl^- concentrations on P_o in a single Cl^- channel. The value of P_o at each of the indicated *cis* Cl^- concentration is indicated in the figure. The *trans* solution contained 50 mM Cl^-. Note that the differences in current amplitude at the three different *cis* Cl^- concentrations occurred because of variations in E_{Cl}, and consequently in $(V_H - E_{Cl})$, the electrochemical gradient

energy for Cl^- channel activity when added to *cis*, but not *trans* solutions. Furthermore, in the presence of *cis* PGO, reducing the *cis* Cl^- concentration had no effect on gating charge, but altered the voltage-independent energy for channel opening. A preliminary report of some of these findings has appeared elsewhere [15].

Materials and Methods

The procedure for preparing basolaterally enriched vesicles from rabbit renal outer medulla, and the enzymatic characteristics of these vesicles, have been described previously [13]. For the present studies, these vesicles were suspended in 250 mM sucrose and 30 mM histidine (pH 7.4) at a protein concentration of 10–20 mg/ml. The vesicles were used immediately or stored at a temperature of −70°C for up to a week without noticeable changes in the characteristics of the Cl^- channels.

Lipid bilayer membranes were formed by painting a lipid solution over a 0.2–0.3 mm aperture in the wall of a polystyrene Mueller-Rudin cup [11]. The solutions used to form bilayers were 1 : 1 mixtures of phosphatidylserine and phosphatidylethanolamine in decane (20 mg lipid/ml). Formation and thinning of the bilayers were monitored electrically. The aqueous phases (volume ≈ 3 ml) initially contained varying concentrations of KCl, 1 mM $CaCl_2$ and 5 mM HEPES (pH 7.4) solutions in both the *cis* and *trans* chambers. The specific KCl concentrations, as well as varying buffer compositions, are indicated in individual experiments. $CaCl_2$, 1 mM, was uniformly present in all aqueous phases.

Channels were incorporated into lipid bilayers as described previously [13]. Fusion of membrane vesicles added to the *cis* chamber was promoted by an osmotic gradient [4, 13] across the bilayer (*cis* = 320 mM KCl, *trans* = 50 mM KCl). After detection of a Cl^- channel in the bilayer, additional changes in the solutions were made by addition or by perfusing the *cis* chamber with a controlled infusion pump (Gilson Minipulse II). Samples obtained during perfusion were analyzed for Cl^- concentrations electrotitrametrically [12].

The bilayers were voltage clamped using a patch-clamp amplifier (Dagan 8900) connected to the bilayer chambers by Ag-AgCl electrodes in 3 M KCl agar bridges. Voltages were expressed as *cis* with respect to *trans* chamber; the *trans* chamber was grounded. The output of the clamp amplifier was digitized at 44 kHz, recorded on VHS tape (PCM-2, Medical Systems), and simultaneously displayed on an oscilloscope (Hitachi VC6020). Records were replayed, filtered by an 8-pole Bessel filter (Model 902LPF, Frequency Devices), digitized (System 570, Keithley) and analyzed by computer as described previously [13]. Records were filtered at 200 Hz (−3 dB cutoff) and sampled at 2 kHz.

The data in this paper are presented using the following conventions. The bilayer voltages are referenced to the *trans* chamber, which was grounded. Movement of Cl^- from *cis* to *trans* chambers was taken to be a negative current, which appeared as a downward deflection in current traces. All results are expressed as mean values ± SEM for the indicated number of experiments. A single bilayer was taken to be $n = 1$.

Results

Effect of Varying *cis* Cl^- Concentrations

Figure 1 shows the results of paired measurements in a single bilayer membrane where we evaluated the effect of varying *cis* Cl^- concentrations on P_o while holding the *trans* Cl^- concentration constant at 50 mM. Clearly, reducing the *cis* Cl^- concentration produced a progressive decline in P_o. The tracing shown in Fig. 1 also indicates that, as the *cis* Cl^- concentration was reduced, bursts of channel activity were separated by increasingly long periods during which the channel was quiescent.

Figure 2 illustrates the results of paired observations in 11 different bilayer membranes on the relation between P_o and *cis* Cl^- concentrations when the *trans* Cl^- concentration was held constant at 50 mM. The experiments reported in Fig. 2, as well as

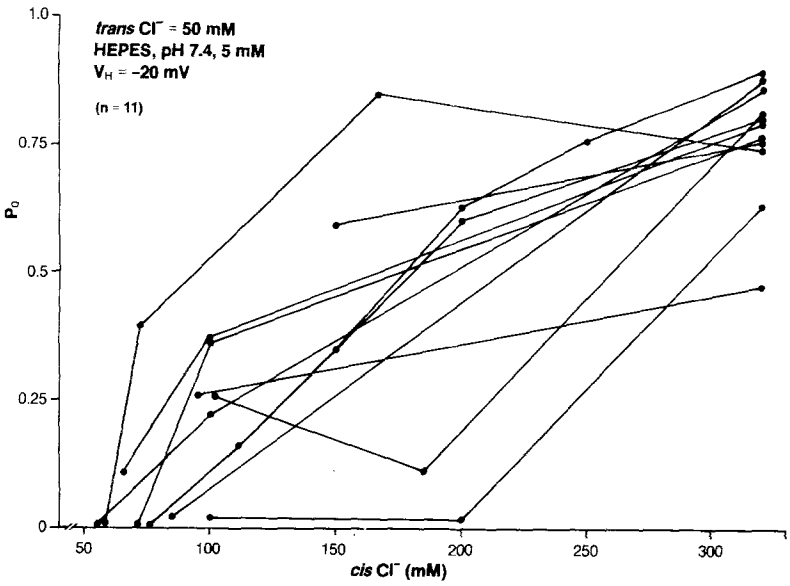

Fig. 2. The relation between P_o and *cis* Cl^- concentrations. The lines connect measurements in individual bilayers (n = 11). The *trans* Cl^- concentration was 50 mM, and both aqueous phases contained 5 mM HEPES, pH 7.4. The holding voltage (V_H, mV) was uniformly −20 mV

all other comparisons, were carried out by paired experimental maneuvers on the same bilayer.

Two observations are especially pertinent. First, in 10 of 11 instances, there was a monotonic decline in P_o as *cis* Cl^- concentrations were reduced; and in all instances, reducing *cis* Cl^- concentrations to ≈70 mM produced virtually complete channel inactivation. Second, the results in Fig. 2 show that there was considerable variation in the values of P_o at a *cis* Cl^- concentration of 320 mM. Thus the P_o data from Fig. 2, at varying *cis* Cl^- concentrations, were expressed as the percentage of the paired P_o values at a *cis* Cl^- concentration of 320 mM (Fig. 3, filled circles). The results were rationalized by a linear fit, with a 50% reduction in P_o occurring at a *cis* Cl^- concentration of ≈175 mM (Fig. 3, solid lines).

It was pertinent to exclude the possibility that the relation between P_o and *cis* Cl^- concentrations was affected by the *trans* Cl^- concentration. Accordingly, we also evaluated, in paired experiments like those shown in Fig. 2, the effect of varying *cis* Cl^- concentrations on P_o when the *trans* Cl^- concentration was held constant at 320 mM (Fig. 3, open circles). The dashed line in Fig. 3 is the linear regression of the latter data. The experimental data shown in Fig. 3, as well as the two regression lines for the two sets of data, show clearly that the relation between varying *cis* Cl^- concentrations and P_o was virtually the same whether the *trans* solution contained 50 or 320 mM KCl.

It has been observed that, in other tissues, both HEPES [18] and SO_4^{2-} [17] can block Cl^- channels. Since the experiments reported in Figs. 1–3 were

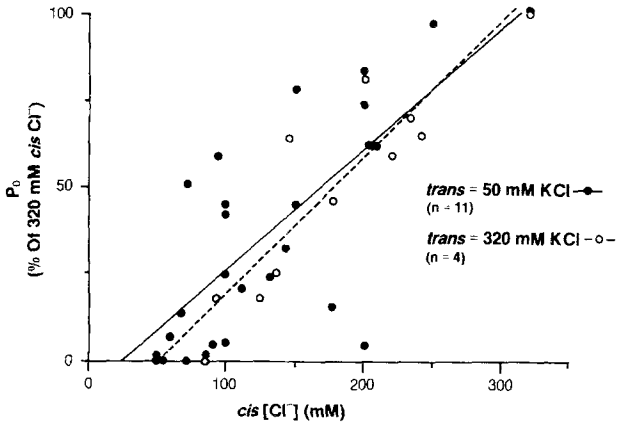

Fig. 3. The filled circles express the data from Fig. 1 as individual P_o values, at a given *cis* Cl^- concentration, as a percentage of control values at a *cis* Cl^- concentration of 320 mM. The solid line is the linear regression for these data (r = 0.84). In the experiments shown as open circles, measurements like those shown in Fig. 1 were carried out when the *trans* Cl^- concentration was 320 mM. The dashed line is the linear regression (r = 0.94) of the latter data. The slopes and intercepts of solid and dashed lines were statistically indistinguishable

carried out with a HEPES buffer, we wished to exclude the possibility that the inactivation of P_o at low *cis* Cl^- concentrations was referable to an unmasking of inhibition of Cl^- channel activity by HEPES. We also wished to evaluate whether SO_4^{2-} might alter inactivation at reduced *cis* Cl^- concentrations. The experiments reported in Table 1 show, in this connection, that reducing *cis* Cl^- concentrations to 70 mM virtually abolished channel activity when the aqueous buffer was either Tris-

Table 1. Effect of varying *cis* Cl^- on P_o using various aqueous buffers

Buffer	P_o	
	cis Cl^- = 320 mM	*cis* Cl^- = 75 mM
Tris-SO_4	85.8 ± 0.4	5.7 ± 4.7
	(n = 4)	
Tris-Cl	84.8 ± 10.4	5.5 ± 3.5
	(n = 3)	

Open probability (P_o) of the chloride channel was determined in the two different indicated buffers (all pH 7.4) with paired meaurements in the same Cl^- channel in the same bilayer under control (320 mM KCl *cis*) and experimental (75 mM KCl *cis*) conditions. All data were obtained at a holding voltage of −20 mV and with 50 mM KCl in the *trans* solution. The results are expressed as mean values ± SEM for the indicated numbers of bilayers.

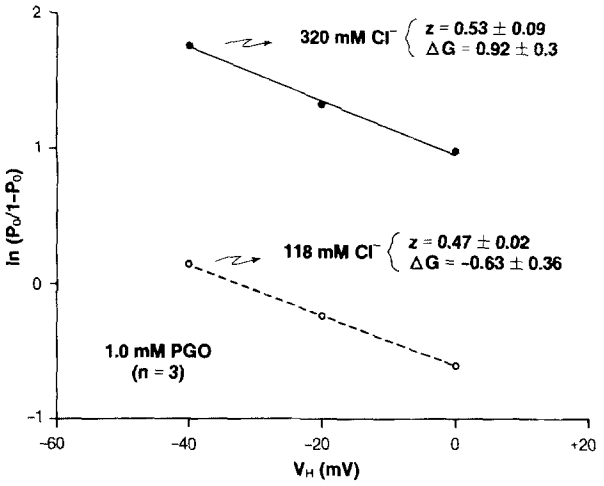

Fig. 4. Paired measurements of the relations between $\ln(P_o/1 - P_o)$ and V_H at the two indicated *cis* Cl^- concentrations. The symbols are the mean values of $\ln(P_o/1 - P_o)$ at the indicated V_H values for the indicated number of bilayers. The lines are the mean regressions for the two different conditions. The indicated values of Z and ΔG were obtained from the slopes and the observed values of $\ln(P_o/1 - P_o)$ at a zero V_H, respectively

SO_4 or Tris-Cl (Table 1), in close accord with the results obtained using HEPES (Figs. 1–3).

It was also pertinent to assess the relation between the holding voltage (V_H, mV), varying *cis* Cl^- concentrations and determinants of channel activity. The Boltzmann distribution for a simple two-state model may be expressed as:

$$\ln(P_o/1 - P_o) = (ZF/RT)\, V_H + \Delta G \qquad (1)$$

where Z is the gating charge; ΔG is the voltage-independent free energy change for channel opening; and F, R and T have their usual meanings. Thus an estimate of Z is provided by the slope of the relation between $\ln(P_o/1 - P_o)$ and V_H; and the value of $\ln(P_o/1 - P_o)$ at a zero V_H provides an index to ΔG. We note in this regard that, at any given Z, the measured value of $\ln(P_o/1 - P_o)$ at zero V_H will be rather sensitive to the control value of P_o. But as noted in Fig. 2, control values of P_o, at a *cis* Cl^- concentration of 320 mM, vary appreciably. As a consequence, ΔG values may also vary appreciably. Thus in our view, an evaluation of the effects of a given maneuver on either Z or ΔG in terms of Eq. (1) requires, for these Cl^- channels, paired observations in the same bilayer.

Accordingly, in order to assess the interactions between Z, ΔG and *cis* Cl^- concentrations, we evaluated the relations between P_o and V_H in paired experiments when the *cis* solution was either: 320 mM KCl, the control condition; or 125 mM KCl, which is, from the data in Fig. 2, a *cis* Cl^- concentration where P_o was ≈0.25 at V_H = −20 mV. At *cis* Cl^- concentrations below 100 mM, the values of P_o at V_H values greater than −20 mV were too small to permit assessment of the relation between P_o and V_H.

The results presented in Fig. 4 show clearly that, under paired experimental conditions, reducing the *cis* Cl^- concentration from 320 to 125 mM had no effect on the gating charge Z, which was approximately 1.4 in both cases. However, the measured values of $\ln(P_o/1 - P_o)$ at a zero V_H were clearly different at the two different *cis* Cl^- concentrations. In short, reducing *cis* Cl^- concentrations from 320 to 125 mM altered the voltage-independent, but not the voltage-dependent, determinants of channel activity. It should also be noted that the Z and ΔG values shown in Fig. 4 for 320 mM *cis* Cl^- agree closely with those reported previously for the same condition [13].

Effect of Varying *trans* Cl^- Concentrations

Figure 5 shows the results of a representative experiment in a single bilayer in which we evaluated the effect of varying the *trans* Cl^- concentration on channel activity (P_o) while the *cis* Cl^- concentration was held constant at 320 mM. The results presented in Fig. 5 show clearly that reducing the *trans* Cl^- concentrations from 320 to 50 mM produced only a modest fall in P_o, while reducing the *trans* Cl^- from 50 to 10 mM produced a considerably greater reduction in P_o. The tracing shown in Fig. 5 also shows that, when the *trans* Cl^- concentration was reduced,

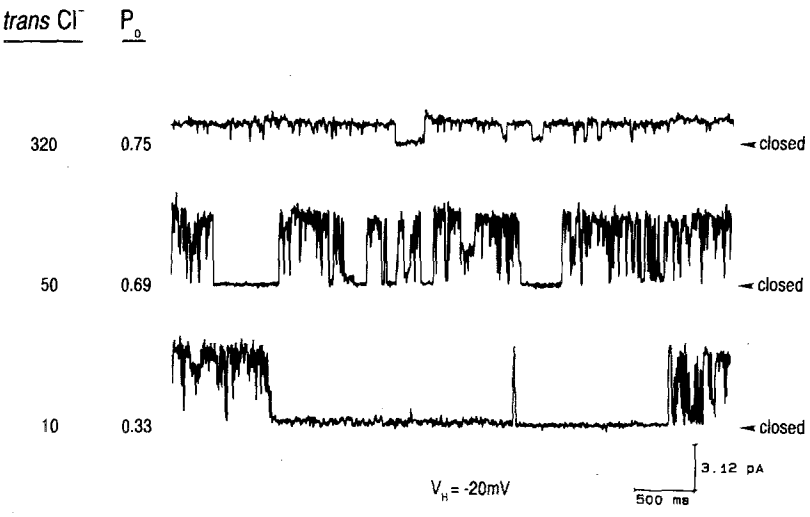

Fig. 5. A representative experiment showing the effect of varying *trans* Cl^- concentrations on P_o in a single Cl^- channel. The value of P_o at each of the indicated *trans* Cl^- concentrations is indicated in the figure. The *cis* solution contained 320 mM KCl

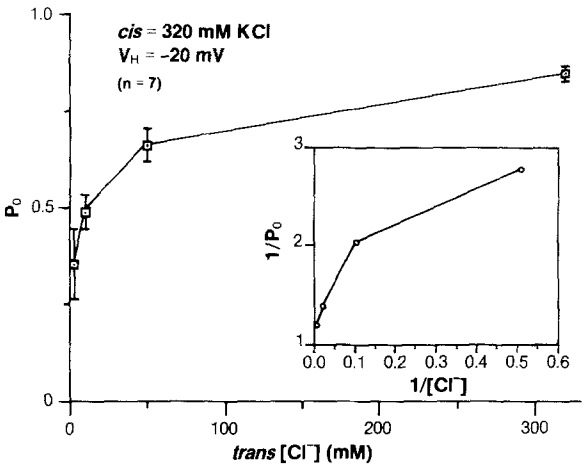

Fig. 6. The relation between P_o and *trans* Cl^- concentrations. Paired measurements of P_o at each of the indicated *trans* Cl^- concentrations were carried out in each of the indicated number of bilayers. The *cis* Cl^- was 320 mM KCl and both aqueous phases contained 5 mM HEPES, pH 7.4. The holding voltage (V_H, mV) was −20 mV. The results are expressed as mean values ± SEM. The lines connecting these values were drawn by eye. The inset contains a Lineweaver-Burk plot for the mean data

bursts of channel activity were separated by increasingly long intervals when the channel was quiescent.

Figure 6 presents the results of paired experiments in a number of bilayers which evaluated the effect of varying *trans* Cl^- concentrations on P_o, while the *cis* Cl^- concentration remained constant at 320 mM. As in earlier studies, V_H was −20 mV, where P_o is approximately 0.8 at a *trans* Cl^- concentration of 320 mM [13]. The results presented in Fig. 6, like those shown in Fig. 5, indicate that reducing the *trans* Cl^- concentration from 320 to 50 mM had only a slight effect on P_o, while subsequent reductions in *trans* Cl^- concentrations produced greater reductions in P_o. Thus the relation between P_o and *trans* Cl^- was nonlinear. P_o was reduced to 50% of control values at a *trans* Cl^- of 10 mM, but appreciable channel activity occurred even at a *trans* Cl^- concentration of 2 mM, where P_o was 0.32.

The inset in Fig. 6 shows a Lineweaver-Burk plot of the mean data presented in Fig. 6. The Lineweaver-Burk plot was not linear over the range of *trans* Cl^- concentrations tested, but rather was convex upward, and thus consistent with negative cooperativity.

It is also pertinent to note in this connection that, in our earlier studies [13], we observed that reducing *trans* Cl^- concentrations from 320 to 50 mM had no effect on the gating charge Z, but altered appreciably the $\ln(P_o/1 - P_o)$ at a zero V_H. Thus varying the *trans* Cl^- concentration appears to affect voltage-independent determinants of channel function, but not gating charge.

Anion Selectivity

To evaluate the anion selectivity sequence for these Cl^- channels, we measured I/V relations and zero-current reversal voltages (V_r, mV) when *cis* solutions contained 320 mM KCl and *trans* solutions contained 50 mM KCl plus 270 mM K^+ salts of the different test anions. The results are shown in Fig. 7 and Table 2.

The data presented in Fig. 7 show clearly, in accord with our earlier findings [13], that the I/V relations were linear for V_H in the range ±40 mV for each of the anions tested. Table 2 shows the zero-current reversal voltages (V_r, mV) for the anions indicated in Fig. 7 with respect to Cl^-, and the per-

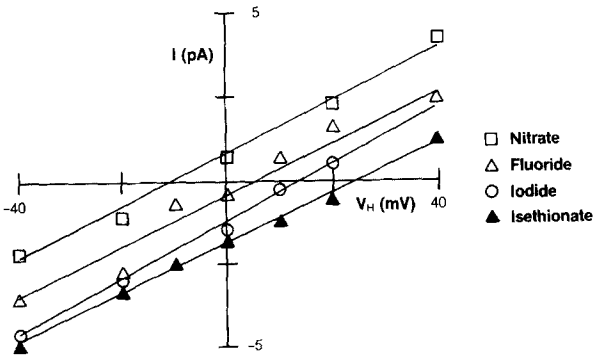

Fig. 7. Evaluation of anion selectivity. Current-voltage (I/V) relations were carried out when the *cis* solutions contained 320 mM KCl and the *trans* solutions contained 50 mM KCl plus 270 mM of the K^+ salt of each of the indicated anions. The results are expressed as mean values for the number of bilayers indicated for each test anion in Table 2

Table 2. Anion selectivity of Cl^- channels estimated from reversal voltages

Anion	V_r (mV)	n	P_x/P_{Cl}
NO_3^-	−10.1 ± 2.8	3	1.65
F^-	4.9 ± 1.2	4	0.78
I^-	13.9 ± 0.7	3	0.43
Isethionate	25.4	2	0.17

The zero-current reversal voltage (V_r, mV) was measured when the *cis* solution contained 320 mM KCl and the *trans* solution contained 50 mM KCl plus 270 mM KX, where X is each of the indicated anions. The results are expressed as mean values ± SEM for the indicated number of bilayers.

meability ratio P_x/P_{Cl}, where x is the test anion, computed from the V_r data.

The anion selectivity sequence estimated from V_r (Table 2) was NO_3>Cl>F>I>isethionate. The NO_3^-/Cl^- selectivity ratio in Table 2 is the opposite of that determined from $^{36}Cl^-$ fluxes in intact rabbit renal medullary vesicles [1] and in porcine outer medullary vesicles [2]. The present data provide no insights into the factors responsible for this difference.

Effect of Permeant or Impermeant *cis* Anions on P_o

Figures 8 and 9 present the results of a series of experiments evaluating the effect on P_o when the *cis* K^+ concentration was 320 mM, and 270 mM of the *cis* Cl^- concentration was replaced either by the relatively impermeant anion isethionate (Fig. 8) or by the relative permeant anion F^-(Fig. 9). The results shown in Fig. 6 indicate clearly, in accord with our earlier findings [13], that the channel inactivation which occurred when the *cis* Cl^- concentration was reduced from 320 to 50 mM was reversed when the *cis* Cl^- concentration was again raised to 320 mM.

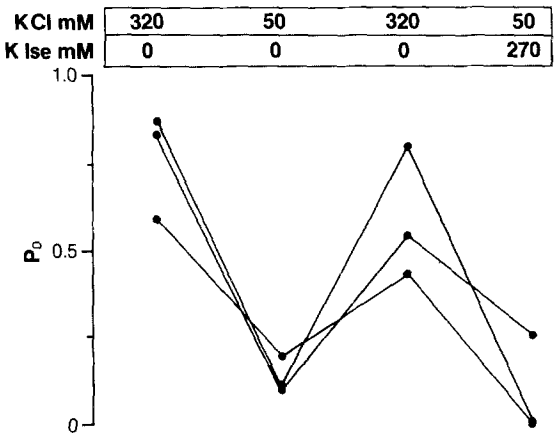

Fig. 8. The effect of varying *cis* KCl and/or K isethionate concentrations on P_o. The *trans* solution was 50 mM KCl; both solutions contain 5 mM HEPES, pH 7.4. The lines connect paired measurements in the same bilayer

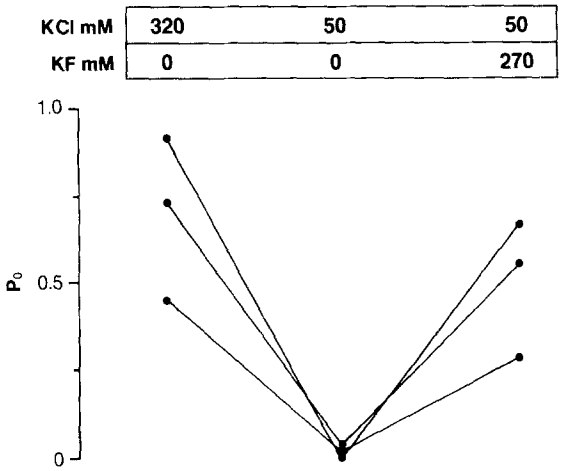

Fig. 9. The effect of varying *cis* KCl and/or KF concentrations on P_o. The *trans* solutions are the same as listed in Fig. 6. The lines connect paired measurements in the same bilayer

The results in Fig. 8 also show that, when 270 mM *cis* Cl^- was replaced by an equimolar concentration of isethionate, a relatively impermeant anion (Table 2), the channels were again inactivated. Thus the inactivation of Cl^- channels which occurred when *cis* Cl^- concentrations were reduced to 50 mM was not referable to variations in ionic strength and/or osmolality, but rather to the reduction in the concentration of the permeant species Cl^-. Finally, the

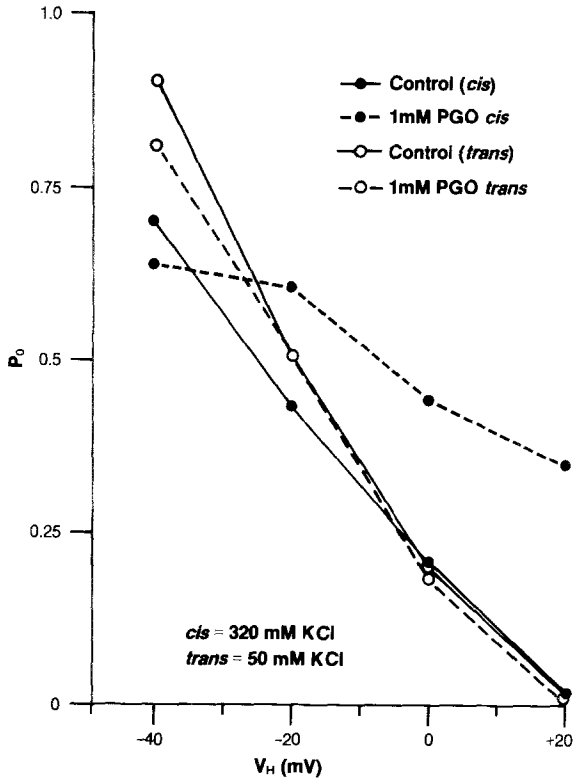

Fig. 10. Paired observations on the effects of 1 mM PGO on the relation between P_o and V_H. PGO was added to either the *cis* (filled symbols) or *trans* (open symbols) solutions. The solid and dashed lines connect control measurements and, in the same bilayer, measurements with PGO, respectively. The *cis* and *trans* solutions contained 320 mM KCl and 50 mM KCl, respectively

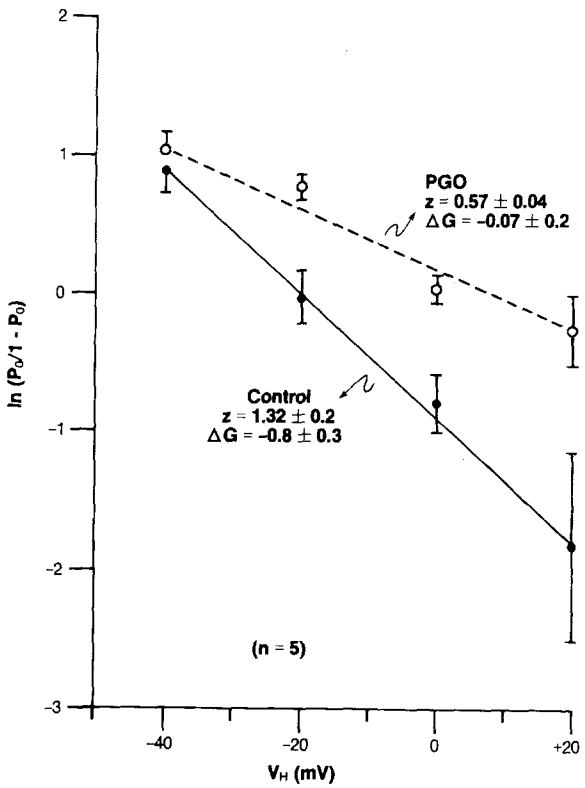

Fig. 11. Effect of 1 mM *cis* PGO on the relation between P_o and V_H expressed according to Eq. (1). The *cis* and *trans* solutions contained 320 mM KCl and 51 mM KCl, respectively. The control (filled symbols) and experimental (*cis* PGO, open symbols) data are paired values (mean ± SEM for the indicated number of bilayers) at each of the indicated values of V_H. The indicated values of Z (mean ± SEM) were computed from the linear slopes according to Eq. (1). The indicated values of ΔG are the experimental values of $\ln(P_o/1 - P_o)$ at a zero V_H

results shown in Fig. 9 indicate that, when F^- replaced Cl^- in *cis* solutions, channels inactivated by Cl^- deletion were reactivated. Thus the data in Figs. 8 and 9 indicate that relatively permeant, but not relatively impermeant, anionic species in *cis* solutions are required for channel activity.

Effect of PGO

The relatively hydrophilic molecule PGO is an arginyl- and lysyl-specific reagent [8, 9, 16] which inactivates Na^+ channels in toad urinary bladder by interacting with a guanido group of arginine presumed to be near the mouth of the channel [5]. Virtually all voltage-sensitive channels studied to date contain arginine-rich domains [3], and it is reasonable to suppose that the anion selectivity of these Cl^- channels might also depend in part on positively charged moieties such as dibasic amino acids. Thus in the present experiments, we evaluated the effects of PGO on channel activity and on the determinants of channel activity.

Figure 10 shows the results of paired experiments evaluating the effect of adding 1 mM PGO to either *cis* (filled symbols) or *trans* (open symbols) solutions on the relation between P_o and V_H. The solid lines and dashed lines connect control measurements and paired measurements with PGO, repectively. The *cis* and *trans* solutions contained 320 and 50 mM KCl, respectively. The results presented in Fig. 10 indicate that *cis* PGO addition caused a significant displacement of the relation between P_o and V_H, while *trans* PGO addition had no effect on this relation. Although the data are not shown in Fig. 10, neither *cis* nor *trans* PGO addition had a significant effect on g_{Cl}, the single-channel Cl^- conductance.

In order to evaluate quantitatively the effects of *cis* PGO addition on the determinants of channel activity, we tested the relation between P_o and V_H, expressed according to a Boltzmann distribution (Eq. (1)), in paired observations in a number of bilayers. The results are presented in Fig. 11. The

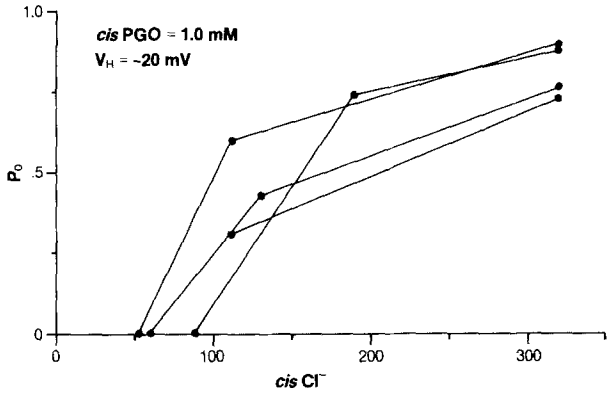

Fig. 12. The effect of varying *cis* Cl^- on P_o when 1 mM PGO was uniformly present in *cis* solution. As in the experiments shown in Fig. 6, isethionate provided an equimolar replacement for Cl^- when the latter was less than 320 mM. The *trans* solution contained 50 mM Cl^-; 5 mM HEPES, pH 7.4, was added to *cis* and *trans* solutions. V_H was −20 mV. Lines connect paired measurements in the same bilayer

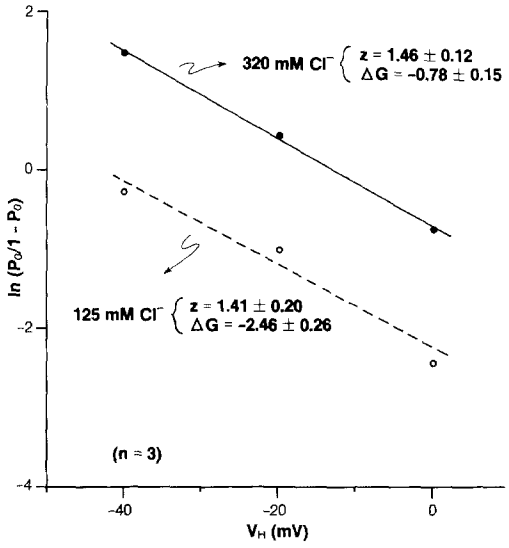

Fig. 13. Paired measurements of the relation between P_o and V_H, expressed in terms of Eq. (1), when *cis* solutions contained either 320 mM Cl^- (filled symbols, solid line) or 118 mM Cl^- (open symbols, dashed line). The *cis* solutions uniformly contained 1 mM PGO. The data are mean values for the indicated number of bilayers. The values of Z and ΔG for the paired conditions were computed as described in Figs. 3 and 9

control values of Z and ΔG, that is, without PGO, are closely comparable to those reported for the same conditions in Fig. 4 and in our earlier studies [13]. The results in Fig. 11 also indicate that *cis* PGO reduced the gating charge Z from 1.32 ± 0.2 to 0.57 ± 0.04. Moreover, the value of $\ln(P_o/1 - P_o)$ at a zero V_H was −0.8 ± 0.3, without PGO, and −0.07 ± 0.2, with PGO. In other words, the results presented in Figs. 10 and 11 show clearly that *cis* but not *trans* PGO altered two determinants of channel activity. That is, *cis* PGO reduced the gating charge for channel opening and altered the voltage-independent energy charge (ΔG, Eq. (1)) for channel opening.

Interaction Between *cis* PGO and Reduced *cis* Cl^-

In Na^+ channels from toad urinary bladder, increasing external Na^+ concentrations provide partial protection against channel inactivation by PGO [5]. Accordingly, we assessed the interactions between varying *cis* Cl^- concentrations, PGO, channel activity and the determinants of channel activity.

The data shown in Fig. 12 are the results of paired experiments in which the *cis* Cl^- concentration was reduced while 1 mM PGO was uniformly present in *cis* solutions. There was equimolar substitution of isethionate for Cl^- (*see* Fig. 8), so that ionic strength and osmolality were both constant. A comparison of the results in Figs. 2 and 12 indicates that the magnitude of Cl^- channel inactivation by reducing *cis* Cl^- concentrations was approximately the same with or without *cis* PGO.

As noted previously, either a variation in *cis* Cl^- concentration (Fig. 4) or *cis* PGO addition (Fig. 11) affects the value of ΔG. Thus we evaluated, in paired experiments, the effects of reducing *cis* Cl^- concentrations on the determinants of channel activity when 1 mM PGO was uniformly present in *cis* solutions (Fig. 13). A comparison of the data in Figs. 11 and 13 indicates that comparable values of Z, approximately 0.5, obtained under the same conditions, that is, with 320 mM *cis* Cl− and 1 mM *cis* PGO. Significantly, and in keeping with the results in Fig. 4, the data in Fig. 13 indicate that, when *cis* PGO was present, reducing *cis* Cl^- from 320 to 118 mM had no significant effect on Z, but altered significantly the value of ΔG.

Discussion

We proposed previously that the *cis*, or extracellular, and *trans*, or intracellular, faces of these Cl^- channels were asymmetric [13]. The results in this paper provide added support for this view. Thus the *cis*, but not the *trans*, faces of the channels contained PGO-sensitive determinants of channel activity. Moreover, a comparison of the results in Figs. 2, 3 and 6 indicates, in accord with our earlier findings [13], that dramatic reductions in P_o with reducing aqueous Cl^- concentrations occurred over a rather lower range of Cl^- concentrations in the *trans* with respect to *cis* solutions.

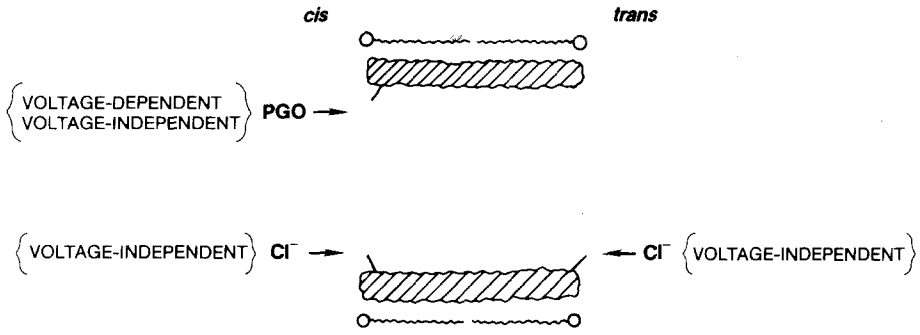

Fig. 14. A tentative model for these Cl^- channels, as discussed in the text

The present experiments permit a limited number of conclusions about the *trans*, or intracellular, faces of these Cl^- channels. The results in Fig. 6 indicate, for the intracellular faces of these Cl^- channels, the steepest variation of P_o with Cl^- occurred with *trans* Cl^- concentrations in the vicinity of 10 mM. This finding may have particular pertinence to the determinants of basolateral Cl^- conductance (g^b_{Cl}, mS cm^{-2}) in intact thick ascending limb (TALH) cells. Thus the measured intracellular Cl^- concentrations in in vitro microperfused rabbit TALH cells are in the range 10–25 mM [6]. Likewise, calculations from intracellular voltage data indicate that, in microperfused mouse medullary TALH, antidiuretic hormone (ADH) raises intracellular Cl^- activity from approximately 16 to 25 mM; *pari passu*, the basolateral membrane Cl^- conductance g^b_{Cl} rises from approximately 65 mS cm^{-2} to 142 mS cm^{-2} [10].

We have proposed [7, 10] that this ADH-dependent rise in g^b_{Cl} is secondary to a rise in intracellular Cl^- activity; and that the latter, in turn, is the consequence of a primary hormone effect on apical membranes [14]. Since $\bar{g}_{Cl}$, the time-averaged conductance of a Cl^- channel, is given by the product P_o g_{Cl} [13], the findings presented in Fig. 6 are consistent with the argument [7, 10] that ADH-dependent increases in intracellular mTALH Cl^- activity may be primarily responsible for significant hormone-dependent increases in g^b_{Cl}.

Our earlier observations [13] indicated that, with a *cis* Cl^- concentration of 320 mM, reducing *trans* Cl^- from 320 to 50 mM yielded Z values of 1.11 and 1.15, respectively, and ΔG values of 1.5 and -0.5, respectively. These values of Z and ΔG at 50 mM *trans* Cl^- correspond closely to values reported in the present studies for the same conditions (Figs. 3 and 9). Thus the *trans* faces of these Cl^- channels may contain Cl^--sensitive site(s), which modulate open-time probability (Fig. 6) by affecting the voltage-insensitive determinants of channel activity, but not gating charge [13].

The present results also permit certain conclusions about the *cis*, or extracellular, faces of these Cl^- channels. The results in Figs. 2 and 3 and Table 1 indicate that reducing *cis* Cl^- concentrations produced a linear decline in P_o which was independent of either the aqueous buffer used or the Cl^- concentration in the *trans* solution. Reducing the *cis* Cl^- concentration also altered the voltage-independent but not the voltage-dependent energy required for channel opening (Fig. 4). Moreover, the maintenance of channel activity, for a given *cis* anion concentration, was appreciably greater with Cl^- and the relatively permeant species F^- than with the relatively impermeant species isethionate (Table 1, Figs. 8 and 9).

Accordingly, it is reasonable to conclude that the *cis* faces of these Cl^- channels also contain anion-sensitive site(s) which modulate P_o (Figs. 2 and 3) by affecting the voltage-independent determinant of channel activity, but not gating charge (Fig. 4). Judging by the results in Figs. 8 and 9, relatively large anions such as isethionate are sterically hindered from interacting with Cl^--sensitive sites.

The present results also indicate that the *cis* but not the *trans* faces of these channels contained PGO-sensitive site(s), presumably arginine or lysine residues [5, 8, 9, 16], which modulate P_o by affecting both the voltage-dependent and voltage-independent determinants of channel activity (Figs. 10 and 11). We infer that the PGO-sensitive sites on the *cis* faces of the Cl^- channels are in a hydrophilic region of the channel mouth, since *trans* PGO addition did not affect the channels (Fig. 10).

Moreover, at least three lines of argument indicate that PGO-sensitive sites on the *cis* faces of these channels may be different from *cis* Cl^--sensitive sites determining channel activity. First, as noted above, *cis* PGO altered voltage-sensitive and voltage-insensitive determinants of channel activity (Fig. 11) while varying *cis* Cl^- concentrations altered ΔG but not gating charge (Fig. 4). Second, *cis* PGO produced no appreciable change in the relation

between *cis* Cl^- concentration and P_o (Figs. 2 and 12). Finally, reducing the *cis* Cl^- concentration affected ΔG but not Z in channels where both ΔG and Z had been modulated by PGO addition (Figs. 11 and 13).

Thus when taken together, the present results are consistent with the model for these Cl^- channels presented in Fig. 14. The *cis* face of the channel is pictured as containing a PGO-sensitive site, possibly an arginine guanido group or a lysine residue [5, 8, 9, 16], which modulates both voltage-dependent and voltage-independent determinants of channel activity (Figs. 10 and 11). It is noteworthy in this connection that both voltage-sensitive K^+ channels and Na^+ channels contain arginine-rich domains which are responsible for voltage sensitivity [3]. The *cis* face of the channel is also pictured as having a separate Cl^--sensitive site from which isethionate is sterically hindered, which modulates voltage-independent determinants of channel activity, but not gating charge (Figs. 2–4, 8, 9, 11, and 13 and Table 2). Moreover, judging by the present (Fig. 6) and prior [13] results, the *trans* face of the channel also contains a Cl^--sensitive site which affects ΔG but not gating charge.

We acknowledge the able technical assistance of Steven D. Chasteen. Clementine M. Whitman provided her customary excellent secretarial assistance. This work was supported by a grant from the National Institutes of Health (5-R01-DK 25540). W.B.R. is a Squibb-American Heart Association Clinician-Scientist.

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Received 6 March 1990; revised 29 May 1990