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Cl^- Channels in Basolateral Renal Medullary Membranes: VII. Characterization of the Intracellular Anion Binding Sites

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Abstract. A unique property of basolateral membrane Cl^- channels from the mTAL is that the Cl^- concentration facing the intracellular aspects of these channels is a determinant of channel open time probability (P_o). The $K_{1/2}$ for maximal activation of P_o by Cl^- facing intracellular domains of these channels is 10 mM Cl^-. The present experiments evaluated the nature of these Cl^--interactive sites. First, we found that the impermeant anion isethionate, when exposed to intracellular Cl^- channel faces, could augment P_o with a $K_{1/2}$ in the range of 10 mM isethionate without affecting conductance (g_{Cl}, pS). Second, pretreatment of the solutions facing the intracellular aspects of the channels with either 1 mM phenylglyoxal (PGO), an arginine-specific reagent, or the lysine/terminal amine reagent trinitrobenzene sulfonic acid (TNBS, 1 mM), prevented the activation of P_o usually seen when the Cl^- concentration of solutions facing intracellular channel domains was raised from 2 to 50 mM. However, when the Cl^- channel activity was increased by first raising the Cl^- concentration bathing intracellular channel faces from 2 to 50 mM, subsequent addition of either PGO or TNBS to solutions bathing intracellular Cl^- channel faces had no effect on P_o. We conclude that the intracellular aspects of these Cl^- channels contain Cl^--interactive loci (termed $[Cl]_i$) which are accessible to impermeant anions in intracellular fluids and which contain arginine- and lysine-rich domains which can be inactivated, at low ambient Cl^- or isethionate concentrations, by interactions with PGO or TNBS.

Key words: Cl^- channels — Cl^--interactive loci — PGO — TNBS — Anion binding sites

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Introduction

This paper provides a partial characterization of the Cl^- binding sites of the intracellular faces of Cl^- channels from basolaterally enriched vesicles from outer rabbit medulla. The latter have virtually identical properties to Cl^- channels obtained from highly enriched basolateral vesicles prepared from 95–97% pure suspensions of mouse medullary thick ascending limbs [18].

These Cl^- channels, when fused into planar lipid bilayers under osmotic gradient conditions, orient such that the extracellular and intracellular channel surfaces face the relatively hypertonic (*cis*) and relatively hypotonic (*trans*) solutions, respectively. Among other observations considered in detail elsewhere [11, 16–18], one result supporting this argument is that channel open time probability (P_o) is quite sensitive to variations in nanomolar Ca^{2+} concentrations in *trans* but not *cis* solutions [11]. Also consistent with this view of channel orientation is the observation that, under appropriate conditions (*see below*), the catalytic subunit of cAMP-dependent protein kinase A (C-PKA) increases P_o when added to *trans* solutions, while in paired experiments on the same Cl^- channels, *cis* C-PKA has no effect on P_o in channels inactivated by exposure to low (50 mM, *see* ref. 18) *cis* Cl^- concentrations. The latter observation also supports the notion that these channels are from basolateral membranes rather than from intracellular organelles [18].

A particularly interesting property of these Cl^- channels is the interplay between two variables, the *trans* Cl^- concentrations bathing intracellular channel faces and C-PKA activity, in modulating P_o. Raising *trans* (intracellular) Cl^- concentrations from 2 to 50 mM approximately doubles P_o; the $K_{1/2}$ for

this effect is 10 mM Cl^- [16]. Moreover, C-PKA augments P_o when *trans* Cl^- concentrations are 2 mM, but has no effect on P_o when *trans* Cl^- concentrations are 50 mM [17]. Now since the calculated [10] and measured [4, 12] intracellular Cl^- concentrations of intact mTAL segments during net NaCl absorption are in the range 15–30 mM, we have proposed that C-PKA activation of Cl^- channel activity may be a biologically redundant mechanism for modulating P_o in basolateral mTAL segments [17].

This argument may be unique to the latter. For example, Cl^- channels fused from apically enriched vesicles from either rabbit jejunum or bovine trachea show no dependence of P_o on *trans* Cl^- concentrations in the range 2–270 mM, while with 270 mM *trans* Cl^- concentrations, C-PKA increases channel number in Cl^- channels from apical jejunal vesicles, and reactivates totally inert Cl^- channels from apical tracheal vesicles [18].

In the present experiments, we evaluated the nature of the intracellular sites for Cl^- modulation of basolateral mTAL Cl^- channel activity. It is reasonable to suppose, on a priori grounds, that these anion interaction sites might be positively charged. Furthermore, the proposed membrane topology for the chloride channel of *Torpedo* [6] and mammalian skeletal muscle chloride channel [14] has demonstrated several positively charged domains containing arginine and lysine on intracellular surfaces. Thus in the present experiments, we evaluated the effects of the arginine-specific reagent phenylglyoxal (PGO) [3, 7, 8, 15] and the lysine/terminal amine reagent trinitrobenzene sulfonic acid (TNBS) [2, 8, 13] on channel activity and the determinants of channel activity.

The results show the pretreatment of the Cl^- channel from the *trans* surface with PGO or TNBS prevented the increase in P_o by raising *trans* Cl^- concentrations from 2 to 50 mM. However, neither PGO nor TNBS had any effect on either P_o or the determinants of channel activity when P_o had been increased by raising *trans* Cl^- concentrations from 2 to 50 mM. Thus, the Cl^--dependent increase in basolateral mTAL Cl^- channel activity produced by raising intracellular Cl^- concentrations may be referable to anion interactions with lysine-arginine regions on the intracellular surfaces of these channels. A preliminary report of these data has been presented elsewhere [19].

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 [1]. The methods for vesicle incorporation into lipid bilayers and subsequent analysis of channel currents were used also as described previously [11, 16–18]. In the present studies, *cis* and *trans* chambers uniformly contained 1 mM $CaCl_2$ and 5 mM HEPES, pH 7.4; the KCl concentrations in the *cis* and *trans* solutions in each experiment are indicated in Results. Lipid bilayers were painted from a 1:1 mixture of phosphatidyl ethanolamine and phosphatidyl serine in decane (20 mg/ml) (Figs. 1–6; Tables 1, 2), or pure phosphatidyl choline in decane (20 mg/ml) (Fig. 7), and membrane vesicles were added to the *cis* chamber. PGO and TNBS were each added from 100 mM stock solutions, pH 7.4, to the *trans* chamber as indicated in the results.

As in prior experiments [16–18], all experiments were paired, that is, experimental maneuvers were preceded by a control period such that each channel served as its own control, and data were recorded for 5–10 min before and after the addition of different agents. Data were filtered at 200 Hz and sampled at 2 kHz for computer analysis. Openings and closings of the channel were defined by a 50% threshold discriminator. All results were expressed as mean values ± SEM for the indicated number of experiments. A single bilayer was taken to be $n = 1$.

Sign Convention and Definitions

In all of our prior studies [11, 16–18], the Cl^- channels were fused into bilayers from vesicles added to *cis* solutions while *cis* and *trans* solutions contained relatively hypertonic and hypotonic solutions, respectively. All voltages were reported as *cis* solutions with respect to *trans* solutions. However, as indicated in the Introduction, as well as previously [11], it is likely that the intracellular aspects of these Cl^- channels face *trans* solutions when the latter are relatively hypotonic. In intact microperfused mTAL segments, all reported voltages have been expressed for intracellular compartments with respect to extracellular fluids (*see*, for example, refs. 4, 5, 9, 10, 12). Thus, to establish a greater degree of congruence between bilayer data and data from intact tubule segments, all voltages reported in this paper are expressed for *trans* solutions with respect to *cis* solutions. The sign convention in this paper is therefore the opposite of that reported in our prior studies [11, 16–18].

The terminology used in this paper is identical to that used previously [11, 16–18]. Specifically, V_H (mV) is used to indicate the holding voltage in a given experiment. The term g_{Cl} (pS) is the individual conductance of a given channel, computed as described in detail previously [11, 16–18]. The terms Z and ΔG refer to the gating charge and to the voltage-independent determinants of channel activity, respectively, computed, as described previously [11, 16–18], from the slope (Z) and zero-voltage intercept (ΔG) of paired experiments in a given bilayer using the Boltzmann expression:

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

where F, R and T have their usual meanings.

Results

Effects of Varying *trans* Isethionate

Our prior observations [16] have indicated that these Cl^- channels are impermeant to isethionate, and that, when the channels were inactivated by reduc-

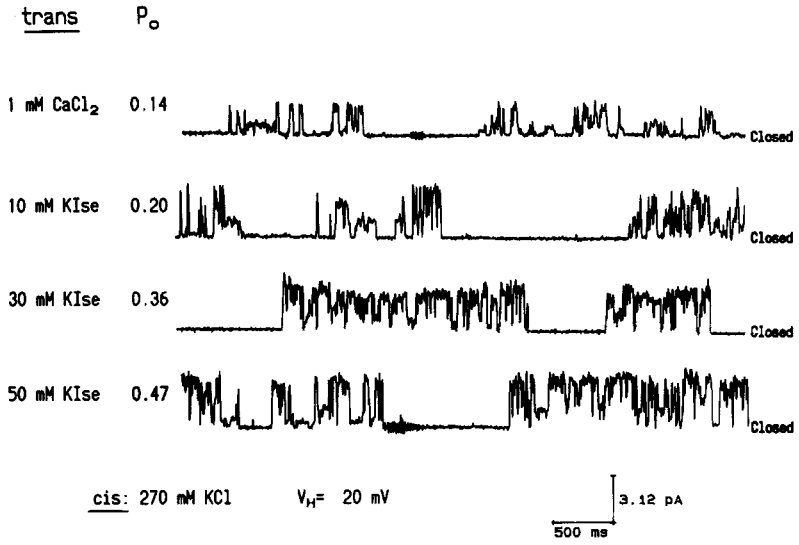

Fig. 1. A representative tracing in a single Cl^- channel illustrating the effect of varying *trans* isethionate concentrations on P_o. The holding voltage V_H (mV) is for the *trans* with respect to *cis* solutions. As indicated in Materials and Methods, *cis* and *trans* solutions uniformly contained 1 mM $CaCl_2$ and 5 mM HEPES, pH 7.4. The same conditions apply to the experiments shown in Figs. 2–7 and Tables 1 and 2.

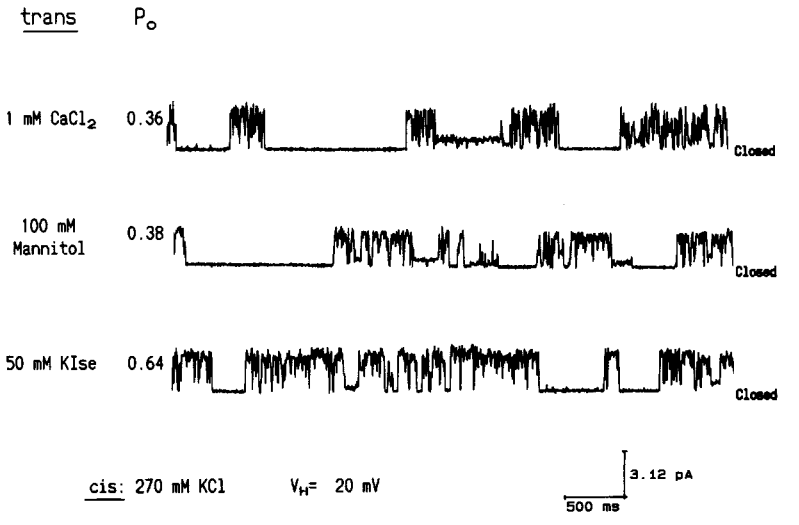

Fig. 2. A representative experiment illustrating the effects of increasing *trans* osmolality with mannitol on P_o.

ing *cis* Cl^- concentrations from 320 to 50 mM, subsequent *cis* addition of 270 mM isethionate had no effect on either P_o or single channel conductance (g_{Cl}, pS). We concluded from these data that the Cl^--interactive sites on these Cl^- channels which modulated P_o were beyond, or deep to, the selectivity filter on the extracellular faces of these channels. However, as indicated in Figs. 1–3 and Table 1, the effects of isethionate as a substitute for Cl^- in modulating P_o were quite different for the intracellular faces of these channels.

A representative experiment in a single Cl^- channel illustrating the effect of varying *trans* isethionate concentrations on P_o is illustrated in Fig. 1. Clearly, P_o rose monotonically as the *trans* isethionate concentrations were increased in stepwise fashion from 10 to 50 mM. The results presented in Fig. 2, all from a single Cl^- channel, show clearly that this effect of isethionate on P_o was not referable to osmolality, since P_o remained constant when identical increments in *trans* osmolality were produced with mannitol instead of isethionate. Figure 3 presents a paired comparison of the effects of Cl^- with respect to isethionate$^-$ on channel activation. Clearly, at a given *trans* anion concentration, Cl^- was a more effective agent than isethionate in augmenting P_o. But the results in Fig. 3 also show clearly that isethionate reproducibly augmented P_o in a pattern qualitatively similar to that obtained with Cl^-.

Table 1 presents a paired comparison of the effects of varying either *trans* isethionate or *trans* Cl^- concentrations on g_{Cl} and on the voltage-dependent (Z, gating charge) and voltage-independent (ΔG) determinants of channel activity. The data with increasing *trans* Cl^- concentrations confirm our earlier observations [16–18], that is: g_{Cl} rose

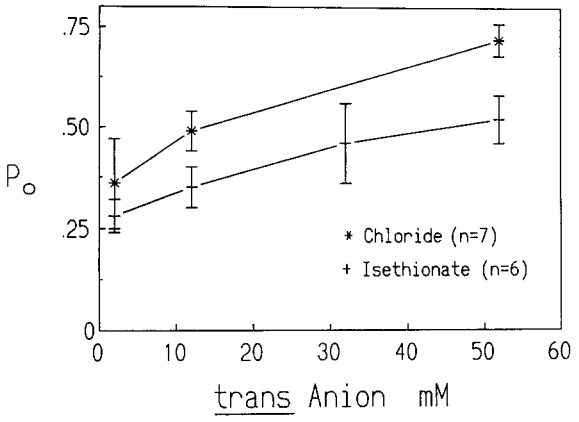

Fig. 3. The relation between P_o and *trans* anion concentrations. Paired measurements of P_o at each of the indicated *trans* Cl^- concentrations (*) and *trans* isethionate concentrations (+) were carried out in each of the indicated number of bilayers. The *cis* Cl^- was 270 mM KCl and both aqueous phases contained 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4. The holding voltage (V_H, mV) was +20 mV. A paired comparison of the data indicate that the P_o values at 50 mM *trans* Cl^- ($P < 0.01$) or 50 mM *trans* isethionate ($P < 0.05$) were clearly greater than the P_o values with these anions of 10 mM *trans* concentrations.

Table 1. Effect of varying *trans* salt concentrations on the determinants of channel activity

trans (mM)		g_{Cl} (pS)	Z	ΔG
0 Ise^-		59.8 ± 4.0	1.32 ± 0.35	−2.24 ± 0.44
50 Ise^-		60.3 ± 4.0	1.08 ± 0.28	−1.01 ± 0.42
	(n = 6)	(NS)	(NS)	($P < 0.03$)
2 Cl^-		67.8 ± 4.0	1.07 ± 0.14	−1.97 ± 0.35
50 Cl^-		83.3 ± 1.0	1.02 ± 0.22	−0.70 ± 0.18
	(n = 5)	($P < 0.05$)	(NS)	($P < 0.03$)

All of the data are paired comparisons on a given bilayer for the indicated experimental circumstances. The values for the determinants of channels gating activity (Z = gating charge; ΔG = chemical determinant of bond energy) were obtained from Boltzmann plots of $\ln P_o/(1 - P_o)$ vs. V_H (mV, *trans* with respect to *cis*) over the V_H range zero to +40 mV, as in prior studies.

significantly; and P_o rose primarily because of a change in ΔG rather than Z. The results with increasing *trans* isethionate show clearly, in accord with our earlier observations using increasing *cis* isethionate concentrations [16], that g_{Cl} did not change, or stated alternatively, that isethionate was an impermeant species. The data in Table 1 also indicate that increasing *trans* isethionate concentrations augmented P_o primarily by increasing ΔG, in accord with the data obtained using varying *trans* Cl^- concentrations.

In this connection, it will be noted that the analysis of the experiments described in Fig. 1 and Table 1 were carried out using a 50% threshold discriminator, which is the usual practice for channel analysis in this laboratory [11, 16–18]. It will also be noted from Fig. 1 that subconductance states for the channel were occasionally observed. To exclude the possibility that the effect of varying *trans* isethionate concentrations on P_o might be referable to altering subconductance states not detected by a 50% threshold discriminator, we utilized both 50 and 15% discriminator thresholds to analyze the data from the bilayers reported in Table 1, where the *trans* isethionate concentration was raised from zero to 50 mM. With zero *trans* isethionate, the P_o values were 0.20 ± 0.07 and 0.26 ± 0.08 using 50 and 15% discriminator thresholds, respectively. With 50 mM *trans* isethionate, the P_o values were 0.54 ± 0.06 and 0.65 ± 0.08 using 50 and 15% discriminator thresholds, respectively. Thus, for a given *trans* isethionate (i.e., either zero or 50 mM), the P_o values were statistically indistinguishable whether threshold discrimination was set at either 15 or 50%. In other words, the P_o of subconductance states for the experiments reported in Table 1 was statistically negligible and thus had no effect on the conclusions deduced from the data in Table 1.

Conversely, at a given threshold discriminator setting (i.e., either 15 or 50%), raising *trans* isethionate concentrations from zero to 50 mM had a highly significant ($P < 0.001$) effect on P_o. We argue from these results that the effects of increasing *trans* isethionate concentrations on P_o (i.e., Figs. 1–3; Table 1) were due primarily to increasing the open time probability of channels having a conductance of approximately 60 pS (*see* Table 1) rather than to increasing the fractional contribution of subconductance states to total open time probability.

It should also be noted in this connection (Fig. 5, ref. 11) that, with Cl^- in *cis* and *trans* solutions, frequency histograms indicate that one of two states, closed or fully open, accounts quantitatively for a minimum of 85% of total channel events. Likewise, as indicated previously (Figs. 2 and 3, ref. 18), the linear Boltzmann plots obtained when *trans* Cl^- concentrations were either 2 or 50 mM are consistent with the view that, for both Cl^- concentrations, the channel existed primarily in one of two states, closed or fully open.

Effect of *trans* PGO or TNBS Addition

In earlier experiments [16], we found that *trans* addition of 1 mM PGO did not alter P_o when *trans* solutions contained 50 mM KCl. The data from the paired

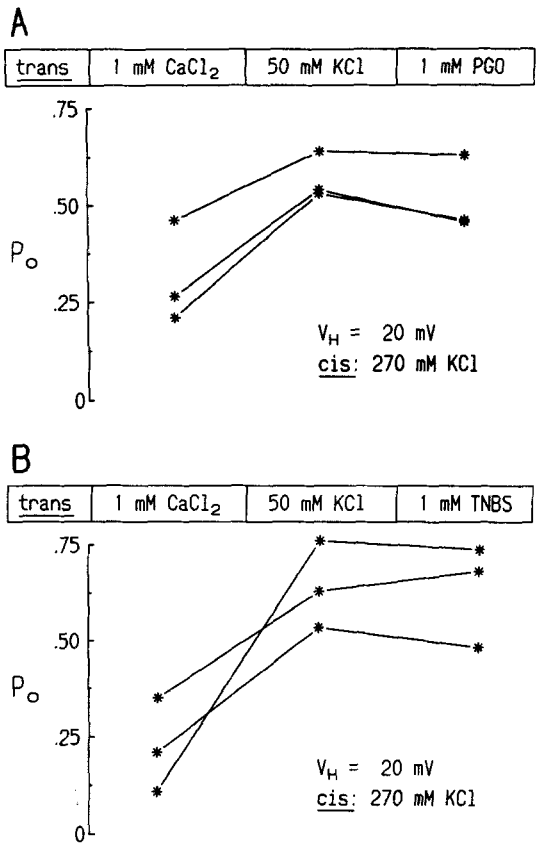

Fig. 4. Paired experiments indicating the effects of adding either 1 mM *trans* PGO (*A*) or 1 mM *trans* TNBS (*B*) on P_o when the *trans* solution Cl^- concentration had been raised from 2 to 50 mM to augment P_o. The experimental conditions were identical to those in Fig. 3 except for the addition of 1 mM PGO (*A*) or 1 mM TNBS (*B*) to *trans* solutions.

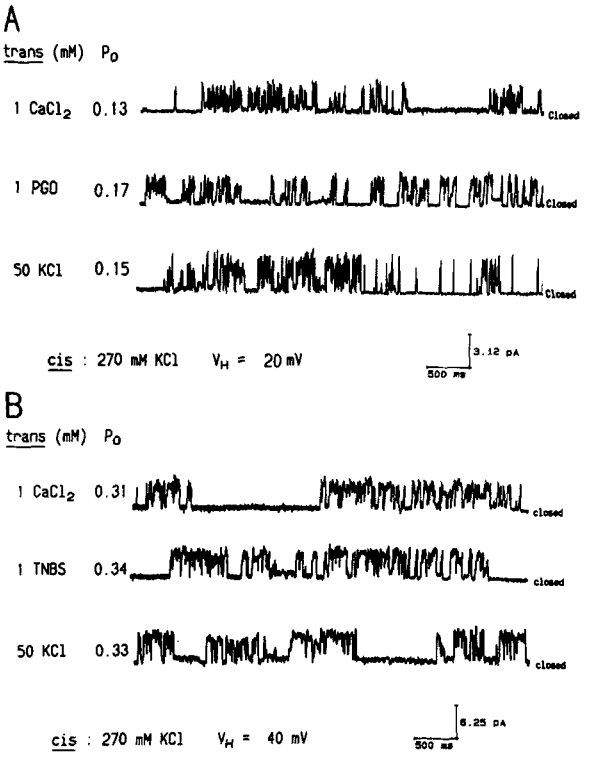

Fig. 5. Representative experiments illustrating that, with 2 mM *trans* Cl^-, pretreatment with 1 mM PGO (*A*) or 1 mM TNBS (*B*) blunts the activation of P_o by adding 50 mM Cl^- to *trans* solutions.

Table 2. Effect of *trans* PGO or TNBS additional and varying *trans* salt concentrations on the determinants of channel activity

trans (mM)	g_{Cl} (pS)	Z	ΔG
1 $CaCl_2$	58.0 ± 2.4	1.44 ± 0.06	−2.79 ± 0.16
1 PGO	56.0 ± 3.7	1.23 ± 0.14	−2.68 ± 0.36
50 KCl	91.1 ± 11.4*	1.51 ± 0.12	−3.08 ± 0.33
(n = 5)			*($P < 0.03$)
1 $CaCl_2$	60.6 ± 4.6	1.59 ± 0.18	−3.12 ± 0.45
1 TNBS	62.0 ± 3.9	1.48 ± 0.13	−3.03 ± 0.31
50 KCl	90.5 ± 3.2**	1.50 ± 0.14	−3.20 ± 0.29
(n = 5)			**($P < 0.01$)

All data represent paired observations on a given bilayer for the indicated experimental circumstances. The values of g_{Cl}, Z and ΔG were computed as described in Table 1.

experiments presented in Fig. 4*A* confirm these observations. Likewise, the paired data presented in Fig. 4*B* show that, under comparable conditions with 50 mM *trans* KCl, 1 mM TNBS did not alter P_o. The first two panels of Fig. 4*A* and *B* show, in accord with the results presented in Figs. 1–3 and previously [16], the activation of P_o produced by raising *trans* Cl^- concentrations from 2 to 50 mM.

However, the results presented in Figs. 5 and 6 and Table 2 show that *trans* addition of either 1 mM PGO or 1 mM TNBS produced rather different effects on P_o when the initial *trans* Cl^- concentrations were 2 mM. Figure 5*A* presents the results of a representative experiment in a single Cl^- channel which shows clearly that, when *trans* solutions initially contained 2 mM Cl^-, addition of 1 mM PGO blocked the activation of P_o produced by raising *trans* Cl^- concentrations to 50 mM (e.g., Figs. 1–3; Table 1). The results presented in Fig. 5*B* show that, in a representative experiment on another Cl^- channel, pretreatment with 1 mM TNBS also blocked the activation of P_o produced by raising *trans* Cl^- concentrations from 2 to 50 mM.

Figure 6*A* and *B* present the results of a number of such paired experiments in individual Cl^- channels. In all cases, we obtained results identical to those shown in Fig. 5*A*, that is, a complete blockade of the P_o increase produced by raising *trans* Cl^- or

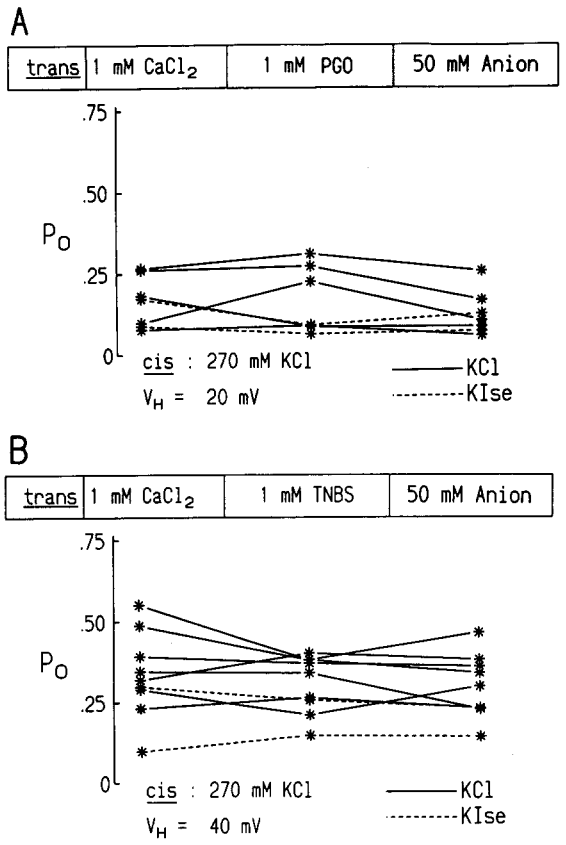

Fig. 6. Paired experiments in a number of Cl^- channels illustrating the effects of pretreatment of *trans* solutions with 1 mM PGO (*A*) or 1 mM TNBS (*B*) on the activation of P_o by either 50 mM *trans* Cl^- or 50 mM *trans* isethionate. The lines connect measurements in individual bilayers.

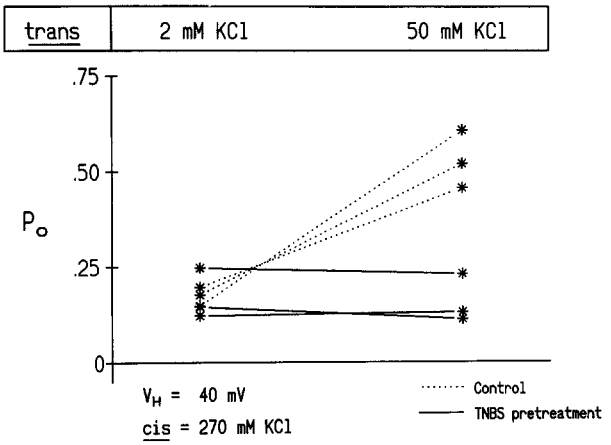

Fig. 7. Paired observations on the effects of increasing *trans* Cl^- concentrations from 2 to 50 mM on P_o when 1 mM TNBS was either absent (dotted lines) or present (unbroken lines) in *trans* solutions. The bilayers were formed from lipid solutions containing pure phosphatidyl choline (20 mg/ml) in decane. The lines connect measurements in individual bilayers.

isethionate concentrations from 2 to 50 mM when either 1 mM PGO (Fig. 6*A*) or 1 mM TNBS (Fig. 6*B*) was present in *trans* solutions. Likewise, the results presented in Table 2 indicate that prior exposure to either 1 mM *trans* PGO or 1 mM *trans* TNBS, with *trans* 2 mM Cl^-, abolished the effects of raising *trans* Cl^- concentrations on the determinants of P_o, that is, Z or ΔG. It should also be noted from the data in Table 2 that neither 1 mM PGO nor 1 mM TNBS blocked the increase in g_{Cl} produced by raising *trans* Cl^- concentrations from 2 to 5 mM.

It is relevant to note in this context that TNBS is not a wholly specific reagent for the primary amino groups of lysine, since TNBS can also react with terminal amines [2, 9, 13]. The experiments reported in Figs. 1–6 and Tables 1–2 were carried out using bilayers containing a 1 : 1 mixture of phosphatidyl ethanolamine and phosphatidyl serine (*see* Materials and Methods). Accordingly, it was pertinent to exclude the possibility that the TNBS effects described in Figs. 4–6 and Table 2 might be secondary to modification of primary amino groups of phosphatidyl serine or phosphatidyl ethanolamine. Since TNBS is unreactive with t-alkylamines [8], we repeated the experiment shown in Figs. 4–6 using phosphatidyl choline bilayers. Figure 7 illustrates paired experiments using phosphatidyl choline bilayers showing the effect of *trans* pretreatment with 1 mM TNBS on subsequent *trans* addition of 50 mM KCl. In three control experiments, *trans* addition of 50 mM KCl increased P_o (0.17 ± 0.02 to 0.53 ± 0.06, $P < 0.03$). But when *trans* solutions were pretreated with 1 mM TNBS, there was no rise in P_o when *trans* KCl concentrations were raised to 50 mM.

Discussion

A unique property of basolateral mTALH Cl^- channels is that, in the absence of C-PKA, P_o rises monotonically when increasing Cl^- concentrations, in the range 2–50 mM, are added to *trans* solutions ([17]; Figs. 4, 7). This range of *trans* Cl^- concentration is within the range of intracellular Cl^- concentrations in intact mTALH segments during net NaCl absorption [4, 10, 12]. The present experimental results provide additional insights into the channel site or sites which interact with Cl^- in modulating P_o.

The present data, together with our earlier studies [16], are consistent with the view that these Cl^- channels contain at least two sites, or groups of sites, which interact with Cl^- to modify P_o: one vicinal to the extracellular channel face and one vicinal to the intracellular channel face [16]. While both modulate P_o by affecting the voltage-independent

determinants of channel activity ([16]; Table 1), the two sites differ significantly in other respects.

Our prior data indicate that there is a Cl^--interactive site, or sites, facing the extracellular face of the channel [11, 16, 18]. For convenience, we denote this site, or sites, as the $[Cl]_e$ locus. The $[Cl]_e$ locus has a $K_{1/2}$ of 175 mM Cl^- for maximal activation of P_o [16], and appears to be located sufficiently deep within the channel that the impermeant anion isethionate cannot substitute for Cl^- in activating P_o [16]. Stated in another way, the extracellular faces of these channels contain a selectivity filter which prevents the access of isethionate to the $[Cl]_e$ locus. Clearly, in the case of the $[Cl]_e$ locus, the effect is related to a specific anionic effect rather than to ionic strength. Thus, when channels were inactivated by reducing *cis* KCl concentrations to 50 mM, adding either 270 mM KCl or KF to *cis* solutions produced complete channel reactivation while adding 270 mM K isethionate to *cis* solutions had no effect on channel activity.

The results in this paper, as well as prior studies [16], indicate that a different site, or sites, facing intracellular solutions modulates P_o by interacting with Cl^- in *trans* solutions. For convenience, we denote this site or sites as the $[Cl]_i$ locus. The $K_{1/2}$ for maximal activation of P_o by *trans* Cl^- interaction with the $[Cl]_i$ locus is 10 mM ([16]; Fig. 3). As indicated in the results presented in Figs. 1, 3 and Table 1, the impermeant anion isethionate has access to the $[Cl]_i$ site. Thus isethionate, when added to *trans* solutions, activates P_o in a manner qualitatively similar to that observed with identical concentrations of Cl^- (Fig. 3). But while increases in *trans* Cl^- also produce increases in single channel conductance, increases in *trans* isethionate concentrations have no detectable effect on single channel conductance (Table 1). These data are therefore consistent with the notion that the $[Cl]_i$ sites of these Cl^- channels appear to be directly accessible to interaction with either permeable (Cl^-) or impermeable (isethionate) anions in intracellular fluids. The results presented in this paper do not indicate whether the effect of raising *trans* KCl or K isethionate concentrations on P_o was referable to a specific anion effect on $[Cl]_i$ or to an ionic strength effect on $[Cl]_e$.

The results presented in this paper and in earlier studies [16] indicate that the interactions of PGO, or TNBS, with the $[Cl]_e$ and $[Cl]_i$ loci also differ. In prior studies [16], we noted that 1 mM PGO, when added to *cis* solutions, altered P_o by modifying both Z and ΔG, and that PGO plus increasing *cis* Cl^- concentrations, from 125 to 320 mM, had additive effects on activating P_o. In other words, those data were consistent with the possibility that, on extracellular faces of these Cl^- channels, the PGO-sensitive sites and the $[Cl]_e$ loci differed.

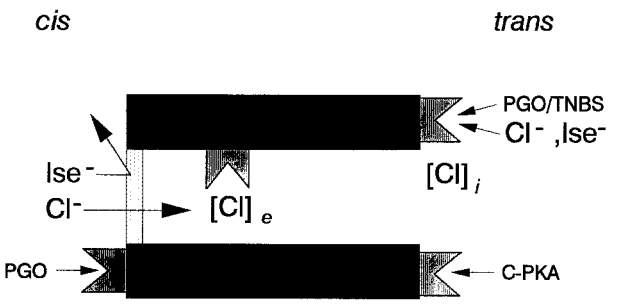

Fig. 8. A tentative functional model for basolateral mTAL Cl^- channels.

Alternatively, the data presented in Figs. 4–7 and Table 2 indicate that both PGO and TNBS interacted with $[Cl]_i$. In our view, an explanation for the results presented in Figs. 4–7 and Table 2 is that $[Cl]_i$ loci contained arginine- and/or lysine-rich domains. The latter, when screened electrostatically by increasing *trans* Cl^- or isethionate concentrations (Fig. 3; [11, 16–18]), resulted in both an increase in P_o and in an inaccessibility of these loci to PGO or TNBS (Fig. 4). The reasons for such an inaccessibility are not clear from the present data, although steric and/or electrostatic hindrances are reasonable possibilities.

It is also plausible to argue that, when the $[Cl]_i$ loci were not screened electrostatically by relatively high *trans* Cl^- concentrations, both PGO and TNBS interacted, presumably covalently [2, 3, 7, 8, 13, 15], with the arginine- and/or lysine-rich domains of $[Cl]_i$. According to this view, one could argue that, under the latter conditions, electrostatic interactions between $[Cl]_i$ and increased *trans* Cl^- or isethionate concentrations were minimal (Fig. 5), and that, as a consequence, P_o was not affected appreciably.

Figure 8 presents a tentative model for basolateral mTAL Cl^- channels which illustrates these conclusions schematically. Our data to date on the PGO-interactive site or sites on the extracellular faces of these channels do not allow any conclusions about whether this site or sites are external or internal to the selectivity filter. We assume, given the molecular weight of PGO (134.1 D), that this PGO-sensitive locus may be external to the selectivity filter. But clearly, the answer to this question, as well as a direct analysis of the proposals set forth in this paper, will depend on isolating, cloning and sequencing these Cl^- channels. As a first step in this process, we [20] have recently been able to express Cl^- channel activity in *Xenopus* oocytes using 1.8–3.2 kb fractions of mRNA from rabbit outer medulla.

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