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Cl^- Channels in Basolateral Renal Medullary Membrane Vesicles: IV. Analogous Channel Activation by Cl^- or cAMP-Dependent Protein Kinase

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Summary. We examined the interactions of cAMP-dependent protein kinase and varying aqueous Cl^- concentrations in modulating the activity of Cl^- channels obtained by fusing basolaterally enriched renal outer medullary vesicles into planar lipid bilayers. Under the present experimental conditions, the *cis* and *trans* solutions face the extracellular and intracellular aspects of these Cl^- channels, respectively. Raising the *trans* Cl^- concentration from 2 to 50 mM increased the channel open-time probability, raised the unit channel conductance, and affected the voltage-independent determinant (ΔG) of channel activity but not the gating charge (Winters, C.J., Reeves, W.B., Andreoli, T.E. 1990. *J. Membrane Biol.* **118**:269–278). With 2 mM *trans* KCl, *trans* addition of the catalytic subunit of PKA (C-PKA) plus ATP increased channel open-time probability and altered the voltage-independent determinant of channel activity without affecting either unit channel conductance or gating charge. The effect was ATP specific, did not occur with (C-PKA plus ATP) addition to *cis* solutions, and was abolished by denaturing C-PKA. Finally, (C-PKA plus ATP) activation of channel activity was not detected with relatively high (50 mM) *trans* Cl^- concentrations. These data indicate that (C-PKA plus ATP) might modulate Cl^- channel activity by phosphorylation at or near the Cl^--sensitive site on the intracellular face of these channels.

Key Words Cl^- channels/bilayers · Cl^- channels/vesicles · thick ascending limb · channel conductance · cAMP-dependent protein kinase

Introduction

This paper describes the interactions of cAMP-dependent protein kinase (PKA) and varying aqueous Cl^- concentrations in modulating the activity of Cl^- channels fused from basolaterally enriched renal medullary vesicles into planar lipid bilayer membranes [1, 15, 23]. The general rationale for these experiments depends on the following considerations.

In certain secretory epithelia, cAMP-dependent PKA regulates the activity of apically situated Cl^- channels [5, 11, 12, 20, 21]. Secretory diarrheas mediated by cholera toxin and like agents are due in part to apical Cl^- channel activation in small intestine cells [5, 12]. Alternatively, in the trachea, a failure of PKA to activate apical Cl^- channel activity is one of the physiologic aberrancies in cystic fibrosis [11, 20].

In the mammalian medullary thick ascending limb of Henle (mTALH), antidiuretic hormone (ADH)-dependent increases in the rate of net salt absorption [6–8, 18] or furosemide-mediated reductions in the rate of net salt absorption [2–4, 10, 13] are accompanied by increases or decreases in transcellular electrical conductance (G_c, mS cm^{-2}), respectively. It is clear that these changes in G_c are referable primarily to changes in basolateral Cl^- conductance (g_b^{Cl}, mS cm^{-2}) [4, 13]. But there are at least two different explanations for such variations in g_b^{Cl}, especially those variations referable to ADH.

On the one hand, Schlatter and Greger [18] found that, in mouse mTALH segments, cAMP and ADH elicited a fall in the fractional resistance of basolateral membranes when cell Cl^- activity was kept constant by blocking apical Cl^- entry with furosemide. These workers thus proposed that, in mTALH segments, cAMP activated basolateral Cl^- channels in a manner analogous to the activation of apical Cl^- channels by PKA in small intestine and in trachea [5, 11, 20, 21]. Paulais and Teulon [14] found that cAMP increased the activity of patch-clamped basolateral Cl^- channels from cortical thick limbs of mouse kidney. However, since ADH does not augment strikingly NaCl absorption in the cTALH [7, 24], the relevance of these latter data to ADH-dependent g_b^{Cl} changes in the mTALH is unclear.

Alternatively, we [13] have found that pretreatment of mTALH segments with luminal furosemide

reduces dramatically ADH-dependent increases in G_c; and that, in apical renal medullary vesicles, PKA activates directly K^+ channels [16]. We [9, 13] have proposed that, in mTALH segments, ADH might enhance the activity of apical K^+ channels and apical $Na^+/K^+/2Cl^-$ cotransport units; and that the attendant rise in intracellular Cl^- activity might be a major factor regulating the magnitude of g_b^{Cl}.

It is pertinent to note in this connection that Cl^- channels are fused from basolaterally enriched renal medullary vesicles into planar bilayers by adding vesicles to a *cis* chamber containing a salt solution relatively hypertonic to the *trans* solution. As indicated previously [15], our working assumption is that volume flow from *trans* to *cis* chambers produces osmotic lysis and fusion of vesicles in the *cis* chamber with the bilayer. The Cl^- channels orient such that the intracellular aspects of the channels face *trans* solutions [23]; and the open-time probability for channel opening (P_o) is exquisitely sensitive to *trans* Cl^- concentrations in the range 2–50 mM. This variation of P_o with *trans* Cl^- concentrations confers on these Cl^- channels a rather greater voltage dependence than would be expected from Goldman-Hodgkin-Katz (GHK) formalism [15, 23]. Likewise, in intact mTALH segments, the fall in g_b^{Cl} with basolateral membrane hyperpolarization is considerably more than expected from GHK formalism [4, 13].

Given these considerations, the present experiments were designed to evaluate the interactions of the catalytic subunit of PKA (C-PKA) with varying aqueous Cl^- concentrations in modulating the activity of Cl^- channels fused from basolaterally enriched renal medullary vesicles into planar bilayers. The key experimental observations were that *trans* (C-PKA plus ATP) increased P_o without affecting unit channel conductance (g_{Cl}, pS). This effect was ATP specific and did not occur with C-PKA addition to *cis* solutions. Moreover, while this ATP-specific C-PKA effect occurred at relatively low (2 mM) *trans* Cl^- concentrations, C-PKA activation of channel activity was not detected with relatively high (50 mM) *trans* Cl^- concentrations. Thus, these data imply that, in mTALH segments, the interplay of both intracellular Cl^- activity and PKA with intracellular sites on basolateral Cl^- channels may modulate g_b^{Cl}. A preliminary report of some of these findings has appeared elsewhere [22].

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]. 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 −70°C for up to a week without noticeable changes in the characteristics of the Cl^- channels.

The catalytic subunit of cAMP-dependent protein kinase (C-PKA) was prepared from fresh beef heart using the procedure of Reimann and Beham [17]. The activity of the protein kinase was determined by phosphorylation of histone protein as described by Schlender and Reiman [19]. A unit of activity was defined as the incorporation of 1 pM of phosphate into histone per minute at 30°C [19].

The bilayer apparatus, electronics, and the methods for bilayer formation, vesicle incorporation and data processing were identical to those described previously [15, 23]. In the present experiments, lipid bilayers were painted from a 1 : 1 mixture of phosphatidyl ethanolamine and phosphatidyl serine in decane (20 mg/ml). The *cis* solutions contained 270 mM KCl while the *trans* solutions contained either 2 or 50 mM KCl. All solutions contained 1 mM $CaCl_2$ and were buffered with 5 mM HEPES, pH 7.4, and all experiments were performed at room temperature (23 ± 1.0°C). In experiments testing the effects of C-PKA, 830 units of C-PKA (0.4 μM) were added to either the *cis* or *trans* chamber (volume ≈ 3 ml) along with 1 mM ATP or ADP.

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 chloride from the *cis* to *trans* chamber is indicated as a negative current which appears as a downward deflection in current traces. All results were expressed as mean values ± SEM for the indicated number of experiments. A single bilayer was taken to be $n = 1$.

Results

Effect of Varying *trans* Cl^- Concentrations

It is likely that Cl^- channels fused from these basolaterally enriched medullary vesicles into planar bilayers are oriented such that the intracellular aspects of these channels face *trans* solutions [15, 23]. For example, the *trans* but not *cis* faces of these channels are exquisitely sensitive to variations in ionic Ca^{2+} concentrations, in the range 10–50 nM [15].

Moreover, reducing *cis* Cl^- concentrations from 270 to 175 mM reduces by 50% the channel open-time probability. Alternatively, *trans* Cl^- concentration variations in the range 50–250 mM do not affect P_o [23], but at a single holding voltage (V_H, mV) of −20 mV, reducing *trans* Cl^- concentrations from 50 to 2 mM reduces P_o [23]. Since the present experiments included an evaluation of the interactions of C-PKA and these two *trans* Cl^- concentrations in modulating channel activity, it was pertinent to consider the effects of varying *trans* Cl^- concentrations on P_o and on single-channel conductance over a range of holding voltages.

The experimental data are presented in Table 1 and Fig. 1. The results presented in Table 1 show

Table 1. Effect of varying V_H and *trans* Cl^- concentrations on P_o

V_H (mV)	P_o *trans* Cl^- 50 mM	P_o *trans* Cl^- 2 mM	ΔP_o	
−40	0.70 ± 0.06	0.45 ± 0.05	0.25 ± 0.08	($P < 0.05$)
−20	0.52 ± 0.05	0.30 ± 0.04	0.21 ± 0.06	($P < 0.05$)
0	0.34 ± 0.04	0.14 ± 0.04	0.20 ± 0.06	($P < 0.05$)
	(n =5)			

The open-time probability (P_o) was measured in each bilayer at each of the indicated values of V_H and at the two indicated *trans* Cl^- concentrations. The *cis* Cl^- concentration was 270 mM. The results are expressed as mean values ± SEM.

Table 2. Effects of (C-PKA plus ATP) on P_o with 2 mM *trans* Cl^-

V_H (mV)	P_o (C-PKA + ATP) −	P_o (C-PKA + ATP) +	ΔP_o	
−40	0.47 ± 0.03	0.65 ± 0.04	0.17 ± 0.05	($P < 0.04$)
−20	0.32 ± 0.04	0.49 ± 0.04	0.20 ± 0.07	($P < 0.04$)
0	0.19 ± 0.04	0.35 ± 0.03	0.16 ± 0.05	($P < 0.04$)
		(n = 5)		

Open-time probability was measured, in paired fashion, at each V_H with and without (C-PKA plus ATP) addition to *trans* solutions as described in Materials and Methods. The *cis* and *trans* solutions contained 270 mM Cl^- and 2 mM Cl^-, respectively. The data are expressed as mean values ± SEM.

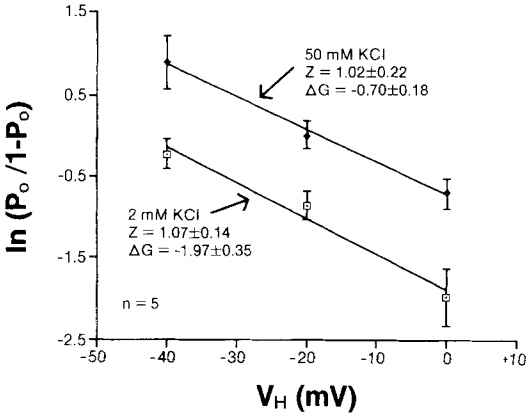

Fig. 1. A Boltzmann plot of the data presented in Table 1. The symbols are the mean values of $\ln(P_o/1 - P_o)$ at the indicated V_H values for the indicated number of bilayers, all from the data in Table 1. The lines are the mean regressions for the two different conditions. The indicated values of Z and ΔG were obtained from the slopes and the observed values of $\ln(P_o/1 - P_o)$ at zero V_H, respectively

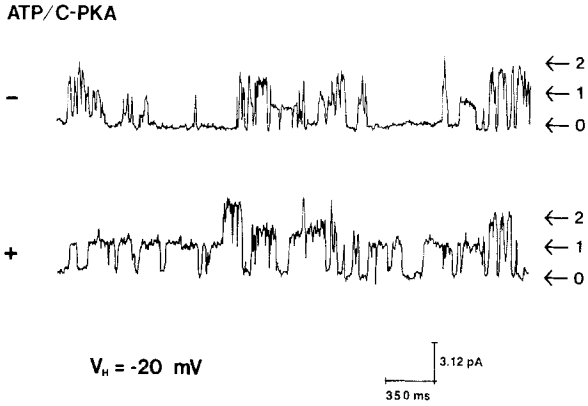

Fig. 2. Representative continuous tracing of a single lipid bilayer containing two Cl^- channels before and after the addition of (C-PKA plus ATP) to the *trans* solution. The *cis* and *trans* solutions contained 270 mM Cl^- and 2 mM Cl^-, respectively. The closed state is represented by *0*, while *1* and *2* represent one- and two-channel openings in the bilayer

clearly that, in paired observations, P_o at a given V_H was detectably smaller at 2 mM *trans* Cl^- than at 50 mM *trans* Cl^-. The unit channel conductances were greater with 50 mM *trans* Cl^- than with 2 mM *trans* Cl^-. From a GHK analysis of the current-voltage relations at the three V_H values indicated in Table 1, we obtained, using a P_{Cl}/P_K ratio of 10 [15], slope conductances at $V_h = -40$ mV of 83.3 ± 1.1 pS, with 50 mM *trans* Cl^-, and 67.8 ± 4.1 pS with 2 mM *trans* Cl^- ($n = 5$; $P < 0.05$).

In Fig. 1, the results from Table 1 are plotted according to a Boltzmann distribution, that is:

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

where Z is the gating charge and ΔG is the voltage-independent determinant of channel activity. The results shown in Fig. 1 indicate that, over the V_H range tested, the data could be expressed according to the Boltzmann relation. In keeping with our earlier findings [23], reducing *trans* Cl^- concentrations affected the voltage-independent determinant of channel activity, but not the gating charge. The gating charge values shown in Fig. 1 are quite similar to those reported previously for similar experimental conditions [15, 23].

Effects of *trans* (C-PKA Plus ATP) with 2 mM *trans* Cl^-

The relevant observations on the effects of adding (C-PKA plus ATP) to *trans* chambers containing 2 mM Cl^- are shown in Table 2 and Figs. 2–6. Figure 2 shows a continuous tracing from a representative bilayer containing two Cl^- channels. It is clear that, with a holding voltage of −20 mV, adding (C-PKA

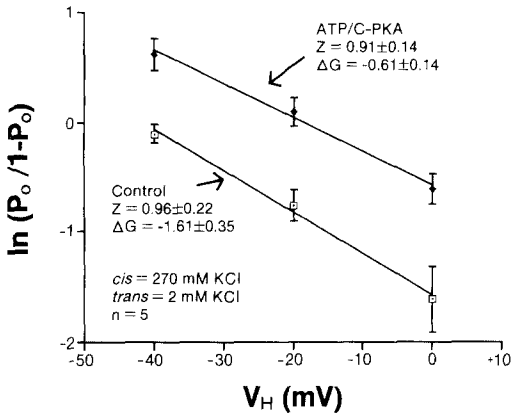

Fig. 3. A Boltzmann plot of the experimental data presented in Table 2. The data were plotted as indicated in Fig. 1

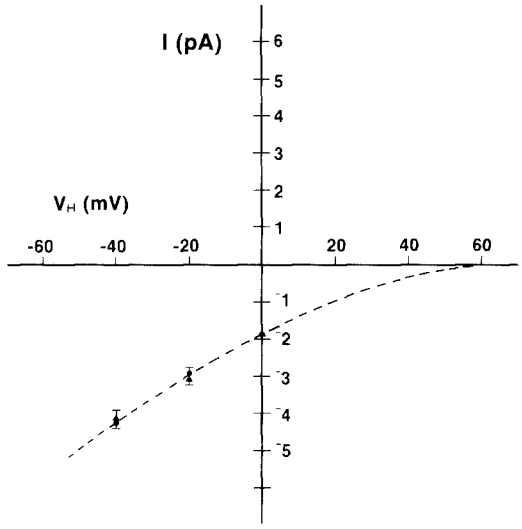

Fig. 4. Current-voltage data for the results presented in Table 2. The dashed line is the GHK relation for these experimental conditions using a P_{Cl}/P_K ratio of 10, as reported previously [15]

plus ATP) to the *trans* solution increased perceptibly the open-time probability for each channel. Clearly, from the tracing illustrated in Fig. 2, the dominant effect of *trans* (C-PKA plus ATP) addition was to increase mean open-time probability. Experiments currently in progress are intended to evaluate how this effect of (C-PKA plus ATP) on open-time probability modifies channel-gating kinetics. It should also be noted that, at this V_H, the magnitude of current fluctuations for each channel was unaffected (*see* Fig. 4).

Table 2 and Fig. 3 show the paired comparisons of *trans* addition of (C-PKA plus ATP) on P_o over a range of holding voltages and with 2 mM Cl^- uniformly present in *trans* solutions. A comparison of Tables 1 and 2 and Figs. 1 and 3 illustrates several noteworthy characteristics. First, without (C-PKA plus ATP), the control values of P_o, Z and ΔG at 2 mM *trans* Cl^- were quite similar in the two sets of experiments. When (C-PKA plus ATP) were added to *trans* solutions: the values of P_o, at each V_H, were quite similar to those observed when the *trans* Cl^- concentration was increased from 2 to 50 mM without (C-PKA plus ATP) addition (Tables 1 and 2). Moreover, ΔG fell to a value similar to that observed by raising *trans* Cl^- concentrations from 2 to 50 mM, while gating charge was unaffected (Figs. 1 and 3). In short,there was analogous activation of these Cl^- channels either by adding (C-PKA plus ATP) to *trans* solutions with 2 mM Cl^-, or by raising the *trans* Cl^- concentration from 2 to 50 mM.

Figure 4 compares the current-voltage relations for these Cl^-channels with 2 mM *trans* Cl^- and with or without *trans* addition of (C-PKA plus ATP). The data points are for the experiments presented in Table 2 and Fig. 3. The curvilinear relation is from GHK formalism using a P_{Cl}/P_K ratio of 10 which is, as noted previously [15], the Cl^-/K^+ permselectivity ratio for these Cl^- channels. The data shown in Fig. 4 indicate that adding (C-PKA plus ATP) to *trans* solutions had no detectable effect on the P_{Cl}/P_K ratio or on unit channel conductance. At $V_H = -40$ mV, the limiting slope conductances were: 66.1 ± 4.1 pS, without (C-PKA plus ATP); and 66.5 ± 4.2 pS, with (C-PKA plus ATP). These two values are statistically indistinguishable and virtually identical to the g_{Cl} values at $V_H = -40$ mV noted with 2 mM *trans* Cl^- in the experiments reported in Table 1. Thus we conclude that (C-PKA plus ATP) addition to *trans* solutions clearly increased P_o and altered ΔG (Table 2, Fig. 3), but did not alter g_{Cl}.

Figure 5 shows the results of experiments intended to assess aspects of the specificity of the augmenting effect on P_o obtained by adding (C-PKA plus ATP) to *trans* solutions containing 2 mM Cl^-. For convenience, control and experimental observations were carried out at a single holding voltage (−20 mV). The results presented in Fig. 5 show that, in eight different bilayers, adding (C-PKA plus ATP) to *trans* solutions increased P_o from 0.31 ± 0.03 to 0.50 ± 0.04 ($P < 0.01$). However, in paired observations in three bilayers, the combination (C-PKA plus ADP) had no detectable effect on P_o. Similarly, in two bilayers, the combination of (boiled C-PKA plus ATP) had no detectable effect on P_o. In other words, the effect of *trans* (C-PKA plus ATP) addition on P_o and ΔG at 2 mM *trans* Cl^- was ATP specific and required undenatured C-PKA.

Finally, Fig. 6 shows the results of paired experiments which evaluated the sidedness of (C-PKA plus

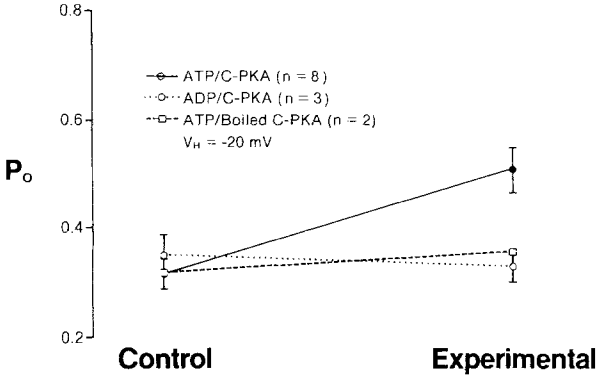

Fig. 5. The effect of *trans* addition of (C-PKA plus ATP), (C-PKA plus ADP), or (boiled C-PKA plus ATP) on P_o. All comparisons were paired at a holding voltage of −20 mV and a *trans* Cl^- concentration of 2 mM. The lines connect mean paired measurements under the same experimental conditions

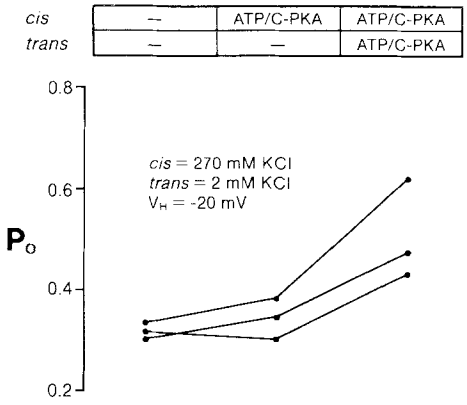

Fig. 6. Paired comparisons of the effects of adding (C-PKA plus ATP) to *cis* or *trans* solutions. The *cis* and *trans* solutions contained 270 mM Cl^- and 2 mM Cl^-, respectively; V_H was −20 mV. The lines connect paired measurements in individual bilayers

ATP) addition on P_o. The *cis* and *trans* solutions contained 270 mM Cl^- and 2 mM Cl^-, respectively; V_H was −20 mV. It is clear from the paired data shown in Fig. 6 that, for these conditions, (C-PKA plus ATP) increased P_o only when added to *trans* solutions.

Effects of *trans* (C-PKA Plus ATP) Addition with 50 mM *trans* Cl^-

The relevant observations on *trans* (C-PKA plus ATP) addition with 50 mM *trans* Cl^- are presented in Fig. 7, which shows the results of paired measurements of the Boltzmann distribution of P_o with and without (C-PKA plus ATP) addition to *trans* solutions when the latter contained 50 mM Cl^-. Clearly,

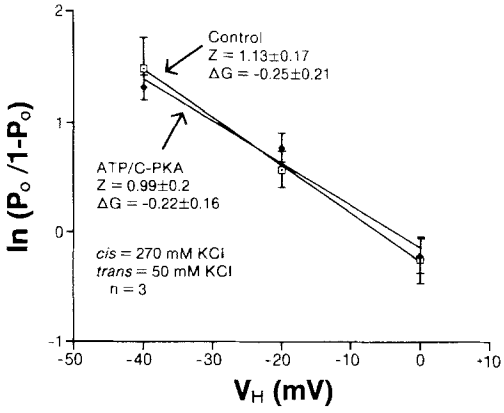

Fig. 7. Paired measurements of the relations between P_o and V_H, expressed in terms of the Boltzmann equation, before and after the addition of (C-PKA plus ATP) to the *trans* chamber, with 50 mM *trans* Cl^-. The data are mean values for the indicated number of bilayers. The values of Z and ΔG for the paired conditions were computed as described in Fig. 1

in these paired observations, *trans* (C-PKA plus ATP) addition had no detectable effect on the determinants of channel activity. From the current-voltage relations for the data shown in Fig. 7, we obtained, using a P_{Cl}/P_K ratio of 10 [15], slope conductances at −40 mV of 86.4 ± 8.9 pS, without (C-PKA plus ATP), and 87.2 ± 6.2 pS, with (C-PKA plus ATP).

Finally, in paired measurements in nine bilayers with 50 mM *trans* Cl^-, the P_o values were 0.62 ± 0.04 and 0.60 ± 0.05 (P = NS) without and with *trans* (C-PKA plus ATP), respectively. Thus, these control P_o values were similar to those reported in Table 1, with 50 mM *trans* Cl^-; and in accord with the data in Fig. 7, adding (C-PKA plus ATP) to the *trans* solutions had no detectable effect on P_o when the *trans* solutions contained 50 mM Cl^-.

Discussion

The experiments reported in this paper are consistent with the view that, in these basolaterally enriched renal medullary vesicles, *trans* (intracellular) addition of (C-PKA plus ATP) enhanced open-time probability when the *trans* solutions contained 2 mM KCl (Figs. 2–3), but had no effect on g_{Cl} (Fig. 4). This effect was specific for both ATP and for undenatured C-PKA (Fig. 5). Under the conditions used in our experiments, channel activation occurred when (C-PKA plus ATP) were added to *trans* but not to *cis* solutions (Fig. 7). We recognize in this regard that, in the experiments reported in Fig. 6, the *cis* KCl concentration of 270 mM was in excess of 170

mM, which is the *cis* KCl required for a half-maximal reduction in P_o, while the *trans* KCl concentration of 2 mM was less than 10 mM, the *trans* KCl concentration required for a half-maximal reduction in P_o. Accordingly, the present experiments do not exclude the possibility that, at lower *cis* KCl concentrations, (C-PKA plus ATP) addition to *cis* (extracellular) faces of these channels might affect channel activity.

The present results also show, in accord with our earlier findings [15, 23], that increasing the *trans* KCl concentration increased P_o appreciably. This effect was associated with a significant increase in single-channel conductance and with a perceptible change in the voltage-independent determinants of channel activity (Fig. 1). As noted in Fig. 1, however, increasing the *trans* KCl concentration from 2 to 50 mM had no effect on channel-gating charge.

The activation of open-time probability by *trans* (C-PKA plus ATP) addition was, in many respects, analogous to that observed by raising *trans* KCl concentration. Thus, the magnitude of the increase in P_o at the different holding voltages tested was quite similar either with *trans* (C-PKA plus ATP) addition or by raising *trans* KCl concentrations (Tables 1 and 2). Likewise, *trans* (C-PKA plus ATP) addition produced approximately the same magnitude change in ΔG, without effect on Z, as did raising *trans* KCl concentrations from 2 to 50 mM (Figs. 1 and 3). However, in contrast to the increase in single-channel conductance, which occurred when *trans* KCl concentrations were increased, *trans* (C-PKA plus ATP) addition did not affect g_{Cl} while increasing P_o (Fig. 4). This latter result is comparable to the effects of PKA on apical Cl^- channel activity in other epithelia [5, 11, 20, 21].

Clearly, the experiments reported in this paper provide no explicit information about the locus of Cl^- channel phosphorylation by *trans* (C-PKA plus ATP) addition. However, given the quantitative similarities between *trans* addition of (C-PKA plus ATP) and increases in *trans* KCl concentrations on P_o (Figs. 1 and 3; Tables 1 and 2), it is plausible to speculate that *trans* (C-PKA plus ATP) addition might modulate Cl^- channel activity at or near the *trans* Cl^--sensitive site which affects ΔG but not Z ([23]; Fig. 1). Evidently, added data are required to evaluate this possibility.

The present data may have some pertinence to interpreting the ADH-dependent increase in g^b_{Cl} which occurs in intact mTALH segments [13, 18]. Specifically, g^b_{Cl} might be modulated by at least two processes. Clearly, an increase in intracellular Cl^- activity referable to increased apical NaCl admittance [6, 13] might, as noted previously [15], increase time-averaged Cl^- channel conductance by increasing P_o (*see* Fig. 1). The data presented in this paper (Fig. 3) are also consistent with the view that, when intracellular Cl^- is reduced—for example, with luminal furosemide [18]—cAMP-dependent PKA might increase time-averaged Cl^- channel conductance directly. But the significance of this latter effect on the magnitude of g^b_{Cl} in intact mTALH segments [13, 18] may diminish appreciably at higher intracellular Cl^- activities ([4, 13, 18]; Figs. 3 and 7).

Finally, as noted in our prior publications about these channels [15, 23], the basolaterally enriched medullary vesicles used to fuse Cl^- channels into bilayer membranes are not homogenous. Hence added experimental data will be required to verify the origin of these channels as from basolateral membranes of mTALH segments.

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