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Chloride Channels in Basolateral TAL Membranes. XVIII. Phenylglyoxal Induces Functional mcClC-Ka Activity in Basolateral MTAL Membranes

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Abstract. Cultured mouse MTAL cells contain more mRNA encoding the Cl^- channel mcClC-Ka, which mediates CTAL Cl^- absorption, than mRNA encoding the Cl^- channel mmClC-Ka, which mediates MTAL Cl^- absorption. mmClC-Ka and mcClC-Ka have three functional differences: 1) mmClC-Ka open time probability, P_o, increases with increasing cytosolic Cl^-, but variations in cytosolic Cl^- do not affect P_o in mcClC-Ka; 2) mmClC-Ka is gated by (ATP + PKA), while (ATP + PKA) have no effect on P_o in mcClC-Ka; and 3) mmClC-Ka channels have single-ion occupancy, while mcClC-Ka channels have multi-ion occupancy. Using basolateral vesicles from MTAL cells fused into bilayers, we evaluated the effects of 1 mM cytosolic phenylglyoxal (PGO), which binds covalently to lysine or arginine, on Cl^- channels. With PGO pretreatment, Cl^- channels were uniformly not gated either with increases in cytosolic-face Cl^- or with (ATP + PKA) at 2 mM cytosolic-face Cl^-; and they exhibited multi-ion occupancy kinetics typical for mcClC-Ka channels. Thus, in basolateral MTAL membranes, blockade of Cl^- access to arginine or lysine residues on mmClC-Ka by PGO results in Cl^- channels having the functional characteristics of mcClC-Ka channels.

Key words: MTAL — Cl^- channels — mmClC-Ka — mcClC-Ka — Phenylglyoxal

Introduction

This paper and the succeeding paper [37] are intended to evaluate why cultured mouse MTAL cells, which contain greater levels of mRNA encoding the Cl^- channel mcClC-Ka than mRNA encoding the Cl^- channel mmClC-Ka [7, 16, 23], utilize mmClC-Ka channels as the principal Cl^- conductance pathway across basolateral membranes [20, 30, 33, 38]. Cultured mouse MTAL and CTAL cells have Cl^- influx and efflux properties similar to those of microperfused MTAL and CTAL segments, respectively [7, 9, 10, 11, 22]. For example, in cultured mouse MTAL cells [20, 31, 33], $^{36}Cl^-$ uptake is bumetanide-sensitive and enhanced either by forskolin, dibutyryl adenosine 3′,5′-cyclic monophosphate (db-cAMP) or vasopressin. The same adenylate cyclase effect on Cl^- uptake occurs in microperfused MTAL segments [9, 10, 11, 22]. In contrast, in cultured mouse CTAL cells [33] and in microperfused CTAL segments [7] the adenylate cyclase cascade has no effect on $^{36}Cl^-$ uptake.

The mmClC-Ka channel from cultured mouse MTAL cells [33] is the homologue of human ClC-Ka [13] and of rat ClC-K2 [1, 14]. The mcClC-Ka channel obtained from cultured mouse CTAL cells [33] is the homologue of human ClC-Kb [13] and rat ClC-K1 [1]. The mmClC-Ka and mcClC-Ka proteins have molecular weights of ~75 kDa and share at least 95% homology [33]. When mmClC-Ka or mcClC-Ka channels are fused into bilayers from basolateral vesicles prepared from cultured mouse MTAL or CTAL cells, respectively [20, 31, 38], each channel has the same unit conductance, approximately 100–110 pS in symmetrical 320 mM Cl^- solutions. And, as noted previously, [19, 26, 27, 28], frequency histograms as well as Boltzmann analyses indicate that these channels exist primarily in two states, open or closed, with infrequent occurrences of subconductance states.

Our prior studies indicate [20, 33, 38] that, under normal circumstances, Cl^- conductance across basolateral membranes of MTAL and CTAL cells

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utilizes mmClC-Ka and mcClC-Ka channels, respectively. Several lines of evidence, presented in detail previously [20, 31, 33], support this contention. Stated briefly, some of the results consistent with this view are as follows. When cultured mouse MTAL cells are transfected with an antisense oligonucleotide specific for the cDNA encoding mmClC-Ka, there is virtual abolition of Cl^- channel incorporation into bilayers from basolateral vesicles prepared from the transfected MTAL cells [33]. And the antisense oligonucleotide specific for the cDNA encoding mmClC-Ka suppresses bumetanide-insensitive $^{36}Cl^-$ efflux in cultured mouse MTAL cells but not in cultured mouse CTAL cells [33]. Furthermore, using a polyclonal antibody to mmClC-Ka and mcClC-Ka [33, 38], Western blots of basolateral vesicles from these transfected MTAL cells have a striking reduction in the signal for the 75 kDa band, that is, the molecular weight of either mmClC-Ka or mcClC-Ka [33]. However, this 75 kDa band is unaffected when CTAL cells are transfected with the antisense oligonucleotide specific for the cDNA encoding mmClC-Ka.

In cultured mouse CTAL or MTAL cells transfected with an antisense oligonucleotide specific for the cDNA encoding mcClC-Ka, $^{36}Cl^-$ efflux is suppressed in cultured CTAL cells but not in cultured MTAL cells [33]. Western blots from basolateral vesicles obtained from these transfected CTAL cells show a striking reduction in the 75 kDa band. But Western blots using basolateral vesicles from MTAL cells transfected with the antisense specific for mcClC-Ka show no suppression of the 75 kDa marker. Finally, as will be noted below, excised inside-out patch-clamp studies of basolateral membranes of MTAL or CTAL cells show the same physiologic characteristics as observed in Cl^- channels fused into bilayers from basolateral vesicles of cultured mouse MTAL or CTAL cells, respectively [20, 31].

The mmClC-Ka and mcClC-Ka channels also have a number of physiologic characteristics that differ significantly [31, 32, 33, 34]. First, mmClC-Ka channels exhibit single-ion occupancy first-order kinetics for Cl^- conductance [32] while mcClC-Ka channels have multi-ion occupancy kinetics for Cl^- conductance [5, 12, 18, 21, 34]. Second, in mmClC-Ka channels, the open time probability P_o rises with increasing cytosolic-face Cl^- concentrations, with a $K_{1/2}$ = 10 mM Cl^-, as long as (ATP + PKA) are absent [20, 27, 28, 38]. This dependence of P_o on cytosolic-face Cl^- concentrations in mmClC-Ka channels is absent in mcClC-Ka channels from cultured CTAL cells [31]. Third, in mmClC-Ka channels, cytosolic-face (ATP + PKA) augment P_o at low cytosolic-face Cl^- concentrations, 2 mM, but have no effect on P_o when cytosolic-face Cl^- concentrations are raised to 25 mM [20, 29]. This complex interplay between cytosolic-face Cl^- concentrations and (ATP + PKA) in modulating P_o does not occur in mcClC-Ka channels of cultured CTAL cells [20, 31]. Importantly, in excised inside-out patches of basolateral membranes from cultured MTAL and CTAL cells, the above-described interplay between cytosolic Cl^- and (ATP + PKA) in modulating P_o is present in patches from MTAL cells but absent in patches from CTAL cells [20, 31]. Put differently, the differing physiologic characteristics of mmClC-Ka and mcClC-Ka channels observed in bilayers [27, 28, 32, 33, 34, 38] are also present in basolateral patch-clamp experiments [20, 31] from cultured mouse MTAL and CTAL cells, respectively. Thus, when taken together, these disparate characteristics of mmClC-Ka and mcClC-Ka channels provide a way of distinguishing mmClC-Ka from mcClC-Ka when a given channel is fused into a lipid bilayer.

In prior studies [29], we found that, when mmClC-Ka channels were fused into bilayers, pretreatment of cytosolic-face solutions with phenylglyoxal (PGO), which binds specifically to arginine or lysine residues [29], prevented gating of P_o by raising cytosolic-face Cl^- concentrations. Our working assumption [29] was, and is, that PGO, by prior covalent binding to arginine or lysine residues, prevented access of cytosolic-face Cl^- to Cl^--sensitive sites on mmClC-Ka responsible for gating the latter.

In the present studies, we found that, when mmClC-Ka channels were incorporated into bilayers, PGO addition to cytosolic-face solutions blocked (ATP + PKA) channel gating at low cytosolic-face Cl^- concentrations; and, in the absence of (ATP + PKA), blocked activation of mmClC-Ka channels due to raised cytosolic-face Cl^-. We also found that, in mmClC-Ka channels from basolateral MTAL membranes, PGO pretreatment converted the kinetics of Cl^- conductance from a single-occupancy, first-order process to the multi-ion occupancy, single-file kinetic process [5, 12, 18, 21] characteristic of mcClC-Ka channels from CTAL cells [34]. Thus, for mmClC-Ka channels fused into bilayers, PGO, by blocking Cl^- access to Cl^- binding sites required to gate mmClC-Ka, resulted in Cl^- channels having the functional characteristics of mcClC-Ka channels. Preliminary reports of the results in this and the succeeding paper have appeared in abstract form [35, 36].

Materials and Methods

The procedure for preparing basolaterally enriched vesicles from cultured mouse MTAL cells [20, 32, 38], suspensions of mouse MTAL segments [28], and rabbit outer medulla [19, 26, 27], and the enzymatic characteristics of these vesicles have been described previously [2]. Whole frozen rabbit kidneys were obtained from Pel Freez (Rogers, AR). The cultured mouse MTAL cells used in the present studies shared, as shown previously [33], and noted in the Introduction, many of the key physiologic properties of microperfused mouse MTAL segments [9, 10, 11, 22].

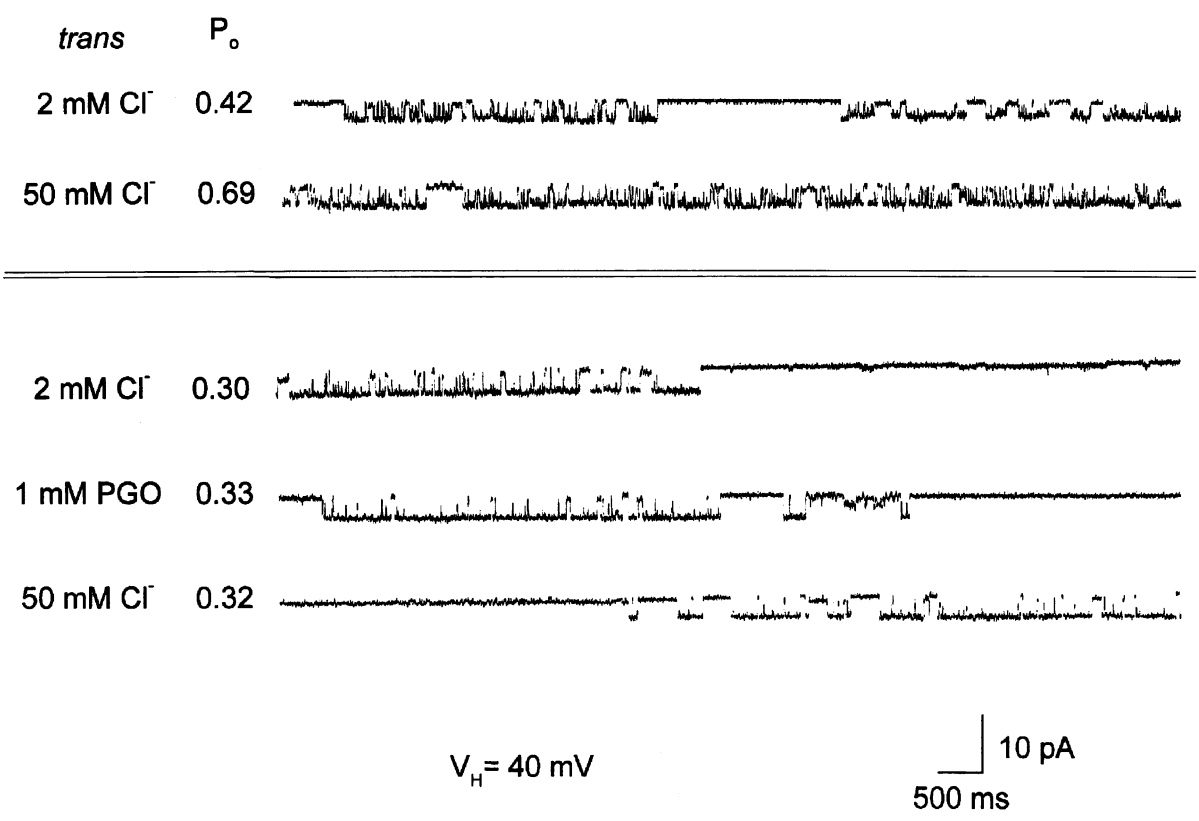

Fig. 1. Representative paired experiments illustrating the effect of PGO on P_o and g_{Cl} in single Cl^- channels fused into bilayers from basolateral vesicles of cultured MTAL cells. The cis chambers contained 270 mM KCl.

Lipid bilayer membranes were formed as described previously [19] by painting a lipid solution over a 0.2–0.3 mM aperture in the wall of a polystyrene Mueller-Rudin cup. The solutions used to form bilayers were a 1:1 mixture of phosphatidylserine and phosphatidylethanolamine in decane (20 mg lipid/ml). As described previously, the bilayers were voltage-clamped using a patch-clamp amplifier (Dagan 8900) connected to the bilayer chambers via silver electrodes in 3 M KCl agar bridges. The records were stored and analyzed by computer using pclamp 5.5 (Axon Instrumental, Foster City, CA), and filtered at 200 Hz (−3 dB cutoff) and sampled at 2 kHz. Openings and closings of the channel were defined by a 50% threshold discriminator.

All voltages reported in this paper are expressed for trans solutions with respect to cis solutions. As in prior studies [19, 26, 27], the trans and cis solutions bathed the intracellular and extracellular channel faces, respectively.

The methods for vesicle incorporation into lipid bilayers were identical to those described previously [19]. 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 the Results. Phenylglyoxal (PGO; Sigma, St. Louis, MO) was added directly to trans solutions at a final concentration of 1 mM, then removed from the trans chamber five minutes later by trans-chamber perfusion [29]. The catalytic subunit of protein kinase A (PKA) used in the present experiments was identical to that described previously [27]. When present, the aqueous concentrations of ATP and PKA were 1 mM Mg ATP and 1 μg/ml PKA. In all cases, (ATP + PKA) were added to trans solutions.

All results were expressed as mean values ±SEM for the indicated number of experiments. A single bilayer was taken to be $n = 1$. Curve fitting and linear regressions were done on computer, using Origin 4.1 (Microcal Software, Northampton, MA).

Results

PGO Pretreatment Abolishes mmClC-Ka Activation either by Cytosolic (ATP + PKA) or by Increases in Cytosolic Cl^- Concentrations

Figure 1 illustrates representative paired experiments showing the effect of PGO on P_o and on Cl^--channel conductance in single Cl^- channels from basolateral vesicles from cultured MTAL cells. The upper panel of Fig. 1 shows, in a paired experiment on the same Cl^- channel, that raising cytosolic-face Cl^- from 2 mM to 50 mM increased P_o from 0.42 to 0.69, that is, by approximately 65%, while the single-channel Cl^- conductance (g_{Cl}, pS), rose slightly, by 13%. The lower panel of Fig. 1 indicates, in a paired experiment on another Cl^- channel, that when 1 mM PGO was added to cytosolic solutions, the subsequent addition of 50 mM Cl^- to cytosolic solutions had no effect either on P_o or on g_{Cl}. Thus the data presented in Fig. 1 confirm findings reported previously by us using basolateral vesicles from rabbit outer medulla [29]. It

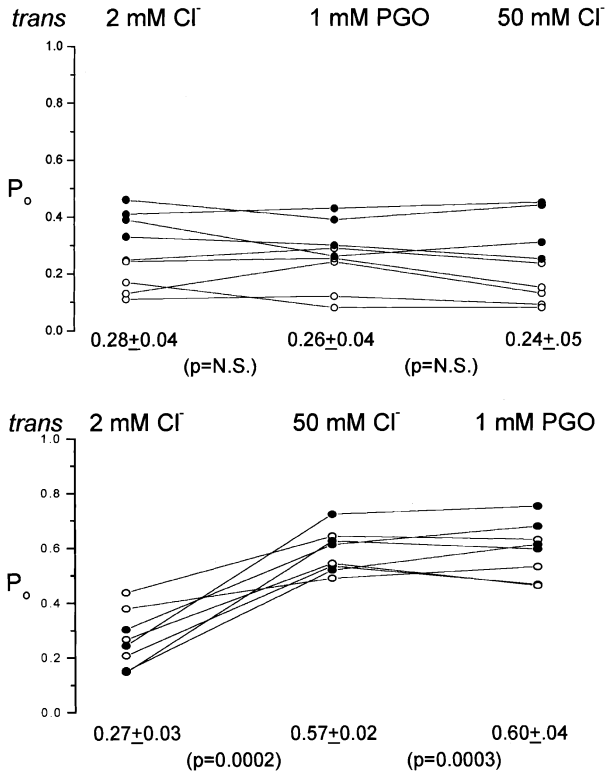

Fig. 2. The effect of pretreatment with 1 mM PGO on the response of P_o to raising cytosolic-face (trans) Cl^- concentrations in Cl^- channels from basolateral vesicles either from rabbit outer medulla (*empty circles*) or from cultured mouse MTAL cells (*filled circles*). The cytosolic-face (trans) solutions initially contained 2 mM Cl^-; the extracellular (cis) solutions contained 270 mM Cl^-. The *p* values with either 50 mM Cl^- or 50 mM Cl^- plus PGO are with respect to cytosolic-face 2 mM Cl^-. The lines connect measurements in the same bilayer. The data using vesicles from rabbit outer medulla (*empty circles*) are from [29]. The data using vesicles from cultured mouse MTAL cells were obtained using the same vesicles used in the studies shown in Figs. 5 and 6.

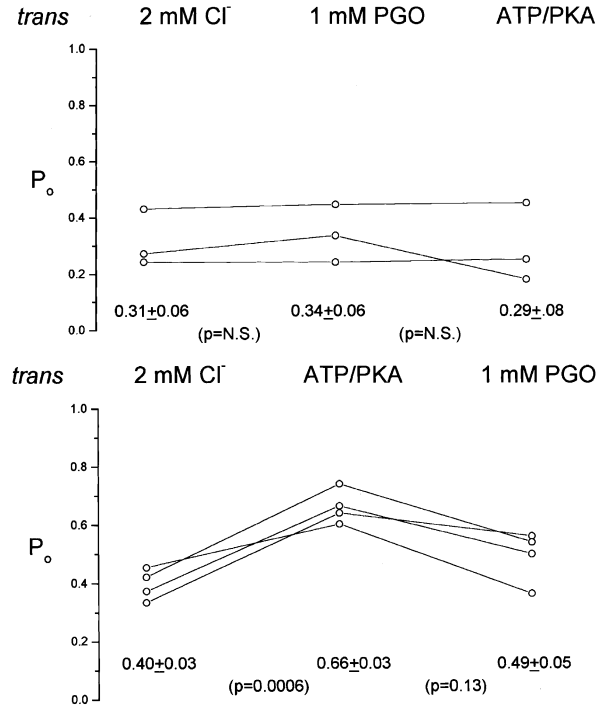

Fig. 3. The effect of pretreatment of trans solutions with 1 mM PGO on the response of P_o to (ATP + PKA) in Cl^- channels incorporated into bilayers from basolateral vesicles from rabbit outer medulla. The cytosolic-face (trans) solutions contained 2 mM Cl^- and the extracellular (cis) solutions contained 270 mM Cl^-. When present, the trans solutions contained 1 mM ATP plus 1 μg/ml PKA. The *p* values with either PGO or PGO and (ATP + PKA) are with respect to cytosolic-face 2 mM Cl^-. The lines connect paired measurements in the same bilayer.

should also be noted from the recordings in Fig. 1 that, as indicated previously [19, 26, 27, 28] and noted in the Introduction, mmClC-Ka channels, either with or without PGO, exist primarily in open or closed states, with no consistently detectable subconductance states.

Figure 2 illustrates paired experiments using either Cl^- channels from basolateral vesicles from rabbit outer medulla, described previously [29], or Cl^- channels from the basolateral vesicles of cultured mouse MTAL cells used in the experiments shown in Figs. 5 and 6. Thus the data shown in Fig. 2 provided an internally consistent set of data with respect to the results shown in Figs. 1, 5 and 6. The upper panel of Fig. 2 shows that, using basolateral vesicles either from cultured mouse MTAL cells or from rabbit outer medulla, prior PGO addition to cytosolic-face solutions abolished completely the increase in P_o, which occurs when cytosolic-face Cl^- concentrations are increased from 2 mM to 50 mM and no PGO is present in cytosolic-face solutions. The lower panel of Fig. 2 shows that increasing cytosolic-face Cl^- concentrations produced the expected [20, 27, 29, 38] increases in P_o for mmClC-Ka Cl^- channels derived either from rabbit vesicles or from cultured mouse MTAL cells. And as noted previously [29], this increase in P_o was not affected by subsequent addition of PGO to cytosolic-face solutions.

Figure 3 shows the results of paired experiments using the same set of basolateral vesicles from MTAL cells as the source for Cl^- channels. In the upper panel, 1 mM PGO was added to trans solutions prior to trans addition of (ATP + PKA). In the lower panel of Fig. 3, (ATP + PKA) addition to trans solutions preceded trans PGO addition. In both cases, cytosolic-face trans solutions contained 2 mM Cl^-. The upper panel of Fig. 3 shows that pre-treatment with 1 mM cytosolic-face PGO abolished the augmentation of P_o when (ATP + PKA) were subsequently added to cytosolic-face solutions. The results in the lower panel of Fig. 3, without cytosolic-face PGO addition, show results typically obtained with mmClC-Ka channels under these experimental conditions [20, 27, 38], namely, a highly significant

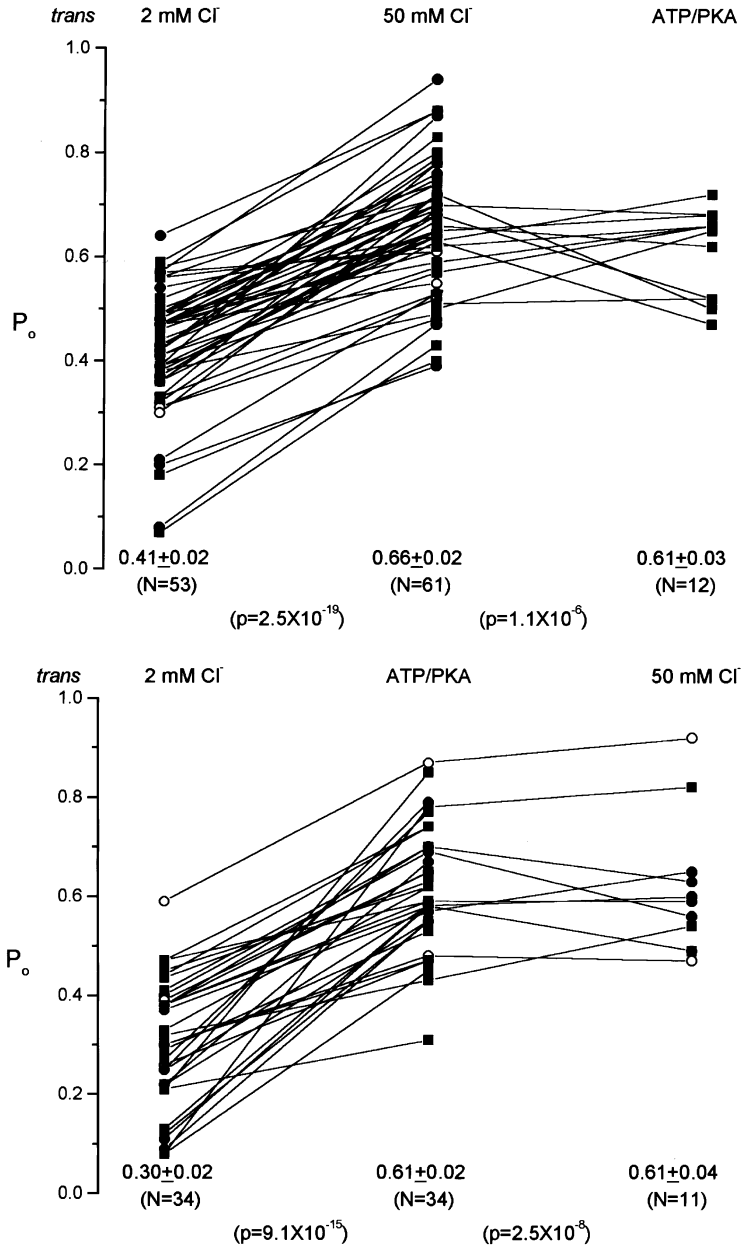

Fig. 4. A summary of all data, prior and present, obtained in our laboratory on the effects on P_o of raising either cytosolic-face Cl^- concentrations from 2 mM to 50 mM, and/or of adding (ATP + PKA) to cytosolic-face Cl^- solutions. The data were obtained using Cl^- channels obtained from basolateral vesicles either from rabbit outer medulla (*filled squares*, [26, 27, 29]) suspensions of mouse MTAL tubules (*empty circles*, [28]) or from cultured mouse MTAL cells (*filled circles*; present studies and [20, 31, 38]). The lines connect paired measurements in the same bilayer. The *p* values with 50 mM Cl^- and/or (ATP + PKA) are all with respect to the P_o values with 2 mM cytosolic-face Cl^-.

and reproducible increase in P_o with the addition of (ATP + PKA) to cytosolic-face solutions. The subsequent addition of PGO blunted this response.

Figure 4 shows a historical summary of all the data obtained previously and currently in our laboratory in paired experiments on the activation of rbClC-Ka channels from basolateral vesicles from rabbit outer medulla [19, 26, 27, 29], or mmClC-Ka channels from basolateral vesicles obtained from suspensions of mouse MTAL tubules [28] or cultured mouse MTAL cells [20, 33, 38] either by increasing cytosolic Cl^- from 2 mM to 50 mM or by the addition of (ATP + PKA) at 2 mM cytosolic-face Cl^-. In a large number of bilayers, increasing cytosolic-face Cl^- concentrations from 2 mM to 50 mM increased P_o virtually uniformly, with subsequent addition of (ATP + PKA) to cytosolic-face solutions having no further effect on P_o. Likewise, in a large number of paired bilayer experiments, (ATP + PKA) addition with 2 mM cytosolic-face Cl^- concentrations increased P_o virtually uniformly, with subsequent increases of cytosolic-face Cl^- to 50 mM having no further effect on P_o. Thus, when viewed in the context of the large body of experimental data presented in Fig. 4, the results shown in Figs. 1–3 indicate clearly that pre-addition of PGO to cytosolic-face solutions abolished, both qualitatively and quantitatively, these two properties of mmClC-Ka channels, each of which is absent in mcClC-Ka channels from mouse CTAL cells [31].

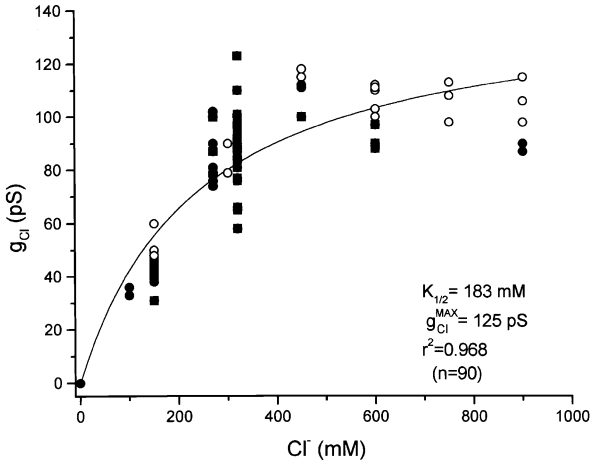

Fig. 5. Michaelis plot of all data obtained in our laboratory on the relation of g_{Cl} to external Cl^- concentrations. The data were obtained using Cl^- channels obtained from basolateral vesicles either from rabbit outer medulla (*filled squares*, [26, 27, 29]) or from cultured mouse MTAL cells (*filled circles*, [20, 32, 38]). The data indicated by empty circles were obtained from basolateral vesicles of mouse MTAL cells cultured contemporaneously with the mouse MTAL cells used in the experiments shown in Fig. 6.

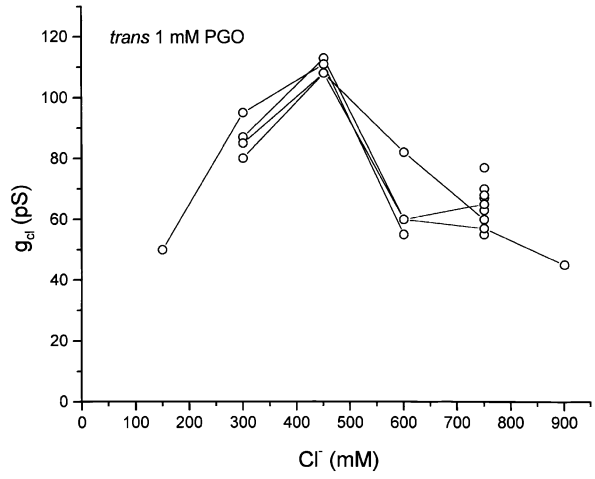

Fig. 6. The relation of g_{Cl} to external Cl^- concentrations in bilayers containing Cl^- channels from basolateral vesicles from cultured mouse MTAL cells. The cytoplasmic-face (trans) solutions contained 1 mM PGO. The lines connect paired measurements in the same bilayer.

Effect of PGO on the Kinetics of Cl^- Conductance

Figure 5 shows, for mmClC-Ka channels, a plot of the dependence of g_{Cl} on symmetrical increases in external Cl^- concentrations fitted according to traditional Michaelis kinetics. The filled squares and filled circles are control data from prior studies [20, 27, 28, 32, 38], fitted as described previously [32] by the Michaelis equation. The data signified by empty circles were obtained using vesicles from the same mouse MTAL cells used in the studies presented in Fig. 6. That is, the empty-circle data presented in Fig. 5 served both as controls for the experimental data to be presented in Fig. 6 and for comparison with data obtained previously [20, 27, 28, 32, 38] with mmClC-Ka channels in bilayers. Clearly, the current data shown in Fig. 5, like the data described in prior studies [20, 27, 28, 32, 38], were fitted easily by Michaelis first-order kinetics, consistent with channels having saturating single-ion occupancy [12, 15]. Moreover, the $K_{1/2}$ and g_{Cl}^{max} values shown in Fig. 5 are in close accord with those reported previously [32].

Figure 6 shows the effect of 1 mM cytosolic-face PGO addition on the kinetics of Cl^- conductance, using the same basolateral vesicles from cultured mouse MTAL cells illustrated by the empty circles shown in Fig. 5. The data presented in Fig. 6 indicate that, with 1 mM cytoplasmic-face PGO, g_{Cl} declined monotonically when external Cl^- concentrations exceeded 450 mM, that is, g_{Cl} exhibited self-block typical of multi-ion occupancy [12, 18, 21, 34]. Figure 7 presents a Hill plot of these data, using as g_{Cl}^{max} the highest g_{Cl} values observed in the studies presented in Fig. 6, i.e., g_{Cl} = 110 pS at 450 mM external Cl^-. The results presented in Fig. 7 show a positive slope of 2.29 at log Cl^- concentrations $\leq$2.65, and a negative slope of -3.4 at log Cl^- concentrations $\geq$2.65. In short, when the mmClC-Ka channels fused into bilayers were exposed to 1 mM cytosolic-face PGO, they exhibited self-block virtually identical to the kinetic behavior, without PGO exposure, of mcClC-Ka channels from basolateral membranes of cultured mouse CTAL cells [34].

Discussion

The results presented in this paper show clearly that, without cytosolic-face PGO addition, the MTAL basolateral vesicles used in the present studies, when fused into bilayers, yielded Cl^- channels having the same physiologic characteristics (Figs. 1, 2, 5) as those observed in our prior studies either with mmClC-Ka channels fused into bilayers from MTAL basolateral vesicles [20, 32, 38] or in excised inside-out patch-clamp studies [20] on basolateral membranes of cultured mouse MTAL cells [20, 27, 28, 32, 38], namely: augmentation of P_o by an increase in cytosolic-face Cl^- concentrations in the absence but not the presence of (ATP + PKA) (Figs. 1, 2); augmentation of P_o by cytosolic-face addition of (ATP + PKA) at 2 mM cytosolic-face Cl^- (Fig. 3); and, in basolateral MTAL vesicles fused into bilayers, first-order conductance kinetics with single-ion occupancy for Cl^- conductance (Fig. 5). However, when bilayers containing the same basolateral MTAL vesicles were

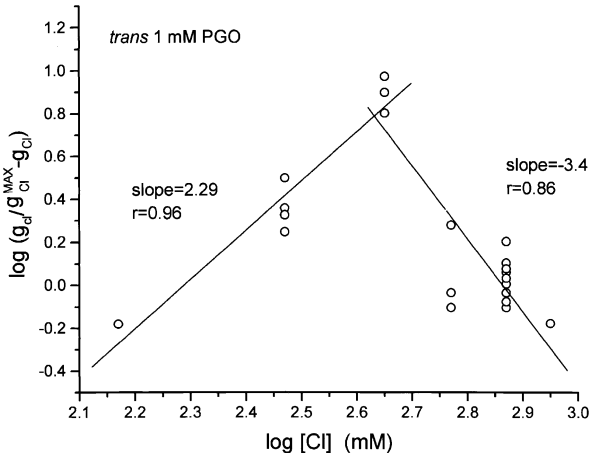

Fig. 7. A Hill plot of the data from Fig. 6. As in our prior studies [34], g_{Cl}^{max} was taken to be the maximum g_{Cl} observed in the experiments shown in Fig. 6, that is, 115 pS.

first exposed to cytosolic-face PGO, the observed physiologic characteristics of the channels were virtually identical to those observed previously in mcClC-Ka channels from basolateral membrane vesicles of cultured CTAL cells [31, 34] or in patch-clamp studies on basolateral membranes of cultured CTAL cells [31], that is: no augmentation of P_o either by an increase in cytosolic-face Cl^- concentrations (Figs. 1, 2) or by adding (ATP + PKA) to cytosolic-face solutions (Fig. 3); and, in basolateral CTAL vesicles fused into bilayers, self-block with increasing the external Cl^- concentrations above 450 mM (Figs. 6, 7) typical of multi-ion occupancy with single file kinetics.

Clearly, these PGO effects occurred in Cl^- channels already fused into bilayers. Moreover, the aggregated results presented in Figs. 4 and 5 show convincingly that, without cytosolic-face PGO addition, virtually all of the channels we have studied using basolateral vesicles from rabbit outer medulla, suspensions of mouse MTAL segments, or cultured mouse MTAL cells had the physiologic characteristics typical of mmClC-Ka channels [20, 26, 29, 31]. In contrast, with PGO addition to cytosolic solutions, the Cl^- channels had the physiologic characteristics typical of mcClC-Ka channels [31, 34; *see* Introduction].

At least two classes of explanations might account for the findings reported in this paper. First, PGO pretreatment of mmClC-Ka channels fused into bilayers may have altered the properties of the latter. That is, covalent PGO binding to lysine or arginine residues on mmClC-Ka may have resulted in a conformational change in mmClC-Ka channels, which altered the transport properties of these channels to those of mcClC-Ka channels. This possibility is plausible since mmClC-Ka channels and mcClC-Ka channels share ~95% homology [33]. Now, since (ATP + PKA) generally phosphorylate serine or threonine residues [6, 8], the fact that PGO interaction with arginine or lysine residues in mmClC-Ka also blocked P_o activation by (ATP + PKA) at low cytosolic Cl^- concentrations (Fig. 3), indicates that serine or threonine phosphorylation by (ATP + PKA) may require unencumbered arginine or lysine residues in various regions of the mmClC-Ka molecule. We note in this context that the primary sequences described previously [33] for mmClC-Ka and mcClC-Ka show that the only cytoplasmic arginine and lysine residues present in mmClC-Ka but not mcClC-Ka, are lysine residues in positions 13, 386 and 595, and an arginine residue at position 457. Evidently, the role of these four residues interacting with PGO as a mechanism for altering the conductance properties of mmClC-Ka remains to be evaluated.

Second, Miller [17] predicted that ClC-0 channels are double-barreled. Weinreich and Jentsch [25] found that mixed ClC-0/ClC-1 dimers and ClC-0/ClC-2 dimers each exhibited two separate conductances, indicating that each subunit functions as a pore. And Dutzler et al. [3] showed that ClC channels in prokaryotic cells from *Salmonella enterica* and *E. coli* contain identical homodimeric pores, each subunit of which can function as an independent pore. Accordingly, the possibility that basolateral MTAL membranes contain mmClC-Ka/mcClC-Ka heterodimers warrants evaluation.

In this connection, as noted in the Introduction, the antisense oligonucleotide data reported previously by us [33] are consistent with the view that, in cultured MTAL cells, the Cl^--conductive species is mmClC-Ka; and likewise, in cultured CTAL cells, that the Cl^--conductive species is mcClC-Ka. A similar conclusion derives from patch-clamp experiments with basolateral membranes of cultured MTAL and CTAL cells, where the Cl^- channels have the physiologic characteristics of mmClC-Ka and mcClC-Ka, respectively [20, 31]. Finally, as indicated previously [19, 26, 27, 28], in the Introduction and in Fig. 1, neither mmClC-Ka channels in MTAL cells nor mcClC-Ka channels in CTAL cells exhibit subconductance states. Thus, the possibility that MTAL cells might contain mmClC-Ka/mcClC-Ka heterodimers is not supported by the results of the present studies.

Finally, Estivez et al. [4] have recently reported that barttin, an integral membrane protein, serves as an essential β-subunit for human ClC-Ka Cl^- channels. And Waldegger et al. [24] have indicated that barttin is a functional activator of ClC-Ka and ClC-Ka channels. Obviously, additional experiments are required to evaluate specifically whether barttin is required for the channel activity observed when mouse mmClC-Ka and mcClC-Ka channels are incorporated into bilayers (Figs. 1–7; Refs. 20, 31, 32, 34, 38).

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