BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **246,** 59–63 (1998)
ARTICLE NO. RC988571

pI_{Cln} Can Regulate Swelling-Induced Cl^- Currents in Either Layer of Rabbit Ciliary Epithelium

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Received March 30, 1998

Swelling-induced Cl^- currents were investigated in freshly prepared non-pigmented epithelial (NPE) and pigmented epithelial (PE) cells of the rabbit ciliary body using the whole-cell patch clamp technique. Exposure of both NPE and PE cells to hypotonic stress induced Cl^- currents that exhibited outward rectification and were insensitive to Ca^{+2}. We found that swelling-induced Cl^- currents in PE cell are observed shortly after isolation. The swelling-induced Cl^- current showed little or no inactivation at positive membrane voltages and was sensitive to 100 μM NPPB and 100 μM DIDS. Injection of cRNA encoded rabbit pI_{Cln} into Xenopus oocytes produced an outwardly rectifying Cl^- current displaying features consistent with the swelling-induced Cl^- current in epithelium. pI_{Cln} is ubiquitous in the ciliary epithelium. It participates in the equilibration of short term tonicity alterations, a phenomenon underlying mechanisms with larger and slower amplitudes for aqueous secretion by these cells.

Extraction of water and solute from the blood and secretion of these components of the aqueous humor into the posterior chamber of the eye is accomplished by the bilayered ciliary epithelium (1, 2). A great deal of attention has been given to the role of volume regulation via Cl^- channels as part of the process of ciliary secretion and/or reabsorption (3, 4). pI_{Cln} (5), P-glycoprotein (6), CIC-2 (7), and CIC-3 (8) are reported to be volume activated Cl^- channels/channel regulators in most epithelial cell types. In ciliary epithelium the activation of Cl^- currents as a response to the swelling and subsequent reduction in volume of these cells (RVD) after exposure to hypotonic solutions has been noted (3, 4, 9). Regulatory volume increases have also been studied. (10, 11). Volume-sensitive Cl^- currents have been reported in both bovine NPE and PE cells. They are mediated by different Cl^- channels, P-glycoprotein in bovine cultured NPE cells (4), later shown sensitive to PKC (9). pI_{Cln} and CIC-3 were reported present in a cultured transformed human NPE cell line (3, 12) and the properties of swelling-activated Cl^- currents recorded were consistent with pI_{Cln}. pI_{Cln} was also detectable by Northern analysis from this cell line (3). Previously, by in situ hybridization, we reported that there is pI_{Cln} in both NPE and PE cells in rabbit (13). Botchkin and Matthews (14) found swelling-induced Cl^- currents in rabbit NPE cells. PE cells were not characterized nor have swelling induced currents been characterized in freshly prepared cells. The purpose of the present study was to examine the electrophysiologic properties of the swelling-induced Cl^- channel/channel regulatory protein, pI_{Cln}, [previously cloned from human NPE cell line (3), and, more recently, from rabbit ciliary epithelium (13)], to characterize these in separated and freshly prepared cell layers, and, finally, to confirm these properties utilizing recordings from Xenopus oocytes injected with pI_{Cln} cRNA. Our new finding include swelling-induced Cl^- currents observed in freshly prepared PE as well as NPE from the rabbit. In addition, Xenopus oocytes, injected with cRNA encoded rabbit pI_{Cln}, produced an outwardly rectifying Cl^- current displaying features consistent with the swelling-induced Cl^- current seen in both NPE and PE cells.

MATERIALS AND METHODS

All procedures conformed with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research as adopted from the National Institutes of Health. Pigmented rabbits usually weighing between 2∼3 kg were anesthetized by intravenous with a 1:1 mixture of ketamine hydrochloride and xylazine hydrochloride, then heparinized with a excess of sodium heparin, usually 1000∼2500 U/kg. Both eyes were quickly enucleated and transferred to cold physiological buffered salt solution (BSS). For isolating NPE and PE cells, we adopted a previously described method (15, 16).

Whole-cell currents were recorded with a Dagan 3900A patch-clamp amplifier (Dagan, MN, USA) from single NPE and PE cells. Recording pipettes were fabricated from borosilicate glass pulled in a two-stage micropipette puller (Narishige PP-83, Tokyo, Japan), heat-polished under a microscope (Narishige MF-83, Tokyo, Japan). The electrode resistance was 4∼7 MO when filled with the pipette solution. Test solutions were applied locally to patches using a 4-channel gravity-feed microperfusion system combined with simultaneous bath perfusion. Connection to the external reference was made

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with a 3M KCl-agar-filled electrode to reduce changes of liquid junction potentials with the various bath solutions. The junction potential between the standard pipette and bath solution ranged up to 6 mV and corrections in current voltage relationships toward the hyperpolarizing direction were made accordingly (17). Currents were recorded using a Dagan 3900A integrating patch-clamp amplifier (Dagan, MN, USA), and conjunction with an A/D, D/A converter (Labmaster TL-1 DMA interface, Axon Instruments, Foster City, CA, USA). Currents were low-pass Bessel filtered at 2 kHz and digitized at 10 kHz. The filtered currents were displayed on an oscilloscope (Hitachi digital storage oscilloscope VC-6025, Japan) and stored in a hard disk for later analysis (pCLAMP v.6.0.3. Axon Instruments, Foster City, CA).

Cloning of pI_{Cln} has been previously described (13). Macroscopic patch currents were measured 3∼4 days after pI_{Cln} cRNA injection into Xenopus oocytes by standard inside-out configuration at room temperature. The electrode resistance was 7∼9 MO when filled with the pipette solution. The internal and external solutions both contained (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.4 (ND 96). The final free $Ca^{2+} \leq$ 100 mM ($CaCl_2$:EGTA = 1.8:5∼10), approximated the normal intracellular Ca^{2+} concentration. Procedures were as described above.

The composition of the bathing solution (BSS) used for the ciliary epithelium (NPE and PE cells) was as follows (mM): Na^+ 142.2, K^+ 4.7, Mg^{2+} 1.2, Ca^{2+} 1.8, $C1^-$ 118.4, HCO_3^- 33.2, $H_2PO_4^-$ 1.2, Glucose 6.9. BSS was aerated with 5% CO_2 and 95% O_2. The pH of BSS equilibrated with 5% CO_2 was about 7.4∼7.5. For Ca^{2+}, Mg^{2+}-free BSS, Ca^{2+} and Mg^{2+} were simply omitted from BSS. The pipette solution contained (in mM) 110 N-Methyl-D-Glutamate chloride (NMDG-Cl), 1 $MgCl_2$, 1 EGTA, 10 HEPES, 2 ATP, 80 D-Mannitol. The standard test solutions used in the present experiments are the same as the pipette solution except for 2 mM $CaCl_2$ and 1 mM ATP. In comparing the effects of isotonic (300 mosmol l^{-1}) and hypotonic (220 mosmol l^{-1}) suspension the ionic strength was kept constant, and the osmolality was varied by adding or omitting 80 mM D-mannitol.

All chemical reagents used in the present experiments were purchased from Sigma. (St. Louis, MO), and LC Laboratories (Woburn, MA). All drug solutions used were prepared fresh daily.

Experimental values are reported as mean ± S.E.M (n = number of patches). Statistical differences were evaluated by Student's t test.

RESULTS

NPE cell swelling induced Cl^- currents. Using the whole-cell patch clamp technique, all experiments were conducted with symmetrical Cl^- (116 mM), and K^+-free in both the pipette and bath solutions. Onset of current activation was not immediate, typically occurring 1∼2 min after solution exchange. The current reached its peak after 3∼5 minutes of hypotonic stress and did not run down during about 30-40 min of continued hypotonic stress. Figure 1 shows a typical trace of the whole-cell currents following the changes in holding membrane potential between −80 and +80 mV (Fig. 1A) and their current-voltage relationship (Fig. 1B). The currents showed little or no inactivation at positive membrane voltages and exhibited outward rectification. The current was carried by Cl^- because replacement of all extracellular Na^+ with $NMDG^+$ (under these conditions, the major current carrying ion is Cl^-) did not significantly affect the reversal potential of the swelling-induced Cl^- current [−0.2 ± 0.09 mV, (3)]. These observations were further complemented by measuring the reversal potential at two different extracellular Cl^- concentrations. When external Cl^- was reduced from 116 mM to 6 mM by replacement with gluconate, and the intracellular Cl^- concentration was kept constant at 116 mM, the outward current was reduced (decreased Cl^- influx) and the reversal potential shifted from −0.7 ± 0.05 mV (5) to 31.3 ± 0.7 mV (5) (Fig. 2B). This shift in the expected direction, was close to the theoretical value for a Cl^--selective channel. Ion permeabilities relative to Cl^- were calculated from the Goldman-Hodgkin-Katz equation and the permeability ratio of $P_{gluconate}/P_{Cl}$ was 0.26 (3). A similar value was observed for the pI_{Cln}-associated Cl^- current

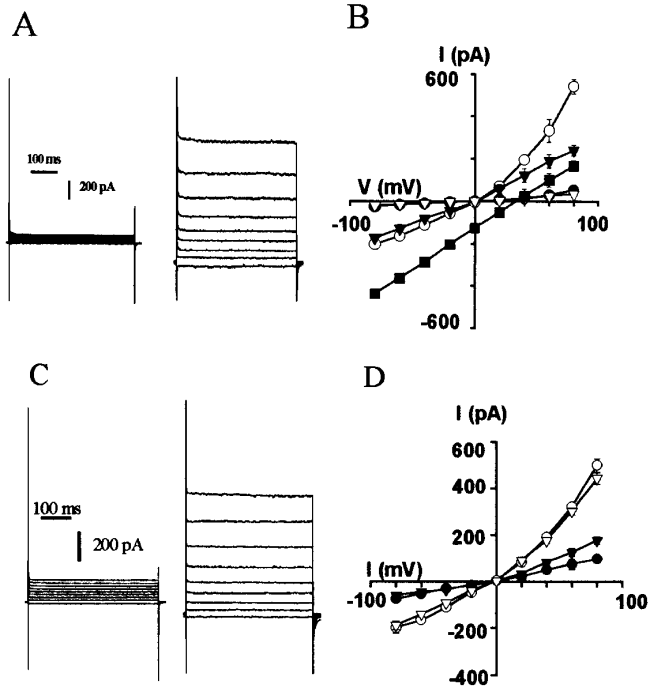

FIG. 1. Swelling-induced Cl^- current in NPE and PE cells. (A) Whole-cell currents recorded from an NPE cell. Recordings were done on several NPE cells. Data shown are from one NPE cell. From a holding potential of −70 mV, pulses from −80 to +80 mV in 20 mV steps were applied for 400 ms in isotonic solution (first panel) or hypotonic solution (second panel). (B) Current-voltage relationship of swelling-induced currents. Before (●) and during exposure to hypotonic solution (○); hypotonic bath solution with NPPB 100 μM (▽); hypotonic bath solution with DIDS 100 μM (▼); external 110 mM Cl^- by replacement with 110 mM $gluconate^-$ (■). The amplitudes of the currents were measured 50 ms after stepping to different voltages. Points and bars are the mean ± s.e.m. from 5 ∼10 cells, respectively. (C) Whole-cell recording performed on the same PE cell. Background isotonic current (first panel) was determined with the pulsing protocol used in A. The current was increased by hypotonic exposure (second panel). Recorded were done on several cells. Data shown are from one cell. (D) The current-voltage relationship of swelling-induced current is blocked by NPPB. In isotonic bath solution (●); hypotonic solution induced currents (○); hypotonic solution with 100 μM NPPB (▼); washout (in hypotonic bath solution without NPPB) (▽).The amplitudes of the currents were measured 50 ms after stepping to the different voltages. Points and bars are the mean ± s.e.m. (5). from 5 ∼10 cells, respectively.

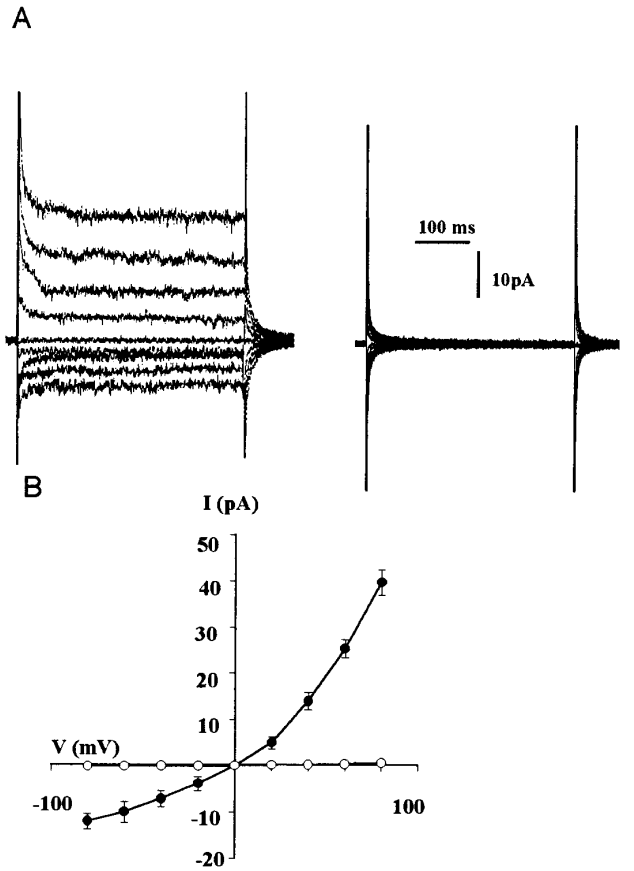

FIG. 2. pI_{Cln}-associated Cl^- current. (A) Inside-out patch clamp recording. After injection of Xenopus oocytes with pI_{Cln} cRNA (left panel). Control oocytes injected with 50 nl water (right panel). Holding potential, 0 mV. Voltage steps (400 ms) were made from −80 mV to +80 mV in 20 mV steps. ND 96 on both sides. (B) Current-voltage relationship (●) or control (○). The amplitudes of the currents were measured 50 ms after stepping to the different voltages. Points and bars are mean ± s.e.m. from 5∼ 10 patches, respectively.

in oocytes (18, 19). The activity of the swelling-induced Cl^- currents was not noticeably affected by omitting calcium and/or adding the calcium chelating agent EGTA (5∼10 mM) to the both solutions. The peak currents averaged 541.9 ± 34.9 pA [control 544.4 ± 32.7 pA, (5)] at +80 mV and −207 ± 24.9 pA [control −198.2 ±25.1 pA, (5)] at −80 mV, $p > 0.5$. Therefore this current was Ca^{2+} insensitive. The swelling-induced Cl^- currents were sensitive to Cl^- channel/transport blockers. 100 μM NPPB rapidly blocked the currents (Fig. 1B), and this effect was almost fully reversible. The nonselective anion transporter antagonist 100 μM DIDS also suppressed the swelling-induced Cl^- current (Fig. 1B), but this effect was usually not reversible. Addition of 2 mM cAMP in the extracellular solution, also suppressed the swelling-induce Cl^- currents (Fig. 3), but this effect was usually reversible after washout. A similar result was observed in the presence of 10 mM ATP (Fig. 3).

Consistent swelling-induced chloride current in PE cells. Under the same experimental conditions used for NPE cells, we have consistently found swelling-induced Cl^- currents in PE cells. The swelling-induced Cl^- current began to increase 1∼2 min after exposure to of hypotonic solution. The Cl^- current did not run down for the duration of the exposure to hypotonic solution and sustained for 30-40 min. Figure 1C and 1D shown the current profile and current-voltage relationship for the swelling-induced Cl^- current in PE cells. Although the current did not display prominent inactivation, it exhibited outward rectification. Current was blocked by 100 μM NPPB (Fig. 3), and this effect was almost full reversible. 100 μM DIDS also suppressed the swelling-induced Cl^- currents in PE cells, but this effect was usually not reversible (data not shown). The external nucleotides, 2 mM cAMP or 10 mM ATP, also suppressed the swelling-induced Cl^- current (Fig. 3).

pI_{Cln} expressed in Xenopus oocytes. Injection of pI_{Cln} cRNA into Xenopus oocytes produced an outward rectifying current measured by two-electrode voltage clamp without an osmotic challenge (13). The pI_{Cln}-associated current was further investigated using the inside-out patch clamp technique in Xenopus oocytes. 3∼4 days after injection of ∼50 ng of pI_{Cln} cRNA into oocytes, a macroscopic pI_{Cln}-associated Cl^- current was detected by patch clamp recording. This macroscopic pI_{Cln}-associated Cl^- current is consistent with that shown in previous studies (13). Xenopus oocytes injected with pI_{Cln}

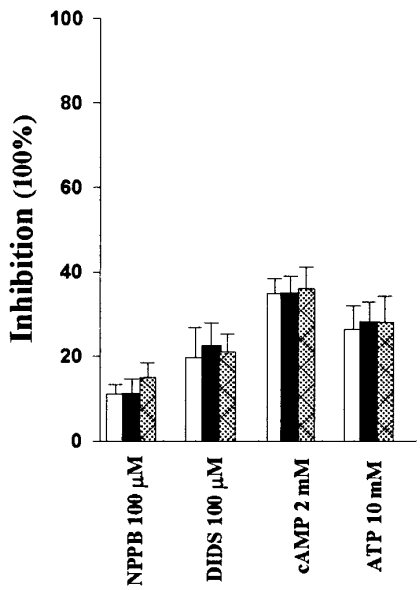

FIG. 3. Effects of various agents on pI_{Cln}-associated Cl^- current. Effects of application of 100 μM NPPB, 100 DIDS μM, 2 mM cAMP or 10 mM ATP on the swelling-induced Cl^- current: NPE cells (□), PE cells (■), Xenopus oocytes injected with pI_{Cln} cRNA (▨). The percent inhibition of the amplitude of the peak currents Cl^- (at +40 mV) before (control) and after application of the compound is displayed. Bars show mean ± s.e.m. from 5∼10 cells, respectively.

cRNA generated ~10 fold larger outwardly rectifying currents in the absence of osmotic challenge (Fig. 2A left panel), in contrast to the oocytes injected with water [Fig. 2B right panel, the peak currents averaged -0.9 ± 0.4 pA at -80 mV and 1.2 ± 2.8 pA at $+80$ mV, (7)], $p<0.01$. The macroscopic pI_{Cln}-associated Cl^- currents averaged -12.1 ± 1.4 pA at -80 mV and 39.67 ± 2.8 pA at $+80$ mV (5), and reversed at 0.3 ± 0.1 mV (5) with symmetrical Cl^- (ND 96). Replacing NaCl in the bath solution (the internal face of the membrane) with $NMDG^+$ did not affect the current profile or the reversal potential significantly [reversed at 0.2 ± 0.2 mV, (3)]; therefore, the current must be carried by Cl^-. Although the absolute amplitude of the pI_{Cln}-associated Cl^- current differed from the swelling-induced Cl^- current observed from ciliary epithelium, the profile of pI_{Cln}-associated Cl^- current was consistent with the swelling-induced Cl^- current recorded in ciliary epithelium (Fig. 2). This pI_{Cln}-associated Cl^- current was also sensitive to the Cl^- channel/transport blockers, 100 μM NPPB and 100 μM DIDS. DIDS (100 μM) blockade was only partially reversible whereas NPPB (100 μM) blocked reversed almost fully. 2 mM cAMP or 10 mM ATP to the pipette solution (ND 96) (the external face of the membrane), produced a smaller pI_{Cln}-associated current (Fig. 3). These blocking profiles are consistent with the behavior of the swelling-induced Cl^- current recorded from ciliary epithelium (Fig. 3).

DISCUSSION

Currents are induced in both NPE and PE cells of the rabbit eye after exposure to hypotonic stress. These swelling-induced Cl^- currents display an outward rectification, and little or no inactivation at positive membrane voltages (Fig. 1). In our experiments, the NPE and PE cells generated >10 fold Cl^- current in hypotonic stress, in contrast with currents found under isotonicity. Swelling-induced Cl^- currents dissipated when isotonic conditions were reestablished. The Cl^- channel/transporter blocks, 100 μM NPPB and 100 μM DIDS, blocked the Cl^- currents, and prevented the recovery from swelling. The pI_{Cln}-associated Cl^- current has been reported to be Ca^{+2} insensitive and blocked by external nucleotides (5, 18). The swelling-induced Cl^- current reported here was also found insensitive to Ca^{+2} and to be blocked by the external nucleotide, 10 mM ATP, or 2 mM cAMP (Fig. 3). pI_{Cln} has been characterized by its ability to bind nucleotides (5). Functionally, nucleotides have been shown to inhibit swelling-induced Cl^- currents (5, 18, 19). For example, Ackerman et al. (18) found that swelling-induced Cl^- currents were attenuated by extracellularly-applied cAMP with an IC_{50} value of 3.5 mM, and, by similar concentrations of ATP. But the addition of membrane permeant 1 mM dibutryl cAMP did not suppress swelling-induced Cl^- currents beyond that observed for cAMP suggesting an extracellular site of action. In our experiments as well, pI_{Cln}-associated Cl^- currents show sensitivity only to extracellular ATP or cAMP.

Swelling-induced Cl^- currents have been reported from human NPE cell lines (3), bovine ciliary epithelium (3, 4, 9), and rabbit NPE cells (14), displaying outward rectification, sensitive to the Cl^- channel blocker, NPPB. The properties of these swelling-induced Cl^- currents appear similar by electrophysiologic technique. Data from molecular biology indicate that there are at least three kinds of Cl^- channels or possible Cl^- channel regulators in ciliary epithelium: pI_{Cln} (3), P-glycoprotein, the product of the multidrug resistance (MDR1) gene (4), and CIC-3 (12). Although there are similarities among them, no sequence homology is found (5, 6, 20). It is suggested that MDR1 might perform the function of an ATP-dependent drug efflux pump and a volume-sensitive Cl^- channel or possible Cl^- channel regulator (6, 21, 22). In our experiments, the character of the swelling-induced Cl^- currents recorded from rabbit NPE and PE cells is consistent with a pI_{Cln}-associated Cl^- current.

Xenopus oocytes are commonly used for heterologous expression of proteins, particularly those involved in ion transport (23, 24, 25, 26). Several proteins involved in Cl^- conductance have been expressed in oocytes, including pI_{Cln} (5, 19). Voets et al. (19) could only find endogenous swelling-induced Cl^- currents in manually defolliculated or follicle-enclosed oocytes. Voets et al. (19) injected human pI_{Cln} RNA into oocytes. They have reported that pI_{Cln} was readily expressed in collagenase-defolliculated oocytes, but was not modulated by extracellular hypotonicity. In our experiments, only collagenase-defolliculated oocytes were used. Cl^- currents were recorded from oocytes after injection of pI_{Cln} cRNA [-12.1 ± 1.4 pA at -80 mV and 39.67 ± 2.8 pA at $+80$ mV (7)], with only small background currents from controls [uninjected or water injected into oocytes], -0.9 ± 0.4 pA at -80 mV and 1.2 ± 2.8 pA at $+80$ mV (7), $p<0.01$, as noted before (13). The outward rectifying Cl^- current measured in oocytes injected with pI_{Cln} cRNA was consistent with the swelling-induced Cl^- current measured from both NPE and PE cells (Fig. 3). The results agree with pI_{Cln}-associated currents previously reported (5, 18, 19). The Cl^- currents could not be changed by applying suction to isolated patches of the oocyte membrane. Thus pI_{Cln} may be a channel/channel regulator in NPE and PE cells, but may, as Voets et al. (19) concluded, be different from endogenous swelling-induced Cl^- currents in oocytes.

We have described the electrophysiological characteristics of the cAMP-activated Cl^- channel in rabbit NPE cells and dog NPE cells during isotonic conditions (15, 16). In NPE the pI_{Cln}-associated Cl^- current was clearly different from the cAMP-activated Cl^- current, but was never observed in rabbit PE cells (S. Chen & M. Sears, unpublished observations). The cAMP-activated

Cl^- current showed slow activation at positive membrane voltages and displayed a nearly linear current-voltage relationship, but the pI_{Cln}-associated Cl^- current showed little or no inactivation at positive membrane voltages and exhibited an outward rectification. The pI_{Cln}-associated Cl^- current was actually suppressed by cAMP applied to the extracellular surface.

It is clear that a variety of Cl^- channels exist in both NPE and PE cells (27). What is the relationship between the different Cl^- channels and the secretion of aqueous humor? It is not surprising that secretion of chloride into the posterior chamber of the eye is accomplished through multiple pathways. At the present, there does not seem to be a close link between pI_{Cln} and cAMP-activation. pI_{Cln} expression is more abundant in NPE than PE cells (13), but present in both as in the case it MDR1 (4). On the other hand, a low conductance Cl^- channel in low abundance is present only in NPE cells, on their basolateral membranes, and is sensitive to cAMP (10, 15, 16). This cAMP sensitive Cl^- channel, present on the aqueous side of NPE cells, the secretory exit, and probably serves as a key modulator of aqueous secretion from the ciliary epithelium. With a low open probability, the latter may be rate limiting for aqueous production. Therefore, the ubiquitous pI_{Cln} and/or may be implicated in quick or short term local changes in tonicity of both the PE and NPE, perhaps with somewhat different volume regulators for each layer, as indicated by the work of Edelman et al. (28) and Mitchell et al. (9). Swelling or shrinkage of the ciliary epithelium initiates transduction of several signals and complex transport mechanisms (11, 12), likely different for each layer (11, 28, 29), and some, like the pI_{Cln} reaction described here, may be the same. These short term tonicity equilibrations in these cell layers provide a base upon which the larger and slower changes in aqueous secretion induced by the beta adrenergic system and its modulators are imposed.

ACKNOWLEDGMENTS

This work was supported by United States Public Service Grants EY08879 and EY00785. We thank Fred J. Sigworth for helpful discussions and a critical review of the manuscript and Yang Yang Yan for assistance with oocyte injection.

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