J. Membrane Biol. 150, 197–208 (1996)

The Journal of
Membrane
Biology

Association of ClC-3 Channel with Cl^- Transport by Human Nonpigmented Ciliary Epithelial Cells

M. Coca-Prados[1], J. Sánchez-Torres[1], K. Peterson-Yantorno[2], M.M. Civan[2,3]

[1]Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, CT;
[2]Department of Physiology, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104-6085
[3]Department of Medicine, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104-6085

Received: 26 June 1996/Revised: 11 December 1995

Abstract: Electrophysiologic and volumetric evidence link the swelling-activated Cl^- channels [gCl(Vol)] of nonpigmented ciliary epithelial (NPE) cells with the Cl^--channel/Cl^--channel regulator protein pI_{Cln}. However, inhibitors (verapamil and dideoxyforskolin) of another Cl^- channel/regulator (MDR1) have been found to inhibit the volume-activated transport response [the regulatory volume decrease (RVD)] of bovine NPE cells. We have addressed the possible molecular basis for the NPE Cl^- channels by volumetric measurements of ODM human NPE cells in hypotonic and isotonic test solutions, and by polymerase chain reaction (PCR) cloning and Northern analyses of the same cells.

Verapamil and dideoxyforskolin did inhibit the RVD. However, at a concentration (100 μM) which blocks >90% of the MDR1-associated Cl^- currents, forskolin had no effect on the volume-activated Cl^- channels or on the inhibition of those channels by protein kinase C. High concentrations of ATP (3.5 and 10 mM) and niflumic acid (IC_{50} ~ 200 μM) also block [gCl(Vol)]. The RVD is inhibited by 9-phenylanthranilic acid (DPC) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), unaffected by anthracene-9-carboxylic acid (9-AC), and stimulated by ionomycin. The Cl^--channel blockers NPPB, niflumic acid, DPC and 9-AC, and the Ca^{2+}-ionophore ionomycin had qualitatively similar effects on the rate of staurosporine-activated isotonic cell shrinkage.

These results support the concept that the volume-sensitive protein pI_{Cln} regulates the Cl^- channels, and that the same conduits subserve volume- and staurosporine-activated Cl^- release. Of the cloned and sequenced Cl^- channels, ClC-3 uniquely conforms to the stationary currents and PKC sensitivity of the NPE Cl^- channels. PCR amplifications of human cDNA libraries from ciliary body, NPE cells and retina with primers based on human ClC-3 and ClC-4 cDNA, and Northern analyses using the products generated indicated that ciliary epithelial cells express transcripts for ClC-3 (but not ClC-4). We suggest that ClC-3 provides the same conduit for both volume-activated and isotonically staurosporine-activated Cl^- channels of human nonpigmented ciliary epithelial cells.

Key words: Cl^- channels — pI_{Cln} — RVD — PKC — ClC-3 — ClC-4 — Cultured NPE cells

Introduction

The aqueous humor is secreted by the ciliary epithelium (Cole, 1977), a bilayer of nonpigmented (NPE) and pigmented (PE) ciliary epithelial cells whose apical faces are apposed. Based on the composition of the aqueous humor (Caprioli, 1987), the principal anion secreted is Cl^-. According to current models of aqueous humor formation, the final step in Cl^- secretion is the translocation from the NPE cells into the aqueous humor through Cl^- channels (Wiederholt & Zadunaisky, 1986; Carré et al., 1992; Edelman, Sachs & Adorante, 1994; Coca-Prados et al., 1995). The activity of the Cl^- channels is likely to be a rate-limiting factor in aqueous humor secretion (Coca-Prados et al., 1995). Cl^- channel activity of cultured NPE cells can be markedly stimulated either by inhibiting protein kinase C (PKC) activity (Civan et al., 1994; Coca-Prados et al., 1995) or by hypotonically swelling the cells (Yantorno et al., 1992; Civan et al., 1994; Anguíta et al., 1995); PKC inhibition also en-

Correspondence to: M.M. Civan

hances the response to cell swelling. Hypotonic swelling *per se* produces a very large activation of Cl^- (Yantorno et al., 1992; Edelman, Sachs & Adorante, 1994; Coca-Prados et al., 1995) and a much smaller activation of K^+ channels (Coca-Prados et al., 1995), leading to the primary release of KCl and the secondary release of water (Farahbakhsh & Fain, 1988; Yantorno et al., 1989; Edelman, Sachs & Adorante, 1994). This response to swelling is termed the regulatory volume decrease (RVD). This volume activation of the Cl^- channels is manifest in volumetric, as well as electrophysiologic measurements.

The molecular identity of the Cl^- channels in NPE cells is uncertain. Of the 12 nonsynaptic Cl^- channels/channel regulatory thus far cloned and sequenced, three [pI_{Cln} (Paulmichl et al., 1992), MDR1 (Valverde et al., 1992) and ClC-2 (Gründer et al., 1992)] have been reported to be volume sensitive. The largely voltage-independent kinetics of the Cl^- currents in cultured human NPE cells (Yantorno et al., 1992) and their association with a conserved human homologue of the rat pI_{Cln} point towards pI_{Cln} (Coca-Prados et al., 1995; Anguíta et al., 1995) as underlying the volume-activated Cl^- currents of the NPE cells. As well discussed by Jentsch (1994), it is currently uncertain whether pI_{Cln} is a novel membrane-spanning channel with a pore formed by an eight-stranded β-barrel (Paulmichl et al., 1992) or a cytoplasmic regulatory protein (Krapivinsky et al., 1994). However, in addition to pI_{Cln}, a recent preliminary report has also implicated MDR1 (Wu & Jacob, 1994) in the volume-activated Cl^- currents of bovine NPE cells in primary culture. It is also unclear whether MDR1 is a channel or a channel regulator (Jentsch, 1993). One aim of the current study was to examine whether the pharmacologic profile of the volume-activated NPE Cl^- movements conforms more closely to that reported for pI_{Cln} or MDR1. A second aim was to explore whether the modulation of NPE Cl^--channel activity by PKC could be direct, by phosphorylating a Cl^--channel component. The present results obtained from volumetric and Northern analyses support the concept that both the volume-sensitive Cl^--channel/channel regulator pI_{Cln}, and the PKC-sensitive Cl^- channel ClC-3 (Kawasaki et al., 1994*a*) are involved in Cl^- release by the NPE cells.

Materials and Methods

Cellular Model

The cells studied (ODM) originated from a primary culture of human nonpigmented ciliary epithelium (Martin-Vasallo, Ghosh & Coca-Prados, 1989). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, #11965-027, Gibco BRL, Grand Island, NY and 51-43150, JRH Biosciences, Lenexa, KS) with 10% fetal bovine serum (FBS, A-1115-L, HyClone Laboratories, Logan, UT) and 50 μg/ml gentamycin (#15750-011, Gibco BRL, Grand Island, NY), at 37°C in 5% CO_2 (Yantorno et al., 1989). The medium had an osmolality of 328 mOsm. Cells were passaged every 6–7 days and, after reaching confluence, were suspended in solution for study within 6–10 days after passage.

After harvesting of a single T-75 flask by a widely-used trypsinization approach (Yantorno et al., 1989), a 0.5-ml aliquot of the cell suspension in DMEM (or in Cl^- and HCO_3^--free medium, where appropriate), was added to 20 ml of each test solution. In each case, the pH was adjusted to 7.4. Four aliquots of cells were always studied. Usually, one aliquot served as a control, with the other three aliquots being exposed to different experimental conditions. On occasion, the four aliquots were divided into paired control and experimental samples under both hypotonic and isotonic conditions. Unless otherwise stated, comparisons were drawn only between experimental and control aliquots harvested from the same flask on the same day. Furthermore, the sequence of study of the suspensions was always randomized to preclude any systematic time-dependent artifacts.

Data Acquisition and Reduction

Cell volume was measured with a Coulter Counter (model ZBI-Channelyzer II), using a 100-μm aperture (Civan et al., 1992). As previously described (Yantorno et al., 1989), the cell volume (v_c) of the suspension was taken as the peak of the distribution function. The time course of cell shrinkage in the hypotonic test solution was fit with the monoexponential function:

$$v_c = v_\infty + (\Delta v) \cdot (e^{-kt}) \quad (1)$$

where v_∞ is the steady-state cell volume, Δv is the maximal swelling, and k is the rate constant of the RVD. The rate constant and, to a lesser extent, v_∞ varied largely as a function of passage number, so that parallel controls were always conducted during each experiment. Unless otherwise stated, experimental effects were quantified by measuring the percent change in the rate constant k (Table 2).

Values are presented as the means ± 1 SE. The number of experiments is indicated by the symbol N. The probability (P) of the null hypothesis has been calculated using Student's t-test.

Solutions and Chemicals

The compositions of the iso- and hypotonic test solutions are presented in Table 1. In comparing the effects of iso- and hypotonic suspension, the ionic strength was maintained constant, and the osmolality was varied by adding or omitting sucrose (Yantorno et al., 1989; Civan et al., 1992, 1994).

All chemicals were reagent grade. Niflumic acid, ATP and forskolin were purchased from Sigma Chemical (St. Louis, MO), anthracene-9-carboxylic acid (9-AC) from Aldrich Chemical (Milwaukee, WI), 9-phenylanthranilic acid (DPC) from Fluka (Ronkonkoma, NY), 4,4′-diisothiocyano-2,2′-disulfonic acid (DIDS) from Molecular Probes (Eugene, OR), and verapamil from Knoll Pharmaceutical (Whippany, NJ). Dideoxyforskolin was bought from Calbiochem (San Diego, CA) and staurosporine from Biomol Research Laboratories (Plymouth Meeting, PA). NPPB [5-nitro-2-(3-phenylpropylamino)-benzoate] was a gracious gift from Prof. Rainer Greger (Albert-Ludwigs-Universität, Freiburg, FRG).

Polymerase Chain Reaction (PCR) Amplification and Sequencing of Human ClC-3 and ClC-4

The PCR method of Saiki et al. (1985) was used to anneal primers based on the human retina ClC-3 cDNA nucleotide sequence (Borsani

Table 1. Compositions of solutions (in mM or mOsm)

Component	S1	S2	S3	S4
NaCl	110.0		55.0	40.0
$NaCH_3O_4S$		135.0		
HEPES (acid)	15.0	10.0	7.5	7.5
HEPES (Na^+)		5.0		
$CaCl_2$	2.5		1.2	1.2
$CaSO_4$		2.5		
$MgCl_2$	1.2		0.6	0.6
$MgSO_4$		1.2		
KCl	4.7		2.4	2.4
KCH_3O_4S		4.7		
KH_2PO_4	1.2	1.2	0.6	0.6
$NaHCO_3$	30.0		15.0	15.0
Glucose	10.0	10.0	10.0	10.0
Sucrose			146.0*	146.0*
Osmolality (in mOsm)				
Isosmotic	295–306	306–307	305–315	319–323
Hyposmotic			155–162	164–172

S1 and S2 Ringer's solutions contain full ionic strength with or without Cl^- and HCO_3^-, respectively. S3 and S4 Ringer's solutions are at half-ionic strength, differing only in the concentration of NaCl. S3 and S4 were prepared in isotonic and hypotonic forms by including or omitting sucrose (*).

et al., 1995) (accession number X78520) to DNA from three human cDNA libraries: ciliary body (Escribano, Ortego & Coca-Prados, 1995), ODM-2 cells (Escribano et al., 1994) and retina (Swaroop & Xu, 1993). The forward primer (nucleotides 907–930): 5′-GGGCACTGGCCGGATTAATAGACA-3′; and reverse primer (nucleotides 1450–1473): 5′-GTGCACCAAAAGCTACAGAAACCC-3′ were selected according to the Primer Select program (DNASTAR, Madison, WI). Reaction mixtures consisted of 100 μl-volumes in MicroAmp tubes (Perkin Elmer, Emeryville, CA), containing (in mM): 10 Tris-Cl (pH 9), 50 KCl, 0.1% Triton X-100, and 1.5 $MgCl_2$ as buffering components, and DNA from 10^8 pfu of λ Uni-ZAP, 200 μM (each) of desoxynucleoside triphosphates, each oligonucleotide primer at 1 μM, and 5 U of Taq polymerase (Gibco) as reaction components. PCR was performed in a Perkin Elmer DNA Thermal Cycler (#480, Perkin Elmer Cetus, Norwalk, CT). Samples were inserted into the machine during an initial incubation of the lambda phage particles at 94°C for 2 min. Each cycle consisted of a denaturation step at 94°C for 1 min, 1 min of annealing at the optimal temperature (59°C), and 1 min of polymerization at 72°C. This cycle was repeated for 35 cycles; the final polymerization step was extended by an additional 5 min at 72°C. Five percent of the resulting PCR product was size-fractionated by electrophoresis on 1% agarose-SeaKem LE gels (FMC BioProducts, Rockland, ME) (*see* Fig. 6*A*). The 567-bp PCR product for human ClC-3 (Fig. 6*A*) was directly sequenced using a new method based on a Sequenase PCR sequencing kit (United States Biochemical, Cleveland, OH). The nucleotide sequence showed 100% homology with the human ClC-3 nucleotide sequence recently published (Borsani et al., 1995).

Based on the human retina ClC-4 cDNA nucleotide sequence (van Slegtenhorst et al., 1994) (accession number X77197), we designed a set of primers and hybridized to DNA from the three libraries indicated above. The forward primer (nucleotides 684–707): 5′-GGGGTCTGCCTGT-CTGCCTTCTGG-3′; and reverse primer (nucleotides 1121–1144): 5′-TCCCGCCTCTTGCC-CTCATTCTTG-3′ were selected (Fig. 5) according to the Primer Select program (DNASTAR, Madison, WI). The conditions for PCR amplification were as indicated above for ClC-3 except for the annealing temperature, which was 61°C. An expected 461-bp PCR product was obtained in the retina library, but not in the ciliary body or cell line libraries (*see* Fig. 6*B*). The PCR product was directly sequenced and displayed 100% homology with the human ClC-4 (van Slegtenhorst et al., 1994) (*data not shown*).

RNA Isolation and Northern Analysis

RNA was isolated from human ciliary body, human retina and the ODM-2 cell line by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). For Northern analyses, 20 μg of total RNA were subjected to agarose gel electrophoresis and transferred to Nytran plus (Schleicher & Schuell, Keene, NH). After transfer, the membranes were crosslinked with a UV crosslinker apparatus (Stratagene), prehybridized, sequentially hybridized with the 567-bp fragment of human ClC-3 and the 461-bp fragment of ClC-4 generated by PCR, and then washed to high stringency as previously described (Escribano et al., 1994).

Results

Regulatory Volume Decrease

Cl^--channel Inhibitors

The Cl^--channel inhibitors NPPB and DIDS have been found to block both the Cl^--channel/Cl^--channel regulator pI_{Cln} (Paulmichl et al., 1992) and the RVD of the ODM line of NPE cells (Yantorno et al., 1989; Civan et al., 1992). On the other hand, the Cl^--channel blocker niflumic acid has been reported to have no effect on pI_{Cln}-associated Cl^- currents (Ackerman, Wickman & Clapham, 1994) at concentrations <50 μM, while modestly inhibiting the RVD of NPE cells at 50–100 μM (Civan et al., 1994). This apparent discrepancy is resolved by the dose-response results presented in Fig. 1 and Table 2 (entry A). Niflumic acid can clearly inhibit the RVD by about two-thirds, but the concentration needed for half-maximal inhibition can be estimated to be ~200 μM, fourfold higher than the largest concentration applied by Ackerman et al. (1994).

Table 2 also presents the results obtained with two additional widely used Cl^--channel inhibitors, 9-AC and DPC. These experiments were conducted in the presence of 5 μM gramicidin, an ionophore which provides an exit path for K^+, so that the RVD is limited largely by the Cl^--channel activity (Civan et al., 1994). Under these conditions, the 9-AC had no significant effect on the RVD of the NPE cells (entry B), but DPC reduced the rate constant by 30 ± 10% (entry C, $P < 0.05$). Comparable data have not yet been reported for pI_{Cln}-associated Cl^- currents in other cells, and are therefore unavailable for comparison.

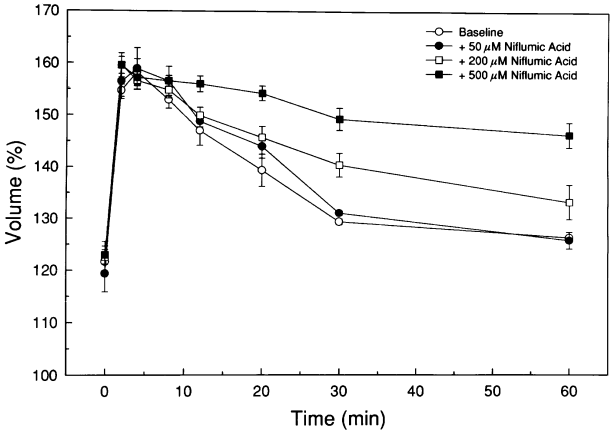

Fig. 1. Effect of niflumic acid on the regulatory volume decrease (RVD). Following hypotonic swelling, the cell volume spontaneously returned towards the initial isotonic value (100%). Increasing concentrations of niflumic acid slowed the regulatory response with an IC_{50} of ~200 μM (entry A, Table 2).

Nucleotides

pI_{Cln}-associated Cl^- currents have been reported to be inhibited by a wide range of nucleotides, generally in high concentration (Paulmichl et al., 1992; Ackerman, Wickman & Clapham, 1994). For example, 1-mM levels of cAMP and cGMP were found to inhibit the Cl^- currents of *Xenopus* by ~70% (Paulmichl et al., 1992). At this concentration, these cyclic nucleotides do not affect volume-activated Cl^- release by NPE cells (Civan et al., 1994). On the other hand, the results of Fig. 2 and Table 2 (entry D) demonstrate that another nucleotide, ATP, inhibits the RVD by two-thirds at 3.5–10 mM. These results are consistent with the observation that ATP partially blocks pI_{Cln} at 1–10 mM (Ackerman, Wickman & Clapham, 1994).

Inhibitors of MDR1

Wu and Jacob (1994) have recently described pharmacologic properties of the volume-activated Cl^- currents of bovine NPE cells in primary culture. Two of the agents which inhibited the currents, dideoxyforskolin and verapamil, are known to inhibit MDR1, as well (Valverde et al., 1992), but their actions on the other cloned and sequenced nonsynaptic Cl^- channels and Cl^--channel regulators are unknown.

At 100 μM, verapamil did not affect the rate constant of the RVD (entry E, Table 2). However, this agent is also known to interact with other channels (Miller, 1987), so that the apparent unresponsivity might have reflected a complex interaction of multiple effects. This possibility was addressed by repeating the experiment in the presence of 5 μM gramicidin, so that the K^+ channels could play only a minor role in the regulatory volume response (Civan et al., 1994). Under these conditions, verapamil significantly inhibited the RVD by 35 ± 8% (entry E, Table 2).

Verapamil's action could have been direct, or indirect by blocking L-type Ca^{2+} channels (Miller, 1987). The latter possibility was supported by the observation that D-diltiazem, another Ca^{2+}-channel blocker (Miller, 1987) also inhibited the RVD (entry F, Table 2), whereas the Ca^{2+} ionophore ionomycin stimulated the RVD (entry G, Table 2). This point was pursued by reexamining the effects of verapamil in the presence of 2 μM ionomycin, as well as gramicidin. Even when Ca^{2+} entry was facilitated by the ionophore, verapamil still significantly inhibited the RVD. We conclude that verapamil directly inhibits the volume-activated Cl^- channels of the ODM cells. As indicated in Table 2 (entry H), 100 μM dideoxyforskolin also significantly inhibited the RVD, either under baseline conditions or in the presence of gramicidin.

Thus, we have verified (with ODM cells) the preliminary report by Wu and Jacob (1994) (using cultured bovine cells) that verapamil and dideoxyforskolin inhibit the RVD of nonpigmented ciliary epithelial cells. However, both these inhibitors are known to block a number of different channels (Miller, 1987; Nishizawa et al., 1990). To further test the potential role of MDR1, we have used forskolin, which nearly abolishes MDR1-associated Cl^- currents at a concentration of 100 μM (Fig. 3, Valverde et al., 1992). Rather than inhibiting the RVD, forskolin increased the rate constant (entry I, Table 2). Forskolin also stimulates cAMP production by activating the catalytic subunit of adenylyl cyclase. Since cAMP is known to stimulate K^+ channels of NPE cells (Civan et al., 1994), these experiments were repeated in the presence of gramicidin. Under these conditions, forskolin had no effect on the RVD (Fig. 3; entry J, Table 2).

Regulation of the RVD

The preceding volumetric results are in accord with the hypothesis that pI_{Cln} underlies the volume-sensitivity of the Cl^- channels. One of the major modulators of the RVD of the NPE cells is protein kinase C (PKC). Activation of PKC with the diacylglycerol DiC_8 blocks, and inhibition of PKC with staurosporine enhances, the RVD (Civan et al., 1994). However, pI_{Cln} in the NPE cells does not display a consensus site for PKC phosphorylation (Paulmichl et al., 1992), so that the effects of PKC must be mediated either directly by another Cl^--channel/Cl^--channel inhibitor or indirectly by phosphorylation of another modulator. The Cl^- channel or Cl^--channel regulator MDR1 has been reported to play such a role in transfected HeLa cells (Hardy et al., 1995). However,

Table 2. Effects of Cl^--channel inhibitors and ionomycin on rate constant of RVD

Conditions	Concentration	% Change in RVD	*N*	*P*
A. *Niflumic acid:*	50 μM	−10.6 ± 14.2	3	>0.5
	200 μM	−57.0 ± 4.0	3	***<0.005***
	500 μM	−83.0 ± 3.1	3	***<0.001***
B. *9-AC* (*Gramicidin present*)	1 mM	−14.3 ± 9.6	4	>0.1
C. *DPC* (*Gramicidin present*)	1 mM	−29.6 ± 10.5	4	***<0.05***
D. *ATP* (*Gramicidin present*)	3.5 mM	−60.6 ± 6.4	3	***<0.005***
	10 mM	−67.6 ± 3.8	3	***<0.001***
E. Verapamil				
(*Baseline*)	100 μM	−29.3 ± 13.0	7	>0.05
(*Gramicidin present*)	100 μM	−34.6 ± 8.1	4	***<0.02***
(*Gramicidin + Ionomycin*)	100 μM	−47.4 ± 5.8	4	***<0.005***
F. *Diltiazem*	20 μM	+3.4 ± 10.1	8	>0.7
	200 μM	−23.0 ± 5.8	8	***<0.005***
	300 μM	−44.0 ± 7.6	8	***<0.001***
	300 μM	−53.8 ± 20.4	4	***<0.025***
	1 mM	−82.9 ± 22.7	8	***<0.005***
G. *Ionomycin*				
(*Baseline*)	2 μM	+82.8 ± 17.5	10	***<0.001***
(*Gramicidin present*)	2 μM	+39.2 ± 16.1	4	***<0.05***
H. *Dideoxyforskolin*				
(*Baseline*)	100 μM	−73.9 ± 3.2	4	***<0.001***
(*Gramicidin present*)	100 μM	−35.7 ± 10.1	4	***<0.05***
I. *Forskolin* (*Baseline*)	10 μM	+54.2 ± 22.5	4	>0.05
	50 μM	+60.2 ± 22.4	4	***<0.05***
	100 μM	+57.8 ± 19.3	4	***<0.025***
J. *Forskolin* (*Gramicidin*)	10 μM	+13.8 ± 11.1	4	>0.2
	50 μM	−0.6 ± 9.6	4	>0.95
	100 μM	+8.2 ± 15.0	4	>0.6
K. DiC_i				
(*Baseline*)	250 μM	−45.6 ± 8.4	4	***<0.02***
(*100 mM Forskolin*)*	250 μM	−60.2 ± 8.0	4	***<0.01***

Gramicidin and ionomycin were used in concentrations of 0.3 μM and 5 μM, respectively.
* Forskolin itself (at 100 μM) accelerated the RVD by 56.5 ± 21.3% ($P < 0.05$).

the results presented above (Fig. 3, entries I–J, Table 2) argue against the participation of MDR1 in the regulatory volume decrease. Furthermore, the activation of PKC by adding 250 μM DiC_8 markedly inhibits the RVD, whether or not MDR1 is blocked with 100 μM forskolin (entry K, Table 2). Thus, as considered in the Discussion, the only nonsynaptic Cl^- channel thus far sequenced which displays the requisite electrophysiologic and biochemical properties and which could be operative in the NPE cells studied is ClC-3 (Kawasaki et al., 1994*a*,*b*). The ClC-3 channel has not yet been reported to be volume-sensitive. Therefore, we have also conducted measurements of cell volume in isosmotic suspensions, to determine if the pharmacologic properties of the Cl^- channels of the NPE cells are similar under isotonic and hypotonic conditions.

Time Course of Cell Volume under Isosmotic Conditions

Figure 4 presents the time courses of the cell volumes in isotonic test solutions. Panels *A* and *B* present the time courses under baseline and experimental conditions. The magnitudes of the rate and extent of spontaneous cell shrinkage depended in part upon the isotonic solution

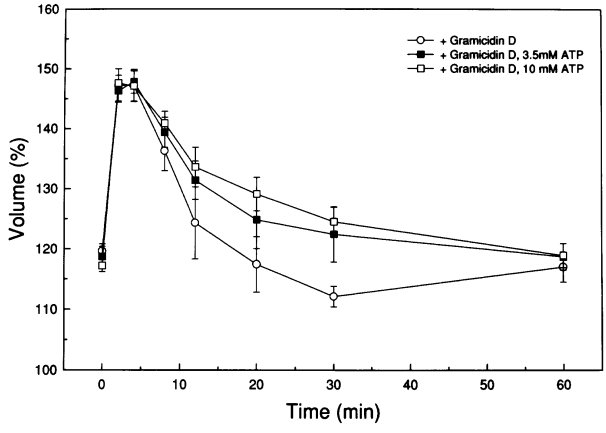

Fig. 2. Effect of external ATP on the regulatory volume decrease. Gramicidin (5 μM) was included in the test solution so that the rate of the RVD was not limited by K^+ release. Under these conditions, external ATP slowed the RVD by two-thirds (entry D, Table 2).

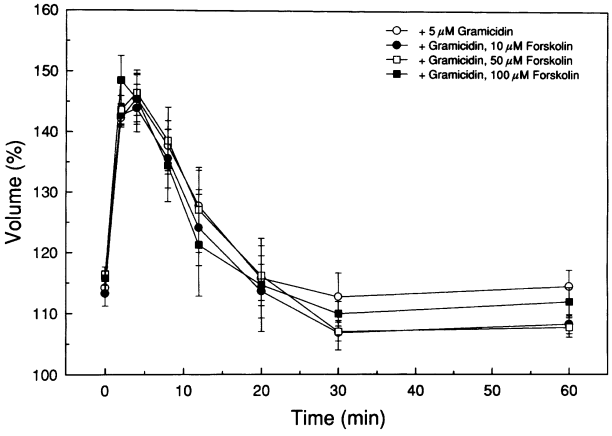

Fig. 3. Effect of forskolin on the RVD. Addition of 5 μM gramicidin produced a characteristic speeding of the RVD. Under these conditions, increasing concentrations of forskolin had no significant effect on the RVD (entry J, Table 2).

chosen as test solution (Table 1), and upon the passage number. This complexity likely reflected the fact that a number of different molecules (including both nonelectrolytes and electrolytes) were released by the cells upon transfer from DMEM to the test solutions (*see* Materials and Methods), each with characteristic kinetics. Given the complexity of baseline solute release and the small magnitude of the isosomotic shrinkage (*cf.* Figs. 1–3 with Fig. 4), it is not surprising that fitting the time course with a single exponential was less satisfactory than for the RVD. On the other hand, the complex release of multiple solutes is expected to be common to both the control and experimental isosmotic suspensions. Therefore the time courses for the differences between the experimental and baseline volumes were not unexpectedly found to be satisfactorily fit by a single exponential. The rate constants for the time courses of these point-by-point differences are itemized in Table 3, and the time courses for some of these volume differences are displayed in panel *C*, Fig. 4.

At a concentration of 0.3 μM, staurosporine markedly increased the rate constant and extent of the spontaneous cell shrinkage (entry A, Table 3), both under baseline conditions (open triangles, Fig. 4*A*, and open squares, Fig. 4*C*) and in the presence of 5 μM gramicidin. The staurosporine-triggered effect was almost completely abolished by the nominal removal of Cl^- and HCO_3^- from the test solution (filled squares, Fig. 4*C*). The staurosporine-induced acceleration of cell shrinkage was largely blocked by the Cl^--channel inhibitor NPPB (100 μM) (entry B, Table 3), both in the absence (closed symbols, Fig. 4*A*) and presence (filled rhomboids, Fig. 4*B*, and open hexagons, Fig. 4*C*) of gramicidin. Not only NPPB, but also niflumic acid (entry C, Table 3) and DPC (entry D, Table 3) inhibited the rate of cell shrinkage under conditions where the rate of K^+ release was not likely to be rate-limiting. The inhibitor 9-AC had no significant effect (entry E, Table 3). The Ca^{2+} ionophore ionomycin (2 μM) produced a small but significant increase in cell shrinkage (entry F, Table 3).

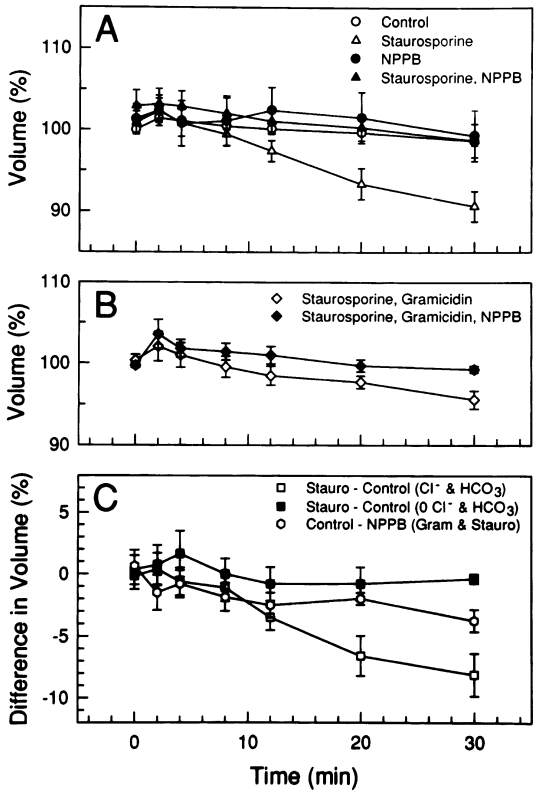

Fig. 4. Measurements of the time courses of cell volumes in isotonic suspensions (S1 solution, Table 1). Panels *A* and *B* display absolute time courses, and panel *C* presents the time courses of the point-by-point differences between several of the experimental and control measurements.

Staurosporine (0.3 μM) markedly accelerated the rate of spontaneous cell shrinkage, both under baseline conditions (open triangles, panel *A*, and open squares, panel *C*) and in the presence of 5 μM gramicidin (open rhomboids, panel *B*). The staurosporine-triggered effect was almost completely abolished by the nominal removal of Cl^- and HCO_3^- from the test solution (filled squares, panel *C*). The staurosporine-induced acceleration of cell shrinkage was largely blocked by the Cl^--channel inhibitor NPPB (100 μM), both in the absence (closed triangles, panel *A*) and presence (closed rhomboids, panel *B*, and open hexagons, panel *C*) of gramicidin.

Thus, the results of Fig. 4 and Table 3 indicate that staurosporine activated Cl^- channels under isosmotic conditions. The Cl^--channel inhibitors (NPPB, niflumic acid and DPC) which blocked the RVD also slowed the rate of isosmotic shrinkage. The Cl^--channel inhibitor (9-AC) which had no effect on the RVD also had no effect under isotonic conditions. These results are consistent with the electrophysiologic report that hypotonic

Table 3. Time course of differences in volume of cells in isosmotic solution between experimental and baseline suspensions

Experimental conditions	$k(RVD)$ ($10^{-3} \cdot min^{-1}$)	Δv_∞ (%)	N	P
A. *Stauro*				
(*No Gram*)	−23.8 ± 2.1	−15%	8	***<0.001***
(*Gram*)	−205.3 ± 11.9	−5%	4	***<0.001***
B. *NPPB*				
(*30 μM, Stauro, No Gram*)	−164.1 ± 11.3	−6.4%	4	***<0.001***
(*100 μM, Stauro, No Gram*)	−156.3 ± 21.7	−8.5%	16	***<0.005***
(*100 μM, Stauro, Gram*)	−154.1 ± 17.9	−4.4%	9	***<0.001***
C. *Niflumic*				
(*100 μM, Stauro, Gram*)	−315.0 ± 82.0	−4.5%	5	***<0.05***
(*NPPB, Same cond., prepn*)	−227.5 ± 14.8	−5.4%	5	***<0.001***
D. *DPC*				
(*1 mM, Stauro, Gram*)	−133.9 ± 29.3	−3.4%	5	***<0.025***
E. *9-AC*				
(*1 mM, Stauro, Gram*)	15.4 ± 73.7	−2.4%	5	>0.8
(*Same cond., prepn as D*)				
F. *Iono*				
(*No Gram*)	−53.0 ± 11.7	−1.7%	3	***<0.01***
G. *Stauro*				
(*No DMA*)	−46.9 ± 3.3	−6%	4	***<0.001***
(*DMA*)	−37.0 ± 14.1	−6%	4	***<0.05***
H. *Stauro*				
(*No Bumet*)	−69.8 ± 9.0	−4.6%	3	***<0.002***
(*Bumet*)	−79.1 ± 6.1	−8.8%	3	***<0.001***
I. *Stauro*				
(*No Stroph*)	−130.1 ± 43.3	−6.1%	4	***<0.05***
(*Stroph*)≠	−110.4 ± 30.0	−4.1%	4	***<0.05***

''Stauro,'' ''Gram'' and ''Iono'' refer to 0.3 μM staurosporine, 5 μM gramicidin and 2 μM ionomycin, respectively. The approximate magnitude of the steady-state shrinkage is entered under ''Δv_∞.'' (≠) Strophanthidin itself (at 100 μM) had no significant effect on the rate constant over the period of measurement, reducing the isotonic volume by 1.5% ($N = 4$, $P > 0.2$).

swelling and isotonic application of the PKC inhibitor staurosporine activate functionally similar Cl^- channels (Coca-Prados et al., 1995). These observations raise the possibility that the same Cl^- channel may underlie Cl^- release under both hypotonic and isotonic conditions.

As indicated above, the Cl^--channel/Cl^--channel regulator MDR1 does not appear to regulate Cl^- currents in the NPE cells of the present study. The only other cloned and sequenced Cl^- channel known to be down-regulated by PKC is ClC-3 (Kawasaki et al., 1994*a*). In addition, ClC-4 displays consensus sites for phosphorylation by PKC, although ClC-4-mediated Cl-currents have not yet been functionally expressed (van Slegtenhorst et al., 1994). To determine if ClC-3 and/or ClC-4 is in fact expressed by the nonpigmented ciliary epithelial cells, probes were generated by PCR amplification for purposes of Northern analysis.

PCR Amplification of Human DNA Homologous to ClC-3 and ClC-4

Using a set of oligonucleotide primers based on the nucleotide sequences of the human ClC-3 (Borsani et al., 1995) and the human ClC-4 (van Slegtenhorst et al., 1994) (Fig. 5), we amplified DNA by PCR from three human cDNA libraries, including ciliary body, ODM-2 cells and retina, as described in Material and Methods. Two expected DNA fragments of 567-bp and 461-bp were obtained for ClC-3 and ClC-4, respectively (Fig. 6). Both DNA products were sequenced directly without subcloning, using a Sequenase PCR sequencing kit (United States Biochemical, Cleveland, OH). The nucleotide sequence obtained from each PCR product confirmed that the DNA products amplified were 100% homologous to the nucleotide sequences for the human ClC-3 (Borsani et al., 1995) and the human ClC-4 (van Slegtenhorst et al., 1994), respectively (*data not shown*). The human retina library amplified DNA products for both ClC-3 and ClC-4. However, the ciliary body and ODM-cDNA libraries amplified only ClC-3.

Expression of Human ClC-3 Transcripts in the Human Ocular Tissues

To verify the expression of ClC-3 transcripts in ocular tissues, we probed a Northern blot containing RNA from

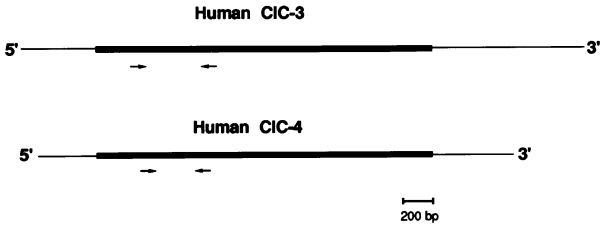

Fig. 5. Diagrams of the human ClC-3 and human ClC-4 cDNAs with coding regions (black box). Arrows indicate the relative positions of pairs of oligonucleotide primers selected to amplify by PCR. The primers used successfully in PCR were derived from oligonucleotides 907–930 (forward) and 1450–1473 (reverse) of the human ClC-3 cDNA sequence (accession number X78520) and 684–707 (forward) and 1121–1144 (reverse) of the human ClC-4 sequence (accession number X77197).

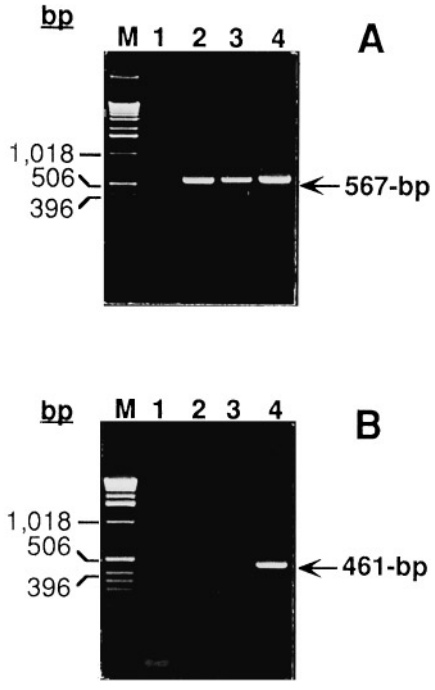

Fig. 6. PCR amplification of ClC-3 and ClC-4. (*A*). Human ClC-3 primers were annealed to recombinant lambda DNA from three directional cDNA libraries: no DNA or control (lane 1); ciliary body (lane 2); cell line (lane 3) and retina (lane 4). DNA products were resolved on a 1.5%-agarose gel. (*B*). Human ClC-4 primers were annealed to DNA from libraries, as in *A*. Conditions for PCR amplification are indicated in Materials and Methods. Marks to the left of gels *A* and *B* indicate the size (in base pairs, bp) of several DNA fragments of a DNA standard marker.

the cell line, the ciliary body and human retina (as positive control), with a radiolabeled human ClC-3 DNA probe (567-bp) obtained by PCR. Figure 7 shows that the human DNA probe hybridized to two major RNA species, one ranging from 6.0- to 6.5-kb and the other from 3.5- to 4.0-kb RNA from both tissues and from the

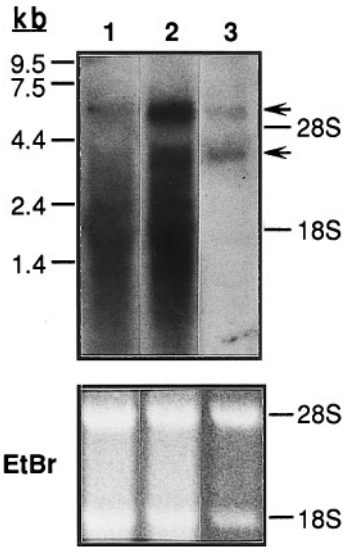

Fig. 7. Expression of ClC-3 transcripts in human ciliary body (lane 1), retina (lane 2) and ODM-2 NPE cells (lane 3). Equal amounts of RNA (20 μg) were size separated and hybridized with a radiolabeled DNA probe (567 bp) for human ClC-3 generated by PCR. Arrows at the right of the top gel indicate the positions of two major transcripts. At the bottom is the ethidium-bromide (EtBr) staining of the top gel showing the 28S and 18S markers.

cell line. These two mRNA species are in agreement with the sizes found in human retina and human nonocular tissues (Borsani et al., 1995). When the Northern blot was re-probed with the 461-bp DNA PCR product corresponding to ClC-4, only the retina RNA hybridized to an RNA of the same size as that for ClC-3. No hybridization signal (as expected from the PCR results) was obtained with RNA from either the cell line or the ciliary body (*data not shown*).

Potential Roles of Other Transporters in Mediating the Response to Staurosporine

Staurosporine has been documented to activate Cl^- channels in the NPE cells of the present study (Coca-Prados et al., 1995). However, much of the observed staurosporine-induced effects on volume could possibly have been mediated by stimulation or inhibition of three other transporters known to be present in these cells: a Na^+/H^+ antiport (Civan, Coca-Prados & Peterson-Yantorno, 1996), a $Na^+/K^+/2Cl^-$ symport (Mito, Delamere & Coca-Prados, 1993; Crook & Polansky, 1994), and the Na^+/K^+-exchange pump (Krupin et al., 1984; Martin-Vasallo et al., 1989). To address this possibility, three additional sets of experiments were performed with isotonic suspensions. In each case, the time courses of the cell volumes were monitored in the presence or absence of the 0.3 μM staurosporine. An inhibitor of one of the three

transporters was added at the time of the test suspension to half of the aliquots of cells. In particular, the Na^+/H^+ antiport was blocked with 20 μM dimethylamiloride, the $Na^+/K^+/2Cl^-$ symport with 0.5 mM bumetanide, and the Na^+,K^+-exchange pump with 100 μM strophanthidin. We have found these concentrations to provide effective blockage in previous measurements (Civan et al., 1996). In each case, staurosporine produced a significant cell shrinkage, whether or not the antiport, symport or pump was blocked (entries G–I, Table 3). We conclude that the documented isotonic effect of staurosporine on the Cl^- channels of these cells (Coca-Prados et al., 1995) is likely to be the major mediator of the isotonic response of cell volume to staurosporine.

Discussion

Regulatory Volume Decrease

Anisosmotic swelling of NPE cells triggers release of intracellular solute leading to the secondary loss of water (Farahbakhsh & Fain, 1988; Yantorno et al., 1989; Edelman, Sachs & Adorante, 1994). This spontaneous return towards the initial isotonic volume is termed the regulatory volume decrease (RVD), and is characteristic of most vertebrate cells (Hoffmann, 1987). The specific solute released by NPE cells is KCl, with K^+ and Cl^- leaving through parallel ion channels (Yantorno et al., 1989, 1992; Civan et al., 1992, 1994; Edelman, Sachs & Adorante, 1994; Anguíta et al., 1995). Both functional and molecular biological results suggest that the molecular basis for the volume-activated Cl^- release is pI_{Cln} (Coca-Prados et al., 1995; Anguíta et al., 1995), but recent pharmacologic observations raise the possibility that the Cl^--channel/Cl^--channel regulator MDR1 may also be playing a role (Wu & Jacob, 1994).

The present results provide a further functional characterization of the volume-activated Cl^- channels of nonpigmented ciliary epithelial cells. The Cl^--channel blocker niflumic acid had previously been noted to produce a small inhibition of the RVD (by 18 ± 4%) at 50–100 μM, while having no effect in electrophysiological measurements of pI_{Cln} in amphibian oocytes at <50 μM (Ackerman, Wickman & Clapham, 1994). The current data (Fig. 1, entry A, Table 2) verify that niflumic acid inhibits the volume-activated Cl^- channels of NPE cells, but at an IC_{50} of ~200 μM, at least fourfold higher than the concentration previously applied in electrophysiological studies. The Cl^--channel blocker DPC also inhibited the rate constant of the RVD by ~30% (entry C, Table 2), whereas 9-AC had no significant effect (entry B, Table 2).

Thus, the RVD of NPE cells is inhibited by at least four Cl^--channel inhibitors [NPPB (Civan et al., 1992), DIDS (Civan et al., 1992), niflumic acid, and DPC], and is unaffected by a fifth inhibitor (9-AC). These data are consonant with published information concerning the effects of Cl^--channel inhibitors on pI_{Cln}.

The current work also documents that external ATP inhibits the RVD of NPE cells (Fig. 2; entry D, Table 2). pI_{Cln}-associated Cl^- currents are also known to be inhibited by a wide range of nucleotides (Paulmichl et al., 1992; Ackerman, Wickman & Clapham, 1994), but the concentrations required for half-maximal inhibition seem to strongly depend on the preparation. For example, the IC_{50} of cAMP was found to be 3.5 mM for the volume-activated native Cl^- currents of *Xenopus* oocytes (Ackerman, Wickman & Clapham, 1994) and was 1–2 orders of magnitude lower for overexpressed pI_{Cln}-associated Cl^- currents in those oocytes (Paulmichl et al., 1992).

We have confirmed the report by Wu and Jacob (1994) that both verapamil and dideoxyforskolin inhibit the volume-activated Cl^- channels of NPE cells (entries E and H, Table 2). Both agents do block MDR1-regulated Cl^- channels (Valverde et al , 1992), but both inhibitors are known to block other channels as well (Miller, 1987; Nishizawa et al., 1990). As a further test of the possible role of MDR1, we have applied forskolin, which has been reported to inhibit MDR1-associated Cl^- currents by >90% with an IC_{50} of <50 μM (Fig. 3, Valverde et al., 1992). In contrast, forskolin in concentrations of 10–100 μM had no significant effect on the volume-activated Cl^- pathway of the current cells (Fig. 3; entry J, Table 2). We conclude that MDR1 cannot subserve the Cl^- release in the ODM NPE cells of this study. Instead, the foregoing evidence indicates that the volume-sensitive Cl^- channels of NPE cells are identical with, or regulated by, pI_{Cln}.

As noted above, activation of PKC inhibits the RVD (entry K, Table 2) and blocking of PKC with staurosporine enhances the RVD (Civan et al., 1994) of these cells. PKC cannot modify the RVD by acting directly on pI_{Cln}, since this protein does not display consensus sites for PKC phosphorylation (Paulmichl et al., 1992). Instead, three other types of mechanisms are possible: (i) PKC could phosphorylate an intermediary target, which in turn regulates transmembrane conduits formed by pI_{Cln}; (ii) Parallel conduits for Cl^- movement could be provided by the volume-sensitive pI_{Cln} and a separate PKC-sensitive Cl^- channel; and (iii) The volume-sensitive pI_{Cln} serves strictly as a regulator of a separate PKC-sensitive Cl^- channel. Possibilities (i) and (ii) are brought into doubt because of the concerns (Krapivinsky et al., 1994; Jentsch, 1994) whether pI_{Cln} itself forms a membrane-spanning conduit. The results of the current study bring further information to bear on differentiating possibilities (ii) and (iii). In particular, we have monitored cell volume in isotonic suspensions to examine whether the volume-sensitive and PKC-sensitive Cl^- conduits display different [as expected for possibility

(ii)] or the same pharmacologic profiles [as expected for possibility (iii)].

Isosmotic Shrinkage

Both electrophysiologic (Coca-Prados et al., 1995) and volumetric measurements (Civan et al., 1994) have indicated that inhibition of endogenous PKC activity with staurosporine leads to activation of Cl^- channels in ODM cells under isosmotic conditions. In the current study, we have characterized the pharmacologic profile of the staurosporine-activated Cl^- channels. As can be appreciated from comparing Tables 2 and 3, the same Cl^--channel blockers (NPPB, niflumic acid and DPC) which inhibit the volume-activated Cl^- channels also inhibit the staurosporine-activated Cl^- channels. The Cl^--channel blocker (9-AC) which has no effect on the volume-activated Cl^- channels (entry B, Table 2) also has no effect on the staurosporine-activated Cl^- channels (entry E, Table 3). Furthermore, 2 μM ionomycin stimulates both the volume-activated and the staurosporine-activated Cl^- channels (entry G, Table 2, and entry F, Table 3). Thus, our current volumetric measurements support our earlier conclusion (based on electrophysiologic evidence) (Coca-Prados et al., 1995) that the two sets of Cl^- channels (volume-activated and staurosporine-activated) are identical. Despite these pharmacologic similarities, it was possible that a large part of the staurosporine-induced effects on cell volume could have been mediated by actions on three other transporters known to be present in NPE cells. However, even when the Na^+/H^+ antiport, $Na^+/K^+/2Cl^-$ symport and Na^+,K^+-exchange pump were blocked, staurosporine still produced cell shrinkage in isotonic suspensions (entries G–I, Table 3). We conclude that staurosporine and swelling likely affect cell volume in either isotonic or hypotonic suspensions by acting on a single set of Cl^- channels. These results are consistent with possibility (iii) of the foregoing paragraph, suggesting that pI_{Cln} is acting as a regulator of a PKC-sensitive Cl^- channel.

Potential Role of ClC-3

The identity of the PKC-sensitive Cl^- channel was unclear. In principle, MDR1 might have fulfilled that role, but blockage of MDR1 with 100 μM forskolin neither affected the swelling-activated Cl^- channels (*as noted above*) nor altered the volume response to staurosporine (entry K, Table 2). Among the remaining 11 nonsynaptic Cl^--channels/Cl^--channel regulators thus far cloned and sequenced, the Cl^- channel ClC-3 appears unique in being inhibited by PKC and in subserving stationary Cl^- currents (neither activated nor inactivated by voltage) (Kawasaki et al., 1994*a*,*b*). Both properties are also displayed by the volume- and staurosporine-activated Cl^- currents of ODM NPE cells (Yantorno et al., 1992; Coca-Prados et al., 1995; Anguíta et al., 1995). Attempts by two other groups to express ClC-3 regulated Cl^- currents have not yet been successful (Borsani et al., 1995; Jentsch et al., 1995), but Kawasaki et al. (1995) have reported expression of such currents by stably transfecting CHO cells with ClC-3. Furthermore, another member of the ClC family (ClC-4) displays consensus sites for phosphorylation by PKC, but ClC-4 regulated currents have not yet been expressed (van Slegtenhorst et al., 1994).

The putative roles of ClC-3 and ClC-4 were examined by generating ClC-3 and ClC-4 DNA probes by PCR amplification of DNA from human cDNA libraries obtained from ciliary body, NPE cells and retina, using primers based on the human cDNA nucleotide sequences. Analysis of the PCR amplification products and of the Northern hybridizations using those products indicates that transcript for only ClC-3, and not ClC-4, is expressed by human ciliary epithelial cells.

Model

We suggest that the hypothesis presented in Fig. 8 is the simplest and most direct interpretation accommodating the data. ClC-3 is considered to provide the pore for Cl^- translocation, so that activators and inhibitors of this channel will produce qualitatively similar effects on the volume- and staurosporine-activated Cl^- currents. The volume-sensitive pI_{Cln} is considered to regulate movement through the ClC-3 channel by as yet unidentified mechanisms, and is tethered in the vicinity of the channel through actin-binding sites (Paulmichl et al., 1992). The ClC-3 channel is known to possess consensus sites for phosphorylation by PKC (at Ser-51 and Ser-362) and is directly downregulated by PKC (Kawasaki et al., 1994*a*). The model also incorporates the observation that ClC-3 displays consensus sites for phosphorylation by calcium-calmodulin (Ca/CaM)-activated kinase (at Thr-655 and also possibly at Ser-51) (Kawasaki et al., 1994*a*). Several lines of evidence indicate that Ca^{2+} certainly plays a role in supporting activation of the NPE Cl^- channels: (i) increasing the cytosolic Ca^{2+} activity enhances the volume-activated Cl^- channels (Civan et al., 1994); (ii) decreasing cytosolic Ca^{2+} activity has the opposite effect (Civan et al., 1992); and (iii) inhibiting Ca/CaM with trifluoperazine partially blocks the volume-activated Cl^- channels (Civan et al., 1992, 1994). However, we have not regarded an increase in cytosolic Ca^{2+} activity as the primary trigger in activating the Cl^- channels since hypotonicity activates the channels even when the intracellular Ca^{2+} activity is buffered with 5 mM BAPTA (Yantorno et al., 1992) and since transiently elevating the cytosolic Ca^{2+} activity with thapsigargin does not trigger cell shrinkage in isotonic medium (Civan et al., 1994). On the other hand, the more sustained increase in Ca^{2+} activity which can be produced by ionomycin does lead

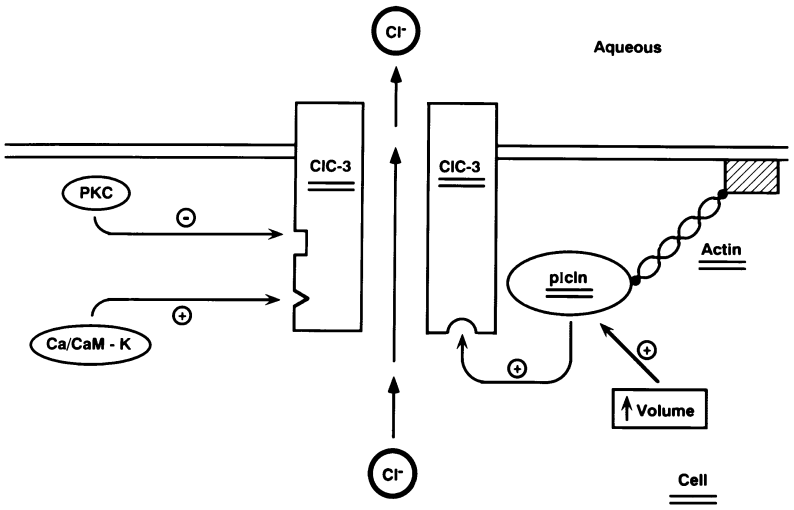

Fig. 8. Working hypothesis of basis for Cl^- secretion by NPE cells. Cl^- is considered to be released through ClC-3 channels, which are directly regulated by Ca/CaM-activated kinase by PKC. The ClC-3 channels are also pictured as being regulated by the volume-sensitive Cl^--channel regulator pI_{Cln}, which is considered tethered near the plasma membrane by interactions with actin.

to a small degree (<2%) of cell shrinkage (entry F, Table 3), possibly by enhancing the baseline level of ClC-3 phosphorylation by Ca/CaM-activated kinase.

Supported in part by research grants from the National Institutes of Health [EY08672, EY08343 and both EY00785 and EY01583 for core facilities]. J.S.-T. was supported in part by an ARVO/Alcon Laboratories Research Fellowship Grant. We thank Prof. Rainer Greger (Albert-Ludwigs-Universität, Freiburg, FRG) for providing the NPPB, and David A. Carré and Michael L. Chalfant (University of Pennsylvania) for their critical readings of the manuscript.

References

Ackerman, M.J., Wickman, K.D., Clapham, D.E. 1994. Hypotonicity activates a native chloride current in *Xenopus* oocytes. *J. Gen. Physiol.* **103:**153–179

Angúita, J., Chalfant, M.L., Civan, M.M., Coca-Prados, M. 1995. Molecular cloning of the human volume-sensitive chloride conductance regulatory protein, pl_{Cln}, from ocular ciliary epithelium. *Biochem. Biophys. Res. Commun.* **208:**89–95

Borsani, G., Rugarli, E.I., Taglialatela, M., Wong, C., Ballabio, A. 1995. Characterization of a human and murine Gene (CLCN3) sharing similarities to voltage-gated chloride channels and to a yeast integral membrane protein. *Genomics* **27:**131–141

Caprioli, J. 1987. The ciliary epithelia and aqueous humor. *In:* Adler's Physiology of the Eye: Clinical Application. R.A. Moses and W.M. Hart Jr., editors. pp. 204–222. C.V. Mosby, St. Louis

Carré, D.A., Tang, C.-S.R., Krupin, T., Civan, M.M. 1992. Effect of bicarbonate on intracellular potential of rabbit ciliary epithelium. *Curr. Eye Res.* **11:**609–624

Chomczynski, P., Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:**156–159

Civan, M.M., Coca-Prados, M., Peterson-Yantorno, K. 1994. Pathways signalling the regulatory volume decrease of cultured nonpigmented ciliary epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **35:**2876–2886

Civan, M.M., Coca-Prados, M., Peterson-Yantorno, K. 1996. Regulatory volume increase of human nonpigmented ciliary epithelial cells. *Exp. Eye Res. (in press)*

Civan, M.M., Peterson-Yantorno, K., Coca-Prados, M., Yantorno, R.E. 1992. Regulatory volume decrease by cultured non-pigmented ciliary epithelial cells. *Exp. Eye Res.* **54:**181–191

Coca-Prados, M., Anguíta, J., Chalfant, M.L., Civan, M.M. 1995. PKC-sensitive Cl^- channels associated with ciliary epithelial homologue of pI_{Cln}. *Am. J. Physiol.* **268:**C572–C579

Cole, D.F. 1977. Secretion of the aqueous humour. *Exp. Eye Res.* **25**(Suppl):161–176

Crook, R.B., Polansky, J.R. 1994. Stimulation of Na^+, K^+, Cl^- cotransport by forskolin-activated adenylyl cyclase in fetal human nonpigmented epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **35:**3374–3383.

Edelman, J.L., Sachs, G., Adorante, J.S. 1994. Ion transport asymmetry and functional coupling in bovine pigmented and nonpigmented ciliary epithelial cells. *Am. J. Physiol.* **266:**C1210–1221

Escribano, J., Hernando, N., Ghosh, S., Crabb, J., Coca-Prados, M. 1994. cDNA from human ocular ciliary epithelium homologous to βig-h3 is preferentially expressed as an extracellular protein in the corneal epithelium. *J. Cell Physiol.* **160:**511–521

Escribano, J., Ortego, J., Coca-Prados, M. 1995. Isolation and characterization of cell-specific cDNA clones from a subtractive library of the ocular ciliary body of a single normal human donor: Transcription and synthesis of plasma proteins. *J. Biochem.* **118:**921–931

Farahbakhsh, N.A., Fain, G.L. 1988. Volume regulation of nonpigmented cells from ciliary epithelium. *Invest. Ophthalmol. Vis. Sci.* **28:**934–944

Gründer, S., Thiemann, A., Pusch, M., Jentsch, T.J. 1992. Regions involved in the opening of ClC-2 chloride channel by voltage and cell volume. *Nature* **360:**759–762

Hardy, S.P., Goodfellow, H.R., Valverde, M.A., Gill, D.R., Sepúlveda, F.V., Higgins, C.F. 1995. Protein kinase c-mediated phosphorylation of the human multidrug resistance p-glycoprotein regulates cell volume-activated chloride channels. *EMBO J.* **14:**68–75

Hoffmann, E.K. 1987. Volume-regulation in cultured cells. *Curr. Top. Membr. Transp.* **30:**125–180

Jentsch, T.J. 1993. Chloride channels. *Curr. Opinion Neurobiol.* **3:**316–321

Jentsch, T.J. 1994. Molecular biology of voltage-gated chloride channels. *Curr. Top. Membr.* **42:**35–57

Jentsch, T.J., Günther, W., Pusch, M., Schwappach, B. 1995. Properties of voltage-gated chloride channels of the ClC gene family. *J. Physiol.* **482.P:**19S–28S

Kawasaki, M., Suzuki, M., Uchida, S., Sasaki, S., Marumo, F. 1995.

Stable and functional expression of the ClC-3 chloride channel in somatic cell lines. *Neuron* **14:**12885–1291

Kawasaki, M., Uchida, S., Monkawa, T., Miyawaki, A., Mikoshiba, K., Marumo, F., Saskai, S. 1994*a*. Cloning and expression of a protein kinase C-regulated chloride channel abundantly expressed in rat brain neuronal cells. *Neuron* **12:**597–604

Kawasaki, M., Uchida, S., Sasaki, S., Marumo, F. 1994*b*. Single-channel currents of cloned ClC-3 channels in stably transfected cells. *J. Am. Soc. Nephrol.* **5:**288

Krapivinsky, G.B., Ackerman, M.J., Gordon, E.A., Krapivinsky, L.D., Clapham, D.E., 1994. Molecular characterization of a swelling-induced chloride conductance regulatory protein, pI_{Cln}. *Cell* **76:**439–448

Krupin, T., Reinach, P.S., Candia, O.A., Podos, S.M. 1984. Transepithelial electrical measurements of the isolated rabbit iris-ciliary body. *Exp. Eye. Res.* **38:**115–123

Martin-Vasallo, P., Ghosh, S., Coca-Prados, M. 1989. Expression of Na,K-ATPase alpha subunit isoforms in the human ocular ciliary body and nonpigmented ciliary epithelial cell lines. *J. Cell Physiol.* **141:**243–252

Miller, R.J. 1987. Multiple calcium channels and neuronal function. *Science* **235:**46–52

Mito, T., Delamere, N.A., Coca-Prados, M. 1993. Calcium-dependent regulation of cation transport in cultured human nonpigmented ciliary epithelial cells. *Am. J. Physiol.* **264:**C519–C526

Nishizawa, Y., Seamon, K.B., Daly, J.W., Aronstam, R.S. 1990. Effects of forskolin and analogues on nicotinic receptor-mediated sodium flux, voltage-dependent calcium flux, and voltage-dependent rubidium efflux in pheochromocytoma PC12 cells. *Cell. Molec. Neurobiol.* **10:**351–368

Paulmichl, M., Li, Y., Wickman, K., Ackerman, M., Peralta, E., Clapham, D. 1992. New Mammalian chloride channel identified by expression cloning. *Nature* **356:**238–241

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230:**1350–1354

Swaroop, A., Xu, J. 1993. cDNA libraries from human tissues and cell lines. *Cytogenet. Cell Genet.* **64:**292–294

Valverde, M.A., Díaz, M., Sepúlveda, F.V., Gill, D.R., Hyde, S.C., Higgins, C.F. 1992. Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein. *Nature* **355:**830–833

van Slegtenhorst, M.A., Bassi, M.T., Borsani, G., Wapenaar, M.C., Ferrero, G.B., de Conciliis, L., Rugarli, E.I., Grillo, A., Franco, B., Zoghbi, H.Y., Ballabio, A. 1994. A gene from Xp22.3 region shares homology with voltage-gated chloride channels. *Hum. Mol. Genet.* **3:**547–5528

Wiederholt, M., Zadunaisky, J.A. 1986. Membrane potentials and intracellular chloride activity in the ciliary body of the shark. *Pfluegers Arch.* **407:**S112–S115

Wu, J., Jacob, T.J.C. 1994. Small conductance, volume-activated chloride channels in isolated nonpigmented ciliary epithelial cells of bovine eye. *Invest. Ophthalmol. Vis. Sci.* **35:**1454

Yantorno, R.E., Carré, D.A., Coca-Prados, M., Krupin, T., Civan, M.M. 1992. Whole-cell patch clamping of ciliary epithelial cells during anisosmotic swelling. *Am. J. Physiol.* **262:**C501–509

Yantorno, R.E., Coca-Prados, M., Krupin, T., Civan, M.M. 1989. Volume regulation of cultured, transformed, non-pigmented epithelial cells from human ciliary body. *Exp. Eye Res.* **49:**423–437