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Functional and Molecular Characterization of a Volume-activated Chloride Channel in Rabbit Corneal Epithelial Cells

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Abstract. We characterized the functional and molecular properties of a volume-regulated anion channel (VRAC) in SV40-immortalized rabbit corneal epithelial cells (tRCE), since they mediate a robust regulatory volume decrease (RVD) response during exposure to a hypotonic challenge. Whole-cell patch clamp-monitored chloride currents and light-scattering measurements evaluated temporal cell-volume responsiveness to hypoosmotic challenges. Exposure to 200 mOsm medium elicited an outwardly-rectifying current (VACC), which was reversible upon reperfusion with isotonic (300 mOsm) medium. VACC and RVD were chloride-dependent because either chloride removal or application of NPPB (100 μM) suppressed these responses. VACC behavior exhibited voltage-dependent inhibition in the presence of DIDS (500 μM), whereas inhibition by both NPPB (100 μM) and niflumic acid (500 μM) was voltage-independent. VACC was insensitive to glibenclamide (250 μM), verapamil (500 μM) or removal of extracellular calcium. Phorbol dibutyrate, PDBu, (100 nM) had no effect on activated VACC. However, preincubation with PDBu prior to hypotonic challenge prevented VACC and RVD responses as well as prolonged characteristic time. An inactive phorbol ester analogue had no effect on RVD behavior. Moreover, Northern blot analysis verified expression of ClC-3 gene transcripts. The presence of ClC-3 transcripts along with the correspondence between the effects of known ClC-3 inhibitors on VACC and RVD suggest that ClC-3 activation underlies these responses to hypotonic-induced cell swelling.

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Key words: Volume-regulated anion channel — VRAC — Corneal epithelial cells — Regulatory volume decrease — Patch clamp

Introduction

Corneal epithelial barrier function is essential to the maintenance of normal vision because it prevents noxious agents and infection from damaging the three different corneal tissue layers (Klyce, 1972). Sustaining this function relies on close cell-to-cell apposition, which can affect tight junction integrity. Under environmental conditions, it is possible that cell volume changes can occur from exposure to an anisosmotic condition. In all likelihood, in vivo cell volume shrinkage or swelling is transient because cultured corneal epithelial cells can undergo regulatory volume responses to such challenges (Wu et al., 1997; Bildin et al., 2000, 2003). These responses include activation of either a regulatory volume decrease (RVD) or regulatory volume increase (RVI) during exposure to an anisosmotic challenge. Their activation results in either the appropriate gain or loss of sufficient osmolytes to restore isotonic volume, despite continued exposure to an anisosmotic challenge. The ionic pathways underlying the responses to a hypotonic challenge in corneal epithelial cells are relatively unknown.

Ample evidence in other epithelia suggests that the pathways activated by a hypotonic challenge elicit net loss of KCl (Strange, Emma & Jackson, 1996). In epithelia mediating Cl^- secretion, this can occur because each of these ions is maintained above its electrochemical value, due to net inward transport of KCl. Their uptake depends on parallel basolateral

Na:K pump and Na:K:2Cl cotransporter activity (Haas, 1994). In corneal epithelial cells, these two transporters elicit net Cl^- transport from the stroma towards the tears and Cl^- efflux across the apical membrane by electrodiffusion into the tears (Klyce & Wong, 1977; Patarca, Candia & Reinach, 1983; Reuss et al., 1983). In rabbit corneal epithelial cells, net Cl^- transport is stimulated by rises in intracellular cAMP levels (Klyce, Neufeld & Zadunaisky, 1973). Similarly, rises in intracellular Ca^{2+} levels have comparable effects and are requisite to elicit an RVD response during a hypotonic challenge (Candia, Montoreano & Podos, 1977; Wu et al., 1997). The increases in cAMP-mediated Cl^- transport are associated with rises in apical membrane Cl^- and Na:K:2Cl cotransport activity, but they have an opposite effect on basolateral membrane K^+ permeability (Wolosin & Candia, 1987; Klyce & Wong, 1977; Al-Nakkash & Reinach, 2001; Yang et al., 2001; Candia & Zamudio, 2002). One apical membrane cAMP-sensitive Cl^- pathway in tRCE cells is the cystic fibrosis transmembrane conductance regulator protein, CFTR (Al-Nakkash & Reinach, 2001). In addition, there is evidence of a voltage-dependent outwardly rectifying Cl^- channel in freshly isolated rabbit and rat corneal epithelial cells (Marshall & Hanrahan, 1991). The contributory role of a VRAC in these cells in mediating RVD has not been described.

In many other Cl^--transporting epithelia, interest has focused on identifying the protein mediating VACC. In particular, two members of the ClC voltage-gated family of Cl^- channel proteins are potential molecular candidates for the VRAC (Jentsch et al., 2002; Nilius & Droogmans, 2003), underlying swelling or VACC (Duan et al., 1997; Wang, Chen & Jacob, 2000; Nilius & Droogmans, 2003; Sardini et al., 2003): ClC-2, an inwardly rectifying Cl^- channel (Thiemann et al., 1992) and ClC-3, an outwardly rectifying Cl^- channel (Kawasaki et al., 1994; Duan et al., 1997). Recent studies using an Sf9 expression system indicate that ClC-2 may be involved in RVD (Xiong et al., 1999). In human corneal epithelial cells, the cDNA encoding the Ca^{2+}-activated Cl^- channel subtype ClCA2 was identified (Itoh et al., 2000). However, its role was not identified in regulatory volume behavior. The specific Cl^- channel activated by cell swelling may be tissue specific because in human gastric epithelial cells, ClC-3 has been proposed to be the channel responsible for this current and cell volume regulation (Jin et al., 2003). Accordingly, it is not possible to deduce from studies in other tissues the role of a specific Cl^--channel subtype in mediating swelling-activated currents without making a direct assessment in a tissue of interest.

Normal vision depends on the ability of the cornea to maintain constant volume during exposure to decreased extracellular osmolarity, thereby maintaining close cell-to-cell apposition without compromising tight junctional integrity. These attributes protect all three layers of the cornea from damage by infectious agents. Characterization of the specific channel subtype underlying an RVD response to a hypotonic challenge may provide novel approaches to restoring epithelial barrier function in pathophysiological conditions. Therefore, we used SV40-immortalized rabbit corneal epithelial cells (tRCE) to better understand the biophysical and physiological events underlying RVD in this tissue. Using the patch-clamp technique, we describe the biophysical properties of the VRAC that elicit Cl^- efflux. ClC-3 appears to be the same protein that mediates both VACC and RVD (each of these two functions exhibit similar drug inhibitory profiles). Such agreement is consistent with the identification of the ClC-3 gene transcript in these cells and suggests that the ClC-3 channel is a likely candidate for VACC and RVD.

Materials and Methods

Cell Culture

SV40-immortalized rabbit corneal epithelial cells, tRCE (Araki-Sasaki et al., 1995), were grown on plastic culture dishes, perpetuated under standard tissue culture conditions (95% air-5% CO_2, 37°C) and maintained in Dulbecco's modified Eagle's medium (DMEM/F12) containing: 10% fetal bovine serum, insulin (5 ng/ml), EGF (5 ng/ml) and gentamicin (10 mg/ml).

Whole-Cell Patch-Clamp Studies

Cell suspensions were prepared as described previously (Al-Nakkash & Reinach, 2001). Pipette electrodes were made from Corning 7056 capillary tubing (Warner Instrument, USA), with a two-stage vertical puller (Narishige, Japan). Pipette tips (resistances ~3 MΩ in bath solution) were fire-polished with a microforge (Narishige, Japan). VACC currents were recorded at room temperature (~22°C) using an EPC-9 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany). Membrane potential was held at 0 mV. Current voltage (*I-V*) relationships were generated using Igor software (Wavemetrics, Lake Oswego, OR). Current traces in response to voltage pulses (± 100 mV, in 20 mV increments, 200 ms duration) were filtered at 1 kHz, digitized at 2 kHz through an ITC-16 interface (Instrutech, Port Washington, NY). All recordings were made at steady-state: VACC activation ~10 minutes and inhibitor effects ~3 minutes. Pipette solution contained (in mM): 130 CsCl, 20 tetraethylammonium-Cl, 5 MgATP, 5 EGTA, 5 HEPES and 0.1 Tris-GTP, and pH 7.2. Isotonic bath solution contained (in mM): 140 NaCl, 5.4 CsCl, 0.5 $MgCl_2$, 2.5 $CaCl_2$,11 glucose, 5.5 HEPES (pH 7.4). Hypotonic bath solution contained (in mM): 90 NaCl, 5.4 CsCl, 0.5 $MgCl_2$, 2.5 $CaCl_2$, 11 glucose, 5.5 HEPES (pH 7.4).

RNA Isolation and Northern Analysis

RNA was isolated from tRCE cells by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). For Northern analysis, 20 μg total RNA was agarose-gel

electrophoresed, transferred to Nytran plus (Schleicher and Schuell, Keene, NH); membranes were crosslinked (UV crosslinker apparatus, Stratagene), prehybridized, sequentially hybridized with the 567-bp fragment of human C1C-3 generated by PCR (*data not shown*) and washed as previously described (Coco-Prados et al., 1996).

Regulatory Volume Measurements

Low-angle light scattering identified relative changes in cell volume (70% confluent cultures, seeding density: 10^5–10^6 cells) to osmotic shifts by the characteristic time of the osmotic transients (τ_{osm}, s). The methodology and deconvolution procedure to resolve the osmotic and regulatory volume contributions from one another to the overall hypotonic-induced volume transients were as described previously (Wu et al., 1997; Iserovich et al., 1998).

Data Analysis and Statistics

Mean steady-state current amplitudes were calculated with Igor software from steady-state VACC. Data are presented as mean ± SEM.

Statistical analyses (*t*-tests) were performed using Sigmaplot or Sigmastat software (Jandel Scientific, San Rafael, CA). Significance was determined at $P < 0.05$.

Reagents

All chemicals were purchased from Sigma.

Results

Biophysical Properties of the Volume-activated Chloride Current

Figure 1*A* shows a typical *I-V* relationship obtained by perfusing tRCE cells with an isotonic (300 mOsm), then hypotonic bath (200 mOsm). This VACC was outwardly-rectifying and reversible upon reperfusion with an isotonic bath. Average time to reach peak steady-state VACC in hypotonic bath was 13.60 ± 0.89 minutes ($n = 51$). Figure 1*B* shows summary data of the mean peak VACC. Under isotonic conditions these cells possessed a small current of 5.32 ± 0.79 pA/pF at +100 mV and −0.65 ± 0.74 pA/pF at −100 mV ($n = 51$). Following perfusion with hypotonic solution, the Cl^- current increased to 91.57 ± 9.07 pA/pF at +100 mV and −46.26 ± 5.02 pA/pF at −100 mV ($n = 51$).

Pharmacological Properties of the Volume-activated Chloride Current

Several known Cl^- channel blockers (DIDS, NPPB and niflumic acid) have previously been shown to inhibit VACC in other cell types, e.g., pancreatic ducts, rat brain endothelial cells, mouse medullary collecting duct, *Xenopus* oocytes (Ackerman, Wickman & Clapham, 1994; Verdon et al., 1995; von Weikersthal, Barrand & Hladky, 1999; Boese et al., 2000). Application of DIDS (500 μM) voltage-dependently inhibited peak steady-state VACC, (Fig. 2*A*) by 87.88 ± 2.82% ($n = 7$, $P < 0.05$) and 44.00 ± 5.86% ($n = 7$, $P < 0.05$) at ±100 mV respectively. In contrast, NPPB (100 μM) voltage-independently inhibited the VACC (Fig. 2*B*) by 85.32 ± 2.36% ($n = 6$, $P < 0.05$) and 90.50 ± 2.76% ($n = 6$, $P < 0.05$) at ±100 mV respectively. Similarly, niflumic acid (500 μM) voltage-independently inhibited the VACC (Fig. 2*C*) by 77.15 ± 8.58% ($n = 6$, $P < 0.05$) and 73.82 ± 4.79% ($n = 6$, $P < 0.05$) at ±100 mV, respectively. The effects of DIDS, NPPB and niflumic acid were reversible upon washout of agonists (*data not shown*). In addition, we found no effect of either glibenclamide (250 μM) or verapamil (500 μM) on peak steady-state VACC at ± 100 mV ($n = 3$ and $n = 4$, respectively).

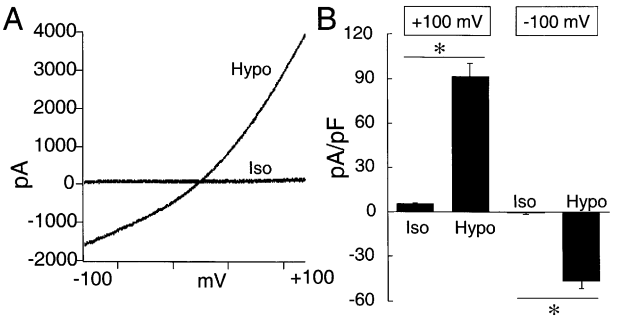

Fig. 1. Effect of hypotonic bath on whole-cell current in tRCE cells. (*A*.) Representative *I-V* relationship obtained over the range of voltage pulses tested (±100 mV). Perfusing with isotonic bath (*Iso*, 300 mOsm) generates little current and perfusing with hypotonic bath (*Hypo*, 200 mOsm) generates a large outwardly rectifying current. (*B*) Average whole-cell current from similar experiments at ±100 mV ($n = 51$); * denotes significance at $P < 0.05$.

Regulation of the Volume-activated Chloride Current by Extracellular Calcium

We examined the necessity for extracellular Ca^{2+} on VACC activation. Figure 3*A* demonstrates the requirement for extracellular Ca^{2+} on VACC development. In the same cells (at +100 mV), a reduced Ca^{2+} hypotonic bath generated Cl^- currents of 6.96 ± 3.06 pA/pF, increasing to 41.53 ± 12.29 pA/pF upon perfusion with hypotonic bath containing Ca^{2+} ($n = 5$, $P < 0.05$). VACC was measured in each bath condition after 10 minutes. In addition, we examined the importance of extracellular Ca^{2+} in maintaining VACC, by removing bath Ca^{2+} once VACC was activated. Figure 3*B* shows that VACC generated by perfusion with a Ca^{2+}-containing hypotonic bath (74.52 ± 10.95 pA/pF, $n = 5$, at +100 mV) was unchanged by perfusion with a Ca^{2+}-free hypotonic bath (77.93 ± 14.22 pA/pF, $n = 5$, at

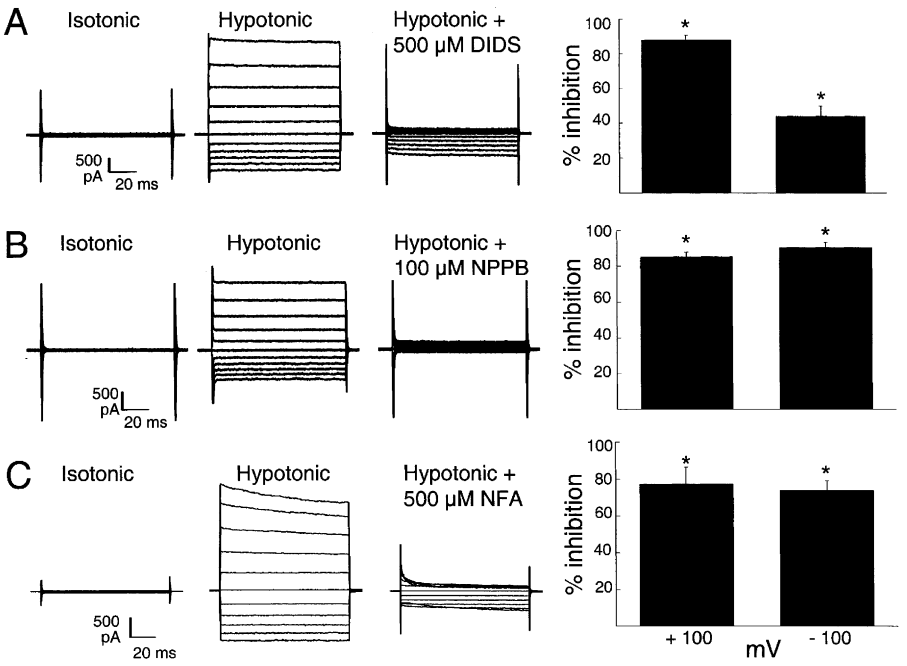

Fig. 2. Effect of DIDS, NPPB and niflumic acid on the volume-activated chloride current in tRCE cells. (*A*) The left panels show the whole-cell currents in response to voltage pulses applied under isotonic, hypotonic, hypotonic + DIDS (500 μM) bath conditions. Right panel shows the average percent inhibition by DIDS ($n = 7$). (*B*) Left panels show the whole-cell currents in response to voltage pulses applied under isotonic, hypotonic, hypotonic + NPPB (100 μM) bath conditions. Right panel shows the average percent inhibition by NPPB ($n = 6$). (*C*) Left panels show the whole-cell currents in response to voltage pulses applied under isotonic, hypotonic, hypotonic + niflumic acid (500 μM) bath conditions. Right panel shows the average percent inhibition by niflumic acid ($n = 6$). Voltage pulses applied between ±100 mV, 20 mV steps. Data are mean ± SEM.; * denotes significance at $P < 0.05$.

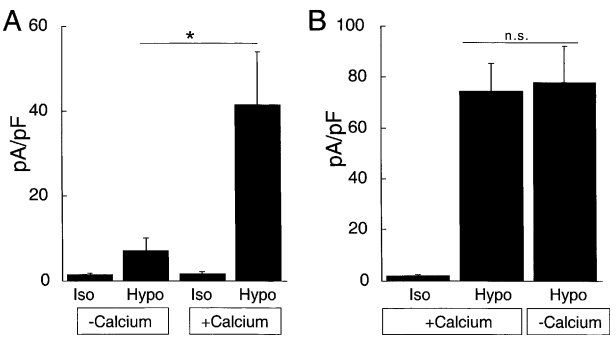

Fig. 3. Effect of extracellular calcium on the volume-activated chloride current in tRCE cells.(*A*) Average data shows the effect of Ca^{2+} removal from hypotonic bath upon activation of VACC. The presence of Ca^{2+} in hypotonic bath generates VACC that are significantly increased compared to currents generated in the absence of Ca^{2+} ($n = 5$). (*B*) Average data shows that there is no effect of Ca^{2+}-free hypotonic bath on VACC that are already activated ($n = 5$). Currents were measured at +100 mV and normalized to cell capacitance; * denotes significance at $P < 0.05$.

+100 mV). These data suggest that extracellular Ca^{2+} is required for VACC activation, but is not essential to the maintainance of VACC.

Stretch-activated cation channels are a possible route for Ca^{2+} influx during cell swelling. In epithelial cells, these channels are blocked by gadolinium (Filipovic & Sackin, 1991). We found activation of VACC in the presence of 100 μM $GdCl_3$. At +100 mV, VACC generated in hypotonic bath containing $GdCl_3$ (159.83 ± 29.19 pA/pF, $n = 4$) was similar to that in $GdCl_3$-free bath (91.57 ± 9.07 pA/pF, $n = 51$). Furthermore, there was no effect of $GdCl_3$ on VACC once activated; addition of $GdCl_3$ to hypotonic bath generated a Cl^- current that was 99.25 ± 13.31% of that seen in $GdCl_3$-free, $n = 4$). These data suggest that Ca^{2+} influx during cell swelling is not via stretch-activated cation channels.

Regulation of the Volume-activated Chloride Current by PKC

Activation of PKC by the phorbol ester PDBu has been shown to inhibit ClC-3-mediated Cl^- conductances and is thus used as a criterion with which to establish the molecular identity of VACC (Duan et al., 1999). External application of PDBu (100 nM) had no effect on isotonic current (Fig. 4*A*, 96.83 ± 27.38% of the current without BDBu, $n = 6$). PDBu had no effect on pre-generated VACC (Fig. 4*A* and *B*); VACC in hypotonic bath plus PDBu was 98.72 ± 6.49% that of hypotonic bath alone ($n = 7$). However, if the cells were exposed to PDBu

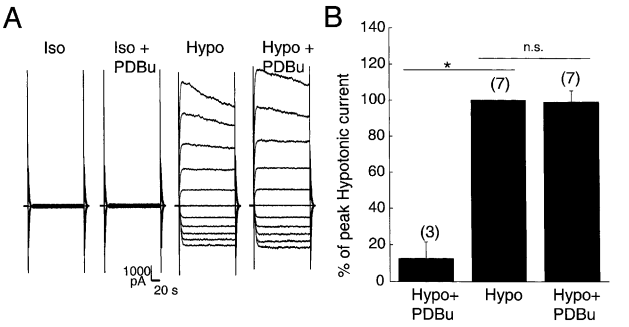

Fig. 4. Effect of PDBu on the volume-activated chloride current in tRCE cells. (*A*) Typical whole-cell experiment demonstrating no effect of PDBu (100 nM) on isotonic bath or on VACC that are already activated (voltage pulses applied between ±100 mV, 20 mV steps).(*B*) Average data shows the lack of effect of 100 nM PDBu on VACC that are already activated and the inhibitory effect of PDBu on VACC when exposed before or concomitantly with hypotonic bath. Numbers in parentheses are numbers of experiments; *denotes significance at $P < 0.05$.

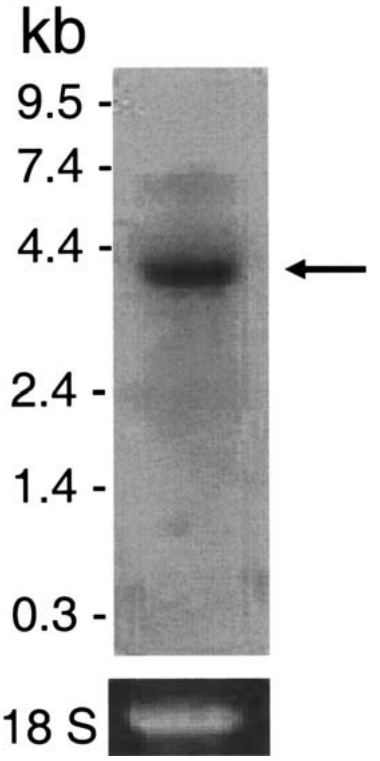

Fig. 5. Expression of ClC-3 transcript in tRCE cells. RNA (20 μg) was hybridized with a radiolabeled DNA probe (567 bp) for human ClC-3 generated by PCR. The standard RNA ladder marker is shown to the left. The arrow to the right denotes the position of the major transcript. At the bottom of the figure is ethidium-bromide staining, showing the ribosomal RNA 18S marker of the agarose gel before transferring into the Northern blot.

before or concomitantly with hypotonic bath, then the hypotonic challenge was ineffective (Fig. 4*B*) and removal of PDBu resulted in generation of VACC. In similar experiments, Cl^- current generated by hypotonic bath plus PDBu was similar to the current in isotonic conditions and only 12.45 ± 9.25% ($n = 3$, $P < 0.05$) of that obtained by removal of PDBu from hypotonic bath (100.00 ± 0.0%, $n = 3$). These results are consistent with the published effects of PDBu on VACC in other cell types such as rat brain endothelial cells and mouse renal inner medullary collecting duct cell line (Boese et al., 2000; von Weikersthal et al., 1999).

Expression of CLC-3 Transcripts in the tRCE Cells

To verify expression of ClC-3 transcripts in the tRCE cells, we probed a Northern blot containing RNA with a radiolabeled human ClC-3 DNA probe (567 bp) obtained by PCR. Figure 5 shows that the human DNA probe hybridized to a major RNA species, ranging from 3.5 to 4.0 kb RNA (some hybridization occurs with a second RNA species ranging from 6.0 to 6.5 kb). These two mRNA species are in agreement with the sizes found in other tissues: human retina (Borsani et al., 1995) and human nonpigmented ciliary epithelial cells (Coco-Prados et al., 1996).

Dependence of Regulatory Volume Decrease on Cl^- Transport

There is a large body of evidence in other tissues that RVD behavior elicited by exposure to hypotonic challenge is mediated by net loss of KCl. Such loss requires the cells to accumulate intracellular K^+ and Cl^- to concentrations above their electrochemical equilibrium. For example, in corneal epithelium, their accumulation to levels at least 2-fold above electrochemical equilibrium depends on the concerted parallel activity of the Na:K pump and the Na:K:2Cl cotransporter (Reuss et al., 1983; Riordan, Forbush & Hanrahan, 1994). To determine the relative importance of Cl^- egress to the RVD response in tRCE cells, we characterized in a Cl^--free medium the magnitude and kinetics of this response. As evident in Fig. 6, the RVD response was markedly attenuated by removal of Cl^- from the medium. Under control conditions, recovery and characteristic time, τ_{vr}, in NaCl Ringer were 101 ± 2% and 2.8 ± 0.4 min, respectively. In Cl^--free medium (Cl^- substituted with cyclamate), recovery and τ_{vr} were 46 ± 3% and 1.3 ± 0.1 min, respectively. Since our results indicate that NPPB (50 μM) inhibited VACC, we determined its effect on RVD. Similar to the effect of Cl^- removal, in 10 paired experiments the RVD response diminished to 36 ± 3% and the τ_{vr} was 1.9 ± 0.5 min. These declines in RVD suggest that Cl^- efflux mediates a significant portion of this response to hypotonic challenge.

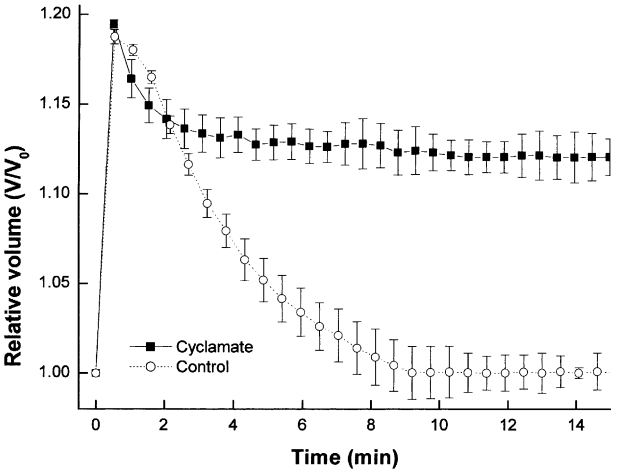

Fig. 6. Cl^- removal suppresses RVD response to hypotonic challenge. Cl^--free solution was obtained by substitution of Cl^- with cyclamate in both isotonic and hypotonic solutions. Light scattering data was fitted and deconvoluted ($n = 6$).

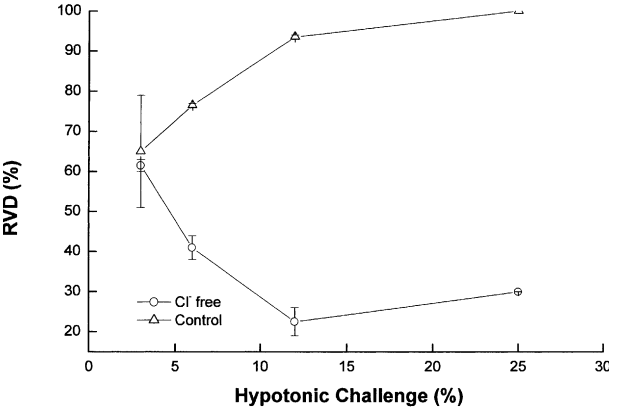

Fig. 7. Effect of extent of dilution of bathing solution on amplitude of RVD response in the presence and absence of Cl^- in the medium. Light scattering data was fitted and deconvoluted. Extent of recovery evaluated based on R/A ratio $\times$ 100%. R= extent of regulation and A = amplitude of osmometric response ($n = 6$).

We determined the relationship between the magnitude of hypotonic challenge and the requirement for Cl^- to elicit an RVD response. As is seen in Fig. 7, RVD is unaffected by Cl^- removal for challenges less than 2.5%. However, the importance of Cl^- presence increased up to a 12% hypotonic challenge. At higher dilutions, the Cl^- dependence remained constant. Therefore, in all RVD studies with these cells the hypotonic challenge was a 25% dilution of the medium (higher than the threshold value). This suggests a critical dilution is required for activation of RVD. Interestingly, in a Cl^--free medium recovery was significantly blunted at all dilutions, indicating that swelling-activated Cl^- efflux is an important component of the overall RVD response.

Since our electrophysiological data suggested that ClC-3 is a likely Cl^--channel candidate mediating VACC, we sought evidence for its role in eliciting RVD during exposure to hypotonic challenge (comparable to that used to activate VACC). To make this evaluation, we characterized the individual effects of an active and inactive phorbol ester analogue on RVD behavior. Accordingly, in five paired experiments with each of the analogues, the cells were initially challenged in the absence of an analogue to a 225 mOsm medium. Following stabilization of RVD recovery during such challenge, the bathing solution was substituted with isotonic medium. As a result, the cells mediated a post RVD-RVI response due to the increased bathing solution osmolality. Subsequent to reaching another stabilized condition, the cells were exposed to either 0.1 μM PDBu or its inactive analogue 4α phorbol didecanoate (PDD) for 30 min followed by re-exposure to 225 mOsm challenge. The time-dependent effects of each of these analogues on relative cell volume are provided in Fig. 8. The initial rise in relative cell volume in the presence of each analogue was comparable. However, the kinetics and extent of recovery towards the isotonic volume during the challenge were altered. With PDBu, recovery was delayed and reached 48 ± 2% of the recovery in the absence of this analogue. On the other hand, with 4α PDD there was complete recovery to the isotonic level (i.e., 99%), even though its kinetics were slightly prolonged relative to the control value.

Discussion

We used a two-pronged approach to obtain functional and molecular evidence for the role of Cl^- channel activation in enabling corneal epithelial cells to maintain constant cell volume during exposure to hypotonic challenge. The presence of ClC-3 gene transcripts coupled with the pharmacological profiles for inhibition of VACC and RVD suggest the possibility that ClC-3 activity could underlie these responses to hypotonic challenge.

Our biophysical results clearly show that increases in Cl^- fluxes occur through VRAC. Evidence of VACC in these cells is based on data shown in Fig. 1, whereby exposure to hypotonic challenge activated outwardly-rectifying current. Furthermore, VRAC activity is shown by the inhibitory pharmacological profiles to known blockers of its activity. In particular, activity was markedly inhibited by DIDS, NPPB and niflumic acid, as described in a number of other tissues where VACC activity was documented. On the other hand, negative evidence for either efflux elicited via CFTR or p-glycoprotein multi-drug resistance (MDR) was obtained based on the failure of glibenclamide or verapamil to alter hypotonic-in-

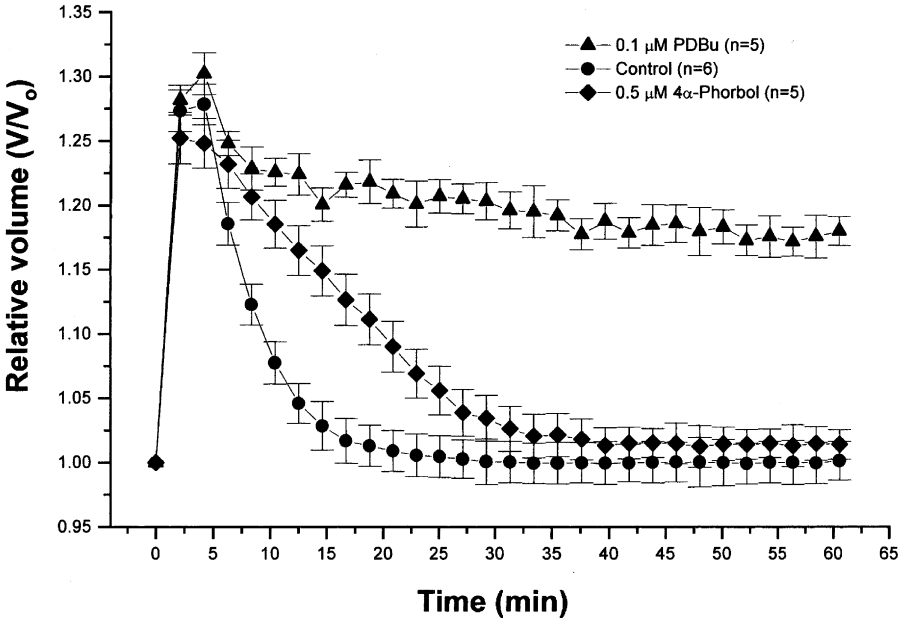

Fig. 8. PKC stimulation suppresses RVD response to hypotonic challenge. Both phorbol ester analogues were applied 30 min prior to exposure to the 225 mOsm hypotonic challenge. PKC stimulation was obtained with the active phorbol ester analogue, PDBu. Its inactive counterpart is 4α phorbol didecanoate. Both drugs were dissolved in DMSO and reached a final concentration following dilution in bathing solution of 0.01% DMSO. This vehicle concentration was also used in the control experiment ($n = 5$). Light scattering data was fitted and deconvoluted.

duced efflux. Other evidence indicating VACC activity is shown by the dependence of its activation on extracellular Ca^{2+} or exposure to a protein kinase C activator. As described in other cell systems, we showed that VRAC was unaffected by Ca^{2+} removal or exposure to PDBu if hypotonic exposure preceded either manipulation. On the other hand, VRAC was markedly inhibited if Ca^{2+} removal or exposure to PDBu preceded exposure to hypotonic challenge. Finally, these large increases in hypotonic-induced currents were not a result of the stimulation of stretch-activated channel activity since gadolinium had no effect on their activity.

The parallel functional approach showed that VACC activity underlies RVD behavior whose functionality depends on net Cl^- efflux. The evidence that such activity mediates RVD is based on the data shown in Figs. 6 and 7. Figure 6 shows that hypotonic exposure elicited a smaller and delayed RVD recovery during hypotonic challenge, if this was imposed in Cl^--free medium. Convincing evidence for the dependence of the RVD response on bath Cl^- is our demonstration that activation of RVD is related to bath solution osmolality. The results shown in Fig. 7 indicate that a 2.5% decline in osmolality from the isotonic condition activates an RVD response, provided the bathing solution contains Cl^-. There is a steep dependence on Cl^- for recovery and a 12.5% dilution fully activated the RVD response. Further dilution to 25% did not affect RVD. However, in a Cl^--free bath, the RVD recovery was retarded and only 20% of that observed in the presence of Cl^-.

Emerging evidence in different cell types suggests that the molecular identity of the Cl^--channel subtype underlying VACC can be either ClC-2 or ClC-3. Several distinguishing criteria differentiate between these two potential subtypes underlying VACC activity (Verdon et al., 1995; von Weikersthal et al., 1999). Our results support the notion that ClC-3, rather than ClC-2, underlie VRAC and RVD behavior in tRCE cells. This is indicated by the inhibitory effects of DIDS, NPPB and niflumic acid on VRAC activity (effects not associated with the inwardly-rectifying ClC-2).

Significant pharmacological evidence to support the idea that ClC-3 is a likely channel subtype eliciting VACC, comes from our finding that stimulation of Ca^{2+}-dependent protein kinase C activity with PDBu prior to a hypotonic challenge inhibited both VACC and the RVD response. This assessment was required since ClC-3 channels are inhibited by activation of PKC by PDBu (Boese et al., 2000; Duan et al., 1999). Our results show that pre-exposure to 100 nM PDBu blocked VACC elicited by a hypotonic challenge (by 90%). Furthermore, it is known that such channels are unaffected by PKC activation if PKC stimulation occurs concomitantly with exposure to the hypotonic challenge. We found that pre-exposure to PDBu also blocked RVD, whereas its inactive analogue was ineffective. This correspondence between the inhibitory effects of PKC activa-

tion on VACC and on RVD strengthens the notion that the ClC-3 channel subtype likely underlies VACC in these cells. Finally, our Northern blot analysis identifying ClC-3 RNA further strongly suggests that the ClC-3 Cl^- channel is a molecular candidate for VACC. Our suggestion of ClC-3 involvement in VACC activity in tRCE is in agreement with its possible role in human nonpigmented ciliary epithelial cells (Coco-Prados et al., 1996).

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