J. Membrane Biol. 166, 119–132 (1998)

The Journal of
Membrane
Biology

Protein Kinase C and Regulatory Volume Decrease in Mudpuppy Red Blood Cells

D.B. Light, M.R. Adler, J.K. Ter Beest, S.A. Botsford, R.T. Gronau
Department of Biology, Ripon College, Ripon, WI 54971, USA

Received: 20 January 1998/Revised: 2 September 1998

Abstract. This study examined whether protein kinase C (PKC) stimulates K^+ efflux during regulatory volume decrease (RVD) in *Necturus maculosus* (mudpuppy) red blood cells (RBCs). The limit of osmotic fragility increased with the general protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7, 10 μM), but not with the cyclic nucleotide-dependent kinase antagonists N-(2′-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004, 10 μM) and N-2-(methylamino)ethyl-5-isoquinoline-sulfonamide (H-8, 5 μM). Consistent with these results, osmotic fragility also increased with the PKC antagonists bisindolylmaleimide I (GF-109203X or bis I, 100 nM), bisindolylmaleimide II (bis II, 100 nM), and chelerythrine (10 μM). The effect of these three antagonists and H-7 was reversed with gramicidin (5 μM in a choline Ringer), indicating PKC was linked to K^+ efflux (gramicidin is a cationophore that was used to ensure a high K^+ permeability). We also measured cell volume recovery from hypotonic shock (0.5× Ringer) with a Coulter counter and estimated cell volume from the hematocrit. The percent RVD compared to control decreased with H-7 (10 μM), sphingosine (100 nM), chelerythrine (10 μM), bis I (100 nM), and bis II (100 nM), but not with HA-1004 (10 μM) nor H-8 (5 μM). Inhibition of RVD by H-7, chelerythrine, bis I, and bis II was reversed with gramicidin (5 μM). Furthermore, using the patch clamp technique, we found H-7 (10 μM) reduced a whole cell conductance that was activated during cell swelling. In addition, a conductance responsible for K^+ efflux during cell swelling was inhibited by bis I (100 nM) and bis II (100 nM). These results indicate that a conductive pathway mediating K^+ loss during RVD is regulated, at least in part, by protein kinase C.

Correspondence to: D.B. Light

Key words: Volume regulation — Calcium — Patch clamp — Potassium channel — Bisindolylmaleimide — Chelerythrine — *Necturus maculosus*

Introduction

Exposure of vertebrate cells to a hypotonic solution results in a rapid increase in cell volume followed by a slower, spontaneous regulatory volume decrease (RVD) close to the original size [5, 11, 17, 21, 33]. Because water is in thermodynamic equilibrium across the plasma membrane, the intracellular solute content may be used to regulate cell volume. This is accomplished by selectively increasing the permeability of the plasma membrane during cell swelling to allow for efflux of specific intracellular osmolytes, thereby decreasing the driving force for water influx [5, 11, 17, 21, 33]. Most vertebrate cells lose K^+ and Cl^- during RVD [5, 11, 17, 21, 33]. This may occur by electroneutral ion transport pathways [17] or by the separate activation of K^+ and anion channels [3, 5, 8, 17, 21, 30, 37, 38]. Loss of organic anions and osmolytes also may occur during RVD [13, 33].

The cellular mechanisms that activate and regulate permeability pathways during RVD are not completely understood, and appear to differ between cell types. For example, in some instances the activation mechanism for an RVD response is Ca^{2+} independent [12, 15, 22, 23, 29]. In contrast, calcium appears to play a role during cell volume regulation in several cells types [2, 3, 21, 24, 27, 28, 30, 39]. In addition, although it has been suggested that Ca^{2+} directly activates ion channels during RVD [8, 21, 37], there also is evidence that several Ca^{2+}-dependent intracellular messengers and enzymes (e.g., calmodulin, phospholipase A_2, eicosanoids, and protein kinases) are involved with cell volume regulation [3, 11, 17, 19, 21, 28, 35]. Recent studies in our laboratory indicate that RVD in mudpuppy (*Necturus maculosus*)

red blood cells (RBCs) depends on a quinine-inhibitable K^+ conductance that is regulated during cell swelling, in part, by a Ca^{2+}-calmodulin-dependent mechanism [3] and by a 5-lipoxygenase metabolite of arachidonic acid [19].

Protein kinase C is a calcium- and phospholipid-dependent enzyme that has a crucial role in signal transduction for a variety of biological processes [24, 26]. Although several studies have shown that protein phosphorylation/dephosphorylation is important for cell volume regulation [6], there is a paucity of data on the role of PKC in this process. Furthermore, the few reports concerning PKC and cell volume regulation reach different conclusions. For example, several studies, have shown that PKC regulates swelling-induced currents [22, 24, 27, 30]. Moran and Turner [24] reported that K^+ channels in a human salivary ductal cell line are activated by PKC during RVD [25], and O'Neil and Leng [27] showed that PKC regulates an osmomechanically sensitive Ca^{2+} influx channel in renal epithelial cells. In addition, Rubera et al. [30] concluded that PKC regulates swelling-induced Cl^- currents in primary cultures of rabbit distal bright convoluted tubules, and Mitchell et al. [22] reported that inhibitors of PKC completely prevented activation of a volume-sensitive chloride current in pigmented ciliary epithelial cells.

In contrast to the studies cited above, Bender et al. [2] concluded that PKC inhibits RVD because PKC antagonists caused cell shrinkage under isosmotic conditions and activation of PKC potentiated cell swelling induced by hypo-osmotic stress. In addition, Leaney et al. [15] reported that a swelling-activated chloride current in rat sympathetic neurones was unaffected by inhibition of PKC, and Grinstein et al. [6] states that there is increasing evidence against the involvement of PKC in cell volume regulation. Larsen et al. [16], on the other hand, reported that PKC is activated during both hypertonic and hypotonic conditions in Ehrlich mouse ascites tumor cells. Finally, MacLeod et al. [20] found that PKC does not activate a Cl^- conductance that is used for cell shrinkage following hypotonic swelling, yet PKC activates a Cl^- conductance required for volume regulation during Na^+-nutrient cotransport. Thus, the role of PKC in volume recovery from hypo-osmotic stress is still unclear.

In view of these uncertainties, the purpose of this study was to investigate whether PKC regulates K^+ efflux during RVD in mudpuppy RBCs. The basis of this study stemmed from our earlier work that demonstrated RVD in mudpuppy RBCs depends on Ca^{2+}, and that H-7 increases the limit of osmotic fragility (LOF) in this cell type [3]. In addition, Musch and Goldstein [25] reported that hypotonicity generates diacylglycerol in erythrocytes; diacylglycerol is formed by phospholipase C breakdown of phosphatidylinositol-4,5-bisphosphate and increases the affinity of PKC for Ca^{2+} [26]. In addition, in a recent report we show that phospholipase A_2 (PLA_2) plays a key role in cell volume recovery by mudpuppy RBCs [19], whereas others have shown that several fatty acid products of phosphatidylcholine hydrolysis by PLA_2 potentiate the activation of PKC [26]. Thus, it seemed appropriate to determine whether PKC is important for RVD. To this end, we used three different approaches: (i) hemolysis studies to determine the limit of osmotic fragility, (ii) a Coulter counter and the hematocrit to measure the volume of osmotically stressed cells, and (iii) the whole-cell patch clamp technique to study membrane currents.

Materials and Methods

Animals

Mudpuppies (*Necturus maculosus*) were obtained from a local vendor (Lemberger, Oshkosh, WI) and kept in well-aerated, aged tap water at 5–10°C for no more than 6 days prior to use. They were anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, 1%) and sacrificed by decapitation. Blood was obtained from a mid-ventral incision and collected into heparin (10,000 units/ml) coated tubes. Immediately following esanguination, the blood was spun in a centrifuge (Hermel-Z230, National Labnet, Woodbridge, NJ) at 1000 rpm for 1 min. The supernatant was aspirated and replaced with an equal volume of amphibian Ringer. This process of spinning and washing the cells was repeated twice.

Osmotic Fragility

The LOF in hypotonic solutions was determined by finding the lowest osmolality a suspension of RBCs could be bathed in without lysing. Cell lysis (hemolysis) was determined by observing a turbidity shift (cloudy to clear) that occurs when the integrity of the plasma membrane is compromised. This was detected with a spectrophotometer (Spectronic 20D, Milton Roy Co) 10, 15, or 20 min after blood (30–50 μl) was added to saline solutions (3 ml) of different osmolalities and compositions. Spectrophotometric experiments were conducted at 625 nm; this wavelength provided the greatest difference in optical density (OD) between intact and lysed cells [3].

A hemolytic index (%) was determined using the formula: HI(%) = (OD of Test Compound − OD of Negative Control)/(OD of Positive control − OD of Negative Control) × 100, where the positive control was blood in distilled water, the negative control referred to blood in diluted Ringer, and the test compound was blood in diluted Ringer containing a specific pharmacological agent. All reported hemolytic indices were calculated using the concentration of Ringer that provided the limit of osmotic fragility for the negative control.

Hematocrit

Hematocrit was used to estimate the volume of RBCs exposed to solutions of different osmolalities. We assumed cells remained isomorphic and packed equally regardless of their volume. Blood was collected in heparinized capillary tubes (Labcraft Brand, Red-Tip) and spun for 60 sec by a microhematocrit centrifuge (model MB, International Equipment, Needham Hts., MA). Hematocrit was measured by a micro-capillary reader (Int. Equip., Needham Hts., MA).

As described by others (12), a percent volume recovery at X min after hypotonic exposure was calculated as $[(V_{max} - V_{Xmin})/(V_{max} - V_0)] \times 100$, where V_{max} is the peak relative cell volume, V_0 is the initial relative volume or one, and V_{xmin} is the relative cell volume measured X min following hypotonic exposure. We also used the peak relative volume for the control when assessing the effect of a pharmacological agent added at 0 min. A percent regulatory volume decrease was calculated as [(percent recovery$_{experimental}$)/(percent recovery$_{control}$)] × 100, where maximal recovery in hypotonic Ringer is 100%.

Coulter Counter

Cell volume distribution curves were obtained by electronic sizing using a Coulter counter model Z2 with channelyzer (Coulter Electronics, Hialeah, FL). Cell volume was taken as the mean volume of the distribution curves. The diameter of the aperture tube orifice was 200 μM and the metered volume was 0.5 ml. Absolute cell volumes were obtained using polystyrene latex beads (20.13 μM diameter or 4.271×10^3 fl volume) as standards (Coulter). Experiments with the latex beads showed that measured volumes were unaffected by changes in osmolality and ionic composition within the ranges used for this study. Cell suspensions were diluted 4000-fold with amphibian Ringer or 2000-fold with amphibian Ringer followed by a 1-fold dilution with distilled water to give a final cell density of approximately 5,000 cells per ml.

Patch Clamp

Patch pipettes were fabricated from Kovar sealing glass (Corning model 7052, 1.50 mm OD, 1.10 mm ID; Garner Glass, Claremont, CA) using of a two-pull method (Narishige PP-7). Pipette tips were fire polished (Narishige MF-9) to give a direct current resistance of approximately 5–8 MΩ in symmetrical 100 mM KCl solutions. All pipette solutions were filtered immediately before use with a 0.22 μm membrane filter (Millex-GS, Bedford, MA), and the pipettes were held in a polycarbonate holder (E.W. Wright, Guilford, CT). Membrane currents were measured with a 10^{10} Ω feedback resistor in a headstage (CV-201A, Axon Instruments, Foster City, CA) with a variable gain amplifier set at 1 mV/pA (Axopatch 200A, Axon Instruments, Foster City, CA). The current signals were filtered at 1 kHz through a 4-pole low-pass Bessel filter and digitized at 5 kHz with an IBM-486 computer.

Acquisition and analysis of data were conducted with P-Clamp® (version 6, Axon Instruments, Foster City, CA). Data were acquired during 100 msec voltage pulses and the command potential was set to −15 mV (close to the resting potential for RBCs) for 100 msec between each pulse. All voltage measurements refer to the cell interior.

RBCs, attached to glass coverslips (5 mm diam., Bellco Biotech., Vineland, NJ) with poly-D-lysine (150,000–300,000; 1 mg/ml), were placed in a specially designed open-style chamber (250 μl volume, Warner Instruments, Hamden, CT). The bath solution could be changed by a six-way rotary valve (Rheodyne, Cotati, CA). The whole-cell configuration was achieved following formation of a gigaohm seal (cell-attached configuration) by applying suction to disrupt the patch of membrane beneath the pipette or by applying a large voltage (>200 mV) to the patch. A sudden increase in the capacitance current transient accompanied disruption of the membrane.

Solutions

Amphibian Ringer solution consisted of (in mM): 110 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES (titrated to pH 7.4 with NaOH), and 5 glucose. A low Na^+ Ringer was prepared by substituting choline chloride for NaCl (used for all experiments with gramicidin), and a 0.5× Ringer was obtained by mixing equal volumes of Ringer and distilled water. A stock solution of gramicidin was dissolved in methanol, whereas stock solutions of A23187 (calcium ionophore calcimycin), bisindolylmaleimide I (GF-109203X, 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(1*H*-indol-3-yl)), bisindolylmaleimide II (2-{1-[2-(methylpyrrolidinyl)ethyl]indol-3-yl}-3-(1*H*-indol-3-yl)maleimide), and ONO-RS-082 (2-(p-amylcinnamoyl)amino-4-chlorbenzoic acid) were prepared with dimethylsulfoxide (DMSO) or ethanol, and chelerythrine (1,2-dimethoxy-N-methyl-[1,3]benzodioxolo[5,6-*c*]phenanthridinium) and sphingosine were dissolved in DMSO. All the above nonaqueous stock solutions were mixed at 1000× the final concentration and then diluted 1000× to give an appropriate working concentration, thereby diluting the vehicle an equivalent amount. All stock aqueous solutions (e.g., H-7, H-8, and HA-1004) were diluted 100× to give an appropriate final concentration.

Patch pipettes were filled with an intracellular Ringer solution containing (in mM): 100 KCl, 3.5 NaCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 2.0 EGTA, 5.0 HEPES (pH 7.4 with KOH), 5.0 glucose, 1.0 Mg-ATP, and 0.5 GTP. During seal formation, the extracellular solution contained (in mM): 105 NaCl, 2.5 KCl, 1.8 $CaCl_2$, O.5 $MgCl_2$, 10.0 HEPES (pH 7.4), and 5 glucose. An isosmotic high K^+ bath contained (in mM): 105 KCl, 2.5 NaCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10.0 HEPES (pH 7.4), and 5 glucose. A hypotonic (0.5×) high K^+ bath contained (in mM): 2.5 NaCl, 50 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES (pH 7.4), and 5 glucose.

For hemolysis experiments, cells were incubated with a pharmacological agent or its vehicle for 1–10 min prior to experimentation. For cell volume studies, pharmacological agents were added with hypotonic exposure (0 min) or at peak cell volume (5 min after hypotonic stress). Osmolality of solutions was measured with a vapor pressure osmometer (#5500, Wescor, Logan, UT). Chemicals were purchased from Sigma Chemical (St. Louis, MO), Alexis Biochemicals (San Diego, CA), and ICN (Costa Mesa, CA). All experiments were conducted at room temperature (21–23°C).

Statistics

Data are reported as mean ± SE. The statistical significance of an experimental procedure was determined by a paired Student's *t*-test or least significant difference test with paired design of analysis of variance (ANOVA)/multivariate ANOVA (MANOVA), as appropriate (Data Desk® software, Ithaca, NY). A $P < 0.05$ was considered significant. Each animal served as its own control, and cell volumes at specific times were tested against each other. For patch clamp studies, each cell served as its own control.

Results

Osmotic Fragility Studies

Although the LOF depends on several factors, we first established this limit as one assessment of a cell's ability to regulate volume in a hypotonic medium. The OD, measured at the lowest osmolality RBCs could tolerate without hemolysis (20.7 ± 1.3 mosmol/Kg H_2O), was 0.023 ± 0.001 ($n = 7$ experiments, Fig. 1). To determine whether the LOF depended on a protein kinase, we used the general protein kinase antagonist 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7, 10 μM [ref. 10]). In

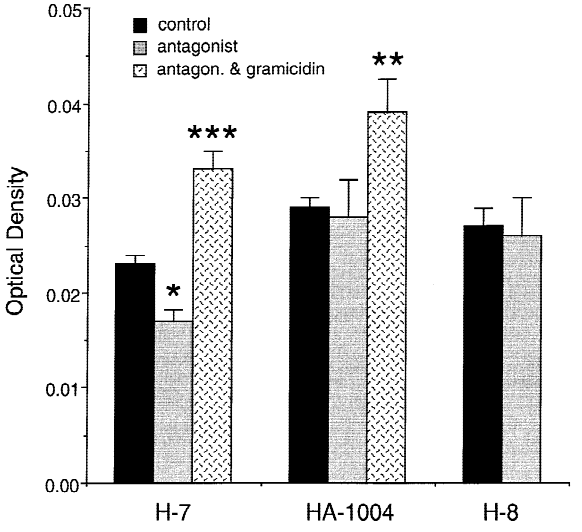

Fig. 1. Inhibition of a protein kinase increased the osmotic fragility of mudpuppy RBCs. Cells were incubated for 1–10 min in isosmotic Ringer with 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7, 10 μM, $n = 7$), N-(2′-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004, 10 μM, $n = 6$), or N-2-(methylamino)ethyl-5-isoquinolinesulfonamide (H-8, 5 μM, $n = 8$) before being diluted. The *control solution* was diluted amphibian (high Na^+) Ringer, the *antagonist solution* was diluted Ringer with pharmacological agent, and the *antagonist & gramicidin solution* was a choline Ringer that contained both the antagonist and ionophore (5 μM). Vehicle for gramicidin (methanol, diluted 1:1000, $n = 8$) and replacing Na^+ with choline ($n = 8$) had no effect on osmotic fragility. Data are reported as the mean ± SE. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

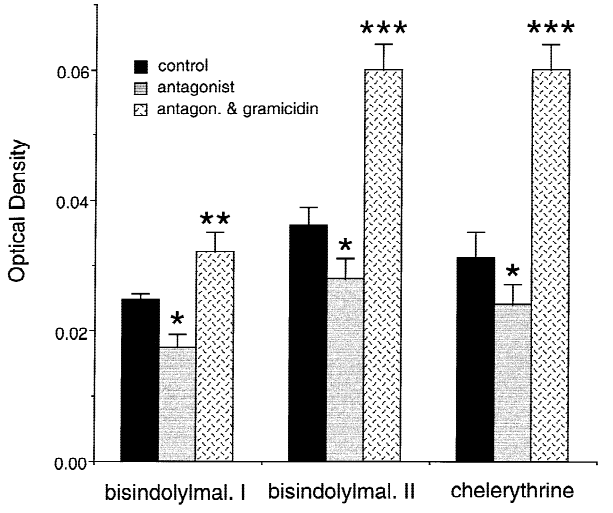

Fig. 2. Inhibition of protein kinase C increased the limit osmotic fragility. Cells were incubated for 1–10 min in isosmotic Ringer with bisindolylmaleimide I (bis I, 100 nM, $n = 10$), bisindolylmaleimide II (bis II, 100 nM, $n = 10$), or chelerythrine (10 μM, $n = 8$) before dilution. The *control solution* was diluted amphibian (high Na^+) Ringer, the *antagonist solution* was diluted Ringer with inhibitor, and the *antagonist & gramicidin solution* was a choline Ringer that contained both antagonist and ionophore (choline Ringer). Vehicle for bis I (ethanol), bis II (ethanol), chelerythrine (DMSO), and gramicidin (methanol) were diluted 1000× and had no effect on osmotic fragility ($n = 8$ for each). Substitution of choline for Na^+ also had no effect. Data reported as mean ± SE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

this case, the OD decreased to 0.017 ± 0.001 ($n = 7$, $P < 0.05$, Fig. 1), giving a hemolytic index of 26%. As illustrated in Fig. 1, the cationic ionophore gramicidin (5 μM in a choline Ringer), which was used to maintain a high K^+ permeability, reversed the inhibitory effect of H-7, increasing the OD to 0.033 ± 0.002 ($n = 7$, $P < 0.001$ compared to control, Fig. 1). In contrast to H-7, the cyclic-nucleotide kinase inhibitors N-(2′-guanidinoethyl)-5-isoquinolinesulfonamide (HA-1004, 10 μM, $n = 6$ [10]) and N-2-(methylamino)ethyl-5-isoquinolinesulfonamide (H-8, 5 μM, $n = 8$ [10]) had no significant effect on the OD (Fig. 1).

Based on the results illustrated in Fig. 1, we next examined whether three specific inhibitors of PKC would effect the LOF (9, 36). Bisindolylmaleimide I (GF-109203X or bis I, 100 nM) decreased the OD from 0.025 ± 0.001 to 0.017 ± 0.002 ($n = 10$, $P < 0.05$, Fig. 2), giving a hemolytic index of 32%. As shown in Fig. 2, gramicidin (5 μM) reversed the inhibitory effect of bis I, bringing the optical density to 0.032 ± 0.003 ($n = 10$, $P < 0.01$ compared to control). In addition, bisindolymaleimide II (bis II, 100 nM) increased the LOF. It decreased the OD from 0.036 ± 0.003 to 0.028 ± 0.003 ($n = 10$, $P < 0.05$, Fig. 2), resulting in a hemolytic index of 22%. Gramicidin (5 μM) also reversed the inhibitory effect of bis II, bringing the optical density to 0.060 ± 0.004 ($n = 10$, $P < 0.001$, Fig. 2). Chelerythrine (10 μM) had a similar effect on osmotic fragility as bis I and bis II. It decreased the optical density from 0.031 ± 0.004 to 0.024 ± 0.003 ($n = 8$, $P < 0.05$, Fig. 2), causing a hemolytic index of 23%. The inhibitory effect of chelerythrine was reversed with gramicidin, which increased the optical density to 0.060 ± 0.004 ($n = 8$, $P < 0.001$, Fig. 2).

Cell Volume Studies

The hematocrit of whole blood was 20.4 ± 4.7 ($n = 40$), a value close to that previously reported for this species [3]. When RBCs were placed in a hypotonic (0.5×) Na^+ Ringer, they quickly swelled, which was then followed by a slower, spontaneous decrease in volume (Fig. 3)[1].

[1] Cells behaving as perfect osmometers should double their size in this solution. However, we did not observe this amount of swelling. One possible explanation for this phenomenon is that there was a relatively large volume of cells compared to the extracellular volume. This could have resulted in a significant shift of water from the bath into the cells during hypotonic shock (sufficient enough to increase the extracellular osmolality), thereby decreasing the expected level of swelling.

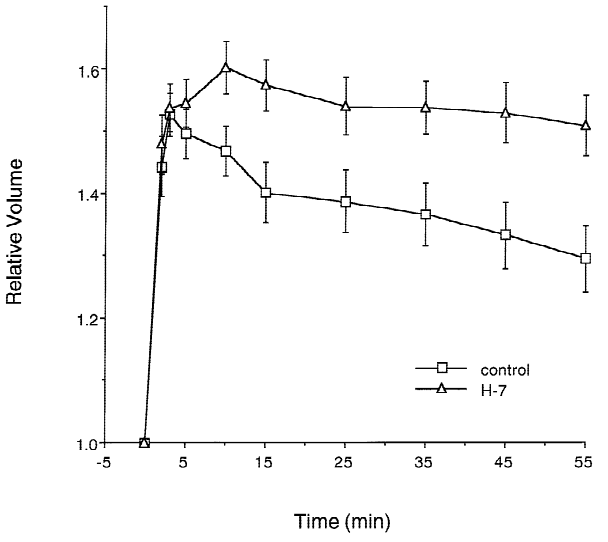

Fig. 3. Inhibition of a protein kinase by H-7 reduced the recovery from cell swelling (estimated from the hematocrit). At time 0, cells were abruptly exposed to a hypotonic (0.5×) high Na^+ Ringer, which caused a rapid initial increase in relative volume followed by a gradual recovery toward basal values, despite the continued presence of hypotonic buffer (□). Recovery was inhibited by addition of H-7 (10 μM, $n = 7$, △) to the extracellular medium at 0 min. Data are reported as the mean ± SE.

As illustrated in Fig. 3, the relative volume with H-7 (10 μM) was significantly higher than the control for all measurements beyond 5 min ($n = 7$, $P < 0.05 > 5$ min), and H-7 decreased the percent RVD at 55 min to 32% of control values (Table 1). In contrast to H-7, H-8 (5 μM, $n = 12$) and HA-1004 (10 μM, $n = 6$) had no effect on RVD (*not shown*). However, the PKC inhibitor sphingosine (100 nM, ref. 7) decreased percent RVD at 35 min to 50% of control values (Table 1), and the relative volume with sphingosine was significantly higher than the control after 5 min ($n = 6$, $P < 0.05 \geqslant 10$ min, Fig. 4).

To determine whether Ca^{2+} was involved in the activation of RVD, we used the Ca^{2+} ionophore A23187 (0.5–1 μM). When added at time 0, the relative cell volume with ionophore was significantly lower than the control for all measurements following hypotonic shock ($n = 6$, $P < 0.001$, Fig. 5). In this case, the percent RVD of the control at 25 min was only 56% that of A23187 (Table 1). As illustrated in Fig. 6, the calcium ionophore had a similar effect on RVD when added to the extracellular medium 5 min after hypotonic shock, a time when cells were maximally swollen ($n = 6$, $P < 0.001$ between 10 and 30 min, $P < 0.01$ between 50 and 70 min). At 20 min, the percent RVD of the control was only 38% that of A23187 (Table 2). Further, the Ca^{2+} ionophore induced a decrease in cell volume when added under isosmotic conditions. As shown in Fig. 7, there was a significant reduction in cell volume 2 min after the addition of A23187, which was followed by a slower, spontaneous recovery ($n = 9$, $P < 0.001$ between 6 and 10 min following A23187, $P < 0.01$ between 4 and 20 min after ionophore, $P < 0.05$ between 2 and 50 min after A23187).

Consistent with the hemolysis studies, the PKC antagonist chelerythrine (10 μM) inhibited cell volume recovery following hypotonic shock ($n = 6$, $P < 0.05 > 0$ min, Fig. 8), resulting in a percent RVD that was only 76% of the control (Table 1). However, A23187 (1 μM) did not reverse the inhibitory effect of chelerythrine ($n = 6$, Fig. 8). In this case, the relative cell volume with A23187 and chelerythrine together was not different than chelerythrine alone for all time periods after hypotonic shock.

We next examined whether PKC regulates K^+ efflux during RVD. As illustrated in Fig. 9, H-7 reduced cell volume recovery ($n = 6$, $P < 0.05 > 10$ min), giving a percent RVD of 77% control values (Table 2). Further, this effect was reversed with gramicidin (5 μM, choline Ringer, $n = 6$, Fig. 9). Mean values for relative cell volume with both H-7 and gramicidin in the extracellular medium were significantly below control values for all measurements following hypotonic shock ($P < 0.001$). In this case, the percent RVD of the control at 90 min was only 62% that of gramicidin (Table 2).

Gramicidin also reversed the inhibitory effect of chelerythrine. As shown in Fig. 10, chelerythrine (10 μM) inhibited cell volume recovery following hypotonic shock ($n = 6$, $P < 0.05 > 0$ min), reducing percent RVD at 60 min to 73% of control values (Table 1). Gramicidin (5 μM) reversed the antagonist effect of chelerythrine on RVD ($n = 6$), such that the relative volume with gramicidin and chelerythrine together was significantly less than chelerythrine alone ($P < 0.001 > 0$ min). At 60 min, the percent RVD of control was only 77% that of gramicidin (Table 1).

Consistent with the results described above, the PKC antagonist bis I (100 nM) reduced RVD ($n = 10$, $P < 0.05 > 20$ min, Fig. 11), and decreased percent RVD to 65% of control values at 90 min (Table 2). In addition, mean values for relative cell volume with both bis I and gramicidin in the extracellular medium were significantly below control values for all measurements following hypotonic shock ($P < 0.001$, Fig. 11). In this case, the percent RVD of the control at 90 min was only 47% that of gramicidin (Table 2). Furthermore, the PKC inhibitor bis II (100 nM) reduced RVD in a manner similar to bis I ($n = 6$, $P < 0.05 \geqslant 15$ min, Fig. 12), giving a percent RVD only 46% of control values (Table 1). As with the other PKC antagonists, gramicidin (5 μM) reversed the inhibitory effect of bis II, such that mean values for relative cell volume with both bis II and gramicidin were significantly below control values for all measurements after hypotonic exposure ($P < 0.001$, Fig. 12). In this case, percent RVD of the control was only 65% that of gramicidin (Table 1).

Table 1. Cell volume recovery in a hypotonic Ringer estimated from the hematocrit

Pharmacological agent	Percent volume recovery*				No.	Time (min)	Figure in text
	Control	Pharm. ag.	Pharm. ag. with gramicidin	Pharm. ag. with A23187			
H-7	51.2 ± 7.5	16.3 ± 6.3			7	55	3
Sphingosine	55.8 ± 7.6	27.9 ± 6.3			6	35	4
A23187	48.2 ± 8.2	86.1 ± 6.8			6	25	5
Chelerythrine	41.0 ± 4.9	31.1 ± 3.0		31.2 ± 3.1	6	60	8
Chelerythrine	86.3 ± 5.4	62.8 ± 10.1	112.7 ± 1.6		6	60	10
Bisindolylmaleimide II	59.0 ± 5.2	27.2 ± 6.6	91.0 ± 1.8		6	45	12

* Values for percent volume recovery are means ± SE (*see* Methods for details). No. indicates number of experiments; time refers to the point after hypotonic shock used to calculate percent volume recovery.

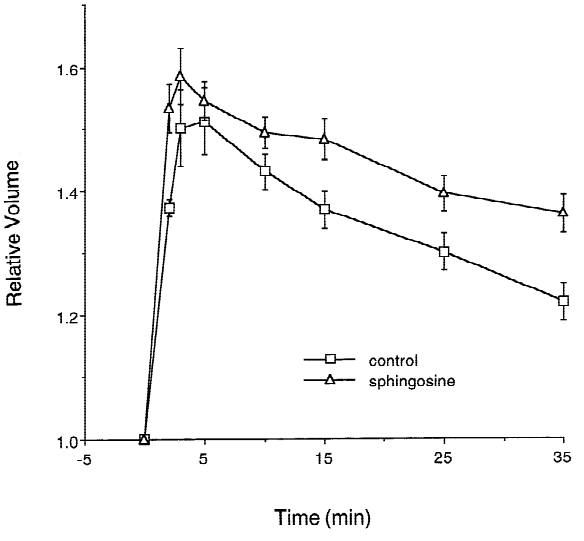

Fig. 4. Inhibition of protein kinase C by sphingosine reduced recovery from cell swelling (estimated from the hematocrit). At time 0, cells were abruptly exposed to a hypotonic (0.5×) Ringer, which caused a rapid initial increase in relative volume followed by a gradual recovery toward basal values (□). Recovery was inhibited by addition of sphingosine (100 nM, $n = 6$, △) to the extracellular medium at 0 min. Vehicle (DMSO, diluted 1000×, $n = 6$) had no effect on cell volume recovery. Data are reported as the mean ± SE.

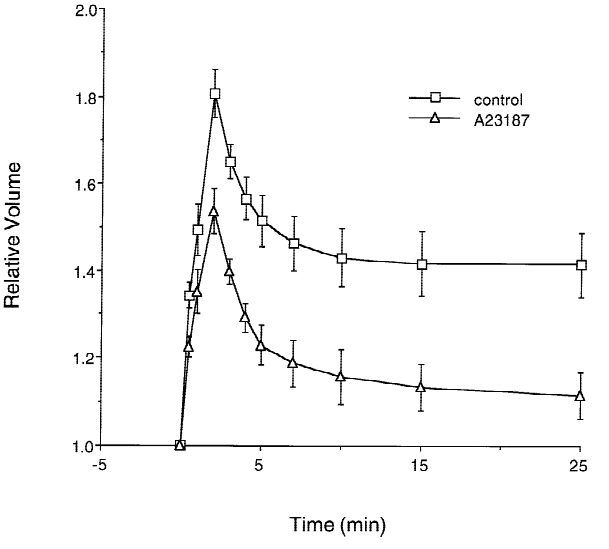

Fig. 5. The calcium ionophore A23187 enhanced recovery from cell swelling (estimated from the hematocrit). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer, which caused a rapid initial increase in relative volume (□). Recovery was enhanced by addition of A23187 (0.5 μM, $n = 6$, △) to the extracellular medium at 0 min. Vehicle (ethanol, diluted 1000×, $n = 8$) had no effect on cell volume recovery. Data are reported as the mean ± SE.

To further examine whether PKC was important for K^+ efflux during RVD, we added gramicidin to the extracellular medium 5 min after hypotonic shock; a point at which the cells were maximally swollen and when it appeared endogenous K^+ channels were activated. As illustrated in Fig. 13, application of gramicidin (5 μM) at this point still reversed the inhibitory effect of bis I (100 nM, $n = 6$, $P < 0.05 > 10$ min). In this case, the percent RVD of control at 90 min was only 50% that of gramicidin (Table 2). In addition, we examined the effect of gramicidin on cells bathed in an isosmotic choline Ringer. As shown in Fig. 7, cells quickly decreased in volume 2 min after addition of gramicidin (5 μM, $n = 9$, $P < 0.001 > 2$ min after gramicidin).

We recently reported that PLA_2 is involved with RVD by this cell type, and that arachidonic acid (AA) stimulates an RVD response [19]. It has been shown by others that cis unsaturated fatty acids, including AA, which are produced from phospholipids by the action of PLA_2, enhance the diacylglycerol-dependent activation of protein kinase C [26]. Accordingly, we examined whether there is a connection between PLA_2, AA, and PKC during RVD. As shown in Fig. 14, the PLA_2 inhibitor ONO-RS-082 (10 μM) had a greater inhibitory effect on RVD than the PKC inhibitor bis I (100 nM,

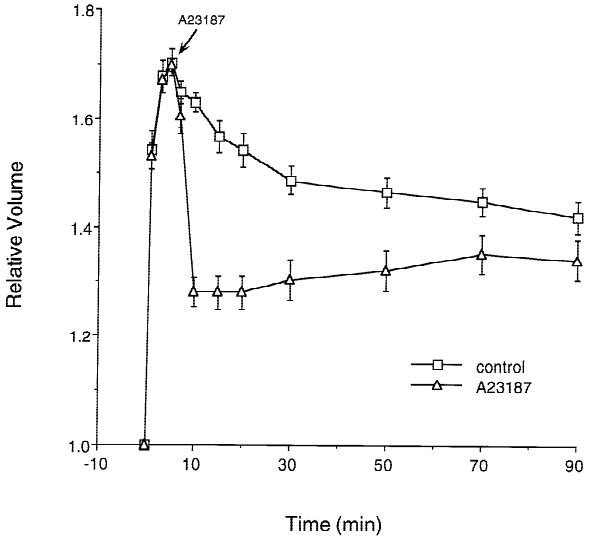

Fig. 6. The calcium ionophore A23187 enhanced recovery from cell swelling when added at peak cell volume (measured with a Coulter counter). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer, which caused a rapid initial increase in relative volume (□). Recovery was enhanced by addition of A23187 (0.5 μM, $n = 6$, △) to the extracellular medium at 5 min (indicated by arrow). Data are reported as the mean ± SE.

$n = 8$). The percent RVD with bis I at 90 min was 70% of control (Table 2). In contrast, it was only 22% of control with ONO-RS-082. In addition, a combination of ONO-RS-082 and bis I together was not significantly different than ONO-RS-082 alone ($n = 8$, Fig. 14). Further, in preliminary studies AA (1 μM) enhanced cell volume recovery, such that percent RVD for the control was only 64% that of AA at 40 min ($n = 3$). However, mean values with bis I (100 nM) and AA (1 μM) together were not significantly different from control ($n = 3$).

Patch Clamp Studies

The membrane of mudpuppy RBCs formed gigaohm seals without enzymatic treatment (~15% success rate). After a gigaohm seal was formed (cell-attached patch), negative pressure or voltage was used to form the whole-cell configuration (in approximately 20% of the patches, a whole-cell configuration formed spontaneously shortly after obtaining a cell-attached patch). Previously we described a whole-cell K^+ conductance that was inhibited by quinine and activated by a Ca^{2+}-calmodulin dependent mechanism [3] and by a 5-lipoxygenase metabolite of arachidonic acid [19] during cell swelling. Below we describe the role of protein kinase C in regulating this channel.

We first examined whether activation of whole-cell currents during cell swelling depended on a protein kinase. The general protein kinase antagonist H-7 (10–100 μM) inhibited a whole-cell conductance when cells were bathed in a 0.5× high K^+ Ringer ($n = 10$, Fig. 15). After initial addition of H-7 to the extracellular bath, the conductance gradually decreased until a maximum inhibition occurred by approximately 3–5 min. No decrease in current was observed in control cells over a similar time period. The whole-cell conductance was reduced by 51% with H-7, from 14.6 ± 2.2 nS to 7.2 ± 1.7 nS ($n = 8$, $P < 0.001$). Further, this antagonist shifted the reversal potential (E_{rev}) away from E_K and towards E_{Cl}, from −6.9 ± 3.1 mV to −3.0 ± 3.6 mV ($n = 8$, Fig. 14); however, this change was not statistically significant. In contrast to H-7, neither H-8 (5 μM) nor HA-1004 (10 μM) had an effect on whole-cell currents ($n = 6$, not shown).

The PKC antagonist bis II had a similar affect on whole-cell currents as did H-7. Three to five min after addition of bis II (100 nM) to the bath solution, the whole-cell conductance was reduced by 41%, decreasing from 33.9 ± 7.1 nS to 20.0 ± 7.9 nS ($n = 7$, $P < 0.01$, Fig. 16). Further, this antagonist shifted the reversal potential (E_{rev}) from −8.9 ± 5.2 mV to −2.0 ± 2.0 mV ($n = 7$, $P < 0.05$, Fig. 16). The only major ions in these solutions were K^+ and Cl^-, and the equilibrium potentials for perfectly cation- and anion-selective conductances were −16.2 mV and +14.7 mV, respectively. The shift in E_{rev} by bis II was therefore away from E_K and towards E_{Cl}. From E_{rev} and the Goldman-Hodgkin-Katz equation, we calculated a change in the K^+ permeability-to-Cl^- permeability (P_K:P_{Cl}) ratio from 3.7:1 to 1.3:1. This indicated that inhibition of protein kinase C by bis II blocked a K^+ conductance. In addition, bis I (100 nM) inhibited whole-cell conductance, reducing it by 52%, from 25.2 ± 1.9 nS to 12.2 ± 5.2 nS ($n = 4$, $P < 0.05$). Bis I also shifted E_{rev}, changing the P_K:P_{Cl} ratio from 2.8:1 to 1.4:1 ($n = 4$, $P < 0.05$).

Discussion

The major finding of this study was that RVD in mudpuppy red blood cells is regulated, at least in part, by protein kinase C. A dependence of cell volume regulation on PKC was demonstrated by bathing cells with various inhibitors of this kinase, and all five of the antagonists we used consistently increased the LOF and reduced percent RVD. We also chose antagonists that block the action of PKC by different mechanisms. Chelerythrine, a tetracyclic aromatic naturally occurring alkaloid, acts as a competitive inhibitor of the catalytic domain of PKC with respect to the phosphate acceptor and as a noncompetitive inhibitor with respect to ATP [9]. Although chelerythrine has a low IC_{50} for protein kinase C (0.7 μM), it has a high IC_{50} for cAMP-

Table 2. Cell volume recovery in a hypotonic Ringer measured with a Coulter counter

Pharmacological Agent	Percent Volume Recovery*				No.	Time (min)	Figure in text
	Control	Pharm. Ag.	Pharm. Ag. with Gramicidin	Pharm. Ag. with A23187			
A23187 (5 min)	23.1 ± 4.0	60.8 ± 3.0			6	20	6
H-7	61.4 ± 2.6	47.4 ± 2.0	99.6 ± 0.2		6	90	9
Bisindolylmaleimide I	44.8 ± 3.2	29.1 ± 3.5	95.1 ± 2.3		10	90	11
Bisindolylmaleimide I	45.6 ± 2.3	34.6 ± 3.0	91.4 ± 3.4		6	90	13
Bisindolylmaleimide I & ONO-RS-082†	44.1 ± 3.3	30.8 ± 2.5 (Bis I)	9.6 ± 4.5 (ONO)	5.9 ± 3.7 (Bis I & ONO)	8	90	14

* Values for precent volume recovery are means ± SE (*see* Methods for details). No. indicates number of experiments; time refers to the point after hypotonic shock used to calculate percent volume recovery. †In this case, gramicidin and A23187 were not used, and the experimental conditions are shown in the parentheses below each percent volume recovery value (*see* text for details).

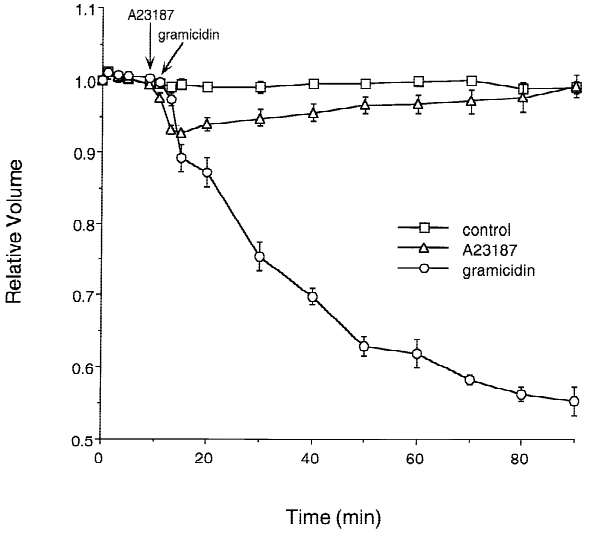

Fig. 7. Effect of A23187 and gramicidin on relative cell volume for RBCs in an isosmotic Ringer (measured with a Coulter counter). In these experiments, cells remained in an isosmotic Ringer for 90 min (□). Addition of A23187 (0.5 μM, $n = 9$, △) at 9 min (indicated by arrow) caused a rapid initial decrease in relative volume, followed by a gradual recovery toward basal values. Addition of gramicidin (5 μM, $n = 9$, ○) at 11 min caused a rapid decrease in relative volume (a lack of volume recovery with gramicidin was most likely due to the choline Ringer, which could inhibit a regulatory volume increase). Data are reported as mean ± SE.

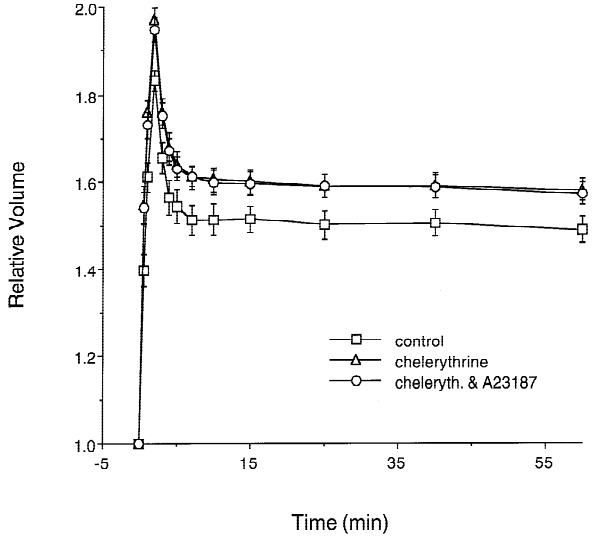

Fig. 8. Calcium ionophore A23187 did not reverse the inhibitory effect of chelerythrine (estimated from the hematocrit). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer (□). Recovery was inhibited by addition of chelerythrine (10 μM, $n = 6$, △) to the extracellular medium at 0 min. A combination of chelerythrine and A23187 (0.5 μM, $n = 6$, ○) was not significantly different from chelerythrine alone. Vehicle (DMSO or ethanol, diluted 1000×, $n = 8$) had no effect on cell volume recovery. Data are reported as the mean ± SE.

dependent protein kinase (PKA, 70 μM), tyrosine protein kinase (100 μM), and calcium/calmodulin-dependent protein kinase (CAM-kinase, >100 μM [9]). Bis I and bis II are cell permeable, synthetic derivatives of staurosporine that display competitive binding kinetics with ATP by interacting with the ATP-binding site on the catalytic domain of PKC [36]. Like chelerythrine, they have a low IC_{50} (10–13 nM) for PKC, and a relatively high IC_{50} for PKA (2 μM), phosphorylase kinase (0.75 μM), and tyrosine kinase (>50 μM) [9, 36].

Isoquinolinesulfonamide derivatives, such as H-7 and H-8, bind to the kinase molecule and compete for ATP binding with the ATP binding site on the enzyme [10]. The general kinase antagonist H-7 inhibits PKC, PKA, and PKG with a similar potency (IC_{50} values of 3–6 μM) [9, 10]. However, given H-7 mimicked the block of cell volume recovery caused by chelerythrine, bis I, and bis II, it is most likely that this isoquinoline inhibited RVD by antagonism of PKC. This was further supported by using HA-1004 and H-8 as negative controls, which had no effect on the LOF fragility and RVD

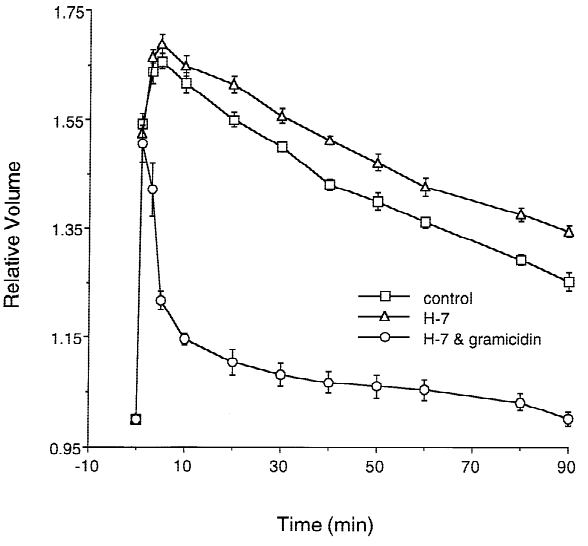

Fig. 9. Gramicidin reversed the inhibitory effect of H-7 (measured with a Coulter counter). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer (□). Recovery was inhibited by addition of H-7 (10 μM, $n = 6$, △) to the extracellular medium at 0 min. In contrast, recovery was enhanced with a combination of H-7 and gramicidin (5 μM, $n = 6$, ○). Data are reported as the mean ± SE.

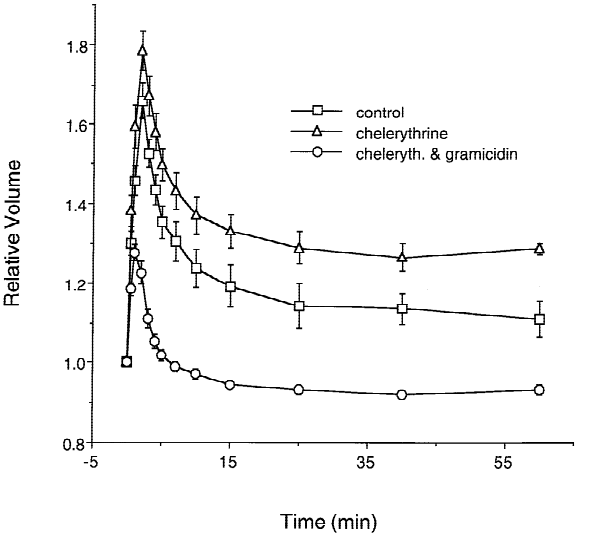

Fig. 10. Gramicidin reversed the inhibitory effect of chelerythrine (estimated from the hematocrit). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer (□). Recovery was inhibited by addition of chelerythrine (10 μM, $n = 6$, △) to the extracellular medium at 0 min. In contrast, recovery was enhanced with a combination of chelerythrine and gramicidin (5 μM, $n = 6$, ○). Data are reported as the mean ± SE.

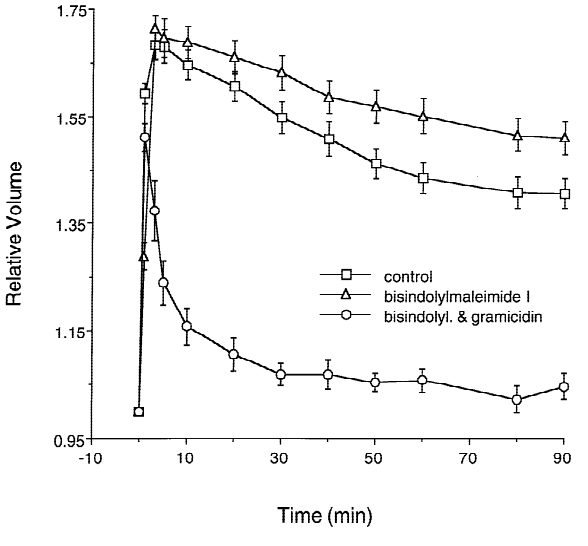

Fig. 11. Gramicidin reversed the inhibitory effect of bisindolymaleimide I (measured with a Coulter counter). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer (□). Recovery was inhibited by addition of bis I (100 nM, $n = 10$, △) to the extracellular medium at 0 min. In contrast, recovery was enhanced with a combination of bis I and gramicidin (5 μM, $n = 10$, ○). Data are reported as the mean ± SE.

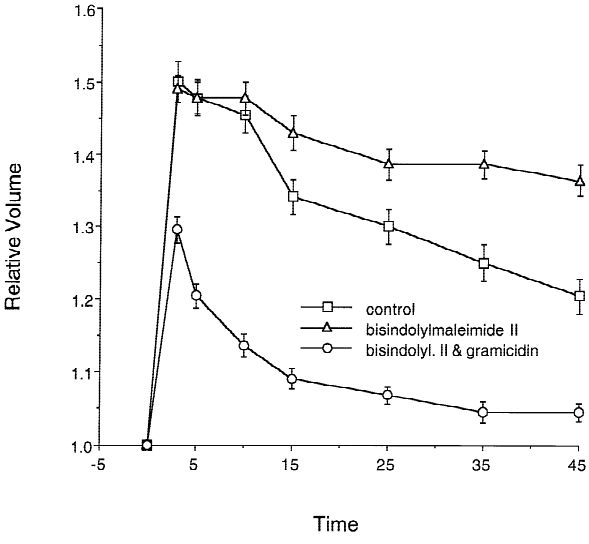

Fig. 12. Gramicidin reversed the inhibitory effect of bisindolylmaleimide II (estimated from the hematocrit). At time 0, cells were abruptly exposd to hypotonic (0.5×) Ringer (□). Recovery was inhibited by addition of bis II (100 nM, $n = 6$, △) to the extracellular medium at 0 min. In contrast, recovery was enhanced with a combination of bis I and gramicidin (5 μM, $n = 6$, ○). Data are reported as the mean ± SE.

when used at concentrations below the K_i for PKC, but above the K_i for PKA and PKG [9, 10]. Thus, an inhibition of cell volume recovery by H-7, chelerythrine, bis I, bis II, and sphingosine, coupled with a lack of an effect by HA-1004 and H-8, indicates that cell volume recovery following hypotonic shock was associated with protein kinase C.

The dependence of cell volume regulation on PKC has been reported for several other cell types. For in-

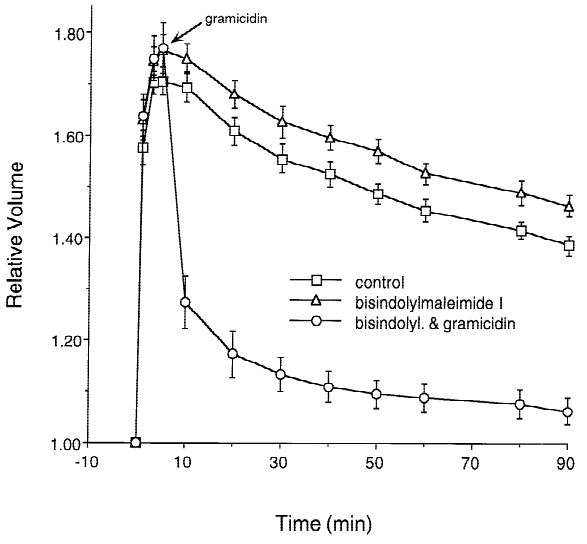

Fig. 13. Gramicidin reversed the inhibitory effect of bisindolylmaleimide I when added at peak relative volume (measured with a Coulter counter). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer (□). Recovery was inhibited by addition of bis I (100 nM, $n = 6$, △) to the extracellular medium at 0 min. In contrast, recovery was enhanced with a combination of bis I and gramicidin (5 μM, $n = 6$, ○), where the ionophore was added 5 min after hypotonic shock (indicated by arrow). Data are reported as the mean ± SE.

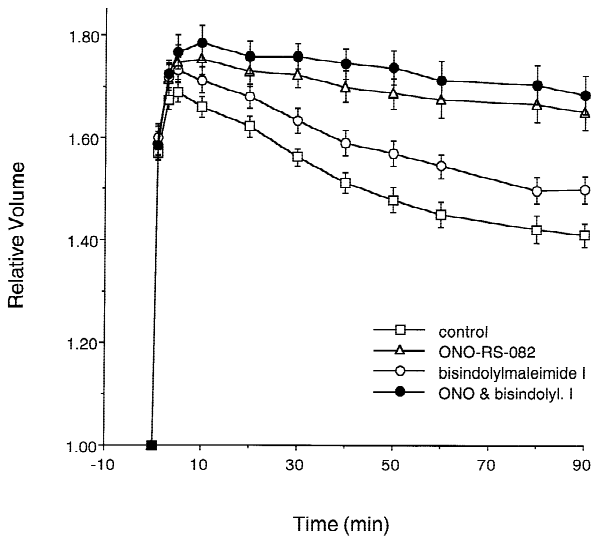

Fig. 14. Effect of inhibition of PKC and phospholipase A_2 on recovery from cell swelling (measured with a Coulter counter). At time 0, cells were abruptly exposed to hypotonic (0.5×) Ringer (□). Recovery was inhibited by addition of the phospholipase A_2 antagonist ONO-RS-082 (10 μM, $n = 8$, △) and the PKC inhibitor bis I (100 nM, $n = 8$, ○) to the extracellular medium at 0 min. A combination of ONO-RS-082 and bis I ($n = 8$, ●) was not significantly different from ONO alone. Data are reported as the mean ± SE.

stance, using phorbol ester, an activator of PKC, Moran and Turner [24] demonstrated that carbachol-induced RVD in human salivary ductal cells involves K^+ channels that are activated by PKC. Rubera et al. [30] found that staurosporine completely inhibits the development of swelling-induced Cl^- currents in primary cultures of rabbit distal bright convoluted tubules, implicating PKC in RVD. In addition, O'Neil and Leng [27] reported that PKC regulates an osmomechanically sensitive Ca^{2+} channel in cultured rabbit proximal tubule cells. Similar to our results, they found that regulation of the osmosensitive channel did not occur through a PKA pathway. Larsen et al. [16] reported hypotonic swelling in Ehrlich mouse ascites tumor cells results in late activation of PKC concomitant with late activation of the Na^+, K^+, $2Cl^-$ cotransporter, whereas MacLeod et al. [20] found that PKC activates a Cl^- conductance required for volume regulation during Na^+-nutrient cotransport. Further, Mitchell et al. [22] showed that inhibitors of PKC completely prevent activation of a volume-sensitive chloride current in pigmented ciliary epithelial cells. However, they concluded that although phospholipase C (PLC) and PLA_2 mediate RVD, the PLC pathway regulates the PLA_2 pathway via a PKC-dependent phosphorylation of PLA_2. Finally, activation of PKC by phorbol ester potentiates RVD in clam RBCs [28], whereas inhibitors of PKC block RVD and a swelling-activated whole cell Cl^- current in a cortical collecting duct cell line [32, 34].

In contrast to our finding that PKC enhances RVD in mudpuppy RBCs, several studies have shown that RVD is independent of this kinase, or even inhibited by PKC. For example, Leaney et al. [15] demonstrated that a swelling-activated chloride current in rat sympathetic neurones is unaffected by bis I. Bender et al. [2] reported that activation of PKC in cultured astrocytes potentiates cell swelling induced by hypoosmotic stress, indicating PKC antagonizes RVD. This was further supported by showing PKC inhibitors lead to cell shrinkage under isosmotic conditions [2].

It was recently shown in our labortory that RVD by mudpuppy RBCs depends on K^+ efflux through a conductance that is activated during cell swelling by a Ca^{2+}-calmodulin dependent process [3] and by a 5-lipoxygenase metabolite of arachidonic acid [19]. The time course for activation of this K^+ current during cell swelling coincided with the time course for volume recovery from hypotonic shock [3, 19]. In addition, the rate of RVD was accelerated in the presence of gramicidin, indicating that the K^+ permeability was a rate-limiting process and that Cl^- permeability was high in swollen cells [3]. This cell type also displayed a high Cl^- permeability under isosmotic conditions [3].

In this study we demonstrated both pharmacologically and electrophysiologically that the K^+ conductance also is modulated by PKC. A dependence of K^+ efflux on PKC was shown pharmacologically using the cationophore gramicidin in a choline Ringer solution. In this case, the only two permeable ions of significance were

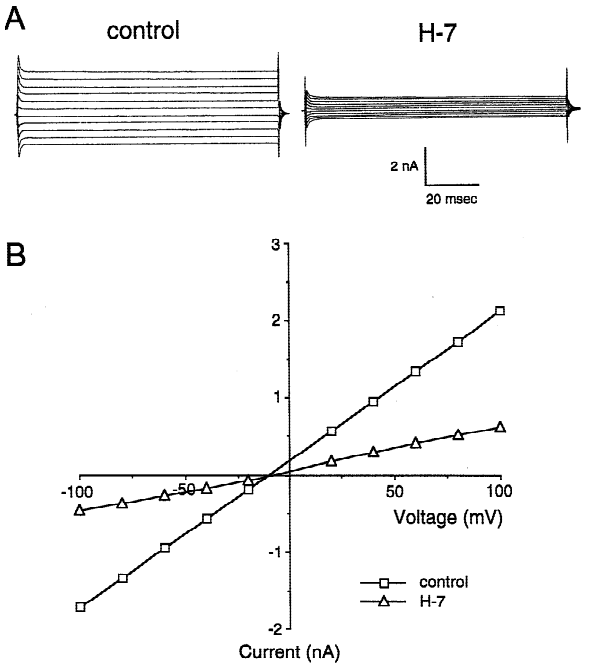

Fig. 15. Inhibition of a protein kinase blocked a whole-cell conductance. Cell was maintained at a holding potential of −15 mV and stepped to potentials between −100 to +100 mV in 20 mV intervals. (*A*) whole-cell currents for a RBC exposed to a hypotonic (0.5×) high K^+ bath and inhibition of these currents by adding H-7 (10 μM) to the bath. (*B*) corresponding current-voltage (*I-V*) relationship. The conductance decreased by 51% ($n = 8$, $P < 0.001$) with H-7. Although E_{rev} shifted away from E_K and towards E_{Cl}, from −6.9 ± 3.1 mV to −3.0 ± 3.6 mV ($n = 8$), this change was not statistically significant. A single representative experiment is illustrated, however, statistical analysis was based on replicate experiments.

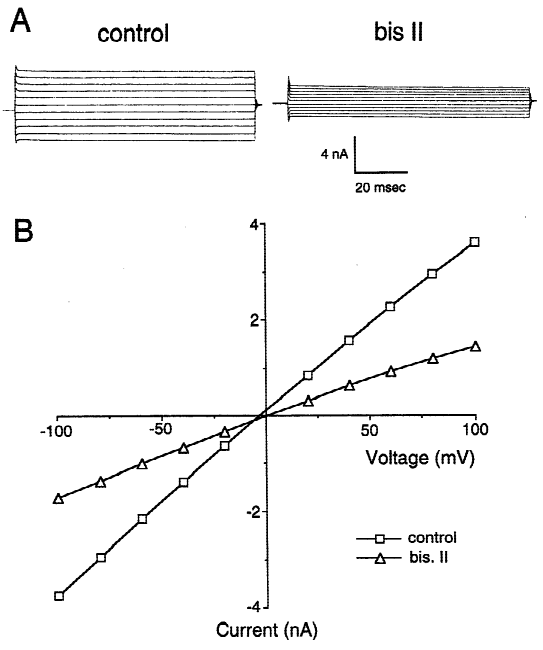

Fig. 16. Inhibition of protein kinase C with bis II blocked a whole-cell K^+ conductance. Cell was maintained at a holding potential of −15 mV and stepped to potentials between −100 to +100 mV in 20 mV intervals. (*A*) whole-cell currents for a RBC exposed to a hypotonic (0.5×) high K^+ bath and inhibition of these currents by adding bis II (100 nM) to the bath. (*B*) corresponding current-voltage (*I-V*) relationship. The conductance decreased by 41% ($n = 7$, $P < 0.05$) with bis II, and the P_K:P_{Cl} shifted from 3.7:1 in the control to 1.3:1 with the antagonist ($n = 7$, $P < 0.05$). A single experiment is illustrated, however, statistical analysis was based on replicate experiments.

K^+ and Cl^-, and addition of gramicidin ensured a high K^+ permeability. For both the limit of osmotic fragility studies and cell volume recovery experiments, gramicidin consistently reversed the inhibitory effect of H-7, bis I, bis II, and chelerythrine. In addition, for the cell volume experiments, it did not matter whether gramicidin was added at 0 min or at 5 min. The reason for examining the effect of gramicidin at 5 min is because that point in time corresponded with maximum cell swelling, indicating it took several minutes for endogenous K^+ channels to activate following hypotonic stress. Thus, percent volume recovery increased regardless of whether K^+ permeability was artificially enhanced with gramicidin at the time of hypotonic stress or at 5 min, even in the presence of PKC antagonists. In addition, gramicidin caused cells to shrink under isosmotic conditions. This is consistent with these cells having a low K^+ permeability under normal conditions, and an elevated K^+ permeability during hypotonic stress [3].

A similar comparison was made using the Ca^{2+} ionophore A23187. That is, it increased the percent volume recovery regardless of whether it was added at 0 min or 5 min, indicating RVD was sensitive to the level of calcium. Further, A23187 caused cells to shrink under isosmotic conditions. Presumably, the ionophore elicited a change in cells mimicking the response that occurs when cells are stimulated by hypotonic exposure. However, we cannot rule out the possibility that A23187 caused an RVD-type response that was fundamentally different from the swelling-induced response. Interestingly, A23187 did not reverse the inhibitory effect of chelerythrine, as did gramicidin. These observations are consistent with a rise in intracellular Ca^{2+} preceding activation of PKC, and K^+ efflux occurring after PKC stimulation. It is worth noting that RBCs exposed to gramicidin or A23187 stabilized their volume at a much lower size than control cells. It is possible that under the conditions of our study, control cells lacked a sufficient rise in intracellular Ca^{2+} or an adequate increase in K^+ permeability to display a full RVD response. Alternatively, these cells may naturally reach a level of cell volume recovery that is less than what occurred with A23187 and gramicidin.

There was an obvious difference between control and experimental cell volumes at 5 min in many of the relative volume figures. This discrepancy depended on

what point in time pharmacological agents were added and how much the pharmacological agent effected cell volume. For example, the differences between Figs. 5 and 6 can be explained by A23187 being added at 0 min for Fig. 5, but not until peak volume for Fig. 6. In addition, there was a difference in the overall shape of the relative volume curves depending on whether cell volume was estimated from the hematocrit or measured with a Coulter counter.

A dependence of K^+ efflux on PKC was shown electrophysiologically using the whole-cell patch clamp technique. Both bis I and bis II blocked a K^+ conductance that is activated by cell swelling in a hypotonic Ringer. In fact, with bis I or bis II, the K^+ conductance in a hypotonic bath was reduced to near the levels found for control cells in isosmotic conditions [3]. Further, the inhibition of both RVD and the K^+ conductance with PKC antagonists was similar to the inhibition we previously reported with quinine, a specific inhibitor of K^+ channels [3]. For example, the decrease in whole-cell conductance and the change in P_K:P_{Cl} with PKC inhibitors was similar to the reduction with quinine [3]. In addition, HA-1004 and H-8 had no effect on whole-cell currents; that is, they neither altered whole-cell conductance nor whole-cell reversal potential. Taken together, these results can best be explained by protein kinase C stimulation of a K^+ conductance during cell swelling.

These findings are consistent with other reports that have shown ion channels may be regulated by PKC. For example, phorbol esters and exogenous PKC activate a maxi Cl^- channel in a rabbit cortical collecting duct cell line [32]. Phorbol esters also stimulate a K^+ channel in a human salivary duct cell line [24] and a dihydropyridine-sensitive calcium influx channel in rabbit proximal tubule cells [27]. Our finding that PKC stimulates an ion conductance is different, however, from what was reported for several other types of channels. For example, phorbol ester inhibits GABA-gated Cl^- currents in rhol-expressing oocytes [14] and inhibits a Ca^{2+}-permeable cation channel in liver cells [4].

Interestingly, H-7 reduced whole-cell conductance without significantly altering E_{rev}. We do not have a definitive explanation for this, however, it is consistent with concomitant inhibition of K^+ and Cl^- currents. It also is consistent with preliminary studies we have conducted using HA-1077 (1-(5-isoquinolinesulfonyl)*homo*-piperazine) to inhibit a Ca^{2+}/calmodulin-dependent protein kinase [18, 31]. In this case, HA-1077 increased the LOF and partially inhibited the rate of cell volume recovery; both of these effects were not reversed with gramicidin [18]. Further, HA-1077 inhibited a whole-cell Cl^- conductance in swollen cells [18]. These preliminary results suggest that a chloride channel, activated by a Ca^{2+}/calmodulin-dependent protein kinase, may augment chloride efflux during RVD. Consequently, it is possible that H-7 not only inhibited PKC, but also blocked a Ca^{2+}/calmodulin-dependent protein kinase, which has a K_i of approximately 80 μM for H-7 [9]. This, in turn, would simultaneously reduce both K^+ and Cl^- currents, thereby reducing whole-cell conductance without necessarily altering the reversal potential. In contrast to H-7, experiments with bis I and bis II, which are more specific for PKC, caused the reversal potential to shift towards E_{Cl}, indicting inhibition of a K^+ conductance.

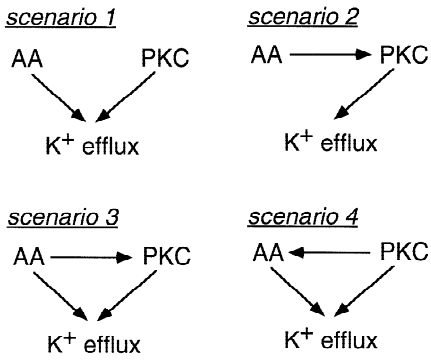

Fig. 17. Four scenarios describing potential interactions between arachidonic acid (AA), protein kinase C (PKC), and K^+ efflux during RVD. Arrows refer to directions of stimulatory signals (N.B., arrows imply directionality only, and may represent direct interactions or a series of steps). Although not explicitly illustrated, it is assumed that PKC increases AA levels by stimulation of PLA_2 (scenario 4). Our results are most consistent with scenario three (see text for details).

Finally, we have previously shown that PLA_2 is involved with RVD by this cell type, and that eicosanoid products of arachidonic acid stimulate an RVD response [19]. For example, inhibition of PLA_2 and 5-lipoxygenase reduces both RVD and a K^+ conductance, whereas addition of AA under isosmotic conditions stimulates a K^+ conductance [19]. Although it is possible that AA and PKC independently stimulate K^+ efflux, it has been shown by others that AA, which is produced by the action of PLA_2, enhances diacylglycerol-dependent activation of PKC [26]. In addition, and alternatively, it is possible that PLA_2 is regulated by PKC-dependent phosphorylation [23a]. With this in mind, we tested four scenarios describing potential interactions between AA, PKC, and K^+ efflux (Fig. 17).

We attempted to distinguish between these scenarios by using an inhibitor of PLA_2 (ONO-RS-082) and protein kinase C (bis I) independently of each other and together (Fig. 14). We found ONO consistently had a greater inhibitory effect on percent RVD than bis I. In addition, bis I and ONO together were significantly greater in their inhibition of cell volume recovery than bis I alone, however, the combination of the two drugs were not significantly different than ONO alone. Taken together, these results are most consistent with scenario 3 (Fig. 17), however, we cannot definitively rule out other

possibilities. Further our preliminary observation that AA enhances RVD, and that AA and bis I together give the same percent recovery as the control, also is most consistent with scenario 3 (Fig. 17). Interestingly, this scenario is different from the model described by Mitchell et al. [22] where PKC modulates the activity of PLA_2. Our findings also are different from what is observed in medullary thick ascending limbs of rat kidney, where apical Na^+,K^+(NH_4^+),$2Cl^-$ cotransport is stimulated by PKC, but inhibited by activation of PLA_2 [1].

In conclusion, inhibition of PKC increases the limit of osmotic fragility, reduces cell volume recovery following hypotonic stress, and blocks a conductance that mediates K^+ efflux during cell swelling. We therefore propose that PKC modulates a K^+ channel during hypotonic shock, thereby helping in the recovery of cell volume during RVD in mudpuppy RBCs.

We thank Tracy Capes (Ripon College) for providing some data used in the isosmotic studies, and Sharon Provinzano (Ripon College) and Erica Smith (Ripon College) for their technical assistance in running the laboratory. We also thank Dr. Fiona L. Stavros (Texas Biotechnology Corporation, Houston, TX) for developing the hemolytic index we used, and Dr. Robert L. Wallace (Ripon College) for helpful discussions and suggestions on the manuscript. Research support was provided by the National Science Foundation (MCB-9603568). Portions of this study were presented in abstract form at *Experimental Biology* '96, Washington, D.C., April, 1996 and *Experimental Biology* '98, San Francisco, April, 1998.

References

1. Amlal, H., Legoff, C., Vernimmen, C., Paillard, M., Bichara, M. 1996. $Na^+K^+(NH_4^+),2Cl^-$ cotransport in medullary thick ascending limb: control by PKA, PKC, and 20-HETE. *Am. J. Physiol.* **271:** C455–C463
2. Bender, A.S., Neary, J.T., Blicharska, J., Norenberg, L.-O.B., Norenberg, M.D. 1992. Role of calmodulin and protein kinase C in astrocytic cell volume regulation. *J. Neurochem.* **58:**1874–1882
3. Bergeron, L.J., Stever, A.J., Light, D.B. 1996. Potassium conductance activated during regulatory volume decrease by mudpuppy red blood cells. *Am. J. Physiol.* **270:**R801–810
4. Fitz, J.G., Sostman, A.H., Middleton, J.P. 1994. Regulation of cation channels in liver cells by intracellular calcium and protein kinase C. *Am. J. Physiol.* **266:**G677–G684
5. Grinstein, S., Foskett, J.K. 1990. Ionic mechanisms of cell volume regulation in leukocytes. *Annu. Rev. Physiol.* **52:**399–414
6. Grinstein, S., Furuya, W., Bianchini, L. 1992. Protein kinases, phosphatases, and the control of cell volume regulation. *NIPS* **7:**232–237
7. Hannun, Y.A., Bell, R.M. 1989. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* **243:**500–507
8. Hazama, A., Okada, Y. 1988. Ca^{2+} sensitivity of volume-regulatory K^+ and Cl^- channels in cultured human epithelial cells. *J. Physiol.* **402:**687–702
9. Herbert, J.M., Augereau, J.M., Gleye, J., Maffrand, J.P. 1990. Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **172:**993–999
10. Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y. 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23:**5036–5041
11. Hoffman, E.K., Dunham, P.B. 1995. Membrane mechanisms and intracellular signaling in cell volume regulation. *Intern. Rev. Cytol.* **161:**173–262
12. Jorgensen, N.K., Christensen, S., Harbak, H., Brown, A.M., Lambert, I.H., Hofmann, E.K., Simonsen, L.O. 1997. On the role of calcium in the regulatory volume decrease (RVD) response in Ehrlich mouse ascites tumor cells. *J. Membrane Biol.* **157:**281–299
13. Kirk, K., Stange, K. 1998. Functional properties and physiological roles of organic solute channels. *Annu. Rev. Physiol.* **60:**719–739
14. Kusama, T., Sakurai, M., Kizawa, Y., Uhl, G.R., Murakami, H. 1995. GABA rhol receptor: inhibition by protein kinase C activators. *Europ. J. Pharmacol.* **21:**431–434.
15. Leaney, J.L., Marsh, S.J., Brown, D.A. 1997. A swelling-activated chloride current in rat sympathetic neurones. *J. Physiol.* **501(3):**555–564
16. Larsen, A.K., Jensen, B.S., Hoffmann, E.K. 1994. Activation of protein kinase C during cell volume regulation in Ehrlich mouse ascites tumor cells. *Biochimica et Biophysica Acta* **1222:**477–482
17. Lewis, S.A., Donaldson, P. 1990. Ion channels and cell volume regulation: chaos in an organized system. *NIPS* **5:**112–119
18. Light, D., Botsford, S. 1996. Role of protein kinase C and CAM-kinase in regulatory volume decrease. *FASEB J.* **10:**A86
19. Light, D.B., Mertins, T.M., Belongia, J.A., Witt, C.A. 1997. 5-Lipoxygenase metabolites of arachidonic acid regulate volume decrease by mudpuppy red blood cells. *J. Membrane Biol.* **158:**229–239
20. MacLeod, R.J., Lembessis, P., Hamilton, J.R. 1992. Effect of protein kinase C inhibitors on Cl^- conductance required for volume regulation after L-alanine cotransport. *Am. J. Physiol.* **262:**C950–C955
21. McCarty, N.A., O'Neil, R.G. 1992. Calcium signaling in cell volume regulation. *Physiol. Rev.* **72:**1037–1061
22. Mitchell, C.H., Zhang, J.J., Wang, L, Jacob, T.J.C. 1997. Volume-sensitive chloride current in pigmented ciliary epithelial cells: role of phospholipases. *Am. J. Physiol.* **272:**C212–C222
23. Montrose-Rafizadeh, C., Guggino, W.B. 1991. Role of intracellular calcium in volume regulation by rabbit medullary thick ascending limb cells. *Am. J. Physiol.* **260:**F402–F409
24. Moran, A., Turner, R. 1993. Secretagogue-induced RVD in HSY cells is due to K^+ channels activated by Ca^{2+} and protein kinase C. *Am. J. Physiol.* **265:**C1405–C1411
25. Musch, M.W., Goldstein, L. 1990. Hypotonicity stimulates phosphatidylcholine hydrolysis and generates diacylglycerol in erythrocytes. *J. Biol. Chem.* **265:**13055–13059
26. Nishizuka, Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258:**607–614
27. O'Neil, R.G., Leng, L. 1997. Osmo-mechanically sensitive phosphatidylinositol signaling regulates a Ca^{2+} influx channel in renal epithelial cells. *Am. J. Physiol.* **273:**F120–F128
28. Pierce, S.K., Politis, A.D. 1990. Ca^{2+}-activated cell volume recovery mechanisms. *Annu. Rev. Physiol.* **52:**27–42
29. Ross, P.E., Cahalan, M.D. 1995. Ca^{2+} influx pathways mediated by swelling or stores depletion in mouse thrombocytes. *J. Gen. Physiol.* **106:**415–444
30. Rubera, I., Tauc, M., Poujeol, C., Bohn, M.T., Bidet, M., De Renzis, G., Poujeol, P. 1997. Cl^- and K^+ conductances activated by cell swelling in primary cultures of rabbit distal bright convoluted tubules. *Am. J. Physiol.* **273:**F680–F697
31. Sasaki, Y., Sasaki, Y. 1990. Inhibition of myosin light chain phosphorylation in cultured smooth muscle cells by HA 1077, a new type of vasodilator. *Biochem. Biophys. Res. Commun.* **171:**1182–1187
32. Schwiebert, E.M., Karlson, K.H., Friedman, P.A., Dietl, P., Spiel-

man, W.S., Stanton, B.A. 1992. Adenosine regulates a chloride channel via protein kinase C and a G protein in a rabbit cortical collecting duct cell line. *J. Clin. Invest.* **89:**834–841

33. Strange, K. (Editor) 1994. Cellular and Molecular Physiology of Cell Volume Regulation. CRC Press. Boca Raton, FL
34. Strange, K., Emma, F., Jackson, P.S. 1996. Cellular and molecular physiology of volume-sensitive anion channels. *Am. J. Physiol.* **270:**C711–C730
35. Tilly, B.C., Edixhoven, M.J., Van Der Berghe N., Bot, A.G.M., De Jonge, H.R. 1994. Ca^{2+}-mobilizing hormones potentiate hypotonicity activation of ionic conductances in intestine 407 cells. *Am. J. Physiol.* **267:**C1271–C1278
36. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D., Kirilovsky, J. 1991. The bisindolylmaleimide GF-109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266:**15771–15781
37. Ulb, J., Murer, H., Kolb, H.A. 1988. Hypotonic shock evokes opening of Ca^{2+}-activated K^+ channels in opossum kidney cells. *Pfluegers Arch.* **412:**551–553
38. Virkki, L.V., Nikinmaa, M. 1995. Regulatory volume decrease in lamprey erythrocytes: mechanisms of K^+ and Cl^- loss. *Am. J. Physiol.* **268:**R590–R597
39. Wong, S.M.E., Debell, M.C., Chase, H.S., Jr. 1990. Cell swelling increases intracellular free [Ca] in cultured toad bladder cells. *Am. J. Physiol.* **258:**F292–F296