Cell Volume Regulation in Response to Hypotonicity Is Impaired in HeLa Cells Expressing a Protein Kinase Ca Mutant Lacking Kinase Activity* 

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The chloride conductance (GCl,swell) that participates in the regulatory volume decrease process triggered by osmotic swelling in HeLa cells was impaired by removal of extracellular Ca²⁺, deletion of intracellular Ca²⁺ stores with thapsigargin, or by preloading the cells with BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid). Furthermore, overnight exposure to the phorbol ester tetradecanoyl phorbol acetate and acute incubation with inhibitors of the conventional protein kinase C (PKC) isoforms bisindolylmaleimide I and Gö6976 inhibited GCl,swell. Treatment of HeLa cells with U73122, a phospholipase C inhibitor, also prevented GCl,swell. Hypotonicity induced selective PKCα accumulation in the membrane/cytoskeleton fraction in fractionation experiments and translocation of a green fluorescent protein-PKCα fusion protein to the plasma membrane of transiently transfected HeLa cells. To further explore the role of PKCs in hypotonicity-induced GCl,swell, HeLa clones stably expressing either a kinase-dead dominant negative variant of the Ca²⁺-dependent PKC isoform α (PKCα K386R) or of the atypical PKC isoform ξ (PKCζ K275W) were generated. GCl,swell was significantly reduced in HeLa cells expressing the dominant negative PKCα mutant but remained unaltered in cells expressing dominant negative PKCζ. These findings strongly implicate PKCα as a critical regulatory element that is required for efficient regulatory volume decrease in HeLa cells.

Cells undergo dynamic changes in volume in response to different stimuli, including variations in extracellular tonicity. Upon cell swelling by exposure to hypotonic conditions, transport pathways for organic osmolytes as well as K⁺ and Cl⁻ channels are activated and accompanied by movement of water to the external medium in a process known as regulatory volume decrease (RVD)† (1, 2). In mammalian tissues most of the osmotic luminal fluid. Even at constant extracellular osmolarity cell volume is compromised by alterations of intracellular osmolarity. Several metabolic pathways lead to cellular formation or degradation of osmotically active substances. Furthermore, transport across the cell membrane modifies cellular osmolarity and, thus, cell volume (for review, see Ref. 2).

In addition, cell volume changes have important physiological roles as integral components of the signal transduction events involved in diverse processes such as proliferation, apoptosis, migration, exocytosis, and neuronal excitability (3, 4). Under pathological conditions cell swelling has been also observed, namely during correction of extracellular acidosis in the course of the treatment of diabetic ketoacidosis (5). Although the precise mediators and pathways that connect cell swelling to the activation of GCl,swell and, thus, RVD remain largely unidentified. Possible mechanisms involved include cytosolic changes in ion strength, macromolecular crowding (6, 7), and variations in intracellular Ca²⁺ concentration (8). In addition to Ca²⁺ other intracellular components, such as ATP (9–11), have been implicated as mediators of GCl,swell in certain cell types. Protein phosphorylation also plays a role in the modulation of GCl,swell. Activation of GCl,swell by tyrosine kinases has been shown to occur in some cells, such as cardiac myocytes (12), H4IE hepatoma cells (13), and 1407 cells (14). In various cell types protein kinase Cs (PKCs) appear involved in modulating GCl,swell (15–17). In Ehrlich ascites tumor cells PKCs were found to be activated in the later phases of RVD, and it was suggested that PKCs may play a role in terminating the RVD response, presumably by modulating the Na⁺-K⁺-2Cl⁻ transporter (18).

The PKC family includes at least 11 members that are generally classified in three categories based on cofactor requirements for activation and sequence homologies. Conventional Ca²⁺-dependent PKCs (cPKCs) are activated by DAG in the presence of Ca²⁺, whereas novel PKCs are Ca²⁺-independent. Both groups are, however, activated by phorbol esters. In contrast, atypical PKCs are neither activated by DAG nor by phorbol esters (19–24).

HeLa cells have been extensively used as a model to study the actions of PKCs. Despite this, little is known about the specific role and distribution of PKC isoforms in these cells. In particular, conventional PKCs (α, β, γ, and δ) are considered important regulators of acute cellular responses, whereas novel PKCs (ε, η, ι, θ, μ, κ, ζ, ξ) have been identified in certain cell types.

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RVD (25–27) and, thus, are well suited to address questions concerning the role of PKCs and the signal transduction cascades involved in G_{C,lowell} activation. Here, we examined the effect of PKCs on G_{C,lowell} by monitoring in intact cells the time course of activation of the volume-activated Cl⁻ conductance. Because conditions under which patch clamp whole-cell recordings are carried out are significantly different from intact cell experiments, we have pursued this question using an intact-cell technique (27). Experiments described here were performed on endogenously expressed intracellular Ca²⁺ for G_{C,lowell} activation in HeLa cells. In addition, PKCs were depleted by pharmacological manipulation with inhibitors, and a role for PKCα was inferred from translocation experiments. To specifically determine the contribution of PKCa to G_{C,lowell} activation, HeLa cell lines stably expressing either dominant negative PKCa (K386R) or PKCζ (K275W) were generated and studied. Hypotonicity-induced G_{C,lowell} was found to be severely impaired in all lines expressing dominant negative PKCa (K386R), whereas the presence of PKCζ (K275W) had no effect. These results implicate PKCa as an important regulatory element controlling RVD in HeLa cells.

EXPERIMENTAL PROCEDURES

Cells—HeLa cells were grown at 37°C in a 5–95% CO₂-air atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 80,000 IU/liter penicillin, and 50 μg/liter streptomycin.

Western Blot Analysis—The presence of the different wild type PKC isotypes and expression of the dominant negative PKCa (K386R) and PKCζ (K275W) mutants was determined by Western blot analysis using either PKC isoform-specific or His-tag-specific antibodies, respectively. Cells were lysed and sonicated in a minimal volume of lysis buffer (containing 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM phenylmethylsulfonyl fluoride, and 50 mM Heps, pH 7.5) and centrifuged. Protein content of cell lysates was determined by the Bradford method (28). Proteins in whole-cell lysates were dissolved in sample buffer (29), separated by SDS-PAGE in 8% gels, and subsequently transferred to nitrocellulose as described (30). Nonspecific binding sites of the membrane were blocked using 5% nonfat milk in 0.05M Tris-buffered saline, pH 7.2. PKC isoforms in HeLa cells were identified using different dilutions of mouse monoclonal antibodies against PKC α, δ, ε (Transduction Laboratories, KY) or rabbit polyclonal antibodies against PKC β₁, β₂, γ, and ζ (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described (31). A mouse polyclonal antibody against a His₆-tagged salmon fusion protein (BiosChile, Santiago, Chile) was used to identify expression of PKC mutant isoforms in transfected HeLa cells. Non-specifically bound antibody was removed by washing with Tris-buffered saline containing 0.1% Tween 20. Specific PKC and His₆ reactivity were determined using either peroxidase-conjugated goat-anti mouse or goat-anti rabbit IgG antibodies (Jackson ImmunoResearch Laboratories). The peroxidase activity was detected using the ECL chemiluminescence reagent (PerkinElmer Life Sciences) and revealed by exposure to x-ray films (Eastman Kodak Co.). Immunoreactive bands present on the films were scanned. Intensity values were obtained after scanning densitometry of individual bands with Scion-Image (NIH) software.

Down-regulation of PKC—In PKC down-regulation studies cells were incubated for 24 h with 1 μM TPA in the culture medium. Control cells were incubated with the vehicle Me₆SO (0.1%) for 24 h.

Subcellular Fractionation and Preparation of Cell Extracts—For subcellular fractionation studies, about 10⁷ cells were exposed to 30% hypotonic medium for 5 min. Cells were collected by centrifugation at 1500 g for 5 min, washed with phosphate-buffered saline, and resuspended in 1 ml of buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.3% β-mercaptoethanol, and a mixture of protease and phosphatase inhibitors (leupeptin, NaF, pepstatin, Na₂VO₃, benzamidine, Na₃P₂O₅, and phenylmethylsulfonyl fluoride).

Analysis of PKC Translocation by Cell Fractionation—Cells were homogenized by pipetting 30 times through a 25-gauge needle and centrifuged at 100,000 g for 10 min to sediment the cytosol. The P₂ receptor binding assay medium was then centrifuged at 100000 × g for 1 h. The resulting soluble supernatant (cytosolic) fraction was stored at −80°C, whereas the residual pellet was resuspended in the same buffer supplemented with 1% Triton X-100, sonicated for 1 min, and stored at −80°C (particulate fraction). Before Western blot analysis the protein concentration was determined using the BCA assay. Equal amounts of protein (10 μg) were loaded per lane and separated by SDS-PAGE as described above.

In Situ Analysis of PKC Translocation Using GFP-tagged Proteins—HeLa cells were plated on glass bottom dishes (MatTek Corp., Ashland, MA) 1 day before liposome-mediated transfection with LipofectAMINE 2000 according to the manufacturer’s instructions (Invitrogen). The PKCα GFP or PKCζ GFP construct (BD Biosciences and Clontech, Palo Alto, CA, respectively). Half a microgram of each plasmid and 3 μl of LipofectAMINE 2000 were used for 5.5 × 10⁶ cells. After 4 h in the presence of LipofectAMINE, cells were transferred to normal medium for 24–28 h. Cells were then washed with phosphate-buffered saline and maintained in isosmotic solution. The fluorescence-imaging experiments were performed at room temperature (20°C). Images were obtained using a laser-scanning confocal system coupled to a 510 Zeiss microscope with an immersion objective (40×, 1.2 numerical aperture) and subsequently analyzed with the software ImageJ, version 1.30p (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, rsb.info.nih.gov/ij/) to determine relative PKC-GFP intensity distribution. Cells were imaged either under isosmotic conditions (control) after exposure to 30% hypotonicity or after TPA treatment.

Construction of HeLa Cell Lines Stably Expressing either PKCa or PKCζ Dominant Negative Mutants—HeLa cells were stably transfected with a bovine PKCα Lys/Arg³⁶⁶ and PKCζ Lys/Trp⁷⁷ DNA mutant containing a His tag at the COOH terminus subcloned into the cytomegalovirus expression vector pEF-neo (32). Cells were transfected using LipofectAMINE as indicated by the manufacturer (Invitrogen), and clones were selected with 200 μg/ml G418 in the culture medium.

Cell Volume Measurements—Changes in cell water volume of individual cells were assessed by measuring variations in the concentration of an intracellularly trapped fluorescent dye as previously described (27, 33). Briefly, HeLa cells were loaded with 5 μM calcine-AM (Molecular Probes, Eugene, OR) for 5 min and then superfused with an iso-osmotic solution for 15 min before subjecting the cells to hypotonicity. Changes in fluorescence were monitored using a Zeiss LSM 410 confocal microscope as previously described. Images were obtained at 10-s intervals, and the fluorescence of a 10-μm² area in the center of a cell was measured as V/V₀. The data are presented as V/V₀ and V/V₀ was calculated from the fluorescence intensity ratio F₁/F₀, as described (33).

Cell Calcium Measurements—Relative changes in intracellular Ca²⁺ were assessed by measuring variations in the fluorescence of the Ca²⁺ sensitive dye Rhod-2 (Molecular Probes) (34). Cells attached to glass coverslips were loaded for 20–40 min at 37°C with salmine containing the acetoxyethyl ester of the dye (Rhod-2AM, final concentration, 5.2 μM and 0.1% pluronic acid). Coverslips were placed at the bottom of a 1-ml capacity chamber and submitted initially in an iso-osmotic solution. Fluorescence changes at emission wavelength 570 nm, as a consequence of exposure to hypoosmotic conditions, were measured using a Zeiss LSM 410 confocal microscope and an excitation wavelength of 543 nm. Images were scanned every 10 s. The images were analyzed off-line with ImageJ, version 1.30p. The relative fluorescence intensity ratio (F₁/F₀) is expressed as a function of time.

Solutions—The iso-osmotic solution contained 100 mM/liter NaCl, 1 mmol/liter MgCl₂, 2 mmol/liter CaCl₂, 10 mmol/liter glucose, and 10 mmol/liter HEPES, adjusted to pH 7.4 with Tris, and mannitol to yield a final osmolality of 310 mosmol/liter. The hypotonic NaCl solution had the same composition as the iso-osmotic solution without mannitol (210 mosmol/liter). The hypotonic NMDG-Cl solution contained 100 mM/liter NMDG-Cl, 1 mmol/liter MgCl₂, 2 mmol/liter CaCl₂, 10 mmol/liter HEPES, and 10 mmol/liter HEPES, adjusted to pH 7.4 with Tris. Gramicidin (10 μM) (Sigma) was added to the NMDG-Cl hypotonic solution as previously described (27). Mannitol was omitted, yielding an osmolality of 210 mosmol/liter.

Statistical Analysis—Statistical differences of the data were evaluated by unpaired Student’s t test and considered significant at p < 0.05.

References and Treatment—Distilled water (31°C) was used as the isosmotic control solution. Biphasic NMDG-Cl solution was composed of 310 mosmol/liter NMDG-Cl, 100 mM/liter MgCl₂, 2 mmol/liter CaCl₂, 10 mmol/liter HEPES, and 10 mmol/liter HEPES, adjusted to pH 7.4 with Tris. BAPTA-AM (calculated from the fluorescence intensity ratio F₁/F₀) is expressed as a function of time.

Solutions—The iso-osmotic solution contained 100 mM/liter NaCl, 1 mmol/liter MgCl₂, 2 mmol/liter CaCl₂, 10 mmol/liter glucose, and 10 mmol/liter HEPES, adjusted to pH 7.4 with Tris, and mannitol to yield a final osmolality of 310 mosmol/liter. The hypotonic NaCl solution had the same composition as the iso-osmotic solution without mannitol (210 mosmol/liter). The hypotonic NMDG-Cl solution contained 100 mM/liter NMDG-Cl, 1 mmol/liter MgCl₂, 2 mmol/liter CaCl₂, 10 mmol/liter HEPES, and 10 mmol/liter HEPES, adjusted to pH 7.4 with Tris. Gramicidin (10 μM) (Sigma) was added to the NMDG-Cl hypotonic solution as previously described (27). Mannitol was omitted, yielding an osmolality of 210 mosmol/liter.

Statistical Analysis—Statistical differences of the data were evaluated by unpaired Student’s t test and considered significant at p < 0.05.
GCl\(_{\text{swell}}\) in HeLa Cells Is Ca\(^{2+}\)-dependent and Blocked by TPA—Calcium has been shown to be an intracellular mediator of hypotonicity-induced GCl\(_{\text{swell}}\) in some cells, in particular epithelial cells (1, 3). Thus, the effect of Ca\(^{2+}\) on GCl\(_{\text{swell}}\) was explored next. Under control conditions, cells rapidly swelled after exposure to a 30% hypotonic NaCl solution (Fig. 2). GCl\(_{\text{swell}}\) was monitored by switching to a hypotonic NMDG-Cl solution containing 10 \(\mu\)M of the cation-selective ionophore nigericin (27). The rationale of this experimental procedure is to make Cl\(^{-}\) conductance rate-limiting for net ion transport across the plasma membrane. This is achieved by replacement of all monovalent cations in the medium by the impermeant cation NMDG and the addition of gramicidin. Under these conditions the conductance of the membrane is dominated by the K\(^{+}\) permeability and, should a Cl\(^{-}\) conductance be present, the cell will shrink at a rate proportional to anion permeability (27). Upon exposure to a 30% hypotonic solution the cells immediately swelled, indicating that cell volume was inadequately monitored in these experiments. Under these experimental conditions, cells typically displayed excessive volume recovery (control trace in Fig. 2), a fact that may be explained by the absence of an appropriate ion gradient for the Na\(^{-}\)-K\(^{-}\)-2Cl\(^{-}\) transporter in Na\(^{-}\)-free medium. HeLa cells were preloaded with 100 \(\mu\)M BAPTA-AM to deplete bulk cytoplasmic free Ca\(^{2+}\). Under these conditions GCl\(_{\text{swell}}\) was markedly retarded as compared with control cells (Fig. 2 and Table I). Different sets of experiments were conducted to identify the origin of Ca\(^{2+}\). The participation of extracellular Ca\(^{2+}\) in GCl\(_{\text{swell}}\) was studied by removal of the divalent cation from the medium. As depicted (Fig. 2), this treatment impaired GCl\(_{\text{swell}}\) to a similar degree as seen in the BAPTA-loaded cells. The role of intracellular Ca\(^{2+}\) was addressed by long term preincubation of cells with 1 \(\mu\)M thapsigargin in the presence or absence of extracellular Ca\(^{2+}\). These conditions (Table I) reduced GCl\(_{\text{swell}}\) to a similar extent as seen when Ca\(^{2+}\) was omitted from the medium, indicating that Ca\(^{2+}\) of intra- and extracellular origin regulate the rate of hypotonicity-induced GCl\(_{\text{swell}}\) in HeLa cells.

**RESULTS**

**Expression of Protein Kinase C Isoforms in HeLa Cells**—PKC isoforms expressed in HeLa cells were detected as bands of the predicted molecular weights by Western blot analysis of whole cell lysates using isotype-specific antibodies against PKC\(\alpha\), \(\beta_2\), \(\delta\), \(\varepsilon\), \(\eta\), \(\theta\), and \(\zeta\) (Fig. 1). Bands observed in HeLa cell extracts comigrated with the corresponding bands detected in tissue extracts employed as controls for each PKC isotype (brain for \(\alpha\), \(\beta_2\), \(\delta\), and \(\zeta\); skeletal muscle for \(\varepsilon\), \(\eta\), and \(\theta\)). The isozyme PKC\(\theta\) was not detected in HeLa cell lysates under these experimental conditions. These results indicate that at least one isozyme of each PKC subgroup (cPKCs, novel PKCs, and atypical PKCs) was expressed in HeLa cells.

GCl\(_{\text{swell}}\) time course in control conditions (Control), in Ca\(^{2+}\)-free extracellular medium (no Ca\(^{2+}\) added; Ca\(^{2+}\)-free medium), after preloading cells with 100 \(\mu\)M BAPTA-AM (BAPTA-AM), and after overnight incubation with 1 \(\mu\)M TPA (TPA) are shown. Results are the averages of values obtained from 8–10 cells and are representative of three independent experiments with similar outcome. A statistical analysis of these results is shown in Table I.

**TABLE I**

| Treatment            | Maximal \(V/V_0\) at 10 min | \(V/V_0\) at 10 min |
|----------------------|-----------------------------|---------------------|
|                      | Control cells               | Treated cells       |
| Ca\(^{2+}\)-free extracellular medium |                     |                     |
| BAPTA (100 \(\mu\)M) | 1.36 ± 0.06                 | 0.96 ± 0.04         |
| TPA (1 \(\mu\)M)     | 1.40 ± 0.03                 | 0.93 ± 0.05         |
| Thapsigargin (1 \(\mu\)M) | 1.38 ± 0.04              | 0.91 ± 0.07         |
| Thapsigargin (1 \(\mu\)M) | 1.41 ± 0.01              | 0.89 ± 0.07         |
| BIM-1 (0.5 \(\mu\)M) | 1.30 ± 0.02                 | 0.99 ± 0.05         |
| G66976 (0.1 \(\mu\)M) | 1.32 ± 0.01                 | 0.95 ± 0.06         |
| U73122 (1 \(\mu\)M)  | 1.32 ± 0.02                 | 1.04 ± 0.03         |
| 2-APB (50 \(\mu\)M)  | 1.39 ± 0.04                 | 0.99 ± 0.05         |
| PKC\(\alpha\)-K3686  | 1.37 ± 0.03                 | 0.97 ± 0.01*        |
| PKC\(\varepsilon\)-K275W | 1.38 ± 0.04              | 1.06 ± 0.02         |

* Without extracellular Ca\(^{2+}\).
The results described above prompted us to examine intracellular Ca\(^{2+}\) changes induced by hypotonicity under the different experimental conditions. A 30% decrease in extracellular hypotonicity in a Ca\(^{2+}\)-containing hypo-osmotic medium triggered a biphasic Ca\(^{2+}\) response. The rapid more transient component terminated within 5–6 min, whereas the slower sustained component was maintained up to 10–12 min. Upon removal of extracellular Ca\(^{2+}\), only the faster transient component was retained but displayed slightly slower kinetics and a smaller amplitude. After a 30-min exposure to 1 \(\mu\)M thapsigargin in the presence or absence of extracellular Ca\(^{2+}\), hypotonicity failed to produce any significant variation in intracellular Ca\(^{2+}\) (Fig. 3). Peak fluorescence intensity of the rapid component in the presence of extracellular Ca\(^{2+}\) was 1.54 ± 0.02, compared with 1.31 ± 0.01 in the absence of Ca\(^{2+}\). Fluorescence intensity for the slower component was 1.28 ± 0.05 in the presence of extracellular Ca\(^{2+}\) compared with 1.01 ± 0.02 in the absence of Ca\(^{2+}\) (inset in Fig. 3).

Long-term incubation with TPA has been shown previously to down-regulate PKC and novel PKC protein levels by promoting proteolytic degradation. This often occurs at different rates for individual isoforms (19). Therefore, we investigated the effect of overnight exposure of HeLa cells to 1 \(\mu\)M TPA on \(G_{\text{Cl,swell}}\). As suspected, cell volume recovery was significantly delayed in TPA-treated cells upon hypotonic challenge (Fig. 2 and Table I), suggesting that TPA-sensitive PKC(s) regulated \(G_{\text{Cl,swell}}\) in HeLa cells. Western blot analysis of HeLa cells after overnight incubation with TPA revealed a significant decrease in PKCa protein levels. Scanning densitometry analysis of PKCa before and after TPA treatment confirmed that PKCa levels were reduced by at least 50%, whereas PKC\(\xi\) levels remained essentially unaltered (Fig. 4, A and B).

Hypotonicity Induced Translocation of PKCα to the Particulate Fraction of HeLa Cells—PKC activation is often associated with PKC translocation to the plasma membrane. To investigate this possibility cells were initially fractionated as described, and the presence of PKCs in the particulate (membrane) fraction was assayed by Western blotting. Brief (5 min) exposure of HeLa cells to hypotonic conditions resulted in an increase by 47.5 ± 4.7% of PKCα in the particulate fraction, whereas no changes were observed for PKC\(\xi\) (Fig. 5, A and B).

Hypotonicity Induced Translocation of GFP-PKCa to the Plasma Membrane of HeLa Cells—The results above demonstrated that PKCα specifically altered its intracellular distribution in response to hypoosmotic cell swelling and accumulated in a preparation that included the plasma membrane. To implicate PKCα more directly in a model for RVD control in HeLa cells, we anticipated that PKCα should translocate to the plasma membrane to induce changes in \(G_{\text{Cl,swell}}\) activity. To test this model translocation of GFP-tagged wild-type PKCα and PKC\(\xi\) were compared in transiently transfected HeLa cells. GFP-PKCa- or GFP-PKCa\(\xi\)-transfected cells maintained in either isotonic medium or after exposure to 30% hypotonicity for 4 min were examined by laser-scanning confocal microscopy (Fig. 6, A and B, respectively). In Fig. 6, A and B, a representative experiment is depicted showing cells for which dimensions in the \(x, y\) plane were conserved upon exposure to hypotonicity. Hence, swelling of these cells occurs along the \(z\) axis only. Cells in which dimensions in the \(x, y\) plane were not conserved upon swelling are also shown (Fig. 6A, inset). Only cells in which swelling occurred along the \(z\) axis were chosen for statistical analysis. This analysis was performed by taking a section across the cell that included the portion of the membrane showing fluorescence in iso- and hypotonicity. Then, the area under the fluorescence intensity profile corresponding to the membrane in isotonicity was subtracted from the profile obtained in hypotonicity. GFP-PKCa fluorescence increased from 1.04 ± 0.07 to 1.32 ± 0.09 \((p < 0.05, n = 12)\) in hypotonicity, whereas GFP-PKCa\(\xi\) fluorescence changed from 1.01 ± 0.09 to 1.11 ± 0.06 \((p > 0.05, n = 12)\) under the same conditions. The differences in relative fluorescence intensity (\(\Delta\)), calculated by subtracting the fluorescence detected in hypotonicity minus isotonicity, is depicted (bottom panels, Fig. 6, A and B). In the case of GFP-PKCa, arrows indicate fluorescence
spots associated with plasma membrane regions in which the relative fluorescence intensity was higher after hypotonic stimulus and, thus, indicative of translocation to the plasma membrane. In the case of GFP-PKC\(\alpha\)/H9256 no difference was observed. As an additional control, cells transiently transfected with GFP-PKC\(\alpha\)/H9251 were exposed to 20 nM TPA (Fig. 6C) for 4 min in isotonicity. The difference in relative fluorescence intensity indicated significant translocation of GFP-PKC\(\alpha\) to the plasma membrane. As expected, TPA treatment of GFP-PKC\(\zeta\)-expressing HeLa cells did not promote translocation of GFP-PKC\(\zeta\) (data not shown). Quantitative analysis of such translocation experiments indicated that PKC\(\alpha\) but not PKC\(\zeta\) accumulated in plasma membrane proximity under hypotonic conditions (Fig. 6D).

**Pharmacological Inhibition of PKC, PLC, or the Endoplasmic Reticulum Ca-ATPase Impaired \(G_{\text{Cl,swell}}\)**—To implicate further PKCs and specifically PKC\(\alpha\)/H9251 in the control of RVD, HeLa cells were treated with BIM-1 and Go\(\delta\)6976, two compounds known to be fairly selective inhibitors for cPKC isoforms when used at appropriate concentrations (35). As depicted in Table I, \(G_{\text{Cl,swell}}\)-elicited (by 30%) extracellular hypotonicity was significantly impaired when cells were pretreated with either BIM-1 or Go6976, whereas maximal \(V/V_0\) was comparable in inhibitor and Me2SO-treated cells. Given that both BIM-1 and Go6976 are PKC kinase domain inhibitors with some preference for the PKC\(\alpha\) isoform (35), these results further implicated PKC\(\alpha\)/H9251 in controlling \(G_{\text{Cl,swell}}\) in HeLa cells.

DAG, in conjunction with Ca\(^{2+}\), is a physiologically relevant activator of cPKCs in numerous cellular systems (21). The results presented so far supported the notion that RVD in HeLa cells was dependent on increased intracellular Ca\(^{2+}\), partially generated by intracellular Ca\(^{2+}\) mobilization, perhaps via an IP\(_3\)-dependent mechanism. To test this, HeLa cells were treated with the IP\(_3\) receptor blocker 2-aminoethoxydiphenyl borate. This treatment significantly impaired \(G_{\text{Cl,swell}}\) (Table I), thus implicating further this second messenger in the signaling pathways controlling \(G_{\text{Cl,swell}}\). In addition, inhibition of the endoplasmic reticulum Ca-ATPase with thapsigargin significantly blunted \(G_{\text{Cl,swell}}\) (Table I). Because PLCs cleave membrane phospholipids to yield DAG and IP\(_3\), the role of PLCs in swelling-induced \(G_{\text{Cl,swell}}\) in HeLa cells was assessed in the...
Regulatory Volume Decrease in HeLa Cells Is Modulated by PKCa

Fig. 6. Hypotonicity-induced translocation of GFP-PKCs to the plasma membrane of HeLa cells. HeLa cell lines stably transfected with plasmids permitting constitutive expression of either GFP-PKCa or GFP-PKCa were analyzed by confocal microscopy as described. HeLa cells expressing GFP-PKCa (A) or GFP-PKCa (B) in isotonic medium and after 4 min in hypotonic conditions are shown. The lowermost panels in A, B, and C indicate the difference (Δ) in relative fluorescence after image subtraction (hypotonic = isotonicity). As a control for translocation, HeLa cells transiently transfected with GFP-PKCa (C) are depicted in isotonicity (Control) and after exposure to 20 nM TPA for 4 min (scale bar, 5 μm). The insets in A depict representatives cells for which the dimensions in the x→y plane were not conserved after cell swelling. For quantitative analysis, cells were considered where swelling occurred along the z axis only (A and B). Values shown indicate GFP-PKCa levels at the plasma membrane before and after treatment (D). The cells shown (A, B, and C) are representative of results obtained analyzing four cultures per experiment and 3 independent experiments. Quantitative data were obtained by averaging results from 4 different experiments and a total of 16 cells. Normalized mean values ± S.E. are shown (D). Statistically significant increases in plasma membrane proximity were detected for PKCa only upon exposure to hypotonicity.

Volume regulation is considered a crucial physiological mechanism required for adequate cell functioning. RVD subsequent to cell swelling occurs due to the activation of channels and transporters that allow the efflux of KCl and organic osmoles. The mechanisms that regulate these processes are complex and differ among various cell types (36). For instance, extracellular hypotonicity has been shown to mobilize intracellular Ca2+ that was partially required to sustain K+ efflux in astrocytes (37). Also, PKC-dependent activation of volume-sensitive chloride channels has been suggested to occur in HT-3 cells (16, 17). In general, however, the processes that modulate cell volume recovery are not well understood. Here, using HeLa cells as a model system, intra- and extracellular Ca2+ mobilization, PLC activation, and most importantly, unequivocal evidence implicating PKCa acti-

presence of U73122 (1 μM) and absence of extracellular Ca2+ before hypotonic challenge. Immediately after hypotonic exposure, U73122-treated and control cells rapidly increased their volume to values observed in nontreated cells (compare Fig. 2 and Table I). However, in U73122-treated cells G\sub{Cl,swell} was substantially slower (Table I). These results indicate that a PLC isoform participates in the intracellular signaling cascade leading to RVD in HeLa cells and possibly activates cPKCa via DAG and IP3-dependent calcium release.

Expression of Dominant Negative PKCa and PKCa Mutants—Pharmacological inhibitors are important tools for implicating signal transduction effector proteins in specific cellular processes. However, because such inhibitors have additional, often unknown, intracellular targets, HeLa cell lines stably expressing either a dominant negative PKCa (K368R) or PKCa (K275W) mutant were generated. Five different PKCa (K368R)- and four different PKCa (K275W)-expressing HeLa clones were characterized and yielded similar results. The immunoreactivity pattern of PKCa and PKCa from representative HeLa clones overexpressing the PKC isoforms is shown. Scanning densitometric analysis revealed a 4.6-fold increase in the expression of PKCa (K368R) and a 3-fold increase for PKCa (K275W) (Fig. 7A). As expected, PKCa and PKCa were detected in homogenates of parental HeLa cells and transfected clonal populations (Fig. 7A). In addition, a band of the same size as the corresponding PKC was detected using a His6-specific antibody in PKCa (K368R)- and PKCa (K275W)-transfected cells, whereas that was not the case for parental (wt) and mock-transfected (mock) HeLa cells (Fig. 7B).

Hypotonicity-induced G\sub{Cl,swell} Is Impaired in HeLa Cells Expressing the Dominant Negative PKCa Mutant—Hypotonicity-induced G\sub{Cl,swell} response was studied using these stably transfected cell lines expressing either PKCa (K386R) or PKCa (K275W). RVD was significantly impaired in PKCa (K386R) HeLa cells as compared with PKCa (K275W) transfected cells (Fig. 8 and Table I). These results demonstrate that cPKCa is a critical component in signaling mechanisms that control G\sub{Cl,swell} in HeLa cells.

DISCUSSION

Volume regulation is considered a crucial physiological mechanism required for adequate cell functioning. RVD subsequent to cell swelling occurs due to the activation of channels and transporters that allow the efflux of KCl and organic osmoles. The mechanisms that regulate these processes are complex and differ among various cell types (36). For instance, extracellular hypotonicity has been shown to mobilize intracellular Ca2+ that was partially required to sustain K+ efflux in astrocytes (37). Also, PKC-dependent activation of volume-sensitive chloride channels has been suggested to occur in HT-3 cells (16, 17). In general, however, the processes that modulate cell volume recovery are not well understood. Here, using HeLa cells as a model system, intra- and extracellular Ca2+ mobilization, PLC activation, and most importantly, unequivocal evidence implicating PKCa acti-
viation in the sequence of events controlling cell volume recovery after osmotic swelling are provided.

A significant decrease in $G_{\text{Cl,swell}}$ was observed after exposure to Ca$^{2+}$-free hypotonic medium (Fig. 2). This observation indicates that hypotonicity may activate Ca$^{2+}$ influx through different membrane pathways, including stretch-activated Ca$^{2+}$ channels (38, 39). Moreover, it has been shown that several types of cells undergo RVD only when Ca$^{2+}$ influx is derived from the extracellular compartment takes place (8).

Suppression of swelling-associated intracellular Ca$^{2+}$ increases with BAPTA-AM significantly reduced $G_{\text{Cl,swell}}$ in HeLa cells as did also thapsigargin and 2-aminoethoxydiphenylborate treatment (Fig. 2 and Table I), suggesting that Ca$^{2+}$ release from intracellular stores via an IP$_3$-dependent mechanism is critical for volume recovery in these cells. This conclusion is in agreement with results obtained in astrocytes where Ca$^{2+}$ mobilized from the endoplasmic reticulum was suggested to be the source of Ca$^{2+}$ required for hypotonicity-stimulated K$^+$ release (37, 40). In addition, swelling has been associated in a wide variety of cells with intracellular Ca$^{2+}$ increases that activate K$^+$ and Cl$^-$ efflux via channels, loss of water, and as a consequence, recovery of cell volume (8, 41). Nonetheless, this mechanism is not generic, even among epithelial cell lines. For example, the volume-activated K$^+$ current in Ehrlich ascites tumor cells is activated even under conditions where intracellular Ca$^{2+}$ is buffered (42).

The results obtained by measuring relative intracellular Ca$^{2+}$ variations after cell exposure to hypotonicity (Fig. 3) coincide well with the findings showing Ca$^{2+}$ dependence of $G_{\text{Cl,swell}}$ in HeLa cells (Fig. 2). In addition, they underscore the importance of Ca$^{2+}$ mobilization from thapsigargin-sensitive intracellular stores in generating the Ca$^{2+}$ signal responsible for $G_{\text{Cl,swell}}$. After long term (30 min) exposure to thapsigargin, intracellular Ca$^{2+}$ variations in response to hypotonicity were not detectable even in the presence of extracellular Ca$^{2+}$ (Fig. 3). Similar results to those described here have been obtained in renal A6 cells (43). Depletion of internal Ca$^{2+}$ stores by acute treatment with thapsigargin would be expected to trigger Ca$^{2+}$ influx via store-activated plasma membrane channels and, hence, augment cytosolic Ca$^{2+}$ observed in response to hypotonicity. However, in experiments conducted here cells were preincubated for 30 min with thapsigargin. Clearly, this protocol depleted intracellular stores such that Ca$^{2+}$ transients were no longer detectable upon exposure to hypotonicity (Fig. 3), and as a consequence, $G_{\text{Cl,swell}}$ was blocked (Table I).

Because of the above findings the participation of phospholipases was investigated. Indeed, marked reduction of $G_{\text{Cl,swell}}$ was observed with the PLC inhibitor U73122 in the absence of extracellular Ca$^{2+}$ (Table I). A role for PLCs in the regulation of osmo-sensitive K$^+$ release has been reported (44), and calcium mobilization triggered by hepatocellular swelling was more recently associated with PLC activation via tyrosine kinases (41). Activation of tyrosine kinases during RVD has been demonstrated in cardiac myocytes (12), H4IIE hepatoma cells (13), and I407 cells (14), and in Ehrlich ascites tumor cells the tyrosine kinase inhibitor genistein inhibits the RVD response (3). Consistent with the notion that tyrosine phosphorylation plays a critical role in RVD in HeLa cells, possibly via

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**Fig. 7.** Expression of dominant negative PKCa (K386R) and PKCζ (K275W) mutants in stably transfected HeLa cell lines. Extracts were prepared from stably transfected cell lines as described, and 50 μg of total protein from parental (wt) and mock- and PKC-transfected cells were analyzed. A, representative immunoblots of parental (wt) and mock and dominant negative His6-tagged PKC proteins were revealed using a specific monoclonal antibody against PKCa and a polyclonal antibody against PKCζ. B, the same samples were revealed using an anti-His$_6$ antibody. His$_6$-tagged PKCa (K386R) or PKCζ (K275W) protein bands (82 and 72 kDa, respectively) were detected exclusively in the transfected clones. A 34-kDa His$_6$-tagged salmon fusion protein (control) was used as a positive control for the His$_6$ antibody. Molecular weights are indicated to the left of individual panels.

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**Fig. 8.** Impaired $G_{\text{Cl,swell}}$ activation in cells expressing a dominant negative PKCa (K386R) mutant. Time course of swelling-activated Cl$^-$ conductance in HeLa cells expressing PKCa (K386R) or PKCζ (K275W). Arrows indicate the same solution changes as described for Fig. 2. Results shown for one experiment were averaged from 8–12 cells and are representative of the outcome in three independent experiments. A statistical analysis of these results is shown in Table I.
PLC activation, the Src-family kinase inhibitor PP-1 was also found in preliminary experiments to block swelling-induced taurine efflux (53).

The role of serine/threonine protein kinases in the regulation of cell volume recovery is less well understood, although they have been implicated in the late phase of RVD in Ehrlich ascites tumor cells (18). Also, a tight connection between calmodulin activation and organic osmolyte fluxes evoked by hypotonicity has been reported in some epithelial cells (45, 46). Furthermore, evidence suggesting a role for PKC in the activation of the swelling-dependent Cl\(^-\) conductance was provided in HTC hepatoma and Mz-ChA-1 cells (17). In those experiments reagents such as chelerythrine, calphostin C, and phorbol esters, all currently known to display modest selectivity for PKCs (47–51), were employed. Also, upon microinjection of HT-3 cells with anti-PKC\(\alpha\) antibodies at high concentrations, RVD was partially blocked at times greater than 10 min, and the time course of RVD was prolonged (16). Finally, chelerythrine, a highly controversial PKC inhibitor, as mentioned above (50, 51), was found to decrease the RVD response in Ehrlich ascites tumor cells (3). These experiments all provided suggestive, but not conclusive, evidence that PKCs were involved in events leading to \(G_{Cl,swell}\) activation.

Hence, we were interested in investigating the importance of PKCs as potential downstream effectors required for \(G_{Cl,swell}\) activation in HeLa cells as well as in identifying unequivocally the PKC isoform involved. Indeed, initial experiments with BIM-1 and Go6976, compounds known to be relatively selective cPKC inhibitors, blocked \(G_{Cl,swell}\) very efficiently (Table I). Although pharmacological inhibitors are valuable experimental tools in helping to identify relevant cell signaling pathways and proteins, most are known to have multiple intracellular targets, as has been discussed for chelerythrine, calphostin C, and phorbol esters. To implicate further PKC\(\alpha\)s and, specifically, cPKC\(\alpha\)s in the control of the swelling-activated Cl\(^-\) conductance and, hence, RVD in HeLa cells, additional experimental approaches were employed. Experiments analyzing translocation of PKCs in response to hypotonicity indicated that this isoform altered its distribution and accumulated in the particulate fraction (Fig. 5), as has been described previously in HTC hepatoma cells (17). No such changes were seen for PKC\(\delta\) employed as a control (Fig. 5). Furthermore, selective translocation of GFP-tagged PKC\(\alpha\), but not PKC\(\epsilon\), to the plasma membrane upon exposure of HeLa cells to hypotonicity was observed. Thus, our experiments demonstrate that in response to hypotonicity PKC\(\alpha\)s translocate to the subcellular site (i.e. the plasma membrane), where its activation is expected to control the swelling-sensitive Cl\(^-\) conductance.

Direct confirmation that PKC\(\alpha\) is critically involved in controlling \(G_{Cl,swell}\) in HeLa cells was obtained using HeLa cells lines stably expressing either dominant negative PKC\(\alpha\) (K368R) or PKC\(\zeta\) (K275W). These mutations in the ATP binding site preclude catalytic activity of the respective isoforms (32). Only in the case of PKC\(\alpha\) (K368R) was \(G_{Cl,swell}\) significantly delayed (Fig. 8). The control using a corresponding PKC\(\zeta\) mutant had no consequence on \(G_{Cl,swell}\) thereby eliminating the possibility of a promiscuous effect, for instance, due to sequestration of upstream kinases such as PDK1, essential for activation of several PKCs (52). It should be noted that the PKC\(\zeta\) mutant displayed slower \(G_{Cl,swell}\) activation in comparison to non-transfected cells (see Fig. 2). Although these observations suggest that the transfection/selection procedure per se altered \(G_{Cl,swell}\) activation in HeLa cells, an additional effect due to the presence of PKC\(\alpha\) (K368R), but not with PKC\(\zeta\) (K275W), was clearly distinguishable.

In conclusion, the data presented support a model in which hypotonicity triggers extracellular Ca\(^{2+}\) influx, PLC activation, and intracellular Ca\(^{2+}\) mobilization. These events are crucial to the onset of the swelling-activated Cl\(^-\) conductance and, thus, RVD in HeLa cells. Downstream of this initial sequence, translocation of cPKCs to the plasma membrane occurs, and activity of this isoform is required to mediate some of the processes leading to hypotonicity-induced cell volume regulation in HeLa cells.

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Cell Volume Regulation in Response to Hypotonicity Is Impaired in HeLa Cells Expressing a Protein Kinase C α Mutant Lacking Kinase Activity
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