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Hypotonic Activation of Volume-sensitive Outwardly Rectifying Anion Channels (VSOACs) Requires Coordinated Remodeling of Subcortical and Perinuclear Actin Filaments

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Abstract. Cell volume regulation requires activation of volume-sensitive outwardly rectifying anion channels (VSOACs). The actin cytoskeleton may participate in the activation of VSOACs but the roles of the two major actin pools remain undefined. We hypothesized that structural reorganization of both subcortical and perinuclear actin filaments (F-actin) contributes to the hypotonic activation of VSOACs. Hypotonic stress of pulmonary artery smooth muscle cells (PASMCs) was associated with reorganization of both peripheral and perinuclear F-actin, and with activation of VSOACs. Preincubation with cytochalasin D caused prominent dissociation of perinuclear, but not of subcortical F-actin. Cytochalasin D failed to induce isotonic activation and delayed the hypotonic activation of VSOACs. F-actin stabilization by phalloidin delayed both the hypotonic stress-induced dissociation of membrane-associated actin filaments and the activation kinetics of VSOACs. PKCε, which was proposed to phosphorylate and inhibit VSOACs, colocalized primarily with F-actin and the net kinase activity remained unchanged during hypotonic cell swelling. In conclusion, normal hypotonic activation of VSOACs requires disruption of peripheral F-actin but intact perinuclear F-actin; interference with this pattern of actin reorganization delays the activation kinetics of VSOACs. The cell swelling-induced peripheral actin dissociation may underlie the observed translocation of PKCε, which leads to a net decrease of PKCε inhibitory activity in submembranous sites. Thus, reorganization of actin and PKCε may establish conditions for mechano- and/or signal transduction-mediated activation of VSOACs.

Key words: Volume-sensitive outwardly rectifying anion channels (VSOACs) — PKCε — Subcortical F-actin — Perinuclear F-actin — PASMCs — Hypotonic cell swelling

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Introduction

Regulation of cell volume is a complex and well-coordinated process, which requires activation of membrane ion transporters including volume-sensitive outwardly rectifying anion channels (VSOACs) [25, 32, 45]. These channels are expressed and function in a variety of cell types, but their molecular identity and regulation are controversial [14, 45]. For example, the actin cytoskeleton has been proposed to play a regulatory role in the activation of VSOACs by either generating the volume signal, being a part of the volume sensor, activating actin-binding proteins and signal-transduction molecules, or serving as a scaffold of signal transduction and/or protein complexes [20, 22, 34]. However, data defining a role of the cytoskeleton is based primarily on observations that osmotic changes of the cell volume are associated with alteration of the F-actin content and/or assembly. Mechanistic approaches aimed at establishing a link between the actin cytoskeleton and VSOACs consider a role for Rho GTPases (i.e., Rho, Rac or Cdc42), which are activated upon cellular osmotic stress and function as regulators of the peripheral actin filament assembly [18, 24]. This raises the possibility that by reorganization of subcortical actin filaments, Rho GTPases regulate ion flux through membrane ion channels, and thus play a major role in the actin cytoskeleton-dependent regulation of the cell volume [7, 8, 15].

The cytosolic actin filaments are compartmentalized and exhibit site-specific properties [12]. The actin filament network immediately under the plasma membrane consists of short and branched filaments, while the actin filaments in the contractile

domain, which include stress fibers and focal adhesions, are longer and rarely branched [2, 47]. Also, the subcortical actin filaments are more dynamic, and reorganization of the branched peripheral network is spatially and temporally regulated by a group of actin-binding proteins with high concentration in subcortical areas, which nucleate (Arp2/3 and Actin Binding Protein, ABP 280), depolymerize (cofilin), sever (cofilin and gelsolin), or extend filaments by incorporation of actin monomers in uncapped barbed filament ends (profilin) [5]. Stress fibers, on the other hand, contain actin filament-stabilizing proteins that are not found in subcortical areas, including tropomyosin, caldesmon, α-actinin and villin: these proteins protect from severing and branching and hence suppress dynamic remodeling of stress fibers [6, 12]. While both the subcortical and the contractile actin filaments may play roles in the maintenance of physiological cell volume, the underlying mechanisms remain undefined [34, 37].

We undertook this study to assess the role of the peripheral and the contractile actin filaments for the activation of VSOACs in canine cultured PASMCs, by testing the hypothesis that structural reorganization of both the subcortical and contractile actin filaments is required for activation of VSOACs. Our results suggest that hypotonic activation of VSOACs requires disruption of peripheral actin filaments and intact intracellular actin structures. Factors that interfere with this pattern of actin reorganization during hypotonic cell swelling delay the onset and the activation kinetics of VSOACs.

Material and Methods

Materials

D-Mannitol was purchased from Sigma (St. Louis, MO), cell culture basal medium Eagle (BME), culture medium M199 and newborn calf serum (NCS) were from Gibco (Gibco BRL, Gaithersburg, MD). Polyclonal anti-PKCε antibody and a kit for PKCε activation assay were purchased from Upstate Biotechnology (UBI, Lake Placid, NY). Alexa Fluor 594 phalloidin and a second anti-rabbit Alexa Fluor 488 fluorescence antibody were from Molecular Probes (Eugene, OR); cytochalasin D and latrunculin A were purchased from A.G. Scientific; actin rabbit polyclonal antibody was obtained from Cytoskeleton (Denver, CO).

Cell Culture

Canine lung was obtained from adult mongrel dogs of either sex euthanized by barbiturate overdose. Lung was dissected to isolate second pulmonary artery branches, which were used to disperse and culture vascular myocytes as previously described [3].

Cell Treatment Protocol and Separation of S100 and P100 Fractions

First-passage cells were growth-arrested in M199/0.1% NCS for 36–48 hours prior to the assays. This M199 culture medium has an osmolarity of about 290 mOsm. The medium was diluted with sterile DI water to obtain 230 mOsm (23% hypotonic) solutions for use in our experiments. This culture medium was then supplemented with D-mannitol to increase osmolarity back to the isotonic range (290–300 mOsm). All PASMCs were treated with these solutions for the time span and conditions outlined in the individual experimental protocols. Cells were then washed twice with ice-cold PBS in mM: 10 Na_2HPO_4, 1.8 KH_2PO_4, 2.6 mM KCl and 137 NaCl, pH 7.4, and lysed with a buffer composed of (in mM) 20 MOPS (pH 7.2), 25 β-glycerophosphate, 1 sodium orthovanadate and 1 dithiothreitol. Lysates were passaged 20 times through a 25-gauge needle and incubated on ice for 1 h to increase protein solubilization. Unless otherwise specified, cell lysates were centrifuged at $1{,}000 \times g$ for 3 min to remove cell debris and nuclei, and then at $100{,}000 \times g$ for 60 min to obtain soluble (cytosolic, S100) and cell membrane-rich (P100) fractions. Protein concentrations were assayed by the Bradford assay (BioRad kit, Hercules, CA), and the fractions were stored frozen at −20°C until use.

PKCε Activation Assay

PKCε activation was assayed by phosphorylation of a synthetic peptide substrate (ERMRPRKRQGSVRRRV) in vitro, using a PKCε assay kit and protocol of Upstate Biotechnology, Inc. The phosphorylation reaction took place at 30°C for 60 min in a reaction volume of 40 μl, containing 30 μl S100 or P100 fractions, 10 μg substrate peptide (SP), lipid activator and [γ-^{32}P] ATP as recommended by the manufacturer. Phosphorylation reactions were stopped by cooling on ice and 10 μl reaction were spotted onto P81 filter strips (Whatman, Maidstone, UK). Excess radioactivity was removed by 3 × 10-min washes with 0.85% orthophosphoric acid, followed by 1 × 1-min wash with 95% ethanol. The phosphate incorporated in the substrate peptide was quantitated by radiography and densitometry. The individual filters were then separated and subjected to scintillation counting. Nonspecific radioactive signal was assayed in control reactions, lacking enzyme or substrate, and was then subtracted from the signal of substrate-containing reactions, in order to determine the net PKCε-dependent peptide phosphorylation. Finally, PKCε activation of osmotically stressed cells was calculated and presented relative to cell controls, incubated in isotonic medium.

Western Immunoblot Analysis

Western immunoblot analysis was used to assay the content of PKCε in supernatant and pellet, obtained upon centrifugation of lysed PASMCs at $100{,}000 \times g$ (S100 and P100 fractions, respectively). Equal amount of total protein (usually 30 μg) was resolved by SDS-PAGE and transferred onto nitrocellulose membranes for 1.5 h at 24V, 4°C (Genie blotter, Idea Scientific Company, Minneapolis, MN). The membranes were blocked for 2 hours with 5% skim milk in PBS. PKCε levels were assayed with a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.), diluted 1:200 in 5% milk/PBS. Incubation took place for 2 hours at room temperature, or overnight at 4°C. Excess primary antibody was removed by 3 × 5-min washes with TNT buffer (100 mM Tris, pH 7.5, 0.1% Tween-20,150 mM NaCl), followed by a 1-h incubation with secondary alkaline phosphatase-conjugated antibody, diluted 1:10,000 with 5% milk/PBS. Excess secondary antibody was removed by 3 × 5-min washes with TNT, and color was developed with the

5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate. Immunoblots were scanned to obtain images and the immunoreactive bands of PKCε were analyzed by scanning densitometry, using the Quantity One software (BioRad, Hercules, CA).

Fluorescent Labeling of F-Actin and PKCε

PASMCs, plated on glass cover slips at 50–60% confluence, were growth-arrested for 48 hours and treated according to the experimental protocol. Cells were rinsed twice with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. PFA solution was removed, cells were rinsed with PBS, incubated for 5 min with 10 mM sodium tetrahydroborate to quench excess PFA, and permeabilized with 0.1% Triton X-100 in PBS. After blocking with 1% BSA in PBS, cells were incubated for 1 hour at room temperature with a primary rabbit polyclonal PKCε antibody (Santa Cruz Biotechnology), diluted 1:10 in 1% BSA/PBS. The secondary anti-rabbit Alexa-Fluor 488-coupled IgG (Molecular Probes, Portland, OR) was diluted 1:10,000 and incubation took place for 45 min in the dark. Excess secondary antibody was removed by 3 × 5-min washes with 0.1% Triton X-100 in PBS, and then cellular F-actin was labeled by 1-h incubation with Alexa Fluor 594-phalloidin (final concentration 0.165 μM, Molecular Probes, Portland, OR). Excess label was washed twice with 1% BSA/PBS, and then the cover slips were mounted on slides with Aqua Mount (PolySciences, PA). Fluorescence microscopy was performed using a Nikon Eclipse TE 300 inverted fluorescence microscope (Nikon, USA). Images were acquired with a Spot Slider RT CCD camera (Nikon Corp., CA) and Spot software (Diagnostic Instruments, MI). Images were analyzed for fluorescence intensity using the imaging processing and analysis module of Compix Simple PCI software (Compix, PA). Final images were assembled using Adobe Photoshop software (Wacom, WA).

Quantification of F-Actin

Quantification of F-actin was estimated essentially as described by Carton et al. [7] with slight modifications. In brief, cells were grown to confluence in 12-well culture plates and triplicate wells were treated according to the experimental protocol. Cells were fixed by 15-min incubation with 4% PFA at room temperature. Excess PFA was washed with PBS, quenched with 10 mM sodium tetrahydroborate for 10 min, and then permeabilized/blocked for 10 min with a solution containing 0.1% saponin and 1% BSA in PBS. F-actin was labeled for 1 h with 0.165 μM Alexa Fluor 594-phalloidin in 1% BSA/PBS. The excess label was washed 3 × 5 min with PBS, and F-actin-bound label was extracted by two 45-min incubations with 130 μl methanol. The extracts were pooled together, 200 μl were transferred into wells of 96-well fluorescence plates (Corning Costar, Corning, NY) and fluorescence was read on a plate reader at 594 and 630-nm excitation and emission wavelengths, respectively. The triplicate readings were averaged, the fluorescence of the methanol blanks was subtracted, and the fluorescence of treated cells was expressed relative to the fluorescence of untreated cell controls.

Assay of cytosolic, Membrane and Cytoskeletal Actin Content

PASMCs were fractionated into cytosolic, membrane and cytoskeletal fractions as previously described [4]. Following treatment, cytosolic fraction was extracted by a 10-min agitation with ice-cold cytosolic buffer (in mM): 10 PIPES (pH 6.8), 300 sucrose, 100 NaCl, 3 $MgCl_2$, 5 EDTA, 5 μM phallacidin and protease and phosphatase inhibitors. Membrane fraction was extracted by a 20-min agitation with buffer composed of 0.5% Triton X-100, (in mM) 10 PIPES (pH 7.4), 300 mM sucrose, 100 NaCl, 3 $MgCl_2$, 3 EDTA, 5 μM phallacidin and protease and phosphatase inhibitors. Remaining nuclear/cytoskeletal protein was scraped with SDS-containing buffer: 0.5% Triton X-100, 0.5% SDS, 10 mM Tris-HCl (pH 6.8) and inhibitors, then sonicated and boiled for 5 min. Equal amounts of total protein were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and actin was assayed by immunoblotting.

Electrophysiology

For the electrophysiological measurements, primary cultured cells were trypsinized, transferred onto glass cover slips and allowed to attach overnight at 37°C, in M199 culture medium without NCS. Cell membrane currents were measured using a whole-cell voltage-clamp technique. Patch pipettes were made from borosilicate glass capillaries (Sutter Instrument, CO) and had a tip resistance of 1.5–2.5 MΩ when filled with pipette solutions. The bath and pipette solutions were connected via Ag/AgCl wires to a patch-clamp amplifier (3900A Integrating Patch Clamp, Dagan Corporation, Minneapolis, MN). A 3 M KCl-agar salt bridge between the bath and Ag/AgCl reference electrode was used to minimize changes in liquid junction potential. To follow the time course of change in membrane currents, repetitive voltage-clamp steps to ±80 mV were applied every 30 s from a holding potential of −40 mV. Current densities were calculated by dividing the whole-cell membrane current by cell capacitance. All bath and pipette solutions were chosen to facilitate VSOAC current recording. The hypotonic bath solution contained (in mM): 112 *N*-methyl-D-glucamine, 112 HCl, 1.5 $MgCl_2$, 0.5 $CdCl_2$, 0.05 $GdCl_3$,10 glucose, and 10 HEPES, (pH 7.4, 230 mOsm). Appropriate amounts of D-mannitol were added to make the isotonic (310 mOsm) and hypertonic (350 mOsm) bath solutions. The pipette solution contained (in mM): 96 CsCl, 20 TEA-Cl, 5 ATP-Mg, 5 EGTA, 60 D-mannitol and 5 HEPES (pH = 7.2, 300 mOsm). External solution with osmolarity 230 mOsm has previously been shown to elicit significant increases in PASMC volume [52]. For the experiments with cytochalasin D and phalloidin, PASMCs were first incubated with 1 μM cytochalasin D for 1 h or with 10 μM phalloidin overnight. Then membrane currents were measured with patch electrodes filled with pipette solutions supplemented with 1 μM cytochalasin D or 10 μM phalloidin, respectively. All experiments were conducted at room temperature (22–24°C).

Statistical Methods

Results are presented as the mean ± SEM. The *n* values refer to the number of parallel experiments. Student's *t*-test for paired and unpaired data, or one-way ANOVA was applied to test for differences between treatment means, as appropriate. Values of $P < 0.05$ were considered statistically significant.

Results

Hypotonic Swelling of PASMCs Is Associated with Peripheral Actin Reorganization and Activation of VSOACs

Previous studies have demonstrated that osmotic cell volume changes are associated with reorganization of the actin microfilament structure in areas adjacent to

the sarcolemmal cell membranes [20, 35]. To identify the type of changes that occur with the actin structures in peripheral regions of PASMCs, we exposed cells to hypotonic solutions, then fixed and permeabilized cells, and stained F-actin with phalloidin, coupled to the red fluorescent dye Alexa Fluor 594. We have previously reported that PKCε plays an obligatory role for hypotonic activation of VSOACs in PASMCs [54]. To assess the intracellular localization of PKCs, we also stained the kinase with a primary rabbit antibody and a secondary antibody, coupled to the Alexa Fluor 488 marker that produces green fluorescence. Resting PASMCs displayed a characteristic pattern of actin filament arrays parallel to the longitudinal cell axis (Fig. 1, panel *A1*). Pixel density distribution plots indicated that actin filaments were denser in subcortical areas and more transparent around the cell nucleus (Fig. 1, panel *A2*). PKCε staining was diffuse and uniform throughout the cell (Fig. 1, panels *A3–A4*). After exposure to hypotonic medium (230 mOsm, 23% hypotonic) the intracellular distribution of both proteins visibly altered: the density of the actin filament network in the cell periphery decreased at 5 and 10 min of hypotonic stress, while the density of perinuclear actin increased (Fig. 1, panels *B1–B2*, and *C1–C2*). PKCs staining also transitioned from diffuse and uniform to more intense in perinuclear actin-rich areas (Fig. 1, panels *B3–B4*, and *C3–C4*). The perinuclear localization of F-actin and PKCε appeared not as strong at 20 min compared to 5 and 10 min of hypotonic exposure (Fig. 1, panels *D1–D4*); there appeared to be dissociation between localization of F-actin and PKCε, possibly due to activation of mechanisms leading to regulatory volume decrease (RVD). The observed hypotonic activation of VSOACs (*see* Fig. 3*A*) is consistent with activation of such mechanisms. In agreement with our previous studies [50, 54], the present results indicate that hypotonic swelling of PASMCs is associated with changes in the actin architecture, concomitant translocation of PKCs and activation of VSOACs.

To test whether the decreased peripheral actin filament density is the result of filament dissociation, we incubated cells with hypotonic medium (230 mOsm, 10 min), then fixed and permeabilized cells, and stained F-actin with phalloidin-Alexa Fluor 594. Then we extracted label with methanol and quantified fluorescence by spectrofluorometry. Compared to control cells incubated in isotonic medium (0.86 ± 0.06, arbitrary fluorescent units), the fluorescence intensity in hypotonically stressed cells was reduced to 0.80 ± 0.05 ($n = 4$, ~7% decrease). Together, the pattern of intracellular actin cytoskeletal distribution (Fig. 1) and the quantitative assay of F-actin indicate that although hypotonic swelling is associated with dissociation of actin filaments, the dominant event was intracellular reorganization of the actin assembly. Since the fluorescence of both F-actin and PKCε underwent similar translocation pattern in hypotonically stressed PASMCs, the intracellular distribution of F-actin and PKCε may be interrelated.

Cytochalasin D Disrupts Actin Stress Fibers and Delays Hypotonic Activation of VSOACs

Because hypotonic activation of VSOAC was associated with decreased density of peripheral actin filaments, we tested the hypothesis that disruption of the actin cytoskeleton under isotonic conditions is sufficient for activation of VSOACs. For this purpose we used cytochalasin D, a naturally occurring cell-permeable compound that caps and prevents filament growth [39], as well as depolymerizes and disrupts actin filaments [26, 38]. After 1 h of incubation with cytochalasin D, cells were permeabilized and actin filaments were stained with phalloidin-Alexa Fluor 594. Images of stained cells demonstrated moderate change of the actin assembly after 30 min incubation with 1 μM cytochalasin D (*not shown*), but the actin remodeling was significant at 60 min. However, the pattern of actin remodeling differed from the pattern in hypotonically swollen cells: cytochalasin D caused prominent disruption of actin stress fibers, while actin structures at the cell membrane remained largely intact (Fig. 2, panel *B1*). To test whether these changes reflect actin reorganization or filament dissociation, we extracted phalloidin-Alexa Fluor 594 from cells treated and labeled in a similar fashion. Spectrophotometrical quantification of the extracted fluorescence revealed that 1-h incubation with 1 μM cytochalasin D caused a significant decrease of the F-actin content from 0.86 ± 0.06 to 0.72 ± 0.04 fluorescent units ($P < 0.05$, $n = 4$, ~16% decrease). These results indicate that cytochalasin D produces a different pattern of actin reorganization, which involves primarily dissociation of actin filaments in the cell body, rather than actin remodeling in the cell periphery, observed in hypotonically stressed cells. Incubation of PASMCs with latrunculin A, a chemical that sequesters G-actin monomers and hence causes shortening of actin filaments [10], decreased the density of perinuclear F-actin, similar to cytochalasin D (Fig. 2, panel *C*). PKCε-like immune staining colocalized with F-actin in the cell periphery and residual perinuclear actin filaments, and was low in areas of significant actin filament disruption (Fig. 2, panels *A2* and *B2*), suggesting that the intracellular localization pattern of PKCε may be determined by the presence of intact actin filaments.

To test how hypotonic stress-induced changes of the actin assembly affected activation of VSOACs, we incubated PASMCs with 1 μM cytochalasin D for 1 h prior to whole-cell voltage clamping with pipettes containing 1 μM cytochalasin D to ensure a

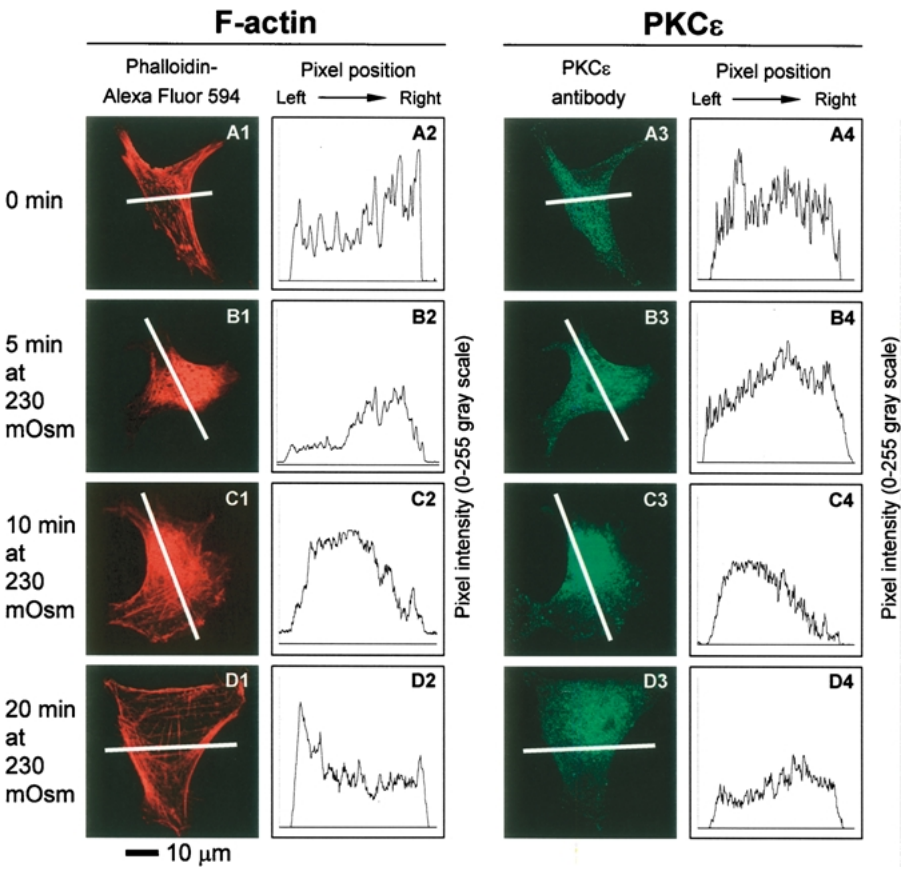

Fig. 1. Hypotonic swelling of PASMCs changes the intracellular organization of actin filaments and intracellular localization of PKCε. Cells were exposed to hypotonic medium (230 mOsm), then were fixed with paraformaldehyde and permeabilized. Actin filaments were stained with phalloidin-Alexa Fluor 594 (*red*) and PKCε was stained with a rabbit polyclonal primary antibody, followed by a secondary antibody coupled to Alexa Fluor 488 (*green*). In isotonic medium, cells exhibit parallel actin filament arrays along the cell axis (panel *A1*); pixel intensity plots show denser actin filaments in submembranous areas (panel *A2*). PKCε staining revealed a uniform and diffuse intracellular distribution of the kinase (panels *A3* and *A4*). Hypotonic swelling was associated with decrease of peripheral actin network (panels *B1* and *B2* at 5 min, and *C1* and *C2* at 10 min) and translocation of PKCε toward perinuclear areas (panels *B3* and *B4* at 5 min, and *C3* and *C4* at 10 min). The perinuclear localization of F-actin and PKCε decreased at 20 min of hypotonic exposure (Fig. 1, panels *D1–4*). This experiment was repeated 3 times with similar results.

saturating concentration of cytochalasin D is present intracellularly throughout the experiment. Membrane currents were elicited by voltage pulses spanning to ±80 mV. Incubation with cytochalasin D failed to induce isotonic activation of VSOACs and did not change the maximal amplitude of VSOACs in hypotonically stressed PASMCs (Fig. 3, panels *B* and *D*). However, cytochalasin D pretreatment significantly reduced the activation kinetics of VSOACs in PASMC perfused with hypotonic solution (230 mOsm). As a result, the membrane currents reached steady state in about 20 min, compared to 10 min in control cells: accordingly, the activation half-time increased from 5.22 ± 0.23 in controls to 9.82 ± 1.49 min in cytochalasin D-treated PASMCs (Fig. 3, panels *B* and *E*). A similar delay of the activation kinetics was observed in cells incubated with latrunculin A prior to exposure to hypotonic media (*not shown*).

To test whether the slower activation of Cl^- current in cells preincubated with cytochalasin D was due to cytochalasin D-induced delay in dissociation of peripheral F-actin, we incubated cells for different times in hypotonic media, then fractionated cells into membrane, cytosolic and cytoskeletal fractions, and assayed the actin content by immunoblotting. As expected, incubation with cytochalasin D decreased the cytoskeletal, and increased the cytosolic actin content, but subsequent hypotonic stress produced little additional change (Fig. 4, panel *A*). Incubation with cytochalasin D, however, had little effect on the stability of membrane-associated actin filaments, which was demonstrated by the parallel decrease of the membrane actin content between non-treated and cytochalasin D-treated PASMCs (Fig. 4, panel *B*). These observations indicate that the delay in Cl^- current activation may be due to the cytochalasin D-induced disruption of stress fibers. Thus, an intact

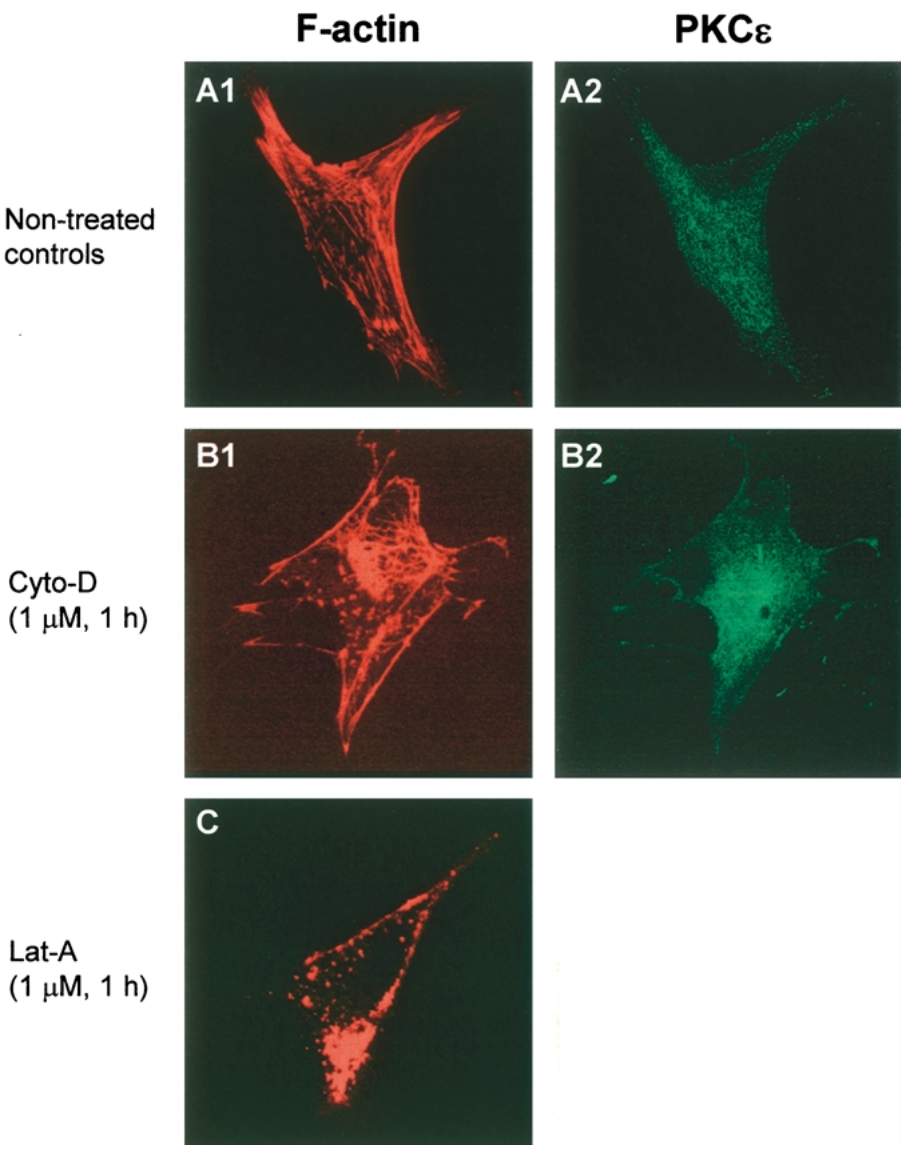

Fig. 2. Incubation with cytochalasin D (panel *B1*) and latrunculin A (panel *C*) caused dramatic disruption of perinuclear actin filaments and stress fibers, and a modest decrease of subcortical actin filaments, compared to control PASMCs (panel *A1*). PKCε colocalized with intact stress fibers or subcortical actin filaments (panels *A2* and *B2*). Cells were incubated for 1 h with 1 μM cytochalasin D (panel *B*) or 1 μM latrunculin A (panel *C*), then fixed and permeabilized. F-actin was stained with phalloidin-Alexa Fluor 594 (*red*) and PKCε was stained with a primary polyclonal antibody and a secondary antibody coupled to Alexa Fluor 488 (*green*). The experiment was repeated 3 times with similar results.

perinuclear F-actin network may be required for normal hypotonic activation of VSOAC.

Phalloidin Stabilizes the Subcortical Actin Cytoskeleton and Delays Hypotonic Activation of VSOACs

If the cytochalasin D-mediated delay of the hypotonic activation of VSOACs is due to disruption of perinuclear actin filaments, then agents that stabilize the actin filamentous network should potentiate the hypotonic activation of VSOACs. To test this hypothesis, we incubated PASMCs with non-labeled phalloidin (10 μM, 18 hours), then fractionated cell content into cytosolic, membrane and cytoskeletal fractions, and assayed the actin content of control and osmotically stressed cells. Alone, incubation with phalloidin resulted in no apparent change of the G-actin content in control cells incubated in isotonic medium. Phalloidin failed to induce isotonic activation of VSOACs of PASMCs, dialyzed with 10 μM phalloidin through the patch pipette as well (Fig. 3). In control cells not incubated with phalloidin, hypotonic swelling produced insignificant alteration of the cytosolic and cytoskeletal actin content; however, the content of the membrane-associated actin was markedly decreased (Fig. 5, top inset and bar graph): this decrease is consistent with dissociation of peripheral actin filament also observed in hypotonically swollen cells by immunocytochemistry (*see* Fig. 1, panels *B1* and *C1*). As in control cells, incubation with phalloidin produced no change of the cytosolic and cytoskeletal actin content in hypotonically stressed cells, but slowed the disruption time course of membrane actin filaments (Fig. 5, bottom inset and bar graph). Moreover, incubation with phalloidin caused an increase of the activation half-time of VSOACs from 5.22 ± 0.23 to 9.98 ± 1.75

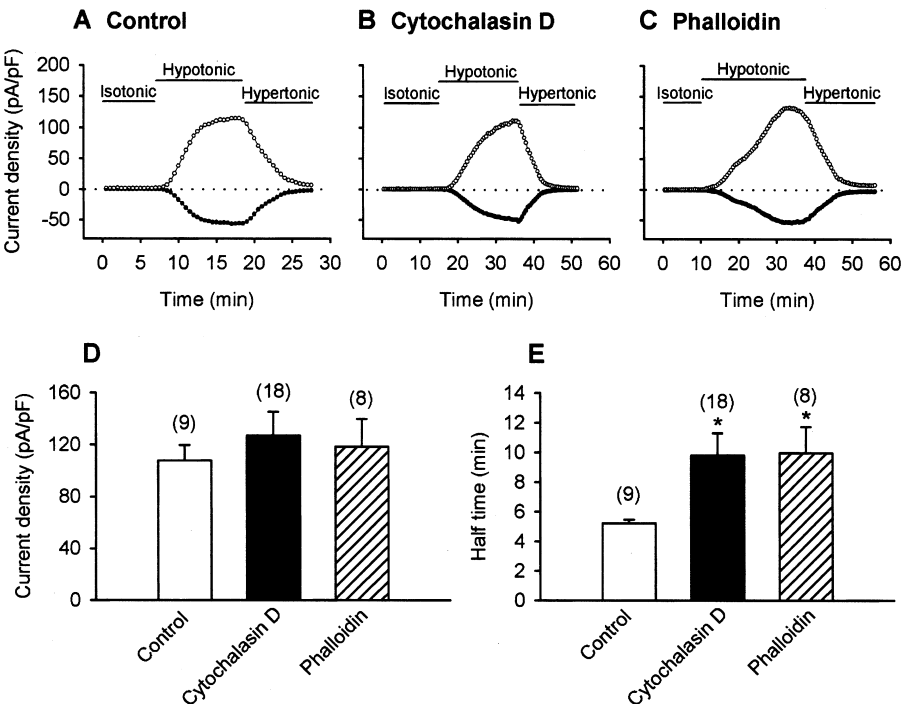

Fig. 3. VSOACs were activated by hypotonic stress of canine PASMCs (panel *A*). Incubation with cytochalasin D and phalloidin delayed the activation velocity of VSOACs (panels *B*, *C* and *E*) compared to control PASMCs (panel *A*). Cells were preincubated with either 1 μM cytochalasin D for one hour or 10 μM phalloidin overnight, and then dialyzed with the same concentrations of cytochalasin D or phalloidin through the patch pipettes. VSOACs were measured by voltage-clamping the membrane potential to ±80 mV from a holding potential of −40 mV every 30 seconds. Panels *A–C* depict representative time courses of change in membrane currents measured at +80 (*empty circles*) and −80 mV (*filled circles*) under isotonic, hypotonic and hypertonic conditions in a control cell (panel *A*) and in cells treated with either cytochalasin D (panel *B*) or phalloidin (panel *C*). Note that the time scale in panel *A* is different from that in panel *B* and *C*. Panels *D* and *E* summarize the effects of cytochalasin D and phalloidin on maximal VSOACs current densities (panel *D*) and the time for half maximal activation of VSOACs currents (panel *E*) measured at +80 mV under hypotonic conditions. Cell numbers used in each group are shown in parentheses above the respective bar. *, $P < 0.05$ versus respective controls.

min, without changing the maximal amplitude (Fig. 3, panels *C – E*). These results indicate that the phalloidin-induced delay of the hypotonic activation of VSOACs may be secondary to the delayed disruption of peripheral actin filaments. Longer exposure to hypotonic media eventually surmounts the stabilizing effects of phalloidin and allows maximal activation of VSOACs. These results again suggest that timely subcortical actin dissociation is a required step for normal hypotonic activation of VSOACs in PASMCs.

Hypotonic Translocation of PKGε Depends on Actin Reorganization

As suggested by previous studies, presence of basal PKCε activity proximal to sarcolemmal cell membrane may maintain low basal VSOAC activity in isotonic medium [13, 54]. Since activation of PKCε requires association with membrane phospholipids, hypotonic swelling-induced activation of VSOACs may not involve activation of PKCε, but only kinase translocation from submembranous to perinuclear areas. To test this hypothesis, we lysed 10-min hypotonically stressed PASMCs and used differential centrifugation to separate cytosolic (supernatant, SI00) and membrane-rich (P100) fractions, the latter containing also F-actin and associated PKCε. For positive controls, we stimulated PASMCs with 100 nM PMA for 10 min and separated cytosolic and particulate fractions. All S100 and P100 fractions were split into two: one for assay of PKCε activation by phosphorylation of a synthetic peptide substrate in vitro; and the other, for assay of protein levels by immunoblot analysis. As expected, incubation with PMA enhanced the kinase activity of PKCε to 1.98 ± 0.26-fold the basal in S100 (Fig. 6, panel *A*, bar graph) and 3.31 ± 0.32-fold the basal in PI00 fractions (Fig. 6, panel *B*, bar graph), and PMA-dependent activation was abolished by cell preincubation with the PKC inhibitor Rö318425 (Ro, Fig. 6, panels *A–D*). PKCs activation was associated with a decrease in the immunoreactivity in S100 fractions, compared to non-stimulated controls, and increase of the immunoreactivity in the particulate P100 fraction (Fig. 6, panel *A*, insets). This is consistent with

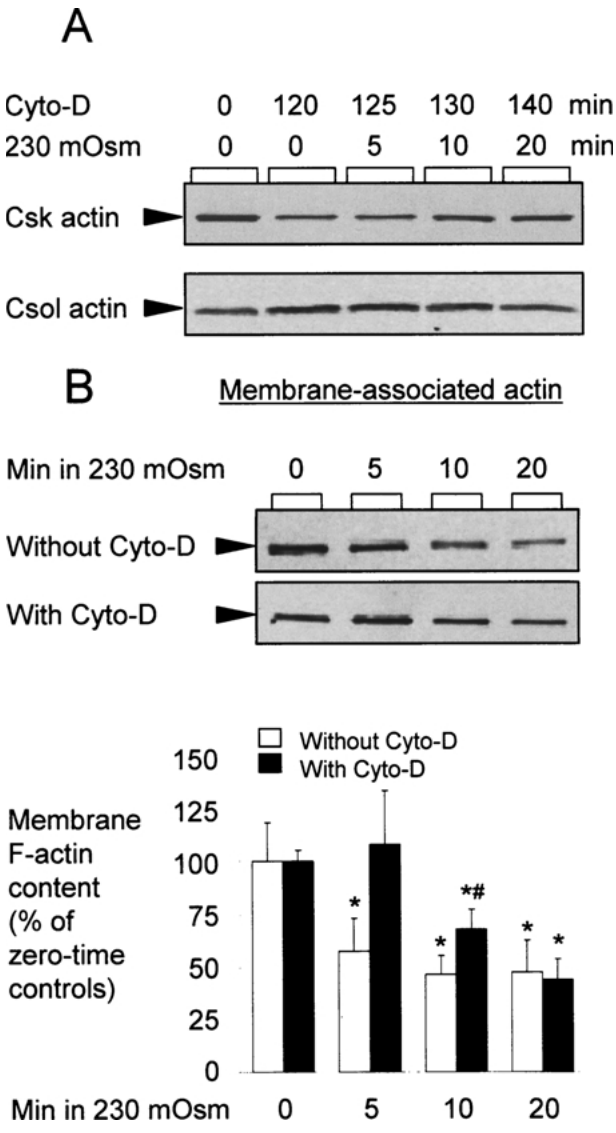

Fig. 4. Incubation with cytochalasin D in isotonic media decreased the content of actin stress fibers and increased the cytosolic actin content (panel *A*), but did not affect the hypotonic stress-induced dissociation of membrane-associated actin filaments (panel *B* and bar graph). PASMC cells were incubated with 1 μM cytochalasin D (*Cyto-D*) for 1 h prior to exposure to hypotonic medium (230 mOsm). Actin was fractionated into cytoskeletal (*Csk*), cytosolic (*Csol*) and membrane fractions, and quantified by immunoblotting and scanning densitometry (panels *A* and *B*, insets, $n = 3$). Bar graph: *, $P < 0.05$ compared to the series' zero-time controls; #, $P < 0.05$ compared to the respective time point of cytochalasin D-untreated cells.

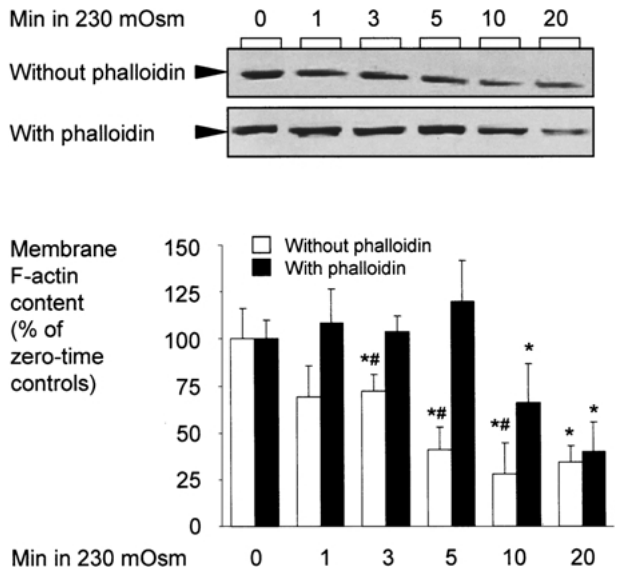

Fig. 5. Incubation with phalloidin delayed the hypotonic swelling-induced disruption of subcortical actin filaments of canine PASMCs. Cells were incubated with phalloidin (10 μM, 18 h), followed by exposure to hypotonic medium (230 mOsm). Actin was fractionated into cytosolic, cytoskeletal and membrane fractions as described in the Methods section. The membrane-associated actin content was quantified in control (*top inset*) and phalloidin-treated cells (*bottom inset*) by immunoblotting and scanning densitometry (bar graph). *, $P < 0.05$ compared to the series' zero-time controls; #, $P < 0.05$ compared to the respective time point of phalloidin treated cells ($n = 4$).

translocation of PKCε from the cytosol to cell membranes, which is required for activation, and possibly to actin filaments, which bind active PKCε [37, 43]. In contrast, in hypotonically stressed cells, PKCε activity (1.68 ± 0.31) and immunoreactivity increased in parallel in S100 (Fig. 6*A*, inset), and decreased in parallel in the P100 fractions (Fig. 6, panel *B*, inset). Since kinase activation and immunoreactivity exhibited different trends in the PMA and hypotonically stressed PASMCs, we normalized kinase activation (*bars*) to the respective density of the PKCε immunoreactive bands (Fig. 6, *insets*). The results reveal that the normalization eliminated the hypotonic activation of PKCs in the S100 fractions (Fig. 6, panel *C*) and the decreased activity in the P100 fractions (Fig. 6, panel *D*). These observations suggest that the apparent hypotonic swelling-induced changes in PKCε activity are due to intracellular redistribution, rather than to a net alteration of the catalytic activity. All cell lysates in this group of experiments were prepared after a 10-min treatment with PMA or hypotonic medium and hence may not reflect the maximal enzyme activation. To test whether activation of PKCs was time-dependent, we assayed kinase activity at different times, using total cell lysates centrifuged at 1000 × *g* for 5 min to remove only cell nuclei. The remaining cell lysate, containing cytosolic and particulate PKCε, was used as an enzyme source in an in vitro kinase assay. Hypotonic swelling caused insignificant fluctuation of PKCε activity around the basal level, while incubation with PMA produced a significant increase of the total PKCε activity (Fig. 6, panel *E*). Together these data indicate that the hypotonic translocation of PKCε is not the result of kinase activation or inactivation. It

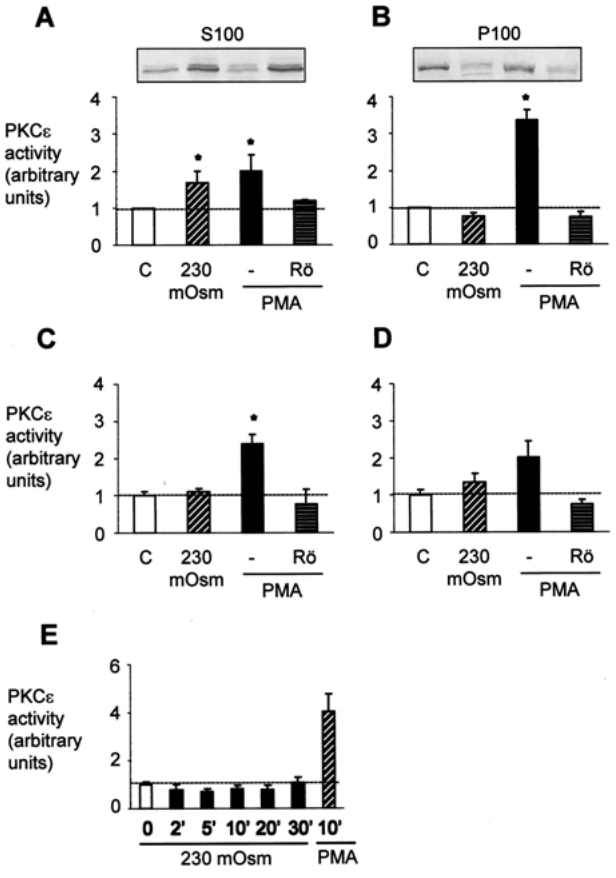

Fig. 6. Hypotonic swelling-induced translocation is independent of the PKCε activity. PASMCs were incubated with hypotonic medium (230 mOsm) for 10 min, then lysed and centrifuged at 100,000 $\times g$ to obtain supernatant (S100) and particulate (P100) fractions. PKCε activity and protein levels were assayed in these fractions by phosphorylation of a synthetic peptide substrate in vitro, and by Western immunoblot analysis, respectively. The apparent hypotonic increase of PKCε activity in S100 and decrease in P100 fractions (panels *A* and *B*, bar graphs) was paralleled by similar changes of protein levels (panels *A* and *B*, insets). Normalization of kinase activity (*bars*) to protein levels (*insets*) showed lack of a net change of PKCε activity at 10 min (panels *C* and *D*) or during 30-min incubation in hypotonic solution (panel *E*). For positive controls, cells were stimulated with PMA without or after 30-min preincubation with the kinase inhibitor Rö318425 (*Rö*). Kinase activation is expressed relative to the non-treated cell controls. *, $P < 0.05$, Dunnett's test.

appears, therefore, that the subcellular redistribution of PKCε may be driven by reorganization of the actin network during hypotonic swelling of PASMCs.

Discussion

The intracellular actin cytoskeleton not only serves as a supporting network to maintain cell shape, but may also play an important role in the regulation of cell volume by participating in signal or mechanotransduction [34]. Dynamic structural reorganization of F-actin during osmotic perturbations has been observed in various cell types, and pharmacological disruption of F-actin by cytochalasin D has been shown to interfere with the ability of cells to undergo regulatory volume decrease (RVD) or regulatory volume increase (RVI) [21]. Although a series of studies has demonstrated that the actin cytoskeleton undergoes dynamic structural changes in response to osmotic cell swelling, serious discrepancies exist with regard to the pattern of actin cytoskeletal remodeling. For example, in Ehrlich ascites tumor cells [36] and B-lymphocytes [26], hypotonic cell swelling is associated with decrease of the cellular F-actin content, while in human Intestine 407 and C6 glial cells hypotomc swelling causes actin polymerization and/or reorganization [30, 48]. In shark rectal gland cells, hypotonic challenge causes reorganization without changes of the F-actin content [21]. In contrast to most other cell types, human endothelial cells displayed no swelling-induced rearrangement of the actin cytoskeleton [33]. While these results indicate that the response of actin cytoskeleton to hypotonic challenges may depend upon the cell type, they also indicate that the functional integrity of F-actin is required for volume regulation in most cell types. Our present study provides additional support of this notion by showing that interference with the hypotonic swelling-induced rearrangement of the actin network can alter the activation kinetics of VSOACs and potentially the time course of RVD. Moreover, our results indicate that the two major actin compartments, i.e., peripheral actin filaments and stress fibers, differ in their stability during hypotonic swelling and may play distinct roles in activation of VSOACs. For example, hypotonic swelling of PASMCs seems to cause a more dramatic decrease of the density of peripheral actin filaments than of perinuclear actin filaments and/or stress fibers (Fig. 1 and Fig. 4). This observation could be explained by the presence of actin-binding proteins, including caldesmon and tropomyosin, which overlay filaments and provide more stability and protection of stress fibers against severing [17, 27, 28]. Because caldesmon and tropomyosin are excluded from the short and highly branched peripheral actin filaments, the latter are prone to severing and depolymerization. The distinct structural properties of the two actin pools suggest that upon hypotonic swelling they may play different, and possibly functionally coordinated roles in the activation of VSOACs and hence in counteracting cell volume perturbations. This notion is consistent with our demonstration that cytochalasin D and phalloidin, agents that interfere with the hypotonic reorganization of the central and the peripheral F-actin pools, delay the hypotonic activation of VSOACs.

Surprisingly, incubation with cytochalasin D or latrunculin A per se was insufficient for isotonic

activation of VSOACs in PASMCs (Fig. 3) and B-lymphocytes [26], but induced isotonic activation of VSOACs in *Xenopus ocytes* [1], PC12 cells [9], cervical cancer HT-3 cells [41] and human embryonic skeletal muscle cells [19]. Moreover, while cytochalasin D delayed the hypotonic activation without altering the maximal amplitude of VSOACs in PASMCs (*see* Fig. 3), it suppressed the maximal VSOAC activity in cultured chick cardiac myocytes [53], renal proximal tubule cells [46] and HSG cells [16]. In contrast, cytochalasin D increased the rate of hypotonic activation of VSOACs in B-lymphocytes [26]. Although the reasons are presently unclear, these discrepancies may reflect cell type-specific signaling pathways involved in the regulation of VSOACs activity and/or existence of diverse VSOACs subtypes. The lack of isotonic activation of VSOACs in canine PASMCs could perhaps be explained by the abundance and the high intrinsic flexibility of actin filaments. Such actin configuration is likely to underlie a significant potential for compensatory remodeling of the actin assembly in cells incubated with cytochalasin D and/or phalloidin, and thus prevent unnecessary activation of cell VSOACs in isotonic environment.

Although the exact mechanism of regulation of VSOACs by the actin cytoskeleton remains undefined, it is plausible that coordination between peripheral actin remodeling and signal transduction are necessary for activation of VSOACs. Phosphorylation by PKCε was proposed to inhibit membrane VSOACs in resting PASMCs, while hypotonic swelling was shown to cause translocation of PKCε away from cell membranes and channel activation [13, 54]. This model is based on the assumption that sufficient PKCε activity is present at the channel site in resting cells, and is consistent with mutagenesis and pharmacological data demonstrating that inhibition of PKCε mediates channel phosphorylation [13, 54]. However, the mechanism whereby PKCε is translocated from subcortical to perinuclear areas during cell swelling remained elusive. Our present results suggest that hypotonic swelling-induced remodeling of peripheral actin filaments reduces the number of PKCε binding sites in the proximity of membrane VSOACs. Our observations thus suggest that the net decrease of the PKCε activity in submembranous sites proximal to VSOACs may develop secondary to the cell swelling-induced peripheral actin dissociation and loss of binding sites for active PKCε (RACK) [29].

The physiological functions of VSOACs in the pulmonary circulation remain undefined, although PASMCs express VSOACs and respond to hypotonic stress with significant activation of these channels [50, 52, 54]. In healthy pulmonary arteries, activation of VSOACs may be involved in maintenance of physiological cell volume and in counteracting cell volume fluctuations during abrupt deviations of the normal intracellular osmolyte content due to metabolic processes [32, 40]. VSOACs may be further involved in the regulation of contractility of pulmonary arteries [31]. However, the role of VSOACs may be even more important during inflammatory processes, leading to development of atherosclerosis and pulmonary artery hypertension [23]. Inflammation is associated with remodeling of the actin cytoskeleton of PASMCs, which is manifested by an enhanced flexibility of submembranous actin filaments that is required for the increased motility and proliferation of PASMCs [6, 44, 51]. Increased flexibility of peripheral actin filaments, therefore, is one mechanism that may facilitate the activation of VSOACs and Cl^- currents. Activation of VSOACs may be further complemented by inflammation-induced expression of volume-sensitive channels; we have shown previously that this mechanism may apply for at least one member of the VSOAC family, ClC-3 [11]. These data suggest an important role of VSOAC for critical cellular processes that include progression through the cell cycle [42, 49] and better survival capability [11] of SMCs in inflamed pulmonary arteries. Thus, inflammation-associated remodeling of the actin cytoskeleton and activation of VSOACs may be sequential events of a broader scheme that enables cell adaptation under pathophysiological conditions.

In summary, the results of the present study demonstrate that the actin cytoskeleton mediates the hypotonic activation of VSOACs by participating in signal and/or mechanotransduction. Both actin cytoskeletal compartments, i.e., short subcortical actin assembly and stress fibers, may be well synchronized and equally important for normal activation of VSOACs and RVD during hypotonic stress. Interference with the actin reorganization during hypotonic swelling delays the activation kinetics of VSOACs in PASMCs. It remains to be determined whether the hypotonic activation of VSOACs depends solely upon the release of the inhibitory effects of PKCε following its perinuclear translocation during hypotonic cell swelling. It is also conceivable that reorganization of the subcortical actin network serves as a mechanical trigger for conformational changes, causing and/or facilitating the hypotonic activation of VSOACs.

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