Regulation of volume-sensitive outwardly rectifying anion channels in pulmonary arterial smooth muscle cells by PKC

Juming Zhong, Ge-Xin Wang, William J. Hatton, Ilia A. Yamboliev, Michael P. Walsh, and Joseph R. Hume

Center of Biomedical Research Excellence, Department of Pharmacology, University of Nevada, Reno, Nevada 89557-0046; and Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada T2N 4N1

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Regulation of volume-sensitive outwardly rectifying anion channels in pulmonary arterial smooth muscle cells by PKC. We previously tested the hypothesis that PKC regulates the activation state of native volume-sensitive organic osmolyte and anion channels (VSOACs) in acutely dispersed canine pulmonary artery smooth muscle cells (PASMC). Hypotonic cell swelling activated native volume-regulated Cl− currents (ICl.vol) which could be reversed by exposure to phorbol 12,13-dibutyrate (0.1 μM) or by hypertonic cell shrinkage. Under isotonic conditions, calphostin C (0.1 μM) or Ro-31–8425 (0.1 μM), inhibitors of both conventional and novel PKC isoforms, significantly activated ICl.vol and prevented further modulation by subsequent hypertonic cell swelling. Bisindolylmaleimide (0.1 μM), a selective conventional PKC inhibitor, was without effect. Dialyzing acutely dispersed and cultured PASMC with eV1–2 (10 μM), a translocation inhibitory peptide derived from the V1 region of εPKC, activated ICl.vol under isotonic conditions and prevented further modulation by cell volume changes. Dialyzing PASMC with bC2–2 (10 μM), a translocation inhibitory peptide derived from the C2 region of βPKC, had no detectable effect. Immunohistochemistry in cultured canine PASMC verified that hypotonic cell swelling is accompanied by translocation of εPKC from the vicinity of the membrane to cytoplasmic and perinuclear locations. These data suggest that membrane-bound εPKC controls the activation state of native VSOACs in canine PASMC under isotonic and anisotonic conditions. chloride channels; cell volume; protein kinase C

Address for reprint requests and other correspondence: J. R. Hume, Center of Biomedical Research Excellence, Dept. of Pharmacology/318, Univ. of Nevada, School of Medicine, Reno, NV 89557-0046 (E-mail: joeh@med.unr.edu).

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leimide; BIM) and stimulation (phorbol esters), respectively, of endogenous PKC. Moreover, phosphatase inhibitors such as okadaic acid and calyculin A also inhibited I_{gpClC-3}, and mutation of an amino terminal PKC phosphorylation site (S51A) completely eliminated the response of expressed I_{gpClC-3} to cell swelling, resulting in a constitutively active channel under isotonic conditions which was insensitive to PKC activation and phosphatase inhibition. These results indicated that an important mechanism linking cell swelling to activation of PKC isozymes in PASMC is altered by changes in cell volume. A preliminary report describing these results has been published (41).

METHODS

Cell preparation. Mongrel dogs were anesthetized with pentobarbital sodium (45–50 mg kg$^{-1}$, iv). Segments of pulmonary artery were removed, and vascular smooth muscle cells were enzymatically dispersed as previously described (9, 39). Freshly dispersed cells were kept in the refrigerator and used within 10 h. The animal use protocol was reviewed and approved by the Animal Care and Use Committee of the University of Nevada.

Electrophysiological recordings. Membrane currents were measured from canine PASMC at room temperature (22–24°C) by the tight-seal, whole cell, voltage-clamp technique (16). Patch pipettes were made from borosilicate glass capillaries and had a tip resistance of 2–5 MΩ. Ag-AgCl wires were immersed in the bath and pipette solutions and connected to a patch-clamp amplifier (Axopatch-200A, Axon Instruments, Foster City, CA). A 3 M KCl-agar salt bridge between the bath and Ag-AgCl reference electrode was used to minimize changes in liquid junctional potential. To obtain Cl$^{-}$ current-voltage relations, whole cell currents were recorded during voltage pulses (150 ms) applied from the holding potential (~10 mV). The time courses of changes in membrane currents were monitored by the application of repetitive voltage-clamp steps to ±80 mV applied every 30 s. Membrane currents were filtered at a frequency of 1 kHz and digitized online at 5 kHz using a Pentium III processor and pCLAMP 8 software (Axon Instruments).

Solutions and reagents. All bath and pipette solutions were chosen to facilitate Cl$^{-}$ current recording. The standard isotonic bath solution contained (in mM) 107 N-methyl-D-glucamine, 107 HCl, 1.5 MgCl$_2$, 2.5 MnCl$_2$, 10 glucose, 70 D-mannitol, and 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, pH 7.4, 300 mosmol/kgH$_2$O). D-mannitol was removed from this solution to make the standard hypotonic solution (230 mosmol/kgH$_2$O), and 140 mM D-mannitol was included to make the standard hypertonic solution (370 mosmol/kgH$_2$O). GdCl$_3$ (0.05 mM) was routinely included in all bath solutions to prevent possible contamination of membrane currents by activation of nonspecific cation channels. The pipette solution contained (in mM) 95 CsCl, 20 tetraethylammonium chloride, 5 ATP-Mg, 5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 80 D-mannitol, and 5 HEPES (pH 7.2, 300 mosmol/kgH$_2$O).

Fig. 1. Phorbol 12,13-dibutyrate (PDBu) inhibits hypotonic swelling-induced volume-sensitive Cl$^{-}$ currents (I_{Cl.vol}) in acutely dispersed canine pulmonary artery smooth muscle cells (PASMC). Currents were recorded at every 30 s when the membrane potential was stepped to −80 mV (●) followed by +80 mV (○) from a holding potential of −10 mV. A: time course of currents measured from a cell without any drug application. Inset shows representative currents recorded at the times indicated in A. The cell was superfused with different bath solutions as marked by horizontal bars. B: average current density from a group of cells under different conditions. C: time course of currents measured from a cell exposed to PDBu (100 nM). PDBu was added to the bath solution after the currents reached a steady state in the hypotonic solution. D: average current density from a group of cells exposed to PDBu. Values in B and D represent means ± SE (n = 5 in each group of cells). *Significantly different from the values under isotonic conditions (P < 0.05).
The PKC translocation inhibitory peptides βC2–2 (MD-PNGLSDPVYKVL, corresponding to residues 186–198 of βPKC), εV1–2 (EAVSLKPT, corresponding to residues 14–21 of εPKC), and scrambled εV1–2 (LSETKPAV) were synthesized in the University of Calgary Peptide Synthesis Core Facility using a Beckman model 990B automated peptide synthesizer and purified by preparative reverse-phase HPLC. Phorbol 12,13-dibutyrate (PDBu), calphostin C, Ro-31 8425, and BIM were purchased from Calbiochem (La Jolla, CA). These drugs were first dissolved in dimethylsulfoxide (DMSO) and then further diluted in the solution to a final concentration of DMSO of <0.2%. DMSO alone at 0.2% had no effect on Cl− currents. In Figs. 5–7, the onset of membrane rupture and intracellular dialysis is indicated at time 0.

Immunohistochemistry. PASMCs were dispersed and cultured as previously described (40). Special culture dishes were incorporated to aid with imaging. Holes were cut (23 mm) in the base of 35-mm culture dishes, and 25-mm glass coverslips were then bonded to the base of the dish. Cultured PASMCs (48 h) were then exposed to isotonic (300 mosmol/kgH2O) and hypotonic (230 mosmol/kgH2O) solutions for 5, 10, and 20 min. After treatment, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 10 min at 4°C. After fixation, cells were washed in PBS (4 × 30 min), and nonspecific antibody binding was reduced by blocking with 10% bovine serum albumin in PBS containing 0.03% Triton X-100 for 1 h and then incubated with a polyclonal anti-PKCε antibody raised in rabbit (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:200 with a polyclonal anti-PKCε antibody raised in rabbit (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:200 with a polyclonal anti-PKCε antibody raised in rabbit (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:200 with a polyclonal anti-PKCε antibody raised in rabbit (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:200 with a polyclonal antikgG secondary antibody (Molecular Probes) at 5 μg/ml. Secondary incubations were performed for 1 h at room temperature. After secondary incubation, cells were washed in PBS (3 × 15 min). For negative control cells, the primary antibody was omitted and substituted with PBS. Cells were examined using a Nikon Eclipse TE 300 inverted fluorescent microscope with excitation wavelength appropriate for Alexa 488 (488 nm). Digital micrographs were acquired using a Spot RT digital camera, and final images were prepared using Adobe Photoshop software.

For immunofluorescence quantitation, images were acquired of cultured PASMCs subjected to isotonic (n = 11) or hypotonic (n = 10) bath solutions for 20 min, keeping image acquisition parameters constant. Simple PCI software (Compix, Cranberry Township, PA) was used to select regions of interest (ROIs) and calculate pixel intensities. The initial ROIs chosen spanned the entire cell diameter for each image (see Fig. 8, A and C). The pixel intensity across the ROI was then computed, and data were plotted graphically (see Fig. 8, B and D). The ROI was a standard width, with length standardized to account for differences in cell size by having the ROI “overshoot” the membrane on either side of the cell by a fixed amount (2.5 μm). Additional, multiple, smaller ROIs in each cell were sampled in 2 × 4-μm areas juxtaposed either close to the sarcolemmal membrane or close to the nuclear membrane, and mean pixel intensities in these areas were computed to compare differences between these regions in cells exposed to isotonic or hypotonic bath solutions.

Data were expressed as means ± SE (n, no. of cells). Statistical analysis was made by paired t-tests and analysis of variance where appropriate. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Effect of PDBu on native I\textsubscript{Cl.vol} in canine PASMC. I\textsubscript{Cl.vol} in canine PASMC are outwardly rectifying membrane currents, with an anion permeability sequence of SCN− > I− > Br− > Cl− > aspartate−, and are inhibited by DIDS, extracellular ATP, and the anti-estrogen compound tamoxifen (39). Membrane currents are typically very small in isotonic solutions (300 mosmol/kgH2O) but increase after cell swelling induced by exposure to hypotonic solutions (230 mosmol/kgH2O, Fig. 1A). Cell swelling-induced increases in I\textsubscript{Cl.vol} were completely reversed by perfusion of cells with a hypertonic (370 mosmol/kgH2O) solution. Figure 1B illustrates that, in a group of cells, current densities measured at ±80 mV were more than doubled after hypotonic challenge and returned to the basal level in response to exposure to hypertonic bath solutions. In another group of cells, when the activation of I\textsubscript{Cl.vol} in hypotonic solutions reached a steady state, 100 nM PDBu was applied to the hypotonic bath solution. As

![Image](http://ajpcell.physiology.org/Downloadedfrom/10.220.33.2onOctober20,2017)
shown in Fig. 1, C and D, activation of PKC by PDBu effectively reversed the hypotonic cell swelling-induced current increase. The currents after PDBu application under hypotonic conditions were no longer different from the values under isotonic conditions, although hypotonic cell swelling alone significantly increased the currents. These data suggest that, similar to native \( I_{\text{Cl,vol}} \) in cardiac myocytes, \( \text{Xenopus} \) oocytes, and expressed CIC-3 channels in NIH 3T3 cells (5, 32), native \( I_{\text{Cl,vol}} \) in PASMC is strongly inhibited by endogenous PKC activation.

Effects of PKC inhibitors on native \( I_{\text{Cl,vol}} \) in canine PASMC. We next tested whether inhibition of endogenous PKC would cause activation of \( I_{\text{Cl,vol}} \) in PASMC under isotonic conditions, as previously demonstrated for native \( I_{\text{Cl,vol}} \) in cardiac myocytes, \( \text{Xenopus} \) oocytes, and expressed CIC-3 channels in NIH 3T3 cells (5, 32). Figure 2 shows the effect of the PKC inhibitor, BIM at 100 nM, on \( I_{\text{Cl,vol}} \). BIM at this concentration is a more potent inhibitor of conventional (c)PKCs than novel (n)PKCs (20). Unlike native \( I_{\text{Cl,vol}} \) in cardiac myocytes and expressed CIC-3 channels in NIH 3T3 cells (5), exposure to 100 nM BIM failed to activate \( I_{\text{Cl,vol}} \) under isotonic conditions in PASMC. Current amplitudes after BIM exposure were not significantly different from the values without BIM (Fig. 2B). \( I_{\text{Cl,vol}} \) in cells treated with BIM were similar to those in control cells, being significantly increased under hypotonic conditions and reversed by exposure to hypertonic solutions. On the other hand, exposure of PASMC to another PKC inhibitor, calphostin C, which at 100 nM is reported to be an equally potent inhibitor of cPKCs and nPKCs (14), increased \( I_{\text{Cl,vol}} \) under isotonic conditions (Fig. 3). Mean current densities in a group of cells was more than doubled by addition of calphostin C to the isotonic bath solution, whereas the current-voltage relationship of the calphostin C-induced current was unchanged by switching to hypotonic solutions (Fig. 3B). Superfusion of cells with hypertonic solutions after treatment with calphostin C failed to further increase the current densities. Similarly, another equally potent inhibitor of cPKCs and nPKCs, Ro-31 8425 (14), also increased \( I_{\text{Cl,vol}} \) densities under isotonic conditions and prevented further activation of the channels by subsequent hypotonic cell swelling (Fig. 4).

These results strongly suggest that, similar to several other cell types, PKC-catalysed phosphorylation and dephosphorylation of PKC-phosphorylated sites play a crucial role in VSOAC regulation by changes in cell volume in canine PASMC. These results also suggest that the isoform of PKC involved in the volume regulation of these channels in PASMC may differ from other cell types. For instance, for native \( I_{\text{Cl,vol}} \) in guinea pig cardiac myocytes, \( \text{Xenopus} \) oocytes, and recombinant CIC-3 Cl– channels expressed in NIH 3T3 cells, regulation by changes in cell volume may involve cPKCs, because currents were activated under isotonic conditions by exposure to BIM (5, 32), whereas in PASMC regulation of native \( I_{\text{Cl,vol}} \) appears to involve nPKCs.

Effects of isozyme-specific PKC translocation inhibitory peptides on native \( I_{\text{Cl,vol}} \) in canine PASMC. To further test the hypothesis that nPKCs are responsible for the cell volume sensitivity of native \( I_{\text{Cl,vol}} \) in PASMC, we examined the effects of different isozyme-specific PKC translocation inhibitory peptides on these channels. eV1–2 is a short peptide derived from the V1 region of ePKC.
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In another test, we examined the effects on I_{Cl,vol} in PASMC of βC2–2, a synthetic peptide derived from the C2 region of βPKC, which inhibits translocation of conventional (Ca^{2+}-dependent) PKC isozymes (cPKCs) (22). As shown in Fig. 6, intracellular dialysis with βC2–2 peptide (10 μM) did not evoke any significant change in I_{Cl,vol} under basal isotonic conditions, nor did it prevent I_{Cl,vol} regulation by cell volume changes. Current densities measured at 1 and 10 min of isotonic superfusion were not different in cells dialyzed with βC2–2. Subsequent exposure of these cells to hypotonic solutions significantly increased current density, an effect which was reversed by hypertonic superfusion. Taken together, results with PKC translocation inhibitory peptides are consistent with those with different PKC inhibitors and further confirmed that ePKC, a member of the nPKC family, plays a crucial role in cell volume regulation of native I_{Cl,vol} in PASMC.

Alterations in cellular distribution of ePKC in response to cell swelling. Although several different PKC isoforms have been shown to translocate in response to agonist stimulation in canine PASMC (4), the effects of changes in cell volume on PKC isoform cellular distribution have not been previously tested. We performed immunohistochemistry on cultured canine PASMC to examine ePKC-like immunoreactivity in cells exposed to isotonic extra-cellular solutions and after hypertonic cell swelling. Before performing these experiments, we first confirmed that dialyzing cultured PASMC with the εV1–2 peptide produced similar effects on I_{Cl,vol} as previously observed in acutely dispersed PASMC (Fig. 7). Figure 8A illustrates the typical pattern of immunoreactivity of a canine PASMC under isotonic conditions. ePKC-like immunoreactivity showed a diffuse, uniform cytosolic and membrane disposition. This is illustrated in Fig. 8B in the plot of pixel intensity over the region of interest illustrated (rectangular box) in Fig. 8A. In contrast, there appeared to be a consistent decrease in near-membrane immunoreactivity (horizontal dashed lines) and a marked increase in cytoplasmic and perinuclear ePKC-like immunoreactivity in cells exposed to hypotonic extra-cellular solutions (Fig. 8D). ePKC-like immunoreactivity in regions near the sarcolemmal membrane were significantly lower in cells exposed to hypotonic solutions compared with similar regions in cells exposed to isotonic solutions. There also was a significant increase in ePKC-like immunoreactivity in perinuclear regions in cells exposed to hypotonic solutions compared with similar regions in cells exposed to isotonic solutions. These data suggest that hypotonic cell swelling is associated with a translocation of ePKC from the vicinity of the sarcolemmal membrane to the cytoplasm and perinuclear regions.

Figure 4. Ro-31 8425 activates I_{Cl,vol} in isotonic solutions in acutely dispersed PASMC and prevents further volume regulation of the current. A: time course of currents measured at +80 mV (●) followed by −80 mV (○) from a cell under different experimental conditions as indicated by horizontal bars. B: average current density from a group of cells tested under different experimental conditions. Values represent means ± SE (n = 3). *Significantly different from the values under isotonic conditions (P < 0.05).

Figure 5 shows the effect of εV1–2 on native I_{Cl,vol} in PASMC. Cells were dialyzed with εV1–2 (10 μM) under isotonic conditions and subsequently exposed to hypotonic and then hypertonic solutions. Dialyzing the cells with εV1–2 significantly increased I_{Cl,vol} under isotonic conditions and abolished further regulation by either hypotonic cell swelling or hypertonic cell shrinkage (Fig. 5, A and B). The mean current densities in cells dialyzed with εV1–2 were increased 239 ± 16% at +80 mV and 100 ± 8% at −80 mV under isotonic conditions, and the current densities measured at 10 min of intracellular dialysis in isotonic solutions were not significantly different from the values after superfusion with hypotonic or hypertonic solution. On the other hand, in cells dialyzed with the scrambled εV1–2 peptide (10 μM), current amplitudes were unchanged under isotonic conditions. In addition, cells dialyzed with scrambled εV1–2 peptide responded in the usual manner to hypotonic and hypertonic solutions (Fig. 5, C and D).
DISCUSSION

For $I_{\text{Cl,vol}}$ in native cardiac myocytes, *Xenopus* oocytes, and recombinant ClC-3 Cl$^{-}$/H11002 channels expressed in NIH 3T3 cells, channels can be activated under isotonic conditions by exposure of cells to 100 nM BIM, a highly selective inhibitor of cPKCs (20), whereas stimulation of endogenous PKC activity by phorbol esters strongly inhibits channel activity (5, 32). These data suggest that $I_{\text{Cl,vol}}$ in native cardiac myocytes, *Xenopus* oocytes, and recombinant ClC-3 Cl$^{-}$/H11002 channels expressed in NIH 3T3 cells are regulated primarily by cPKCs, in a manner consistent with a model recently proposed which links changes in cell volume to changes in protein kinase-dependent phosphorylation of the channel (18). Translocation of cPKC away from the channel during cell swelling would allow the phosphorylation/dephosphorylation equilibrium of the channel to favor dephosphorylation and channel opening, whereas translocation of cPKC toward the vicinity of the channel during cell shrinkage would allow the phosphorylation/dephosphorylation equilibrium of the channel to favor phosphorylation and channel closure. This equilibrium would also be expected to be strongly influenced by the activity of protein phosphatases, but
the possible influence of cell volume changes on phosphatase activity or distribution remains to be determined. Because $I_{\text{Cl,vol}}$ in canine PASMC is also strongly inhibited by phorbol esters (Fig. 1), it appears that the same general model might account for cell volume-induced changes in native $I_{\text{Cl,vol}}$ in PASMC as well. However, the observed inability of BIM exposure to activate $I_{\text{Cl,vol}}$ under isotonic conditions in these cells suggests the possibility that different PKC isoforms may be responsible for regulation of $I_{\text{Cl,vol}}$ in PASMC.

The possible role of nPKCs in the regulation of $I_{\text{Cl,vol}}$ in PASMC was tested using two PKC-specific inhibitors, calphostin C and the compound Ro-318425, and two PKC isozyme-specific translocation inhibitory peptides, $\epsilon V1-2$ and $\beta C2-2$. Inhibition of endogenous PKC activity by either calphostin C or Ro-318425 caused activation of native $I_{\text{Cl,vol}}$ in PASMC under isotonic conditions and prevented further modulation of $I_{\text{Cl,vol}}$ by anisotonic-induced changes in cell volume. A similar effect was observed after intracellular dialysis of the $\epsilon V1-2$ peptide, but not with dialysis of the $\beta C2-2$ peptide. These results are consistent with involvement of nPKC (most likely ePKC), not cPKC isoforms, in the regulation of $I_{\text{Cl,vol}}$ in canine PASMC. Thus dialysis with the inhibitory peptide $\epsilon V1-2$ may displace endogenous PKC from its anchoring protein, thus promoting dephosphorylation and channel activation. Indeed, immunohistochemistry of the subcellular distribution of PKC isoforms in canine PASMC provided evidence that hypotonic cell swelling is accompanied by translocation of ePKC away from the membrane to cytoplasmic and perinuclear locations.

Fig. 7. $\epsilon V1-2$ peptide dialysis activates $I_{\text{Cl,vol}}$ in isotonic solutions in cultured canine PASMC and prevents further volume regulation of the current. A–D, same as in Fig. 5. Values in B ($n = 4$) and D ($n = 4$) represent means ± SE. *Significantly different from the values under isotonic conditions ($P < 0.05$).
thus favoring dephosphorylation and channel activation. Such interactions between PKC and F-actin or caveolin may also play a role in the ability of some cellular enzymes and second messengers, known to be altered during cell volume regulation, to effect changes in $I_{\text{Cl,vol}}$, because PKC isoforms are believed to represent upstream effectors for several of these pathways as well.

Despite cell-specific differences in PKC isoform regulation of native $I_{\text{Cl,vol}}$ in canine PASMC compared with native $I_{\text{Cl,vol}}$ in cardiac cells and Xenopus oocytes, and recombinant CIC-3 channels expressed in NIH 3T3 cells (5), these currents all exhibit a similar general sensitivity to regulation by PKC activation and inhibition. In contrast, native $I_{\text{Cl,vol}}$ in rabbit portal vein smooth muscle cells exhibits the opposite regulation by PKC, in that currents are stimulated by activation of endogenous PKC (10, 42). Diversity in the properties of native VSOACs across different tissues and cell types is well established (27, 34), and future experiments are required to determine whether differences in PKC regulation reflect different volume-sensitive intracellular signaling pathways or different molecular entities responsible for VSOACs in different cell types. The existence of subtypes of VSOACs may explain the apparent unaltered properties of native VSOACs in two cell types examined (pancreatic cells and hepatocytes) from mice with disrupted CIC-3 (Clcn$^{-/-}$ knockout) (33).
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Present address of J. Zhong: Dept. of Anatomy, Physiology, and Pharmacology, Auburn University College of Veterinary Medicine, Auburn, AL 36849.

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