Hypotonic swelling stimulates L-type Ca\(^{2+}\) channel activity in vascular smooth muscle cells through PKC

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Ding, Yanfeng, Dean Schwartz, Philip Posner, and Juming Zhong. Hypotonic swelling stimulates L-type Ca\(^{2+}\) channel activity in vascular smooth muscle cells through PKC. Am J Physiol Cell Physiol 287: C413–C421, 2004. First published April 7, 2004; 10.1152/ajpcell.00537.2003.—It has been suggested that L-type Ca\(^{2+}\) channels play an important role in cell swelling-induced vasoconstriction. However, there is no direct evidence that Ca\(^{2+}\) channels in vascular smooth muscle are modulated by cell swelling. We tested the hypothesis that L-type Ca\(^{2+}\) channels in rabbit portal vein myocytes are modulated by hypotonic cell swelling via protein kinase activation. Ba\(^{2+}\) currents (I\(_{\text{Ba}}\)) through L-type Ca\(^{2+}\) channels were recorded in smooth muscle cells freshly isolated from rabbit portal vein with the conventional whole cell patch-clamp technique. Superfusion of cells with hypotonic solution reversibly enhanced Ca\(^{2+}\) channel activity, but did not alter the voltage-dependent characteristics of Ca\(^{2+}\) channels. Bath application of selective inhibitors of protein kinase C (PKC), Ro-31–8245 or Go-6983, prevented I\(_{\text{Ba}}\) enhancement by hypotonic swelling, whereas the specific protein kinase A (PKA) inhibitor KT-5720 had no effect. Bath application of phorbol 12,13-dibutyrate (PDBu) significantly increased I\(_{\text{Ba}}\) under isotonic conditions and prevented current stimulation by hypotonic swelling. However, PDBu did not have any effect on I\(_{\text{Ba}}\) when cells were first exposed to hypotonic solution. Furthermore, downregulation of endogenous PKC by overnight treatment of cells with PDBu prevented current enhancement by hypotonic swelling. These data suggest that hypotonic cell swelling can enhance Ca\(^{2+}\) channel activity in rabbit portal vein smooth muscle cells through activation of PKC.

The cellular and molecular mechanisms that link changes in cell volume to the activation of different ion channels remain of great interest. In guinea pig cardiac myocytes and canine pulmonary arterial smooth muscle cells, cell swelling-induced activation of Cl\(^{-}\) channels is thought to result from inhibition of protein kinase C (PKC) or enhancement of phosphatase. In rabbit pulmonary arterial myocytes, activation of Ca\(^{2+}\)-activated K\(^{+}\) channels appears to occur by a direct effect of stretch on the channels. Modulation of L-type Ca\(^{2+}\) channels in rabbit cardiac cells by osmotic cell swelling and by cell swelling has been reported to elicit vasoconstriction, which was blunted by a reduction of extracellular Ca\(^{2+}\) concentration. However, until now there has been no direct measurement of Ca\(^{2+}\) channel activity in these cells exposed to a hypotonic condition.

The purpose of the present study was to delineate the mechanism underlying the effect of hypotonic cell swelling on L-type Ca\(^{2+}\) channels in rabbit portal vein smooth muscle cells. Using the conventional whole cell technique and various protein kinase activators and inhibitors, we have demonstrated that exposure of cells to a hypotonic bath solution significantly increases L-type Ca\(^{2+}\) channel activity. Furthermore, PKC appears to play an important role in the hypotonic swelling-induced activation of these channels.

MATERIALS AND METHODS

Isolation of rabbit portal vein smooth muscle cells. Myocyte isolation was performed as previously reported. Male albino rabbits (2.0–3.0 kg) were killed with an intravenous overdose of pentobarbital sodium (50 mg/kg), and the portal vein was rapidly removed and cleaned of connective tissue in ice-cold Krebs solution (in mM: 125 NaCl, 4.2 KCl, 1.2 MgCl\(_2\), 1.8 CaCl\(_2\), 11 glucose, 1.2 K\(_2\)HPO\(_4\), 23.8 NaHCO\(_3\), and 11 HEPES; pH 7.4 with NaOH). The portal vein was then cut into small segments and preincubated for 60 min in a shaking water bath at 35°C in a dispersion solution (enzyme free; in mM: 90 NaCl, 1.2 MgCl\(_2\), 1.2 K\(_2\)HPO\(_4\), 20 glucose, 50 taurine, and 5 HEPES; pH 7.2 with NaOH). The segments were then incubated in the dispersion solution containing 2 mg/ml collagenase.
type I (Sigma, St. Louis, MO), 0.5 mg/ml protease type XXVII (Sigma), and 2 mg/ml bovine serum albumin for 10–14 min at 35°C. After the digestion period, the segments were rinsed with enzyme-free dispersion solution, and cells were separated by gentle trituration with a wide-tipped fire-polished Pasteur pipette. After dispersion, cells were stored in enzyme-free dispersion solution containing 100 μM CaCl₂ at 4°C. Cells were used within 10 h except in the experiments with overnight phorbol 12,13-dibutyrate (PDBu) treatment. The animal use protocol was approved by the Institutional Animal Use and Care Committee at Auburn University.

Electrophysiology. Inward Ba²⁺ current (I₉₆) in vascular myocytes was measured with the whole cell patch-clamp technique at room temperature (40°C). A drop of cell suspension was added to a small recording chamber mounted on the stage of an inverted microscope (Nikon TS-100). Cells in the chamber were superfused by gravity at a constant rate (~1–2 ml/min). Patch electrodes were made from borosilicate glass pulled with a micropipette puller (PP-830, Narishige) and fire-polished with a microforge (MF-830, Narishige). Pipette resistance was 3–5 MΩ when filled with the appropriate solution. After the whole cell configuration was established, membrane capacitance and series resistance were recorded with a 20-mV hyperpolarization potential and were partially compensated. Inward current was elicited by stepping voltage from the holding potential of ~70 mV to 0 mV at 30-s intervals with an Axopatch 200B patch-clamp amplifier and pCLAMP 8 (Axon Instruments). The leak currents at both isotonic and hypotonic states were not subtracted. The standard isotonic bath solution (~290 mosmol/kgH₂O) used to record inward I₉₆ in portal vein cells was composed of (in mM) 80 NaCl, 10 tetraethylammonium chloride (TEA-Cl), 5 BaCl₂, 0.5 MgCl₂, 5.5 glucose, 5 CsCl, 10 HEPES, and 70 d-mannitol, pH 7.40 with NaOH. Both TEA-Cl and CsCl were used to block K⁺ currents. The standard hypotonic solution was made from the isotonic bath solution by removing d-mannitol (~220 mosmol/kgH₂O). The standard hyperosmotic bath solution was made from the isotonic solution by adding 70 mM d-mannitol (total d-mannitol 140 mM, ~360 mosmol/kgH₂O). The pipette solution contained (in mM) 80 CsCl, 20 TEA-Cl, 5 glucose, 2 MgCl₂, 5 ATP, 1 GTP, 5 EGTA, and 80 d-mannitol, pH 7.2 with CsOH (40).

PKC-ε mRNA expression. RT-PCR was used to evaluate the expression of PKC-ε mRNA. Total RNA was isolated by a monophasic solution of phenol and guanidine isothiocyanate with TRIzol according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). Rabbit portal vein smooth muscle rings were homogenized in TRIzol. Phase separation was carried out with chloroform (0.2 ml chloroform/ml TRIzol) and centrifuged at 12,000 g at 4°C. Cells were used within 10 h except in the experiments with overnight phorbol 12,13-dibutyrate (PDBu) treatment. The animal use protocol was approved by the Institutional Animal Use and Care Committee at Auburn University.

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One-step RT-PCR was performed with the cMaster RTplus PCR System (Eppendorf, Westbury, NY). Total RNA (0.5 μg) was incubated with RTplus PCR buffer containing Mg²⁺ (2.5 mM), dNTPs (200 μM each), cMaster RT enzyme (0.15 U/μl), cMaster PCR enzyme (0.05 U/μl) and PKC-ε forward and reverse primers (400 nM) in a total volume of 20 μl. The PCR primer sequences were PKC-ε (forward) 5’-GGCTCTGGCGCCGAAACACCCTTAT-3’ and PKC-ε (reverse) 5’-GATGCTGCTGGACGCCCTCCTT-3’. Primers were derived from the human PKC-ε gene (GenBank accession no. NM005400) and produced an amplification product of 440 bp. Control reactions were performed in the absence of RT. RT-PCR products were carried out in a Bio-Rad iCycler. The RT step was performed at 50°C for 45 min. The PCR reaction was carried out at 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, and 68°C for 45 s. Primer extension was carried out at 68°C for 3 min. RT-PCR amplification products were analyzed on a 1.2% agarose gel and stained with ethidium bromide. The gels were visualized by ultraviolet light and photographed with a Bio-Rad Fluor S Multilimage. To confirm the identity of the PCR product, the band was cut out and eluted with the PerfectPrep Gel Cleanup Kit (Eppendorf) and TA cloned into pCR2.1 with the Original TA Cloning Kit (Invitrogen, Carlsbad, CA). One Shot chemically competent cells (INVOTET; Invitrogen) were transformed, and plasmid DNA was isolated for sequencing. Sequencing was performed at the Auburn University Genomic and Sequencing Laboratory.

Drugs and reagents. PDBu, Ro-31-8425, Go-6983, and KT-5720 were purchased from Calbiochem (La Jolla, CA). Isoproterenol, nicardipine, niflumic acid, and DIDS were purchased from Sigma. Those drugs not soluble in water were first dissolved in dimethyl sulfoxide (DMSO) and then further diluted in the appropriate solution with the final concentration of DMSO <0.2%. DMSO alone at 0.2% had no effect on Ca²⁺ currents. The PKC translocation inhibitory peptides βC2-4 (SLNPEWNET, corresponding to residues 218–226 of PKCθ, εV1-2 (EAVSLKPT, corresponding to residues 14–21 of PKC-ε), and scrambled εV1-2 (LSETKPAV) were purchased from Calbiochem.

Statistical analysis. Values are reported as means ± SE, and n is the number of cells studied. Single-point data between control and treated cells were compared with the two-tailed unpaired Student’s t-test. Comparisons between multiple groups were done with a two-way ANOVA with a Student-Newman-Kuels posttest. P values <0.05 were considered significantly different.

![Fig. 1. Hypotonic swelling increased Ba²⁺ current. A: time course of peak current measurements from a cell under isotonic and hypotonic conditions. Currents were recorded every 30 s when the membrane potential was stepped to 0 mV from a holding potential of ~70 mV. Inset, representative current traces recorded at 5 (1), 10 (2), and 20 (3) min under different conditions. B: % increase of peak Ba²⁺ currents under various hypotonic conditions. Cells were first superfused with isotonic bath solution, followed by gradual reduction in osmolarity of bath solutions (n = 8).](http://ajpcell.physiology.org/content/287/3/C414/F1)

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RESULTS

Hypotonic superfusion increased inward currents through L-type Ca\(^{2+}\) channels. To test the effect of osmolarity change on L-type Ca\(^{2+}\) channels in vascular smooth muscle cells, rabbit portal vein myocytes were first superfused with isotonic solution (~290 mosmol/kgH\(_2\)O) while \(I_{Ba}\) was recorded continuously. When steady-state current amplitude was obtained under isotonic conditions (~3 min), the superfusate was switched to the standard hypotonic solution (~220 mosmol/kgH\(_2\)O). Exposure of cells to the hypotonic solution did not significantly change the access resistance of the pipettes (isotonic 4.9 ± 0.3 mΩ, hypotonic 5.2 ± 0.3 mΩ; \(P = 0.56, n = 18\)). However, exposure to the hypotonic solution increased cell size within 3 min as assessed roughly by measuring the two-dimensional nominal cell length and width under the microscope. The mean length and width of cells were 131 ± 7 and 7.9 ± 0.4 μm, respectively, under isotonic conditions and 146 ± 9 and 9.3 ± 0.7 μm, respectively, after superfusion with hypotonic solution (\(n = 6\)). Concomitant with the cell swelling, \(I_{Ba}\) was increased in all cells examined. As shown in Fig. 1A, switching the superfusate from an isotonic solution to the standard hypotonic solution significantly increased \(I_{Ba}\) within 5 min (34 ± 7%, \(n = 20\)), which could be reversed by switching the superfusate back to the isotonic solution. The increase of \(I_{Ba}\) was closely proportional to the degree of reduction in osmolarity. Figure 1B demonstrates the gradual increase of peak \(I_{Ba}\) in response to a proportional reduction of osmolarity. The degree of hyposmolarity was adjusted by adding various amounts of d-mannitol into the standard hypotonic solution.

To evaluate whether the currents recorded under our experimental conditions were through L-type Ca\(^{2+}\) channels in rabbit portal vein smooth muscle cells, we tested the effect of the L-type Ca\(^{2+}\) channel blocker nicardipine (Nic) on the inward currents recorded under isotonic or hypotonic conditions. Nic (10 μM) completely abolished the inward current when applied in the isotonic bath solution and further prevented any detectable change of the current when cells were exposed to the hypotonic solution (Fig. 2A; \(n = 7\)). Furthermore, when cells were first exposed to hypotonic solution, Nic also completely abolished the increased inward current (Fig. 2B; \(n = 6\)).

In rabbit portal vein smooth muscle cells, a volume-regulated Cl\(^{-}\) current (\(I_{Cl-swell}\)) has been identified that is completely eliminated by either DIDS or niflumic acid (13). To prevent possible contamination of \(I_{Ba}\) by \(I_{Cl-swell}\) in our experiments, we set the test potential at 0 mV, which is the theoretical equilibrium potential for \(I_{Cl-swell}\) under our experimental conditions (13). In addition, we tested the effects of DIDS and niflumic acid on the currents recorded under our experimental conditions. Nicardipine (Nic) completely abolished, whereas volume-regulated Cl\(^{-}\) current (\(I_{Cl-swell}\)) inhibitors had no effect on, currents under either isotonic or hypotonic conditions. A: time course of peak current measurements from a cell in the presence of Nic (10 μM). Nic was applied before cell exposure to hypotonic solution, as indicated by the horizontal lines. B: time course of peak current measurements from a cell in the presence of Nic (10 μM). Nic was applied after cell exposure to hypotonic solution, as indicated by the horizontal lines. C: time course of peak current measurements from a cell in the presence of DIDS (100 μM). DIDS was added to the bath solutions as indicated by the horizontal lines. D: time course of peak current measurements from a cell in the presence of niflumic acid (NFA, 100 μM). NFA was added into the bath solutions as indicated by the horizontal lines.

Fig. 2. Nicardipine (Nic) completely abolished, whereas volume-regulated Cl\(^{-}\) current (\(I_{Cl-swell}\)) inhibitors had no effect on, currents under either isotonic or hypotonic conditions. A: time course of peak current measurements from a cell in the presence of Nic (10 μM). Nic was applied before cell exposure to hypotonic solution, as indicated by the horizontal lines. B: time course of peak current measurements from a cell in the presence of Nic (10 μM). Nic was applied after cell exposure to hypotonic solution, as indicated by the horizontal lines. C: time course of peak current measurements from a cell in the presence of DIDS (100 μM). DIDS was added to the bath solutions as indicated by the horizontal lines. D: time course of peak current measurements from a cell in the presence of niflumic acid (NFA, 100 μM). NFA was added into the bath solutions as indicated by the horizontal lines.

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conditions. Bath application of DIDS (100 μM) or niflumic acid (100 μM) had no detectable effect on either basal currents under isotonic conditions or current enhanced by hypotonic swelling. Peak inward currents were elevated 34 ± 5% by hypotonic swelling in the presence of DIDS (Fig. 2C; n = 8) and 33 ± 4% in the presence of niflumic acid (Fig. 2D; n = 11). Thus 100 μM DIDS was routinely added into the bath solutions in subsequent experiments.

To examine the effect of hypotonic cell swelling on the voltage-dependent characteristics of L-type Ca2+ channels, we compared the current-voltage (I-V) relationships and the steady-state inactivation under isotonic and hypotonic conditions. The I-V relationship was measured when the test membrane potentials were stepped between −60 and +60 mV by increments of 10 mV from the holding potential of −70 mV in the presence of either DIDS or niflumic acid. As shown in Fig. 3A, peak Iba was significantly higher at test potentials between −40 and +30 mV after hypotonic exposure in the presence of DIDS. The test potentials for threshold current and the maximal peak current were also shifted toward the left after hypotonic exposure in the presence of DIDS. Similarly, exposure of cells to hypotonic solution significantly increased the peak currents at the test potentials between −30 and +30 mV in the presence of niflumic acid. However, hypotonic exposure did not change the voltage-dependent patterns of the current in this group of experiments (Fig. 3B). The left shift of the I-V relationship by hypotonic exposure in the first set of experiments suggested possible contamination of Iba by ICl-swell at the negative test potentials in the presence of DIDS, as DIDS is more potent to block ICl-swell at positive membrane potentials (13). On the other hand, niflumic acid has been reported to be equally potent in blocking ICl-swell at all membrane potentials (13). Our data suggest that hypotonic exposure did not change the shape of the I-V relationship, whereas it significantly enhanced Iba at most test potentials.

The effect of hypotonic cell swelling on steady-state inactivation was tested with a two-pulse protocol (40). The resting membrane potential was held at −70 mV. The conditioning prepulses ranged from −60 to +40 mV in 10-mV increments and were applied for 500 ms. The test pulses were stepped to 0 mV for 200 ms. The two pulses were separated by an interpulse resting interval of 5 ms. The relative availability of peak Iba was calculated by dividing the peak Iba by the peak Iba,max (peak Iba/peak Iba,max), where the peak Iba is the peak Iba measured at the test pulse after different prepulses and the peak Iba,max is the peak Iba measured at the test pulse with a prepulse of −60 mV. Increasing the potential of the conditioning prepulse reduced Iba elicited by the following test pulse in cells. However, hypotonic swelling did not affect the relative availability of peak Iba under different prepulse potential conditions (Fig. 3C). These data suggest that although hypotonic cell swelling significantly increased the activity of Ca2+ channels it did not change the voltage-dependent characteristics of these channels.

PKC plays an important role in hypotonic swelling-induced activation of Ca2+ channels. In vascular smooth muscle cells, both PKA and PKC are able to stimulate L-type Ca2+ channels, whereas protein kinase G (PKG) has an inhibitory effect (for review, see Ref. 16). To examine the mechanism underlying the hypotonic swelling-induced stimulation of Ca2+ channel activity in vascular smooth muscle cells, we first tested whether PKA plays a role in the signal transduction pathway. Cells were treated with a specific PKA inhibitor, KT-5720, before and during exposure to hypotonic solution. A previous report (41) indicated that KT-5720 has no detectable effect on basal Ca2+ channel currents but eliminated the stimulatory effect of PKA in rabbit portal vein smooth muscle cells. Figure 4A indicates that when the maximal concentration needed to inhibit PKA activity KT-5720 (200 nM) did not prevent the increase of Iba induced by hypotonic exposure. The increases in Iba induced by hypotonic exposure were 31 ± 3% and 29 ± 4%, respectively, in the absence (n = 16) and presence (n = 11) of KT-5720. In another group of experiments, effects of the
β-adrenergic receptor agonist isoproterenol (Iso) on $I_{Ba}$ were examined after cell exposure to hypotonic solution. As shown in Fig. 4B, exposure to hypotonic solution increased peak $I_{Ba}$ by 30%. Application of Iso (1 μM) under hypotonic conditions further increased peak $I_{Ba}$ by 30%. Peak $I_{Ba}$ in response to hypotonic exposure plus Iso was 158 ± 7% of that under isotonic conditions without Iso treatment ($n = 8$). When these experiments were repeated in the presence of KT-5720, exposure of cells to hypotonic solution significantly increased peak $I_{Ba}$. However, the stimulatory effect of Iso on $I_{Ba}$ under hypotonic condition was completely prevented (Fig. 4C; $n = 7$). These data suggest that PKA is not involved in the cell swelling-induced increase of $I_{Ba}$.

To evaluate the involvement of PKC in the stimulation of vascular $Ca^{2+}$ channels by hypotonic swelling, we treated cells with specific PKC inhibitors before and during exposure to hypotonic bath solution. Application of the PKC inhibitor Go-6983 (200 nM) did not have any detectable effect on $I_{Ba}$ under isotonic conditions. However, Go-6983 prevented the hypotonic swelling-induced increase of $I_{Ba}$ without affecting the cell size change. Cell length and width were 126 ± 5 and 7.6 ± 0.4 μm, respectively, under isotonic conditions and 144 ± 8 μm and 9.4 ± 0.8 μm, respectively, under hypotonic conditions in the presence of Go-6983 ($n = 5$). Similarly, the structurally different PKC inhibitor Ro-31-8425 (200 nM) also blocked the hypotonic swelling-induced increase of $I_{Ba}$. Figure 5A depicts representative recordings of peak $I_{Ba}$ in the presence or absence of Ro-31-8425 before and after cell exposure to hypotonic solution. Figure 5B demonstrates the effects of different PKC inhibitors on the hypotonic swelling-induced increase in $I_{Ba}$.
In rabbit portal vein smooth muscle cells, three isoforms of PKC, including PKC-\(\alpha\)/H9251, -\(\beta\)/H9280, and -\(\gamma\)/H9264, have been reported (3). To determine which isoform of PKC is involved in the activation of Ca\(^{2+}\)/H11001 channels after hypotonic swelling, we tested the effects of selective PKC isozyme inhibitor peptides on the hypotonic swelling-induced increase of Ca\(^{2+}\) channel current. Cells were dialyzed with \(\alpha\)/V1-2, \(\alpha\)/V1-2S, or \(\alpha\)/C2-4 before exposure to hypotonic solution. \(\alpha\)/V1-2 is a short peptide derived from the V1 region of PKC-\(\alpha\) that inhibits translocation of PKC-\(\alpha\). \(\alpha\)/V1-2S is a scrambled peptide of \(\alpha\)/V1-2 and served as a negative control. \(\alpha\)/C2-4 is a short peptide derived from the C2 domain of PKC-\(\alpha\) and inhibits translocation of PKC-\(\alpha\) and -\(\beta\) (24, 42). Dialyzing cells with either \(\alpha\)/V1-2S or \(\alpha\)/C2-4 had no effect on hypotonic swelling-induced activation of Ca\(^{2+}\) channels. On the other hand, dialyzing cells with \(\alpha\)/V1-2 prevented the current increase in response to exposure to hypotonic solution (Fig. 5B). Furthermore, RT-PCR analysis identified the expression of PKC-\(\alpha\) mRNA in portal vein smooth muscle cells (Fig. 5C). The cloned DNA fragment showed 90% and 88% similarity, respectively, with human and mouse PKC-\(\alpha\).

Involvement of PKC in the hypotonic swelling-induced stimulation of \(I_{\text{Ba}}\) was further evaluated with the PKC activator PDBu (200 nM). Application of PDBu under isotonic conditions significantly increased \(I_{\text{Ba}}\), which reached a steady state in ~5 min. Switching the superfusate from isotonic to hypotonic solution in the presence of PDBu did not further change the amplitude of inward current (Fig. 6, A and B). In another set of experiments, cells were treated with PDBu after hypotonic exposure. Again, peak \(I_{\text{Ba}}\) was significantly increased when cells were exposed to hypotonic solution. Application of PDBu under hypotonic conditions did not further increase Ca\(^{2+}\) channel activity (Fig. 6, C and D).

Long-term exposure of cells to phorbol esters is a common method used to downregulate endogenous PKC activity and an alternative method to test possible involvement of PKC in a signaling pathway (31, 41). We also used this method to further confirm the involvement of PKC in hypotonic swelling-induced stimulation of Ca\(^{2+}\) channels in vascular smooth muscle cells. Cells were pretreated with phorbol ester (PDBu, 200 nM) or its vehicle (time-matched control) and stored at 4°C in the enzyme-free dispersion solution (100 \(\mu\)M CaCl\(_2\)) for \(\geq 18\) h before \(I_{\text{Ba}}\) recording. Pretreatment of cells with PDBu did not have any significant effect on the basal currents. Peak \(I_{\text{Ba}}\) under isotonic conditions in cells pretreated with PDBu (-201 ± 12 pA; \(n = 7\)) was comparable to that in time-matched control

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Fig. 6. Hypotonic swelling prevented phorbol 12,13-dibutyrate (PDBu) stimulation of \(I_{\text{Ba}}\). A: peak currents measured from a cell treated with PDBu before and after exposure to hypotonic bath solution as indicated by the horizontal lines. B: averaged % increase of peak \(I_{\text{Ba}}\) by PDBu before and after hypotonic swelling (\(n = 9\)). Values are means ± SE. C: peak currents measured from a cell treated with PDBu after exposure to hypotonic bath solution. The cell was superfused with isotonic solution followed with hypotonic bath solution as indicated by the horizontal lines. PDBu was added into the hypotonic bath solution when peak \(I_{\text{Ba}}\) reached a steady state. D: averaged % increase of peak \(I_{\text{Ba}}\) by hypotonic swelling with or without PDBu (\(n = 15\)). Values are means ± SE.

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cells ($-196 \pm 13 \text{ pA}; n = 6$). Exposure of time-matched control cells to hypotonic solution increased $I_{Ba}$ (Fig. 7). On the other hand, overnight pretreatment of cells with PDBu eliminated the $I_{Ba}$ response to cell exposure to hypotonic solution (Fig. 7) as well as the $I_{Ba}$ response to an acute application of PMA (200 nM) under isotonic conditions ($n = 8$; data not shown). These data further confirmed that PKC plays an important role in the activation of Ca$^{2+}$ channels by hypotonic cell swelling.

**DISCUSSION**

Activation of ion channels by hypotonic cell swelling has been reported in a wide range of cell types. However, whether and how hypotonic cell swelling affects L-type Ca$^{2+}$ channel activity in vascular smooth muscle cells is not well understood. The present study demonstrates that in rabbit portal vein smooth muscle cells L-type Ca$^{2+}$ channels are stimulated by hypotonic cell swelling through a PKC-dependent pathway. In the present study, contamination of recorded currents by K$^+$ channels was eliminated by using Cs$^+$ and TEA-Cl in bath and pipette solutions, and the possible activation of $I_{Cl-swell}$ was prevented with DIDS. In addition, the specific L-type Ca$^{2+}$ channel blocker Nic completely abolished the inward currents recorded in our experiments and prevented the further increase of current induced by hypotonic superfusion. These data indicate that hypotonic cell swelling activates an inward current through L-type Ca$^{2+}$ channels. Whether the increased channel activity induced by hypotonic swelling is associated with an increase in number of functional channels, channel opening probability, or channel conductance was not tested in the present study and deserves further evaluation.

Hypotonicity of the extracellular environment occurs in several situations including reduced extracellular Na$^+$ concentration seen in normal pregnancy (11), overhydration following intense exercise (1, 12), and treatment with citalopram (15). Other hyposmotic states are found in situations involving deficits in plasma proteins secondary to hepatic dysfunction and nutritional deficits. L-type Ca$^{2+}$ channels play a central role in the excitation-contraction coupling in vascular smooth muscle cells, and it has been suggested that activation of L-type Ca$^{2+}$ channels is responsible for the vasoconstriction induced by osmotic swelling (2). Ca$^{2+}$ channels are also activated by membrane depolarization through volume-regulated Cl$^-$ channels or stretch-activated nonselective cation channels (4, 19, 23, 26, 37). Our data demonstrated a proportional increase in peak $I_{Ba}$ in response to gradual reductions in osmolality. These data are consistent with the reports that graded decreases of extracellular osmolarity lead to a proportional increase in the tension of guinea pig aortic strips (19) and rat portal vein rings (2). Our data are also consistent with other reports demonstrating that L-type Ca$^{2+}$ channels in vascular smooth muscle cells are stimulated by inflating cells with positive pressure through a pipette electrode (4, 20, 23). Thus activation of Ca$^{2+}$ channels may play an important role in the myogenic response under physiological and pathophysiological conditions. Although the 25% reduction of extracellular osmolarity used in this study may not occur under normal physiological conditions, it has been used as a common experimental procedure for the study of volume-regulated channels by many research groups (5, 13, 21, 37, 42).

Previous studies on various volume-regulated anion channels in different cell types demonstrated that PKA activation plays an important role in the cell volume change-induced modulation of these channels (14, 6, 10, 34). In the present study, we evaluated the possible involvement of PKA in the modulation of L-type Ca$^{2+}$ channels in vascular smooth muscle cells by hypotonic cell swelling. Exposure of cells to hypotonic bath solution did not prevent, but rather blunted, the stimulatory effects of Iso on $I_{Ba}$. In addition, pretreatment of cells with the PKA inhibitor KT-5720 did not prevent the increase in $I_{Ba}$ induced by hypotonic swelling but prevented further increase of $I_{Ba}$ by Iso under hypotonic conditions. These data are consistent with previous reports that Iso induces a >50% increase in Ca$^{2+}$ channel currents in both rabbit and rat portal vein smooth muscle cells through activation of both PKA and PKC (36, 41). Thus it is unlikely that hypotonic swelling stimulates Ca$^{2+}$ channel activity through activation of PKA in vascular smooth muscle cells.

Modulation of volume-regulated ion channels by PKC has also been reported. For example, PKC inhibitors enhanced the basal $I_{Cl-swell}$ under isotonic conditions and further abolished the swelling-induced activation of $I_{Cl-swell}$ in canine pulmonary artery smooth muscle cells (42) and guinea pig cardiac myocytes (8). In contrast, phorbol esters dose-dependently increased the amplitude of $I_{Cl-swell}$ in canine atrial myocytes (7). More pertinent to our study is a recent report demonstrating that in rabbit portal vein smooth muscle cells phorbol esters increased, whereas PKC inhibitors decreased, the amplitude of $I_{Cl-swell}$ (10). Thus hypotonic swelling may activate $I_{Cl-swell}$

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**Fig. 7.** Overnight pretreatment with PDBu prevented hypotonic swelling-induced stimulation of Ca$^{2+}$ channels. A: peak currents measured from a cell pretreated with 200 nM PDBu for >18 h and a time-matched control cell. The cell was exposed to isotonic and hypotonic solutions as indicated by the horizontal lines. B: averaged % increase after hypotonic exposure. Values are means ± SE for PDBu-pretreated cells ($n = 7$) and time-matched control cells ($n = 6$). *Significantly different from values under isotonic conditions ($P < 0.05$).
through activation (7, 10) or inhibition (8, 42) of PKC in different cells. The discrepancy in the PKC-dependent modulation of \( I_{\text{Cl,swell}} \) might be related to species variation. In the present study, stimulation of endogenous PKC by PDBu strongly increased peak \( I_{\text{Na}} \) under isotonc conditions and prevented further current enhancement by hypotonic swelling, whereas PKC inhibitors completely abolished \( I_{\text{Na}} \) stimulation by hypotonic cell swelling. Long-term treatment of cells with PDBu to downregulate endogenous PKC activity also abolished \( I_{\text{Na}} \) stimulation induced by hypotonic cell swelling. These data strongly suggest that a hypotonic-induced cell volume change can stimulate L-type \( Ca^{2+} \) channels by activating PKC in rabbit portal vein cells. Furthermore, our results demonstrated that the selective inhibitory peptide against PKC-\( \epsilon \), \( \epsilon V1-2 \), was able to prevent the activation of \( Ca^{2+} \) channels by hypotonic swelling, whereas neither the scrambled \( \epsilon V1-2 \) nor the selective conventional PKC inhibitor \( \epsilon C2-4 \) was effective. Thus PKC-\( \epsilon \) may play an important role in the hypotonic swelling-induced activation of \( Ca^{2+} \) channels.

Although our results do not directly answer the question as to how cell swelling stimulates PKC activity in this cell type, results from other groups have demonstrated a PKC isozyme-specific interaction with F-actin (29, 30) and caveolae (27, 32). In addition, possible redistribution or reorganization of F-actin and caveolar microdomains during cell swelling has been suggested (25, 28). If F-actin and caveolin serve as essential anchoring proteins for specific PKC isoforms, alteration of F-actin or caveolin during cell swelling may change PKC translocation and activity to its specific targets. Whether this same mechanism can account for cell swelling-induced stimulation of L-type \( Ca^{2+} \) channels in rabbit portal vein smooth muscle cells has not yet been elucidated and deserves further study. In addition, further studies should also answer the question of whether the cell swelling-induced increase of \( I_{\text{Na}} \) through these channels is related to the increase of single-channel conductance, open channel probability, or number of functional channels.

In summary, results from the present study demonstrate for the first time that hypotonic cell swelling can enhance L-type \( Ca^{2+} \) channel activity in rabbit portal vein smooth muscle cells. Furthermore, PKC, but not PKA, plays an important role in the cell swelling-induced stimulation of L-type \( Ca^{2+} \) channels. Thus stimulation of \( Ca^{2+} \) channels as well as stimulation of volume-regulated \( Cl^{-} \) channels by hypotonic cell swelling may subsequently enhance the contractility of blood vessels and be a mechanism for modulating afterload in arteriolar vessels or preload (capacitance) in venous vessels.

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