PI3-kinase/Akt modulates vascular smooth muscle tone via cAMP signaling pathways

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PI3-kinase/Akt modulates vascular smooth muscle tone via cAMP signaling pathways. J Appl Physiol 91: 1819–1827, 2001.—Phosphatidylinositol 3-kinase (PI3-kinase) activates protein kinase B (also known as Akt), which phosphorylates and activates a cyclic nucleotide phosphodiesterase 3B. Increases in cyclic nucleotide concentrations inhibit agonist-induced contraction of vascular smooth muscle. Thus we hypothesized that the PI3-kinase/Akt pathway may regulate vascular smooth muscle tone. In unstimulated, intact bovine carotid artery smooth muscle, the basal phosphorylation of Akt was higher than that in cultured smooth muscle cells. The phosphorylation of Akt decreases in a time-dependent manner when incubated with the PI3-kinase inhibitor, LY-294002. Agonist (serotonin)-, phorbol ester (phorbol 12,13-dibutyrate; PDBu)-, and depolarization (KCl)-induced contractions of vascular smooth muscles were all inhibited in a dose-dependent fashion by LY-294002. However, LY-294002 did not inhibit serotonin- or PDBu-induced increases in myosin light chain phosphorylation or total O2 consumption, suggesting that inhibition of contraction was not mediated by reversal or inhibition of the pathways that lead to smooth muscle activation and contraction. Treatment of vascular smooth muscle with LY-294002 increased the activity of cAMP-dependent protein kinase and increased the phosphorylation of the cAMP-dependent protein kinase substrate heat shock protein 20 (HSP20). These data suggest that activation of the PI3-kinase/Akt pathway in unstimulated smooth muscle may modulate vascular smooth muscle tone (allow agonist-induced contraction) through inhibition of the cyclic nucleotide/HSP20 pathway and suggest that cyclic nucleotide-dependent inhibition of contraction is dissociated from the myosin light chain contractile regulatory pathways.

serotonin; phorbol ester; myosin light chains; cAMP-dependent protein kinase

THE SERINE-THEONINE KINASE Akt was identified as the product of the oncogene v-akt in the lymphomagenic murine retrovirus AKT8 (7). Because of the structural homology to protein kinase A and protein kinase C, Akt is also referred to as protein kinase B and RAC-PK (related to the A and C kinases) (11). Various growth factors activate Akt. Akt is a direct downstream target of phosphatidylinositol 3-kinase (PI3-kinase) and is involved in mediating cell survival and protection from apoptosis (17). Akt has been implicated in other biological actions such as meiosis in oocytes (1), myogenic differentiation (18), and differentiation of adipocytes (20) and several actions of insulin (29). A specific inhibitor of PI3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002) (31), has been used to study the involvement of Akt in various biological functions. Wortmannin, structurally unrelated to LY-294002, also inhibits PI3-kinase (2). However, wortmannin is less specific in that it also inhibits myosin light chain kinase (MLCK) (25) and the phosphorylation of the myosin regulatory light chains [myosin light chain 20 (MLC20)] in vivo (36).

Physiological substrates of Akt include the glycogen synthase kinase 3β (12), BAD [one of the Bcl2 family of proteins (14)], phosphofructose-2-kinase (15), and a cyclic nucleotide phosphodiesterase 3B (PDE3B) (19). Akt phosphorylates PDE3B on serine-273 in response to insulin and activates it, leading to decreases in cAMP levels in 3T3-L1 adipocytes (19). Cyclic nucleotide phosphodiesterases are a family of structurally related enzymes that hydrolyze the nucleotides cAMP and cGMP, thus inactivating the cyclic nucleotides. Phosphodiesterase 3 (PDE3) activity represents a substantial percentage of total cAMP phosphodiesterase activity in heart, blood vessels, and platelets (8). Therapeutically, the PDE3 inhibitors are used as positive inotropes, vasodilators, and inhibitors of platelet aggregation (6, 8). Molecular cloning has identified two distinct genes that encode PDE3 activity, yielding isoforms PDE3A and PDE3B. These isoforms are expressed in several tissues, including heart, aorta, liver,

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kidney, epididymal fat, and vascular smooth muscle (23).

cAMP and cGMP are second messengers that mediate several biological functions, including vascular smooth muscle relaxation (22, 24) and inhibition of smooth muscle contraction (38). cAMP and cGMP mediate cellular processes through activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG), respectively. Because the activation of smooth muscle contraction is thought to occur through increases in intracellular Ca\(^{2+}\), activation of MLCK, and increases in the phosphorylation of the MLC\(_{20}\), many investigators have focused on mechanisms by which activation of cyclic nucleotides would lead to relaxation by reversing or inhibiting this pathway. However, the existing data suggest that cyclic nucleotide-dependent relaxation is not simply a reversal or inhibition of pathways that activate contraction (27, 38). We and others (4, 5, 27, 38) have recently determined that the small heat shock-related protein, HSP20, is a substrate of PKA and PKG that may directly mediate smooth muscle relaxation, independent of the Ca\(^{2+}\)/MLC\(_{20}\) regulatory pathways. Thus we hypothesized that Akt might activate PDE3B in vascular smooth muscle maintaining low levels of cyclic nucleotides. This would facilitate agonist-induced contraction. On the other hand, inhibition of Akt would lead to increases in the levels of cyclic nucleotides, increases in the phosphorylated isoform of HSP20, and inhibition of agonist-induced contraction of vascular smooth muscle.

**EXPERIMENTAL PROCEDURES**

**Materials.** 12-Deoxyphorbol 13-isobutyrate (PDBu) and se-rotonin (5-HT) were purchased from LC Services (Woburn, MA). Forskolin and 1,2-bis[2-aminoethoxy]ethane-N\(_2\),N\(_4\),N\(_7\),N\(_9\)-tetraacetic acid (BAPTA-AM) were purchased from Calbiochem (La Jolla, CA), and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY-294002) was purchased from Alexis Biochemicals (San Diego, CA). HEPES was obtained from American Bioanalytical (Natick, MA). Urea, SDS, glycine, and Tris were from Research Organics (Cleveland, OH). Coomassie brilliant blue was from ICN Biomedicals (Aurora, OH). EGTA, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), EDTA, polyoxyethylene-sorbitan mono-laurate (TWEEN 20), and all other reagent-grade chemicals were from Sigma Chemical (St. Louis, MO). The reagents used for electrophoretic analysis were purchased from Bio-Rad (Hercules, CA). Rabbit polyclonal anti-MLC\(_{20}\) antibodies were a gift from Dr. James Stull (University of Texas, Galveston, TX), and rabbit polyclonal anti-HSP29 antibodies were kindly provided by Dr. Kanefusa Kato (Aichi Human Service Center, Aichi, Japan). Anti-Akt and anti-phospho-Akt antibodies were obtained from New England Biolabs (Beverly, MA). Goat anti-rabbit secondary antibodies were from Jackson Immunochemical (West Grove, PA). Protein was estimated by the modified Bradford assay kit from Pierce (Rockford, IL).

**Preparation of bovine carotid artery smooth muscle and physiological measurements.** Bovine carotid arteries were dissected from fetal calves at a local abattoir (Shaprio's, Augusta, GA), placed directly in HEPES buffer (in mM: 140 NaCl, 4.7 KCl, 1.0 MgSO\(_4\), 1.0 NaH\(_2\)PO\(_4\), 1.5 CaCl\(_2\), 10 glucose, and 10 HEPES; pH 7.4), and stored at 4°C for 24–72 h. The carotid vessels were dissected free from the adventitia and were opened longitudinally. The endothelium was removed by rubbing the intima with a cotton-tipped applicator. Complete denudation of endothelial cells with this technique has been previously confirmed with scanning electron microscopy (39). Transverse strips, 1.0 mm in width, were cut, and each end was tied to a loop of 3-0 silk. In some cases, rings of bovine carotid artery vessel were cut and endothelium was removed by gently denuding the intimal surface with forceps. The muscle strips were suspended in a muscle bath containing a bicarbonate buffer (in mM: 120 NaCl, 4.7 KCl, 1.0 MgSO\(_4\), 1.0 NaH\(_2\)PO\(_4\), 10 glucose, 1.5 CaCl\(_2\), and 25 Na\(_2\)HCO\(_3\); pH 7.4), equilibrated with 95% O\(_2\)-5% CO\(_2\), at 37°C. The strips were fixed at one end to a stainless steel wire and attached to a Kent Scientific (Litchfield, CT) force transducer (TRN001) interfaced with a DT2801 analog-to-digital board (Data Translation, Marlboro, MA). Data were acquired with Dasylab software (Dasytec, Amherst, MA). For experiments in Ca\(^{2+}\)-free conditions, the muscles were equilibrated in bicarbonate buffer without CaCl\(_2\) and containing 4 mM EGTA (an extracellular Ca\(^{2+}\) chelator) and 0.1 mM BAPTA-AM (an intracellular Ca\(^{2+}\) chelator). Depletion of extracellular Ca\(^{2+}\) under these conditions has been previously confirmed by failure of the muscles to contract in response to high extracellular KCl (34). All tissues were allowed to equilibrate for 1 h before the experiment. The strips were contracted with 110 mM KCl (with equimolar replacement of NaCl in bicarbonate buffer), the maximal tension obtained was taken as 100%, and tension obtained with agents (agonists, inhibitors) was determined. Force was converted to stress (10\(^5\) N/m\(^2\); force (g) \times 0.0987/area, where area is equal to the wet weight [mg/length (mm at maximal length)] divided by 1.055. Agonists and inhibitors were added directly to the muscle baths.

**Immunoblotting for Akt and phospho-Akt.** Strips of smooth muscle were treated with 50 \(\mu\)M LY-294002 for different time intervals and frozen in liquid N\(_2\) and pulverized. The tissue was solubilized in urea-CHAPS buffer consisting of 9 M urea, 2% CHAPS, and 100 mM dithiothreitol (DTT) followed by centrifugation (14,000 g) to remove insoluble material. Fifty micrograms of protein were resolved by SDS-PAGE using 10% polyacrylamide gels. In a control experiment, the proteins were transferred to nitrocellulose membrane and blocked with 5% milk in Tris-buffered saline (TBS)-0.5% Tween 20. Separate blots were incubated with antibodies to Akt or phospho-Akt (1:1,000 dilution in TBS-3% bovine serum albumin) overnight at 4°C. The blots were washed with TBS-Tween 20 five times and incubated with goat anti-rabbit IgG-peroxidase secondary antibodies for 1 h at room temperature. The specific binding was detected using Supersignal chemiluminescent substrate (Pierce) and exposed to film (Kodak XAR-5). Akt and phospho-Akt bands (~59 kDa) were identified by comparisons to the markers provided in the antibody kit and were quantitated densitometrically.

**Determination of myosin light chain phosphorylation.** The strips of bovine carotid artery were equilibrated in a muscle bath as described above and treated with the appropriate agonists. The strips were snap frozen in dry ice-acetone, and the frozen tissue was pulverized under liquid N\(_2\). The frozen samples were placed in a frozen slurry of precipitating solution consisting of 90% acetone, 10% trichloroacetic acid, and 10 mM DTT and then allowed to melt to room temperature. The precipitating solution was removed, and the tissues were washed three times with 90% acetone and 10 mM DTT. The samples were dried, and the pellets were suspended in urea-CHAPS buffer consisting of 9 M urea, 2% CHAPS, and 100 mM DTT and then vortexed to solubilize the proteins.
muscle MLC20 (26). After a brief rinsing with TBS, the bovine serum albumin) raised to bovine tracheal smooth at 4°C with antiserum (1:12,000 dilution in TBS with 3% blot was blocked in 5% nonfat milk in TBS (150 mM NaCl 2-min sampling window. Stress measurements are reported as the force generated and normalized for ring cross-sectional 2.40% and 22.4 6 3.41% KCl response; Fig. 2 A). Pretreatment of the muscle (25.3 6 2.40%) also significantly inhibited contraction (109.14 6 3.41% KCl response at 15 and 30 min, respectively; data not shown). It has been demonstrated significantly inhibited the magnitude of contraction (25.3 6 2.40% and 22.4 6 1.90% response, 6 6.31% and 6 6.12% KCl response). Inhibition of smooth muscle contraction by LY-294002. Treatment of bovine carotid artery smooth muscle with 5-HT (1 µM) led to a rapid, sustained contraction (109.14 6 5.39% of the active stress KCl response, n = 4; Fig. 2A). Pretreatment of the muscle strip with LY-294002 (50 µM, 30 min) before 5-HT significantly inhibited the magnitude of contraction (4.52 6 3.41% KCl response; Fig. 2A) in a dose-dependent fashion (Fig. 2, B and C). Addition of LY-294002 (30 µM) followed by 5-HT (1 µM) also significantly inhibited contraction (25.3 6 2.40% and 22.4 6 1.90% KCl response at 15 and 30 min, respectively; data not shown). The effect of LY-294002 was reversible in that the muscle strips contracted to 5-HT (99.08 6 6.31% of KCl to deliver 100 mM KCl response at 15 and 30 min, respectively, Fig. 1 C). Pretreatment of the muscleבול arbeidad cardiovascular constricción por LY-294002. Tratamiento de la arteria carótida bovina de las tiras musculares con 5-HT (1 µM) condujo a una contricción rápida e inmediata (109.14 ± 5.39% de la respuesta de control con KCl, n = 4; Fig. 2A). Pretratamiento de la tiras musculares con LY-294002 (50 µM, 30 min) antes de 5-HT significativamente inhibió la magnitud de la contricción (4.52 ± 3.41% de la respuesta a KCl; Fig. 2A) a una dosis-dependiente de su contenido (Fig. 2, B y C). Adición de LY-294002 (30 µM) seguido de 5-HT (1 µM) también significativamente inhibió la contricción (25.3 ± 2.40% y 22.4 ± 1.90% de la respuesta a KCl a 15 y 30 min, respectivamente; dato no mostrado). El efecto de LY-294002 era reversible en que las tiras musculares contraídas a 5-HT (99.08 ± 6.31% de la respuesta a KCl) fueron reversibles en que las tiras musculares contraídas a 5-HT (99.08 ± 6.31% de la respuesta a KCl) fueron reversibles en que las tiras musculares contraídas a 5-HT (99.08 ± 6.31% de la respuesta a KCl) fueron reversibles en que las tiras musculares contraídas a 5-HT (99.08 ± 6.31% de la respuesta a KCl).
was inhibited (6.26 ± 2.05% KCl response) by LY-294002 (Fig. 5C).

Smooth muscle contraction can also be elicited by depolarization of the membrane with high extracellular Ca\(^{2+}\), activation of MLCK, and in the phosphorylation of the regulatory MLCK, we conducted experiments to determine whether inhibition of PI3-kinase led to changes in MLC\(_{20}\) phosphorylation. Stimulation of smooth muscle strips with 5-HT (1 \(\mu\)M for 2 min) led to increases in MLC\(_{20}\) phosphorylation (0.50 ± 0.05 mol P/mol MLC\(_{20}\); Fig. 5, A and B). However, preincubation of the smooth muscle strips with LY-294002 (50 \(\mu\)M, 30 min) before stimulation with 5-HT did not significantly inhibit (0.51 ± 0.03 mol P/mol MLC\(_{20}\)) the MLC\(_{20}\) phosphorylation (Fig. 5, A and B). Thus 5-HT-induced contractile force was inhibited by LY-294002, but MLC\(_{20}\) phosphorylation was not (Fig. 5C). Similarly, PDBu stimulation induced significant MLC\(_{20}\) phosphorylation to 0.26 ± 0.03 and 0.27 ± 0.03 mol P/mol MLC\(_{20}\) in the presence and absence of LY-294002, respectively (Fig. 5, A and B).

**Effect of PI3-kinase inhibition on oxygen consumption.** Due to the relatively slow time course of smooth muscle contraction and the limited phosphocreatine and glycogen stores, there is a tight association between increased energy utilization and metabolic recovery (16). One of the more unique properties of smooth muscle is an energetic behavior that is well correlated with the extent of myosin light chain phosphorylation (28). We studied the energetic state of the carotid artery, oxygen consumption, and the stress in carotid artery rings stimulated with 5-HT in the absence and presence of LY-294002. The magnitude of the \(J_O2\) increase with 5-HT stimulation was similar in the presence (25.7 ± 8.6 nmol O\(_2\)/min \(-1\)·g\(^{-1}\)) or absence of the LY-294002 (26.4 ± 9.1 nmol O\(_2\)/min \(-1\)·g\(^{-1}\)), suggesting an equivalent total energetic cost for 5-HT activation of this tissue.

**Activation of PKA by LY-294002.** One reported substrate of Akt is PDE3 (19). To determine whether the inhibition of PI3-kinase inhibited smooth muscle contraction by increases in cyclic nucleotide levels, we measured the activity of PKA in the presence and absence of LY-294002 (100 \(\mu\)M). PKA activity was significantly increased in smooth muscle strips treated with LY-294002 (Fig. 6).

**HSP20 phosphorylation.** Both relaxation and inhibition of contraction of carotid artery smooth muscle is associated with increases in the phosphorylation of the small heat shock-related protein, HSP20 (5, 27, 38). HSP20 is also phosphorylated in response to insulin and insulin antagonists in rat skeletal and smooth muscle.
muscle (32, 33). Because treatment of carotid artery smooth muscle with LY-294002 leads to activation of PKA and PKA phosphorylates HSP20, we examined HSP20 phosphorylation in response to LY-294002. Treatment with LY-294002 led to significant increases in the phosphorylation of HSP20. These increases were comparable to those seen after treatment with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) and the PKA activator forskolin (10 μM; Fig. 7).

**DISCUSSION**

In this study, we observed that there was a significant level of basal phosphorylation of Akt in intact vascular smooth muscles compared with cultured vascular smooth muscle cells (Fig. 1), suggesting a high basal activity of PI3-kinase in intact vascular smooth muscle. Results from this study demonstrated that inhibition of this basal activity by the PI3-kinase inhibitor LY-294002 inhibited the ability of bovine carotid artery smooth muscle to contract in response to various contractile stimuli, even though the stimuli did not involve activation of PI3-kinase. Consequently, we designed experiments to study the effect of LY-294002 on the basal activity of the downstream substrate of PI3-kinase, Akt, and subsequent changes in signaling events downstream of Akt activation.

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Fig. 2. Inhibition of serotonin (5-HT)-induced contraction by LY-294002 in bovine carotid artery. Bovine carotid artery smooth muscle strips were prepared as described in EXPERIMENTAL PROCEDURES and equilibrated in bicarbonate buffer in a muscle bath for 1 h. Muscle strips were then treated with 110 mM KCl (K⁺), and tension was recorded. Muscle strips were washed 3 times (wash) and equilibrated in bicarbonate buffer for 30 min and then with LY-294002 (0, 3, 10, 30, or 100 μM) for 30 min followed by 1 μM 5-HT, and the force generated was recorded and converted to stress. A: representative tracing of high KCl contraction (KCl) followed by 5-HT (1 μM). B: representative tracings of force generated when muscle strips were incubated with 0 (a), 3 (b), 10 (c), 30 (d), or 100 (e) μM LY-294002 for 30 min followed by 5-HT (1 μM). C: averaged data (means ± SE for 3 independent experiments), expressed as relative stress with respect to KCl response in which stress value obtained with KCl was defined as 100%. *P < 0.05.

Fig. 3. Inhibition of 12-deoxyphorbol 13-isobutyrate (PDBu)-induced contraction by LY-294002 in bovine carotid artery. Bovine carotid artery smooth muscle strips were prepared as described in EXPERIMENTAL PROCEDURES and equilibrated in bicarbonate buffer in a muscle bath for 1 h. Muscle strips were then treated with 110 mM KCl (K⁺, stoichiometric substitution of KCl for NaCl in the bicarbonate buffer), and tension was recorded and converted to stress. Muscle strips were washed three times (wash) and equilibrated with bicarbonate buffer without CaCl₂, containing 4 mM EGTA and 0.1 mM BAPTA-AM (extracellular and intracellular Ca²⁺ chelator, respectively) for an additional 1 h. Depletion of Ca²⁺ was confirmed by stimulation of the muscle strips with 5-HT (1 μM). Muscle strips were then treated with LY-294002 (0, 10, 30, or 100 μM) for 30 min followed by PDBu (0.7 μM), and tension was recorded at different time intervals. A: representative tracing showing the stress values for KCl and PDBu (0.7 μM). B: representative tracings of stress values when muscle strips in Ca²⁺-free conditions were incubated with 0 (a), 10 (b), 30 (c), or 100 (d) μM LY-294002 for 30 min followed by 0.7 μM PDBu. C: averaged data (means ± SE for 3 independent experiments), expressed as relative stress with respect to KCl response in which stress value obtained with KCl was defined as 100%. *P < 0.05, compared with force generated in the absence of LY-294002.
The basal level of phosphorylation of Akt was inhibited by the PI3-kinase inhibitor, LY-294002 (Fig. 1; Ref. 31). Recently, Akt was determined to activate a phosphodiesterase (19), leading to a decrease in intracellular cyclic nucleotide levels. Because increases in intracellular cyclic nucleotides inhibit contraction of vascular smooth muscle, we examined the effect of LY-294002 on physiological contractile responses and the activation of contractile signaling pathways. Treatment of intact vascular smooth muscles with LY-294002 inhibited agonist (5-HT)-, phorbol ester (PDBu)-, and depolarization (KCl)-induced contractions in a dose-dependent manner (Figs. 2–4). LY-294002 had a maximal effect on 5-HT- and PDBu-induced contractions and partial effects on KCl-induced contractions. This differential effect could be partially explained by the fact that these agents initiate contraction by different mechanisms and depolarization-induced contraction is blocked by cyclic nucleotides less effectively than agonist-induced contraction. 5-HT causes vascular smooth

Fig. 4. Inhibition of KCl contraction by LY-294002. Bovine carotid rings were prepared as described in EXPERIMENTAL PROCEDURES and equilibrated in MOPS-based buffer (physiological saline solution) for 1 h. A: mean active stress response of bovine carotid artery rings to K+ physiological saline solution (Na+ substitution) and 100 mM KCl (bolus addition) before and after 30-min preincubation of graded concentrations of LY-294002 (3–100 μM). Reported are mean active stresses of 4 different arteries. Standard errors are not reported for clarity. B: dose-response curve for the impact of preincubation of LY-294002 (3–100 μM) on KCl-induced active stress generation. Reported are means ± SE of 4 arteries. *P < 0.05 compared with relative KCl response in which active stress obtained with KCl was defined as 100%.

Fig. 5. Contraction and myosin light chain phosphorylation in the presence of LY-294002, 5-HT, and 12-deoxyphorbol 13-isobutyrate. Bovine carotid artery smooth muscle strips were prepared as described in EXPERIMENTAL PROCEDURES and equilibrated in bicarbonate buffer in a muscle bath for 1 h. Muscle strips were then treated with 110 mM KCl, and tension was recorded. Muscle strips were washed 3 times, equilibrated in bicarbonate buffer for 30 min, and then stimulated with 5-HT (1 μM), PDBu (0.7 μM), LY-294002 (LY294; 50 μM) for 30 min followed by 5-HT or PDBu, or 50 μM LY-294002. The muscle strips were snap frozen after 2 (5-HT) or 40 (PDBu) min, and myosin light chain 20 (MLC20) phosphorylation was determined as described in EXPERIMENTAL PROCEDURES. A: representative immunoblot of the urea-glycerol gel with MLC20 and phosphorylated (P) MLC20. B: quantitation of the densitometric analysis of the immunoblot, expressed as mol P/mol MLC20. *P < 0.05 (n = 3), compared with the respective basal phosphorylation. C: averaged data (means ± SE; n = 3–5) for contraction measurement during stimulation of the muscle strips with the agonists expressed as relative stress with respect to KCl response in which stress obtained with KCl was defined as 100%. *P < 0.05, compared with contraction in the absence of LY-294002.
muscle contraction by interacting with cell surface receptors and activating signaling pathways that lead to increases in intracellular Ca\(^{2+}\) and activation of protein kinase C (21). Banes et al. (3) reported that LY-294002 inhibits 5-HT\(\text{-}\)-induced contraction in rat aorta. However, they attributed the inhibition of contraction to the possibility of LY-294002 being a 5-HT\(\text{-}\text{2A}\) receptor antagonist. Phorbol esters do not require receptor activation and directly activate protein kinase C. We determined that LY-294002 inhibits contractions elicited by the phorbol ester PDBu. Most investigators have focused on the role of the Ca\(^{2+}\), MLCK, and regulatory myosin light chain phosphorylation pathway of muscle contraction. Interestingly, LY-294002 inhibited contractions induced by depolarization with high K\(^+\), which leads to increases in intracellular Ca\(^{2+}\). In addition, LY-294002 also inhibited phorbol ester-induced contractions under conditions in which intracellular Ca\(^{2+}\) is chelated. Taken together, these data suggest that LY-294002 is inhibiting muscle contraction by activating pathways independent of increases in intracellular Ca\(^{2+}\) and myosin light chain phosphorylation.

To directly determine the effect of LY-294002 on the MLC\(_{20}/\)MLCK pathway, we measured the phosphorylation of the regulatory myosin light chains. Increases in MLC\(_{20}\) phosphorylation were similar in response to 5-HT and PDBu in the presence and absence of LY-294002 (Fig. 5). Thus there were similar increases in MLC\(_{20}\) phosphorylation under conditions in which stress was present (agonist alone) and absent (LY-294002 before agonist). Finally, we examined the energetic response of the bovine carotid smooth muscle during 5-HT stimulation in the presence and absence of LY-294002. The \(J_{\text{O}_2}\) served as an indirect measure of the activation state of the tissue reflecting a combination of cross-bridge ATPase activity and the oxidative energy necessary to activate the tissue (e.g., regulatory myosin light chain phosphorylation/dephosphorylation) (37). This suggests that the energetic consequences of 5-HT stimulation are equivalent in both the presence and absence of LY-294002. When viewed with the myosin light chain phosphorylation measurements, we postulate that the impaired force generation ability seen in the presence of LY-294002 relies on a mechanism that does not impair the tissue activation pathway that utilizes light chain phosphorylation and thus cross-bridge cycling. Because energy demands for cross-bridge ATPase activity and myosin light chain phosphorylation can be nearly equivalent at high levels of phosphorylation (37), the substantial energetic
cost seen here could account for the moderate levels of phosphorylation. This would be expected if the proposed relaxation mechanism were working by a process independent of myosin phosphorylation and crossbridge cycling.

To determine whether inhibition of the PI3-kinase/Akt pathway in vascular smooth muscle inhibits contractile responses through the cyclic nucleotide-dependent signaling pathway, we measured the activity of PKA. Treatment of vascular smooth muscle with LY-294002 increased PKA activity (Fig. 6). Because the only known activators of PKA are cyclic nucleotides, this provides indirect evidence that inhibition of PI3-kinase leads to increases in intracellular cyclic nucleotide concentrations. It is likely that the increase in basal concentration of cyclic nucleotides in response to LY-294002 also leads to activation of PKG, since cross-activation of PKG by cAMP during vascular smooth muscle relaxation is possible and has been demonstrated previously by several investigators (22). The specific mechanisms by which activation of cyclic nucleotide-dependent protein kinases leads to vasorelaxation and inhibition of contraction are not known. However, recent attention has been focused on HSP20. HSP20 is phosphorylated in vascular smooth muscle in response to activation of cyclic nucleotide-dependent signaling pathways (5). In addition, HSP20 is not phosphorylated in a muscle that is uniquely refractory to cyclic nucleotide-dependent relaxation, the human umbilical smooth muscle (9). HSP20 can be phosphorylated in vitro by both PKA and PKG (5). The physiologically relevant site of phosphorylation on HSP20 appears to be serine-16 (4). Transient permeabilization of vascular smooth muscle and the introduction of phosphopeptide analogs of HSP20 inhibit agonist-induced contractions of the muscles (4). In this study, we demonstrate that inhibition of PI3-kinase with LY-294002 also leads to increases in the phosphorylation of the PKA substrate HSP20 (Fig. 7).

Taken together, these data suggest that the basal activity of PI3-kinase is necessary for contraction of vascular smooth muscle by contractile agents. The physiological consequences of this pathway appear to be the lowering of intracellular cyclic nucleotide levels to allow agonist-induced contraction to occur (Fig. 8). Finally, the results of this study suggest that inhibition of the basal activation of PI3-kinase/Akt pathway leads to activation of the cyclic nucleotide-dependent signaling pathway with subsequent increases in the phosphorylation of HSP20. The cyclic nucleotide pathway inhibits agonist-induced contraction in a manner that is dissociated from the MLCK/MLC20 pathway in that force generation is inhibited but myosin light chain phosphorylation and energy consumption are not. Although the mechanisms by which phosphorylated HSP20 inhibits the generation of force are not known, it is interesting to speculate that HSP20 may be directly interacting with specific, but as yet not defined, elements of the contractile machinery. Because PDE3 inhibitors are used as positive inotropes, vasodilators, and inhibitors of platelet aggregation, manipulation of PDE activity and cAMP levels through this pathway may have therapeutic potential in the treatment of vasospastic disorders.

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