Phosphatidylinositol 3-kinase modulates vascular smooth muscle contraction by calcium and myosin light chain phosphorylation-independent and -dependent pathways

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Su, Xiaoling, Elaine M. Smolock, Kristi N. Marcel, and Robert S. Moreland. Phosphatidylinositol 3-kinase modulates vascular smooth muscle contraction by calcium and myosin light chain phosphorylation-independent and -dependent pathways. Am J Physiol Heart Circ Physiol 286: H657–H666, 2004. First published October 9, 2003; 10.1152/ajpheart.00497.2003.—Regulation of smooth muscle contraction involves a number of signaling mechanisms that include both kinase and phosphatase reactions. The goal of the present study was to determine the role of one such kinase, phosphatidylinositol (PtdIns)3-kinase, in vascular smooth muscle excitation-contraction coupling. Using intact medial strips of the swine carotid artery, we found that inhibition of PtdIns3-kinase by LY-294002 resulted in a concentration-dependent decrease in the contractile response to both agonist stimulation and membrane depolarization-dependent contractions and a decrease in Ca2+-dependent myosin light chain (MLC) phosphorylation, the primary step in the initiation of smooth muscle contraction. Inhibition of PtdIns3-kinase also depressed phorbol dibutyrate-induced contractions, which are not dependent on either Ca2+ or MLC phosphorylation but are dependent on protein kinase C. To determine the Ca2+-dependent site of action of PtdIns3-kinase, we determined the effect of several inhibitors of calcium metabolism on LY-294002-dependent inhibition of contraction. These inhibitors included nifedipine, SK&F-96365, and caffeine. Only SK&F-96365 blocked the LY-294002-dependent inhibition of contraction. Interestingly, all compounds blocked the LY-294002-dependent inhibition of MLC phosphorylation. Our results suggest that activation of PtdIns3-kinase is involved in a Ca2+- and MLC phosphorylation-independent pathway for contraction likely to involve protein kinase C. In addition, our results also suggest that activation of PtdIns3-kinase is involved in Ca2+-dependent signaling at the level of receptor-operated calcium channels.

LY-294002; phorbol dibutyrate; SK&F-96365; caffeine; nifedipine; Akt

CONTRACTION OF VASCULAR SMOOTH MUSCLE is initiated by an increase in cellular [Ca2+], activation of myosin light chain (MLC) kinase, and the resultant phosphorylation of the 20-kDa regulatory MLC (11). However, it has become clear that other modulatory and regulatory pathways play an important role in determining the characteristics of most contractile events (10, 28). In fact, one of the most interesting and potentially important aspects of smooth muscle physiology is the tremendous number of signaling pathways present within the cell. Organ-specific differences in these pathways may be important in allowing a single cell type to perform properly over the wide range of physiological functions that involve smooth muscle.

One signaling molecule that has been implicated in several aspects of smooth muscle function is phosphatidylinositol (PtdIns)3-kinase. PI3-kinase is activated by several cellular stimulants including both growth factors and contractile agonists (20, 35) and has been shown to be involved in many aspects of smooth muscle function. Most notable are the pathways involved in cell growth and migration (7, 13, 40), but, more recently, inhibition of PI3-kinase activity has been shown to decrease contractile activity (12, 18). PI3-kinase is a multifunctional, heterodimer enzyme composed of a catalytic subunit and a regulatory subunit with both lipid and protein kinase activity (3). The PI3-kinase family is subdivided into three classes defined by substrate specificity, structure, and means of regulation. Class I PI3-kinases consist of either an α-, β-, or γ-catalytic subunit that, when activated, interacts with GTP-bound Ras. Class II and III PI3-kinases are important in calcium-independent lipid binding and vesicle formation, respectively. Inhibitors of PI3-kinase are targeted against the catalytic domain. Wortmannin works predominantly by inhibiting the p110γ-catalytic subunit, which is activated through a G protein-coupled receptor. LY-294002 (36) acts by competitively inhibiting the ATP binding domain of PI3-kinase (for a review, see Ref. 5).

How PI3-kinase alters smooth muscle contractility is not known. Brophy and colleagues (12) have suggested a unique role for this enzyme. They proposed that, in contrast to a direct positive effect on contraction, PI3-kinase inhibits the vasorelaxant cyclic nucleotide-dependent pathway allowing agonist binding to initiate a contraction. Other groups have presented evidence to link PI3-kinase activity to opening of voltage-gated and receptor-coupled calcium channels (14, 21). Watts’ group (18) has suggested that an increase in PI3-kinase activity is associated with an increase in spontaneous activity in the aorta from hypertensive rats. Thus it appears fairly well documented that PI3-kinase modulates smooth muscle contraction, but how this occurs is not as well understood.

The goal of this study was to answer the following questions: Does PI3-kinase modulate the activity of the swine carotid artery, and, if so, what are the potential mechanisms responsible? We were interested in determining whether inhibition of PI3-kinase decreased contraction by membrane depolarization and/or receptor- and G protein-mediated pathways and whether PI3-kinase alters contractile activity directly or

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through changes in stimulation-induced levels of Ca$^{2+}$. Our results are consistent with the hypothesis that PI3-kinase activity is increased during all modes of contractile stimulation. PI3-kinase increases contraction by increasing calcium flux through receptor-operated channels and by activation of a pathway(s) that is apparently not dependent on either calcium or MLC phosphorylation. PI3-kinase does not appear to increase contraction by pathways involved in either voltage-gated calcium channels or release of intracellular calcium stores. Of particular interest to us were the findings that suggest that inhibition of PI3-kinase activity decreases MLC phosphorylation levels, but reversal of this inhibitory effect does not increase the levels of force developed. All of the results to be presented in this study point to a mechanism by which PI3-kinase activity increases contraction that does not involve either calcium or MLC phosphorylation.

MATERIALS AND METHODS

**Tissue preparation.** Swine carotid arteries were obtained from a local slaughterhouse and transported to the laboratory in ice-cold MOPS-buffered physiological salt solution (PSS). PSS contained (in mM) 40 NaCl, 4.7 KCl, 1.2 MgSO$_4$, 1.6 CaCl$_2$, 1.2 Na$_2$HPO$_4$, 2 MOPS (pH 7.4), 5 mg glucose, and 0.02 EDTA. Arteries could be stored with daily solution changes for a minimum of 3 days. We and others (9, 16, 23) have previously reported that no change in any parameter of contractility is evident within this storage time as long as the pH of the storing solution is maintained at 7.4 and a large volume of solution relative to arteries is refreshed daily. Arteries were cleaned of connective tissue and then dissected free of both intima and adventitia, leaving a thin medial layer for experimentation. Intact medial strips of swine carotid artery (7 × 0.7 mm) were suspended between a Grass FT.03 force transducer and a stationary clip in water-jacketed organ baths. The strips were equilibrated in PSS at 37°C, pH 7.4, and bubbled with 100% O$_2$ for 90–120 min. A passive force of ~2 g was applied to all tissues. This passive force sets the muscle at a length that approximates the length for maximal active force development ($L_0$). During the equilibration period, tissues were maximally contracted with 110 mM KCl (equimolar substitution for NaCl) several times until similar levels of force were attained.

**Determination of MLC phosphorylation levels.** Muscle tissues that were used for determination of MLC phosphorylation levels were treated identically to those used for force measurement. The tissues were rapidly frozen, at rest or after stimulation, in an acetone-dry ice slurry containing 6% TCA and 10 mM DTT. The tissues were slowly brought to room temperature and rinsed in acetone-containing 10 mM DTT. The tissues were then air dried, weighed, and homogenized on ice in a solution containing 2% SDS, 2 mM DTT, and 10% glycerol. Homogenized samples were clarified by centrifugation, and the supernatants were assayed for total protein using a Bio-Rad kit (Richmond, CA) based on the Bradford technique. Five micrograms of protein from each sample were then subjected to two-dimensional gel electrophoresis, followed by transfer to nitrocellulose membranes as previously described (17). Proteins were visualized using colloidal gold (Amersham Bioscience; Piscataway, NJ) and quantified using scanning densitometry (model GS-800, Bio-Rad). MLC phosphorylation levels were calculated by taking the densitometric analysis of phosphorylated MLC as a percentage of the sum of the densitometric analysis of both the phosphorylated and unphosphorylated forms of MLC.

**Determination of MLC kinase activity.** MLC kinase activity was measured by monitoring the incorporation of $^{32}$P into purified MLC in small pieces of tubes warmed to 37°C. The solutions contained 0.1 mM CaCl$_2$, 10 mM MgCl$_2$, 50 mM HEPES (pH 7.0), 1 mM DTT, 1 mM ATP, 0.4 μM calmodulin, 9 μM MLC, 1 mM Mg$_2$ATP, and 40 μCi [γ-$^{32}$P]ATP. In addition, each reaction mixture contained DMSO, LY-29402, or wortmannin. The reaction was initiated by the addition of MLC kinase and allowed to proceed for 15 or 30 min. The reaction was stopped by adding an aliquot of the solution to an Eppendorf tube containing 2% SDS, 50 mM Tris (pH 6.8), 10 mM DTT, and 10% sucrose. The samples were then subjected to one-dimensional SDS-PAGE and stained with Coomassie blue. The stained gels were dried, and radioactivity in the gels was quantified using a Molecular Dynamics phosphorimagener (Sunnyvale, CA).

**Determination of Akt and phospho-Akt levels.** Phospho-Akt levels were measured as an index of PI3-kinase activation. Total Akt and phospho-Akt levels were measured using anti-Akt and anti-phospho-Akt antibodies. Free-floating swine carotid arterial strips were stimulated with 1 μM histamine for 10 min alone or after a 20-min incubation in 40 μM LY-29402. The tissues were then homogenized, subjected to 10% SDS-PAGE, and then transferred to nitrocellulose membranes. Blots were saturated with 5% milk proteins in Tris-buffered saline solution (TBS) and incubated overnight in TBS-5% BSA containing a polyclonal antibody directed against either total Akt (1:15,000 anti-Akt, Cell Signaling Technology; Beverly, MA) or phospho-Akt (1:2,000 anti-phospho-Akt, Cell Signaling Technology) and actin (1:2,500,000 clone no. 1A4, Sigma; St. Louis, MO). The actin antibody allowed for total Akt and phospho-Akt levels to be normalized within tissues to account for any differences in tissue loading and transfer efficiency. The membranes were washed three times in TBS-1% Tween 20 and incubated with a secondary antibody, anti-IgG conjugated to horseradish peroxidase (1:5,000). Immunoreactive bands were visualized by enhanced chemiluminescence. Quantitation of the bands on the X-ray film was performed using a Bio-Rad GS-800 densitometer.

**Inhibition of various sources of activator calcium.** To determine which, if any, sources of activator calcium are modulated by PI3-kinase, the inhibitory effect of LY-29402 on a histamine-induced contraction was tested in the presence and absence of several different compounds. The compounds chosen were ones that are known to effect different sources of stimulation-induced activator calcium. These included nifedipine to block the dihydropyridine voltage-sensitive calcium channels, caffeine to deplete sarcoplasmic reticular calcium stores, thapsigargin and cyclopiazonic acid (CPA) to inhibit sarcoplasmic reticular Ca$^{2+}$-ATPase activity, and SK&F-96365 to inhibit receptor-operated calcium channels. The effect of LY-29402 was tested on a contraction in response to 1 μM histamine alone and then in the presence of one of the listed inhibitors. The data were normalized as a percentage of the maximal force in response to 1 μM histamine alone and as a percentage of the maximal force in response to 1 μM histamine in the presence of the appropriate inhibitor. To determine whether any specific source of calcium was involved in the LY-29402-dependent inhibition of contraction, the percent inhibition of contraction by LY-29402 in the absence of a single inhibitor was compared with the percent inhibition in the presence of the inhibitor.

**Drugs and statistics.** All chemicals were analytic grade or better and obtained from Fisher Chemical (Pittsburgh, PA). All electrophoresis chemicals were obtained from Bio-Rad Laboratories, CPA, LY-29402, thapsigargin, and wortmannin were purchased from Calbiochem-Novabiochem (San Diego, CA). SK&F-96365 was purchased from Bio-Mol (Plymouth Meeting, PA). Caffeine and nifedipine were obtained from Sigma. Purified MLC, MLC kinase, and calmodulin were generously provided by Drs. C.-L. Albert Wang and Zenon Grabarek of the Boston Biomedical Research Institute (Watertown, MA).

Statistical significance was determined using Student’s t-test. A P value of <0.05 was taken as significant.

RESULTS

LY-29402 has been reported to be a specific inhibitor of the enzyme PI3-kinase (36). We tested the effect of this kinase inhibitor on contractions of the medial strip of the swine carotid artery.
carotid artery. Figure 1 shows the results of these experiments. LY-294002 (20-min incubation, 1–40 μM) inhibited membrane depolarization-induced (50 mM KCl) contractions of the vascular preparation in a concentration-dependent fashion. The IC50 for LY-294002-dependent inhibition of a 50 mM KCl-induced contraction was 12.8 ± 1.4 μM. Contractions in the presence of appropriate concentrations of DMSO were similar to those in the absence of solvent and would represent (if shown) a straight line without variability along the 0% inhibition versus [LY-294002] axis. Thus inhibition of PI3-kinase has a significant inhibitory effect on vascular smooth muscle contraction.

The signaling steps involved in contraction of smooth muscle are different depending on the mode of stimulation. Classically, two forms of contractile stimulation have been described: electromechanical coupling and pharmacomechanical coupling (29). Although there are several sites of overlap in signaling initiated by these two routes of activation (27), they can be examined semi-independently to determine whether any distinct step in the regulation of contraction is specific for one route or general for both. We began our investigation on the role of PI3-kinase in these two excitation pathways with membrane depolarization-induced contractions. Figure 2. A and B, shows the effects of inhibition of PI3-kinase by LY-294002. Figure 2A shows the time course of a contraction in response to 50 mM KCl in the presence and absence of 10 μM LY-294002 added 20 min before stimulation. At all time points during the contractile event, inhibition of PI3-kinase activity reduced levels of stress (force/cross-sectional area). Cumulative concentration-response curves in response to KCl stimulation were also generated in the presence and absence of 10 μM LY-294002 added 20 min before stimulation; the results are shown in Fig. 2B. Inhibition of PI3-kinase significantly depressed KCl-induced contractions at every concentration tested with the exception of the lowest, 10 mM KCl. Thus inhibition of PI3-kinase alters membrane depolarization-induced force across the whole spectrum of stimulation levels.

The next series of experiments was to determine whether inhibition of PI3-kinase also affects agonist-induced contractions of swine carotid media. Figures 3, A and B, and 4 show the results obtained. Inhibition of PI3-kinase by incubation of the arterial tissues for 20 min in 10 μM LY-294002 reduced contraction during both the early stage of force development (1-min stimulation; Fig. 3A) and the later stage of force
maintenance (10-min stimulation; Fig. 3B) in response to histamine (1.0–30.0 μM). In contrast, inhibition of PI3-kinase by LY-294002 had no effect on the early peak (1 min) levels of MLC phosphorylation but did significantly inhibit the later steady-state (10 min) levels (Fig. 4). To ensure that the effects of LY-294002 were specific for PI3-kinase and not other cellular kinases, we tested varying concentrations of wortmannin against a 1 μM histamine-induced contraction (data not shown). Wortmannin inhibited the histamine-induced contraction in a concentration-dependent manner with a negligible effect at 100 nM, 15% inhibition at 500 nM, 64% inhibition at 1 μM, and >90% inhibition at 2 μM. Thus similar results were obtained using two chemically distinct inhibitors of PI3-kinase, suggesting a specificity of effect.

The data shown in Figs. 1–4 clearly demonstrate that LY-294002 inhibits contraction of the swine carotid artery. By inference, this would suggest that PI3-kinase is involved in vascular smooth muscle contraction and that inhibition of this enzyme reduces the contractile response. To provide more direct results demonstrating not only that histamine increases PI3-kinase activity in the swine carotid artery but that LY-294002 inhibits this activity, we measured phospho-Akt levels. As outlined in detail in MATERIALS AND METHODS, we measured total Akt and phospho-Akt levels, a generally accepted index of PI3-kinase activation (3, 5, 12, 20), in response to 10 min of 1 μM histamine stimulation in the presence and absence of 40 μM LY-294002 (20-min incubation before histamine stimulation). These data are shown in Fig. 5. Histamine significantly increased phospho-Akt levels (Fig. 5, A and C), which were blocked by the addition of LY-294002 (Fig. 5, B and C), consistent with the hypothesis that the effects of LY-294002 are due to inhibition of PI3-kinase activity.

On the basis of the results shown in Figs. 1–5, PI3-kinase plays a role in both membrane depolarization and agonist-induced contractions of vascular smooth muscle. The result, which is quite intriguing, is that force development in response to histamine was reduced by LY-294002, but peak MLC phosphorylation levels were not. At steady state, both force and MLC phosphorylation levels were reduced. Therefore, we performed a series of experiments in an attempt to elucidate the mechanism(s) by which LY-294002 inhibits contraction and, by extension, how PI3-kinase augments contraction. The apparent differential effect of LY-294002 on force and MLC phosphorylation suggests that PI3-kinase may modulate contraction via calcium-independent as well as calcium-dependent pathways. We began by examining a calcium-independent pathway for contraction of vascular smooth muscle. We (4) have previously shown that phorbol dibutyrate (PDBu)-in-
duced contractions are not dependent on either calcium or MLC phosphorylation. The effect of 40 μM LY-294002 on PDBu-induced contractions is shown in Fig. 6. LY-294002 inhibited PDBu-induced contractions of the swine carotid artery during normal, calcium-containing conditions as well as after the arteries had been exhaustively depleted of intracellular calcium and placed in a calcium-free PSS (Fig. 6A) as previously described (4). MLC phosphorylation levels were not increased above basal during any condition (Fig. 6B). This strongly suggests that the effect of LY-294002 on vascular contraction is mediated at least in part by inhibition of a calcium-independent pathway and therefore that PI3-kinase is one step in the activation of this pathway.

A simpler explanation for the LY-294002-dependent inhibition of contraction is nonspecific inhibition of MLC kinase. To test this possibility, we incubated purified MLC kinase, MLC, calmodulin, appropriate salts, 0.1 mM CaCl2, and [γ-32P]ATP in the presence of either DMSO, 10 μM LY-294002, or 10 μM wortmannin at 37°C for 15 and 30 min. Reactions were

Fig. 6. Effect of LY on phorbol dibutyrate (PDBu)-induced contractions of the swine carotid artery. A, left: swine carotid arteries, replete with calcium and bathed in normal calcium-containing PSS, were contracted in response to 1 μM PDBu in the presence (solid bars) and absence (open bars) of 10 μM LY. Right, swine carotid arteries, depleted of intracellular calcium and bathed in calcium-free PSS, were contracted in response to 1 μM PDBu in the presence (solid bars) and absence (open bars) of 10 μM LY. B: corresponding MLC phosphorylation values in each experimental condition described in A. Values shown are means ± SE for at least 5 determinations. *P < 0.05.
stopped as described in MATERIALS AND METHODS, and the proteins were subjected to SDS-PAGE and analyzed using a phosphorimager. Representative images of several experiments are shown in Fig. 7. The images shown are those of phosphorylated MLC. High levels of MLC kinase activity, as evidenced by high levels of MLC phosphorylation, are seen in the control lanes (DMSO). There were no significant differences in the density of phosphorylated MLC in the presence of 10 μM LY-294002 compared with control for either the 15- or 30-min incubation periods (203 ± 23 arbitrary units, 15-min control; 228 ± 52 arbitrary units, 30-min control; 186 ± 31 arbitrary units, 15-min LY-294002; 219 ± 41 arbitrary units, 30-min LY-294002). In contrast, 10 μM wortmannin completely inhibited MLC kinase-catalyzed MLC phosphorylation (8 ± 7 arbitrary units, 15-min wortmannin; 7 ± 5 arbitrary units, 30-min wortmannin). These results demonstrate that our assay is able to detect a decrease in MLC kinase activity and, more importantly, that LY-294002 does not inhibit MLC kinase activity.

To examine potential calcium-dependent sites of action of PI3-kinase, we implemented a protocol designed to dissect different sources of activator calcium. As described in MATERIALS AND METHODS, we compared the inhibitory effect of a 20-min incubation of 40 μM LY-294002 against a 1 μM histamine-induced contraction in the presence and absence of a variety of reasonably specific inhibitors of various sources of activator calcium. We assumed that if any source of calcium affected by LY-294002 was already blocked, then the LY-294002-dependent decrease in contraction would be abolished or at least blunted. The results of these experiments are shown in Fig. 8, A–C. Inhibition of voltage-dependent calcium channels by 1 μM nifedipine had no effect on the LY-294002-dependent inhibition of a histamine-induced contraction (Fig. 8A). Similarly, depletion of intracellular calcium stores by 10 μM caffeine had no effect on the LY-294002 inhibitory action on contraction (Fig. 8B). Qualitatively similar effects were obtained with 1 μM ryanodine, 1 μM thapsigargin, and 10 μM CPA (data not shown). In contrast, inhibition of receptor-operated calcium channels by 30 μM SK&F-96365 (8) significantly attenuated the inhibitory effect of LY-294002 (Fig. 8C).

Steady-state MLC phosphorylation levels were measured in all of the tissues used to generate the data shown in Fig. 8, A–C. These values are shown in Fig. 9. The first two bars show the expected 40 μM LY-294002-dependent decrease in MLC phosphorylation levels during the steady-state (10 min) force response to histamine. The next three sets of bars show the results of similar experiments in the presence of 1 μM nifedipine, 10 μM caffeine, or 30 μM SK&F-96365. All three compounds abolished the LY-294002-dependent decrease in histamine-stimulated MLC phosphorylation levels.

**DISCUSSION**

In the present study, we have presented results from experiments designed to examine the mechanism(s) by which PI3-kinase alters vascular smooth muscle contraction. We have shown that inhibition of PI3-kinase, by the reasonably specific inhibitor LY-294002 (36), significantly depressed both membrane depolarization and receptor-dependent forms of contractile activation. Considering it is safe to assume that a decrease in contraction during inhibition of a specific kinase allows the interpretation that that particular kinase was involved in increasing the contraction, PI3-kinase is an integral part of smooth muscle excitation-contraction coupling. This finding is consistent with others who have presented a similar conclusion (12, 18, 30, 42). We have also shown that two distinct mechanisms appear to be responsible for the PI3-kinase-dependent increase in contractile activity. One pathway, which is apparently the more minor of the two based on the magnitude of reversal of the LY-294002 effect, involves activation of receptor-operated calcium channels and the resultant increase in MLC phosphorylation levels. The second and major pathway does not involve an increase in either Ca^{2+} or MLC phosphorylation and most likely is important in the protein kinase C-dependent pathway for contraction.

The simplest explanation to account for inhibition of a smooth muscle contraction is a decrease in stimulation-dependent increases in activator [Ca^{2+}]. To test whether this simpler explanation could account for all or part of the LY-294002-dependent inhibition of force, we performed the experiments shown in Fig. 8, A–C. As discussed in RESULTS, we assumed that if PI3-kinase increased cytosolic calcium through one of the many physiologically relevant routes of calcium entry, then...
inhibition of this pathway would abolish the LY-294002-dependent inhibition of contraction. Only inhibition of receptor-mediated calcium channels decreased the inhibitory effect of LY-294002. No inhibitor of intracellular stores of calcium or of voltage-dependent calcium channels affected the LY-294002-dependent decrease in force. One obvious caveat to this interpretation is the potential for nonspecific effects of the inhibitors used. However, the fact that in most cases we used...
The most direct evidence suggesting that PI3-kinase alters smooth muscle contraction independent of calcium and MLC phosphorylation is the effect of LY-294002 on a PDBu-induced contraction. PDBu-induced contractions of the swine carotid artery have been shown previously by us (4) to be MLC phosphorylation and calcium independent. A later study (23) suggested that PDBu increases force in the swine carotid artery with a concomitant increase in MLC phosphorylation levels. We agree with this finding, but would point out that if calcium is removed, PDBu produces similar levels of force as in the presence of calcium, although no increase in MLC kinase-catalyzed MLC phosphorylation is evident (4). Therefore, we believe it is well established that in many smooth muscles, including the swine carotid artery, contraction in response to phorbol esters is not dependent on MLC phosphorylation (2, 4, 34, 37, 43). Our present results confirm this information and demonstrate that inhibition of PI3-kinase significantly reduces the magnitude of PDBu-induced, calcium- and MLC phosphorylation-independent contractions. Assuming that PDBu directly activates protein kinase C, then one may assume that PI3-kinase is downstream from protein kinase C in the cascade of events leading to contraction. Where PI3-kinase activity is involved downstream from protein kinase C is an open and important question.

Given that PDBu is not an endogenous compound, the most physiologically relevant results demonstrating a dual role for PI3-kinase in the regulation/modulation of a vascular smooth muscle contraction comes from studies using inhibitors of various sources of activator calcium. LY-294002 produced a consistent decrease in steady-state levels of histamine-induced force and MLC phosphorylation. The addition of nifedipine, caffeine, or SK&F-96365 abolished the inhibitory effect of LY-294002 on steady-state MLC phosphorylation levels. However, only SK&F-96365 reversed the inhibitory effect of LY-294002 on force. Because LY-294002 does not inhibit MLC kinase directly, this suggests two interesting phenomenon. First, it provides additional evidence that PI3-kinase modulates contraction independently of effects, indirect or direct, on MLC phosphorylation. Second, the small decreases in MLC phosphorylation in the presence of nifedipine and less so in the presence of SK&F-96365 translated to fairly large decreases in force. This is in agreement with previous work demonstrating a tight relationship between force and MLC phosphorylation in a preparation devoid of other signaling molecules (25).

Most studies examining PI3-kinase and smooth muscle function have been directed toward its role in migration and growth. PI3-kinase has been shown to be important for growth and migration of airway smooth muscle cells (13) and vascular smooth muscle cells (7, 40), chemotactic responses in colonic smooth muscle cells (34), and for medial growth after vascular injury (24). Moreover, in many of these studies, PI3-kinase has been linked to MAPK activity (26, 31, 41). We have previously presented evidence to suggest that at least in the swine carotid artery, inhibition of p42/p44 MAPK activity has no effect on the regulation or modulation of a contraction (9). PI3-kinase has also been shown to regulate p38 MAPK activity (41). p38 MAPKs have been implicated in cytoskeletal rearrangement and potentially in contraction (6); however, we have no evidence concerning this pathway in the swine carotid artery.
A potential role for PI3-kinase in regulation of force development has also been previously suggested (12, 18, 42). PI3-kinase increased calcium influx through voltage-gated calcium channels in response to ANG II stimulation (14, 21). This may be related more to the hypertrophic actions of ANG II compared with its contractile activity. Our results did not support a role for PI3-kinase in nifedipine-sensitive calcium channels. This difference could be due to agonist specificity as we used histamine, which does not have hypertrophic properties, compared with ANG II or PDGF (14, 21). In colonic smooth muscle cells, PI3-kinase has been linked to endothelin-induced contraction (30). Interestingly, this and other studies have provided evidence suggesting that different isoforms of PI3-kinase may have different upstream regulators as well as downstream effects (22, 30). This could account for the two distinct roles for PI3-kinase in smooth muscle growth and contraction.

As discussed in the introduction, Brophy and colleagues (12) have proposed a unique role for PI3-kinase in the regulation of contraction. These investigators suggest that PI3-kinase is required to reverse the tonic inhibition of vascular contraction by a cyclic nucleotide/heat shock protein (HSP)20 pathway. Although our results do not directly assess this pathway, our study is in agreement with one important aspect of this hypothesis. Neither our study nor that of Brophy’s study showed a role for MLC phosphorylation in the PI3-kinase-dependent regulation of contraction. These findings are in contrast to work presented by Begum et al. (1), who demonstrated that PI3-kinase-dependent inactivation of Rho kinase results in an increase in MLC phosphae activity and therefore a decrease in MLC phosphorylation levels. A serial connection between PI3-kinase and activation of Rho A activity in smooth muscle function has also been suggested (15, 38). A decrease in PI3-kinase-dependent Rho A activation was associated with a decrease in force, although no direct mechanism at the contractile protein level was proposed (15). Wang and Bitar (38) proposed a role for PI3-kinase in the activation of Rho and enhancement of force but, interestingly, they suggested the end point to involve HSP27 and cytoskeletal remodeling. PI3-kinase also has been implicated in the pathology of vascular smooth muscle. Watts and co-workers (18) and Yang et al. (42) have shown that PI3-kinase is important in hypertension-induced vascular hypersensitivity and ethanol-induced cerebral contractions, respectively.

An intriguing speculation on how PI3-kinase may modulate contraction of smooth muscle comes from studies linking PI3-kinase activity to integrin receptors (19) and p38 MAPKs (41). Gunst and colleagues (32, 33, 39) have proposed a model by which phosphorylation of proteins associated with the attachment plaques in smooth muscle can alter the transduction of the force signal from the contractile apparatus to the cell membrane. If PI3-kinase is activated by receptors associated with these attachment plaques or phosphorylates proteins within the plaques, it is possible that the coupling between the contractile apparatus and the cell membrane will change in such a way that more force per unit myosin ATPase activity will be developed.

In summary, we have demonstrated that inhibition of PI3-kinase reduces force in response to either membrane depolarization or receptor activation. The reduction in steady-state force but not the development of force is accompanied by a concomitant decrease in MLC phosphorylation levels. Our studies suggest that PI3-kinase has at least two distinct roles in the regulation and/or modulation of force. The first is by a minor component whereby PI3-kinase increases the influx of calcium through receptor-operated calcium channels resulting in an increase in MLC phosphorylation levels. The second major pathway is potentially protein kinase C dependent and enhances force independently of changes in either [Ca^{2+}] or MLC phosphorylation levels.

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