Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho kinase

LUKE J. JANSSEN, HWA LU-CHAO, AND STUART NETHERTON
Asthma Research Group, Firestone Institute for Respiratory Health, and Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

Received 22 June 2000; accepted in final form 25 October 2000

Janssen, Luke J., Hwa Lu-Chao, and Stuart Netherton. Excitation-contraction coupling in pulmonary vascular smooth muscle involves tyrosine kinase and Rho kinase. Am J Physiol Lung Cell Mol Physiol 280: L666–L674, 2001.—We investigated the mechanisms that underlie the responses to norepinephrine (NE) and thromboxane (Tx) A₂ (TxA₂) in the canine pulmonary vasculature with fura 2 fluorimetric, intracellular microelectrode, and force transduction techniques. KCl, caffeine, and cyclopiazonic acid elevated intracellular Ca²⁺, whereas voltage-dependent Ca²⁺ release currents were subthreshold for voltage-dependent Ca²⁺ release. However, contractions evoked by NE or the TxA₂ mimetic U-46619 were unaffected by nifedipine or by omitting external Ca²⁺ and were reduced only partially by depleting the internal Ca²⁺ store; furthermore, NE-evoked depolarization was subthreshold for voltage-dependent Ca²⁺ currents. Agonist-evoked contractions were insensitive to inhibitors of protein kinase C (calphostin C and chelerythrine), mitogen-activated protein kinase (PD-98059), and p38 kinase (SB-203580) but were abolished by the tyrosine kinase inhibitor genistein and the Rho kinase inhibitor Y-27632. We conclude that, although Ca²⁺ influx and Ca²⁺ release are sufficient for contraction, they are not necessary for adrenergic or TxA₂ contractions. Instead, excitation-contraction coupling involves the activation of tyrosine kinase and Rho kinase, leading to enhanced Ca²⁺ sensitivity of the contractile apparatus.

mitogen-activated protein kinase; norepinephrine; thromboxane A₂; intracellular calcium; protein kinase C; myosin light chain kinase; myosin light chain phosphatase

THE SIGNALING PATHWAYS THAT UNDERLIE contraction in smooth muscle have been the focus of intense study, and several mechanisms have been identified. Ultimately, contraction in smooth muscle is triggered by phosphorylation at Ser¹⁹ of the regulatory light chain of myosin (13). Generally, this is mediated by the Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK), which, in turn, is activated by the influx of Ca²⁺ across the plasmalemma or the release of Ca²⁺ from an internal store. More recently, it has come to be appreciated that excitation-contraction (E-C) coupling can also involve increased Ca²⁺ sensitivity of the contractile apparatus such that even basal levels of intracellular Ca²⁺ concentration ([Ca²⁺]₁) are sufficient to trigger contraction (14). Some studies (13) found that this latter phenomenon involves protein kinase C (after agonist-mediated activation of membrane receptors, cytosolic G proteins, and phospholipase C, with subsequent generation of diacylglycerol). Others (14) found that increased Ca²⁺ sensitivity involved tyrosine kinases and mitogen-activated protein (MAP) kinases, which, in turn, act on a variety of intracellular targets. For example, Rho kinase seems to phosphorylate one of the subunits of myosin light chain phosphatase (MLCP), thereby suppressing the activity of the latter and leading to a net increase in myosin light chain phosphorylation and contraction (14). Extracellular signal-regulated kinase MAP kinases, on the other hand, phosphorylate caldesmon, thereby removing its inhibitory effect on actin- and thin filament-mediated regulation of actomyosin ATPase activity (4).

Although E-C coupling has been investigated in many smooth muscles, there still remains a general paucity in our understanding of the mechanisms that operate in pulmonary vascular smooth muscles, particularly those in the vein (almost all studies of pulmonary vascular function use pulmonary arteries, even though there are many documented differences between these and pulmonary veins). Neurogenic regulation of the pulmonary vasculature is mediated almost exclusively by adrenergic innervation acting on adrenoceptors on the smooth muscle (8, 9). Also, many arachidonic acid metabolites excite the pulmonary vasculature via action on thromboxane-selective prostanoid receptors including thromboxane A₂ (TxA₂) itself, PGF₂α, and PGD₂ (10), and isoprostanes (15); levels of these autacoids are markedly elevated under pathophysiological conditions such as hypertension, inflammation, acute lung injury, and oxidative stress (2, 16).

In this study, we sought to examine the E-C coupling mechanisms that underlie responses to the adrenergic agonist norepinephrine (NE) and the TxA₂ mimetic U-46619 in canine pulmonary arteries and veins.

METHODS

Preparation of tissues. Whole lobes of lung were obtained from dogs that had been euthanized with pentobarbital so-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.


dium (100 mg/kg); these were pinned out in physiological saline, and ring segments (3- to 4-mm long) of pulmonary artery and pulmonary vein (outer diameter 2–8 mm; third- to fifth-division vessels) were excised. Tissues were either used immediately or stored at 4°C for use the next day; we found no functional differences in tissues that were studied immediately compared with those used after 24 h of refrigeration.

Muscle bath technique. Ring segments were mounted in 3-ml muscle baths with stainless steel hooks inserted into the lumen; care was taken not to damage the endothelium while the hooks were inserted. One hook was fastened to a Grass FT03 force transducer with silk thread (Ethicon 4-0); the other was attached to a Plexiglas rod that served as an anchor. Tissues were bathed in Krebs-Ringer buffer (for composition, see Solutions and chemicals) containing 10 μM indomethacin, bubbled with 95% O2-5% CO2, and maintained at 37°C; preload tension was ~1 g (determined to allow maximal responses). Isometric changes in tension were digitized (2 samples/s) and recorded with an on-line program (DigiMed System Integrator; MicroMed, Louisville, KY). Tissues were superfused at a rate of 2–3 ml/min.


gently triturated. Cells were studied with the membrane-permeant form of fura 2 (fura 2-AM; 2 mM for 30 min at 37°C) and then exposed to pharmacological inhibitors or experimental conditions (e.g., nominally Ca2+-free bathing medium) for 20–30 min before the experiments were begun. Some tissues were dried and weighed to express the contractile responses as grams of tension developed per milligram of dry tissue weight.

Fura 2 flurometry. Ring segments (0.5–1.0 g wet wt) of the pulmonary vasculature were minced and transferred to dissociation buffer (for composition, see Solutions and chemicals) containing 2.7 U/ml of collagenase (type IV), 12.5 U/ml of elastase (type IV), and 1 mg/ml of BSA, then were either used immediately or stored at 4°C for use the next day. We have previously found that cells used immediately and those used after refrigeration exhibit similar functional responses (7). To isolate single cells, tissues in enzyme-containing solution were incubated at 37°C for 60–120 min and then gently triturated. Cells were studied with a filter-based photometer-driven system (DeltaScan, Photon Technology International, South Brunswick, NJ). After being settled on a glass coverslip mounted on a Nikon TMD inverted microscope, the cells were loaded with the membrane-permeant form of fura 2 (fura 2-AM; 2 μM for 30 min at 37°C) and then were superfused continuously with Ringer buffer (2–3 ml/min). The cells were alternately illuminated (0.5 Hz) at the excitation wavelengths and the emitted fluorescence (measured at 510 nm) induced by 340-nm excitation and that induced by 380-nm excitation were measured with a photo multiplier tube assembly. The fluorescence ratio was converted to [Ca2+], with previously published methods (3). The fluorescence ratio values under saturating and Ca2+-free conditions (Rmax and Rmin, respectively) were obtained previously; the Ca2+-fura 2 dissociation constant (Kd) was assumed to be 224 nM (3). Agonists were applied by pressure ejection from a puffer pipette (Picospritzer II, General Valve, Fairfield, NJ).

Intracellular microelectrodes. Intact tissues were carefully pinned out in a chamber with a bath volume of ~10 ml; Krebs-Ringer buffer (for composition, see Solutions and chemicals) was bubbled with 95% O2-5% CO2, heated to 37°C, and superfused over the tissues at a rate of ~3 ml/min. Conventional microelectrodes (with a tip resistance of 30–80 MΩ when filled with 3 M KCl) were pulled from borosilicate capillary tubes and used to impale single smooth muscle cells. Membrane potential changes were observed on a dual-beam oscilloscope (Tektronix D13; 5A22N differential amplifier; 5B12 dual-time base) and recorded on 0.25-inch magnetic tape with a Hewlett-Packard instrumentation recorder.

Portions of the recorded data were played back, digitized (Digitdata 1200), sampled at 500 kHz with pCLAMP 6 software (Axon Instruments, La Jolla, CA), and then filtered with the use of pCLAMP 6 and/or exported to SigmaPlot (Jandel, Corte Madera, CA) for graphic presentation.

Solutions and chemicals. Dissociation buffer (in mM) 125 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 0.25 EDTA, 10 d-glucose, and 10 taurine, pH 7.0. Single cells were studied in Ringer buffer containing (in mM) 130 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 20 HEPES, and 10 d-glucose, pH 7.4. Intact tissues were studied with Krebs-Ringer buffer containing (in mM) 116 NaCl, 4.2 KCl, 2.5 CaCl2, 1.6 NaH2PO4, 1.2 MgSO4, 22 NaHCO3, and 11 d-glucose, bubbled to maintain pH at 7.4. Indomethacin (10 μM) was also added to the latter to prevent generation of cyclooxygenase metabolites of arachidonic acid.

Chemicals were obtained from Sigma, with the exception of Y-27632 (kindly provided by A. Yoshimura, Welfide, Osaka, Japan). Stock solutions (10−2 M) of cyclopiazonic acid (CPA), genistein, daidzen, PD1885, SB-203580, calphostin C, and chelerythrine were made up in DMSO; and nifedipine and Y-27632 were made up in 95% absolute ethanol. All other agents were prepared as aqueous solutions.

Data analysis. Responses are reported as means ± SE; n refers to the number of animals. Statistical comparisons were made with Student’s t-test or ANOVA (followed by Student-Newman-Keuls test for pairwise comparisons) as appropriate. P < 0.05 was considered significant.

RESULTS

Mechanical responses to NE and to a Tx mimetic. NE evoked contractions in a dose-dependent fashion in both pulmonary arterial smooth muscle (PASM) and pulmonary venous smooth muscle (PVSM; Fig. 1). PVSM was significantly more sensitive than PASM to NE (log ED50 values were −7.5 ± 0.1 and −6.7 ± 0.1 μM, respectively), but it was not significantly more responsive (when normalized for tissue dry weight, peak responses were 2.2 ± 0.4 and 1.6 ± 0.2 g/mg, respectively; n = 5 dogs). These two vascular tissues also differed in their sensitivity to the Tx mimetic U-46619, with PASM being essentially unresponsive, whereas PVSM exhibited large dose-dependent contractions even at submicromolar concentrations of U-46619 (Fig. 1). The log ED50 value for U-46619 was −8.2 ± 0.2.

The contractions evoked by NE and by U-46619 were also notably different with respect to the rate at which they developed, the latter developing much more slowly than the adrenergic responses. Figure 1C highlights the different rates of rise of contractions to U-46619 and NE at concentrations that achieved relatively similar peak heights (i.e., both reached ~50% of the KC1 response obtained during the equilibration period and approximately one-third of the peak response to the agonist at the end of the experiment); the adrenergic response was considerably faster than that triggered by U-46619. We determined the moment-by-moment rates of rise for responses to the entire range of concentrations of U-46619 and NE used and found the mean peak rate of rise for NE to be markedly and significantly greater (78 ± 10 mg/s at 10−6 M NE; n = 10 dogs) than that for U-46619 (12 ± 10 mg/s at 10−6 M U-46619; n = 19 dogs).

Downloaded from http://ajplung.physiology.org/ by 10.220.33.2 on March 30, 2017
The differences in the actions of NE and U-46619 would suggest that these two agonists act through different intracellular signaling pathways. We therefore sought to elucidate the mechanisms that underlie E-C coupling for these two agonists.

Role of electromechanical coupling mechanisms in contraction of the pulmonary vasculature. KCl (60 mM) elevated $[Ca^{2+}]_{i}$ and evoked dose-dependent contractions in both PASM and PVSM (Fig. 2). The KCl-evoked change in $[Ca^{2+}]_{i}$ was slower and smaller in magnitude than that evoked by caffeine (32.6 $\pm$ 14.9%; 17 cells; $n = 5$ dogs). In addition, this change was generally sustained, although “oscillations” in $[Ca^{2+}]_{i}$ were seen in one cell (Fig. 2A, right). These findings indicate that membrane depolarization and voltage-dependent $Ca^{2+}$ influx alone are sufficient to trigger contraction and might mediate the responses to NE and U-46619.

Pulmonary vascular tissues were pretreated with 10$^{-6}$ M nifedipine (a blocker of voltage-dependent $Ca^{2+}$ channels) or bathed in nominally $Ca^{2+}$-free medium (0.1 mM EGTA) to prevent voltage-dependent $Ca^{2+}$ influx. Neither nifedipine nor removal of external $Ca^{2+}$ had any significant effect on the dose-response relationship for NE or U-46619 in either PASM or PVSM (Fig. 3), suggesting that voltage-dependent $Ca^{2+}$ influx is not necessary to produce these responses.

We also examined the electrophysiological responses to adrenergic stimulation in intact pulmonary tissues; these have been shown elsewhere (8, 9) to be mediated almost exclusively by adrenergic mechanisms. At rest, the mean membrane potential was $-54.3 \pm 3.3$ mV ($n = 10$ dogs), and there was no evidence of oscillations, action potentials, or phasic activity. Electrical stimulation of the tissues evoked excitatory junction potentials, which reached a peak of 15.6 $\pm$ 7.4 mV ($n = 10$ dogs) within 1–2 s after the onset of stimulation and then resolved back to prestimulation levels within 10 s after electrical stimulation ceased (Fig. 4). These depolarizations never exceeded a potential of $-20$ mV (i.e., the threshold at which L-type $Ca^{2+}$ channels open) (11) and were never accompanied by action potentials. Likewise, exogenous NE evoked sustained membrane depolarization to $-43.8 \pm 8.1$ mV ($10^{-6}$ M NE) or to $-30.3 \pm 15$ mV ($10^{-5}$ M NE). Taken together, these findings indicate that electromechanical coupling mechanisms alone are not absolutely necessary for agonist-evoked contraction in the pulmonary vasculature. Next, we examined the contribution made by the internal $Ca^{2+}$ stores in mediating these contractions.

Release of internal $Ca^{2+}$ is sufficient to evoke contractions. Caffeine (5 mM) evoked large $Ca^{2+}$ transients in PASM and PVSM (Fig. 5A). These reached a mean peak magnitude of 364 $\pm$ 24 nM ($n = 26$ dogs) within 1–2 s after the application of caffeine and then decayed almost as quickly to a plateau value of 96 $\pm$ 81 nM, a value that was relatively sustained. When the application of caffeine ended, $[Ca^{2+}]_{i}$ fell back to prestimulation levels; in some cases, $[Ca^{2+}]_{i}$ even briefly exceeded prestimulation levels, creating an “undershoot.”

In intact tissues, caffeine evoked transient contractions that were considerably larger in PVSM compared with PASM (Fig. 5B), with a mean magnitude of 0.2 $\pm$ 0.1 and 1.1 $\pm$ 0.2 g/mg dry wt, respectively. The non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine ($10^{-5}$ M) evoked no significant change
in basal tone (n = 4 dogs; data not shown), indicating that the effects of caffeine are not secondary to changes in cyclic nucleotide levels.

CPA also elevated [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 5C), but these responses had a much slower time course, reaching a peak ~60 s after the application of CPA and resolving over the course of 5–10 min, and were smaller (mean magnitude of 80 ± 18 nM) than those evoked by caffeine. CPA evoked no change in tone in the majority of intact canine tissues studied (>90 tissues; data not shown).

Fig. 2. KCl-evoked excitatory responses. A: fluorimetric responses recorded in 2 different fura 2-loaded PASM cells exposed to 60 mM KCl (K\textsuperscript{+}). Responses to 10 mM caffeine (caff) are also shown for reference. \(\frac{F_{340}}{F_{380}}\), fluorescence ratio. B: tension recording in a PASM cell exposed to 60 mM KCl.

Fig. 3. Role of external Ca\textsuperscript{2+} in NE- and U-46619-evoked contractions. A: original traces from PASM strips bathed in normal medium (left), in the presence of nifedipine (+ nifed; middle), or in the absence of Ca\textsuperscript{2+} (0 Ca\textsuperscript{2+}; right) and stimulated with NE. Nos. at top, log molar concentrations. B: mean dose-response relationships for NE and U-46619 in control tissues and tissues bathed in Ca\textsuperscript{2+}-free medium (plus 0.1 mM EGTA) and in the presence of nifedipine (10\textsuperscript{-6} M) as indicated. Data are means ± SE; n = 5 dogs/group.
shown). In those tissues that did exhibit a contractile response on application of CPA (1 artery, 14 veins), tension increased relatively slowly, with a time course similar to the fluorimetric responses described in *Role of electromechanical coupling mechanisms in contraction of the pulmonary vasculature*, and were never >10% of the magnitude of the response evoked by 60 mM KCl.

Because caffeine and CPA both act on the internal Ca\(^{2+}\) store and neither activates G proteins directly, these findings indicate that release of internally sequestered Ca\(^{2+}\) may be sufficient to trigger contraction. We therefore sought to examine the role of the sarcoplasmic reticulum in mediating the responses to autacoids.

**Agonist-evoked contractions are mediated in part by release of internal Ca\(^{2+}\).** In single PASM and PVSM cells, NE (10\(^{-5}\) M) evoked a transient elevation of [Ca\(^{2+}\)]\(_i\), with a mean magnitude (301 ± 32 nM; \(n = 18\) dogs) and time course that were similar to those of the caffeine-evoked responses (Fig. 6A). In *Role of electromechanical coupling mechanisms in contraction of the pulmonary vasculature*, we showed that these responses were accompanied by contraction (Fig. 1). It is worth pointing out, however, that although the fluorimetric responses to NE and caffeine appeared somewhat similar with respect to time course (both rising and falling within minutes; compare Figs. 5A and 6A), the mechanical responses to NE were sustained (Fig. 1), which was in stark contrast to the transient contractions triggered by caffeine (Fig. 5).

The Ca\(^{2+}\) transients and contractions evoked by NE differed in their sensitivity to CPA (3 \(\times\) 10\(^{-5}\) M). Whereas the fluorimetric responses were reversibly abolished (to 6 ± 5% of the control value; \(n = 7\) dogs; Fig. 6A), substantial contractions could still be evoked in tissues pretreated with CPA (Fig. 6B); the mean tension evoked by 10\(^{-5}\) M NE in the presence of CPA (plus 10 mM caffeine to promote Ca\(^{2+}\) store depletion) was 68 ± 5% of that evoked in the absence of CPA (\(n = 7\) dogs; Fig. 6B). Caffeine alone (10 mM) had no significant effect on the NE dose-response relationship (data not shown).

U-46619 (10\(^{-7}\) M), on the other hand, did not evoke any change in [Ca\(^{2+}\)]\(_i\) in 9 of 16 PVSM cells tested, even though all 16 cells exhibited substantial responses to caffeine (data not shown). PASM cells were not tested because they do not exhibit a mechanical response to U-46619 (Fig. 1). The remaining seven PVSM cells showed variable fluorimetric responses to U-46619, ranging from 0 to 250 nM (mean of 92 ± 88 nM). In addition, contractions evoked by U-46619 were completely unaffected by CPA (\(n = 5\) dogs; Fig. 6B).

**Roles of various kinases in agonist-evoked contractions.** We examined the effects of several protein kinase inhibitors on the contractile responses evoked by the \(\alpha\)-adrenergic agonist phenylephrine (PE) and by U-46619; mean dose-response relationships are given...
in Fig. 7, and statistical analysis of the effects of these antagonists on peak contractions is given in Table 1. Calphostin C (1 \( \mu \text{M} \)) and chelerythrine (1 \( \mu \text{M} \)), inhibitors of protein kinase C with differing mechanisms of action, reduced PE contractions slightly but had no effect on U-46619-evoked contractions. The tyrosine kinase inhibitor genistein (100 \( \mu \text{M} \)), on the other hand, abolished adrenergic responses and markedly attenuated responses to U-46619. Daidzen (the less active analog of genistein; 100 \( \mu \text{M} \)) exerted only a fraction of the inhibitory effect of genistein. To ascertain which MAP kinases might be mediating the tyrosine kinase-sensitive contractions, we tested the effects of the p38 kinase inhibitor SB-203580 (25 \( \mu \text{M} \)), the MAP kinase kinase inhibitor PD-98059 (50 \( \mu \text{M} \)), and the Rho kinase inhibitor Y-27632 (10 and 100 \( \mu \text{M} \)). The inhibitory effects of genistein on PE and U-46619 contractions were mimicked by Y-27632, whereas SB-203580 and PD-98059 were largely ineffective.

DISCUSSION

In this study, we sought to characterize the intracellular pathways that underlie E-C coupling in pulmonary arteries and pulmonary veins, particularly those involved in the responses to adrenergic and TxA2 stimulation.

Substantial contractions could be evoked in these tissues by KCl, caffeine, and CPA. These agents act by mobilizing Ca\(^{2+}\) in a G protein-independent fashion, suggesting that elevation of [Ca\(^{2+}\)]\(_{i}\) alone, as a result of either increased Ca\(^{2+}\) influx (KCl) or release from the sarcoplasmic reticulum (caffeine, CPA), is sufficient for contraction. Despite this, we obtained several lines of evidence that suggest that electromechanical coupling and Ca\(^{2+}\) mobilization are not necessary for mechanical responses to adrenergic or TxA2 stimulation.

First, although NE triggers a Ca\(^{2+}\) response, the contractions produced were at best marginally affected by the blocking of Ca\(^{2+}\) influx with nifedipine or omitting external Ca\(^{2+}\) and were reduced only partially by depletion of the internal Ca\(^{2+}\) store with CPA. This had previously been shown for pulmonary arteries (6) but not for pulmonary veins. Likewise, U-46619 did not evoke a change in [Ca\(^{2+}\)]\(_{i}\), and the mechanical response that it evoked was unaffected by nifedipine, Ca\(^{2+}\) removal, or CPA.

Second, there was no correlation between the fluorimetric and mechanical responses evoked by these agonists. Caffeine and NE evoked Ca\(^{2+}\) transients with a similar magnitude and time course, yet the caffeine contractions were very small and transient in the pulmonary arteries but relatively large and transient in the pulmonary veins, whereas the NE contractions in both tissues were large and sustained. Likewise, U-46619 evoked little or no Ca\(^{2+}\) response but did evoke large and sustained contractions. Previous studies comparing U-46619-evoked responses to NE responses in rabbit pulmonary smooth muscle (5) or to carbachol responses in porcine epicardial coronary arteries (1) also found that the adrenergic and cholinergic responses were accompanied by a substantial Ca\(^{2+}\) transient, whereas U-46619 acted without elevating [Ca\(^{2+}\)]\(_{i}\).
Third, the membrane depolarization evoked by nerve-released and exogenously added NE rarely exceeded −40 mV and never exceeded −20 mV; voltage-dependent Ca$^{2+}$ currents are not discernible in this voltage range (11).

Collectively, these data indicate that electromechanical coupling and Ca$^{2+}$ mobilization are sufficient for contraction in general but are not necessary for contractions evoked by these two physiologically relevant agonists.

Table 1. Effect of various kinase inhibitors on adrenergic- and U46619-evoked contractions

<table>
<thead>
<tr>
<th></th>
<th>Pulmonary Artery</th>
<th>Pulmonary Vein</th>
<th>U-46619</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PE</td>
<td>PE</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83 ± 7° (11)</td>
<td>82 ± 9 (16)</td>
<td>257 ± 24 (10)</td>
</tr>
<tr>
<td>+ Calphostin C (10$^{-6}$ M)</td>
<td>80 ± 6 (5)</td>
<td>60 ± 7 (5)</td>
<td>242 ± 27 (4)</td>
</tr>
<tr>
<td>+ Chelerythrine (10$^{-6}$ M)</td>
<td>94 ±10 (5)</td>
<td>71 ± 12 (5)</td>
<td>ND</td>
</tr>
<tr>
<td>+ Genistein (10$^{-4}$ M)</td>
<td>11 ± 3° (4)</td>
<td>10 ± 6° (10)</td>
<td>25 ± 14° (5)</td>
</tr>
<tr>
<td>+ Daidzen (10$^{-4}$ M)</td>
<td>ND</td>
<td>25 ± 7° (4)</td>
<td>188 ± 30 (4)</td>
</tr>
<tr>
<td>+ SB-203580 (2.5 × 10$^{-5}$ M)</td>
<td>104 ± 9 (4)</td>
<td>90 ± 7 (6)</td>
<td>230 ± 15 (4)</td>
</tr>
<tr>
<td>+ PD-98059 (5 × 10$^{-5}$ M)</td>
<td>79 ± 13 (4)</td>
<td>73 ± 10 (6)</td>
<td>171 ± 22 (4)</td>
</tr>
<tr>
<td>+ Y-27632 (10$^{-6}$ M)</td>
<td>15 ± 9° (5)</td>
<td>8 ± 7° (4)</td>
<td>203 ± 18 (5)</td>
</tr>
<tr>
<td>+ Y-27632 (10$^{-4}$ M)</td>
<td>24 ± 10° (4)</td>
<td>1 ± 3° (5)</td>
<td>113 ± 15° (5)</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as percent response to 60 mM KCl of peak contractions evoked by either 10$^{-4}$ phenylephrine (PE) or 10$^{-7}$ M U-46619; nos. in parentheses, no. of dogs. ND, not done. *Significantly different from control, P < 0.05 by ANOVA.
Instead, the data suggest that NE and the Tx mimetic act primarily through enhanced Ca\(^{2+}\) sensitivity of the contractile apparatus. Other groups have investigated this phenomenon in various smooth muscle preparations and found it to involve protein kinase C or tyrosine kinase-triggered MAP kinases (14). We found that the protein kinase C inhibitors calphostin C and chelerythrine had no effect against U-46619 contractions and reduced NE contractions only partially, whereas genistein abolished the response to both agonists. We went on to study which MAP kinase(s) might be involved, finding adrenergic and U-46619 contractions to be essentially insensitive to specific inhibitors of MAP kinase kinase (PD-98509) or of p38 kinase (SB-203580), whereas the Rho kinase inhibitor Y-27632 reproduced the near total inhibitory effect of genistein. Thus E-C coupling in the pulmonary vasculature appears to be mediated largely by agonist-induced activation of tyrosine kinase, which, in turn, activates Rho kinase; the target of the latter is not clear from these data, but Somlyo and Somlyo (14) found that Rho kinase inhibits MLCP activity, leading to a net increase in myosin light chain phosphorylation and, hence, contraction. The contractions that remain in the presence of these tyrosine and Rho kinase inhibitors may be a product of Ca\(^{2+}\) mobilization.

The interpretation that these agonists act primarily by increasing the Ca\(^{2+}\) sensitivity of the contractile apparatus does not eliminate the importance of Ca\(^{2+}\) mobilization in E-C coupling. That is, suppression of MLCP activity alone will not lead to contraction; there must also be some ongoing level of kinase activity to lead to a net increase in the phosphorylation state of myosin. Thus agonists such as NE act in part by increasing MLCK activity while simultaneously suppressing MLCP activity. This would explain the slower rate of rise of U-46619-evoked contractions compared with those evoked by NE (Fig. 1). Although both act to suppress MLCP activity, NE (but not U-46619) also triggers a substantial Ca\(^{2+}\) transient that would greatly increase MLCK activity.

These findings are important from a clinical viewpoint: knowing that the physiologically relevant agonists tested here act in the pulmonary vascular bed primarily via a mechanism that does not involve Ca\(^{2+}\) mobilization, it should not be surprising that only a small percentage of patients with pulmonary hypertension respond favorably to Ca\(^{2+}\) channel blockers (12). Our data suggest that it would be much more effective to somehow target tyrosine and/or Rho kinases. Delivery via inhalation of candidate therapeutic agents may minimize the potential of undesirable side effects related to affecting such kinases in other tissues. Prostacyclin, which is currently used to treat primary pulmonary hypertension, may act through functional antagonism of the signaling pathway, which we describe in this study.

In this study, as in a previous one by our laboratory (7), we obtained further evidence for important functional differences between PASM and PVSM. For example, these two tissues differ substantially with respect to the mechanical responses evoked by NE, U-46619, and caffeine. Previously, we found a marked difference in their sensitivity to electric field stimulation (which evokes an adrenergic response) and to the nitric oxide donor S-nitroso-N-acetylpenicillamine. In ongoing studies, we have also found that they differ in the manner in which they regulate [Ca\(^{2+}\)]. Collectively, our findings emphasize that conclusions made with the use of pulmonary arteries (which are the tissues usually studied) cannot be extrapolated to apply to pulmonary veins and that a thorough understanding of pulmonary vascular physiology requires further studies with pulmonary veins. Moreover, our finding that PVSM is much more sensitive than PASM to several spasmogens is important from the point of view of edema formation, given that the latter is in part determined by the transcapillary bed pressure gradient.

We conclude that although Ca\(^{2+}\) mobilization (via voltage-dependent Ca\(^{2+}\) influx and/or release of internally sequestered Ca\(^{2+}\)) is sufficient to trigger contraction in the pulmonary vasculature, it is not strictly necessary for the mechanical responses evoked by NE and TxA\(_2\). Instead, these spasmogens act primarily through tyrosine kinase and Rho kinase, which likely suppress MLCP activity and thereby increase the Ca\(^{2+}\) sensitivity of the contractile apparatus.

We gratefully acknowledge the gift of Y-27632 from Dr. A. Yoshimura (Welfide, Osaka, Japan) and the technical assistance of Matt Ostrowski and Kai Mardi, who performed the intracellular microelectrode recordings. These studies were supported by a grant from the Medical Research Council of Canada, a Career Award (to L. J. Janssen) from the Pharmaceutical Manufacturers Association of Canada, and the Medical Research Council of Canada.

REFERENCES


