Mechanisms of endothelin-1-induced contraction in pulmonary arteries from chronically hypoxic rats

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Mechanisms of endothelin-1-induced contraction in pulmonary arteries from chronically hypoxic rats. Am J Physiol Lung Cell Mol Physiol 290: L284–L290, 2006. First published September 9, 2005; doi:10.1152/ajplung.00449.2004.—Endothelin-1 (ET-1), a potent vasoconstrictor, is believed to contribute to the pathogenesis of hypoxic pulmonary hypertension. Previously we demonstrated that contraction induced by ET-1 in intrapulmonary arteries (IPA) from chronically hypoxic (CH) rats occurred independently of changes in intracellular Ca2+ concentration ([Ca2+]i), suggesting that ET-1 increased Ca2+ sensitivity. The mechanisms underlying this effect are unclear but could involve the activation of myosin light chain kinase, Rho kinase, PKC, or tyrosine kinases (TKs), including those from the Src family. In this study, we examined the effect of pharmacological inhibitors of these kinases on maximum tension generated by IPA from CH rats (10% O2 for 21 days) in response to ET-1. Experiments were conducted in the presence of nifedipine, an L-type Ca2+ channel blocker, to isolate the component of contraction that occurred without a change in [Ca2+]i. The mean change in tension caused by ET-1 (10−8 M) expressed as a percent of the maximum response to KCl was 184.0 ± 39.0%. This response was markedly inhibited by the Rho kinase inhibitors Y-27632 and HA-1077 and the TK inhibitors genistein, tyrphostin A23, and PP2. In contrast, staurosporine and GF-109203X, inhibitors of PKC, had no significant inhibitory effect on the tension generated in response to ET-1. We conclude that the component of ET-1-induced contraction that occurs without a change in [Ca2+]i in IPA from CH rats requires activation of Rho kinase and TKs, but not PKC.

PULMONARY HYPERTENSION ASSOCIATED with prolonged alveolar hypoxia has been attributed to both persistent active vasoconstriction and increased muscularity of the pulmonary vasculature. In early studies, administration of vasodilators only partially reversed the increase in pulmonary arterial pressure secondary to chronic hypoxia (18, 26), suggesting a large fixed component of hypoxic pulmonary hypertension due to intimal proliferation of smooth muscle. However, recent evidence has shown that increased smooth muscle cell tone may play a more prominent role than remodeling in the elevation of pulmonary vascular resistance and arterial pressure secondary to chronic hypoxia. For example, increased pulmonary arterial pressure in chronically hypoxic (CH) rats has been shown to be acutely reversible by inhibitors of Rho kinase, a mediator of myofilament contractility (9, 22). Moreover, studies in resistance-sized pulmonary arteries showed that the proliferation of smooth muscle cells had no effect on lumen diameter (13), providing more evidence against increased pulmonary vascular resistance due to luminal narrowing secondary to structural remodeling.

The mechanisms leading to vasoconstriction in response to chronic hypoxia and the subsequent development of pulmonary hypertension remain unclear, but may be due, in part, to the release of endothelium-derived contracting factors. One such agent, endothelin-1 (ET-1) is a potent vasoactive peptide that is abundant in the pulmonary vasculature. Exposure to both acute and chronic hypoxia has been shown to increase ET-1 gene expression and plasma ET-1 levels (5, 7). Moreover, treatment with ET-1 receptor antagonists has been shown to prevent and reverse pulmonary hypertension in rat models of chronic hypoxia (4, 5). Thus it has become widely accepted that ET-1 contributes to the pathogenesis of hypoxic pulmonary hypertension.

After prolonged exposure to hypoxia, ET-1 sensitivity is increased, endothelin-A receptors are upregulated, and ET-1-induced vasodilation is altered (6, 20, 28), suggesting modifications in ET-1-mediated regulation of pulmonary vascular tone. One alteration induced by chronic hypoxia involves activation of an ET-1 contractile pathway that is distinct from that which is activated during normoxia. Under normoxic conditions, ET-1 causes release of Ca2+ from intracellular stores, PKC-dependent inhibition of voltage-gated K+ (Kv) channels, and subsequent membrane depolarization and Ca2+ influx through voltage-dependent calcium channels (VDCCs) (30, 33, 34). This increase in intracellular calcium concentration ([Ca2+]i), due to both influx and release, activates myosin light chain kinase (MLCK), resulting in phosphorylation of myosin light chains and contraction (39, 40). Indeed, ET-1-induced contraction in intrapulmonary arteries (IPA) from normoxic animals can be markedly inhibited by treatment with nifedipine, a VDCC antagonist (35), as well as ryanodine (41), confirming the roles of Ca2+ influx through L-type Ca2+ channels and Ca2+ release from sarcoplasmic reticulum (SR) as essential for the development of contraction. In contrast, in pulmonary artery smooth muscle isolated from CH rats, the ET-1-induced rise in [Ca2+]i is markedly reduced and is due entirely to influx through VDCCs, as no change in [Ca2+]i in response to ET-1 is observed in cells pretreated with nifedipine (35). Moreover, pretreatment with a concentration of nifedipine that completely blocked KCl-induced contraction inhibited maximum contraction in response to ET-1 by only 20% (35). These data suggest that after exposure to chronic hypoxia, 80% of ET-1-induced contraction occurred independently of a change in [Ca2+]i. This phenomenon is known as Ca2+ sensitization of the contractile apparatus.

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Signaling pathways responsible for Ca\(^{2+}\) sensitization in smooth muscle remain unclear. Investigation has shown that PKC may play a role in Ca\(^{2+}\)-independent contraction in systemic vascular smooth muscle (12, 21, 24). ET-1 also activates tyrosine kinases (TKs), which have been shown to participate in the regulation of smooth muscle cell tone (38). The c-Src family of TKs has generated particular interest and may play a role in Ca\(^{2+}\) sensitization, as c-Src phosphorylation increased in the presence of ET-1 (37). Most recently, investigation into the mechanisms responsible for Ca\(^{2+}\) sensitization has focused on the Rho kinase signaling pathway. Studies in porcine coronary arteries (24), rat IPA (29), and isolated, perfused arteries were divided into 3- to 4-mm segments. The endothelium was disrupted by rubbing the luminal surface with the wooden end of a cotton swab, and each segment was suspended between two stainless steel stirrups in organ chambers filled with modified KRB solution heated to 37°C and gassed with 16% O2-5% CO2 to maintain a pH of 7.4. One stirrup was connected to a strain gauge (model FY03, Grass Instruments), and tension was recorded using a PowerLab computer system from ADInstruments (Colorado Springs, CO).

In this study, we examined various signaling pathways that may be involved in generating [Ca\(^{2+}\)]\(_i\)-independent contraction in response to ET-1 in pulmonary arterial smooth muscle from CH rats. Using arteries mounted for isometric tension recording, we examined the effect of pharmacological inhibitors of MLCK, TK, PKC, and Rho kinase on ET-1-induced contraction and compared these results with effects on KCl-induced (nonreceptor-mediated) contraction.

**METHODS**

**Hypoxic exposure.** All protocols were approved by the Johns Hopkins Animal Care and Use Committee. Adult male Wistar rats (150–200 g) were placed in a Plexiglas chamber continuously gassed with a mixture of air plus N\(_2\) (10% O\(_2\)) for 3 wk. Normoxic animals were kept in room air. Oxygen levels were measured with a gas analyzer (ProOx 110; Biospherix, Redfield, NY), and the animals were allowed free access to food and water. Rats were removed from the chamber for <5 min twice weekly to replenish food and water supplies and to clean cages. We have previously demonstrated the development of pulmonary hypertension in this model (35, 36). At the end of the exposure period, the animals were heparinized (500 units ip) and anesthetized with pentobarbital sodium (130 mg/kg ip), and the heart and lungs were removed en bloc. Isometric tension recording. IPAs (200–600 μm) were dissected from the left upper and right lower lobes. The arteries were placed in oxygenated Krebs-Ringer bicarbonate (KRB) solution containing (in mM): 118 NaCl, 4.7 KCl, 0.57 MgSO\(_4\), 1.18 KH\(_2\)PO\(_4\), 25.0 NaHCO\(_3\), 10.0 dextrose, and 2.5 CaCl\(_2\). After removal of connective tissue, arteries were divided into 3- to 4-mm segments. The endothelium was disrupted by rubbing the luminal surface with the wooden end of a cotton swab, and each segment was suspended between two stainless steel stirrups in organ chambers filled with modified KRB solution heated to 37°C and gassed with 16% O2-5% CO2 to maintain a pH of 7.4. One stirrup was connected to a strain gauge (model FY03, Grass Instruments), and tension was recorded using a PowerLab computer-linked system from ADInstruments (Colorado Springs, CO).

The arteries were stretched to a resting tension of 2 g in 0.5-g increments over a period of 40 min. The viability of each segment was tested by adding KCl (80 mM) to the bath solution, and exposure to phenylephrine (3 × 10\(^{-7}\) M) followed by acetylcholine (10\(^{-6}\) M) was used to verify endothelium disruption. Arteries that diluted >20% to acetylcholine or did not contract to KCl were discarded. A second KCl exposure (80 mM) to block Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels. We previously demonstrated that this concentration of nifedipine completely blocked the increase in [Ca\(^{2+}\)]\(_i\) induced by ET-1 (10\(^{-8}\) M) in pulmonary arterial smooth muscle cells from CH rats (35). Under these conditions, ET-1 (10\(^{-8}\) M) caused a sustained increase in isometric tension in IPAs isolated from CH rats (Fig. 1A) that generally stabilized in 10 min. Maximum contraction in response to ET-1 was measured at 25 min after beginning the ET-1 challenge and normalized to the increase in tension induced in the same artery by 80 mM KCl (KCl\(_{\text{max}}\)). In IPAs from CH rats, the mean change in tension in response to KCl was 0.55 ± 0.05 g, and ET-1-induced contraction in the presence of nifedipine was found to be 106.4 ± 11.8% of KCl\(_{\text{max}}\) at 25 min.

Effect of MLCK inhibition on contractile responses to ET-1 and KCl. The Ca\(^{2+}\)/calmodulin-mediated MLCK signaling pathway is an essential component of ET-1-induced contraction under normoxic conditions (1). To test the involvement of this pathway in contraction in response to ET-1 during chronic hypoxia, the MLCK inhibitor ML-9 was used. ML-9 decreased baseline tension by 4.87 ± 2.51% (Table 1), suggesting that activation of MLCK contributed to active sustained contraction.
under basal conditions during chronic hypoxia. In arteries pretreated with 50 μM ML-9, a concentration within the range found to inhibit MLCK activity and contractile responses (14), ET-1-induced contraction was significantly reduced to 41.5 ± 10.8% of KClmax (Fig. 1A; *P < 0.05 by ANOVA). Because ET-1-induced contraction was only partially reduced when MLCK was inhibited, we tested the efficacy of ML-9 in our preparation by examining its effect on contraction in response to KCl (Fig. 1B). Indeed, pretreatment with ML-9 nearly abolished KCl-induced contraction (P < 0.05 by ANOVA), reducing generated tension to 7.1 ± 18.6% of maximum (Fig. 1C).

**Table 1. Effect of pharmacological inhibitors on baseline tension**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>%Change in Baseline Tension</th>
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<tbody>
<tr>
<td>Y-27632, 10 μM</td>
<td>0.89±0.26</td>
</tr>
<tr>
<td>HA-1077, 10 μM</td>
<td>0.11±0.08</td>
</tr>
<tr>
<td>Genistein, 100 μM</td>
<td>0.13±0.08</td>
</tr>
<tr>
<td>Daidzein, 100 μM</td>
<td>−5.41±1.71*</td>
</tr>
<tr>
<td>Tyrphostin A23, 100 μM</td>
<td>−5.21±1.8</td>
</tr>
<tr>
<td>PP2, 100 μM</td>
<td>0.25±0.19</td>
</tr>
<tr>
<td>Staurosporine, 1 nM</td>
<td>−6.6±3.3</td>
</tr>
<tr>
<td>GF-109203X, 30 nM</td>
<td>−1.0±1.5</td>
</tr>
<tr>
<td>ML-9, 10 μM</td>
<td>−4.87±2.51*</td>
</tr>
</tbody>
</table>

Percent change in initial baseline tension in rat IPAs after treatment with pharmacological inhibitors of Rho kinase (n = 6 for Y-27632; n = 3 for HA-1077), tyrosine kinases (n = 3 for genistein; n = 4 for tyrphostinA23; n = 4 for daidzein; n = 4 for PP2), PKC (n = 3 for staurosporine; n = 4 for GF-109203X), and myosin light chain (n = 3 for ML-9). *P < 0.05 from control (by paired Student’s t-test).

**Fig. 2.** A: average maximum tension (left) generated by rat IPAs in response to ET-1 in the absence and presence of Y-27632 (Y; 10 μM) and HA-1077 (HA; 10 μM) (n = 17 for control, n = 6 for Y, n = 3 for HA). Right: bar graph representing the percent of control ET-1-induced response measured at 25 min after beginning challenge in the presence of Y or HA. B: effect of HA and Y on average maximum tension generated in response to KCl (left) and percent of control maximum KCl-induced response at 25 min (right) (n = 7 for control, n = 3 for Y, and n = 3 for HA). C: effect of HA and Y on average maximum tension generated in response to KCl in endothelium-intact arteries (n = 3 for control, n = 3 for HA, n = 6 for Y). *Significant difference from control (P < 0.05 by unpaired Student’s t-test).
To determine whether the effect of Rho kinase inhibition on ET-1-induced contraction was specific for this agonist, the effect of Y-27632 and HA-1077 on KCl-induced contraction was also examined. In contrast to the marked inhibitory effect on ET-1-induced contraction, pretreatment with Y-27632 had minimal effect on the tension generated in response to KCl (Fig. 2B), with a slowed time course of contraction \((P < 0.05\) by ANOVA) and a small but statistically significant reduction in maximum tension \((-10.5 \pm 2.9\%\)). HA-1077 had no significant effect on either the time course or the maximum KCl-induced contraction \((-2.9 \pm 12.7\%).\) Although the minimal effects of HA-1077 and Y-27632 on KCl responses were consistent with our hypothesis that activation of Rho was specific for ET-1, these results seemed to be at odds with previous studies in endothelium-intact pulmonary arteries and isolated perfused lungs, which indicated that Rho kinase inhibition also inhibited KCl-induced contraction (9, 22). We speculated that this difference in results might be due to endothelial influences. To test this possibility, pulmonary arteries with intact endothelium, verified by \(>50\%\) dilation of phenylephrine-induced contraction in response to ACh, were pretreated with either Y-27632 or HA-1077 before KCl challenge (Fig. 2C). We found that HA-1077 inhibited maximum KCl-induced contraction by 46.3 \(\pm\) 9.6\% in endothelium-intact vessels, whereas Y-27632 had no consistent significant effect \((17.6 \pm 22.1\%\) increase from control).

Effect of TK inhibitors on contraction in response to ET-1 and KCl. To assess the role of TKS in the contractile response to ET-1 that occurred independently of a change in [Ca\(^{2+}\)], arteries from CH rats were pretreated with nifedipine and challenged with ET-1 in the presence or absence of the TK inhibitors. Addition of the general TK inhibitors GEN or TA23 had no effect on baseline tension (Table 1). In the presence of 100 \(\mu\)M GEN, a concentration shown to inhibit contraction in response to both phenylephrine and U-46619 in canine pulmonary arteries (16), ET-1-induced contraction only reached 12.1 \(\pm\) 11.5\% of KCl\(_{\text{max}}\), an 88.6 \(\pm\) 10.8\% decrease in tension compared with that generated in the absence of TK inhibition (Fig. 3A). A similar reduction \((62.7 \pm 10.82\%)\) was observed in arteries pretreated with TA23, whereas DZ, the inactive form of GEN, had no effect. Whereas GEN and TA23 are nonselective TK inhibitors, we also used PP2, a putative antagonist of c-Src, a TK thought to play a role in Ca\(^{2+}\) nonselective TK inhibitors, we also used PP2, a putative selective inhibitor of Ca\(^{2+}\)sensitization (38). Initial experiments with 10 \(\mu\)M PP2, a concentration demonstrated to effectively inhibit agonist-induced vasoconstriction (2), had no effect on contraction in response to ET-1 in our preparation (data not shown). At a concentration of 100 \(\mu\)M, pretreatment with PP2 had no significant effect on baseline tension and was less effective than GEN or TA23 in reducing the ET-1-induced response. In the presence of 100 \(\mu\)M PP2, maximum tension generated in response to ET-1 was 48.8 \(\pm\) 16.4\%, a decrease of 54.2 \(\pm\) 15.3\% (Fig. 4A).

The specificity of GEN, TA23, and PP2 for ET-1-induced contractile pathways was determined by testing the effect of these inhibitors on KCl-induced responses (Fig. 3B and 4B). PP2 had no significant inhibitory effect on the maximum KCl-induced increase in tension, causing a 1.1 \(\pm\) 17.7\% decrease in the response to KCl (Fig. 4C); however, the time course of contraction was slowed \((P < 0.05\) by ANOVA). In contrast, GEN and TA23 reduced maximum KCl-induced contraction by 46.5 \(\pm\) 14.2\% and 70.4 \(\pm\) 6.0\%, respectively.

Effect of PKC inhibitors on ET-1-induced tension. Activation of PKC has often been associated with Ca\(^{2+}\) sensitization (12, 21, 24). To examine the role of PKC in the nifedipine-resistant ET-1-induced contraction in CH IPAs, two PKC inhibitors were used: STAuro, a relatively nonselective PKC inhibitor, and GFX, a putative selective inhibitor of Ca\(^{2+}\) sensitive PKC isoforms. Neither STAuro (1 nM, Fig. 5A) nor GFX (30 nM, Fig. 5B), at concentrations we had previously used to inhibit PKC-dependent ET-1 responses (33), altered baseline tension or had a significant inhibitory effect on the maximum tension generated in response to ET-1, causing decreases of 5.8 \(\pm\) 18.4\% and 15.7 \(\pm\) 18.2\%, respectively (Fig. 5C).

**DISCUSSION**

After exposure to chronic hypoxia, the majority of the contractile response of IPAs to ET-1 occurs as a result of increased myofilament Ca\(^{2+}\) sensitization, since nifedipine, which completely blocked any change in [Ca\(^{2+}\)], in response to ET-1, only inhibited 20\% of the contractile response (35). The goal of this study was to extend those previous findings and explore signaling pathways that may mediate Ca\(^{2+}\) sensitization during ET-1 challenge in IPAs from CH rats. We found that nifedipine-resistant ET-1-induced contraction in CH rat IPAs appears to require the activation of, and complex interaction between, Rho kinase, tyrosine kinases, and MLCK. PKC, however, appears to be uninvolved.
Under normoxic conditions, ET-1-induced vasoconstriction occurs via a Ca\textsuperscript{2+}-dependent mechanism. ET-1 stimulates both release of Ca\textsuperscript{2+} from intracellular stores and Ca\textsuperscript{2+} influx through VDCCs, which occurs secondary to K\textsubscript{v} channel inhibition and membrane depolarization (30, 33, 34), resulting in contraction via Ca\textsuperscript{2+}/calmodulin-induced activation of MLCK (1). After exposure to chronic hypoxia, however, contraction in response to ET-1 occurred with only a small increase in [Ca\textsuperscript{2+}], due entirely to influx through VDCCs (35). Moreover, treatment with nifedipine, an inhibitor of VDCCs that prevented any change in [Ca\textsuperscript{2+}], in response to ET-1 in pulmonary arterial smooth muscle cells (PASMCs) from CH rats, reduced ET-1-induced contraction by only 20% (35), suggesting that ~80% of the contraction remained despite the absence of a change in [Ca\textsuperscript{2+}].

Whereas ET-1-induced contraction in IPA from CH rats appeared to occur without a change in [Ca\textsuperscript{2+}], removal of extracellular Ca\textsuperscript{2+} was shown to decrease ET-1-induced contraction (35). In this study, contraction in response to ET-1, in the presence of nifedipine, was only partially inhibited by ML-9. The inhibition of ET-1-induced contraction by ML-9 was similar in magnitude to that observed following removal of extracellular Ca\textsuperscript{2+} (35). It is interesting to note that maintenance of elevated basal [Ca\textsuperscript{2+}], in PASMCs from CH rats required continuous Ca\textsuperscript{2+} influx (35) and that both ML-9 and removal of extracellular Ca\textsuperscript{2+} caused a decrease in baseline tension in IPAs after exposure to chronic hypoxia. These data suggest the possibility that during chronic hypoxia an elevation in resting [Ca\textsuperscript{2+}], may lead to activation of MLCK and an increase in basal tone. The activation of MLCK may be required for subsequent interaction with Ca\textsuperscript{2+}-independent ET-1-induced signaling pathways. Alternatively, generation of ET-1-induced contraction in pulmonary vascular smooth muscle may result from mechanisms independent of Ca\textsuperscript{2+}/calmodulin-induced activation of MLCK after exposure to chronic hypoxia.

Contraction that occurs without a change in [Ca\textsuperscript{2+}], also known as Ca\textsuperscript{2+} sensitization, is a well-studied phenomenon that has been observed in a variety of vascular beds under normoxic conditions (8, 31, 32). Ca\textsuperscript{2+} sensitization also occurs in the pulmonary vasculature after exposure to chronic hypoxia (9, 22, 28). The mechanisms responsible for Ca\textsuperscript{2+} sensitization are not fully understood and are likely to be vascular bed and/or agonist dependent. Distinct signaling pathways that promote an increase in phosphorylated myosin light chains without activation of Ca\textsuperscript{2+}/calmodulin, such as Rho kinase, TKs, or PKC, are activated in response to different agonists (11, 22, 32). For example, in isolated perfused lungs from rats with hypoxic pulmonary hypertension, Ca\textsuperscript{2+} sensitization was mediated by the Rho kinase signaling pathway, and, to a lesser degree, by PKC, whereas TK appeared to be uninvolved (22). In contrast, in coronary arteries, Ca\textsuperscript{2+} sensitization in response to ET-1 involved activation of both PKC and TK (31).

In this study, we have identified several signaling pathways involved in nifedipine-resistant ET-1-induced contraction (Ca\textsuperscript{2+} sensitization) in CH rat IPAs. We found that inhibition of Rho kinase with two structurally dissimilar antagonists, Y-27632 and HA-1077, nearly abolished contraction in response to ET-1. These results are consistent with other studies that have shown that Rho kinase mediates agonist-induced Ca\textsuperscript{2+} sensitization in isolated CH rat lungs (22) and that Ca\textsuperscript{2+} desensitization in pulmonary arterial smooth muscle after exposure to chronic hypoxia occurs through inhibition of the Rho

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**Fig. 4.** A: average maximum tension generated by rat IPAs in response to ET-1 in the absence and presence of PP2 (100 nM) (n = 17 for control; n = 4 for PP2). The effect of PP2 on average maximum tension generated in response to KCl (B) and percent of control maximum KCl-induced response after 25 min (C) (n = 9 for control; n = 3 for PP2) is shown. *Significant differences from control (P < 0.05 by unpaired Student’s t-test).

**Fig. 5.** Average maximum tension generated by rat IPAs in response to ET-1 in the absence and presence of staurosporine (A, STAUCRO: 1 nM) and GF-109203X (B, GFX: 30 nM). C: percent of maximum ET-1-induced tension in IPAs in the presence of STAUCRO and GFX (n = 17 for control; n = 3 for STAUCRO; n = 4 for GFX).
mechanism of ET-induced contraction during CH. The effects of Y-27632 and HA-1077 appear to be specific for ET-1-induced contraction as, in our hands, treatment with these inhibitors had no effect on KCl-induced generation of tension, which is receptor independent and occurs via membrane depolarization and Ca\(^{2+}\) influx through VDCCs. These results appear to be at odds with previous studies in the lung (9, 22) and in systemic arteries in which Y-27632 was found to inhibit KCl-induced responses. The reason for this discrepancy is unclear. We initially hypothesized that the difference represented an endothelial cell-dependent influence; however, in subsequent experiments in endothelium-intact arteries, HA-1077, but not Y-27632, reduced KCl-induced contraction. These results suggest possible differences between potency and/or action of the two inhibitors and that the explanation may be more complex than simply the presence or absence of endothelium. Thus determining whether the differences between our results and other studies represent variations in endothelial cell influences or vascular beds will require further investigation.

The mechanism by which activation of Rho kinase causes contraction is an area of intense study, and several possibilities exist. For example, Rho kinase phosphorylates myosin light chain phosphatase, resulting in decreased phosphatase activity and a buildup of phosphorylated myosin light chains (27, 40). Rho kinase has also been demonstrated to directly phosphorylate myosin light chains independently of MLCK and phosphatase activity (3). Alternatively, Rho kinase may be involved in the regulation of actin-binding proteins, which regulate availability of actin-binding sites and, consequently, the interaction between actin and myosin (19).

Pretreatment of IPAs from CH rats with both GEN and TA23, relatively nonselective inhibitors of TKs, caused reductions in nifedipine-resistant ET-1-induced contraction comparable to that of the Rho kinase inhibitors. However, PP2, an inhibitor of the c-Src family of TKs, implicated previously in ET-1-induced contraction (37), was less effective. One explanation for these results is that the inhibitory effects of the general TK inhibitors are simply due to nonspecific actions of the drugs. This is unlikely, however, given that DZ, the inactive form of GEN, had no effect on either ET-1- or KCl-induced contraction. Another possibility is that although the TK signaling pathway plays a significant role in nifedipine-resistant ET-1-induced contraction, its action is only partially mediated through c-Src and may instead involve other classes of TKs. Consistent with this possibility, both GEN and TA23 markedly inhibited KCl-induced contraction, whereas PP2 had no significant effect.

It is likely that Rho kinase and TK may be in the same signaling pathway, but it is unclear which is activated first. While some studies have found that TKs function as upstream effectors of Rho kinase (23, 25), others place TKs downstream of Rho (10). Because ET-1-induced contraction was completely blocked by the Rho kinase inhibitors Y-27632 and HA-1077, but only partially inhibited by the c-Src antagonist PP2, it seems likely that under our experimental conditions, Rho kinase acts upstream of c-Src. However, if TKs other than c-Src are involved in ET-1-induced Ca\(^{2+}\) sensitization, they may be required for activation of Rho kinase, as other evidence has suggested (10).

Previous studies indicate that PKC plays a role in ET-1-induced Ca\(^{2+}\) sensitization (31). In this study, however, we found that PKC inhibitors had no effect on the response to ET-1. The concentrations used in this study had been previously shown to block ET-1-induced inhibition of K\(_{\text{c}}\) channels as well as ET-1-induced contraction in isolated pulmonary arteries (33). GFX is a more selective inhibitor of PKC, specific for classic or Ca\(^{2+}\)-dependent isoforms. Although a lack of effect of GFX could thus be attributed to activation of isoforms that are nonresponsive to GFX (i.e., atypical and Ca\(^{2+}\)-independent), STAuro, a general PKC antagonist, should have inhibited ET-1-induced contraction if these isoforms were involved. Instead, our results suggest that although PKC plays a role in ET-1-induced contraction under normoxic conditions by mediating K\(_{\text{c}}\), channel inhibition, depolarization, and Ca\(^{2+}\) influx, exposure to chronic hypoxia alters the mechanism of action of ET-1 such that PKC is no longer involved. Consistent with this possibility, the PKC-dependent effects of ET-1 on K\(_{\text{c}}\) channels and membrane potential are absent in PASMCs from CH rats (33).

In summary, our results indicate that in IPAs isolated from rats exposed to chronic hypoxia, ET-1-induced contraction appears to be mediated by the activation of Rho kinase and TK, but not PKC. Moreover, although contraction occurs without a change in [Ca\(^{2+}\)]\(_{\text{i}}\), elevated resting [Ca\(^{2+}\)]\(_{\text{i}}\) is required, perhaps leading to basal activation of MLCK. Subsequent interactions between MLCK, Rho, and TK lead to enhanced Ca\(^{2+}\) sensitivity of the myofilament and contraction.

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