α_1 -Adrenoceptor-mediated phosphorylation of myosin in rat-tail arterial smooth muscle

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The mechanism of α_1 -adrenoceptor-mediated contraction was investigated in helical strips of the rat-tail artery. Muscle strips with the endothelium removed contracted in response to the α_1 -adrenoceptor agonist cirazoline, with half-maximal contraction at 0.23 μ M. The contractile response to a submaximal concentration of cirazoline (0.3 μ M) was biphasic, with a rapid phasic component peaking at approx. 30 s, followed by sustained tonic contraction. Phosphorylation of the 20 kDa light chain of myosin (LC₂₀) in response to 0.3 μ M cirazoline was also biphasic and closely matched the time-course of contraction. Resting LC₂₀ phosphorylation levels were 0.22 ± 0.06 mol of P_i/mol of LC₂₀ ($n = 3$) and reached a maximum of 0.58 ± 0.08 mol of P_i/mol of LC₂₀ ($n = 3$). Phosphopeptide mapping and phospho-

amino acid analysis revealed that LC₂₀ phosphorylation occurred exclusively at serine-19. The sustained phase of contraction was eliminated by removal of extracellular Ca²⁺ and the phasic response was eliminated by depletion of endogenous Ca²⁺ stores. Both phases of the contractile response were restored by re-addition of Ca²⁺ to the bathing medium. LC₂₀ phosphorylation and both phases of the contractile response to 0.3 μ M cirazoline were inhibited by the myosin light-chain kinase inhibitor ML-9 (30 μ M). Resting LC₂₀ phosphorylation, however, was unaffected by ML-9. Finally, both phasic and tonic responses to 0.3 μ M cirazoline were partially inhibited by chloroethylclonidine (50 μ M), suggesting the involvement of both α_{1A} and α_{1B} adrenoceptors in these contractile responses.

INTRODUCTION

Four mechanisms have been identified whereby contractile agonists can induce vascular smooth-muscle contraction: (i) depolarization of the sarcolemma, which activates voltage-gated Ca²⁺ channels and entry of extracellular Ca²⁺ into the sarcoplasm, (ii) activation of Ca²⁺-permeable channels (including L-type Ca²⁺ channels) without a change in membrane potential, (iii) release of Ca²⁺ from the sarcoplasmic reticulum into the sarcoplasm, and (iv) increased Ca²⁺ sensitivity of contraction [1]. The first three mechanisms all involve an increase in sarcoplasmic free [Ca²⁺]. As a result, calmodulin (CaM) becomes saturated with Ca²⁺ (four Ca²⁺ ions bind per CaM molecule) and undergoes a conformational change with exposure of hydrophobic sites of interaction with the target enzyme myosin light-chain kinase (MLCK) [2,3]. The interaction of Ca²⁺₄/CaM with MLCK activates the kinase that catalyses transfer of the terminal phosphoryl group of MgATP²⁻ to serine-19 of the two 20 kDa light chains of myosin (LC₂₀). This simple phosphorylation reaction triggers cycling of myosin cross-bridges along actin filaments and forces development or shortening of the muscle. Myosin phosphorylation is necessary and sufficient for contraction of smooth muscle in general. However, several instances have been reported of dissociation of myosin phosphorylation and force, e.g. [4–9].

In this study, we have investigated the mechanism whereby an α_1 -adrenoceptor agonist induces contraction of de-endothelialized rat-tail arterial smooth muscle, in particular the relationship between LC₂₀ phosphorylation and contraction.

MATERIALS AND METHODS

Materials

[³²P]P_i (10 mCi/ml) and [γ -³²P]ATP (> 5000 Ci/mmol) were purchased from Amersham (Oakville, Ontario, Canada). 1-(5-Chloronaphthalene-1-sulphonyl)homopiperazine (ML-9) hydrochloride was purchased from Calbiochem (Windsor, Ontario, Canada), chloroethylclonidine dihydrochloride from ICN Biomedicals (Aurora, OH, U.S.A.) and okadaic acid from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The following proteins were purified as described previously: CaM [10], MLCK [11] and LC₂₀ [12].

Preparation of muscle strips and tension measurement

The endothelial layer and adventitial tissue were removed mechanically from tail arteries of male Sprague–Dawley rats (250–350 g). Helical strips [0.5 mm $\times$ (6–7) mm] were cut and stored in Hepes/Tyrode (H-T) solution (137 mM NaCl/2.7 mM KCl/1.8 mM CaCl₂/1 mM MgCl₂/5.6 mM glucose/10 mM Hepes, pH 7.4). Muscle strips were mounted horizontally between two hooks and immersed in a pool of solution (0.8 ml), which was rounded by surface tension on a rotation plate of 6 cm diameter. One hook was connected to a force/displacement transducer mounted on a micromanipulator and the other to a second micromanipulator. Solution changes were effected rapidly by rotation of the plate. A resting tension of 75 mg was experimentally determined to give a maximal contractile response to 40 mM KCl and was used for all experiments. Once mounted,

Abbreviations used: CaM, calmodulin; H-T, Hepes/Tyrode; LC₂₀, 20 kDa light chain of smooth-muscle myosin; ML-9, 1-(5-chloronaphthalene-1-sulphonyl)homopiperazine; MLCK, myosin light-chain kinase.

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muscle strips were held at this resting tension in H-T solution at room temperature (20 °C) for 45 min before transfer to the test solutions indicated in the text. Sequential treatments with cirazoline separated by periods of relaxation elicited identical contractile responses in the same muscle strip. All solutions were bubbled thoroughly with 100% O₂.

Quantification of myosin light-chain phosphorylation

Tissue samples were frozen at selected times following agonist stimulation in 10% (w/v) trichloroacetic acid/20 mM dithiothreitol in dry ice/acetone for 10 min and kept at -80 °C for 1 h. Frozen muscle strips were then washed with 10 mM dithiothreitol in acetone at room temperature. The tissues were freeze-dried for 2 h and homogenized in extraction buffer (6 M deionized urea/20.1 mM Tris/22.2 mM glycine/10 mM dithiothreitol/10 mM EGTA/1 mM EDTA/1 mM PMSF/0.6 M KI/0.15 mM Bromophenol Blue) for 90 min. After filtration and centrifugation, phosphorylated and unphosphorylated LC₂₀ were separated by urea/glycerol gel electrophoresis [13] and transblotted to nitrocellulose membrane (0.2 µm; Bio-Rad, Mississauga, Ontario, Canada) in 10 mM sodium cyclohexylaminopropane-sulphonic acid, pH 11, in a mini transblot cell (Bio-Rad) at 27 V and 5 °C for 16 h. LC₂₀ was detected using a polyclonal antibody raised in rabbits against purified chicken gizzard LC₂₀. The antibody was purified from antiserum using HiTrap Protein A (Pharmacia, Baie d'Urfe, Québec, Canada) and was used at a dilution of 1:1000 with the Boehringer-Mannheim Chemiluminescence Western Blotting Kit, as described by the manufacturer. Phosphorylated and unphosphorylated LC₂₀ bands were quantified by densitometric scanning using a Pharmacia Image Master Desktop Scanning System.

Radiolabelling of muscle strips

De-endothelialized rat-tail arterial muscle strips were incubated for 16 h at room temperature (20 °C) in 10 ml of carrier-free [³²P]orthophosphate (0.1 mCi/ml) in H-T solution. Excess radiolabel was removed by washing 3 times in H-T solution. Force was recorded and muscle strips processed for urea/glycerol gel electrophoresis as described above. Phosphorylated LC₂₀ was detected by autoradiography and was cut out of the gel for phosphopeptide mapping and phosphoamino acid analysis.

Phosphopeptide mapping and phosphoamino acid analysis

LC₂₀ in the gel slices was digested with trypsin and the peptides were separated by two-dimensional thin-layer electrophoresis/chromatography as described by Colburn et al. [14]. Phosphopeptides were detected by autoradiography and this region of the plate was scraped off. The phosphopeptide was extracted and subjected to acid hydrolysis and two-dimensional phosphoamino acid analysis using the method of Cooper et al. [15], as described by Colburn et al. [14], except that hydrolysis was effected in 250 µl of 6 M HCl at 110 °C for 2 h, and first-dimension electrophoresis was carried out for 80 min and second-dimension electrophoresis for 70 min.

Phosphorylation of LC₂₀ *in vitro*

Purified chicken-gizzard LC₂₀ (0.1 mg/ml) was phosphorylated at serine-19 by incubation for 5 min at 30 °C in 25 mM Tris/HCl (pH 7.5)/60 mM KCl/0.1 mM CaCl₂/10 mM MgCl₂/10 mM dithiothreitol/1 µM okadaic acid/0.2 mM [γ -³²P]ATP (490 c.p.m./pmol) with CaM (10 µg/ml) and MLCK (10 µg/ml). Phosphorylated LC₂₀ was purified by SDS/PAGE [16] before tryptic digestion and phosphopeptide mapping as described above.

RESULTS

Helical strips of endothelium-free rat-tail arterial smooth muscle contracted in response to cirazoline (an α_1 -adrenoceptor agonist; see the Discussion section) in a concentration-dependent manner, with an EC₅₀ of 0.23 µM (results not shown). The contractile response to a submaximal concentration (0.3 µM) of cirazoline was biphasic (Figure 1): an initial phasic contraction, which peaked 30 s after the addition of cirazoline, was followed by a sustained tonic contraction that reached a plateau 5 min after the addition of agonist. LC₂₀ phosphorylation levels were quantified by Western blotting of urea/glycerol gels with enhanced chemiluminescence detection, as described in the Materials and methods section (Figure 1). LC₂₀ phosphorylation levels, like contraction, increased rapidly in response to cirazoline from a

Figure 1 Biphasic contraction and myosin light-chain phosphorylation in response to 0.3 µM cirazoline

De-endothelialized rat-tail arterial smooth-muscle strips were held at resting tension for 45 min, then transferred to a solution containing 0.3 µM cirazoline, and the time course of force development (○; expressed as a percentage of the maximal force developed in response to 0.3 µM cirazoline) and LC₂₀ phosphorylation (△; expressed as mol of P/mol of LC₂₀) were quantified as described in the Materials and methods section. Values are means ± S.E.M. ($n = 4$ for force data and $n = 3$ for phosphorylation data). Maximal force was 360.9 ± 45.9 mg.

Figure 2 Two methods of analysis of myosin light-chain phosphorylation in response to 0.3 µM cirazoline

(A) De-endothelialized rat-tail arterial smooth-muscle strips were equilibrated with [³²P]orthophosphate and treated with 0.3 µM cirazoline, and LC₂₀ phosphorylation was analysed by urea/glycerol gel electrophoresis and autoradiography as described in the Materials and methods section. A representative autoradiograph is shown. Lane 1, resting muscle; lanes 2–4, 15 s, 1 min and 5 min respectively following addition of cirazoline. The phosphorylated light chain is indicated by P-LC₂₀. (B) LC₂₀ phosphorylation was quantified by densitometric scanning of Western blots (such as that shown) following separation of unphosphorylated and phosphorylated LC₂₀ by urea/glycerol gel electrophoresis. This method was used to obtain the quantitative data shown in Figures 1 and 9.

Figure 3 Phosphopeptide mapping of LC_{20} phosphorylated in response to cirazoline

LC_{20} bands (see Figure 2A) were cut out of the gel, the light chain was digested with trypsin and the tryptic peptides were separated by two-dimensional thin-layer electrophoresis/chromatography (TLE, TLC) as described in the Materials and methods section. Phosphopeptides were detected by autoradiography. (A) Resting muscle; (B)–(D) 15 s, 1 min and 5 min respectively following addition of cirazoline; (E) tryptic digest of purified LC_{20} phosphorylated *in vitro* by MLCK; (F) a mixture of tryptic digests (equal c.p.m.) of purified LC_{20} phosphorylated *in vitro* by MLCK and LC_{20} from tissue stimulated with 40 mM KCl for 15 s; (G) a mixture of tryptic digests (equal c.p.m.) of LC_{20} from tissues stimulated with 0.3 μ M cirazoline for 15 s or 40 mM KCl for 15 s.

resting level of 0.22 ± 0.06 mol of P_i /mol of LC_{20} to a peak of 0.54 ± 0.05 mol of P_i /mol of LC_{20} 15 s after agonist addition. LC_{20} phosphorylation then declined slightly before increasing again to a sustained level of 0.58 ± 0.08 mol of P_i /mol of LC_{20} . The biphasic contraction followed very closely the change in myosin phosphorylation. The biphasic nature of myosin phosphorylation was confirmed by urea/glycerol gel electrophoresis and autoradiography of tissue strips pre-equilibrated with [32 P]orthophosphate and stimulated with cirazoline. Results of a representative experiment are shown in Figure 2(A). Western blotting following separation of unphosphorylated and phosphorylated LC_{20} enabled determination of the phosphorylation stoichiometry (Figure 2B).

Phosphopeptide mapping and phosphoamino acid analysis indicated that all the phosphate incorporation was into serine-

Figure 4 Phosphoamino acid analysis of tryptic phosphopeptides of LC_{20} phosphorylated in response to 0.3 μ M cirazoline

The radiolabelled tryptic phosphopeptides identified in Figures 3(A)–3(D) were scraped from the thin-layer plates, extracted and acid-hydrolysed, and the phosphoamino acids separated by two-dimensional thin-layer electrophoresis as described in the Materials and methods section. Phosphoamino acid standards (added to the acid hydrolysate) were detected by ninhydrin staining and radiolabelled phosphoamino acids were detected by autoradiography. (A) Resting muscle; (B)–(D) 15 s, 1 min and 5 min respectively following addition of cirazoline. In the autoradiograph of panel D, approx. one-third of the amount of radioactivity was applied to the thin-layer plate compared with the other panels. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

19. A single tryptic phosphopeptide was detected in resting muscle and at 15 s, 1 min and 5 min following addition of cirazoline (Figures 3A–3D). This phosphopeptide contained only phosphoserine (Figure 4) and it co-migrated with the tryptic phosphopeptide obtained from muscle strips contracted with 40 mM K^+ (Figure 3G). The latter phosphopeptide also co-migrated with the tryptic phosphopeptide derived from purified chicken-gizzard LC_{20} phosphorylated *in vitro* by MLCK, which is known to occur at serine-19 (Figures 3E and 3F). Figure 5 confirms the exclusive phosphorylation of serine-19 in muscle strips stimulated with 40 mM KCl. Phosphoamino acid analysis

Figure 5 Phosphopeptide mapping of LC_{20} , phosphorylated in response to K^+ depolarization

The experimental protocol was as described in the legend to Figure 3 and the Materials and methods section, except that contraction was elicited by 40 mM KCl rather than 0.3 μ M cirazoline. (A) Resting muscle; (B)–(D) 15 s, 1 min and 5 min respectively following addition of KCl. TLE, thin-layer electrophoresis.

Figure 6 Effects of removal of extracellular Ca^{2+} and depletion of intracellular Ca^{2+} stores on cirazoline-induced contraction

De-endothelialized rat-tail arterial smooth-muscle strips were held at resting tension for 45 min in H-T solution and then transferred sequentially to the following solutions: (i) 0.3 μ M cirazoline in H-T solution for 5 min, (ii) H-T solution for 60 min, (iii) 0.3 μ M cirazoline in H-T solution for 15 min, (iv) H-T solution for 60 min, (v) Ca^{2+} -free H-T solution + 2 mM EGTA for 5 min, (vi) 0.3 μ M cirazoline in Ca^{2+} -free H-T solution + 2 mM EGTA for 15 min, (vii) Ca^{2+} -free H-T solution + 2 mM EGTA for 60 min, (viii) 0.3 μ M cirazoline in Ca^{2+} -free H-T solution + 2 mM EGTA for 15 min, (ix) H-T solution for 60 min and (x) 0.3 μ M cirazoline in H-T solution. (O) Initial contractile response to 0.3 μ M cirazoline, (●) contractile response following treatment with Ca^{2+} -free/EGTA solution for 5 min to remove extracellular Ca^{2+} , (□) contractile response following continued incubation in Ca^{2+} -free/EGTA solution for 60 min to prevent refilling of depleted intracellular Ca^{2+} stores, (Δ) contractile response following treatment with Ca^{2+} -free/EGTA solution for another 5 min, followed by Ca^{2+} -containing solution for 60 min. Data are means $\pm$ S.E.M. ($n = 4$). Maximal force was 417.6 ± 48.4 mg.

Figure 7 Verification that incubation in Ca^{2+} -free/EGTA solution and repeated exposure to sarcoplasmic-reticulum Ca^{2+} -releasing agents prevents refilling of the Ca^{2+} stores

A de-endothelialized rat-tail arterial smooth-muscle strip was induced to contract for 5 min with 0.3 μ M cirazoline (CIR) and washed for 60 min in H-T solution. The strip was then incubated for 5 min in Ca^{2+} -free/EGTA solution, following which 20 mM caffeine induced a normal phasic contraction. The strip was then incubated for 60 min in Ca^{2+} -free/EGTA solution, following which 20 mM caffeine failed to trigger a significant contraction.

Figure 8 Effect of ML-9 on cirazoline-induced contraction

(A) Pre-equilibrated de-endothelialized rat-tail arterial smooth-muscle strips were transferred sequentially to the following solutions: (i) 0.3 μ M cirazoline in H-T solution for 15 min, (ii) H-T solution for 45 min, (iii) ML-9 (30 μ M) in H-T solution for 30 min, and (iv) 0.3 μ M cirazoline in H-T solution containing ML-9 (30 μ M). (O) Initial contractile response to 0.3 μ M cirazoline; (●) contractile response following treatment with 30 μ M ML-9. Maximal force was 412.7 ± 37.6 mg. (B) The small contractile response in the presence of ML-9 is shown on an expanded force scale. Data are means $\pm$ S.E.M. ($n = 4$).

Figure 9 Effect of ML-9 on cirazoline-induced myosin light-chain phosphorylation

(●) LC₂₀ phosphorylation levels were quantified (mol of P_i/mol of LC₂₀) by the Western blotting procedure described in the Materials and methods section, in de-endothelialized rat-tail arterial smooth-muscle strips pre-incubated with ML-9 (30 μ M) for 30 min and then treated with cirazoline (0.3 μ M) in the presence of ML-9 (denoted time zero). (□) The resting level of LC₂₀ phosphorylation in the absence of ML-9 was also determined. Values are means of two independent experiments.

indicated that the tryptic phosphopeptide in each case contained phosphoserine but not phosphothreonine or phosphotyrosine (results not shown).

To determine the relative importance of extracellular versus intracellular Ca²⁺ stores in the two phases of the cirazoline-induced contraction, control contractions were compared with contractions elicited in muscle strips treated (i) with Ca²⁺-free solution containing 2 mM EGTA for 5 min, (ii) continued incubation in Ca²⁺-free solution containing 2 mM EGTA for 60 min, and (iii) with Ca²⁺-free solution containing 2 mM EGTA for another 5 min, followed by Ca²⁺-containing solution for 60 min. Brief treatment to remove extracellular Ca²⁺ resulted in loss of the sustained contractile response to cirazoline without affecting the phasic response (Figure 6). When intracellular Ca²⁺ stores were depleted by repeated treatment with cirazoline in the absence of Ca²⁺, the contractile response to cirazoline was almost completely abolished. The biphasic contraction was restored following refilling of intracellular Ca²⁺ stores and provision of extracellular Ca²⁺. Figure 7 confirms that the phasic contraction remaining in the absence of extracellular Ca²⁺ is due to release of Ca²⁺ from the sarcoplasmic reticulum. Thus following a 5-min incubation in Ca²⁺-free solution, caffeine elicited a normal phasic contraction but failed to do so after continued incubation in Ca²⁺-free solution.

The MLCK-selective membrane-permeant inhibitor ML-9 (30 μ M) almost completely abolished both phases of the contractile response to cirazoline (Figure 8A). Only a very small component of the phasic response was retained in the presence of ML-9 (Figure 8B). Figure 9 indicates that ML-9 completely prevented the increase in LC₂₀ phosphorylation due to cirazoline. Interestingly, ML-9 did not reduce the resting level of LC₂₀ phosphorylation.

Finally, the involvement of α_1 -adrenoceptor subtypes in the cirazoline-induced contraction was investigated using chloroethylclonidine, an irreversible inhibitor of α_{1B} and α_{1D} adrenoceptors that is without effect on α_{1A} adrenoceptors [17,18]. Figure 10 shows that both the phasic and tonic components of the cirazoline-induced contraction were partially inhibited by chloroethylclonidine, the phasic component by $36.1 \pm 2.9\%$ and the tonic component by $43.6 \pm 6.7\%$ ($P < 0.01$ in each case).

Figure 10 Effect of chloroethylclonidine on cirazoline-induced contraction

De-endothelialized rat-tail arterial smooth-muscle strips were held at resting tension for 45 min in H-T solution and then transferred sequentially to the following solutions: (i) 0.3 μ M cirazoline in H-T solution for 2 min, (ii) H-T solution for 60 min, (iii) 0.3 μ M cirazoline in H-T solution for 15 min, (iv) H-T solution for 60 min, (v) 50 μ M chloroethylclonidine in H-T solution for 30 min, with a solution change every 10 min, (vi) H-T solution for 30 min, and (vii) 0.3 μ M cirazoline in H-T solution for 15 min. Contractile response to 0.3 μ M cirazoline (○) before and (●) after incubation with 50 μ M chloroethylclonidine. Force development is expressed as a percentage of the maximal response to 0.3 μ M cirazoline under control conditions. Data are means $\pm$ S. E. M. ($n = 4$). Maximal force was 229.4 ± 34.3 mg.

DISCUSSION

Three pharmacological tools were used in this study of α_1 -adrenoceptor-mediated activation of LC₂₀ phosphorylation and contraction of rat-tail arterial smooth muscle: the α_1 -adrenoceptor agonist cirazoline, the MLCK inhibitor ML-9 and the α_{1B} - and α_{1D} -adrenoceptor antagonist chloroethylclonidine.

There is strong evidence in the literature that cirazoline behaves as an α_1 -adrenoceptor agonist when applied to rat-tail arterial smooth muscle. Thus Li and Triggle [19] showed that the contractile responses of rat-tail arterial rings to cirazoline are sensitive to prazosin at concentrations appropriate for an α_1 -adrenoceptor-mediated response. Furthermore, Li et al. [20] reported that treatment of isolated, single, rat-tail arterial smooth-muscle cells with prazosin greatly depressed responses to cirazoline. Finally, we have shown that prazosin also inhibits cirazoline-induced contraction of rat-tail arterial-muscle helical strips (L. Weber and M. P. Walsh, unpublished result).

ML-9 has been reported to be a selective inhibitor of MLCK [21] and was shown to inhibit the KCl-stimulated increase in LC₂₀ phosphorylation and force development in rabbit mesenteric artery [22,23]. This inhibition by ML-9 was not affected by treatment with adrenergic or cholinergic antagonists. Moreover, ML-9 exhibited non-competitive inhibition with noradrenaline (norepinephrine)-, 5-hydroxytryptamine-, angiotensin II-, histamine- and CaCl₂-induced contractions, suggesting that the mechanism of action of ML-9 does not involve blocking of a membrane receptor. In all cases, inhibition of contraction by ML-9 could be explained by its ability to inhibit MLCK-catalysed phosphorylation of LC₂₀.

Three α_1 -adrenoceptor subtypes have been identified: α_{1A} , α_{1B} and α_{1D} [18]. The α_{1A} -adrenoceptor is resistant to covalent modification and inactivation by chloroethylclonidine, whereas α_{1B} - and α_{1D} -adrenoceptors are alkylated and inactivated by chloroethylclonidine. Although mRNA for the α_{1D} -adrenoceptor has been detected in the rat-tail artery (and all other arteries examined) [24], this subtype does not appear to play a role in mediating contractile responses in rat-tail, mesenteric and renal

arteries, but does appear to play a role in rat aorta and iliac artery [25].

The following principal conclusions are reached from this study. (i) The contractile response to a submaximal concentration of cirazoline is biphasic, consisting of a rapid phasic contraction followed by a sustained tonic contraction. (ii) The time course of LC₂₀ phosphorylation, which occurs exclusively at serine-19, closely parallels that of force. (iii) The phasic component of the contraction is due to the release of Ca²⁺ from the sarcoplasmic reticulum and the tonic component to the entry of extracellular Ca²⁺. (iv) Both phases of the contraction and LC₂₀ phosphorylation are abolished by pre-treatment with the membrane-permeant MLCK inhibitor ML-9. (v) Resting LC₂₀ phosphorylation levels (approx. 0.2 mol of P_i/mol of LC₂₀) are unaffected by ML-9. (vi) α_{1A} and α_{1B} adrenoceptors are involved in both the phasic and tonic components of cirazoline-induced contraction. These results support a mechanism whereby α_1 -adrenoceptor agonist stimulation triggers both Ca²⁺ release from the sarcoplasmic reticulum and Ca²⁺ entry from the extracellular space, leading to LC₂₀ phosphorylation specifically at serine-19 and force development. Force maintenance also correlates with high levels of LC₂₀ phosphorylation. In this tissue, under the conditions described, we detected no latch state, i.e. reduction of LC₂₀ phosphorylation during force maintenance [26]. Higher concentrations of cirazoline (10 μ M), however, triggered a monophasic contractile response with a transient increase in LC₂₀ phosphorylation, i.e. force was maintained following a decline in LC₂₀ phosphorylation to resting levels (results not shown).

Using a different α_1 -adrenoceptor agonist, phenylephrine, Chen and Rembold [27] concluded that a low concentration of the agonist (0.1 μ M) induced contraction of rat-tail arterial smooth-muscle rings primarily via depolarization of the sarcolemma with entry of extracellular Ca²⁺ via voltage-gated Ca²⁺ channels, and via increased Ca²⁺ sensitivity, with a minor contribution due to entry of extracellular Ca²⁺, independently of changes in membrane potential (principally via L-type Ca²⁺ channels). Release of Ca²⁺ from intracellular stores was only observed at high concentrations of phenylephrine (10–100 μ M). The situation with cirazoline-induced contraction is clearly quite different since a submaximal concentration elicited a rapid phasic contraction due entirely to the release of intracellular stored Ca²⁺, and a sustained contraction due to the entry of extracellular Ca²⁺. It has been postulated that α_{1A} adrenoceptors act via Ca²⁺ influx and α_{1B} adrenoceptors via intracellular Ca²⁺ release [28,29]. However, partial overlap between the signal-transduction mechanisms of these two α_1 -adrenoceptor subtypes has been indicated [30–32]. Our results with chloroethylclonidine would support such overlap. Under the conditions used in this study there is a clear temporal distinction between intracellular Ca²⁺ release and extracellular Ca²⁺ entry. A plausible explanation would be that the submaximal concentration of cirazoline first of all triggers G-protein-mediated activation of phosphatidylinositol-specific phospholipase C via α_{1A} - and α_{1B} -adrenoceptors, resulting in Ins(1,4,5)P₃ production, release of Ca²⁺ from the sarcoplasmic reticulum and rapid contraction due to LC₂₀ phosphorylation at serine-19. The increase in [Ca²⁺]_i could then increase Cl[−] channel activity [33], resulting in depolarization of the sarcolemma and Ca²⁺ influx via voltage-gated Ca²⁺ channels. Since 1,2-diacylglycerol is also generated by phosphatidylinositol-specific phospholipase C, protein kinase C may be involved in the mechanism of activation of Ca²⁺ entry.

Finally, the observation that the resting level of LC₂₀ (serine-

19) phosphorylation is unaffected by ML-9 suggests that this phosphorylation is not catalysed by Ca²⁺/CaM-activated MLCK. Possibilities include MLCK autophosphorylated at threonine-803 (which exhibits Ca²⁺/CaM-independent activity) [34], Rho-associated kinase [35], Ca²⁺/CaM-dependent protein kinase II (an autophosphorylated form exhibits Ca²⁺/CaM-independent activity) [36] or mitogen-activated-protein-kinase-activated protein kinase-2 [37]. Further studies will be required to identify the kinase involved.

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