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Augmentation of α_1-Adrenoceptor-Mediated Contraction by Warming Without Increased Phosphorylation of Myosin in Rat Caudal Arterial Smooth Muscle

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Abstract. We previously reported the relationship between α_1-adrenoceptor-mediated contraction and phosphorylation of 20-kDa myosin light chain (LC_{20}) in de-endothelialized rat caudal arterial smooth muscle at room temperature (Mita M, Walsh MP. Biochem J. 1997;327:669–674). We now describe the effect of increasing the temperature to 37°C on this relationship. The EC_{50} value (76.6 ± 18.2 nM) for cirazoline (α_1-adrenergic agonist)-induced contraction of the strips at room temperature (23°C) was significantly greater than that (14.5 ± 1.9 nM) at 37°C. The initial rate of the contraction to a sub-maximal concentration of cirazoline (0.3 μM) was similar at the two temperatures. However, cirazoline-induced maximal force at 37°C was approximately 1.8 times that at room temperature. LC_{20} phosphorylation in response to cirazoline at room temperature and 37°C closely matched the time courses of contraction, but values were not significantly different at the two temperatures: resting phosphorylation levels were 0.09 ± 0.04 mol P_i/mol LC_{20} at 37°C and 0.22 ± 0.06 mol P_i/mol LC_{20} at room temperature; maximal cirazoline-stimulated LC_{20} phosphorylation levels were 0.58 ± 0.08 mol P_i/mol LC_{20} at room temperature and 0.49 ± 0.05 mol P_i/mol LC_{20} at 37°C. We conclude, therefore, that the enhanced cirazoline-induced contraction at 37°C is not due to increased LC_{20} phosphorylation.

Keywords: vascular smooth muscle, contraction, myosin phosphorylation, temperature dependence, cirazoline

Introduction

Receptor-operated activation of vascular smooth muscle contraction by various agonists involves mechanisms that elevate cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) through its release from intracellular stores and transmembrane influx from the extracellular fluid (1, 2). A rise in $[Ca^{2+}]_i$ is the most important stimulus for force production in smooth muscle and leads to activation of Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) and hence phosphorylation of the 20-kDa light chains of myosin (LC_{20}) at serine19 (3, 4). This simple phosphorylation reaction triggers cycling of myosin crossbridges along actin filaments and force development or shortening of the muscle. Myosin phosphorylation is necessary and sufficient for contraction of smooth muscle in general. Relaxation generally follows a return of $[Ca^{2+}]_i$ to resting levels, resulting in dissociation of Ca^{2+} from CaM, inactivation of MLCK, and dephosphorylation of myosin catalyzed by myosin light chain phosphatase (MLCP), a myosin-associated type 1 protein serine/threonine phosphatase (5). However, several instances have been reported of dissociation of myosin phosphorylation and force (6 – 11). Dillon et al. originally described tension maintenance during prolonged stimulation while myosin was dephosphorylated (6) and ascribed this "latch state" to a slow rate of detachment of attached, dephosphorylated crossbridges (12).

We previously investigated the relationship between LC_{20} phosphorylation and contraction in de-endothelial-

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ized rat caudal arterial smooth muscle at room temperature (13) and reached the following principal conclusions: i) The contractile response to a sub-maximal concentration of the α_1-adrenoceptor agonist cirazoline at room temperature is biphasic, consisting of a rapid phasic contraction followed by a sustained tonic contraction; ii) The phasic component of the contraction is due to the release of Ca^{2+} from the sarcoplasmic reticulum and the tonic component to the entry of extracellular Ca^{2+}; iii) The time course of LC_{20} phosphorylation, which occurs exclusively at serine19, closely parallels that of force, such that force maintenance correlates with high levels of LC_{20} phosphorylation catalyzed by MLCK. Therefore, no latch state was detected in this tissue in response to α_1-adrenoceptor activation at room temperature.

Temperature is an important determinant of membrane fluidity (14, 15) and a critical determinant of myogenic contraction in vascular smooth muscle (16, 17). Moreover, temperature has been shown to affect many stages in the excitation-contraction coupling process: lowering temperature decreases the maximal shortening velocity and the rate of force development (18 – 23) and slows the rates of phosphorylation and dephosphorylation of myosin by decreasing the activities of both MLCK and MLCP, but with a more potent effect on the latter in chemically skinned smooth muscle (24). This was associated with increased LC_{20} phosphorylation and greater force developed by the tissue at lower temperature at the same $[Ca^{2+}]_i$ (24). Therefore, we hypothesized that cirazoline-activated LC_{20} phosphorylation during force maintenance at room temperature (13) remains high due to the low temperature and therefore low MLCP activity, and increasing the temperature to 37°C would activate MLCP more than MLCK and, therefore, result in a reduction in LC_{20} phosphorylation and force. Hence we compared the relationship between LC_{20} phosphorylation and contraction in de-endothelialized rat caudal arterial smooth muscle in response to α_1-adrenoceptor stimulation at room temperature and 37°C.

Materials and Methods

Materials

Cirazoline was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dithiothreitol (DTT) was from Wako Pure Chemical Industries, Ltd. (Osaka). Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) was from Dojindo Laboratories (Kumamoto). BM Chemiluminescence Western Blotting Kit was from Roche (Mannheim, Germany). All other chemicals were of reagent grade.

Preparation of muscle strips and tension measurement

Male Sprague-Dawley rats (250 – 450 g) were sacrificed by a blow on the neck and exsanguination as approved by the Institutional Ethics Committee for Animal Research at Meiji Pharmaceutical University. Caudal arterial smooth muscle strips were isolated as previously described (13, 25). The endothelial layer and adventitial tissue were removed mechanically. Helical strips (0.5 mm × 6 – 7 mm) were cut and stored in Hepes-Tyrode (H-T) solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM Hepes, pH 7.4 at room temperature or 37°C). Muscle strips were mounted horizontally between two hooks and immersed in a pool of solution (750 μl), which had a rounded surface, on a rotation plate placed on a temperature-controlled stage. One hook was connected to a force displacement transducer (TB-612T; Nihon Kohden, Tokyo) 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, muscle strips were held at this resting tension in H-T solution at room temperature (23°C) or 37°C for 45 min prior to transfer to the test solution. 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% O_2.

Quantification of myosin light chain phosphorylation

Quantification of LC_{20} phosphorylation was achieved as previously described (13, 25). Tissue samples were frozen in 10% TCA, 10 mM DTT in dry ice/acetone for 10 min at selected times following agonist stimulation. Frozen muscle strips were then washed with 10 mM DTT in acetone at room temperature. The tissues were lyophilized overnight and stored at −80°C until LC_{20} extraction. Proteins were extracted from tissue strips in 30 μl of extraction buffer (6 M deionized urea, 20.1 mM Tris, 22.2 mM glycine, 10 mM DTT, 10 mM EGTA, 1 mM EDTA, 1 mM PMSF, 0.6 M KI and 0.15 mM bromophenol blue) by constant rotation in a microcentrifuge tube for 90 min at room temperature. After filtration and centrifugation, phosphorylated and unphosphorylated LC_{20} were separated by urea/glycerol gel electrophoresis and transblotted to a nitrocellulose membrane (BioRad, Mississauga, Ontario, Canada) in 10 mM Na-cyclohexylaminopropanesulfonic acid, pH 11 in a Mini Transblot cell (BioRad) at 27 V and 5°C for 16 h. LC_{20} was detected using a polyclonal antibody raised in rabbits against purified chicken gizzard LC_{20} with enhanced chemiluminescence detection (13). Phos-

phorylated and unphosphorylated LC_{20} bands were quantified by densitometric scanning with a Pharmacia Image Master Desktop Scanning System using Image Master 1D software with data storage on a Pharmacia Biotech NEC Image 466es equipped with a Sharp JX-330 scanner. LC_{20} phosphorylation levels were calculated by dividing the chemiluminescence signal (optical density × area) of the phosphorylated LC_{20} peak by the total chemiluminescence signal of the phosphorylated and unphosphorylated LC_{20} peaks. We observed no diphosphorylated LC_{20} in any experiment, that is, LC_{20} phosphorylated at both serine19 and threonine18.

Statistical analyses

All statistical analyses were performed using StatView-J5.0 (SAS Institute Inc., Cary, NC, USA). Student's paired or unpaired *t*-tests were performed as appropriate and $P<0.05$ was considered significant. Data represent the mean ± S.E.M. Values of n indicate the numbers of strips utilized.

Results

Contractile sensitivity to cirazoline at room temperature and 37°C

Helical strips of de-endothelialized rat caudal arteries contracted in response to the α_1-adrenoceptor agonist cirazoline in a concentration-dependent manner with EC_{50} values of 76.6 ± 18.2 and 14.5 ± 1.9 nM at room temperature (23°C) and 37°C, respectively (Fig. 1). These values are statistically significantly different. The maximal forces developed in response to cirazoline were 471.0 ± 64.1 mg at room temperature and 535.0 ± 47.5 mg at 37°C, respectively, but not significantly different.

Time courses of cirazoline-induced contraction at room temperature and 37°C

The contractile response to a sub-maximal concentration (0.3 μM) of cirazoline at room temperature was biphasic (Fig. 2 and Ref. 13): an initial phasic contraction, which peaked 30 s after the addition of cirazoline, was followed by a sustained tonic contraction that reached a plateau approximately 5 min after the addition of cirazoline. On the other hand, the contractile response to 0.3 μM cirazoline at 37°C was monophasic with a sustained tonic contraction (Fig. 2). The contraction at 37°C reached a plateau approximately 6 min after the addition of cirazoline. Maximal force in response to 0.3 μM cirazoline was 263.8 ± 18.4 and 464.6 ± 31.6 mg at room temperature and 37°C, respectively, indicating that 0.3 μM cirazoline-induced maximal force at 37°C was approximately 1.8 times that at room temperature.

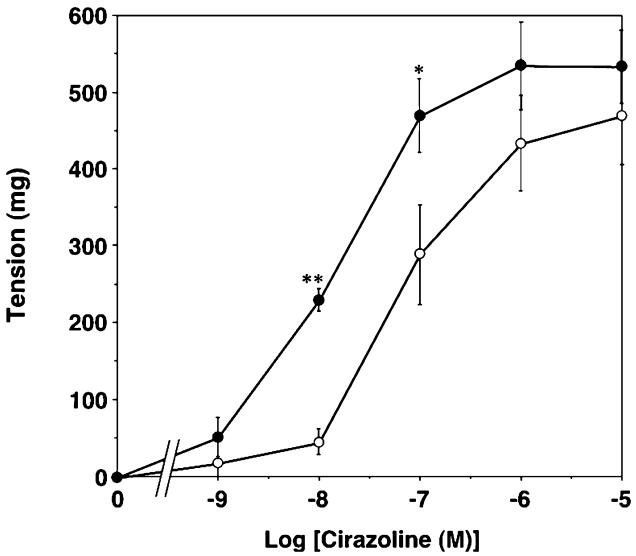


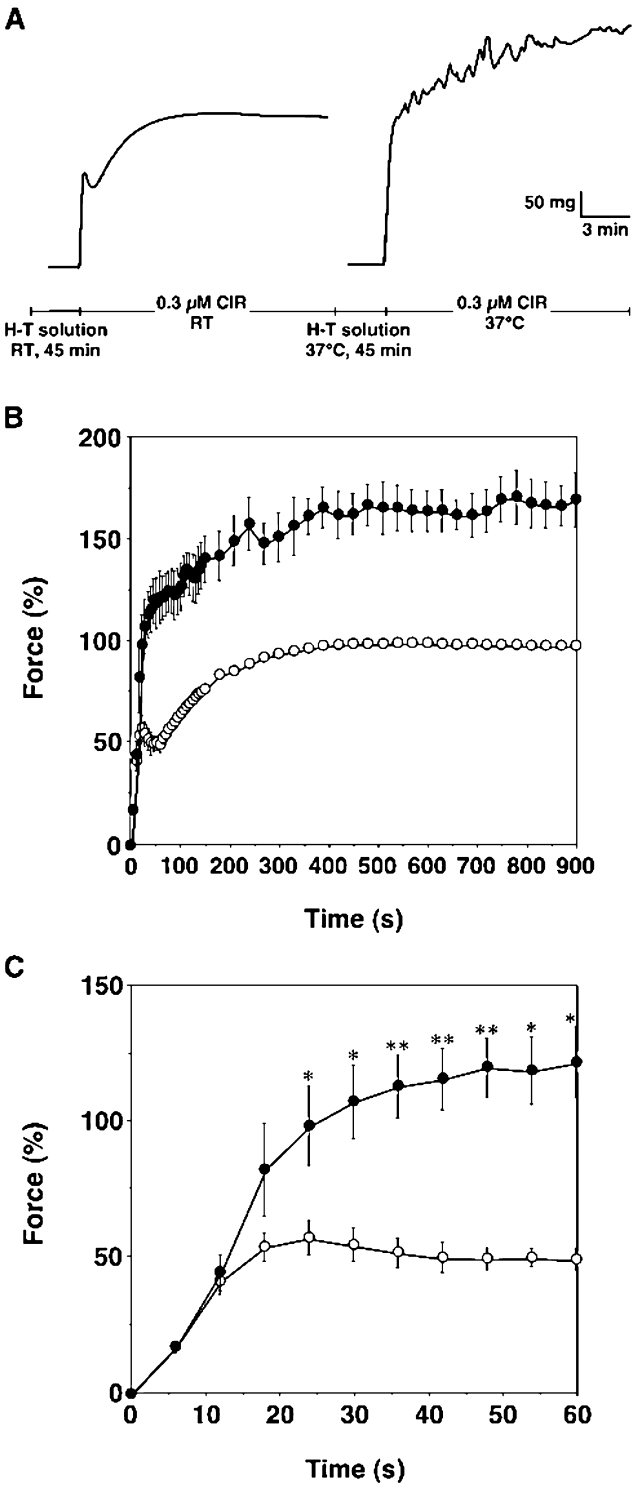
Fig. 1. Concentration dependence of cirazoline-induced contraction of rat caudal arteries at room temperature and 37°C. De-endothelialized rat caudal arterial smooth muscle strips were held at resting tension for 45 min at room temperature (23°C, open circles) or 37°C (closed circles) and then transferred to solution containing various concentrations of cirazoline. Values represent the mean ± S.E.M. (n = 4). *$P<0.05$, **$P<0.005$: significantly different from the corresponding values at room temperature by Student's *t*-test. The concentration of cirazoline required to produce 50% of the maximal contraction (EC_{50}) was 76.6 ± 18.2 nM at room temperature and 14.5 ± 1.9 nM at 37°C; the difference between these values was statistically significant ($P<0.05$).

Quantification of LC_{20} phosphorylation in response to cirazoline treatment at room temperature and 37°C

LC_{20} phosphorylation (monophosphorylation) levels at 37°C increased rapidly in response to cirazoline from a resting level of 0.09 ± 0.04 mol P_i/mol LC_{20} to a peak and an approximate plateau of 0.47 ± 0.06 mol P_i/mol LC_{20} 30 s after cirazoline addition, and they closely matched the time course of contraction (Fig. 3). LC_{20} phosphorylation appeared to decline slightly 1 min after cirazoline addition, but this value was not significantly different from that 30 s after cirazoline addition. Moreover, LC_{20} phosphorylation was maintained at this level for at least 15 min in the continued presence of cirazoline with maximal LC_{20} phosphorylation stoichiometry of 0.49 ± 0.05 mol P_i/mol LC_{20}. Force maintenance stimulated by 0.3 μM cirazoline at 37°C, therefore, correlates with maintenance of a high level of LC_{20} phosphorylation.

LC_{20} phosphorylation (monophosphorylation) levels at room temperature also increased rapidly in response to cirazoline from a resting level of 0.22 ± 0.06 mol P_i/mol LC_{20} to a peak of 0.54 ± 0.05 mol P_i /mol LC_{20} 15 s after cirazoline addition (Fig. 4). LC_{20} phosphorylation then declined significantly 30 s and

1 min after cirazoline addition, before increasing again to a sustained level of 0.58 ± 0.08 mol P_i/mol LC_{20}. We previously reported these results (13). The biphasic contraction followed very closely the change in LC_{20} phosphorylation and force maintenance correlated with maintenance of this high level of LC_{20} phosphorylation.



Resting LC_{20} phosphorylation levels and the time courses of increased LC_{20} phosphorylation did not differ significantly at room temperature and 37°C (Fig. 4), indicating that cirazoline-stimulated LC_{20} phosphorylation was unaffected by temperature.

Relationship between LC_{20} phosphorylation and force at room temperature and 37°C

From the data of Figs. 2B and 4, the tension-LC_{20} monophosphorylation relationship was determined at room temperature and 37°C. This relationship was shifted to the left with little difference in slope at 37°C compared to room temperature, indicating that greater force is generated at a given level of LC_{20} phosphorylation at the physiological temperature (Fig. 5).

Discussion

The phosphorylation of LC_{20} triggers the cycling of myosin crossbridges along actin filaments, leading to force development or contraction of smooth muscle. This activation mechanism, however, cannot explain all the regulatory properties of smooth muscle contraction, and dissociation of force and myosin phosphorylation has frequently been observed. Nevertheless, we previously reported that cirazoline-induced LC_{20} phosphorylation in rat caudal arterial smooth muscle closely matched the time course of contraction at room temperature (13), that is, we detected no latch state (reduction of LC_{20} phosphorylation during force maintenance (12)). To determine whether the latch state is temperature-dependent, we compared the relationship between LC_{20} phosphorylation and contraction induced in de-endothelialized rat caudal arterial smooth muscle by cirazoline at room temperature and at physiological temperature. Alterations of temperature are known to

Fig. 2. Comparison between contractions in response to cirazoline at room temperature and 37°C. De-endothelialized rat caudal arterial smooth muscle strips were held at resting tension for 45 min at room temperature or 37°C and then transferred to solution containing 0.3 μM cirazoline. A: A typical trace showing 0.3 μM cirazoline (CIR)-induced contraction of rat caudal arterial smooth muscle at room temperature (RT) and 37°C. The trace is representative of four experiments. B: Cumulative data showing the contractile responses at room temperature (open circles) and at 37°C (closed circles). C: The contractile responses induced by 0.3 μM cirazoline are shown on an expanded time scale between 0 and 60 s. Force development was expressed as a percentage of the maximal force developed in response to 0.3 μM cirazoline at room temperature. Maximal force in response to 0.3 μM cirazoline was 263.8 ± 18.4 and 464.6 ± 31.6 mg at room temperature and 37°C, respectively. Values represent the mean ± S.E.M. (n = 4). *P<0.05, **P<0.005: significantly different from the value for the contractile response at room temperature by Student's t-test.

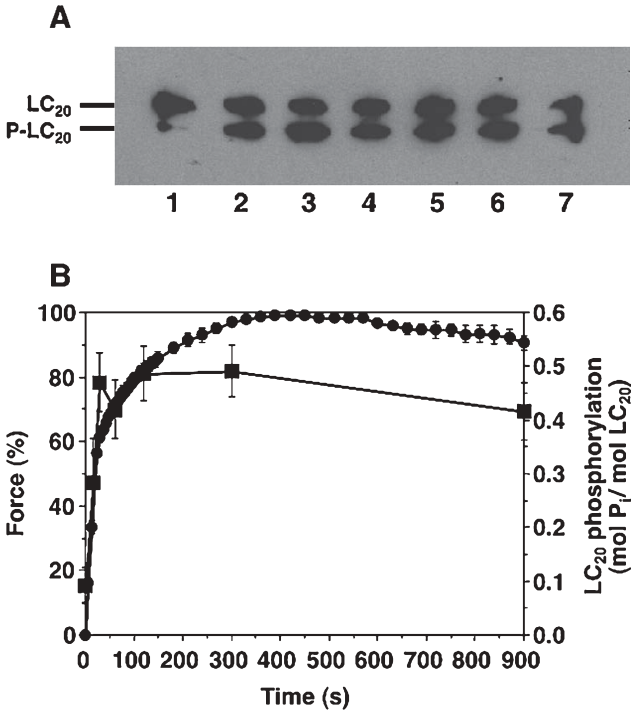


Fig. 3. Contraction and myosin light chain phosphorylation (monophosphorylation) in response to cirazoline treatment at 37°C. De-endothelialized rat caudal arterial smooth muscle strips were held at resting tension for 45 min and then transferred to solution containing 0.3 μM cirazoline at 37°C. Tissues were frozen at the indicated times and LC_{20} phosphorylation was quantified, as described in the Materials and Methods section. A: Representative Western blots showing separation of phosphorylated (P-LC_{20}) and unphosphorylated LC_{20} (LC_{20}) by urea/glycerol gel electrophoresis. Lane 1: resting conditions at 37°C. Lanes 2 – 7: 15 s, 30 s, 1 min, 2 min, 5 min, and 15 min, respectively, following addition of cirazoline. B: The time courses of force development (closed circles, expressed as a percentage of the maximal force developed in response to 0.3 μM cirazoline at 37°C) and LC_{20} phosphorylation (closed squares, expressed as mol P_i/mol LC_{20}) were quantified. Values represent the mean ± S.E.M. (n = 4 for force and phosphorylation data). Maximal force was 476.0 ± 34.5 mg. Resting and maximal LC_{20} phosphorylation levels were 0.09 ± 0.04 and 0.49 ± 0.05 mol P_i/mol LC_{20}, respectively.

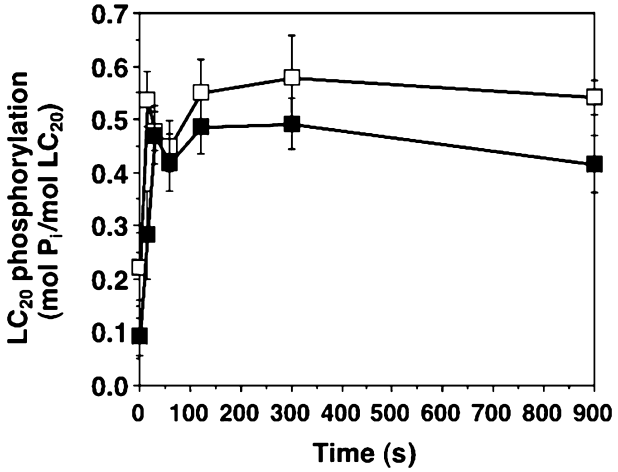


Fig. 4. Time courses of myosin light chain phosphorylation (monophosphorylation) in response to cirazoline treatment at room temperature and 37°C. De-endothelialized rat caudal arterial smooth muscle strips were held at resting tension for 45 min and then transferred to solution containing 0.3 μM cirazoline at room temperature or 37°C. Tissues were frozen at the indicated times and LC_{20} phosphorylation was quantified [n = 3 for the results at room temperature (open squares) and n = 4 for the results at 37°C (closed squares)]. Values are expressed as mol P_i/mol LC_{20} and represent the mean ± S.E.M. The LC_{20} phosphorylation data at 37°C are the same as shown in Fig. 3. Resting and maximal LC_{20} phosphorylation levels were 0.22 ± 0.06 and 0.58 ± 0.08 mol P_i/mol LC_{20} at room temperature and 0.09 ± 0.04 and 0.49 ± 0.05 mol P_i/mol LC_{20} at 37°C, respectively. The values at room temperature and 37°C were not statistically significantly different.

have a strong modulatory influence on the mechanical activity of different types of smooth muscle. However, there is little definitive information concerning the fundamental mechanisms underlying the effect of changes in temperature, which vary considerably between different smooth muscles. In intact smooth muscles, lowering temperature was reported to have both potentiating (24, 26 – 28) and inhibitory effects (18, 20, 29, 30), depending on the method of stimulation, experimental conditions (31, 32), and species (19, 23).

In rat caudal arterial smooth muscle, the concentration-response relationship of contraction induced by cirazoline at 37°C was shifted to the left as compared

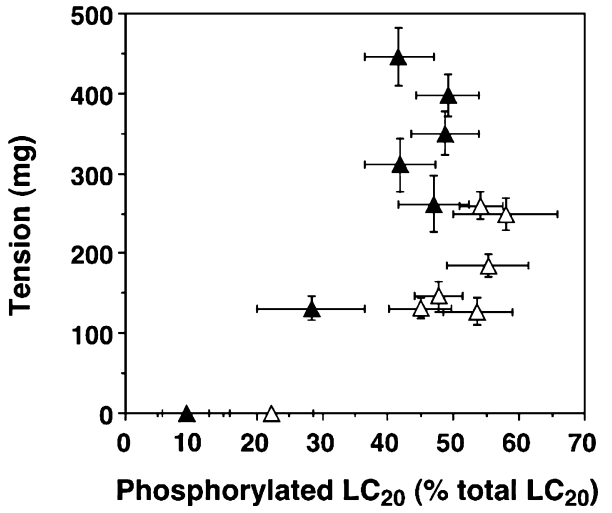


Fig. 5. Relationships between contraction and myosin light chain phosphorylation (monophosphorylation) in response to cirazoline treatment at room temperature and 37°C. From the data in Figs. 2B and 4, the relationships between tension and LC_{20} monophosphorylation were determined at room temperature (open triangle) and at 37°C (closed triangle). Tension in response to 0.3 μM cirazoline treatment was expressed in mg and phosphorylated LC_{20} was expressed as a percentage of the total LC_{20}. Values represent the mean ± S.E.M. (n = 4).

with that at room temperature (Fig. 1), indicating that the sensitivity to cirazoline is affected by the change in temperature. Furthermore, cirazoline-induced maximal force at 37°C was approximately 1.8 times that at room temperature (Fig. 2). However, the initial rate of force development at 37°C was similar to that at room temperature (Fig. 2C), indicating that the rate of contraction was temperature insensitive.

The time course of LC_{20} monophosphorylation, which occurred exclusively at serine19 (13), closely paralleled that of force at both room temperature and 37°C. Furthermore, force maintenance correlated with maintenance of high levels of LC_{20} monophosphorylation at both temperatures (Fig. 4), that is, we obtained no evidence for formation of a latch state either at room temperature or at 37°C. Moreover, the tension-LC_{20} monophosphorylation relationship was shifted to the left at 37°C with little difference in the slope of the relationship (Fig. 5). In vitro studies have indicated that MLCK and MLCP exhibit very different temperature dependencies. MLCP has an unusually high Q_{10} (5.2) compared to that of MLCK (1.7) (24). This would suggest that increasing the temperature from 23°C to 37°C would decrease LC_{20} phosphorylation, resulting in a decrease in the rate of contraction and/or steady-state force generated. The rate of contraction, however, was found to be similar at each temperature and steady-state force was significantly greater at 37°C (Fig. 2). Our results suggest that the anticipated greater increase in MLCP activity than that of MLCK in going from 23°C to 37°C is balanced by enhanced inhibition of MLCP at the higher temperature by the protein kinase C (PKC)/CPI-17 pathway and/or the RhoA/Rho-associated kinase pathway (2). The fact that the time courses of LC_{20} phosphorylation were quantitatively similar at the two temperatures (Fig. 4) is consistent with the similar rates of contraction, and indicates that the greater steady-state force at 37°C is not due to an increase in LC_{20} phosphorylation stoichiometry.

The thin filament-associated proteins calponin and caldesmon could account for the augmentation of force at the higher temperature. Calponin colocalizes with actin and tropomyosin in smooth muscle and inhibits the actin-activated Mg^{2+}-ATPase activity of phosphorylated smooth muscle myosin (33). Actin binding and ATPase inhibition are alleviated by phosphorylation of calponin by PKC or Ca^{2+}/CaM-dependent protein kinase II (3). Caldesmon, which is expressed in smooth muscle and nonmuscle cells and is also localized to actin filaments, has also been implicated in the regulation of crossbridge cycling (34). It has been shown to inhibit actomyosin ATPase activity and the movement of actin filaments over phosphorylated myosin in the in vitro motility assay. Pfitzer et al. (35) previously reported that addition of caldesmon to chemically skinned smooth muscle strips causes a rightward shift in the force-LC_{20} phosphorylation relationship. Caldesmon is phosphorylated by the mitogen-activated protein kinase, which reduces its affinity for actin, leading to loss of inhibition of the actomyosin ATPase and contraction (36). Enhanced phosphorylation of calponin and caldesmon could, therefore, explain the increased steady-state force at 37°C in response to cirazoline treatment. We previously attributed the plateau phase of cirazoline-induced contraction to maintained Ca^{2+} entry via voltage-gated Ca^{2+} channels since it was abolished by removal of extracellular Ca^{2+} or treatment with the L-type Ca^{2+} channel blocker nicardipine (13). Burdyga and Wray (18, 19) demonstrated that in guinea-pig and rat ureteric smooth muscles, the rate of rise or amplitude of the Ca^{2+} transient are little influenced by temperature and do not underlie its effects on force, suggesting that temperature does not affect Ca^{2+} entry through L-type Ca^{2+} channels from the extracellular space or Ca^{2+} release from the sarcoplasmic reticulum. However, it may not be possible to extrapolate from these species and phasic smooth muscle tissues to the tonic rat caudal artery. Increased temperature might, therefore, enhance Ca^{2+} entry during the sustained phase of contraction of the rat caudal artery, leading to an increase in MLCK activity. Since Ca^{2+}-dependent myosin phosphorylation during the sustained contraction is comparable at room temperature and 37°C, the increase in MLCK activity would have to be balanced by a greater increase in MLCP activity at the higher temperature. However, the fact that augmentation of α_1-adrenoceptor-mediated contraction by warming occurs without increased LC_{20} phosphorylation, it is necessary to consider factors other than Ca^{2+} dependency, in particular, Ca^{2+}-insensitive mechanisms of force production. We conclude, therefore, that the augmentation of force induced by cirazoline at higher temperature may be attributed to Ca^{2+}-insensitive mechanisms involved in force production such as calponin or caldesmon phosphorylation.

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