Membrane depolarization-induced RhoA/Rho-associated kinase activation and sustained contraction of rat caudal arterial smooth muscle involves genistein-sensitive tyrosine phosphorylation

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Abstract

Rho-associated kinase (ROK) activation plays an important role in K⁺-induced contraction of rat caudal arterial smooth muscle (Mita et al., Biochem J. 2002; 364: 431–40). The present study investigated a potential role for tyrosine kinase activity in K⁺-induced RhoA activation and contraction. The non-selective tyrosine kinase inhibitor genistein, but not the src family tyrosine kinase inhibitor PP2, inhibited K⁺-induced sustained contraction (IC₅₀ = 11.3 ± 2.4 µM). Genistein (10 µM) inhibited the K⁺-induced increase in myosin light chain (LC₂₀) phosphorylation without affecting the Ca²⁺ transient. The tyrosine phosphatase inhibitor vanadate induced contraction that was reversed by genistein (IC₅₀ = 6.5 ± 2.3 µM) and the ROK inhibitor Y-27632 (IC₅₀ = 0.27 ± 0.04 µM). Vanadate also increased LC₂₀ phosphorylation in a genistein- and Y-27632-dependent manner. K⁺ stimulation induced translocation of RhoA to the membrane, which was inhibited by genistein. Phosphorylation of MYPT1 (myosin-targeting subunit of myosin light chain phosphatase) was significantly increased at Thr855 and Thr697 by K⁺ stimulation in a genistein- and Y-27632-sensitive manner. Finally, K⁺ stimulation induced genistein-sensitive tyrosine phosphorylation of proteins of ~55, 70 and 113 kDa. We conclude that a genistein-sensitive tyrosine kinase, activated by the membrane depolarization-induced increase in [Ca²⁺], is involved in the RhoA/ROK activation and sustained contraction induced by K⁺.

Key words: Ca²⁺ sensitization, myosin light chain phosphatase, RhoA, Rho-associated kinase, tyrosine kinase
Introduction

Contraction of smooth muscle is regulated not only by electromechanical coupling and cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{c}), but also by membrane potential-independent, pharmacomechanical coupling (1). Force development and muscle shortening elicited by either electromechanical or pharmacomechanical coupling mechanisms are regulated by phosphorylation and dephosphorylation of the 20 kDa light chains of myosin (LC\(_{20}\)) catalysed by Ca\(^{2+}\)/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), respectively (2, 3). One of the most important recent developments in this field has been the identification of secondary mechanisms of regulation that can modify the activities of MLCK and MLCP (1–3).

Much attention has been focused recently on the molecular mechanisms underlying regulation of force independent of changes in [Ca\(^{2+}\)]\text{c}, referred to as Ca\(^{2+}\) sensitization (1). Ca\(^{2+}\) sensitizing agents include agonists that activate receptors coupled to heterotrimeric G proteins, agents that directly activate G proteins, activators of conventional and novel protein kinase Cs (PKCs), and arachidonic acid (1). Signaling pathways involved in Ca\(^{2+}\) sensitization converge on an increase in LC\(_{20}\) phosphorylation, and analyses of kinase and phosphatase activities have indicated that Ca\(^{2+}\) sensitization is mediated predominantly via inhibition of MLCP, leading to an increase in LC\(_{20}\) phosphorylation (1). The monomeric GTPase, RhoA, plays a major role in Ca\(^{2+}\) sensitization of smooth muscle contraction: RhoA-GTP activates Rho-associated kinase (ROK), which subsequently phosphorylates the myosin-targeting subunit of MLCP (MYPT1), thereby inactivating the phosphatase and leading to enhanced LC\(_{20}\) phosphorylation and smooth muscle contraction (1, 3). The activation of RhoA/ROK is, therefore, a major downstream pathway of receptor-dependent, G protein-mediated Ca\(^{2+}\) sensitization (1, 4).

Electromechanical coupling operates through changes in membrane potential, which affect [Ca\(^{2+}\)]\text{c}. Stimulation by K\(^+\) induces depolarization of the cell membrane, which opens voltage-gated Ca\(^{2+}\) channels causing Ca\(^{2+}\) influx, increased [Ca\(^{2+}\)]\text{c}, binding of Ca\(^{2+}\) to CaM, activation of MLCK, phosphorylation of myosin, activation of cross-bridge cycling and contraction (1, 5). It was assumed that K\(^+\)-induced contraction could be accounted for entirely by this signal transduction pathway. However, we reported that the ROK inhibitors Y-27632 and HA-1077 inhibited K\(^+\)-induced LC\(_{20}\) phosphorylation and sustained contraction of endothelium-free rat caudal arterial smooth muscle in a concentration-dependent manner, without affecting the K\(^+\)-induced elevation of [Ca\(^{2+}\)]\text{c} (6). Moreover, this contractile response to K\(^+\)-induced membrane depolarization was found to be absolutely dependent on the influx of Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels, since it could be blocked by a Ca\(^{2+}\) channel blocker or by removal of extracellular Ca\(^{2+}\). These results suggested that ROK activation plays an important role in K\(^+\)-induced contraction of endothelium-free rat caudal arterial smooth muscle and specifically that the tonic phase of K\(^+\)-induced contraction is mediated via inhibition of MLCP activity by Ca\(^{2+}\)-dependent ROK activation (6). Physiological elevations of [Ca\(^{2+}\)]\text{c}, and consequent MLCK activation are not sufficient to cause a maximal force response unless GTPase-dependent pathways leading to MLCP inhibition are simultaneously activated. Maintained contraction in response to membrane depolarization as well as agonists depends on both MLCK and ROK activities. Several more recent studies have shown that RhoA/ROK activation plays an important role in K\(^+\)-induced contraction of a variety of smooth muscle types (7–13). However, the signal transduction pathway underlying RhoA/ROK activation in response to K\(^+\) depolarization remains incompletely defined.

The involvement of protein kinase C (PKC), tyrosine kinase and ROK in Ca\(^{2+}\) sensitization has been reported in intact and permeabilized smooth muscle (1). In addition, cross-talk between these different kinase pathways may be a key signaling event of Ca\(^{2+}\) sensitization of the contractile apparatus during agonist-induced
contractile activation of vascular smooth muscle (14). Tyrosine kinase inhibitors have been shown to attenuate agonist-induced contraction and LC\textsubscript{20} phosphorylation in intact smooth muscle (15) and genistein was found to inhibit agonist-induced augmentation of Ca\textsuperscript{2+}-induced contraction in permeabilized smooth muscle (16). Furthermore, tyrosine kinase activation in response to receptor stimulation has been implicated in Ca\textsuperscript{2+} sensitization via activation of the RhoA/ROK pathway in various smooth muscle tissues (16–19). Tyrosine kinases, therefore, may play an important role in RhoA-mediated Ca\textsuperscript{2+} sensitization of smooth muscle contraction. In this study we examined the involvement of tyrosine phosphorylation in K\textsuperscript+-induced contraction and RhoA/ROK activation of rat caudal arterial smooth muscle.

### Materials and Methods

**Materials**

Prazosin, DL-propranolol, genistein, genistin and creatine kinase were purchased from Sigma-Aldrich (St. Louis, MO, USA), dithiothreitol (DTT) from Wako Pure Chemical Industries (Osaka, Japan), Hepes from Dojin Laboratories (Kumamoto, Japan) and sodium orthovanadate from Calbiochem-Novabiochem (San Diego, CA, USA). Y-27632 was generously provided by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). All other chemicals were of reagent grade. Stock solutions were prepared in water for prazosin, propranolol, and Y-27632, and in dimethylsulphoxide (DMSO) for genistein and genistin. The following proteins were purified from chicken gizzard smooth muscle as previously described: LC\textsubscript{20} (20), CaM (21) and MLCK (22).

**Force measurements in intact muscle strips**

Male Sprague-Dawley rats (300–400 g) were anaesthetized and killed by exsanguination as approved by the Institutional Ethics Committee for Animal Research at Meiji Pharmaceutical University. De-endothelialized caudal arterial smooth muscle strips were prepared for force measurements as previously described (6, 23). All buffers were at room temperature and were pre-oxygenated with 100% O\textsubscript{2}. 60 mM K\textsuperscript{+} solution was prepared by replacing the NaCl in Hepes-Tyrode (H-T) solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5.6 mM glucose, 10 mM Hepes, pH 7.4) with equimolar KCl. All measurements of 60 mM K\textsuperscript{+}-induced contraction were carried out in the presence of 1 µM prazosin and 0.1 µM propranolol in order to block the \(\alpha\textsubscript{1}\)- and \(\beta\)-adrenergic effects of noradrenaline, which is released from nerve terminals by depolarization (6).

**Force and LC\textsubscript{20} phosphorylation measurements in skinned muscle strips**

Male Sprague-Dawley rats (300–350 g) were killed by exsanguination following halothane inhalation as approved by the Animal Care Committee of the Faculty of Medicine, University of Calgary and conforming to the standards of the Canadian Council on Animal Care. De-endothelialized caudal arterial smooth muscle strips were skinned (demembranated) with Triton X-100 and prepared for force measurements as previously described (24). Skinned tissues were washed three times for 5 min each in pCa 9 solution (20 mM TES, 4 mM K\textsubscript{2}EGTA, 5.83 mM MgCl\textsubscript{2}, 7.56 mM potassium propionate, 3.9 mM Na\textsubscript{2}ATP, 0.5 mM dithioerythritol (DTE), 16.2 mM phosphocreatine, 15 units/ml creatine kinase, pH 6.9) followed by incubation for 15 min in pCa 4.5 solution (20 mM TES, 4 mM CaEGTA, 5.66 mM MgCl\textsubscript{2}, 7.53 mM potassium propionate, 3.9 mM Na\textsubscript{2}ATP, 0.5 mM DTE, 16.2 mM phosphocreatine, 15 units/ml creatine kinase, pH 6.9) to elicit a sustained Ca\textsuperscript{2+}-induced contraction. Skinned muscle strips were then relaxed by incubation in pCa 9 solution for 10 min and washed for 2 × 5 min in pCa 9 solution. At selected times before or after stimulation, tissues were rapidly frozen in 10% (w/v) trichloroacetic acid (TCA), 10 mM DTT in dry ice/acetone. The residual TCA was washed out with 3
× 1 ml washes of dry ice-cold 10 mM DTT/acetone and tissues were lyophilized for 16 h and stored at –80°C until LC20 extraction. Quantification of LC20 phosphorylation was achieved by urea/glycerol-polyacrylamide gel electrophoresis and western blotting as previously described (6, 23). Phosphorylation stoichiometry was calculated from the following equation: mol Pi/mol LC20 = (x + 2z)/(x + y + z), where x, y and z are the signal intensities of unphosphorylated, mono- and di-phosphorylated LC20 bands, respectively.

**Measurement of changes in [Ca2+]i**

Measurement of [Ca2+]i in fura-2-loaded smooth muscle strips was achieved as described by Mita et al. (6). Muscle strips were incubated with H-T solution containing 16 µM fura 2-AM for 4 h in the dark at room temperature (23 °C). The fluorescence of mounted muscle strips was recorded at an emission wavelength of 500 nm with excitation at 340 and 380 nm using a Jasco CAF-100 spectrofluorimeter (Jasco, Tokyo, Japan). The fluorescence ratio \( \frac{F_{340}}{F_{380}} \) was monitored before (resting state) and during stimulation with 60 mM K+ for 20 min in the absence and presence of genistein. Separate tissues were used to evaluate the effect of genistein on the [Ca2+]i response to 60 mM K+ and to compare them with controls since the fluorescence fades gradually over the long time periods that would be required to carry out control and drug treatments on the same tissue.

**Analysis of RhoA translocation**

Separation of particulate and cytosolic fractions was achieved by the method of Gong et al. (25). Six small strips (0.5 mm × 6 mm) of rat caudal arterial smooth muscle were used to provide sufficient protein for reliable separation of cytosolic and particulate fractions. Strips were homogenized in ice-cold homogenization buffer (10 mM Tris, pH 7.5, 5 mM MgCl2, 2 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM 4-(2-aminoethyl) benzensulfonyl fluoride, 20 µg/ml leupeptin and 20 µg/ml aprotinin) and centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatant was collected as the cytosolic fraction. Pellets were resuspended, and membrane proteins were extracted by incubation for 30 min in homogenization buffer containing 1% Triton X-100 and 1% sodium cholate. The extract was centrifuged at 800 × g for 10 min and the supernatant was collected as the membrane fraction. Samples (17 µl) of cytosolic fraction and membrane fraction were subjected to SDS-PAGE (15% acrylamide). After transfer to polyvinylidene difluoride (PVDF) membrane, the membranes were blocked with 1% Blocking Reagent (Roche, Mannheim, Germany) in 50 mM Tris, 150 mM NaCl, pH 7.5 for 16 h at 4 °C and then incubated with primary antibody for 3 h and secondary antibody for 1 h at room temperature. RhoA was detected with the enhanced BM Chemiluminescence Western Blotting Kit (Roche) and quantified by densitometry using Image Master 1D software and a Pharmacia Biotech NEC Image 466es equipped with a Sharp JX-330 scanner. The percentage of RhoA in the particulate fraction was calculated according to: \[
\frac{\text{particulate RhoA}}{\text{particulate + cytosolic RhoA}} \times 100\%.
\]

Monoclonal anti-RhoA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 1 : 100 dilution.

**Assay of MLCK activity**

The reaction mixture (0.16 ml) contained 20 mM Tris-HCl, pH 7.5, 60 mM KCl, 4 mM MgCl2, 0.1 mM CaCl2, 0.5 nM MLCK, 1 µM CaM, 10 µM LC20 and 0–100 µM genistein or genistin. Reaction mixtures were pre-incubated at 30°C for 2 min and reactions were initiated by the addition of [γ-32P]ATP to a final concentration of 0.2 mM (240 cpm/pmol). Samples (20 µl) of reaction mixtures were withdrawn at 1, 2, 3, 4, 5, 7.5 and 10 min and spotted onto Whatman P81 phosphocellulose paper discs, which were washed and counted as previously described (22). Reaction time courses were linear under these conditions.
Western blotting of MYPT1

Extraction of proteins was achieved by the method of Wilson et al. (26). Protein was extracted from freeze-dried tissues by addition of 200 µl of 50 mM Tris-HCl, pH 6.8, containing 1 % SDS and 1 mM diisopropylfluorophosphate. Samples were heated to 95 °C for 5 min, and then mixed for 60 min. Samples (20 µl) were subjected to SDS-PAGE (7.5 % acrylamide). After transfer to nitrocellulose membrane, the membranes were blocked with 1 % Blocking Reagent (Roche) in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) for 16 h at 4 °C and then incubated with primary antibody for 4 h and secondary antibody for 1 h at room temperature. Signals were detected with the enhanced BM Chemiluminescence Western Blotting Kit (Roche) and quantitated by densitometry using Image J (v. 1.32) software and stored on a PhotoStudio4 equipped with a Canon 9950F scanner (Canon Inc., Tokyo, Japan). The level of phosphorylation of MYPT1 at Thr697 and Thr855 was calculated according to P-Thr697 MYPT1 or P-Thr855 MYPT1/total MYPT1. Anti-MYPT1 rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 1 : 250 dilution. Anti-[phosphoThr697]-MYPT1 rabbit polyclonal antibody (Upstate Cell Signaling Solutions, Charlottesville, VA, USA) was used at 1 : 3,500 dilution. Anti-[phosphoThr855]-MYPT1 rabbit polyclonal antibody (Upstate Cell Signaling Solutions) was used at 1 : 3,000 dilution.

Identification of tyrosine-phosphorylated proteins

Tissue samples were frozen at selected times following 60 mM K+ stimulation in the absence or presence of 10 µM genistein by immersion in 10 % (w/v) TCA/10 mM DTT in dry ice/acetone for 10 min. The residual TCA was washed out with 10 mM DTT/acetone and tissues were lyophilized for 16 h. Protein was extracted from freeze-dried tissues by addition of 175 µl of sample buffer (60 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 0.02 % bromophenol blue, 0.1 M DTT containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Sigma-Aldrich)). Samples were heated to 95 °C for 10 min, and then mixed for 16 h at 4 °C. Protein samples (45 µl for tyrosine-phosphorylation or 22.5 µl for actin) were then subjected to SDS-PAGE (5 % or 7.5 % acrylamide). After electrophoresis, proteins were transferred to a PVDF membrane at 140 V for 1 h. Firstly, phosphorylated proteins on the PVDF membrane were detected by Phos-tagTM BTL-104 (NARD Institute Ltd., Hyogo, Japan) (27) according to the manufacturer’s instructions. This biotin-pendant Zn2+-Phos-tag reagent enables the chemiluminescence detection of proteins phosphorylated on serine, threonine or tyrosine residues using horseradish peroxidase-conjugated streptavidin. After complete removal of the Phos-tag by incubating with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2 % SDS and 1.4 % 2-mercaptoethanol), membranes were blocked using 1 % Blocking Reagent (Roche) in 50 mM Tris, 150 mM NaCl, pH 7.5 for 1 h and then incubated overnight with an anti-phosphotyrosine mouse monoclonal antibody (AG10, Upstate, NY, USA) or an anti-actin goat polyclonal antibody (Santa Cruz) at 4 °C and secondary antibody for 2 h at room temperature. Immunoreactive bands were detected with the enhanced BM Chemiluminescence Western Blotting Kit (Roche) and quantified by Lightcapture (ATTO, Tokyo, Japan) using CS Analyzer software. The protein tyrosine phosphorylation levels were normalized to actin. Anti-phosphotyrosine mouse monoclonal antibody and anti-actin goat polyclonal antibody were used at 1 : 900 and 1 : 800 dilution, respectively.

Statistical analysis

Data represent the mean ± standard error of the mean (S.E.M.). Values of n indicate the numbers of smooth muscle strips utilized. Student’s t test was used for statistical comparisons. One-way ANOVA followed by Tukey-Kramer multiple-comparisons test was used to compare three or more groups. P values<0.05 were considered to be statistically significant. These analyses were performed using JMP-5J (SAS Institute Japan, Tokyo, Japan).
**Results**

**Effects of tyrosine kinase inhibitors on K⁺- and ionomycin-induced contractions**

Membrane depolarization of de-endothelialized rat caudal arterial smooth muscle with 60 mM K⁺ induces a rapid increase in force, which then declines to a steady-state level that is significantly greater than resting force (Mita et al. (6) and controls in Figs. 1C and D). The initial phasic component of the contractile response is attributable to an increase in [Ca²⁺], activation of MLCK and phosphorylation of LC₂₀, while the sustained tonic component involves activation of the RhoA/ROK pathway leading to inhibition of MLCP. We used tyrosine kinase inhibitors to test the hypothesis that tyrosine phosphorylation plays a role in depolarization-induced contraction. When added after steady-state 60 mM K⁺-induced force had been achieved, the tyrosine kinase inhibitor genistein evoked concentration-dependent relaxation to the basal level of force with an IC₅₀ of 11.3 ± 2.4 µM (Fig. 1A). The inactive analogue genistin had no effect on K⁺-induced sustained contraction (Fig. 1A). The src family tyrosine kinase inhibitor PP2, on the other hand, had a slight inhibitory effect on K⁺-induced sustained contraction at 10 µM (to 91.0 ± 7.7% of control force) and a greater effect at 100 µM (to 53.7 ± 8.0% of control force) (Fig. 1B), likely due to a non-specific effect given the high potency of PP2 for inhibition of src family kinases (IC₅₀ ~5 nM (28)). Pre-treatment of rat caudal arterial strips with genistein (3–30 µM) (Fig. 1C) reduced both the phasic and tonic components of 60 mM K⁺-induced contraction in a concentration-dependent manner. Pre-treatment with genistin, on the other hand, had no effect on the tonic contractile response to KCl and actually caused a small (~20%) increase in the phasic response (Fig. 1D). Moreover, pre-treatment with genistein (10 µM) or the ROK inhibitor Y-27632 (1 µM) almost completely abolished the contractile response induced by the Ca²⁺ ionophore ionomycin (40 µM) (Fig. 1E).

**Effect of genistein on [Ca²⁺]**

In fura 2-AM-loaded rat caudal arterial smooth muscle strips, the time course of the change in [Ca²⁺], in response to K⁺ depolarization closely matched the time course of contraction. Stimulation of arterial strips with 60 mM K⁺ led to a transient increase in [Ca²⁺], which settled at a sustained level significantly above resting [Ca²⁺] (Fig. 2A). The F₃₄₀/F₃₈₀ ratio under resting conditions was unaffected by 10 or 30 μM genistein (Fig. 2B). The peak and sustained increases in [Ca²⁺] induced by 60 mM K⁺ were unaffected by 10 µM genistein, but were slightly decreased by 30 µM genistein (Fig. 2B). Therefore, we used 10 µM genistein in the following experiments.

**Effect of genistein on K⁺-induced LC₂₀ phosphorylation**

The time courses of the changes in [Ca²⁺], (Fig. 2A), LC₂₀ phosphorylation (Fig. 3) and contraction (Fig. 1C) in response to 60 mM K⁺ were similar and consistent with the phasic component of the contractile response being due to Ca²⁺ entry, leading to CaM-dependent activation of MLCK. Under control conditions, LC₂₀ phosphorylation increased rapidly in response to 60 mM K⁺ from a resting level of 0.12 ± 0.04 mol P/mol LC₂₀ to a peak of 0.54 ± 0.03 mol P/mol LC₂₀ at 15 s after K⁺ addition. LC₂₀ phosphorylation then declined to a steady-state level of 0.35 ± 0.08 mol P/mol LC₂₀ at 15 min after K⁺ addition. In the presence of genistein (10 µM), LC₂₀ phosphorylation levels increased rapidly in response to K⁺ from a resting level of 0.19 ± 0.04 mol P/mol LC₂₀ to a peak of 0.28 ± 0.05 mol P/mol LC₂₀ at 15 s after 60 mM K⁺ addition and then declined to 0.23 ± 0.05 mol P/mol LC₂₀ at 15 min after 60 mM K⁺ addition. 10 µM genistein, therefore, reduced the transient increase in LC₂₀ phosphorylation, and abolished the sustained elevation of LC₂₀ phosphorylation. The effects of 10 µM genistein on the contractile response to membrane depolarization (Fig. 1C) can be explained, therefore, by inhibition of LC₂₀ phosphorylation (Fig. 3) without affecting the Ca²⁺ transient (Fig. 2).
Effects of genistein and Y-27632 on vanadate-induced contraction and LC20 phosphorylation

The tyrosine phosphatase inhibitor vanadate (30 µM) (29, 30) induced a slow, sustained contraction of rat caudal arterial smooth muscle, which reached a maximum after ~3 h. The steady-state force evoked by vanadate was 368.4 ± 20.1 mg (n=12), which was significantly greater than that elicited by 60 mM K+ (188.5 ± 9.2 mg (n=12); P<0.005). Genistein inhibited vanadate-induced contraction and caused a concentration-dependent
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relaxation to the basal level of force with an IC\textsubscript{50} of 6.5 ± 2.3 µM (Figs. 4A and B). The inactive analogue genistin had a weak inhibitory effect on vanadate-induced contraction at high concentrations (Fig. 4B). The ROK inhibitor Y-27632 also inhibited vanadate-induced contraction to the basal level with an IC\textsubscript{50} of 0.27 ± 0.04 µM (Fig. 4C). LC\textsubscript{20} phosphorylation levels increased significantly in response to vanadate from a resting level of 0.15 ± 0.03 mol P\textsubscript{i}/mol LC\textsubscript{20} to 0.36 ± 0.02 mol P\textsubscript{i}/mol LC\textsubscript{20} at the maximal contraction (Figs. 4D and E). Vanadate-induced LC\textsubscript{20} phosphorylation was significantly reduced by pre-treatment with genistein (10 µM) or Y-27632 (1 µM) to 0.22 ± 0.03 mol P\textsubscript{i}/mol LC\textsubscript{20} and 0.26 ± 0.02 mol P\textsubscript{i}/mol LC\textsubscript{20}, respectively (Figs. 4D and E). Thus, genistein and Y-27632 reduced the sustained elevation of LC\textsubscript{20} phosphorylation induced by vanadate.

Effects of genistein on Ca\textsuperscript{2+}- and microcystin-induced contractions of Triton-skinned smooth muscle, and on MLCK activity in vitro

To evaluate the possibility that genistein acts on kinases capable of evoking LC\textsubscript{20} phosphorylation, i.e. MLCK, integrin-linked kinase (ILK) and zipper-interacting protein kinase (ZIPK) (26, 31, 32), we investigated the effect of genistein on Ca\textsuperscript{2+}- and phosphatase inhibitor (microcystin)-induced contractions of Triton-skinned smooth muscle; Ca\textsuperscript{2+}-induced contraction is mediated by MLCK whereas Ca\textsuperscript{2+}-independent, microcystin-induced contraction is mediated by ILK and/or ZIPK (26, 31, 32). Neither genistein (100 µM) nor the inactive analogue genistin (100 µM) had an effect on steady-state force induced by treatment of Triton-skinned smooth muscle strips with 60 mM K\textsuperscript{+} or 1 µM genistein (100 µM) or the inactive analogue genistin (100 µM) had an effect on steady-state force induced by treatment of Triton-skinned smooth muscle strips with 60 mM K\textsuperscript{+} or 1 µM genistein (100 µM) or the inactive analogue genistin (100 µM).

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Fig. 2. Effect of genistein on K\textsuperscript{+}-induced changes in [Ca\textsuperscript{2+}].

Intact rat caudal arterial smooth muscle strips were loaded with fura 2-AM. The F\textsubscript{340}/F\textsubscript{380} ratio was recorded following stimulation with 60 mM K\textsuperscript{+} in the absence or presence of genistein. Genistein was present 20 min prior to, and during, the K\textsuperscript{+}-induced contraction. (A) Representative traces showing the change in F\textsubscript{340}/F\textsubscript{380} ratio induced by K\textsuperscript{+} in the absence (a) and presence of 10 µM (b) or 30 µM (c) genistein. (B) The F\textsubscript{340}/F\textsubscript{380} ratio for resting, peak and sustained components in response to K\textsuperscript{+} in the absence (open bars; n=9) and presence of 10 µM (hatched bars; n=6) or 30 µM (filled bars; n=7) genistein. The fluorescence intensity of the sustained component was measured 20 min after K\textsuperscript{+} addition. Results are expressed as the mean ± S.E.M. F\textsubscript{340}/F\textsubscript{380} values with 10 µM genistein were not significantly different from those without genistein; however, those with 30 µM genistein were significantly different from those without genistein (* P<0.005).
rat caudal arterial smooth muscle strips with pCa 4.5 (Figs. 5A and C) or microcystin (1 µM) at pCa 9 (Figs. 5B and C). Likewise, neither compound had a significant effect on the time to half-maximal contraction (t_{1/2}) of Ca^{2+}-induced or Ca^{2+}-independent, microcystin-induced contraction (Fig. 5D).

At pCa 4.5, only mono-phosphorylation of LC_{20} was detected (Fig. 5E), consistent with phosphorylation of LC_{20} by MLCK exclusively at Ser19. However, at pCa 9 in the presence of microcystin, both mono- and di-phosphorylation of LC_{20} were detected (Fig. 5E), consistent with Ca^{2+}-independent contraction in response to MLCP inhibition being due to phosphorylation of LC_{20} by ILK and/or ZIPK (26, 31–33). Neither genistein (100 µM) nor the inactive analogue genistin (100 µM) had any effect on LC_{20} phosphorylation in Triton-skinned muscle strips at pCa 4.5 or at pCa 9 in the presence of microcystin (Fig. 5E), indicating that the inhibitory effect of genistein on K^{+}-induced contraction cannot be attributed to inhibition of MLCK, ILK or ZIPK.

Furthermore, to ensure that the inhibition of K^{+}-induced contraction by genistein was not due to inhibition of MLCK, the activity of purified MLCK in vitro was assayed in the presence of various concentrations...
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of genistein or the inactive analogue genistin. Genistein had no effect on the activity of purified MLCK at concentrations below 50 µM, but did cause significant inhibition at 50 and 100 µM, whereas genistin had no inhibitory effect over the entire concentration range from 0–100 µM (Fig. 5F). Thus, endogenous MLCK activity is unlikely to be affected at the concentration (10 µM) of genistein used in the present study.
Effect of genistein on K⁺-induced translocation of RhoA

RhoA activation involves its translocation from the cytosol to the plasma membrane (25). To investigate the possibility that membrane depolarization activates a tyrosine kinase that induces RhoA translocation, RhoA was quantified in the cytosolic and particulate fractions obtained from rat caudal arterial smooth muscle.
and the effect of pre-treatment with genistein on RhoA translocation examined (Fig. 6). In the absence of K⁺ stimulation, the particulate fraction contained 20.3 ± 2.8% of the total RhoA. This was not significantly different (21.8 ± 5.3%) 15 s after 60 mM K⁺ addition. However, the amount of RhoA in the particulate fraction was significantly increased (to 40.0 ± 5.2%) at 15 min after stimulation (Fig. 6). Pre-treatment with genistein (10 µM) prevented the translocation (activation) of RhoA: the particulate fraction under these conditions contained 27.9 ± 6.2% of total RhoA at 15 min after stimulation, which was not significantly different from the value (27.7 ± 2.2%) under resting conditions in the presence of genistein (Fig. 6).

Fig. 6. Effect of genistein on K⁺-induced translocation of RhoA
Muscle strips were pre-incubated for 20 min without or with 10 µM genistein. RhoA was quantified in the cytosolic and particulate fractions as described in the Materials and Methods section. (A) Representative western blots showing the translocation of RhoA from the cytosolic to the particulate fraction in the absence or presence of 10 µM genistein. (B) Cumulative data (n=4–5) indicating the proportion of RhoA recovered in the particulate fraction in the absence (open bars) or presence (filled bars) of genistein. Values represent the mean ± S.E.M. * P<0.05; significantly different from the value under resting conditions without genistein.

Effects of Y-27632 and genistein on K⁺-induced MYPT1 phosphorylation at Thr697 and Thr855

Phosphorylation of MYPT1 at Thr697 and Thr855 in rat caudal arterial smooth muscle in response to 60 mM K⁺ was examined using phosphospecific antibodies. Basal phosphorylation was detected at Thr697 and Thr855 in unstimulated tissue (Fig. 7A). 60 mM K⁺ stimulation for 15 s did not significantly change the phosphorylation at Thr697 or Thr855. However, treatment with 60 mM K⁺ for 15 min caused a significant increase in phosphorylation at Thr697 and Thr855 (Fig. 7A).

Basal levels of phosphorylation of MYPT1 in the presence of 3 µM Y-27632 or 10 µM genistein were not significantly different from those in the absence of inhibitors (Figs. 7B and C). 60 mM K⁺ stimulation for 15 s in the presence of Y-27632 or genistein did not affect the level of phosphorylation at Thr697 or Thr855 (Figs. 7B and C). Furthermore, the significant increase in phosphorylation of MYPT1 at Thr697 and Thr855 induced
by 60 mM K+ stimulation for 15 min was not observed in the presence of Y-27632 or genistein (Figs. 7B and C).

**K+**-induced tyrosine-phosphorylated proteins

Proteins phosphorylated on tyrosine residues in response to membrane depolarization with 60 mM K+ were detected by SDS-PAGE and western blotting. Tyrosine-phosphorylated proteins were identified by overlap of bands detected with an anti-phosphotyrosine antibody with those that bound biotinylated Phos-tag™ BTL-104 (27). 60 mM K+ caused an increase in tyrosine phosphorylation of proteins of ~55, 70 and 113 kDa following 2–5 min of stimulation (Fig. 8). The increase in phosphorylation of MYPT1 at Thr697 and Thr855 after 15 min, but not 15 s. The increase in phosphorylation of MYPT1 at Thr697 and Thr855 at 15 min after addition of 60 mM K+ was not observed in the presence of Y-27632 or genistein. (b) Cumulative data of P-Thr697 MYPT1 (open bars) and P-Thr855 MYPT1 (hatched bars) in the absence (n=5–7) or presence (n=4–5) of Y-27632 or genistein. The data are expressed as the ratio of signal intensities of phosphorylated MYPT1: total MYPT1. Values represent the mean ± S.E.M. * P<0.05; significantly different from the value under resting conditions.

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**Discussion**

We demonstrated previously that vascular smooth muscle membrane depolarization activates the RhoA/ROK pathway via the influx of extracellular Ca2+ (6). Protein tyrosine phosphorylation has been implicated in the regulation of [Ca2+], and the Ca2+ sensitivity of vascular smooth muscle contraction in response to receptor stimulation (14–19, 34–36). The primary objective of the current study was to determine if a tyrosine kinase mediates the depolarization-induced activation of RhoA/ROK and contraction. We found that the phasic and tonic components of the contractile response of rat caudal arterial smooth muscle induced by membrane depolarization with 60 mM K+ were inhibited by a well-known inhibitor of a broad range of tyrosine kinases, genistein (37), in a concentration-dependent manner, but not by genistin, which lacks tyrosine kinase inhibitory activity. Genistin actually caused a small (~20%) increase in the phasic K+−induced force response, confirming the specificity of the inhibitory response to genistein. The IC50 for inhibition of sustained force by genistein...
was 11.3 ± 2.4 µM (Fig. 1A), i.e. similar to the reported value for inhibition of tyrosine kinase activity (2.6 µM) (37). These results suggest that the inhibitory effect of genistein on K⁺-induced contraction is due to its tyrosine kinase inhibition. On the other hand, the PKC inhibitors calphostin C and chelerythrine, and the MAP kinase kinase (MEK) inhibitor PD98059, had no significant effect on K⁺-induced contraction (data not shown), ruling out the possibility that K⁺-induced contraction requires PKC and MEK activities. Moreover, the src family tyrosine kinase inhibitor PP2 (IC₅₀ ~5 nM (28)) exhibited partial inhibition of K⁺-induced sustained contraction only at a very high concentration (100 µM) (Fig. 1B), consistent with the observations of Nakao et al. (19) who reported that another src family tyrosine kinase inhibitor (PP1) had no effect on K⁺-induced contraction of porcine coronary arterial smooth muscle. We conclude, therefore, that protein tyrosine phosphorylation induced by a genistein-sensitive tyrosine kinase, which is not a src family member, is involved in both the phasic and tonic contractions induced by 60 mM K⁺ in rat caudal arterial smooth muscle.
Genistein has been reported to inhibit voltage-operated Ca\(^{2+}\) currents in vascular smooth muscle cells isolated from rabbit ear artery in a concentration-dependent manner (38). Our results, however, cannot be explained by inhibition of voltage-operated Ca\(^{2+}\) currents by genistein. The Ca\(^{2+}\) transient in response to K\(^+\) was unaffected by 10 µM but not 30 µM genistein (Fig. 2). Furthermore, genistein inhibited Ca\(^{2+}\) ionophore (ionomycin)-induced contraction (Fig. 1E), whereas the Ca\(^{2+}\) channel blocker nicardipine (30 nM), which abolished 60 mM K\(^+\)-induced contraction, did not inhibit ionomycin-induced contraction (data not shown). These results suggest that 10 µM genistein does not act as a Ca\(^{2+}\) channel blocker to reduce LC20 phosphorylation and contraction, and furthermore, physiological elevations of [Ca\(^{2+}\)], appear to activate a genistein-sensitive tyrosine kinase. On the other hand, it was reported that tyrosine kinases such as c-Src and focal adhesion kinase (FAK) regulate Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels in smooth muscle (39). However, 10 µM genistein had no effect on the Ca\(^{2+}\) transient stimulated by K\(^+\) depolarization, suggesting that K\(^+\) stimulation does not activate Src or FAK.

Seok et al. (13) reported that KCl-induced activations of RhoA, LC20 phosphorylation and contraction of rat aortic rings were inhibited by the isoflavone tyrosine kinase inhibitor genistein and daidzein, which lacks tyrosine kinase inhibitory activity. They concluded, therefore, that the observed inhibitory effects of genistein could not be attributed to tyrosine kinase inhibition. The inhibitory effect of daidzein on rat aortic smooth muscle contraction was confirmed by Je and Sohn (40). In contrast, we have shown that genistin, an isoflavone analog of genistein that lacks tyrosine kinase inhibitory activity, has no effect on KCl-induced sustained contraction of rat caudal arterial smooth muscle (Fig. 1A) supporting the conclusion that, in this tissue, membrane depolarization activates the RhoA/ROK pathway via activation of a tyrosine kinase.

Genistein (10 μM) inhibited K\(^+\)-induced LC20 phosphorylation (Fig. 3) without affecting the K\(^+\)-induced increase in [Ca\(^{2+}\)], (Fig. 2), which correlated well with the force response to K\(^+\) in the presence of this concentration of genistein (Fig. 1C). The possibility that the inhibition of force development and LC20 phosphorylation by genistein could be due to inhibition of MLCK, ILK or ZIPK, which are capable of phosphorylating LC20 directly (26, 31–33), had to be considered. Therefore, we examined the effects of genistein on contractions of Triton-skinned tissue elicited by activation of MLCK (by Ca\(^{2+}\)) or ILK and ZIPK (by microcystin via phosphatase inhibition). ILK and ZIPK phosphorylate LC20 in a Ca\(^{2+}\)-independent manner at Ser19 and Thr18 (26, 31) whereas MLCK phosphorylates LC20 exclusively at Ser19 (32, 33). Genistein (100 µM) had no effect on Ca\(^{2+}\)-induced contraction or LC20 mono-phosphorylation or on Ca\(^{2+}\)-independent force development or LC20 mono- and di-phosphorylation induced by microcystin (Fig. 5). Moreover, genistein had no effect on the activity of purified MLCK \textit{in vitro} at concentrations below 50 µM (Fig. 5F). These results indicate that inhibition of K\(^+\)-induced contraction of rat caudal arterial smooth muscle by genistein (10 µM) is not due to an inhibitory effect of the compound on MLCK, ILK or ZIPK.

The tyrosine phosphatase inhibitor vanadate induces smooth muscle contraction (30, 36, 41), which is associated with increased tyrosine phosphorylation of several proteins (16, 41). We found that vanadate induced a slow, sustained contraction of rat caudal arterial smooth muscle that was inhibited by genistein with an IC\(_{50}\) of 6.5 ± 2.3 µM (Fig. 4), which is not significantly different from the IC\(_{50}\) for inhibition of K\(^+\)-induced contraction (Fig. 1). The ROK inhibitor Y-27632 also inhibited vanadate-induced contraction, with an IC\(_{50}\) of 0.27 ± 0.04 µM (Fig. 4), similar to the IC\(_{50}\) value (0.41 ± 0.02 µM) for inhibition of K\(^+\)-induced contraction (6). Vanadate also increased LC20 phosphorylation, which was inhibited by both genistein and Y-27632 (Figs. 4D and E). These results suggest that protein tyrosine phosphorylation induces contraction of rat caudal arterial smooth muscle via the activation of ROK, consistent with previous studies showing that vanadate-induced contraction and LC20 phosphorylation of ileal smooth muscle was inhibited by pre-treatment with genistein or Y-27632 (30).
Membrane-bound RhoA represents the GTP-bound, active form of RhoA, and α₁-adrenoceptor activation, GTPγS, and high \([\text{Ca}^{2+}]\), have been shown to induce translocation of RhoA from the cytosol to the membrane (25). We confirmed the translocation of RhoA in rat caudal arterial smooth muscle in response to membrane depolarization, which was observed after 15 min but not 15 s of K⁺ stimulation, and was inhibited by genistein (Fig. 6). Our results suggest that K⁺ depolarization, leading to a sustained increase in \([\text{Ca}^{2+}]\), causes RhoA translocation via activation of a genistein-sensitive tyrosine kinase. It was previously reported that in rabbit renal artery, rabbit aorta, and rat aorta, translocation of RhoA is induced by K⁺ stimulation (8, 9, 13) and the K⁺-induced activation of RhoA is inhibited by a CaM inhibitor, suggesting that \([\text{Ca}^{2+}]\) and CaM may be involved in RhoA activation (8). However, the molecular mechanism by which genistein and a CaM inhibitor inhibit RhoA activation remains to be elucidated.

K⁺ stimulation increased the phosphorylation of MYPT1 at the two ROK sites, Thr697 and Thr855, after 15 min but not 15 s (Fig. 7). In other words, K⁺-induced phosphorylation of MYPT1 at Thr697 and Thr855 increased only during the tonic phase of K⁺-induced contraction. These results are consistent with the time course of translocation of RhoA stimulated by K⁺ (Fig. 6) and our previous results showing that the ROK inhibitors Y-27632 and HA-1077 abolished the tonic component of K⁺-induced contraction but not the phasic component (6). Furthermore, these increases in MYPT1 phosphorylation were inhibited by Y-27632 and genistein. These results suggest that K⁺-induced inhibition of MLCP activity is caused by phosphorylation of MYPT1 at both Thr697 and Thr855 by ROK via activation of a genistein-sensitive tyrosine kinase and RhoA. This leads to an increase in LC₂₀ phosphorylation and contraction. Our results indicate that RhoA/ROK activation via a genistein-sensitive tyrosine kinase plays an important role in the tonic component of K⁺-induced contraction and LC₂₀ phosphorylation. However, pre-treatment of caudal arterial strips with genistein also reduced the phasic component of the K⁺-induced contraction. The inhibitory effect of genistein on the phasic component of the contraction must, therefore, involve a mechanism distinct from the tyrosine kinase-mediated activation of the RhoA/ROK pathway. Genistein (10 μM) reduced the initial increase in LC₂₀ phosphorylation (Fig. 3), which would explain the genistein-induced reduction in the phasic contractile response. It is unclear, however, how genistein inhibits LC₂₀ phosphorylation since it had no effect on: (i) the \([\text{Ca}^{2+}]\) transient in intact rat caudal artery (Fig. 2), (ii) \([\text{Ca}^{2+}]\)-induced contraction of Triton-skinned tissue (Figs. 5A and C), (iii) \([\text{Ca}^{2+}]\)-induced LC₂₀ phosphorylation in Triton-skinned tissue (Fig. 5E), or (iv) the activity of purified MLCK (Fig. 5F). The present study suggests that genistein attenuates K⁺-induced sustained contraction of rat caudal arterial smooth muscle through inhibition of the RhoA/ROK signaling pathway. Seok and co-workers also reported that the inhibitory targets of genistein with respect to rat aortic smooth muscle contraction include components of the RhoA/ROK signaling pathway (13). What is the link between tyrosine kinase activation in response to K⁺ stimulation and RhoA/ROK activation? Since RhoA activation is effected by a Rho-guanine nucleotide exchange factor (Rho-GEF) and reversed by a Rho-GTPase activating protein (RhoGAP) (1), one possibility arises that RhoGEF or RhoGAP, which are known to be tyrosine phosphorylated (42–45), may be phosphorylated in response to the sustained increase in \([\text{Ca}^{2+}]\) upon membrane depolarization, leading to activation of RhoGEF or inhibition of RhoGAP. It has been reported that p115RhoGEF, tyrosine-phosphorylated in response to angiotensin II, activates RhoA in vascular smooth muscle (45). The molecular weight of p115RhoGEF (103 kDa in rat) is similar to that of one of the tyrosine-phosphorylated proteins detected in this study (Fig. 8A). Ying et al. (46) have also suggested that the non-receptor tyrosine kinase PYK2 and PDZ-RhoGEF may link \([\text{Ca}^{2+}]\) signaling to RhoA. In addition, it was suggested that a phosphatidylinositol 3-kinase (PI3K) class II α-isoform is essential for \([\text{Ca}^{2+}]\)-dependent RhoA activation in vascular smooth muscle (47) and this pathway is augmented in the spontaneously hypertensive rat (48). In the present study, we detected 3 proteins of ~55, 70 and 113 kDa, which
were tyrosine-phosphorylated in response to 60 mM K⁺ and were inhibited by genistein (Fig. 8). Furthermore, analysis of the time-course of K⁺-stimulated protein tyrosine phosphorylation suggests that it may occur prior to RhoA activation and MYPT1 phosphorylation. Tyrosine phosphorylation of these proteins by a genistein-sensitive tyrosine kinase may, therefore, be involved in RhoA activation through interaction with RhoGEFs, RhoGAPs or PI3K in response to K⁺ stimulation. Several proteins with molecular weights ranging from 40 to 120 kDa were shown to be tyrosine-phosphorylated by K⁺ depolarization of PC12 cells in a Ca²⁺-dependent manner (49), while K⁺ depolarization failed to induce any increase in protein tyrosine phosphorylation in the omental artery (50). These proteins activated by K⁺ stimulation remain unidentified and their participation in the contractile response and RhoA/ROK activation requires further investigation. Nevertheless, our results support a role for tyrosine phosphorylation by a genistein-sensitive and Ca²⁺-activated tyrosine kinase in depolarization-induced activation of rat caudal arterial smooth muscle contraction.

In conclusion, we have demonstrated that the sustained phase of the membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves activation of a genistein-sensitive and Ca²⁺-dependent tyrosine kinase, leading to activation of RhoA and ROK. Taken together with information from the literature concerning RhoA activation and the regulation of smooth muscle contraction by tyrosine phosphorylation, we suggest the following signaling pathway to explain the involvement of tyrosine kinase activation in K⁺-induced contraction of rat caudal arterial smooth muscle: Membrane depolarization opens voltage-gated Ca²⁺ channels leading to an influx of Ca²⁺ from the extracellular space, elevation of [Ca²⁺]ᵢ and activation of a Ca²⁺- and genistein-sensitive tyrosine kinase, which phosphorylates proteins of ~55, 70 and 113 kDa. These tyrosine-phosphorylated proteins induce the translocation of RhoA to the plasma membrane and activation of ROK. Activated ROK phosphorylates MYPT1 at Thr697 and Thr855, resulting in a decrease in MLCP activity. Ca²⁺ also activates CaM-dependent MLCK, and the combination of MLCK activation and MLCP inhibition results in increased LC₂₀ phosphorylation, cross-bridge cycling and contraction. Future studies will focus on identification of the putative Ca²⁺-dependent tyrosine kinase and the mechanism of regulation of RhoA activation by this upstream tyrosine phosphorylation event. The non-receptor proline-rich tyrosine kinase 2 (PYK2) is a candidate (46, 51).

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