

## 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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Submitted January 26, 2013; Accepted in final form May 23, 2013

### Abstract

Rho-associated kinase (ROK) activation plays an important role in K<sup>+</sup>-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<sup>+</sup>-induced RhoA activation and contraction. The non-selective tyrosine kinase inhibitor genistein, but not the src family tyrosine kinase inhibitor PP2, inhibited K<sup>+</sup>-induced sustained contraction (IC<sub>50</sub> = 11.3 ± 2.4 μM). Genistein (10 μM) inhibited the K<sup>+</sup>-induced increase in myosin light chain (LC<sub>20</sub>) phosphorylation without affecting the Ca<sup>2+</sup> transient. The tyrosine phosphatase inhibitor vanadate induced contraction that was reversed by genistein (IC<sub>50</sub> = 6.5 ± 2.3 μM) and the ROK inhibitor Y-27632 (IC<sub>50</sub> = 0.27 ± 0.04 μM). Vanadate also increased LC<sub>20</sub> phosphorylation in a genistein- and Y-27632-dependent manner. K<sup>+</sup> 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<sup>+</sup> stimulation in a genistein- and Y-27632-sensitive manner. Finally, K<sup>+</sup> 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 [Ca<sup>2+</sup>]<sub>i</sub>, is involved in the RhoA/ROK activation and sustained contraction induced by K<sup>+</sup>.

**Key words:** Ca<sup>2+</sup> sensitization, myosin light chain phosphatase, RhoA, Rho-associated kinase, tyrosine kinase

Abbreviations used: [Ca<sup>2+</sup>]<sub>i</sub>, cytosolic free Ca<sup>2+</sup> concentration; CaM, calmodulin; DMSO, dimethylsulphoxide; DTE, dithioerythritol; DTT, dithiothreitol; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; H-T, Hepes-Tyrosine solution; ILK, integrin-linked kinase; LC<sub>20</sub>, 20 kDa light chain of myosin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin-targeting subunit of MLCP; PKC, protein kinase C; PVDF, polyvinylidene difluoride; ROK, Rho-associated kinase; TCA, trichloroacetic acid; ZIPK, zipper-interacting protein kinase.

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## Introduction

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Contraction of smooth muscle is regulated not only by electromechanical coupling and cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), 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 ( $\text{LC}_{20}$ ) catalysed by  $\text{Ca}^{2+}$ /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  $[\text{Ca}^{2+}]_i$ , referred to as  $\text{Ca}^{2+}$  sensitization (1).  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  sensitization converge on an increase in  $\text{LC}_{20}$  phosphorylation, and analyses of kinase and phosphatase activities have indicated that  $\text{Ca}^{2+}$  sensitization is mediated predominantly via inhibition of MLCP, leading to an increase in  $\text{LC}_{20}$  phosphorylation (1). The monomeric GTPase, RhoA, plays a major role in  $\text{Ca}^{2+}$  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  $\text{LC}_{20}$  phosphorylation and smooth muscle contraction (1, 3). The activation of RhoA/ROK is, therefore, a major downstream pathway of receptor-dependent, G protein-mediated  $\text{Ca}^{2+}$  sensitization (1, 4).

Electromechanical coupling operates through changes in membrane potential, which affect  $[\text{Ca}^{2+}]_i$ . Stimulation by  $\text{K}^+$  induces depolarization of the cell membrane, which opens voltage-gated  $\text{Ca}^{2+}$  channels causing  $\text{Ca}^{2+}$  influx, increased  $[\text{Ca}^{2+}]_i$ , binding of  $\text{Ca}^{2+}$  to CaM, activation of MLCK, phosphorylation of myosin, activation of cross-bridge cycling and contraction (1, 5). It was assumed that  $\text{K}^+$ -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  $\text{K}^+$ -induced  $\text{LC}_{20}$  phosphorylation and sustained contraction of endothelium-free rat caudal arterial smooth muscle in a concentration-dependent manner, without affecting the  $\text{K}^+$ -induced elevation of  $[\text{Ca}^{2+}]_i$  (6). Moreover, this contractile response to  $\text{K}^+$ -induced membrane depolarization was found to be absolutely dependent on the influx of  $\text{Ca}^{2+}$  through voltage-gated  $\text{Ca}^{2+}$  channels, since it could be blocked by a  $\text{Ca}^{2+}$  channel blocker or by removal of extracellular  $\text{Ca}^{2+}$ . These results suggested that ROK activation plays an important role in  $\text{K}^+$ -induced contraction of endothelium-free rat caudal arterial smooth muscle and specifically that the tonic phase of  $\text{K}^+$ -induced contraction is mediated via inhibition of MLCP activity by  $\text{Ca}^{2+}$ -dependent ROK activation (6). Physiological elevations of  $[\text{Ca}^{2+}]_i$  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  $\text{K}^+$ -induced contraction of a variety of smooth muscle types (7–13). However, the signal transduction pathway underlying RhoA/ROK activation in response to  $\text{K}^+$  depolarization remains incompletely defined.

The involvement of protein kinase C (PKC), tyrosine kinase and ROK in  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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<sub>20</sub> phosphorylation in intact smooth muscle (15) and genistein was found to inhibit agonist-induced augmentation of Ca<sup>2+</sup>-induced contraction in permeabilized smooth muscle (16). Furthermore, tyrosine kinase activation in response to receptor stimulation has been implicated in Ca<sup>2+</sup> 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<sup>2+</sup> sensitization of smooth muscle contraction. In this study we examined the involvement of tyrosine phosphorylation in K<sup>+</sup>-induced contraction and RhoA/ROK activation of rat caudal arterial smooth muscle.

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## Materials and Methods

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### *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 Dojindo 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<sub>20</sub> (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<sub>2</sub>. 60 mM K<sup>+</sup> solution was prepared by replacing the NaCl in Hepes-Tyrode (H-T) solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.6 mM glucose, 10 mM Hepes, pH 7.4) with equimolar KCl. All measurements of 60 mM K<sup>+</sup>-induced contraction were carried out in the presence of 1 μM prazosin and 0.1 μM propranolol in order to block the α<sub>1</sub>- and β-adrenergic effects of noradrenaline, which is released from nerve terminals by depolarization (6).

### *Force and LC<sub>20</sub> 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 (demembrated) 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<sub>2</sub>EGTA, 5.83 mM MgCl<sub>2</sub>, 7.56 mM potassium propionate, 3.9 mM Na<sub>2</sub>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<sub>2</sub>, 7.53 mM potassium propionate, 3.9 mM Na<sub>2</sub>ATP, 0.5 mM DTE, 16.2 mM phosphocreatine, 15 units/ml creatine kinase, pH 6.9) to elicit a sustained Ca<sup>2+</sup>-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  $-80^{\circ}\text{C}$  until LC<sub>20</sub> extraction. Quantification of LC<sub>20</sub> 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:  $\text{mol P}_i/\text{mol LC}_{20} = (y + 2z)/(x + y + z)$ , where x, y and z are the signal intensities of unphosphorylated, mono- and di-phosphorylated LC<sub>20</sub> bands, respectively.

### ***Measurement of changes in $[\text{Ca}^{2+}]_i$***

Measurement of  $[\text{Ca}^{2+}]_i$  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  $\mu\text{M}$  fura 2-AM for 4 h in the dark at room temperature ( $23^{\circ}\text{C}$ ). 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 ( $F_{340}/F_{380}$ ) was monitored before (resting state) and during stimulation with 60 mM  $\text{K}^+$  for 20 min in the absence and presence of genistein. Separate tissues were used to evaluate the effect of genistein on the  $[\text{Ca}^{2+}]_i$  response to 60 mM  $\text{K}^+$  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  $\text{MgCl}_2$ , 2 mM EGTA, 250 mM sucrose, 1 mM DTT, 1 mM 4-(2-aminoethyl) benzensulfonyl fluoride, 20  $\mu\text{g}/\text{ml}$  leupeptin and 20  $\mu\text{g}/\text{ml}$  aprotinin) and centrifuged at  $100,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ , 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  $800 \times g$  for 10 min and the supernatant was collected as the membrane fraction. Samples (17  $\mu\text{l}$ ) 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  $4^{\circ}\text{C}$  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:  $[(\text{particulate RhoA})/(\text{particulate} + \text{cytosolic RhoA})] \times 100\%$ . 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  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , 0.5 nM MLCK, 1  $\mu\text{M}$  CaM, 10  $\mu\text{M}$  LC<sub>20</sub> and 0–100  $\mu\text{M}$  genistein or genistin. Reaction mixtures were pre-incubated at  $30^{\circ}\text{C}$  for 2 min and reactions were initiated by the addition of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  to a final concentration of 0.2 mM (240 cpm/pmol). Samples (20  $\mu\text{l}$ ) 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  $\mu$ l 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  $\mu$ l) 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<sup>+</sup> stimulation in the absence or presence of 10  $\mu$ M 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  $\mu$ l 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  $\mu$ l for tyrosine-phosphorylation or 22.5  $\mu$ l 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-tag<sup>TM</sup> BTL-104 (NARD Institute Ltd., Hyogo, Japan) (27) according to the manufacturer's instructions. This biotin-pendant Zn<sup>2+</sup>-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  $\pm$  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<sup>+</sup>- and ionomycin-induced contractions*

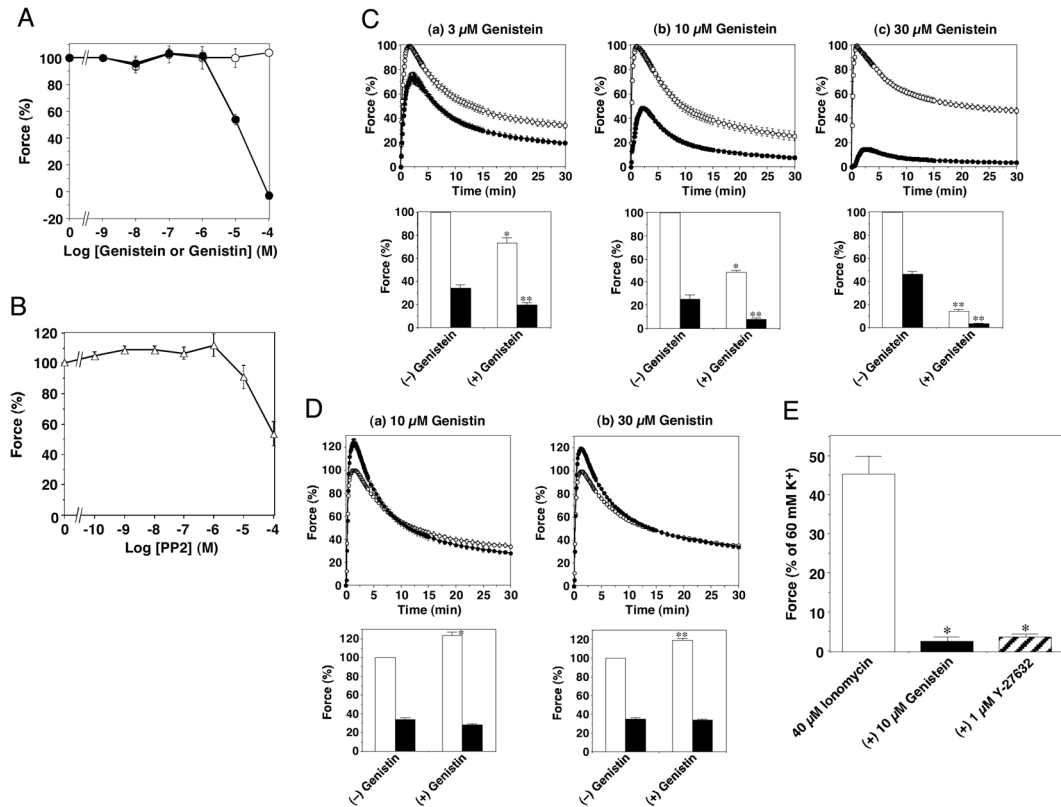
Membrane depolarization of de-endothelialized rat caudal arterial smooth muscle with 60 mM K<sup>+</sup> induces a rapid increase in force, which then declines to a steady-state level that is significantly greater than resting force (Mita et al. (6) and controls in Figs. 1C and D). The initial phasic component of the contractile response is attributable to an increase in [Ca<sup>2+</sup>]<sub>i</sub>, activation of MLCK and phosphorylation of LC<sub>20</sub>, 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<sup>+</sup>-induced force had been achieved, the tyrosine kinase inhibitor genistein evoked concentration-dependent relaxation to the basal level of force with an IC<sub>50</sub> of 11.3 ± 2.4 μM (Fig. 1A). The inactive analogue genistin had no effect on K<sup>+</sup>-induced sustained contraction (Fig. 1A). The src family tyrosine kinase inhibitor PP2, on the other hand, had a slight inhibitory effect on K<sup>+</sup>-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 (IC<sub>50</sub> ~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<sup>+</sup>-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 Ca<sup>2+</sup> ionophore ionomycin (40 μM) (Fig. 1E).

### *Effect of genistein on [Ca<sup>2+</sup>]<sub>i</sub>*

In fura 2-AM-loaded rat caudal arterial smooth muscle strips, the time course of the change in [Ca<sup>2+</sup>]<sub>i</sub> in response to K<sup>+</sup> depolarization closely matched the time course of contraction. Stimulation of arterial strips with 60 mM K<sup>+</sup> led to a transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, which settled at a sustained level significantly above resting [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 2A). The  $F_{340}/F_{380}$  ratio under resting conditions was unaffected by 10 or 30 μM genistein (Fig. 2B). The peak and sustained increases in [Ca<sup>2+</sup>]<sub>i</sub> induced by 60 mM K<sup>+</sup> 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<sup>+</sup>-induced LC<sub>20</sub> phosphorylation*

The time courses of the changes in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 2A), LC<sub>20</sub> phosphorylation (Fig. 3) and contraction (Fig. 1C) in response to 60 mM K<sup>+</sup> were similar and consistent with the phasic component of the contractile response being due to Ca<sup>2+</sup> entry, leading to CaM-dependent activation of MLCK. Under control conditions, LC<sub>20</sub> phosphorylation increased rapidly in response to 60 mM K<sup>+</sup> from a resting level of 0.12 ± 0.04 mol P<sub>i</sub>/mol LC<sub>20</sub> to a peak of 0.54 ± 0.03 mol P<sub>i</sub>/mol LC<sub>20</sub> at 15 s after K<sup>+</sup> addition. LC<sub>20</sub> phosphorylation then declined to a steady-state level of 0.35 ± 0.08 mol P<sub>i</sub>/mol LC<sub>20</sub> at 15 min after K<sup>+</sup> addition. In the presence of genistein (10 μM), LC<sub>20</sub> phosphorylation levels increased rapidly in response to K<sup>+</sup> from a resting level of 0.19 ± 0.04 mol P<sub>i</sub>/mol LC<sub>20</sub> to a peak of 0.28 ± 0.05 mol P<sub>i</sub>/mol LC<sub>20</sub> at 15 s after 60 mM K<sup>+</sup> addition and then declined to 0.23 ± 0.05 mol P<sub>i</sub>/mol LC<sub>20</sub> at 15 min after 60 mM K<sup>+</sup> addition. 10 μM genistein, therefore, reduced the transient increase in LC<sub>20</sub> phosphorylation, and abolished the sustained elevation of LC<sub>20</sub> phosphorylation. The effects of 10 μM genistein on the contractile response to membrane depolarization (Fig. 1C) can be explained, therefore, by inhibition of LC<sub>20</sub> phosphorylation (Fig. 3) without affecting the Ca<sup>2+</sup> transient (Fig. 2).

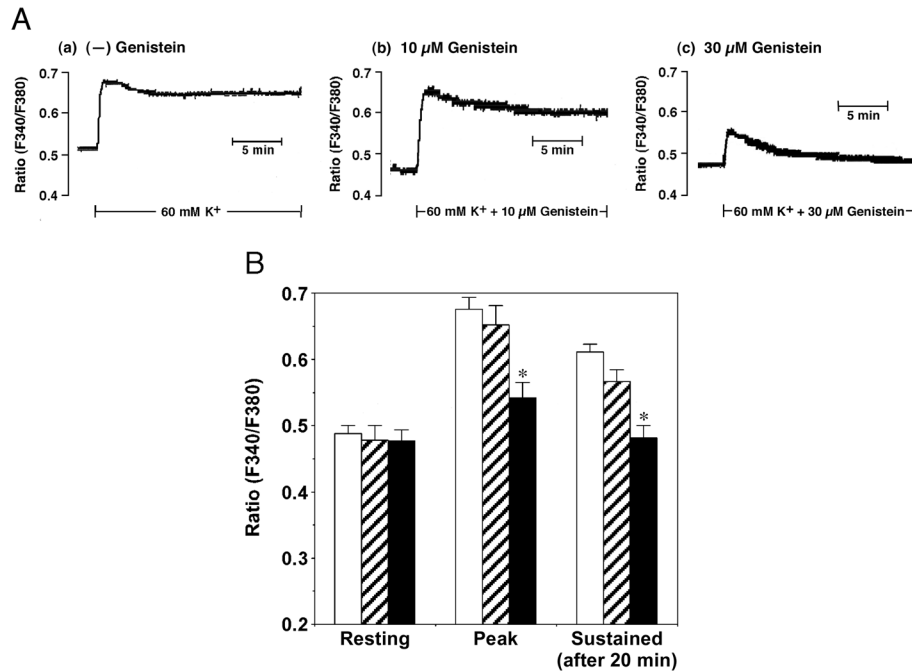


**Fig. 1.** Effects of tyrosine kinase inhibitors on K<sup>+</sup>- and ionomycin-induced contractions of rat caudal arterial smooth muscle

Effects of (A) genistein (●;  $n=4$ ) and the inactive analogue genistin (○;  $n=4$ ) and (B) the src family tyrosine kinase inhibitor PP2 (△;  $n=4$ ) on 60 mM K<sup>+</sup>-induced sustained contraction of rat caudal arterial smooth muscle. Compounds were added once K<sup>+</sup>-induced contraction became stable. Force is expressed as a percentage of the sustained contraction in response to K<sup>+</sup> before the addition of drugs. Values represent the mean  $\pm$  S.E.M. (C and D) Effect of pre-treatment with genistein (C) or genistin (D) on K<sup>+</sup>-induced contraction of rat caudal arterial smooth muscle. Upper panels show the time courses of K<sup>+</sup>-induced contraction without (○) or with (●) genistein (C) or genistin (D). Lower panels show the effects of genistein (C) or genistin (D) on the phasic and tonic components of K<sup>+</sup>-induced contraction. (C) (a) – (c) 3, 10 and 30  $\mu$ M genistein pre-treatment, respectively; (D) (a) and (b) 10 and 30  $\mu$ M genistin pre-treatment, respectively. Force is expressed as a percentage of the maximal force of the phasic contraction induced by K<sup>+</sup> without genistein or genistin. Open bars indicate the phasic contraction in response to K<sup>+</sup> and filled bars the tonic contraction (30 min after K<sup>+</sup> addition). Values represent the mean  $\pm$  S.E.M. ( $n=4$ ). \*  $P<0.05$  in (C) and  $P<0.01$  in (D), \*\*  $P<0.005$  in (C) and (D); significantly different from the value of the force without genistein or genistin. (E) Effects of genistein and Y-27632 on ionomycin-induced contraction of rat caudal arterial smooth muscle. After stable K<sup>+</sup>-induced contraction was attained, strips were pre-incubated for 20 min with 10  $\mu$ M genistein or 1  $\mu$ M Y-27632, and then incubated with 40  $\mu$ M ionomycin in the presence of 10  $\mu$ M genistein or 1  $\mu$ M Y-27632. The changes in tension were observed for 60 min. Force development is expressed as a percentage of the maximal force developed in response to K<sup>+</sup>. Values represent the mean  $\pm$  S.E.M. ( $n=8$  for the data in the absence of genistein or Y-27632;  $n=4$  for the data in the presence of genistein or Y-27632). \*  $P<0.005$ ; significantly different from the value of the force without inhibitor.

### **Effects of genistein and Y-27632 on vanadate-induced contraction and LC<sub>20</sub> phosphorylation**

The tyrosine phosphatase inhibitor vanadate (30  $\mu$ M) (29, 30) induced a slow, sustained contraction of rat caudal arterial smooth muscle, which reached a maximum after  $\sim$ 3 h. The steady-state force evoked by vanadate was  $368.4 \pm 20.1$  mg ( $n=12$ ), which was significantly greater than that elicited by 60 mM K<sup>+</sup> ( $188.5 \pm 9.2$  mg ( $n=12$ );  $P<0.005$ ). Genistein inhibited vanadate-induced contraction and caused a concentration-dependent



**Fig. 2.** Effect of genistein on  $K^+$ -induced changes in  $[Ca^{2+}]_i$

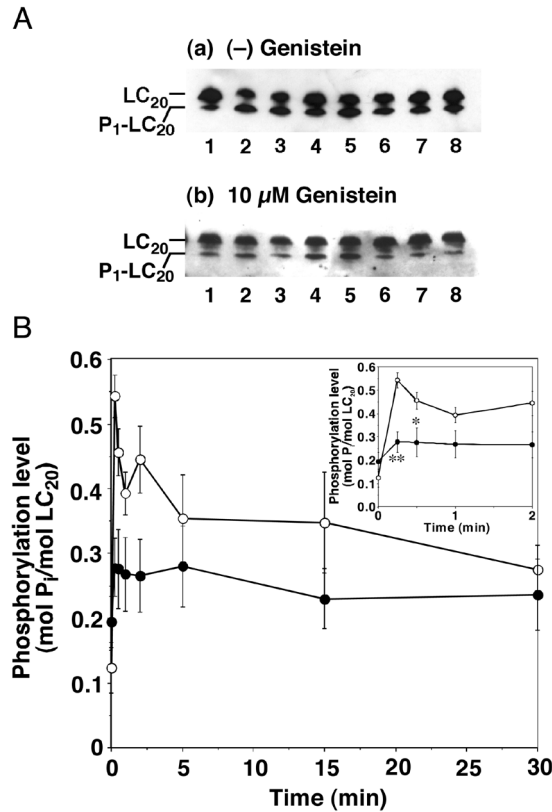
Intact rat caudal arterial smooth muscle strips were loaded with fura 2-AM. The  $F_{340}/F_{380}$  ratio was recorded following stimulation with 60 mM  $K^+$  in the absence or presence of genistein. Genistein was present 20 min prior to, and during, the  $K^+$ -induced contraction. (A) Representative traces showing the change in  $F_{340}/F_{380}$  ratio induced by  $K^+$  in the absence (a) and presence of 10  $\mu$ M (b) or 30  $\mu$ M (c) genistein. (B) The  $F_{340}/F_{380}$  ratio for resting, peak and sustained components in response to  $K^+$  in the absence (open bars;  $n=9$ ) and presence of 10  $\mu$ M (hatched bars;  $n=6$ ) or 30  $\mu$ M (filled bars;  $n=7$ ) genistein. The fluorescence intensity of the sustained component was measured 20 min after  $K^+$  addition. Results are expressed as the mean  $\pm$  S.E.M.  $F_{340}/F_{380}$  values with 10  $\mu$ M genistein were not significantly different from those without genistein; however, those with 30  $\mu$ M genistein were significantly different from those without genistein (\* $P<0.005$ ).

relaxation to the basal level of force with an  $IC_{50}$  of  $6.5 \pm 2.3$   $\mu$ M (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  $IC_{50}$  of  $0.27 \pm 0.04$   $\mu$ M (Fig. 4C).  $LC_{20}$  phosphorylation levels increased significantly in response to vanadate from a resting level of  $0.15 \pm 0.03$  mol  $P_i$ /mol  $LC_{20}$  to  $0.36 \pm 0.02$  mol  $P_i$ /mol  $LC_{20}$  at the maximal contraction (Figs. 4D and E). Vanadate-induced  $LC_{20}$  phosphorylation was significantly reduced by pre-treatment with genistein (10  $\mu$ M) or Y-27632 (1  $\mu$ M) to  $0.22 \pm 0.03$  mol  $P_i$ /mol  $LC_{20}$  and  $0.26 \pm 0.02$  mol  $P_i$ /mol  $LC_{20}$ , respectively (Figs. 4D and E). Thus, genistein and Y-27632 reduced the sustained elevation of  $LC_{20}$  phosphorylation induced by vanadate.

### ***Effects of genistein on $Ca^{2+}$ - 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  $LC_{20}$  phosphorylation, i.e. MLCK, integrin-linked kinase (ILK) and zipper-interacting protein kinase (ZIPK) (26, 31, 32), we investigated the effect of genistein on  $Ca^{2+}$ - and phosphatase inhibitor (microcystin)-induced contractions of Triton-skinned smooth muscle;  $Ca^{2+}$ -induced contraction is mediated by MLCK whereas  $Ca^{2+}$ -independent, microcystin-induced contraction is mediated by ILK and/or ZIPK (26, 31, 32). Neither genistein (100  $\mu$ M) nor the inactive analogue genistin (100  $\mu$ M) had an effect on steady-state force induced by treatment of Triton-skinned





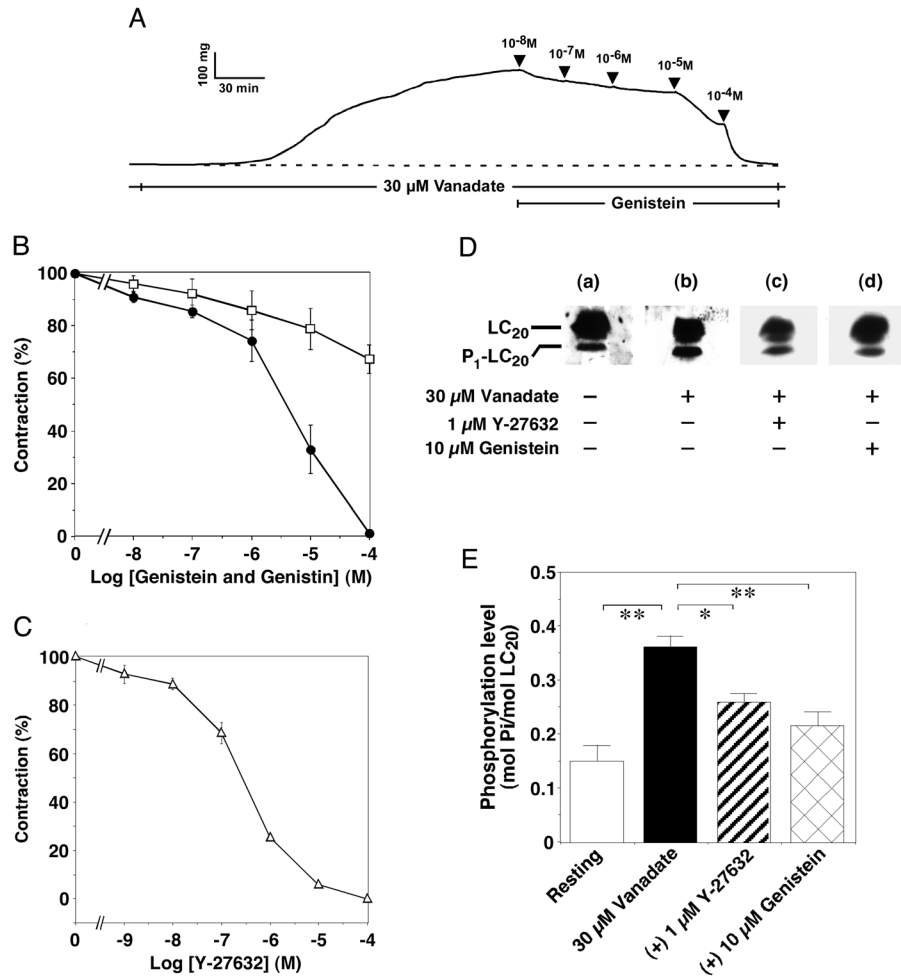
**Fig. 3.** Effect of genistein on K<sup>+</sup>-induced LC<sub>20</sub> phosphorylation

Muscle strips were pre-incubated for 20 min without or with 10 μM genistein and treated with 60 mM K<sup>+</sup> in the absence or presence of 10 μM genistein for the indicated times at which the tissue was frozen and LC<sub>20</sub> phosphorylation quantified as described in the Materials and Methods section. (A) Representative western blots of LC<sub>20</sub> showing separation of the phosphorylated from the unphosphorylated species by urea-glycerol gel electrophoresis. Lane 1: resting conditions without (a) or with (b) 10 μM genistein; lanes 2–8: 0.25, 0.5, 1, 2, 5, 15 and 30 min, respectively, following addition of 60 mM K<sup>+</sup> in the absence or presence of 10 μM genistein. We observed no di-phosphorylated LC<sub>20</sub> in any experiment using intact strips. (B) Cumulative data ( $n=5$ ). LC<sub>20</sub> phosphorylation was expressed as mol P<sub>1</sub>/mol LC<sub>20</sub> in the absence (○) and presence (●) of 10 μM genistein. The inset shows the data for the first 2 min on an expanded time scale. Values represent the mean ± S.E.M. \*  $P<0.05$ , \*\*  $P<0.005$ ; significantly different from the value without genistein. The control data in the absence of genistein were reported previously (6).

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<sup>2+</sup>-induced or Ca<sup>2+</sup>-independent, microcystin-induced contraction (Fig. 5D).

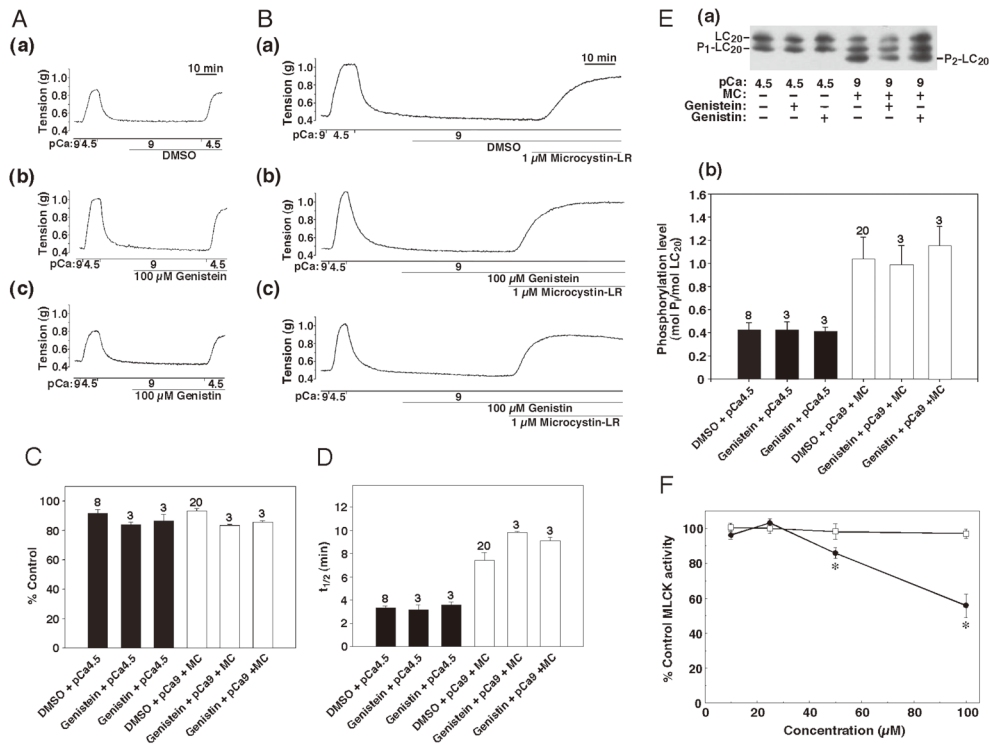
At pCa 4.5, only mono-phosphorylation of LC<sub>20</sub> was detected (Fig. 5E), consistent with phosphorylation of LC<sub>20</sub> by MLCK exclusively at Ser19. However, at pCa 9 in the presence of microcystin, both mono- and di-phosphorylation of LC<sub>20</sub> were detected (Fig. 5E), consistent with Ca<sup>2+</sup>-independent contraction in response to MLCP inhibition being due to phosphorylation of LC<sub>20</sub> by ILK and/or ZIPK at both Ser19 and Thr18 (26, 31–33). Neither genistein (100 μM) nor the inactive analogue genistin (100 μM) had any effect on LC<sub>20</sub> 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<sup>+</sup>-induced contraction cannot be attributed to inhibition of MLCK, ILK or ZIPK.

Furthermore, to ensure that the inhibition of K<sup>+</sup>-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



**Fig. 4.** Effects of genistein, genistin and Y-27632 on vanadate-induced contraction and LC<sub>20</sub> phosphorylation (A) A typical trace showing the inhibitory effect of genistein on 30 μM vanadate-induced sustained contraction of rat caudal arterial smooth muscle. When vanadate-induced contraction became stable, genistein was added. (B) Effects of genistein and genistin on 30 μM vanadate-induced sustained contraction of rat caudal arterial smooth muscle. When vanadate-induced contraction became stable, genistein (●) or genistin (□) was added. Force is expressed as a percentage of the tonic contraction in response to vanadate before the addition of genistein or genistin. Values represent the mean ± S.E.M. (*n*=6 for genistein and *n*=7 for genistin). (C) Effect of Y-27632 on vanadate-induced sustained contraction of rat caudal arterial smooth muscle. When vanadate-induced contraction became stable, Y-27632 was added. Force is expressed as a percentage of the tonic contraction in response to vanadate before the addition of Y-27632. Values represent the mean ± S.E.M. (*n*=6). (D) Representative western blots showing LC<sub>20</sub> phosphorylation stimulated by vanadate. When vanadate-induced contraction became stable, 10 μM genistein or 1 μM Y-27632 was added. The tissue was frozen under resting conditions (lane a), at the maximal contraction in response to vanadate (lane b), and at the maximal relaxation to Y-27632 (lane c) or genistein (lane d). LC<sub>20</sub> phosphorylation was quantified as described in the Materials and Methods section. (E) Cumulative data (*n*=6). LC<sub>20</sub> phosphorylation was expressed as mol P<sub>1</sub>/mol LC<sub>20</sub>. Values represent the mean ± S.E.M. \* *P*<0.01, \*\* *P*<0.005; significantly different from the value of LC<sub>20</sub> phosphorylation under resting conditions or following vanadate treatment.

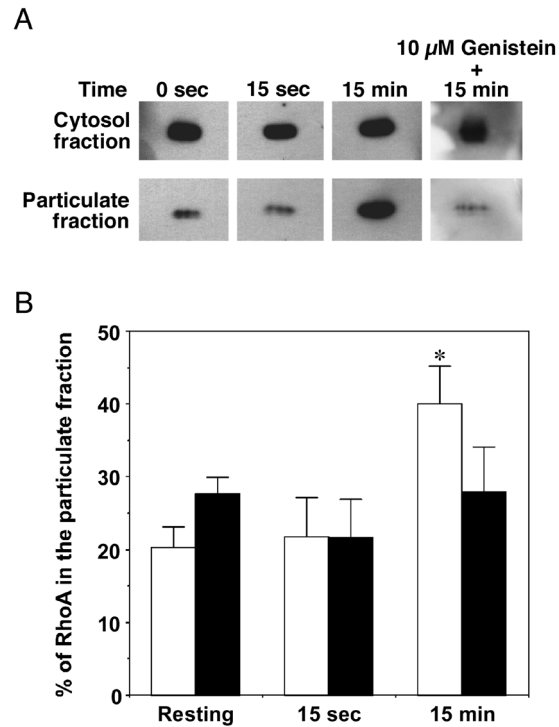
of genistein or the inactive analogue genistin. Genistein had no effect on the activity of purified MLCK at concentrations below 50 μM, but did cause significant inhibition at 50 and 100 μM, whereas genistin had no inhibitory effect over the entire concentration range from 0–100 μM (Fig. 5F). Thus, endogenous MLCK activity is unlikely to be affected at the concentration (10 μM) of genistein used in the present study.



**Fig. 5.** Effects of genistein and genistin on Ca<sup>2+</sup>- and microcystin-induced (Ca<sup>2+</sup>-independent) contractions and LC<sub>20</sub> phosphorylation of Triton-skinned rat caudal arterial smooth muscle, and on MLCK activity *in vitro* (A) Effects of genistein and genistin on Ca<sup>2+</sup>-induced contraction of Triton-skinned rat caudal arterial smooth muscle. Triton-skinned strips were relaxed in pCa 9 solution. Contraction was initiated by application of pCa 4.5 solution. Relaxation followed the return to pCa 9 solution. Tissues were then incubated in vehicle (DMSO; a), genistein (100 μM; b) or genistin (100 μM; c) at pCa 9 for 30 min prior to re-administration of pCa 4.5. (B) Effects of genistein and genistin on microcystin-induced contraction of Triton-skinned rat caudal arterial smooth muscle in the absence of Ca<sup>2+</sup>. Following a control Ca<sup>2+</sup>-induced contraction-relaxation cycle, tissues were incubated in vehicle (DMSO; a), genistein (100 μM; b) or genistin (100 μM; c) at pCa 9 for 30 min prior to addition of the phosphatase inhibitor microcystin-LR (1 μM). (C) Cumulative data showing the effects of genistein and genistin on steady-state force induced by pCa 4.5 or microcystin at pCa 9. Values are expressed as a percentage of the force elicited in the initial Ca<sup>2+</sup>-induced contraction. (D) Cumulative data showing the effects of genistein and genistin on the time to half-maximal contraction (t<sub>1/2</sub>). Values in panels C and D are the mean ± S.E.M. with *n* values indicated above each histogram. (E) Tissues from experiments carried out as described above were quick frozen in TCA/acetone, and LC<sub>20</sub> phosphorylation analysed by urea/glycerol polyacrylamide gel electrophoresis and western blotting with anti-LC<sub>20</sub> antibody as described in the Materials and Methods section. (a) Representative western blots showing the separation of unphosphorylated (LC<sub>20</sub>), mono-phosphorylated (P<sub>1</sub>-LC<sub>20</sub>) and di-phosphorylated (P<sub>2</sub>-LC<sub>20</sub>) myosin light chains. (b) Cumulative data. LC<sub>20</sub> phosphorylation was expressed as mol P<sub>i</sub>/mol LC<sub>20</sub>. Phosphorylation stoichiometry was calculated as described in the Materials and Methods section. Values are the mean ± S.E.M. with *n* values indicated above each histogram. At pCa 4.5, only mono-phosphorylated LC<sub>20</sub> was detected; at pCa 9 in the presence of microcystin, both mono- and diphosphorylated LC<sub>20</sub> were detected as previously reported (26, 31–33). (F) Effects of genistein and genistin on MLCK activity *in vitro*. Genistein (●) had a significant inhibitory effect on MLCK activity at 50 and 100 μM (\* *P* < 0.05), whereas genistin (□) had no inhibitory effect over the entire concentration range from 0–100 μM. Values are the mean ± S.E.M. (*n* = 3). 100% activity was 4.3 μmol P<sub>i</sub>/min/mg MLCK.

### Effect of genistein on K<sup>+</sup>-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



**Fig. 6.** Effect of genistein on  $K^+$ -induced translocation of RhoA

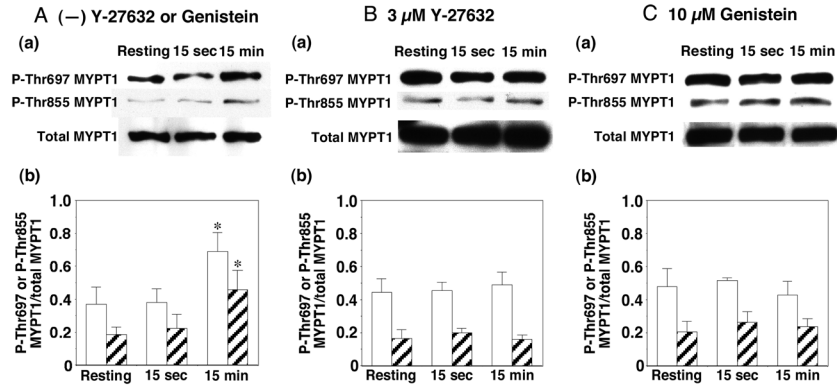
Muscle strips were pre-incubated for 20 min without or with 10  $\mu$ M genistein. RhoA was quantified in the cytosolic and particulate fractions as described in the Materials and Methods section. (A) Representative western blots showing the translocation of RhoA from the cytosolic to the particulate fraction in the absence or presence of 10  $\mu$ M 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  $\pm$  S.E.M. \*  $P < 0.05$ ; significantly different from the value under resting conditions without genistein.

and the effect of pre-treatment with genistein on RhoA translocation examined (Fig. 6). In the absence of  $K^+$  stimulation, the particulate fraction contained  $20.3 \pm 2.8\%$  of the total RhoA. This was not significantly different ( $21.8 \pm 5.3\%$ ) 15 s after 60 mM  $K^+$  addition. However, the amount of RhoA in the particulate fraction was significantly increased (to  $40.0 \pm 5.2\%$ ) at 15 min after stimulation (Fig. 6). Pre-treatment with genistein (10  $\mu$ M) prevented the translocation (activation) of RhoA: the particulate fraction under these conditions contained  $27.9 \pm 6.2\%$  of total RhoA at 15 min after stimulation, which was not significantly different from the value ( $27.7 \pm 2.2\%$ ) under resting conditions in the presence of genistein (Fig. 6).

#### ***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  $\mu$ M Y-27632 or 10  $\mu$ M 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



**Fig. 7.** Effects of Y-27632 and genistein on  $K^+$ -induced phosphorylation of MYPT1 at Thr697 and Thr855. Muscle strips were pre-incubated for 20 min with 3  $\mu$ M Y-27632 or 10  $\mu$ M genistein and then treated with 60 mM  $K^+$  in the absence (A) or presence of 3  $\mu$ M Y-27632 (B) or 10  $\mu$ M genistein (C) for the indicated times. Tissues were then frozen, and MYPT1 phosphorylation quantified as described in the Materials and Methods section. (a) Representative western blots showing 60 mM  $K^+$ -induced phosphorylation of MYPT1 at Thr697 and Thr855 in the absence or presence of 3  $\mu$ M Y-27632 or 10  $\mu$ M genistein. 60 mM  $K^+$  stimulation caused significant increases 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  $\pm$  S.E.M. \*  $P<0.05$ ; significantly different from the value under resting conditions.

by 60 mM  $K^+$  stimulation for 15 min was not observed in the presence of Y-27632 or genistein (Figs. 7B and C).

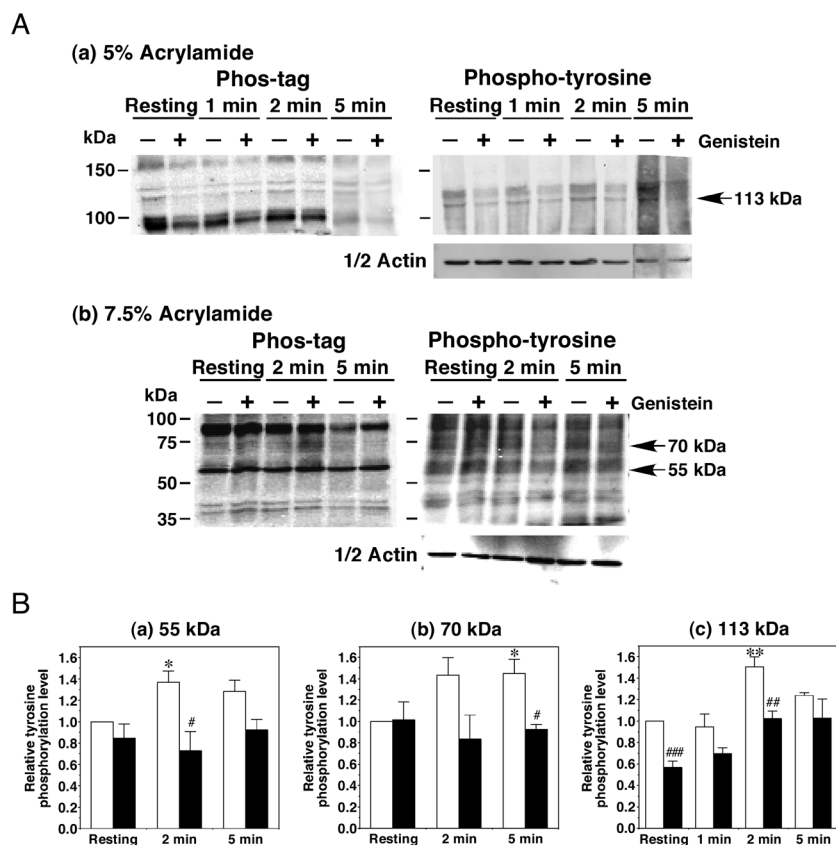
### *$K^+$ -induced tyrosine-phosphorylated proteins*

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<sup>TM</sup> 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 increases in tyrosine phosphorylation of these proteins were significantly inhibited by pre-treatment with 10  $\mu$ M genistein (Fig. 8).

## Discussion

We demonstrated previously that vascular smooth muscle membrane depolarization activates the RhoA/ROK pathway via the influx of extracellular  $Ca^{2+}$  (6). Protein tyrosine phosphorylation has been implicated in the regulation of  $[Ca^{2+}]_i$  and the  $Ca^{2+}$  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  $IC_{50}$  for inhibition of sustained force by genistein





**Fig. 8.** Genistein-sensitive protein tyrosine phosphorylation in response to  $K^+$ . Muscle strips were pre-incubated for 20 min with 10  $\mu$ M genistein or vehicle and then treated with 60 mM  $K^+$  in the absence or presence of 10  $\mu$ M genistein for the indicated times at which the tissues were frozen and tyrosine phosphorylation quantified as described in the Materials and Methods section. (A) Representative western blots of phosphorylated proteins detected with biotinylated Phos-tag<sup>TM</sup> and tyrosine-phosphorylated proteins detected with an anti-phosphotyrosine antibody. Protein samples were subjected to SDS-PAGE (5 % (a) or 7.5 % (b) acrylamide). Tyrosine phosphorylation levels in proteins of ~55, 70 and 113 kDa were increased in response to  $K^+$  stimulation and these increases were inhibited by genistein. (B) Cumulative data ( $n=3-6$ ) for tyrosine phosphorylation levels in the absence (open bars) or presence (closed bars) of genistein. Tyrosine phosphorylation levels were normalized to actin and are expressed as the ratio to signal intensities of tyrosine-phosphorylated proteins in unstimulated strips in the absence of genistein. Values represent the mean  $\pm$  S.E.M. \*  $P < 0.01$ , \*\*  $P < 0.001$ ; significantly different from the value under resting conditions. #  $P < 0.05$ , ##  $P < 0.01$ , ###  $P < 0.001$ ; significantly different from the value without genistein.

was  $11.3 \pm 2.4 \mu$ M (Fig. 1A), i.e. similar to the reported value for inhibition of tyrosine kinase activity (2.6  $\mu$ M) (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 ( $IC_{50} \sim 5$  nM (28)) exhibited partial inhibition of  $K^+$ -induced sustained contraction only at a very high concentration (100  $\mu$ M) (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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  currents by genistein. The  $\text{Ca}^{2+}$  transient in response to  $\text{K}^+$  was unaffected by 10  $\mu\text{M}$  but not 30  $\mu\text{M}$  genistein (Fig. 2). Furthermore, genistein inhibited  $\text{Ca}^{2+}$  ionophore (ionomycin)-induced contraction (Fig. 1E), whereas the  $\text{Ca}^{2+}$  channel blocker nicardipine (30 nM), which abolished 60 mM  $\text{K}^+$ -induced contraction, did not inhibit ionomycin-induced contraction (data not shown). These results suggest that 10  $\mu\text{M}$  genistein does not act as a  $\text{Ca}^{2+}$  channel blocker to reduce  $\text{LC}_{20}$  phosphorylation and contraction, and furthermore, physiological elevations of  $[\text{Ca}^{2+}]_i$  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  $\text{Ca}^{2+}$  influx through L-type  $\text{Ca}^{2+}$  channels in smooth muscle (39). However, 10  $\mu\text{M}$  genistein had no effect on the  $\text{Ca}^{2+}$  transient stimulated by  $\text{K}^+$  depolarization, suggesting that  $\text{K}^+$  stimulation does not activate Src or FAK.

Seok et al. (13) reported that KCl-induced activations of RhoA,  $\text{LC}_{20}$  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  $\mu\text{M}$ ) inhibited  $\text{K}^+$ -induced  $\text{LC}_{20}$  phosphorylation (Fig. 3) without affecting the  $\text{K}^+$ -induced increase in  $[\text{Ca}^{2+}]_i$  (Fig. 2), which correlated well with the force response to  $\text{K}^+$  in the presence of this concentration of genistein (Fig. 1C). The possibility that the inhibition of force development and  $\text{LC}_{20}$  phosphorylation by genistein could be due to inhibition of MLCK, ILK or ZIPK, which are capable of phosphorylating  $\text{LC}_{20}$  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  $\text{Ca}^{2+}$ ) or ILK and ZIPK (by microcystin via phosphatase inhibition). ILK and ZIPK phosphorylate  $\text{LC}_{20}$  in a  $\text{Ca}^{2+}$ -independent manner at Ser19 and Thr18 (26, 31) whereas MLCK phosphorylates  $\text{LC}_{20}$  exclusively at Ser19 (32, 33). Genistein (100  $\mu\text{M}$ ) had no effect on  $\text{Ca}^{2+}$ -induced contraction or  $\text{LC}_{20}$  mono-phosphorylation or on  $\text{Ca}^{2+}$ -independent force development or  $\text{LC}_{20}$  mono- and di-phosphorylation induced by microcystin (Fig. 5). Moreover, genistein had no effect on the activity of purified MLCK *in vitro* at concentrations below 50  $\mu\text{M}$  (Fig. 5F). These results indicate that inhibition of  $\text{K}^+$ -induced contraction of rat caudal arterial smooth muscle by genistein (10  $\mu\text{M}$ ) 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  $\text{IC}_{50}$  of  $6.5 \pm 2.3 \mu\text{M}$  (Fig. 4), which is not significantly different from the  $\text{IC}_{50}$  for inhibition of  $\text{K}^+$ -induced contraction (Fig. 1). The ROK inhibitor Y-27632 also inhibited vanadate-induced contraction, with an  $\text{IC}_{50}$  of  $0.27 \pm 0.04 \mu\text{M}$  (Fig. 4), similar to the  $\text{IC}_{50}$  value ( $0.41 \pm 0.02 \mu\text{M}$ ) for inhibition of  $\text{K}^+$ -induced contraction (6). Vanadate also increased  $\text{LC}_{20}$  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  $\text{LC}_{20}$  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  $\alpha_1$ -adrenoceptor activation, GTP $\gamma$ S and high  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$ , 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  $Ca^{2+}$  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  $LC_{20}$  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  $LC_{20}$  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  $\mu$ M) reduced the initial increase in  $LC_{20}$  phosphorylation (Fig. 3), which would explain the genistein-induced reduction in the phasic contractile response. It is unclear, however, how genistein inhibits  $LC_{20}$  phosphorylation since it had no effect on: (i) the  $Ca^{2+}$  transient in intact rat caudal artery (Fig. 2), (ii)  $Ca^{2+}$ -induced contraction of Triton-skinned tissue (Figs. 5A and C), (iii)  $Ca^{2+}$ -induced  $LC_{20}$  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 (RhoGEF) 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  $[Ca^{2+}]_i$  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  $Ca^{2+}$  signaling to RhoA. In addition, it was suggested that a phosphatidylinositol 3-kinase (PI3K) class II  $\alpha$ -isoform is essential for  $Ca^{2+}$ -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  $Ca^{2+}$ -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  $Ca^{2+}$ -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  $Ca^{2+}$ -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  $Ca^{2+}$  channels leading to an influx of  $Ca^{2+}$  from the extracellular space, elevation of  $[Ca^{2+}]_i$  and activation of a  $Ca^{2+}$ - 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.  $Ca^{2+}$  also activates CaM-dependent MLCK, and the combination of MLCK activation and MLCP inhibition results in increased LC<sub>20</sub> phosphorylation, cross-bridge cycling and contraction. Future studies will focus on identification of the putative  $Ca^{2+}$ -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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## Acknowledgments

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This work was supported by grants from the High-Tech Research Center Project (S0801043) to S.M. and M.M., the Meiyaku Open Research Project to M.M., the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, the Naito Scientific Research Foundation, a special grant from Meiji Pharmaceutical University to M.M., and by a grant (MOP-111262) to M.P.W. from the Canadian Institutes of Health Research. M.P.W. is an Alberta Innovates - Health Solutions Scientist and recipient of a Canada Research Chair (Tier 1) in Vascular Smooth Muscle Research.

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