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Tyrosine phosphorylation as a convergent pathway of heterotrimeric G protein- and *rho* protein-mediated Ca^{2+} sensitization of smooth muscle of rabbit mesenteric artery

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1 The aim of this study was to determine whether different signal transduction mechanisms underlie the Ca^{2+} sensitizing effects of guanosine 5′-*O*-(3-thiotriphosphate) ($GTP_{\gamma}S$) and receptor agonists on *β*-escin-skinned smooth muscle of rabbit mesenteric artery.

2 In the homogenate of the *β*-escin-skinned arterial strip, C3 exoenzyme of *Clostridium botulinum* catalyzed the [^{32}P]-ADP-ribosylation of only one protein that had the same molecular mass as the protein detected in Western blots with anti-*rho* p21 antibody. Pretreatment of preparations with C3 resulted in great inhibition of $GTP_{\gamma}S$-induced Ca^{2+} sensitization, although the effect of $GTP_{\gamma}S$ at higher concentrations ($\geqslant 30\ \mu M$) was not completely blocked by this treatment. In contrast, the enhancement by phenylephrine and histamine, in the presence of guanosine 5′-triphosphate, of the Ca^{2+}-induced contraction was not affected by C3 pretreatment.

3 The protein kinase C (PKC) inhibitors calphostin C and staurosporine completely eliminated the enhancement by phorbol ester 12,13-dibutyrate of the Ca^{2+}-induced contraction. However, these PKC inhibitors had no effect on $GTP_{\gamma}S$- and receptor agonist-induced Ca^{2+} sensitization.

4 The tyrosine kinase inhibitors genistein and tyrphostin 25 caused an irreversible and complete block of the enhancement by $GTP_{\gamma}S$ of the Ca^{2+}-induced contraction without affecting this Ca^{2+} contraction. The inactive genistein analogue daidzein did not modify the effect of $GTP_{\gamma}S$. The Ca^{2+} sensitizing effects of phenylephrine and histamine were also blocked by these tyrosine kinase inhibitors.

5 These results suggest that *rho* p21 predominantly mediates $GTP_{\gamma}S$-induced Ca^{2+} sensitization of *β*-escin-skinned smooth muscle of rabbit mesenteric artery, while the Ca^{2+} sensitizing actions of heterotrimeric G protein-coupled receptor agonists do not involve this small G protein. However, it seems that tyrosine phosphorylation, but not PKC activation, plays an important role in both of the *rho* p21 protein- and heterotrimeric G protein-mediated Ca^{2+} sensitization mechanisms.

Keywords: Ca^{2+} sensitization; small G protein *rho* p21; heterotrimeric G protein-coupled receptor agonists; protein kinase C; tyrosine phosphorylation; vascular smooth muscle

Introduction

Smooth muscle contraction is primarily regulated by phosphorylation of the 20 kDa myosin light chain (MLC_{20}) (Hartshorne, 1987). MLC_{20} is specifically phosphorylated by the Ca^{2+}/calmodulin-dependent MLC_{20} kinase, which then activates actomyosin ATPase (Hartshorne, 1987). Thus, the greater tension development is associated with an increase in MLC_{20} phosphorylation (Rembold & Murphy, 1988; Kitazawa *et al*., 1991; Hori *et al*., 1992), and a rise in cytosolic Ca^{2+} increases the MLC_{20} kinase/phosphatase activity ratio by activation of the kinase (Hartshorne, 1987; Kamm & Stull, 1989). However, the cytosolic Ca^{2+} level is not always parallel with the contraction level (Bradley & Morgan, 1987). Indeed, receptor agonists cause greater contraction than high K^+-depolarization at a given cytosolic Ca^{2+} level (Karaki, 1989; Somlyo & Himpens, 1989). In smooth muscle permeabilized with bacterial α-toxin, receptor agonists have been found to lower the Ca^{2+} concentrations necessary for the contraction, suggesting that the agonists enhance Ca^{2+} sensitivity of contractile apparatus ('Ca^{2+} sensitization') (Nishimura *et al*., 1988; Kitazawa *et al*., 1989). Because receptor agonists stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate through activation of phospholipase C and result in diacylglycerol as well as inositol 1,4,5-trisphosphate, and diacylglycerol activates protein kinase C (PKC) (Nishizuka, 1986), and because phorbol esters that activate PKC also enhance the Ca^{2+} sensitivity of myofilaments in permeabilized smooth muscle preparations (Chatterjee & Tejada, 1986; Fujiwara *et al*., 1988; Nishimura & van Breemen, 1989; Itoh *et al*., 1994), it has been suggested that PKC plays a role in agonist-induced Ca^{2+} sensitization (Lee & Severson, 1994).

Guanosine 5′-triphosphate (GTP) or guanosine 5′-*O*-(3-thiotriphosphate) ($GTP_{\gamma}S$) can mimic the Ca^{2+} sensitizing effects of receptor agonists (Nishimura *et al*., 1988; Fujiwara *et al*., 1988; Kitazawa *et al*., 1989). Recently, it has been shown that $GTP_{\gamma}S$-induced Ca^{2+} sensitization is inhibited by pretreatment with C3 exoenzyme from *Clostridium botulinum*, which is known to selectively inactive *rho* p21, a small G protein (Takai *et al*., 1992), in permeabilized smooth muscle of rabbit mesenteric artery (Hirata *et al*., 1992), guinea-pig ileum (Itagaki *et al*., 1995), and guinea-pig vas deferens (Fujita *et al*., 1995). These findings suggest that *rho* p21, rather than

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heterotrimeric G proteins, is involved in $GTP_{\gamma}S$-induced Ca^{2+} sensitization. However, the important question of whether the Ca^{2+} sensitizing effects of heterotrimeric G protein-coupled receptor agonists are also regulated by this small G protein remains unresolved. Thus, to address this question, we initially decided to compare the influence of C3 pretreatment on the Ca^{2+} sensitizing effects of $GTP_{\gamma}S$ and receptor agonists, phenylephrine and histamine, in β-escin-skinned smooth muscle of rabbit mesenteric artery. If different G proteins were involved in $GTP_{\gamma}S$- and agonist-induced Ca^{2+} sensitization, the signalling events for these two Ca^{2+} sensitizing actions might differ each other. A number of recent reports have shown that pharmacological inhibitors of tyrosine kinases inhibit contractions by agonists such as angiotensin II and norepinephrine in vascular smooth muscle (Di Salvo *et al*., 1993; Hollenberg, 1994; Filipeanu *et al*., 1995; Jinsi *et al*., 1996), suggesting that tyrosine phosphorylation, rather than PKC activation, may be a key signalling event for Ca^{2+} sensitization of contractile apparatus during agonist-induced contractile activation of vascular smooth muscle. Therefore, the goal of this study was to ask the question what plays a signaling role in each of $GTP_{\gamma}S$- and agonist-induced Ca^{2+} sensitization of contractile apparatus of vascular smooth muscle. For this purpose, we assessed the contribution of PKC and tyrosine kinases to the actions of $GTP_{\gamma}S$ and receptor agonists on Ca^{2+}-induced contractions in rabbit arterial skinned muscle using a pharmacological approach with the respective relatively selective inhibitors.

Methods

Preparation and tension measurement

Male New Zealand White rabbits, weighing 2–2.5 kg, were anaesthetized with pentobarbitone sodium (35 mg kg^{-1}, i.v.) and then exsanguinated. The mesenteric artery of the ileum region (diameter around 1 mm) was removed immediately and placed in oxygenated physiological salt solution at room temperature. The solution contained (mM) NaCl 130, KCl 4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, Tris-HCl 10 (pH 7.4) and glucose 10. The artery was cleaned of adhering fat and connective tissue under a dissection microscope. Endothelial cells were removed by gentle rubbing of the intimal surface of the artery with a razor blade. A small bundle of muscle fibres (1.5–2.0 mm length, 0.4–0.5 mm width) were tied with monofilament silk to the fire tips of two tungsten needles, one of which was connected to a force transducer (TB 651-T, Nihon Kohden). The preparation was immersed in one of wells (0.75 ml volume each) engraved in a plastic plate. The bundles were stretched to about 1.2 times their resting length. Solutions were rapidly changed by sliding the plate to an adjacent well. The skinning of bundles was achieved using β-escin. The methods used for skinning and the composition of the solutions have been described elsewhere (Tomita *et al*., 1997). Briefly, the preparations were treated with 50 μM β-escin (Sigma) for 30 min in a relaxing solution. The composition of the relaxing solution was: 87 mM potassium methanesulphonate, 20 mM PIPES, 5.1 mM $Mg(methanesulphonate)_2$, 4.2 mM ATP, 10 mM phosphocreatine, 0.5 mg ml^{-1} creatine phosphokinase and 10 mM EGTA; the solution was set to pH 7.0 with KOH. After the skinning procedure, the preparations were washed several times to remove β-escin by replacing the normal relaxing solution.

To determine the contractile response of the skinned muscle to Ca^{2+}, various concentrations of Ca^{2+} were applied by adding appropriate amounts of $Ca(methanesulphonate)_2$ to the relaxing solution. The pH of the solution was adjusted to 7.0 with KOH and the ionic strength was standardized at 0.2 M by changing the amount of potassium methanesulphonate added. To avoid spurious effects due to Ca^{2+} release from intracellular storage sites in the skinned muscle, 1 μM ionomycin (Sigma) was applied. To prevent deterioration of the Ca^{2+}-induced contraction, 0.1 μM calmodulin (Wako) was applied throughout the experiments. The amplitude of contraction induced by each of various interventions was normalized with respect to that induced by 30 μM Ca^{2+} in the same preparation. All experiments were carried out at room temperature (22–25°C).

ADP-ribosylation of proteins with C3 exoenzyme

Some skinned muscle strips were incubated with 1 μg ml^{-1} C3 exoenzyme for 30 min at room temperature in relaxing solution containing 10 μM nicotinamide adenine dinucleotide (NAD). Native and recombinant C3 exoenzymes, which were originated from *Clostridium botulinum* type D strains South African and from *Escherichia coli* containing the C3 gene, respectively, as previously described (Moriishi *et al*., 1991, 1993), were used. After the incubation, the strips were washed several times with the relaxing solution. As control preparations, the strips incubated without C3 but otherwise handled in the same manner as the C3-treated preparations were used.

The strips were placed in 100 mM Tris-HCl (pH 7.6) containing 1 mM EGTA and 0.5 mM dithiothreitol and ground manually with a micro homogenizer (NS-310E, Nition). Homogenates (50 μg of protein) were added to 200 μl of reaction mixture containing 100 mM Tris-HCl (pH 7.6), 10 μM [α-^{32}P]-NAD (10 μCi per assay; 800 Ci $mmol^{-1}$, New England Nuclear), 10 mM thymidine and 1 μg ml^{-1} C3. After incubation for 60 min at room temperature, the reactions were stopped by adding 22 μl of 100% (w/v) trichloroacetic acid, and the mixtures were centrifuged at $3{,}000 \times g$ for 5 min. The resulting pellets were washed three times with ether to remove trichloroacetic acid, and then solubilized in sodium dodecyl sulphate (SDS) solution containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.5 mM dithiothreitol, 5% 2-mercaptoethanol and 0.05% bromophenol blue. The solubilized proteins were subjected to SDS–PAGE using a 12.5% polyacrylamide gel followed by autoradiography.

*Immunoblotting with anti-*rho *protein antibody*

The skinned muscle strips pretreated with or without C3 in the presence of NAD were placed in lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 2.5 mM ethylenediaminetetraacetic acid, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride, 1% Triton X-100, 0.1% SDS, 10% glycerol and 10 μg ml^{-1} aprotinin on ice for 30 min. Lysates were then homogenized and centrifuged at $6{,}000 \times g$ for 20 min to pellet any insoluble material. The protein concentration of the supernatant was determined by the method of Bradford (1976). Samples (20 μg) run on SDS–PAGE using a 12.5% polyacrylamide gel, and electrotransferred to polyvinylidine difluoride filter (PVDF) membrane. To reduce non-specific binding, the PVDF was blocked for 30 min at room temperature in Tris-buffered saline (TBS: 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 3% gelatin. Thereafter, the PVDF was washed for 5 min three times in TBS-Tween buffer (TTBS: 20 mM Tris-HCl, 500 mM

NaCl, 0.05% Tween 20, pH 7.5) and incubated overnight with mouse monoclonal anti-*rho* A antibody (Santa Cruz Biotechnology) at 1 : 1,000 dilution in TTBS containing 1% gelatin. After extensive washing with TTBS, the PVDF was incubated with horseradish peroxidase conjugated anti-mouse antibody (Bio-Rad) diluted at 1 : 3,000 in TTBS containing 1% gelatin at room temperature for 120 min. Then, the PVDF was washed for 5 min twice in TTBS and washed for 5 min in TBS. The blots were visualized using the enhanced chemiluminescence method according to the manufacturer's (Bio-Rad) instructions.

Drugs

$GTP_{\gamma}S$, guanosine 5′-*O*-(2-thiodiphosphate) ($GDP_{\beta}S$), calphostin C, staurosporine, phorbol 12,13-dibutyrate (PDBu), genistein, daidzein, *l*-phenylephrine hydrochloride were purchased from Sigma Chemical Co. Tyrphostin 25 was purchased from Calbiochem, and histamine dihydrochloride was from Merck Research Laboratories. All materials for SDS–gel electrophoresis were obtained from Bio-Rad Laboratories or Wako Pure Chemical Industries. Other chemicals used in this study were of the highest purity available from Sigma or Wako.

Statistical analysis

All values are presented in terms of the means ± s.e.mean. Student's paired *t*-test or two way analysis of variance (ANOVA) followed by Scheffé's multiple comparison test was used to test the significance of differences between different series. A P value <0.05 was considered statistically significant.

Results

Influence of pretreatment with C3 exoenzyme

In the homogenate of the rabbit mesenteric arterial strip permeablized with β-escin, C3 exoenzyme catalyzed the [^{32}P]-ADP-ribosylation of the substrate which was detected as a single band corresponding to a 23-kDa protein on autoradiograph (Figure 1a). This ADP-ribosylated protein is most likely to be *rho* p21, because Western blots of the homogenate with anti-*rho* p21 antibody showed a band at about 23-kDa that overlayed the band on autoradiograph (Figure 1b). Pretreatment of the β-escin-permeabilized strip with 1 μg ml^{-1} C3 in the presence of 10 μM NAD for 30 min eliminated the level of C3-catalyzed [^{32}P]-ADP-ribosylation (Figure 1a), indicating that C3 pretreatment of the strip entirely catalyzed ADP-ribosylation of *rho* p21 in the presence of NAD, rendering *rho* p21 unavailable for subsequent *in vitro* [^{32}P]-ADP-ribosylation on exposure to C3 and [^{32}P]-NAD.

Figure 2a shows a representative tracing of the concentration-dependent effect of $GTP_{\gamma}S$ on the Ca^{2+}-induced contraction in β-escin-skinned smooth muscle of rabbit mesenteric artery. The contraction induced by 0.3 μM Ca^{2+} (pCa 6.5) was enhanced by $GTP_{\gamma}S$ in a concentration-dependent manner. $GDP_{\beta}S$ (1 mM) reversibly blocked this enhancement of the Ca^{2+}-induced contraction (data not shown), indicating that G proteins play a role in this Ca^{2+} sensitization. Pretreatment with C3 in the presence of NAD did not affect the contraction induced by 0.3 μM Ca^{2+}, but greatly inhibited the $GTP_{\gamma}S$-induced enhancement of the Ca^{2+} contraction (Figure 2b). As summarized in Figure 2c, C3 pretreatment caused a marked downward shift of the concentration-response curve for $GTP_{\gamma}S$-induced Ca^{2+} sensitization. However, the Ca^{2+}-

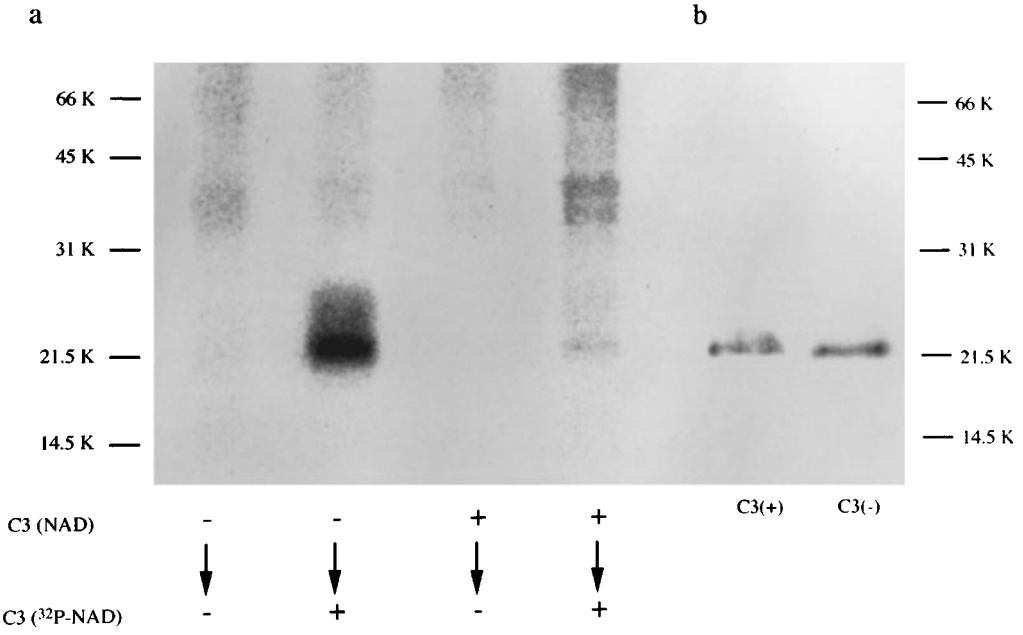

Figure 1 ADP-ribosylation by C3 of the protein (a) and immunoblot of *rho* p21 (b) in β-escin-permeabilized rabbit mesenteric artery. (a) The permeabilized strip preparation was incubated with or without 1 μg ml^{-1} C3 and 10 μM NAD for 30 min in the relaxing solution. After homogenization, the samples were incubated with [^{32}P]-NAD in the absence or presence of C3, subjected to SDS–PAGE and autoradiography as described in Methods. A single substrate for C3 was identified: Mr = 23,000. Note that pretreatment of the strip with C3 induced an identical reduction in the amount of substrate available for subsequent *in vitro* ADP-ribosylation. (b) The strip homogenate was resolved by SDS–PAGE, transferred to PVDF, and incubated with anti-*rho* p21 antibody. Then, blots were processed with anti-rabbit horseradish peroxidase-labelled antibody as described in Methods. The anti-*rho* p21 antibody identified one single protein band with a molecular mass of 23 kDa, regardless of whether the strip was pretreated with C3.

sensitizing effects elicited by GTP$_\gamma$S at higher concentrations (30 and 100 μM) were not completely eliminated by this treatment.

As shown in Figure 3a, GTP (10 μM), when applied during the tension development induced by 0.3 μM Ca^{2+}, produced an additional small increase in tension. Subsequent application of 1 μM phenylephrine or 10 μM histamine caused further contraction. Phenylephrine and histamine increased the amplitude of contraction by $47\pm3\%$ ($n=3$) and $42\pm5\%$ ($n=4$) of maximal force at pCa 4.5,

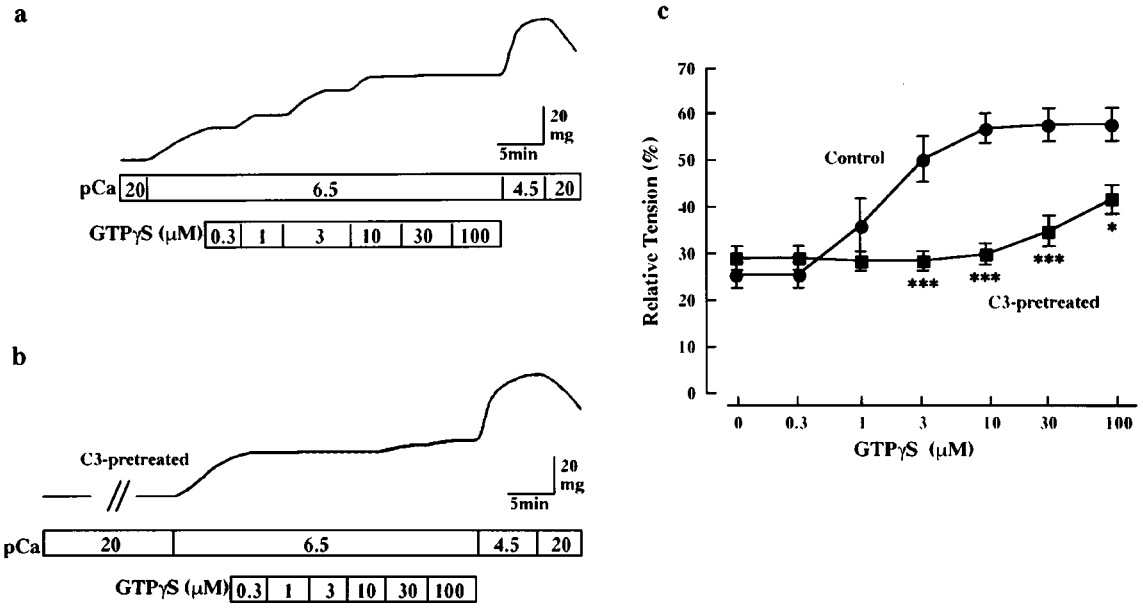

Figure 2 Inhibition by C3 pretreatment of GTP$_\gamma$S-induced enhancement of the Ca^{2+} contraction in β-escin-skinned smooth muscle of rabbit mesenteric artery. The skinned preparations were treated with 1 μg ml^{-1} C3 in the presence of 10 μM NAD for 30 min. (a) A representative tracing of the concentration-dependent effect of GTP$_\gamma$S on the contraction obtained at pCa 6.5. (b) A representative tracing of the effect of C3 pretreatment on GTP$_\gamma$S-induced enhancement of the contraction at pCa 6.5. (c) Concentration-response curves for GTP$_\gamma$S-induced enhancement of the contraction at pCa 6.5 in control and C3-pretreated muscle. Points are the means$\pm$s.e.mean of 5–6 experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs the corresponding control value.

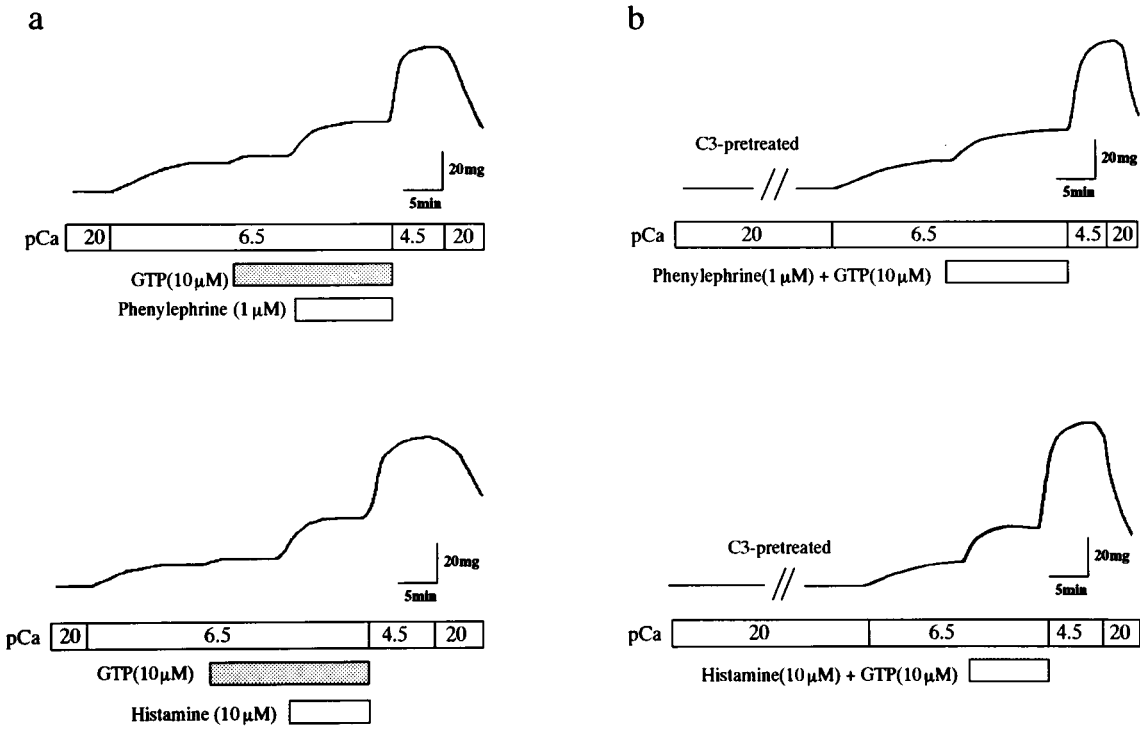

Figure 3 Phenylephrine- and histamine-induced Ca^{2+} sensitization in β-escin-skinned smooth muscle of rabbit mesenteric artery untreated and treated with C3 exoenzyme. (a) The preparations were first contracted at pCa 6.5. After steady-state contraction developed, 10 μM GTP was applied. Then, when GTP-induced contraction became constant, 1 μM phenylephrine (upper trace) or 10 μM histamine (lower trace) was applied. (b) The preparations were treated with 1 μg ml^{-1} C3 in the presence of 10 μM NAD for 30 min. After steady-state contraction was obtained at pCa 6.5, the effect of 1 μM phenylephrine or 10 μM histamine was tested in the simultaneous presence of 10 μM GTP.

respectively. The extent of the enhancement by these agonists of the Ca^{2+}-induced contraction was unaltered by C3 pretreatment (Figure 3b). Thus, the phenylephrine- and histamine-induced increases in the amplitude of contraction were $39\pm4\%$ ($n=4$) and $35\pm3\%$ ($n=4$) of maximal Ca^{2+}-induced contraction in C3-treated arteries, values which were not significantly different from those obtained in untreated arteries. Figure 4 shows the concentration-response curves for the effects of phenylephrine and histamine on the contraction induced by 0.3 μM Ca^{2+} in untreated and C3-treated arteries. The Ca^{2+}-sensitizing effects of phenylephrine and histamine in C3-treated arteries were virtually identical with those in untreated arteries through a wide range of their concentrations.

Influences of PKC inhibitors

The PKC activator PDBu (100 nM) increased the contractile force at pCa 6.5 from 18 ± 3 to $49\pm4\%$ ($n=10$) of maximum force at pCa 4.5. The PKC inhibitors calphostin C (1 μM) and staurosporine (20 nM) completely blocked the enhancement by PDBu of the Ca^{2+}-induced contraction without affecting the Ca^{2+} contraction alone (Figure 5).

The enhancement by 100 μM GTP$_\gamma$S of the 0.3 μM Ca^{2+}-induced contraction was apparently unaffected by 1 μM calphostin C (Figure 6a, b). The Ca^{2+}-sensitizing effects of 3 and 100 μM GTP$_\gamma$S were substantially similar in the absence and presence of calphostin C (Figure 8). Staurosporine also did not modify the enhancement by GTP$_\gamma$S of the Ca^{2+}-induced contraction (Figure 6c). The addition of 100 μM GTP$_\gamma$S increased the contractile force at pCa 6.5 from 22 ± 2 to $61\pm3\%$ ($n=14$) of maximum force at pCa 4.5 in control and from 27 ± 5 to $57\pm3\%$ ($n=4$) in the presence of 20 nM staurosporine.

In the presence of 10 μM GTP, 1 μM phenylephrine and 10 μM histamine enhanced the contraction induced by 0.3 μM Ca^{2+} regardless of whether 1 μM calphostin C was present (Figure 7). The Ca^{2+}-sensitizing effects of phenylephrine (0.1 and 1 μM) and histamine (1 and 10 μM) in the presence of calphostin C were not significantly different from those in its

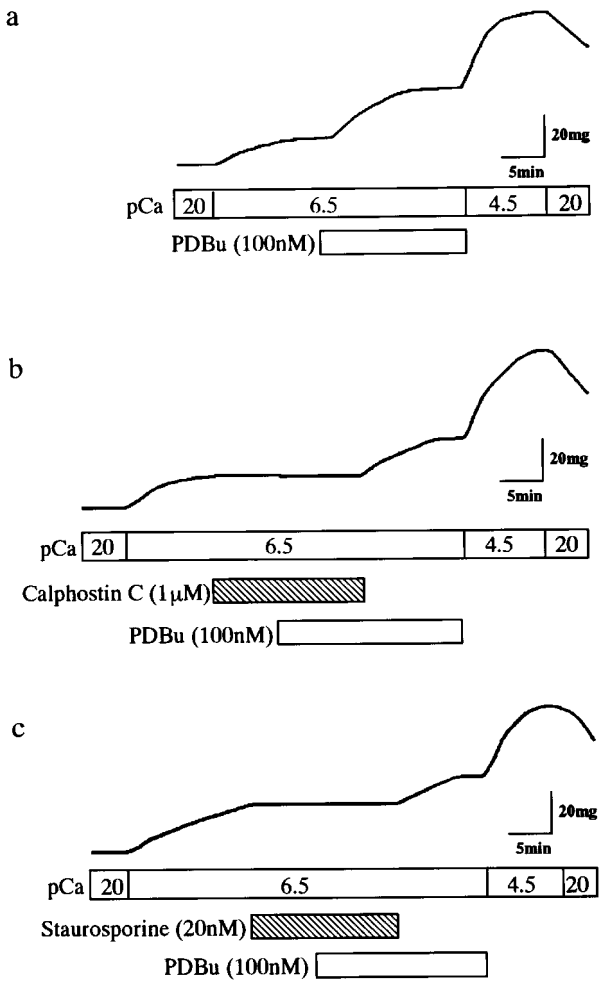

Figure 5 Blocking effect of PKC inhibitors on PDBu-induced enhancement of the Ca^{2+} contraction in β-escin-skinned smooth muscle of rabbit mesenteric artery. PDBu (100 nM) enhanced the contraction obtained at pCa 6.5 (a). Application of the PKC inhibitors 1 μM calphostin C (b) and 20 nM staurosporine (c) reversibly blocked this enhancement by PDBu.

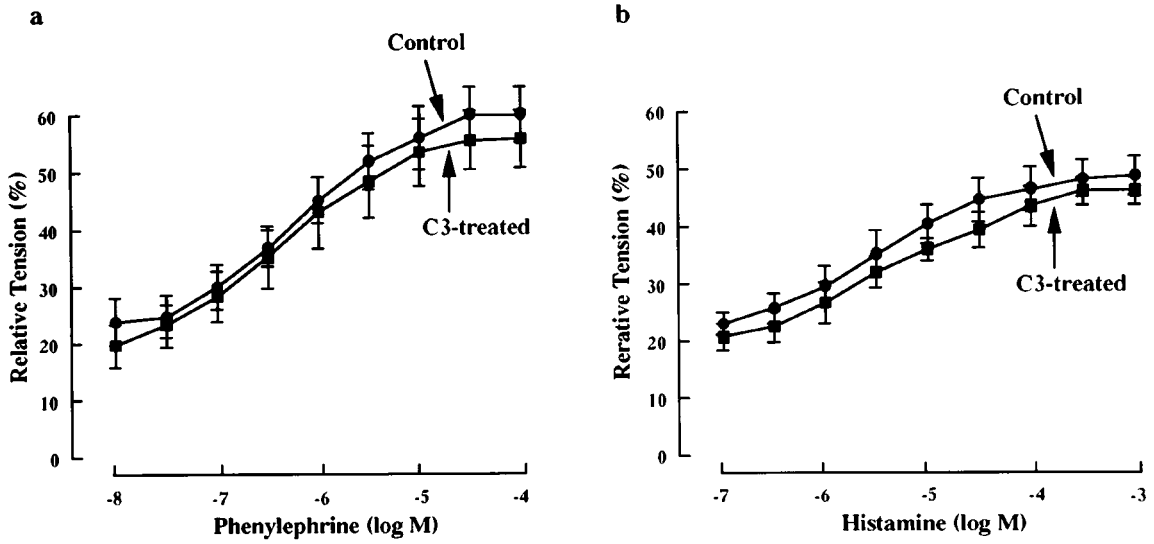

Figure 4 Concentration-response curves for the effects of phenylephrine (a) and histamine (b) on the contraction at pCa 6.5 in β-escin-skinned smooth muscle of rabbit mesenteric artery untreated and treated with C3 exoenzyme. All experiments were performed in the presence of 10 μM GTP. Relative tension is expressed as a percentage of the tension obtained at pCa 4.5 in the absence of any drug. Points are the means$\pm$s.e.mean of four experiments.

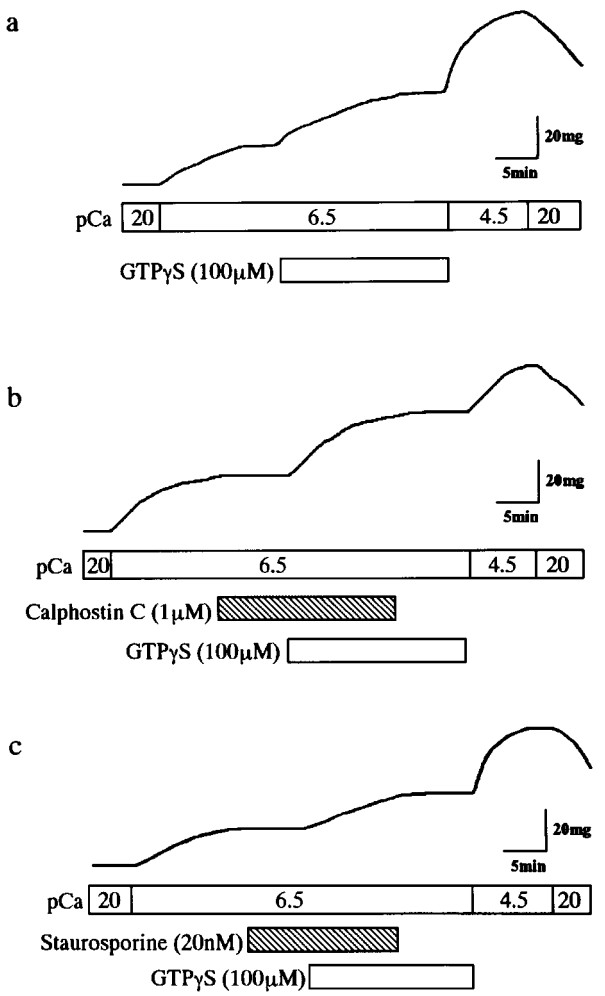

Figure 6 Lack of effect of PKC inhibitors on $GTP_{\gamma}S$-induced enhancement of the Ca^{2+} contractions in β-escin-skinned smooth muscle of rabbit mesenteric artery. $GTP_{\gamma}S$ (100 μM) enhanced the contraction obtained at pCa 6.5 (a). Application of the PKC inhibitors 1 μM calphostin C (b) and 20 nM staurosporine (c) affected neither the Ca^{2+}-induced contraction nor the enhancement by $GTP_{\gamma}S$ of this contraction.

absence (Figure 8). Staurosporine (20 nM) also had no effect on the enhancement by these agonists of the Ca^{2+} contraction (data not shown).

Influences of tyrosine kinase inhibitors

The tyrosine kinase inhibitor genistein (100 μM) irreversibly blocked the enhancement by 100 μM $GTP_{\gamma}S$ of the 0.3 μM Ca^{2+}-induced contraction without affecting this Ca^{2+} contraction alone (Figure 9a). Similarly, 100 μM tyrphostin 25, another tyrosine kinase inhibitor, which is structurally different from genistein, caused an irreversible block of the $GTP_{\gamma}S$-induced enhancement of the Ca^{2+} contraction (Figure 9b). On the other hand, 100 μM daidzein, an inactive analogue of genistein, had little inhibitory effect on $GTP_{\gamma}S$-induced Ca^{2+} sensitization (Figure 9c). The results of these experiments are summarized in Figure 9d.

Genistein (100 μM) also caused an irreversible and complete inhibition of the phenylephrine- and histamine-induced enhancement of the 0.3 μM Ca^{2+}-induced contraction (Figure 10). Similar results were found with 100 μM tyrphostin 25 (data not shown).

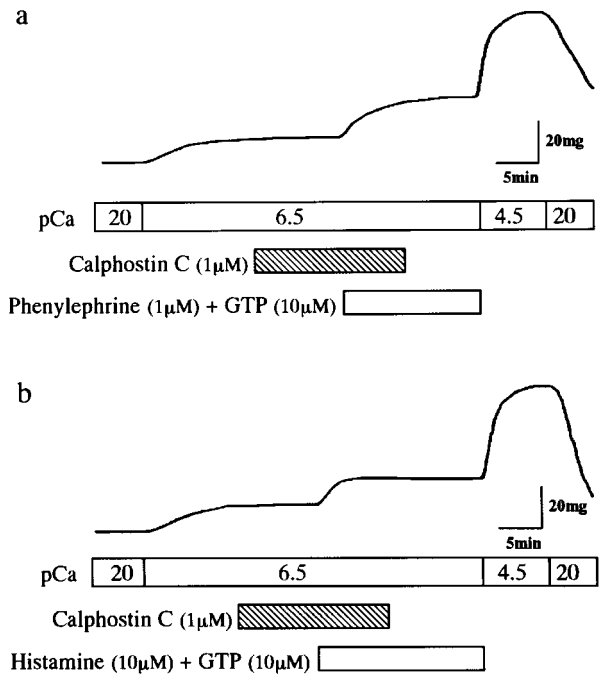

Figure 7 Lack of effect of calphostin C on phenylephrine- and histamine-induced enhancement of the Ca^{2+} contraction in β-escin-skinned smooth muscle of rabbit mesenteric artery. The Ca^{2+} sensitizing effects of 1 μM phenylephrine (a) and 10 μM histamine (b) obtained in the simultaneous presence of 10 μM GTP were not modified by 1 μM calphostin C.

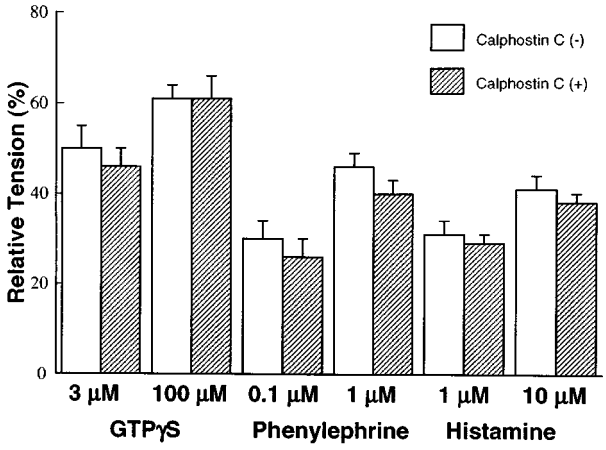

Figure 8 Enhancement of the contraction at pCa 6.5 by $GTP_{\gamma}S$ (3 and 100 μM), phenylephrine (0.1 and 1 μM) and histamine (1 and 10 μM) in the absence and presence of 1 μM calphostin C in β-escin-skinned smooth muscle of rabbit mesenteric artery. The experiments with phenylephrine and histamine were performed in the presence of 10 μM GTP. Relative tension is expressed as a percentage of the tension obtained at pCa 4.5 in the absence of any drug. Bars are the means $\pm$ s.e.mean of 4–14 experiments.

Neither genistein nor tyrphostin 25 modified the enhancement by 100 nM PDBu of the 0.3 μM Ca^{2+}-induced contraction (data not shown).

Discussion

There are two major superfamilies of G proteins involved in signal transduction; one is a superfamily of heterotrimeric G

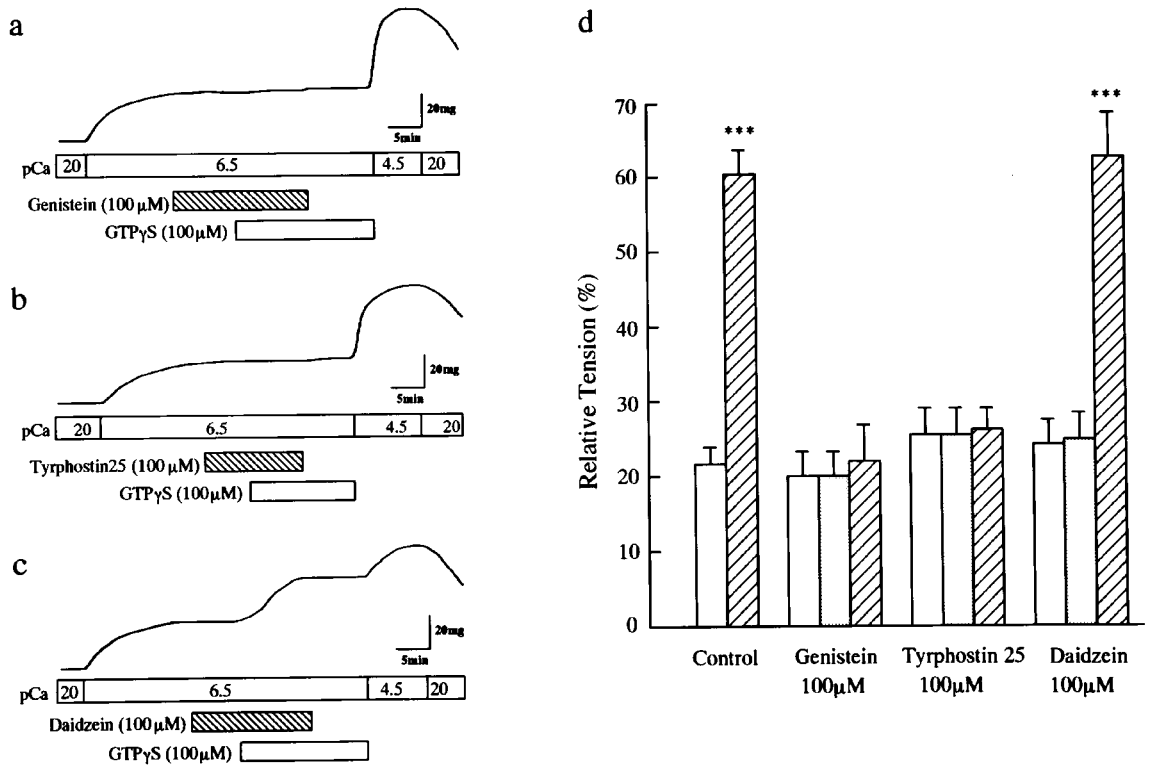

Figure 9 Effects of tyrosine kinase inhibitors on $GTP_{\gamma}S$-induced enhancement of the Ca^{2+} contractions in β-escin-skinned smooth muscle of rabbit mesenteric artery. (a, b) Application of the tyrosine kinase inhibitors 100 μM genistein and 100 μM tyrphostin 25 irreversibly and completely blocked the enhancement by 100 μM $GTP_{\gamma}S$ of the contraction at pCa 6.5 without affecting this Ca^{2+} contraction. (c) The inactive genistein analogue daidzein (100 μM) did not modify the enhancement by $GTP_{\gamma}S$. (d) Summarized data. Open and dotted bars indicate the contractions obtained at pCa 6.5 before and after application of tyrosine kinase inhibitors, respectively, and hatched bars show the contractions at pCa 6.5 in the presence of 100 μM $GTP_{\gamma}S$ in each case. Relative tension is expressed as a percentage of the tension obtained at pCa 4.5 in the absence of any drug. Bars are the means$\pm$s.e.mean of 4–14 experiments. ***$P<0.001$ vs the respective value obtained at pCa 6.5 alone.

proteins serving as transducers for membrane receptors and the other is a superfamily including small G proteins more than 40 members (Takai *et al*., 1992). C3 exoenzyme is known to ADP-ribosylation only the *rho* p21 family among many small G proteins (Takai *et al*., 1992). In the present study, we confirmed that C3 apparently ADP-ribosylated only one protein in β-escin-permeabilized smooth muscle of rabbit mesenteric artery, the protein having the same molecular mass as the protein detected in Western blots with anti-*rho* p21 antibody. We found that pretreatment with C3 significantly inhibited the $GTP_{\gamma}S$-induced enhancement of the Ca^{2+}-induced contractions in this arterial preparation. This result is essentially consistent with the notion reported by Hirata *et al*. (1992) that activation of *rho* p21 is involved in the $GTP_{\gamma}S$-induced increase in myofilament Ca^{2+} sensitivity in rabbit vascular smooth muscle. However, it should be emphasized as a novel finding in this study that C3 pretreatment did not completely suppress Ca^{2+} sensitization induced by $GTP_{\gamma}S$ at higher concentrations ($\geqslant 30$ μM). Since the finding that $GDP_{\beta}S$ caused a complete block of $GTP_{\gamma}S$-induced Ca^{2+} sensitization suggests that G proteins play a role in this sensitization, it is likely that heterotrimeric G proteins could contribute, at least in part, to the increase in myofilament Ca^{2+} sensitivity produced by higher concentrations of $GTP_{\gamma}S$. Thus, small G proteins such as *rho* p21 may have a greater intrinsic affinity for guanine nucleotide analogues compared to heterotrimeric G proteins. If the Ca^{2+} sensitizing effect of higher concentrations of $GTP_{\gamma}S$ is indeed mediated partly by heterotrimeric G proteins, this suggests that stimulation of heterotrimeric G protein-coupled receptors may cause Ca^{2+} sensitization through a mechanism independent of activation of *rho* p21. In support of this suggestion, we found that C3 pretreatment failed to inhibit Ca^{2+} sensitization induced by phenylephrine and histamine. Similarly, Gong *et al*. (1996) have demonstrated that ADP-ribosylation of *rho* p21 with epidermal cell differentiation inhibitor (EDIN) does not inhibit phenylephrine-induced Ca^{2+} sensitization in β-escin-permeabilized rabbit mesenteric artery, although the Ca^{2+} sensitizing effects of carbachol and endothelin could be suppressed by EDIN. However, our findings are in contrast to those of Kokubu *et al*. (1995) who have found that C3 pretreatment abolishes Ca^{2+} sensitization produced by activation of the α_{1A}-adrenoceptor subtype with norepinephrine or clonidine in β-escin-permeabilized smooth muscle of rabbit aorta. This apparent discrepancy may be due to tissue differences with respect to whether agonist-induced Ca^{2+} sensitization requires activation of *rho* p21. Alternatively, we cannot exclude the possibilty that different G protein-coupled receptor agonists may cause Ca^{2+} sensitization through different signal transduction pathways. Nevertheless, we interpret the present data to indicate that *rho* p21 is involved in Ca^{2+} sensitization induced by $GTP_{\gamma}S$, but not that by receptor agonists, in smooth muscle of rabbit mesenteric artery.

The role of PKC in Ca^{2+} sensitization is well recognized by the potentiating effects of phorbol esters on the Ca^{2+}-induced contractions in permeabilized smooth muscle preparations (Chatterjee & Tejada, 1986; Fujiwara *et al*., 1988; Nishimura & van Breemen, 1989; Itoh *et al*., 1994). It has been believed that

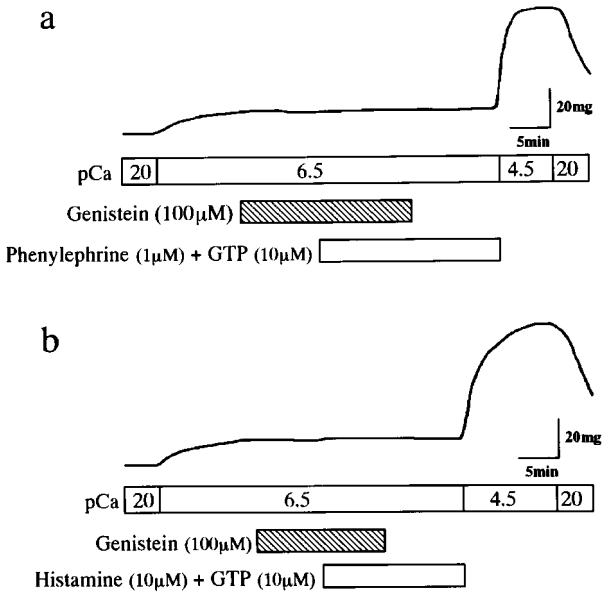

Figure 10 Effect of genistein on phenylephrine- and histamine-induced enhancement of the Ca^{2+} contraction in β-escin-skinned smooth muscle of rabbit mesenteric artery. The Ca^{2+} sensitizing effects of 1 μM phenylephrine (a) and 10 μM histamine (b) obtained in the simultaneous presence of 10 μM GTP were irreversibly and completely blocked by 100 μM genistein.

both receptor agonists and $GTP_{\gamma}S$ activate PKC through the production of diacylglycerol and then increase the sensitivity of the contractile proteins to Ca^{2+} in vascular smooth muscle (Lee & Severson, 1994). This has been supported by the finding that the PKC inhibitor H-7 inhibits the enhancement of the Ca^{2+}-induced contractions induced by $GTP_{\gamma}S$ and some receptor agonists (Nishimura *et al*., 1988; Satoh *et al*., 1994). By contrast, the present study demonstrated that neither calphostin C nor staurosporine did affect $GTP_{\gamma}S$-induced Ca^{2+} sensitization in β-escin-permeabilized smooth muscle of rabbit mesenteric artery, whereas they completely inhibited PDBu-induced Ca^{2+} sensitization. This discrepancy may be related to the selectivity of PKC inhibitors used; H-7 is known to have nonspecific actions, especially at high concentrations, such as inhibition of MLC kinase and cyclic nucleotide-dependent kinases (Hidaka *et al*., 1984), resulting in nonselective inhibition of smooth muscle contractions. In agreement with our results, Itoh *et al*. (1994) have reported that PKC_{19-36}, a peptide inhibitor of PKC, inhibited the Ca^{2+} sensitizing effect of PDBu, but not that of $GTP_{\gamma}S$ in vascular smooth muscle. Furthermore, $GTP_{\gamma}S$ is capable of further potentiating the Ca^{2+}-induced contraction even in the presence of saturated concentrations of PDBu (Kitazawa & Somlyo, 1991; Itoh *et al*., 1994), suggesting that $GTP_{\gamma}S$ and PDBu induce Ca^{2+} sensitization through activation of different signaling pathways. It is thus reasonable to conclude that PKC does not contribute to $GTP_{\gamma}S$-induced Ca^{2+} sensitization of smooth muscle of rabbit mesenteric artery. In similar to $GTP_{\gamma}S$, phenylephrine and histamine enhanced the Ca^{2+}-induced contractions without being modified by the PKC inhibitors. While the possibility cannot ruled out that some receptor agonists may generate enough diacylglycerol to activate PKC to a level similar to that activated by phorbol esters and result in Ca^{2+} sensitization through the PKC-dependent pathway, our conclusion that PKC has only a minor role, if any, in agonist-induced Ca^{2+} sensitization is consistent with the results obtained in PKC-downregulated rat aorta (Hori *et al*., 1993). Therefore, it seems likely that phenylephrine and histamine also caused Ca^{2+} sensitization through a PKC-independent pathway.

In the present study, we showed that the putative tyrosine kinase inhibitors genistein and tyrphostin 25 caused an irreversible and complete inhibition of $GTP_{\gamma}S$-induced Ca^{2+} sensitization. The fact that the same effect was produced by the two structurally unrelated tyrosine kinase inhibitors, which act through different mechanisms (Akiyama *et al*., 1987; Gazit *et al*., 1989), confirms that the inhibition was the result of a specific action on tyrosine kinases. This conclusion is supported by the finding that the inactive genistein analogue daidzein was ineffective in inhibiting the Ca^{2+} sensitizing effect of $GTP_{\gamma}S$. Furthermore, the lack of effect on the Ca^{2+}-induced contraction and PDBu-induced Ca^{2+} sensitization provides evidence that the inhibitors did not produce their effects nonspecifically by inhibiting the contractile process directly. Hence, we suggest that tyrosine kinases play a major role in $GTP_{\gamma}S$-induced Ca^{2+} sensitization of smooth muscle of rabbit mesenteric artery.

In view of the above-mentioned conclusion that *rho* p21 is predominantly involved in $GTP_{\gamma}S$-induced Ca^{2+} sensitization, we assume that *rho* p21 may activate a tyrosine kinase cascade in vascular smooth muscle cells which could eventually lead to Ca^{2+} sensitization of contractile apparatus. There is evidence from a number of cell types that *rho* p21 mediates tyrosine phosphorylation of multiple substrates including p125 focal adhesion kinase and paxillin (Seckl *et al*., 1995; Imamura *et al*., 1996). Paxillin is a regulatory component of the complex of cytoskeletal proteins that link the actin cytoskeleton to the plasma membrane. Wang *et al*. (1996) have demonstrated that an increase in the tyrosine phosphorylation of paxillin is associated with active force development in canine tracheal smooth muscle. They have also observed that MLC phosphorylation could be reduced by genistein in proportion to the reduction in force in smooth muscle. These findings suggest that phosphotyrosine-containing proteins may be involved in signal transduction processes for the regulation of force development in smooth muscle. It is therefore likely that the large part of $GTP_{\gamma}S$-induced Ca^{2+} sensitization in vascular smooth muscle is closely related to *rho* p21-mediated protein tyrosine phosphorylation.

The present study revealed that the tyrosine kinase inhibitors irreversibly and completely inhibited Ca^{2+} sensitization induced by phenylephrine and histamine. This suggests that the protein tyrosine phosphorylation process may also be a key step of signal transduction in Ca^{2+} sensitization caused by these receptor agonists in vascular smooth muscle. Indeed, a recent study has demonstrated that histamine increases phosphorylation of a number of proteins on tyrosine residues in swine carotid artery (Rembold & Weaver, 1997). However, whether heterotrimeric G protein-coupled receptors are directly or indirectly linked to tyrosine kinases is not known. The lack of effect of C3 pretreatment on agonist-induced Ca^{2+} sensitization suggests that *rho* p21 is not a component of the signal transduction pathway linking heterotrimeric G protein-coupled receptors with tyrosine phosphorylation, but other small G proteins may work as a link between them. The precise mechanism(s) linking the receptors with tyrosine phosphorylation of the proteins involved in Ca^{2+} sensitization of contractile apparatus warrents further investigation.

In conclusion, using β-escin-permeabilized smooth muscle of rabbit mesenteric artery, we demonstrate that protein tyrosine phosphorylation, but not PKC, plays a critical role in both of the Ca^{2+} sensitizing effects of $GTP_{\gamma}S$ and receptor agonists. Since *rho* p21 predominantly mediated the effect of

$GTP_{\gamma}S$, at least anyone of tyrosine kinases involved in the protein phosphorylation could act in the downstream of *rho* p21, thereby leading to $GTP_{\gamma}S$-induced Ca^{2+} sensitization. On the other hand, *rho* p21 does not appear to lie in the upstream of tyrosine phosphorylation associated with receptor agonist activation.

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