Effect of a peptide inhibitor of protein kinase C on G-protein-mediated increase in myofilament Ca^{2+} -sensitivity in rabbit arterial skinned muscle

¹Takeo Itoh, Akito Suzuki & Yoshimasa Watanabe

Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

1 To investigate the role of protein kinase C in the increase mediated by guanosine 5'-triphosphate (GTP)-binding proteins (G-proteins) in the sensitivity of the contractile proteins to Ca^{2+} in vascular smooth muscle, the effect of a novel peptide inhibitor of protein kinase C (PKC_{19-36}) on Ca^{2+} -induced contraction and myosin light chain (MLC) phosphorylation was studied in the presence and absence of guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}_\gamma\text{S}$) in β -escin-skinned smooth muscle strips of rabbit mesenteric artery. For comparison, the effects were also observed of PKC_{19-36} on the action of phorbol 12,13-dibutyrate (PDBu, an activator of PKC) on the two Ca^{2+} -induced responses.

2 In β -escin-skinned strips treated with ionomycin, Ca^{2+} ($0.1\text{--}3\text{ }\mu\text{M}$) concentration-dependently produced contraction in parallel with an increase in MLC-phosphorylation. $\text{GTP}_\gamma\text{S}$ ($10\text{ }\mu\text{M}$) and PDBu ($0.1\text{ }\mu\text{M}$) each shifted both the Ca^{2+} -force and Ca^{2+} -MLC-phosphorylation relationships to the left without a significant change in either maximum response. The relationship between force and MLC-phosphorylation was not modified by either $\text{GTP}_\gamma\text{S}$ or PDBu, indicating that the sensitivity of MLC-phosphorylation to Ca^{2+} is enhanced by both $\text{GTP}_\gamma\text{S}$ and PDBu.

3 PKC_{19-36} itself modified neither the contraction nor MLC-phosphorylation induced by Ca^{2+} but it did block the PDBu-induced enhancement of these two Ca^{2+} -induced responses. By contrast, PKC_{19-36} did not modify the $\text{GTP}_\gamma\text{S}$ -induced enhancement of the two Ca^{2+} -induced responses. Guanosine 5'-O-(2-thiodiphosphate) (GDP_βS) attenuated the $\text{GTP}_\gamma\text{S}$ -induced enhancement of the Ca^{2+} -induced contraction.

4 These results suggest that $\text{GTP}_\gamma\text{S}$ increases Ca^{2+} -induced MLC-phosphorylation through the activation of a PKC-independent mechanism and thus causes an increase in the sensitivity of the contractile proteins to Ca^{2+} in β -escin-skinned smooth muscle of rabbit mesenteric artery.

Keywords: G-proteins; protein kinase C; myosin phosphorylation; vascular smooth muscle

Introduction

It is generally believed that a Ca^{2+} -mobilizing hormone (agonist) binds to its receptor and promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) through activation of heterotrimeric ($\alpha\beta\gamma$) G-proteins, and that this is achieved by catalysing the replacement of the guanosine 5'-diphosphate (GDP) bound to the α -subunit by guanosine 5'-triphosphate (GTP) (Bourne *et al.*, 1990). Following the hydrolysis of PIP_2 , inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) are produced: the former releases Ca^{2+} from the cellular storage sites and the latter activates protein kinase C (PKC). It is also known that treatment with GTP or guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}_\gamma\text{S}$) increases the contraction, phosphorylation of myosin light chain (MLC) and unloaded shortening velocity induced by low concentrations of Ca^{2+} through activation of unidentified G-proteins in chemically skinned smooth muscle (Nishimura *et al.*, 1988; Fujiwara *et al.*, 1989; Kitazawa *et al.*, 1989; Itoh *et al.*, 1992; Kubota *et al.*, 1992). It was recently suggested that agonists acting together with GTP or $\text{GTP}_\gamma\text{S}$ increase MLC-phosphorylation through inhibition of MLC-phosphatases (Kitazawa *et al.*, 1991; Kubota *et al.*, 1992), but the precise mechanism for this is not well understood.

It has been reported that $\text{GTP}_\gamma\text{S}$ itself activates heterotrimeric G-proteins and thus increases the activity of phospholipase C, causing production of IP_3 in vascular smooth muscle (Sasaguri *et al.*, 1985). This suggests that, following application of $\text{GTP}_\gamma\text{S}$, DAG (a co-product of IP_3 via hydrolysis of PIP_2) may also be produced, causing activation of PKC in vascular smooth muscle. In saponin-skinned smooth

muscle, we have previously reported that 12-O-tetradecanoyl-13-acetate (TPA), a potent activator of PKC, increased the contraction, MLC-phosphorylation and unloaded shortening velocity in the presence of $0.3\text{ }\mu\text{M}$ Ca^{2+} (Itoh *et al.*, 1986b; Fujiwara *et al.*, 1988). Similar effects of phorbol esters on Ca^{2+} -induced contraction were also reported in Triton X-100-treated skinned vascular smooth muscle (Chatterjee & Tejada, 1986). These results suggest that PKC, activated by an application of $\text{GTP}_\gamma\text{S}$, may play a primary role in the $\text{GTP}_\gamma\text{S}$ -induced enhancement of Ca^{2+} -induced contractile responses in vascular smooth muscle. Indeed, Nishimura *et al.* (1988) reported that H-7, an inhibitor of protein kinase C, inhibited the $\text{GTP}_\gamma\text{S}$ -induced enhancement of the Ca^{2+} -induced contraction, and suggested that PKC may play an important role in the $\text{GTP}_\gamma\text{S}$ -induced Ca^{2+} -sensitization in vascular tissue. However, since H-7 inhibits PKC at its ATP-binding site, a region with a high degree of sequence homology in most kinases (Hidaka *et al.*, 1984; Edelman *et al.*, 1987), this agent may have some non-specific actions, especially at high concentrations, such as inhibition of guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent protein kinase, adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase and MLC-kinase (Hidaka *et al.*, 1984). In preliminary experiments, on β -escin-skinned smooth muscle, we found that H-7, at concentrations over $10\text{ }\mu\text{M}$, concentration-dependently inhibited both the contraction and MLC-phosphorylation induced by $10\text{ }\mu\text{M}$ Ca^{2+} with no change in the relationship between force and MLC-phosphorylation, suggesting that high concentrations of H-7 may inhibit MLC-kinase as well as PKC. Thus, the contribution of PKC to the $\text{GTP}_\gamma\text{S}$ -induced enhancement of Ca^{2+} -induced contraction needs further examination, using more specific PKC inhibitors.

¹ Author for correspondence.

PKC₁₉₋₃₆, which corresponds to the autoinhibitory region of PKC, has been recently introduced and shown to inhibit selectively PKC (House & Kemp, 1987; Gong *et al.*, 1992). In order to investigate the contribution of PKC to the mechanisms by which G-proteins mediate an enhancement of the contraction induced by Ca²⁺, we studied the effects of PKC₁₉₋₃₆ on Ca²⁺-induced contraction and MLC-phosphorylation in ionomycin-treated, β -escin-skinned smooth muscle. For comparison, we also studied the effects of PKC₁₉₋₃₆ on the action of PDBu (an activator of PKC) on the same Ca²⁺-induced responses.

Methods

Male albino rabbits, weighing 1.9–2.5 kg, were anaesthetized with pentobarbitone sodium (40 mg kg⁻¹, i.v.) and then exsanguinated. A segment of the third branch of the mesenteric artery distributing to the region of the ileum (diameter around 70 μ m) was excised immediately and cleaned by removal of connective tissue in Krebs solution at room temperature. Endothelial cells were removed by gentle rubbing of the internal surface of the vessels with small knives. The fine circularly-cut muscle strip (0.2–0.3 mm length, 0.04–0.05 mm width, 0.02–0.03 mm thickness) was transferred to a chamber of 0.1 ml volume and mounted horizontally, the resting force being adjusted to obtain a maximal contraction in 128 mM K⁺.

Experiment on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were made using β -escin (Kobayashi *et al.*, 1989; Itoh *et al.*, 1992). The methods used for skinning and the composition of the solutions have been described elsewhere (Itoh *et al.*, 1986b). Briefly, skinned muscle preparations were obtained by treatment with β -escin (30 μ M) in Ca²⁺-free solution containing EGTA and adenosine 5'-triphosphate (ATP) ('relaxing solution') for 25 min. When the Ca²⁺-force relationship was to be determined, the concentration of EGTA in the solution was 4 mM and 1 μ M ionomycin was applied to avoid spurious effects due to Ca²⁺ release from intracellular storage sites in the skinned muscle. To prevent deterioration of the Ca²⁺-induced contraction, 0.1 μ M calmodulin was applied throughout the experiments, as described previously (Itoh *et al.*, 1986a). Various concentrations of Ca²⁺ were applied cumulatively from low to high concentration. The amplitude of contraction induced by each of the various concentrations of Ca²⁺ was normalized with respect to that induced by 10 μ M Ca²⁺ in the same strip.

Measurements of myosin light chain phosphorylation

Muscle strips (0.2–0.3 mm length, 1.2 mm width, 0.02–0.03 mm thickness) were suspended in a relaxing solution for over 10 min. Then, the tissues were skinned with a relaxing solution containing 30 μ M β -escin with 1 μ M ionomycin for 25 min and washed again with the relaxing solution (containing no β -escin). Each skinned muscle strip was then suspended in solutions containing various concentrations of Ca²⁺ for 15 min in the presence or absence of either 10 μ M GTP_S or 0.1 μ M PDBu. The strips were quick-frozen with 10% trichloroacetic acid (TCA) in acetone-dry ice containing 10 mM dithiothreitol and allowed to reach room temperature. The strips were then washed three times with acetone to remove residual TCA. Protein, including myosin light chain (MLC), was extracted in a lysing solution containing 8 M urea, 20 mM tris(hydroxymethyl)aminomethane (Tris), 23 mM glycine, 10 mM dithiothreitol, 0.004% bromophenol blue and saturated sucrose (pH 8.6) (Persechini *et al.*, 1986).

Nonphosphorylated, monophosphorylated and diphosphorylated forms of MLC were separated by urea/glycerol-polyacrylamide gel electrophoresis followed by electrophoretic

transfer of the proteins to nitrocellulose paper and quantitation of the relative amounts of each form by an immunoblot procedure, as reported previously (Persechini *et al.*, 1986). Antibody against bovine tracheal MLC was used as a first antibody and biotin-labelled goat anti-rabbit IgG (Histofine, Seikagaku Kogyo, Japan) as a second antibody. Alkaline phosphatase-labelled streptavidine was then applied. Regions containing MLC were visualized as dark blue bands after incubation with the colour development reagents, 5-bromo-4-chloro-3-indolyl phosphate toluidine and *p*-nitroblue tetrazolium chloride (Alkaline phosphatase substrate kit, Vector Laboratories, CA, U.S.A.). Relative amounts of nonphosphorylated, monophosphorylated and diphosphorylated MLC were quantified densitometrically by scanning the blot using a chromatoscanner (CS-930, Shimadzu, Japan). The phosphorylation of MLC was expressed in mol PO₄ mol⁻¹ MLC.

Calculation of Hill coefficient

The slope of the concentration-response relationship for the effect of Ca²⁺ on force and MLC-phosphorylation is shown by the Hill coefficient (*n*) and mid-point position (*pK* = $-\log K$), where *K* is the dissociation constant). These parameters were obtained by fitting the data points for each curve to eqn.(1) by a non-linear least-squares method.

$$F/F_0 = (C/K)^n / [1 + (C/K)^n] \quad (1)$$

Where *C* represents the concentration of Ca²⁺, *F* is the amplitude of contraction at any given concentration of Ca²⁺, and *F*₀ is the maximum response evoked by 10 μ M Ca²⁺ expressed as a relative force of 1.0.

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na⁺ 137.4, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 2.6, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134, glucose 11.5. The concentration of K⁺ was modified by replacing NaCl with KCl, isosmotically. The solutions were bubbled with 95% O₂ and 5% CO₂ and their pH maintained at 7.3–7.4.

For experiments on skinned muscle, the composition of the 'relaxing solution' was: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-*N,N'*-bis-(2-ethanesulphonic acid) (PIPES), 5.1 mM Mg(methanesulphonate)₂, 5.2 mM adenosine 5'-triphosphate (ATP), 5 mM phosphocreatine and 4 mM ethyleneglycol-bis-(β -aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA). Various Ca²⁺ concentrations were prepared by adding appropriate amounts of Ca(methanesulphonate)₂ to 4 mM EGTA, based on the calculation reported previously (Itoh *et al.*, 1986a). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength was standardized at 0.2 M by changing the amount of KMS added.

Drugs

Drugs used were EGTA, PIPES and HEPES (Dojin, Japan), PKC₁₉₋₃₆ (Peninsula Laboratories), calmodulin, β -escin and GTP_S (Sigma) and ionomycin (free acid; Calbiochem). The antibody against bovine tracheal MLC was provided by Dr J.T. Stull (Department of Physiology and Pharmacology, University of Texas Southwestern Medical Center, TX, U.S.A.).

Statistics

The values recorded were expressed as mean $\pm$ s.d. and statistical significance determined by a paired or unpaired Student's *t* test. Probabilities less than 5% (*P* < 0.05) were considered significant.

Results

GTP γ S and PDBu both increase the sensitivity of contractile proteins to Ca $^{2+}$ in β -escin-skinned smooth muscle

The effects were observed of GTP γ S and PDBu on the contraction and phosphorylation of myosin light chain (MLC) induced by various concentrations of Ca $^{2+}$ in ionomycin-, β -escin-skinned smooth muscle strips (Figure 1). In the skinned muscle, the minimum concentration of Ca $^{2+}$ needed to produce contraction was 0.1 μ M and the maximum contraction was obtained at 3 μ M Ca $^{2+}$. Figure 1a shows the effects of applying 10 μ M GTP γ S or 0.1 μ M PDBu on contractions induced by various concentrations of Ca $^{2+}$ (0.03–10 μ M). GTP γ S and PDBu each shifted the Ca $^{2+}$ -force-relation curve to the left with no change in the maximum amplitude of contraction induced by 10 μ M Ca $^{2+}$. The threshold concentration of Ca $^{2+}$ needed to produce contraction was slightly lower in the presence of either GTP γ S (0.03 μ M) or PDBu (0.03 μ M) than in control (0.1 μ M). The concentration of Ca $^{2+}$ required for half-maximum force (ED $_{50}$) was 0.61 ± 0.18 μ M in control ($n = 12$), 0.18 ± 0.13 μ M in the presence of GTP γ S ($n = 6$) and 0.14 ± 0.07 μ M in PDBu ($n = 6$) and the ED $_{50}$ values obtained in the presence of GTP γ S or PDBu were significantly smaller than that in the corresponding control ($P < 0.05$). The Hill coefficient was 2.2 ± 0.3 in control, 1.3 ± 0.2 in the presence of GTP γ S and 1.6 ± 0.3 in PDBu.

Figure 1 Effects of GTP γ S and PDBu on relationships between Ca $^{2+}$ -force (a) and Ca $^{2+}$ -myosin light chain (MLC) phosphorylation (b) in β -escin-skinned smooth muscle strips. Ca $^{2+}$ at any given concentration (0.03–10 μ M) was applied for 15 min in a relaxing solution containing 4 mM EGTA in the absence (●) or presence of either 10 μ M GTP γ S (▲) or 0.1 μ M PDBu (Δ). In (a), the maximum amplitude of contraction induced by 10 μ M Ca $^{2+}$ was normalized as a relative force of 1.0 for each strip. The curves for the effects of Ca $^{2+}$ on force and MLC-phosphorylation were obtained by fitting the data points to eqn.(1) by a non-linear least-squares method (see Methods). Results shown are each the mean of 6–12 observations with s.d. For abbreviations, see text.

Figure 1b shows the effects of applying 10 μ M GTP γ S or 0.1 μ M PDBu on the MLC-phosphorylation induced by various concentrations of Ca $^{2+}$ (0.03–10 μ M). In Ca $^{2+}$ -free solution containing 4 mM EGTA, MLC-phosphorylation was 0.04 ± 0.04 mol PO $_4$ mol $^{-1}$ MLC ($n = 6$) and Ca $^{2+}$ (0.03–10 μ M) concentration-dependently increased the MLC-phosphorylation. Under these conditions, Ca $^{2+}$ (0.03–10 μ M) mainly increased the monophosphorylated form of MLC (over 95%) whether in the presence or absence of GTP γ S or PDBu. GTP γ S and PDBu each shifted the relationship between Ca $^{2+}$ and MLC-phosphorylation (monophosphorylated form) to the left without affecting the maximum level of MLC-phosphorylation obtained with 10 μ M Ca $^{2+}$. The ED $_{50}$ values of Ca $^{2+}$ for monophosphorylated form of MLC were 0.88 ± 0.06 μ M in control ($n = 6$), 0.53 ± 0.08 μ M in the presence of GTP γ S ($n = 6$) and 0.31 ± 0.08 μ M in PDBu ($n = 6$). The ED $_{50}$ value obtained in the presence of GTP γ S or PDBu was significantly smaller than that in control ($P < 0.05$).

Figure 2 shows the relationship between relative force and the monophosphorylated form of MLC in the presence of various concentrations of Ca $^{2+}$ with and without GTP γ S or PDBu. Neither GTP γ S nor PDBu changed this relationship.

GTP γ S increases Ca $^{2+}$ -induced contraction and MLC-phosphorylation by a PKC-independent mechanism

PKC $_{19-36}$, a synthetic peptide corresponding to autoinhibitory domain of PKC, inhibits PKC with a K_i value of 0.2 μ M when assayed with a synthetic peptide, Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, as a substrate (House & Kemp, 1987).

PKC $_{19-36}$ (0.1 mM) modified neither the contraction (1.1 ± 0.2 times control) nor the MLC-phosphorylation induced by 0.3 μ M Ca $^{2+}$ alone. The MLC-phosphorylation induced by 0.3 μ M Ca $^{2+}$ was 0.16 ± 0.06 mol PO $_4$ mol $^{-1}$ MLC in control ($n = 6$) and 0.17 ± 0.03 mol PO $_4$ mol $^{-1}$ MLC in the presence of 0.1 mM PKC $_{19-36}$ ($n = 4$). The effects of PKC $_{19-36}$ were then observed on the contraction induced by 0.3 μ M Ca $^{2+}$ in the presence of either 10 μ M GTP γ S or 0.1 μ M PDBu (Figure 3). PKC $_{19-36}$ (10–100 μ M) concentration-dependently attenuated the PDBu-induced enhancement of

Figure 2 The relationship between relative force and myosin light chain (MLC) phosphorylation in the presence of various concentrations of Ca $^{2+}$ (0.03–10 μ M) with and without either GTP γ S or PDBu in β -escin-skinned smooth muscle strips: (●) control; (▲) in the presence of 10 μ M GTP γ S; (Δ) in 0.1 μ M PDBu. The curve was obtained by fitting the data points to eqn.(1) by a non-linear least-squares method according to the equation: $F/F_0 = (C/K)^n / [1 + (C/K)^n]$. n , Hill coefficient; K , dissociation constant. C and F/F_0 represent MLC-phosphorylation (mol PO $_4$ mol $^{-1}$ MLC) and relative force, respectively. The fitted parameters of n and K were 2.5 and 0.28 mol PO $_4$ mol $^{-1}$ MLC, respectively ($r = 0.99$). The data shown in Figure 1 were used for this analysis. Results shown are each the mean of 4–8 observations with s.d. For abbreviations, see text.

the Ca^{2+} -induced contraction and, at 0.1 mM blocked the action of PDBu (Figure 3b). The PKC peptide (0.1 mM) also blocked the PDBu-induced enhancement of the MLC-phosphorylation induced by $0.3 \mu\text{M}$ Ca^{2+} (Figure 3c). By contrast, PKC₁₉₋₃₆ failed to modify the GTP _{γ} S-induced enhancement of either contraction or MLC-phosphorylation induced by $0.3 \mu\text{M}$ Ca^{2+} (Figures 3 and 4). PKC₁₉₋₃₆ did not seem to change the relationship between relative force and MLC-

Figure 3 Effects of a protein kinase C inhibitor, PKC₁₉₋₃₆, on contraction and myosin light chain (MLC) phosphorylation induced by $0.3 \mu\text{M}$ Ca^{2+} in the presence of GTP _{γ} S or PDBu in β -escin-skinned smooth muscle strips. (a) Ca^{2+} ($0.3 \mu\text{M}$) was applied first. Then, GTP _{γ} S ($10 \mu\text{M}$) was applied on the steady state of contraction induced by $0.3 \mu\text{M}$ Ca^{2+} . Subsequently, PKC₁₉₋₃₆ (0.1 mM) was applied during the tonic phase of the contraction induced by Ca^{2+} in the presence of GTP _{γ} S. Following washout of PKC₁₉₋₃₆ by a solution containing Ca^{2+} with GTP _{γ} S, $10 \mu\text{M}$ Ca^{2+} was finally applied to obtain the maximum Ca^{2+} -induced contraction for each strip. Each agent was applied as indicated by the bar. (b) Concentration-dependent effects of PKC₁₉₋₃₆ on contraction induced by $0.3 \mu\text{M}$ Ca^{2+} in the presence of $10 \mu\text{M}$ GTP _{γ} S or $0.1 \mu\text{M}$ PDBu. Solid column, the contraction induced by $0.3 \mu\text{M}$ Ca^{2+} alone; open column, in the presence of $0.1 \mu\text{M}$ PDBu; hatched column, in $10 \mu\text{M}$ GTP _{γ} S. PKC₁₉₋₃₆ was applied cumulatively from low to high concentration on the steady state contraction induced by $0.3 \mu\text{M}$ Ca^{2+} in the presence of either PDBu or GTP _{γ} S. The maximum amplitude of contraction induced by $10 \mu\text{M}$ Ca^{2+} was normalized as a relative force of 1.0 for each strip. (c) Effects of PKC₁₉₋₃₆ on MLC-phosphorylation induced by $0.3 \mu\text{M}$ Ca^{2+} in the presence or absence of either $10 \mu\text{M}$ GTP _{γ} S or $0.1 \mu\text{M}$ PDBu. Open column, control; hatched column, in the presence of $0.1 \mu\text{M}$ PKC₁₉₋₃₆. Results shown are each the mean of 4 observations with s.d. *Significant difference from the corresponding control. For abbreviations, see text.

phosphorylation in the presence of either GTP _{γ} S or PDBu (Figure 4).

GDP _{β} S (1 mM) slightly inhibited the contraction induced by $0.3 \mu\text{M}$ Ca^{2+} and greatly attenuated the GTP _{γ} S-induced enhancement of this contraction (Figure 5a and b). Neomycin interacts with polyphosphoinositides and inhibits the activation of phospholipase C (Cockcroft & Gomperts, 1985; Kobayashi *et al.*, 1989), but at 0.1 mM it did not modify the contraction induced by $0.3 \mu\text{M}$ Ca^{2+} in the presence or absence of GTP _{γ} S (Figure 5c). The extent of the GTP _{γ} S-induced enhancement of the Ca^{2+} -induced contraction was 2.2 ± 0.2 times control in the presence of neomycin ($n = 4$) and 2.2 ± 0.3 times control in its absence ($n = 4$).

The effects of $1 \mu\text{M}$ PDBu and $30 \mu\text{M}$ GTP _{γ} S were observed on the contraction induced by $0.3 \mu\text{M}$ Ca^{2+} in the presence of either $0.1 \mu\text{M}$ PDBu or $30 \mu\text{M}$ GTP _{γ} S. PDBu ($1 \mu\text{M}$) did not modify the amplitude of Ca^{2+} -induced contraction in the presence of either $0.1 \mu\text{M}$ PDBu (1.03 ± 0.04 times control, $n = 3$) or $30 \mu\text{M}$ GTP _{γ} S (1.04 ± 0.05 times control, $n = 4$). While, GTP _{γ} S slightly enhanced the amplitude of Ca^{2+} -induced contraction in the presence of PDBu (1.27 ± 0.08 times control, $n = 4$). The maximum amplitude of contraction induced by $0.3 \mu\text{M}$ Ca^{2+} in the presence of $30 \mu\text{M}$ GTP _{γ} S (2.98 ± 0.42 times the amplitude of contraction induced by $0.3 \mu\text{M}$ Ca^{2+} alone, $n = 3$) was similar to that obtained in the presence of GTP _{γ} S with PDBu (2.78 ± 0.38 times the amplitude of contraction induced by $0.3 \mu\text{M}$ Ca^{2+} alone, $n = 4$).

Discussion

Myosin light chain (MLC) phosphorylation by the Ca^{2+} -calmodulin-MLC-kinase complex plays a pivotal role in the initiation of smooth muscle contraction (Hartshorne, 1987; Itoh *et al.*, 1989). In the last few years, many reports have indicated that agonists increase the sensitivity of the contractile proteins to Ca^{2+} in intact and skinned vascular smooth muscle (Morgan & Morgan, 1984; Nishimura *et al.*, 1988; Kitazawa *et al.*, 1989; Rembold, 1990; Hori *et al.*, 1992). Agonist-induced Ca^{2+} -sensitization of the contractile proteins requires GTP and is inhibited by GDP _{β} S in chemically skinned smooth muscle, suggesting that G-proteins may play a role in this sensitization (Nishimura *et al.*, 1988; Kitazawa *et*

Figure 4 Effects of PKC₁₉₋₃₆ on the relationship between relative force and myosin light chain (MLC) phosphorylation in the presence and absence of either GTP _{γ} S or PDBu in β -escin-skinned smooth muscle strips. (●) Ca^{2+} ($0.3 \mu\text{M}$) alone; (▲) in the presence of $10 \mu\text{M}$ GTP _{γ} S without (▲) or with (△) 0.1 mM PKC₁₉₋₃₆; (□) in $0.1 \mu\text{M}$ PDBu without (■) or with (□) 0.1 mM PKC₁₉₋₃₆. The data shown in Figure 3 were used for this analysis and the curve was obtained by fitting the data points by a non-linear least-squares method using the parameters obtained in Figure 2 ($r = 0.98$). The maximum amplitude of contraction induced by $10 \mu\text{M}$ Ca^{2+} was normalized as a relative force of 1.0 for each strip. Results shown are each the mean of 4 observations with s.d. For abbreviations, see text.

Figure 5 Effects of GDP β S and neomycin on contraction induced by 0.3 μ M Ca $^{2+}$ in the presence of GTP γ S in β -escin-skinned smooth muscle strips. After the muscle strip had been skinned by an application of 30 μ M β -escin for 25 min, 0.3 μ M Ca $^{2+}$ was applied. In (a) 10 μ M GTP γ S was then applied on the steady state of the contraction induced by 0.3 μ M Ca $^{2+}$. Finally, 10 μ M Ca $^{2+}$ was applied to obtain the maximum Ca $^{2+}$ -induced contraction for that strip. In (b) GDP β S (1 mM) was present for 8 min before application of 10 μ M GTP γ S in a solution containing 0.3 μ M Ca $^{2+}$. GTP γ S was then applied in a solution containing 0.3 μ M Ca $^{2+}$ with or without GDP β S. In (c) 0.1 mM neomycin was applied on the Ca $^{2+}$ -induced steady state contraction and 10 μ M GTP γ S then applied in the presence of 0.3 μ M Ca $^{2+}$ and neomycin. Each agent was applied as indicated by the bar. The results shown are typical traces and were reproducible in another 2 strips. For abbreviations, see text.

et al., 1989). It was recently hypothesized that, in smooth muscle, agonists acting together with GTP or GTP γ S inhibit MLC-phosphatases through the activation of unidentified G-proteins and thus enhance Ca $^{2+}$ -induced MLC-phosphorylation, causing an increase in the amplitude of the Ca $^{2+}$ -induced contraction (Kitazawa *et al.*, 1991; Kubota *et al.*, 1992). In the present experiments on β -escin-skinned smooth muscle, GTP γ S increased both the contraction and the MLC-phosphorylation induced by low concentrations of Ca $^{2+}$ (0.03–1 μ M), but not by the highest concentration of Ca $^{2+}$ (10 μ M), and this action of GTP γ S was attenuated by GDP β S. Since the relationship between force and MLC-phosphorylation was not modified by GTP γ S, these results suggest that GTP γ S activates G-proteins and consequently increases the MLC-phosphorylation that occurs in response to Ca $^{2+}$, thus causing the increase in the Ca $^{2+}$ -induced contraction in β -escin-skinned smooth muscle of the rabbit mesenteric artery.

It is known that agonists induce hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP $_2$) through activation of heterotrimeric G-proteins, leading to generation of inositol 1,4,5-trisphosphate (IP $_3$) and diacylglycerol (DAG). The former contributes to the agonist-induced Ca $^{2+}$ release from

the Ca $^{2+}$ -storage sites and the latter activates protein kinase C (PKC). For the following reasons it seems likely that GTP γ S activates PKC through the production of DAG and then increases the sensitivity of the contractile proteins to Ca $^{2+}$: (1) GTP γ S itself produces hydrolysis of PIP $_2$ through activation of heterotrimeric G-proteins in vascular smooth muscle (Sasaguri *et al.*, 1985). (2) Phorbol esters (TPA and PDBu), activators of PKC (Nishizuka, 1984), increase the contraction, MLC-phosphorylation and unloaded shortening velocity in the presence of 0.3 μ M Ca $^{2+}$ (Chatterjee & Tejada, 1986; Itoh *et al.*, 1986b; Fujiwara *et al.*, 1988). (3) PDBu shifts both the Ca $^{2+}$ -force and Ca $^{2+}$ -MLC-phosphorylation curves to the left in β -escin-skinned muscle strips (in the present experiments). In the present experiments, PKC $_{19-36}$ (a novel peptide inhibitor of PKC, House & Kemp, 1987) itself modified neither the contraction nor the MLC-phosphorylation induced by 0.3 μ M Ca $^{2+}$, but it completely inhibited the PDBu-induced enhancement of both the Ca $^{2+}$ -induced responses, suggesting that PKC $_{19-36}$ selectively inhibits PKC, but not MLC-kinase in β -escin-skinned smooth muscle. Under our experimental conditions, PKC $_{19-36}$ failed to modify the GTP γ S-induced enhancement of either the Ca $^{2+}$ -induced contraction or MLC-phosphorylation, suggesting that PKC may not play a primary role in the GTP γ S-induced enhancement of responses induced by Ca $^{2+}$ in β -escin-skinned smooth muscle of the rabbit mesenteric artery. This hypothesis is also supported by the finding that neomycin did not modify the GTP γ S-induced enhancement of the contraction induced by 0.3 μ M Ca $^{2+}$ in the skinned strips. Since neomycin interacts with polyphosphoinositides and inhibits the production of both IP $_3$ and DAG through its inhibitory action on phospholipase C (Cockcroft & Gomperts, 1985; Kobayashi *et al.*, 1989), this agent would be expected to inhibit the GTP γ S-action if PKC activated by the synthesized DAG played a role in the GTP γ S-induced response in the skinned muscles. Indeed, in preliminary experiments, we found that under the same experimental conditions, neomycin completely blocked the endothelin-1-induced increase in the sensitivity of the contractile proteins to Ca $^{2+}$. However, we cannot deny the possibility that our results with β -escin-skinned smooth muscle may underestimate the role of PKC in the GTP γ S-induced enhancement of the Ca $^{2+}$ -induced responses if some unidentified proteins that contribute to the action of GTP γ S leak out from skinned muscle cells.

GTP γ S slightly enhanced the contraction induced by 0.3 μ M Ca $^{2+}$ in the presence of PDBu, but PDBu did not modify the Ca $^{2+}$ -induced contraction in the presence of GTP γ S. The maximum amplitude of Ca $^{2+}$ -induced contraction in the presence of 30 μ M GTP γ S was similar to that in the presence of GTP γ S with PDBu, indicating that GTP γ S is a more potent stimulant than PDBu in enhancing the Ca $^{2+}$ -induced contraction and there is no possible additivity on the actions between PDBu and GTP γ S. These results suggest that PDBu and GTP γ S may act on common targets through activations of different signalling pathways.

There are two major superfamilies of G-proteins involved in signal transduction: one is a superfamily of heterotrimeric G-proteins acting as transducers for membrane receptors and the other is the superfamily including ras p21 and its related small G-proteins (Bourne *et al.*, 1990). It was recently suggested that small G-proteins, like rho p21 (Hirata *et al.*, 1992) and/or ras p21 (Pfitzer & Satoh, 1993), may contribute to the agonist-induced increase in the sensitivity of the contractile proteins to Ca $^{2+}$. Since neomycin did not modify the GTP γ S-induced enhancement of the Ca $^{2+}$ -induced contraction in β -escin-skinned muscles, the present results may also suggest that small G-proteins, rather than heterotrimeric G-proteins, contribute to the GTP γ S-induced increase in myofilament Ca $^{2+}$ -sensitivity in skinned smooth muscle.

Recently, Steusloff *et al.* (1993) reported that in α -toxin-skinned longitudinal smooth muscle of the guinea-pig ileum, carbachol together with GTP enhances the contraction induced by submaximal concentration of Ca $^{2+}$ with corresponding

increase in tyrosine phosphorylation of unidentified proteins. The result suggests that the other serine/threonine kinases, such as mitogen-activated protein (MAP) kinase, may also play a role on GTP_S-induced enhancement of Ca²⁺-induced contraction. This remains to be clarified.

The phosphorylation of heavy meromyosin by PKC does not stimulate actin-activated myosin MgATPase activity *in vitro*, but rather reduces the rate of phosphorylation of MLC by MLC-kinase causing a decrease in the actin-activated MgATPase activity of heavy meromyosin which has been pre-phosphorylated by MLC-kinase (Nishikawa *et al.*, 1984). In glycerin-treated porcine carotid artery, the phosphorylation of MLC by PKC neither provokes contraction nor modifies the contraction induced by Ca²⁺-calmodulin (Sutton & Haeblerle, 1990), suggesting that MLC-phosphorylation by PKC inhibits rather than enhances Ca²⁺-induced smooth muscle contraction. In the present experiments, PDBu enhanced both the Ca²⁺-induced contraction and MLC-phosphorylation without a change in the relationship between force and MLC-phosphorylation in β -escin-skinned muscle. Under the conditions of our experiments, Ca²⁺ (0.03–1 μ M) increased the monophosphorylated form of MLC in a concentration-dependent manner. Further, a peptide inhibitor of PKC, PKC_{19–36}, inhibited the PDBu-induced enhancement of both the Ca²⁺-induced contraction and MLC-phosphorylation without a change in their relationship. Furthermore, *in vitro* phosphorylation of MLC-kinase by PKC causes a

decrease in its affinity for the Ca²⁺-calmodulin complex (Ikebe *et al.*, 1985). Although we did not examine whether or not PKC directly phosphorylates MLC, all these results suggest that PKC may indirectly activate the phosphorylation of MLC by MLC-kinase, possibly through an inhibition of MLC-phosphatases.

It was recently hypothesized that PKC may increase Ca²⁺-induced contractions though a phosphorylation of caldesmon (Walsh, 1990) and/or calponin (Nakamura *et al.*, 1993) which are thin-filament-linked regulatory proteins in smooth muscle. However, we found that PDBu enhanced the Ca²⁺-induced contraction in parallel with an increase in the phosphorylation of MLC. Thus, the present result does not support the above hypothesis, although we cannot deny the possibility of a minor contribution of these thin-filament-linked regulator proteins to the PDBu-induced response.

In conclusion, GTP_S increases the phosphorylation of MLC induced by lower concentrations of Ca²⁺ through the activation of a PKC-independent mechanism and thus enhances the Ca²⁺-induced contraction.

We thank Dr R.J. Timms for the language editing. We thank Dr J.T. Stull (Univ. of Texas, Dallas, TX, U.S.A.) for providing an antibody against bovine tracheal myosin light chain. This work was partly supported by a Grant-In-Aid from the Ministry of Education of Japan.

References

- BOURNE, H.R., SANDERS, D.A. & MCCORMICK, F. (1990). The GTPase superfamily: a conserved switch for reverse cell functions. *Nature*, **348**, 125–132.
- CHATTERJEE, M. & TEJADA, M. (1986). Phorbol ester-induced contraction in chemically skinned vascular smooth muscle. *Am. J. Physiol.*, **251**, C356–C361.
- COCKCROFT, S. & GOMPERTS, B.D. (1985). Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature*, **314**, 534–536.
- EDELMAN, A.M., BLUMENTHAL, D.K. & KREBS, E.G. (1987). Protein serine/threonine kinase. *Annu. Rev. Biochem.*, **56**, 567–613.
- FUJIWARA, T., ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1988). Actions of a phorbol ester on factors regulating contraction in rabbit mesenteric artery. *Circ. Res.*, **63**, 893–902.
- FUJIWARA, T., ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1989). Effects of guanosine nucleotides on skinned smooth muscle tissue of the rabbit mesenteric artery. *J. Physiol.*, **408**, 535–547.
- GONG, M.C., FUGLSANG, A., ALESSI, D., KOBAYASHI, S., COHEN, P., SOMLYO, A.V. & SOMLYO, A.P. (1992). Arachidonic acid inhibits myosin light chain phosphatase and sensitizes smooth muscle to calcium. *J. Biol. Chem.*, **267**, 21492–21498.
- HARTSHORNE, D.J. (1987). Biochemistry of the contractile process in smooth muscle. In *Physiology of the Gastrointestinal Tract*, ed 2. vol. 1, ed. Johnson, L.R. pp. 423–482. New York: Raven Press.
- HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry*, **23**, 5036–5041.
- HIRATA, K., KIKUCHI, A., SASAKI, T., KURODA, S., KAIBUCHI, K., MATSUURA, Y., SEKI, H., SAIDA, K. & TAKAI, Y. (1992). Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J. Biol. Chem.*, **267**, 8719–8722.
- HORI, M., SATO, K., SAKATA, K., OZAKI, H., TAKANO-OHMURO, H., TSUCHIYA, T., SUGI, H., KATO, I. & KARAKI, H. (1992). Receptor agonists induce myosin phosphorylation-dependent and phosphorylation-independent contractions in vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **261**, 506–512.
- HOUSE, C. & KEMP, B.E. (1987). Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science*, **238**, 1726–1728.
- IKEBE, M., INAGAKI, M., KANAMARU, K. & HIDAKA, H. (1985). Phosphorylation of smooth muscle myosin light chain kinase by Ca²⁺-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.*, **260**, 4547–4550.
- ITO, T., IKEBE, M., KARGACIN, G.J., HARTSHORNE, D.J., KEMP, B.E. & FAY, F.S. (1989). Effects of modulators of myosin light-chain kinase activity in single smooth muscle cells. *Nature*, **338**, 164–167.
- ITO, T., KAJIKURI, J. & KURIYAMA, H. (1992). Characteristic features of noradrenaline-induced Ca²⁺ mobilization and tension in arterial smooth muscle of the rabbit. *J. Physiol.*, **457**, 297–314.
- ITO, T., KANMURA, Y. & KURIYAMA, H. (1986a). Inorganic phosphate regulates the contraction-relaxation cycle in skinned muscles of the rabbit mesenteric artery. *J. Physiol.*, **376**, 231–252.
- ITO, T., KANMURA, Y., KURIYAMA, H. & SUMIMOTO, K. (1986b). A phorbol ester has dual actions on the mechanical response in the rabbit mesenteric and porcine coronary arteries. *J. Physiol.*, **375**, 515–534.
- KITAZAWA, T., GAYLINN, B.D., DENNEY, G.H. & SOMLYO, A.P. (1991). G-protein-mediated Ca²⁺ sensitization of smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.*, **266**, 1708–1715.
- KITAZAWA, T., KOBAYASHI, S., HORIUTI, K., SOMLYO, A.V. & SOMLYO, A.P. (1989). Receptor-coupled, permeabilized smooth muscle: role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca²⁺. *J. Biol. Chem.*, **264**, 5339–5342.
- KOBAYASHI, S., KITAZAWA, T., SOMLYO, A.V. & SOMLYO, A.P. (1989). Cytosolic heparin inhibits muscarinic and α -adrenergic Ca²⁺ release in smooth muscle. *J. Biol. Chem.*, **264**, 17997–18004.
- KUBOTA, Y., NOMURA, M., KAMM, K.E., MUMBY, M.C. & STULL, J.T. (1992). GTP_S-dependent regulation of smooth muscle contractile elements. *Am. J. Physiol.*, **262**, C405–C410.
- MORGAN, J.P. & MORGAN, K.G. (1984). Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *J. Physiol.*, **351**, 155–167.
- NAKAMURA, F., MINO, T., YAMAMOTO, J., NAKA, M. & TANAKA, T. (1993). Identification of the regulatory site in smooth muscle calponin that is phosphorylated by protein kinase C. *J. Biol. Chem.*, **268**, 6194–6201.
- NISHIKAWA, M., SELLERS, J.R., ADELSTEIN, R.S. & HIDAKA, H. (1984). Protein kinase C modulated *in vitro* phosphorylation of the smooth muscle heavy meromyosin by myosin light chain kinase. *J. Biol. Chem.*, **259**, 8808–8814.

- NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTP- γ S increase myofilament Ca^{2+} sensitivity in α -toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.*, **157**, 677–683.
- NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693–698.
- PERSECHINI, A., KAMM, K.E. & STULL, J.T. (1986). Different phosphorylated forms of myosin in contracting tracheal smooth muscle. *J. Biol. Chem.*, **261**, 6293–6299.
- PFITZER, G. & SATOH, S. (1993). Modulation of calcium sensitivity of vascular smooth muscle myofilaments. *Pflügers Arch.*, **422**, Suppl. 1, R11.
- REMBOLD, C.M. (1990). Modulation of the $[\text{Ca}^{2+}]$ sensitivity of myosin phosphorylation in intact swine arterial smooth muscle. *J. Physiol.*, **429**, 77–94.
- SASAGURI, T., HIRATA, M. & KURIYAMA, H. (1985). Dependence on Ca^{2+} of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-triphosphate phosphatase in smooth muscles of the porcine coronary artery. *Biochem. J.*, **231**, 497–503.
- STEUSLOFF, A., PFITZER, G., KOLQUIST, K. & DI SALVO, J. (1993). Is tyrosine phosphorylation (TP) involved in the regulation of smooth muscle Ca^{2+} responsiveness? *Pflügers Arch.*, **422** (Suppl. 1), R80.
- SUTTON, T.A. & HAEBERLE, J.R. (1990). Phosphorylation by protein kinase C of the 20,000-dalton light chain of myosin in intact and chemically skinned vascular smooth muscle. *J. Biol. Chem.*, **265**, 2749–2754.
- WALSH, M.P. (1990). Smooth muscle caldesmon. In *Frontiers in Smooth Muscle Research*, ed. Sperelakis, N. & Wood, J.D. pp. 127–140. New York: Alan R. Liss, Inc.

(Received June 28, 1993
 Revised September 7, 1993
 Accepted September 23, 1993)