A Novel Mechanism for the Ca^{2+}-sensitizing Effect of Protein Kinase C on Vascular Smooth Muscle: Inhibition of Myosin Light Chain Phosphatase

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ABSTRACT Mechanisms of Ca^{2+} sensitization of both myosin light chain (MLC) phosphorylation and force development by protein kinase C (PKC) were studied in permeabilized tonic smooth muscle obtained from the rabbit femoral artery. For comparison, the Ca^{2+} sensitizing effect of guanosine 5'-O-(γ-thiotriphosphate) (GTPγS) was examined, which had been previously shown to inhibit MLC phosphatase in phasic vascular smooth muscle. We now report that PKC activators (phorbol esters, short chain synthetic diacylglycerols and a diacylglycerol kinase inhibitor) and GTPγS significantly increase both MLC phosphorylation and force development at constant [Ca^{2+}]. Major phosphorylation site occurring in the presence of phorbol-12,13-dibutyrate (PDBu) or GTPγS at constant [Ca^{2+}] is the same serine residue (Ser-19) as that phosphorylated by MLC kinase in response to increased Ca^{2+} concentrations. In an ATP- and Ca^{2+}-free solution containing 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), to avoid the kinase activity, both PDBu and GTPγS significantly decreased the rate of MLC dephosphorylation to half its control value. However, PDBu inhibited the relaxation rate more than did GTPγS. In the presence of microcystin-LR to inhibit the phosphatase activity, neither PDBu nor GTPγS affected MLC phosphorylation and force development. These results indicate that PKC, like activation of GTP binding protein, increases Ca^{2+} sensitivity of both MLC phosphorylation and force production through inhibition of MLC phosphatase.

INTRODUCTION

In smooth muscle, phosphorylation of the 20,000 D myosin light chain (MLC) leading to contraction is primarily regulated by cytoplasmic Ca^{2+}. A rise in Ca^{2+}

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increases the MLC kinase/phosphatase activity ratio by activation of the kinase (reviewed in Hartshorne, 1987, and Kamm and Stull, 1989). However, the Ca^{2+} sensitivity of MLC phosphorylation and force development is strongly modulated by physiological regulatory mechanisms (Morgan and Morgan, 1984; also see Kitazawa, Gaylinn, Denney, and Somlyo, 1991*a*, for references). In addition to Ca^{2+} release from the sarcoplasmic reticulum, α_1-adrenergic (Kitazawa et al., 1991*a*) and muscarinic agonists (Kitazawa and Somlyo, 1990) can increase both MLC phosphorylation and force at constant Ca^{2+} in *Staphylococcus aureus* α-toxin-permeabilized vascular and intestinal smooth muscle, respectively. These increases in MLC phosphorylation and force by agonists are mimicked by GTPγS and inhibited by GDPβS, indicating that G protein(s) couple excitatory receptors to a Ca^{2+}-sensitizing system (Nishimura, Kobler, and van Breemen, 1988; Fujiwara, Itoh, Kubota, and Kuriyama, 1989; Kitazawa, Kobayashi, Horiuti, Somlyo, and Somlyo, 1989). Recent studies on permeabilized phasic vascular smooth muscle further demonstrated that this Ca^{2+}-sensitizing action of agonist and GTPγS was mediated through inhibition of MLC phosphatase, thereby increasing the MLC kinase/phosphatase activity ratio (Kitazawa, Masuo, and Somlyo, 1991*b*). Similar inhibition of MLC phosphatase by GTPγS was also shown in tracheal homogenates (Kubota, Nomura, Kamm, Mumby, and Stull, 1992). The molecular mechanisms of signal transduction between G protein activation and phosphatase inhibition, however, remain to be identified.

G protein-coupled activation of phospholipase C results in production of two distinct transmitters, inositol -1,4,5-trisphophate and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (Berridge, 1987; Nishizuka, 1984). The former transmitter acts as a second messenger for fast (less than a second) Ca^{2+} release from the sarcoplasmic reticulum of smooth muscle, but by itself has no effect on Ca^{2+} sensitivity (Kitazawa and Somlyo, 1991). Therefore, the hypothesis that DAG activating PKC acts as a second messenger for the slow (several seconds) Ca^{2+} sensitization of the regulatory/contractile apparatus, seems attractive (Rasmussen, Takuwa, and Park, 1987; Jiang and Morgan, 1987; Nishimura and van Breemen, 1989). In fact, phorbol ester, a direct activator of PKC, initiates slowly developing and sustained contractions (Rasmussen, Forder, Kojima, and Scriabine, 1984; Itoh, Kabota, and Kuriyama, 1988; Rembold and Murphy, 1988; Ruzycky and Morgan, 1989; Ozaki, Ohyama, Sato, and Karaki, 1990; Singer, 1990*a*), but their relationship to intracellular Ca^{2+} concentration in intact smooth muscles is controversial. Phorbol esters likewise increase the contractile Ca^{2+} sensitivity of permeabilized preparations (Chatterjee and Tejada, 1986; Itoh, Kubota, and Kuriyama, 1988; Miller, Hawkins, and Wells, 1986; Fujiwara, Itoh, Kubota, and Kuriyama, 1988; Nishimura and van Breemen, 1989). PKC phosphorylates MLC at Ser-1 and -2 and Thr-9 distinct from MLC kinase sites (Ser-19 and Thr-18) (Ikebe, Hartshorne, and Elzinga, 1987*a;* Bengur, Robinson, Apella, and Sellers, 1987). Phosphorylation of PKC-sites, however, inhibits or has no effect on either actin-activated myosin ATPase (Nishikawa, Hidaka, and Adelstein, 1983; Bengur et al., 1987; Ikebe et al., 1987*a*) or on contraction of glycerol-permeabilized smooth muscle strips (Sutton and Haeberle, 1990). Recent quantitative measurements also have revealed that agonist stimulation induces phosphorylation of MLC at MLC kinase-specific, but not at PKC-specific sites (Singer, Oren, and Benscoter, 1989; Kamm, Hsu, Kubota, and Stull, 1989). These

observations strongly suggest that additional mechanism(s) other than direct phosphorylation of MLC by PKC must be operative in situ through which PKC activators can increase the sensitivity of the regulatory/contractile apparatus to Ca^{2+}.

The purpose of the present study was (*a*) to examine whether a variety of PKC activators can increase both MLC phosphorylation and force at a given Ca^{2+} concentration as has been seen with GTPγS and agonists in *Staphylococcus aureus* α-toxin permeabilized smooth muscle (Kitazawa et al., 1991*a*); and, if so, (*b*) to identify using phosphopeptide analysis, whether MLC kinase or PKC mainly phosphorylates MLC during the Ca^{2+} sensitization by PKC activation; and (*c*) to determine whether the mechanism involved in Ca^{2+} sensitization of MLC phosphorylation by PKC activation is inhibition of MLC dephosphorylation (in a manner similar to the action of GTPγS and agonists), or activation of MLC phosphorylation (analogous to the action of Ca^{2+}), or both. We now document a novel mechanism for the Ca^{2+} sensitizing action of PKC through inhibition of MLC dephosphorylation in the signal transduction systems. A preliminary report of some of these findings has been already presented (Kitazawa, Reardon, Ikebe, and Masuo, 1993).

MATERIALS AND METHODS

Methods

Tissue preparation and force measurement. The femoral arteries were dissected from male albino rabbits (2–3 kg). The arterial strips were carefully freed from connective tissue and their endothelium was removed by rubbing with a razor blade. The smooth muscle strips 50–60 μm thick, 500–600 μm wide and 2.5–3 mm long were used for measurements of both force and phosphorylation. The strips were then tied with silk monofilament to the fine tips of two tungsten needles, one of which was connected to a force transducer (AM801, SensoNor, Horten, Norway), and mounted in a well on bubble plate (Horiuti, 1988) to allow either moderately rapid (within a second) solution exchange or freezing, as described (Kitazawa et al., 1991*a*). All experiments were carried out at 20°C.

Solutions. The normal relaxing solution contained (in millimolar): potassium methanesulfonate, 74.1; Mg^{2+}, 2; MgATP, 4.5; EGTA, 1; creatine phosphate, 10; piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 30, neutralized to pH 7.1 with KOH at 20°C. In the activating solution, 10 mM EGTA was used and a specified amount of calcium methanesulfonate was added to give a desired concentration of free Ca^{2+} ions (Horiuti, 1988). Ionic strength was kept constant at 0.2 M by adjusting the concentration of potassium methanesulfonate.

Cell permeabilizations. We used three distinct cell-permeabilization techniques depending on the purpose of each experiment; α-toxin, β-escin, and saponin. α-toxin produces 2–3-nm diam pores in the plasma membrane that permit the passage of only low molecular weight solutes (e.g., Ca^{2+}, ATP, GTP, InsP3, phorbol esters), so that soluble cytosolic proteins such as calmodulin, kinases and phosphatases are retained in the cell at their physiological concentrations (Kitazawa et al., 1989). Because receptor/G protein-coupled as well as Ca^{2+}-activated responses are well preserved (Kitazawa et al., 1989), most of the studies were performed using this preparation. Microcystin-LR (M_r = 995) applied to the extracellular space to inhibit MLC phosphatase, however, does not readily penetrate into α-toxin permeabilized cells. β-escin, on the other hand, can make cell membranes permeable to much higher molecular weight solutes such as microcystin-LR and calmodulin. These β-escin-permeabilized smooth muscles also retain coupled receptors (Kobayashi, Kitizawa, Somlyo, and Somlyo, 1989). However, the "run-down" of contractions is somewhat greater than that of α-toxin permeabilized prepara-

tions. Finally, to examine the direct effects of guanine nucleotides and drugs on the regulatory/contractile apparatus of smooth muscle, we used receptor/G protein-uncoupled preparations permeabilized with high concentrations of saponin (Kitazawa et al., 1991*b*).

After measuring contractions induced by high K^+ (154 mM) and by phenylephrine (100 μM), the strips were incubated at room temperature (20–23°C) in the relaxing solution for several minutes. For permeabilization with α-toxin, the strips were treated for 25–35 min with 5,000 U/ml of purified *Staphylococcus aureus* α-toxin at pCa 6.7, buffered with 10 mM EGTA, at 30°C. To maintain cytoplasmic Ca^{2+} constant, the sarcoplasmic reticulum was depleted of calcium by further treating each strip with 10 μM A23187, a Ca^{2+}-ionophore for 20–25 min in the relaxing solution.

To determine the time course of MLC phosphorylation in the presence of microcystin-LR, smooth muscle strips were incubated in normal relaxing solution for several minutes and treated for 45 min with 40 μM β-escin at 5°C. The temperature was subsequently increased to 30°C, and 10 μM A23187 was added. Permeabilization was continued for a total of 60 min. The initial treatment with β-escin at a low temperature did not significantly permeabilize the membrane, but it was required to obtain subsequent homogeneous permeabilization at a higher temperature (30°C) over a short period, suggesting slow penetration and/or binding of β-escin to the surface membrane of the strips.

In some experiments, muscles were extensively permeabilized with 200 μg/ml saponin at 25°C for 20 min in relaxing solution to which was added 10 μM A23187, 1 μM calmodulin, 100 μM leupeptin and 5 mM dithiothreitol.

Two-dimensional gel electrophoresis. For measuring MLC phosphorylation, permeabilized preparations were rapidly frozen with liquid N_2-cooled chlorodifluoromethane (Freon 22; Atochem, Philadelphia, PA) at intervals indicated in the figures. Force was monitored throughout the experiment. MLC was separated by two-dimensional isoelectric focusing/SDS gel electrophoresis and stained with colloidal gold (Kitazawa et al., 1991*a*). The percentage of MLC phosphorylation was calculated by dividing the density of the mono- and diphosphorylated spots by the combined density of un-, mono-, and diphosphorylated spots. The amount of the di-phosphorylated smooth muscle MLC was compensated by subtracting the average value (5.6% of the total MLC) of unphosphorylated nonmuscle MLC that comigrated with diphosphorylated smooth muscle MLC (Kitazawa et al., 1991*a*).

Phosphopeptide analysis. Arterial smooth muscle strips permeabilized with α-toxin were incubated in the ATP-free, 10 mM EGTA-containing solution at 25°C for 30 min. After washing with additional EGTA- and ATP-free solution, the strips were incubated in a solution containing 4 mM [γ-^{32}P]ATP and the appropriate Ca^{2+} concentration buffered with 10 mM EGTA in the presence of 4 μM PDBu or 100 μM GTPγS or in the absence of both for 15 min at 20°C. The specific radioactivity of [γ-^{32}P] ATP was 10^6 cpm/nmol. The reaction was stopped by the addition of trichloroacetic acid to a 5% final concentration. The muscle strips were then dissolved in a solution containing 4% SDS, 0.5 M $NaHCO_3$ and 1% β-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. The gel was in contact with Kodak X-Omat film overnight and the 20,000 D MLC band was excised from the gel.

The excised gel was cut into small pieces which were washed three times with 25% propanol and then three additional times with 10% methanol. The gels were lyophilized and rehydrated with 9 M urea solution containing 50 mM Tris-HCl (pH 8.0) and incubated at 37°C for 1 h. The urea concentration was decreased to 2 M with H_2O and 0.1 mg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma Chemical Co., St. Louis, MO) was added. After 3 h, an additional 0.1 mg/ml of TPCK-trypsin was added and the mixture was incubated for ~12 h at 37°C. The gel was removed and the extracted peptide mixture had its pH adjusted to 2.2 and was then subjected to C18 reverse-phase HPLC column chromatography (Brownlee Spheri5 ODS column). The peptides were eluted using a linear gradient to 5%

CH_3CN at 30 min and to 100% CH_3CN at 60 min in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The radioactivity of each fraction was monitored by a Beckman LS5000 scintillation counter using Cerenkov counting. The recovery of ^{32}P-labeled peptides from the gel slices ranged between 30–50%.

Phospho-amino acid analysis was done according to the methods of Ikebe and Hartshorne (1985).

As a control, isolated MLC was phosphorylated either by MLC kinase or PKC and treated as described above. MLC kinase was prepared from turkey gizzard (Ikebe, Stepinska, Kemp, Means, and Hartshorne, 1987*b*) and PKC from bovine brain (Ikebe and Reardon, 1990).

Statistical significance was evaluated by an unpaired two-tailed t test. A value of $P < 0.05$ was taken as significant. All values are expressed in mean ± SE for 4–12 experiments.

Chemicals

α-toxin, PDBu, 4α- and 4β-PDBu, and 4α- and 4β-PMA were from Gibco BRL (Gaithersburg, MD); β-escin, microcystin-LR, diC_6, diC_8, 1,3-diC_8 and hexokinase from Sigma Chemical Co.; DAG kinase inhibitor (R59022), GTPγS, and GDPβS from Boehringer Mannheim Corp. (Indianapolis, IN); ML-9 and A23187 from Calbiochem Corp. (San Diego, CA). All other chemicals were of reagent grade purity.

RESULTS

The Effect of Phorbol Ester on Contraction in Intact Rabbit Femoral Artery Smooth Muscle

To compare the phorbol ester-induced responses of our preparations with previous reports, we performed measurements of contractile responses on intact rabbit femoral artery smooth muscle strips. Under resting conditions, 0.3 μM PDBu produced a contraction equal to 49 ± 12% of α_1-agonist (100 μM phenylephrine)-induced contraction with a half time (time required to reach 50% of maximum force induced by the given stimulant) of 5.0 ± 0.7 min. Stimulation by high (154 mM) K^+ induced a phasic contraction which developed rapidly and returned to a sustained lower level (24 ± 8% of phenylephrine-induced contraction) after peak. During the sustained contractions induced by high K^+, PDBu more rapidly (half-time of 2.9 ± 0.1 min) evoked a larger contraction (106 ± 6% of phenylephrine-induced contraction) than those evoked by PDBu in resting state. In the absence of Ca^{2+}, PDBu-induced contractions were significantly smaller (28 ± 5% of phenylephrine-induced contraction in the presence of Ca^{2+}) and slower (6.3 ± 0.3 min) even in high K^+ solutions than those occurring in the presence of Ca^{2+}. These results suggest that the rapidity and amplitude of phorbol ester-induced contractions in unpermeabilized smooth muscle are dependent upon the level of cytoplasmic Ca^{2+}.

The Ca^{2+}-sensitizing Effect of Various PKC Activators on Contraction in α-Toxin Permeabilized Arterial Smooth Muscle

Fig. 1 *A shows a representative tracing of the G protein-dependent contractile* response of α-toxin permeabilized, A23187-treated femoral artery smooth muscle. An α1-agonist (100 μM phenylephrine) in the presence of 10 μM GTP moderately enhanced contractile response in the presence of submaximal Ca^{2+} (pCa 7 buffered with 10 mM EGTA). GDPβS (1 mM) reversibly inhibited agonist-induced enhance-

ment of contraction at constant Ca^{2+}, indicating G protein-mediated regulation of Ca^{2+} sensitivity of arterial smooth muscle contraction, as previously described (Kitazawa et al., 1989, 1991*a*). At pCa 7, a saturating concentration of nonhydrolyzable GTP analog, GTPγS (100 μM) increased the contractile force from 4 ± 0.9% to 64 ± 1.8% of the maximum force obtained at pCa 5.

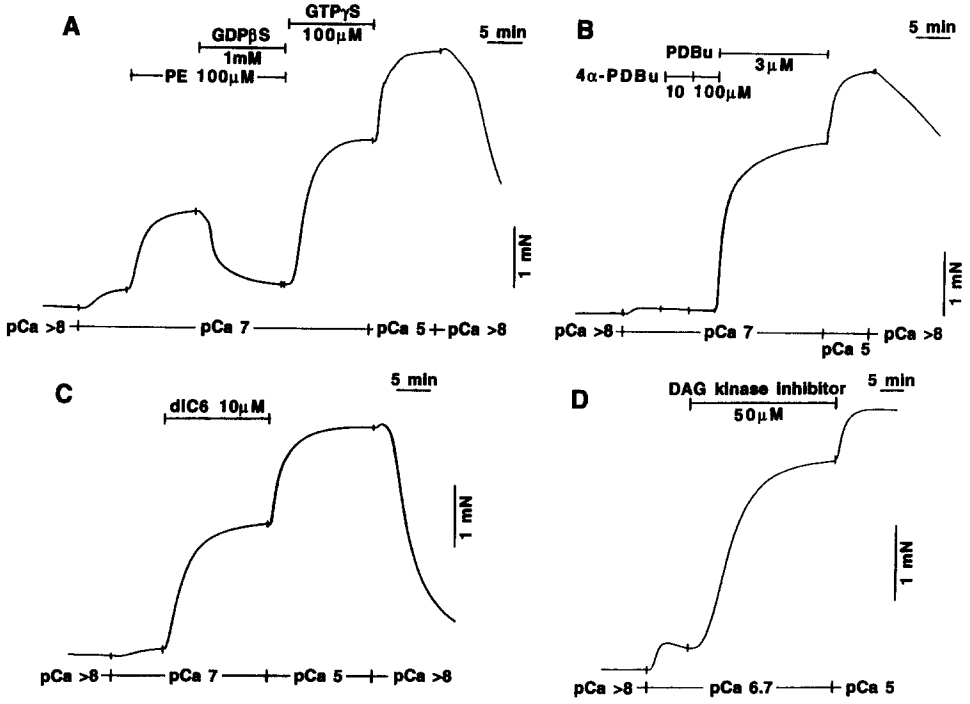

FIGURE 1. Potentiating effect of various PKC activators on contraction activated by submaximal Ca^{2+} in α-toxin-permeabilized rabbit femoral artery smooth muscle in comparison with those of agonist and GTPγS. Ca^{2+} release from intracellular stores was eliminated by treatment of permeabilized smooth muscle with 10 μM A23187. The muscles were incubated in Ca^{2+}-free (pCa > 8), 1 mM EGTA-containing solution, followed by increase in Ca^{2+} to pCa 7 and 5 with 10 mM EGTA-containing solutions. (*A*) 100 μM α_1-agonist, phenylephrine (PE)-potentiated contraction at constant Ca^{2+} (pCa 7) was inhibited by 1 mM GDPβS. All solutions contained 10 μM GTP. The nonhydrolyzable GTP analog, GTPγS evoked much larger contraction than agonists. pCa 5 evoked maximal contraction that was not affected by GTPγS and PDBu. (*B*) Potentiation of contraction at constant Ca^{2+} by 3 μM PDBu, but not by even 100 μM 4α-PDBu, an inactive isomer. (*C* and *D*) Potentiation of contraction by 10 μM diC_6 at pCa 7 (*C*) and 50 μM DAG kinase inhibitor, R59022 at pCa 6.7 (*D*).

The effects of different classes of PKC activators were next examined in these preparations. These included two classes of direct activators: (*a*) phorbol esters such as 4β-phorbol 12,13-dibutyrate (PDBu) and 4β-phorbol 12-myristate 13-acetate (PMA); and (*b*) synthetic short chain 1,2-*sn*-diacylglycerols such as 1,2-*sn*-dihexanoylglycerol (diC_6) and 1,2-*sn*-dioctanoylglycerol (diC_8) (Lapetina, Reep, Ganong, and

Bell, 1985). In addition, R59022, an inhibitor of DAG kinase which can inhibit the metabolism of 1,2-DAG to phosphatidic acid so as to lead to an accumulation of DAG (de Chaffoy de Courcelles, Roevens, and Van Belle, 1985), was examined.

As shown in Fig. 1 *B*, PDBu was capable of markedly enhancing the contraction of α-toxin permeabilized femoral artery smooth muscle under Ca^{2+}-clamped conditions (pCa 7) at 20°C in a manner similar to that produced by GTPγS (Fig. 1 *A*). The maximum stimulation of force development by PDBu (from 2 ± 0.4% to 61 ± 0.9% of maximum force obtained at pCa 5) was seen at a level of 0.1 μM, and increasing the concentrations of this activator to between 0.3 and 3 μM had no further increase in amplitude. The EC_{50} of PDBu on amplitude of contraction at pCa 7 was 10 nM. The half-time of force development by PDBu even at concentrations ranging between 0.1 and 3 μM was, however, dose-dependently decreased; 10.9 ± 0.6 min at 0.1 μM, 6.2 ± 0.4 min at 0.3 μM and 4.1 ± 0.1 min at 3 μM. An increase in Ca^{2+} to pCa 6.5 significantly decreased the half-time of 0.3 μM PDBu-induced force development to 2.5 ± 0.4 min. PMA, on the other hand, was found to induce only very slow potentiation of the contractile force (half time of 1.5 ± 0.2 h) even at 50 μM although the steady state force levels could reach close to that observed with PDBu. Similar slow responses to PMA have also been observed with saponin-permeabilized, much thinner vascular smooth muscle preparations (Itoh, Kanmura, Kuriyama, and Sumimoto, 1986). The phorbol ester isomers, which have no activating effect on PKC (Castagna, Takai, Kaibuchi, Sano, Kikkawa, and Nishizuka, 1982), such as 4α-PDBu (or 4α-PMA) produced no potentiating effects on the contractile properties at concentrations up to 100 μM at either a pCa of 7 or 5 (Fig. 1 *B*).

The response of the α-toxin permeabilized muscle to 10 μM diC_6, a synthetic DAG is shown in Fig. 1 *C*. At pCa 7, the contractile force was enhanced from 2 to 50 ± 2.5% of maximum with a half time of 3.6 ± 0.3 min. The EC_{50} of diC_6 on amplitude of contraction at pCa 7 was 1 μM. In the case of diC_8, similar stimulation of the contractile force (49 ± 2.4%) was observed at pCa 7 over the concentration range of 30–100 μM. However, the half time of 7.0 ± 0.4 min for diC_8-induced contraction at pCa 7 was significantly longer than that in the presence of diC_6. This may be attributable to a slower penetration into the muscle because diC_8 has a longer chain length. A slight but significant increase in force from 2 to 13% at pCa 7 was also observed with the inactive stereoisomer of diC_8, 1,3-dioctanoylglycerol (100 μM) (Boni and Rando, 1985), but this is most likely due to a small degree of contamination by the active 1,2-isomer.

Because 1,2-DAGs can be either phosphorylated by DAG kinase to phosphatidic acid or hydrolyzed by DAG lipase, the effect of DAG kinase inhibitor, R59022, on these preparations was also examined. R59022 (50 μM) at pCa 7 increased steady state force from 2 to 35 ± 3.7% of maximum force at pCa 5. An increase in Ca^{2+} to pCa 6.7 resulted in a much greater potentiation of contraction from 7 ± 1.0% to 62 ± 3.2% with a half time of 8.7 ± 0.3 min (Fig. 1 *D*). These results were consistent with the increased accumulation of DAG in smooth muscle.

PKC activators also markedly shifted the Ca^{2+} sensitivity of the regulatory/contractile apparatus in α-toxin-permeabilized femoral artery smooth muscle (Fig. 2; also see Nishimura and van Breemen, 1989). The steady state pCa-force relationship was obtained by cumulatively increasing Ca^{2+} concentrations. PDBu (3 μM) and diC_6

(30 μM) increased the Ca^{2+} sensitivity (defined as a reciprocal of the concentration of free Ca^{2+} required for 50% of maximum contraction) by five- and threefold, respectively, and evoked a significant force (5–10% of the maximum force in either case) even at pCa >8 (10 mM EGTA and no added calcium). At pCa 5, neither PDBu nor diC_6 affected the maximum force (Fig. 2) for at least 60 min. This observation was similar to that with GTPγS which shifted the Ca^{2+}-force relationship, but did not increase the maximum force in tonic smooth muscle (Kitazawa et al., 1991*a*).

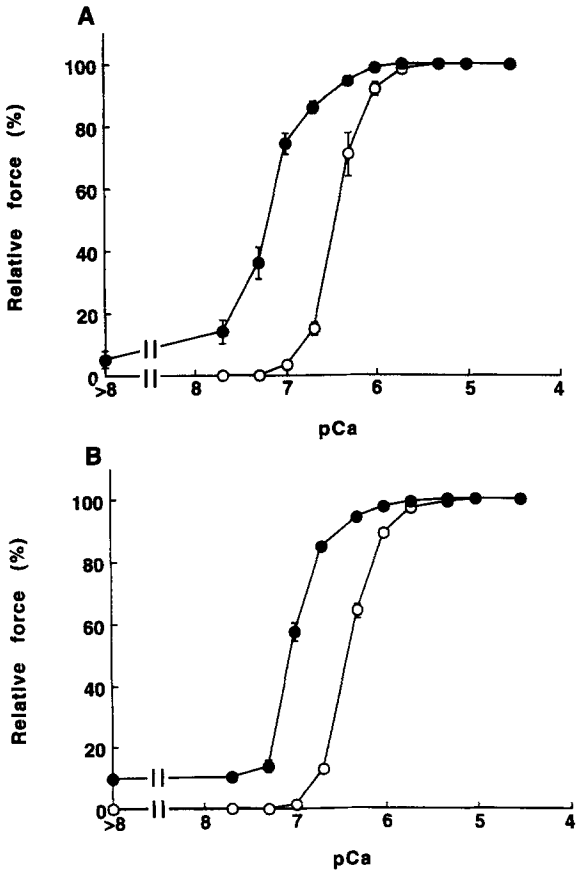

FIGURE 2. Increase in Ca^{2+} sensitivity of the regulatory/contractile apparatus of α-toxin-permeabilized smooth muscle by 3 μM PDBu (*A*) and 30 μM diC_6 (*B*) at pH 7.1 and 20°C. The steady state Ca^{2+}-force relationship was obtained by cumulatively increasing Ca^{2+} in the presence (*solid circle*) or in the absence (*open circle*) of saturating concentrations of either PDBu or diC_6. Force was normalized to the force produced at pCa 5. Each value is mean ± SE of seven experiments.

To examine whether or not the observed Ca^{2+} sensitization by PKC activators is a direct effect on the regulatory/contractile apparatus in smooth muscle, saponin (0.2 mg/ml)-permeabilized smooth muscle preparations were used because in phasic smooth muscle such preparations exhibit normal Ca^{2+} dependence but show little or no receptor/G protein-coupled responses (Kitazawa et al., 1991*b*). Addition of a saturating concentration of PDBu (3 μM) and of GTPγS (100 μM) at pCa 6.7 resulted in a negligible ($P > 0.1$) increase in force from 3 ± 0.6% to 5 ± 0.9% and to 6 ± 0.9% of maximum at pCa 5, respectively. 1 μM calmodulin was added to these preparations because the endogenous calmodulin can diffuse out during and

following saponin permeabilization. On the other hand, microcystin-LR (10 μM), a direct inhibitor of protein phosphatase, rapidly increased force at pCa6.7 to 96 ± 1.5% of maximum, indicating the presence of active MLC kinase and phosphatase in this preparation. This result suggests that phorbol ester-induced Ca^{2+} sensitization, like that of GTPγS, either requires membrane-associated and/or soluble protein(s), or that vigorous permeabilization with saponin inactivates the pathway activated by phorbol ester in the receptor/G protein-coupled preparations.

The Effects of PKC Activators and GTPγS on MLC Phosphorylation

Table I shows MLC phosphorylation in the presence and absence of GTPγS and various PKC activators at saturating concentrations. To measure MLC phosphorylation, strips were rapidly frozen at the incubation time required for ~80% of the maximum potentiation of force after each activator was applied. GTPγS markedly increased MLC phosphorylation at pCa 7 (Table I) with increased force development

TABLE I

Effect of GTPγS and Various Protein Kinase C Activators on MLC Phosphorylation in α-toxin Permeabilized Rabbit Femoral Artery at 20°C

pCa, substance, incubation time	Control	+ Activators	*P*
	Percent of total MLC ± SEM (n)		
pCa 7, 100 μM GTPγS, 5 min	23 ± 2.6 (5)	56 ± 3.4 (4)	<0.01
pCa 7, 3 μM PDBu, 5 min	23 ± 2.6 (5)	49 ± 4.4 (5)	<0.01
pCa 7, 100 μM 4α-PDBu, 5 min	23 ± 2.6 (5)	17 ± 5.7 (4)	NS*
pCa 7, 0.1 μM PDBu, 25 min	15 ± 2.1 (5)	31 ± 1.0 (5)	<0.01
pCa 7, 50 μM PMA, 150 min	16 ± 2.0 (3)	32 ± 1.5 (4)	<0.01
pCa 7, 10 μM diC_6, 5 min	23 ± 2.6 (5)	35 ± 4.4 (6)	<0.05
pCa 6.7, 50 μM DAGKI‡, 15 min	25 ± 4.6 (5)	50 ± 3.4 (6)	<0.01

*NS; not significant. ‡DAGKI; DAG kinase inhibitor (R59022).

MLC phosphorylation was assayed with two-dimensional isoelectric focusing/SDS gel electrophoresis (Kitazawa et al., 1991*a*). Values are expressed as a percent of the total of phosphorylated plus unphosphorylated MLC. PKC activators were added after 10-min incubation in pCa 7- or pCa 6.7- containing solution. Unpaired *t* test compared MLC phosphorylation in the presence of GTPγS or PKC activators with controls.

(Fig. 1). All PKC activators significantly increased MLC phosphorylation under Ca^{2+}-clamped conditions. PDBu (3 μM) at pCa 7 markedly increased MLC phosphorylation to a level similar to that induced by GTPγS at pCa 7 (Table I) or by pCa 6.3 alone (57 ± 2.5%). 4α-PDBu at 100 μM, which is inactive toward PKC, increased neither MLC phosphorylation (Table I) nor force development. It should be noted, however, that 0.1 μM PDBu or 50 μM PMA significantly increased MLC phosphorylation, but the phosphorylation level was significantly lower than that in the presence of 3 μM PDBu or 100 μM GTPγS. As mentioned above, 0.1 μM PDBu or 50 μM PMA increased force to the same steady state level as those of 3 μM PDBu or 100 μM GTPγS, but the rate of force development with the former was much slower than that with the latter.

PDBu (3 μM) at pCa 7 significantly increased not only monophosphorylation of MLC (from 23 ± 2.6% for the control to 44 ± 4.2%) but also diphosphorylation from 0 ± 0.1 to 5 ± 1.9%. The total for both phosphorylations in the presence of PDBu

was 49 ± 4.4% (Table I). However, the level of increased diphosphorylation was not significantly ($P > 0.1$) different from the values obtained at pCa 6.3 alone (4 ± 1.3%) or in the presence of GTPγS at pCa 7 (8 ± 1.9%). At maximum Ca^{2+} (pCa 5), however, PDBu did not significantly increase either mono- (from 77 ± 4.5 to 78 ± 1.7% of total MLC) or diphosphorylation of MLC (from 13 ± 2.4 to 18 ± 0.9% of total MLC).

To determine whether or not phorbol ester-induced increases in MLC phosphorylation corresponding to force development are responses unique to α-toxin perme-

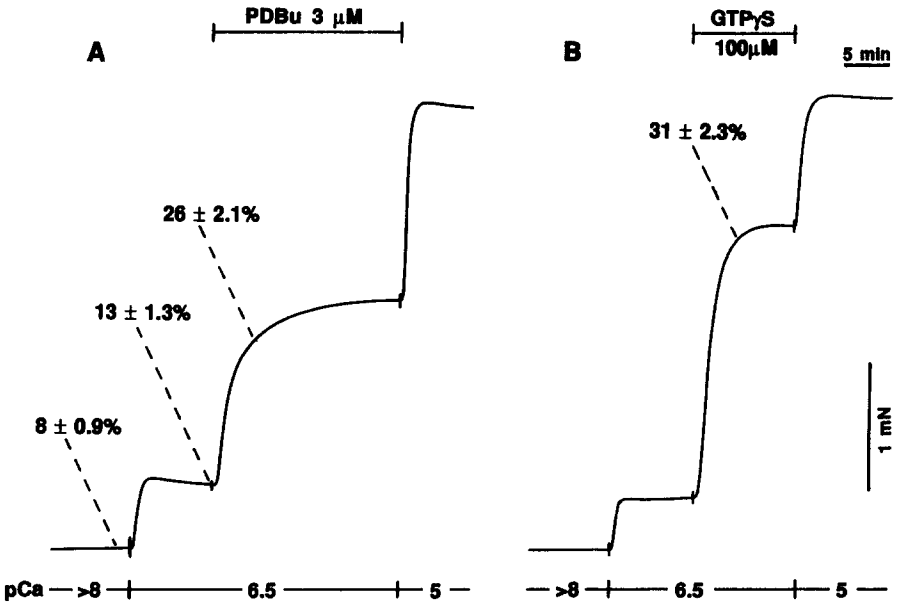

FIGURE 3. PDBu- (*A*) and GTPγS-induced (*B*) increase in MLC phosphorylation and force at constant Ca^{2+} in β-escin-permeabilized smooth muscle. Femoral artery smooth muscle strips were permeabilized with β-escin as described in the Methods and incubated in the relaxing solution (pCa > 8) followed by stimulation with submaximal Ca^{2+} (pCa 6.5) in the absence of calmodulin for 10 min. PDBu (3 μM) or GTPγS (100 μM) was added in the pCa 6.5-containing solution. Strips were last stimulated with pCa 5 in the absence of stimulants to obtain a maximum force. Values inserted in the figure are the average phosphorylation (percent of total MLC ± SE of 4–5 experiments) at each indicated point.

abilized preparations, we used β-escin permeabilized smooth muscle preparations that also retain receptor/G protein responses (Kobayashi et al., 1989). At 5 min after stimulation at pCa 6.5 in the absence of added calmodulin, PDBu (3 μM) and GTPγS (100 μM) significantly ($P < 0.01$) increased the amount of phosphorylated MLC from 13 ± 1.3% to 26 ± 2.1% and to 31 ± 2.3% of total MLC, respectively (Fig. 3). Although levels of MLC phosphorylation under these conditions were lower in β-escin permeabilized preparations than those in α-toxin permeabilized prepara-

tions, the percent increase in the phosphorylation by PDBu or GTPγS as compared with control values was similar in both preparations.

Analysis of the Phosphopeptides of MLC

It is known that smooth muscle MLC can be phosphorylated at various sites in vitro by MLC kinase and PKC (Ikebe, Hartshorne, and Elzinga, 1986, 1987*a*; Bengur et al., 1987). Phosphopeptide analyses were performed to identify the sites of phosphorylation increased by PDBu or GTPγS at constant Ca^{2+}. The α-toxin permeabi-

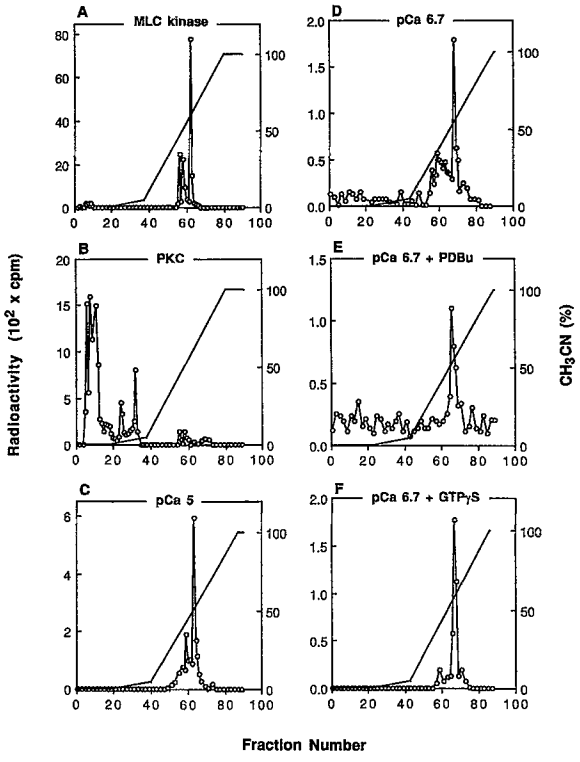

FIGURE 4. Reverse phase chromatography of phosphopeptides of MLC. Trypsin-digested, ^{32}P-labeled phosphopeptides were applied to C18 reverse phase column and eluted with CH_3CN gradients (—) as described in the Materials and Methods. The radioactivity of each fraction was detected by Cerenkov counting (—○—). (*A*) chromatography of radioactive fractions from isolated MLC phosphorylated by turkey gizzard MLC kinase; (*B*) from isolated MLC phosphorylated by bovine brain PKC; (*C*) from MLC of α-toxin permeabilized smooth muscle strips activated by pCa 5 alone; (*D*) from MLC of permeabilized strips activated by pCa 6.7 alone; (*E*) from MLC of permeabilized strips activated by pCa 6.7 and 4 μM PDBu; (*F*) from MLC of permeabilized strips activated by pCa 6.7 and 100 μM GTPγS.

lized strips were incubated for 15 min in solutions containing 4 mM total [γ-^{32}P]ATP at various Ca^{2+} concentrations under essentially the same conditions used for force measurements except creatine phosphate. MLC, isolated by SDS-polyacrylamide gel electrophoresis, was completely digested by trypsin and subjected to phosphopeptide analysis using C18 reverse phase column (see Materials and Methods). As a control, isolated MLC was phosphorylated either by MLC kinase (to 1.2 mol P/mol MLC) or by PKC (to 1.4 mol P/mol MLC) and also subjected to phosphopeptide analysis (Fig. 4, *A* and *B*). The peptides phosphorylated by MLC kinase (at Ser-19 alone or at both

Ser-19 and Thr-18; see Ikebe et al., 1986) were eluted at ~45–55% of CH_3CN (Fig. 4 *A*), while the peptides containing PKC-induced phosphorylation (Fig. 4 *B*) were eluted much earlier in the acetonitrile gradient. One was eluted in the flow-through fraction and the other at 2–4% CH_3CN. Using the methods of Ikebe et al. (1987*a*), it was confirmed that the flow-through fraction of an acetonitrile gradient contained phosphothreonine while the fraction eluted at 2–4% acetonitrile contained phosphoserine as determined by phospho-amino acid analysis. Phosphorylation sites of MLC from permeabilized strips activated by Ca^{2+} alone were the same as those of MLC phosphorylated by MLC kinase (Fig. 4, *C* and *D*). Neither PDBu (Fig. 4 *E*) nor GTPγS (Fig. 4 *F*) at pCa 6.7 significantly induced phosphorylation at PKC sites, and only the peptides phosphorylated by MLC kinase (see Fig. 4 *A*) were detected. It should be noted that some of the phosphorylated MLC was lost during the gel washing steps and the total counts that appeared in the C18 reverse phase chromatography do not represent the original total ^{32}P incorporation. Phospho-amino acid analyses of the peptides of MLC from permeabilized strips revealed that the predominant phosphorylation was on the serine residue although minor phosphorylation (~10%) on the threonine site was also observed.

The Effect of Phorbol Ester on the Time Course of MLC Phosphorylation and Force Development

An increase in both MLC phosphorylation and force induced by phorbol esters may result from either stimulation of MLC kinase and/or inhibition of MLC phosphatase. In a previous study (Kitazawa et al., 1991*b*), adenosine 5′-[γ-thio]triphosphate (ATPγS) was used as a substrate for MLC kinase to determine the effect of GTPγS on the time course of MLC thiophosphorylation, because MLC thiophosphorylation cannot be removed by MLC phosphatase (Cassidy, Hoar, and Kerrick, 1979). In the present study, however, ATPγS was not used because PKC does not utilize it as a substrate (unpublished observation; Parente, Walsh, Kerrick, and Hoar, 1992). Instead, microcystin-LR, a potent and specific phosphatase inhibitor (MacKintosh, Beattie, Klumpp, Cohen, and Codd, 1990) was used to block MLC dephosphorylation and measure the rate of MLC phosphorylation. Effect of PDBu on the rate of MLC phosphorylation was measured in the presence of ATP and a saturating concentration (10 μM) of microcystin-LR. This phosphatase inhibitor rapidly increased force development maximally in saponin-permeabilized arterial smooth muscle (also see Kitazawa et al., 1991*b*), but it did not affect a submaximally Ca^{2+}-activated contraction of α-toxin-permeabilized preparations. This indicates that the inhibitor does not penetrate the latter preparations. Okadaic acid, another well known phosphatase inhibitor, is not suitable for our experiments, because although it is known that this inhibitor is membrane permeable, the potentiating effect on α-toxin-permeabilized smooth muscle contraction was slow and the sensitivity to this inhibitor of protein phosphatase type 1, including smooth muscle MLC phosphatase (Gong, Cohen, Kitazawa, Ikebe, Masuo, Somlyo, and Somlyo, 1992*a*), is very low. Therefore, another receptor/G protein-coupled, β-escin-permeabilized preparation (Kobayashi et al., 1989) permeable to relatively large molecules such as calmodulin, was used for rapid introduction of microcystin-LR into cells. In β-escin-permeabilized smooth muscle, 3 μM PDBu and 100 μM GTPγS in the absence of additional

calmodulin markedly increased force at pCa 6.7 from 0% to 39 ± 1.0% and to 36 ± 2.6%, respectively, of maximum force at pCa 5 (Fig. 3), indicating the existence of both PKC- and G protein-coupled pathways in this preparation. However, both potentiating effects were somewhat smaller than those found in α-toxin permeabilized preparations (Fig. 3).

After permeabilization with 40 μM β-escin, strips were incubated in the rigor (ATP-free), 1 mM EGTA-containing solution with 2 mM glucose and 29 U/ml hexokinase for 20 min at 20°C to deplete ATP remaining in the cells. Then, the fibers were transferred to the rigor solution containing 10 μM microcystin-LR with or without either PDBu (3 μM) or GTPγS (100 μM) for 10 min. Under these conditions, both protein kinases and phosphatases were inactive toward MLC because both ATP and Ca^{2+} were depleted and the phosphatases were blocked by high concentration of the inhibitor. MLC phosphorylation reactions were initiated by quickly transferring the strips to the pCa 6.7 (buffered with 10 mM EGTA) solution containing 4.5 mM MgATP, 10 mM creatine phosphate and 10 μM microcystin-LR with or without PDBu or GTPγS. Under these conditions where kinases but not phosphatases were active, the rates of both force development and MLC phosphorylation were Ca^{2+} dependent. MLC phosphorylation at 20°C was increased by pCa 6.7 from 9 ± 1.5% to 22 ± 3.7% of total MLC at 1 min after addition of ATP and reached to 52 ± 2.0% at pCa 5, indicating the presence of intact Ca^{2+}-calmodulin-activated MLC kinase system in these preparations. As shown in Fig. 5, *A* and *B*, neither the rate of MLC phosphorylation nor force development was significantly affected by a saturating concentration of PDBu. GTPγS also did not influence either MLC phosphorylation rate or force development under conditions inhibiting the dephosphorylation in rabbit tonic (femoral artery) smooth muscle. This confirms the previous results (Kitazawa et al., 1991*b*) using phasic smooth muscle (rabbit portal vein) that the guanine nucleotides have no effect on the rate of MLC thiophosphorylation. The half time of force development was 2.8 ± 0.07 min for the control, 3.0 ± 0.12 for PDBu and 2.7 ± 0.11 for GTPγS. In addition, 30 μM diC_6 did not significantly ($P > 0.4$) affect the time course of force development in the presence of 10 μM microcystin-LR. These results indicate that neither PKC activators nor GTPγS affect protein kinase(s) activity toward MLC.

In another set of control experiments, omitting microcystin-LR from both rigor and ATP-containing solutions led to marked difference depending on whether PDBu (3 μM) was present. Upon the addition of ATP, a large contraction was seen only in the presence of PDBu. In another experiment, however, the time course of force development induced by addition of microcystin-LR and Ca^{2+} (pCa 6.7) to the relaxing solution that contained ATP was not affected by the presence of 3 μM PDBu. These results suggest that the presence of protein phosphatase inhibitor abolishes the potentiating effect of PDBu as well as GTPγS.

The Effect of Phorbol Ester on the Time Course of MLC Dephosphorylation and Relaxation

To examine the second possibility, i.e., the increase in MLC phosphorylation by PKC activation results from inhibition of MLC phosphatase, the effect of PDBu on the rate of dephosphorylation of MLC was measured at 20°C in α-toxin-permeabilized tonic

(femoral artery) smooth muscle. The fibers were first preactivated by maximum Ca^{2+} (pCa 5) for 10 min and then the dephosphorylation kinetics was monitored by incubating the strips in Ca^{2+}-free (10 mM EGTA-containing) and ATP-free (also creatine phosphate-free) solution containing 100 μM 1-(5-chloronaphthalene-1-sulfonyl)-1*H*-hexahydro-1,4-diazepine (ML-9), a MLC kinase inhibitor with weak activity toward PKC (Saitoh, Ishikawa, Matsushima, Naka, and Hidaka, 1987). Under these conditions, no MLC kinase activity is expected. Thereafter, strips were rapidly frozen at the indicated intervals, as previously described (Kitazawa et al., 1991*b*).

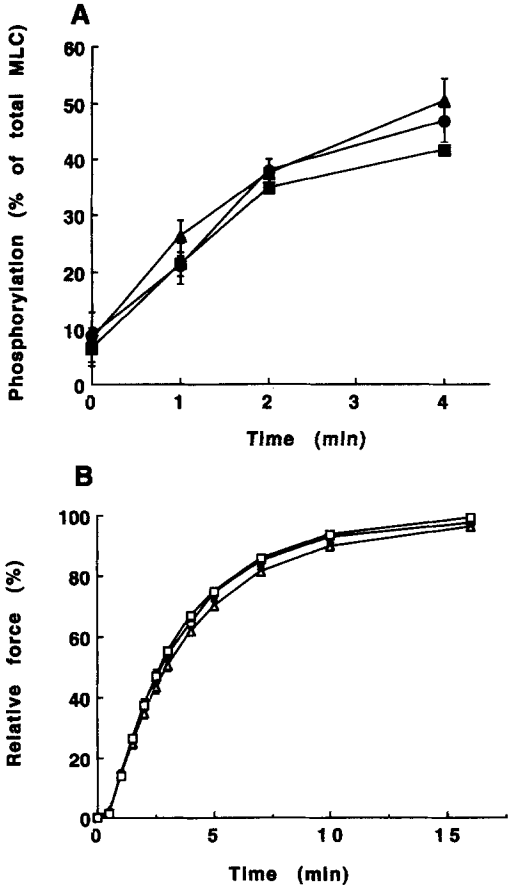

FIGURE 5. Lack of effect of 3 μM PDBu and 100 μM GTPγS on the time course of MLC phosphorylation (*A*) and force development (*B*) in β-escin-permeabilized femoral artery smooth muscle. MgATP (4.5 mM) plus creatine phosphate (10 mM) and Ca^{2+} (pCa 6.7) buffered with 10 mM EGTA were added to start MLC phosphorylation (*A*) and force development (*B*) in the presence of 10 μM microcystin-LR, a potent protein phosphatase inhibitor, after a 30-min incubation in the ATP-free, Ca^{2+}-free (1 mM EGTA) rigor solution. 3 μM PDBu (*triangle*) or 100 μM GTPγS (*square*) with 10 μM microcystin-LR was applied 10 min before ATP addition. All solutions contained 100 μM leupeptin and 1 mM phenylmethanesulfonyl fluoride. Circles represent control values. Each value is mean ± SE of four to eight experiments.

Fig. 6*A* shows the effect of PDBu and GTPγS on the time course of MLC dephosphorylation under the conditions where protein kinase activity toward MLC was inhibited. PDBu (3 μM) or GTPγS (100 μM) was added to both the activating solution and a Ca^{2+}-free, ATP-free solution containing ML-9. GTPγS significantly increased the half-time of MLC dephosphorylation from 1.2 to 2.0 min. This 1.72-fold increase in the half time in tonic smooth muscle is similar to the guanine nucleotide effect on the phasic smooth muscle, although the rate of dephosphorylation in the absence of GTPγS was much slower in tonic than phasic smooth muscle

(Gong et al., 1992*a*). PDBu also significantly increased the half-time of MLC dephosphorylation from control value to 2.3 min (Fig. 6 *A*). This 1.96-fold increase was similar to that of GTPγS.

The effects of 3 μM PDBu and 100 μM GTPγS on the relaxation rate were also determined under the same conditions as those for measurement of MLC dephosphorylation, except that all solutions contained ATP. As shown in Fig. 6 *B*, the relaxation was much slower in the presence of GTPγS or PDBu than in the absence of

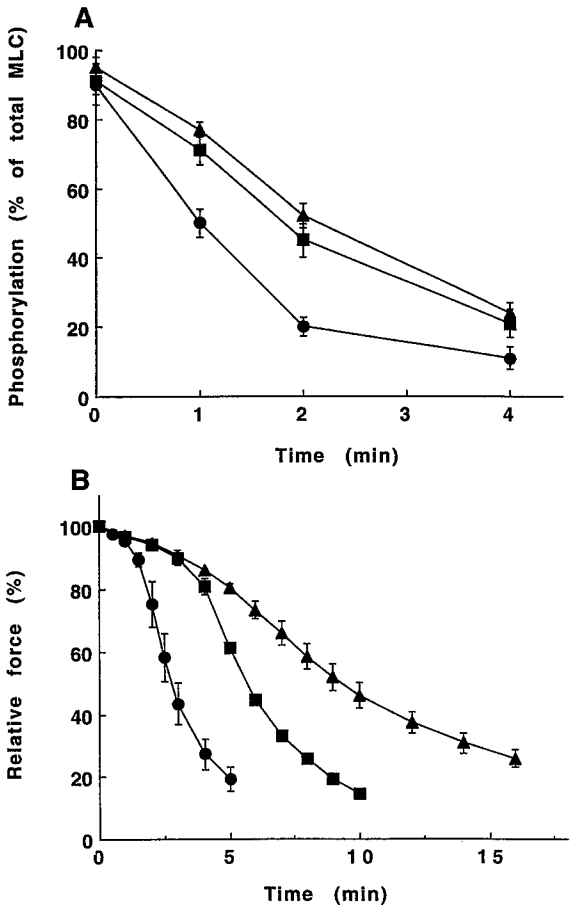

FIGURE 6. Time courses of MLC dephosphorylation (*A*) and relaxation (*B*) in α-toxin-permeabilized smooth muscle with and without PDBu and GTPγS. Permeabilized strips preactivated by maximum Ca^{2+} (pCa 5) for 10 min were incubated in a Ca^{2+}-free (10 mM EGTA) and ATP-free solution containing 100 μM ML-9 to block the protein kinase activity toward MLC. 3 μM PDBu (*triangle*) or 100 μM GTPγS (*square*) was present in both activating solution and Ca^{2+}-free, ATP-free solution. Circles represent control values. Each value is mean ± SE of five to eight experiments.

either agent. This result supports the notion that these agents act by inhibiting the rate of MLC dephosphorylation. The half time of relaxation was prolonged by GTPγS from 2.5 ± 0.2 to 5.3 ± 0.2 min. This 2.12-fold increase in the half time of relaxation in tonic smooth muscle is similar to that (2.13-fold increase) of phasic smooth muscle as described previously (Kitazawa et al., 1991*b*) and also similar to the increase in the half time of MLC dephosphorylation by the guanine nucleotide analog. On the other hand, PDBu markedly (3.96-fold) increased the half-time of the

relaxation to 9.9 ± 0.5 min. Relaxation in the presence of PDBu was significantly slower than in the presence of GTPγS although the inhibitory effect of the same concentration of phorbol ester on MLC dephosphorylation was not significantly different from that of the guanine nucleotide analog (Fig. 6 *A*). In addition, 10–30 μM diC_6 also significantly increased the half time of relaxation to 6.2 ± 0.2 min, which was significantly ($P < 0.05$) longer than that of GTPγS.

DISCUSSION

The present study clearly demonstrates that agents known to activate PKC, but not their inactive isomers, increase both MLC phosphorylation at MLC kinase-specific site and force development at constant Ca^{2+} due to inhibition of MLC dephosphorylation rather than stimulation of MLC phosphorylation. The inhibition of MLC phosphatase activity would increase MLC kinase/phosphatase activity ratio which results in the sensitization of MLC phosphorylation and force development to Ca^{2+}. This conclusion is supported by the results of phospho-peptide and phospho-amino acid analyses that revealed the main phosphorylation site to be Ser-19 (MLC kinase-specific site) during Ca^{2+} sensitization by phorbol ester. At least in part, the inhibition of protein phosphatase may be the mechanism of synergistic interaction of PKC activation and Ca^{2+} mobilization in smooth muscle (Park and Rasmussen, 1985) and perhaps, in other cell systems (Nishizuka, 1986; Berridge, 1987).

It has been shown in phasic vascular (Kitazawa et al., 1991*b*) and tracheal (Kubota et al., 1992) smooth muscle, that GTPγS, an activator of G proteins, increases MLC phosphorylation associated with increased force through the inhibition of MLC phosphatase. This was confirmed in tonic vascular smooth muscle in the present study. These results further suggest that the inhibition of MLC phosphatase by G protein is mediated through the activation of PKC by DAG formed by G protein-coupled phospholipases (Berridge, 1987; Nishizuka, 1984; Takuwa, Takuwa, and Rasmussen, 1986). This is further supported by the fact that inositol trisphosphate and its metabolites have no effect on the Ca^{2+} sensitization (Kitazawa and Somlyo, 1991). However, GTPγS-induced Ca^{2+} sensitization may be complex and there are several possibilities that the Ca^{2+} sensitization might be mediated by multiple pathways. For examples, in phasic smooth muscle, GTPγS further potentiates contraction even in the presence of saturated concentrations of PDBu (Kitazawa and Somlyo, 1991), suggesting that GTPγS induces a more complex cascade than does PKC. Alternatively, PDBu may cause incomplete activation of PKC isoforms in phasic smooth muscle. Recently, it was demonstrated that arachidonic acid directly, not through PKC activation, inhibits MLC phosphatase and slowly increases contractile Ca^{2+} sensitivity (Gong, Fuglsang, Alessi, Kobayashi, Cohen, Somlyo, and Somlyo, 1992*b*). Small G protein, rho p21 might be involved in GTPγS-induced Ca^{2+} sensitization in smooth muscle as well (Hirata, Kikuchi, Sasaki, Kuroda, Kaibuchi, Matsumura, Seki, Saida, and Takai, 1992). It should also be mentioned that, relaxation in the presence of PDBu or diC_6 with inhibited MLC kinase activity was more delayed than that expected in the presence of GTPγS (Fig. 6 *B*). Although relaxation is not simply determined by the rate of MLC dephosphorylation (Chatterjee and Murphy, 1983; Kuhn, Tewes, Gagelmann, Guth, Arner, and Ruegg, 1990), this suggests that PKC activation has an additional effect on cross-bridge detachment

of dephosphorylated MLC. Thus, the physiological significance of PKC activation remains obscure.

Specific phosphorylation of MLC (Ser-19) by the Ca^{2+}/calmodulin-dependent MLC kinase is thought to be a primary mechanism of smooth muscle contraction (Hartshorne, 1987 and Kamm and Stull, 1989). In addition, PKC can also directly phosphorylate MLC at residues (Ser-1 and -2 and Thr-9) distinct from MLC kinase-sites in vitro. As a result, however, actin-activated myosin ATPase is inhibited (Ikebe et al., 1987*a;* Bengur et al., 1987). Furthermore, in skinned preparations, PKC activators have no contractile effects (this study) and phosphorylation of MLC by purified or constitutively active PKC does not activate contraction and does not potentiate Ca^{2+}-activated contraction (Inagaki et al., 1987; Sutton and Haeberle, 1990; Parente et al., 1992). We observed, however, a significant increase in MLC phosphorylation associated with increased force by PKC activators in the receptor/G protein-coupled permeabilized preparations. This force/phosphorylation ratio in the presence of 3 μM PDBu (Fig. 1 and Table I) was not significantly different from those in the presence of GTPγS (at pCa 7) or Ca^{2+} (pCa 6.3) alone. In fact, a major phosphorylation site of MLC in permeabilized preparations activated by PDBu or by GTPγS (see also Kubota et al., 1992) was localized at the same MLC kinase-specific site as that activated by Ca^{2+} alone (Fig. 4). These findings suggest that PKC activators lead to an increase in MLC phosphorylation, albeit indirectly, at the MLC kinase-sites without an increase in cytoplasmic Ca^{2+}. This is consistent with the observation that PKC activators inhibit MLC phosphatase. However, the mechanism by which PKC inhibits MLC phosphatase remains to be clarified. Similar Ca^{2+} sensitizing effects of PKC activators have been observed on secretory cells (Knight, von Grafenstein, and Athayde, 1989), suggesting that the inhibition of protein phosphatase by PKC is a more general mechanism rather than specific to smooth muscle. Likewise, PKC may regulate the phosphorylation/dephosphorylation reaction of other protein kinase systems that are active under these conditions.

Activated PKC phosphorylates purified MLC up to three mol of P/mol of MLC (Bengur et al., 1987; Ikebe et al., 1987*a*). Less than 2 mol of P/mol of MLC are phosphorylated for in situ MLC in glycerinated smooth muscle by PKC (Sutton and Haeberle, 1990). In the present α-toxin permeabilized preparations, however, PKC activators in the presence of a protein phosphatase inhibitor did not significantly increase the rate of MLC phosphorylation and force development (Fig. 5). Furthermore, under conditions where MLC kinase was almost fully activated at pCa 5, the presence of PDBu tended to, but did not significantly, increase the ratio of di-phosphorylation (from 13 to 18% of total MLC). This diphosphorylation can be mainly caused by the phosphorylation at Thr-18 ($\sim$10% in phospho-amino acid analysis), but not at PKC-specific sites. Phorbol ester causes only a small increase in phosphorylation of PKC-sites of MLC in intact smooth muscle (Singer et al., 1989; Kamm et al., 1989; Barany et al., 1990, 1992; Sutton and Haeberle, 1990). The phosphopeptides containing PKC-specific phosphorylation sites of MLC during Ca^{2+} sensitization by PDBu were not detectable in our phosphopeptide analysis (Fig. 4). This implies that PKC-induced phosphorylation of MLC at PKC-specific sites is minimal or nonexistent. These results indicate that the PKC-sites of MLC may be well

protected from phosphorylation by in situ-PKC, presumably due to the specific localization of the kinase in intact, β-escin- or α-toxin-permeabilized preparations.

In some types of intact vascular smooth muscle, phorbol esters induce a large contraction associated with an increase in both cytoplasmic Ca^{2+} and phosphorylation of MLC (Rembold and Murphy, 1988; Sato, Hori, Ozaki, Takanoohmura, Tsuchiya, Sugi, and Karaki, 1992; Barany et al., 1992). Their analysis of the relationship between Ca^{2+} and force development or between Ca^{2+} and MLC phosphorylation indicated that Ca^{2+} sensitivity of force development and MLC phosphorylation during phorbol ester-induced contraction was similar to those during agonist-induced stimulation but higher than those during high K^+-induced stimulation. In permeabilized smooth muscle, phorbol esters have been shown to increase Ca^{2+} sensitivity of force development (Itoh et al., 1988; Nishimura and van Breemen, 1989) as well as MLC phosphorylation (Fujiwara et al., 1988; the present study). This type of Ca^{2+} sensitization by phorbol esters can be well explained by the inhibition of MLC phosphatase by PKC activators. In some types of intact vascular smooth muscle, however, only small or insignificant elevation of Ca^{2+} and/or MLC phosphorylation was observed during phorbol ester-induced contraction (Park and Rasmussen, 1986; Jiang and Morgan, 1987, 1989; Kamm et al., 1989; Singer, 1990*a,b;* Barany et al., 1990; Sato et al., 1992). In skinned preparations, high force development by phorbol ester was associated with low MLC phosphorylation (Chatterjee and Tejada, 1986), suggesting MLC phosphorylation-independent contraction. This type of Ca^{2+} sensitization by phorbol ester might be correlated to the observation that phorbol ester markedly maintained a high force level with minimal MLC phosphorylation (Fig. 6).

In summary, at constant Ca^{2+}, PKC activators enhance MLC phosphorylation at MLC kinase-specific, but not PKC-specific, sites. This Ca^{2+} sensitization is mainly due to indirect inhibition of MLC dephosphorylation, similar to that of G protein-mediated Ca^{2+} sensitization.

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