J. Physiol. (1986), **375**, *pp.* 515–534
With 9 text-figures
Printed in Great Britain

A PHORBOL ESTER HAS DUAL ACTIONS ON THE MECHANICAL RESPONSE IN THE RABBIT MESENTERIC AND PORCINE CORONARY ARTERIES

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(*Received 18 July 1985*)

SUMMARY

1. To clarify the role of protein kinase C in the mechanical response, the effects of 12-*o*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, were investigated on intact and skinned smooth muscle preparations of the rabbit mesenteric artery.

2. TPA (0·1 μM) showed dual actions (initial enhancement followed by inhibition during long exposure) on the K^+-induced contraction. The enhancement was marked in the presence of 39 mM-K^+ but inhibition was the predominant effect in the presence of 128 mM-K^+.

3. Addition of 2·6 mM-Ca^{2+} to a Ca^{2+}-free solution containing 2 mM-EGTA following application of A23187 (1 μM), produced contraction. TPA showed the same dual actions on this Ca^{2+}-induced contraction.

4. In chemically skinned muscles, TPA increased the amplitude of Ca^{2+}-induced contractions evoked by low concentrations of Ca^{2+} (0·1–0·3 μM), but reduced those evoked by high concentrations of Ca^{2+} (1–10 μM). Both actions of TPA were facilitated in the presence of phosphatidylserine (PS).

5. TPA with PS had no effect on the Ca^{2+}-independent contraction evoked in relaxing solution containing 10 mM-EGTA and 4 mM-Mg ATP following application of adenosine-5-*o*-3-thiotriphosphate ($ATP_\gamma S$) and 0·3 μM-Ca^{2+}.

6. The amount of Ca^{2+} stored in cells estimated from the amplitude of the caffeine-induced contraction was not modified by application of TPA with PS in skinned or intact muscle tissues.

7. The effects of TPA were investigated on the Ca^{2+} transient measured from the intensity of fluorescence of quin-2 in dispersed cell suspensions prepared from the porcine coronary artery. TPA had no effect on the Ca^{2+} transient in high K^+ but enhanced the amplitude of the contraction.

8. Amplitudes of the tonic response evoked by 39 mM-K^+ in intact muscle tissues and the contraction induced by 0·3 μM-Ca^{2+} in skinned muscle were much the same. TPA with PS enhanced the amplitudes of both contractions to the same extent.

9. From the above results, we concluded that TPA shows dual actions on the contractile machinery and may act on the regulatory systems of contractile proteins.

Both excitatory and inhibitory actions of TPA depended on the concentration of Ca^{2+}. However, the physiological action of protein kinase C as estimated from the action of TPA seems to be related to an excitatory action on the contractile machinery.

INTRODUCTION

The contraction–relaxation cycles are mainly dependent on the intracellular free Ca^{2+} concentration in vascular smooth muscles, and Ca^{2+}–calmodulin-dependent phosphorylation of the myosin light chain (MLC) catalysed by myosin light chain kinase (MLCK) which is the major regulation system of contractile proteins (see for review, Hartshorne & Mrwa, 1982; Kuriyama, Ito, Suzuki, Kitamura & Itoh, 1982). The contractile system in vascular smooth muscles is also regulated by second messenger systems, such as cyclic nucleotides, through phosphorylation of MLCK (Conti & Adelstein, 1980; Pfitzer, Hofmann, Disalvo & Ruegg, 1984; Itoh, Kanmura, Kuriyama & Sasaguri, 1985*b*).

Recently, Naka, Nishikawa, Adelstein & Hidaka (1983) and Nishikawa, Hidaka & Adelstein (1983) showed that the Ca^{2+}-activated and phospholipid-dependent enzyme, protein kinase C phosphorylated the MLC at a different site from that phosphorylated by MLCK and inhibited the actin-activated ATPase in heavy meromyosin of smooth muscles. Thus, activation of protein kinase C may inhibit the contraction, in contrast to the function of this kinase as described in non-muscle cells (see for review, Nishizuka, 1984). However, the function of this kinase on contractile systems in living vascular smooth muscle has not been investigated.

Protein kinase C is widely distributed in many tissues, including vascular smooth muscles (Kuo, Andersson, Wise, Mackerlova, Salomonsson, Brackett, Katoh, Shoji & Wrenn, 1980) and has a crucial role in signal transduction for physiologically active substances (for example, neurotransmitters or hormones) in non-muscle cells (Nishizuka, 1984). A 1,2-diacylglycerol (DG), which is produced in the membrane during the agonist-induced breakdown of phosphatidylinositol (PI) or phosphatidylinositol 4,5-bisphosphate (PI-P_2), activates the protein kinase C in the presence of acidic phospholipid (for example, phosphatidylserine; PS). In addition, PI-P_2 is hydrolysed to inositol 1,4,5-trisphosphate ($InsP_3$) as a co-product of DG by activation of neurohumoral receptors in smooth muscles (Berridge & Irvine, 1984). $InsP_3$ released Ca^{2+} from non-mitochondrial intracellular store sites (presumably, sarcoplasmic reticulum) in smooth muscles of the porcine coronary artery (Suematsu, Hirata, Hashimoto & Kuriyama, 1984). However, the action of DG also remains unclear in vascular smooth muscles.

Kikkawa, Takai, Tanaka, Miyake & Nishizuka (1983) reported that a most powerful tumour-promoting ester, 12-*o*-tetradecanoylphorbol-13-acetate (TPA), was intercalated into the cell membrane and substituted for actions of DG, permanently activating protein kinase C. Since then, numerous data have been accumulated using TPA instead of unstable DG for investigations of the role of protein kinase C in non-muscle cells (Nishizuka, 1984).

We attempt to clarify the physiological roles of protein kinase C on the contraction in intact and skinned smooth muscle cells of the rabbit mesenteric artery. To activate protein kinase C, we used TPA instead of DG.

METHODS

Male albino rabbits (1·8–2·2 kg) were given sodium pentobarbitone (40 mg/kg I.V.), exsanguinated and the mesenterium of the iliac region was removed and placed in a dissecting chamber filled with Krebs solution. The mesenteric artery was carefully excised and thin circular strips (0·3–0·5 mm in length, 0·05–0·1 mm in width and 0·02–0·03 mm in thickness) were prepared, under a binocular microscope. To avoid untoward responses, the tunica interna, adventitia and tunica externa were carefully removed.

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+, 137·4; K^+, 5·9; Mg^{2+}, 1·2; Ca^{2+}, 2·6; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; Cl^-, 134·4; glucose, 11·5. The solution was bubbled with 97% O_2 and 3% CO_2 and the pH of the solution was adjusted to 7·4. High-K^+ solution was prepared by replacing NaCl with KCl, isosmotically.

In skinned muscles, the following relaxing solution was used (mM): K methanesulphonate (KMs), 114; Tris maleate, 20; $Mg(Ms)_2$, 5·1; adenosine 5′-triphosphate (ATP), 5·2; and ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(Ms)_2$ to 10 mM-EGTA (Harafuji & Ogawa, 1980; Itoh, Kuriyama & Suzuki, 1983). The rigor-producing solution had the following composition (mM): KMs, 114; Tris maleate, 20; $Mg(Ms)_2$, 1·1 and EGTA, 4. The composition of the adenosine-5-*o*-3-thiotriphosphate ($ATP_\gamma S$) with 0·3 μM-Ca^{2+}-containing solution was as follows (mM): KMs, 127; Tris maleate, 20; $Mg(Ms)_2$, 2·6; $ATP_\gamma S$, 2·0; EGTA, 4; and $Ca(Ms)_2$, 0·9.

Recordings of mechanical activity

Mechanical activity of intact and skinned muscles was measured by attaching a circular strip to a strain gauge (UL-2, Shinko Co., Tokyo, Japan) in a chamber with a capacity of 0·9 ml. The solution was changed by perfusing rapidly from one end and siphoning off simultaneously with a water pump from the other end. Temperature of perfusate was kept at 25 °C.

Mechanical responses evoked by high external K^+ concentrations or Ca^{2+} in the presence of 1 μM-A23187 in intact preparations were measured in the presence of 1 μM-tetrodotoxin (TTX) and 3 μM-guanethidine to prevent the release of noradrenaline (NA) from nerve terminals. In order to estimate the Ca^{2+} released from the intracellular store, the contractions induced by 10 mM-caffeine were measured after rinsing for several minutes in Ca^{2+}-free, 2 mM-EGTA-containing solution.

Skinned muscle preparations were obtained by using saponin (25 μg/ml) for 20 min in relaxing solution (Itoh *et al.* 1983). To compare the results obtained in intact muscle, the amplitude of the Ca^{2+}-induced contraction was represented relative to the amplitude of the contraction induced by 128 mM-K^+. TPA with or without PS was applied during the Ca^{2+}-induced contraction after the level of tension was constant. Action of TPA on Ca^{2+}-independent contraction was observed after the following procedures: ATP-free and Ca^{2+}-free solution containing 4 mM-EGTA (rigor-producing solution) was applied to deplete ATP in the bath and possibly in muscle tissues, and 2 mM-$ATP_\gamma S$ with 0·3 μM-Ca^{2+}-containing solution was applied to skinned muscles for 30 s or 5 min following application of rigor-producing solution for 3 min. Finally 4 mM-MgATP containing Ca^{2+}-free (10 mM-EGTA) solution was applied (Cassidy, Hoar & Kerrick, 1979). To estimate the amount of Ca^{2+} stored within the skinned muscles, the amplitude of the contraction evoked by 25 mM-caffeine was measured in the presence of 0·5 mM-EGTA after application of 3 μM-Ca^{2+} buffered with 2 mM-EGTA for 1 min.

Ca^{2+}-transient measurement

Preparation of isolated single smooth muscle cells was essentially the same as described previously by Ueno (1985). In order to obtain a sufficient number of single cells, we used the porcine coronary artery. After enzymatic dispersal with collagenase, cells in each batch (2×10^5 cells/ml) were suspended in Krebs solution for 30 min for equilibration. Several batches of cells were incubated with 100 μM quin-2-acetoxymethylester (quin-2/AM) for 120 min. Uptake of quin-2/AM into the cells and the hydrolysis of quin-2/AM to quin-2 by esterases in the cell were examined by the shift in maximum emission from 430 to 490 nm and this process was virtually complete after 120 min incubation as determined fluorometrically (Tsien, Pozzan & Rink, 1982). After centrifugation at 1000 revs/min for 1 min to remove the extracellular quin-2/AM, the quin-2-loaded

single cell suspension was inserted into a thermostatted cuvette in a spectrofluorometer (Hitachi 650-40 type, Hitachi Co., Tokyo, Japan). Cells were suspended to a final volume of 2 ml modified Krebs solution (140 mM-NaCl, 4 mM-KCl, 1·0 mM-KH_2PO_4, 1·0 mM-$CaCl_2$, 1·0 mM-$MgCl_2$, 10 mM-glucose, 20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) at pH 7·4) and transferred to 1 cm square quartz cuvettes and continuously stirred by an inserted stirrer. After replacement of modified Krebs solution with 80 mM-K^+-containing solution, the fluorescence change was continuously recorded for up to 30 min at an excitation wave-length of 339 nm (5 nm slit) and an emission wave-length of 490 nm (10 nm slit). The fluorometer output signal was recorded on a pen recorder (057 type $X-Y$ recorder, Hitachi Co., Japan). After the Ca^{2+} transient was constant in the high-K^+ solution, TPA was applied (the volume was less than 0·2 %) and stirred in the cuvette. The temperature of all solutions was maintained at 32 ± 1 °C. Values of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (in nM) were estimated from the observed fluorescence intensity of intracellular quin-2, F, and the intensity of Ca^{2+}-saturated dye, F_{max}, according to

$$[Ca^{2+}]_i = 115\ [(F - F_{min})/(F_{max} - F)],$$

where 115 nM is the apparent K_d for Ca^{2+}–quin-2 in cytoplasmic ionic conditions (Tsien *et al.* 1982), and F_{min} is the fluorescence intensity of the Mn^{2+}–quin-2 complex (Hesketh, Smith, Moore, Taylor & Metcalfe, 1983). F_{max} was determined by rapidly saturating intracellular quin-2 with Ca^{2+} by permeabilizing the cells with 0·2 % Triton X-100 in normal Ca^{2+}-containing medium.

To compare the results obtained from the Ca^{2+}-transient experiment, we observed the effect of TPA on the tonic contraction induced by 80 mM-K^+ in small strips of the same porcine coronary artery (0·3 mm in length, 0·05 mm in width and 0·03 mm in thickness). The experimental procedures and apparatus were the same as those in the case of mesenteric artery.

Drugs

Chemicals used were 12-*o*-tetradecanoylphorbol-13-acetate (TPA; Sigma Chem. Co., St. Louis, U.S.A.), phosphatidylserine (PS, beef brain; Serdary Research Laboratories, Ontario, Canada), A23187 (free acid; Calbiochemicals, CA, U.S.A.), caffeine (Wako Pharmac. Co., Tokyo, Japan), saponin (ICN Pharmac. Inc., Cleveland, OH, U.S.A.), adenosine-5-*o*-3-thiotriphosphate ($ATP_\gamma S$; Boehringer Mannheim; Yamanouchi Co., Tokyo, Japan), guanethidine HCl (Tokyo Kasei Co., Tokyo, Japan), tetrodotoxin (TTX; Sigma), ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA; Dozin Lab., Kumamoto, Japan), quin-2-acetoxymethylester (quin-2/AM; Dozin), 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES, Dozin).

Statistics

The measured value was expressed as the mean ± S.D. and number of observations. The statistical significance was assessed using Student's t test. P values less than 0·05 were considered significant.

RESULTS

Effects of TPA on mechanical responses in intact muscles

Fig. 1 shows the effects of 0·1 μM-TPA on the contraction evoked by 39 mM-K^+ or 128 mM-K^+-containing solution. In the presence of 3 μM-guanethidine and 0·1 μM-TTX, the amplitude of the contraction evoked by 39 mM-K^+ or 128 mM-K^+ at 10 min intervals remained the same for over 120 min. TPA (0·1 μM) transiently enhanced the amplitude of contractions induced by 39 mM-K^+ (in the presence of 3 μM-guanethidine and 0·1 μM-TTX) and then gradually reduced the amplitude of contractions with a long time course (up to 120 min). The resting tone was gradually but consistently elevated time dependently (Fig. 1*A*). The minimum concentration of TPA required to elevate the resting tone and to enhance the contraction was below 10 nM. Since the actions of TPA appeared very slowly in low concentrations of TPA, 0·1 μM-TPA was used throughout the following experiments. When 0·1 μM-TPA was applied, the initial enhancement of the contractions induced by 128 mM-K^+ appeared

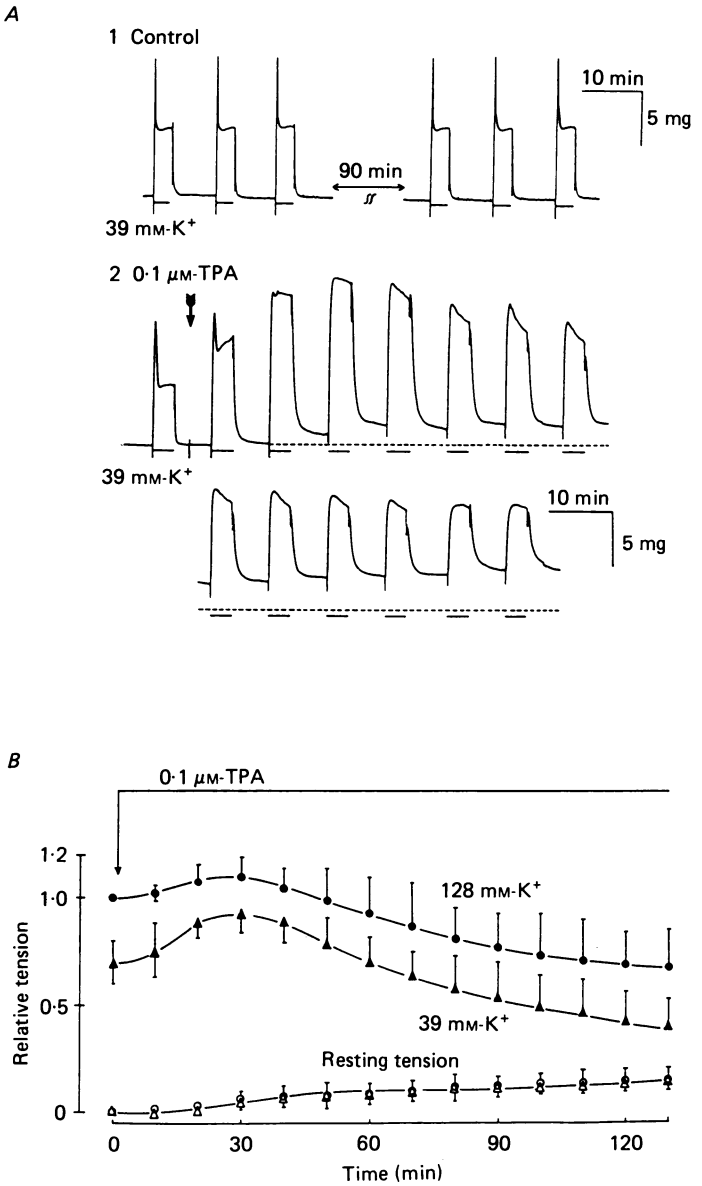

Fig. 1. Effects of 0·1 μM-TPA on the contraction evoked by 39 mM-K^+ or 128 mM-K^+ in the rabbit mesenteric artery. *A*1, control, 39 mM-K^+ was applied for 3 min every 10 min; *A*2, shows the typical example of TPA action on the contraction induced by 39 mM-K^+. TPA was applied at the arrow. *B*, effects of TPA on the amplitude of contraction evoked by 39 mM-K^+ and 128 mM-K^+ and on resting tonus. The maximum amplitude of the contraction was normalized as that evoked by 128 mM-K^+ in the absence of TPA ($n = 10$). When 128 mM-K^+ or 39 mM-K^+-containing solution was applied the same amplitudes of contraction were obtained for over 120 min. Vertical scale shows the relative tension. Horizontal scale shows the time after TPA application. TPA was applied at time zero. To prevent neuronal effects, guanethidine (3 μM) and TTX (0·1 μM) were present throughout the experiments. Vertical bars indicate S.D. ($n = 5$).

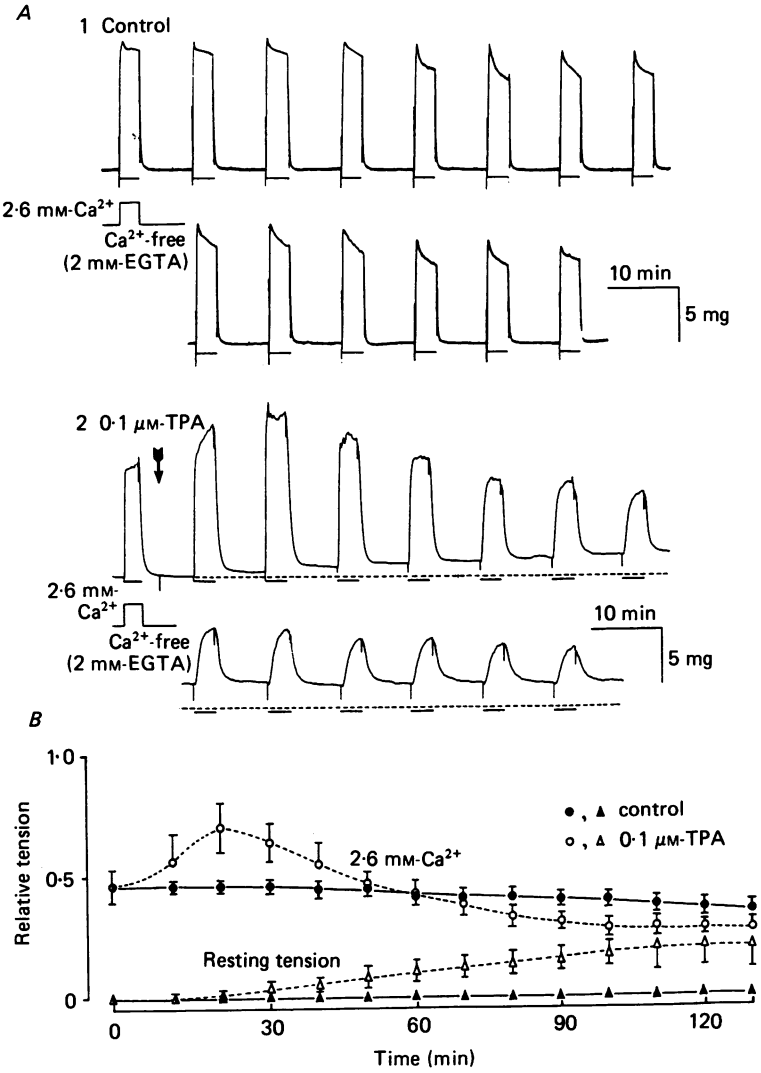

Fig. 2. Effects of 0·1 μM-TPA on contractions induced by 2·6 mM-Ca^{2+} in Ca^{2+}-free, 2 mM-EGTA-containing solution, in the presence of 1 μM-A23187 (guanethidine and TTX were also present). *A*1, control, 2·6 mM-Ca^{2+} was applied for 3 min at 7 min intervals in Ca^{2+}-free solution containing 2 mM-EGTA; *A*2, shows the typical pattern of TPA action on Ca^{2+}-induced contractions in the presence of A23187. Arrow indicates the application of TPA. *B*, effect of TPA on Ca^{2+}-induced contraction (○) and resting tonus (△) in A23187-treated muscle. ● and ▲ represent control responses. The maximum amplitude of contraction was normalized as that evoked by 128 mM-K^+ in the absence of TPA. Vertical bars indicate S.D. ($n = 5$).

to a lesser extent and delayed inhibition was more pronounced than those for contractions induced by 39 mM-K^+. The elevation of the resting tone was similar for both concentrations of K^+ (Fig. 1*B*).

In the presence of a high concentration of A23187 (1 μM), in Ca^{2+}-free solution containing 2 mM-EGTA, application of 2·6 mM-Ca^{2+} produced contraction, due to an

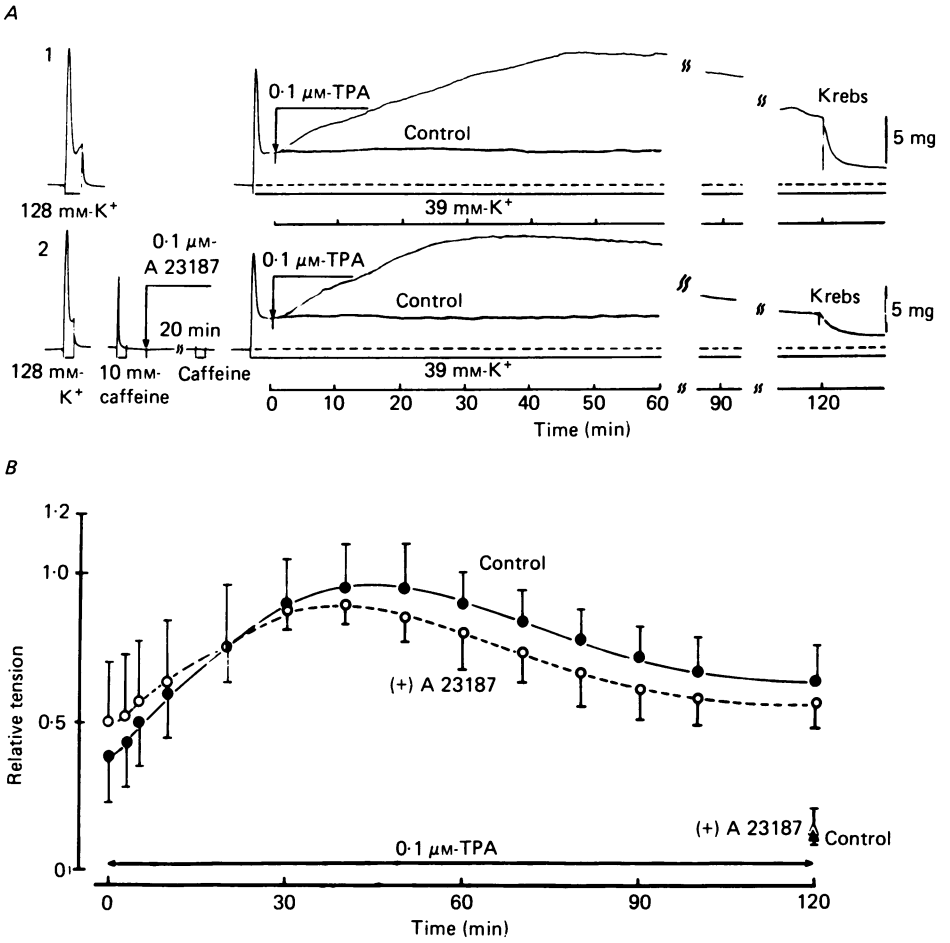

Fig. 3. Effects of 0·1 μM-TPA on tonic contractions evoked by 39 mM-K^+ in the presence or absence of 0·1 mM-A23187. *A*, shows the typical example of TPA action on the tonic response induced by 39 μM-K^+ in the absence (1) or presence (2) of A23187. Control shows the tonic response evoked by 39 mM-K^+ in the absence of TPA and dotted lines indicate the level of the resting tension. Arrow shows the application of TPA. *B*, effect of TPA on tonic responses induced by 39 mM-K^+ in the presence (○) and absence (●) of 0·1 μM-A23187. △ and ▲ represents the level of resting tonus in the presence and absence of A23187, respectively. TPA was applied at time zero. Vertical bars indicate S.D. ($n = 5$).

ionophore action of A23187 at the plasma membrane (Itoh, Kanmura & Kuriyama, 1985*a*) and the amplitudes of these contractions were almost the same for 60 min, but slightly decayed for over 90 min (Fig. 2*A*). The amplitude of the contraction induced by 2·6 mM-Ca^{2+} was $0{\cdot}46 \pm 0{\cdot}07$ ($n = 5$) times the contraction induced by 128 mM-K^+. Increased concentrations of Ca^{2+} over 2·6 mM did not further increase the amplitude of contractions. When 0·1 μM-TPA was applied, the effects on the contractions induced by 2·6 mM-Ca^{2+} were the same as those observed for the contraction induced by 39 mM-K^+, but the delayed inhibition appeared more rapidly and was more marked (Fig. 2). An elevation of the resting tone was consistently observed in the presence of 1 μM-A23187 and TPA, but not in the absence of TPA (Fig. 2*B*).

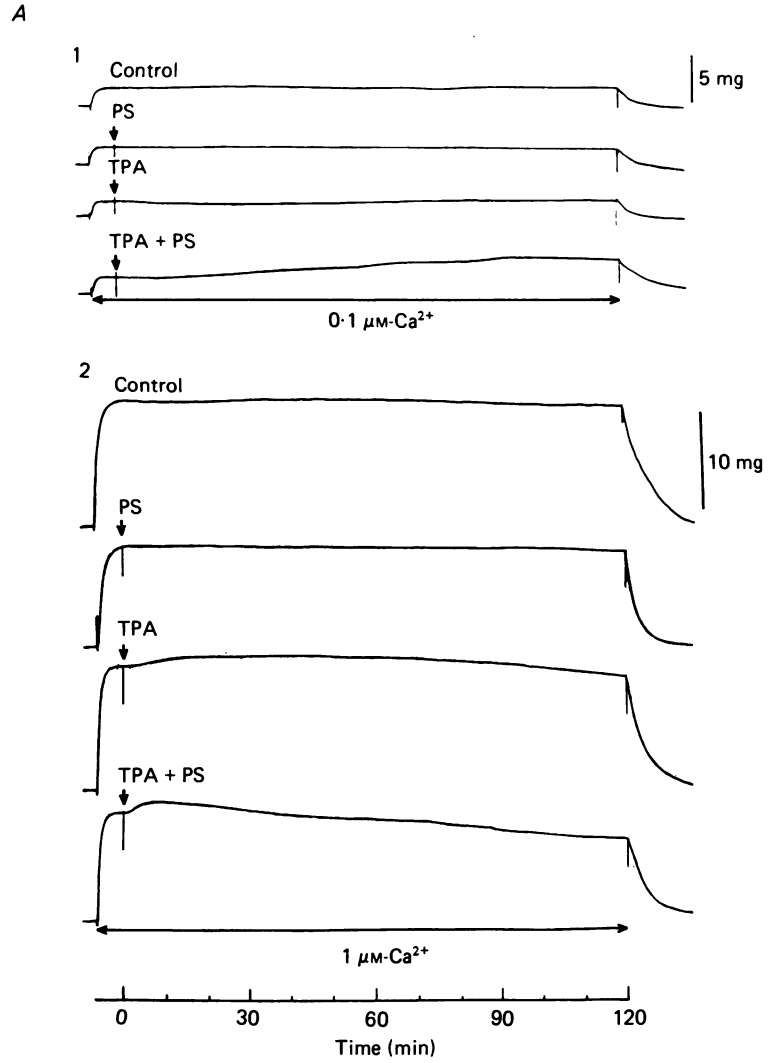

Fig. 4*A*. For legend see opposite.

Following the treatment of 0·1 μM-A23187, the vascular tissues could not store Ca^{2+} in cells due to leakage of Ca^{2+}, but Ca^{2+} influx through the sarcolemma was not affected (Itoh *et al.* 1985*a*). The effects of TPA on the tonic contraction induced by 39 mM-K^+ in the presence or absence of 0·1 μM-A23187 are shown in Fig. 3. After application of 0·1 μM-A23187 for 20 min the contraction induced by 10 mM-caffeine ceased (Fig. 3*A*2). The tonic contraction evoked by 39 mM-K^+ persisted at an almost constant level for over 60 min in the presence of A23187. TPA (0·1 μM) had almost the same effect on the tonic contraction and resting tension in the presence or absence of A23187. This indicates that the elevation of the resting tone and contraction induced by TPA has no causal relation to the Ca^{2+} store site.

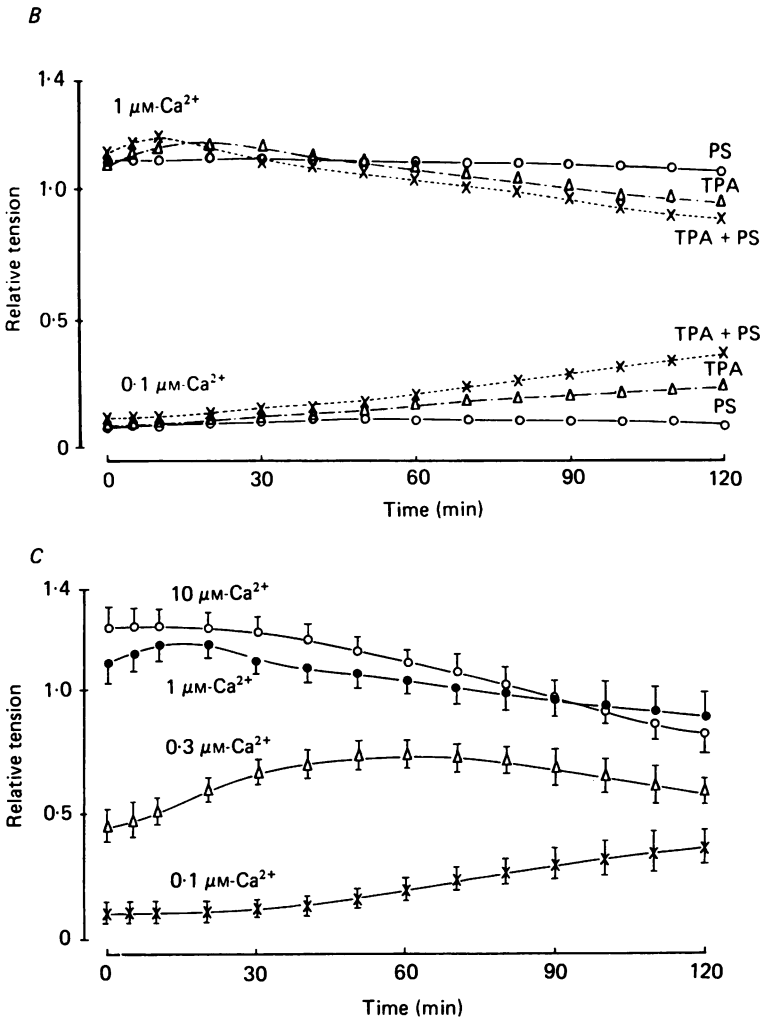

Fig. 4. Effects of 0·1 μM-TPA on the Ca^{2+}-induced contraction in chemically skinned muscle of rabbit mesenteric artery. *A*1, effect of 50 μg PS/ml, 0·1 μM-TPA and TPA with PS on contractions induced by 0·1 μM-Ca^{2+}. The agents were applied at the arrow. *A*2, effect of the above agents on the contraction induced by 1 μM-Ca^{2+}. Horizontal scale indicates the time after application of these agents. The agents were applied at time zero. *B*, effects of PS (50 μg/ml; ○), TPA (0·1 μM; △) or TPA with PS (×) on two different concentrations of Ca^{2+}-induced contraction (0·1 μM- and 1 μM-Ca^{2+}). When 0·1 μM-Ca^{2+} or 1 μM-Ca^{2+} was applied, the amplitude of the Ca^{2+}-induced contraction remained the same for over 120 min. The marks represent the mean value of five preparations. After the Ca^{2+}-induced contraction reached a steady level the agents were applied at time zero. The amplitudes of the Ca^{2+}-induced contractions in skinned muscle are expressed relative to the contraction induced by 128 mM-K^+ in intact muscle ($n = 5$). *C*, effects of TPA (0·1 μM) with PS (50 μg/ml) on contractions induced by various concentrations of Ca^{2+}. The amplitudes of contraction evoked by various concentrations of Ca^{2+} used in this study remained constant for over 120 min in the absence of TPA ($n = 5$ for each Ca^{2+} concentration). ×, 0·1 μM-Ca^{2+}; △, 0·3 μM-Ca^{2+}; ●, 1 μM-Ca^{2+}; ○, 10 μM-Ca^{2+}. Vertical bar represents S.D. ($n = 5$).

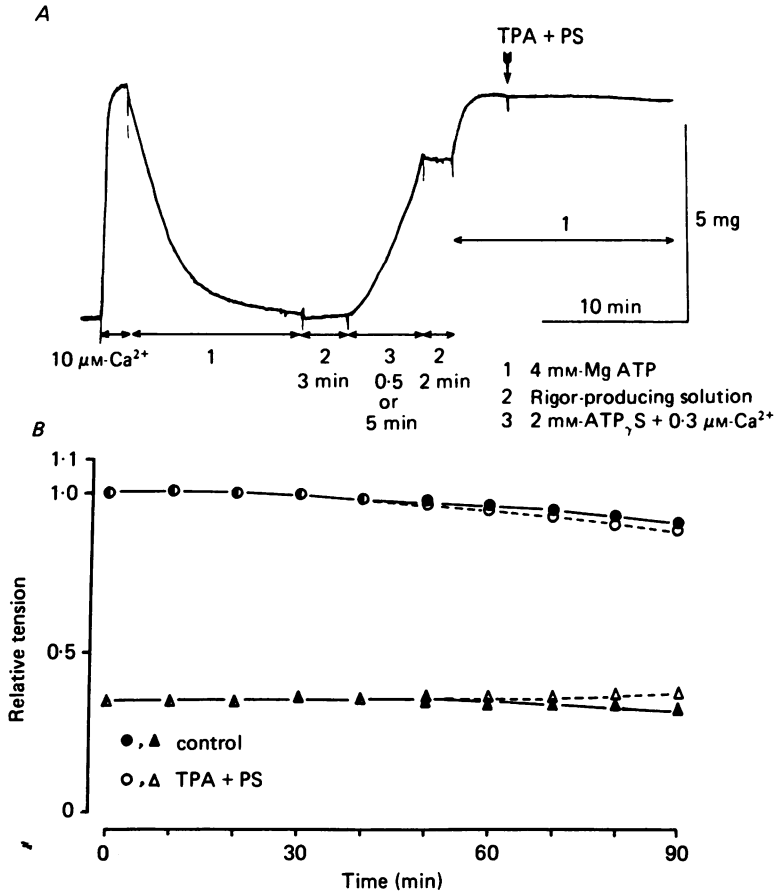

Fig. 5. Effects of 0·1 μM-TPA with 50 μg PS/ml on Ca^{2+}-independent contraction. *A*, shows the experimental procedures. A contraction was induced in 10 μM-Ca^{2+}. The tissue was then rinsed in relaxing solution and put in ATP-free and Ca^{2+}-free (4 mM-EGTA) solution (rigor-producing solution) for 3 min. After application of 2 mM-$ATP_{\gamma}S$ with 0·3 μM-Ca^{2+} for 30 s or 5 min in ATP-free and Ca^{2+}-free (4 mM-EGTA) solution (rigor-producing solution), the tissue was rinsed with rigor-producing solution for 2 min and 4 mM-MgATP containing Ca^{2+}-free solution (10 mM-EGTA) was applied (see text). When the tension reached a steady level TPA with PS was applied. *B*, effects of 0·1 μM-TPA with 50 μg PS/ml on the Ca^{2+}-independent contraction following application of $ATP_{\gamma}S$ and Ca^{2+} for 30 s (△, ▲) and 5 min (○, ●). The amplitude of contraction was normalized relative to the contraction induced by 10 μM-Ca^{2+}. Marks represent mean value ($n = 3$).

Effects of TPA on contractile proteins in skinned muscles

After skinning the tissue, effects of TPA on the Ca^{2+}-induced contraction were observed. The minimum concentration of Ca^{2+} required to produce contraction was 0·1 μM and increased concentration of Ca^{2+} enhanced the amplitude of contraction. The maximum amplitude of the Ca^{2+}-induced contraction was obtained at 10 μM-Ca^{2+} ($1{\cdot}25 \pm 0{\cdot}08$ times the contraction induced by 128 mM-K^+ in the intact muscle, $n = 5$). Fig. 4*A* shows the effects of TPA (0·1 μM) or TPA with PS (50 μg/ml) on the contraction evoked by two different concentrations of Ca^{2+}. When 0·1 μM- or 1 μM-Ca^{2+}

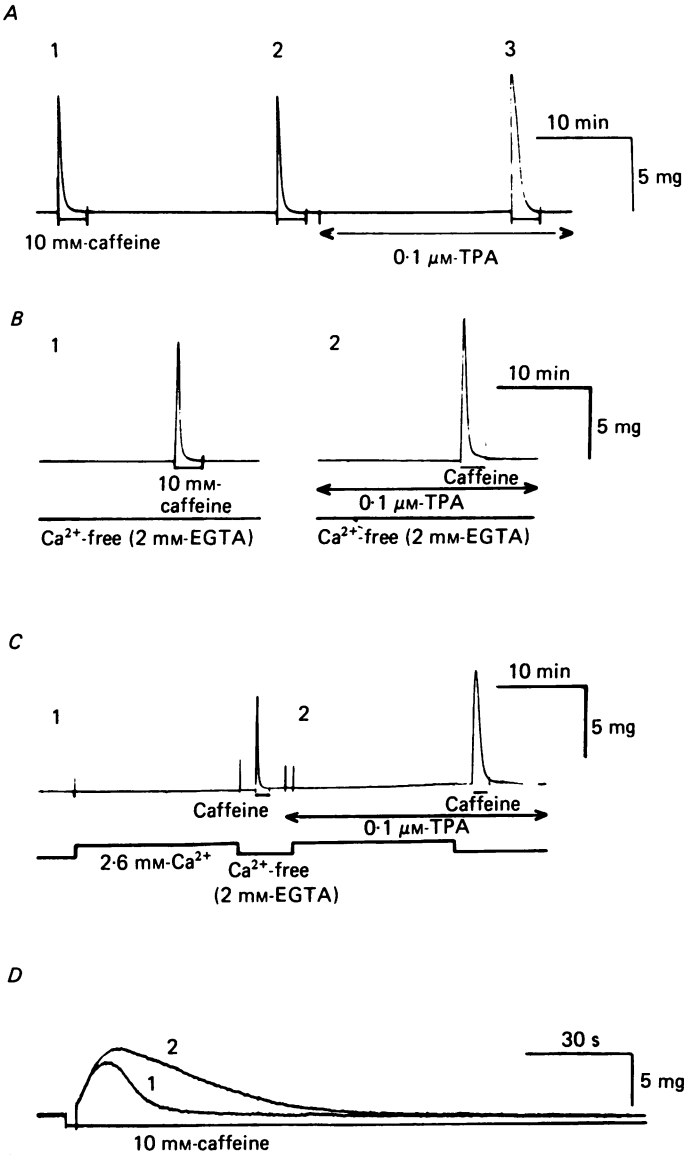

Fig. 6. Effects of 0·1 μM-TPA on the contraction induced by 10 mM-caffeine in intact muscle. *A*, in Krebs solution. 1 and 2, control; 3, in the presence of 0·1 μM-TPA. Caffeine was applied for 3 min in every 20 min. *B*, in Ca^{2+}-free, 2 mM-EGTA-containing solution. 1, control; 2, in the presence of 0·1 μM-TPA. After the Ca^{2+} store had been emptied by applying caffeine in Ca^{2+}-free, 2 mM-EGTA-containing solution, 2·6 mM-Ca^{2+} was applied for 20 min, the tissue was then bathed with Ca^{2+}-free EGTA-containing solution for 20 min in the presence or absence of 0·1 μM-TPA and 10 mM-caffeine was subsequently applied. *C*, TPA was applied during application of 2·6 mM-Ca^{2+} and during the following procedures. Experimental procedures were the same as in *B*, but the duration of application of Ca^{2+}-free EGTA-containing solution before caffeine application was less (2 min). 1, control; 2, in the presence of 0·1 μM-TPA. *D*, change of shape of caffeine contraction in Krebs solution with or without 0·1 μM-TPA. 1, control; 2, in the presence of TPA. TPA was applied for 20 min before caffeine application.

was applied to skinned muscles, the amplitude of contraction remained the same for over 120 min (Fig. 4*A*). The same phenomena were also observed by application of 0·3 μM- or 10 μM-Ca^{2+}. After the Ca^{2+}-induced contraction had reached a steady amplitude, TPA alone or TPA and PS was added in the Ca^{2+}-containing solution. When TPA was applied during the contraction induced by 0·1 μM-Ca^{2+}, it enlarged the amplitude of the contraction; however, in the presence of 1 μM-Ca^{2+}, TPA transiently elevated and then gradually reduced the amplitude of contraction. PS alone did not modify Ca^{2+}-induced contractions. When PS was applied with TPA, both actions of TPA were facilitated; Fig. 4*C* shows this dual effect on the contraction induced by 1 μM-Ca^{2+}. TPA with PS caused an initial enhancement and then delayed inhibition of the contraction induced by 1 μM-Ca^{2+}. Enhancement of the contraction induced by TPA with PS was also apparent in the contraction induced by 0·1 μM- or 0·3 μM-Ca^{2+} (120 min). The inhibitory action of TPA with PS on the Ca^{2+}-induced contraction appeared without the initial enhancement in the presence of 10 μM-Ca^{2+}. These results indicate that the concentration of Ca^{2+} or the amplitude of the contraction is important in determining whether TPA with PS only enlarges or also inhibits the amplitude of contraction.

To further investigate the mechanism of action of TPA with PS, the effects of these agents on the Ca^{2+}-independent contraction were observed (Fig. 5). The experimental procedure is shown in Fig. 5. After the contraction was evoked by application of 10 μM-Ca^{2+}, the tissue was rinsed with the relaxing solution until the tissue relaxed completely. Following application of the rigor-producing solution (see Methods) for 3 min, a small contraction was evoked. When 2 mM-$ATP_\gamma S$ with 0·3 μM-Ca^{2+} was applied (for 30 s or 5 min), the tissue produced a rapidly developing contraction. Subsequently applied rigor-producing solution halted the contraction at the level reached at the termination of application of $ATP_\gamma S$ and Ca^{2+}. (This result indicates the presence of two rigor states in skinned arterial smooth muscles, depending on whether or not the muscle was activated by treatment with Ca^{2+} and ATP before removal of ATP, as suggested by Kawai & Brandt (1976) in skinned crayfish muscles). Subsequently, when relaxing solution (a Ca^{2+}-free solution containing 10 mM-EGTA and 4 mM-MgATP) was applied to the tissue, the amplitude of contraction was enhanced further and sustained at the same amplitude for over 60 min (Ca^{2+}-independent contraction). When 0·1 μM-TPA with 50 μg PS/ml were applied during the contraction in this Ca^{2+}-free solution, the amplitude of contraction was not affected whether $ATP_\gamma S$ with 0·3 μM-Ca^{2+} was applied for 30 s or 5 min (Fig. 5*B*). This means that dual actions of TPA appear only on the Ca^{2+}-induced contraction.

Effects of TPA on the caffeine-induced contraction in intact and skinned muscles

Caffeine releases Ca^{2+} stored in cells. When caffeine, in concentrations over 10 mM, is applied, contraction in Ca^{2+}-free EGTA-containing solution only occurs once due to depletion of Ca^{2+} stored in cells (Itoh *et al.* 1983). To clarify the site of actions of TPA further, the effects of TPA on the Ca^{2+}-releasing mechanism were investigated. When 10 mM-caffeine was applied to the intact muscle for 3 min in every 20 min in Krebs solution, a constant amplitude of contraction was obtained (0·68 $\pm$ 0·12 times the contraction induced by 128 mM-K^+, $n = 5$). TPA (0·1 μM) marginally enlarged the caffeine-induced contraction in the presence or absence of Ca^{2+} (Fig. 6*A* and *B*:

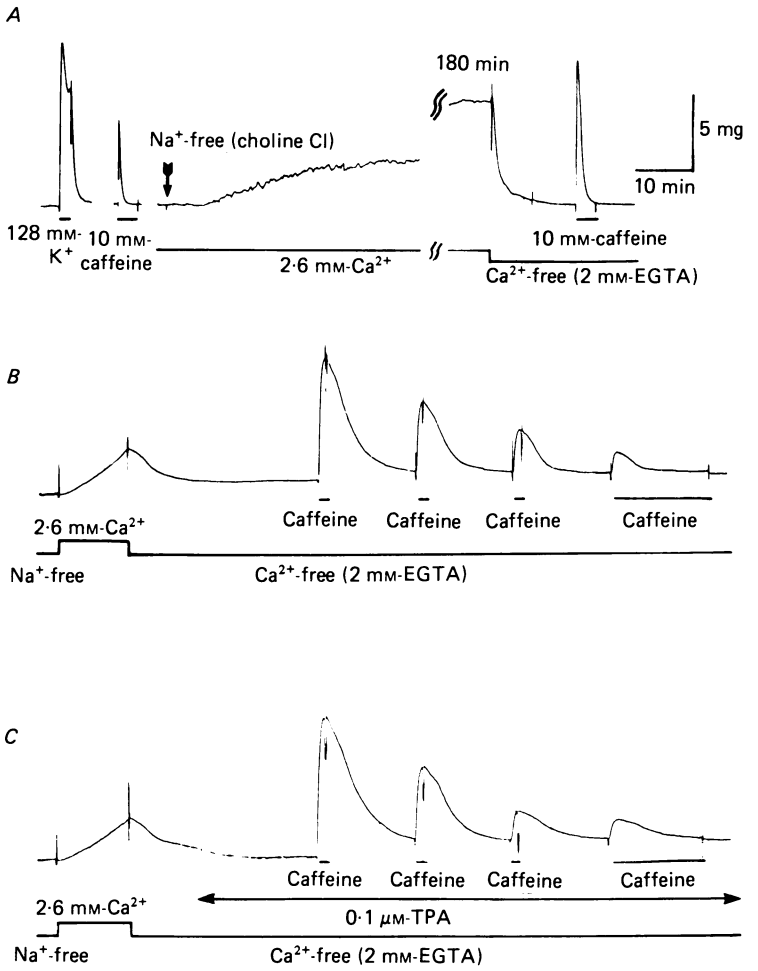

Fig. 7. Effect of 0·1 μM-TPA on the contractions generated by the repeated application of 10 mM-caffeine in Na^+-free and Ca^{2+}-free solution. *A*, contractions evoked by 128 mM-K^+ and caffeine (10 mM). Na^+-free solution (choline with 1 μM-atropine) was then applied for 3 h and the solution replaced with Ca^{2+}-free (2 mM-EGTA) solution; Na^+-free solution and caffeine were again applied. *B*, after loading the stores in 2·6 mM-Ca^{2+}, caffeine was repetitively applied for 10 s in every 10 min in Ca^{2+}-free, Na^+-free solution. *C*, 0·1 μM-TPA was added 20 min before the first caffeine application and was present for the rest of the experiment.

1·19 $\pm$ 0·05 times the control in the presence of Ca^{2+}; $n = 5$ and 1·20 $\pm$ 0·05 times the control in Ca^{2+}-free solution, $n = 5$). The effects of TPA on the amount of Ca^{2+} accumulated into the cells were observed from the amplitude of the caffeine-induced contraction (Fig. 6*C*). When Ca^{2+} was applied in the presence of TPA, the resulting caffeine-induced contraction was increased to 1·25 $\pm$ 0·08 times the control ($n = 3$). Fig. 6*D* shows shapes of the contraction induced by 10 mM-caffeine in Krebs solution in the presence or absence of TPA. TPA (0·1 μM) enlarged the amplitude and prolonged the duration of the caffeine-induced contraction.

Fig. 7 shows the effects of TPA on the contraction evoked by repetitively applied caffeine (10 mM) in Na^+- and Ca^{2+}-free solution in intact muscle (Na^+ was substituted by choline and 1 μM-atropine; 3 μM-guanethidine and 0·1 μM-TTX were added). When the tissue was immersed in Na^+-free solution, a small tonic contraction

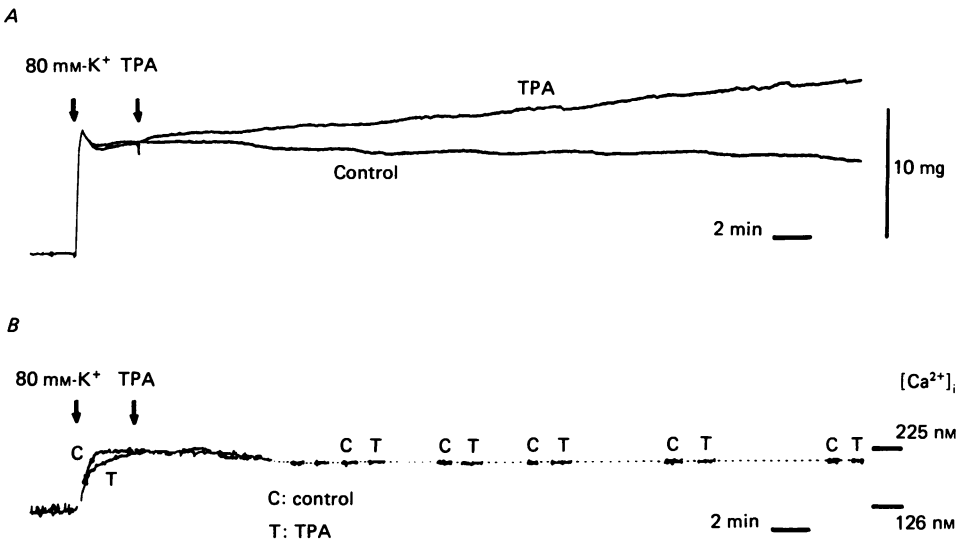

Fig. 8. Effects of 0·1 μM-TPA on the contraction evoked by 80 mM-K^+ in small strips of the porcine coronary artery (*A*) and on quin-2 fluorescence evoked by 80 mM-K^+ in single cells of the same artery (*B*). *A*, 80 mM-K^+ and TPA were applied at the arrows. *B*, TPA was applied after the fluorescence was constant in high-K^+ solution. Experimental procedures as described in Methods and Results.

developed within 30 min. Application of caffeine in Na^+- and Ca^{2+}-free solution produced a larger contraction than that evoked in Na^+-containing Ca^{2+}-free solution (Fig. 7*A*). Repetitive application of caffeine (10 mM) for 10 s produced contractions which steadily decayed in amplitude (Fig. 7*B*). When TPA was applied in the Na^+- and Ca^{2+}-free solution, the amplitude of the first caffeine-induced contraction was increased to 1·15 times the control, but the slope of the decay of amplitude was parallel to that observed in the absence of TPA (Fig. 7*C*). The relaxation of the tissue after removal of caffeine became slow in the presence of TPA (Fig. 7*C*). This means that the action of TPA has no relation to the influx of Ca^{2+} nor to the amount of Ca^{2+} stored in cells.

To observe the effects of TPA with PS on the Ca^{2+} accumulation into the store site in skinned muscles, the following procedure was used; after skinning the tissue, 0·3 μM-Ca^{2+} buffered with 2 mM-EGTA was applied for 1 min (recording the resulting Ca^{2+}-induced contraction), then the tissue was rinsed successively with a Ca^{2+}-free solution containing 4 mM-EGTA for 15 s and a Ca^{2+}-free solution containing 0·2 mM-EGTA for 4 min. Finally 25 mM-caffeine was applied in Ca^{2+}-free solution containing 0·5 mM-EGTA and recording of the resulting caffeine-induced contraction was made. In the above experimental procedures, the amplitudes of the contraction induced by

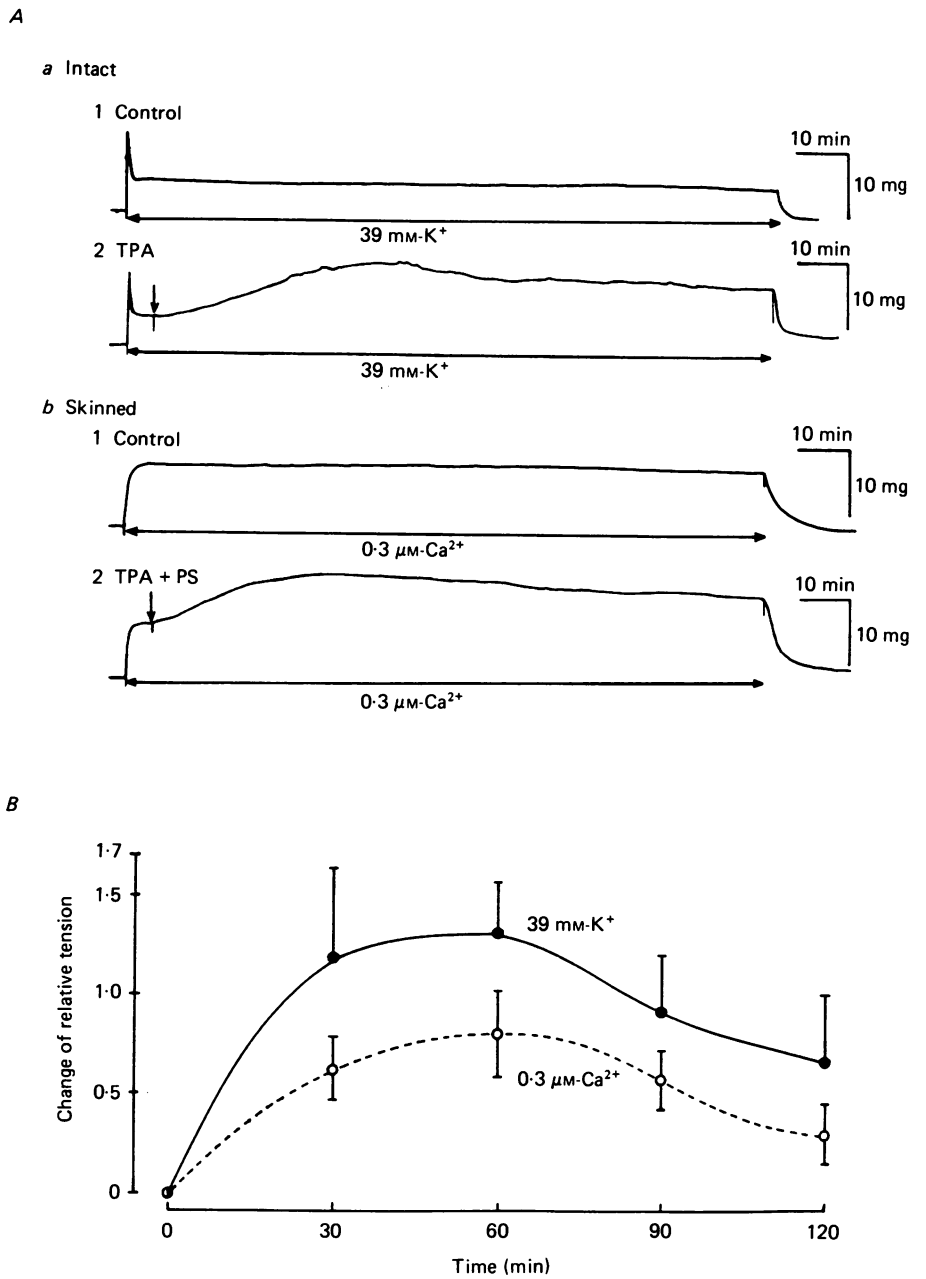

Fig. 9. Comparative study of TPA action on contraction in intact and in saponin-treated skinned muscle of rabbit mesenteric artery. *A*, shows actual records. *a*, the contraction induced by 39 mM-K^+ in intact muscle in the absence (1) or presence (2) of 0·1 μM-TPA. *b*, the contraction induced by 0·3 μM-Ca^{2+} in skinned muscle in the absence (1) or presence (2) of TPA with PS. TPA (0·1 μM) or TPA with PS (50 μg/ml) was applied at the arrow. *B*, changes in amplitude of the contraction after application of TPA are shown (the amplitude of contraction evoked in the presence of TPA was subtracted from that evoked in the absence of TPA (control)). Experiments were all done on the same arteries and animals ($n = 4$). Vertical bars represent S.D.

0·3 μM-Ca^{2+} and the caffeine-induced contraction were almost the same. With addition of 0·1 μM-TPA with 50 μg PS/ml, the amplitudes of the Ca^{2+}- and caffeine-induced contraction were increased in parallel. This means that enhancement of the amplitudes of the caffeine-induced and of the Ca^{2+}-induced contractions is due to a similar mechanism and this is not due to an increased accumulation of Ca^{2+} into the store site.

$[Ca^{2+}]_i$ measurement using quin-2

To prove directly whether TPA modulates the contraction by increasing the Ca^{2+}-influx or not, we measured the $[Ca^{2+}]_i$ using quin-2 fluorescence in dispersed single cells of the porcine coronary artery. At 1·0 mM-external Ca^{2+}, the $[Ca^{2+}]_i$ was 115–130 nM (125 $\pm$ 10 nM; $n = 5$) in the resting condition. Reduction of external Na^+ from 137 mM to 80 mM by replacement of Na^+ with Tris did not significantly alter the intensity of quin-2 fluorescence (K. Sumimoto, unpublished observation), but when Na^+ was replaced by 80 mM-K^+, the intensity of fluorescence provoked by quin-2 was increased, due to activation of voltage-dependent Ca^{2+} influx. TPA did not change the intensity of fluorescence elevated by 80 mM-K^+. However, TPA increased the amplitude of the contraction evoked by 80 mM-K^+ in the same artery. This means that TPA increases the contraction without changing the $[Ca^{2+}]_i$ (Fig. 8). Moreover, TPA also did not modify the quin-2 fluorescence signal in resting conditions (5 mM-extracellular K^+), but the resting tension was consistently increased as described previously.

The effects of TPA on the influx of Ca^{2+} as estimated from the intact and skinned muscles

Since the contraction induced by 39 mM-K^+ (tonic response) had almost the same amplitude as the contraction induced by 0·3 μM-Ca^{2+} in skinned muscles, we compared the effects of TPA on the mechanical response from the same artery. As shown in Fig. 9, when 0·1 μM-TPA with 50 μg PS/ml was applied after the contraction reached a steady level, the contraction was enhanced to the same extent with almost the same time course in both tissues (Fig. 9*A* and *B*).

DISCUSSION

Effects of TPA on the mechanical response

In the rabbit mesenteric artery, TPA showed initial enhancement and delayed inhibition of the contractions evoked by excess K^+ or 2·6 mM-Ca^{2+} in Ca^{2+}-free (2 mM-EGTA) solution in the presence of A23187 in intact muscles and by application of Ca^{2+} in saponin-treated skinned muscles. When small contractions were generated, TPA always elevated the contraction which was followed by a decay upon long exposure in intact and skinned muscles. On the other hand, TPA inhibited large contractions with or without generation of the transient increase in tension. Since the effects of TPA appeared to be the same in intact and skinned muscles, it may suggest that $[Ca^{2+}]_i$ or tension level is a key step on the mode of TPA actions. Larger inhibitory actions of TPA on contractions were observed in 2·6 mM-Ca^{2+} in the presence of A23187 (1 μM) than in 39 mM-K^+. Since A23187 at high concentrations activates protein kinase C (Nishizuka, 1984), TPA may activate protein kinase C more in the presence of Ca^{2+} and A23187 than 39 mM-K^+.

In skinned muscles, the dual actions of TPA were facilitated by application of PS. Since the activation of protein kinase C is dependent on the presence of acidic phospholipid, the TPA action on contractions may be due to activation of protein kinase C (Nishizuka, 1984).

Effects of TPA on the Ca^{2+}-independent contraction

Ca^{2+}-independent contraction was obtained by application of 10 mM-EGTA and 4 mM-MgATP-containing solution following application of $ATP_{\gamma}S$ with Ca^{2+} to the skinned muscle tissue. This Ca^{2+}-independent contraction is due to the irreversible thio-phosphorylation of MLC by MLCK in the presence of $ATP_{\gamma}S$ and Ca^{2+} (Cassidy *et al.* 1979). Since the thio-phosphorylated myosin is not dephosphorylated by phosphatase, Ca^{2+}-independent contractions are maintained. The amplitude of the Ca^{2+}-independent contraction depended on the duration of application of $ATP_{\gamma}S$ and 0·3 μM-Ca^{2+}. TPA with PS did not modify the contraction evoked for any duration of application. Since the action of TPA or DG on activation of protein kinase C is dependent on the concentration of Ca^{2+} in the presence of phospholipid (Takai, Kishimoto, Kikkawa, Mori & Nishizuka, 1979; Castaga, Takai, Kaibuchi, Sano, Kikkawa & Nishizuka, 1982; Mcphail, Clayton & Synderman, 1984) the mode of TPA action on the contraction is closely related to the presence of Ca^{2+}.

Nishikawa *et al.* (1983) found that applications of the protein kinase C activator, diolein (one of the 1,2-diacylglycerols) and PS, phosphorylated myosin, markedly decreased its affinity for MLCK, thus inhibiting actin-activated myosin ATPase in the presence of Ca^{2+} in cell-free systems. If that is the case in the mesenteric artery, activated protein kinase C would decrease the contractile activity, but in the present experiment, TPA and PS not only inhibited the contraction but also enhanced it. It remains unknown whether these dual actions of TPA on the mesenteric artery are due to phosphorylation of one protein or to many protein phosphorylations by activation of protein kinase C. Protein kinase C has a broad substrate specificity for many endogenous proteins and the physiological and biological significance of the covalent modification of these proteins has not been shown (Nishizuka, 1984).

Effects of TPA on the Ca^{2+} store site

In the mesenteric artery, Ca^{2+} stored in cells (presumably, sarcoplasmic reticulum) plays an essential role in the regulation of the contraction–relaxation cycle (Itoh *et al.* 1983, 1985*a*). The amplitude of the caffeine-induced contraction in the presence or absence of Ca^{2+} can be used as a useful indicator of the amount of Ca^{2+} stored in cells.

Application of TPA with PS, before or after addition of Ca^{2+} in intact muscles, did not modify the amplitude of subsequent caffeine-induced contractions. Furthermore, when the tissue was superfused in Na^{+}-free solution for over 30 min, Ca^{2+} extrusion was inhibited and short applications of caffeine could repeatedly produce contractions in Ca^{2+}-free solution with a small decay of the amplitude (Itoh *et al.* 1983). If TPA activates Ca^{2+} uptake into store sites or Ca^{2+} extrusion through the plasma membrane, the second or third application of caffeine would elicit a contraction of reduced amplitude. However, TPA with PS did not modify the decay in amplitude of contractions evoked by repetitive application of caffeine in Na^{+}-free solution. The ineffectiveness of TPA on the Ca^{2+} uptake into the store sites was also observed in skinned muscles. Therefore, TPA may not act primarily on the Ca^{2+}-uptake system

in the mesenteric artery in contrast to cardiac muscle (Iwasa & Hosey, 1984; Movsesian, Nishikawa & Adelstein, 1984). This conclusion was also supported from the effects of low concentrations of A23187 in relation to actions of TPA, i.e. the enhancement of the contraction induced by application of TPA in 39 mM-K^+-containing solution was almost the same as that observed in the presence or absence of 0·1 μM-A23187. Therefore, the action of TPA on the Ca^{2+} store site may be ruled out.

TPA extrudes intracellular Ca^{2+}, presumably due to activation of a Ca^{2+} pump at the plasma membrane in guinea-pig and human neutrophils (Lagast, Pozzan, Waldvogel & Lew, 1984). The Ca^{2+} transient in porcine coronary artery observed from the quin-2 fluorescence in high-K^+ solution was not modified in the presence of TPA. Therefore, even if the extrusion mechanism is activated by TPA, it does not play a major role in TPA-induced muscle relaxation in vascular smooth muscle. It has also been shown that TPA activates the Na^+–H^+ exchange mechanism and lowers the intracellular pH (Moolenaar, Trentoolen & Laat, 1984; Besterman, May, Levine, Cragoe & Cuatrecasas, 1985). However, in the mesenteric artery, this mechanism does not play a major role, because TPA also acts on contractions evoked in Na^+-free conditions.

Physiological significance of protein kinase C in contraction in vascular tissues as estimated from the action of TPA

Protein kinase C is widely distributed in tissues including vascular smooth muscles and $InsP_3$ and DG are synthesized through hydrolysis of PI-P_2 by agonist stimulation (Kuo *et al.* 1980; Baron, Cunningham, Strauss & Coburn, 1984; Suematsu *et al.* 1984; Hashimoto, Hirata & Ito, 1985). In vascular tissues, the physiological range of intracellular Ca^{2+} regulating the contraction–relaxation cycle is 0·1–1 μM (Itoh *et al.* 1983; Ueno, 1985). In the rabbit mesenteric artery, application of NA reduced the amount of PI-P_2, increased the amount of phosphatidic acid (indicates synthesis of DG) and also $InsP_3$ (direct measurement) (Hashimoto, Hirata, Itoh, Kanmura & Kuriyama, 1986). This means that NA leads to synthesis of $InsP_3$ and DG. DG may activate protein kinase C, thus producing dual actions on the contractile machinery as seen with TPA. However, actions of NA in relation to the mechanical response seem to be related to the initial enhancement of the mechanical response rather than the delayed inhibition. For example, the inhibition of the contraction induced by TPA with PS required more than 60 min in the presence of Ca^{2+} below 0·3 μM, and in increased concentrations of Ca^{2+} these agents reduced the onset of the inhibition to 10 min. On the other hand, PI-P_2 breakdown and actions of $InsP_3$ appeared within 10 s. In the rabbit mesenteric artery, Kanamaru, Inagaki, Ishikawa & Hidaka (1985) reported that TPA phosphorylated MLC through activation of protein kinase C. Presumably this action of TPA may be related to the delayed inhibition of contractions rather than the initial enhancement.

In conclusion, TPA initially enhanced and later inhibited contraction in the rabbit mesenteric artery. These actions were dependent on the Ca^{2+} concentration. In physiological Ca^{2+} concentration ranges, TPA and Ca^{2+} may act synergistically to cause activation of contractile proteins rather than inhibition. The underlying mechanisms involved in the excitatory actions of protein kinase C in relation to

synthesis of DG and also the synergistic actions of protein kinase C with $InsP_3$ on mechanical properties need clarification.

We thank Dr Kate Creed for her help in preparing the manuscript.

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