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EFFECTS OF A PHORBOL ESTER ON ACETYLCHOLINE-INDUCED Ca^{2+} MOBILIZATION AND CONTRACTION IN THE PORCINE CORONARY ARTERY

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SUMMARY

1. The effects of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, have been investigated on intact and chemically skinned muscle strips of the porcine coronary artery.

2. In the presence or absence of extracellular Ca^{2+}, TPA (0·1–1 nM) slightly enhanced the amplitude of ACh (10 μM)-induced contractions but at 100 nM, inhibited the contractions by approximately 50%.

3. ACh (10 μM) reduced the amount of [^{32}P]phosphatidylinositol 4,5-bisphosphate (PIP_2) and increased the amount of [^{32}P]phosphatidic acid (PA) in the presence or absence of Ca^{2+}. TPA (over 1 nM) dose-dependently inhibited the hydrolysis of PIP_2 induced by ACh.

4. ACh (over 0·1 μM) dose-dependently increased the intensity of fura-2 fluorescence in dispersed single-cell suspensions. TPA (over 1 nM) dose-dependently inhibited the increase of the Ca^{2+} transient evoked by ACh, but it did not modify the ionomycin-induced Ca^{2+} transient or the resting fluorescence. These inhibitory effects of TPA occurred over a similar dose range to that which inhibited ACh-induced PIP_2 break-down.

5. When the relationship between ACh-induced contraction amplitude and Ca^{2+} transient was observed in the presence or absence of 10 nM-TPA, TPA greatly reduced the Ca^{2+} transient but did not modify the amplitude of contraction.

6. In saponin-treated skinned muscle strips, TPA (10 nM) or 1,2-diolein (50 μg/ml) with phosphatidylserine (PS; 50 μg/ml) increased the amplitude of contraction evoked by various concentrations of Ca^{2+} (0·1–1·0 μM) without any change in the maximum amplitude of the Ca^{2+}-induced contraction.

7. TPA (10 nM) with PS (50 μg/ml) increased the amplitude of contraction evoked by 10 μM-inositol 1,4,5-trisphosphate in chemically skinned muscle strips.

8. It is concluded that TPA inhibits the ACh-induced $[Ca^{2+}]_i$ increase by inhibiting the hydrolysis of PIP_2, but that it enhances the Ca^{2+} sensitivity of the contractile proteins. These results indicate that ACh-induced contractions are controlled by negative feed-back regulation of PIP_2 hydrolysis together with a positive feed-back regulation of the Ca^{2+} sensitivity of the contractile proteins. This may depend on the on-going level of protein kinase C activation.

INTRODUCTION

In vascular smooth muscle cells, the production of inositol 1,4,5-trisphosphate ($InsP_3$) is stimulated by several Ca^{2+}-mobilizing agonists such as vasopressin (Nabika, Velletri, Lovenberg & Beaven, 1985), angiotensin II (Griendling, Rittenhouse, Brock, Ekstein, Gimbrone & Alexander, 1986), noradrenaline (NA) (Hashimoto, Hirata, Itoh, Kanmura & Kuriyama, 1986) and acetylcholine (ACh) (Sasaguri, Hirata, Itoh, Koga & Kuriyama, 1986). Evidence obtained from saponin-treated chemically skinned muscle has shown that $InsP_3$ triggers a contraction by releasing $^{45}Ca^{2+}$ from non-mitochondrial intracellular storage sites (Suematsu, Hirata, Hashimoto & Kuriyama, 1984; Somlyo, Bond, Somlyo & Scarpa, 1985; Yamamoto & van Breemen, 1985; Hashimoto *et al.* 1986; Sasaguri *et al.* 1986). Thus, it has been proposed that $InsP_3$ acts as a second messenger of Ca^{2+}-mobilizing hormones for the release of Ca^{2+} from intracellular stores in vascular smooth muscle cells.

In contrast, diacylglycerol (DG), a co-product of $InsP_3$ synthesis from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), activates the Ca^{2+}-activated and phospholipid-dependent enzyme, protein kinase C, and has a crucial role in signal transduction of physiologically active substances in non-muscle cells (for reviews see Berridge & Irvine, 1984; Nishizuka, 1984, 1986; Abdel-Latif, 1986). Protein kinase C is, however, widely distributed in many cells, including those of vascular smooth muscle (Kuo, Andersson, Wise, Mackerlova, Salomonsson, Brackett, Katoh, Shoji & Wrenn, 1980; Minakuchi, Takai, Yu & Nishizuka, 1981). Tumour-promoting phorbol esters bind to and permanently activate protein kinase C and mimic the effects of Ca^{2+}-mobilizing hormones on the phosphorylation of cytosolic proteins (Nishizuka, 1984, 1986). In intact smooth muscle, several phorbol esters activate protein kinase C and elevate the basal tone (Danthuluri & Deth, 1984; Rasmussen, Forder, Kojima & Scriabine, 1984) and/or increase the amplitude of contractions induced by either high K^+ or agonists (Baraban, Gould, Peroutka & Snyder, 1985).

In chemically skinned rabbit mesenteric artery, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a most powerful tumour promoter, enhanced Ca^{2+}-induced contractions (Itoh, Kanmura, Kuriyama & Sumimoto, 1986*b*). Furthermore, it has been shown that phorbol 12,13-didecanoate (PDD) or phorbol 12,13-dibutyrate (PDBu), but not its inactive isomer 4α-PDD, dose-dependently enhance contractions evoked by Ca^{2+} in skinned porcine carotid and coronary arteries (Chatterjee & Tejada, 1986; Miller, Hawkin & Wells, 1986). These findings suggest that protein kinase C acts synergistically with Ca^{2+} to elicit full cellular responses in vascular tissues.

Recently, it has been proposed that protein kinase C may also exert a negative feed-back control on several hormone receptors coupled to PIP_2 break-down in non-muscle cells (Nishizuka, 1986). Such a feed-back control mechanism has also been reported on the angiotensin II receptor (Brock, Rittenhouse, Powers, Ekstein, Gimbrone & Alexander, 1985), 5-hydroxytryptamine receptor (Roth, Nakaki, Chuang & Costa, 1986), and on the NA receptor (Colucci, Gimbrone & Alexander, 1986; McMillan, Chernow & Roth, 1986) in vascular smooth muscle cells. Thus, the physiological roles of protein kinase C on agonist-induced responses in vascular smooth muscle cells remain unclear.

In the present study, the ability of TPA to modify ACh-induced PIP_2 hydrolysis, Ca^{2+} transients and contractions has been examined in porcine coronary artery. In addition, the effects of TPA or 1,2-diolein on Ca^{2+}-induced contractions in skinned porcine coronary artery have also been studied. Using this approach, it was hoped to clarify the physiological role of protein kinase C in agonist-induced mechanical responses in vascular smooth muscle cells.

METHODS

Tissue preparation

Fresh porcine hearts were obtained from a local slaughterhouse and transported in ice-cold oxygenated Krebs solution to the laboratory. The left descending coronary artery (diameter 0·5–1·0 mm) was carefully dissected and opened longitudinally after connective tissue and endothelium had been removed as described previously (Itoh *et al.* 1986*b*). A circular strip (0·3 mm in length, 0·05–0·07 mm in width and 0·025–0·03 mm in thickness) was prepared by cutting transversly.

Force measurement and recording

Mechanical responses were measured by attaching a circular strip to a strain gauge (UL-2, Shinko Co., Tokyo, Japan). The tissue was superfused in a chamber with a capacity of 0·5 ml filled with Krebs solution. The perfusate was changed rapidly from one end, while the solution already present was simultaneously aspirated off with a water pump from the other end. The resting tension was adjusted to obtain the maximal contraction in 128 mM-K^+ and was not greater than 15 μN. To suppress sympathetic nerve activity, 0·3 μM-tetrodotoxin (TTX) and 3 μM-guanethidine were present in the Krebs solution throughout the experiments. All experiments were performed at 25 °C.

Skinned muscle preparations were obtained by treatment with saponin (25 μg/ml) in a relaxing solution for 20 min. To prevent deterioration of the Ca^{2+} sensitivity of the contractile proteins, 0·1 μM-calmodulin was added throughout the experiment (Itoh *et al.* 1986*b*). The tension-pCa relationship was obtained by cumulative application of solutions containing various Ca^{2+} concentrations buffered with 4 mM-EGTA, in ascending concentration steps. To measure $InsP_3$-induced contractions in skinned muscle, the concentration of EGTA was reduced to 0·2 mM to reduce its Ca^{2+}-chelating action (Hashimoto *et al.* 1986; Itoh *et al.* 1986*b*).

Preparation of fura-2-loaded single smooth muscle cells

Fura-2-loaded dispersed single smooth muscle cells were prepared by essentially the same methods as described before (Sumimoto & Kuriyama, 1986). The large branches of the right and left porcine coronary arteries (2–3 mm diameter) were dissected and the adventitia, connective tissue and endothelium were carefully removed. The muscle strips were fixed at a slightly extended length to a plastic plate in Ca^{2+}-free HEPES-buffered solution (EGTA not present), equilibrated for 1 h at 37 °C, then placed in a Ca^{2+}-free HEPES-buffered solution containing collagenase 1 mg/ml (Worthington Biochem, Freehold, NJ, U.S.A. Type III) and bovine serum albumin 2 mg/ml. Following digestion for 1 h at 37 °C, loosened muscle strips were chopped into small pieces and suspended in a polyethylene tube with the same solution. Fura-2-acetoxymethylester (1·5 μM) was added to the tube and the digestion was continued for a further 1·5 h at 37 °C. The suspension containing single cells and undigested residue was filtered through double nylon gauze and the residue was trapped on the gauze. The single cells were collected by centrifugation at 30 *g* for 5 min. The final fura-2-loaded cell pellet was washed twice, resuspended to give approximately 10^6 cells/ml in fresh HEPES-buffered solution and equilibrated for 30 min at room temperature. To measure cytosolic free Ca^{2+} concentration, the intensity of the fluorescence of fura-2-loaded cells was monitored at 25 °C in a Hitachi 650–40 fluorescence spectrophotometer with a thermostatically controlled cell holder, using an excitation wavelength of 340 nm (slit width 4 nm) and emission wavelength of 490 nm (slit width 10 nm). The concentration of cytosolic free Ca^{2+} was calculated as previously described by Grynkiewicz, Poenie & Tsien (1985).

Assays of phosphatidylinositol 4-monophosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA)

The amounts of ^{32}P-labelled [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA in muscle strips of the porcine coronary artery were measured before and after application of 10 μM-ACh. The left descending coronary artery was prepared, as described above. Muscle strips were labelled in phosphate-free Krebs solution containing 40 μCi $^{32}P_i$/ml (specific radioactivity 30–40 Ci/mmol; Japan Atomic Energy Research Institute) at 37 °C for 3 h. The labelled strips were then washed three times with the above solution without $^{32}P_i$ and incubated for 40 min at 25 °C in the presence or absence of TPA (1–100 nM). ACh was applied in the presence or absence of TPA for various times 1 min after removal of Ca^{2+} and addition of 2 mM-EGTA. The reaction were halted at each time by adding an ice-cold solvent containing chloroform, methanol and concentrated HCl (100:200:2, v/v), and strips were then homogenized in a glass homogenizer. Crude phospholipid extracts in the solvent were chromatographed on Silica Gel-60 plates (Merck), according to Billah & Lapetina (1982). The plates were then autoradiographed using Fuji X-ray film for 12 h. The fractions corresponding to PIP_2, PIP and PA on the plates were excised, and counted for radioactivity in a liquid scintillation counter (Hashimoto *et al.* 1986).

Solutions

Krebs solution. The Krebs solution contained (mM): Na^+, 137; K^+, 5·9; Mg^{2+}, 1·2; Ca^{2+}, 2·6; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; Cl^-, 134; 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. A 128 mM-K^+ solution was prepared by replacing all NaCl with KCl, isosmotically. In Ca^{2+}-free solutions, the $CaCl_2$ was replaced with $MgCl_2$ and 2 mM-EGTA was added. The HEPES-buffered Krebs solution contained (mM): Na^+, 140; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1; HPO_4^{2-}, 1; glucose, 10; HEPES, 20 (pH 7·4).

Relaxing and Ca^{2+}-containing solutions. The relaxing solution contained (mM): potassium methanesulphonate, 90; piperazine-*N*-*N*′-bis-(2-ethanesulphonic acid) (PIPES), 20; magnesium methanesulphonate, 5·1; ATP, 5·2; ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 4. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of calcium methanesulphonate to 4 mM-EGTA. The binding constants used in this experiment have been previously reported (Itoh, Kanmura & Kuriyama, 1986*a*). The pH of the solutions was adjusted to 6·8 at 25 °C with KOH and the ionic strength was standardized at 0·17 M by changing the amount of potassium methanesulphonate added.

Drugs

The chemicals used were acetylcholine chloride (ACh), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1-(5-isoquinoline sulphonyl)-2-methylpiperazine (H-7), tetrodotoxin (TTX) and adenosine 5′-triphosphate disodium salt (Na_2ATP; Sigma Chemical Co., St Louis, U.S.A.), ionomycin (Wako Chemical Industries Ltd, Osaka, Japan), phosphatidylserine (PS, beef brain dissolved in chloroform) and 1,2-diolein (dissolved in hexane; Serdary Research Laboratories, Ontario, Canada), saponin (ICN Pharmaceuticals Inc., Cleveland, OH, U.S.A.), guanethidine (Tokyo Kasei Co., Tokyo, Japan), ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), fura-2-acetoxymethylester (fura-2/AM), piperazine-*N*-*N*′-bis-(2-ethanesulphonic acid) (PIPES) and 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) (Dozin Lab., Kumamoto, Japan). All other chemicals were of the highest reagent grade. TPA was dissolved in 100% dimethyl sulphoxide (DMSO, Nakarai Chemical Co., Kyoto, Japan) to give a stock concentration of 8·1 mM and stored at −80 °C in the dark. For experiments using intact muscles, the final concentration of TPA was prepared by dilution with Krebs solution and sonicated (Branson sonifier cell disruptor 200). Turbidity did not occur up to 0·1 μM-TPA and DMSO (0·001%) itself did not modify mechanical responses in intact muscle tissues. For the skinned muscle experiments, the hexane (solvent for diolein) and chloroform (solvent for PS) were removed using a stream of N_2 gas, and the residues were suspended in ice-cold distilled water with sonication for 5 min at 0 °C, as described by Nishikawa, Sellers, Adelstein & Hidaka (1984). Finally, TPA and PS or diolein and PS were mixed and were again sonicated for 3 min. The appropriate amounts of these mixtures was added to the relaxing or Ca^{2+}-containing solutions. Ionomycin was dissolved in 100% DMSO and the final concentration was diluted to 0·001% as described in the case of TPA. Inositol 1,4,5-trisphosphate ($InsP_3$) and 1-(5-isoquinoline sulphonyl)-2-methylpiperazine (H-7) were kindly

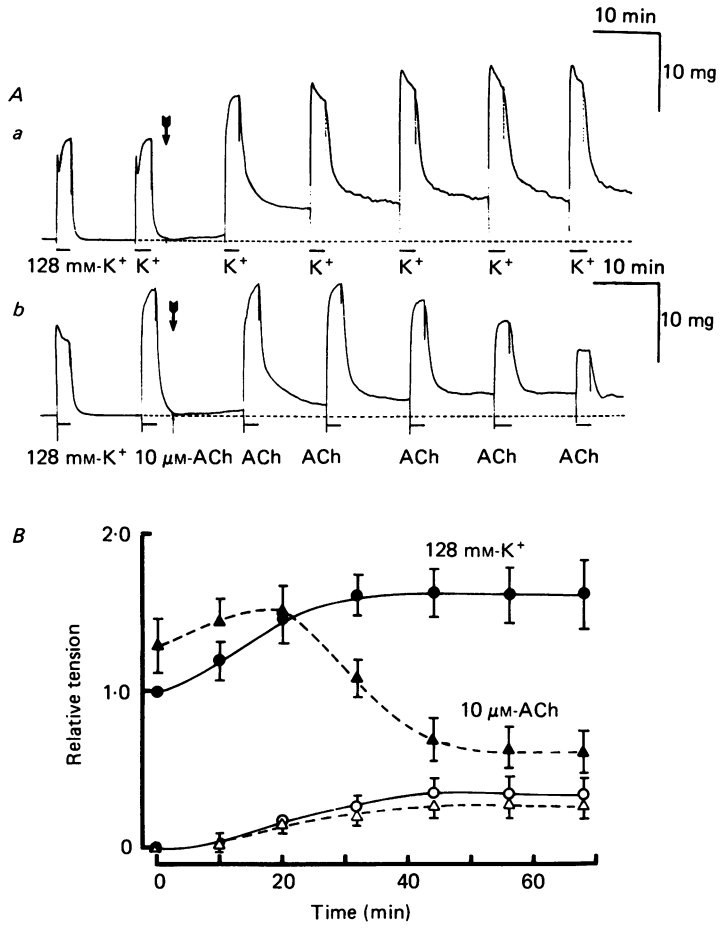

Fig. 1. Effects of 100 nM-TPA on the contraction evoked by 128 mM-K^+ or 10 μM-ACh in the porcine coronary artery. *A*, typical examples of TPA action on the contractions evoked by 128 mM-K^+ (*Aa*) or 10 μM-ACh (*Ab*). 128 mM-K^+ or 10 μM-ACh was applied for 3 min at 10 min intervals. In *Ab*, to compare the amplitudes of contraction induced by 128 mM-K^+ or by 10 μM-ACh, high-K^+ solution was first applied in both series of experiments. *B*, the effects of 100 nM-TPA on the amplitude of contraction evoked by 128 mM-K^+ and 10 μM-ACh and on resting tension. The maximum amplitude of the contraction was defined as that evoked by 128 mM-K^+ in the absence of TPA ($n = 6$). The amplitudes of contraction evoked by either stimulant were constant over 120 min in the absence of TPA. Vertical scale shows the relative tension. Horizontal scale shows the time after TPA application. To prevent neuronal effects, guanethidine (3 μM) and TTX (0·3 μM) were present throughout the experiments. Vertical bars indicate S.D. ($n = 6$).

provided by Dr M. Hirata, Department of Biochemistry, Faculty of Dentistry, Kyushu University and Professor H. Hidaka, Mie University, respectively.

Statistics

The measured values were expressed as the mean $\pm$ S.D. and the number of observations. The statistical significance was assessed using Student's t test for paired or unpaired values. P values less than 0·05 were considered significant.

RESULTS

Effects of TPA on mechanical responses in intact muscles

Figure 1 shows the effects of 100 nM-TPA on the contraction evoked by repetitive application of 128 mM-K^+ or 10 μM-ACh. The amplitude of contraction evoked by 10 μM-ACh was larger ($1{\cdot}31 \pm 0{\cdot}18$ times, $n = 15$) than that of 128 mM-K^+ in the presence of 3 μM-guanethidine and 0·3 μM-TTX. When 10 μM-ACh or 128 mM-K^+ was applied for 3 min at 10 min intervals, the amplitude of contractions evoked by either

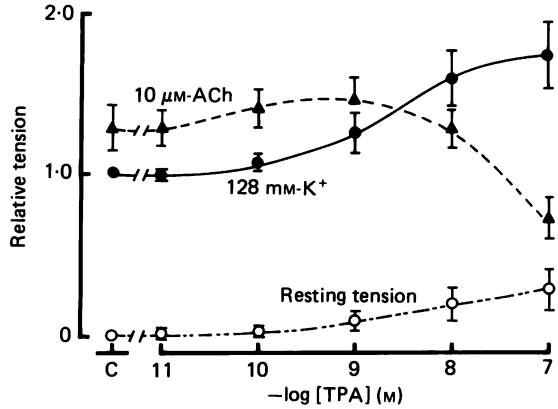

Fig. 2. Dose–response relationship of TPA on resting tension and on contractions evoked by 128 mM-K^+ or 10 μM-ACh. The maximum amplitude of contraction was defined as that evoked by 128 mM-K^+ in the absence of TPA. 128 mM-K^+ or 10 μM-ACh was applied for 3 min. The effects of TPA on the resting tension in the ACh experimental series are shown but almost the same effects were observed in the 128 mM-K^+ experiments. TPA was applied 40 min before application of either ACh or K^+. Vertical bars indicate S.D. ($n = 6$). C represents the control (before application of TPA).

stimulant remained the same for over 120 min (in the presence of 3 μM-guanethidine and 0·3 μM-TTX). In the presence of 100 nM-TPA, the amplitude of the contraction evoked by 128 mM-K^+ gradually increased in a time-dependent manner and a steady level was obtained 45 min after application of 100 nM-TPA ($1{\cdot}63 \pm 0{\cdot}13$ times the control, $n = 8$). TPA (100 nM) initially enhanced the amplitude of contraction induced by 10 μM-ACh (within 20 min) but subsequently inhibition of the ACh response was observed. Since the maximum effects of TPA were obtained after about 40 min exposure to this substance, a 40 min pre-incubation period was used in subsequent experiments.

Figure 2 shows the effects of various concentrations of TPA on the contractions evoked by 10 μM-ACh or 128 mM-K^+. TPA (> 10 nM) dose-dependently enhanced the amplitude of the high-K^+-induced contraction and elevated the resting tension. On the other hand, TPA showed dual actions on the ACh-induced contraction. In the concentration range 0·1–1 nM, 40 min incubation with TPA enhanced the response to ACh whilst at higher concentrations (> 10 nM), contractions to ACh were reduced.

In Ca^{2+}-free solution containing 2 mM-EGTA, ACh produces a contraction due to the release of Ca^{2+} from intracellular storage sites in the porcine coronary artery (Itoh, Kajiwara, Kitamura & Kuriyama, 1982). To investigate the role of intra- and

extracellular Ca^{2+} on the actions of TPA, ACh was applied in Ca^{2+}-free solution containing 2 mM-EGTA in the presence or absence of various concentrations of TPA.

Figure 3 shows the effects of TPA on the contraction evoked by 10 μM-ACh and on the resting tension in Ca^{2+}-free solution containing 2 mM-EGTA. After constant-

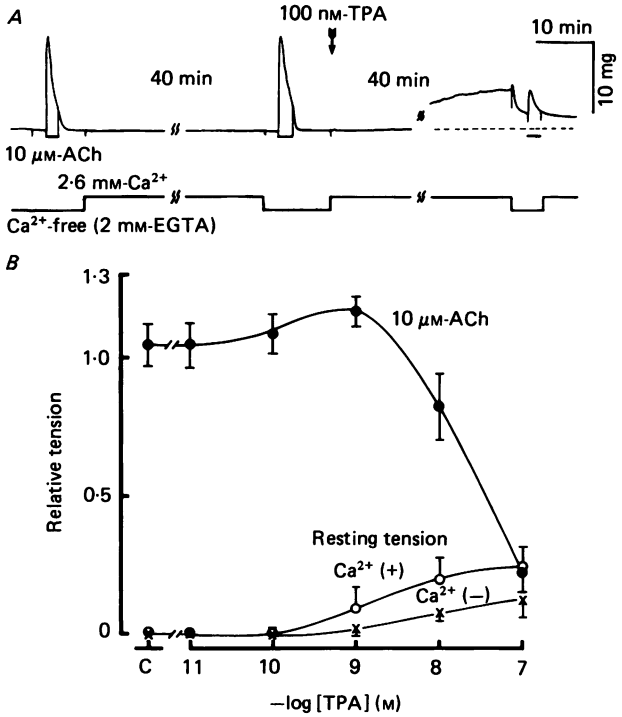

Fig. 3. Effects on TPA on the contraction evoked by 10 μM-ACh in Ca^{2+}-free solution containing 2 mM-EGTA. *A*, typical example of the effect of 100 nM-TPA on the ACh-induced contraction. Following incubation in Krebs solution (containing 2·6 mM-Ca^{2+}) for 40 min, ACh (10 μM) was applied for 2 min after 2 min removal of Ca^{2+}. When 128 mM-K^+ was applied using the same protocol, a contraction was not provoked. After the contraction to ACh was constant in size, 100 nM-TPA was applied (arrow). *B*, dose-dependent effects of TPA on the contraction evoked by 10 μM-ACh in Ca^{2+}-free solution containing 2 mM-EGTA and on resting tension in the presence or absence of Ca^{2+}. Only one concentration of TPA was applied to each muscle strip. The maximum amplitude of the contraction evoked by 128 mM-K^+ in the absence of TPA was defined as unity. C represents the control. Vertical bars indicate S.D. ($n = 6$).

amplitude contractions to 10 μM-ACh had been obtained in Krebs solution, the solution was replaced with Ca^{2+}-free solution for 2 min and then 10 μM-ACh was re-applied. The amplitude of contraction evoked by 10 μM-ACh in Ca^{2+}-free solution was reproducible over the duration of an experiment. When the response to ACh in Ca^{2+}-free solution was normalized to that of 128 mM-K^+ or 10 μM-ACh in Krebs solution, it was $1{\cdot}05 \pm 0{\cdot}07$ times ($n = 15$) or $0{\cdot}80 \pm 0{\cdot}05$ times ($n = 18$) the control, respectively. TPA had dual actions on the ACh-induced contraction in Ca^{2+}-free solution similar to those exerted on the ACh-induced contractions in the presence of

Ca^{2+}. At low concentrations (0·1–1 nM), TPA enhanced but at high concentrations (over 10 nM), it inhibited the ACh contractions. These inhibitory actions of TPA in the absence of Ca^{2+} were greater than those observed in the presence of Ca^{2+}. The TPA-induced increase in resting tension was observed in both the presence and absence of Ca^{2+}, but it was greater in the presence of Ca^{2+}.

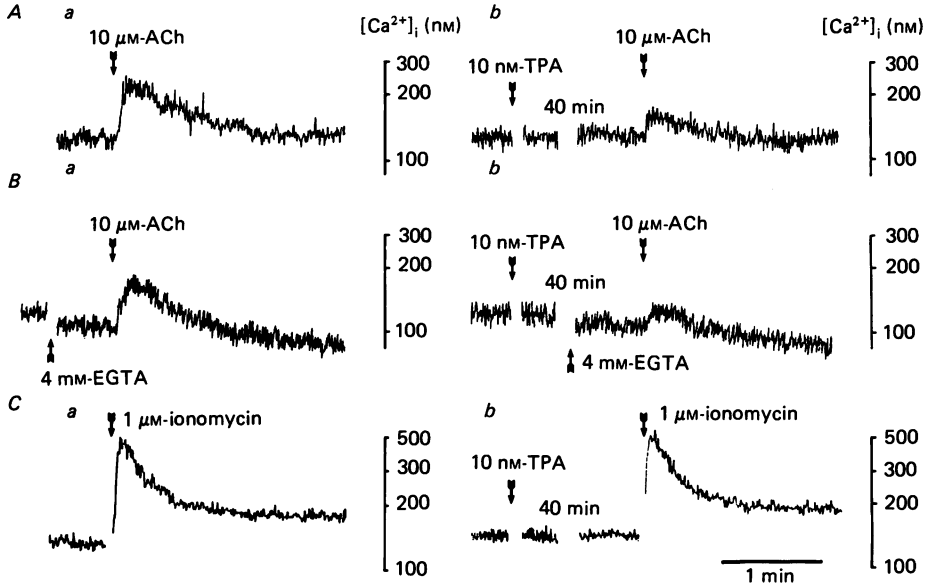

Fig. 4. Effects of 10 nM-TPA on the Ca^{2+} transient evoked by 10 μM-ACh or 1 μM-ionomycin in the presence or absence of Ca^{2+}. The Ca^{2+} transient was measured using the intensity of the fura-2 fluorescence in dispersed single-cell suspensions of porcine coronary artery at 25 °C. Left panel shows the control response and right panel shows the effect of TPA. TPA (10 nM) was applied for 40 min prior to application of 10 μM-ACh or 1 μM-ionomycin. *Aa* and *Ab*, in a solution containing 1 mM-Ca^{2+}. *Ba* and *Bb*, in a solution containing 1 mM-Ca^{2+} and 4 mM-EGTA. *Ca* and *Cb*, 1 μM-ionomycin was applied in a solution containing 1 mM-Ca^{2+}. When 10 μM-ACh or 1 μM-ionomycin was applied after 40 min incubation in each condition, the control response was not significantly different. All agents were added at arrows.

Effects of TPA on the ACh-induced Ca^{2+} transient

To investigate the possible mechanisms responsible for the TPA-induced inhibition of ACh-induced contractions, the intracellular concentration of Ca^{2+}, $[Ca^{2+}]_i$, was measured using fura-2 fluorescence in dispersed single cells of the porcine coronary artery. At rest, $[Ca^{2+}]_i$ was 127·7 ± 13·4 nM ($n = 5$) in a solution containing 1 mM-Ca^{2+}. When 4 mM-EGTA was added in the presence of 1 mM-Ca^{2+} (the calculated free Ca^{2+} concentration in the solution was 23 nM and the pH was adjusted by NaOH to 7·4), $[Ca^{2+}]_i$ was rapidly reduced to 105·3 ± 9·1 nM within 5 s ($n = 5$, $P < 0·05$) and was followed by a further gradual decrease, reaching a minimum at 3 min (at 3 min, it was 93·5 ± 5·1 nM, $n = 5$, $P < 0·05$). When 10 μM-ACh was applied to the bath, the intensity of fluorescence provoked by the fura-2–Ca^{2+} complex was transiently increased within 10 s (in the presence of 1 mM-Ca^{2+}, the value was 227·7 ± 16·7 nM,

$n = 5$, and in the solution containing 1 mM-Ca^{2+} with 4 mM-EGTA, it was 176·4 ± 13·0 nM, $n = 5$). This was followed by a gradual decay in both solutions; in Ca^{2+}-containing solution, the steady level was slightly higher than the basal level (Fig. 4, the value was 145·6 ± 4·5 nM, $n = 5$, $P < 0·05$).

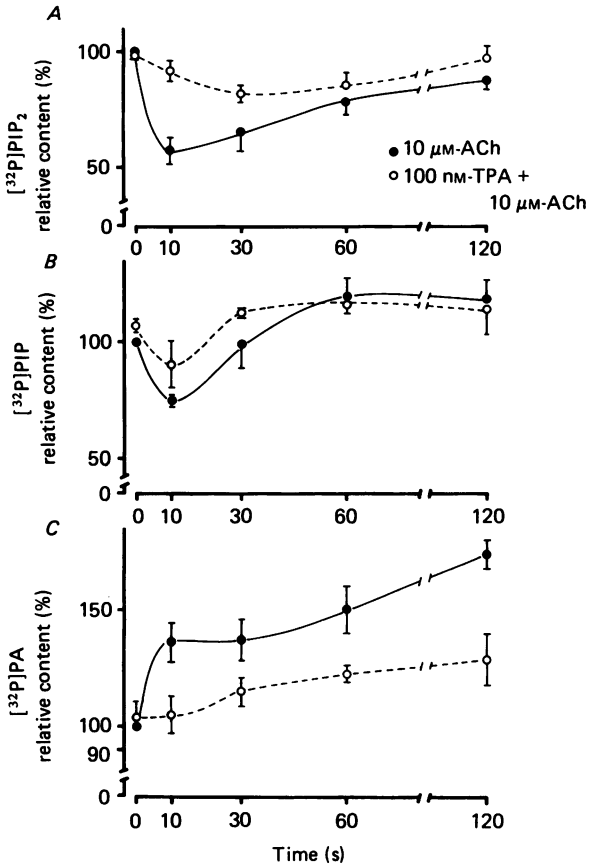

Fig. 5. Effects of 100 nM-TPA on changes in the amount of ^{32}P-labelled phospholipids induced by 10 μM-ACh in Ca^{2+}-free solution containing 2 mM-EGTA. TPA (100 nM) was applied for 40 min prior to application of ACh. *A*, time-dependent changes in the amount of ^{32}P-labelled phosphatidylinositol 4,5-bisphosphate ([^{32}P]PIP_2) induced by 10 μM-ACh in Ca^{2+}-free solution. *B* and *C*, changes in the amount of ^{32}P-labelled phosphatidylinositol 4-monophosphate ([^{32}P]PIP) and ^{32}P-labelled phosphatidic acid ([^{32}P]PA), respectively. ACh (10 μM) was applied at time zero 1 min after removal of Ca^{2+} in the presence (○) or absence (●) of 100 nM-TPA. The amounts of each phospholipid prior to application of 100 nM-TPA were each defined as 100 %. The assay methods are described in the Methods. Vertical bars indicate the mean ± S.D. ($n = 5$).

TPA (10 nM) did not modify fluorescence intensity in the resting condition but it markedly inhibited the increased fluoresence provoked by 10 μM-ACh in either the presence or absence of Ca^{2+} (in the presence of 10 nM-TPA and in 1 mM-Ca^{2+}-containing solution the $[Ca^{2+}]_i$ evoked by 10 μM-ACh was 171·2 ± 8·3 nM, $n = 5$, and in 1 mM-Ca^{2+} with 4 mM-EGTA-containing solution, it was 142·1 ± 12·3 nM, $n = 5$).

When 1 μM-ionomycin was applied to a solution containing 1 mM-Ca^{2+}, the intensity of the fluoresence increased rapidly and was gradually followed by a decay to a steady level. The peak $[Ca^{2+}]_i$ in the presence of 1 μM-ionomycin was $501{\cdot}1 \pm 92{\cdot}8$ nM ($n = 5$). TPA (10 nM) did not modify the ionophore-induced increase in the intensity of fluorescence (Fig. 4*C*).

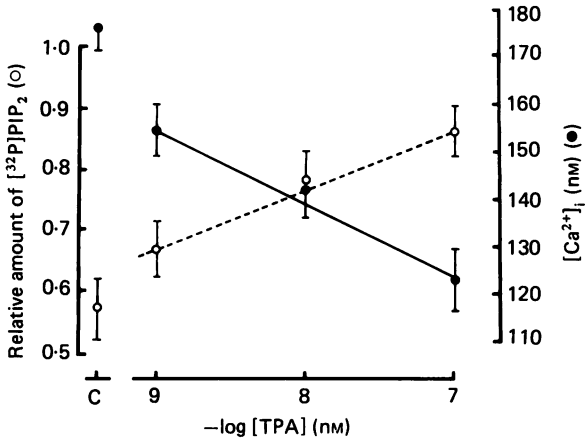

Fig. 6. Effects of TPA on changes induced by 10 μM-ACh in the amount of $[^{32}P]PIP_2$ measured in smooth muscle strips and on the increase of $[Ca^{2+}]_i$ measured in dispersed muscle cells in Ca^{2+}-deficient solution. The amount of $[^{32}P]PIP_2$ (○) was measured 10 s after application of 10 μM-ACh in the presence or absence (control; C) of TPA 1 min after replacement with Ca^{2+}-free solution containing 2 mM-EGTA. To measure the Ca^{2+} transient, a similar experimental protocol was used except for using Ca^{2+}-free solution containing 1 mM-Ca^{2+} and 4 mM-EGTA. The amount of $[^{32}P]PIP_2$ without application of ACh and TPA was normalized as 1·0. The maximum increase of the Ca^{2+} transient induced by 10 μM-ACh (●) was measured in the presence or absence (control; C) of TPA. The $[Ca^{2+}]_i$ was calculated from the peak intensity of the fura-2 fluorescence in the presence of 10 μM-ACh as described in Methods and values were expressed as the absolute values. TPA was applied for 40 min prior to application of ACh. Vertical bars indicate the mean ± S.D. ($n = 4$–6).

Effects of TPA on the change of [^{32}P]phosphoinositides in the presence of ACh

To investigate further the actions of TPA on mechanical responses, the effects of TPA on the amounts of $[^{32}P]PIP_2$, $[^{32}P]PIP$ and $[^{32}P]PA$ induced by ACh in Ca^{2+}-free solution were determined. The amounts of total radioactivity per milligram of protein of individual phospholipids measured 1 min after removal of Ca^{2+} from the tissue in the absence of TPA and ACh were normalized as 1·0. The absolute values were 71776 ± 6196 c.p.m./mg protein for $[^{32}P]PIP_2$, 16067 ± 2488 c.p.m./mg protein for $[^{32}P]PIP$ and 16452 ± 225 c.p.m./mg protein for $[^{32}P]PA$ ($n = 5$). When 10 μM-ACh was applied to the muscle strip for 10 s after removal of Ca^{2+} for 1 min, the amount of $[^{32}P]PIP_2$ was reduced to $0{\cdot}57 \pm 0{\cdot}06$ times the control ($n = 5$). After 30 s, the amount of $[^{32}P]PIP_2$ was $0{\cdot}653 \pm 0{\cdot}08$ times the control ($n = 5$), whilst after 120 s, the amount was $0{\cdot}87 \pm 0{\cdot}04$ times the control ($n = 5$). When the amount of $[^{32}P]PIP$ was measured at various times after application of 10 μM-ACh, the time-dependent changes showed a biphasic pattern, i.e. after application of ACh, the

amount of [^{32}P]PIP was transiently decreased (at 10 s, 0·74 ± 0·04 times the control) and then slightly enhanced (at 60 s, 1·12 ± 0·08 times the control, $n = 5$). When the amount of [^{32}P]PA was measured at various times after application of 10 μM-ACh, it was immediately increased to 1·37 ± 0·09 times the control at 10 s ($n = 5$) and gradually increased (at 30 s, 1·40 ± 0·10 times the control, $n = 5$, and at 120 s, 1·74 ± 0·06 times, $n = 5$).

When muscle strips were treated with 100 nM-TPA for 40 min, the amount of [^{32}P]PIP was slightly increased (1·07 ± 0·03 times the control, $n = 5$), but the amounts of [^{32}P]PIP_2 and [^{32}P]PA were not significantly changed (0·98 ± 0·02 times the control for PIP_2, $n = 5$, and 1·04 ± 0·07 times for PA, $n = 5$). Figure 5 shows the effects of 100 nM-TPA on the changes in the quantities of [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA after application of 10 μM-ACh in Ca^{2+}-free solution containing 2 mM-EGTA. TPA inhibited the rate of ACh-induced [^{32}P]PIP_2 break-down (in the presence of 100 nM-TPA, 0·85 ± 0·05 times the control at 10 s, $n = 5$, $P < 0·05$, and 0·78 ± 0·03 times the control at 30 s, $n = 5$, $P < 0·05$), but the values obtained after 120 s exposure to ACh were not significantly different from those of the control ($n = 5$, $P > 0·05$). TPA inhibited the increase in the amount of [^{32}P]PA produced by ACh at any given measured time up to 120 s. TPA inhibited the ACh-induced reduction of [^{32}P]PIP, but had no effect on its elevation. Figure 6 shows the dose-dependent effects of TPA on the hydrolysis of [^{32}P]PIP_2 in muscle strips and on the increase in $[Ca^{2+}]_i$ induced by 10 μM-ACh in dispersed smooth muscle cells of the porcine coronary artery.

Effects of TPA on the contractile proteins in intact and chemically skinned muscles

Figure 7 shows the effects of TPA on the ACh-induced contraction in the porcine coronary artery. ACh (> 0·1 μM) produced a dose-dependent contraction which was maximal at a concentration of 10 μM. When 1 nM-TPA was applied, the ACh concentration-effect relationship was shifted to the left with an increase in the maximum response. TPA, in concentrations over 10 nM, elevated the resting tension but reduced the amplitude of ACh-induced contractions (Fig. 7*A*).

The relationship between the amplitude of ACh-induced contractions in coronary muscle strips and the Ca^{2+} transient in suspensions of single muscle cells in the presence or absence of 10 nM-TPA is shown in Fig. 7*B*. TPA (10 nM) did not significantly modify the amplitude of contraction evoked by any given concentration of ACh ($P > 0·05$). As shown in Fig. 7*B*, however, 10 nM-TPA increased the resting tension without any change in $[Ca^{2+}]_i$ measured by fura-2 fluorescence ($[Ca^{2+}]_i$ was 128·8 ± 15·3 nM, $n = 15$, in the absence of TPA and it was 131·9 ± 18·2 nM, $n = 15$, following application of 10 nM-TPA for 40 min), and it significantly inhibited the increase in $[Ca^{2+}]_i$ evoked by ACh (> 1 μM; $P < 0·05$). These results suggest that in the presence of TPA, the contractile proteins of the smooth muscle cells may be sensitized to Ca^{2+}.

To study the action of TPA on contractile proteins more directly, its effects on Ca^{2+}-induced contractions were observed in skinned muscle strips. To produce a contraction, various concentrations of Ca^{2+} buffered with 4 mM-EGTA were cumulatively applied in a step-wise manner. A23187 (3 μM) was applied prior to the application of Ca^{2+} to avoid spurious effects due to release of Ca^{2+} from the store (Itoh, Kanmura & Kuriyama, 1985). Since the action of TPA on the contractile

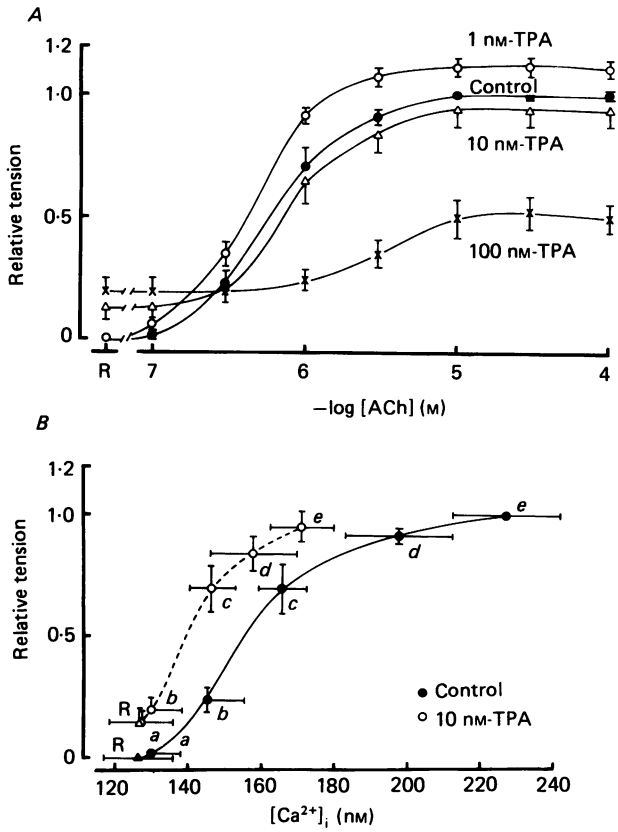

Fig. 7. Effects of TPA on the contraction and on the Ca^{2+} transient provoked by ACh. *A*, ACh was applied for 2 min at 8 min intervals. After the control response had been recorded, TPA was applied 40 min before and during application of ACh. The maximum response evoked by 10 μM-ACh in the absence of TPA was defined as unity. Vertical bars represent mean ± S.D. ($n = 6$). *B*, effects of 10 nM-TPA on the contraction and the Ca^{2+} transient provoked by ACh. The Ca^{2+} transient was measured using the fura-2-loaded single-cell suspension and the tension was measured using small muscle strips in the presence (○) or absence (●) of 10 nM-TPA. The times taken to reach the peak amplitude of contraction and the peak of the Ca^{2+} transient induced by ACh were about 30–90 s and 5–10 s, respectively. The relative tension data are reproduced from Fig. 7*A*. R represents resting tension. *a*–*e* indicate the concentrations of ACh: *a*, 0·1 μM; *b*, 0·3 μM; *c*, 1 μM; *d*, 3 μM; *e*, 10 μM. Bars indicate mean ± S.D. ($n = 6$).

proteins is enhanced in the presence of phosphatidylserine (PS) (Itoh *et al.* 1986*b*), TPA was applied in the presence of 50 μg PS/ml. The experimental procedures employed are shown in Fig. 8*A*, i.e. after skinning the tissues, the first cumulatively evoked contractions were provoked by various concentrations of Ca^{2+} (the 1st trial). The muscle strip was then allowed to relax and subsequently exposed to 0·1 μM-Ca^{2+} for 40 min in the presence or absence of 10 nM-TPA with 50 μg PS/ml. When Ca^{2+}-induced contractions were subsequently provoked (the 2nd trial), the response of control tissues (in the absence of TPA with PS), was the same as that observed at

the 1st trial. However, exposure to TPA with PS enhanced the contractions evoked by low concentrations of Ca^{2+} (0·1–1 μM) without any effect on the maximum Ca^{2+}-induced response (Fig. 8*B*). PS (50 μg/ml) did not modify the contraction induced by any given concentration of Ca^{2+} (0·3–10 μM). 1,2-diolein (50 μg/ml), a natural DG, had similar but lesser effects on the Ca^{2+}-induced contraction in the presence of PS

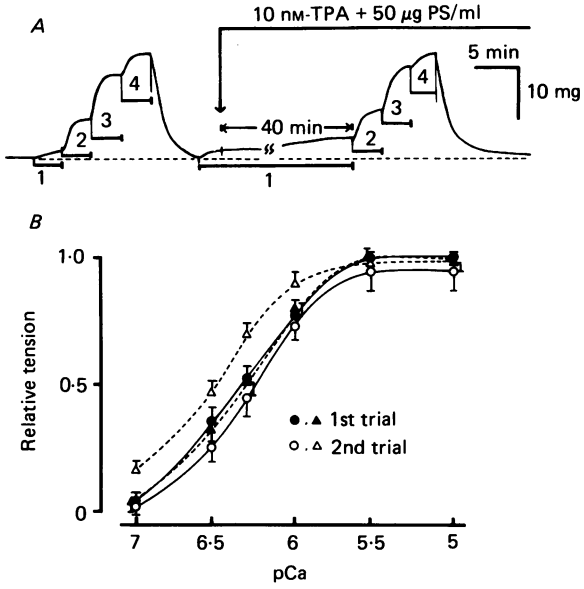

Fig. 8. Effects of 10 nM-TPA with 50 μg PS/ml on the Ca^{2+}-induced contraction in chemically skinned muscle strips of the porcine coronary artery. *A*, the experimental protocol and a typical example of the action of TPA with PS on the Ca^{2+}-induced contraction. 1, 0·1 μM-Ca^{2+}; 2, 0·3 μM-Ca^{2+}; 3, 1 μM-Ca^{2+}; 4, 10 μM-Ca^{2+}. *B*, effects of TPA with PS on the pCa–tension relationship in chemically skinned muscle strips. After skinning the tissue, various concentrations of Ca^{2+} were cumulatively applied in a step-wise manner from low to high (the 1st trial; ●, ▲). Subsequently, 0·1 μM-Ca^{2+} was applied for 40 min in the presence (△) or absence (○) of TPA with PS after the muscle had been completely relaxed by wash-out of the applied Ca^{2+}. Various concentrations of Ca^{2+} were again applied (the 2nd trial). The results of a second trial in the absence of TPA with PS were similar to the first one. Calmodulin (0·1 μM) was applied throughout the experiment. Vertical bars indicate the mean ± S.D. ($n = 3$).

(50 μg/ml). Furthermore, this action of DG analogues with PS was inhibited by 50 μM-1-(5-isoquinoline sulphonyl)-2-methylpiperazine (H-7), a potent inhibitor of protein kinase C (not shown). These results indicate that simultaneous application of TPA and PS may activate protein kinase C and enhance the sensitivity of the contractile proteins to Ca^{2+}.

Figure 9 shows the effects of 10 nM-TPA with 50 μg PS/ml on the contraction evoked by 10 μM-$InsP_3$ in chemically skinned muscle strips. In a solution containing 0·3 μM-Ca^{2+} buffered with 0·2 mM-EGTA, successive exposures to $InsP_3$ at 20 min intervals produced repeated contractions. These were abolished by prior application of 3 μM-A23187 (due to depletion of stored Ca^{2+}) or by exposure to a solution containing 0·3 μM-Ca^{2+} buffered with 4 mM-EGTA (due to a strong buffering effect on

the released Ca^{2+}) as previously reported (Itoh *et al.* 1985; Kanmura, Itoh & Kuriyama, 1987). This result shows that the $InsP_3$-induced contraction is due to the release of Ca^{2+} from the cellular storage sites. When 10 nM-TPA with 50 μg PS/ml was applied, the amplitudes of contraction induced by 0·3 μM-Ca^{2+} and by 10 μM-$InsP_3$ were gradually enhanced in a time-dependent manner. After 40 min application of TPA with PS, the amplitude of $InsP_3$-induced contractions was enhanced to 1·7

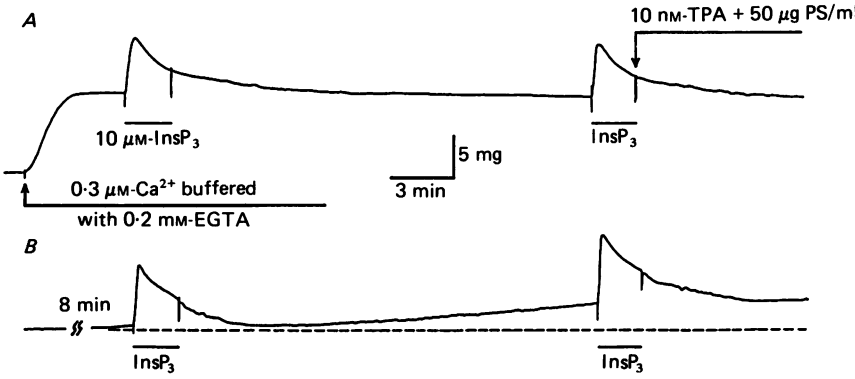

Fig. 9. Effects of 10 nM-TPA with 50 μg PS/ml on the contraction induced by 10 μM-$InsP_3$ in saponin-treated skinned muscle strips. To evoke contraction, a solution containing 0·3 μM-Ca^{2+} buffered with 0·2 mM-EGTA was applied. $InsP_3$ (10 μM) was applied for 2 min at 20 min intervals during the contraction induced by a 0·3 μM-Ca^{2+} solution buffered with 0·2 mM-EGTA. After the control response had been recorded (*A*), TPA with PS was applied followed by exposure to 10 μM-$InsP_3$ as described in *A* (*B*). *A* and *B* are continuous records.

times the control ($n = 3$). PS (50 μg/ml) itself did not modify the contraction induced by 0·3 μM-Ca^{2+} and 10 μM-$InsP_3$. Since $InsP_3$ (10 μM) did not itself modify the pCa–tension relationship when observations were made in a solution containing various concentrations of Ca^{2+} (0·1–10 μM) buffered with 4 mM-EGTA (not shown), a direct action of $InsP_3$ on the contractile apparatus seems unlikely. These results may indicate, therefore, that TPA with PS enhances the $InsP_3$-induced contraction due to an increase in the Ca^{2+} sensitivity of the contractile proteins.

DISCUSSION

Effects of TPA on ACh-induced hydrolysis of PIP_2

In porcine coronary artery, ACh produces an initial phasic and a later tonic contraction with no change in either membrane potential or membrane resistance (Ito, Kitamura & Kuriyama, 1979; Itoh *et al.* 1982). Such a phenomenon has been termed pharmaco-mechanical coupling (Somlyo & Somlyo, 1968). At least the phasic component of this response is thought to be due to release of Ca^{2+} from intracellular stores, because it persists in Ca^{2+}-free solution (Itoh *et al.* 1982). Recently, Sasaguri *et al.* (1986) reported that ACh rapidly hydrolyses PIP_2 with production of PA and $InsP_3$ in porcine coronary artery. $InsP_3$ has been shown to release Ca^{2+} from non-mitochondrial stores (Suematsu *et al.* 1984; Smith, Smith & Higgins, 1985;

Yamamoto & van Breemen, 1985) and to produce a contraction due to release of Ca^{2+} from stores in chemically skinned vascular smooth muscle (Somlyo *et al.* 1985; Hashimoto *et al.* 1986). PA is produced by rapid phosphorylation of DG which is synthesized through agonist-induced hydrolysis of PIP_2 (Nishizuka, 1984; Abdel-Latif, 1986; Takuwa, Takuwa & Rasmussen, 1986), and DG activates protein kinase C and enhances Ca^{2+}-induced contractions in chemically skinned muscle (Chatterjee & Tejada, 1986; Miller *et al.* 1986; Itoh *et al.* 1986*b*; Kanmura *et al.* 1987). These findings suggest that pharmaco-mechanical coupling may be associated with the production of $InsP_3$ and DG from the hydrolysis of polyphosphoinositides.

The present results have confirmed those of previous reports (Sasaguri *et al.* 1986; Sumimoto & Kuriyama, 1986). They have shown that ACh rapidly hydrolysed PIP_2, elevated $[Ca^{2+}]_i$ and produced a contraction in the presence or absence of Ca^{2+}. TPA (100 nM) inhibited all these ACh-induced responses in the presence or absence of Ca^{2+} but did not affect the ionomycin-induced Ca^{2+} transient. Ca^{2+} ionophores (ionomycin and A23187) act directly on Ca^{2+} stores and release Ca^{2+} through a mechanism different from that of $InsP_3$ (Berridge & Irvine, 1984; Itoh *et al.* 1985; Suematsu *et al.* 1985; Arslan, Di Virgilio, Beltrame, Tsien & Possan, 1986). As estimated from the effects of TPA on ACh-induced PIP_2 break-down and Ca^{2+} transient, both responses were similarly inhibited by TPA. Therefore, TPA may inhibit the ACh-induced Ca^{2+} transient due to a reduction in the synthesis of $InsP_3$ by inhibiting the hydrolysis of PIP_2. Furthermore, TPA enhanced the $InsP_3$- and Ca^{2+}-induced contractions in chemically skinned porcine coronary artery, as did DG. These results further support the postulate that in this tissue, $InsP_3$ and DG may have an essential role in ACh-induced pharmaco-mechanical coupling.

Similar inhibitory effects of TPA on agonist-induced PIP_2 break-down have also been reported in thrombin-stimulated human platelets (De Chaffoy de Courcelles, Roevens & Van Belle, 1984; Halenda & Feinstein, 1984; Watson & Lapetina, 1985) and these actions are inhibited by H-7, a potent inhibitor of protein kinase C in platelets (Inagaki, Kawamoto & Hidaka, 1984; Tohmatsu, Hattori, Nagao, Ohki & Nozawa, 1986). These results suggest that TPA may activate protein kinase C and directly or indirectly inhibit the ACh-activated phospholipase C activity in smooth muscle cells of the porcine coronary artery. Thus, protein kinase C may exert a negative feed-back control on ACh-induced PIP_2 break-down (synthesis of $InsP_3$ and DG) and the subsequent increase in $[Ca^{2+}]_i$.

Effects of TPA on contractile proteins

Using fura-2 fluorescence, TPA (> 1 nM) consistently inhibited the ACh-induced increase in $[Ca^{2+}]_i$ in a dose-dependent manner. However, the ACh-induced contraction was enhanced by low concentrations of TPA (0·1–1 nM) but inhibited by higher concentrations of TPA (100 nM). TPA enhanced the contraction evoked by 80 mM-K^+ without any marked change in $[Ca^{2+}]_i$ in intact muscles of the porcine coronary artery (Itoh *et al.* 1986*b*). A possible explanation for these findings is that low concentrations of TPA sensitize the contractile apparatus to Ca^{2+}, thus enhancing the contraction, whilst at higher TPA concentrations, the inhibitory effect on the Ca^{2+} transient becomes dominant, with consequent inhibition of contraction. This hypothesis is supported by the results obtained from chemically skinned muscle

strips. In these experiments TPA with PS increased the amplitude of contraction induced by low concentrations of Ca^{2+} (0·1–1·0 μM) without any change in the amplitude of the maximal Ca^{2+}-induced contraction, as does 1,2-diolein. These findings were consistent with results obtained in the rabbit coronary artery (Kanmura *et al.* 1987).

TPA activates Na^+–H^+ exchange and causes cytoplasmic alkalinization in various secretory cells (Moolenaar, Trentoolen & Laat, 1984; Besterman, May, Levine, Cragoe & Cuatrecasas, 1985; Swann & Whitaker, 1985). However, this mechanism does not seem to play a major role in the action of TPA on the contractile apparatus, because TPA enhanced contractions induced by high K^+ in Na^+-free conditions in intact muscle (unpublished observations). Furthermore, TPA increased the Ca^{2+} sensitivity of the contractile apparatus in chemically skinned muscle, under well-buffered conditions. The possible role of this exchange system in the ACh-induced hydrolysis of PIP_2 and/or the Ca^{2+} transient in vascular tissues should be clarified by further investigations.

In the present experiments, ACh only transiently increased $[Ca^{2+}]_i$ and this was followed by a gradual decay of fluorescence to near-basal levels within 1 min. In contrast, the ACh contraction was maintained for several minutes. Since fura-2 is a Ca^{2+}-chelating agent (Grynkiewicz *et al.* 1985) and the estimated concentration of this agent in the cell is 0·1–0·3 mM, the results may not indicate the absolute Ca^{2+} movement or Ca^{2+} concentration, especially if these changes are localized to restricted zones in the cells. However, using the Ca^{2+}-sensitive photoprotein aequorin (a weaker Ca^{2+} buffer than fura-2), Morgan & Morgan (1984) reported that in the ferret portal vein, agonists only transiently increased the $[Ca^{2+}]_i$ whilst the contraction was maintained. Furthermore, in porcine coronary artery, ACh-induced phosphorylation of myosin was also a transient phenomenon (unpublished observations). Therefore, in the presence of ACh, Ca^{2+} may be only transiently mobilized and the contraction maintained by an increase in the Ca^{2+} sensitivity of the contractile mechanism.

Physiological significance of protein kinase C on agonist-induced contractions as estimated from the action of TPA

Vascular smooth muscles are rich in protein kinase C (Kuo *et al.* 1980; Minakuchi *et al.* 1981), and agonist action stimulates the synthesis of $InsP_3$ and DG through the hydrolysis of PIP_2 (Brock *et al.* 1985; Hashimoto *et al.* 1986; Takuwa *et al.* 1986; Sasaguri *et al.* 1986). Park & Rasmussen (1986) reported that in the bovine tracheal smooth muscle, 12-deoxyphorbol 13-isobutyrate, another phorbol ester, phosphorylates the same cytosolic and contractile proteins as does carbachol when these agents were applied for relatively long periods. In the present study, the role of protein kinase C activated by DG has been estimated using TPA. TPA was found to have dose-related dual actions on the ACh-induced contractions, a factor which may be related to the on-going level of protein kinase C activation. It seems reasonable to conclude, therefore, that in physiological conditions, protein kinase C may act synergistically with Ca^{2+} to maintain contraction, but that under highly activated conditions, this enzyme may act as a feed-back controller of processes mediated by the muscarinic receptor. Further experiments are required to clarify whether the

precise site of action of TPA is the GTP-binding protein, phospholipase C, or the receptor itself.

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