Br. J. Pharmacol. (1993), **110**, 875–881

Eicosanoid-induced Ca^{2+} release and sustained contraction in Ca^{2+}-free media are mediated by different signal transduction pathways in rat aorta

Ryuichi Kurata, [1]Issei Takayanagi & [2]Tetsuhiro Hisayama

Department of Chemical Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1, Miyama, Funabashi, Chiba 274, Japan

1 The effects of 12-*O*-tetradecanoyl 4β-phorbol 13-acetate (β-TPA) on the inositol 1,4,5-trisphosphate (IP_3) production, Ca^{2+} release from the intracellular Ca^{2+} stores and sensitization of contractile apparatus, induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and U46619, a thromboxane A_2-mimetic, were studied, using fura-2-loaded and -unloaded rat thoracic aortic strips.

2 Both eicosanoids had characteristic patterns of responses in Ca^{2+}-free, 2 mM EGTA-containing solution (Ca^{2+}-free solution). They induced transient increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) without corresponding transient contraction, but produced delayed, sustained contraction, where $[Ca^{2+}]_i$ was returned to the basal level.

3 Treatment with β-TPA for 60 min reduced the eicosanoids-induced IP_3 production, suggesting that the treatment inhibits PIP_2 breakdown.

4 The treatment also attenuated $[Ca^{2+}]_i$ transient induced by the eicosanoids, but not by caffeine (an IP_3-independent releaser of stored Ca^{2+}), in fura-2-loaded preparations incubated in Ca^{2+}-free solution.

5 In contrast in the presence of β-TPA, the sustained contractions evoked by the eicosanoids in Ca^{2+}-free solution were potentiated, suggesting that the sites of actions of β-TPA and the eicosanoids may differ from each other.

6 $PGF_{2\alpha}$ and U46619 utilize different and parallel signal transduction pathways to release Ca^{2+} by IP_3 produced by PIP_2 breakdown (β-TPA-sensitive), and to increase the sensitivity of contractile apparatus, in which protein kinase C may not be involved (β-TPA-insensitive).

Keywords: Vascular smooth muscle; prostaglandin $F_{2\alpha}$; U46619; calcium; fura-2; Ca stores; protein kinase C; phorbol ester; inositol trisphosphate

Introduction

Activation of Ca^{2+}-mobilizing receptors, has been shown to stimulate PIP_2 breakdown, catalyzed by phospholipase C. This reaction results in an increased formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), which it has been suggested, contributes to vascular smooth muscle contraction by releasing Ca^{2+} from the sarcoplasmic reticulum (SR; Berridge, 1989), stimulating Ca^{2+} entry (Litten *et al.*, 1987) and sensitizing contractile apparatus by activating protein kinase C (PKC; Rasmussen *et al.*, 1987; Ruzycky & Morgan, 1989; Karaki, 1989). One approach to monitor the IP_3-induced intracellular Ca^{2+} release and the PKC-mediated increased sensitivity of contractile elements is to study the muscle responses to receptor agonists under Ca^{2+}-free conditions. For example, transient and sustained contractions in Ca^{2+}-free media have been assumed to be due to the released Ca^{2+} and changes in Ca^{2+}-sensitivity of the contractile apparatus (Bradley & Morgan, 1987; Karaki, 1989).

We previously reported that under Ca^{2+}-free conditions, prostaglandins $F_{2\alpha}$ ($PGF_{2\alpha}$) elicited an immediate transient increase in $[Ca^{2+}]_i$ without any corresponding contraction. On the other hand, the large size of the sustained contraction which follows occurred without any increase in $[Ca^{2+}]_i$ (Hisayama *et al.*, 1990). Similar observation has been reported by other researchers (Bradley & Morgan, 1987; Heaslip & Sickels, 1989; Ozaki *et al.*, 1990). However, the α_1-adrenoceptor stimulant, phenylephrine caused a transient increase in $[Ca^{2+}]_i$ accompanied by a corresponding transient contraction. This was followed by a small, sustained contraction where $[Ca^{2+}]_i$ was reduced to the basal level (Hisayama *et al.*, 1990). The $PGF_{2\alpha}$-like actions are shared by endothelin-1 (Huang *et al.*, 1990a). These results seem to point to three issues intimately involved in receptor-mediated signal transduction, Ca^{2+}-mobilization and contractile mechanisms: (1) the $[Ca^{2+}]_i$ transient does not necessarily produce a transient contraction; (2) in contrast, contraction that is sustained in nature can be produced without any appreciable increase in $[Ca^{2+}]_i$; (3) the relative predominance between induction of the transient contraction by released Ca^{2+} from the SR and the sustained contraction brought about by increased responsiveness of contractile apparatus to Ca^{2+}, depends on the type of the receptor stimulated. The third case seems to be important, because it suggests that the signal transduction utilized by Ca^{2+} mobilizing receptors could not be accounted for by a unitary system, such as PIP_2 breakdown.

The present work was undertaken to obtain insight mainly into the third point. It is well known that short-term treatment with phorbol esters results in inhibition of receptor-mediated PIP_2 breakdown (Orellana *et al.*, 1985; Leeb-Lundberg *et al.*, 1985; Roth *et al.*, 1986; Litten *et al.*, 1987; Slivka & Insel, 1988; Kaya *et al.*, 1989; Araki *et al.*, 1989). This providues us with a possible method of addressing this point, i.e., to learn which of the functional response(s) could be induced by the PIP_2 breakdown reaction. Thus, the effects of 12-*O*-tetradecanoyl 4β-phorbol 13-acetate (β-TPA) on IP_3 production, $[Ca^{2+}]_i$ transient and sustained contraction in response to thromboxane A_2 receptor agonists (U46619 and $PGF_{2\alpha}$) were investigated. In addition, regarding the second point, we also studied the relationship of the sustained contraction induced by the eicosanoids with PKC.

[1] Author for correspondence.

[2] Present address: Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokushima, 1-78-1, Shomachi, Tokushima 770, Japan.

Methods

Tissue preparation

Male 8 to 12-week-old Wistar rats weighing 200–300 g were stunned and bled. The thoracic aorta was dissected out and spiral strips, 2–3 mm wide and 7–10 mm long, were prepared. The endothelium was removed by gently rubbing the intimal surface with filter paper moistened with normal physiological saline solution (PSS).

Measurement of muscle tension

The aortic strips were suspended in siliconized organ baths filled with a normal PSS which contained (mM): NaCl 145, KCl 45, $CaCl_2$ 2.5, $MgCl_2$ 1.2, N-2-hydroxyethylpiperazine *N'* -2-ethanesulphonic acid (HEPES) 20, N,N'-tetrakis(2-pyridylmethyl)ethylenedeiamine (TPEN) 10, glucose 11 and ethylenediamine-N,N'-tetraacetic acid (EDTA) Na_2 10 μM (pH 7.4 at 37°C). The solution was gassed with 100% O_2 and maintained at 37°C. Isometric tension development was recorded under an initial tension of 0.5 g. After equilibration in normal PSS for 60 min, the strips were challenged with 1 μM phenylephrine three times. Tension generated by the last challenge was used as a reference value to normalize the size of the response of the eicosanoids. In some experiments, we used Ca^{2+}-free PSS (made by omitting Ca^{2+} from normal PSS and adding 2 mM [ethylene *bis*(oxyethylenenitrilo)] N,N'-tetraacetic acid (EGTA) or high K^+ PSS (made by replacing NaCl with an equimolar amount of KCl).

Measurement of endogenous IP_3 content

Segments of aorta were equilibrated in normal PSS at 37°C bubbled with 100% O_2 for 2 h. After treatment with appropriate drugs and/or solutions, if necessary, U46619 (1 μM) and $PGF_{2\alpha}$ (10 μM) were added at timed intervals. The preparations were quickly frozen in liquid nitrogen and homogenized in ice-cold 6% trichloroacetic acid (TCA). The homogenate was centrifuged, and the resulting supernatant used for determination of IP_3 levels. TCA was extracted with water-saturated ethyl ether and the solution was then neutralized by addition of $NaHCO_3$. After concentration of the samples by lyophilization, IP_3 was measured by protein binding assay with commercially available [^{3}H]-IP_3 assay system. Each experimental group consisted of 4 to 5 preparations from different animals.

Measurement of $[Ca^{2+}]_i$ in fura-2-loaded preparation

The experiment was carried out as described previously (Sato *et al.*, 1988; Hisayama *et al.*, 1990). The strips were incubated with 5 μM fura-2/AM in normal PSS for 3–4 h at room temperature in the presence of 0.2% Cremophor EL, then rinsed with the solution for 15 min. Thereafter, experiments were performed with a double wavelength excitation fluorimeter (CAF 100, Japan spectroscopic Co., Tokyo, Japan) where the fura-2-loaded strips was fixed horizontally in a bath that was bubbled with 100% O_2 at 37°C. The mechanical activity was monitored isometrically. Simultaneously, 500 nm fluorescence emitted by 340 nm and 380 nm excitation (F_{340} and F_{380}, respectively) were measured by successive alternating illuminations (48 Hz), and the ratio ($R_{340/380}$) of F_{340} to F_{380} was automatically calculated. In the muscle strips that were successfully loaded with fura-2, the increase in $[Ca^{2+}]_i$ resulted in a symmetrical increase in F_{340} and decrease in F_{380}, and an increase in $R_{340/380}$. Relative changes of $[Ca^{2+}]_i$ were determined by measuring the $R_{340/380}$. (Himpens & Somlyo, 1988; Sato *et al.*, 1988; Hisayama *et al.*, 1990). Changes in $R_{340/380}$ and muscle tension were expressed as a percentage of those seen with caffeine (30 mM) and phenylephrine (1 μM) in normal PSS, respectively.

Treatment with β-TPA

IP_3 determination After equilibration in normal PSS for 2–3 h, the preparations were treated with β-TPA (5 μM) for 60 min in the same buffer, followed by incubation with U46619 (1 μM) or $PGF_{2\alpha}$ (10 μM) for 15 and 45 s, respectively.

$[Ca^{2+}]_i$ determination The fura-2 loaded aortae were treated with β-TPA (5 μM) first in the normal PSS for 55 min, followed by incubation in the Ca^{2+}-free PSS for 5 min. After incubation in the Ca^{2+}-free medium in the continuing presence of β-TPA for 5 min, the prostanoids were applied and the change in $[Ca^{2+}]_i$ level was monitored.

Muscle tension measurement As shown in Figure 4b, the Ca^{2+} released from the intracellular Ca^{2+} store sites is unlikely to contribute to the development of the sustained contraction induced in Ca^{2+}-free PSS by the eicosanoids. However, to determine the effect of β-TPA on the sustained contractions, we carried out the experiment according to the following protocol which was somewhat different from that used in the $[Ca^{2+}]_i$ determination experiment, so that any interference in the contractions by the released Ca^{2+}, or by a possible buffering action of fura-2 for Ca^{2+} was excluded completely. The fura-2-*unloaded* preparations were incubated in Ca^{2+}-free PSS before and during treatment with β-TPA (5 μM), if necessary, for 5 min and 60 min, respectively, for the contraction to reach a steady-state level and for the stored Ca^{2+} to be depleted. The prostanoids were then added and the size of the resulting contractions measured.

Statistics

Numerical results are presented as the mean ± s.e. with the number of the observations in parentheses. Tests for significance were made by Student's two-tailed, unpaired *t* test or Duncan's new multiple range test, a *P* value less than 0.05 being considered significant.

Drugs

The following drugs were used: U46619 ((15)S-hydroxy-11α,9α-(epoxymethano)-prosta-5Z,13E-dienoic acid) (Cayman Chemical Co., Ann Arbor, MI, U.S.A.), prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical Co., Ltd., Osaka, Japan), 1-phenylephrine hydrochloride, caffeine, flurbiprofen (Sigma Chemical Co., St. Louis, MO, U.S.A.), fura-2/AM, EDTA Na_2, TPEN, HEPES, EGTA (Dojindo Laboratories, Kumamoto, Japan), Cremophor EL (Nacalai Tesque, Kyoto, Japan), 4α and 4β isomers of 12-*O*-tetradecanoylphorbol 13-acetate (α- and β-TPA; Funakoshi Co. Ltd., Tokyo, Japan). The [^{3}H]-IP_3 assay system was purchased from Amersham Japan (Tokyo, Japan). Other chemicals used were of analytical grade.

Results

Effect of short-term treatment with β-TPA on endogenous IP_3 formation by U46619 and $PGF_{2\alpha}$

Figure 1 shows the endogenous IP_3 levels between 0 (= unstimulated) and 15 min following incubation of rat aortic segments with U46619 and $PGF_{2\alpha}$ at concentrations of 1 and 10 μM, which produced maximum contractions, respectively (for example, see Figure 3 and Table 2). Both eicosanoids induced transient increases in IP_3 production, which were observed 15 and 45 s after incubation with U46619 and $PGF_{2\alpha}$, respectively. Thereafter, the IP_3 levels declined rapidly to the basal values.

The IP_3 levels, which were increased by U46619 and $PGF_{2\alpha}$ from the basal values of 34.1 ± 4.1 to 69.1 ± 4.7 and 66.3 ± 4.3 pmol mg^{-1} wet wt., were significantly reduced by the

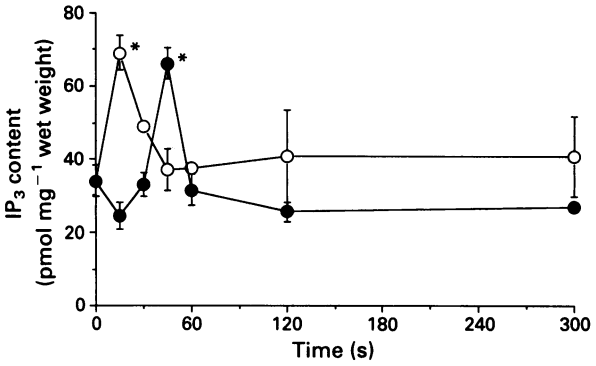

Figure 1 Time course of changes in the contents of endogenous IP_3 in the presence of $PGF_{2\alpha}$ and U46619 in rat aortic strips. The concentrations of $PGF_{2\alpha}$ (●) and U46619 (○) applied were 10 and 1 μM, respectively. Abscissa scale: time after addition of each eicosanoid; ordinate: IP_3 contents. Values are the means ± s.e. of 4–8 preparations. *$P<0.05$ versus the control value (= 0 time point).

Table 1 Effect of prior treatment with β-TPA on the IP_3 content determined in the presence of $PGF_{2\alpha}$ and U46619 in rat aortic strips

	IP_3 content (pmol mg^{-1} wet weight)	n
Basal	34.1 ± 4.1	8
β-TPA	30.1 ± 1.0	4
$PGF_{2\alpha}$	66.3 ± 4.3*	7
U46619	69.1 ± 4.7*	8
$PGF_{2\alpha}$ + β-TPA	48.7 ± 6.0†	5
U46619 + β-TPA	48.5 ± 2.8†	4

After the 60 min treatment with β-TPA (5 μM), the preparations were incubated with $PGF_{2\alpha}$ (10 μM) and U46619 (1 μM) for 45 and 15 s, respectively. Figures are the means of 4–8 preparations with s.e.
*$P<0.05$ versus the basal value (= 0 time point); †$P<0.05$ versus the values obtained with each eicosanoid alone.

60-min treatment with β-TPA (5 μM) to 48.5 ± 2.8 and 48.7 ± 6.0 ($n = 4-5$; $P<0.05$), respectively (Table 1). The inactive isomer, α-TPA had no effect on the IP_3 formation induced by these eicosanoids (data not shown).

Contractile responses to U46619 and $PGF_{2\alpha}$ under Ca^{2+}-free conditions

In the absence of external Ca^{2+}, U46619 (Figure 2) and $PGF_{2\alpha}$ induced only relatively large sustained contractions which were not accompanied by any transient contraction. When the amplitude of the phenylephrine (1 μM)-induced contraction in the normal PSS was taken as the reference (= 100%), the maximum contractions induced by U46619 (1 μM) and $PGF_{2\alpha}$ (10 μM) were 154.4 ± 2.1 ($n = 8$) and 127.8 ± 4.7% ($n = 5$) in the normal PSS, and 30.8 ± 2.9 ($n = 8$) and 25.4 ± 1.6% ($n = 5$) in the Ca^{2+}-free PSS, respectively (Figure 3 and Table 2).

Changes in intracellular Ca^{2+} level induced by U46619 and $PGF_{2\alpha}$ under Ca^{2+}-free conditions

As shown in Figure 4a, in the fura-2-loaded preparations, deprivation of extracellular Ca^{2+} resulted in a rapid decrease in $[Ca^{2+}]_i$, which reached the steady-state level in about 5 min. At this point, U46619 (1 μM) and $PGF_{2\alpha}$ (10 μM) evoked transient increases in $[Ca^{2+}]_i$, which were 23.6 ± 1.0 ($n = 5$) and 21.1 ± 2.1% ($n = 5$) respectively of that induced by 30 mM caffeine in the normal PSS. However, the $[Ca^{2+}]_i$ transients were not accompanied by any corresponding contractions, as shown in Figure 2 with the fura-2-unloaded preparations. In the falling phase of the transient increases in $[Ca^{2+}]_i$, the contractions began to develop, and were well sustained where the $[Ca^{2+}]_i$ was returned to the resting level. U46619, added 40 min after Ca^{2+} depletion, induced only the sustained contractions and not the $[Ca^{2+}]_i$ transient (Figure 4b).

Effect of short-term treatment with β-TPA on Ca^{2+} release and sustained contraction induced by U46619 and $PGF_{2\alpha}$

β-TPA, applied at 5 μM in the normal PSS, produced a slowly developing large contraction, which reached a plateau in about 60 min, with a slight increase in $[Ca^{2+}]_i$: the amplitude of the contraction was 117.4 ± 10.2% ($n = 5$) of that induced by phenylephrine (1 μM), and the increase in $[Ca^{2+}]_i$

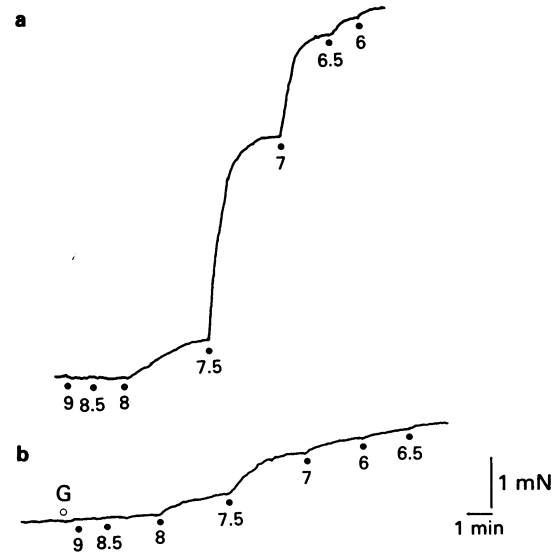

Figure 2 Typical tracing of contractions of rat aortic strips induced by cumulative application of U46619 in normal (a) and Ca^{2+}-free, 2 mM EGTA-containing (Ca^{2+}-free; b) solutions. G marks the change from normal solution to Ca^{2+}-free solution. Numbers show the negative logarithm of molar concentration of U46619 in the muscle bath. Vertical and horizontal bars show 1 mN and 1 min, respectively.

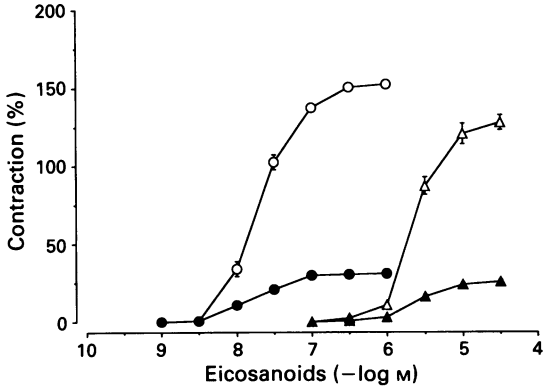

Figure 3 Concentration-response curves for $PGF_{2\alpha}$ and U46619 in normal and Ca^{2+}-free, 2 mM EGTA-containing solutions in rat aortic strips. The contractions induced by $PGF_{2\alpha}$ (△, ▲) and U46619 (○, ●) in the absence (closed symbols) and presence (open symbols) of extracellular Ca^{2+}, are shown in relation to the phenylephrine (1 μM)-evoked contraction in normal solution which was taken as 100%. Abscissa scale; concentrations of eicosanoids; ordinate scale; contraction. Values are the means ± s.e. of 6 preparations.

Table 2 Values of $-\log EC_{50}$ and intrinsic activities of $PGF_{2\alpha}$ and U46619 in normal and Ca^{2+}-free, 2 mM EGTA-containing solutions

	$-\log EC_{50}$	*Intrinsic activity*	n
In normal solution			
$PGF_{2\alpha}$	5.65 ± 0.02	1.28 ± 0.05	5
U46619	7.70 ± 0.07	1.54 ± 0.02	8
In Ca^{2+}-free solution			
$PGF_{2\alpha}$	5.64 ± 0.02	0.25 ± 0.02	5
U46619	7.77 ± 0.03	0.31 ± 0.03	8

Figures are the means of 5–8 preparations with s.e.

Table 3 Effect of prior treatment with β-TPA on $[Ca^{2+}]_i$ transients induced by $PGF_{2\alpha}$, U46619 and caffeine in Ca^{2+}-free, 2 mM EGTA-containing solution in fura-2-loaded rat aortic strips

	$[Ca^{2+}]_i$ transient	n
$PGF_{2\alpha}$	20.1 ± 1.1	5
U46619	19.8 ± 2.6	5
$PGF_{2\alpha}$ + β-TPA	11.3 ± 0.9*	6
U46619 + β-TPA	12.6 ± 1.9*	6

The preparations were incubated with and without β-TPA (5 μM) for 55 min in normal solution. $PGF_{2\alpha}$ (10 μM) and U46619 (1 μM) were then applied 5 min after changing medium to Ca^{2+}-free, 2 mM EGTA-containing solution. Experimental details are as for Figure 5. Values were normalized in relation to the $[Ca^{2+}]_i$ transient evoked by caffeine (30 mM) in normal solution which was taken to be 100%. Figures are the means of 5–6 preparations with s.e. *$P < 0.05$ versus the values obtained with the corresponding eicosanoid alone.

was 10.7 ± 3.0% ($n = 5$) of that by caffeine (30 mM). Neither stress nor $[Ca^{2+}]_i$ was changed by the same concentration of α-TPA.

To study the effect of β-TPA on the Ca^{2+} release and contractions induced by the eicosanoids, the fura-2-loaded preparations were treated for 60 min. Ca^{2+} deprivation resulted in a rapid decrease in the $[Ca^{2+}]_i$ level below the resting one observed in the normal PSS with a slight reduction of the β-TPA-induced contraction. Five minutes later, application of $PGF_{2\alpha}$ or U46619 resulted in the development of a larger contraction than that produced by either eicosanoid alone, while inducing the attenuated amplitude of the $[Ca^{2+}]_i$ transient (Figure 5). As shown in Table 3, the increases in $[Ca^{2+}]_i$ level by U46619 and $PGF_{2\alpha}$ were significantly reduced by prior treatment with β-TPA ($P < 0.05$). On the other hand, there was no difference between the caffeine (30 mM)-induced $[Ca^{2+}]_i$ transients in the absence of $[Ca^{2+}]_o$ observed with the control and β-TPA-treated preparations [24.1 ± 1.8 ($n = 6$) and 20.3 ± 2.9% ($n = 6$) of that evoked by caffeine (30 mM) in normal PSS, respectively]. The $[Ca^{2+}]_i$ transient induced by the eicosanoids was not affected by the same concentration of α-TPA (data not shown).

In contrast, Figure 5b shows that β-TPA had no antagonizing effect on the sustained contraction. After changing the medium to Ca^{2+}-free PSS, the (fura-2-unloaded) preparation was treated for 60 min with β-TPA, if necessary, and then U46619 or $PGF_{2\alpha}$ was applied. The size of contraction induced by β-TPA, U46619 or $PGF_{2\alpha}$ applied alone was 43.1 ± 2.0 ($n = 4$), 31.9 ± 1.7 ($n = 6$) or 12.9 ± 1.0% ($n = 4$) of that induced by 1 μM phenylephrine in normal PSS (the reference contraction), respectively. After treatment with β-TPA, additional contractions by U46619 and $PGF_{2\alpha}$ [= (the total size of contraction by each eicosanoid with β-TPA) − (the size of the β-TPA-induced contraction just before addition of the eicosanoid)] were 50.2 ± 5.1 ($n = 4$) and 25.5 ± 2.7% ($n = 5$) of the reference contraction, respectively. These values were significantly larger than that induced by the corresponding eicosanoid alone ($P < 0.05$; Table 4).

Effects of flurbiprofen and quinacrine on Ca^{2+} release and the sustained contraction induced by U46619 and $PGF_{2\alpha}$

Prior treatment with a potent cyclo-oxygenase inhibitor, flurbiprofen (3 μM) or quinacrine (100 μM) for 30 min had no effect on the Ca^{2+} transients or sustained contractions by EC_{50} concentrations of U46619 (0.03 μM) and $PGF_{2\alpha}$ (3 μM) (Table 5).

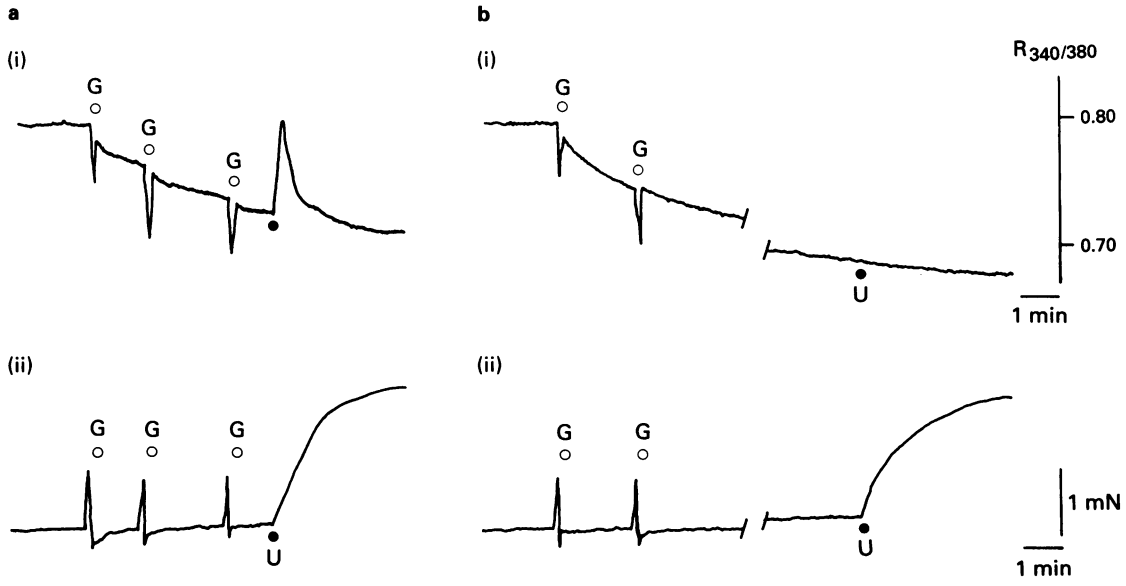

Figure 4 Typical tracings of U46619-induced $[Ca^{2+}]_i$ transients (i) and contractions (ii) obtained 5 (a) and 40 min (b) after incubation of fura-2-loaded rat aortic strips in Ca^{2+}-free, 2 mM EGTA-containing solution (Ca^{2+}-free solution). G, change of the medium from normal to Ca^{2+}-free solution; U, addition of U46619 (1 μM). Vertical and horizontal bars show $R_{340/380}$ (i) and 1 mN (ii), and 1 min, respectively.

Table 4 Effect of prior treatment with β-TPA on sustained contractions induced by $PGF_{2\alpha}$, U46619 in Ca^{2+}-free, 2 mM EGTA-containing solution in rat aortic strips

	Δ *Contraction*	n
$PGF_{2\alpha}$	12.9 ± 1.0	4
U46619	31.9 ± 1.7	6
$PGF_{2\alpha}$ + β-TPA	25.5 ± 2.7*	5
U46619 + β-TPA	50.2 ± 5.1*	4

Five minutes after deprivation of extracellular Ca^{2+}, the preparation was incubated in the absence and presence of β-TPA (5 μM) for 1 h, and $PGF_{2\alpha}$ (10 μM) or U46619 (1 μM) was then applied. Values are shown according to the following formula: (the amplitude of contraction by each eicosanoid) − (the tension level obtained just before addition of the eicosanoid), and normalized in relation to the phenylephrine (1 μM)-induced contraction (100%). Figures are the means of 4–6 preparations with s.e.
*$P<0.05$ versus the values obtained with the corresponding eicosanoid alone.

Table 5 Effect of flurbiprofen and quinacrine on the $PGF_{2\alpha}$- and U46619-induced sustained contractions in Ca^{2+}-free, 2 mM EGTA-containing solution in rat aortic strips

	Contraction	n
$PGF_{2\alpha}$ + flurbiprofen	7.6 ± 0.4	4
U46619 + flurbiprofen	20.6 ± 1.2	4
$PGF_{2\alpha}$ + quinacrine	8.6 ± 1.5	6
U46619 + quinacrine	23.7 ± 3.8	4

The preparations were incubated with and without flurbiprofen (1 μM) or quinacrine (100 μM) for 25 min in normal solution. $PGF_{2\alpha}$ (3 μM) and U46619 (30 nM) were then applied 5 min after changing medium to Ca^{2+}-free, 2 mM EGTA-containing solution with and without flurbiprofen or quinacrine. Figures are normalized for the contraction by each eicosanoid alone to be 100%, and are the means of 4–6 preparations with s.e.

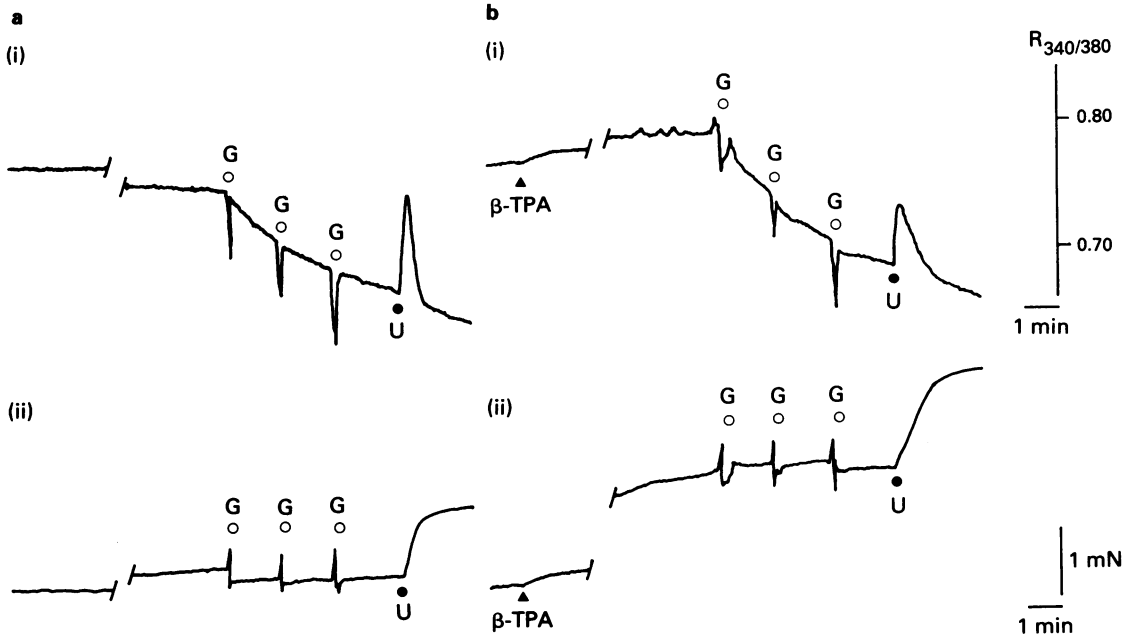

Figure 5 Typical tracings of $[Ca^{2+}]_i$ transients (a) and contractions (b) induced by U46619 observed 5 min after incubation in Ca^{2+}-free, 2 mM EGTA-containing solution (Ca^{2+}-free solution) in fura-2-loaded rat aortic strips which had been incubated in the absence (a) and presence (b) of β-TPA. β-TPA (5 μM) was added 55 min before change of the medium from normal solution to Ca^{2+}-free solution, and present throughout. β-TPA, start of incubation with β-TPA; G, change of the medium from normal to Ca^{2+}-free solution; U, addition of U46619 (1 μM). Vertical and horizontal bars show $R_{340/380}$ (i) and 1 mN (ii), and 1 min, respectively.

Discussion

In the present study, we monitored PIP_2 breakdown by measuring the content of IP_3, which is one of the products of the reaction. The rat aortic strips responded to $PGF_{2\alpha}$ and U46619, both of which act on the thromboxane A_2 receptor in this tissue (Kennedy *et al.*, 1982; Hanasaki *et al.*, 1988), with an increased production of endogenous IP_3. The response was transient in nature, as observed with α_1-adrenoceptor agonists (Langlands & Diamond, 1990; Huang *et al.*, 1990b). Prior short-term treatment with β-TPA greatly reduced the eicosanoid-induced production of IP_3. The effect of β-TPA seems to be specific, since α-TPA, the biologically-inactive isomer, did not affect the IP_3 contents.

It is well known that stimulated PIP_2 breakdown mediated by activation of Ca^{2+} mobilizing receptors is inhibited by short-term treatment with active phorbol esters in many cell types (Orellana *et al.*, 1985; Leeb-Lundberg *et al.*, 1985; Litten *et al.*, 1987; Slivka & Insel, 1988; Kaya *et al.*, 1989; Chardonnens *et al.*, 1990), including vascular smooth muscle cells (Roth *et al.*, 1986; Reynolds *et al.*, 1989; Araki *et al.*, 1989). Our results showing that the 60-min treatment with β-TPA reduced the increased production of IP_3 by $PGF_{2\alpha}$ and U46619 are consistent with these reports, and suggest that the IP_3-and DG-mediated responses to these eicosanoids should be diminished by prior treatment with the phorbol ester. The experiments were carried out under Ca^{2+}-free conditions, since the transient and sustained contractions under such conditions have been assumed to be due to the Ca^{2+} released by IP_3 and changes in Ca^{2+}-sensitivity of contractile apparatus by PKC, respectively (Bradley & Morgan, 1987; Karaki, 1989; Huang *et al.*, 1990b; Hisayama *et al.*, 1990).

In the absence of extracellular Ca^{2+}, both eicosanoids produced transient increases in $[Ca^{2+}]_i$, most probably by releasing Ca^{2+} from the intracellular Ca^{2+} stores or the SR. However, the transients were not accompanied by corresponding transient contractions. In the falling phase of the transient increases in $[Ca^{2+}]_i$, the contractions began to develop

and were well sustained, even though the $[Ca^{2+}]_i$ had then returned to the resting level. Prior short-term treatment with β-TPA, while having no significant effect on the $[Ca^{2+}]_i$ transient induced by caffeine which releases Ca^{2+} through an IP_3-independent mechanism (Endo, 1977), significantly reduced the amplitude of the $[Ca^{2+}]_i$ transient evoked by $PGF_{2\alpha}$ and U46619, as it did on IP_3 production, suggesting that the Ca^{2+} release is triggered by IP_3 produced by PIP_2 breakdown. The reduction of $[Ca^{2+}]_i$ transient by the phorbol ester was, however, smaller than that of IP_3 production. We have no explanation about this point, but it might be that other than IP_3-independent Ca^{2+} release is induced by the eicosanoids.

In contrast, the sustained contractions induced in Ca^{2+}-free media were not inhibited but augmented by the prior short-term treatment with β-TPA. This suggests that the signal-transducing pathway utilized for development of the sustained contraction is different from that for the intracellular Ca^{2+} release, and further that at least DG derived from PIP_2 is not involved in the sustained contraction. Rather, the extra contraction of the β-TPA-treated preparations by the eicosanoid [(the total size of contraction by each eicosanoid with β-TPA) − (the size of the β-TPA-induced contraction just before addition of the eicosanoid)] was larger in size than that of the sustained contraction by either eicosanoid alone: synergism between β-TPA and the eicosanoids was observed in inducing the sustained contraction under Ca^{2+}-free conditions. This would raise the possibility that the mechanism involved in the eicosanoid-induced sustained contractions is unrelated to the PKC that is activated by application of β-TPA. Similar synergism with β-TPA was obtained with urotensin II (Itoh *et al.*, 1991).

On the other hand, the prior short-term treatment with β-TPA resulted in attenuation of IP_3 production and Ca^{2+} release, but no effect on the sustained contraction in response to endothelin-1 (Huang *et al.*, 1990a,b). Although the pathway used by endothelin-1 for Ca^{2+} release also differs from that for sustained contraction, the mechanism involved in the latter is somewhat different from that in the eicosanoid-induced contraction, because the effects of β-TPA and endothelin-1 were additive in inducing the sustained contraction. This result was recently confirmed (Itoh *et al.*, 1991). Therefore, mechanistically, the receptor-mediated sustained contraction obtained under Ca^{2+}-free conditions may be of at least two types.

It has been reported that the prior short-term treatment with phorbol esters, while inhibiting PIP_2 breakdown, potentiates the phospholipase A_2 activity which is stimulated by some drugs, such as endothelin-1 (Reynolds *et al.*, 1989), vasopressin (Chardonnens *et al.*, 1990), and bradykinin (Slivka & Insel, 1988). One might consider whether or not the synergistic effect of β-TPA on the eicosanoid-induced sustained contraction shown in the present study is the result of potentiated phospholipase A_2 activity by the phorbol ester, which resulted in the increase in the effective concentration of constrictor eicosanoids due to their accumulation in the organ bath during prolonged incubation with $PGF_{2\alpha}$ or U46619. This situation has been demonstrated with endothelin-1 (Reynolds & Mok, 1990). However, in our case, this possibility can be excluded, since quinacrine and flurbiprofen had no inhibitory effect on the contraction induced by the eicosanoids, the concentrations of which were around their EC_{50} values to make it easier to detect any inhibition.

The present study strongly suggests that in the rat aorta, $PGF_{2\alpha}$ and U46619 utilize different and parallel signal transduction pathways to release Ca^{2+} by IP_3 produced by PIP_2 breakdown, and to increase the sensitivity of contractile apparatus in which PKC may not be involved. Work is in progress to determine whether the two independent pathways derive from different receptor types or coupling of one type of receptor with different effector systems.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (No. 04304030).

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(Received January 21, 1993
Revised May 25, 1993
Accepted June 7, 1993)