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INVOLVEMENT OF INOSITOL 1,4,5-TRIPHOSPHATE AND PROTEIN KINASE C IN THROMBIN-INDUCED CONTRACTION OF PORCINE PULMONARY ARTERY

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Abstract—The role of the intracellular messengers inositol 1,4,5-triphosphate (IP_3) and protein kinase C (PKC) in the thrombin (3 U/mL)-induced contraction of endothelium-denuded porcine pulmonary arteries was investigated. Thrombin induced a sustained contractile response with an initial transient increase in IP_3 to about 160% of the unstimulated control. Omission of extracellular Ca^{2+} or preincubation with verapamil (10 μmol/L) reduced the maximum of contraction without significantly affecting the thrombin-induced increase in IP_3. To evaluate the role of PKC for the contractile response, the PKC was activated directly by phorbol 12,13-dibutyrate (PDBu, 50 nmol/L). The phorbol ester produced a slowly increasing tonic contraction without any changes in the basal IP_3 level. There was a moderate inhibition of PDBu-induced contractions in Ca^{2+}-free solution, while they were not inhibited after preincubation with verapamil. Preincubation with the PKC inhibitor staurosporine (50 nmol/L) significantly reduced the PDBu-induced contraction (by about 80%). In thrombin-stimulated vessels staurosporine only inhibited the tonic phase of the contractile response whereas the increase in IP_3 and the phasic component of contraction were still evident. These results suggest that IP_3 and PKC are involved in the thrombin-induced contraction. The phasic component of contraction is associated with the generation of IP_3; the tonic component might be due to the activation of PKC.

Key words: porcine pulmonary artery; thrombin; vasocontraction; inositol 1,4,5-triphosphate; protein kinase C; staurosporine

The contractile response of vascular smooth muscle to agonists such as thromboxane A_2 [1] and serotonin [2] was found to be mediated via receptor-coupled activation of PLC†. The subsequent hydrolysis of PIP_2 leads to the generation of IP_3, which releases calcium from intracellular stores, and DAG, which activates PKC [3, 4]. The IP_3-mediated rise in intracellular Ca^{2+} seems to be associated with the initial fast "phasic" component of smooth muscle contraction [3, 5] whereas the following slower and more sustained "tonic" component is attributed to the activation of PKC [5, 6]. Tumour-promoting phorbol esters directly activate PKC such as DAG [4, 7] and are therefore a useful tool in investigating the physiological role of PKC. In isolated vessels phorbol esters induce a slowly developing sustained contraction [7–9]. In some smooth muscle preparations the activated PKC was found to cause Ca^{2+} influx through voltage dependent Ca^{2+} channels [10, 11]. Phorbol esters are also able to induce contractions without increasing intracellular Ca^{2+} [12]. In previous studies we have shown that thrombin causes a long-lasting contractile response of rabbit aorta and porcine pulmonary artery [13, 14]. An increase in IP_3 formation preceded thrombin-induced contraction in porcine pulmonary arteries [15]. The question arises whether activation of PKC is involved in the sustained contractile processes induced by thrombin. In various cell types such as platelets and endothelial cells thrombin stimulates PKC [16, 17]. To evaluate the role of PKC in the thrombin-induced contractile response of porcine pulmonary arteries, the effect of the PKC inhibitor staurosporine was investigated. Staurosporine, a microbial alkaloid, is a potent but non-specific PKC inhibitor [18, 19]. For comparison purposes, the inhibitory potency of staurosporine on the contractile response of porcine pulmonary arteries to a phorbol ester was studied. To obtain more information about the contribution of external Ca^{2+} to thrombin- or phorbol ester-induced contractile responses experiments were conducted in the presence of verapamil or in Ca^{2+}-free solution.

MATERIALS AND METHODS

Materials. Krebs–Henseleit solution (mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11); thrombin (bovine thrombin, sp. act. 1187 U/mg, Arzneimittelwerk, Dresden, Germany); $PGF_{2\alpha}$, PDBu and substance P (Serva, Heidelberg, Germany); 4α-phorbol 12,13-didecanoate and staurosporine (Sigma, Deisenhofen, Germany); verapamil (Falicard®, Fahlberg-List, Magdeburg, Germany); D-myo-[2-^{3}H]inositol 1,4,5-triphosphate ([^{3}H]IP_3, 18.3 Ci/mmol) and D-myo-

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† Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; PDBu, phorbol 12,13-dibutyrate; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-biphosphate; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$

inositol 1,4,5-triphosphate (IP_3, Amersham Buchler, Braunschweig, Germany). PDBu and staurosporine were dissolved in DMSO to prepare stock solutions (10 mmol/L). Further dilution was done with distilled water.

Measurement of vasoconstriction. Pig lungs were obtained from a slaughter house. Small branches of the pulmonary artery were dissected and cut into rings of 2–3 mm in length. The endothelium was removed by gently rubbing the intimal surface with a rough glass stick. Each arterial ring was attached to L-shaped platinum hooks and placed in a 10 mL organ bath containing Krebs–Henseleit solution (pH 7.4) which was continuously gassed with 5% CO_2 in oxygen at 37°. To investigate the influence of external Ca^{2+} in some experiments $CaCl_2$ was omitted and EGTA (1 mmol/L) was added. A resting tension of 2 g was maintained throughout the experiments. Changes in tension were measured isometrically by strain gauge transducers. During the equilibration period of 60 min the bath solution was changed every 15 min. Contractions were induced at 45 min intervals. The ring segments were initially contracted by KCl (30 mmol/L), and contractions were then elicited by $PGF_{2\alpha}$ three to four times until the contractile response remained constant. In endothelium-denuded rings the substance P (10 nmol/L)-induced relaxation of $PGF_{2\alpha}$-precontracted vessels was lower than 10%. Because thrombin induces rapid receptor desensitization, only one thrombin concentration could be investigated on one vessel segment. PDBu was applied at a concentration of 50 nmol/L. The contractile effect occurring 60 min after the addition of PDBu was assessed. The antagonists were added 20–30 min before the agonist or when the plateau of the contraction was reached.

Measurement of IP_3. The vessel segments were preincubated in oxygenated Krebs–Henseleit solution containing 10 mmol/L of LiCl at 37° for 10 min. After various periods of exposure time to the agonists the arterial segments were frozen in liquid nitrogen. The frozen tissues were homogenized in 0.5 mL 10% $HClO_4$. After centrifugation at 2000 *g* for 10 min, 100 μL of EDTA (10 mmol/L, pH 7.0) were added to 400 μL of the supernatant. The samples were neutralized by the addition of 0.5 mL of a mixture (1:1, v/v) of freon and tri-*n*-octylamine, shaken vigorously and centrifuged at 2000 *g* for 2 min. The upper phase of the resulting three phases contained the water-soluble compounds and was used for the determination of IP_3.

The binding of [^{3}H]IP_3 to rat cerebellar membranes was assayed as described by Bredt *et al.* [20] with some modifications. Two hundred microlitres of the cerebellar membrane preparation (0.12 mg protein) were mixed with 1.0 nmol/L [^{3}H]IP_3 (22,000 dpm), and 50 μL tissue extract in a total volume of 275 μL buffer B (50 mmol/L Tris–HCl, pH 8.4, 1 mmol/L EDTA, 1 mmol/L 2-mercaptoethanol). The samples were incubated at 4° for 10 min; thereafter the reaction was stopped by centrifugation at 10,000 *g* for 5 min. Scintillation was counted in the pellet after solubilization in 500 μL water. To calculate the IP_3-values in the tissues a standard curve of IP_3 (1–100 nmol/L) was used. The non-specific binding was defined as binding not displaced by 2 μmol/L IP_3.

Results are expressed as means ± SD of N experiments from different lung preparations. The significance of differences was evaluated by Student's *t*-test. For comparison of one control with several experimental groups the modified *t*-test according to Bonferroni was applied. The values were considered statistically significant at $P < 0.05$.

RESULTS

Effect of staurosporine on thrombin-induced contraction and IP_3-formation in porcine pulmonary arteries

In deendothelialized vessels thrombin (3 U/mL) induced a sustained contractile response of 1.54 ± 0.4 g (N = 10) (Fig. 2). Maximum contraction was reached within 12 ± 4 min (Fig. 1A). The concentration of IP_3 in thrombin-stimulated vessels

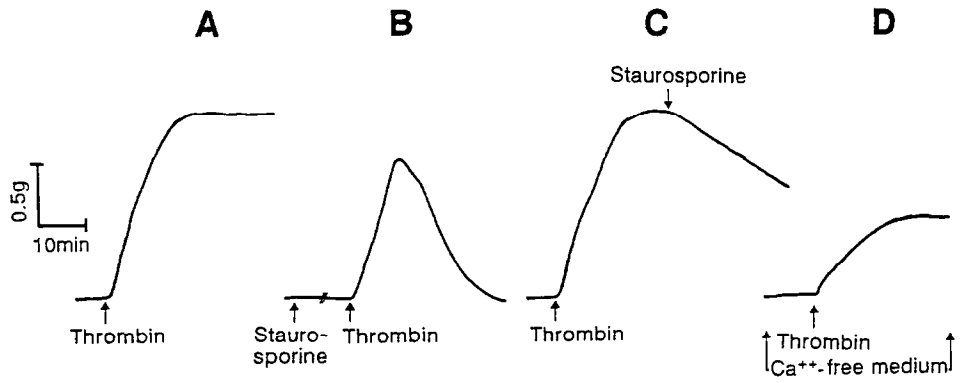

Fig. 1. Thrombin (3 U/mL)-induced contractile response of endothelium-denuded ring segments from porcine pulmonary artery. Results are representative of four to five separate experiments. (A) control; (B) preincubation with staurosporine (50 nmol/L; for 25 min before the addition of thrombin); (C) addition of staurosporine (50 nmol/L) to the precontrated vessel; (D) contraction after incubation of the vessels in Ca^{2+}-free medium for 10 min.

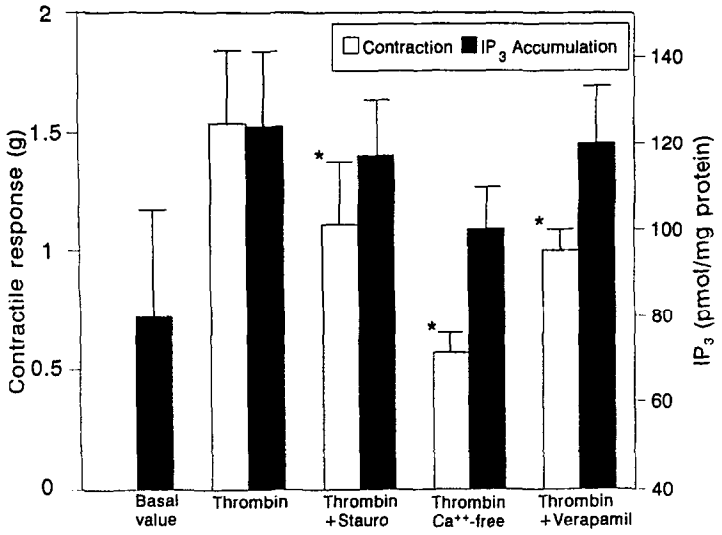

Fig. 2. Influence of staurosporine (50 nmol/L), verapamil (10 μmol/L) or omission of extracellular Ca^{2+} on thrombin (3 U/mL)-induced contractile responses and IP_3 formation (maximum values are given) in endothelium-denuded ring segments from porcine pulmonary artery. Verapamil and staurosporine were given 20 and 25 min, respectively, before thrombin was added. The vessels were incubated in Ca^{2+}-free medium for 10 min prior to the addition of thrombin. Means ± SD, N = 4–6, *P < 0.05.

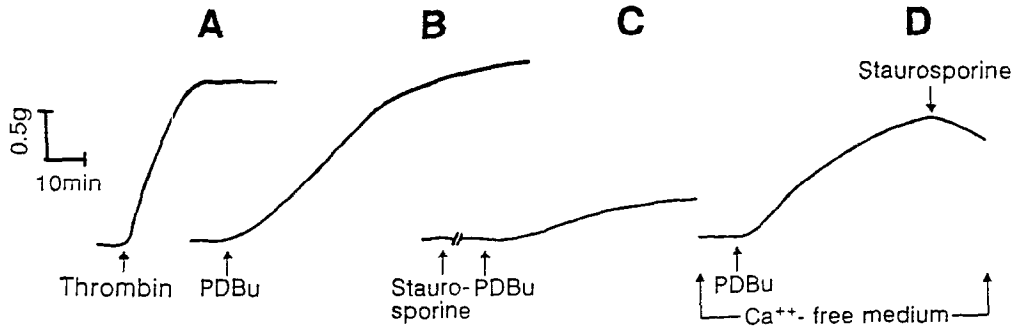

Fig. 3. PDBu (50 nmol/L)-induced contractile response of endothelium-denuded ring segments from porcine pulmonary artery. Results are representative of four to six separate experiments. (A) thrombin (3 U/mL) control; (B) PDBu control; (C) preincubation with staurosporine (50 nmol/L); 25 min before the addition of DPBu); (D) contraction after preincubation in calcium-free medium for 10 min and addition of staurosporine (50 nmol/L) at maximum contraction.

increased to an average of 160% compared to unstimulated segments. Maximum IP_3 formation was reached after 2 min; within the following 8 min IP_3 decreased to values not significantly different from the control.

After preincubation of the arterial rings with the PKC inhibitor staurosporine (50 nmol/L; for 25 min) the maximum phasic component of contraction was diminished by 28 ± 8% (N = 4); the onset of contraction was identical to that of the control. The sustained tonic phase of contraction was completely suppressed by the PKC inhibitor (Fig. 1B). The formation of IP_3 in the staurosporine-treated vessels did not differ significantly from that in the thrombin control (Fig. 2). The addition of staurosporine to thrombin-precontracted vessels resulted in a decline in tone (Fig. 1C). Pretreatment with verapamil diminished maximum contraction by 35 ± 5% (N = 6) without affecting the IP_3 level (Fig. 2) and the slope of contraction. In calcium-free medium the contractile response was slower in onset (Fig 1D) and was reduced to 37 ± 3% of the thrombin control ($P < 0.05$) (Fig. 2). In contrast, the IP_3 values were not significantly diminished (Fig. 2) and reached a maximum level after 2 min, as in the thrombin control.

Influence of staurosporine on PDBu-induced contraction and IP_3 formation in porcine pulmonary arteries

PDBu, a direct activator of PKC, induced a slowly

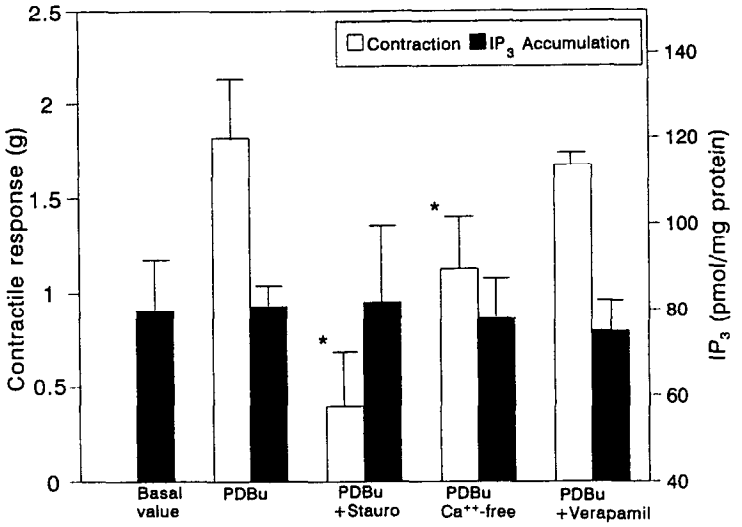

Fig. 4. Influence of staurosporine (50 nmol/L), verapamil (10 μmol/L) or omission of external Ca^{2+} on PDBu (50 nmol/L)-induced contractile responses and IP_3 formation (maximum values are given) in endothelium-denuded porcine pulmonary artery. Verapamil and staurosporine were given 20 and 25 min, respectively, before PDBu was added. The vessels were incubated in calcium-free medium for 10 min prior to the addition of PDBu. Means ± SD, N = 4, *P < 0.05

developing and sustained contraction at 50 nmol/L (Fig. 3B). Maximum tension (1.82 ± 0.4 g; N = 10) was usually reached after 60 min. Inactive 4α-phorbol 12,13-didecanoate (1 μmol/L) did not induce any contractile effect. In PDBu-stimulated vessels the IP_3 level corresponded to basal values in unstimulated vascular preparations (Fig. 4). When the vessels were incubated in Ca^{2+}-free medium for 10 min PDBu-induced contraction was inhibited by 38 ± 7% (Figs 3D, 4). In contrast, preincubation with verapamil (10 μmol/L, for 20 min) did not exert an inhibitory effect (Fig. 4).

Pretreatment of arteries with staurosporine (50 nmol/L; for 25 min) caused an inhibition of PDBu-induced contraction by 78 ± 15% (Figs 3C, 4). When staurosporine (50 nmol/L) was added to PDBu-precontracted vessels vascular tone decreased continuously (not shown). In the absence of external Ca^{2+} staurosporine caused a decline in tone of PDBu-contracted arteries as well (Fig. 3D). In all experiments IP_3 concentration was in the same range as in unstimulated vessels (Fig. 4).

Effect of staurosporine on KCl*-induced contraction in porcine pulmonary arteries*

Since staurosporine is not a highly specific PKC inhibitor, its effect on KCl-induced contraction, which does not depend on PKC activation, was investigated. After preincubation with staurosporine (50 nmol/L, for 25 min) maximum KCl-induced contraction was reduced by 23 ± 8% (N = 4); Fig. 5 shows a representative experiment. When staurosporine was added to KCl-precontracted arterial segments it failed to decrease tension up to a concentration of 0.1 μmol/L (Fig. 5C).

DISCUSSION

The present studies confirm that thrombin induces a sustained contraction of de-endothelialized porcine pulmonary arteries [14, 15]. To elucidate the mechanism of the thrombin-mediated contractile response, the roles of IP_3 and PKC were investigated. In thrombin (3 U/mL)-stimulated vessels a transient increase in IP_3 concentration with a maximum after 2 min was found. The rise in IP_3 preceded the maximum contractile effect which occurred after 10–15 min. In studies on $PGF_{2\alpha}$-stimulated rat aorta IP_3 concentration reached a peak more quickly than contractile force. The increase in IP_3 was followed by an initial phasic increase in cytosolic-free calcium important for the development of contraction [9].

In calcium-free solution the contractile effect of thrombin was slower in onset and considerably weaker than in calcium-containing solution. Since the thrombin-induced increase in IP_3 was only slightly diminished the reduced contraction may not be due to reduced PLC activity. The remaining contraction in calcium-free medium is obviously due to the release of intracellular calcium by IP_3 which seems to be responsible for the initiation of thrombin contraction. For the development of maximum contraction the influx of extracellular Ca^{2+} is necessary. After preincubation of the vessels with verapamil maximum contraction was significantly less inhibited than under Ca^{2+}-free conditions; the increase in IP_3 and the slope of contraction were not affected. These results indicate that the Ca^{2+}-dependency of the thrombin-induced contraction is due only in part to Ca^{2+} influx through verapamil-sensitive voltage-operated channels (L-type Ca^{2+}

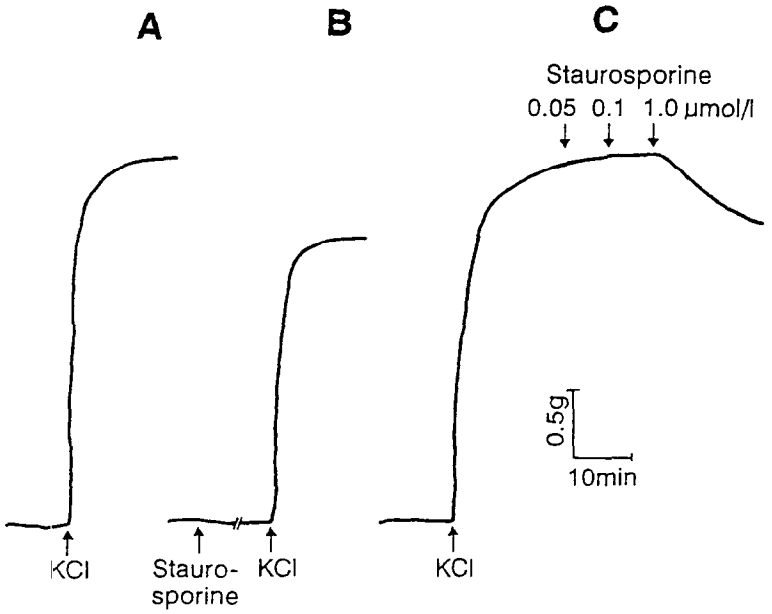

Fig. 5. Influence of staurosporine on the KCl (30 mmol/L)-induced contractile response of endothelium-denuded ring segments from porcine pulmonary artery. Results are representative of four separate experiments. (A) control; (B) preincubation with staurosporine (50 nmol/L; 25 min before addition of KCl; (C) addition of staurosporine to the precontracted vessel.

channels) and that this influx contributes to maximum contraction but not to initial force development.

The activation of PKC is assumed to be an important signal for sustained physiological responses in vascular smooth muscle cells [4]. To demonstrate that PKC activation might generally contribute to contractile responses, PKC was directly activated by PDBu. In porcine pulmonary arteries the phorbol ester evoked a slowly increasing and sustained contraction without any changes in the basal IP_3 level. Preincubation with the PKC inhibitor staurosporine significantly diminished the PDBu-induced slope and extent of contraction. In thrombin-stimulated vessels staurosporine completely inhibited the tonic phase of contraction while the rise in IP_3 and the phasic component of the contraction were still evident. Therefore, we conclude that the thrombin-induced tonic contraction of porcine pulmonary arteries is mainly due to activated PKC. The slope of contraction was not influenced by staurosporine indicating that PKC is not involved in the initial phase of contraction.

Although staurosporine is known to inhibit not only PKC but also other kinases [19], the present experiments support its preferential inhibitory effect on PKC. Staurosporine inhibited PDBu-induced contraction much more effectively than that induced by KCl which acts via voltage-operated calcium channels and independently of phosphoinositide hydrolysis [2]. Similar results were obtained in investigations with rat aorta and mesenteric arteries [21].

In some vascular preparations phorbol esters were found to induce contractions by activating voltage-dependent calcium channels [9, 10]. In the present experiments verapamil had no significant inhibitory effect on PDBu-induced contraction, whereas in calcium-free solution a partial inhibition of the contractile response to PDBu did occur. Thus, in agreement with the findings in porcine carotid arteries [22], the Ca^{2+}-influx through voltage-operated channels did not contribute significantly to the observed moderate calcium dependency of PDBu-induced contractions. Taken together, PKC-mediated contractions of endothelium-denuded porcine pulmonary arteries depended only in part on extracellular calcium.

For the first time the present studies demonstrate that in porcine pulmonary arteries the initial phasic component of thrombin-induced contraction is associated with the generation of IP_3 while the tonic component might be due to PKC activation. In calcium-free medium the remaining contraction is attributed to the release of intracellular calcium by IP_3.

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