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ENDOTHELIN-1 AND PLATELET ACTIVATING FACTOR STIMULATE THROMBOXANE A_2 BIOSYNTHESIS IN RAT VASCULAR SMOOTH MUSCLE CELLS

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Abstract—The effect of endothelin-1 (ET-1) on the release of thromboxane A_2 (TXA_2) was examined in cultured rat vascular smooth muscle cells (VSMC). ET-1 (10^{-11} to 10^{-6} M) significantly stimulated the release of thromboxane B_2 (TXB_2), a stable metabolite of TXA_2. These effects of ET-1 were blocked by a cyclooxygenase inhibitor (indomethacin), a TXA_2 synthetase inhibitor (CV-4151) and a specific platelet activating factor (PAF) antagonist (CV-6209). Additionally, PAF (10^{-11} to 10^{-6} M) stimulated the TXB_2 release. Pretreatment with the phospholipase A_2 inhibitor dexamethasone potently inhibited both ET-1 and PAF-induced elevation of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in fura-2-loaded VSMC. These results clearly demonstrate that both ET-1 and PAF stimulate TXA_2 biosynthesis in cultured rat VSMC, and TXA_2 may contribute to the elevation of $[Ca^{2+}]_i$ induced by ET-1 or PAF in VSMC. Furthermore, the stimulation of TXA_2 biosynthesis may be a result of PLA_2 activation by not only ET-1 but also PAF.

Endothelial cells are now known to be capable of releasing vasoactive substances which mediate vasodilatation [1, 2] as well as vasoconstriction [3–5]. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide, recently isolated and purified from the supernatant of cultured porcine aortic endothelial cells [6]. ET-1 is a 21 amino acid peptide containing two internal disulfide bridges and produced from a 38-(human) or 39-(porcine) amino acid precursor (big endothelin) by an unusual proteolytic processing between Trp 21 and Val 22. ET-1 potently constricts a variety of blood vessels from various species including human coronary arteries. Vasoconstrictive effects of ET-1 are dependent on extracellular Ca^{2+} [6, 7]. Moreover, the vasoconstriction was induced primarily by elevation of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) [8]. However, the mechanisms of ET-1-induced increase in $[Ca^{2+}]_i$ of vascular smooth muscle cells (VSMC) have not been fully elucidated.

We recently reported the possible involvement of thromboxane A_2 (TXA_2) and platelet activating factor (PAF) in the mechanism of ET-1-induced mobilization of Ca^{2+} in cultured rat VSMC [9]. TXA_2, a product of arachidonic acid (AA) metabolism, is a potent inducer of platelet aggregation and also a vasoconstrictor [10, 11]. Furthermore, TXA_2 increases $[Ca^{2+}]_i$ in vascular smooth muscles [12, 13]. In endothelial cells, TXA_2 is released in response to various chemical stimuli such as calcium ionophore, AA and prostaglandin H_2 (PGH_2) [14]. However, biosynthesis of TXA_2 in VSMC has not been reported.

To elucidate the possible interactions among ET-1, PAF and TXA_2, the present study was designed to examine the effects of ET-1 and PAF on the release of TXA_2 from cultured rat VSMC. Furthermore, in view of the potentially important role of phospholipase A_2 (PLA_2) activity in the interactions among ET-1, PAF and TXA_2, we also investigated the effect of a glucocorticosteroid PLA_2 inhibitor (dexamethasone) on the ET-1 and PAF-induced increase in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Vascular smooth muscle cell culture. Rat VSMC were obtained from 7-week-old male Sprague–Dawley rats according to the method described by Ross [15]. These cells were plated on square petri dishes (90 × 90 × 15 mm, Falcon) containing 16 rectangular glass cover slides (10 × 40 mm, Matsunami) or 25-cm^2 tissue culture flasks (Corning). Cells were cultured to confluency in the alpha modification of Eagle's minimum essential medium (MEM) (Nissui, No. 1) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, U.K.), glutamine (3 mg/mL), and incubated with 95% air and 5% CO_2 at 37°. Media were replaced at 24 hr and thereafter every 2 days. At confluency, the media were removed and the cells were incubated for 1 min with 0.05% ethylenediamine-*N*,*N*′-diacetic acid in Dulbecco's phosphate-buffered saline containing 0.5% trypsin (Difco Laboratories, Detroit, MI, U.S.A.). Plates or flasks were washed with MEM, and cells were collected by centrifugation at 400 *g* for 2 min. In this study cultured VSMC were used between the 5th to 10th passages.

Stimulation of cells and extraction of TXB_2 and 6-keto-$PGF_{1\alpha}$. Confluent rat VSMC monolayers in 25-cm^2 tissue culture flasks (Corning) were gently washed

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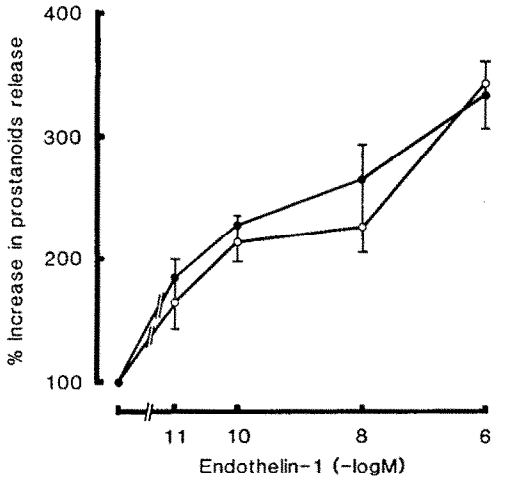

Fig. 1. Effect of endothelin (ET)-1 on the biosynthesis of thromboxane B_2 (●) and 6-keto-prostaglandin $F_{1\alpha}$ (○) in rat vascular smooth muscle cells (VSMC) as a function of concentration. Confluent rat VSMC (2×10^6 cells) in 1 mL of HEPES-buffered PSS supplemented with 0.05% bovine serum albumin were incubated with various doses of ET-1 for 10 min at 37°. The basal levels of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ were 99 ± 28 pg/10^6 cells and 126 ± 18 pg/10^6 cells, respectively. Values represent the means ± SE of three experiments.

three times with HEPES-buffered physiological salt solution (PSS: 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 25 mM glucose, 25 mM HEPES), pH 7.2. The cells in 1 mL of HEPES-buffered PSS supplemented with 0.05% bovine serum albumin were incubated with various doses of ET-1 (10^{-11}–10^{-6} M) or PAF (10^{-11}–10^{-6} M) for 10 min at 37°. At the end of the incubation period, the pH was adjusted to pH 3 with 1 N HCl, cells were dislodged with a rubber policeman (costar) and then extracted with 4 mL ethyl acetate. The ethyl acetate layer was collected by centrifugation at 1200 *g* for 10 min and stored at −20° until the TXB_2 and 6-keto-$PGF_{1\alpha}$ measurements.

Measurement of TXB_2 and 6-keto-$PGF_{1\alpha}$. The amounts of TXB_2 and 6-keto-$PGF_{1\alpha}$ were determined by radioimmunoassay according to a previously described method [16]. The minimum detectable dose was 10 pg/tube for both prostanoids. Cross reactivity of TXB_2 antiserum with other prostaglandins was less than 0.1% for PGE_1 and PGE_2, 0.6% for $PGF_{2\alpha}$, 0.1% for 6-keto-$PGF_{1\alpha}$ and 1.1% for PGD_2. Those of 6-keto-$PGF_{1\alpha}$ antiserum were less than 0.1% for PGA_2, PGD_2 and TXB_2, 1.8% for PGE_1, 1.3% for PGE_2, 4.5% for $PGF_{2\alpha}$, 0.4% for 6, 15-diketo-$PGF_{1\alpha}$ and 0.3% for 6, 15-diketo-13, 14-dihydro-$PGF_{1\alpha}$.

Treatment of cells with dexamethasone. Rat VSMC were grown on glass cover slides for 24 hr after seeding in 15 mL of fresh MEM supplemented with 10% fetal calf serum. The cells were then pretreated for various incubation times (0.5–24 hr) with dexamethasone (10^{-11}, 10^{-9}, 10^{-7}, 10^{-5} and 10^{-3} M). Dexamethasone was dissolved in dimethylsulfoxide (DMSO) and added to the media at a dilution of 1:1000. This level of DMSO had no effect on the cells.

Measurement of $[Ca^{2+}]_i$ using fura-2. Rat VSMC on glass cover slides were washed with HEPES-buffered PSS. Fura-2 acetoxymethyl ester was added to cells at a final concentration of 4 μM in HEPES-buffered PSS and incubated for 80 min at 15°. After loading, cells were washed and incubated for an additional 20 min. A glass cover slide coated with fura-2 loaded VSMC was inserted diagonally into a quartz cuvette that contained 2.5 mL HEPES-buffered PSS supplemented with 0.05% bovine serum albumin. Ca^{2+}-fura-2 fluorescence was measured at 37° in a Hitachi F-4000 spectrofluorimeter equipped with a thermostated cuvette holder and a stirring apparatus. Wavelengths were set at 340 and 380 nm for excitations and 505 nm for emission. At the end of each experiment, maximum and minimum fluorescences were estimated after addition of ionomycin (2 μM) as calcium ionophore and glycoletherdiamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (8 mM) as Ca^{2+}-chelator, respectively. $[Ca^{2+}]_i$ values were calculated according to the method of Grynkiewicz *et al.* [17].

Chemicals. Endothelin-1 was purchased from Peptide Institute Inc. (Osaka, Japan). 1-*O*-Octadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine (PAF), CV-6209 (2-[*N*-acetyl-*N*-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)aminomethyl]-1-ethylpyridinium chloride) [18] and CV-4151 [(*E*)-7-phenyl-7-(3-pyridyl)-6-heptenoic acid] [19] were synthesized and antiserum for TXB_2 and 6-keto-$PGF_{1\alpha}$ [16] were prepared at Takeda Chemical Industries. [^{3}H]TXB_2 (sp. act. 150 Ci/mmol) and [^{3}H]6-keto-$PGF_{1\alpha}$ (sp. act. 100 Ci/mmol) were purchased from New England Nuclear Co. (Boston, MA, U.S.A.). Indomethacin and dexamethasone were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Ionomycin was purchased from Calbiochem (La Jolla, CA, U.S.A.). Glycoletherdiamine-*N*,*N*,*N*′,*N*′-tetraacetic acid and fura-2 acetoxymethyl ester were purchased from Dojin Chemical (Kumamoto, Japan). All other reagents were of the highest grade commercially available.

RESULTS

Effect of ET-1 on the production of prostanoids in rat vascular smooth muscle cells

The extracts from rat VSMC which received various doses of ET-1 for 10 min were subjected to radioimmunoassay for TXB_2, a stable metabolite of TXA_2. As shown in Fig. 1, ET-1 at concentrations of 10^{-11} M to 10^{-6} M increased TXB_2 in rat VSMC in a dose-dependent manner. ET-1 at the highest concentration of 10^{-6} M increased TXB_2 production to 334 ± 28% (basal TXB_2 release is 100%). ET-1 at concentrations of 10^{-11} M to 10^{-6} M also increased 6-keto-$PGF_{1\alpha}$, a stable metabolite of PGI_2 in rat VSMC in a dose-dependent manner. ET-1 at the highest concentration of 10^{-6} M increased 6-keto-$PGF_{1\alpha}$ production to 345 ± 17% (basal 6-keto-$PGF_{1\alpha}$ release is 100%). The pretreatment of rat VSMC with indomethacin (10^{-6} M) for 10 min inhibited completely the 10^{-6} M ET-1-stimulated release of both TXB_2 and 6-keto-$PGF_{1\alpha}$ (data not shown).

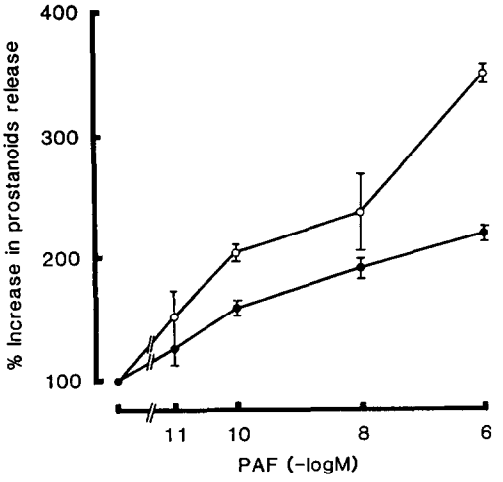

Fig. 2. Effect of PAF on the biosynthesis of thromboxane B_2 (●) and 6-keto-prostaglandin $F_{1\alpha}$ (○) in rat vascular smooth muscle cells (VSMC) as a function of concentration. Confluent rat VSMC (2×10^6 cells) in 1 mL of HEPES-buffered PSS supplemented with 0.05% bovine serum albumin were incubated with various doses of PAF for 10 min at 37°. The basal levels of thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ were 128 ± 13 pg/10^6 cells and 121 ± 17 pg/10^6 cells, respectively. Values represent the means ± SE of three experiments.

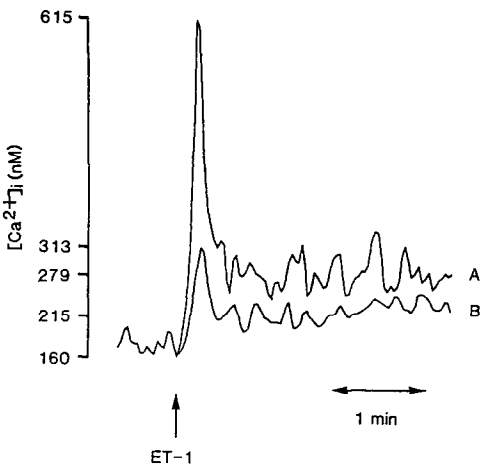

Fig. 3. Ca^{2+}-fura-2 fluorescence by endothelin (ET)-1 in cultured rat vascular smooth muscle cells (VSMC) and inhibitory effect of dexamethasone on ET-1-induced increase in $[Ca^{2+}]_i$. The trace reflects changes in $[Ca^{2+}]_i$ as evaluated from changes in fluorescence of fura-2-loaded rat VSMC cultured directly on glass cover slides. Each trace is representative of three similar experiments. (A) Control response at 10^{-9} M ET-1; (B) pretreatment with 10^{-7} M dexamethasone for 4 hr. The values of $[Ca^{2+}]_i$ are indicated on the ordinates.

A TXA_2 synthetase inhibitor CV-4151 (10^{-6} M) completely inhibited the TXB_2 release (data not shown). A specific PAF antagonist CV-6209 (10^{-6} M) reduced the TXB_2 release by 66 ± 8% (N = 3) and the 6-keto-$PGF_{1\alpha}$ release by 83 ± 9% (N = 3).

Effect of PAF on the production of prostanoids in rat vascular smooth muscle cells

As shown in Fig. 2, PAF at concentrations of 10^{-11} M to 10^{-6} M also increased rat VSMC TXB_2 and 6-keto-$PGF_{1\alpha}$ concentration in a dose-dependent manner. PAF at the highest concentration of 10^{-6} M increased TXB_2 production to 220 ± 2% and 6-keto-$PGF_{1\alpha}$ production to 351 ± 5% (basal TXB_2 and 6-keto-$PGF_{1\alpha}$ releases are 100%), respectively.

Effect of dexamethasone on Ca^{2+} mobilization induced by ET-1 and PAF

ET-1 caused a two phase increase of $[Ca^{2+}]_i$ in rat VSMC, characterized by a rapid (15–20 sec) increase followed by a sharp decline to the sustained phase which was approximately 20% of maximal $[Ca^{2+}]_i$ and which lasted for ~10–15 min (Fig. 3, curve A).

Pretreatment of rat VSMC with dexamethasone (10^{-7} M) for 4 hr inhibited the transient increase and the sustained increase in $[Ca^{2+}]_i$ induced by ET-1 by 66 and 54%, respectively (Fig. 3, curve B).

This inhibitory effect of dexamethasone was time-dependent for the first 4 hr and constant at times beyond 4 hr (Table 1). The transient increase and the sustained increase in $[Ca^{2+}]_i$ induced by ET-1 were only slightly inhibited after 0.5 hr of 10^{-3} M dexamethasone pretreatment (by 47 and 33%, respectively). However, after 4 hr pretreatment, the IC_{50} (concentration that causes 50% inhibition) values for both transient and sustained increase in $[Ca^{2+}]_i$ induced by ET-1 were lower than 10^{-8} M.

Table 1. Inhibitory effect of dexamethasone on endothelin-1-induced transient and sustained increases in $[Ca^{2+}]_i$ of rat vascular smooth muscle cells (VSMC) for various treatment times

	IC_{50} (M)	
Incubation time	Transient increase	Sustained increase
30 min	$> 10^{-3}$	$> 10^{-3}$
4 hr	$(6.6 \pm 0.7) \times 10^{-9}$	$(9.6 \pm 1.6) \times 10^{-9}$
15 hr	$(1.8 \pm 0.6) \times 10^{-9}$	$(7.6 \pm 1.2) \times 10^{-9}$
24 hr	$(8.1 \pm 1.9) \times 10^{-10}$	$(5.0 \pm 0.2) \times 10^{-9}$

Rat VSMC (3.2×10^5) were pretreated with dexamethasone in the culture medium for the desired incubation time. After the dexamethasone-treated VSMC were loaded with fura-2, endothelin-1 was added to a final concentration of 10^{-9} M. IC_{50} (concentration for 50% inhibition) values were determined from dose–response curves of inhibition by dexamethasone.

Values are means ± SE from three experiments.

PAF induced a transient elevation of $[Ca^{2+}]_i$ (30–40 sec), but it was not sustained (Fig. 4, curve A). The PAF-induced elevation of $[Ca^{2+}]_i$ was also strongly inhibited by dexamethasone after 15 hr (Fig. 4, curve B) and 24 hr of pretreatment. IC_{50} values for 15 and 24 hr of dexamethasone pretreatment were $(1.7 \pm 0.5) \times 10^{-9}$ M and $(1.5 \pm 0.9) \times 10^{-9}$ M (N = 3), respectively.

DISCUSSION

We previously reported the possible involvement

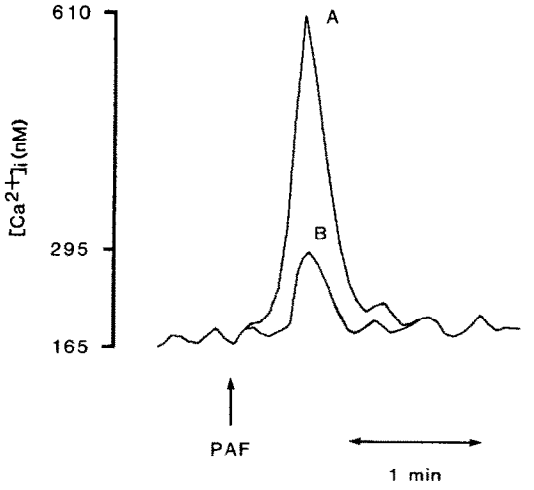

Fig. 4. Ca^{2+}-fura-2 fluorescence by PAF in cultured rat vascular smooth muscle cells (VSMC) and inhibitory effect of dexamethasone on PAF-induced increase in $[Ca^{2+}]_i$. The trace reflects the increase in $[Ca^{2+}]_i$ as evaluated from the changes in fluorescence of fura-2-loaded rat VSMC cultured directly on glass cover slides. Each trace is representative of three similar experiments. (A) Control response at 4×10^{-8} M PAF; (B) pretreatment with 10^{-7} M dexamethasone for 15 hr. The values of $[Ca^{2+}]_i$ are indicated on the ordinates.

of TXA_2 in ET-1-induced elevation of $[Ca^{2+}]_i$ in cultured rat VSMC [9]. de Nucci *et al.* [20] reported that ET-1 stimulates the release of TXA_2 and PGI_2 in guinea-pig and rat isolated lungs. Thus, the present study was designed to investigate whether ET-1 stimulates the biosynthesis of these eicosanoids in cultured rat VSMC or not. The biosynthesis of PGI_2 was also stimulated by ET-1. However, CV-4151 (TXA_2 synthetase inhibitor) and AA-2414 (TXA_2 receptor antagonist) inhibited the ET-1-induced elevation of $[Ca^{2+}]_i$ [9]. Thus, TXA_2 but not PGI_2 mediates ET-1-induced elevation of $[Ca^{2+}]_i$. In preliminary experiments, U-46619, a TXA_2/PGH_2 agonist, induced elevation of $[Ca^{2+}]_i$ in cultured rat VSMC (unpublished data). A lowering effect of PGI_2 on $[Ca^{2+}]_i$ in platelets [21] has been reported, but the effect of PGI_2 on $[Ca^{2+}]_i$ mobilization in rat VSMC remains to be clarified.

PAF also stimulated the biosynthesis of TXA_2 and PGI_2 in cultured rat VSMC (Fig. 2). Furthermore, not only the cyclooxygenase inhibitor indomethacin but also a specific PAF antagonist CV-6209 potently inhibited this ET-1-induced biosynthesis of TXA_2 and PGI_2. CV-6209 is a specific PAF antagonist; CV-6209 does not inhibit cyclooxygenase, TXA_2 synthetase, PGI_2 synthetase or contraction of rabbit aorta induced by KCl [18]. Therefore, inhibition of biosynthesis of both eicosanoids by CV-6209 may not be due to a direct effect of CV-6209 on TXA_2 synthetase and PGI_2 synthetase. Thus, it is strongly suggested that PAF stimulates the key enzyme of arachidonic acid cascade, PLA_2, through PAF receptor mediated mechanisms. Since TXA_2 related drugs (indomethacin, CV-4151 and AA-2414) also inhibit the elevation of $[Ca^{2+}]_i$ induced by PAF, TXA_2 is a responsible mediator for PAF-induced mobilization of $[Ca^{2+}]_i$ in rat VSMC. The pharmacological evidence suggests that ET-1-induced elevation of $[Ca^{2+}]_i$ is mediated through the production of PAF and subsequently TXA_2.

As previously reported [9], PAF antagonist (CV-6209) inhibits the ET-1 (10^{-9} M)-induced elevation of $[Ca^{2+}]_i$ in a dose-dependent manner, and at a higher concentration of this PAF antagonist the inhibition is complete. Therefore, at least in this experimental condition, the PAF pathway contributes to the total action of 10^{-9} M ET-1 in increasing $[Ca^{2+}]_i$ by 100%.

However, the $[Ca^{2+}]_i$ mobilization is qualitatively different between ET-1 and PAF; ET-1 caused a biphasic increase in $[Ca^{2+}]_i$ (Fig. 3), whereas PAF induced a monophasic one (Fig. 4). At present, though the precise reason for the qualitative difference is unknown, the existence of an intracellular PAF receptor [22] could explain the difference. Endogenous PAF produced by ET-1 at first would bind to the intracellular PAF receptor, which links to the initial phase of increase in $[Ca^{2+}]_i$. Unbound PAF would diffuse or be carried to the extracellular space, and the "extracellular PAF" would traverse the cell membrane and bind to the intracellular PAF receptor, which links to the second phase. Exogenous PAF as well as the diffused extracellular PAF would traverse the cell membrane and reach the PAF receptor, which causes a monophasic increase. Thus, ET-1 causes biphasic $[Ca^{2+}]_i$ mobilization through the endogenous PAF action, whereas exogenous PAF may produce the monophasic $[Ca^{2+}]_i$ mobilization. Furthermore, there are a large number of high-affinity binding sites for ET-1 in rat VSMC [8]. Tight association of ET-1 with its receptor would cause this long-lasting sustained increase.

We previously described that a putative PLA_2 inhibitor chlorpromazine inhibits both ET-1 and PAF-induced elevation of $[Ca^{2+}]_i$ [9]. The present study was also designed to clarify the role of PLA_2 in $[Ca^{2+}]_i$ mobilization induced by ET-1 or PAF using dexamethasone. Dexamethasone was reported to be a typical glucocorticoid having the ability to induce synthesis of the PLA_2 inhibitory proteins, lipocortins. The dexamethasone treatment required time to effect $[Ca^{2+}]_i$ mobilization (Table 1). Exposure of cells to dexamethasone for 0.5 hr before application of ET-1 resulted in only slight inhibition of $[Ca^{2+}]_i$ elevation. However, more than 4 hr pretreatment of rat VSMC with dexamethasone potently inhibited the ET-1-induced increase in $[Ca^{2+}]_i$. These results suggest that dexamethasone induces the synthesis of lipocortins, which inhibit the elevation of $[Ca^{2+}]_i$ via the inhibition of PLA_2. Moreover, dexamethasone also inhibited the PAF-induced elevation of $[Ca^{2+}]_i$ in rat VSMC.

These results can be explained by the following model. ET-1 binds to its receptors on the plasma membrane of rat VSMC. This causes biosynthesis of PAF, and PAF stimulates the PLA_2 through the receptor mediated mechanism, and subsequently activate AA cascade especially TXA_2. Finally, TXA_2 causes both Ca^{2+} release from intracellular Ca^{2+}

stores and Ca^{2+} influx possibly through the Ca^{2+} channels in the plasma membrane.

It is concluded that both ET-1 and PAF stimulate TXA_2 biosynthesis in cultured rat VSMC and that the activities of PLA_2 may play a key role in the interactions among ET-1, PAF and TXA_2.

Acknowledgements—After submission of this paper, Reynolds and Mok reported that ET-1 stimulates biosynthesis of TXA_2 and role of TXA_2 in ET-1-induced contraction in rat aorta [23].

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