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RECEPTOR-MEDIATED MITOGENIC EFFECT OF THROMBOXANE A_2 IN VASCULAR SMOOTH MUSCLE CELLS

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Abstract—The effects of thromboxane A_2 (TXA_2) on the proliferation of vascular smooth muscle cells (VSMC) were examined using primary cultures of VSMC from rat aorta. U46619, a stable TXA_2 mimetic, stimulated DNA synthesis of VSMC only in the presence of insulin. The effect was concentration-dependent with a half-maximal effect obtained at approximately 1×10^{-8} M. The mitogenic effect of U46619 was larger than that of endothelin, another mitogen derived from endothelium. Among several TXA_2/PGH_2 analogs, the proliferative activity was detected only in the agonists, and not in the antagonists or in the metabolite of TXA_2. A series of TXA_2/PHG_2 receptor antagonists completely suppressed the U46619-stimulated DNA synthesis as well as the [^{3}H]SQ29,548 binding to the TXA_2/PGH_2 receptors in VSMC. The rank order of binding affinities to the receptors among the respective antagonists correlated well with the potencies for suppression of the proliferative effects of U46619. The mitogenic effects of U46619 were also attenuated by the presence of calcium antagonists. U46619 caused activation of phospholipase C with the production of inositol trisphosphate, leading to increases in the intracellular free Ca^{2+} concentration as measured with the fluorescent indicator fura-2. These results suggest that TXA_2 induces mitogenic effects on VSMC through binding to its specific receptors. This effect of TXA_2 on the proliferation of VSMC may be related to the development of atherosclerosis.

Thromboxane A_2 (TXA_2†), the predominant metabolite of arachidonic acid in platelets, is an exceptionally potent inducer of platelet aggregation and constrictor of vascular smooth muscles [1], and thus has been implicated as an important pathophysiologic mediator of a variety of cardiovascular and renal diseases [2, 3]. A number of stable agonists and antagonists have been synthesized as tools for investigating the nature of putative thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) receptors and the mechanisms of TXA_2 action [4, 5]. The specific receptors for TXA_2/PGH_2 have been characterized recently in both platelets [6–9] and vascular smooth muscles [10–12] by binding studies using several radioligands. In platelets, TXA_2 is known to stimulate the hydrolysis of plasma membrane inositol phospholipids through binding to its receptors, resulting in the generation of inositol trisphosphate (IP_3) and diacylglycerol, which serve as second messengers for intracellular Ca^{2+} mobilization and protein kinase C activation respectively [13–15]. However, the biochemical signals generated by TXA_2 in vascular smooth muscles remain to be clarified.

Gryglewski *et al.* [16] have previously reported enhanced synthesis of TXA_2 in platelets during the development of experimental atherosclerosis in rabbits. Antagonism of the TXA_2/PGH_2 receptor with specific receptor antagonists has been shown recently to reduce the deposition of cholesterol in the aortic wall and retard plaque formation in coronary arteries in hypercholesterolemic rabbits [17]. These observations have led to the proposal of involvement of TXA_2 in the promotion of atherosclerosis. The abnormal proliferation of vascular smooth muscle cells (VSMC) has been established as a crucial event in the development of vascular lesions by atherosclerosis [18]. Furthermore, activated platelets are known to release several mitogenic factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), which may act to stimulate the growth of VSMC leading to atherosclerotic plaque formation [19, 20]. Thus, one possible mechanism by which TXA_2 participates in the progression of atherosclerosis may involve TXA_2-induced platelet activation and another is TXA_2 having a direct effect on VSMC proliferation. In an attempt to test our hypothesis, we used primary cultures of smooth muscles derived from rat aorta, which possess the intrinsic TXA_2/PGH_2 receptors corresponding to the pharmacological responses [10].

The present study provides evidence for the mitogenic effects of TXA_2 on rat VSMC through binding to the TXA_2/PGH_2 receptors. TXA_2 is also found to induce phospholipase C activation leading to the production of IP_3, which appears to induce the mobilization of intracellular Ca^{2+} in VSMC.

EXPERIMENTAL PROCEDURES

Materials. [5,6-^{3}H]SQ29,548 (40.0 Ci/mmol) and

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† Abbreviations: TXA_2 (B_2), thromboxane A_2 (B_2); PGH_2 ($F_{2\alpha}$), prostaglandin H_2 ($F_{2\alpha}$); VSMC, vascular smooth muscle cells; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; IP_3, inositol 1,4,5-triphosphate; and EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

[*methyl*-^{3}H]thymidine (85.6 Ci/mmol) were purchased from Du Pont-New England Nuclear, and *myo*-[2-^{3}H]inositol (10–20 Ci/mmol) was obtained from the Amersham Corp. U46619, $PGF_{2\alpha}$, forskolin. transferrin, insulin and bovine serum albumin (BSA) were from Sigma. Verapamil and diltiazem were from Wako Chemicals, Japan; endothelin was from Peptide Research Laboratories, Japan; PDGF, TXB_2, CTA_2 and PTA_2 were from Funakoshi, Japan; and fura-2/AM was purchased from Dojin, Japan. S-145 as well as its (+)-isomer and (−)-isomer, STA_2, SQ29,548 and ONO3708 were synthesized at the Shionogi Research Laboratories [21]. Other materials and chemicals were obtained from commercial sources.

Cell culture. Vascular smooth muscle cells were isolated from medial explants of rat thoracic aorta (7-week-old Sprague–Dawley male rats) by the method of Ross [22]. The resulting cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°. Cells were passaged by harvesting with 0.125% trypsin–0.01% EDTA. After serial subculture, VSMC harvested between the fifth and eighth passages were used in the experiments.

Binding experiments. The [^{3}H]SQ29,548 binding assay was carried out according to the method of our previous papers [10, 11]. Briefly, confluent VSMC were washed twice with phosphate-buffered saline and then harvested with 0.125% trypsin–0.01% EDTA. After washing twice with serum-free DMEM, VSMC were suspended in Hanks' medium, pH 7.6, containing 0.1% BSA (buffer A). The binding study was performed by incubating the cells (1.5×10^6) with 4.2 nM [^{3}H]SQ29,548 in 0.5 mL buffer A at 24° for 40 min. Specific binding is defined as the differences between binding in the presence and absence of 10 μM unlabeled SQ29,548. After incubation, ice-cold 0.9% NaCl (3 mL) was added to each tube, and the reaction mixture was filtered immediately by suction through a Whatman GF/C glass filter, which was then washed four times with cold 0.9% NaCl, and the radioactivity was measured. The specific binding of [^{3}H]SQ29,548 (4.2 nM) represented 83 ± 3% of the total binding to rat VSMC.

Measurement of DNA synthesis. The assay for DNA synthesis was carried out by the method of Huang and Ives [23] with slight modification. Confluent cultures of VSMC were grown in 24-well plates and growth-arrested by placing them in DMEM containing 20 mM Hepes (pH 7.4), 5 μg/mL transferrin and 0.5 mg/mL BSA for 48 hr. After exchanging medium for fresh DMEM, the quiescent cells were stimulated with various agents for 24 hr at 37°. [^{3}H]Thymidine (1 μCi/well) was added during the final 6 hr. The cells were fixed with trichloroacetic acid (TCA), and TCA-insoluble radioactivity was measured as described [24].

Measurement of intracellular Ca^{2+} *concentration.* Confluent VSMC were dispersed by 0.125% trypsin–0.01% EDTA treatment. The harvested cells (2×10^7 cells) were washed twice with phosphate-buffered saline, suspended in 10 mL of Hanks' medium containing 10 mM Hepes (pH 7.4) and 0.25% BSA (buffer B), and then incubated for 40 min at room temperature with 1 μM fura-2/AM, which was dissolved in dimethyl sulfoxide (final concentration 0.1%). After loading, the cells were washed twice and resuspended in buffer B at a concentration of approximately 1×10^6 cells/mL. The fluorescence ratio, obtained by dividing the fluorescence at 340 nm by that at 380 nm, was determined using a CAF-100 Ca^{2+} analyzer (Japan Spectroscopic Co., Ltd., Japan) while stirring at 37°. The emission wavelength was 500 nm. Changes in the fluorescence ratio were calibrated to changes in intracellular Ca^{2+} concentrations using the method of Grynkiewicz *et al.* [25].

Measurement of the production of inositol phosphates. The assay for the formation of inositol phosphates was performed according to the method of Araki *et al.* [26] with slight modifications. After VSMC were grown to confluence in 35-mm dishes, the culture medium in each dish was replaced with 2 mL of serum- and inositol-free medium containing 5 μCi/mL of [^{3}H]inositol, and the cells were incubated for 48 hr at 37°. The labeled cells were washed three times with a warm balanced salt solution containing 20 mM Hepes (pH 7.4), 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.5 mM $CaCl_2$, and incubated in the same solution containing additionally 10 mM LiCl, 10 mM glucose and 0.1% BSA for 10 min, and finally stimulated as indicated in the figure. The reactions were stopped by replacement with 5% TCA, and the acid-soluble inositol phosphates were separated by anion exchange chromatography as described by Berridge *et al.* [27].

Statistical analysis. Linear regression of the data was performed according to the standard method [28]. Statistical analysis was done by Student's *t*-test, and a P value of less than 0.05 was used as the criterion for statistical significance.

RESULTS

Effect of U46619 on DNA synthesis in rat VSMC. Figure 1 shows the effect of U46619, a stable TXA_2/PGH_2 mimetic agonist [29], on [^{3}H]thymidine incorporation into acid-insoluble DNA in rat VSMC. When quiescent cells were incubated with U46619 alone for 24 hr, DNA synthesis of VSMC was not altered significantly. However, U46619 stimulated DNA synthesis in a concentration dependent manner in the presence of 1 μM insulin, which by itself showed only minimal mitogenic activity. The concentration required to evoke half-maximal proliferation was 1×10^{-8} M, and maximal stimulation was obtained at 3×10^{-7} M. These concentrations corresponded well with those required for inducing constriction of rat aorta [10, 30]. The cells, which were treated with 3×10^{-7} M U46619 plus insulin for 1 min and then incubated in the insulin-containing medium after thorough washing, showed a significant increase in [^{3}H]thymidine incorporation (20 ± 3% increase compared to the cells continuously treated with U46619 for 24 hr). The treatment of VSMC with 3×10^{-7} M U46619 in the presence of insulin under serum-free conditions

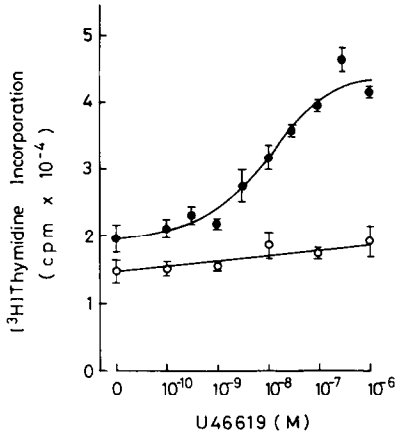

Fig. 1. Stimulation of DNA synthesis by U46619 in cultured VSMC. The quiescent cells were incubated with various concentrations of U46619 in the presence (●) or absence (○) of 1 μM insulin for 24 hr at 37°. [^{3}H]Thymidine incorporation into DNA was measured as described under Experimental Procedures. Each point is the mean ± SE of three experiments.

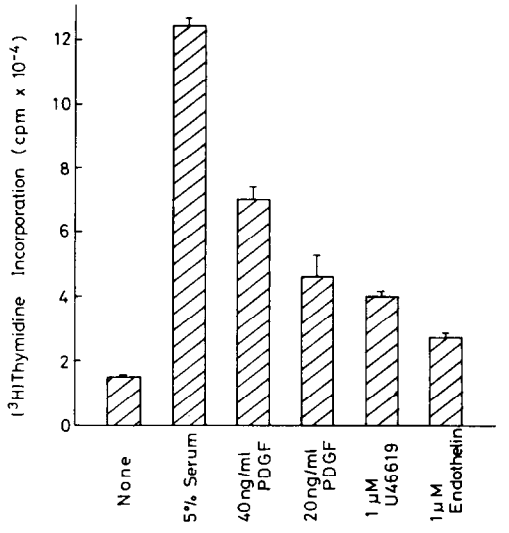

Fig. 2. Stimulation of DNA synthesis by various compounds in cultured VSMC. The quiescent cells were incubated with fetal bovine serum (5%), PDGF (20 or 40 ng/mL), U46619 (1 μM) plus insulin (1 μM) or endothelin (1 μM) plus insulin (1 μM) for 24 hr at 37°. [^{3}H]Thymidine incorporation into DNA was measured as described under Experimental Procedures. Each point is the mean ± SE of three experiments.

for 2 days resulted in a significant increase in cell number (control: $5.0 \pm 0.01 \times 10^4$ cells/cm^2; U46619 treatment; $8.0 \pm 0.51 \times 10^4$ cells/cm^2; N = 3). As shown in Fig. 2, the maximal effects by 1 μM U46619 were, respectively, about 38 and 58% compared with those caused by 5% FBS and 40 ng/mL of PDGF, and almost comparable to that of 20 ng/mL of PDGF. Endothelin, an endothelium-derived vasoconstrictor peptide, also exhibited mitogenic effects on VSMC, but its maximal level at 1 μM was smaller than that of U46619. To assess the specificity of U46619 effects, a variety of TXA_2 analogs were examined for their capacities to induce proliferation of VSMC. As shown in Fig. 3, STA_2 and CTA_2, both of which are known as TXA_2/PGH_2 receptor agonists in vasculature [31, 32], significantly enhanced [^{3}H]thymidine incorporation into VSMC. On the other hand, TXA_2/PGH_2 receptor antagonists [(+)-S-145, SQ29,548 ONO3708 and PTA_2], as well as a stable TXA, metabolite (TXB_2), had no effects on DNA synthesis, suggesting that U46619 induces the mitogenic effects via the TXA_2/PGH_2 receptors.

Inhibition of U46619-stimulated DNA synthesis in VSMC. A number of structurally dissimilar compounds were tested for their abilities to suppress the DNA synthesis stimulated by U46619. Figure 4 depicts the inhibition curves for these compounds in VSMC. Six TXA_2/PGH_2 receptor antagonists inhibited the DNA synthesis in a concentration-dependent fashion, and the potency series of the IC_{50} values was found to be (+)-S-145 > (±)-S-145 > (−)-S-145 ≧ SQ29,548 > ONO3708 = PTA_2. Forskolin, a compound reported to increase intracellular cAMP levels in rat VSMC [33], also blocked the U46619 effects at an IC_{50} of 3×10^{-6} M. Calcium antagonists, such as verapamil and diltiazem, were inhibitory only at considerably higher concentrations. Neither TXB_2 not $PGF_{2\alpha}$ suppressed the U46619-induced proliferation at 10 μM. (+)-S-145 did not inhibit the DNA synthesis stimulated by

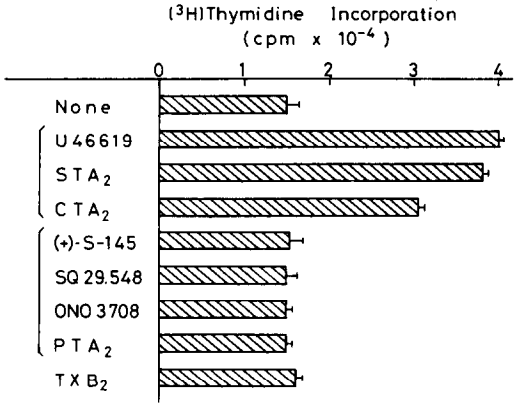

Fig. 3. Effect of various TXA_2 analogs on DNA synthesis in cultured VSMC. The quiescent cells were incubated with various TXA_2 analogs at a concentration of 1 μM in the presence of 1 μM insulin for 24 hr at 37°. [^{3}H]Thymidine incorporation into DNA was measured as described in Experimental Procedures. Data are the means ± SE of three experiments.

PDGF (40 ng/mL) and endothelin (1 μM) (data not shown). As shown in Fig. 5, all of the TXA_2/PGH_2 receptor antagonists completely displaced the specific binding of [^{3}H]SQ29,548 to the TXA_2/PGH_2 receptors in VSMC. The potency for inhibition was found to be (+)-S-145 > (±)-S-145 > SQ29,548 ≧ (−)-S-145 > ONO3708 = PTA_2. Two calcium blockers also inhibited the [^{3}H]SQ29,548 binding at 1 μM, but complete suppression was not observed even at 10 μM. TXB_2 and forskolin scarcely

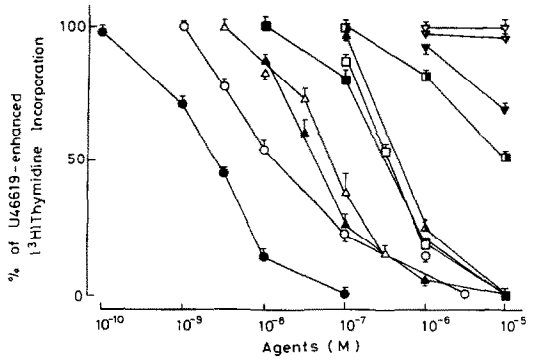

Fig. 4. Inhibition of U46619-stimulated DNA synthesis by various compounds in cultured VSMC. The quiescent cells were incubated with 100 nM U46619 plus 1 μM insulin in the presence of various concentrations of (+)-S-145 (●), (±)-S-145 (○), (−)-S-145 (▲), SQ29,548 (△), ONO3708 (□), PTA_2 (■), TXB_2 (▽), $PGF_{2\alpha}$ (▼), forskolin (Δ), verapamil (▼) and diltiazem (◨) for 24 hr. [^{3}H]Thymidine incorporation into DNA was measured as described in Experimental Procedures. The control value (25,500 ± 700 cpm) was defined as the DNA synthesis level stimulated by U46619 in the absence of these compounds. Each point is the mean ± SE of three experiments.

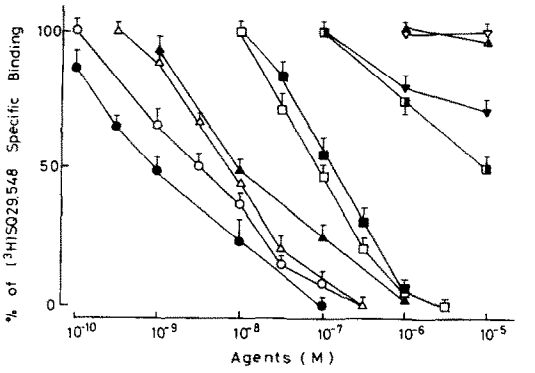

Fig. 5. Displacement of specific [^{3}H]SQ29,548 binding to cultured VSMC by various compounds. The cells were incubated with 4.2 nM [^{3}H]SQ29,548 in the presence of various concentrations of (+)-S-145 (●), (±)-S-145 (○), (−)-S-145 (▲), SQ29,548 (△), ONO3708 (□), PTA_2 (■), TXB_2 (▽), forskolin (Δ), verapamil (▼) and diltiazem (◨) at 24° for 40 min. The specific binding was found by subtracting non-specific binding obtained with 10 μM SQ29,548 from each point. The control value (1800 ± 120 dpm) was defined as the specific binding of [^{3}H]SQ29,548 in the absence of these compounds. Each point is the mean ± SE of three experiments.

caused displacement of the ligand binding. The [^{3}H]SQ29,548 binding characteristics were not altered in the presence of 1 μM insulin (data not shown). Using the IC_{50} values and the K_d value for [^{3}H]SQ29,548 (1.72 nM, derived from previous data [10]), K_i values for the receptor antagonists were approximated according to the Cheng–Prusoff equations [34]. The negative logarithms of K_i values for six TXA_2/PGH_2 receptor antagonists against the [^{3}H]SQ29,548 binding correlated highly with the corresponding IC_{50} values against the U46619-provoked DNA synthesis (Fig. 6, $r = 0.97$). S-145 suppressed both responses in a stereospecific manner and (+)-S-145 was found to be the most potent antagonist among those used in the present study. Further evidence for the relationships of the inhibitory potencies between the binding and proliferation was obtained in the following experiments. As shown in Table 1, pretreatment of VSMC with 1 μM (+)-S-145 for 24 hr, followed by washing of the cells, resulted in *ca.* a 40% decrease, compared to the control cells, in the activity not only for [^{3}H]SQ29,548 binding but also for U46619-induced DNA synthesis. In contrast, no changes were observed in both responses of VSMC pretreated with 1 μM ONO3708 or SQ29,548. These data agree well with the previous observation that S-145 has a much smaller dissociation rate constant than other antagonists in the VSMC TXA_2/PGH_2 receptors [11]. Thus, U46619 may exert its mitogenic effects through binding to the TXA_2/PGH_2 receptors.

Effect of U46619 on intracellular free Ca^{2+} *concentration.* The TXA_2-induced Ca^{2+} mobilization in VSMC was studied by measuring the changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), using fura-2 as a Ca^{2+} indicator. Addition of U46619 (100 nM) provoked a rapid increase in

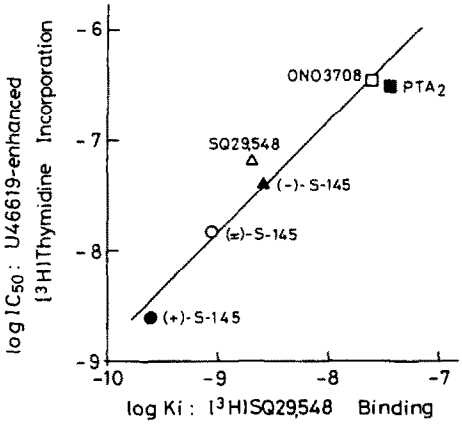

Fig. 6. Correlation between the potencies of several TXA_2/PGH_2 receptor antagonists to displace [^{3}H]SQ29,548 binding and to inhibit U46619-stimulated DNA synthesis in cultured VSMC ($r = 0.97$, slope = 0.96). The data represent a log–log plot of K_i ([^{3}H]SQ29,548 binding to VSMC) versus IC_{50} (U46619-stimulated [^{3}H]thymidine incorporation) values employing data from Figs. 5 and 4, respectively. The K_i values were calculated from the Cheng–Prusoff equation [34]. Each point is the mean of three experiments.

$[Ca^{2+}]_i$, followed by a gradual decline to the baseline value (data not shown). Figure 7 shows the concentration–response relationships for the U46619-evoked $[Ca^{2+}]_i$ rise. The half-maximal effects occurred at approximately 1×10^{-8} M U46619. Pretreatment with 1 μM SQ29,548 caused complete abolition of the U46619-induced elevation of $[Ca^{2+}]_i$. When extracellular Ca^{2+} was lowered by adding 2 mM EGTA, U46619 still provoked the increase in

Table 1. Effect of pretreatment with TXA_2/PGH_2 receptor antagonists on U46619-stimulated DNA synthesis and [^{3}H]SQ29,548 binding in VSMC

Pretreatment	U46619-stimulated [^{3}H]thymidine incorporation (%)	[^{3}H]SQ29,548 binding (%)
Control	100	100
ONO3708	101 ± 2.3	103 ± 4.6
SQ29,548	105 ± 5.6	97.1 ± 9.1
(+)-S-145	53.2 ± 3.2	59.8 ± 12.3

For the assay of DNA synthesis, the quiescent cells were pretreated with 1 μM ONO3708, SQ29,548 or (+)-S-145 for 24 hr at 37°. After washing five times with DMEM, the cells were incubated with 100 nM U46619 in the presence of 1 μM insulin for 24 hr, and the incorporation of [^{3}H]thymidine was measured as described under Experimental Procedures. For the binding assay, the cells were pretreated with 1 μM ONO3708, SQ29,548 or (+)-S-145 for 24 hr at 37°, and then the specific binding activity of [^{3}H]SQ29,548 was measured after thorough washing, as described under Experimental Procedures. The control value (100%) was defined as the level in the nontreated VSMC ([^{3}H]thymidine incorporation = 22,000 ± 500 cpm, [^{3}H]SQ29,548 binding = 2,100 ± 130 dpm). Data are the means ± SE of four experiments.

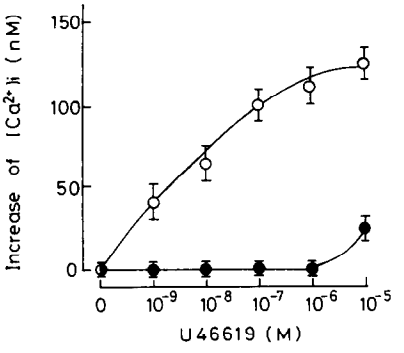

Fig. 7. Effect of SQ29,548 on U46619-stimulated increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured VSMC. Fura-2-loaded cells were pretreated with (●) or without (○) 10 μM SQ29,548 for 2 min at 37°, and then exposed to various concentrations of U46619. $(Ca^{2+})_i$ was measured with a fluorescent indicator, fura-2, and peak $[Ca^{2+}]_i$ values were determined. Each point is the mean ± SE of three experiments.

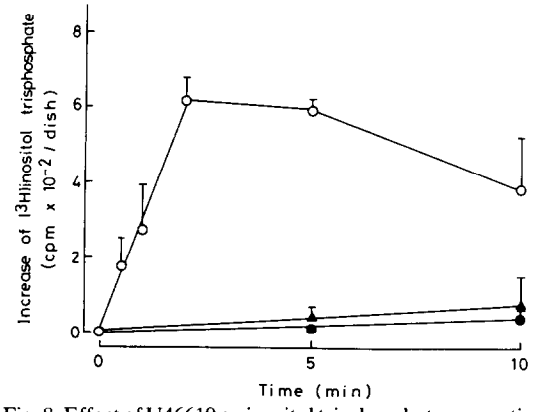

Fig. 8. Effect of U46619 on inositol trisphosphate generation in cultured VSMC. The [^{3}H]inositol-labeled cells were incubated for 10 min, and then stimulated with vehicle (▲) or U46619 (1 μM) in the presence (●) or absence (○) of 1 μM (+)-S-145 for indicated time periods at room temperature. Increases of IP_3 over the level at the zero time point (1203 ± 56 cpm) are represented. Each point is the mean ± SE of three experiments.

$[Ca^{2+}]_i$ (data not shown), suggesting that U46619 induces the mobilization of Ca^{2+} from intracellular pools.

Effect of U46619 on the formation of inositol phosphates in VSMC. The activation of phospholipase C during stimulation with U46619 was examined by measuring the increase of [^{3}H]IP_3 in the [^{3}H]inositol-prelabeled VSMC. As shown in Fig. 8, 1 μM of U46619 evoked a significant increase of IP_3, an effect which was blocked completely by the addition of the TXA_2/PGH_2 receptor antagonist, (+)-S-145. U46619 also stimulated the formation of inositol monophosphate as well as inositol bisphosphate, and still induced IP_3 generation even in the presence of 2 mM EGTA (data not shown).

DISCUSSION

TXA_2 has been known as a potent constrictor of vascular smooth muscles [1]. The present study demonstrated that TXA_2 also stimulates mitogenesis in vascular smooth muscle cells through binding to its specific receptors. We utilized cultured VSMC from rat thoracic aorta for the *in vitro* model, as these cells possess intrinsic TXA_2/PGH_2 receptors corresponding to the contractile responses [10], and also retain the capacity for mobilizing intracellular Ca^{2+} by stimulation with a TXA_2/PGH_2 agonist [35].

Although U46619 alone could not stimulate DNA synthesis in the growth-arrested VSMC (Fig. 1), it showed proliferative effects in the presence of a progression factor, insulin, which stimulates G_1 stage cells to progress to the S stage of the cell cycle [36]. These results suggest that TXA_2 may function as a competence factor, which stimulates G_0 stage cells

to enter the G_1 stage [36]. The stimulatory effect of a stable TXA_2 analog on the 10% fetal calf serum-induced DNA synthesis has also been reported in rat VSMC [37, 38]. These characteristics resemble those of endothelin, which also displays potent vasoconstrictive and mitogenic effects on VSMC [39, 40].

Among the variety of TXA_2 analogs used in the present study, the mitogenic effects were observed only in the agonists for the TXA_2/PGH_2 receptors (Fig. 3), demonstrating that U46619 stimulates proliferation through binding to the TXA_2/PGH_2 receptors. Several lines of evidence support this conclusion. First, six TXA_2/PGH_2 receptor antagonists completely suppressed the DNA synthesis stimulated by U46619 (Fig. 4). Furthermore, the IC_{50} values of these antagonists for inhibition of U46619 effects correlated linearly with the K_i values for the [^{3}H]SQ29,548 binding (Fig. 6). Second, in the S-145-pretreated VSMC subjected to thorough washing, the mitogenic activity of U46619 decreased in parallel with the decrease in the binding activity for the TXA_2/PGH_2 receptors, whereas no effects were observed in the SQ29,548- or ONO3708-pretreated VSMC (Table 1). The differences among these receptor antagonists may be explained by the discrepancies in the rate of dissociation from the receptors [9]. Long-lasting antagonism of S-145 against the U46619 effects may be due to the much smaller dissociation rate constant (K_{-1}) of this agent [11].

The growth-promoting effects could be detected only in the 1-min treatment with U46619, indicating that TXA_2 may act as a physiological mitogen in VSMC despite its inherent instability [1]. The EC_{50} value of U46619 for the induction of mitogenic effects (*ca.* 10 nM) was closely comparable with its EC_{50} value of contraction provocation in rat aorta [10, 30] as well as the K_d value of the high-affinity binding site of U46619 in cultured VSMC [12]. Moreover, the rank order of inhibitory potencies of several TXA_2/PGH_2 receptor antagonists against the U46619-stimulated DNA synthesis (S-145 > SQ29,548 > ONO3708) was identical with that against the U46619-induced constriction [10]. These data strongly suggest that TXA_2 causes VSMC proliferation via the same receptors as those that induce vasoconstriction. Two types of VSMC have been proposed by Campbell and Campbell: one is a contractile type and the other is a synthetic type [41]. The synthetic type of VSMC was stimulated to proliferate by growth factors, whereas the contractile type was not. The migration of VSMC from the medial layer to the intima may cause the cells to change functionally from being "contractile" to "synthetic," thus leading to abnormal VSMC proliferation. The cultured cells used in the present study were regarded as the synthetic type [41]. It is therefore tempting to speculate that, through binding to the same receptor, TXA_2 provokes contractile effects on VSMC in the medial layer, but induces mitogenic effects in the intima. Further studies are needed to examine this proposal.

Two calcium antagonists, verapamil and diltiazem, suppressed the U46619-stimulated DNA synthesis only at micromolar concentrations (Fig. 4), but they also caused a partial displacement of the [^{3}H]SQ29,548 bindings in the same concentration range (Fig. 5). These data indicate a cross-reactivity of calcium blockers on the vascular TXA_2/PGH_2 receptors to exert inhibitory effects in high doses, which may be the same mechanism operating in the inhibition as in the competitive antagonism in the platelet TXA_2/PGH_2 receptors [42]. However, the interpretation of their suppressive effects is complicated, because these agents can reduce the proliferative activity of other growth factors by lowering the intracellular Ca^{2+} availability [43, 44]. Forskolin also blocked the mitogenic effects of U46619 without preventing the TXA_2/PGH_2 receptor binding, which may be due to the increase of intracellular cAMP caused by this agent [45].

The present study also offers evidence for the coupling of the TXA_2/PGH_2 receptors with inositol lipid signaling pathways in vascular smooth muscles. Stimulation of rat VSMC by a TXA_2 agonist caused the activation of phospholipase C with the production of IP_3 (Fig. 8), leading to a Ca^{2+} mobilization from the intracellular store (Fig. 7). These mechanisms are also operative in the TXA_2-induced platelet activation [13, 14]. The same extent of IP_3 formation by U46619 was observed even in the absence of extracellular Ca^{2+}, suggesting that the TXA_2-evoked activation of phospholipase C does not result from Ca^{2+} influx from outside the cells. It has been shown that IP_3 could induce Ca^{2+} release and vasoconstriction in permeabilized rabbit pulmonary artery [46]. Therefore, TXA_2 may provoke contraction of vascular smooth muscles through an IP_3-dependent mechanism. PDGF and endothelin have also been reported to activate phospholipase C, leading to increased protein kinase C activity and to mobilized Ca^{2+} signals, thereby inducing VSMC growth as well as contraction [47, 48]. The present observation on the mitogenic effects of TXA_2 would further support the concept that contraction and cell growth involve a shared signaling mechanism [47].

The present study demonstrated that TXA_2 may be responsible for stimulating VSMC proliferation as a competence factor. Although insulin has been shown to possess weak progression activity, other potent progression factors such as somatomedin C are present *in vivo* in circulating blood [36]. Furthermore, TXA_2 is synthesized in platelets, VSMC, vascular endothelial cells and monocytes-macrophages [49], all of which are thought to play a crucial role in atherosclerosis [18]. Thus, one might propose paracrine mechanisms where degranulated platelets, infiltrating macrophages and damaged or activated endothelial cells secrete TXA_2 which, in turn, stimulates VSMC proliferation. As VSMC themselves synthesize TXA_2, autocrine-stimulated growth of VSMC could also be considered. In addition to these direct effects on VSMC, TXA_2 may provoke platelet activation resulting in the release of several growth factors (PDGF, EGF, etc.), which stimulate the migration and proliferation of VSMC [19, 20]. The increased generation of TXA_2 was observed in platelets during the development of experimental atherosclerosis in rabbits [16, 50]. Therefore, blockade of the TXA_2/PGH_2 receptors in both platelets and vascular

smooth muscles may exert beneficial effects on atherosclerosis. In fact, Osborne and Lefer [17] have reported recently the antiatherogenic effect of BM13505, a specific TXA_2/PGH_2 receptor antagonist, in hyperchoresterolemic rabbits.

In conclusion, we present here evidence for the receptor-mediated mitogenesis by TXA_2 in vascular smooth muscles. This suggests that TXA_2, as one of the platelet-derived growth factors, has an important role in the development of atherosclerosis.

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