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Characterization of the thromboxane (TP-) receptor subtype involved in proliferation in cultured vascular smooth muscle cells of rat

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1 The effects of the thromboxane A_2 (TxA_2)-mimetic, U-46619, on the proliferation of vascular smooth muscle cells (VSMCs) were examined in a clonal smooth muscle cell line, A10, which was derived from foetal rat aorta.

2 [^{3}H]-U-46619 bound to A10 cells of passages 18-20 (p18-20) with two classes of sites. The high affinity site showed a B_{max} of 3.0 ± 1.8 fmol mg^{-1} protein with a K_D value 1.0 ± 0.1 nM, while the low affinity site showed a B_{max} of 43.0 ± 6.0 fmol mg^{-1} protein and K_D value of 129.0 ± 7.9 nM. However, [^{3}H]-U-46619 bound to A10 cells from passages 28-30 (p28-30) at a single class of site with a B_{max} 111.0 ± 9.0 fmol mg^{-1} protein and a K_D value of 175.4 ± 22.0 nM.

3 Cinnamophilin and SQ29548 inhibited specific [^{3}H]-U-46619 binding to p18-20 A10 cells in a concentration-dependent manner with K_i values of 390.0 ± 3.2 and 4.6 ± 1.0 nM, respectively at a high affinity site, and 2.6 ± 0.2 μM and 310.0 ± 6.4 nM, respectively at the low affinity site.

4 U-46619 produced isometric contractions of rat aorta in a concentration-dependent manner with an EC_{50} 7.0 ± 1.2 nM. Cinnamophilin and SQ29548 antagonized U-46619-induced aortic contractions with pA_2 values 6.3 ± 0.1 and 8.2 ± 0.2, respectively.

5 U-46619 increased [^{3}H]-thymidine incorporation into DNA of p18-20 and p28-30 A10 cells in a concentration-dependent manner with EC_{50} values 362.7 ± 27.0 and 302.5 ± 20.1 nM, respectively. The U-46619-induced increase of [^{3}H]-thymidine incorporation into DNA of p28-30 A10 cells was potentiated by PDGF (1 ng ml^{-1}) and FCS (1%) and was inhibited by cinnamophilin (10 μM) and SQ29548 (1 μM) with estimated pK_B values 5.4 ± 1.2 and 6.3 ± 0.9, respectively.

6 Cell cycle analysis revealed that U-46619-increased cell cycle progression was primarily due to a rapid transition from the DNA synthetic (S) to the G_2/mitotic (M) phase. Moreover, U-46619 also increased protein synthesis and cell numbers in VSMC. All these effects of U-46619 were inhibited by cinnamophilin and SQ29548.

7 U-46619 caused phosphoinositide breakdown and increased the intracellular Ca^{2+} concentration in VSMC, effects which were blocked by cinnamophilin and SQ29548.

8 These data indicate there are two U-46619 binding sites in A10 VSMC. The high affinity site is correlated to U-46619-induced vasoconstriction while the low affinity site is correlated to U-46619-mediated VSMC proliferation. These data also reveal that U-46619 stimulates the cell cycle progression in VSMC primarily through a rapid transition from S to G_2/M. Since cinnamophilin inhibits TP-receptor-mediated VSMC proliferation, it may thus hold promising potential for the prevention of atherosclerosis or vascular diseases.

Keywords: Thromboxane receptor; proliferation; vascular smooth muscle cells; cinnamophilin; *Cinnamomum philippinense*

Introduction

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 muscle cells (VSMC) (Hamberg *et al.*, 1975). Gryglewski *et al.*, (1978) have previously reported enhanced synthesis of TxA_2 in platelets during the development of experimental atherosclerosis in rabbits. It is also postulated that the vascular wall produces significant amounts of TxA_2. Particularly in some genetic rat models for human hypertension, TxA_2 generation in the vascular wall is enhanced (Uehara *et al.*, 1987; Ishimitsu *et al.*, 1989). In addition, recent studies *in vitro* indicate that TP-agonists enhances VSMC proliferation (Ishimitsu *et al.*, 1988; 1990; Uehara *et al.*, 1988). Antagonism of the TP-receptor with specific receptor antagonists reduces the deposition of cholesterol in the aortic wall and retards plaque formation in coronary arteries in hypercholesterolaemic rabbits (Osborne & Lefer, 1988). Based on these studies, it seems possible that TxA_2 released from the vascular wall as well as aggregated platelets plays some role in cardiovascular diseases including atherosclerosis, hypertension and arterial restenosis after angioplasty (Ross, 1986; Ip *et al.*, 1990).

It is therefore of great interest to investigate the regulatory mechanism of activation of TP-receptors on the proliferation of VSMC. Although, a number of studies have been conducted, the possible roles of TxA_2 in the proliferation of VSMC have not been fully addressed. The continuously cultured clonal smooth muscle cell line, A10, which was derived from foetal rat aorta, has proved to be a valuable tool in studying the properties of vascular smooth muscle (Kimes & Brandt, 1976). We have therefore investigated the mechanism by which the TxA_2-mimetic, U-46619, influences the proliferation of smooth muscle using A10 cells. Since it has been suggested that TP-receptor subtypes exist in various tissues and/or species (Masuda *et al.*, 1991; Borg *et al.*, 1994), the TP-receptor subtype involved in mediated U-46619-induced VSMC proliferation has also been elucidated.

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Cinnamophilin is a new lignan isolated from *Cinnamomum philippinense* which we have previously shown to be a selective TP-receptor antagonist, using functional experiments (Yu *et al.*, 1994). In the present study, its effects on the binding of U-46619 to TP-receptors, and on U-46619-mediated proliferation in VSMC have also been investigated.

Methods

Cell culture

A10 (rat thoracic aortic smooth muscle) cells provided by American Type Cell Collection were cultured at 37°C in a humidified air/CO_2 (95:5) atmosphere in 100 mm dishes. The growth medium was Dulbecco's modified Eagle's medium (DMEM) supplemented wth 10% foetal calf serum (FCS), 100 units of penicillin and 100 μg of streptomycin sulphate ml^{-1}. The cells were characterized as smooth muscle cells by morphology and immunostaining with antibody to smooth muscle α-actin.

Binding of [^{3}H]-U-46619

For binding assay, cells were finally grown in 24-well plates. At confluence, cell monolayers were washed with Krebs-Henseleit solution (KHS) (composition mM: NaCl 117.5, KCl 5.4, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 5.5, HEPES 25.0), pH 7.4, and then 250 μl of various concentrations of [^{3}H]-U-46619 was added to 60 min at 37°C. The incubation was terminated by washing the cell monolayers rapidly with 2 ml of ice-cold KHS three times. Subsequently, 0.5 ml of 1 N NaOH was added to solubilize the cell monolayer. The solubilized cells were counted for bound radioactivity in a liquid scintillation counter (Beckman 5000 TC). The equilibrium saturation binding of [^{3}H]-U-46619 and competition with antagonists were conducted in triplicate. Nonspecific binding was defined as binding in the presence of 200 μM U-46619. Protein content of cells was measured by the method of Lowry *et al.* (1951) with bovine serum albumin used as a standard.

Rat aortic contraction

Rat aortic contractions were performed as previously described (Yu *et al.*, 1994) using 5 mm wide rat thoracic aortic rings without endothelium which were attached to isometric force-displacement transducers in organ baths containing continuously aerated (95% O_2: 5% CO_2) Krebs solution maintained at 37°C plus 10 μM indomethacin to prevent endogenous synthesis of prostaglandins. After equilibration under 1 g of tension, cumulative concentration-response curves were obtained.

[^{3}H]-thymidine incorporation

A10 cells (5×10^4 cells $well^{-1}$) were finally grown in 24-well plates for 2 days, washed twice with 1 ml Krebs-Henseleit solution (KHS), then incubated in 0.5 ml DMEM/FCS-free for 48 h to induce quiescence (cell cycle stopped at Go phase). To investigate the effect of U-46619 on proliferation, quiescent cells were cultured at 37°C for 20 h in medium supplemented with or without 10% FCS, containing or lacking experimental agents. Finally, cells were incubated for 4 h in freshly prepared medium that was additionally supplemented with [^{3}H]-thymidine (1 μCi ml^{-1}) to measure DNA synthesis by thymidine incorporation (Morinelli *et al.*, 1994). The experiments were terminated by washing cells with 1 ml KHS, precipitation of acid-insoluble material with 10% trichloroacetic acid (TCA, 1 ml) and extraction of the DNA with 0.1 N NaOH (0.5 ml). The precipitates were collected on Whatman GF/B filters, and filters were washed twice with 5 ml ice-cold KHS. Filters were cut into pieces and shaken with 3.5 ml scintillation fluid for 24 h before liquid scintillation counting.

Cell cycle analysis

To estimate the proportions of cells in different phases of the cell cycle, cellular DNA contents were measured by flow cytometry as described by March *et al.* (1993). Briefly, cells (2×10^6 cells ml^{-1}) were fixed by 70% ethanol (in PBS) in ice for 30 min and then resuspended in PBS containing 40 μg ml^{-1} propidium iodide (PI) and 0.1 mg ml^{-1} RNAse (Boehringer, Germany). After 30 min at 37°C, 2×10^4 cells were analysed on a FACstar cytofluorometer (Becton-Dickinson; San Jose, U.S.A.), exciting at 488 nm and sensing at 585 nm.

Cell growth

To determine the effect of U-46619 on cell growth, cells were seeded on 12-well plates (density was 2.5×10^3 well) and cultured for 48 h in DMEM supplemented with 10% FCS. Cells were then cultured in FCS-free medium for 48 h to induce quiescence. This procedure was followed by culturing for 1 or 3 days in medium supplemented with various agents. Culture medium was change daily and cell numbers were determined by dissociation of adherent cells with trypsin and counting with a haemocytometer.

Protein assay

Serum-starved cells were incubated with stimulation agent for 24 h, then washed twice with KHS. Cells were lysed on 0.1 N NaOH and the protein contents assayed by protein assay kit (Bio-Rad) with bovine serum albumin used as a standard.

[^{3}H]-inositol monophosphate accumulation

Cells for inositol phospholipid hydrolysis were finally grown in 12-well plates. At confluence, cell monolayers were loaded with [^{3}H]-*myo*-inositol (5 μCi ml^{-1}) for 24 h in inositol-free DMEM. Prelabelled cells were then washed twice with KHS solution and incubated for 15 min in the presence of 10 mM LiCl. U-46619 was added and incubation continued for 60 min. Incubation was terminated by stop buffer (0.1 N NaOH + 0.4% EDTA) and addition of 0.1 N HCl (1:1 v/v). Cells were left a minimum of 30 min at −20°C before isolation of total [^{3}H]-inositol phosphates by anion exchange chromatography (Alexander *et al.*, 1989). Briefly, 800 μl of the supernatant was neutralized by addition of 300 μl of 0.1 N NaOH, 1 ml of 50 mM Tris-HCl and added to an AG1-X8 column. The resin was washed with a total 25 ml of H_2O. The [^{3}H]-inositol monophosphate was then eluted with 3 ml of 0.2 M ammonium formate/0.1 M formic acid. Radioactivity was determined by scintillation counting after addition of scintillation cocktail.

Measurement of intracellular Ca^{2+} level

$[Ca^{2+}]_i$ was measured by loading confluent cells with fura-2/AM as described by Grynkiewicz *et al.* (1985). Individual glass coverslips were placed in 6-well plates at a density of 5×10^4 cells cm^{-2}. When the cells had reached confluence, they were loaded with 5 μM fura-2/AM for 30 min at 37°C. At the end of the loading period, the coverslips were washed three times with the physiological buffer solution (PBS) containing (mM): NaCl 125, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2, NaH_2PO_4 0.5, $NaHCO_3$ 5, HEPES 10 and glucose 10, pH 7.4. The cells were then incubated in the PBS for another 15 min to complete dye de-esterification. Loaded coverslips were then mounted in a specially designed holder which enabled the coverslip to be positioned across the diagonal of the polymethacrylate cuvette. Each cuvette contained 2.4 ml of PBS buffer (drugs were added to the cuvette in 100 μl aliquots) and fluorescent measurements were made at 37°C by using spectrophotometer (CAF-110, Jasco, Japan). The excitation wavelengths were 340 and 380 nm, and emission

wavelength was 510 nm, respectively. The ratio of the fluorescence due to excitation at 340 nm (F_{340}) to that at 380 nm (F_{380}) was calculated from illumination periods and referred to as $R_{340/380}$. $[Ca^{2+}]_i$ was calculated from the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = (R\text{-}R_{min})/(R_{max}\text{-}R) \times K_D \times S$$

Where R is $R_{340/380}$, dissociation constant (K_D) was 224 nM, R_{max} and R_{min} were determined by addition of ionomycin (10 μM) and EGTA (10 mM), respectively. The constant value (S) is the ratio of F_{380} in Ca^{2+}-free solution to that in Ca^{2+}-containing solution in the presence of ionomycin.

Data analysis

Saturation and displacement binding data were analysed by the weighted least-squares iterative curve fitting programme LIGAND (Munson & Rodbard, 1980). The data were fitted to a one- and then a two-site model, and if the residual sums of squares were statistically less for a two-site fit of the data than for a one-site as determined by *F*-test comparison, then the two-site model was accepted.

The experimental results are expressed as the mean ± s.e.mean and accompanied by the number of observations. A one-way analysis of variance (ANOVA) was used for multiple comparison, and if there was significant variation between treatment groups, then the mean values for inhibitors were compared with those for control by Student's *t* test, and *P* values of less than 0.05 were considered to be statistically significant.

Drugs

Cinnamophilin, (**8R**, **8'S**)-4,4'-dihydroxy-3,3'-dimethoxy-7-oxo-8,8'-neolignan, was isolated from the plant *Cinnamomum philippinense* as described previously (Wu *et al.*, 1994). Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), penicillin G, streptomycin sulphate and all other tissue culture reagents were obtained from Grand Island Biological Co. (GIBCO). [^{3}H]-thymidine and *myo*-[2-^{3}H]-inositol were obtained from Amersham (Buckinghamshire, UK) and [^{3}H]-U-46619 (> 10 Ci mmol^{-1}) was obtained from NEN (Boston, U.S.A.). Purified human platelet-derived growth factor (PDGF/BB) was obtained from Biomedical Technologies Inc. Protein dye reagent was obtained from Bio-Rad. U-46619 (9,11-dideoxy-9α, 11α-methanoepoxy-prostaglandin $F_{2\alpha}$), SQ29548 ([1S-[1α,2α(2),3α,4α)]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazine]methyl]-7oxabicyclo[2,2.1]hept-2yl]-5-heptanoic acid), fura-2/AM, trypan blue and other chemical reagents were obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.). The antibody used for immunostaining was anti-smooth muscle α-actin (mouse monoclonal, Dako M851).

Results

Phenotypic change of A10 VSMC in culture

Ultrastructurally, A10 cells of passage 18–20 (p18–20) closely resembled 'contractile state' smooth muscle cells in the intact aortic media with their cytoplasm containing substantial bundles of myofilaments. The A10 cells were ribbon or fusiform in shape. These cells were phase-dense with phase-contrast microscopy, and they responded to mechanical or electrical stimulation or the addition of angiotension II at 0.1 μg ml^{-1} by a slow contraction. However, they underwent a spontaneous change in phenotype to the 'synthetic state' during culture. A10 cells of p28–30 lost thick myosin-containing filaments, and became broader, flatter, and less phase-dense, after which they lost their contractility and began to migrate actively (data not shown). Thus, A10 cells of p18–20 and p28–30 were used, and compared in further experiments.

[^{3}H]-U-46619 binding in A10 VSMC

[^{3}H]-U-46619 at concentrations ranging from 1 to 300 nM was used to label TP-receptors of p18–20 A10 cells. The specific binding of [^{3}H]-U-46619 was 61.0 ± 8.0% of the total binding at 300 nM [^{3}H]-U-46619. Scatchard plots of the binding data were curvilinear, suggesting more than a single class of binding site. LIGAND analysis fitted the data to a two-site model. The high affinity site showed a B_{max} of 3.0 ± 1.8 fmol mg^{-1} protein with a K_D value 1.0 ± 0.1 nM, while the low affinity site showed a B_{max} of 43.0 ± 6.0 fmol mg^{-1} protein and K_D value of 129.0 ± 7.9 nM (Figure 1a). In contrast, [^{3}H]-U-46619 binding to p28–30 A10 cells at a higher concentration-range (25–800 nM) showed a saturable tendency at concentrations of 400–800 nM. The specific binding of [^{3}H]-U-46619 was 65.0 ± 8.0% of total binding at 800 nM [^{3}H]-U-46619. Scatchard plots of the binding data were linear, resulting in a better fit to a one-site model in computerized analysis with a K_D value of 175.4 ± 22.0 nM and a B_{max} of 111.0 ± 9.0 fmol mg^{-1} protein (Figure 1b). Cinnamophilin and SQ29548 displaced specific [^{3}H]-U-46619 binding to p18–20 A10 cells in a concentration-dependent manner (Figure 2) with K_i values 390.0 ± 3.2 and 4.6 ± 1.0 nM (n = 4), respectively at the high affinity site, and 2.6 ± 0.2 μM and 310.0 ± 6.4 nM (n = 4), respectively at the low affinity site (Table 1).

Effect of U-46619 on rat aortic contraction

Our initial experiment was directed toward characterizing the receptor interaction responsible for the contractile effects of the thromboxane-mimetic. U-46619 (1–300 nM) contracted aortic rings in a concentration-dependent manner with EC_{50} 7.0 ± 1.2 nM (n = 7), which was close to the K_D value of [^{3}H]-U-46619 binding to the high affinity site (1.0 ± 0.1 nM) in p18–20

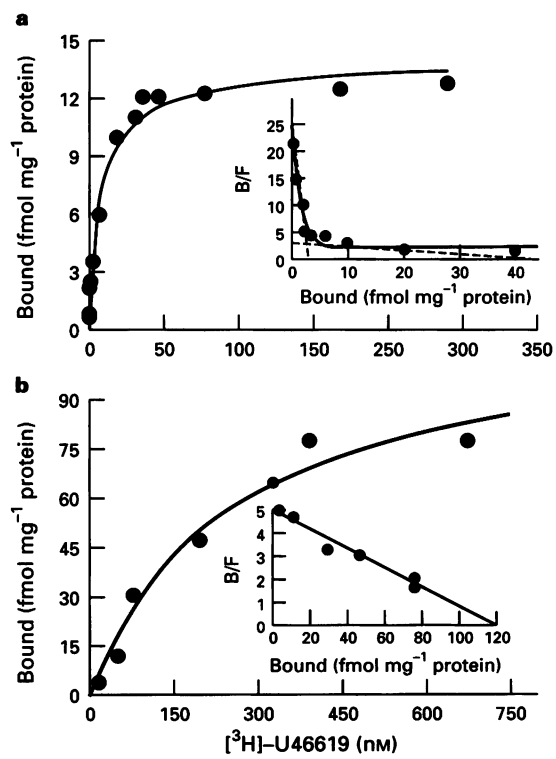

Figure 1 Saturation experimental data and Scatchard plots (insets) of [^{3}H]-U-46619 binding to A10 cells (p18–20, a; p28–30, b). Non-specific binding was defined in the presence of 200 μM unlabelled U-46619 and was substracted from total binding to afford specific binding. The specific binding of 300 nM [^{3}H]-U-46619 was 960 ± 100 d.p.m. mg^{-1} protein in p18–20 A10 cells. The data shown were those from a single experiments of four experiments determined in triplicate in cultured A10 cells.

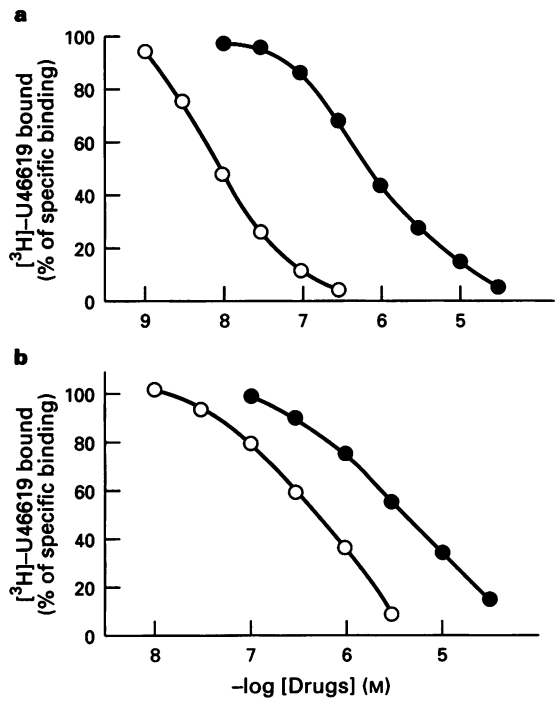

Figure 2 Displacement of [^{3}H]-U-46619 binding from A10 cells by cinnamophilin (●) and SQ29548 (○). The concentrations of [^{3}H]-U-46619 used for high (a) and low (b) affinity sites were 1 and 100 nM, respectively. The figure represents a single experiment for each drug, where each point is the mean of triplicate determinations.

Table 1 Relative potencies of SQ29584 and cinnamophilin against U-46619-mediated responses

Responses	*SQ29548*	*Cinnamophilin*
[^{3}H]-U46619 binding (K_i values)		
High affinity site	4.6 ± 1.0 nM	390.0 ± 3.2 nM
Low affinity site	310.0 ± 6.4 nM	2.6 ± 0.2 μM
Smooth muscle contraction (pA_2 values)	8.2 ± 0.2	6.3 ± 0.1
[^{3}H]-thymidine incorporation (pK_B values)	6.3 ± 0.9	5.4 ± 1.2

A10 cells. Cinnamophilin and SQ29548 antagonized U-46619-induced aortic contractions with pA_2 values 6.3 ± 0.1 and 8.2 ± 0.2 (n = 6), respectively (Table 1).

Effect of U-46619 on DNA synthesis in A10 VSMC

Figure 3 shows the effect of U-46619 on [^{3}H]-thymidine incorporation into acid-insoluble DNA in A10 cells. When quiescent cells were incubated with U-46619 for 24 h, DNA synthesis was stimulated in a concentration-dependent manner. The concentrations required to evoke half-maximal proliferation were 362.7 ± 27.0 and 302.5 ± 20.1 nM, respectively in p18–20 and p28–30 A10 cells. This concentration was also close to the K_D values of [^{3}H]-U-46619 binding to the low affinity site in p18–20 cells and in p28–30 cells. Thus, A10 cells of p28–30 were used in the further studies. Cinnamophilin (10 μM) and SQ29548 (1 μM) caused rightward shifts of the concentration-response curves to U-46619 in p28–30 A10 cells with pK_B values of 5.4 ± 1.2 and 6.3 ± 0.9, respectively (Figure 4 and Table 1), which were close to their K_i values against [^{3}H]-U-46619 binding to the low affinity site in p18–20

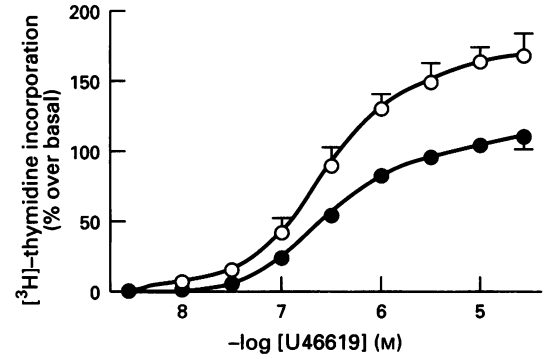

Figure 3 Increase in [^{3}H]-thymidine incorporation in p18–20 (●) and p28–30 (○) A10 cells stimulated with U-46619. [^{3}H]-thymidine was added 20 h after the quiescent cells had been exposed to U-46619 and then incubations were continued for another 4 h. Basal [^{3}H]-thymidine incorporation was 0.9 ± 0.1 d.p.m. × 10^4. Data are expressed as the mean ± s.e.mean (n = 4–5, each in quadruplicate).

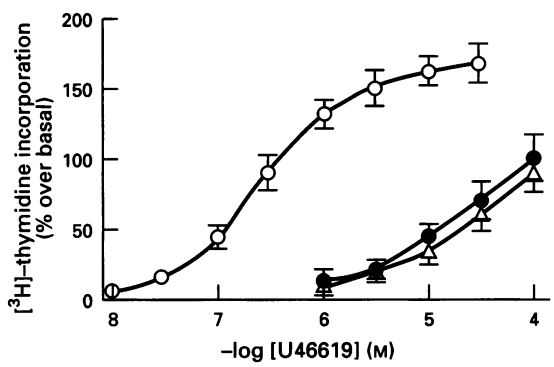

Figure 4 Inhibition of U-46619-stimulated [^{3}H]-thymidine incorporation by cinnamophilin and SQ29548 in p28–30 A10 cells. Quiescent cells were stimulated by U-46619 in the presence of dimethylsulphoxide (0.1% for solvent control, ○), cinnamophilin (10 μM, ●) or SQ29548 (1 μM, △) for 24 h. [^{3}H]-thymidine was added 20 h after the quiescent cells had been exposed to U-46619 and then incubations were continued for another 4 h. Basal [^{3}H]-thymidine incorporation was 1.1 ± 0.2 d.p.m. × 10^4. Antagonist alone had no effect on basal incorporation. Data are expressed as the mean ± s.e.mean (n = 4, each in quadruplicate).

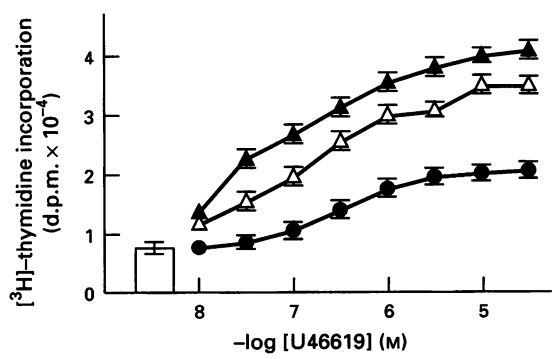

Figure 5 Potentiation by platelet-derived growth factor (PDGF) and foetal calf serum (FCS) of [^{3}H]-thymidine incorporation in p28–30 A10 cells stimulated with U-46619. Quiescent cells were stimulated by U-46619 in the presence of saline (●, control), PDGF (1 ng ml^{-1}, △) or FCS (1%, ▲) for 24 h. Column represents [^{3}H]-thymidine incorporation in unstimulated cells. Data are presented as the mean ± s.e.mean (n = 6, each in quadruplicate). Each data point in the presence of PDGF or FCS is significantly different from control (●).

A10 cells. In contrast, PDGF (1 ng ml^{-1}) and FCS (1%) which alone had no effect on DNA synthesis but markedly potentiated U-46619-induced [^{3}H]-thymidine incorporation into DNA in p28–30 A10 cells (Figure 5).

Effect of U-46619 on cell cycle in synchronized A10 VSMC

To investigate the mechanism of the enhanced cell cycle progression by U-46619, we examined each period in the proliferating cell cycle using flow cytometric techniques. Quiescent p28–30 A10 cells were stimulated with FCS (10%) at time 0 (Figure 6a). DNA synthesis increased slowly, reached its maximum within 16–20 h, and declined soon after. The G_1 phase (pre-DNA synthesis) can be confined to the first 8 h, the S phase (DNA synthesis) between 10 to 20 h, and the G_2/M phase (premitosis/mitosis) between 22–30 h (Figure 6a). U-46619 at 10 μM (the concentration that enhances the cell cycle progression in A10 VSMC) did not alter the initiation time of DNA synthesis but shifted the G_2/M phase was toward the left (Figure 6b). These data strongly suggest that the transition from the Go/G_1 to the S period is not influenced, but the transition from the S to the M period is significantly shortened, by U-46619.

To clarify this difference, we examined alterations in the cell cycle progression, using VSMC synchronized in the G_1/S boundary. The cycling process was stopped at this boundary with hydroxyurea (10 μM) and started again by washout of this reagent with fresh media containing 10% FCS at time 0. DNA content of the G_2/M phase was increased after the washout. This DNA content curve (G_2/M phase) was significantly shifted toward the left when the cells were simultaneously stimulated with 10 μM U-46619 (Figure 7). The effect of U-46619 facilitating the transition from the S to the G_2/M phase was totally inhibited by cinnamophilin (10 μM) and SQ29548 (1 μM) (Figure 7).

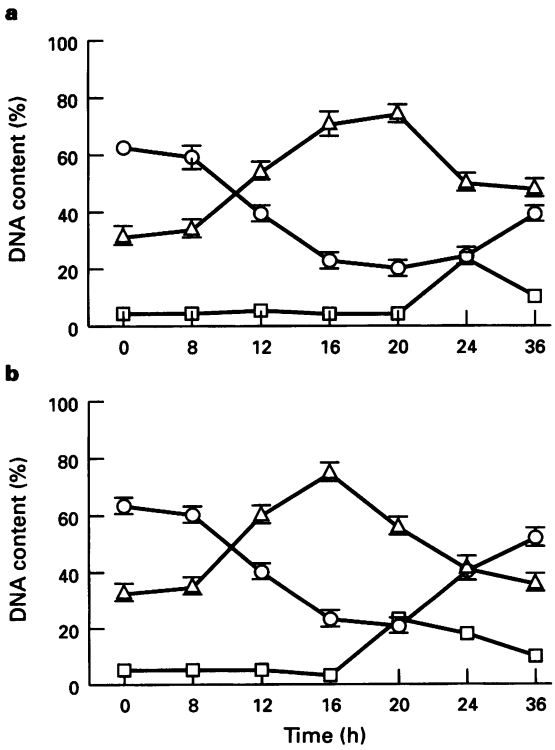

Figure 6 Effect of U-46619 on cell cycle progression in synchronized A10 cells. Cell cycle progression was evaluated by flow cytometric determination of DNA content in synchronized subconfluent A10 cells evaluated at various times. Proliferation of quiescent A10 cells (p28–30) was stimulated by media containing 10% FCS at time 0 in the absence (a) or presence (b) of U-46619 (10 μM). (○), (△) and (□) represent G_o/G_1, S and G_2/M phases, respectively. Data are presented as the mean ± s.e.mean ($n=4$).

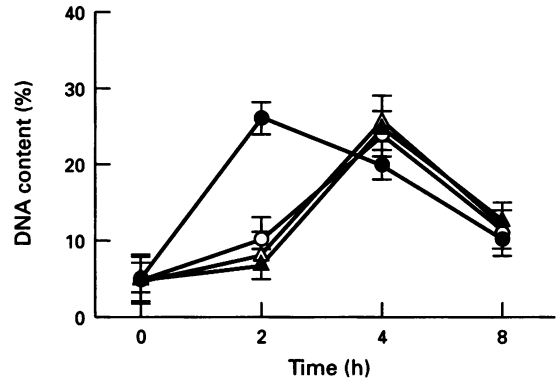

Figure 7 Effects of cinnamophilin and SQ29548 on shortened G_2/M progression by U-46619 in G_1/S boundary synchronized A10 cells. Cells (p28–30) synchronized in G_1/S boundary were stimulated with 10% FCS at time 0 in the absence (○) or presence of U-46619 (10 μM, ●), cinnamophilin (10 μM) + U-46619 (10 μM) (△) or U-46619 (10 μM) + SQ29548 (1 μM) (▲). Data (DNA content of G_2/M phase) are presented as the mean ± s.e.mean ($n=4$).

Effects of U-46619 on cell growth and protein content

Cell counts were used to evaluate the effect of U-46619 on cell growth. A10 cells made quiescent by a 48 h incubation in serum-free DMEM were incubated for 1 or 3 days in fresh media in the presence or absence of U-46619 (10 μM) or 10% FCS. The number of viable A10 cells remained constant during this period in the absence of added serum or growth factor (data not shown). The number of viable cells increased 1.49 ± 0.03 and 3.67 ± 0.16 fold after 1- and 3-day re-exposure of quiescent, serum-starved cells to 10% FCS (Table 2). Thus, even after extended serum starvation, A10 cells divided when full growth medium was reintroduced. In contrast, a 1-day exposure of these cells to U-46619 did not increase cell number, while a 3-day exposure increased cell number 2.42 ± 0.14 fold. The effect of U-46619 was again significantly reduced by cinnamophilin (10 μM) and SQ29548 (1 μM) (Table 2).

Incubation of serum-starved A10 cells with U-46619 (10 μM) or 10% FCS for 24 h significantly increased the pro-

Table 2 Stimulation of cell growth by re-exposure to FCS or U-46619

Treatments	*Cell numbers* (fold)	
	Day 1	*Day 3*
Resting	1.00 ± 0.10	1.16 ± 0.14
FCS (10%)	1.49 ± 0.03[a]	3.67 ± 0.16[a]
U-46619 (10 μM)	1.16 ± 0.07	2.42 ± 0.14[a]
Cinnamophilin (10 μM) + U-46619 (10 μM)	1.04 ± 0.06	1.58 ± 0.17[b]
SQ29548 (1 μM) + U-46619 (10 μM)	0.97 ± 0.06	1.32 ± 0.13[c]

A10 cells (p28–30) were serum-starved for 48 h, then treated with FCS or U46619 in the absence or presence of cinnamophilin or SQ29548 for 1 or 3 days. After specific times, cells were washed and the number counted. Cell numbers are presented as mean ± s.e.mean ($n=4$, each in duplicate). [a] $P<0.001$ as compared with the resting. [b] $P<0.01$; [c] $P<0.001$ as compared with U-46619.

tein content of A10 cells (Figure 8). In four such experiments, U-46619 or FCS increased the amount of protein by 87±27 and 163±31% over the resting level, respectively. Cinnamophilin (10 μM) and SQ29548 (1 μM) markedly inhibited the increase of protein content caused by U-46619 without any significant effect on that increase by FCS.

Effect of U-46619 on the formation of inositol monophosphate and intracellular free Ca^{2+} concentration

The activation of phospholipase C during stimulation with U-46619 was examined by measuring the increase of [^{3}H]-inositol monophosphate accumulation in the [^{3}H]-inositol-prelabelled A10 cells. As shown in Figure 9, 10 μM U-46619 evoked a significant increase of [^{3}H]-inositol monophosphate, which was markedly reduced by cinnamophilin (10 μM) and SQ29548 (1 μM). The U-46619-induced Ca^{2+} mobilization in A10 cells was studied by measuring the changes in intracellular free Ca^{2+} concentration, using fura-2 as a Ca^{2+} indicator. U-46619 (10 μM) induced 109±12 nM (n=4) increase of intracellular Ca^{2+} concentration over the resting level. Again, cinnamophilin (10 μM) and SQ29548 (1 μM) markedly inhibited this U-46619-induced increase of intracellular Ca^{2+} concentration to 27±4 and 18±4 nM (n=4), respectively.

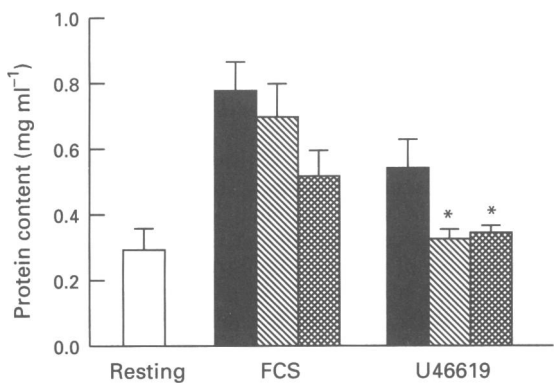

Figure 8 Stimulation of total protein content by FCS and U-46619. A10 cells (p28–30) were serum-starved for 48 h, then stimulated with FCS (10%) or U-46619 (10 μM) in the absence (solid columns) or presence of SQ29548 (1 μM, hatched columns) or cinnamophilin (10 μM, cross-hatched columns) for another 24 h. Protein content of resting cells (open column) is 0.30±0.06 mg ml^{-1}. Data are presented as mean±s.e.mean (n=4, each in triplicate). *P<0.05 as compared with the respective control.

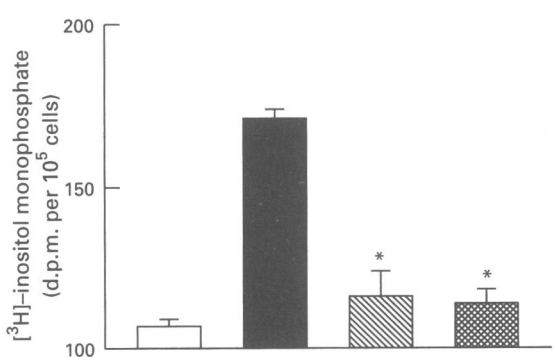

Figure 9 Effects of cinnamophilin or SQ29548 on the formation of [^{3}H]-inositol monophosphate of A10 cells (p28–30) caused by U-46619. [^{3}H]-*myo*-inositol-labelled A10 cells were incubated with DMSO (0.5%), cinnamophilin (10 μM, hatched column) or SQ29548 (1 μM, cross-hatched column) at 37°C for 15 min, then solvent (open column) or U-46619 (10 μM, solid column) was added for another 1 h. Data are presented as mean±s.e.mean (n=4). *P<0.001 as compared with the control (U-46619 alone).

Discussion

Vascular smooth muscle cells (VSMC) alter the phenotype *in vivo* in response to altered functional demands. An example of this is during regeneration and repair of a blood vessel following direct damage or after endothelial denudation. Two types of VSMC have been proposed by Campbell & Campbell (1985): one is a 'contractile' type and the other is a 'synthetic' type. The synthetic type of VSMC is stimulated to proliferate by growth factors, whereas the contractile type is not. The migration of VSMC from medial layer to the intima may cause the cells to change functionally from being 'contractile' to 'synthetic' type, thus leading to abnormal VSMC proliferation. It would appear, therefore, that in most cases phenotypic modulation of VSMC from the contractile toward the synthetic state is a prerequisite for proliferation. The p18–20 and p28–30 cultured A10 cells used in this study were regarded as the 'contractile' and 'synthetic' types, respectively in comparison with their ultrastructural properties and pharmacological responses to those described by Campbell & Campbell (1985). These data indicate that clonal A10 cells are similar to primary VSMC, and change their phenotypes in cultures. Thus, p18–20 and p28–30 A10 cells were used to characterize further the binding properties and the contractile and proliferation effects of thromboxane A_2 (TxA_2).

[^{3}H]-U-46619 bound to p18–20 A10 cells with two classes of sites with K_D values 1.0±0.1 and 129.0±7.9 nM, respectively for high and low affinity sites. These data were consistent with those previously reported by Hanasaki & Arita (1989) using primary culture of rat aortic smooth muscle cells. However, only the low affinity binding site (K_D 175.4±22.0 nM) still existed in p28–30 A10 cells. U-46619 induced contractions of rat aortic smooth muscle with an EC_{50} or 7.0±1.2 nM, which was close to the K_D value of high affinity binding site in p18–20 A10 cells. Furthermore, cinnamophilin and SQ29548, inhibited [^{3}H]-U-46619 binding to this high affinity site with K_i values close to their pA_2 values against U-46619-induced contraction of rat aorta (Table 1). All these data imply that TxA_2-induced VSMC contraction is mediated by the high affinity binding site of VSMC.

U-46619 also stimulated proliferation of A10 cells since it caused [^{3}H]-thymidine incorporation into DNA of A10 cells. The EC_{50} values of U-46619 for the stimulation of proliferation (362.7±2.7 and 302.5±20.1 nM, respectively for p18–20 and p28–30 A10 cells) were closely comparable with the K_D values of the low-affinity binding site of [^{3}H]-U-46619 in p18–20 A10 cells and in p28–30 A10 cells. Moreover, cinnamophilin and SQ29548 antagonized U-46619-induced [^{3}H]-thymidine incorporation into DNA of A10 cells with pK_B values which were similar to their K_i values inhibiting [^{3}H]-U-46619 binding to the low affinity site in p18–20 A10 cells (Table 1). Thus, the growth-promoting effect of U-46619 may be mediated by the low affinity binding site in A10 VSMC. The reason that U-46619 caused more [^{3}H]-thymidine incorporation into DNA of p28–30 than into DNA of p18–20 A10 cell was probably due to the higher receptor numbers present in p28–30 compared with p18–20 A10 cells.

TxA_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) are powerful vasoconstrictor agents in a variety of species and vascular beds. The K_D values of [^{3}H]-U-46619 binding to the low-affinity site of A10 cells were similar to those values for $PGF_{2\alpha}$ (117±13 nM) and PGE_2 (93±18 nM) binding to primary cultures of rat aortic smooth muscle cells (Hanasaki & Arita, 1989). Moreover, the EC_{50} values of U-46619-induced [^{3}H]-thymidine incorporation into DNA of A10 cells were similar to that (230±109 nM) of $PGF_{2\alpha}$-induced protein synthesis (Dorn *et al.*, 1992). However, it is unlikely that U-46619-stimulated VSMC proliferation was due to its action on $PGF_{2\alpha}$/PGE receptor. The first reason is that EC_{50} values for U-46619-, $PGF_{2\alpha}$- and PGE_2-induced VSMC proliferation are not identical whereas their binding affinities (K_D) are identical. The EC_{50} of PGE_2-induced protein synthesis reported by Dorn *et al.* (1992) is one order higher than those of

U-46619 and $PGF_{2\alpha}$. The other reason is that U-46619-induced VSMC proliferation is inhibited by the TP-receptor antagonists, SQ29548 and cinnamophilin, while $PGF_{2\alpha}$- and PGE_2-induced VSMC proliferation is not (Dorn *et al.*, 1992).

The role(s) of TxA_2 in VSMC proliferation is controversial. It was reported that TxA_2 was a competence factor of VSMC proliferation since it caused only protein synthesis and hypertrophy, but not hyperplasia of VSMC (Hanasaki *et al.*, 1990; Dorn *et al.*, 1992). However, others found that it acted as a progression factor for VSMC proliferation since it enhanced the [^{3}H]-thymidine uptake into DNA and shortened the doubling time in randomly cycling VSMC (Ishimitsu *et al.*, 1988; Uehara *et al.*, 1988; Nagata *et al.*, 1992). In the present study, we obtained evidence that TxA_2 may be a progression factor of VSMC proliferation. U-46619 caused protein synthesis, [^{3}H]-thymidine incorporation into DNA and increase of cell number in cultured A10 cells. Cell cycle analysis revealed that the increased cell cycle progression is primary due to a rapid transition from the S to the G_2/M phase. Moreover, the DNA content curve of G_2/M phase was significantly shifted toward the left when the cells synchronized in the G_1/S boundary were stimulated with U-46619. These data also support the notion that U-46619-induced VSMC proliferation is not due to an action on $PGE_{2\alpha}$/PGE_2 receptor since $PGF_{2\alpha}$ and PGE_2 merely stimulate protein synthesis of VSMC (Dorn *et al.*, 1992).

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 and diacyglycerol, which serve as second messengers for intracellular Ca^{2+} mobilization and protein kinase C activation respectively (Nakano *et al.*, 1988; Halushka & Mais, 1989). Phosphoinositide hydrolysis is also the most appealing signalling system to be involved in the control of the cell proliferation (Pouyssegur & Seuwen, 1992). The present study also offers evidence for the coupling of the TP-receptor to the inositol lipid signalling pathways in VSMC proliferation. Stimulation of p28–30 A10 cells by U-46619 caused the activation of phospholipase C with production of [^{3}H]-inositol monophosphate (Figure 9) leading to an increase in intracellular Ca^{2+} concentration. Since a TxA_2 mimetic-induced vasoconstriction was also accompanied with inositol phospholipid hydrolysis (Fukuo *et al.*, 1986; Dorn *et al.*, 1992), the present observation on the proliferative effects of TxA_2 would support the concept that contraction and cell growth involve a shared signalling mechanism (Berk & Alexander, 1989).

The present study demonstrated that TxA_2 may be responsible for stimulating VSMC proliferation as a progression factor. The concentration of the TxA_2-mimetic needed to induce VSMC proliferation are much higher than those to induce vasoconstriction. It may still act as a physiological mitogen in VSMC since PDGF (1 ng ml^{-1}) or FCS (1%) alone had no effect on DNA synthesis but markedly potentiated U-46619-induced [^{3}H]-thymidine incorporation into DNA of VSMC (Figure 5). Furthermore, TxA_2 is a potent inducer of platelet aggregation and activated platelets are known to release several mitogenic factors, such as PDGF and EGF. It was also reported by Hanasaki *et al.* (1990) that the growth-promoting effect could be detected only after 1 min treatment with U-46619. Thus, these data indicate that TxA_2 may act as physiological mitogen in VSMC, and blockade of the TP-receptors in both platelets and vascular smooth muscle may exert beneficial effects on atherosclerosis. In fact, Osborne & Lefer (1988) have reported the anti-atherogenic effect of BM13505, a specific TP-receptor antagonist, in hypercholesterolaemic rabbit. Cinnamophilin, a new thromboxane antagonist isolated from *Cinnamomum philippinense*, inhibited U-46619-induced platelet aggregation and vasoconstriction and proliferation of VSMC *in vitro*. Thus, it may be useful for the treatment of atherosclerosis. However, further investigation of its *in vivo* activity is warranted.

In conclusion, there are two U-46619 binding sites in VSMC. The high affinity site is responsible for thromboxane-induced vasoconstriction while the low affinity site is for thromboxane-mediated VSMC proliferation. TxA_2 may be responsible for stimulating VSMC proliferation as a progression factor. It stimulates the cell cycle progression in VSMC primarily through a rapid transition from the S to the G_2/M phase. The present study also demonstrated that the functional phenotype of VSMC is changed from the contractile to the synthetic type in culture, accompanied by a disappearance of the high affinity thromboxane binding site. Cinnamophilin inhibits [^{3}H]-U-46619 binding to its receptor and U-46619-mediated VSMC proliferation. Its structural novelty may provide an original chemical basis for the development of new TP-receptor antagonist.

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