Antiproliferative effects of A02011-1, an adenylyl cyclase activator, in cultured vascular smooth muscle cells of rat

¹Sheu-Meei Yu, Zhi-Jiao Cheng & *Sheng-Chu Kuo

Department of Pharmacology, Chang Gung Medical College, 259 Wen-Hwa 1st Road, Kwei-San, Tao-Yuan and

*Institute of Pharmaceutical Chemistry, China Medical College, Taichung, Taiwan

1 The effects of A02011-1, a pyrazole derivative, on the proliferation of rat vascular smooth muscle cells (VSMCs) were examined.

2 A02011-1 (1–100 μM) concentration-dependently inhibited [^3H]-thymidine incorporation into DNA in rat VSMCs that were synchronized by 48 h serum depletion and then re-stimulated by addition of foetal calf serum (FCS, 10%), platelet-derived growth factor (PDGF, 10 ng ml $^{-1}$), 5-hydroxytryptamine (10 μM) or ADP (10 μM). The inhibitory effect of A02011-1 was fully reversible. However, FCS-induced [^3H]-thymidine incorporation into rat endothelial cells was unaffected by A02011-1.

3 The concentration of A02011-1 necessary for inhibition of the FCS-induced proliferation was similar to that necessary for adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation. Adenylyl cyclase activity was increased in A02011-1-treated VSMCs, whereas cyclic AMP-specific phosphodiesterase activity was unchanged.

4 A02011-1 was equipotent with forskolin but was more potent than 8-bromo-cyclic AMP against FCS (10%)-induced proliferation.

5 The antiproliferative action of A02011-1 was mimicked by 8-bromo-cyclic AMP, a membrane-permeable cyclic AMP analogue and was antagonized by 2',5'-dideoxyadenosine, an adenylyl cyclase inhibitor and by Rp-cyclic AMPS, a competitive inhibitor of cyclic AMP-dependent protein kinase (PKA) type I and II. 3-Isobutyl-1-methylxanthine (IBMX) caused significant potentiation of the antiproliferative activity of A02011-1. However, Rp-8-bromo-cyclic GMPS and staurosporine did not affect the antiproliferative activity of A02011-1.

6 A02011-1 still inhibited the FCS-induced DNA synthesis even when added 10–18 h after re-stimulation of the serum-starved VSMCs with 10% FCS. Flow cytometry in synchronized cells revealed an acute blockade of FCS-inducible cell cycle progression at a point in the G₁/S phase in A02011-1-treated cells. The inhibition of proliferation by A02011-1 was shown to be independent of cell damage, as documented by several criteria of cell viability.

7 These results indicate that A02011-1 inhibition of VSMC proliferation was mediated by cyclic AMP and was due to a delay in the progression from the G₁ into S phase of the cell cycle. A02011-1 did not cause cell toxicity and may thus hold promising potential for the prevention of atherosclerosis or vascular diseases.

Keywords: A02011-1; adenylyl cyclase activator; cyclic AMP; antiproliferation; vascular smooth muscle cells

Introduction

Cardiovascular disease remains the chief cause of death in the United States and Western Europe, and atherosclerosis, the principal cause of myocardial and cerebral infarction, accounts for the majority of these deaths (Ross, 1986). Vascular smooth muscle cell (VSMC) proliferation is the key event in the development of the advanced lesions of atherosclerosis. Abnormal proliferation of VSMCs is also a major component of vascular disease, including vascular rejection and re-stenosis following angioplasty (Ross & Glomset, 1973; Ross, 1986). Therefore, modulation of VSMC proliferation has critical therapeutic implications for atherosclerosis and vascular disease.

Extracellular signals such as hormones, neurotransmitters and growth factors control the proliferation of their target cells by binding to specific membrane receptors and thus activating a signalling cascade within the cell. Two stimulating pathways have been partly characterized: the phosphatidylinositol-Ca $^{2+}$ cascade and the receptor protein tyrosine kinase pathway (Dumont *et al.*, 1989). Another pathway is the adenylyl cyclase/adenosine 3':5'-cyclic monophosphate (cyclic AMP) system. The role of cyclic

in the regulation of mammalian cell proliferation has been the subject of controversy (Pastan *et al.*, 1975; Dumont *et al.*, 1989). In the yeast *Saccharomyces cerevisiae*, cyclic AMP is the positive signal for growth elicited in response to a sufficient nutrient supply (Dumont *et al.*, 1989). However, cyclic AMP functions as a negative regulator of proliferation in a variety of cell types including VSMCs (Loesberg *et al.*, 1985; Andersson *et al.*, 1994; Bennett *et al.*, 1994). Intracellular concentrations of cyclic AMP are determined by its relative rate of formation, via stimulating adenylyl cyclase, and rate of hydrolysis by phosphodiesterase (PDE) enzymes. Intracellular elevation of cyclic AMP with a subsequent activation of cyclic AMP-dependent protein kinase (PKA) plays a key role in the regulation of DNA replication in a number of cells (Skalhegg *et al.*, 1992).

A02011-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzyl thieno[3,2-c]pyrazole) (Figure 1), a novel adenylyl cyclase activator, was chemically synthesized as described by Yoshina & Kuo (1978). We found previously that A02011-1 possessed vaso-relaxant effects in rat aorta as well as antiaggregatory effects in human platelet via increasing cyclic AMP concentration, both of which protect the vascular wall against arteriosclerotic changes. In this study, A02011-1 is found to inhibit proliferation of rat VSMCs, and an attempt is made to characterize its modes of action.

¹ Author for correspondence.

Figure 1 Chemical structure of A02011-1.

Methods

Cell culture

Vascular smooth muscle cells (VSMCs) were harvested from enzymatically dissociated rat thoracic aorta according to the method of Beasley *et al.* (1991) with some minor modifications. The thoracic aorta was excised, under sterile conditions, from male Wistar rats (300–350 g) and placed in ice cold Dulbecco's phosphate buffered saline (PBS, Ca^{2+} and Mg^{2+} -free) with penicillin (100 unit ml^{-1}), streptomycin (200 $\mu\text{g ml}^{-1}$) and amphotericin B (25 $\mu\text{g ml}^{-1}$). The aorta was washed and transferred to a Petri dish containing Hanks Balanced Salt Solution (HBSS). Fat, connective tissue, and adventitia were removed by blunt dissection. The cleaned aortae were opened and the endothelium carefully scraped off. Fine pieces of aorta were transferred to a 15 ml plastic centrifuge tube and incubated for 90 min at 37°C in HBSS containing CaCl_2 (0.2 mM), HEPES (15 mM), collagenase (type II, 1 mg ml^{-1}), elastase (type III, 0.125 mg ml^{-1}), and bovine serum albumin (2 mg ml^{-1}). The digested tissue was triturated 10 times through an 18-gauge needle, sieved through nylon mesh, and the resulting cell suspension centrifuged at 200 g for 5 min. The cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 unit ml^{-1}), streptomycin (200 $\mu\text{g ml}^{-1}$) and amphotericin B (2.5 $\mu\text{g ml}^{-1}$) and seeded into 75 cm^2 flasks. After reaching confluence, cells were passaged by harvesting with 0.1% trypsin-4 mM EDTA, placed in an equal volume of medium, and centrifuged at 600 g for 5 min. The cells were characterized as smooth muscle cells by morphology and immunostaining with antibodies to smooth muscle α -actin.

[^3H]-thymidine incorporation

VSMCs were finally grown in 24 well plates (2.5×10^4 cells/well), washed twice with 1 ml Krebs-Henseleit solution (KHS) which contained (mM): NaCl 117.5, KCl 5.6, MgSO_4 1.18, NaH_2PO_4 1.2, NaHCO_3 25.0, glucose 5.5, HEPES 25.0 and CaCl_2 2.5, then incubated in 0.5 ml DMEM/FCS-free for 48 h to induce quiescence (cell cycle stopped at G_0 phase) at 37°C. To investigate the effect of A02011-1 on proliferation, quiescent cells were cultured for 20 h in medium supplemented with or without 10% FCS, containing or lacking experimental agents. Finally, cells were incubated for 2 h in freshly prepared medium that was additionally supplemented with [^3H]-thymidine (1 $\mu\text{Ci ml}^{-1}$) to measure DNA synthesis by thymidine incorporation (Grainger *et al.*, 1991; 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) and extraction of the DNA with 0.1 N NaOH. The precipitates were collected on Whatman GF/B filters, and filters were washed twice with 5 ml of ice-cold KHS. Filters were cut into pieces and shaken with 3.5 ml scintillation fluid for 24 h before liquid scintillation counting.

Measurement of cyclic AMP and cyclic GMP

Cyclic AMP and cyclic GMP concentrations in rat VSMCs were assayed as previously described (Karniguian *et al.*, 1982; Kariya *et al.*, 1989). VSMCs were finally grown in 35 mm dishes. At confluence, monolayer cells were washed twice with KHS and incubated with forskolin, sodium nitropruside or A02011-1 for 5 min at 37°C. Incubation was terminated by 0.1 N HCl and then centrifuged at 10,000 g for 5 min. Following extraction with ether (1 ml repeated four times), the supernatant was assayed with cyclic AMP or cyclic GMP ELISA kits (Amersham, England).

Adenylyl cyclase assay of rat VSMCs

VSMCs were finally grown in 35 mm dishes. At confluence, monolayer cells were washed with KHS, treated with 0.1% trypsin/4 mM EDTA, and centrifuged at 600 g for 5 min. Cells were then resuspended in ice-cold 50 mM Tris-HCl buffer (pH 7.4) and sonicated at 4°C for 10 s bursts (Sonicator W-220F). Homogenates were used for adenylyl cyclase determinations. Adenylyl cyclase activity was determined as previously described by Insel *et al.* (1982). The incubation mixture (100 μl total volume) contained 0.5 mM ATP, 1×10^6 c.p.m. of [α - ^{32}P]-ATP, 13 mM MgCl_2 , 1.0 mM cyclic AMP, 10 mM dithiothreitol, 25 mM creatine phosphate, 1.0 mg ml^{-1} of creatine phosphokinase, with or without A02011-1 in a 50 mM Tris-HCl buffer, pH 7.4. Incubations were carried out at 37°C for 10 min with 50 μl (1 mg ml^{-1}) of enzyme preparation. Incubation was terminated by adding 200 μl of 0.5 N HCl and 200 μl of 1 mM imidazole. The tubes were then heated to 100°C for 3 min in an electric heating block and cooled in an ice bath. To each tube was added 1 ml of 50 mM Tris-HCl buffer, pH 7.4. The contents were stirred and centrifuged at 1000 g for 10 min. The supernatant was applied to a neutral alumina column to isolate cyclic AMP for radioassay of ^{32}P and ^3H . Recovery of cyclic AMP was estimated by inclusion of [^3H]-AMP in the reaction mixture. Protein was determined by using Bradford reagent (Bradford, 1976) (Bio-Rad, Germany), with BSA as standard.

Cyclic AMP-specific phosphodiesterase assay

The suspension of VSMCs, prepared as described above, was sonicated at 4°C for 10 s bursts. The VSMC lysate was centrifuged at 30,000 g for 20 min. The supernatant fraction was used for cyclic AMP-specific phosphodiesterase determinations. The phosphodiesterase activity was determined as described previously (Moore *et al.*, 1985). The crude cytosolic enzyme (100 μl , 1.0 mg ml^{-1}) was incubated with cyclic AMP (10 μM , containing 0.1 μCi [^3H]-cyclic AMP) and test compound in a final volume of 0.4 ml (buffer was 50 mM Tris-HCl, 5 mM MgCl_2 , pH 7.4). After 30 min at 25°C, the samples were heated to 100°C for 1 min before cooling. Snake venom (0.1 ml, 1 mg ml^{-1}) (from *Ophiophagus hannah* venom) was then added for 30 min to convert the 5'AMP (the product of the phosphodiesterase enzyme) to the uncharged nucleoside, adenosine; 1 ml of anion-exchange resin (Dowex-1) was added to bind all of the unconverted cyclic AMP. This procedure allows for the separation of excess substrate (negatively charged cyclic AMP, bound to the particulate resin) from the noncomplexed product (now, after snake venom degradation, uncharged adenosine). After centrifugation, an aliquot (0.5 ml) of the supernatant was removed for quantitation in a liquid scintillation counter.

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 $40 \mu\text{g ml}^{-1}$ 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, USA), exciting at 488 nm and sensing at 585 nm.

Cell growth

To determine the effect of A02011-1 on cell growth, cells were seeded on 12 well plates (density was $2.5 \times 10^3/\text{well}$) and cultured for 48 h in DMEM supplemented with 10% FCS. Cells were then cultured for 48 h in FCS-free medium to induce quiescence. This procedure was followed by culturing for 6 days in medium supplemented with 10% FCS, containing or lacking A02011-1. Culture medium was changed daily and cell numbers were determined by dissociation of adherent cells with trypsin and counting with a haemocytometer.

Data analysis

The results are expressed as the mean $\pm$ 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

A02011-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzyl thieno[3,2-c] pyrazole) (Figure 1) was chemically synthesized as described by Yoshina & Kuo (1978). 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).

Figure 2 Increase in [³H]-thymidine incorporation in rat vascular smooth muscle cells (VSMCs) stimulated with foetal calf serum (FCS) (a); platelet-derived growth factor (PDGF) (b); 5-hydroxytryptamine (5-HT) (c) and ADP (d). [³H]-thymidine was added 20 h after the quiescent cells had been exposed to mitogens and then incubations were continued for 2 h. Columns represent [³H]-thymidine incorporation in unstimulated cells. Data are expressed as the mean $\pm$ s.e. mean ($n = 6$, each in quadruplicate).

Figure 3 Inhibition by A02011-1 of [³H]-thymidine incorporation in rat VSMCs (O) or endothelial cells (●) stimulated with foetal calf serum (10%) (a); rat VSMCs (O) stimulated with platelet-derived growth factor (PDGF, 10 ng ml^{-1}) (b); 5-hydroxytryptamine ($10 \mu\text{M}$) (c) or ADP ($10 \mu\text{M}$) (d). Histograms represent [³H]-thymidine incorporation in unstimulated cells (open columns) and cells stimulated with mitogens (solid column). Data are presented as the mean $\pm$ s.e. mean ($n = 6$, each in quadruplicate).

[³H]-thymidine, [³H]-cyclic AMP, [α -³²P]-ATP and cyclic AMP, cyclic GMP ELISA kit were from Amersham (Buckinghamshire, UK). Purified human platelet-derived growth factor (PDGF/BB) from Biomedical Technologies Inc. 2',5'-Dideoxyadenosine from Biomol. Protein dye reagent was from Bio-Rad. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), 8-bromo-cyclic AMP, trypan blue and other chemical reagents were from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). 8-(Aminoethyl)aminoadenosine-3',5'-cyclic monophosphate (8-AHA-cyclic AMP), 8-piperidinoadenosine-3',5'-cyclic monophosphorothioate (Rp-8-PIP-cyclic AMPS), N⁶-benzoyladenine-3',5'-cyclic monophosphate (6-Bnz-cyclic AMP), 8-(chlorophenylthio)-adenosine-3',5'-cyclic monophosphorothioate (Rp-8-CPT-cyclic AMPS) and Rp-8-bromo-cyclic GMPS were obtained from Biolog (Bremen, Germany). The antibody used for immunostaining was anti-smooth muscle α -actin (mouse monoclonal, Dako M851).

Results

Inhibition of mitogen-stimulated [³H]-thymidine incorporation by A02011-1

Initial studies were designed to characterize the capability of mitogens to stimulate incorporation of [³H]-thymidine into DNA as an indicator of proliferation (Nemecsek *et al.*, 1986; March *et al.*, 1993). Foetal calf serum (FCS, 10%) elicited the greatest mitogenic response in rat VSMCs, causing a 5.3 ± 0.6 fold increase in [³H]-thymidine incorporation (Figure 2a). Platelet-derived growth factor (PDGF/BB, 10 ng ml^{-1}), 5-hydroxytryptamine (5-HT, $10 \mu\text{M}$) and ADP ($10 \mu\text{M}$) increased [³H]-thymidine incorporation by 3.6 ± 0.2 , 2.4 ± 0.3 and 2.3 ± 0.1 fold, respectively (Figure 2b–d). A02011-1 (1 – $100 \mu\text{M}$) concentration-dependently inhibited [³H]-thymidine incorporation to DNA in rat VSMCs that were synchronized by 48 h serum depletion and then restimulated by addition of 10% FCS, 10 ng ml^{-1} PDGF/BB, $10 \mu\text{M}$ 5-HT or $10 \mu\text{M}$ ADP, with IC_{50} values of $6.3 \mu\text{M}$, $6.6 \mu\text{M}$, $6.6 \mu\text{M}$ and $5.6 \mu\text{M}$, respectively (Figure 3a–d). The inhibitory effect of A02011-1 was fully reversible, and [³H]-thymidine incorporation recovered within 24 h after the removal of $100 \mu\text{M}$ A02011-1 (data not shown). A02011-1 (1 – $100 \mu\text{M}$) did not inhibit [³H]-thymidine incorporation of 10% FCS-stimulated rat aortic endothelial cells (Figure 3a).

Figure 4 Effects of A02011-1 on cyclic nucleotides formation in rat VSMCs. VSMCs were incubated with various concentrations of A02011-1 for 5 min at 37°C. Cellular cyclic AMP (○) and cyclic GMP (●) were determined by ELISA kit. Basal values of cyclic AMP and cyclic GMP were 3.0 ± 0.2 and $2.0 \pm 0.1 \text{ pmol mg}^{-1} \text{ protein}$, respectively. Data are presented as the mean $\pm$ s.e. mean of six experiments.

Effects of A02011-1 on cyclic AMP and cyclic GMP formation

The concentration of A02011-1 necessary for the inhibition of the FCS-induced proliferation was similar to that necessary for cyclic AMP formation, whereas cyclic GMP formation was not significantly changed by A02011-1 ($100 \mu\text{M}$) (Figure 4). The mechanism of A02011-1-induced cyclic AMP formation was clarified by determining adenylyl cyclase and cyclic AMP-specific phosphodiesterase activity. As shown in Table 1, concentration-dependent increases in adenylyl cyclase activity were observed in A02011-1-treated, or forskolin-treated, VSMCs. The cyclic AMP-specific phosphodiesterase activity was significantly inhibited by 3-isobutyl-1-methylxanthine (IBMX), whereas this enzyme activity remained unchanged by A02011-1 ($100 \mu\text{M}$) in VSMCs (Table 2).

Inhibition of FCS-stimulated [³H]-thymidine incorporation by 8-bromo-cyclic AMP and forskolin

8-Bromo-cyclic AMP (10 – $1000 \mu\text{M}$) and forskolin (1 – $100 \mu\text{M}$) produced concentration-dependent inhibition of 10% FCS-induced [³H]-thymidine incorporation (Figure 5a); A02011-1 was equipotent with forskolin but was more potent than 8-bromo-cyclic AMP.

Effects of 2',5'-dideoxyadenosine, IBMX, Rp-cyclic AMPS, Rp-8-bromo-cyclic GMPS and staurosporine on A02011-1-induced antiproliferation

2',5'-Dideoxyadenosine ($250 \mu\text{M}$), an adenylyl cyclase inhibitor, itself had only a slight effect on 10% FCS-induced proliferation. However, the concentration-response curve for A02011-1-induced inhibition of proliferation was shifted to the right (Figure 5b). IBMX ($100 \mu\text{M}$), a non-selective phosphodiesterase inhibitor, alone caused a 5% decrease of FCS-stimulated [³H]-thymidine incorporation. As shown in Figure 5b, however, it caused significant potentiation of the antiproliferative activity of A02011-1. The antiproliferative action of A02011-1 was also antagonized by Rp-cyclic AMPS ($30 \mu\text{M}$), a competitive inhibitor of cyclic AMP-dependent protein kinase (PKA) type I and II (Figure 5b). 8-Chloroadenosine-3',5'-cyclic monophosphate (8-Cl-cyclic AMP) ($30 \mu\text{M}$), an inhibitor of PKA type II, had only a slight effect on the antiproliferative activity of A02011-1 (Figure 5b). Neither Rp-8-bromo-cyclic GMPS ($10 \mu\text{M}$) nor staurosporine (100 nM) affected the antiproliferative effects of A02011-1 (Figure 5c).

Site-selective cyclic AMP analogues complementing each other in activating either PKA I or PKA II were tested for

Table 1 Effects of A02011-1 and forskolin on adenylyl cyclase activity in rat aortic smooth muscle cells

Treatment	Adenylyl cyclase activity ($\text{pmol min}^{-1} \text{ mg}^{-1} \text{ protein}$)
None (Basal)	5.8 ± 1.9
Forskolin	
$10 \mu\text{M}$	$39.4 \pm 4.9^*$
$30 \mu\text{M}$	$63.5 \pm 7.6^*$
A02011-1	
$3 \mu\text{M}$	$20.9 \pm 3.1^*$
$10 \mu\text{M}$	$35.7 \pm 4.7^*$
$30 \mu\text{M}$	$56.6 \pm 5.9^*$
$100 \mu\text{M}$	$72.9 \pm 5.8^*$

Cell extracts were incubated with the various concentrations of forskolin or A02011-1 for 10 min and then assayed for adenylyl cyclase activity as described in Methods. Data are expressed as the mean $\pm$ s.e. mean ($n = 6$). * $P < 0.001$ as compared with the basal activity.

Table 2 Effects of 3-isobutyl-1-methylxanthine (IBMX) and A02011-1 on cyclic AMP-specific phosphodiesterase activity in rat VSMCs

Treatment	Phosphodiesterase activity (c.p.m. mg ⁻¹ protein)
None (Basal)	10655 ± 599
IBMX 100 µM	8933 ± 413*
500 µM	3888 ± 299*
1000 µM	1000 ± 281*
A02011-1	
30 µM	10600 ± 190
100 µM	10385 ± 100

Cell extracts were incubated with the various concentrations of IBMX or A02011-1 for 10 min and then assayed for enzyme activity as described in Methods. Data are presented as mean ± s.e.mean (*n* = 4). **P* < 0.001 as compared with the basal activity.

their ability to synergize in the inhibition of VSMC proliferation. Proliferating VSMCs co-treated with 8-aminohexylamino-cyclic AMP (8-AHA-cyclic AMP), at increasing concentrations, and the complementary cyclic AMP analogue, 8-piperidino-cyclic AMPs (Rp-8-PIP-cyclic AMPs) at a constant subinhibitory concentration (90 µM), strongly synergized in the inhibition of VSMC proliferation (Figure 6a). This combination shows strong synergism for activation of PKA type I (Skalhegg *et al.*, 1992). In contrast, addition of 8-chlorophenylthio-cyclic AMP (Rp-8-CPT-cyclic AMP) at increasing concentrations, combined with a constant subinhibitory concentration of N⁶-benzoyl-cyclic AMP (6-Bnz-cyclic AMP, 30 µM), showed no such synergism in its inhibitory potency (Figure 6b). This combination shows strong synergism for activation of PKA II (Ogreid *et al.*, 1985).

Effects of A02011-1 on cell cycle in synchronized VSMCs

To ensure that the cells were capable of synchronously re-entering the cell cycle after 48 h serum starvation, the cell stimulation of [³H]-thymidine incorporation was determined. DNA synthesis increased slowly, reached its maximum within 16 h, and declined soon after. The G₁ phase (pre-DNA synthesis) can be confined to the first 8 h, the S phase (DNA synthesis) between 10 to 18 h, and the G₂/M phase (premitosis/mitosis) between 20–26 h. To clarify the mode of antiproliferative action of A02011-1, an examination was made of the relationship between the time of the addition of A02011-1 and its inhibitory action on FCS-induced DNA synthesis. As shown in Figure 7, full inhibition of FCS-induced DNA synthesis still occurred when A02011-1 was present within 10–18 h after the addition of FCS. This result implies that A02011-1 inhibits the FCS-induced proliferation by delaying the progression from the G₁ into S phase of the cell cycle. Furthermore, experimental results in this study have demonstrated that A02011-1 delays the progression from G₁/S phase of cell cycle by using flow cytometry. After a 48 h exposure to a serum-free medium, most of VSMCs stayed at G₀ phase (resting phase)/G₁ phase of the cell cycle (Figure 8a). DNA content in synchronized VSMCs evaluated at 0 h: G₀/G₁, S and G₂/M phase was 48%, 28.6% and 23.4% of the total cells, respectively. After replacement of serum-free medium with DMEM containing 10% FCS, the emergence of cells into and through the S phase was observed by flow cytometry using quantitative DNA staining with propidium iodide (Figure 8b). The majority of cycling cells were found to progress through S phase approximately 18 h after serum repletion (G₀/G₁ = 20.0%, S = 56.6% and G₂/M = 23.7%) (Figure 8b). A02011-1-treated cells showed

Figure 5 (a) Inhibition by 8-bromo-cyclic AMP (Δ), forskolin (●) or A02011-1 (○) of [³H]-thymidine incorporation in VSMCs stimulated with foetal calf serum (FCS, 10%). (b) Effects of 2',5'-dideoxyadenosine, 3-isobutyl-1-methylxanthine (IBMX), Rp-cyclic AMPs and 8-Cl-cyclic AMP on A02011-1-induced antiproliferation. Quiescent VSMCs were stimulated with FCS (10%) in the presence of various concentrations of A02011-1 (○), or A02011-1 combined with 2',5'-dideoxyadenosine (250 µM) (●), IBMX (100 µM) (▲), Rp-cyclic AMPs (30 µM) (Δ) or 8-Cl-cyclic AMP (30 µM) (□) for 20 h. (c) Effects of Rp-8-bromo-cyclic GMPS and staurosporine on A02011-1-induced antiproliferation. Quiescent VSMCs were stimulated with FCS (10%) in the presence of various concentrations of A02011-1 (○), or A02011-1 combined with Rp-8-bromo-cyclic GMPS (●) or staurosporine (Δ) for 20 h. Measurements of [³H]-thymidine incorporation were then performed as described in the Methods section. Results are expressed as percentage of control, defined as [³H]-thymidine incorporation in the presence of 10% FCS: 3.7 ± 0.2 d.p.m. × 10⁻⁴. Data are presented as the mean ± s.e. mean (*n* = 6, each in quadruplicate).

an acute block of cell cycle progression occurring at point G₁/S phase; no increase in DNA content in S phase was observed in cells stimulated by serum in the presence of the A02011-1 (100 µM) (S = 17.2%) (Figure 8c).

Effects of A02011-1 on cell growth

Cell counting was used to evaluate the effects of A02011-1 on cell growth. This revealed growth-inhibition of subconfluent cells by A02011-1 (100 µM) (Figure 9); a lethal effect on the

cell population was discounted as measured by trypan blue exclusion. To examine the possibility that A02011-1 might be toxic to VSMCs the following series of experiments were performed. (a) The number of cells present in the supernatant media was determined daily for 6 days, both in the presence and absence of 100 μM A02011-1. While this concentration of

A02011-1 caused significant inhibition of cell growth (>90%), <5% of cells were found to be present in the supernatant during any portion of the culture period. Thus, detachment and loss of cells did not account for inhibition of cell proliferation. (b) Incubation of VSMCs for 24 h with A02011-1 (100 μM) also caused no significant cell loss: the number of cells was $2.8 \pm 0.2 \times 10^3$ in control wells and $3.0 \pm 0.3 \times 10^3$ in A02011-1 (100 μM)-treated wells. Furthermore, no significant release of lactate dehydrogenase was observed in this experiment, again indicating that in short-term incubations, even high concentrations of A02011-1 did not induce cell damage. (c) Less than 1% of the cells treated with A02011-1 (100 μM) for 24 h stained with trypan blue.

Figure 6 Synergistic activation of cyclic AMP-dependent protein kinase (PKA) I and II using site-selective cyclic AMP analogues in VSMCs. (a) Quiescent VSMCs were stimulated with FCS (10%) in the presence of various concentrations of 8-AHA cyclic AMP (O) or in combination with Rp-8-PIP-cyclic AMPs (90 μM , ●) to activate selectively PKA I. (b) VSMCs were stimulated with FCS (10%) in the presence of Rp-8-CPT-cyclic AMP (O) or in combination with 6-Bnz-cyclic AMP (30 μM , ●) to activate selectively PKA II. Data are presented as the mean $\pm$ s.e. mean ($n = 6$, each in quadruplicate). For abbreviations, see text.

Figure 7 Chronological analysis of effects of A02011-1 on the FCS-induced DNA synthesis. Quiescent VSMCs were stimulated by 10% FCS for 36 h. During this incubation, dimethylsulphoxide (DMSO, 0.05%, control) (O) or A02011-1 (100 μM) (●) was added at the indicated times and incubations were continued for 36 h. ^3H -thymidine was added 2 h before the incubations were terminated. DNA synthesis was assayed by measuring the incorporation of ^3H -thymidine into acid-insoluble materials as described in Methods section. Data are presented as the mean $\pm$ s.e. mean ($n = 6$).

Discussion

The present study has demonstrated that A02011-1, a pyrazole derivative, effectively and reversibly inhibits VSMC

Figure 8 Effect of A02011-1 on cell cycle in synchronized VSMCs. Cell cycle progression was evaluated by flow cytometric determination of DNA content in synchronized subconfluent VSMCs evaluated at 0 h (control) (a) and 18 h after serum repletion in the absence (b) or presence (c) of A02011-1 (100 μM). Individual nuclear DNA content as reflected by fluorescence intensity of incorporated propidium iodide is plotted as a histogram depicting relative cell numbers at each intensity. Each histogram is derived from a data collection of at least 10,000 events.

Figure 9 Inhibition of VSMC growth by A02011-1. VSMCs were seeded on 12 well plates (density was 2.5×10^3 /well) and counted in trypan blue at subsequent time points. Dimethylsulphoxide (○) or A02011-1 (100 μ M) (●) was added and incubated for 6 days in medium supplemented with 10% FCS. Culture media were changed daily and cell numbers were determined. Results are presented as the mean $\pm$ s.e. mean of three separate experiments. Each in quadruplicate, and are expressed as the fold increase of cell number, relative to that on day 0 of the experiments. * $P < 0.001$ as compared with control (Cells not treated with A02011-1).

proliferation, which is a prominent feature of vascular response to mechanical injury (Campbell & Campbell, 1985; Ip *et al.*, 1990). Excessive VSMC proliferation is considered to be a hallmark of atherosclerotic disease (Schwartz *et al.*, 1986) and the experimental removal of endothelium has been associated with proliferation of the underlying VSMCs (Clowes *et al.*, 1983). It has been postulated that one of the functions of endothelial cells is to maintain the mitogenic quiescence of the underlying medial VSMCs (Garg & Hassid, 1989; Assender *et al.*, 1992). This is despite the fact that modulation of VSMC proliferation has crucial therapeutic implications for vascular disease. A prominent therapeutic aim involves inhibiting VSMC proliferation without interfering with endothelial repair (Grainger *et al.*, 1992). A02011-1, which selectively inhibits VSMC proliferation without affecting endothelial cell repair, might therefore be expected to prevent or inhibit progress of vascular diseases. Furthermore, the inhibition of proliferation by A02011-1 occurred with concentrations (1–100 μ M) similar to those causing vasorelaxation and antiplatelet effects (unpublished observation). This property would also contribute towards the vascular protective effect of A02011-1.

Cyclic AMP may be involved in negative, as well as positive, regulation of cellular growth depending on the cell type and culture conditions used (Pastan *et al.*, 1975). In the present study, we demonstrated that cyclic AMP mediated the inhibition of proliferation of rat VSMC. The antiproliferative action of A02011-1 was mimicked by 8-bromo-cyclic AMP, a membrane permeable cyclic AMP analogue, and was antagonized by Rp-cyclic AMPS, a competitive inhibitor of cyclic AMP-dependent protein kinase (PKA) type I and II (Wang *et al.*, 1991). Furthermore, the concentration of A02011-1 necessary for the inhibition of the FCS-induced proliferation was similar to that causing cyclic AMP formation. These experimental results imply that the antiproliferative effect of A02011-1 was likely to be mediated by cyclic AMP as the second messenger. That cyclic AMP formation caused by A02011-1 was likely to be due to activation of adenylyl cyclase was indicated by the decreased potency of A02011-1-induced antiproliferation in the presence of 2',5'-dideoxyadenosine, an adenylyl cyclase inhibitor (Holgate *et al.*, 1980), and by the increased potency of A02011-1 in the presence of IBMX, a phosphodiesterase inhibitor. Moreover, A02011-1 concentration-dependently activated the adenylyl cyclase, but did not affect the phosphodiesterase activity, in rat VSMCs. Although cyclic AMP has been reported to

inhibit the proliferation of VSMC (Blomhoff *et al.*, 1987; Skalhegg *et al.*, 1992), the molecular mechanisms remain to be clarified. Until recently, all known actions of cyclic AMP or its derivatives were assumed to operate via activation of PKA (Beebe & Corbin, 1986). Two major types of mammalian PKA (PKA I and PKA II) are described (Skalhegg *et al.*, 1992). Activation of PKA occurs upon binding of four cyclic AMP molecules, two per regulatory (R) monomer, where the binding takes place on two asymmetric sites (designated A and B) (Rannels & Corbin, 1980). It has been shown that cyclic AMP analogues with selectivity for site A and B, respectively, can complement each other in the activation of PKA (Robinson-Steiner *et al.*, 1984) and that analogue pairs, by synergistic action, also can selectively activate PKA I and II in the intact cell (Lanotte *et al.*, 1991). To what extent PKA I or PKA II mediate cyclic AMP-induced growth-inhibitory effects in VSMC is still unclear. Use of site-selective analogue pairs can to a certain extent distinguish between effects mediated via PKA I and PKA II (Lanotte *et al.*, 1991). Since Rp-8-PIP-cyclic AMPS selects site A of RI and 8-AHA-cyclic AMP selects site B of both RI and RII, they should only synergize in activating PKA I (Skalhegg *et al.*, 1992). Rp-8-CPT-cyclic AMP selects site B of RII and 6-Bnz-cyclic AMP selects sites A of RI and RII, the combination showing strong synergism for activation of PKA II (Ogreid *et al.*, 1985). In the present study, the antiproliferative actions of A02011-1 were antagonized by Rp-cyclic AMPS, a competitive inhibitor of PKA I and II, but not 8-Cl-cyclic AMP, a selective PKA II inhibitor. Furthermore, a cyclic AMP analogue pair directed to activated PKA I (8-AHA-cyclic AMP and Rp-8-PIP-cyclic AMPS) synergized in the inhibition of VSMC proliferation, whereas a PKA II-directed cyclic AMP analogue pair (Rp-8-CPT-cyclic AMPS and 6-Bnz-cyclic AMP) did not. This result indicates that PKA I mediates the inhibitory effects of cyclic AMP in rat VSMCs. At present, the exact mechanism by which the cyclic AMP signal transduction pathway inhibits VSMC proliferation is poorly understood. Several studies have reported that the cyclic AMP reduces the steady-state level of c-myc mRNA in HL60 cells (Schwartz *et al.*, 1991), down-regulates the c-myc mRNA and inhibits PDGF-induced proliferation in fibroblasts and VSMCs (Heldin *et al.*, 1989; Bennett *et al.*, 1994), blocks induction of c-sis mRNA and inhibits the release of PDGF in endothelial cells (Daniel *et al.*, 1987).

To determine whether a protein kinase G (PKG) or protein kinase C (PKC) is involved in the antiproliferative effects of A02011-1, a stable membrane permeant inhibitor of PKG, Rp-8-bromo-cyclic GMPS, and a PKC inhibitor, staurosporine, were used. Neither Rp-8-bromo-cyclic GMPS nor staurosporine had any effect on the antiproliferative effects of A02011-1. This indicated that neither PKG nor PKC is involved in the A02011-1-induced antiproliferative action.

We examined the relationship between the time of the addition of A02011-1 and its inhibitory action on FCS-induced DNA synthesis. Full inhibition of DNA synthesis still occurred when A02011-1 was present within 10–18 h after the re-addition of FCS (Figure 7). A02011-1 is very stable in culture and is not readily degraded. However, A02011-1 had only modest effects in lowering cell number, as assessed over the initial 6 days of the treatment (Figure 9). These results demonstrate that A02011-1 delays rather than prevents the onset of proliferation. Flow cytometry in VSMCs synchronized by serum depletion revealed a delay of FCS-inducible cell cycle progression at the G₁/S phase in A02011-1-treated cells (Figure 8). Cyclic AMP-elevating vasodilators (e.g. forskolin or isoprenaline) also exert their antiproliferative action by delaying the progression from the G₁ into S phase of the cell cycle (Garg & Hassid, 1989; Assender *et al.*, 1992). Our results are also consistent with previous work (Assender *et al.*, 1992) which showed that elevated cyclic AMP concentrations at a latter stage of the G₁ phase of the cell cycle would be necessary to inhibit proliferation.

The antiproliferative effect of A02011-1 was unlikely to be due to cell damage, based on the finding that 100 μ M A02011-1 did not cause the release of lactate dehydrogenase or decrease cell number after 24 h of incubation. Moreover, the inhibition of cell proliferation induced by 100 μ M A02011-1 in long-term experiments was also unlikely to be the result of toxicity and damage to cells as shown by the lack of lactate dehydrogenase release into the extracellular medium, the lack of staining by trypan blue and the lack of cell detachment.

References

- ANDERSSON, K.B., TASKEN, K. & BLOMHOFF, H.K. (1994). Cyclic AMP downregulates c-myc expression by inhibition of transcript initiation in human B-precursor Reh cells. *FEBS Lett.*, **337**, 71–76.
- ASSENDER, J.W., SOUTHGATE, K.M., HALLETT, M.B. & NEWBY, A.C. (1992). Inhibition of proliferation, but not of Ca^{2+} mobilization, by cyclic AMP and GMP in rabbit aortic smooth-muscle cells. *Biochem. J.*, **288**, 527–532.
- BEASLEY, D., SCHWARTZ, J.H. & BRENNER, B.M. (1991). Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J. Clin. Invest.*, **87**, 602–608.
- BEEBE, S.J. & CORBIN, J.D. (1986). Cyclic nucleotide-dependent protein kinase. In *The Enzymes*, vol. XVII. ed. Boyer, P.D. & Krebs, E.G., pp. 43–111. New York: Academic Press.
- BENNETT, M.R., EVAN, G.I. & NEWBY, A.C. (1994). Deregulated expression of the c-myc oncogene abolishes inhibition of proliferation of rat vascular smooth muscle cells by serum reduction, interferon- γ , heparin, and cyclic nucleotide analogues and induces apoptosis. *Circ. Res.*, **74**, 525–536.
- BLOMHOFF, H.K., SMELAND, E.B., BEISKE, K., BLOMHOFF, R., RUUD, E., BJORO, T., PFEIFER-OHLSSON, S., WATT, R., WATT, R., FUNDERUD, S., GODAL, T. & OHLSSON, R. (1987). Cyclic AMP-mediated suppression of normal and neoplastic B cell proliferation is associated with regulation of c-myc and Ha-ras protooncogenes. *J. Cell. Physiol.*, **131**, 426–433.
- BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.*, **72**, 248–254.
- CAMPBELL, G.R. & CAMPBELL, J.H. (1985). Recent advances in molecular pathology. *Exp. Mol. Pathol.*, **42**, 139–162.
- CLOWES, A.W., REIDY, M.A. & CLOWES, M.M. (1983). Kinetics of cellular proliferation after arterial injury. *Lab. Invest.*, **49**, 327–333.
- DANIEL, T.O., GIBBS, V.C., MILFAY, D.F. & WILLIAMS, L.T. (1987). Agents that increase cAMP accumulation block endothelial c-sis induction by thrombin and transforming growth factor- β . *J. Biol. Chem.*, **262**, 11893–11896.
- DUMONT, J.E., JAUNIAUX, J.C. & ROGER, P.P. (1989). The cyclic AMP-mediated stimulation of cell proliferation. *Trends Pharmacol. Sci.*, **14**, 67–71.
- GARG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.*, **83**, 1774–1777.
- GRAINGER, D.J., HESKETH, T.R., METCALFE, J.C. & WEISSBERG, P.L. (1991). A large accumulation of non-muscle myosin occurs at first entry into M phase in rat vascular smooth-muscle cells. *Biochem. J.*, **277**, 145–151.
- GRAINGER, D.J., HESKETH, T.R., WEISSBERG, P.L. & METCALFE, J.C. (1992). Hexamethylenebisacetamide selectively inhibits the proliferation of human and rat vascular smooth-muscle cells. *Biochem. J.*, **283**, 403–408.
- HELDIN, N.-E., PAULSSON, Y., FORSBERG, K., HELDIN, C.-H. & WESTERMARK, B. (1989). Induction of cyclic AMP synthesis by forskolin is followed by a reduction in the expression of c-myc messenger RNA and inhibition of ^3H -thymidine incorporation in human fibroblasts. *J. Cell. Physiol.*, **138**, 17–23.
- HOLGATE, S.T., LEWIS, R.A. & AUSTEN, K.F. (1980). Role of adenylate cyclase in immunologic release of mediators from rat mast cells: agonist and antagonist effects of purine- and ribose-modified adenosine analogs. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 6800–6810.
- INSEL, P.A., STENGEL, D., FERRY, N. & HANOUNE, J. (1982). Regulation of adenylate cyclase of human platelet membranes by forskolin. *J. Biol. Chem.*, **257**, 7485–7490.
- IP, J.H., FUSTER, V., BADIMON, L., BADIMON, J., TAUBMAN, M.B. & CHESEBRO, J.H. (1990). Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. *J. Am. Coll. Cardiol.*, **15**, 1667–1687.
- KARIYA, K.I., KAWAHARA, Y., ARKI, S.I., FUKUZAKI, H. & TAKAI, Y. (1989). Antiproliferative action of cyclic GMP-elevating vasodilators in cultured rabbit aortic smooth muscle cells. *Atherosclerosis*, **80**, 143–147.
- KARNIGUIAN, A., LEGRAND, Y.J. & CAEN, J.P. (1982). Prostaglandins: specific inhibition of platelet adhesion to collagen and relationship with cAMP level. *Prostaglandins*, **23**, 437–457.
- LANOTTE, M., RIVIERE, J.B., HERMOUET, S., HOUGE, G., VINTERMYR, O.K., GJERTSEN, B.T. & DOSKELAND, S.D. (1991). Programmed cell death (apoptosis) is induced rapidly and with positive cooperativity by activation of cyclic adenosine monophosphate-kinase I in a myeloid leukemia cell line. *J. Cell. Physiol.*, **146**, 73–80.
- LOESBERG, C., VAN WIJK, R., ZANBERGEN, J., VAN AKEN, W.G., VAN MOURIK, J.A. & DE GROOT, P.H.G. (1985). Cell cycle-dependent inhibition of human vascular smooth muscle cell proliferation by prostaglandin E_1 . *Exp. Cell. Res.*, **160**, 117–121.
- MARCH, K.L., WILENSKY, R.L., ROESKE, R.W. & HATHAWAY, D.R. (1993). Effects of thiol protease inhibitors on cell cycle and proliferation of vascular smooth muscle cells in culture. *Circ. Res.*, **72**, 413–423.
- MOORE, J.B., FULLER, J.B.L., FALOTICO, R. & TOLMAN, E.L. (1985). Inhibition of rabbit platelet phosphodiesterase activity and aggregation by calcium channel blockers. *Thromb. Res.*, **40**, 401–411.
- MORINELLI, T.A., ZHANG, L.M., NEWMAN, W.H. & MEIER, K.E. (1994). Thromboxane A_2 /prostaglandin H_2 -stimulated mitogenesis of coronary artery smooth muscle cells involves activation of mitogen-activated protein kinase and S6 kinase. *J. Biol. Chem.*, **269**, 5693–5698.
- NEMECEK, G.M., COUGHLIN, S.R., HANDLEY, D.A. & MOSKOWITZ, M.A. (1986). Stimulation of aortic smooth muscle cell mitogenesis by serotonin. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 674–678.
- OGREID, D., EKANGER, R., SHUVA, R.H., MILLER, J.P., STURM, P., CORBIN, J.D. & DOSKELAND, S.O. (1985). Activation of protein kinase isozymes by cyclic nucleotide analogs used singly or in combination. *Eur. J. Biochem.*, **150**, 219–227.
- PASTAN, I., JOHNSON, G.S. & ANDERSON, W.B. (1975). Role of cyclic nucleotides in growth control. *Annu. Rev. Biochem.*, **44**, 491–522.
- RANNELS, S.R. & CORBIN, J.D. (1980). Two different intrachain cAMP binding sites of cAMP-dependent protein kinases. *J. Biol. Chem.*, **255**, 7085–7088.
- ROBINSON-STEINER, A.M., BEEBE, S., RANNELS, S.R. & CORBIN, J.D. (1984). Microheterogeneity of type II cyclic AMP-dependent protein kinase in various mammalian species and tissues. *J. Biol. Chem.*, **259**, 10596–10605.
- ROSS, R. (1986). The pathogenesis of atherosclerosis: an update. *New Engl. J. Med.*, **314**, 488–500.
- ROSS, R. & GLOMSET, J.A. (1973). Atherosclerosis and the arterial smooth muscle cell. *Science*, **180**, 1332–1339.
- SCHWARTZ, E.L., CHAMBERLAIN, H. & BRECHBIIHL, A.-B. (1991). Regulation of c-myc expression by granulocyte-macrophage colony-stimulating factor in human leukemia cells. *Blood*, **77**, 2716–2723.

This work was supported by a research grant of CMRP 377 from Chang Gung Medical Research Foundation and the National Science of the Republic of China (NSC 84-2331-B182-095).

- SCHWARTZ, S.M., CAMPBELL, G.R. & CAMPBELL, J.H. (1986). Replication of smooth muscle cells in vascular disease. *Circ. Res.*, **58**, 427–444.
- SKALHEGG, B.S., LANDMARK, B.F., DOSKELAND, S.O., HANSSON, V., LEA, T. & JAHNSEN, T. (1992). Cyclic AMP-dependent protein kinase type I mediates the inhibitory effects of 3',5'-cyclic adenosine monophosphate on cell replication in human T lymphocytes. *J. Biol. Chem.*, **267**, 15707–15714.
- WANG, L.-Y., SALTER, M.W. & MACDONALD, J.F. (1991). Regulation of kainate receptor by cAMP-dependent protein kinase and phosphates. *Science*, **253**, 1132–1135.
- YOSHINA, S. & KUO, S.C. (1978). Studies on heterocyclic compounds. XXXV. Synthesis of furo [3,2-c]pyrazole derivatives. (2) Electrophilic substitution of 1,3-diphenyl furo [3,2-c]pyrazole. *Yakugaku Zasshi*, **98**, 204–207.

(Received October 3, 1994
Revised November 11, 1994
Accepted November 15, 1994)