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Antiproliferative effects of PCA-4230, a new antithrombotic drug, in vascular smooth muscle cells

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1 In the present study we examined the effects of PCA-4230, a novel antithrombotic agent, on the growth of cultured A10 vascular smooth muscle cells (rat aorta).

2 The action of PCA-4230 on cell proliferation and on serum-induced DNA synthesis was determined by measuring the cell number and the incorporation of the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU), respectively.

3 PCA-4230 reversibly inhibited vascular smooth muscle cell proliferation. The increase in cell number was significantly reduced in the presence of 1 and 50 μM PCA-4230.

4 DNA synthesis was concentration-dependently inhibited by PCA-4230 (0.5 to 50 μM) in A10 cells that were synchronized by 48 h serum starvation and then re-stimulated by serum repletion, with an IC_{50} value of 13 μM. However, serum-induced DNA synthesis in bovine aortic endothelial cells was not significantly affected by PCA-4230. In addition, PCA-4230 (50 μM) caused a significant drop in PDGF-BB-mediated BrdU incorporation in A10 cells.

5 The effect of PCA-4230 on serum-induced DNA synthesis was compared to that elicited by nifedipine, another dihydropyridine-class inhibitor of vascular smooth muscle proliferation. PCA-4230 (10 μM) elicited a degree of inhibition similar to that of nifedipine at equimolar concentration.

6 To define the nature of the cell proliferation inhibition, an evaluation of cell cycle progression was undertaken. Flow cytometry studies of DNA content in synchronized cells revealed a block of the serum-inducible cell cycle progression. This inhibitory effect was markedly reduced when PCA-4230 was added 2 h after serum repletion.

6 Accordingly, PCA-4230 (50 μM) caused a 95 and 90% decrease in the elevation of c-*fos* and c-*jun* proto-oncogenes expression as evaluated by Northern blot analysis of mRNA induced early after serum addition.

7 The present results indicate that PCA-4230 inhibits vascular smooth muscle cell proliferation, in culture, by altering the cell cycle progression. Flow cytometric studies of DNA content and the down regulation of c-*fos* and c-*jun* proto-oncogenes, suggest that the drug is acting at the early G_0/G_1 transition phase. PCA-4230 may hold promising potential for the prevention of structural abnormalities of blood vessels associated with atherosclerosis and vascular diseases.

Keywords: PCA-4230; 1,4-dihydropyridine; antithrombotic agent; antiproliferative effects; vascular smooth muscle cells; atherosclerosis; vascular disease

Introduction

Cardiovascular disease remains the chief cause of death in Europe, U.S.A. and Japan, while atherosclerosis, the principal cause of heart attack, stroke and gangrene of extremities, accounts for the majority of these deaths (Ross *et al*., 1986; Ross, 1993). Therapeutics of cardiovascular disease include different types of drugs; among them are antihypertensive, antithrombotic and antiatherogenic agents.

PCA-4230, 2(1,1,3-trioxo-2,3-dihydro-1,2-benzisothiazol-2-yl)ethyl 2,6-dimethyl-5-(ethoxycarbonyl)-4-methyl-1,4-dihydropyridincarboxylate (Figure 1) is a new drug synthesized by Sunkel and co-workers (Sunkel *et al*., 1988). The compound is a novel agent with antiaggregating and antithrombotic activity showing long lasting although reversible effects (Ortega *et al*., 1992; 1993; Gutierrez-Diaz *et al*., 1992). PCA-4230 was well tolerated after administration to healthy volunteers (Navarro *et al*., 1988; Cillero *et al*, 1991). More recently, in Phase II Clinical Trials PCA-4230 has shown in addition to its antiplatelet action, beneficial effects when administered to patients suffering from atherosclerotic disease of the lower limbs (Dr H.W. Heiss, unpublished observations).

Vascular smooth muscle cell (VSMC) proliferation is the key event in the development of advanced lesions of atherosclerosis. Abnormal proliferation of VSMCs also plays an important role in vascular disease, being involved in vascular rejection and restenosis following angioplasty (Ross, 1993). Therefore, modulation of VSMC growth has critical therapeutic implications.

Proliferation of VSMCs is coordinately regulated by a number of growth-regulatory molecules through different signal transduction pathways, most of which lead ultimately to the activation of transcription factors that, in turn, activate essential genes for cell cycle progression. Immediate-early genes such as the transcription factors c-*fos* and c-*jun* are activated both transcriptionally and post-transcriptionally, in response to numerous growth stimuli (i.e. serum), in a variety of cells and species, including VSMCs (Verma & Sassone, 1987; Sukhatme *et al*., 1988; Komuro *et al*., 1988; Taubman *et al*., 1989; Bobik *et al*, 1990).

In the present study, we investigated whether PCA-4230 has antimitogenic effects on cultured vascular smooth muscle cells (line A10) stimulated to proliferate by the administration of foetal calf serum. We also compared the antiproliferative effects of PCA-4230 to that of another known dihydropyridine-type growth inhibitor. The influence of PCA-4230 on the serum-inducible cell cycle progression was also examined by flow cytometry (FACS analysis). Finally, we looked into the effects of the drug on the mRNA expression of the transcription

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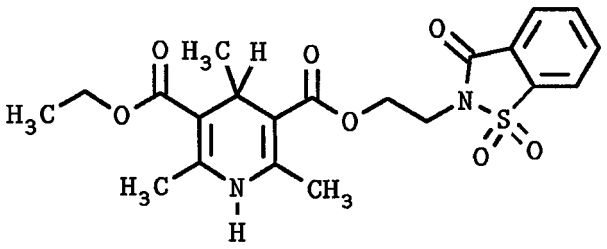

Figure 1 Chemical structure of PCA-4230

factors c-*fos* and c-*jun* as part of the early G_0/G_1 transition induced in quiescent vascular smooth muscle cells by mitogenic stimulation.

Methods

Cell culture and cell number determination

Rat A10 vascular smooth muscle cells were obtained from the American Type Culture Collection (ATCC; A10 CRL 1476). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS supplemented with glutamax I, 100 iu ml^{-1} penicillin G (sodium salt), 100 $\mu g\ ml^{-1}$ streptomycin and 0.25 $\mu g\ ml^{-1}$ amphotericin B (antibiotic-antimycotic solution, Gibco). To follow proliferation in the presence of PCA-4230, cells were seeded at 2.5×10^4 cells/well, detached with trypsin, and counted in a haemocytometer. Throughout the experiments, media were changed daily and cultures were protected from the light. Primary cultures of vascular smooth muscle cells were prepared from enzymatically dissociated rabbit thoracic aorta according to a method previously described (Del Rio *et al*., 1996). The cells were passaged and studied in the same medium as described above for A10 cells, and were characterized as smooth muscle cells by immunostaining with an antibody to smooth muscle α actin. Bovine aortic endothelial cells were harvested according to Salameh *et al*. (1996). The cells were cultured in Iscove's modified Dulbecco's medium: Ham's F12 (1:1) containing 10% FCS supplemented with glutamax I, 100 iu ml^{-1} penicillin G (sodium salt), 100 $\mu g\ ml^{-1}$ streptomycin and 0.25 $\mu g\ ml^{-1}$ amphotericin B (antibiotic-antimycotic solution, Gibco). Cell identity was determined by immunostaining with an antibody to von Willebrand factor. All experiments with endothelial cells were performed with cells of passage 5 or less.

Determination of DNA synthesis

DNA synthesis as assayed by the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into nuclei (Campana *et al*., 1988) was measured in order to study the effects of PCA-4230 on cell growth. Cells were harvested by trypsinization and seeded into glass coverslips in 24-well cluster culture plates at a density of 10^4 cells per well in DMEM with 10% FCS. After 24 h incubation, to allow for cell attachment, the cells were washed with serum-free DMEM with 0.2% bovine serum albumin (BSA) and incubated in the same medium for 48 h to obtain quiescent non-dividing cells. The cultures were then incubated in DMEM with 10% FCS (or 10 ng ml^{-1} platelet-derived growth factor-BB (PDGF-BB)), containing vehicle alone or various concentrations of PCA-4230, for 18 h and then, for 2 h in the same medium containing BrdU (10 μM). BrdU incorporation was visualized by immunocytochemical staining. The cells on coverslips were fixed with acid-alcohol (90% ethanol: 5% acetic acid: 5% water) at room temperature for 30 min, rinsed in phosphate-buffered saline (PBS), incubated for 1 h in a nuclease-containing anti-BrdU monoclonal antibody (Amersham, U.K.) and then washed again in PBS. Finally, the cells were incubated with a peroxidase conjugated anti-mouse IgG antibody (Amersham, U.K.) for an additional hour. Diaminobenzidine solution (0.5 mg ml^{-1} with 0.01% H_2O_2) was used as peroxidase substrate. Coverslips were counterstain and mounted in Eukitt. The ratio of positively stained cell population to total cell was calculated. Black staining at the BrdU-incorporated sites, indicating the cells initiating DNA replication, was detected by light microscopy. At least five hundred nuclei were counted.

Cell cycle analysis

To estimate the proportions of cells in different phases of the cell cycle, cellular DNA contents were measured by flow cytometry. Cells were plated, allowed to attach overnight, and placed in serum-free medium for 48 h as described above. PCA-4230 (50 μM) was added at selected points during serum repletion. At the specified times after serum addition, the cells were harvested by trypsinization, washed with PBS, pelleted and resuspended in PBS containing 0.6% Nonidet P-40 and 100 $\mu g\ ml^{-1}$ propidium iodide (PI), to which RNase was added to a final concentration of 100 $\mu g\ ml^{-1}$ propidium iodide (PI), to which RNase was added to a final concentration 100 $\mu g\ ml^{-1}$. Flow cytometric analyses were done with a FACScan (Becton-Dickinson) flow cytometer equipped with a 15 mW Argon laser emiting at 488 nm. PI fluorescence was recovered through 575/24 BP filter; 10,000 cells were acquired per sample and a Double Discriminator Module was used to detect only single cells.

Oncogene expression

The expression of c-*fos* and c-*jun* mRNA was studied in serum deprived cells that were stimulated with 10% FCS in the presence or absence of PCA-4230 (50 μM) for 30 min. Total RNA was extracted from the cells by acid-phenol procedure (Chomczynski & Sacchi, 1987). Total RNA (20 μg) was separated in a 6% formaldehyde-1.2% agarose gel, blotted onto hybond N^+ membranes in 10× standard saline citrate (SSC) (consisting of 0.15 mM NaCl and 0.015 mM sodium citrate). Membranes were washed in 2× SSC fixed with ultraviolet irradiation and baked at 80°C for 2 h. Hybridization was performed overnight at 65°C in 5× SSC, 0.2% sodium dodecyl sulphate, 50 mM sodium phosphate, 10× Denhardt's solution and 20 $\mu g\ ml^{-1}$ salmon-sperm DNA. Blots were hybridized to a random-primed specific DNA probe for rat c-*fos* and c-*jun*, and then exposed to Kodak X-Omat film for 8 to 17 h at −70°C. Blots

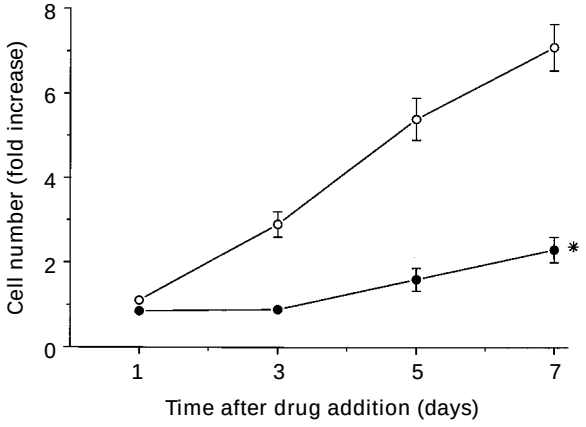

Figure 2 Effect of PCA-4230 (50 μM) on FCS-induced growth (cell number) of A10 rat vascular smooth muscle cells. Vehicle (○) or PCA-4230 (●) was added and incubated for 7 days. Results are presented as the mean ± s.e.mean (vertical lines) of three separate experiments, each in duplicate, and expressed as fold increase in cell number, relative to that on day 0 of the experiment. *Significantly different from the control (vehicle) curve ($P < 0.05$).

were standardized with a complementary DNA probe for β-actin.

Materials

PCA-4230 was dissolved in dimethyl sulphoxide (DMSO) and immediately used at dilution giving a final concentration of less than 0.1% DMSO. Nifedipine was synthesized by Alter, Research Department. BSA, human recombinant PDGF-BB, and antibodies to smooth muscle α actin and von Willebrand factor were purchased from Sigma Biosciences, U.S.A. Foetal calf serum (FCS), DMEM and all other tissue culture reagents were from Gibco, Scotland. BrdU was from Amersham, U.K.

Data analysis

The results are expressed as the mean ± s.e.mean and accompanied by the number of observations. A statistical analysis of the data was done by Student's *t* test. Differences with a P value of less than 0.05 were considered statistically significant.

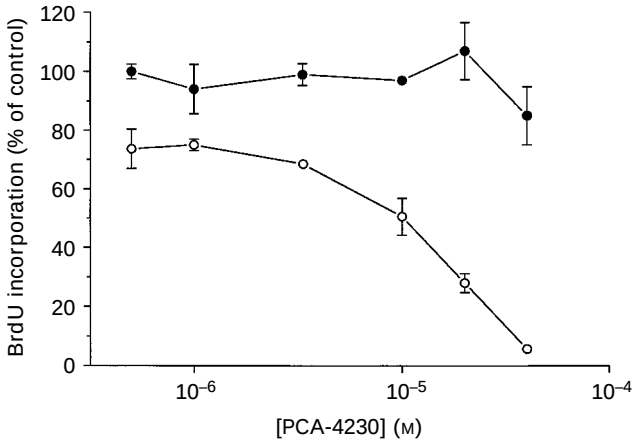

Figure 3 Effect of PCA-4230 on serum-induced BrdU incorporation in A10 vascular smooth muscle cells (○) and endothelial cells (●). Data points represent the mean of five to six determinations each in triplicate, and vertical lines represent the s.e.mean. Results are expressed as percentage of control, defined as BrdU incorporation in the presence of 10% FCS and vehicle.

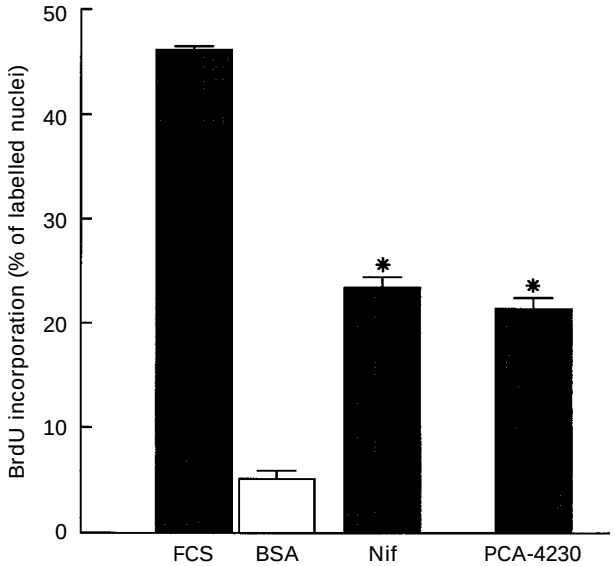

Figure 4 Effect of equimolar concentrations (10 μM) of PCA-4230 and nifedipine on BrdU incorporation in A10 vascular smooth muscle cells stimulated with 10% FCS. Results (mean ± s.e.mean) are from three independent experiments conducted in triplicate. $^*P<0.01$ as compared to vehicle-treated cells.

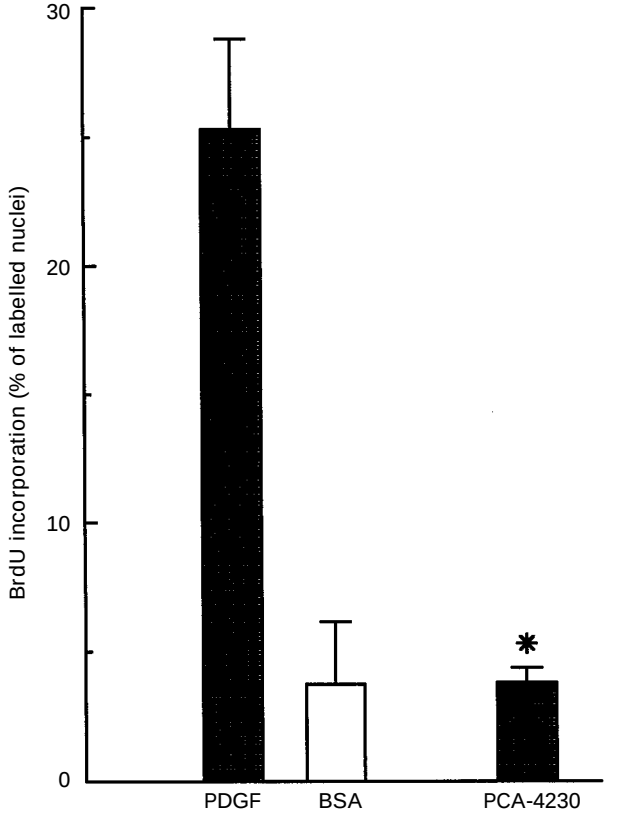

Figure 5 Effect of PCA-4230 (50 μM) on PDGF-BB-induced BrdU incorporation in A10 vascular smooth muscle cells. Results (mean ± s.e.mean) are from three independent experiments conducted in duplicate. $^*P<0.01$ as compared to vehicle-treated cells.

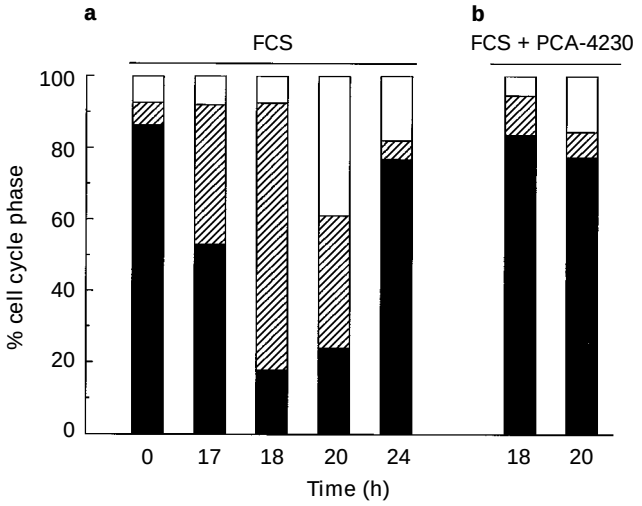

Figure 6 Time-dependence of cell cycle progression in synchronized A10 vascular smooth muscle cells (a). Times given are those transpiring between serum repletion and cell harvesting. Solid areas, % of G_0/G_1; hatched areas, % of S; and open areas, % of G_2/M. (b) Cell distribution in the presence of PCA-4230 (50 μM). Results are given as means of one experiment conducted in duplicate. Similar results were obtained in three other experiments. Individual values differed by less than 15% from means. Cell distribution was evaluated by flow cytometric determination of DNA content.

Results

PCA-4230 and cell proliferation

In the first series of experiments, cell counting was used to test PCA-4230 ability and to alter smooth muscle cell proliferation. Exposure to 50 μM PCA-4230 revealed an important stasis of growth (Figure 2). This inhibitory effect was also observed in the presence of 1 μM PCA-4230. The percentage of inhibition exerted by 1 μM PCA-4230 on day 3, 5 and 7 of the growth curve was 22.0 ± 4.5, 32.0 ± 3.0 and $29.4 \pm 6.0\%$, respectively ($P < 0.05$). The inhibitory effect of PCA-4230 on cell proliferation was reversible and after removal of the drug the proliferation of the cells was resumed (data not shown).

To confirm that the above inhibitory effects were not due to toxicity or damage of the cells, trypan blue viability tests were carried out in cells treated in parallel to growth studies. There was no loss in viability of cells treated with PCA-4230, less than 2% of the cells took up the dye. Furthermore, no floating cells were observed on any particular day for the entire growth curve. Thus, detachment and loss of the cells did not account for the inhibition of cell proliferation.

Effect of PCA-4230 on DNA synthesis

The observed inhibitory effect of PCA-4230 on cell proliferation could have resulted from an inhibition of DNA synthesis. To test this hypothesis, the drug was evaluated for concentration-related effects on DNA synthesis in smooth muscle cells that were synchronized by serum-depletion and then stimulated by the addition of 10% FCS. The concentrations used in these studies were based on the concentrations that have been shown to inhibit platelet function (Sunkel *et al*., 1988). PCA-4230 (0.5 – 50 μM) was inhibitory to serum-stimulated DNA synthesis as measured by the incorporation of the thymidine analogue BrdU, with an IC_{50} value of 13 μM (Figure 3). Moreover, PCA-4230 was found to inhibit serum-stimulated DNA synthesis in primary cultures of smooth muscle cells isolated from rabbit aorta with similar potency to that observed in A10 cells (data not shown). However, BrdU incorporation of 10% FCS-stimulated bovine aortic endothelial cells was not significantly affected by PCA-4230 (0.5 – 50 μM) (Figure 3). Determination of BrdU incorporation in vascular smooth muscle cells after PCA-4230 washout demonstrated the resumption of DNA

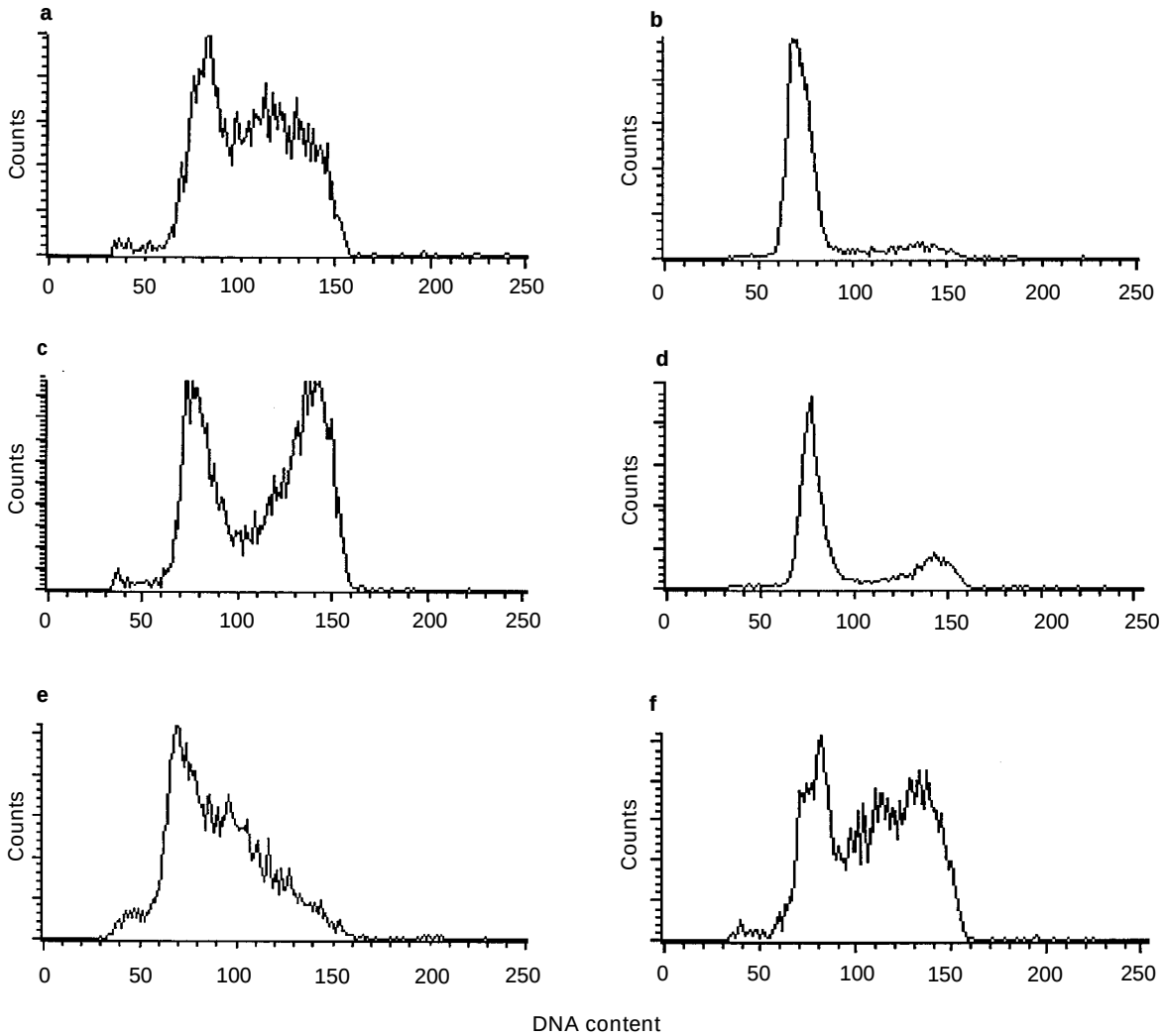

Figure 7 Impact of acute treatment with PCA-4230 (50 μM) on cell cycle progression as evaluated by flow cytometric determination of DNA content in synchronized A10 vascular smooth muscle cells at 18 h (a and b) and 20 h (c and d) after serum repletion in the absence (a and c) or presence (b and d) of PCA-4230. Lower panels show the effect of delay between serum repletion and PCA-4230 addition; (e) corresponding to 2 h and (f) 12 h delay in the addition of PCA-4230. Cells were harvested after 18 h of serum stimulation. Individual nuclear DNA content as reflected by fluorescence intensity of incorporated propidium iodide is plotted as a histogram depicting relative cell number at each intensity. Each histogram is derived from a representative experiment where data from at least 10,000 events were obtained.

synthesis in a majority of the cells preceding substantial increases in total cell numbers. The staining pattern showed that reversal of inhibition did not appear to depend on the selection of a small number of cells resistant to the drug (data not shown).

The effect of PCA-4230 on serum-stimulated DNA synthesis in A10 cells was compared to that exerted by nifedipine. The inhibitory effect of PCA-4230 on BrdU incorporation was found to be of similar magnitude to that of nifedipine at equimolar concentration (Figure 4). In addition, significant reduction of DNA synthesis by PCA-4230 was also observed in A10 cells that were stimulated by the addition of 10 ng ml^{-1} PDGF-BB (Figure 5). The inhibitory effect of PCA-4230 could not be reversed by increasing the PDGF concentration to 50 ng ml^{-1}.

Effects of PCA-4230 on cell cycle in synchronized A10 VSMCs

To ascertain that PCA-4230 indeed affected the transition from G0/G1 to S phase, the effects of the drug on cell cycle progression were also analysed. A10 VSMCs were initially characterized to confirm their synchrony and cell cycle behaviour. After a 48 h exposure to a serum-free medium, essentially no BrdU incorporation into DNA was present (BrdU incorporation in growth-arrested cells was $5.1 \pm 0.8\%$, see Figure 4), and approximately 90% of the cells had a 2n DNA complement consistent with location in the G_0 or G_1 phase of the cell cycle (Figure 6). After replacement of serum-free medium with medium containing 10% FCS, the entrance of cells into the S phase was observed by flow cytometry with quantitative DNA staining with propidium iodide (Figure 5). The majority of the cells was seen to progress through S phase during an interval from approximately 17–20 h after serum repletion. The percentage of cells in S phase increased from 6.0% to 73.4% after 18 h of serum repletion. In contrast, PCA-4230-treated cells showed an acute block of cell cycle progression occurring at a point near to G_0/G_1 - S boundary, since essentially no increase in DNA content was seen in cells stimulated by serum in the presence of PCA-4230 (50 μM) (Figures 5 and 6). Further definition of the PCA-4230-sensitive point of the cell cycle was obtained in experiments in which the drug was added at different times after the mitogenic stimulus. Under these conditions, the inhibitory effect of PCA-4230 was maximal when the drug was added together with the stimulus but was abolished if the drug was added 12 h after serum repletion (Figure 7f). Moreover, the addition of PCA-4230 2 h after cell stimulation had a markedly reduced inhibitory effect (Figure 7e), demonstrating that PCA-4230 must act at the early events of cell cycle to be fully effective against DNA synthesis.

Effects of PCA-4230 on proto-oncogene expression in synchronized A10 VSMCs

In view of the effects of PCA-4230 at early cell cycle entry, the influence of the drug on the expression of c-*fos* and c-*jun*, two genes expressed in the G_0/G_1 transitional phase as part of the early response to mitogenic stimulation, was also studied (Gadeau *et al*., 1991; Rothman *et al*., 1994).

Quiescent cultures (48 h serum starvation) showed no expression of these genes. However, after a 30 min period of stimulation of these synchronized cell populations with serum an important activation of both c-*fos* and c-*jun* genes was observed (Figure 8). Densitometric analysis of the blots revealed that in cells that were stimulated in the presence of PCA-4230, c-*fos* expression was reduced by 95% while c-*jun* was decreased by 90%.

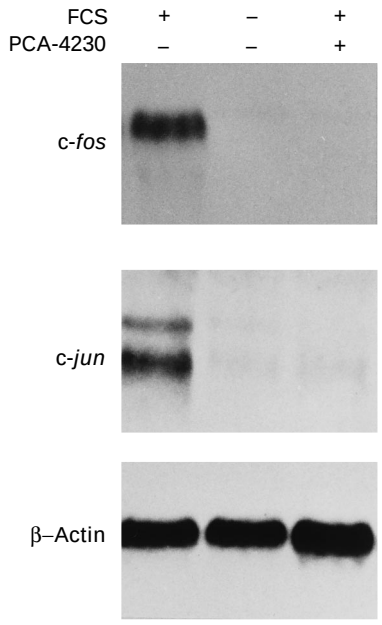

Figure 8 Effect of PCA-4230 (50 μM) on serum-induced expression of c-*fos* and c-*jun* mRNA levels in A10 vascular smooth muscle cells. Total RNA (20 μg) was subject to Northern blot analysis. Hybridization to c-*fos* probe (upper panel) and to c-*jun* probe (middle panel). Rehybridization of the same blot to a β-actin probe is shown in the lower panel. Serum-starved cells (middle lane) were used as reference. Right lane: serum and PCA-4230 treated cells. Left lane: serum and vehicle treated cells. Similar results were obtained in two independent experiments.

Discussion

Abnormal growth of VSMCs is importantly involved in the pathophysiology of vascular disease (Ross *et al*., 1986; Ross, 1993). In the present study we investigated the effects of PCA-4230, a novel dihydropyridine drug with antithrombotic action, on serum-induced VSMC growth. Our results show that PCA-4230 was able to reduce cell number in a concentration-dependent manner. Furthermore, by measuring serum-induced BrdU incorporation we have also demonstrated that the drug concentration-dependently inhibited DNA synthesis. Moreover, this inhibitory effect was of similar magnitude to that elicited by nifedipine, a known dihydropyridine-class growth inhibitor of smooth muscle proliferation (Nilson *et al*., 1985; Ko *et al*., 1993; Patel *et al*., 1995).

Recent studies have emphasized the role of the G_1-S events in regulation of cell proliferation through complex stimulant and inhibitory signals driven by cyclin-dependent kinases and their inhibitors, respectively. At the G_0/G_1 transition, the expression of several transcription factors like *myc*, *myb*, *fos* and *jun* appear to be fundamental (Newmark, 1987; Sibbitt, 1988; Paquet *et al*., 1990; Karin & Smeal, 1992). The growth inhibitory effect of PCA-4230 is likely to have resulted from the fact that the drug delayed the entry of the cells into the S-phase, evidently due to interference with an early step in the mitogenic signalling process. Thus, the addition of PCA-4230 2 h after cell stimulation had a markedly reduced inhibitory effect. Accordingly, the rise in the mRNA levels for the early-immediate genes c-*fos* and c-*jun* was blocked in the presence of PCA-4230. Therefore, the inhibition of proto-oncogene expression in the early G_0/G_1 transitional phase is apparently a necessary prerequisite of the S-phase entry block exerted by PCA-4230 in VSMCs.

Experimental and clinical data support the concept that calcium antagonists may protect against the progression of atherosclerosis and structural changes within the vessel wall in vascular diseases (Henry, 1990; Lichtlen *et al*., 1990). Different studies have shown an inhibitory effect of drugs belonging to the dihydropyridine series on vascular smooth muscle growth (Hirata *et al*., 1989; Jackson & Schwartz, 1992; Ko *et al*., 1993; Del Rio *et al*., 1996). However, there is still no consensus as to

the precise mechanisms underlying the inhibitory action of these drugs on proliferation. Some authors have indicated that the mechanisms by which these drugs exert their effects on vascular smooth muscle cells appear to be independent of a specific blockade of Ca uptake (Hirata *et al*., 1992; Ko *et al*., 1993). In agreement with this hypothesis is the finding that these drugs are also able to act on cells not expressing the voltage-dependent (L-type) calcium channel (Henry, 1988; 1990). PCA-4230 is a drug belonging to a series of 1,4-dihydropyridines that lack the aryl substituent in position 4 of the dihydropyridine ring (Sunkel *et al*., 1988). Because of this modification, PCA-4230 does not exhibit the typical vasodilator and relaxant effects on vascular smooth muscle (Villarroya *et al*., 1990). In addition, PCA-4230 showed a K_i value 500 fold higher than that of nitrendipine in studies performed to measure the affinity of both drugs for the L-type Ca channel (Villarroya *et al*., 1990). Therefore, our data would support the idea of a mechanism that is not completely due to blockade of L-type Ca channels in the antiproliferative effects of dihydropyridines.

From the present data it can be concluded that PCA-4230 could inhibit the proliferation and DNA synthesis induced in vascular smooth muscle cells by the complex mixture of mitogens of serum. Even if the precise mechanism(s) behind the antiproliferative actions of PCA-4230 are not known, flow cytometry studies and evaluation of proto-oncogene expression do suggest that a discrete event of the cell cycle as early as the G_0/G_1 transitional phase can be identified as a site of action for PCA-4230. Inhibition of PDGF-mediated DNA synthesis suggests that PCA-4230 may interfere with a specific step, either at or downstream, of the receptor tyrosine phosphorylation cascade.

The present study has demonstrated that PCA-4230 effectively inhibits VSMC proliferation which is considered to be a hallmark in various vascular disorders. Furthermore, this action appears to be selective for VSMCs, since endothelial cell proliferation was not significantly altered by the drug. Thus, PCA-4230 may be expected to prevent or inhibit the progress of vascular disease without affecting endothelial cell repair, which is a major goal in the treatment of such pathologies (Ross, 1993; Grainger *et al*., 1992). Finally, the inhibition of proliferation by PCA-4230 occurred with concentrations similar to those required to inhibit platelet function (Sunkel *et al*., 1988). In addition, the IC_{50} value of PCA-4230 on collagen-induced aggregation was shown to be approximately 10 fold lower than that of nifedipine, PCA-4230 having an IC_{50} of 10 μM (Sunkel *et al*., 1988). The antiplatelet activity of PCA-4230 may further contribute towards the vascular protective effect of the drug.

In conclusion, the present findings, derived from *in vitro* experiments, show that PCA-4230 may hold promising potential for the treatment of atherosclerosis and vascular diseases. Results of a recent phase II clinical trial seem to confirm the clinical relevance of the present study (Dr H.W. Heiss, unpublished observations).

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