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Inhibition of rabbit aortic smooth muscle cell proliferation by selective inhibitors of protein kinase C

[1]Andrew C. Newby, Karene Lim, Marilyn A. Evans, Nicholas P.J. Brindle & *Robert F.G. Booth

Department of Cardiology, Welsh Heart Research Institute, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN and *Roche Research Centre, Roche Products Ltd., Welwyn Garden City, Herts AL7 3AY

1 We studied the effect of two structurally-related, selective inhibitors of protein kinase C, Ro 31-8220 and Ro 31-7549, on the reinitiation of proliferation in quiescent first passage rabbit aortic smooth muscle cells in response to (a) the direct activator of protein kinase C, phorbol dibutyrate (PDBu), (b) platelet-derived growth factor (PDGF), (c) a combination of PDGF and 5-hydroxytryptamine (5-HT) or (d) serum.

2 Ro 31-8220 and Ro 31-7549 concentration-dependently inhibited proliferation in response to each mitogen. The inhibitory potency (IC_{50}) of Ro 31-8220 and Ro 31-7549, respectively, was similar against proliferation induced by PDBu (0.55 and 1.1 μM), PDGF (0.6 and 0.9 μM), PDGF and 5-HT (0.68 and 1.1 μM), although slightly less against serum (1.7 and 5 μM). The effects of the protein kinase C inhibitors on proliferation could not be ascribed to cytotoxicity. Neither Ro 31-8220 nor Ro 31-7549 (0.3–3 μM) inhibited PDGF receptor tyrosine phosphorylation.

3 The results show that Ro 31-8220 and Ro 31-7549 are potent inhibitors of smooth muscle cell proliferation in response to a direct activator of protein kinase C, the defined growth factors, PDGF and 5-HT, and the complex mixture of mitogens in serum. Protein kinase C activation thus appears to be an important growth transducing mechanism for each of these agents.

Keywords: Arteriosclerosis; vascular smooth muscle, rabbit aorta; protein kinase C; cell proliferation

Introduction

Vascular smooth muscle proliferation is a key process underlying the formation of atherosclerotic plaques (Ross, 1993). It is also the primary factor in reocclusion of arteries after angioplasty or atherectomy (Waller *et al.*, 1991), in the transplanted heart (Rose & Uyr, 1984), and in saphenous vein coronary artery bypass grafts (Angelini & Newby, 1989). There is therefore considerable interest in inhibitors of smooth muscle proliferation. One approach is to develop agents that interfere with growth factor transduction pathways, particularly if they inhibit elements common to diverse agents. Responses to classic growth factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor are thought to be mediated by stimulation of receptor tyrosine kinases followed by activation of phospholipase C-γ (PLC-γ), triggering of calcium mobilization and activation of protein kinase C (PKC) (Meldrum *et al.*, 1991; Rhee, 1991). Vasoconstrictor agents, including 5-hydroxytryptamine (5-HT) and endothelin that act as co-mitogens with PDGF also cause calcium mobilization via PLC-β (Meldrum *et al.*, 1991) and activate PKC (Nishizuka, 1986; 1988). Activation of PKC may therefore contribute to mitogenic responses to both growth factors and vasoconstrictor agents. Phorbol esters that are direct activators of PKC, stimulate vascular smooth muscle proliferation, although to a lesser degree than serum (Kariya *et al.*, 1987a). PKC activating phorbol esters inhibit maximal responses to serum (Kariya *et al.*, 1987a), so that they may be regarded as 'partial agonists' of proliferation. Prolonged exposure to phorbol esters down-regulates PKC α isoforms and abolishes both the stimulatory and inhibitory effects of phorbol esters on proliferation (Kariya *et al.*, 1987a; 1989). Thus, while the effects of phorbol esters on proliferation are clearly mediated by PKC, activation of PKC alone is insufficient to account for the mitogenic responses to serum. Tyrosine phosphorylation of growth factor receptors also leads to activation of the MAP kinase cascade (Egan & Weinberg, 1994) providing an alternative pathway for mitogenic signalling in many cells including vascular smooth muscle (Graves *et al.*, 1993).

This calls into question whether PKC activation is essential at all for mitogenic responses to growth factors and vasoconstrictor agents. In favour of this proposal, inhibitors of PKC including staurosporine and K525a have been found to inhibit smooth muscle proliferation (Kariya *et al.*, 1987b; Tagaki *et al.*, 1988; Matsumoto & Sasaki, 1989; Ohmi *et al.*, 1990), although these agents are poorly-selective and potently inhibit other kinases that may be important for proliferation. For example, K252a and staurosporine are more potent inhibitors of phosphorylase kinase than PKC and both are also potent inhibitors of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) dependent and Ca^{2+}/calmodulin-dependent kinases (Davis *et al.*, 1989; Elliot *et al.*, 1990). To clarify further the role of PKC in proliferation in response to PDGF, 5-HT and the complex mitogen, serum, the effects of two highly selective PKC inhibitors, Ro 31-8220 and Ro 31-7549 (Davis *et al.*, 1989), have been investigated.

Methods

Culture of rabbit aortic explants

This was conducted by a slight modification of the method described previously (Southgate & Newby, 1990; Assender *et al.*, 1992). Briefly, healthy New Zealand White Rabbits (age 9 weeks) were killed by a blow on the neck, the thorax was opened and 1000 units of heparin were injected into the left ventricle of the beating heart. The thoracic aorta was removed and immersed in sterile Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES buffer, pH 7.4, 100 iu ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin. The aorta was opened, the endothelium was removed from

[1] Author for correspondence.

the intimal surface by abrasion, and segments of tunica media were peeled off from the adventitia. The segments of tunica media were then cut up into 1 mm^2 explants with a McIlwain tissue chopper. The explants (approximately 100 per 75 cm^2 flask) were cultured in medium lacking HEPES but containing 25 mM sodium bicarbonate, 8 mM L-glutamine, 1 mM sodium pyruvate and 15% foetal bovine serum for 10 days at 37°C under an atmosphere of air: CO_2 (95:5 v/v). The outgrown cells were then dispersed by incubation for 5 min at 37°C with a 0.05% solution of trypsin, 0.02% EGTA and 154 mM NaCl. The cells were counted in a haemocytometer and replated at a density of 2.5×10^4 cells per 2 cm^2 well of 24 well culture plates. After 24 h, when the cells had attached and spread out, proliferation was arrested by changing to a quiescing medium, which consisted of DMEM and Hams F12 media in a 1:1 (v/v) ratio supplemented with ovalbumin (1 mg ml^{-1}), L-ascorbic acid (25 $\mu g\ ml^{-1}$), sodium selenate (25 ng mg^{-1}) Cohn factor IV (10 $\mu g\ ml^{-1}$), epidermal growth factor (10 ng ml^{-1}), transferrin (10 $\mu g\ ml^{-1}$), hydrocortisone (50 μM), L-glutamine (6 mM), penicillin (1000 units ml^{-1}) and streptomycin (1000 $\mu g\ ml^{-1}$) (Nemecek *et al.*, 1986). The cells were maintained in this medium for 96 hours before growth was re-initiated by adding the same medium containing mitogens, inhibitors (see below) and 1 $\mu Ci\ ml^{-1}$ of [6-^{3}H]-thymidine. Cultures were terminated 24 h later.

Termination of cultures and measurements of ATP, DNA and thymidine incorporation

Cultures were terminated by removing the medium and adding 1 ml of ice cold 10% w/v trichloroacetic acid, scraping off the cells and centrifuging the mixture at 1500 *g* for 5 min at 4°C. The supernatants were neutralised with a solution of 0.5 M tri-n-octylamine in 1,1,2, trichlorotrifluoroethane (Khym, 1975) and the aqueous phase was then separated and retained for measurement of ATP concentration by the firefly luciferase method (Spielmann *et al.*, 1981). The pellet was used for the measurement of DNA concentration (Kissane & Robins, 1958) and thymidine incorporation by liquid scintillation spectrometry as described previously (Southgate & Newby, 1990).

Measurement of tyrosine phosphorylation

Primary rabbit aortic smooth muscle cells were grown from explants and dispersed in trypsin/EDTA as described above. After counting in a haemocytometer, 2.5×10^5 cells were plated out in 29 cm^2 sterile plastic Petri dishes, left to adhere and rendered quiescent for 96 h as described above. The medium was then replaced with 2 ml of HEPES buffered DMEM (see above) supplemented as indicated in the text with 0.1% of dimethylsulphoxide with or without Ro 31-8220 or Ro 31-7549. Cultures were preincubated at 37°C in air for 10 min before the addition of 20 ng ml^{-1} final concentration of PDGFBB. After a further 10 min at 37°C, the medium was removed and replaced with 100 μl of a boiling hot SDS sample buffer (Laemmli, 1970) supplemented with 100 mM dithiothreitol, 10 mM sodium orthovanadate, 10 mM sodium fluoride, 30 mM sodium pyrophosphate and 10 mM EDTA, pH 6.7. The cells were removed with a cell scraper and the solution was boiled for 10 min. After centrifuging for 8 min at 10,000 *g* at room temperature, 100 μl of supernatants were subjected to SDS polyacrylamide gel electrophoresis using 7.5% (w/v) gels (Laemmli, 1970). After electroblotting onto nitrocellulose membranes, the presence of tyrosine phosphorylated proteins was detected as described previously (Brindle *et al.*, 1990). Briefly, membranes were incubated with blocking buffer (25 mM Tris/HCl, pH 7.4, 137 mM NaCl and 5% w/v bovine serum albumin) followed by a 0.5 $\mu g\ ml^{-1}$ solution of anti-phosphotyrosine monoclonal antibody in blocking buffer for 1 h at 25°C. Bound antibodies were detected by with peroxidase-labelled sheep anti-(mouse IgG) (0.2 $\mu g\ ml^{-1}$ in blocking buffer) and the ECL Western blotting detection system as described in the manufacturers instructions. X-ray film (Kodak XAR 5) was exposed for between 1 to 30 s to optimise contrast. The developed film was subjected to densitometric analysis using a UVP (Cambridge, U.K.) GDS 2000 gel documentation and analysis system. The area under the curve for designated peaks was computed above the grey level set from the minimum in each individual track.

Drugs and chemicals

Ro 31-8220 (3-[1-[3-(amidinothio)propyl]-3-indolyl]-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dione methylsulfonate) and Ro 31-7549 (3-[1-(3-hydroxypropyl)-3-indolyl]-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dione acetate) were synthesized as described previously (Davis *et al.*, 1992) and were obtained from Roche Products Ltd, Welwyn Garden City, Herts. The agents were dissolved in dimethylsulphoxide (DMSO) to concentrations ranging from 0.2 to 20 mM and were then diluted into tissue culture media to maintain a constant final concentration of 0.1% DMSO, which was used as control vehicle. [6-^{3}H]-thymidine (20–30 Ci $mmol^{-1}$) was obtained from New England Nuclear, Du Pont (U.K.) Ltd, Stevenage, Herts, or Amersham International, Aylesbury, Bucks. Foetal calf serum (FCS) was obtained from Imperial Laboratories Ltd, Andover, Hants. Recombinant platelet derived growth factor BB homodimer (PDGFBB) was obtained from Boehringer Mannheim, Lewes, Sussex. 5-Hydroxytryptamine (5-HT), phorbol 12,13-dibutyrate (PDBu), anti-phosphotyrosine monoclonal antibodies (clone PT 66) and prestained molecular weight markers were obtained from Sigma Chemical Company, Poole, Dorset. Nitrocellulose membranes (Hybond N), peroxidase-labelled sheep anti-(mouse IgG) and the ECL Wester blotting detection system were supplied by Amersham International, Amersham, Bucks. All tissue culture reagents were obtained from ICN Flow, High Wycombe, Bucks. All other chemicals were obtained from Fisons Scientific Ltd, Loughborough, Leics.

Statistical methods

Values are expressed throughout as mean ± s.e.mean and were compared by Student's *t* test using paired values. For IC_{50} values, the logarithms were subjected to significance testing.

Results

Mitogenic responses

Proliferation of quiescent first passage rabbit aortic smooth muscle cells was stimulated in a concentration-dependent way by PDBu, PDGF and serum (Figure 1a-c). The proliferation response to 0.1 μM PDBu measured in 8 paired experiments, including those shown in Figure 1c amounted to 10 ± 4% of the corresponding response to serum. This partial response agreed with previous work in rabbit aortic smooth muscle cells (Kariya *et al.*, 1987a). We also confirmed in 3 experiments the previous report (Kariya *et al.*, 1987a) that 0.1 μM PDBu inhibited the response to 5% serum by 76 ± 1%. The response to PDGF was potentiated by 5-HT (Figure 1b), being half maximal at 12 ± 1 ng ml^{-1} in the absence and 7 ± 2 ng ml^{-1} in the presence of 5-HT ($P < 0.05$, $n = 4$). The maximal response to 20 ng ml^{-1} of PDGF was not significantly increased, however, by 5-HT (Figure 1b). The maximal responses to 20 ng ml^{-1} PDGF measured in 7 paired experiments, including those in Figure 1b was 52 ± 17% of the response to 5% serum. The response to 20 ng ml^{-1} PDGF and 10 μM 5-HT measured in 9 paired experiments including those in Figure 1b was 61 ± 12% of the response to 5% serum.

Effects of protein kinase C inhibitors

The PKC inhibitors, Ro 31-8220 and Ro 31-7549, concentration-dependently inhibited cell proliferation in response to PDBu, PDGF alone, PDGF plus 5-HT or serum (Figure 2a). This inhibition was almost complete for each mitogen with 3 μM Ro 31-8220 or 10 μM Ro 31-7549 (Figure 2a, Table 1). Ro 31-7549 was consistently slightly less potent than Ro 31-8220, although the difference was statistically significant only for the data with serum (Table 2). The inhibitory potency of Ro 31-8220 or Ro 31-7549 was similar against phorbol dibutyrate, PDGF alone or PDGF plus 5-HT; however, the inhibitors exhibited a 3 or 5 fold reduced potency, respectively, against serum (Figure 2a, Table 2). The effects of Ro 31-8220 (or Ro 31-7549, results not shown) on proliferation were not accompanied by a decrease in cell viability as measured by ATP concentration (Figure 2b).

Neither Ro 31-8220 nor Ro 31-7549 (0.3–3 μM) inhibited the PDGF induced tyrosine phosphorylation of a 180 kDa band, presumed to be PDGF receptor subunits (Figure 3).

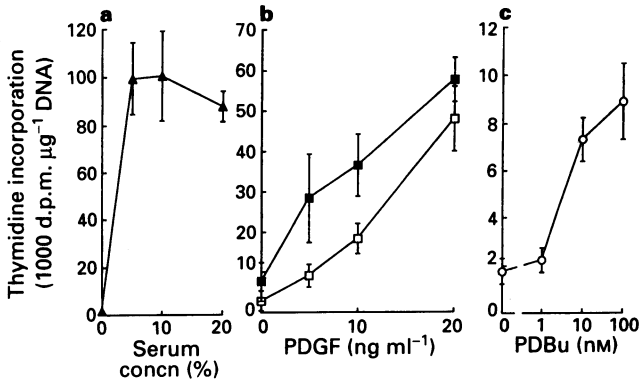

Figure 1 Proliferation responses: incorporation of [^{3}H]-thymidine into the DNA of quiescent rabbit aortic vascular smooth muscle cells was measured in response to stimulation for 24 h with serum (▲, $n = 3$), platelet-derived growth factor (PDGF) (□, $n = 4$), PDGF plus 10 μM 5-hydroxytryptamine (■, $n = 4$) or phorbol dibutyrate (PDBu, ○, $n = 4$). Responses were measured in quadruplicate for the number of aortae (n) shown. Values shown are means ± s.e.mean.

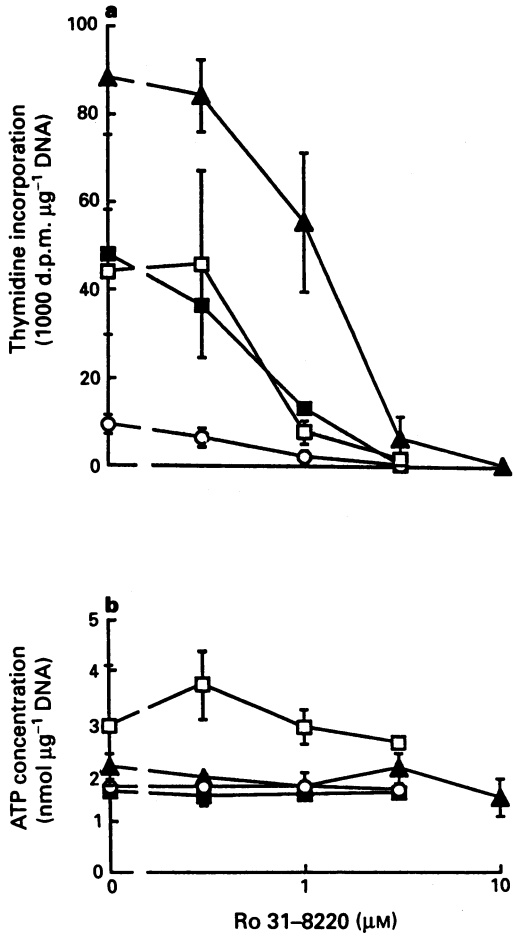

Figure 2 Effect of protein kinase C (PKC) inhibitors on proliferation: (a) The effects of 0.1–3 μM Ro 31-8220 or 0.1–10 μM Ro 31-7459 on incorporation of [^{3}H]-thymidine into DNA were measured in quadruplicate cultures of quiescent rabbit aortic smooth muscle cells restimulated for 24 h with 0.1 μM phorbol dibutyrate (○, $n = 6$), 20 ng ml^{-1} of platelet-derived growth factor (PDGF) alone (□, $n = 3$), PDGF with 10 μM 5-hydroxytryptamine (■, $n = 6$) or 5% (v/v) foetal calf serum (▲, $n = 3$). All cultures also contained vehicle (0.1% DMSO). (b) The concentrations of ATP (a marker of cell viability) were measured in the same cultures as panel (a). Values are means for the number of aortae shown for (a).

Table 1 Effectiveness (% residual proliferation) of protein kinase C (PKC) inhibitors against various mitogens

Mitogen	*PDBu*	*PDGF*	*PDGF + 5-HT*	*FCS*
Ro 31-8220 3 μM	3.7 ± 0.7% (6)	6.7 ± 3.0% (3)	2.2 ± 1.3% (6)	6.8 ± 4.8% (3)
Ro 31-7549 10 μM	1.2 ± 0.5% (3)	5.9 ± 3.0% (3)	4.5 ± 2.5% (4)	0.6 ± 0.3% (5)

The effects of Ro 31-8220 or RO 31-7459 were measured in quadruplicate cultures of quiescent rabbit aortic smooth muscle cells restimulated for 24 h with 0.1 μM phorbol dibutyrate (PDBu), 20 ng ml^{-1} of platelet derived growth factor (PDGF) with or without 10 μM 5-hydroxytryptamine (5-HT) or 5% (v/v) foetal calf serum (FCS). The residual rate of proliferation was then calculated for each experiment as a percentage of the rate in the presence of vehicle (0.1% DMSO) alone. The values shown are the means ± s.e.mean for the number of aortae shown in parentheses.

Table 2 Potency (IC_{50} values) of protein kinase C (PKC) inhibitors against various mitogens

Mitogen	*PDBu*	*PDGF*	*PDGF + 5-HT*	*FCS*
Ro 31-8220 μM	0.55 ± 0.13 (6)	0.6 ± 0.2 (3)	0.68 ± 0.22 (6)	1.7 ± 0.7 (3)
Ro 31-7549 μM	1.1 ± 0.7 (3)	0.91 ± 0.12 (3)	1.1 ± 0.5 (3)	5 ± 1(3)

The effects of 0.1–3 μM Ro 31-8220 or 0.1–10 μM RO 31-7459 were measured in quadruplicate cultures of quiescent rabbit aortic smooth muscle cells restimulated for 24 h with 0.1 μM phorbol dibutyrate (PDBu), 20 ng ml^{-1} of platelet derived growth factor (PDGF) with or without 10 μM 5-hydroxytryptamine (5-HT) or 5% (v/v) foetal calf serum (FCS). The concentrations giving 50% inhibition were then calculated for each experiment. The values shown are the means ± s.e.mean for the number of aortae shown in parentheses.

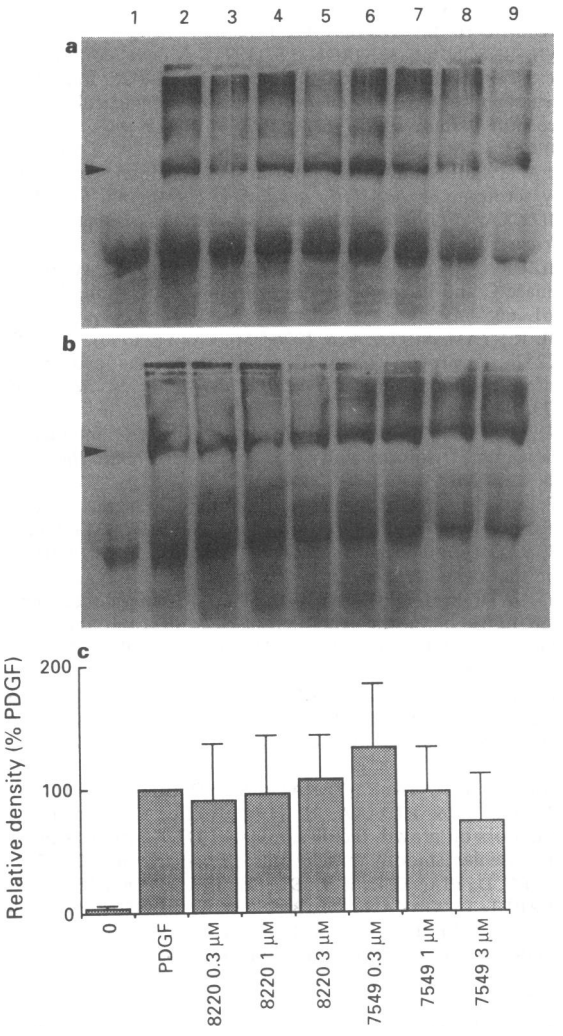

Figure 3 Effect of protein kinase C (PKC) inhibitors on tyrosine phosphorylation: (a) and (b) show 2 representatives of 4 similar experiments conducted on different preparations of rabbit aortic smooth muscle cells. Lane 1 has no additions, lanes 2–9 have 20 ng ml^{-1} of platelet-derived growth factor (PDGF) and lanes 3–9 have 0.1% of dimethylsulphoxide. Ro 31-8220 (lanes 4–6) or Ro 31-7549 (lanes 7–9) were also present at the μM concentrations shown. The arrows show the positions of the 180 kDa molecular weight markers in the two separate blots. Panel (c) shows the results of densitometric analysis of all 4 blots. The value (mean ± s.e.mean) for the other conditions was expressed relative to the optical density of the 180 kDa band in the PDGF alone lane, which was taken as 100%.

Discussion

Ro 31-8220 and Ro 31-7549 are conformationally restricted, substituted bisindolylmaleimides, which possess at least 100 fold greater potency against PKC than against cyclic AMP- or Ca^{2+}/calmodulin-dependent protein kinases (Davis *et al.*, 1989; 1992; Muid *et al.*, 1991). Both compounds act competitively with ATP, exhibiting IC_{50} values of approximately 10 and 80 nM, respectively, in *in vitro* assays using 10 μM ATP as substrate, but much higher values in intact cells, where ATP concentrations are millimolar. For example, both compounds inhibited PKC-induced phosphorylation in intact platelets and PKC-mediated responses in T-lymphocytes with IC_{50} values in the low micromolar range; Ro 31-8220 was approximately 6 fold more potent than Ro 31-7549 (Davis *et al.*, 1989; Muid *et al.*, 1991). We observed similar potencies here (0.5–1 μM) for inhibition of smooth muscle proliferation in response to the direct activator of PKC, PDBu. The mitogenic response to PDBu was small compared to that produced by serum, in agreement with previous studies of PDBu and other PKC activators in rabbit aortic smooth muscle cells, owing to 'partial agonist' behaviour (Kariya *et al.*, 1987a). Nevertheless, the stimulatory effect of PDBu is mediated by PKC, because it is abolished after down-regulation of PKC (Kariya *et al.*, 1987a; 1989). The proliferation response to PDBu is therefore an adequate positive control for the effects of Ro 31-8220 and Ro 31-7549, which takes into account penetration of the agents into smooth muscle cells and competition with intracellular ATP. Ro 31-8220 and Ro 31-7549 completely inhibited proliferation responses to PDBu.

A relatively greater proliferation response was obtained to PDGF than PDBu. The effect of PDGF was potentiated by 5-HT, in agreement with other published work (Araki *et al.*, 1990). Ro 31-8220 and Ro 31-7549 completely inhibited proliferation responses to PDGF alone or PDGF plus 5-HT. The similar inhibitory potencies against responses to PDBu, PDGF and PDGF plus 5-HT imply that inhibition of PKC accounts for the observed effects on proliferation. PKC activation therefore appears essential for responses to all these agents, consistent with previous studies of less specific PKC inhibitors (Kariya *et al.*, 1987b; Tagaki *et al.*, 1988; Ohmi *et al.*, 1990). The reduced inhibitory potency of Ro 31-8220 and Ro 31-7549 against serum, may imply that in this complex mixture there are mitogens that act independently of PKC but other explanations, such as protein-binding of the inhibitors or the need to overcome the larger response to serum, cannot be ruled out.

Based on previous data, it was unlikely that inhibition of a receptor tyrosine kinase rather than PKC accounted for the antiproliferative effects of Ro 31-8220 and Ro 31-7549. These agents exhibit only weak inhibitory activity against $p56^{lck}$, a lymphocyte specific tyrosine kinase or the insulin receptor tyrosine kinase (Welsh & Proud, 1993). Related bisindolylmaleimides also show only weak effects against PDGF, EGF or insulin receptor tyrosine kinases (Toullec *et al.*, 1991). To rule out an effect on the PDGF-receptor tyrosine kinase directly in our cells, we measured tyrosine phosphorylation in response to PDGF and found it to be unaffected by Ro 31-8220 and Ro 31-7549 at antiproliferative concentrations. We cannot, of course, rule out the possibility that Ro 31-8220 and Ro 31-7549 also inhibit one of the many other kinases known to be involved in cell proliferation.

Ro 31-8220 and Ro 31-7549 are potent inhibitors of smooth muscle cell proliferation irrespective of the nature of the mitogenic stimulus. They therefore fulfil one important criterion for use as treatment for vascular pathologies, including angioplasty restenosis. Inhibition by Ro 31-8220 and Ro 31-7459 implies an essential role for PKC in vascular smooth muscle cell proliferation, which needs to be further established by molecular biology methods.

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