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Inhibition of chemotaxis in A7r5 rat smooth muscle cells by a novel panel of inhibitors

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1 Arginine-specific ADP-ribosyltransferase (ART) activity has been implicated in white cell chemotaxis. In this study, we examined the capacity of a panel of structurally unrelated inhibitors and pseudo-substrates of ART to inhibit chemotaxis of A7r5 rat vascular smooth muscle cells in response to PDGF-BB.

2 The IC_{50} values for nicotinamide (12 mM) and novobiocin (165 μM) were similar to those observed for inhibition of chemotaxis by human polymorphonuclear neutrophil leucocytes (PMN), whereas vitamins K_3 ($IC_{50}=22$ μM) and K_1 ($IC_{50}=95$ μM) were less potent than previously described in PMNs. The pseudo-substrates for the enzyme (DEA-BAG, agmatine and arginine-methylester) also inhibited A7r5 chemotaxis, and in addition inhibited cell adhesion at similar concentrations. Vitamin K_3 was unique among the inhibitors of ART, in that it also inhibited cell adhesion.

3 A rat ART1 transcript was amplified by rtPCR from rat skeletal muscle, and was noted to share 94% homology with the mouse ART1 cDNA sequence. No such transcript could be detected in A7r5 cells by Northern blot analysis or rtPCR.

4 Evidence for ART activity on the surface of A7r5 cells was investigated using ^{32}P-NAD$^+$ as substrate, and labelled membrane proteins were observed with MWt values of 116, 100, 90 and 70 kDa. Exposure of the labelled proteins to phosphodiesterase yielded ^{32}P-AMP, and hydrolysis with NaOH yielded ^{32}P-NAD$^+$. These results indicated that the labelled proteins were adducts with NAD$^+$, and not the products of ART activity. The absence of ART catalytic activity in A7r5 cells was confirmed in protocols designed to show ADP-ribosylation of agmatine.

5 We conclude that the chemotactic activity of A7r5 cells is independent of ART activity, and the mechanism whereby the novel panel of inhibitors reduced cell migration remains undefined.

Keywords: Chemotaxis; ADP-ribosyltransferase; smooth muscle; A7r5, PDGF

Introduction

Vascular smooth muscle cell (VSMC) migration is an important component of wound healing, and also has a pathophysiological role in the formation of atheroma and hyperplastic lesions which contribute to the restenosis following angioplasty (Ross 1986; Raines & Ross 1993). A variety of factors, including growth factors, chemokines and other bioregulatory molecules stimulate VSMC migration, the most potent of which is platelet derived growth factor (PDGF) (Abedi & Zachary 1995). Two isoforms of PDGF are secreted by platelets during wound healing, PDGF-A and PDGF-B, which assemble as dimers. PDGF-BB is strongly chemotactic for rat VSMC, whereas PDGF-AA is not. In an *in vivo* rat model, chemotaxis induced by PDGF-BB is thought to play a major role in the formation of the neo-intima in both atherosclerosis (Ross *et al*., 1990) and restenosis following balloon catheter angioplasty (Ferns *et al*., 1991).

The intracellular signalling pathways involved in directed cell movement are unclear. Reorganization of the cytoskeleton and the formation and breaking of contacts with the extracellular matrix must occur, but how these processes are co-ordinated is unknown (Stossel, 1993). In a recent study (Allport *et al*., 1996a, b), a set of structurally unrelated inhibitors of ADP-ribosyltransferase (ART) activity were found to inhibit human polymorphonuclear neutrophil (PMN) receptor-dependent chemotaxis. Inhibition of the enzyme correlated closely with inhibition of both chemotaxis and actin assembly. ART catalyzes the transfer of ADP-ribose from NAD$^+$ to an arginine residue of the target protein. The chemotactic activity in PMNs has been linked with ART1 (MacDermot *et al*., 1997), a glycosylphosphatidylinositol-linked cell surface enzyme also found in skeletal muscle and lymphocytes. The substrates for this enzyme appear to be cell surface integrins, e.g. integrin α7 in skeletal muscle (Zolkiewska & Moss, 1993) and lymphocyte function-activated molecule 1 (LFA-1) in T cells (Nemeto *et al*., 1996). Expression of integrin α7 is restricted to skeletal muscle cells, and it mediates adhesion of the cell to laminin. The use of meta-iodobenzylguanidine, an inhibitor of ART, to block ADP-ribosylation of integrin α7 was followed by inhibition of the fusion of differentiating myoblasts *in vitro* (Kharadia *et al*., 1992). ADP-ribosylation of LFA-1 has been linked to (i) inhibition of inositol phosphate production after exposure of the cells to anti-(LFA-1) antibody, and (ii) inhibition of homotypic cell adhesion in MD17 cells. Similar modifications of an integrin on the surface of VSMCs could perhaps trigger integrin signalling for cytoskeletal re-alignments or affect adhesion of VSMCs to the extracellular matrix.

In the present study we have investigated whether such activity might be found in smooth muscle cells. The A7r5 smooth muscle cell line derived from embryonic rat thoracic aorta was used, and the cells were shown to be chemotactic for PDGF-BB. A panel of inhibitors was tested, and the IC_{50} values compared to those obtained previously for inhibition of chemotaxis in human PMNs. Evidence to support the presence

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of ADP-ribosyltransferase activity was then sought by enzymatic assay and rtPCR.

Methods

Cell line

The A7r5 cell line derived from thoracic aorta of embryonic DBFX rat was obtained from the European collection of cell cultures (ECACC, Cambridge, U.K.). Cells were cultured in DMEM, 10% foetal bovine serum (FBS), supplemented with 2 mM glutamine, at 37°C in a humidified atmosphere containing 5% CO_2.

Migration studies

Confluent A7r5 cells were trypsinised briefly (3–4 min) and added to an equal volume of DMEM containing 10% FBS. The cells were centrifuged, and the cell pellet washed twice with medium. The cells were finally suspended at 1 million cells ml^{-1} in DMEM containing 1% FBS and 20 mM HEPES buffer pH 7.4. Cell migration was studied using a modified micro Boyden chamber (Neuroprobe, Cabin John, MD, U.S.A.) across an 8 μM pore polycarbonate filter. PDGF was added to the lower chamber and (where applicable) inhibitors were added to both the upper and lower chambers. 50,000 cells were added to the upper chamber and incubated at 37°C for the times indicated. The cells that had migrated to the lower surface of the filter were fixed and stained with Diff Quik (Baxter, Thetford, Norfolk, U.K.), and were quantified by counting ten high power fields ($\times$450). The effect of each inhibitor concentration was examined in triplicate, and each experiment was performed with three separate cell passages. Chemotaxis was defined as (the number of cells migrated in the presence of PDGF gradient) minus (the number of cells migrated in the absence of PDGF gradient). In separate experiments inhibition of chemokinesis (the number of cells migrating in the absence of a PDGF-BB gradient, i.e. buffer alone) was also measured.

Cell adhesion

The ability of cells to attach to the polycarbonate filter in the presence of inhibitor was performed using a modification of the migration assay above. 5000 cells were added to the upper chamber in the absence or presence of inhibitor, and the attachment was allowed to proceed for 1 h. Cells were then fixed and stained as above and counted.

Cell viability

The trypan blue dye exclusion assay was performed to assess the cytotoxic effects of the inhibitors tested at concentrations which caused half maximal inhibition of migration. Cells were harvested and incubated with inhibitor for 1 h at 37°C, and then washed and resuspended in 0.5 ml PBS at 0.4 million ml^{-1}. Cells were incubated for 5 min with 0.5 ml of 0.4% (w/v) trypan blue solution before counting in a haemocytometer.

Preparation of smooth muscle cell membranes

Confluent A7r5 cells were harvested by scraping in PBS. The cells were pelleted by centrifugation, washed once with PBS, and then resuspended (1 million cells ml^{-1}) in ice cold 50 mM Tris HCl buffer pH 7.4, 0.25 mM EDTA, 290 mM sucrose, 0.1 mM PMSF, 1 mg ml^{-1} pepstatin, 1 mg ml^{-1} leupeptin. The cells were homogenised at 4°C using a tightly fitting Dounce homogeniser, and the cell debris was removed by centrifugation at 200 g. Membranes were collected from the supernatant by ultracentrifugation at 40,000 g, for 1 h at 4°C. The membranes were then resuspended in 50 mM Tris HCl buffer pH 7.4, 0.25 mM EDTA and stored at −20°C.

Arginine-specific ADP-ribosyltransferase activity

ART activity was measured by a modification of that described by McMahon *et al.* (1993). Intact A7r5 cells were plated on to 35 mm well plates at 500,000 cells per well in DMEM, containing 10% FBS and incubated overnight at 37°C. The medium was removed and 0.5 ml of DMEM added, containing 50 mM Hepes buffer pH 7.4, 300 μM ^{14}C-NAD$^+$ (25–30 μCi μmol^{-1}) and 20 mM agmatine, and the cells were incubated for a further 5 h at 37°C. The product of the reaction, ^{14}C-ADP-ribosylagmatine was separated from ^{14}C-NAD$^+$ by chromatography through Dowex 1X2-400 ion exchange resin, and was quantified by liquid scintillation counting. ART activity was also measured in membrane preparations. The protocol was modified such that a 0.3 ml reaction volume contained 50 μg membrane protein. The reaction was terminated by addition of 1 ml ice cold H_2O, and the membranes were removed by centrifugation at 13,000 g for 10 min.

NAD-glycohydrolase activity

NAD-glycohydrolase activity was measured in A7r5 cells using a modification of the protocol described above. The cells were incubated with [carbonyl-^{14}C]-NAD$^+$ for 1 h at 37°C, and the ^{14}C-nicotinamide released was quantified by scintillation counting following chromatographic separation on Dowex 1X2-400 ion exchange resin.

Transfer of ^{32}P from ^{32}P-NAD$^+$ to membrane proteins

50 μg of membrane proteins were incubated in 50 mM Tris HCl pH 7.4, 1 mM ADP-ribose and 1 μM ^{32}P-NAD$^+$ (30 Ci mmol^{-1}) in a reaction volume of 300 μl at 30°C for the times indicated. The reaction was terminated by the addition of 1 ml ice cold 10% (w/v) trichloroacetic acid (TCA). The proteins were precipitated on ice for 20 min and collected by centrifugation at 13,000 g for 20 min. The precipitate was washed once with 3% (w/v) TCA and once with 100% ethanol, and then allowed to air dry. The precipitate was solubilized by boiling for 5 min in SDS sample buffer [150 mM Tris HCl buffer pH 6.8 containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol]. The proteins were resolved by electrophoresis on 10% polyacrylamide gels and visualized by Coomassie blue staining. The gels were dried and radio-labelled proteins identified by autoradiography.

Analysis of ^{32}P-labelled proteins

(A) Mono-ADP-ribosylated proteins yield AMP when exposed to snake venom phosphodiesterase (Kempsom & Curthoys, 1983). Hence, ^{32}P-labelled proteins were resolved on SDS-PAGE, and transferred to nitrocellulose filters. The location of the labelled proteins was confirmed by autoradiography, and

the excised portion of filter was treated with 1 unit of snake venom phosphodiesterase overnight at 37°C. The supernatant was analysed by t.l.c. on PEI-cellulose following the method of Donnelly *et al*. (1996), and the migration of ^{32}P-labelled product was compared to non-radioactive standards (0.1 mmol NAD^+, 0.1 mmol ADP-ribose and 0.2 mmol AMP) which were located by u.v. quenching.

(B) Mono-ADPribosylated protein yields ADP-ribose when exposed to alkaline solution (Cervantes-Laurean, 1993). Hence, ^{32}P-labelled proteins were again located on nitrocellulose filters, and the excised portions of filter were treated with 0.1 M NaOH, at 56°C for 1 h. The supernatant was analysed by t.l.c. on PEI-cellulose following the method of Donnelly *et al*. (1996).

Reverse transcriptase polymerase chain reaction (rtPCR)

Direct evidence for an ART transcript in A7r5 cells was investigated by rtPCR using primers to ART1 based on cross-species sequence homology regions. ART1 is expressed in human, rabbit and mouse skeletal muscle, however the ART1 coding sequence has not previously been described in the rat. Rat skeletal muscle was therefore used as a likely positive control when seeking evidence for this transcript in rat smooth muscle cells. Total RNA was extracted from 10 million A7r5 cells or 100 mg rat skeletal muscle tissue following the method of Chomczynski and Sacchi (1987). Total RNA (2 μg) was reverse transcribed using oligo-dT primers and mouse Moloney leukaemia reverse transcriptase (Pharmacia). PCR was performed for 30 cycles of 94°C 1 min, 60°C 1 min and 72°C 2 min, with a final extension of 72°C for 7 min. Each reaction contained 1 × PCR buffer, 1.25 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 units of Taq polymerase and 20 pmol of each primer (for ART1 sense GCCTCCTTTGATGACCAGTA and anti-sense CAGGGAAGAAGGAGTA GCC; for rat actin TTGTAACCAACTGGGACGATATG, and GATCTTGATCTTCATGGTGCTAGG). A product of 580 bp for ART1 has been amplified previously from human skeletal muscle cDNA, using these primers. The PCR product for rat actin contains 764 bp, using the primers shown.

The novel 580 bp product (ART1) amplified from rat skeletal muscle was gel purified, and sequenced directly using ABI Taq Dye Deoxy Terminator Cycle Sequencing kit (Perkin Elmer, Bucks, U.K.) with an automated Applied Biosystems DNA sequencer (model 373A).

Northern blotting

The rat skeletal muscle 580 bp product was used to probe a Northern blot of rat A7r5 poly A^+ RNA and rat skeletal muscle poly A^+ RNA. The blot was pre-hybridized in 5 × SSPE, 2% SDS, 10 × Denhardts solution and 0.1 mg ml^{-1} denatured salmon sperm DNA for 3 h and hybridized overnight with ^{32}P-dATP labelled probe at 65°C. The blot was washed with four low stringency washes of 2 × SSC, 0.05% SDS at room temperature and one high stringency wash of 2 × SSC, 0.05% SDS at 65°C for 15 min each. The blot was then exposed to autoradiography film for 24 h at −70°C.

Protein determination

Protein concentrations were estimated using the BCA protein assay kit (Pierce) with BSA as the standard.

Curve fitting

Cell migration data were analysed using non-linear least squares regression (Graphpad prism). The analyses assumed a simple bimolecular interaction between PDGF and a single class of receptors.

Materials

Radiochemicals Nicotinamide [U-^{14}C]-adenine dinucleotide (252 mCi $mmol^{-1}$), [carbonyl-^{14}C]-nicotinamide adenine dinucleotide (35 mCi $mmol^{-1}$) and ^{32}P-dATP (3000 Ci $mmol^{-1}$) were purchased from Amersham (Little Chalfont, Bucks, U.K.), and ^{32}P-NAD^+ (800 Ci $mmol^{-1}$) was puchased from New England Nuclear (Stevenage, Herts, U.K.).

Culture medium Dulbecco's modified Eagle's minimal media (DMEM), trypsin, fetal bovine serum and 1 M Hepes buffer were obtained from Gibco BRL (Paisley, Scotland, U.K.). Polycarbonate PVP free (25 × 80 mm) 8 μM pore filters were purchased from Costar (High Wycombe, Bucks, U.K.). Polyethyleneimine-cellulose t.l.c. plates were obtained from Merck (Northants, U.K.). All other chemicals were obtained from Sigma Chemical Co (Poole, Dorset, U.K.). Diethylamino-(benzidylidineamino)-guanidine (DEA-BAG) was synthesized by Dr S. Murray (Clinical Pharmacology, ICSM, London, U.K.), who generously provided this reagent. The composition of SSPE buffer was: NaCl, 0.3 M; NaH_2PO_4 0.02 M; EDTA 2 mM; pH 7.4. The composition of SSC buffer was: NaCl 0.3 M; sodium citrate, 0.03 M; pH 7.

Results

Chemotaxis of A7r5 cells

The A7r5 cells were grown in continuous culture. The ability of these cells to respond to PDGF was confirmed using a micro Boyden chamber, and chemotaxis to PDGF-BB was linear over a 3–6 h time course. The concentration response curve to PDGF-BB is shown in Figure 1. In the absence of a PDGF gradient, 89 ± 9 cells were counted per high power field. A bell shaped curve, characteristic of a chemotactic response, was observed with maximal migration at 1 nM PDGF-BB and EC_{50} of 160 ± 15 pM (mean ± s.e.m., $n = 3$). A concentration of 400 pM was used in all further studies, which gave a 3 fold stimulation of migration.

Inhibition of migration

Representative curves for inhibition of chemotaxis in a single experiment with each of the inhibitors tested are shown in Figure 2, and the average IC_{50} values determined from three separate experiments are shown in Table 1. Inhibition of chemotaxis was observed in response to all the inhibitors tested. The most potent inhibitor was vitamin K_3 with an IC_{50} of 22 ± 3.4 μM (mean ± s.e.m., $n = 3$). Novobiocin and vitamin K_1 showed similar inhibition patterns with estimated IC_{50} values of 165 ± 29 μM and 95 ± 10 μM respectively. Nicotinamide inhibited chemotaxis at much higher concentrations, with an IC_{50} value of 12 ± 2.5 mM. In separate experiments inhibition of chemokinesis was determined and the average IC_{50} values determined from three separate experiments are shown in Table 1. Vitamin K_3, nicotinamide and novobiocin inhibited chemotaxis and chemokinesis at comparable con-

centrations. Inhibition of chemokinesis was not observed at 200 μM vitamin K_1, the limit of solubility of this compound under these conditions.

The pseudo-substrates of ART (DEA-BAG, agmatine and argininemethylester) all inhibited chemotaxis. Representative curves are shown in Figure 3 and the average IC_{50} values determined from three separate experiments are shown in Table 1. The most potent inhibitor was DEA-BAG with an IC_{50} value of 25 ± 9 μM (mean$\pm$s.e.m., $n=3$), and at 200 μM no cells migrated across the membrane. However this compound was significantly cytotoxic to the cells at its IC_{50} concentration. Agmatine inhibited chemotaxis with an IC_{50} value of 780 ± 36 μM, and the poorest inhibitor was arginine-methylester with an IC_{50} value of 21 ± 3.9 mM. Neither Agmatine nor arginine-methylester inhibited chemokinesis at the maximal concentrations (40 mM) tested.

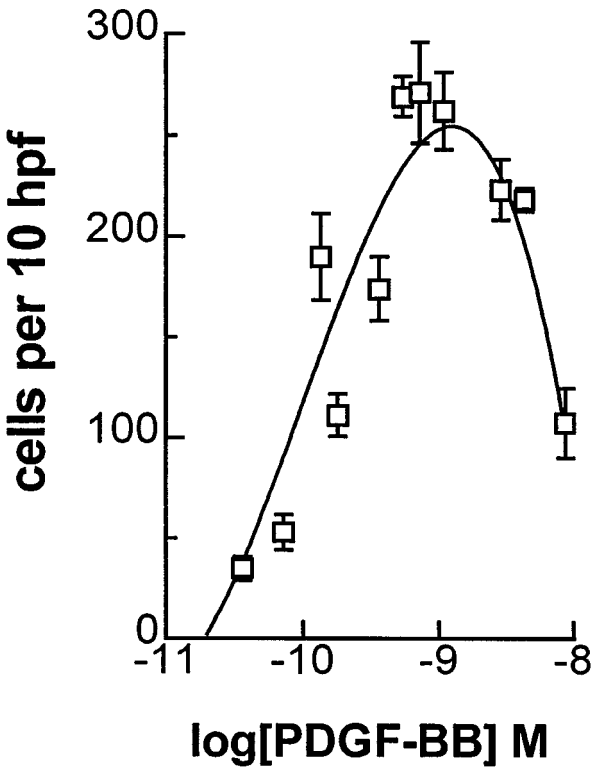

Figure 1 PDGF-BB concentration response curve in A7r5 cells. The experiment was performed as described in the Methods, and the results from one representative experiment is shown (mean$\pm$s.e.m., $n=3$.). Chemokinesis measured in the absence of PDGF-BB was 89 ± 9 cells per ten high power fields (hpf), and the concentration of PDGF required for half maximal migration determined from three separate experiments was 165 ± 15 pM.

Adhesion

The number of cells adherent to the membrane at the IC_{50} concentration for inhibition of chemotaxis by each compound is shown in Table 1. Significant decreases in the number of adherent cells were observed in the presence of the pseudosubstrates or vitamin K_3 at concentrations at which half maximal inhibition of chemotaxis were observed. In contrast, the inhibitors novobiocin, vitamin K_1 and nicotinamide appeared to inhibit migration and adhesion independently. With the exception of DEA-BAG these inhibitory effects were independent of cytotoxic effects upon the cells.

ART activity

Evidence for arginine specific ADP-ribosyltransferase activity on the surface of A7r5 cells similar to that found in human PMNs was sought. Agmatine was used as a substrate for the measurement of ART activity, and the results were

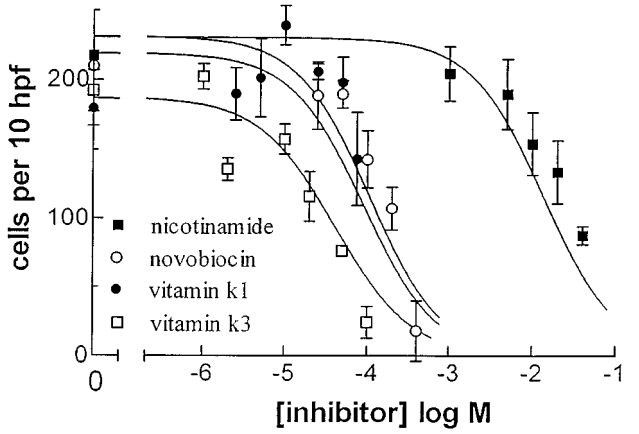

Figure 2 Inhibition of chemotaxis of A7r5 cells by selected ART inhibitors. The number of cells migrated to the underside of the filter in ten random high power fields (hpf) was measured. Representative results for inhibition of the PDGF-BB stimulated chemotaxis from a single experiment (mean$\pm$s.e.m., $n=3$) with each of the inhibitors tested are shown, and are fitted to sigmoidal concentration response curves.

Table 1 Summary of inhibition of migration

Inhibitor	*IC_{50} chemotaxis (μM)*	*IC_{50} chemokinesis (μM)*	*Per cent adhesion at IC_{50} chemotaxis*	*Per cent cell viability at IC_{50} chemotaxis*
Vitamin K1	95 ± 10	>200	97 ± 6	95 ± 4
Vitamin K3	22 ± 3.4	20 ± 2	55 ± 14*	83 ± 6
Novobiocin	165 ± 29	98 ± 13	100 ± 6	89.5 ± 6
Nicotinamide	$12{,}000\pm2{,}500$	$23{,}000\pm4{,}000$	95 ± 9	96 ± 3
Arginine-methylester	$21{,}000\pm3{,}900$	$>40{,}000$	69 ± 9*	92 ± 6
Agmatine	780 ± 36	$>40{,}000$	79 ± 6*	98 ± 2
DEA-BAG	25 ± 9	20 ± 3	5 ± 1*	54 ± 6*

Results for the inhibition of chemotaxis were fitted to sigmoidal dose response curves as shown in Figure 1. Inhibition of chemokinesis (no PDGF) by each of the compounds was also tested in separate experiments. The average IC_{50} values determined for each compound tested from three separate experiments are quoted, mean$\pm$s.e.m. In addition, the affect of the inhibitors on the ability of the cells to adhere to the membrane and cell viability was examined at the concentration required for half maximal inhibition of chemotaxis. In each case inhibitor conditions were compared to vehicle control, and are expressed as a percentage of the control$\pm$s.e.m., $n=3$. (*$P<0.05$ compared to vehicle control, using Mann Whitney non-parametric test).

compared to the activity found in rat skeletal muscle membranes (see Table 2). There was no detectible ART activity in intact A7r5 cells nor in cell membranes. This contrasted with significant levels of ART activity in rat skeletal muscle membranes. NAD-glycohydrolase activity was detected in both membrane fractions and intact A7r5 cells, although at lower levels than those demonstrated in rat skeletal muscle membranes.

Identification of ^{32}P-labelled proteins in A7r5 membranes

A7r5 cell membranes were incubated in the presence of 1 μM ^{32}P-NAD$^+$ (10 μCi) as described in Methods, and a time course for incorporation of the ^{32}P-label into membrane proteins is shown in Figure 4A. After incubation for 90 min, labelled membrane proteins were observed with MWt values of 116, 100, 90 and 70 kDa. The labelled proteins were blotted on to nitrocellulose, and were located by autoradiography and then excised and analysed. The portions of filter containing the individual labelled proteins were exposed to phosphodiesterase, and the supernatants analysed by t.l.c. The ^{32}P-labelled products of phosphodiesterase cleavage co-chromatographed with an AMP standard (a representative result from the 116 kDa band is shown in Figure 4B). This result suggests that the labelled protein was either an ADP-ribosylated protein or an NAD-protein adduct. These two possibilities were examined further by treatment of the individual labelled proteins with 0.1 M NaOH for 1 h. The supernatants were again analysed by t.l.c., and the ^{32}P-labelled products of NaOH hydrolysis co-chromatographed with an NAD$^+$ standard (a representative result from the 116 kDa band is shown in Figure 4B). These results confirm that the ^{32}P-labelled proteins shown in Figure 4 are the result of adduct formation between protein and intact NAD$^+$.

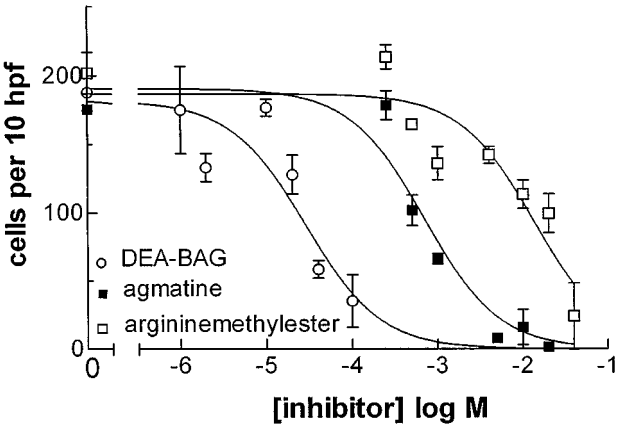

Figure 3 Inhibition of A7r5 chemotaxis by pseudo-substrates of ART. The number of cells migrated to the underside of the filter in ten random high power fields (hpf) was measured. Representative results for inhibition of the PDGF-BB stimulated chemotaxis from a single experiment (mean$\pm$s.e.m., $n=3$) with each of the substrates tested are shown, and are fitted to sigmoidal concentration response curves.

Table 2 ADP-ribosyltransferase and NAD-glycohydrolase activities in A7r5 cells

Sample	*ART activity nmol ADPribosylagmatine h^{-1}*	*NAD-glycohydrolase nmol nicotinamide h^{-1}*
intact A7r5†	<1	7.8 ± 1.3
A7r5 membranes‡	<1	22 ± 3
rat skeletal muscle‡	5.45 ± 0.5	60 ± 5

ADP-ribosyltransferase activity was measured over 5 h and NAD-glycohydrolase activity over 1 h. The amount of radiolabelled product released was quantified as described in Methods. †The activities quoted are per million cells. ‡The activities quoted are per mg membrane protein.

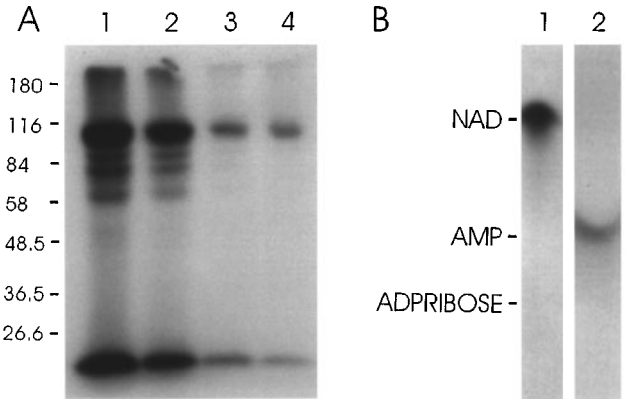

Figure 4 Endogenous ART activity in A7r5 membranes. (A) A7r5 membranes (50 μg) were incubated with ^{32}P-NAD$^+$ as described in the Methods and the reaction was terminated by the addition of 10% (w/v) TCA at given time points lane 1, 120 min, lane 2, 90 min; lane 3, 60 min; lane 4, 30 min, and the precipitated protein resolved by SDS-PAGE. The relative distances migrated by molecular weight standards (kDa) are indicated on the left. The data shown are from a single experiment representative of three separate experiments. (B) The products released from the 116 kDa band by alkaline hydrolysis (lane 1) and treatment with snake venom phosphodiesterase (lane 2) were resolved on PEI-cellulose t.l.c.as described in the Methods. The relative mobility of NAD$^+$, AMP and ADPribose standards (detected by u.v. quenching) are indicated on the left. The result shown is from a single experiment representative of three separate reactions.

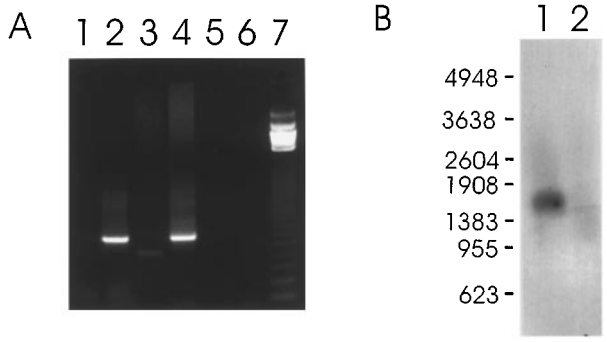

Figure 5 rtPCR and Northern blot analysis of A7r5 RNA. (A) 2 μg total RNA per reaction was used in an rtPCR to amplify actin or ART transcripts from A7r5 or rat skeletal muscle tissue. The products of the reaction were separated on 1% agarose gels, and stained with ethidium bromide. A representative gel, from three similar reactions is shown. Lane 1: A7r5 plus ART primers; lane 2: A7r5 plus actin primers; lane 3: skeletal muscle plus ART primers; lane 4: skeletal muscle plus actin primers; lane 5: no template plus ART primers; lane 6: no template plus actin primers; lane 7: 123 bp DNA ladder. (B) A single Northern blot analysis is shown of 2 μg rat skeletal muscle (lane 1) and A7r5 cells (lane 2) mRNA, probed with the rat 580 bp PCR product. The relative distances migrated by the RNA standards are indicated on the left.

Reverse transcriptase polymerase chain reaction

Measurement of low levels of ART activity in the presence of abundant NAD-glycohydrolase activity may present analytical problems. To examine further the possibility of expression of ART1 in A7r5 cells we sought evidence for the presence of low levels of the ART transcript. rtPCR of both A7r5 and rat skeletal muscle RNA was performed using primers based on homologous regions of the human, rabbit and mouse ART1 sequences. The predicted 580 bp cDNA product was only present in the skeletal muscle sample. Both samples amplified actin from control primers (Figure 5A). Automated DNA sequencing of the 580 bp PCR fragment showed 94% identity with the mouse, 77% with the rabbit and 76% with the human ART1 corresponding sequences. The rat skeletal muscle product was used as a probe in Northern blot analysis of A7r5 and skeletal muscle poly A^+ RNA. A transcript of 1.6 kb was identified in the skeletal muscle sample only (Figure 5B).

Discussion

A7r5 cells grown in continuous culture are chemotactic for PDGF at concentrations similar to those described for primary cultures derived from newborn aorta (Bornfeldt *et al*., 1994). The response to PDGF was inhibited in the presence of both inhibitors and pseudosubstrates of ART. The panel of inhibitors tested are structurally unrelated, and were identified as inhibitors of ART by Banasik *et al*. (1992), however they are not specific inhibitors of this enzyme. Vitamins K_1 and K_3, for instance have known anti-inflammatory properties which may be attributed to their redox potentials (Eichbaum *et al*., 1979, Pitsillides *et al*., 1991). As inhibitors of A7r5 chemotaxis, they were less potent than they were in human PMNs, and this might have reflected differences in the ART catalytic activity of smooth muscle cells or differences in their mechanisms of action. Novobiocin is an antibiotic which was found to be a competitive inhibitor of purified ART from turkey erythrocytes with a K_i value of 280 μM (Banasik *et al*., 1992). This value is close to the IC_{50} value determined for inhibition of A7r5 chemotaxis, and a similar value also was found in human PMNs (Allport *et al*., 1996b). Nicotinamide is a product of the ADP-ribosyltransferase reation, and is a potent inhibitor of (poly ADP-ribosyl)polymerase (PARP) activity, with a K_i value 6 μM, but is only a weak inhibitor of ART, with a K_i value of 3.4 mM (Rankin *et al*., 1989). High concentrations of nicotinamide were required to inhibit A7r5 chemotaxis, and the IC_{50} values were consistent with inhibition of ART, and were in agreement with the IC_{50} values observed in human PMNs.

Small guanidine containing analogues of arginine have been used as pseudo-substrates in assays of ART activity in crude and purified cell fractions. The specificity of the enzymes for the pseudo-substrates varies, and some ART isoenzymes cannot utilise them at all (Okazaki *et al*., 1996). Free arginine is a poor substrate for these enzymes, but it was found that derivatives such as arginine-methylester and agmatine (decarboxyl-arginine) could be used. In purified systems, agmatine has K_m values of 0.6–35 mM (Osborne *et al*., 1985), and inhibition of A7r5 chemotaxis by agmatine was observed at these concentrations. DEA-BAG (Soman *et al*., 1986) was designed as a higher affinity substrate for the rabbit skeletal muscle enzyme ($K_m = 2.8$ μM), and DEA-BAG was the most potent of the pseudo-substrates as an inhibitor of A7r5 chemotaxis. It is intriguing to note in this context that L-arginine has been used successfully to inhibit neointimal formation following balloon injury in rats (Taguchi *et al*., 1993). These effects were however attributed in that study to the nitric oxide generating capacity of arginine.

Some differences in the effects of the compounds tested emerged when their capacity to inhibit chemokinesis and cell adhesion to the filter was examined. The pseudo-substrates and vitamin K_3 significantly inhibited adhesion in parallel with inhibition of chemotaxis. In contrast, there was no inhibition of adhesion by vitamin K_1, novobiocin or nicotinamide. Agmatine and arginine-methylester were unique in inhibiting both chemotaxis and adhesion without affecting chemokinesis at the inhibitor concentrations tested.

There is compelling evidence linking the activity of ART1 to PMN chemotaxis. The capacity of the ART antagonists and pseudo-substrates to inhibit chemotaxis of A7r5 cells in a similar manner suggested initially that ART might fulfil a similar role in VSMC. However the results presented here preclude that possibility. Neither membranes nor intact A7r5 cells had the capacity to ADP-ribosylate agmatine, although exposure of A7r5 membranes to ^{32}P-NAD^+ yielded several ^{32}P-labelled proteins. Hydrolysis of these products with NaOH demonstrated however that the ^{32}P-labelled products were NAD-protein adducts. Similar reactions between NAD^+ and proteins (in the absence or presence of nitric oxide donors) have been described previously (Boyd *et al*., 1993, McDonald & Moss 1993).

The possibility was considered that there might be low level expression of ART1 in A7r5 cells, which might have been masked in the assay by the substantially greater catalytic activity of NAD-glycohydrolase. Evidence in support of the expression of ART1 in rats was obtained by the generation of a cDNA derived from skeletal muscle which had 94% homology with the corresponding region of mouse ART1 cDNA. However rtPCR reactions, based on these same primers failed to yield any cDNA products for A7r5 cells, and in addition, Northern blots of A7r5 mRNA provided no evidence for an ART1 transcript.

In conclusion, we have identified a novel panel of inhibitors of A7r5 chemotaxis in response to PDGF-BB. The preliminary results suggested that their effects might be mediated by inhibition of ART1. However a careful examination demonstrated that this enzyme was not expressed in these cells, and the mechanism(s) whereby these molecules inhibit cell movement has yet to be determined. These data and the observation of Taguchi *et al*. (1993), demonstrating inhibition of smooth muscle cell migration *in vivo* by arginine, suggest a possible role for these compounds to minimise the migration of smooth muscle cells to the neointima following vascular injury and in atherosclerosis.

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