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Growth inhibitory activity of indapamide on vascular smooth muscle cells

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Abstract

Abnormal vascular smooth muscle cell proliferation has a fundamental role in the pathogenesis of vascular diseases. Indapamide is an oral diuretic antihypertensive drug effective for patients with mild or moderate essential hypertension. We now investigated the effects of indapamide on the growth of aortic vascular smooth muscle cells (A10 cell line). Indapamide inhibited cell proliferation as measured by the tetrazolium salt XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) test. The increase in cell number was significantly reduced in the presence of indapamide 10^{-6} and 5×10^{-4} M ($P < 0.05$ $n = 3$ and $P < 0.01$, $n = 3$, respectively). Serum-induced DNA synthesis, determined as the incorporation of 5-bromo-2′-deoxyuridine (BrdU), was concentration-dependently inhibited by indapamide. BrdU incorporation was $47.2 \pm 1.6\%$ (10% foetal calf serum). Indapamide treatment markedly prevented BrdU incorporation ($37.2 \pm 2.1\%$, $29.2 \pm 4.8\%$, $15.0 \pm 1.8\%$, $8.7 \pm 2.1\%$) indapamide 10^{-6}, 10^{-5}, 5×10^{-5} and 5×10^{-4} M, respectively. Cell-cycle progression was also evaluated. Flow cytometry analysis of DNA content in synchronised cells revealed blocking of the serum-inducible cell-cycle progression by indapamide. This inhibition was abolished when the drug was added 2 h after serum repletion, indicating that indapamide must act at the early events of a cell cycle to be fully effective against DNA synthesis. In addition, serum-induced intracellular Ca^{2+} movements and also p44/p42 mitogen-activated protein kinase (MAPK) phosphorylation were studied in the presence or absence of indapamide. Indapamide 10^{-5} and 5×10^{-5} M decreased significantly cytosolic free calcium, and the p44/p42 mitogen-activated protein kinase phosphorylation (5×10^{-5} M) stimulated by 10% foetal calf serum. In accordance with this finding, indapamide (5×10^{-4} M) caused a 95% to 99% decrease in the early elevation of c-*fos* expression as evaluated by northern blot analysis of mRNA induced after serum addition. In conclusion, our results indicate that indapamide reduces vascular smooth muscle cell proliferation by a mechanism which involves a decrease in the intracellular Ca^{2+} movements that might link with the mitogen-activated protein kinase (MAPK) pathway, altering cell-cycle progression. © 2001 Published by Elsevier Science B.V.

Keywords: Indapamide; Vascular smooth muscle cell; Cell growth; DNA synthesis; Ca^{2+}, ERK1/2 (extracellular signal regulated kinase 1/2); MAP (mitogen-activated protein) kinase (p44/p42); Oncogene expression

1. Introduction

Abnormal vascular smooth muscle cell proliferation has a fundamental role in the pathogenesis of vascular diseases, such as atherosclerosis, hypertension and restenosis (Oemar et al., 1995; Schwartz, 1997; Schwartz et al., 1986). Vascular smooth muscle cell proliferation is regulated by both autocrine and paracrine growth factors (Dicorleto and Bowen-Pope, 1983; Seifert et al., 1984; Shimokada et al., 1985; Ross et al., 1986).

Indapamide, 4-chloro-*N*-(2-methyl-1-indoline) 3-sulfamoylbenzamide, is an oral diuretic antihypertensive drug effective for patients with mild or moderate essential hypertension (Thomas, 1985). The diuretic and natriuretic effects are mainly due to the structure of *O*-chlorobenzenesulfonamide, a molecule present in various diuretics. However, a varied side chain gives the drug characteristic properties. Indapamide presents an indolinyl ring and uniquely exhibits free-radical scavenging activity (Tamura

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et al., 1990; Uehara et al., 1990; Breugnot et al., 1992) as well as a direct vasodilator action (Mironneau et al., 1981; Del Rio et al., 1993a). The latter effect has been reported to be caused mainly by its Ca^{2+} channel blocker-like activity (Mironneau, 1988; Del Rio et al., 1993b). In a previous study carried out in our group, we found that indapamide was able to decrease the development of atherosclerotic lesions in cholesterol-fed rabbits, despite the lack of effect on lipid profiles (total cholesterol, triglycerides or phospholipids concentrations) (Del Rio et al., 1995). Moreover, we reported that indapamide had a vasodilator effect in rabbit isolated arteries through a decrease in Ca^{2+} uptake (Del Rio et al., 1993b). Different authors have shown antiproliferative effects of Ca^{2+} channel antagonists on vascular smooth muscle cells (Hérembert et al., 1995; Del Rio et al., 1996). In the present study, we tested whether indapamide had antimitogenic effects on cultured rat vascular smooth muscle cells (A10 cell line) stimulated to proliferate by the administration of foetal calf serum. The influence of indapamide 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 intracellular Ca^{2+}, inhibition of the mitogen-activated protein (MAP) kinase pathway and the mRNA expression of the transcription factor, c-*fos*, as part of the early G_0/G_1 transition induced in quiescent vascular smooth muscle cells by mitogenic stimulation.

2. Materials and methods

2.1. Cell culture and cell number determination

Rat A10 vascular smooth muscle cells were obtained from American Type Culture Collection (ATCC; A10 CRL 1476). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (Gibco) supplemented with glutamax I, (Gibco), 100 IU/ml penicillin G (sodium salt), 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (antibiotic–antimitotic solution, Gibco).

The sodium3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay was used for the quantitative determination of cellular proliferation in the presence of indapamide (10^{-6} and 5×10^{-4}), following the instructions of the manufacturer (Boehringer-Mannheim). The tetrazolium salt, XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate), is especially useful quantifying viable cells (Scudiero et al., 1988). This assay is designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes and is based on the cleavage of the yellow tetrazolium salt, XTT, to form an orange formazan dye by metabolically active cells. Cells were grown in microtiter plates (96 wells), in a final volume of 100 μl culture medium per well in a humidified atmosphere (37 °C, 5% CO_2). After 24 h to allow for cell attachment, the cells were incubated in DMEM with 10% foetal calf serum or 0.4% foetal calf serum containing vehicle alone (controls) or various concentrations of indapamide (10^{-6} and 5×10^{-4}). After the incubation period (7 days), 50 μl of XTT labelling mixture per well was added (final concentration 0.3 mg/ml) and the microtiter plate was incubated for 4 h. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multiwell spectrophotometer enzyme-linked immunosorbent assay (ELISA) reader at 450 nm. The reference wavelength was 690 nm.

2.2. 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 indapamide on cell growth. Cells were harvested by trypsinization and seeded onto glass coverslips in 24-well cluster culture plates at a density of 7000 cells/well in DMEM with 10% foetal calf serum. After 24-h incubation, to allow for cell attachment, the cells were washed with DMEM with 0.4% foetal calf serum and incubated in the same medium for 48 h to obtain quiescent nondividing cells. The cultures were then incubated in DMEM with 10% foetal calf serum, containing vehicle alone or various concentrations of indapamide (10^{-6},10^{-5}, 5×10^{-5} and 5×10^{-4}) for 16 h, then for 2 h in the same medium containing BrdU (10^{-5} M). 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 Intl. Buckinghamshire, UK) and then washed again with physiological buffered saline. Finally, the cells were incubated with a peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (Amersham Intl.) for an additional hour. Diaminobenzidine solution (0.5 mg/ml with 0.01% H_2O_2) was used as peroxidase substrate. Coverslips were counterstained with eosin and mounted. The ratio of positively stained cell population to total cell population was calculated. Black staining at the BrdU-incorporated sites, indicating cells initiating DNA replication, was detected by light microscopy. At least 500 nuclei were counted.

2.3. Cell-cycle analysis

To estimate the proportions of cells in various phases of the cell cycle, cellular DNA contents were measured by flow cytometry (FACS). Cells were plated, allowed to

attach overnight, and placed in DMEM plus 0.4% foetal calf serum for 48 h as described above. Indapamide (10^{-6} or 5×10^{-4} M) was added at selected points during serum repletion. At 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 μg/ml propidium iodide to which RNAse was added to a final concentration of 100 μg/ml. Flow cytometric analysis was carried out with a FACScan (Becton-Dickinson, San Jose, CA, USA) flow cytometer equipped with a 15-mW Argon laser emitting at 488 nm. Propidium iodide fluorescence was recovered through a 575/24 BP filter; 10,000 cells were acquired per sample and a Double Discriminator Module was used only to detect single cells.

2.4. Fluorescence measurements of $[Ca^{2+}]_i$ in A10 rat vascular smooth muscle cells

For $[Ca^{2+}]_i$ determinations, confluent A10 rat vascular smooth muscle cells were pretreated for 16 h with indapamide (10^{-5} M or 5×10^{-5} M) or vehicle in 0.4% foetal calf serum-containing DMEM. Cells were then loaded for 40 min at room temperature with 1 μM fura2-AM (acetomethyl esther) in HEPES buffered Dulbecco's modified Eagle's medium containing 20% foetal calf serum. After loading, the cells were kept in a balanced salt solution (BSS, mM: 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 D-glucose, pH 7.4) containing 1% bovine serum albumin (BSA, w/v) for up to 1 h in the presence of indapamide or vehicle. For single cell photometric $[Ca^{2+}]_i$ measurements, cells were seeded onto round coverslips (24 mm diameter, BDH, UK), which were then mounted onto the stage of an inverted microscope (Diaphot-TMD, Nikon, Japan). The stage was thermostated to maintain a temperature of 37 °C. Gravity perfusion using two lines regulated by electronic valves allowed rapid bath exchange (complete in 10–15 s with laminar flow). Fluorescence was measured as described previously (Jacob, 1991), using a rotating wheel spectrophotometer (Cairn, Sittingbourne, Kent, UK) with excitation at 340, 360 and 380 nm and emission detected at > 500 nm. Autofluorescence and background fluorescence were estimated by quenching the fura2 fluorescence by adding 2 mM Mn^{2+} at the end of an experiment. The ratio of fluorescence at 340 and 380 nm excitation was used as a measure of $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ and serum-induced $[Ca^{2+}]_i$ increases were measured and data were expressed as 340/380 nm ratios or ratios (peak $[Ca^{2+}]_i$ ratio–basal $[Ca^{2+}]_i$ ratio).

2.5. Immunoblotting

Western blotting analysis was performed to establish whether indapamide affects p44/p42 MAP kinase phosphorylation. A10 cells were pretreated with indapamide (5×10^{-5} M) or with vehicle overnight in 0.4% foetal calf serum-containing medium, then serum (9.6% foetal calf serum) was added for 1, 2, 5, 10 and 30 min in the presence or absence of indapamide or vehicle. At the time of harvest, cells were washed twice with ice-cold PBS (containing 0.4 mM Na_3VO4), and lysed on ice with 2000 μl lysis buffer (10% glycerol, 2.3% sodium dodecyl sulphate (SDS), 62.5mM Tris–HCl, pH 7.5, 0.15 M NaCl, 10 mM EDTA, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin, 5 μg ml^{-1} chymostatin, 1 μg ml^{-1} aprotinin, 1mM phenylmethylsulphonyl fluoride) and boiled for 5 min. Equal amounts of protein were run on 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Watford, UK), and blocked for 3 h at room temperature in blocking solution (3% bovine serum albumin (BSA) in PBS, 0.1% v/v Tween 20 (PBS-T). For analysis of phospho p44/p42 MAP kinase, blots were incubated overnight with polyclonal anti-P–p44/p42 antibody (Pierce, Chester, UK) diluted 1:5000 in 0.2% bovine serum albumin in PBS-T with agitation at room temperature. After washing in PBS-T solution, blots were incubated further for 1 h at room temperature with the horseradish peroxidase-conjugated anti-rabbit secondary antibody diluted 1:10,000 (Pierce) in blocking solution. The blots were then washed five times in PBS-T, and antibody-bound protein was visualised with an Enhanced Chemiluminescense (ECL) kit (Amersham Pharmacia Biotech).

2.6. c-fos Expression

The expression of c-*fos* mRNA was studied in serum-deprived cells that were stimulated with 10% foetal calf serum in the presence or absence of indapamide (5×10^{-4} M) for 30 min. Total RNA was extracted from the cells by the acid-phenol procedure (Chomczynsky and 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. Hybridation was performed overnight at 65 °C in 5 × SSC, 0.2% sodium dodecyl sulphate, 50 mM sodium phosphate, 10 × Denhardt's solution and 20 μg/ml salmon-sperm DNA. Blots were hybridized to a random-primed specific DNA probe for a rat c-*fos* and then exposed on Kodak X-Omat film for 8 to 17 h at −70 °C. Blots were standardized with a complementary DNA probe for 7 s.

2.7. Drugs

Indapamide (a gift from Servier Laboratories) was dissolved in dimethyl sulfoxide (DMSO) and immediately

used at dilutions giving a final concentration of less than 0.1% DMSO. Foetal calf serum, Dulbecco's modified Eagle's medium (DMEM) and all other tissue culture reagents were obtained from Gibco (Scotland). BrdU was obtained from Amersham Intl.

2.8. Data analysis

The results are expressed as the means ± S.E.M. and accompanied by the number of observations. Statistical analysis of the data was carried out with Student's *t*-test or by analysis of variance (ANOVA). Differences with a P value of less than 0.05 were considered statistically significant.

3. Results

3.1. Indapamide and cell proliferation

In the first series of experiments, an XTT test was used to study indapamide ability to alter smooth muscle cell proliferation. After 7 days of continuous treatment, a significant inhibition of cell growth was revealed (Fig. 1). The percentage inhibition exerted by indapamide was 33.2 ± 3.0% ($P < 0.05$, $n = 3$, quadruplicated) and 44.4 ± 2.1% ($P < 0.01$, $n = 3$, quadruplicated) with indapamide 10^{-6} and 5×10^{-4} M, respectively. The inhibitory effect of indapamide on cell proliferation was reversible, and after removal of the drug, proliferation of the cells was resumed (data not shown).

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

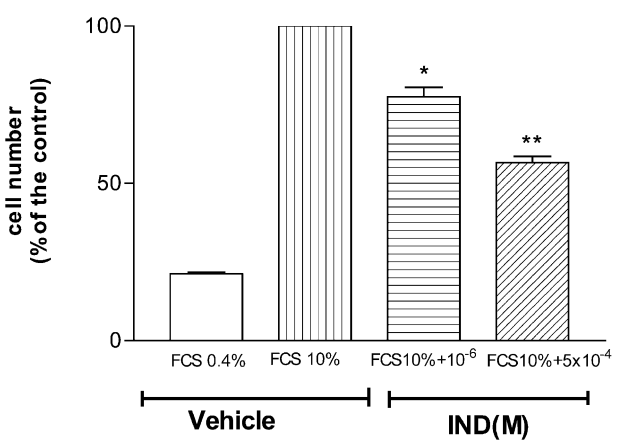

Fig. 1. Effect of indapamide on serum-induced growth of A10 rat vascular smooth muscle cells measured by XTT assay. Indapamide or vehicle alone was added and incubated for 7 days. Results are presented as the means ± S.E.M. (vertical lines) of three separate experiments, each in quadruplicate. The results are expressed as percentages of control, defined as the absorbance ($A_{450\ nm} - A_{690\ nm}$) in the presence of 10% foetal calf serum and vehicle. $^{*}P < 0.05$, $^{**}P < 0.01$.

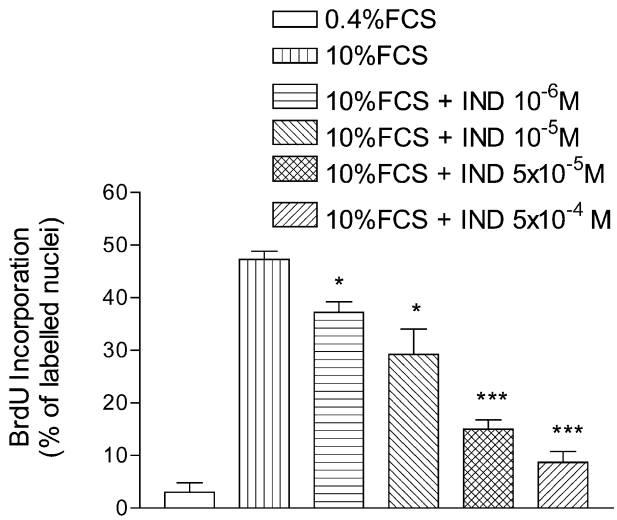

Fig. 2. Effect of indapamide on serum-induced BrdU incorporation in A10 vascular smooth muscle cells. Cells plated on glass coverslips were allowed to attach for 24 h and then serum-starved (0.4% foetal calf serum) for 48 h to obtain quiescent cells. The cultures were then incubated in 10% foetal calf serum-containing DMEM in the presence or absence of various concentrations of indapamide or vehicle for 16 h and then for a further 2 h in the same medium containing BrdU. Cells were then fixed and washed with PBS. BrdU incorporation into the nucleus was assayed by immunocytochemistry (see Methods). Results (means ± S.E.M.) are from four independent experiments, each in duplicate. $^{*}P < 0.05$, $^{***}P < 0.001$.

3.2. Effect of indapamide on DNA synthesis

The inhibitory effect of indapamide on cell proliferation could have resulted from 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 synchronised by 48-h serum starvation and then restimulated by serum repletion. The percentage of BrdU incorporation in control quiescent cells was 3.0 ± 1.8%, while in serum-stimulated cells, this incorporation increased up to 47.2 ± 1.6%. Indapamide treatment partially inhibited serum-induced DNA synthesis, the percentages incorporation being 37.2 ± 2.1% ($P > 0.05$, $n = 4$, duplicated), 29.2 ± 4.8% ($P < 0.05$, $n = 4$, duplicated), 15.0 ± 1.8% ($P < 0.001$, $n = 4$, duplicated) and 8.7 ± 2.1% ($P < 0.001$, $n = 4$, duplicated) with 10^{-6}, 10^{-5}, 5×10^{-5} and 5×10^{-4} M, respectively (Fig. 2). Determination of BrdU incorporation in A10 cells after inda-

pamide washout demonstrated the resumption of DNA synthesis in a majority of the cells. 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).

3.3. Effects of indapamide on cell cycle in synchronised A10 vascular smooth muscle cells

To ascertain whether indapamide indeed affected the transition from G_0/G_1 to S phase, the effects of the drug on cell-cycle progression were also analysed. A10 vascular smooth muscle cells were initially characterised to confirm their synchrony and cell-cycle behaviour (Fig. 3). After 48-h exposure to a serum-free medium, approximately 90% of the cells had a $2n$ DNA complement consistent with location in the G_0 of G_1 phase of the cell cycle (Fig. 3). The percentage of cells in S phase increased from $9.7 \pm 0.2\%$ to $52.0 \pm 5.4\%$ 18 h after serum repletion. In contrast, indapamide-treated cells showed a concentration-dependent block of cell-cycle progression. Indapamide decreased the percentage of cells in S phase to $43.1 \pm 2.0\%$ [$P < 0.05$, $n = 3$, duplicate (indapamide 10^{-6} M)] and to $7.4 \pm 0.4\%$ [$P < 0.001$, $n = 3$, duplicate (5×10^{-4} M)] (Table 1). Further characterisation of the indapamide-sensitive point of the cell cycle was obtained in experiments in which the drug was added when the mitogenic response was already established. Under these conditions, the inhibitory effect disappeared when the cells were analysed after a 2-h delay between serum repletion and indapamide addition (Table 1). The latter finding indicated that indapamide must act at the early events of the cell cycle to be effective against DNA synthesis.

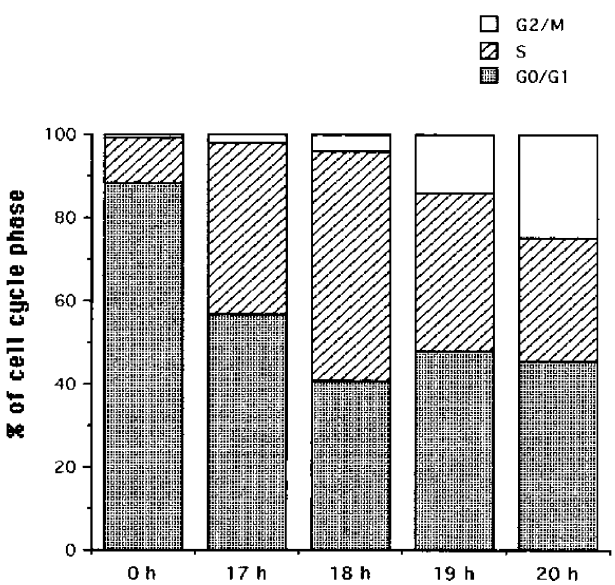

Fig. 3. Time dependence of cell cycle progression in synchronised A10 vascular smooth muscle cells. Cells were plated, allowed to attach and placed in 0.4% foetal calf serum-DMEM for 48 h as described above. Medium was replaced by 10% foetal calf serum-DMEM for the time indicated. Times given are those elapsing between serum repletion and cell harvesting. Open areas, percentage of G_2/M; hatched areas, percentage of S; and solid areas, percentage of G_0/G_1. Results (means ± S.E.M.) are from three independent experiments, each in duplicate.

Table 1
Impact of acute treatment with indapamide on cell cycle progression by flow cytometric determination of DNA content in synchronised A10 vascular smooth muscle cells at 18 h after serum repletion in the presence of vehicle alone or indapamide
Effect of a 2-h delay between serum repletion and indapamide (5×10^{-4} M) addition. Individual nuclear DNA content as reflected by fluorescence intensity of incorporated propidium iodide. Each item is derived from a representative experiment, where data from at least 10,000 events were obtained.

	G_0/G_1 (%)	S (%)	G_2/M (%)
0.4% FCS	83.7	9.7	6.6
10% FCS	44.8	52.1	3.1
IND 10^{-6} M	56.2	43.0	0.8
IND 5×10^{-4} M	83.1	7.4	9.5
IND 5×10^{-4} M (+2 h)	34.78	51.96	13.26

3.4. Effects of indapamide on cytosolic free calcium

Basal Ca^{2+} ratios were unaffected by indapamide pretreatment of cells (Δ ratio; control: 0.45 ± 0.05, $n = 5$ vs. $0.4\ 1 \pm 0.01$, $n = 5$ with indapamide 10^{-5} M and 0.5 ± 0.05 $n = 5$ with indapamide 5×10^{-5} M). When A10 cells were stimulated with 10% foetal calf serum, peak ratio responses were decreased by indapamide (Δ ratio; control: 2.4 ± 0.4, $n = 5$ vs. 1.70 ± 0.08, $n = 5$, $P < 0.05$ with indapamide 10^{-5} M and 0.5 ± 0.05, $n = 5$, $P < 0.01$ with indapamide 5×10^{-5} M). Moreover, indapamide 5×10^{-5} M also significantly decreased the plateau phase of the response (Δ ratio; control: 0.35 ± 0.04, $n = 5$ vs. 0.04 ± 0.04, $n = 5$, $P < 0.001$ with indapamide 5×10^{-5} M). Fig. 4 shows such effects.

3.5. Effects of indapamide on p44 / p42 MAP kinase phosphorylation

To further examine the underlying mechanisms of the antiproliferative effect exerted by indapamide, A10 cells were stimulated with 10% foetal calf serum in the presence or absence of indapamide and the phosphorylation of p44/p42 MAP kinase was assayed. Fig. 5 shows a typical experiment. Serum rapidly induced p44/p42 MAPK phosphorylation after 5-min stimulation. Pretreatment with in-

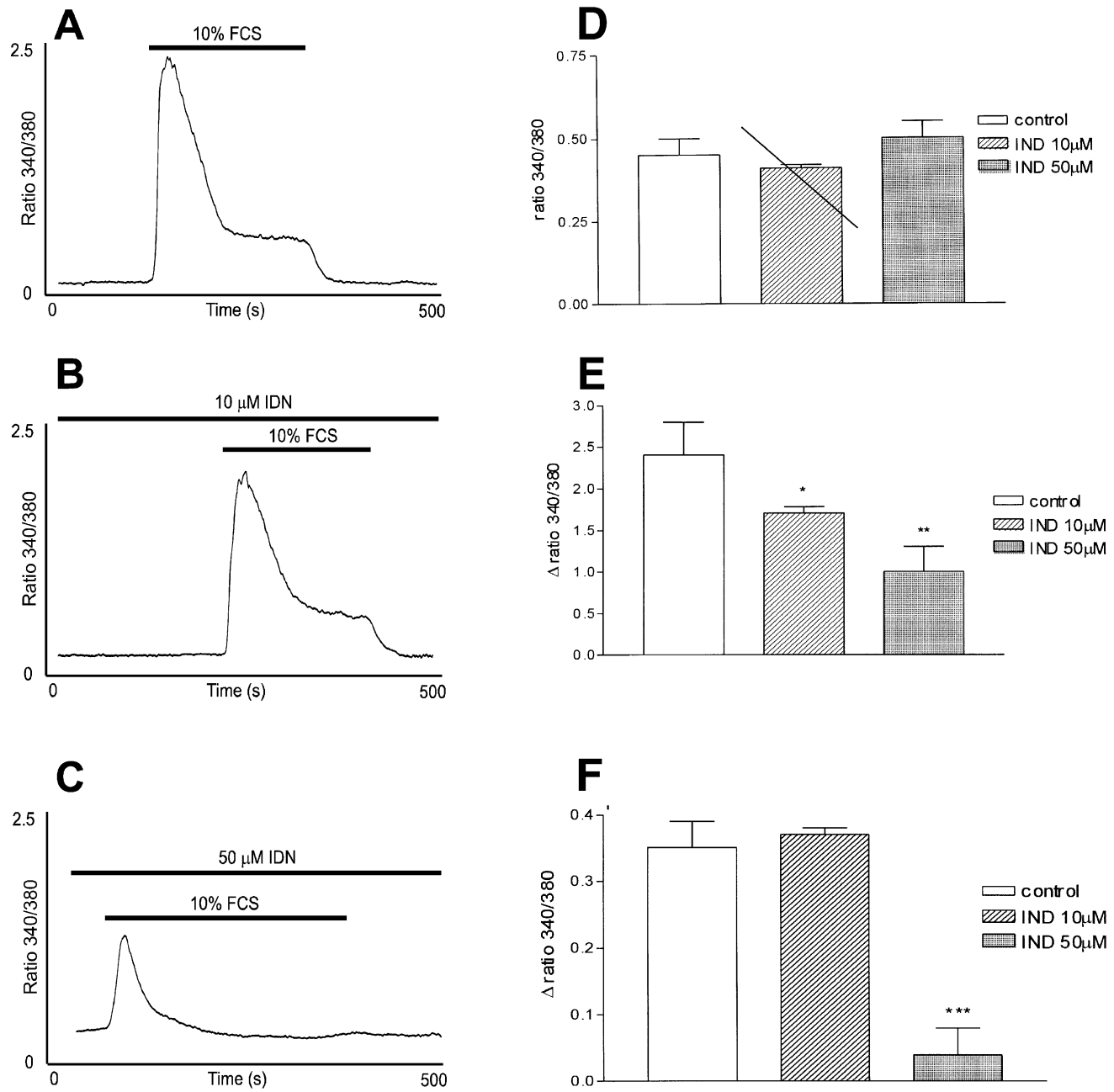

Fig. 4. Effect of indapamide on serum-induced Ca^{2+} store release and influx in rat A10 vascular smooth muscle cells. Rat A10 vascular smooth muscle cells plated on coverslips were pretreated overnight in the presence of indapamide (10 or 50 μM) or vehicle in 0.4% foetal calf serum-containing DMEM. Cells were then loaded for 40 min at room temperature with 1 μM fura2-AM in HEPES buffered Dulbecco's modified Eagle's medium containing 20% foetal calf serum. After loading, cells were kept in a balanced salt solution for up to 1 h in the presence of indapamide or vehicle. Fluorescence was measured as described in Methods. Panels A, B and C represent typical traces. Panel D, Ca^{2+} basal levels. Panel E, Ca^{2+} peak response (store release) and Panel F, plateau response (Ca^{2+} influx). Each bar shows the means ± S.E.M of five experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

dapamide 5×10^{-5} M completely abolished serum-induced p44/p42 MAPK phosphorylation.

3.6. Effect of indapamide on c-fos expression

In view of the effects of indapamide on early cell-cycle entry, the influence of the drug on the expression of c-*fos*, a gene 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; Del Rio et al., 1997). After 30-min serum stimulation, cells that were previously starved for 48 h, showed expression of c-*fos* (Fig. 6). However, when the stimulation was carried out in the presence of indapamide 5×10^{-4} M, activation did not

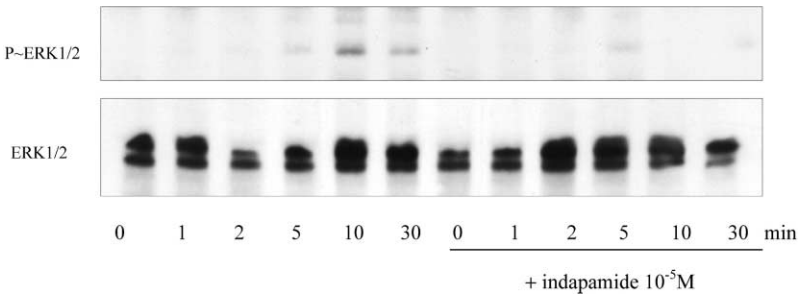

Fig. 5. Effect of indapamide on serum-induced ERK1/2 phosphorylation in A10 cells. Rat aortic smooth muscle cells (A10 cell line) were seeded on P60 dishes in DMEM containing 10% foetal calf serum. Cell monolayers (80–90% confluent) were serum starved (0.4% foetal calf serum) overnight (16 h) in the absence or presence of indapamide 5×10^{-5} M (or vehicle). After the incubation period, 9.6% foetal calf serum was added directly onto the dishes for the time indicated above. Cells were extracted using SDS lysis buffer (10% glycerol, 2.3% SDS, Tris 1 M, pH = 6.8 at 62.5 mM, EDTA 10 mM, ClNa 150 mM, PMSF, leuptin, aprotinin, chymostatin, Na-orthovanadate and FNa). Samples were then boiled at 95 °C and stored at −20 °C. Gels were run in 10% acrylamide. Figure shows a representative blot of 3 different experiments.

occur. Densitometric analysis of the blots revealed that, in cells that were stimulated in the presence of indapamide, c-*fos* expression was reduced by 95% to 99%. Quiescent cultures showed no expression of this gene (data not shown).

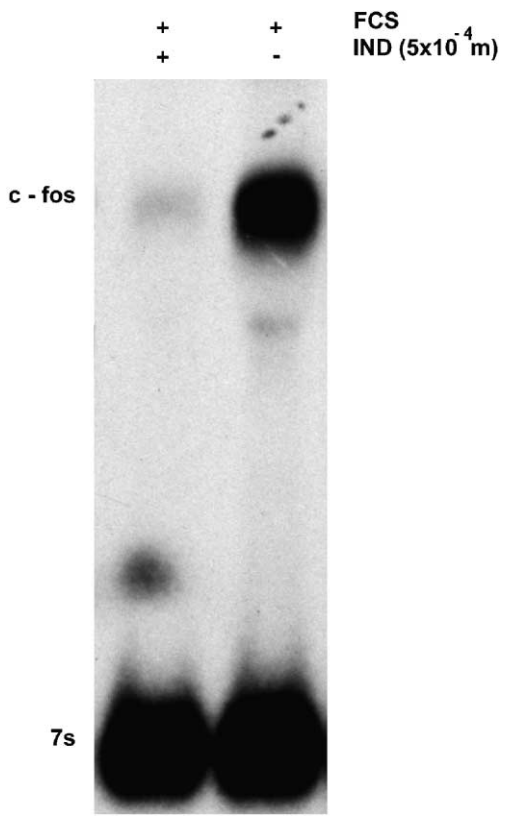

Fig. 6. Effect of indapamide (5×10^{-4} M) on serum-induced expression of c-*fos* mRNA level in A10 vascular smooth muscle cells. Cell monolayers were serum-starved (0.4% foetal calf serum) overnight (16 h) in the absence or presence of indapamide (5×10^{-4} M) (or vehicle). After the incubation period, medium was replaced by 10% foetal calf serum-containing DMEM for 30 min. Total RNA (20 μg) was subject to Northern blot analysis. Left lane: serum- and indapamide-treated cells. Right lane: serum- and vehicle-treated cells. Similar results were obtained in two independent experiments.

4. Discussion

Diuretics are used for long-term treatment of hypertension. Some of them, however, have harmful effects on serum lipoproteins since they increase total serum cholesterol, low-density lipoproteins and triglycerides (Potokar and Scmidt-Dunkar, 1978; Ferrari et al., 1991). In contrast, indapamide, a methylindoline diuretic also currently used for its antihypertensive properties, is devoid of significant effects on serum lipoproteins or lipid levels (Thomas, 1985; Tamura et al., 1990). In addition, in previous work from our laboratory, we had shown that indapamide reduced the development of atherosclerotic lesions produced by diet-induced hypercholesterolemia (Del Rio et al., 1993a, 1995). Vascular smooth muscle proliferation is a key process underlying the formation of atherosclerotic plaques (Ross, 1993). Moreover, it is also the primary factor in reocclusion of arteries after angioplasty (Waller et al., 1991).

We now clearly demonstrated that indapamide reduces serum-induced rat vascular smooth muscle cell growth, acting on early events in DNA synthesis (as established both by a decrease in BrdU incorporation and by a reduction in the S phase as shown by flow cytometry). Moreover, we also reported here that indapamide reduces the increases in intracellular Ca^{2+} induced by serum. Basal calcium ratios were unaffected by indapamide pretreatment, however, peak ratio responses, which represent calcium release from internal stores, (Jacob, 1991), and the plateau phase of the response, which represents Ca^{2+} entry from the extracellular medium (Jacob, 1991) were both decreased when cells were treated with indapamide. In addition, indapamide decreases serum-induced p44/p42 MAPK phosphorylation and c-*fos* expression. These favourable results suggest that indapamide could be used for preventing the progression of vascular complications, such as restenosis after percutaneous transluminal coronary angioplasty.

Recent studies have emphasised the role of G_1–S events in the 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 c-myc, c-myb and c-*fos* appears to be fundamental (Newmark, 1987; Sibbitt, 1988; Paquet et al., 1990; Karin and Smeal, 1992). Flow cytometric analysis showed that indapamide acutely blocked cell-cycle progression. Moreover, the addition of indapamide 2 h after cell stimulation had a markedly reduced inhibitory effect, indicating that the drug might be interfering with an early step in the mitogenic signalling process.

It has been well established that the MAPK superfamily plays a key role in cell proliferation in response to several extracellular stimuli in eukaryotic cells. (Seger and Krebs, 1995). An increasing body of evidence shows that a wide range of growth factors can activate p44/p42 MAPK signal transduction pathways through the GTPase-activating protein of Ras (Ras-GTP), leading to cellular growth by stimulating transcription factors that induce the expression of c-*fos* and others (Daub et al., 1996; Lopez-Ilasaca, 1998). As shown in Fig. 5, indapamide completely abolished the levels of phosphorylated p44/p42 MAPK and, in addition, indapamide did block Ca^{2+} influx and Ca^{2+} store release, both stimulated by foetal calf serum 10%, in the same concentration range that inhibits cell growth. Lucchesi et al. (1996) have shown that angiotensin II stimulates p44/p42 phosphorylation in a Ca^{2+}-dependent manner in rat vascular smooth muscle cells. Our findings may support the possibility that indapamide reduces cellular growth via a decrease in intracellular Ca^{2+}, which may lead to a decrease in the phosphorylation of ERK1/2. This process seems to be crucial in cell proliferation, as has been stated earlier. Accordingly, indapamide also inhibited the serum-induced expression of the early-immediate gene, c-*fos*. It is, therefore, possible that the antiproliferative effect of indapamide results from its ability to block entry of the cells in S-phase due to interference in the early G0/G1 transition phase. In spite of the evidence shown here, we cannot rule out the antioxidant effect of indapamide as an antiproliferative mechanism of action, both since indapamide and its metabolite have been shown to exert antioxidant properties (Vergely et al., 1998) and bearing in mind that a number of antioxidants, such as vitamin E, *N*-acetylcysteine and flavonoids, have been reported to interfere with cell-cycle progression (Chinery et al., 1997; Zi et al., 1998; Stivala et al., 2000).

It is controversial whether the concentration of indapamide used in "in vitro" studies was equivalent to those that would appear in human serum during treatment with this drug (De Wildt and Hillen, 1984). In fact, the concentration of indapamide required to elicit its antiproliferative effects in vitro appears to be high in terms of therapeutic value. However, indapamide has very high binding to blood components, important consequences for distribution of the drug, since more than 40% of the dose is located in the blood compartment 1 h after dosing. Indapamide is able to accumulate in vascular smooth muscle to a concentration 10 times higher than the plasma concentrations (Campbell et al., 1977). Since the mean plasma concentration after a single dose of indapamide (5–10 mg) ranged between 40 and 140 μ/l (10^{-7}–4×10^{-7} M) (Klunk et al., 1983), it is plausible to assume that the concentrations used in our "in vitro" experiments could be reached at indapamide's site of action, the vascular wall. In conclusion, our study showed that indapamide is able to inhibit the proliferation and DNA synthesis induced in vascular smooth muscle cells by the complex mixture of the mitogens of serum. Even if the precise mechanism(s) behind the growth inhibitory effect of indapamide are not known, flow cytometry studies and evaluation of proto-oncogene expression 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 indapamide. The antiproliferative activity of indapamide on vascular smooth muscle may further contribute the vascular protective effect of the drug, due to its well-known beneficial actions on hypertension.

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