Inhibition of proliferation, but not of Ca^{2+} mobilization, by cyclic AMP and GMP in rabbit aortic smooth-muscle cells

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The effects on cellular proliferation and Ca^{2+} mobilization of analogues of cyclic AMP (cAMP) and cyclic GMP (cGMP) and of agents that elevate the intracellular concentrations of cyclic nucleotides were compared in closely similar preparations of first-passage rabbit aortic vascular smooth-muscle cells. Proliferation induced by foetal-bovine serum was inhibited by 78 % by 1 mM-8-bromo cAMP and by 42 % by 1 mM-8-bromo cGMP. In the presence of 100 μM -isobutylmethylxanthine, 100 μM -forskolin increased intracellular cAMP concentration 5-fold and inhibited proliferation by 87 %, but did not affect cGMP concentration or cell viability (ATP concentration). Similarly in the presence of 100 μM -isobutylmethylxanthine, 1 mM-SIN-1 (3-morpholinomethyl-8-phenylthio-1-methyl-7-propionyl-5-oxo-1H-imidazo[4,5-f]quinolin-2-ylidene) elevated cGMP concentration 4-fold and inhibited proliferation by 48 %, but did not affect cAMP or ATP concentration. Isobutylmethylxanthine (1 mM) elevated cAMP concentration by 3-fold and cGMP concentration by 20-fold and inhibited proliferation by 81 %. Concentrations of 8-bromo cAMP, 8-bromo cGMP, forskolin or SIN-1 that inhibited proliferation did not affect the elevation of intracellular free Ca^{2+} concentration caused by 2 % (v/v) foetal-bovine serum, 100 nM-5-hydroxytryptamine or 10 nM-angiotensin II. The results demonstrate that elevation of intracellular cAMP and cGMP concentrations both independently inhibit vascular smooth-muscle cell proliferation, but these effects on proliferation are not mediated by inhibition of Ca^{2+} mobilization.

INTRODUCTION

The cyclic nucleotides cyclic AMP (cAMP) and cyclic GMP (cGMP) have been reported to inhibit the proliferation of several cell types [1,2], although the underlying mechanisms of action are not clearly defined. There is considerable interest in cyclic nucleotides as inhibitors of vascular smooth-muscle cell (VSMC) proliferation, in particular, because cyclic nucleotides have well-established roles in vascular relaxation [3–7]. This implies that there is co-ordinated control of acute VSMC function and long-term development, a principle which might apply to other cells. Furthermore, uncontrolled VSMC proliferation is clinically important in atherosclerosis, angioplasty, re-stenosis and vein graft occlusion [8].

By using isolated cell-culture models, agents that elevate or mimic cAMP [9–13] and cGMP [14–17] have been shown to inhibit smooth-muscle proliferation, but it has not been established whether the cyclic nucleotides act independently. Moreover, the relatively high concentrations of agents necessary to cause effects have cast doubt on their physiological relevance. The low potency of agents in tissue culture may result from instability or down-regulation, so that the elevation of cyclic nucleotide concentrations is not sustained. This is particularly important, since elevation of cyclic nucleotides appears to be required in the late G_1 phase of the cell cycle in order for proliferation to be inhibited [18,19]. The first objective of this study was therefore to clarify the relationship between elevation of intracellular cyclic nucleotide concentrations and inhibition of proliferation. Specifically, we sought conditions under which a sustained and selective elevation of the concentration of one cyclic nucleotide could be achieved and then investigated the effect of these on proliferation.

Vascular relaxation by cAMP- and cGMP-elevating agents is believed to result partly from lowering the cytosolic free Ca^{2+}

concentration $[\text{Ca}^{2+}]_i$ [20–24]. There is also direct evidence for inhibition by cGMP of both inositol 1,4,5-trisphosphate generation [25,26] and protein kinase C activation [27]. We therefore investigated whether inhibition of the elevation of $[\text{Ca}^{2+}]_i$ was also involved in the anti-mitogenic effects of cyclic nucleotides. To do this, we compared the effects of cyclic nucleotide analogues and cyclic-nucleotide-elevating agents on proliferation and $[\text{Ca}^{2+}]_i$ under closely comparable experimental conditions.

MATERIALS AND METHODS

Materials

Fura-2/AM was obtained from Cambridge Bioscience, Cambridge, U.K. SIN-1 (3-morpholinomethyl-8-phenylthio-1-methyl-7-propionyl-5-oxo-1H-imidazo[4,5-f]quinolin-2-ylidene) was a gift from Cassella AG, Frankfurt, Germany. The agents 3-isobutyl-1-methylxanthine (IBMX), forskolin, 8-bromo cyclic AMP (8-Br-cAMP), 8-bromo cyclic GMP (8-Br-cGMP), 5-hydroxytryptamine (serotonin), angiotensin II and human platelet-derived growth factor (PDGF; P8147) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Ovalbumin, L-ascorbic acid, sodium selenate, Cohn factor IV, epidermal growth factor, transferrin and cortisol were also obtained from Sigma. Tissue-culture media and supplements were obtained from ICN Biochemicals, High Wycombe, Bucks., U.K. [^3H]Thymidine (TRA 61; 185 GBq/mmol) was obtained from Amersham International, Aylesbury, Bucks., U.K.

Culture of first-passage VSMC

VSMC were cultured from explants of rabbit aortic tunica media by the method of McMurray *et al.* [28] as modified by Southgate & Newby [13]. Explants (5 per 2 cm² well) were cultured for 10 days in Dulbecco's modification of Eagle's medium (DMEM) containing 15 % (v/v) foetal-bovine serum;

Abbreviations used: cAMP, cyclic AMP; cGMP, cyclic GMP; 8-Br-cAMP, 8-bromo-cAMP; 8-Br-cGMP, 8-bromo-cGMP; VSMC, vascular smooth-muscle cell; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concn.; IBMX, 3-isobutyl-1-methylxanthine; PDGF, platelet-derived growth factor.

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the explants were then removed and the outgrown cells were dispersed with 145 mM-NaCl, 0.6 mM- Na_2EDTA and 0.5 mg of trypsin/ml for 5 min at 37 °C and re-plated at 3×10^4 cells per 2 cm² well. After 24 h, the cells were rendered quiescent by replacing the medium with DMEM and Ham's F-12 medium in a 1:1 ratio (v/v), supplemented with ovalbumin (1 mg/ml), L-ascorbic acid (25 µg/ml), sodium selenate (25 ng/ml), Cohn factor IV (10 µg/ml), epidermal growth factor (10 ng/mg), transferrin (10 µg/ml), cortisol (50 µM), L-glutamine (6 mM), penicillin (100 units/ml) and streptomycin (100 µg/ml) [29]. After 96 h, growth was re-initiated for 24 h by adding 0.5 ml of fresh quiescing medium supplemented with 15% (v/v) serum, 1 µCi of [6-³H]thymidine/ml and the test agents indicated.

Cell extraction and measurement of thymidine incorporation, DNA and ATP concentration

Cells were extracted and assays performed as described in detail by Southgate & Newby [13]. Briefly, ice-cold trichloroacetic acid (final concn. 10%, w/v) was added into the wells without removing the medium. After incubation for 30 min at 4 °C, the wells were scraped and the resulting suspensions were then centrifuged at 1200 *g* for 10 min at 4 °C. The resulting supernatants were neutralized by shaking with 2 vol. of 0.5 M-tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane (Freon) [30] and stored at -20 °C. The resulting pellets were neutralized with a solution of 0.1 M-potassium acetate in ethanol and used for the determination of DNA concentration [31] and [6-³H]thymidine incorporation as previously described [13]. The cAMP and cGMP concentrations in the supernatants were measured with radioimmunoassay kits (Amersham International: RPA 508 and 525 respectively), and the ATP concentration was measured with firefly luciferase [32].

Autoradiography

VSMC were cultured as described above, except that cells were re-plated on to glass slides, and growth was re-stimulated for 48 h in medium containing 2 µCi of [6-³H]thymidine/ml. The slides were then treated with paraformaldehyde (4%, w/v) for 30 min and then coated with photographic emulsion (Amersham LM-1) and exposed for 7 days at room temperature (23 °C). After development, the cells were counterstained in haematoxylin and eosin [33] and viewed under a light microscope.

Measurement of $[\text{Ca}^{2+}]_i$

VSMC were cultured as described above, except that cells were re-plated on to glass coverslips (22 mm × 10 mm) by completely covering them with a suspension of 10^5 cells/ml. The cells were left for 30 min to attach and were then cultured for 36 h to achieve complete coverage, before being rendered quiescent for 96 h, as described above. The Ca^{2+} indicator Fura-2/AM was then added to the medium to a final concentration of 5 µM, and incubation was continued for 30 min at 23 °C, followed by 30 min at 37 °C. The coverslips were then washed and stored in Krebs-Ringer Hepes buffer (110 mM-NaCl, 11 mM- NaHCO_3 , 4.4 mM-KCl, 1.1 mM- KH_2PO_4 , 1.1 mM- Mg_2SO_4 , 11 mM-Hepes/NaOH, pH 7.4, 4.2 mM-D-glucose, 1.2 mM- CaCl_2) at 23 °C. Fluorescence was measured by placing coverslips in a glass cuvette containing 1.5 ml of Krebs-Ringer buffer in a Spex dual-wavelength fluorolog spectrophotometer (Glen Spectra, Stanmore, Middx., U.K.). Excitation wavelengths of 340 and 380 nm were used alternately at 50 Hz and emission at 505 nm was recorded. $[\text{Ca}^{2+}]_i$ was calculated from the ratio of the signals excited by 340 and 380 nm [34], and from measurements of the maximum ratio obtained by subsequently adding 50 µM-

digitonin, and the minimum ratio by subsequently adding 13 mM-EGTA to each coverslip, as previously detailed [35]. In view of the need for accurate correction for autofluorescence [36], final concentrations of serum above 2% (v/v) were avoided.

Statistical methods

Means were compared by the two-tailed Student's *t* test using paired or unpaired data as indicated in the text. When more than five paired experiments were performed, data were also analysed by a non-parametric test, the Wilcoxon signed rank test. The predicted probabilities for the two tests did not differ by more than 2-fold. A *P* value of less than 0.05 in either test was taken to indicate significance.

RESULTS

Time course and serum-dependency of proliferation

Experiments were undertaken to define the optimal time and serum concentration for subsequent studies. Re-addition of serum to quiescent VSMC led to a burst of thymidine incorporation that was just sub-maximal by 24 h (Fig. 1*a*); this time point was therefore chosen. The response was similar to 2 and 15% (v/v) of serum (Fig. 1*b*).

Influence of cAMP on proliferation

The sensitivity of this preparation of VSMC to cAMP was first tested with the lipid-soluble analogue 8-Br-cAMP. 8-Br-cAMP

Fig. 1. Effects of serum on proliferation

Incorporation of [6-³H]thymidine was measured as described in the Materials and methods section. Values are means ± S.E.M. (a) Time course. Initially quiescent VSMC were incubated in quadruplicate for the times shown with 15% (v/v) foetal-bovine serum. **P* < 0.05 versus 0 h (Student's *t* test). (b) Concentration response. VSMC (*n* = 6 preparations) were incubated in quadruplicate for 24 h with the concentrations shown of foetal-bovine serum. **P* < 0.05 versus no serum (Student's *t* test).

Fig. 2. Effects of 8-Br-cAMP and 8-Br-cGMP

Quiescent VSMC ($n = 6$ preparations) were incubated in quadruplicate for 24 h with 15% (v/v) serum and the concentrations shown of 8-Br-cAMP (■) or 8-Br-cGMP (○). Measurements of [^3H]thymidine incorporation were then performed as described in the Materials and methods section. Values are means $\pm$ S.E.M. * $P < 0.05$ versus serum alone (Wilcoxon signed rank test).

(100 μM and above) significantly decreased thymidine incorporation (Fig. 2). Inhibition of proliferation was confirmed by autoradiography, which revealed that 1 mM-8-Br-cAMP decreased the thymidine labelling index from $58 \pm 10\%$ to $13 \pm 6\%$ ($n = 6$; $P < 0.001$, paired Student's t test). Inhibition of proliferation might have been caused by non-specific metabolic effects. To examine this, cellular ATP concentration was measured. ATP concentration was not, however, affected by 1 mM-8-Br-cAMP ($101 \pm 10\%$ of control; $n = 6$).

To elevate the concentration of cAMP stably and selectively, the direct activator of adenylate cyclase, forskolin, was evaluated. Forskolin alone (100 μM) elevated cAMP concentration after 5 min from 8 ± 2 to 46 ± 5 pmol/ μg of DNA ($P < 0.05$ versus control; $n = 4$), but the concentration declined to 12 ± 3 pmol/ μg of DNA after 1 h and to 9 ± 2 pmol/ μg of DNA after 24 h (not significant versus control). The use of IBMX, a non-selective cyclic nucleotide phosphodiesterase inhibitor, was therefore used to sustain the elevation. IBMX (100 μM) alone for 24 h did not increase cAMP concentration (Fig. 3a), but in the presence of 100 μM -IBMX forskolin caused a concentration-dependent increase in cAMP concentration up to 5-fold at 100 μM (Fig. 3a). Incubation with 1 mM-IBMX alone increased cAMP concentration 3-fold, and addition of 100 μM -forskolin caused a further 11-fold increase (Fig. 3a). The concentrations of cGMP were also measured in these experiments to determine the selectivity of the agents. The basal cellular cGMP concentration (0.12 ± 0.07 pmol/ μg of DNA) was much lower than that of cAMP. Incubation with 100 μM -IBMX tended to increase cGMP concentration, but this was not significant (Fig. 3b). Addition of forskolin did not further elevate cGMP concentration (Fig. 3b); rather, it tended to decline. Incubation with 1 mM-IBMX significantly increased cGMP concentration by 20-fold (Fig. 3b), but again addition of forskolin did not further elevate cGMP concentration. Thus although IBMX, particularly at 1 mM, did not cause a selective elevation of cAMP concentration, the additional effects of forskolin were solely on cAMP concentration. As a further control, it was shown that dimethyl sulphoxide (1%, v/v), the vehicle used for forskolin, did not affect cAMP concentration (5 ± 3 pmol/ μg of DNA; $n = 3$) or cGMP concentration (0.05 ± 0.04 pmol/ μg of DNA) after 24 h.

The effects of IBMX and forskolin on thymidine incorporation were investigated in the same preparations of cells (Fig. 3c).

Fig. 3. Effects of forskolin and IBMX

Quiescent VSMC ($n = 6-8$ preparations) were incubated in quadruplicate for 24 h with 15% (v/v) serum and the concentrations shown of the adenylate cyclase activator, forskolin. The phosphodiesterase inhibitor IBMX was also present at either 100 μM (●) or 1 mM (■) except where indicated (0-IBMX). Measurements of cAMP concentration (a), cGMP concentration (b) or [^3H]thymidine incorporation (c) were then performed as described in the Materials and methods section. Values are means $\pm$ S.E.M. * $P < 0.05$ versus serum control, † $P < 0.05$ versus IBMX alone (Wilcoxon signed rank test).

Incubation with 100 μM -IBMX did not significantly decrease thymidine incorporation (Fig. 3b), but addition of forskolin caused a concentration-dependent decrease, up to 87% at 100 μM . Incubation with 1 mM-IBMX decreased thymidine incorporation by 81%, and 100 μM -forskolin caused a further decrease to 97% (Fig. 3c). The inhibition of proliferation by forskolin therefore exactly mirrored the increases in cAMP concentration.

None of the agents used significantly affected the cellular ATP concentration. In detail, ATP concentration expressed as a percentage of that in cells incubated with serum alone was as follows in the presence of the various agents: 1% dimethyl sulphoxide, 108 ± 6 ($n = 4$); 100 μM -IBMX, 90 ± 4 ($n = 9$); 100 μM -IBMX plus 10 μM -forskolin, 104 ± 8 ($n = 8$); 100 μM -IBMX plus 100 μM -forskolin, 94 ± 2 ($n = 8$); 1 mM-IBMX, $93 \pm 10\%$; 1 mM-IBMX plus 10 μM -forskolin, 125 ± 17 ($n = 4$); and 1 mM-IBMX plus 100 μM -forskolin, 82 ± 7 ($n = 4$).

Fig. 4. Effects of SIN-1 and IBMX

Quiescent VSMC ($n = 6$ preparations) were incubated in quadruplicate for 24 h with 15% (v/v) serum and the concentrations shown of the NO-liberating agent SIN-1. The phosphodiesterase inhibitor IBMX was also present at 100 μ M except where indicated (0-IBMX). Measurements of cGMP concentration (■) or [3 H]thymidine incorporation (○) were then performed as described in the Materials and methods section. Values are means $\pm$ S.E.M. * $P < 0.05$ versus serum or IBMX alone (Wilcoxon signed rank test).

Influence of cGMP on proliferation

Incubation with 1 mM-8-Br-cGMP inhibited thymidine incorporation by 42% (Fig. 2), but ATP concentration was unaltered ($104 \pm 12\%$ of control; $n = 6$). The thymidine labelling index in autoradiographs was also decreased by 1 mM-8-Br-cGMP from $58 \pm 10\%$ to $49 \pm 8\%$ ($n = 5$, $P < 0.002$, paired Student's t test).

Incubation for 1 h with 1 mM-SIN-1, a metabolite of molsidomine that rapidly degrades spontaneously to liberate NO [37] elevated cGMP concentration from 0.27 ± 0.08 to 0.8 ± 0.1 pmol/ μ g of DNA ($P < 0.05$ versus control, $n = 4$), but this declined to 0.48 ± 0.14 pmol/ μ g of DNA by 24 h (not significant versus control). IBMX was therefore used in combination with SIN-1. In this series of experiments, 100 μ M-IBMX clearly did not elevate cGMP concentration, but addition of 1 mM-SIN-1 produced a 4-fold increase in cGMP concentration (Fig. 4). In this series of experiments, 100 μ M-IBMX caused a slight, but again not significant, increase in cAMP concentration (1.25 ± 0.39 pmol/ μ g of DNA, versus 0.63 ± 0.16 pmol/ μ g of DNA in control; $n = 6$). The cAMP concentration was not further increased, however, by addition of 10 μ M-SIN-1 (1.24 ± 0.37 pmol/ μ g of DNA), 100 μ M-SIN-1 (1.30 ± 0.23 pmol/ μ g of DNA) or 1 mM-SIN-1 (1.14 ± 0.21 pmol/ μ g of DNA). SIN-1 therefore selectively elevated cGMP concentration.

IBMX did not significantly inhibit proliferation (Fig. 4), but 1 mM-SIN-1 in the presence of IBMX caused a 48% decrease in thymidine incorporation (Fig. 4), which mirrored the elevation of cGMP concentration.

The ATP concentration expressed as a percentage of that in serum alone was not significantly affected by 100 μ M-IBMX (102 ± 14 ; $n = 6$) or 100 μ M-IBMX plus 1 mM-SIN-1 (106 ± 20 ; $n = 6$), although 10 mM-SIN-1 abolished the cellular ATP concentration.

Effects on $[Ca^{2+}]_i$ of serum, platelet-derived growth factor, 5-hydroxytryptamine and angiotensin

VSMC, rendered quiescent, under conditions identical with

Fig. 5. Effect of mitogens and cyclic nucleotides on $[Ca^{2+}]_i$

Quiescent VSMC loaded with Fura-2 were stimulated with: (a) 2% (v/v) of foetal-bovine serum in the presence (broken trace) or absence (continuous trace) of 1 mM-8-Br-cGMP; (b) 100 nM-5-hydroxytryptamine (5-HT) in the presence (broken trace) or absence (continuous trace) of 1 mM-8-Br-cGMP; or (c) 10 nM-angiotensin II (Ang II) in the presence (broken trace) or absence (continuous trace) of 1 mM-8-Br-cAMP. The change in $[Ca^{2+}]_i$ was measured as described in the Materials and methods section. The results in each panel show one trace representative of six experiments.

those used in the proliferation experiments, when loaded with Fura-2 had a resting $[Ca^{2+}]_i$ of 70 ± 20 nM (18 observations from 11 different preparations). Addition of serum (2%, v/v) caused an increase in Ca^{2+} concentration to 1290 ± 310 nM ($n = 18$, $P < 0.001$ versus basal, paired t test) after 10 ± 2 s, which then declined to a plateau value of 190 ± 30 nM ($n = 18$, $P < 0.005$ versus basal) (see Fig. 5a). The time taken for the Ca^{2+} concentration to decline to half its peak value was 12 ± 2 s ($n = 18$). The peak $[Ca^{2+}]_i$ was unaffected in the absence of extracellular Ca^{2+} , although the plateau was abolished (results not shown). PDGF and the co-mitogen 5-hydroxytryptamine are thought to be responsible for a large proportion of the mitogenic action of

Table 1. Paired comparison of $[Ca^{2+}]_i$ in the presence and absence of 8-Br-cAMP and 8-Br-cGMP

Paired slides of VSMC loaded with Fura-2 were preincubated with or without 1 mM-8-Br-cGMP or 1 mM-8-Br-cAMP for 50 s, and basal $[Ca^{2+}]_i$ was measured. Serum (2% v/v), 5-hydroxytryptamine (100 nM) or angiotensin II (10 nM) was then added as shown, and the peak Ca^{2+} concentration and the time taken to decline to half the peak value were also recorded. Values are means $\pm$ S.E.M. for observations on six different batches of cells. No statistically significant effects of 8-Br-cAMP or 8-Br-cGMP were revealed by paired analysis with either parametric or non-parametric tests.

Conditions	$[Ca^{2+}]_i$		Half-time for decline (s)
	Basal	Peak	
Serum	40 $\pm$ 12	670 $\pm$ 90	10 $\pm$ 2
Serum + 8-Br-cAMP	47 $\pm$ 10	610 $\pm$ 210	18 $\pm$ 5
Serum + 8-Br-cGMP	40 $\pm$ 7	730 $\pm$ 350	19 $\pm$ 4
5-Hydroxytryptamine	61 $\pm$ 17	670 $\pm$ 110	6 $\pm$ 1
5-Hydroxytryptamine + 8-Br-cAMP	79 $\pm$ 17	670 $\pm$ 90	11 $\pm$ 4
5-Hydroxytryptamine + 8-Br-cGMP	75 $\pm$ 27	620 $\pm$ 180	6 $\pm$ 1
Angiotensin	37 $\pm$ 25	730 $\pm$ 260	29 $\pm$ 8
Angiotensin + 8-Br-cAMP	80 $\pm$ 36	870 $\pm$ 250	45 $\pm$ 8
Angiotensin + 8-Br-cGMP	69 $\pm$ 7	800 $\pm$ 160	38 $\pm$ 4

serum [38]. Human platelet PDGF (20 ng/ml), which stimulated thymidine incorporation in VSMC from 3300 ± 900 to 16000 ± 5000 d.p.m./ μ g of DNA ($n = 6$, $P < 0.05$), did not affect $[Ca^{2+}]_i$ (190 ± 40 nM before addition versus 180 ± 35 nM after addition; $n = 6$). 5-Hydroxytryptamine (100 nM), by contrast, produced a rapid elevation (Fig. 5b) to 670 ± 120 nM ($n = 6$). The time for $[Ca^{2+}]_i$ to fall to half its peak value was only 6 ± 1 s, significantly less than for serum ($P < 0.005$, unpaired t test). We also tested the effect of another circulating vasoconstrictor substance with putative co-mitogenic action [39], angiotensin II. Angiotensin II (10 nM) produced a similar peak $[Ca^{2+}]_i$ value to serum and 5-hydroxytryptamine (730 ± 260 nM), but the transient was much broader (Fig. 5c). The time for Ca^{2+} concentration to decline to half the peak value was 28 ± 5 s ($n = 8$, $P < 0.02$ versus serum or 5-hydroxytryptamine, unpaired t test).

Effect of cyclic nucleotides on $[Ca^{2+}]_i$

Incubation with concentrations of 8-Br-cAMP or 8-Br-cGMP that inhibited proliferation (1 mM) did not significantly affect basal $[Ca^{2+}]_i$ (Table 1). Similarly, incubation with 8-Br-cAMP or 8-Br-cGMP for 50 s before addition of serum caused no decrease in the peak response (Fig. 5a, Table 1). The plateau concentration was also unaffected (results not shown). The time taken for Ca^{2+} concentration to decline tended to increase, but this was not statistically significant (Table 1). Preincubation with 8-Br-cAMP or 8-Br-cGMP before 5-hydroxytryptamine (Fig. 5b) or angiotensin II (Fig. 5c) also did not decrease the peak Ca^{2+} concentration (Table 1), or the plateau concentration (results not shown), but tended to prolong the transient (Table 1).

Preincubation of cells ($n = 4$) with 100 μ M-forskolin also did not appear to alter basal (70 ± 20 nM) or peak (520 ± 120 nM) Ca^{2+} concentration in response to serum. Incubation of cells ($n = 4$) with 1 mM-SIN-1 produced a small, immediate but transient lowering of basal Ca^{2+} concentration (initially 80 ± 40 nM, versus 130 ± 60 nM before addition), which returned to pre-addition levels within 200 s. Subsequent addition of serum produced a typical Ca^{2+} transient, showing no decrease in peak height (1040 ± 520 nM), although with an increased half-time to decline (20 ± 4 s).

DISCUSSION

This study demonstrates that thymidine incorporation into rabbit aortic VSMC is inhibited by lipid-soluble analogues of cAMP and cGMP, in agreement with previous work [13,16,17]. We have extended previous observations by showing that the proportion of cells that incorporate thymidine (i.e. that enter S-phase) is also decreased by the analogues. The inhibition of proliferation by cyclic-nucleotide-elevating agents at concentrations relatively high compared with vasodilator concentrations [9–12,16,17] was also confirmed. We showed, however, that such higher concentrations were needed to maintain elevated cyclic nucleotide concentrations throughout the 24 h culture period. Reasons for this may include instability of the agents [37] and desensitization of responses. Potencies may also be decreased by binding of agents to components of the culture medium, in particular, serum [40]. Our results are consistent with previous work [18,19] which showed that it was necessary to elevate cyclic nucleotide concentrations at a late stage of the G_1 phase of the cell cycle to inhibit proliferation. In the presence of forskolin and IBMX, proliferation was inhibited by 87% by a 5-fold elevation in cAMP concentration, which is comparable with the 3–9-fold elevation needed to cause relaxation [41]. Similarly, SIN-1 and IBMX inhibited proliferation by 48% and elevated cGMP concentration 4-fold, which is comparable with the 5-fold necessary for vasodilatation [42]. Our experiments therefore demonstrate that the antiproliferative effects of the agents can be obtained at cyclic nucleotide concentrations that appear physiologically attainable. This implies in turn that elevation of cyclic nucleotide concentrations by the endothelium-dependent mediators prostacyclin and NO may indeed have an important role in tonically suppressing VSMC proliferation in arteries *in vivo*. Nevertheless, our present work and that of others [16,17] shows that inhibition of proliferation and vasodilatation by cAMP can be virtually complete, whereas inhibition of proliferation by cGMP appears only partial, for reasons which remain unclear.

Our demonstration that forskolin and SIN-1 elevated only cAMP and cGMP concentrations respectively implies, importantly, that each cyclic nucleotide inhibits proliferation independently. Inhibition of proliferation by IBMX probably results from elevation of both cAMP and cGMP. The existence of distinct initial mechanisms from cAMP and cGMP does not, however, rule out their action through a final common pathway.

The second part of our study sought to investigate the mechanism underlying the inhibition of proliferation based on an hypothesis formulated by analogy with the proposed mechanism of vasodilatation. Receptor-mediated Ca^{2+} mobilization and activation of protein kinase C are known to be central to vascular contraction [43]. Conversely, there is direct evidence that inhibition of Ca^{2+} mobilization [20–26] and protein kinase C [27] accounts for the vasodilator effects of both cAMP- and cGMP-elevating agents. Moreover, protein kinase C [44] and to a lesser extent Ca^{2+} mobilization [45] have been directly implicated in initiating cellular proliferation, including that of VSMC [19,46–48]. Thus the hypothesis that cyclic nucleotides inhibit proliferation by inhibiting receptor-mediated Ca^{2+} mobilization, and hence activation of protein kinase C, was highly plausible.

We first showed that a mitogenic concentration of serum caused Ca^{2+} mobilization in VSMC, as previously demonstrated in fibroblasts [49]. Mitogenic concentrations of PDGF failed, however, to mimic the effect of serum on Ca^{2+} concentration, which implies that it did not stimulate phosphoinositide hydrolysis in this preparation. Ca^{2+} mobilization was stimulated, however, by 5-hydroxytryptamine at concentrations likely to be

present in serum. These concentrations of 5-hydroxytryptamine also stimulate proliferation of rabbit aortic VSMC in the presence of PDGF ([38]; J. W. Assender, M. Evans, K. M. Southgate & A. C. Newby, unpublished work). Angiotensin II, a second putative co-mitogen [39], also caused Ca^{2+} mobilization in these cells.

We clearly demonstrated that cyclic nucleotide analogues and cyclic-nucleotide-elevating agents did not inhibit Ca^{2+} mobilization induced by serum under conditions where they inhibited proliferation. Both cyclic nucleotides also failed to affect Ca^{2+} mobilization in response to 5-hydroxytryptamine and angiotensin. This was surprising, because similar agents have been shown to inhibit profoundly vasoconstrictor-induced $^{45}\text{Ca}^{2+}$ movements in rabbit aortic strips [50–52] and $[\text{Ca}^{2+}]_i$ in strips of rat aorta [21,22]. Moreover, receptor-mediated formation of inositol 1,4,5-trisphosphate [25,26] and activation of protein kinase C [27] are also inhibited by cGMP in aortic strips. Some effects on $[\text{Ca}^{2+}]_i$ have been demonstrated in cultured rat VSMC [23,24]. However, Hassid and colleagues [23] reported effects on basal Ca^{2+} concentration, and Lincoln and colleagues [24] reported that lowering of peak Ca^{2+} concentration by cAMP and cGMP occurred equally in response to vasoconstrictors and membrane depolarization by K^+ . These effects were therefore ascribed to post-receptor events, such as activation of Ca^{2+} uptake into endoplasmic reticulum [24]. The different results obtained with intact strips of muscle and cultured cells suggest that cyclic nucleotide regulation of receptor-mediated Ca^{2+} mobilization may be lost during transformation of contractile VSMC into the proliferative phenotypes. This hypothesis needs now to be tested directly.

The clear conclusions from our data are that cAMP and cGMP independently inhibit VSMC proliferation, and that both cyclic nucleotides act by mechanisms other than inhibition of Ca^{2+} mobilization. Furthermore, cyclic nucleotides inhibit proliferation at concentrations similar to those that cause vascular relaxation. Since only part of the vasodilator response to cyclic nucleotides can be accounted for by decreased $[\text{Ca}^{2+}]_i$ [21,22], vasomotion and proliferation may still be co-ordinately controlled, but the biochemical mechanisms involved have yet to be elucidated.

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