

# Heparin Selectively Inhibits a Protein Kinase C-dependent Mechanism of Cell Cycle Progression in Calf Aortic Smooth Muscle Cells

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**Abstract.** The proliferation of arterial smooth muscle cells (SMCs) plays a critical role in the pathogenesis of arteriosclerosis. Previous studies have indicated that the glycosaminoglycan heparin specifically inhibited the growth of vascular SMCs in vivo and in culture, although the precise mechanism(s) of action have not been elucidated. In this study, we have examined the ability of specific mitogens (PDGF, EGF, heparin-binding growth factors, phorbol esters, and insulin) to stimulate SMC proliferation. Our results indicate that SMCs derived from different species and vascular sources respond differently to these growth factors. We next examined the ability of heparin to inhibit the proliferative responses to these mitogens. In calf aortic SMCs, heparin inhibits a protein kinase C-dependent

pathway for mitogenesis. Detailed cell cycle analysis revealed several new features of the effects of heparin on SMCs. For example, heparin has two effects on the  $G_0 \rightarrow S$  transition: it delays entry into S phase and also reduces the number of cells entering the cycle from  $G_0$ . Using two separate experimental approaches, we found that heparin must be present during the last 4 h before S phase, suggesting a mid-to-late  $G_1$  heparin block. In addition, our data indicate that heparin-treated SMCs, while initially blocked in mid-to-late  $G_1$ , slowly move back into a quiescent growth state in the continued presence of heparin. These results suggest that heparin may have multiple targets for its antiproliferative effect.

**A**BERRANT regulation of cell proliferation plays an important role in many disease processes, including cancer, psoriasis, retinopathies, certain glomerular nephritides, and arteriosclerosis. For example, the hallmark of early atherogenesis is a proliferation of arterial smooth muscle cells (SMCs).<sup>1</sup> Because of this the focus of much of the recent research in the pathobiology of the vascular wall has examined the cellular and molecular mechanisms that promote SMC growth (Ross, 1986). Particular emphasis has been placed on the identification of peptide mitogens which bind to cell surface receptors, and the sequence of events they initiate that results in SMC proliferation. The mitogenic response of murine mesenchymal cells appears to involve at least two postreceptor pathways (Ran et al., 1986; McCaffrey et al., 1987). One pathway is dependent on protein kinase C (PKC) and is activated by PDGF, bombesin, phorbol

esters, and thrombin. The other pathway is dependent on cAMP and calcium, and is activated by EGF.

It is clear, however, that stringent control mechanisms must also exist for regulating the response to mitogenic stimuli, since the proliferation of most cells is tightly controlled (Sager, 1986). Several molecules have been described in the last few years that can inhibit the growth of various cell types, including retinoic acid (Lotan and Nicolson, 1977), interferons (Lengyel, 1987), transforming growth factor- $\beta$  (Roberts et al., 1985), and heparin (Castellot et al., 1987).

Heparin is a structurally complex glycosaminoglycan composed of repeating disaccharide units of alternating glucosamine and uronic acid sugars. Previous work from this laboratory demonstrated that heparin could inhibit the proliferation of SMCs and other selected cell types in vivo (Clowes and Karnovsky, 1977; Guyton et al., 1980) and in vitro (Hoover et al., 1980; Castellot et al., 1981). Heparin has been shown to bind to the SMC surface, and is subsequently internalized (Castellot et al., 1985b). Other effects of heparin include changes in cell cycle kinetics (Castellot et al., 1985a), altered protein synthesis and secretion (Cochran et al., 1985; Majack and Bornstein, 1984; Clowes et al., 1988), inhibition of motility (Majack and Clowes, 1984), and modulation of the antiproliferative activity by extracellular matrix (Her-

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1. *Abbreviations used in this paper:* aFGF and bFGF, acidic and basic fibroblast growth factor, respectively; PKC, protein kinase C; RCEC, rat cervical epithelial cell; SMC, smooth muscle cell; TPA, phorbol 12-myristate 13-acetate.

man and Castellot, 1987). Using BALB/c fibroblasts as a model system, we have recently shown that heparin inhibits a protein kinase C-dependent mitogenic pathway activated by phorbol esters, but has little effect on the EGF-dependent growth pathway (Wright et al., 1989).

We report here a detailed analysis of the effects of heparin on the SMC cell cycle. We have also examined the effect of heparin on the ability of specific mitogens to stimulate the proliferation of different species and sources of SMCs, including human cells. The results indicate that heparin inhibits a PKC-dependent pathway in calf aortic SMCs, and further suggests that heparin may have multiple targets for its antiproliferative mechanism of action.

## Materials and Methods

### Materials

Tissue culture plastics were from Falcon Labware, Becton, Dickinson & Co. (Oxnard, CA) or NUNC, and media and calf serum were from Flow Laboratories, Inc. (McLean, VA). Plasma-derived serum was prepared from human donor blood as previously described (Pledger et al., 1977). Heparin used for these studies was the sodium salt derived from porcine mucosa with a molecular weight of 12,000–18,000 and was obtained from the Institute Choay (Paris, France). Radioisotopes were obtained from New England Nuclear (Boston, MA). Restriction enzymes were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Phorbol 12-myristate 13-acetate (TPA), isobutylmethylxanthine, and molecular weight markers were from Sigma Chemical Co. (St. Louis, MO); EGF (receptor grade) was from Collaborative Research (Lexington, MA); PDGF was from PDGF Inc., Boston, MA; cholera toxin was from Behring Diagnostics American Hoechst Corp. (San Diego, CA); and recombinant human acidic and basic fibroblast growth factor (aFGF and bFGF, respectively) was generously supplied by Drs. S. Thompson, J. Rose, and E. Tischer of California Biotechnology, Inc. (Mountainview, CA).

### Smooth Muscle Cell Culture

All cells were cultured at 37°C in a humidified, 5% CO<sub>2</sub>/95% air atmosphere. All growth media contained 4 mM glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin.

Rat aortic SMCs from Sprague-Dawley rats (CD strain; Charles River Breeding Laboratories, Wellesley, MA) were isolated, cultured, and characterized as previously described (Hoover et al., 1980; Castellot et al., 1982). Briefly, the abdominal segment of the aorta was removed and the fascia was cleaned away under a dissecting microscope. The aorta was cut longitudinally and small pieces of media were carefully stripped from the vessel wall. Two or three such strips were placed under small stainless steel mesh screens in 60-mm tissue culture dishes. Within 1–2 wk, SMCs migrate from the explants; they were capable of being subcultured about a week after the first appearance of cells. They were grown in RPMI 1640 medium containing 20% FCS.

Calf aortic SMCs were isolated from explants of bovine aortas as described by Ross (1971). They were grown in high glucose (4,500 mg/ml) DME containing 10% calf serum.

Human saphenous vein SMCs were kindly provided by Dr. Peter Libby (Tufts University School of Medicine). They were grown in low glucose (1,500 mg/ml) DME buffered with 25 mM Hepes and containing 10% FCS.

All SMC types were identified as smooth muscle cells by the presence of (a) numerous myofilament bundles in the cytoplasm, (b) numerous vesicles near the plasma membrane, (c) SMC-specific myosin, as revealed by indirect immunofluorescence (Larson et al., 1984), and (d) the characteristic "hillocks and valleys" appearance of confluent cultures.

### Growth Arrest of Cells

To growth arrest SMCs, sparsely plated cultures were washed and placed in basal medium (i.e., either RPMI or DME, as described above) containing 0.5% PDS or 0.4% FCS for 72 h. Flow microfluorimetry and determinations of [<sup>3</sup>H]thymidine-labeled nuclei indicated that >95% of the cells were arrested in G<sub>0</sub>(G<sub>1</sub>).

## Cell Proliferation and Inhibition Assays

To assay for the effects of mitogens and heparin on cell growth, 5–10 × 10<sup>3</sup> SMCs were plated into 16-mm multiwell plates in normal growth medium. After 24 h, they were growth arrested as described above. Control SMC cultures were released from G<sub>0</sub> by placing them in their normal growth medium. Replicate cultures were treated with normal growth medium containing heparin. Other SMC cultures were exposed to basal medium + 0.5% PDS containing various mitogens in the presence or absence of heparin. Three methods were used to assess mitogenesis or growth inhibition: cell counting, determination of the fraction of [<sup>3</sup>H]thymidine-labeled nuclei, and DNA synthesis measurements.

Cell numbers were measured in triplicate samples using a counter (Coulter Electronics Inc., Hialeah, FL), and trypsinized cultures were routinely checked by direct microscopic examination to ascertain that the trypsinization procedure had not lysed the cells and to ensure that all cells were removed from the multiwell. The cells were not fed during the experiment. The net growth of SMCs in control cultures was obtained by subtracting the starting cell number (at the time the cells are released from G<sub>0</sub>) from the cell number at the end of the experiment. The net growth of SMCs in heparin-containing medium is computed in a similar fashion. The degree of inhibition is determined from the following relationship:

$$\% \text{ inhibition} = 1 - \frac{\text{net growth in heparin}}{\text{net growth in controls}} \times 100$$

The fraction of labeled nuclei was determined using growth-arrested subconfluent SMCs in 35-mm dishes (5–7 × 10<sup>4</sup>/dish) which were labeled with [<sup>3</sup>H]thymidine (2 µCi/ml; 80 mCi/nmol) in the presence of basal medium + 0.5% PDS containing purified mitogens at 37°C for varying lengths of time, depending upon the experiment. After labeling, the cultures were rinsed extensively in PBS, fixed in 3.7% formaldehyde for 30 min, treated with methanol for 15 min, and air dried, all at room temperature. The cultures were then processed for autoradiography using emulsion (NTB-2; Eastman Kodak Co., Rochester, NY). The percentage of labeled nuclei was determined in triplicate cultures for at least 200 cells per dish.

DNA synthesis was determined by measuring the incorporation of [<sup>3</sup>H]thymidine into TCA-precipitable material as follows: SMCs plated and growth arrested in 16-mm multiwells were exposed to normal growth medium or to basal medium + 0.5% PDS containing mitogens to which 5 µCi/ml [<sup>3</sup>H]thymidine had been added. SMCs were labeled for varying lengths of time, depending on the experiment. At the end of the labeling period, the cells were washed twice with cold PBS. After washing, 1 ml 10% TCA was added to each well for 30 min at 4°C. The TCA was aspirated, and the cells were washed four times with cold 10% TCA. Residual TCA-precipitated label was extracted with 0.2 N NaOH and the level of radioactivity was determined by liquid scintillation counting.

### Flow Microfluorimetry

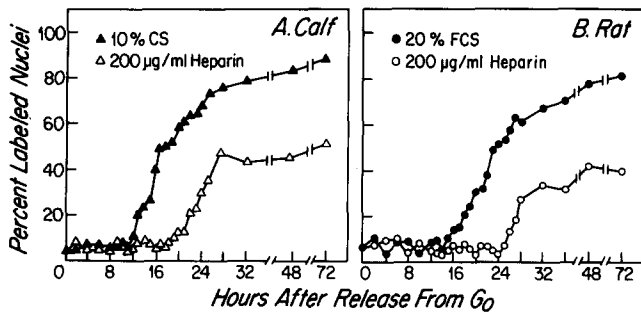
DNA histograms were obtained by flow microfluorimetric analysis of cells stained with a hypotonic solution of propidium iodide (50 µg/ml propidium iodide in 0.1% sodium citrate), according to standard methods (Yen et al., 1977). Cells were washed three times with staining solution, and incubated in the propidium iodide solution for 2 h at 4°C. Nuclei were harvested by gentle pipetting and analyzed within 4 h on a FACS II (Falcon Labware, Becton, Dickinson, & Co., Oxnard, CA).

## Results

### Cell Cycle Analysis

The cell cycle studies described below were designed to answer three questions about the antiproliferative effect of heparin on vascular SMCs: (a) What is the effect of heparin on the kinetics of the G<sub>0</sub>→S transition? (b) Where are the heparin-sensitive points in the cell cycle? and (c) What is the cell cycle distribution of SMCs after exposure to heparin? All of these experiments used subconfluent, low-serum arrested SMCs and examined the first cell cycle traverse after release from G<sub>0</sub>.

**Effects of Heparin on the G<sub>0</sub>→S Transition.** Because previous flow microfluorimetric studies suggested that hepa-



**Figure 1.** Heparin has two effects on the  $G_0 \rightarrow S$  transition. Calf (A) or rat (B) aortic SMCs were growth arrested in low serum for 72 h, as described in Materials and Methods. The cells were then released from the  $G_0$  block by incubating them in medium containing 5  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine and either 10% calf serum or 20% FCS with or without 200  $\mu\text{g/ml}$  heparin. At the indicated times, cells were fixed, processed for autoradiography, and the fraction of labeled nuclei was determined as described in Materials and Methods.

rin altered the kinetics of the  $G_0 \rightarrow S$  transition (Castellot et al., 1985a), we characterized this effect in greater detail (Fig. 1). Growth-arrested rat and calf aortic SMCs were released from the  $G_0$  block by placing them in normal growth medium in the presence or absence of 200  $\mu\text{g/ml}$  heparin, a dose that resulted in 75–85% inhibition of SMC proliferation as measured in cell counting assays on replicate cultures in the same experiment. At this dose of heparin, two effects were observed. Approximately 50% of the cells do not enter S phase; the other half do enter S phase, but enter with an 8-h lag compared to control cells. At lower doses of heparin (10–100  $\mu\text{g/ml}$ ) the delayed entry phenomenon was still observed, although the length of the delay was somewhat more variable at 10  $\mu\text{g/ml}$  (from 4 to 8 h; data not shown). Lower concentrations of heparin also allowed more cells to enter S phase: at 100  $\mu\text{g/ml}$ , 71% of the SMCs entered S phase, whereas at 10  $\mu\text{g/ml}$ , 83% of the cells entered S phase, essentially the same as in control cultures (data not shown). At higher doses of heparin (400  $\mu\text{g/ml}$ ), only 12% of the cells were able to enter S phase. This concentration of heparin resulted in >90% inhibition of SMC proliferation as determined using cell counting assays of replicate cultures in the same experiment. SMCs were still viable after exposure to this heparin concentration as demonstrated by a standard Trypan blue assay. Thus, heparin exerts two effects on cell cycle traverse in SMCs; a reduction in the fraction of cycling cells, and delayed entry into S phase. These data also suggest that heparin may be able to affect SMC growth either at several distinct sites within the cell cycle or that it has two different effects at a single site. Interestingly, these effects may show differing sensitivities to heparin since the ability of heparin to reduce the number of cells entering S phase appears to require a higher concentration than does the delayed entry phenomenon.

**Heparin-sensitive Points in  $G_1$ .** Previous studies on SMC cell cycle kinetics after heparin treatment suggested the presence of a late  $G_1$  block (Castellot et al., 1985a). In contrast, reports of a mid- $G_1$  heparin-sensitive point in human (Seldon, S. C., unpublished data) and primate (Kenagy, R. D.,

and A. W. Clowes, unpublished data) SMCs, and our own observations that rat cervical epithelial cells (RCECs) are blocked in early  $G_1$  by heparin (Wright et al., 1985), and that early  $G_1$  events such as induction of the protooncogenes *c-fos* and *c-myc* in BALB/c fibroblasts are affected by heparin (Wright et al., 1989) suggest the possibility of a very early heparin-sensitive point. We therefore undertook two separate experimental approaches to carefully determine the heparin-sensitive point in the  $G_1$  phase of the SMC cell cycle.

The first approach was to carry out “delayed addition” experiments (Fig. 2). In these studies, quiescent rat and calf SMCs were released from growth arrest by placing them in normal growth medium. At the indicated times after release from  $G_0$ , 200  $\mu\text{g/ml}$  heparin was added to the cultures, and the ability of these cells to synthesize DNA was measured. Cell counting was also performed to ensure that the changes observed in DNA synthesis correlated with reduced cell proliferation. In both rat and calf aortic SMCs, the addition of heparin could be delayed until  $\sim 4$  h before S phase and still inhibit the incorporation of [ $^3\text{H}$ ]thymidine into DNA. If added during the last 4 h of  $G_1$ , heparin became rapidly less effective.

The second approach to determine where in the cell cycle heparin blocks SMCs is based on the work of Campisi and Pardee (1982) to determine the serum-sensitive point in the  $G_1$  phase of BALB/c fibroblasts. The experimental rationale is as follows: if cells require a growth stimulus until a certain point in  $G_1$ , then any cells in an exponential population which are beyond this point should continue to cycle and will enter S phase even after the growth stimulus (for example, serum) is withdrawn, whereas cells which have not yet reached the sensitive point when the stimulus is removed will not traverse the block point and will not begin DNA synthesis. Thus, the fraction of labeled nuclei will continue to increase until all the cells which were past the sensitive point have entered S phase, at which time the labeled nuclei fraction will level off. The length of time that the labeled nuclei fraction continues to increase after withdrawing the growth stimulus represents the temporal distance between the block point and S phase. For serum stimulation of BALB/c fibroblasts, the serum-sensitive point was located  $\sim 2$  h before S phase (Campisi and Pardee, 1982). In our experiments, we added a growth inhibitor (200  $\mu\text{g/ml}$  heparin) to rat aortic SMC to mimic the action of withdrawing a mitogenic stimulus (Fig. 3). For comparison, we also removed serum from these cells. The labeled nuclei fraction continued to increase for 2–4 h after adding heparin. Interestingly, the serum-sensitive point in SMC also appears to be located 2–4 h before S phase. These data corroborate in an independent fashion the finding of a heparin-sensitive point in mid-to-late  $G_1$ , approximately 4 h before the onset of DNA synthesis.

**Cell Cycle Position of SMCs after Exposure to Heparin.** There is little data to indicate the cell cycle position of SMCs which have been exposed to heparin for prolonged periods. Flow microfluorimetry of SMC treated with 100  $\mu\text{g/ml}$  heparin for 72 h indicates that >90% of the cells are in  $G_1$  (Fig. 4, inset; similar data reported in Castellot et al., 1985a). To more carefully determine the cell cycle position of SMCs treated with heparin for prolonged periods, we exposed growth-arrested rat aortic SMCs to normal growth medium

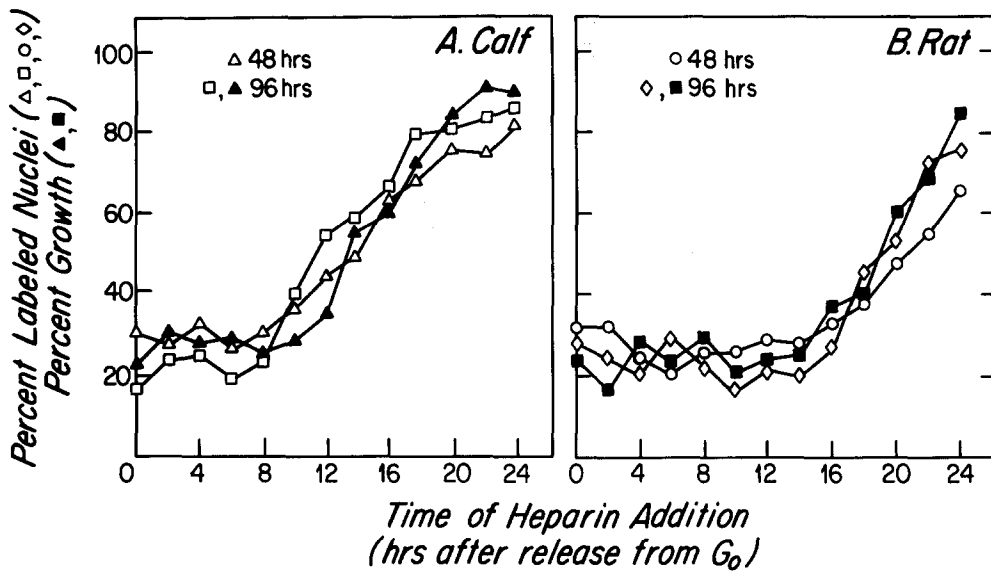


Figure 2. Delayed addition of heparin indicates a heparin-sensitive point in late G<sub>1</sub>. Growth-arrested calf (A) or rat (B) SMCs were placed in normal growth medium containing 5  $\mu$ Ci/ml [<sup>3</sup>H]thymidine. At the indicated times after release from G<sub>0</sub>, 200  $\mu$ g/ml heparin was added to the cultures. The cells were processed for autoradiography at 48 and 96 h for the determination of labeled nuclei. Cell counting assays were performed at 96 h to ensure that the changes observed in DNA synthesis correlated with reduced cell proliferation.

containing 100  $\mu$ g/ml heparin for 24, 48, or 72 h (Fig. 4). At these times, the heparin-containing medium was removed and replaced with normal growth medium containing [<sup>3</sup>H]thymidine without heparin. At the indicated time points, both [<sup>3</sup>H]thymidine incorporation into DNA and the fraction of labeled nuclei were determined. Both approaches yielded similar results; only the DNA synthesis data is shown for simplicity. SMCs exposed to heparin for 24 h entered S phase without any appreciable delay after removal of heparin, as shown in Fig. 1 B and Fig. 4. SMCs treated with heparin for 48 h demonstrated a significantly slower rate of entry into S phase after washing off the heparin-containing medium. After 72 h of exposure to heparin, SMCs required  $\sim$ 16 h to begin DNA synthesis after the heparin was removed. This is essentially the same time required for growth-arrested rat

aortic SMCs to enter S phase in the absence of heparin (Fig. 1 B), and suggests that SMCs do not remain blocked at the heparin-sensitive point 2–4 h before S phase, but move slowly back into the quiescent (i.e., G<sub>0</sub>) state.

It is possible that cells exposed to heparin for 48 or 72 h may escape the late G<sub>1</sub> block, traverse the rest of the cell cycle, and arrest again in G<sub>0</sub>. If this were the case, the SMCs would go through S phase and the fraction of labeled nuclei in heparin-treated cells would slowly increase between 24 and 72 h until the control values were reached

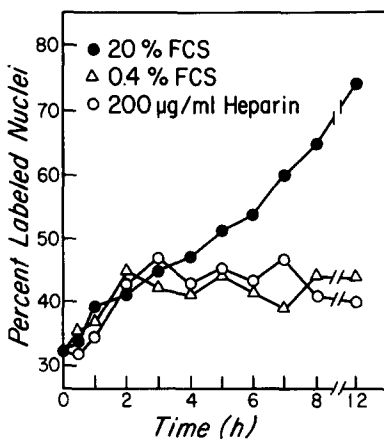


Figure 3. One heparin-sensitive point is 2–4 h before S-phase. Exponentially growing cultures of rat aortic SMCs were incubated in medium containing 20% FCS and 5  $\mu$ Ci/ml [<sup>3</sup>H]thymidine with or without 200  $\mu$ g/ml heparin. At the indicated times, the cells were processed for autoradiography and the fraction of labeled nuclei was determined. For comparison, we also replaced the 20% FCS-containing medium with medium containing 0.4% FCS. Similar results were obtained with calf aortic SMCs (not shown).

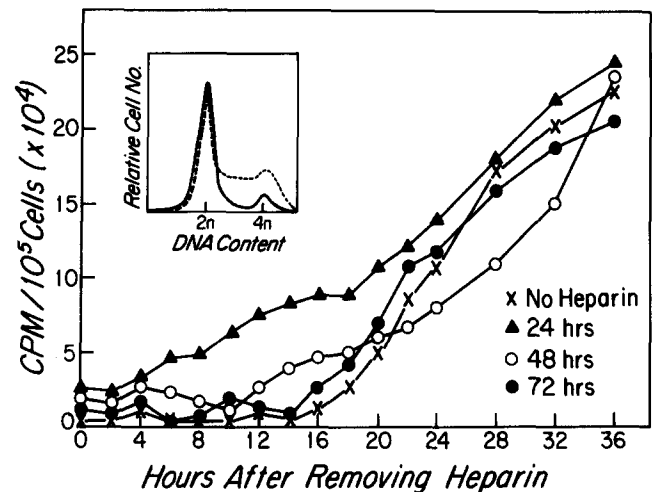
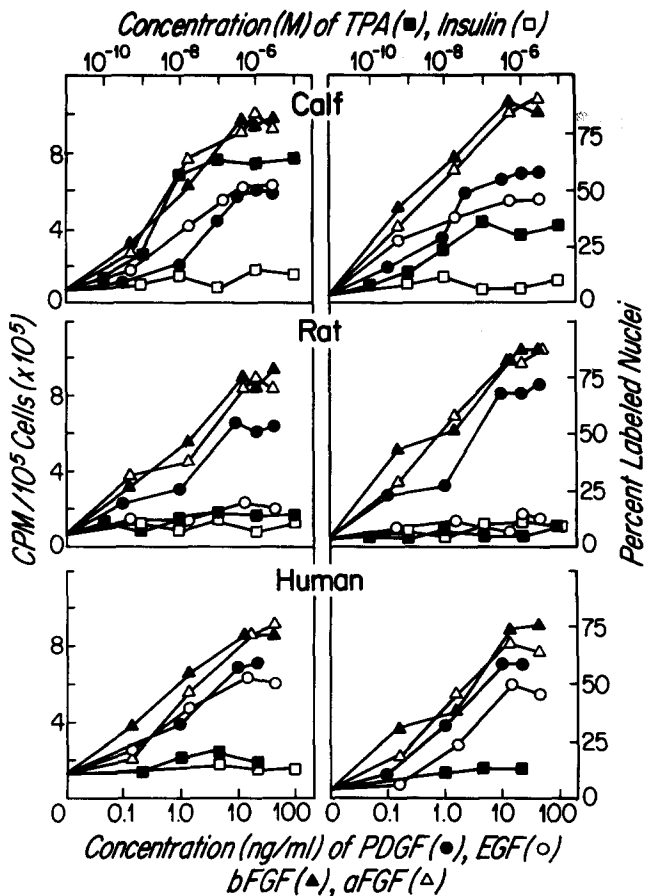


Figure 4. Heparin-treated SMCs move slowly back into G<sub>0</sub>. Growth-arrested rat aortic SMCs were released from G<sub>0</sub> by incubating them in medium containing 20% FCS with (○, ●, ▲) or without (x) 200  $\mu$ g/ml heparin. After 24, 48, or 72 h, the heparin-containing medium was removed and replaced with heparin-free medium containing 20% FCS and 5  $\mu$ Ci/ml [<sup>3</sup>H]thymidine. At the indicated times after adding the radiolabel, cells were precipitated with cold 10% TCA and incorporation of [<sup>3</sup>H]thymidine into DNA determined as described in Materials and Methods. The inset shows the DNA histograms obtained with flow microfluorimetry of SMCs incubated in medium containing 20% FCS with (—) or without (---) 200  $\mu$ g/ml heparin for 72 h.



**Figure 5.** Different species of SMCs respond to different mitogens. Growth-arrested SMCs were incubated in medium containing 0.5% plasma-derived serum and [<sup>3</sup>H]thymidine, along with the indicated concentrations of PDGF, EGF, aFGF, bFGF, insulin, or TPA. After 36 h, the cells were processed for autoradiography or for scintillation counting, and the fraction of labeled nuclei and [<sup>3</sup>H]thymidine incorporation into DNA was determined.

(~80% labeled nuclei; Fig. 1 B). In fact, the data indicate that the fraction of labeled nuclei peaks at 40% by 28–32 h and does not rise significantly after that (72-h data point shown in Fig. 1 B; the same result is obtained at 96 h [not shown]). This clearly demonstrates that cells which initially arrest in G<sub>1</sub> do not escape and traverse the cell cycle.

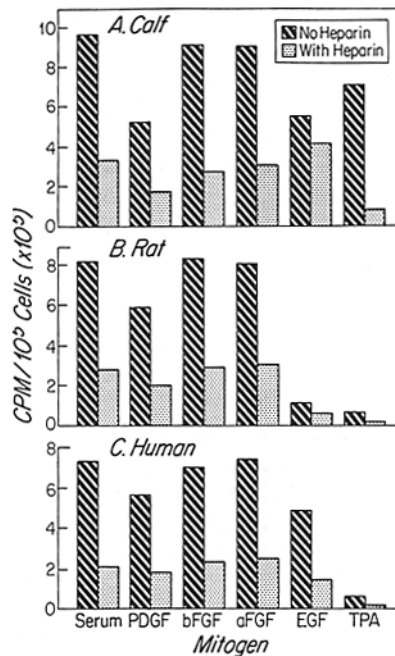
#### Effect of Heparin on SMC Stimulation by Specific Mitogens

It is generally accepted that regulation of the G<sub>0</sub>→S transition is under the positive control of peptide growth factors. Previous work on the stimulation of SMC proliferation by serum or purified PDGF indicated that heparin strongly inhibited growth in response to these mitogenic stimuli (Castellot et al., 1987). However, recent studies in our laboratory on RCECs (Wright et al., 1985) and BALB/c fibroblasts (Wright et al., 1989), and the data of Reilly et al. (1987) on calf aortic SMCs indicated that heparin was not effective in blocking the proliferative stimulus provided by EGF. We therefore undertook a systematic examination of the ability of several purified mitogens to stimulate growth, and the ability of heparin to inhibit the mitogenic responses of vascular SMCs from several different species and sources.

To determine the appropriate concentrations for each mitogen, dose-response experiments were carried out on all the SMC types, using both [<sup>3</sup>H]thymidine incorporation into DNA and autoradiographic measurements of the labeled nuclei fraction. The data for calf aortic SMCs are typical (Fig. 5), and indicate that the optimum concentration of each mitogen is as follows: 30 ng/ml for PDGF, 15 ng/ml for EGF, 15 ng/ml for both aFGF and bFGF, and 10<sup>-7</sup> M for TPA. In experiments with insulin, 5 μM was the concentration used.

**Calf Aortic SMCs.** Calf aortic SMCs were stimulated by PDGF, aFGF, bFGF, EGF, and TPA (Figs. 5 and 6 A). Insulin did not stimulate significant amounts of DNA synthesis in these cells, either alone or in combination with any of the other mitogens. These results were obtained on cells derived from five separate isolation procedures. Heparin (200 μg/ml) inhibited the stimulation by PDGF, aFGF, bFGF, and was particularly effective in blocking the proliferative response to TPA (≥80%). In contrast, heparin only slightly reduced (24%) the EGF-induced growth stimulation.

**Rat Aortic SMCs.** Rat aortic SMC growth was stimulated by PDGF, aFGF, and bFGF (Figs. 5 and 6 B). This result was obtained with cultures derived from seven different isolations. In six out of seven cultures, the cells were not stimulated by EGF (up to 150 ng/ml) or TPA (up to 10<sup>-5</sup> M); SMCs from one of the seven isolations displayed a significant mitogenic response to EGF, but not to TPA. The mechanisms underlying the variation in the response of rat aortic SMCs



**Figure 6.** Heparin inhibits a PKC-dependent proliferation pathway in calf aortic SMCs. The dose-response data shown in Fig. 6 was used to select the optimum concentration for each of the mitogens as follows: 30 ng/ml for PDGF; 15 ng/ml for EGF, aFGF, and bFGF; 10<sup>-7</sup> M for TPA. Growth-arrested SMCs were incubated in 0.5% PDS containing 2 μCi/ml [<sup>3</sup>H]thymidine and the above concentrations of mitogens, in the presence or absence of 200 μg/ml heparin. After 36 h, cells were processed for scintillation counting. All standard errors of the mean are ≤±6%; in calf aortic SMCs, difference between the effect of heparin on EGF- and TPA-stimulated mitogenesis is statistically significant (*P* < 0.001).

to specific mitogens is not known. Insulin had no effect on rat aortic SMCs, either alone or in combination with any of the other mitogens. Heparin was an effective inhibitor of the stimulatory response produced by PDGF and the FGFs.

**Human Saphenous Vein SMCs.** The above results with calf and rat cells suggested that significant differences in growth response pathways might exist between different species or sources of SMC, and we therefore wanted to test human cells (Fig. 5 and 6 C). Human saphenous vein SMC derived from three separate isolation procedures responded mitogenically to PDGF, aFGF, bFGF, and EGF, but did not synthesize DNA in response to TPA (up to  $10^{-5}$  M). Heparin effectively blocked the growth response to all of the stimulatory mitogens, including EGF. This is in distinct contrast to the results obtained with calf aortic SMCs (Fig. 5 A) and the results previously reported with RCECs and BALB/c fibroblasts (Wright et al., 1985, 1989).

### Heparin Inhibits the Protein Kinase C-dependent Pathway in Calf Aortic SMCs

Several studies have shown that there are multiple intracellular signaling pathways by which cells can respond to mitogenic signals. One of these pathways appears to be mediated by PKC, whereas another requires cAMP and calcium and is the pathway activated by EGF. Because TPA directly activates PKC, the results obtained with calf vascular SMCs suggest that heparin can inhibit the PKC-dependent mitogenic response pathway.

To ensure that the stimulation of mitogenesis seen in the presence of TPA was due to its effects on PKC, we tested the growth stimulatory properties of mezerein (a non-phorbol ester activator of PKC) as well as an active and inactive phorbol ester analog (Table I). Mezerein and 4- $\beta$ -phorbol 12,13 didecanoate both stimulate PKC and both were good mitogens for calf aortic SMCs. Heparin was a very effective inhibitor of the mitogenic effects of these two compounds. In contrast, 4- $\alpha$ -phorbol 12,13 didecanoate did not activate PKC and also failed to stimulate DNA synthesis in calf aortic SMCs. These results suggest that TPA stimulates calf aortic SMC mitogenesis via a PKC-dependent pathway, and provide additional evidence that heparin is capable of inhibiting this pathway.

To further characterize the effect of heparin on phorbol ester-stimulated growth, we determined how long an exposure to TPA was required to stimulate calf aortic SMC growth (Fig. 7 A). For these experiments, growth-arrested SMCs were exposed to TPA for various times after which the TPA was removed and the cultures were fed fresh medium containing only 0.5% PDS. [ $^3$ H]Thymidine was present throughout the experiments; mitogenesis was assessed by measuring the incorporation of this radiolabeled precursor into DNA. Half-maximal stimulation of DNA synthesis was achieved after a 15-min exposure to TPA; maximal stimulation required a 1-2-h exposure. To compare the kinetics of heparin inhibition of serum-stimulated growth with TPA-stimulated growth, we carried out the "delayed addition" experiments (Fig. 7 B) described above. TPA was added to quiescent calf aortic SMCs, and at the indicated times after TPA exposure, 200  $\mu$ g/ml heparin was added to the cultures. Heparin addition to TPA-treated calf aortic SMCs could be delayed for up to 8 h, or until  $\sim$ 4 h before S phase, and still inhibit DNA syn-

Table I. Effect of Phorbol Ester Analogs and Mezerein on Calf Aortic SMC Proliferation

	Concentration	cpm/ $10^5$ Cells ( $\times 10^5$ )	
		-HEP	+HEP
	<i>M</i>		
TPA	$10^{-9}$	4.51	0.98
	$10^{-8}$	8.93	1.36
	$10^{-7}$	9.27	1.41
	$10^{-6}$	8.68	1.50
$\beta$ -PDD	$10^{-9}$	2.36	0.89
	$10^{-8}$	5.07	1.31
	$10^{-7}$	7.24	1.47
	$10^{-6}$	7.92	1.38
Mezerein	$10^{-9}$	5.89	1.29
	$10^{-8}$	9.76	1.79
	$10^{-7}$	9.70	1.68
	$10^{-6}$	9.83	1.85
$\alpha$ -PDD	$10^{-9}$	0.72	0.48
	$10^{-8}$	0.80	0.37
	$10^{-7}$	0.85	0.59
	$10^{-6}$	0.69	0.42

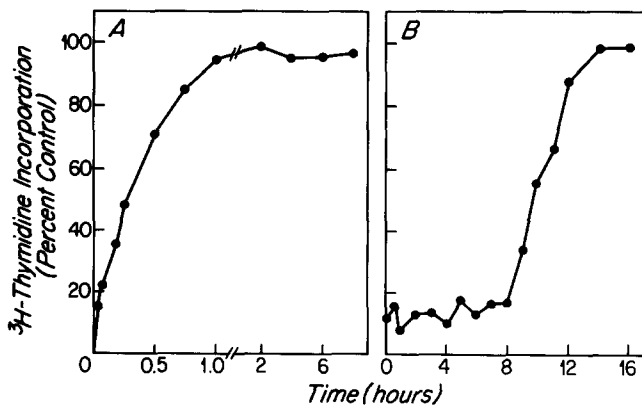
Growth-arrested calf aortic SMCs were exposed to DME + 0.5% PDS containing 2  $\mu$ Ci/ml [ $^3$ H]thymidine and the indicated concentrations of phorbol ester analogs or mezerein in the presence or absence of 200  $\mu$ g/ml heparin. After 36 h, the cells were processed for scintillation counting. SMCs exposed to DME + 0.5% PDS alone incorporated  $0.63 \times 10^5$  cpm/ $10^5$  cells. Cultures exposed to normal growth medium (DME + 10% calf serum) incorporated  $11.4 \times 10^5$  cpm/ $10^5$  cells.  $\alpha$ -PDD, 4- $\alpha$ -phorbol 12,13-didecanoate;  $\beta$ -PDD, 4- $\beta$ -phorbol 12,13-didecanoate.

thesis. This observation parallels the results for serum-stimulated growth (Fig. 2 A).

### Discussion

In this communication, we have analyzed the mitogenic responses of vascular SMCs derived from different species, including human, and have further examined the role of heparin as an inhibitor of SMC proliferation.

In all SMC types, heparin was able to strongly inhibit the mitogenic response to PDGF, aFGF, and bFGF. In calf aortic SMCs, heparin strongly inhibited TPA-stimulated growth, but was much less effective in blocking EGF-stimulated mitogenesis. It is now appreciated that the mitogenic response of cells may follow multiple postreceptor pathways. One growth response pathway is dependent upon protein kinase C, and is activated by PDGF, bombesin, thrombin, and phorbol esters. This pathway appears to be strongly inhibited by heparin in calf aortic SMCs and BALB/c fibroblasts (Wright et al., 1989). The other pathway is dependent upon cAMP and calcium, and is the pathway used by EGF in fibroblasts. This pathway appears to be relatively insensitive to heparin in calf aortic SMCs and BALB/c fibroblasts (Wright et al., 1989). The difference between the effect of heparin on EGF- and TPA-stimulated mitogenesis is statistically highly significant ( $P < 0.001$ ). Further evidence supporting the idea of a pathway-selective effect of heparin is provided by our observations that heparin blocks TPA-stimulated induction of *c-fos* and *c-myc* mRNA in SMCs and in BALB/c fibroblasts, but does not inhibit EGF-stimulated expression of these protooncogene mRNAs (Castellot et al.,



**Figure 7.** Kinetics of TPA-stimulated SMC proliferation. (A) Growth-arrested calf aortic SMCs were incubated in medium containing 0.5% plasma-derived serum,  $2 \mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine, and  $10^{-7}$  M TPA. Control cells were incubated in medium containing 10% calf serum [ $^3\text{H}$ ]thymidine. At the indicated times, the cells were processed for scintillation counting. (B) The "delayed addition" experiment described in Fig. 2 was carried out using TPA as the growth stimulus rather than serum. Growth-arrested calf aortic SMCs were incubated in medium containing 0.5% plasma-derived serum,  $2 \mu\text{Ci/ml}$  [ $^3\text{H}$ ]thymidine, and  $10^{-7}$  M TPA. At the indicated times, 200  $\mu\text{g/ml}$  heparin was added to the cells. After 36 h, the cells were processed for scintillation counting.

1988; Wright et al., 1989; Pukac, L. A., J. J. Castellot, T. C. Wright, B. L. Caleb, M. J. Karnovsky, manuscript submitted for publication). It should be noted that EGF-stimulated mitogenesis is partially inhibited by heparin. One explanation for this observation is that EGF may work via multiple intracellular signaling pathways, only one of which is heparin sensitive. Another possibility is suggested by our cell cycle data; namely, that heparin may block two or more sites within the cell cycle, only one of which is EGF driven.

In rat SMCs, heparin inhibits the mitogenic response to PDGF, aFGF, and bFGF, all of which are thought to activate the PKC-dependent pathway. Molecular analysis has shown that heparin blocks TPA-, but not EGF-stimulated induction of *c-fos* and *c-myc* gene expression (Castellot et al., 1988; Pukac, L. A., J. J. Castellot, T. C. Wright, B. L. Caleb, M. J. Karnovsky, manuscript submitted for publication), indicating pathway-selective inhibition by heparin in this SMC type as well. These observations also suggest that PKC is activated in these cells, but that treatment with TPA or EGF alone is not sufficient to generate a full mitogenic response. The question of whether heparin acts on PKC directly has not been addressed in SMCs. However, in BALB/c cells, heparin does not inhibit PKC directly, but appears to act at a site distal to this enzyme in the mitogenic response pathway (Wright et al., 1989).

An interesting feature of the pathway-specific nature of heparin's antiproliferative effect is that in human SMCs, heparin is able to block the mitogenic stimulus provided by EGF. Similar results have been observed in baboon aortic SMCs (Kenagy, R. D., and A. W. Clowes, unpublished observations). The reasons for this are not clear, but several possibilities exist. For example, as noted above, EGF itself might be able to use several different intracellular signaling pathways, including heparin-sensitive and -insensitive types which are activated preferentially in different cell types. Alternatively, some other property intrinsic to human and ba-

boon SMCs might permit heparin to inhibit the EGF-mediated growth pathway in these cell types.

The experiments with specific mitogens were also designed to resolve a puzzling set of observations. We had reported that the serum-stimulated growth of RCECs was inhibited by heparin, but the EGF-stimulated proliferation of these cells was much less sensitive to heparin (Wright et al., 1985). Rat aortic SMC proliferation was not stimulated by EGF. Subsequently, Reilly et al. (1987) reported that calf aortic SMCs did respond mitogenically to EGF, and that heparin again was relatively ineffective at blocking EGF-stimulated growth. We therefore reasoned that significant differences might exist in the growth factor responsiveness of different species and sources of vascular SMCs, and performed experiments designed to test this possibility. When a panel of purified mitogens were tested for their ability to stimulate the proliferation of SMCs derived from rat, calf, and human vessels, distinct species differences were observed (Fig. 5). Rat aortic SMCs responded mitogenically to PDGF, aFGF, and bFGF; human saphenous vein SMC growth was promoted by PDGF, the FGFs, and EGF; and calf aortic SMC proliferation was enhanced by PDGF, FGFs, EGF, and phorbol esters.

It is important to consider the precise experimental context in which our mitogen studies are carried out. In the present experiments, the growth factors are added to  $G_0$  cells individually and the mitogenic response is determined. Insulin was the only agent tested in combination with other growth factors; it was unable to stimulate DNA synthesis either alone or when combined with any of the other mitogens. It is possible that EGF and TPA are unable to stimulate rat SMC DNA synthesis alone, but could stimulate rat SMC growth in combination with other factors. For example, rat aortic SMCs do not respond to EGF alone, but the combination of EGF and thrombospondin does promote DNA synthesis in this SMC type (our unpublished data), in agreement with the data of Majack et al. (1986). Alternatively, the different responsiveness to mitogens could indicate that different growth pathways are operant in different species or sources of SMCs. Clearly, it will be important to explain the species and vessel source differences observed. Experiments designed to do this are under way.

The cell cycle experiments provide new information and help clarify certain issues raised by previous studies. Our earlier cell cycle kinetic experiments (Castellot et al., 1985a) on rat aortic SMCs in which the addition of heparin was delayed after growth stimulation indicated a late  $G_1$  block, but the 4–6-h time points used allow the heparin-sensitive point to fall within a range of 0 to 6 h before S phase. Using the "delayed addition" approach (with 4-h time points) in baboon aortic SMCs, it was found that the heparin-sensitive point in this species of SMCs was 4–8 h before the onset of DNA synthesis (Kenagy, R. D., and A. W. Clowes, unpublished data). The experiments reported here used 0.5–1.0-h time points in both rat and calf aortic SMCs, allowing greater precision in localizing the heparin-sensitive block point. For both these SMC types, heparin was required only during the last 4 h before S phase, and this was corroborated by an independent experimental approach. The temporal correlation of the late  $G_1$  block by heparin and the serum-sensitive point in calf aortic SMCs (Fig. 3; Campisi and Pardee, 1982) suggests the possibility that heparin may exert its effect in late

G<sub>1</sub> by inhibiting a late serum-dependent step in the mitogenic pathway. These results are in reasonably good agreement with the baboon aortic SMC data of Kenagy and Clowes, especially given that intrinsic species-specific differences in cell cycle kinetics and mitogenic response pathways clearly exist. Other work (Seldon, S. C., unpublished observations) also suggests a late G<sub>1</sub> heparin-sensitive point in human aortic SMCs, but the precise location of the block was not determined.

Our data also indicate that SMCs continuously exposed to heparin do not remain blocked in the latter part of G<sub>1</sub>, but instead move slowly back to G<sub>0</sub>. Thus, if heparin-containing medium is replaced after 72 h with normal growth medium, rat aortic SMCs required 16 h to begin DNA synthesis, the same length of time required by serum-stimulated SMCs never exposed to heparin. Seldon has reported that human aortic SMCs exposed to heparin also return to the G<sub>0</sub> state (unpublished data), although it appears that very short (<1 h) exposures to heparin rapidly induce the cells to return to G<sub>0</sub>. The reasons for this difference in the kinetics of return to quiescence are not clear, but may reflect a species difference or may be due to differences in the experimental protocols used.

The substantial reduction in the fraction of cycling cells seen at higher doses of heparin has been previously observed by several investigators (Castellot et al., 1985a; Kenagy, R. D., and A. W. Clowes, unpublished; Seldon, S. C., unpublished); however, the delayed entry of SMCs into S phase in the presence of heparin (Fig. 1) has not been reported. It should be noted, however, that this observance of a dual effect of heparin on the G<sub>0</sub>→S transition is highly concentration dependent.

Recent results from our laboratories indicate that in addition to blocking growth late in G<sub>1</sub>, heparin also affects very early G<sub>0</sub>→G<sub>1</sub> growth-related events, suggesting that heparin may inhibit proliferation by multiple mechanisms. For example, cell cycle analysis suggests an early G<sub>1</sub> block in RCECs (Wright and Karnovsky, 1987). We also examined the effect of heparin on the expression of *c-fos* and *c-myc*, two "immediate-early" protooncogenes that appear to be critical for cell proliferation (Armelin et al., 1984; Miller et al., 1984; Holt et al., 1986; Heikkila et al., 1987). The results indicate that both *c-fos* and *c-myc* mRNA induction are inhibited by heparin in rat and calf SMCs and BALB/c cells (Wright et al., 1989). The ability of heparin to inhibit protooncogene expression in these three cell types is pathway selective, since induction of *c-fos* and *c-myc* by serum and TPA are inhibited by heparin, but the induction of these genes by EGF is unaffected. These data, in combination with the dual effects of heparin on the G<sub>0</sub>→S transition indicated by the cell cycle kinetic studies, provide further evidence for the possibility of multiple targets for the antiproliferative mechanism of action of heparin.

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