Inhibition of phosphatidylinositol 4-phosphate kinase by heparin

A possible mechanism for the antiproliferative effects of heparin

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Heparin and related glycosaminoglycans are important modulators of vascular smooth muscle cell growth, and may be involved in pathological processes such as atherosclerosis. Since polyphosphoinositide metabolism is a major mechanism for regulating cellular activities, including proliferation, the effects of glycosaminoglycans and polyanionic compounds on the activities of phosphoinositide kinases were characterized. Heparin and heparan sulphate caused dose-dependent inhibitions of rat brain cytosolic phosphatidylinositol 4-phosphate (PIP) kinase activity, with half-maximal inhibitory concentrations of approx. 0.5 and 5 μ M respectively. PIP kinase was also inhibited by several dextran sulphates, but was not sensitive to inhibition by keratin sulphate, chondroitin sulphate or hyaluronic acid. Polynucleotides and acidic polypeptides were only weakly inhibitory. Heparin did not alter either the PIP- or the Mg^{2+} -dependence of PIP kinase. Addition of heparin to brain membranes suppressed PIP kinase activity without affecting phosphatidylinositol (PI) kinase activity. Heparin interfered with the ability of a GTP analogue to stimulate PIP kinase activity in these membranes, suggesting that it uncouples the kinase from an activating guanine-nucleotide-binding protein. In cultured A-10 vascular smooth muscle cells, heparin caused dose- and time-dependent inhibition of [3 H]thymidine incorporation into DNA. Similar treatments with heparin decreased cellular levels of phosphatidylinositol 4,5-bisphosphate (PIP₂) without changing PI and PIP levels. Therefore heparin-mediated inhibition of PIP kinase appears to lead to decreases in PIP₂ levels which may attenuate cellular proliferation.

INTRODUCTION

The uncontrolled proliferation of vascular smooth muscle cells has been clearly implicated as a major pathological process in arteriosclerosis and persistent newborn pulmonary hypertension (reviewed in [1,2]). In addition, a significant fraction of vascular surgical procedures ultimately fail because of vascular occlusion due to hyperproliferation of smooth muscle cells in injured vessels. Because of the extensiveness and seriousness of these problems, understanding the mechanisms for the induction of smooth muscle cell proliferation and for the inhibition of abnormal smooth muscle cell growth is of major concern in current research on cardiovascular disease.

Recent studies have begun to elucidate the roles of growth factors and glycosaminoglycans in the positive and negative regulation of smooth muscle cell proliferation respectively (reviewed in [3]). The highly sulphated glycosaminoglycan heparin acts as a particularly potent modulator of smooth muscle cell function. *In vivo*, heparin inhibits the migration and proliferation of these cells in response to vascular damage [4]. Heparin also inhibits proliferation and promotes the induction of a non-myogenic phenotype in cultured vascular smooth muscle cells [3,5–9]. These effects are specific for heparin, since other glycosaminoglycans, i.e. chondroitin sulphate, dermatan sulphate and keratan sulphate, do not demonstrate similar activities (reviewed in [10]). Physiologically, these effects may be induced by endogenous heparin and heparan sulphate proteoglycans which have been identified in quiescent vascular walls [11–14]. These molecules are synthesized by both endothelial cells and smooth muscle cells, and inhibit the growth of smooth muscle cells.

The mechanisms for the antiproliferative actions of heparin have not been defined. Both anticoagulant and non-anticoagulant forms of heparin are effective in inhibiting smooth muscle cell growth [5], suggesting that the effects are independent of thrombin and other growth factors. Smooth muscle cells demonstrate numerous high-affinity binding sites for heparin, i.e. approx. 100 000 sites/cell with a K_d of 1 nM [15]. Smooth muscle cells from spontaneously hypertensive rats express markedly lower numbers of heparin-binding sites and decreased susceptibility to heparin-mediated inhibition of proliferation than do comparable cells from normotensive control animals [16]. Approximately half of the bound heparin is internalized within 2 h, with the remainder being internalized in 1–2 days [15]. Internalized heparin is subsequently metabolized to low-molecular-mass oligosaccharides [17], and internalization appears to be required for the cellular effects of heparin to occur [18]. The antiproliferative effects of heparin can be overcome by the addition of epidermal growth factor, but not by other mitogens such as platelet-derived growth factor, insulin or fibroblast growth factor [19–21]. Heparin appears to selectively block cell cycling at the G₁-to-S phase transition, or early in the G₁ phase [22–24]. Heparin treatments do not significantly affect the overall rate of protein synthesis, although the synthesis of certain proteins can be modulated [25–27]. These observations are consistent with an intracellular rather than an extracellular site for the inhibitory actions of heparin. Heparin fragments may exert their actions by interacting with cytosolic or membrane-associated components of sensitive cells.

Polyphosphoinositides [phosphatidylinositol 4-phosphate (PIP) and phosphatidyl 4,5-bisphosphate (PIP₂)] are quantitatively minor plasma membrane components, but are enor-

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; IP₃, inositol 1,4,5-trisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethanesulphonyl fluoride.

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mously important in regulating cell function (reviewed in [28–30]). These lipids are synthesized by the sequential phosphorylation of PI and PIP by distinct kinases. Hormone-induced hydrolysis of PIP₂ by phospholipase C generates the intracellular messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP₃), which activate protein kinase C and induce mobilization of intracellular pools of Ca²⁺ respectively. These two processes regulate numerous cellular processes, including cell growth and proliferation. To determine if the actions of heparin may be due to the modulation of these systems, we have examined the effects of heparin on the synthesis of polyphosphoinositides *in vitro* and in intact cells.

EXPERIMENTAL

Materials

Heparin (sodium salt, 170 USP units/mg) was obtained from Sigma Chemical Co. [γ -³²P]ATP and [³H]inositol were purchased from DuPont. Guanine nucleotides were from Boehringer Mannheim. A-10 smooth muscle cells were supplied by the American Tissue Culture Collection, and were grown in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum (Gibco). All other compounds were from Sigma.

Preparation of rat brain subcellular fractions

Subcellular fractions from rat brain were isolated as previously described [31]. Briefly, brains harvested from young adult rats were rapidly cooled in ice-cold 0.32 M-sucrose containing 10 mM-Hepes, pH 7.0, and 10 μ M-phenylmethanesulphonyl fluoride (PMSF). The tissue was washed several times with the same buffer and gently homogenized in 4 vol. of sucrose/Hepes for 60 s with a Teckmar Tissuezizer at 30 % of full speed. The homogenate was filtered through several layers of cheesecloth and centrifuged at 200 *g* for 10 min. The supernatant was then removed and the pellet was resuspended in sucrose/Hepes before being centrifuged again at 200 *g* for 10 min. The combined supernatants were centrifuged at 48 000 *g* for 30 min. The resulting pellet (membranes) was resuspended in 0.25 M-sucrose containing 25 mM-Hepes, pH 7.0, and 10 μ M-PMSF. Cytosol was prepared by centrifuging the supernatant from the previous spin at 100 000 *g* for 60 min. This final supernatant (cytosol) and the resuspended membranes were divided into portions and frozen at –80 °C until used. The lipid kinase activities and the G-protein modulation of PIP kinase are stable for several months under these conditions.

Assay of lipid phosphorylation *in vitro*

Protein concentrations were determined by the method of Bradford [32] using BSA (Sigma) as the standard. In the standard phosphorylation assay, membranes or soluble fractions (50–100 μ g of protein) were preincubated for 1 min at 30 °C with the experimental addition (e.g. heparin or nucleotides) in the presence of 0.2 % Triton X-100, 50 mM-Hepes, pH 7.0, and 4 mM-EGTA. Studies on the soluble fraction included 100 μ g of PIP/ml in the Triton X-100 solution. Phosphorylation reactions were initiated by the addition of an equal volume (50 μ l) of mixture containing 50 mM-Hepes, 100 mM-NaCl, 20 mM-MgCl₂ and 2 mM-ATP plus [γ -³²P]ATP (approx. 1000 c.p.m./pmol). Incubations were continued for 1 min at 30 °C before being terminated by the addition of 2 ml of chloroform/methanol (1:1, v/v). Phospholipids were extracted and analysed as described below. This assay was slightly varied according to the particular experiment, and provides replicates with a coefficient of variation of less than 4 % within each experiment.

Analysis of lipid phosphorylation products

To determine the amounts of [³²P]PIP and [³²P]PIP₂ synthesized, phospholipids were extracted under acidic conditions as described by Schacht [33]. Briefly, 0.5 ml of 2.4 M-HCl was added to the extract and the mixture was thoroughly mixed by vortexing. The samples were centrifuged at 100 *g* for 10 min to separate the phases, and the lower organic phase was carefully transferred to a different tube. The aqueous phase was washed with 1 ml of chloroform, followed by centrifugation as above. The combined lower phases were washed twice with 2 ml of methanol/1 M-HCl (1:1, v/v) by mixing and centrifugation as described above, each time removing and discarding the aqueous phase. Levels of ³²P-labelled PIP and PIP₂ were then determined by affinity chromatography on immobilized neomycin [33]. The amounts of ³²P in the phospholipids can be directly analysed as Cherenkov radiation in a liquid scintillation counter. The method is very rapid and reproducible, and is quantitative over a large range of phospholipid concentrations.

Analysis of phosphoinositide levels in intact cells

A-10 smooth muscle cells in sparse (< 20 % confluent) cultures in 6-well culture plates were incubated with [³H]inositol (10 μ Ci/ml) for 48 h in the absence or the presence of heparin. Cells were lysed by the addition of methanol (2 ml per well), and extracts were placed into test tubes for the addition of an equal volume of chloroform. Phosphoinositides were then extracted and analysed as described above, except that the column eluates were dried before the addition of scintillation fluid and counting of radioactivity.

Assay of cell proliferation

Cells were plated and treated with buffer or heparin for 48 h as described above. The medium was then removed from each well and replaced by 0.5 ml of medium (without serum) containing 1–2 μ Ci of [³H]thymidine. The cultures were incubated for 90 min at 37 °C and then washed three times with phosphate-buffered saline (138 mM-NaCl, 2.7 mM-KCl, 1.1 mM-KH₂PO₄ and 8.1 mM-Na₂HPO₄, pH 7.2). The cells were then lysed and DNA was fixed by the addition of 1 ml of methanol/acetic acid/water (5:1:4, by vol.) for 30 min at room temperature. The lysate was removed by aspiration, and 0.3 ml of 1 % SDS was added to each

Fig. 1. Effects of glycosaminoglycans on cytosolic PIP kinase activity

Brain cytosol was assayed for PIP kinase activity as described in the Experimental section in the presence of the indicated concentrations of heparin (●), heparan sulphate (▲), chondroitin sulphate (□), hyaluronic acid (○) or keratan sulphate (■). Values indicate PIP kinase activity expressed relative to that in the absence of these compounds, and are the means of three similar experiments.

well. The DNA was removed from the plate by scraping with a rubber policeman, and the amount of ^3H in the sample was determined by liquid scintillation counting. The antiproliferative activity of the extracts is manifested as decreased incorporation of [^3H]thymidine into DNA.

RESULTS

Effects of glycosaminoglycans and other polyanions on cytosolic PIP kinase activity

The effects of several polyanionic glycosaminoglycans on cytosolic PIP kinase activity are shown by Fig. 1. Heparin was the most potent glycosaminoglycan tested, causing dose-dependent inhibition of PIP_2 synthesis with an IC_{50} of approx. $1\text{ }\mu\text{g/ml}$. Heparan sulphate was approx. 10-fold less potent, while chondroitin sulphate, hyaluronic acid and keratan sulphate

Fig. 2. Effects of dextran sulphates on cytosolic PIP kinase activity

Brain cytosol was assayed for PIP kinase activity as described in the Experimental section in the presence of the indicated concentrations of dextran sulphates with average molecular masses of 5 (●), 8 (■) or 500 (▲) kDa. Values indicate PIP kinase activity expressed relative to that in the absence of these compounds, and are the means of three similar experiments.

Fig. 3. Effects of polyanions and neomycin sulphate on cytosolic PIP kinase activity

Brain cytosol was assayed for PIP kinase activity as described in the Experimental section in the presence of the indicated concentrations of polyadenosine (●), polyinosine (○), polyaspartate (■), polyglutamate (□) or neomycin sulphate (▲). Values indicate PIP kinase activity expressed relative to that in the absence of these compounds, and are the means of three similar experiments.

Fig. 4. Effects of heparin on the Mg^{2+} -dependence of cytosolic PIP kinase activity

Brain cytosol was assayed for PIP kinase activity as described in the Experimental section in the presence of the indicated concentrations of MgCl_2 with the addition of buffer (●), $0.5\text{ }\mu\text{g}$ of heparin/ml (■) or $3\text{ }\mu\text{g}$ of heparin/ml (▲). Values indicate the c.p.m. incorporated into [^{32}P]PIP₂, and are the means of three similar experiments.

Fig. 5. Effects of heparin on the PIP-dependence of cytosolic PIP kinase activity

Brain cytosol was assayed for PIP kinase activity as described in the Experimental section in the presence of the indicated concentrations of exogenous PIP with the addition of buffer (●) or $0.5\text{ }\mu\text{g}$ of heparin/ml (○). Values indicate the c.p.m. incorporated into [^{32}P]PIP₂, and are the means of three similar experiments.

were virtually inactive. Dextran sulphate molecules with average sizes of 5–500 kDa promoted nearly equivalent inhibitions of PIP kinase activity (Fig. 2). The polynucleotides polyadenosine and polyinosine and the acidic polypeptides polyaspartate and polyglutamate were significantly inhibitory only at doses higher than $10\text{ }\mu\text{g/ml}$ (Fig. 3). The polybasic aminoglycoside neomycin inhibited PIP kinase activity with an IC_{50} of approx. $10\text{ }\mu\text{g/ml}$.

Effects of heparin on the kinetic properties of PIP kinase

Phosphorylation of PIP was optimal in the presence of 10 mM MgCl_2 (Fig. 4). The kinase activity was progressively decreased by heparin; however, the optimal Mg^{2+} concentration was not altered. Similarly, optimal levels of PIP ($100\text{ }\mu\text{g/ml}$), as indicated in Fig. 5, and ATP (1 mM ; results not shown) were not altered by the addition of heparin. Therefore heparin does not appear to compete with any of these substrates for binding to PIP kinase.

Fig. 6. Effects of heparin on polyphosphoinositide synthesis in rat brain membranes

Brain membranes were assayed for PI kinase (●) and PIP kinase (■) activities as described in the Experimental section in the presence of the indicated concentrations of heparin. Values indicate kinase activity expressed relative to that in the absence of heparin, and are the means of three similar experiments.

Fig. 7. Effects of heparin on stimulation by GTP[S] of membrane PIP kinase activity

Brain membranes were assayed for PIP kinase activity as described in the Experimental section in the presence of the indicated concentrations of GTP[S] and no heparin (●), 0.5 μg of heparin/ml (■) or 3 μg of heparin/ml (▲). Values indicate PIP kinase activity expressed relative to that in the absence of heparin, and are the means of three similar experiments.

Effects of heparin on phosphoinositide kinase activity in brain membranes

Brain membranes contain PI kinase and PIP kinase activities of approx. 15 and 6 pmol/min per mg of protein respectively [31]. Addition of heparin to the membranes caused dose-dependent inhibition of PIP kinase activity without affecting the PI kinase activity (Fig. 6). The IC_{50} for heparin in membranes was essentially the same as for cytosolic PIP kinase (1 μg/ml). Synthesis of [^{32}P]PIP₂ was dose-dependently enhanced by the addition of guanosine 5'-[γ-thio]triphosphate (GTP[S]) (Fig. 7). We have previously shown that this response is due to increased PIP kinase activity rather than decreased degradation of PIP₂ [31]. Heparin attenuated the ability of GTP[S] to enhance [^{32}P]PIP₂ synthesis; however, this inhibition was partially overcome by high levels of GTP[S]. High concentrations of heparin,

Fig. 8. Effects of heparin on DNA synthesis in A-10 smooth muscle cells

Sparse cultures of A-10 cells were incubated for 48 h with the indicated concentrations of heparin. The incorporation of [3H]thymidine into DNA was then assayed as described in the Experimental section. Values represent the amount of [3H]thymidine incorporated relative to that in cultures which were not exposed to heparin, and are the means of two similar experiments.

Fig. 9. Effects of heparin on phosphoinositide levels in A-10 smooth muscle cells

Sparse cultures of A-10 cells were incubated with [3H]inositol for 48 h. The indicated concentrations of heparin were then added and cultures were continued for an additional 24 h. Levels of [3H]PI (●), [3H]PIP (■) and [3H]PIP₂ (▲) were then assayed as described in the Experimental section. Values represent the amounts of 3H associated with these lipids relative to levels in cultures which were not exposed to heparin, and are the means of two similar experiments.

e.g. 10 μg/ml and above, strongly inhibited the basal PIP kinase activity and prevented its stimulation by GTP[S] (results not shown).

Effects of heparin on DNA synthesis and phosphoinositide levels in A-10 smooth muscle cells

Treatment of proliferating cultures of A-10 cells with heparin resulted in dose-dependent decreases in their ability to incorporate [3H]thymidine into DNA (Fig. 8). These inhibitory effects of heparin were apparent only after incubations of at least 24 h, suggesting that heparin is blocking an early step in the mitogenic response. Heparin caused dose-dependent decreases in the levels of [3H]PIP₂ in [3H]inositol-labelled cells (Fig. 9). In contrast, heparin elevated the levels of [3H]PIP in these cells, but did not affect levels of [3H]PI. The time courses and IC_{50} values for the decreases in PIP₂ levels and DNA synthetic activity were closely

correlated. Heparin did not alter either DNA synthesis or phosphoinositide levels in NIH 3T3 or neuroblastoma cells (results not shown).

DISCUSSION

The metabolism of phosphoinositides is centrally involved in numerous cellular processes, including secretion, modulation of gene expression and the regulation of proliferation and differentiation. Understanding the molecular mechanisms for regulating phosphoinositide metabolism and the development of biochemical tools for the manipulation of phosphoinositide metabolism are therefore important goals in modern cell biology. The studies reported herein characterize the ability of heparin to modulate polyphosphoinositide synthesis.

Brain cytosolic PIP kinase activity is dose-dependently inhibited by heparin, heparan sulphate and dextran sulphates. The potencies of these compounds appear to be related to their charge densities, i.e. their degree of sulphation, rather than the absolute size of the polyanion. Several other polyanions only weakly inhibit PIP kinase, indicating selectivity for heparin-related molecules. The effects of heparin appear to be due to its interaction with PIP kinase or an associated regulatory protein, since increasing the substrate and cofactor concentrations did not overcome the inhibitions. Furthermore, PIP kinase activity has been found to bind avidly to heparin-agarose (C. D. Smith, D. Wen, S. L. Mooberry & K.-J. Chang, unpublished work).

The inhibitory effects of heparin are selectively directed towards PIP kinase in brain membranes, since PI kinase activity in the same samples was unaffected by heparin. This selectivity is similar to the selective ability of GTP[S] to stimulate PIP kinase in these same membranes [31]. We have previously proposed that this stimulation by GTP[S] is due to activation of a G-protein which enhances PIP kinase activity [31]. We now show that heparin interferes with this activation process. Therefore uncoupling of PIP kinase from the G-protein may mediate the selective inhibitory effects of heparin on polyphosphoinositide synthesis in membranes. It is interesting to note that heparin also appears to uncouple adenylate cyclase from the inhibitory G-protein G_i [34], suggesting that heparin may modulate multiple G-protein-mediated signal transduction systems.

The relationships between modulation of polyphosphoinositide metabolism and cell growth were examined using A-10 rat thoracic smooth muscle cells. It has been recognized for some time that heparin can act as an inhibitor of smooth muscle cell proliferation [3,5-9]; however, the underlying biochemical mechanisms for these effects are not yet defined. Recent studies suggest that phosphoinositide- and protein kinase C-mediated mitogenesis is selectively attenuated in heparin-treated smooth muscle cells [35] and BC3H-1 muscle cells [36,37]. Our present experiments demonstrate that prolonged treatment of these cells with heparin causes parallel decreases in cellular PIP_2 levels and DNA synthesis. As in the isolated membrane system, the effects of heparin are directed only towards PIP_2 synthesis. In fact, levels of PIP are slightly increased, as might be expected for selective inhibition of PIP kinase. The delayed effects of heparin are consistent with a requirement for its internalization and delivery to an intracellular site of action. Similar kinetic and internalization requirements have been established for the antiproliferative effects of heparin [15,18]. It is noteworthy that the antiproliferative effects of heparin appear to be limited to cell types which possess high-affinity binding sites for the glycosaminoglycan, e.g. smooth muscle cells. The effects of heparin on PIP_2 synthesis are also cell-selective, in that decreases in PIP_2 levels were observed in heparin-treated A-10 cells, but not in heparin-treated NIH 3T3 fibroblasts and neuroblastoma cells.

The role of phosphoinositide hydrolysis in regulating cell proliferation has been the subject of considerable interest in recent years [29,30]. Certain growth factors, e.g. insulin and epidermal growth factor, appear to function by pathways which are largely independent of phosphoinositide metabolism. In contrast, strong correlations between phosphoinositide breakdown and proliferative responses to platelet-derived growth factor have been established. Our present results, as well as those reported by others, demonstrate that heparin causes only partial inhibition of DNA synthesis in smooth muscle cells. The residual activity may be due to responses to growth factors which utilize phosphoinositide-independent mechanisms. The A-10 cells appear to provide a useful model system for the resolution of the contributions of specific growth factors, and for analysis of the effects of heparin on these individual growth factors.

The regulation of smooth muscle cell proliferation by growth factors and endogenous heparin and heparin-like molecules appears to be important in the development of vascular diseases such as arteriosclerosis and persistent pulmonary hypertension. We propose that the normal suppression of vascular smooth muscle cell growth may be due to heparin-mediated suppression of PIP_2 synthesis through the constitutive inhibition of PIP kinase. This inhibition may involve uncoupling of PIP kinase from a stimulatory G-protein. Removal of heparin via damage to endothelial cells during vascular injury may release PIP kinase from inhibition, resulting in an elevation of PIP_2 levels and thereby allowing proliferative responses to circulating and locally released growth factors to occur.

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