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Differential Effects of Heparin on Inositol 1,4,5-Trisphosphate Binding, Metabolism, and Calcium Release Activity in the Bovine Adrenal Cortex

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SUMMARY

In a wide variety of cells, inositol-1,4,5-trisphosphate is a second messenger that interacts with specific intracellular receptors and triggers the release of sequestered Ca^{2+} from an intracellular store. We have looked at the influence of heparin on the action and metabolism of inositol-1,4,5-trisphosphate in the bovine adrenal cortex. Heparin blocked inositol-1,4,5-trisphosphate binding with half-maximal efficiency around 10 μg/ml. Scatchard analyses revealed that heparin did not change the affinity but decreased the number of available binding sites. The Ca^{2+}-releasing activity of inositol-1,4,5-trisphosphate was monitored with the fluorescent indicator, fura-2. Heparin blocked this activity with half-maximal effeciency around 10 μg/ml. The effect of heparin could be overcome by a supramaximal dose of inositol-1,4,5-trisphosphate (25 μM). The activity of inositol-1,4,5-trisphosphate-3-kinase from bovine adrenal cortex cytosol was also studied. Heparin inhibited the activity of the kinase with a half-maximal effeciency around 0.4 μg/ml. Lineweaver-Burk plots revealed that this potent effect was noncompetitive. Finally, we observed that heparin is without effect on inositol-1,4,5-trisphosphate-5-phosphatase (at concentrations as high as 2 mg/ml). These results are consistent with the suggestion that the binding sites for inositol-1,4,5-trisphosphate are the intracellular receptors responsible for the Ca^{2+}-mobilizing effects of inositol-1,4,5-trisphosphate. These results also show that the kinase, the phosphatase, and the receptor are three different molecular entities, which are affected in a different manner by heparin.

IP_3 is an intracellular messenger generated from the hydrolysis of phosphatidylinositol-4,5-bisphosphate by phospholipase C in response to Ca^{2+}-mobilizing stimuli (for review, see Refs. 1–4). IP_3 is able to increase the intracellular Ca^{2+} concentration by triggering the release of Ca^{2+} from an intracellular source other than mitochondria (5), thus activating calcium-dependent processes among which calmodulin is the best known. In a wide variety of tissues, it has been demonstrated that IP_3 recognizes and binds to specific receptors through which it initiates the events involved in the mechanism of Ca^{2+} release (6–11). Inside the cell, besides its Ca^{2+} regulation role, IP_3 is also used as substrate for two enzymes, IP_3-phosphatase, which removes the phosphate group from the fifth position (8, 12–14), and IP_3-kinase, which adds a phosphate group to the third position (15–19). It is not clear yet whether these enzymes represent processes for the elimination of IP_3 or for its transformation into other intracellular messengers whose roles are still to be defined (for example, see Refs. 20 and 21).

It has recently been shown that heparin, a mucopolysaccharide known for its anticoagulating effects, can compete with IP_3 for its specific binding sites in brain (10, 22). It was also shown that heparin at micromolar concentrations strongly inhibits the IP_3-promoted Ca^{2+} release from rat liver microsomes (23). The structure of heparin being a sulfated polysaccharide, it was suggested that it might be acting as a structural analogue of IP_3. If this was the case, the analogy could also stand at the level of the metabolizing enzymes and heparin might compete with IP_3 at the level of IP_3-phosphatase and IP_3-kinase.

In the present study, we investigate the effects of heparin on the binding of IP_3 to its receptor, on the IP_3-induced microsomal Ca^{2+} release, and on the activities of IP_3-phosphatase and IP_3-kinase from bovine adrenal cortex (a tissue in which angiotensin II stimulates steroidogenesis by a mechanism involving inositol lipid breakdown). We show that heparin is a very potent and noncompetitive inhibitor of IP_3-kinase and a strong inhibitor of IP_3 binding and IP_3-induced Ca^{2+} release and, finally, that heparin is without effect (at concentrations as high as 2 mg/ml) on IP_3-phosphatase. These results show that IP_3-kinase, IP_3-phosphatase, and IP_3 receptor are three different

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ABBREVIATIONS: IP_3, inositol-1,4,5-trisphosphate; IP_3-phosphatase, inositol-1,4,5-trisphosphate-5-phosphatase; IP_3-kinase, inositol-1,4,5-trisphosphate-3-kinase; EGTA, [ethylenekis(oxgethylenenitrilo)]tetraacetic acid; IP_2, inositol-1,4-bisphosphate; IP_4, inositol-1,3,4,5-tetrakisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

molecular entities that are affected in a different manner by heparin and they also support the notion that the binding sites for IP_3 are the intracellular receptors responsible for the Ca^{2+}-mobilizing effects of IP_3.

Experimental Procedures

Materials. IP_3 and fura-2 (free acid) were obtained from Calbiochem (La Jolla, CA). [^{3}H]IP_3 (20–40 Ci/mmol) was from New England Nuclear (Boston, MA) or Amersham (Arlington Heights, IL). Heparin (ammonium salt, no. H-0880) was from Sigma Chemical Co. (St. Louis, MO). Anion exchange resin AG 1-X 8 (formate form) was from Bio-Rad (Richmond, CA). All other reagents were from Sigma or Boehringer Mannheim (Indianapolis, IN).

Preparation of microsomes. Bovine adrenal cortices (dissected free of medullary tissue) were homogenized with eight strokes of a Dounce homogenizer (loose pestle) in a medium containing 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 25 mM Tris·HCl, pH 7.2, 5 mM KH_2PO_4, 1 mM dithiothreitol, and 2 mM EGTA. After stirring for 5 min and centrifugation at 500 × *g* for 15 min, the supernatant was centrifuged at 35,000 × *g* for 20 min. The pellet was washed with the same medium without EGTA and centrifuged at 35,000 × *g* for 20 min. The pellet was resuspended in the medium without EGTA at a concentration of 20–30 mg of protein/ml. These microsomes were stored at −70° or used immediately for IP_3-phosphatase IP_3-binding or IP_3-induced Ca^{2+} release studies.

Preparation of IP_3-kinase. IP_3-kinase was partially purified from bovine adrenal cortex by the procedure of Hansen *et al.* (16). Bovine adrenal cortices were homogenized (1:10, w/v) in 0.32 M sucrose, 10 mM HEPES/KOH, pH 7.3, 1 mM EGTA, 2 mM $MgCl_2$, and 2 mM dithiothreitol in a Dounce homogenizer (10 strokes of loose pestle). After centrifugation at 25,000 × *g* for 10 min, the supernatant was recentrifuged at 100,000 × *g* for 90 min. The last supernatant was fractionated with ammonium sulfate and a 23–40% ammonium sulfate fraction was dialyzed overnight against 10 mM Tris·HCl, pH 7.0, 2 mM $MgCl_2$, and 2 mM dithiothreitol at 4°.

IP_3-kinase assay. The IP_3-kinase activity was measured in a buffer containing 50 mM Tris·HCl, pH 8.0, 2.5 mM ATP, 5 mM $MgCl_2$, 5 mM sodium pyrophosphate, and 1 mM dithiothreitol. The assay buffer also contained 10^{-8} M $CaCl_2$, which in our system gave the free calcium concentration at which the enzyme was optimally active (24). The partially purified enzyme (25–50 μg of protein) was incubated at 37° for selected periods of time in the presence of tritiated IP_3 and heparin in a final volume of 250 μl. The incubation was stopped by addition of 250 μl of ice-cold perchloric acid (10%), and samples were kept on ice for 30 min and centrifuged at 2000 × *g* for 15 min. Perchloric acid was extracted from the supernatants with a 1:1 mixture of Freon and tri-*n*-octylamine (25). After neutralization, the samples were applied to anion-exchange resin columns (1 ml wet bed volume) and inositol phosphates were sequentially eluted by addition of ammonium (formate/formic acid mixtures of increasing ionic strength (for details, see legend to Fig. 4).

IP_3-phosphatase assay. Bovine adrenal cortex microsomes (10 μg of protein) were incubated at 22° in a buffer containing 25 mM Tris·HCl, pH 7.5, 100 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, tritiated IP_3, and heparin at selected concentrations, in a final volume of 250 μl. At appropriate times, incubations were stopped by addition of 250 μl of ice-cold perchloric acid (10%). Extraction and resolution of inositol phosphates were as described for IP_3-kinase assay.

IP_3-binding assays. Bovine adrenal cortex microsomes (1 mg of protein) were incubated in a medium containing 25 mM Tris·HCl, pH 8.5, 100 mM KCl, 20 mM NaCl, 5 mM KH_2PO_4, 1 mM EDTA, and 0.1% bovine serum albumin. Incubations were performed for 30 min at 0° in a final volume of 1 ml with tritiated IP_3 (≈1 nM) and selected concentrations of heparin. Nonspecific binding was determined in the presence of 1 μM IP_3. Incubations were terminated by vacuum filtration through presoaked glass fiber filters (Whatman GF/B) and rapid washing with 2.5 ml of cold medium. The receptor-bound radioactivity was analyzed by liquid scintillation counting.

IP_3-induced Ca^{2+} release assays. Bovine adrenal cortex microsomes (4–8 mg of protein) were incubated in a medium containing 20 mM Tris·HCl, pH 7.2, 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM phosphocreatine, and 20 unit/ml creatine phosphokinase. Incubations were performed at 37° in a final volume of 2 ml. The free Ca^{2+} concentration of the medium was monitored using fura-2 (free acid, 2.5 μM) in a SLM-Aminco SPF-500C spectrofluorometer. The excitation wavelength was 335 nm (slit 2.5) and the emission was recorded at 500 nm (slit 5.0). Ca^{2+} uptake and release were measured in the presence of oligomycin (2.5 μg/ml) in order to block mitochondrial ATPase. Ca^{2+} uptake was initiated by the addition of 1 mM ATP. Each record was calibrated by the addition of known amount of Ca^{2+} ($CaCO_3$) to the mixture. The actual free Ca^{2+} concentration of the medium was calculated from the F_{max} and F_{min} values obtained by adding excess Ca^{2+} and EGTA, respectively, after treatment with 1 μM ionomycin. The equation used was $[Ca^{2+}] = 224\ nM\ (F-F_{min}/F_{max}-F)$ (26).

Results

Effect of heparin on IP_3 binding. In the absence of heparin, approximately 10% of [^{3}H]IP_3 (0.3 nM; 10,000 cpm) remained specifically bound to bovine adrenal cortex microsomes. The addition of increasing concentrations of heparin diminished IP_3 binding in a dose-dependent manner (Fig. 1, *upper*). IP_3 binding was significantly reduced at 3 μg/ml heparin and almost completely abolished around 100 μg/ml. The IC_{50} value was approximately 10 μg/ml (9.2 ± 3.1 μg/ml; six experiments). When competition binding studies were performed in the presence of heparin (5 μg/ml), a substantial decrease in the binding capacity of the microsomes was observed (Fig. 1, *lower*). Scatchard analyses of these binding data indicated that the affinity of the microsomes for IP_3 was not significantly altered ($K_d \approx 2$ nM) whereas the number of binding sites was significantly reduced (128 fmol/mg of protein in the presence of heparin, as compared with 196 fmol/mg of protein in its absence). In further experiments, microsomes that were incubated for 30 min at 0° in the presence of 50 μg/ml heparin (a concentration high enough to inhibit most IP_3 binding) and washed by centrifugation at 30,000 × *g* for 20 min did not recover any binding activity; if, however, they were diluted 30 times and incubated for 15 min at 37° before centrifugation, then they recovered more than 80% of their binding capacity (data not shown). These results showing that heparin binding is not readily reversible at 0° may explain the reduction of maximal binding capacity observed in Scatchard analyses.

Effect of heparin on IP_3-induced Ca^{2+} release. The activity of IP_3 to mobilize Ca^{2+} from intracellular storage sites in the adrenal cortex was studied. As shown in Fig. 2 (*upper left*), our bovine adrenal microsomal preparation showed high ATP-dependent Ca^{2+}-sequestering activity (downward deflection of the trace) and decreased the ambient Ca^{2+} concentration to about 130 nM. In order to exclude a mitochondrial component of Ca^{2+} uptake, we used oligomycin (2.5 μg/ml) to block mitochondrial ATPase activity. Addition of a submaximal dose of IP_3 (1 μM) caused immediate release of Ca^{2+} (upward deflection of the trace) with a peak at about 5 sec, followed by re-uptake from the medium. Subsequent stimulation with IP_3 evoked a comparable response. The amount of Ca^{2+} released by IP_3 (93 pmol/mg of protein) was calibrated by the addition of 1 nmol of Ca^{2+}. Addition of 1 μM ionomycin (a Ca^{2+} ionophore) immediately released all of the sequestered Ca^{2+}. Fig. 2 (*upper*

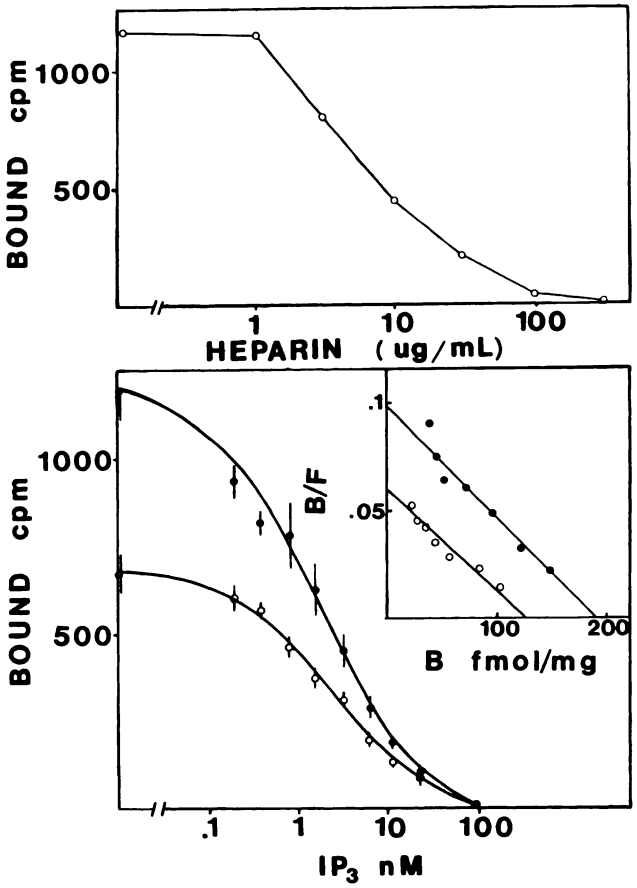

Fig. 1. Effect of heparin on IP_3 binding. *Upper*, Microsomes (1 mg of protein) were incubated at 0° for 30 min in a Tris/phosphate buffer (pH 8.5) in the presence of [^{3}H]IP_3 (1.2 nM; 10,000 cpm) and increasing concentrations of heparin. The binding of IP_3 (cpm) was estimated as described in Experimental Procedures, total binding was 1322 cpm and nonspecific binding was 172 cpm. This experiment performed in duplicate is representative of six similar experiments. *Lower*, microsomes (1 mg of protein) were incubated at 0° for 30 min in the presence of [^{3}H]IP_3 (1.9 nM; 13,500 cpm) and increasing concentrations of unlabeled IP_3. Specific binding of [^{3}H]IP_3 (cpm) was estimated as indicated in Experimental Procedures. The incubation medium contained no heparin (●) or 5 μg/ml heparin (○). *Error bars* represent standard deviations of the means of triplicate determinations. Scatchard plots of the same binding data are shown in the *inset*. The K_d values calculated from the slope of the Scatchard plots were 2.0 nM and 2.1 nM, whereas the B_{max} values calculated from the intercepts on the *abscissa* were 196 fmol/mg of protein and 128 fmol/mg of protein in the absence or presence of 5 μg/ml heparin, respectively. This experiment is representative of five similar experiments.

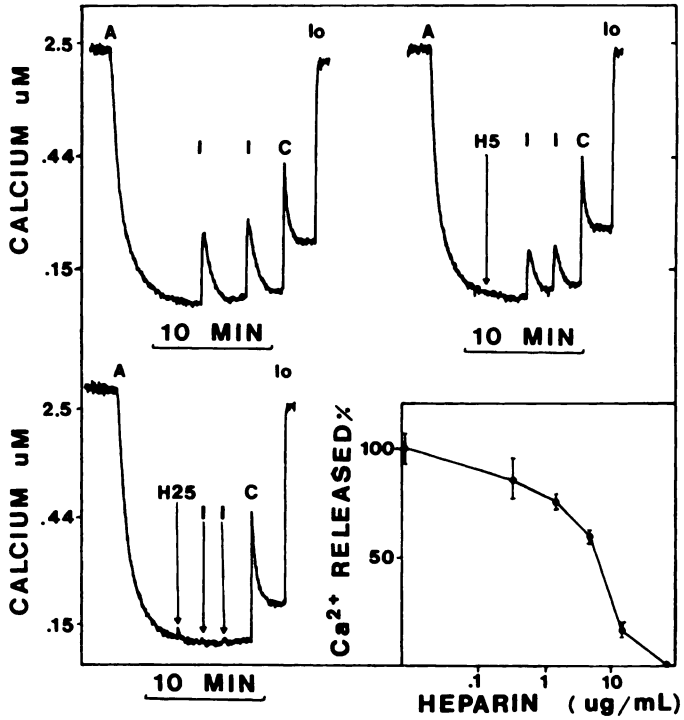

Fig. 2. Effect of heparin on IP_3-induced Ca^{2+} release. Microsomes (6 mg of protein) were incubated at 37° and their Ca^{2+} uptake and release activities were monitored by using fura-2 (free acid) under the conditions described in Experimental Procedures. Typical traces in which the Ca^{2+} taken up by an ATP-dependent process is partially released upon IP_3 addition. *A*, 1 mM ATP; *I*, 1 μM IP_3; *C*, 1 nmol of Ca^{2+}; *Io*, 1 μM ionomycin; *H5*, 5 μg/ml heparin; and *H25*, 25 μg/ml heparin. These typical traces are representative of several such experiments in which the Ca^{2+} movements were studied under different conditions. *Lower right*, the dose-response relationship between heparin and IP_3-induced Ca^{2+} release. *100%* represents the amount of Ca^{2+} released by 1 μM IP_3 in the absence of heparin (98.6 ± 7.4 pmol/mg of protein; triplicate value). This experiment is representative of 4 similar experiments.

right) shows that addition of heparin (5 μg/ml) to Ca^{2+}-loaded microsomes did not produce significant change in the Ca^{2+} concentration. Under these conditions, however, 1 μM IP_3 released only 58 pmol of Ca^{2+}/mg of protein. Fig. 2, *lower left*, shows that 25 μg/ml heparin strongly inhibited the effect of IP_3. The dose-response relationship between heparin and IP_3-induced Ca^{2+} release is shown in Fig. 2 (*lower right*). The threshold effect of heparin was around 1 μg/ml and the IC_{50} was approximately 10 μg/ml (9.7 ± 7.1 μg/ml; four experiments). To better define the nature of the kinetic interaction of heparin on IP_3-mediated Ca^{2+} release, the dose-response effect of IP_3 was evaluated in the presence of different heparin concentrations (Fig. 3). In the absence of heparin, half-maximal Ca^{2+} release occurred at around 0.3 μM IP_3 and maximal release was induced with 4–5 μM IP_3. In the presence of 20 μg/ml heparin, the IP_3 dose-response curve had the same shape but the half-maximal and maximal doses were shifted to 1 μM and about 25 μM, respectively. In the presence of 50 μg/ml heparin, the shift of the curve was more important (half maximal dose around 4 μM IP_3). Fig. 3 shows that the inhibitory effect of heparin could be overcome upon addition of very high doses of IP_3. This is a reflection of a competitive process.

Effect of heparin on IP_3-phosphatase activity. When IP_3 was incubated with adrenal cortex microsomes (10 μg of protein) in the presence of $MgCl_2$, a rapid and extensive degradation occurred, as shown in Fig. 4 (*center*). After 5 min of incubation, about 30% of total IP_3 (10 μM) had been converted to IP_2. It is assumed that this conversion was due to the activity of the enzyme IP_3-phosphatase. A straight line was obtained when the activity of the enzyme was plotted according to the Lineweaver-Burk equation (double-reciprocal plot), consistent with a Michaelis-Menten relationship between the phosphatase and its substrate (Fig. 5). A K_m value of 17 μM and V_{max} of 7.1 nmol min^{-1} mg^{-1} were calculated. These values, which are in good agreement with the values found in other tissues (12–14), did not change even if the experiment was performed in the presence of concentrations of heparin as high as 2 mg/ml. It was concluded that heparin is without effect on IP_3-phosphatase.

Effect of heparin on IP_3-kinase activity. The partially

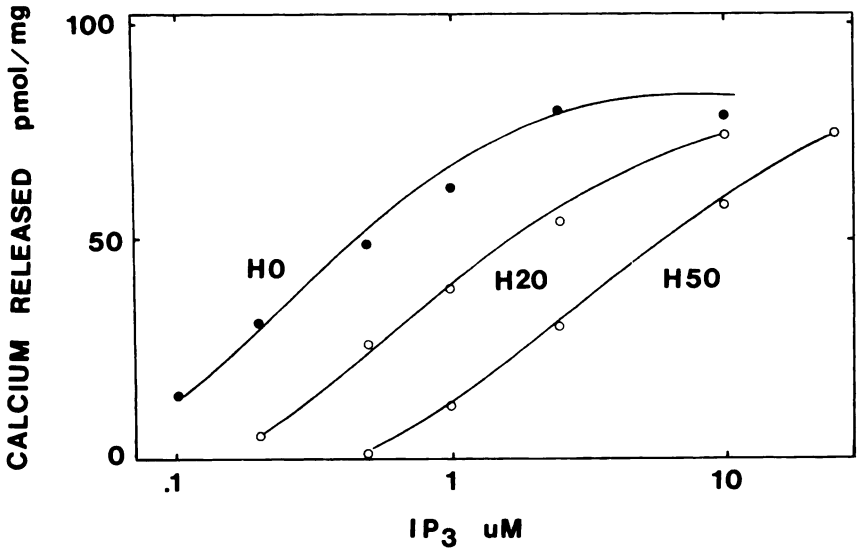

Fig. 3. Competitive effect of heparin on IP_3-induced Ca^{2+} release. Dose-response curves for IP_3-induced Ca^{2+} release were performed in the presence of different concentrations of heparin. Ca^{2+} movements were measured as indicated in Fig. 2. Heparin was present before ATP addition and had no effect on microsomal Ca^{2+} uptake. The amount of Ca^{2+} released (pmol/mg of protein) is indicated on the *ordinate* as a function of IP_3 concentration (μM) on the *abscissa*. *H0*, without heparin; *H20*, with 20 μg/ml heparin; and *H50*, with 50 μg/ml heparin. This experiment was reproduced with two different microsomal preparations.

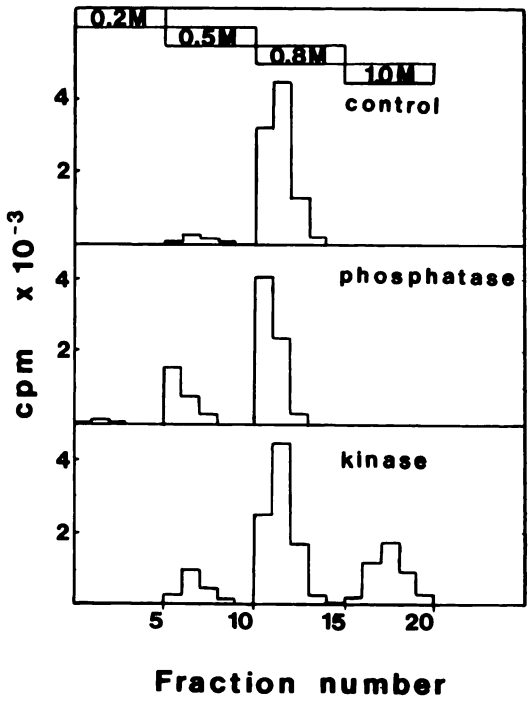

Fig. 4. Separation of inositol phosphates by anion-exchange chromatography. Inositol phosphates were eluted from small AG1 (formate) columns with the following eluants: 0.2 M ammonium formate/0.1 M formic acid (elution of inositol monophosphates); 0.5 M ammonium formate/0.1 M formic acid (elution of inositol bisphosphates); 0.8 M ammonium formate/0.1 M formic acid (elution of inositol trisphosphates); 1.0 M ammonium formate/0.1 M formic acid (elution of inositol tetrakisphosphates). Fractions (3 ml) were collected and counted for radioactivity (tritium). *Upper*, control incubation stopped at time = 0 sec. Center, IP_3 was incubated for 5 min at room temperature with microsomes (10 μg) as described in Experimental Procedures for IP_3-phosphatase assay. *Lower*, IP_3 was incubated for 15 min at 37° with partially purified kinase (25 μg) as described in Experiment Procedures for IP_3-kinase assay. These are typical traces.

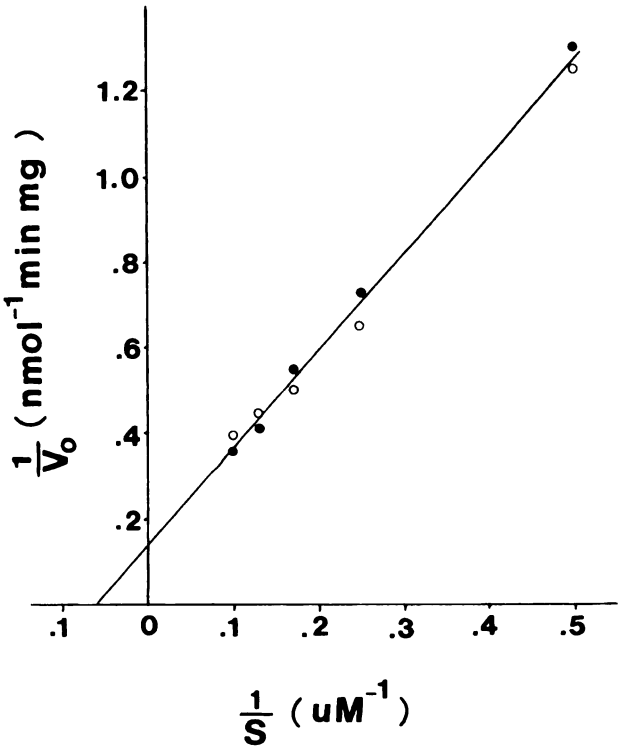

Fig. 5. Effect of heparin on IP_3-phosphatase. Lineweaver-Burk plots of IP_3-phosphatase activity from adrenal cortex; the reciprocal of the initial rate of enzyme activity (nmol of IP_3 hydrolyzed by 1 mg of protein in 1 min) is plotted on the *ordinate* versus the reciprocal of the IP_3 concentration (2–10 μM) on the *abscissa*. The experimental conditions for determining the initial rates were as described in Fig. 3. ●, Control experiment; ○, experiment performed in the presence of 2 mg/ml heparin. This experiment is representative of three similar observations.

purified IP_3-kinase actively converted IP_3 to IP_4 (see Fig. 4, *lower*). The enzyme preparation also contained a small contamination with phosphatase activity, which produced substantial amounts of IP_2. The presence of IP_3-phosphatase activity in the cytosol was not surprising, because in platelets this activity is mostly soluble (14). Fig. 6 shows that heparin was a very potent inhibitor of the kinase activity. A significant effect was observed at 30 ng/ml and the concentration that produced a half-inhibitory effect was 0.4 ± 0.1 μg/ml (three experiments). Lineweaver-Burk plots of the kinase activity (Fig. 7) show that, in the presence of heparin (0.4 μg/ml), the K_m of the kinase was not significantly modified (0.3 ± 0.1 μM; (three experiments), whereas the maximal velocity (V_{max}) was decreased

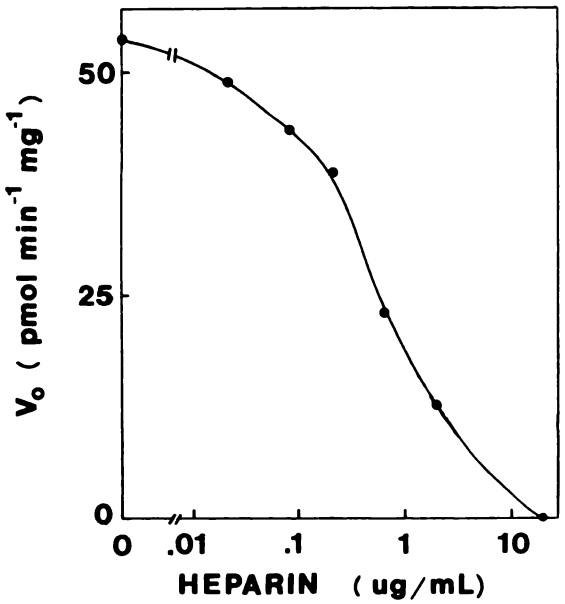

Fig. 6. Effect of heparin on IP_3-kinase. IP_3 (0.5 μM) was incubated for 15 min at 37° with the kinase preparation (25 mg of protein) under the conditions described in Experimental Procedures. The initial velocity of the enzyme is plotted as a function of heparin concentration (0–10 μg/ml). This experiment performed in duplicate is representative of three such observations.

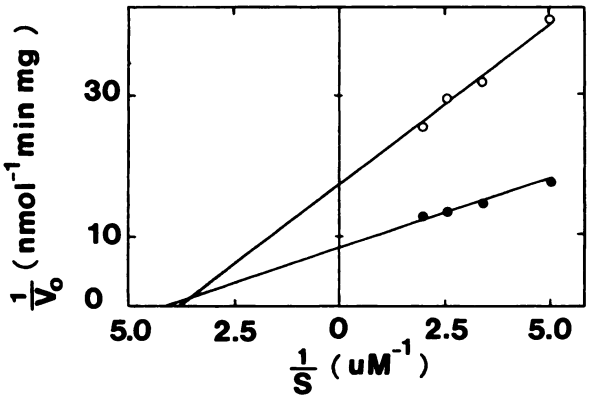

Fig. 7. Noncompetitive inhibition of IP_3-kinase by heparin. Lineweaver-Burk plots of IP_3-kinase activity from adrenal cortex; the reciprocal of the initial rate of enzyme activity (nmol of IP_3 phosphorylated by 1 mg of protein in 1 min) is plotted on the *ordinate* versus the reciprocal of the IP_3 concentration (0.2–0.5 μM) on the *abscissa*. The experimental conditions for determining the initial rates were as described in Fig. 3. ●, Control experiment; ○ experiment performed in the presence of 0.4 μg/ml heparin. This experiment is representative of three similar observations.

from a control value of 125 ± 16 pmol min^{-1} mg^{-1} (three experiments) to 59 ± 15 pmol min^{-1} mg^{-1} (three experiments) in the presence of heparin. These results indicated a noncompetitive type of inhibition.

Discussion

We have shown that heparin is a potent competitive inhibitor of IP_3-induced Ca^{2+} release from bovine adrenal cortex microsomes. This effect is likely due to a structural analogy between heparin and IP_3 inasmuch as binding studies (using the same microsomal preparations) also revealed that heparin could inhibit IP_3 binding. Scatchard analyses revealed that heparin decreases the number of available binding sites. Although this effect could look like a noncompetitive inhibition, we found that heparin binding is not readily reversible in the conditions used for IP_3 binding studies. The likely explanation for the effect of heparin on IP_3 binding is a competitive inhibition in which the decrease of B_{max} is due to a very slowly reversible (quasi-irreversible) process. The similar potency to inhibit both parameters supports our previous hypothesis that the binding sites for IP_3 are the intracellular receptors through which IP_3 triggers Ca^{2+} mobilization.

The most potent effect of heparin was observed on IP_3-kinase (about 10 times more potent than on IP_3 binding or IP_3-induced Ca^{2+} release) and heparin had no effect at all on IP_3-phosphatase. Overall, heparin can be considered as a strong inhibitor of IP_3 action. Indeed, it directly blocks the IP_3 effect on Ca^{2+} release, it strongly inhibits phosphorylation of IP_3 to IP_4 (a putative intracellular messenger), and it does not interfere with IP_3 dephosphorylation (which is presumed to form an inactive product). If the effect of heparin in the present study has any physiological relevance, it implies that heparin has access to the interior of the cell. Recently, it has been suggested that heparin could act intracellularly after uptake by vascular smooth muscle cells (27, 28). The same mechanism might also exist in adrenocortical cells. However, we have no indications so far that support a regulatory role of heparin in this important second messenger system.

The selectivity of heparin is rather poor, inasmuch as, besides its anticoagulating effect, it is known to interfere with a wide variety of physiological processes such as cell proliferation (29, 30), aggregation (31), and interaction (32). Heparin has been shown to alter guanine nucleotide-binding proteins (33, 34) and to interact with extracellular matrix proteins (35). In the present study, we have shown that it interacts with at least two more proteins (IP_3 receptor and IP_3-kinase).

Although its physiological role in the Ca^{2+}-gating system might be questionable, heparin can be regarded as an interesting tool for pharmacological or biochemical studies. In that respect, it has already been used as affinity ligand for the isolation of an IP_3-binding protein from brain tissue (22). Heparin could also serve as prototype in the design of specific drugs that would show selective effects on the binding or the metabolism of IP_3. There is a need for such selective IP_3 analogs.

Acknowledgments

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