Volume 252, number 1,2, 105–108 FEB 07428 July 1989

The effect of heparin on the inositol 1,4,5-trisphosphate receptor in rat liver microsomes

Dependence on sulphate content and chain length

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Received 30 May 1989

Heparin is known to inhibit the binding of inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) to high-affinity binding sites and to inhibit Ins 1,4,5-P_3-induced Ca^{2+} release from intracellular membrane-bound stores [(1987) J. Biol. Chem. 262, 12132–12136; (1987) FEBS Lett. 228, 57–59]. We have performed studies to clarify the structural requirements for this action of heparin in rat liver microsomes. Both *N*- and *O*-linked sulphate groups contribute to binding activity, since de-*N*-sulphated heparin was without effect on the Ins 1,4,5-P_3 receptor whereas a polyxylan bearing only *O*-linked sulphates (pentosan polysulphate) was as active as heparin. Therefore, the density of negative charge contributed by sulphate groups is important for the binding of heparin. Heparins with high and low affinity for antithrombin III both inhibited Ins 1,4,5-P_3 binding. There was a strong dependence on chain length, since binding activity decreased dramatically as the size of the heparin chain was reduced below that of 18–24 monosaccharide units.

Heparin; Fragmin; Pentosan polysulfate; Inositol 1,4,5-trisphosphate receptor; (Rat liver microsome)

1. INTRODUCTION

It is now widely recognised that a large variety of hormones, growth factors and other cell mediators use the breakdown of polyphosphoinositide(s) by an agonist-stimulated phospholipase C as the first step in their signal transduction pathway [3]. Both products of the breakdown of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) are believed to play second messenger roles in cells. 1,2-*sn*-Diacylglycerol is known to activate protein kinase C in a Ca^{2+}-stimulated and phospholipid-dependent manner [4] and Ins 1,4,5-P_3 causes the release of Ca^{2+} from an intracellular membrane-bound store which is non-mitochondrial and may be the endoplasmic reticulum or a specialised region thereof [5].

Several tissues have been demonstrated to contain high-affinity binding sites for Ins 1,4,5-P_3 including adrenal cortex [6], cerebellum [7], neutrophils [8] and liver [8,9]. The relative order of potency of various inositol phosphates to displace Ins 1,4,5-P_3 from these sites is strikingly similar to their ability to cause Ca^{2+} release from intracellular stores, suggesting that the binding sites mediate the Ca^{2+}-releasing effects of Ins 1,4,5-P_3 [10]. Heparin has been shown to inhibit binding of Ins 1,4,5-P_3 to cerebellar membranes [1]. It will also inhibit Ins 1,4,5-P_3-induced Ca^{2+} release from rat liver microsomes [2] and is therefore acting as an Ins 1,4,5-P_3 receptor antagonist. Recent studies have shown this mechanism of inhibition to be of a competitive nature [11].

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
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Heparin is a heterogenous mixture of linear polysaccharide chains of varying lengths which can vary substantially in saccharide composition and degree of *N*-acetylation, *O*- and *N*-sulphation depending on the source of the material [12]. Therefore, it is of considerable interest to determine what structural features of heparin confer on it the ability to act as an Ins 1,4,5-P_3 antagonist. Here, we describe the effects of heparin and related substances on Ins 1,4,5-P_3 binding and Ins 1,4,5-P_3-induced Ca^{2+} release in a rat liver microsomal system.

2. MATERIALS AND METHODS

2.1. *Microsome preparation*

Liver microsomes were prepared from overnight-starved adult male rats (Sprague-Dawley) by the method of Dawson and Irvine [13].

2.2. *[^{32}P]Ins 1,4,5-P_3-binding assays*

Microsomal membranes (approx. 0.5 mg protein) were incubated for 30 min on ice in a total volume of 0.5 ml in a buffer with the following composition: 100 mM KCl, 25 mM Na_2HPO_4, 20 mM NaCl, 1 mg/ml BSA, 0.5 mM EDTA at pH 7.0 and containing ^{32}P-labelled Ins 1,4,5-P_3 (40–185 pM) and D-[2,6-^{3}H]mannose (for correction for trapped volume). Incubations were started by the addition of microsomes to tubes containing radioligand and the substance of interest to be tested and were terminated by dilution with 5 ml of 0.25 M sucrose, 10 mM K_2HPO_4 (pH 7.0) followed by rapid filtration through pre-soaked Whatman GF/C filters using a vacuum apparatus. Two rapid 5-ml washes of the tube and filter were performed using the same buffer. The dilution, filtration and washing procedure took 10–15 s. Filter-associated ^{32}P and ^{3}H radioactivity was measured by dual-label liquid scintillation counting of the filters in 5.0 ml Atomlight scintillation fluid. Non-specific binding was defined as that remaining in the presence of added 8.57 μM non-radioactive Ins 1,4,5-P_3 and was subtracted from all binding results.

2.3. *Ins 1,4,5-P_3-induced Ca^{2+}-release measurement*

The release of Ca^{2+} from rat liver microsomes was measured using Ca^{2+}-selective electrodes according to Dawson and Irvine [13]. Electrodes were manufactured using the Ca^{2+}-selective ligand ETH1001 [14]. After uptake of Ca^{2+} by the microsomes to a steady-state level, GTP was added to give a final concentration of 20 μM to enhance the effect of subsequent Ins 1,4,5-P_3 addition [15]. When testing the effects of substances of interest these were added to microsomes 1 min prior to addition of 0.5 μM Ins 1,4,5-P_3. We have found this to be the maximal concentration for Ca^{2+} release in our hands.

(4,5-^{32}P)-labelled Ins 1,4,5-P_3 (130 Ci/mmol) from New England Nuclear was used in preliminary binding studies but the majority of experiments utilised (5-^{32}P)-labelled Ins 1,4,5-P_3 (1000 €/mmol) obtained from Amersham International. There was no significant difference between results obtained with radioligand from either source. D-[2,6-^{3}H]Mannose and unlabelled Ins 1,4,5-P_3 were also obtained from Amersham. ETH 1001 was purchased from Fluka. Unfractionated heparin (from porcine intestinal mucosa, mean M_r 13000), de-*N*-sulphated heparin (mean M_r 13000) and pentosan polysulphate (M_r 1500–5000) were obtained from Sigma, and Fragmin (mean M_r 5000) was purchased from Kabi-Vitrum. Heparin-derived oligosaccharides were prepared by a process of nitrous acid depolymerisation and repeated gel filtration [17–19]. Fractions were designated high affinity (HA) or low affinity (LA) depending on their behaviour on an antithrombin III affinity column as described in [20]. The numbers assigned to them signify the number of monosaccharide units in the chain. Thus, HA8 is an octasaccharide with high affinity for antithrombin III.

3. RESULTS AND DISCUSSION

We have previously demonstrated that the preparation of rat liver microsomes used in this study contains high-affinity binding sites which are probably the receptors through which Ins 1,4,5-P_3 causes Ca^{2+} release from intracellular stores [10]. Fig.1 shows that Ins 1,4,5-[^{32}P]P_3 binding to this site can be prevented by unfractionated heparin and by Fragmin (a heterogenous low molecular mass heparin fraction with an average molecular mass of 5 kDa). Both polysaccharides exhibit similar affinity for the binding site, having EC_{50} values of 4.8 and 8.0 μg/ml, respectively. It can also be seen that de-*N*-sulphated heparin is ineffective over the same range of concentrations as native heparin. Studies performed on smooth muscle cell microsomes have shown recently that de-*N*-sulphated heparin is unable to antagonise Ins

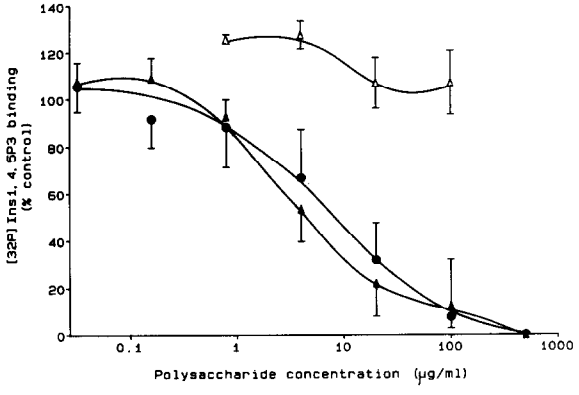

Fig.1. Effect of heparin (▲), de-*N*-sulphated heparin (△) and Fragmin (●) on [^{32}P]Ins 1,4,5-P_3 specific binding to rat liver microsomes. Means ± SE from 3–4 independent experiments are shown.

1,4,5-P_3-induced Ca^{2+} release [11]. We have confirmed this finding in the rat liver microsomes used in the present study (not shown). This suggests that the sulphate groups in the heparin molecule play an important role in the interaction of heparin with Ins 1,4,5-P_3 receptor and raises the possibility that there may be a special role for *N*-sulphate groups. The synthetically *O*-sulphated polyxylan pentosan polysulphate [21], is very effective at competing with Ins 1,4,5-[^{32}P]P_3 binding (fig.2), having an EC_{50} of 6.9 μg/ml. As pentosan polysulphate contains no *N*-linked sulphate groups [22], it is unlikely that the lack of effect of de-*N*-sulphated heparin on the Ins 1,4,5-P_3 receptor is due to a specific lack of *N*-sulphate groups, but is more likely to be due to an overall reduction in density of sulphate groups compared to the parent heparin molecule. It should be noted that the removal of *N*-sulphate substituents will expose positively charged free amino groups. Fig.2 also shows that the ability of pentosan polysulphate to prevent Ins 1,4,5-P_3 binding is accompanied by inhibition of Ins 1,4,5-P_3-induced Ca^{2+} release. The concentration dependence for the displacement of Ins 1,4,5-[^{32}P]P_3 binding by pentosan polysulphate is similar but not identical to that for inhibition of Ca^{2+} release. This is probably due to the difference in conditions of buffer and temperature under which we measure Ca^{2+} release and binding.

Unfractionated heparin consists of heterogeneous polysaccharide chains of varying length and we therefore sought to determine the activity of more well characterised oligosaccharides. Various oligosaccharide fractions of a narrow range in molecular size were separated into two classes by virtue of their affinity for immobilised antithrombin III [20]. The HA fractions contain the specific pentasaccharide sequence which is known to bind to antithrombin III whereas the LA fractions do not [17–19]. The results with HA and LA fractions were essentially similar and in both cases show a clear effect of chain length on the ability of the oligosaccharides to displace bound Ins 1,4,5-[^{32}P]P_3 from the microsomes. Fig.3 shows the ability of each of the oligosaccharide fractions to compete with Ins 1,4,5-[^{32}P]P_3 for binding to the microsomes at a saccharide concentration of 20 μg/ml. The ability of the HA18–24 fraction to compete with labelled ligand is very similar to that of unfractionated heparin and Fragmin (which has a similar mean molecular mass to that of the purified HA oligosaccharide). However, the HA10–14 fraction has substantially lower activity and the HA8 fraction displays none

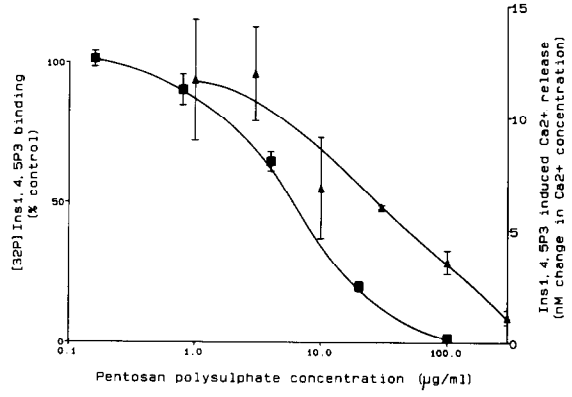

Fig.2. Effect of pentosan polysulphate on [^{32}P]Ins 1,4,5-P_3 specific binding (■) and Ins 1,4,5-P_3-induced Ca^{2+} release (▲) from rat liver microsomes. Data are means ± SE from 4 independent experiments (binding) or means ± range from 2 experiments (Ca^{2+} release).

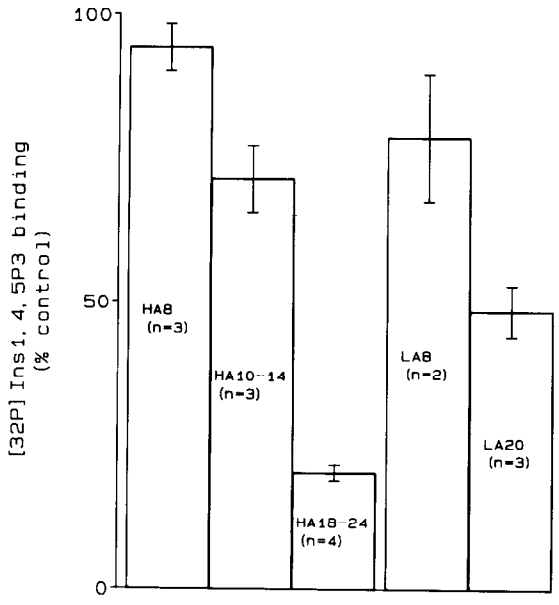

Fig.3. Effect of heparin-derived oligosaccharide fractions (20 μg/ml) on [^{32}P]Ins 1,4,5-P_3 specific binding to rat liver microsomes. Means ± SE or ± range (LA8) are shown. Number of independent experiments given in parentheses. HA, high affinity for antithrombin III; LA, low affinity for antithrombin III. Numbers indicate the number of monosaccharide units per oligosaccharide molecule.

at the concentration tested. Similarly, for the low-affinity fractions, LA20 had substantial displacing activity at 20 μg/ml whereas LA8 had essentially none. These results thus show a clear dependence of activity on chain length. All fractions which had significant displacement activity at that concentration showed a concentration-dependent effect but the complete range of concentrations tested has not been shown for reasons of clarity. It was not possible to perform tests at concentrations higher than 20 μg/ml due to limitations in the amount of material available.

We have shown here that the ability of heparin to act as an Ins 1,4,5-P_3 receptor antagonist is dependent on the presence of sulphate groups and on molecular size. In addition, pentosan polysulphate, which is another linear polymer with a high degree of sulphation but different monomer composition, has been shown to act as an Ins 1,4,5-P_3 receptor antagonist. It is hoped that studies of this type will enable the design of low molecular mass compounds which may be specific Ins 1,4,5-P_3 receptor antagonists. Such compounds would be invaluable experimental tools and may have therapeutic benefit in disease states.

Acknowledgement: We are extremely grateful to Dr Cathy Nieman for setting up the Ca^{2+}-release measurement system.

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