Allotopic antagonism of the non-peptide atrial natriuretic peptide (ANP) antagonist HS-142-1 on natriuretic peptide receptor NPR-A

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The microbial polysaccharide HS-142-1 has been documented as an antagonist of natriuretic peptides. It inhibits activation and peptide binding to both guanylate receptors natriuretic peptide receptor (NPR)-A and NPR-B, but has no effect on the non-cyclase receptor NPR-C. At first sight the effect of HS-142-1 on peptide binding appears to be surmountable, suggesting that it might be competitive despite its chemically divergent nature. We explored its mode of action on wild-type NPR-A (WT), on a disulphide-bridged constitutively active mutant (C423S) and on truncated mutants lacking either their cytoplasmic domain (Δ KC) or both the cytoplasmic and the transmembrane domains (ECD). On the WT, HS-142-1 inhibited atrial natriuretic peptide (ANP) binding with a pK value of 6.51 ± 0.07 ($K_d = 0.31 \mu\text{M}$). It

displayed a similar effect on the C423S mutant ($pK = 6.31 \pm 0.11$), indicating that its action might not be due to interference with receptor dimerization. HS-142-1 also inhibited ANP binding to Δ KC with a pK of 7.05 ± 0.05 ($K_d = 0.089 \mu\text{M}$), but it was inactive on ANP binding to ECD at a concentration of 10^{-4} M, suggesting that the antagonism was not competitive at the peptide-binding site located on the ECD and that the transmembrane domain might be required. HS-142-1 also enhanced dissociation of NPR-A-bound ^{125}I -ANP in the presence of excess unlabelled ANP, implying an allotopic (allosteric) mode of action for the antagonist.

Key words: allosteric, dissociation kinetics.

INTRODUCTION

The natriuretic peptide receptors (NPRs) are a group of single-transmembrane-domain signalling receptors. They are part of the growing family of particulate guanylate cyclases [1,2]. Three main subtypes of NPRs are known. Two of the receptors, NPR-A and NPR-B, are coupled to a guanylate cyclase moiety and respond to natriuretic peptides [atrial natriuretic peptide (1-28) (or natriuretic peptide A; ANP), brain natriuretic peptide (natriuretic peptide B; BNP) and C-type natriuretic peptide (natriuretic peptide C; CNP)] by producing cGMP in the intracellular space. NPR-A responds to ANP and BNP whereas NPR-B responds only to CNP. The third member of the family is the NPR-C receptor, which is not coupled to a guanylate cyclase function. The NPR-C receptor, a disulphide-bridged dimer, is often referred to as the clearance receptor and has a similar affinity for all the natriuretic peptides [3,4]. The NPRs are activated when natriuretic peptide release is triggered by high central venous pressure. cGMP released by NPR-A and NPR-B is believed to mediate the effects of NPRs on diuresis, natriuresis, vasorelaxation and inhibition of the renin-angiotensin-aldosterone system [5,6].

The full-length receptors display the four structural domains typical of the guanylate cyclase receptors. An extracellular domain (ECD) with a ligand-binding domain, a transmembrane domain, a kinase-homology domain and a guanylate cyclase domain [1,2]. A model with several sequential steps has been proposed for the activation of this receptor [1]. The action of the antagonists usually occurs at the first step of binding of the peptide, which induces a conformational change in the receptor. Since it is believed that the receptor is a preformed dimer prior to activation [7], the antagonists could act at two levels: they

could promote the dissociation of the dimeric receptors towards the monomeric form or induce an inactive conformation of the receptors. The typical NPR-A agonist is ANP, which is a 28 amino acid hormone with a loop maintained by a disulphide bridge. Many peptide antagonists are known and they are all variations of the structure of ANP [8]. They can induce a decline in cGMP production which can lead to a decrease in sodium excretion and urine flow and an increase in mean arterial pressure.

HS-142-1 is a novel microbial polysaccharide which was isolated from the culture broth of *Aureobasidium* sp. [9]. HS-142-1 is a mixture of 20–30 kinds of linear β -1,6-glucan esterified by caproic acid, differing in the number of glucose residues (4–14) and the degree of esterification by caproic acid [9]. The structure of HS-142-1 is completely different from that of natural agonists of NPRs. It has been found by other research teams that HS-142-1 is an antagonist of NPR-A and NPR-B but not of NPR-C. In these studies it was found that HS-142-1 inhibited the binding of ^{125}I -ANP to the NPR-A in a dose-dependent manner with an IC_{50} of $0.9 \mu\text{g/ml}$ [9]. In another study they found that HS-142-1 also inhibited the production of cGMP in cells containing NPR-A and NPR-B with an IC_{50} of $1.5 \mu\text{g/ml}$ [10]. With these data at hand they concluded that HS-142-1 is a competitive antagonist of the NPRs. In a study that was done with anaesthetized rats they observed that HS-142-1 could prevent the increase in urine flow and in the urinary excretion of sodium elicited by ANP [11]. The use of HS-142-1 to block the NPR-A receptor has increased in recent years [12,13]. HS-142-1 is used because of its apparently specific antagonism for NPRs.

The main goal of this study was to understand how HS-142-1 works as an antagonist of the NPR-A receptor. Since its structure is so different from those of the natural agonist or the

Abbreviations used: NPR, natriuretic peptide receptor; rNPR-A, rat NPR-A; ECD, extracellular domain; ANP, atrial natriuretic peptide; rANP, rat atrial natriuretic peptide.

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peptide antagonists, we wished to know if HS-142-1 is really a competitive antagonist and what its site of interaction in the receptor is.

MATERIALS AND METHODS

Material and cell growth

HS-142-1 from S. Nakanishi, Kyowa Hakko Kogyo Co., Machida-shi, Tokyo, Japan.

The HEK-293 cell line (A.T.C.C.) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 units of penicillin/streptomycin under an atmosphere of 5% CO₂ in an incubator at 37 °C.

Transient expression in HEK-293 cells

Transient expression of the Δ KC mutant (a truncated mutant lacking the cytoplasmic domain) was obtained by transfection using the CaHPO₄ precipitation method as described elsewhere [14].

Stable expression in HEK-293 cells

The rat NPR-A (rNPR-A), rNPR-A^{C423S} (encoding a disulphide-bridged constitutively active mutant), ECD (encoding a truncated mutant lacking both the cytoplasmic and transmembrane domains) and ECD^{C423S} (encoding a disulphide-bridged truncated mutant) clones were selected in 600 µg/ml G-418 (Boehringer Mannheim) in culture medium. For the control, the clones were selected after transfection with pBK-Neo.

Membrane preparations

Membranes for the binding studies and dissociation kinetics were prepared as follows. The cells were rinsed twice with PBS and lysed in ice-cold homogenization buffer (10 mM Tris/HCl, pH 7.4, and 2 mM EDTA containing 10⁻⁶ M aprotinin, 10⁻⁶ M pepstatin, 10⁻⁶ M leupeptin and 10⁻⁵ M Pefabloc). Cells were homogenized with a polytron homogenizer. The homogenate was centrifuged for 30 min at 35 000 g. The pellet was resuspended and washed twice in the same buffer. Finally the membranes were resuspended in ice-cold freezing buffer (50 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 250 mM sucrose, 1 mM MgCl₂ and protease inhibitors), frozen in liquid nitrogen and stored at -80 °C. For the guanylate cyclase studies membranes were prepared according to a similar procedure, except that the homogenization and freezing were performed in 50 mM Hepes, pH 7.4, containing 20% glycerol, 50 mM NaCl, 10 mM sodium phosphate buffer, 0.1 mM NaF, 1 mM Na₃VO₄ and protease inhibitors. The protein concentration was determined using the BCA protein-assay kit (Pierce).

Purification of secreted rNPR-A extracellular mutant

The ECD mutant was purified from cell-culture medium of a stably expressing clone. For purification, an aliquot of 150 ml was thawed slowly at 4 °C. The sample, to which was added a final concentration of 20 mM imidazole, pH 7.4, was mixed gently overnight with 0.3 ml of Ni²⁺-nitrilotriacetate agarose gel (Qiagen). After the incubation, the gel suspension was packed on to a column and washed with 5 vol. of sodium phosphate buffer, pH 7.4, containing 0.3 M NaCl and 20 mM imidazole. The elution was done with 10 vol. of the same buffer containing 500 mM imidazole.

Receptor-binding assays

¹²⁵I-rANP (rat ANP) was prepared using the lactoperoxidase method [15]. Binding to membranes was performed at 22 °C for 90 min in 1 ml of binding buffer (50 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 5 mM MnCl₂ and 0.5% BSA). Competition experiments were done by incubation of 3–5 µg of membranes with 10 fmol of ¹²⁵I-rANP and increasing concentrations of unlabelled ANP or HS-142-1. Bound ¹²⁵I-rANP was separated from free ligand by filtration on GF/C glass-fibre filters precoated with polyethyleneimine. The binding to purified ECD was performed at 4 °C for 22 h in 0.1 ml of binding buffer (50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, 0.1% BSA and 0.05% lysozyme). For competition studies, 100 fmol of ¹²⁵I-rANP and increasing amounts of unlabelled ANP or HS-142-1 were added to the binding mixture. Bound ¹²⁵I-rANP was separated from the free form by adding 0.1 ml of ice-cold dextran-coated charcoal equilibrated in the same buffer. After vigorous vortexing and centrifugation at 12 000 g for 2 min, 0.1 ml of the supernatant was measured in a gamma counter.

Guanylate cyclase assay

Membranes from the rNPR-A clone were used for the guanylate cyclase assay as described in other studies [16,17]. The membranes (5 µg) were incubated for 10 min at 37 °C in 50 mM Tris/HCl, pH 7.6, with 10 mM theophylline, 2 mM 3-isobutyl-1-methylxanthine, 10 mM creatine phosphate, 10 units of creatine kinase, 1 mM GTP, 1 mM ATP and 4 mM MgCl₂. The stimulation was done with dose-response curves of ANP at four different concentrations of HS-142-1. cGMP was separated from the GTP by chromatography on Alumina and evaluated by radioimmunoassay as reported previously [18].

Dissociation kinetics

NPR-A membranes were incubated with 10 pM ¹²⁵I-rANP for 90 min under the same conditions as the binding assays. The dissociation was started by the addition of an excess of unlabelled ANP (0.1 µM) in absence or presence of 2 µM HS-142-1. Aliquots of the solution were taken at different times and filtered on GF/C filters, pretreated with 1% polyethyleneimine to separate bound from free ligand.

Data analysis

Dose-response curves were analysed with the program AllFit for Windows based on the four-parameter logistic equation [19]. Radioligand-binding data were analysed with the same program based on a model for the law of mass action [20]. Estimates from replicate experiments are expressed as means ± S.E.M. Statistical testing of differences between averaged estimates were performed by Student's *t* test. Drug potency (pA₂) for the antagonist was estimated from concentration-response curves for agonist in the absence and presence of a fixed concentration of antagonist according to the formula pA₂ = log{[antagonist]/(dose ratio - 1)}, where [antagonist] is the concentration of antagonist used and dose ratio is the ratio of the ED₅₀ for the agonist concentration curve in the presence of antagonist divided by that obtained in the absence of antagonist. The affinity constant (*K*) for peptide binding was estimated as *K* = (1/*K*_d), then converted to p*K* = log *K*. Averaging pA₂ and p*K* is known to be statistically better than averaging ED₅₀ and *K*_d because the data for pA₂ and p*K* tend to be more normally distributed.

RESULTS

HS-142-1 behaves like a surmountable antagonist of NPR-A

Since the structure of HS-142-1 is so different from that of the natural peptides that bind to the receptor, we wanted to test the hypothesis that HS-142-1 might be a competitive antagonist specific for the NPR-A and NPR-B receptors. We tested the effect of four different concentrations of HS-142-1 (0 – 10^{-5} M) on the production of cGMP by the NPR-A receptor (Figure 1). With the increasing concentrations of HS-142-1 we observed a shift to the right of the ANP dose-response curves without alteration of the maximal production of cGMP. The pA_2 was calculated to be 6.00 ($ED_{50} = 1 \mu\text{M}$) for HS-142-1. This is typical of a competitive inhibitor but can also be seen in the case of allosteric inhibition by an antagonist [21].

Figure 1 Effects of HS-142-1 on guanylate cyclase in membranes from cells expressing wild-type rNPR-A

Stimulation of the particulate guanylate cyclase of wild-type rNPR-A with different concentrations of HS-142-1. Membranes were prepared as described in the Materials and methods section and were incubated with a regenerating system containing GTP and various agents. The assays were performed at four different concentrations of HS-142-1: 0 M (●), 10^{-6} M (○), 3×10^{-6} M (■) and 10^{-5} M (□). cGMP produced was purified by chromatography on Alumina and evaluated by radioimmunoassay. The pA_2 for HS-142-1 is 6.00 ($ED_{50} = 1 \mu\text{M}$). The experiment was repeated three times with similar results. ANF refers to atrial natriuretic factor (ANP).

Table 1 pK values for ANF and HS-142-1 towards the different NPR-A receptor constructs and the NPR-C receptor

$pK = -\log K_d$, calculated from the binding experiment. Errors are S.E.M. and sample sizes (n) are shown in parentheses.

Receptor construction	pK of the ligand	
	ANF	HS-142-1
Wild-type rNPR-A	10.09 ± 0.03 (3)	6.51 ± 0.07 (3)
Δ KC rNPR-A	$11.08 \pm 0.05^*$ (3)	$7.05 \pm 0.05^*$ (3)
rNPR-A ^{C423S}	$10.87 \pm 0.17^*$ (3)	6.31 ± 0.11 (3)
ECD wild-type rNPR-A	$9.32 \pm 0.32^*$ (3)	< 4.0 (1)
ECD ^{C423S} rNPR-A	9.83 ± 0.10 (3)	< 5.0 (3)
Wild-type rNPR-C	$11.02 \pm 0.15^*$ (3)	< 6.0 (3)

* pK of the receptor is significantly different from that of the wild-type receptor; Dunnett t test, $P < 0.05$.

Figure 2 Effect of HS-142-1 on ANP binding to wild-type rNPR-A

Competition binding curves of rANP (ANF; ●) and HS-142-1 (○) for the binding of ^{125}I -ANP to membranes of HEK-293 stably expressing wild-type rNPR-A (A) or Δ KC mutant (B). Membranes were incubated with 10 pM ^{125}I -ANP and varying concentrations of the competing unlabelled ligands. After 90 min of incubation at 22°C , bound radioligand was separated from the free by filtration as described in the Materials and methods section. The pK values for HS-142-1 for wild-type and Δ KC mutant NPR-A were 6.51 ± 0.07 ($n = 4$) and 7.05 ± 0.05 ($n = 4$) respectively. Each data point represents the mean $\pm$ S.E.M. from duplicate determinations. The results shown are typical of three experiments. The curves were analysed by the AllFit program for Windows based on the law of mass action [20].

The intracellular domain is not required for the antagonistic effect of HS-142-1

In order to assess the structural domain of the receptor required for the effect of HS-142-1 on NPR-A, we first tested its effect on ANP binding to the wild type and to cytoplasmic domain-deleted mutant (Δ KC). The effect of HS-142-1 on the binding of ^{125}I -ANP to the wild-type NPR-A receptor was characterized by an apparent pK of 6.51 ± 0.07 (Table 1; $K_d = 0.3 \mu\text{M}$, Figure 2A). When HS-142-1 was tested on the truncated Δ KC mutant we found an apparent pK of 7.05 ± 0.05 ($K_d = 0.09 \mu\text{M}$; Figure 2B). This higher affinity of the antagonist for the Δ KC mutant, which is significantly different from the one for ANP ($P < 0.001$), paralleled a similar increase for rANP ($pK = 10.09 \pm 0.03$ or $K_d = 81 \text{ pM}$ for wild-type NPR-A compared with 11.08 ± 0.04 or 8.3 pM for Δ KC, Table 1). Therefore the inhibitory effect of HS-142-1 appears to involve either the ectodomain or the transmembrane domain, or both.

HS-142-1 does not bind at the ANP-binding site of the soluble ECD receptor

Binding studies were then performed on the truncated mutant of NPR-A missing both the intracellular domain and the transmembrane domain (the ECD mutant). This mutant has been well-characterized previously by two groups [15,22]. We found that

Figure 3 Competition binding analysis of soluble ECD mutant receptor

Competition binding curves of rANP (ANF; ●) and HS-142-1 (○) for the binding of ^{125}I -ANP to the soluble ECD mutant stably expressed in HEK-293 cells. The purified soluble receptor was incubated with 100 pM ^{125}I -ANP and varying concentrations of the competing unlabelled ligands. After an incubation of 22 h at 4 °C bound radioligand was separated from the free with dextran-coated charcoal. HS-142-1 showed no apparent affinity for the soluble receptor. Each data point represents the mean $\pm$ S.E.M. from duplicate determinations. The results shown are typical of three experiments. The curves were analysed as in Figure 2.

HS-142-1 up to concentration of 10^{-4} M does not compete for ^{125}I -ANP binding to the soluble receptor, whereas ANP could inhibit ^{125}I -ANP binding competitively (Figure 3). From the experiments done on the wild-type, rNPR-A^{C423S} and ΔKC receptors we found a difference of pK of 4.05 ± 0.28 between ANP and HS-142-1 for the receptors (and a K_d ratio of 11 200). From these data and the pK of ANP for the ECD mutant receptor, we would expect the pK of HS-142-1 for the receptor to be 5.76 ($K_d = 1.7 \mu\text{M}$), a prediction which was not confirmed by the results obtained. We also performed experiments on the soluble constitutively dimeric mutant receptor ECD^{C423S} [15] and once again found no competition for ANP binding to this receptor (results not shown). The results thus indicate that HS-142-1 does not bind to the same site as ANP on NPR-A.

Figure 4 Competition binding analysis of wild-type rNPR-C receptor

Competition binding curves of rANP (ANF; ●) and HS-142-1 (○) for the binding of ^{125}I -ANP to membranes of HEK-293 stably expressing wild-type NPR-C. Membranes were incubated with 10 pM ^{125}I -ANP and varying concentrations of the competing unlabelled ligands. After an incubation of 90 min at 22 °C bound radioligand was separated from the free as described in the Materials and methods section. HS-142-1 showed no apparent affinity for the receptor. Each data point represents the mean $\pm$ S.E.M. from duplicate determinations. The results shown are typical of three experiments. The curves were analysed as in Figure 2.

Figure 5 Competition binding analysis of rNPR-A^{C423S} mutant receptor

Competition binding curves of rANP (ANF; ●) and HS-142-1 (○) for the binding of ^{125}I -ANP to membranes of HEK-293 cells stably expressing the mutant rNPR-A^{C423S}. Membranes were incubated with 10 pM ^{125}I -ANP and varying concentrations of the competing unlabelled ligands. After an incubation of 90 min at 22 °C bound radioligand was separated from the free as described in the Materials and methods section. The pK for HS-142-1 was 6.31 ± 0.12 ($n = 3$). Each data point represents the mean $\pm$ S.E.M. from duplicate determinations. The results shown are typical of three experiments. The curves were analysed as in Figure 2.

The antagonist HS-142-1 is specific for the NPR-A and NPR-B receptors

Since the antagonistic effect of HS-142-1 does not appear to interact directly at the ANP-binding site and appears to require membrane anchoring of NPR-A, we wished to confirm that this effect was specific and not due to a non-specific membrane effect. In agreement with previous reports indicating that HS-142-1 has affinity for NPR-A and NPR-B but not for the NPR-C, angiotensin II or endothelin receptors [9], we found no competitive interaction of HS-142-1 with ANP binding to the NPR-C receptor (Figure 4). Since the NPR-C receptor is a dimer devoid of any guanylate cyclase activity, we then tested the role of the dimeric structure of the receptor. When tested on NPR-A^{C423S}, a receptor mutant that is covalently dimeric, HS-142-1 displayed an apparent pK of 6.31 ± 0.12 ($K_d = 0.5 \mu\text{M}$; Figure 5), which is similar to the value for the wild-type NPR-A receptor ($P > 0.1$). Therefore the covalently dimeric state of the receptor does not preclude the antagonistic effect of HS-142-1.

HS-142-1 accelerates the dissociation kinetics of ^{125}I -ANP on NPR-A

One of the main characteristics of an allosteric antagonist is the fact that it promotes the dissociation of the agonist [21]. ^{125}I -ANP was first bound to NPR-A. The dissociation of the radioligand was triggered with the addition of ANP in the absence or presence of HS-142-1. We found that HS-142-1 accelerates the initial dissociation rate of ^{125}I -ANP in the presence of an excess of ANP (Figure 6). The initial rate of dissociation was seven times faster when HS-142-1 was added to the mixture ($0.709 \pm 0.172 \text{ min}^{-1}$ compared with $0.108 \pm 0.009 \text{ min}^{-1}$; $P < 0.05$, $n = 3$). The rate of the secondary phase of dissociation was, however, not altered by the presence of HS-142-1 ($0.00203 \pm 0.0002 \text{ min}^{-1}$ in the presence of HS-142-1 compared with $0.00193 \pm 0.0001 \text{ min}^{-1}$ in its absence; not significant, $n = 3$). Since the unlabelled ANP is in excess and blocks any free receptor, the effect of HS-142-1 must result from its binding at a different site.

Figure 6 Effects of HS-142-1 on the dissociation of rANP from rNPR-A

Membranes were incubated for 90 min with 10 pM ^{125}I -ANP, and then the dissociation was initiated by adding an excess of unlabelled rANP (10^{-7} M) in the absence (○) or presence (▲) of 2×10^{-6} M HS-142-1. Aliquots were taken at different times and bound ^{125}I -ANP was measured as described in the Materials and methods section. Radioligand bound (% B_0) is expressed as a percentage of that bound at the onset of dissociation. The dissociation rate of ^{125}I -ANP in the presence of ANP was accelerated 7-fold when HS-142-1 was added. The data shown are typical of three experiments. The curves are fitted by computer analysis using the sum of two exponential components.

Since excess ANP might be thought to hinder the effect of HS-142-1 on the ^{125}I -ANP dissociation rate allosterically and reciprocally, we performed parallel experiments where the antagonist was added without additional ANP. HS-142-1 still promoted dissociation of ^{125}I -ANP, and at a rate similar to that observed in the presence of ANP (results not shown). An experiment examining the time course of association was also performed in the presence and absence of $2 \mu\text{M}$ HS-142-1. When HS-142-1 was present, the extent of association of ^{125}I -ANP was greatly reduced (results not shown). This reduction (83%) was similar to the reduction seen in the competitive binding experiment with the wild-type receptor (85%) in the presence of $2 \mu\text{M}$ HS-142-1 (Figure 2).

DISCUSSION

The goal of the present study was to investigate the mode of interaction of HS-142-1 with the NPR-A receptor. The results confirmed that HS-142-1 behaved as an antagonist of the NPR-A receptor expressed in HEK-293 cells. With the results obtained, we can say that HS-142-1 is not a competitive antagonist of ANP on the NPR-A receptor but acts rather like an allotropic antagonist towards the NPR-A receptor. We observed that the intracellular domain was not required for the binding of HS-142-1 since its deletion (ΔKC) did not alter its affinity for the receptors. Therefore the antagonist does not act on the cytoplasmic domain of the receptor, which was accessible in the membrane preparation used. However, the binding studies with the soluble ECD receptor showed that the cellular membrane could be crucial for the interaction of HS-142-1 with the receptor. In the absence of the membrane attachment, HS-142-1 has no apparent effect on ANP binding to the receptor. Even if the soluble receptor is a covalent dimer ($\text{ECD}^{\text{C423S}}$), HS-142-1 does not appear to exert an effect. Thus HS-142-1 does not seem to bind to the agonist-binding site on the ECD of the receptor for the natriuretic peptides and therefore is not a competitive antagonist for the receptor. We also tested the binding of HS-142-1 to some covalently dimeric receptors. We documented that the binding of HS-142-1 to a receptor was independent of the dimeric state. HS-142-1 displayed affinity for the covalently

dimeric full-length receptor mutant $\text{NPR-A}^{\text{C423S}}$, but not for the NPR-C receptor nor the soluble covalently dimeric mutant receptor $\text{ECD}^{\text{C423S}}$ (Table 1).

Considering its simple molecular structure, one might ask whether HS-142-1 binds non-specifically to any cellular membrane. In agreement with previous results, the binding studies with the NPR-C receptor confirm that HS-142-1 is specific for some receptors (e.g. NPR-A and NPR-B). Since NPR-C is a covalent dimer, we wonder if the covalently dimeric structure of the receptor might prevent the binding process of HS-142-1. This is ruled out by the binding studies on the covalently dimeric mutant receptor $\text{NPR-A}^{\text{C423S}}$. Since HS-142-1 displays affinity for that mutant, the covalently dimeric state of NPR-C is ruled out as a cause for the lack of affinity of HS-142-1 for that receptor. Nor is the fact that NPR-C is missing an intracellular domain a reason, because the ΔKC mutant, with a truncated intracellular moiety, showed affinity for the antagonist. The fact that HS-142-1 does not bind to the NPR-C receptor is another indication that HS-142-1 does not appear to bind to the same site as the agonist. Our studies with the NPR-C receptor confirm earlier observations that HS-142-1 does not bind to the NPR-C receptor. Morishita et al. [9] reported that 20% of the specific binding of ^{125}I -ANP was not competed by HS-142-1 in a preparation containing all three NPRs. Following purification of the receptor preparation with GTP-agarose, all the HS-142-1-resistant binding disappeared. They concluded that HS-142-1 has little or no effect on ^{125}I -ANP binding to guanylate cyclase-free receptor (i.e. NPR-C). Those results confirm that HS-142-1 appears to require some membrane anchoring in order to interact with the receptor and to antagonize ANP, and that it is specific towards certain receptors. Interestingly, some forms of NPRs analogous to NPR-C are sensitive to HS-142-1. The NPR-D receptor cloned from eel has a hydrophobic trans-membrane region and a short cytoplasmic tail [23]. This structure is reminiscent of the structure of NPR-C. It was found that HS-142-1 showed affinity for eel NPR-D but not for eel NPR-C. These authors concluded that the NPR-D could exist in a tetrameric state and that this conformation could be the key to the affinity for HS-142-1.

The key experiments are related to the dissociation of radio-labelled ANP from the NPR-A receptor. From that experiment, we wanted to know if HS-142-1 produced that effect by acting on the same site as ANP or at a remote site. In that experiment (Figure 6) we added an excess of unlabelled ANP. The excess unlabelled ANP serves two purposes. First it prevents any rebinding of the dissociated radioligand and secondly it blocks any vacant ANP-binding sites. In this experiment, we observed an enhanced initial dissociation rate brought about by HS-142-1. This effect of HS-142-1 cannot be due to binding at the same site as ANP because the excess unlabelled ANP blocks all the sites. The effect is likely to be associated with an allotropic interaction, resulting in reduced affinity of the receptor for ANP. The action of $2 \mu\text{M}$ HS-142-1 on the dissociation rate of ANP affects a smaller proportion (14%) of bound peptide than the effect seen in competitive binding with ANP to the receptor. That difference could be due to the fact that during the dissociation experiment, the antagonist is added after the radioligand, and ANP could first bind the receptor and bring it to a more stable conformation. If that conformation was resistant to the allotropic effect of HS-142-1, it would account for the smaller effect of HS-142-1 when added during the dissociation experiment. This is supported by the fact that during the association experiment where the HS-142-1 was added at the same time as the ANP, we saw an effect of HS-142-1 of a similar amplitude as that seen during the competitive binding experiment. Therefore the effects

of HS-142-1 on the receptors are more potent when the ligand and the antagonist are added at the same time. These new experiments would tend to prove that the inhibition brought about by HS-142-1 on the receptors is not due to competitive antagonism, as previously concluded by others based on competition studies at equilibrium. The experiments also show that the antagonist and ANP mutually hinder each other's binding, as expected for an allosteric mechanism.

There are several reported examples of allotropic or allosteric antagonism. It was documented that major basic protein was an allosteric antagonist of M2 muscarinic receptor [24]. Another example of allotropic antagonism comes from suramin, which could at pharmacological doses prevent the interaction between methylamine-activated $\alpha 2M$ and its receptor. This was done by demonstrating an acceleration of the dissociation of pre-bound $\alpha 2M$ from its receptor [25]. Interestingly, in both cases the allotropic antagonist shared no structural similarity with the natural agonist. The notion that two distinct binding sites for an agonist and an antagonist might exist on the same receptor has been proposed previously, e.g. in a study on angiotensin antagonists [26]. The findings are consistent with an allosteric mechanism of antagonism for the angiotensin antagonist [Sar¹Ile⁸]Ang II. Another case of allotropic inhibition comes from the endothelin receptor [27]. In a study based on the BQ-123 antagonist on endothelin receptors, it was found that BQ-123 could interact allosterically with the receptor. In some binding studies, BQ-123 could produce a rightward shift of the binding curves without altering the $B_{\max}$ values of a specific subtype of endothelin receptors. Dissociation kinetics experiments showed that when dissociation was initiated in the presence of BQ-123, the rate of dissociation was altered. They concluded that BQ-123 acted via a secondary site on the receptor and that the results obtained could arise in the case of allosteric inhibition [27].

An allotropic antagonist binds at a site distinct and removed from the binding domain of the endogenous substrate (agonist) and interferes with the agonist binding due to a protein conformational change induced by the effector (antagonist) [21]. This definition of an allotropic antagonist corresponds to our hypothesis that HS-142-1 binds to a secondary site on the receptor and induces an inactive conformational change, which causes the agonist to leave the binding site faster. Once all the sites are occupied by the antagonist, the effects of HS-142-1 should plateau [21]. Allotropic interactions can be documented by examining dissociation kinetics. An allotropic agent should be able to accelerate the dissociation of the radiolabelled ligand in the presence of excess unlabelled agonist. HS-142-1 did accelerate the initial dissociation of the ligand from its receptor. Other agents appear to act allosterically on the NPR-A receptor. It was reported that ATP was an allosteric modulator of the NPR-A and it accelerated the initial dissociation rate of ¹²⁵I-ANP from its receptor [28], presumably by interacting with the cytoplasmic kinase homology domain. Interestingly, in both cases of HS-142-1 and ATP, the later and slower dissociation phase was not altered by those modulators.

Because of its structure, HS-142-1 is more likely to be an allotropic antagonist of the NPR-A than a competitive one. Agonists for the receptor are 28 amino acids long and are loop-shaped. On the other hand, HS-142-1 is a polysaccharide formed by a linear chain of glucose esterified by caproic acid. Such a structure is hardly likely to binding directly to the same site as the agonists. The mode of action of HS-142-1 with the receptor remains obscure. One possibility is that the antagonist could anchor itself in the membrane or the juxtamembrane region, interact with the receptor and disrupt the conformation needed

for receptor activation by interacting both with the ECD of the protein and with the plasma membrane at a site remote from the peptide-binding domain. Interestingly, the crystal structure of the ECD of NPR-A has been studied [29]. The reported results documented similarities between the ANP receptor-binding domain and the bacterial periplasmic binding fold. One member of the periplasmic binding-protein family is the AmiC-AmiR complex [30]. This complex is modulated allosterically by the binding of amides. When acetamide binds to the complex, motion occurs in the hinge region which causes some re-orientation of the two subdomains. This model might also be applied to the ANP receptor, which could be modulated allosterically by HS-142-1. The binding of the allotropic effector to the NPR-A receptor could cause a motion that would favour the dissociation of ANP. Indeed, Garcia and co-workers [31] have recently reported on the crystal structure of a natriuretic peptide bound to the clearance-type receptor (NPR-C). The results document the interaction of asparagine-linked oligosaccharide with the hinge region of the receptor and the disruption of that interaction upon natriuretic peptide binding. Therefore, these results could illustrate a potential mechanism of allosteric modulation of polysaccharides, i.e. HS-142-1. Further studies will be needed to clarify the site of interaction of HS-142-1 on NPRs.

We would like to thank S. Nakanishi, Kyowa Hakko Kogyo Co., Machida-shi, Tokyo, Japan, for kindly providing us with HS-142-1. We would also like to thank Mr Normand McNicoll for his helping hand and good advice. This work was supported by grants MT-15165 and MT-13753 from the Medical Research Council of Canada. A.D.L. is the recipient of a research chair in pharmacology from Merck Frosst Canada.

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Received 14 August 2001/23 October 2001; accepted 4 December 2001