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Inhibition by HS-142-1, a Novel Nonpeptide Atrial Natriuretic Peptide Antagonist of Microbial Origin, of Atrial Natriuretic Peptide-Induced Relaxation of Isolated Rabbit Aorta through the Blockade of Guanylyl Cyclase-Linked Receptors

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SUMMARY

HS-142-1, a specific nonpeptide antagonist for the atrial natriuretic peptide (ANP) receptor, equally blocked rat ANP (rANP)-, porcine brain natriuretic peptide-, or porcine C-type natriuretic peptide-stimulated GMP production in cultured bovine aortic smooth muscle (BASM) and bovine aortic endothelial (BAE) cells in a concentration-dependent fashion, at concentrations of 1-300 μg/ml. But, even at 300 μg/ml, HS-142-1 only weakly inhibited the specific binding of ^{125}I-rANP to the BASM and BAE cells, where only a small portion of the binding sites are linked to guanylyl cyclase. Further, with BAE cell membranes, HS-142-1 recognized only the 135-kDa ANP receptor, which is thought from ^{125}I-rANP affinity cross-linking studies to be the guanylyl cyclase-linked receptor. HS-142-1 also, if anything, inhibited the labeling of 135-kDa ANP receptors in the affinity cross-linking studies with BASM membranes, suggesting that a major portion of the 135-kDa ANP receptors are HS-142-1 insensitive and only a small portion of the 135-kDa ANP receptors are responsible for the blockade by HS-142-1 of GMP production in BASM cells. At a concentration of 100 μg/ml, HS-142-1 reversibly prevented ANP-induced relaxation of the isolated rabbit thoracic aorta induced to contract with 3×10^{-7} M phenylephrine, but not the relaxation induced by sodium nitroprusside, isoproterenol, or papaverine. These results suggest that HS-142-1 specifically inhibits natriuretic peptide-induced vasorelaxation through the blockade of guanylyl cyclase-linked natriuretic peptide receptors. HS-142-1 thus will be a powerful tool for understanding the physiological roles, in vasculature, of natriuretic peptides, which contribute to the homeostasis of blood pressure and intravascular volume.

The family of natriuretic peptides (ANP, BNP, and CNP) are circulating hormones that regulate body fluid volume and blood pressure (1-6). It is generally assumed that these effects are mediated in part by their vasorelaxant actions (7, 8). Although the intracellular actions through which the natriuretic peptides elicit their diverse biological effects are not known, the action of the peptide hormones is initiated by their binding to the cell membrane of target tissues or cells (9, 10).

At least two functionally and structurally distinct receptors, guanylyl cyclase-linked and guanylyl cyclase-free receptors, with molecular masses of 120-140 kDa and 60-70 kDa, respectively, are present in vascular smooth muscle and endothelial cells, as in other tissues (11-15). Guanylyl cyclase-linked receptor, which is presumed to be the biologically active receptor, contains a guanylyl cyclase domain in its structure (13, 16) and binding of the peptide hormone to an extracellular ligand-binding domain is directly associated with a significant increase in intracellular cGMP accumulation via an activation of the linked guanylyl cyclase (14, 16, 17). The cloning and expression of cDNA encoding a mammalian guanylyl cyclase-linked natriuretic peptide receptor have shown there are two different subtypes of the receptor, A-type (ANP-A or GC-A) and B-type (ANP-B or GC-B) receptors (18-20). Suga *et al.* (21) recently reported that the majority of the guanylyl cyclase-linked receptors expressed in rat aortic smooth muscle and bovine endothelial cells are classified as ANP-B (or GC-B) and ANP-A (or GC-A), respectively. However, recent studies concerning the natriuretic peptides and their receptors have revealed the quite complex nature of the relationships between multiple ligands and multiple receptor subtypes (21, 22). On the other hand,

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ABBREVIATIONS: ANP, atrial natriuretic peptide; BASM, bovine aortic smooth muscle; BAE, bovine aortic endothelial; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; AP-1, atriopeptin I; SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SNP, sodium nitroprusside; ISO, isoproterenol; rBNP, rat brain natriuretic peptide; hBNP, human brain natriuretic peptide; pBNP, porcine brain natriuretic peptide; hANP, human atrial natriuretic peptide; rANP, rat atrial natriuretic peptide; pCNP, porcine C-type natriuretic peptide.

guanylyl cyclase-free receptor (C receptor) is apparently not coupled to guanylyl cyclase and is thought to be specific for storage or clearance of natriuretic peptides, serving as a hormonal buffer system to regulate the plasma level of the peptide hormones (23–25).

Receptor antagonists have traditionally provided powerful tools for helping to elucidate the physiological role of ligands. In the case of the natriuretic peptides, due to the lack of a specific antagonist the true physiological and pathophysiological functions of the peptide hormones are still unclear (26, 27); the development of a specific nonpeptide antagonist for the functional natriuretic peptide receptor would be very useful (5).

Recently, we isolated a novel microbial product, HS-142-1, from the culture broth of *Aureobasidium* sp. (28, 29). HS-142-1 has been proven to be such a specific antagonist for the ANP receptor (28–34). Because of our previous observations (31) that HS-142-1 blocks hypotension as well as diuresis and natriuresis elicited by exogenous ANP in anesthetized rats, it was of interest to determine whether HS-142-1 inhibits the relaxation of isolated rabbit aorta and directly interacts with the guanylyl cyclase-linked receptors of cultured vascular smooth muscle cells. In this paper, we present our results with this compound and detail the intriguing observation that this compound specifically inhibits natriuretic peptide-induced relaxation of aorta through the blockade of guanylyl cyclase-linked ntriuretic peptide receptors. Some of the results presented in this paper have been published in abstract form (35, 36).

Experimental Procedures

Materials. DMEM, penicillin/streptomycin, glutamine, Eagle's minimum essential medium, and Hanks' balanced salt slution were purchased from GIBCO. Fetal calf serum was purchased from Hyclone Laboratories. Other media for cell culture were obtained from Nissui (Tokyo, Japan). Tissue culture dishes were from Corning. Reagents for electrophoresis and molecular standards, 3-isobutyl-1-methylxanthine, L-phenylephrine hydrochloride, L-isoproterenol-D-bitartarate, and AP-1 were purchased from Sigma Chemical Co. rANP, hANP, rBNP, pBNP, hBNP, and pCNP were purchased from the Peptide Institute, Inc. (Osaka, Japan). Purified Triton X-100 and DSS were purchased from Pierce Chemical Co. SNP and papaverine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). (3-[^{125}I]Iodotyrosyl-28)-rANP and ^{125}I-hANP were purchased from Amersham and New England Nuclear, respectively. Radioimmunoassay kits for cGMP measurements were the products of Yamasa Shoyu (Chiba, Japan). BASM and rat aortic smooth muscle cells and BAE cells were prepared according to the methods of Ross (37) and Longenecker *et al.* (38), respectively. HS-142-1 was purified from the fermentation broth of *Aureobasidium* sp. KAC-2383 in our laboratories, as described (29). All other reagents were of analytical grade.

Cell culture. BASM cells were prepared from explants of thoracic aortas obtained from a local slaughterhouse, according to the method of Ross (37). Smooth muscle cells were also prepared from rat aorta, as from bovine aorta. The cultured smooth muscle cells showed a so-called hill-and-valley structure characteristic of smooth muscle cells. The prepared BAE cells showed a typical cobblestone appearance (39) and were characterized by the ability to incorporate acetylated low density lipoprotein (40). Smooth muscle and endothelial cells were maintained in DMEM, pH 7.2, supplemented with 10% fetal calf serum, 2 mM glutamate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml gentamicin. In either case, after reaching confluence the cells were used for experiments at passages 3–8.

cGMP determination. cGMP assays were performed according to the method of Leitman *et al.* (14). BASM and BAE cells in six-well multiwell plates were used when they reached confluence. After the culture medium was removed, the cells were washed twice with DMEM containing 10 mM HEPES. The cells were then incubated for 10 min at 37° with DMEM containing 10 mM HEPES, 0.1% bovine serum albumin, and 0.5 mM 3-isobutyl-1-methylxanthine, in the presence or absence of varying concentrations of HS-142-1 (0.1–100 μg/ml). ANP, BNP, or CNP at 10^{-10} to 10^{-6} M was then added, and the incubation was continued for additional 5 min. After termination of the incubation by acidification with trichloroacetic acid to a final concentration of 6%, the cell contents from each dish were collected by scraping with a rubber policeman. The suspension was frozen and thawed and then agitated on a Vortex mixer. The suspension was immediately centrifuged at 3000 × *g* for 10 min, and the supernatants were extracted four times with water-saturated ether and lyophilized. Each lyophilized sample was dissolved in water and the cGMP was measured by using a Yamasa cGMP radioimmunoassay kit.

Binding assay. Binding assays were performed according to the method of Takayanagi *et al.* (13). BASM and BAE cells were grown to confluence in 24-well multiwell plates. The cells were washed twice with 0.5 ml of buffer A (Hanks' balanced salt solution, pH 7.4, containing 0.2% bovine serum albumin and 10 mM HEPES). The cells were then incubated at 4° for 30 min with 0.5 ml of buffer A in the presence or absence of varying concentrations of HS-142-1, unlabeled rANP, or AP-1. ^{125}I-rANP (specific activity, 2000 Ci/mmol) at 3×10^{-11} M was then added, and the incubation was continued for an additional 60 min. After incubation, the cultures were washed three times with 1 ml of HBSS containing 0.1% bovine serum albumin. The cells were solubilized with 0.5 ml of 0.5 N sodium hydroxide, and radioactivity was determined. Nonspecific binding to cells was determined by incubating parallel culture dishes with ^{125}I-rANP in the presence of 1 μM unlabeled rANP.

Affinity cross-linking study. Affinity cross-linking was performed according to the methods of Martin *et al.* (41) and Fethiere *et al.* (42), with some modifications. Confluent BASM and BAE cells in 150-cm^2 tissue culture flasks were washed three times with Dulbecco's phosphate-buffered saline and mechanically scraped. The cells were then centrifuged at 200 × *g* for 5 min. To prepare mambranes, the BASM or BAE cells were suspended in an ice-cold buffer supplemented with 1 mM $NaHCO_3$, 1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin and were homogenized with a Polytron. To the homogenate was added an equal volume of buffer B (50 mM Tris·HCl buffer, pH 7.4, containing 0.1 mM EDTA, 1 mM $MgCl_2$, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and the mixture was centrifuged at 250 × *g* for 10 min. The supernatant was centrifuged again at 30,000 × *g* for 15 min. The pellet was taken up in buffer B and gently homogenized. The preparation was centrifuged at 30,000 × *g* for 15 min. The final pellet was suspended in 50 mM Tris·HCl buffer, pH 7.4, containing 250 mM sucrose, 0.1 mM EDTA, and 1 mM $MgCl_2$. The suspension was frozen and stored at −80°. The cell membranes thus obtained were preincubated for 20 min at room temperature in the absence or presence of varying concentrations of unlabeled rANP, AP-1, or HS-142-1. ^{125}I-rANP (4×10^{-10} M) was then added and incubation was continued for an additional 120 min. Membranes were then incubated with 0.2 mM DSS for 20 min at room temperature. The reaction was stopped by the addition of an equal volume of quenching buffer (1 M Tris·HCl, pH 6.8, 400 mM EDTA), followed by centrifugation at 18,000 × *g* for 10 min. The pellets were resuspended in SDS sample buffer (62 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) in the presence of 5% 2-mercaptoethanol. After this was boiled for 5 min, the labeled sample mixtures were applied to an SDS-polyacrylamide gel (7.5%) and electrophoresed with prestained markers according to the method of Laemmli (43). The dried gels were exposed to Fuji X-ray RX50 film at −80° for 5 days.

Vasorelaxant assays. The descending thoracic aorta was removed from male Japanese White rabbits (2–4 kg) (Sankyo Labo Service Co., Tokyo, Japan) that had been sacrificed by exsanguination via the

carotid artery. The thoracic aorta was cleaned of adherent fat and connective tissue and then cut into 3–4 mm ring segments. The rings were mounted vertically between stainless steel hooks, in 10-ml organ chambers containing Krebs-Henseleit buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose). The Krebs solution was maintained at 37° and aerated continuously with 95%/$O_2$5% CO_2. Each ring was attached to the lever of a force-displacement transducer (TB-612T, Nihon Koden, Tokyo, Japan, or UM-203, Iwashiya Kishimoto Medical Instrument, Inc., Kyoto, Japan) for isometric tension recording. Aortic rings were equilibrated for 60 min or more under an initial resting tension of 2 g and the buffer was changed every 15 min. After equilibration, contraction of the aortic rings was induced with 3×10^{-7} M phenylephrine and then one of the following relaxants was added to the chambers: hANP or pBNP (3×10^{-11} to 10^{-7} M), SNP (10^{-8} to 10^{-7} M), ISO (10^{-7} M or 3×10^{-7} M), or papaverine (10^{-5} M or 3×10^{-5} M), in the absence or presence of each concentration of HS-142–1 (30–300 μg/ml). Relaxations were calculated as percent reductions in the phenylephrine-induced contraction. In order to examine the reversibility of the inhibition by HS-142–1 of hANP-induced relaxation, the aortic rings initially contracted with phenylephrine were relaxed with hANP and treated with HS-142–1 at different concentrations, and then hANP was added to the organ chamber once again.

Statistical analysis. All data were expressed as the mean ± standard deviation. The data were evaluated with Student's unpaired *t* test. A level of $p < 0.05$ was considered to be significant.

Results

cGMP studies. We examined whether HS-142–1 antagonizes the function of the guanylyl cyclase-linked receptor in cultured vascular BASM and BAE cells. rANP, pBNP, and pCNP equally stimulated the cGMP production by BASM cells in a concentration-dependent manner at concentrations ranging from 10^{-9} M to 10^{-6} M, as reported (14, 44). rANP at 10^{-7} M increased cGMP production about 30–80-fold above the basal value seen in the absence of the peptide in BASM cells (Fig. 1A). pBNP and pCNP stimulated cGMP accumulation with potency almost equal to that of rANP (Fig. 1). HS-142–1 was equally efficacious in inhibiting the increase in cGMP production stimulated by 10^{-7} M rANP, pBNP, or pCNP in a concentration-dependent manner at concentrations ranging from 1 to 300 μg/ml (2.5×10^{-7} to 7.5×10^{-5} M, given that the mean molecular weight is 4000; see Ref. 28) (Fig. 1); the IC_{50} value was calculated to be approximately 3 μg/ml in all cases. At a concentration of 300 μg/ml, HS-142–1 almost completely abolished all natriuretic peptide-induced response in BASM cells (Fig. 1). We next performed a homologous assay with endogenous ANP in combination with primary smooth muscle cells of the same species. HS-142–1 inhibited rANP-stimulated cGMP production in the smooth muscle cells prepared from rat aorta in a dose-dependent manner at concentrations similar to those that inhibited rANP-induced cGMP production in BASM cells; HS-142–1 at 300 μg/ml completely inhibited the stimulation by rANP in rat smooth muscle cells. Because the biological action and molecular form of BNP are known to be markedly divergent among species (45–47), we examined the effects of rBNP and hBNP on cGMP production in BASM cells and compared them with those of rANP. pBNP, rBNP, and hBNP at 10^{-7} M increased cGMP 66-, 24-, and 47-fold above the basal value seen in the absence of the peptides in BASM cells. Although a marked species difference exists in the potency for cGMP production among BNPs in BASM cells, HS-142–1 completely inhibited the stimulation by rBNP or hBNP at concentrations similar to those that inhibited rANP-induced cGMP production (data not shown).

In BAE cells rANP, pBNP, and pCNP caused dose-dependent cGMP accumulation, as in BASM cells (Fig. 2). rANP was equipotent to pBNP in cGMP production in BAE cells. However, it is noteworthy that rANP and pBNP were 60–70-fold more potent in stimulating cGMP formation than was pCNP

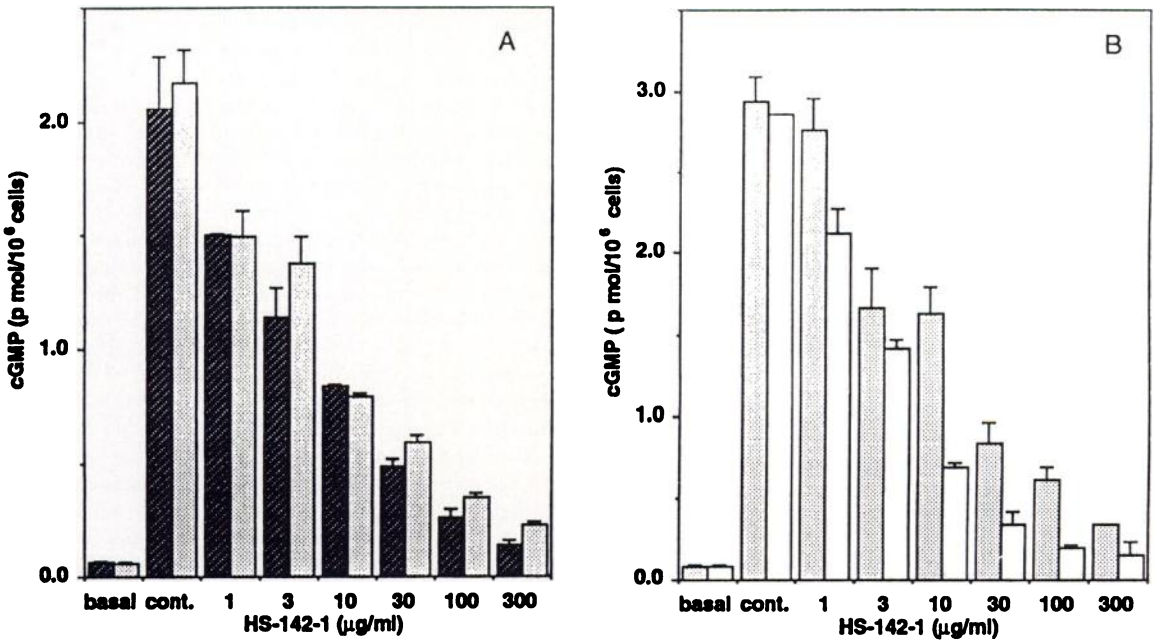

Fig. 1. Effects of HS-142–1 on the elevation of cGMP content induced by rANP, pBNP, or pCNP in BASM cells. cGMP production elicited by 10^{-7} M rANP (▨ in A), pBNP (□ in A and B), or pCNP (□ in B) was measured in the presence of varying concentrations of HS-142–1. cGMP content was measured 5 min after the stimulation with natriuretic peptides, as described in Experimental Procedures. Data are the means ± standard deviations (error bars) for an experiment carried out in triplicate. Basal value seen in the absence of natriuretic peptides is also indicated.

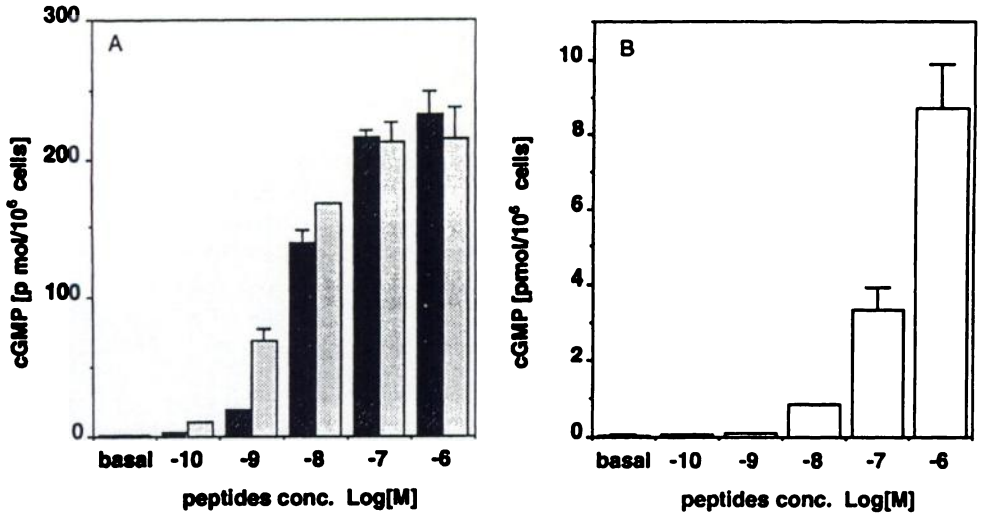

Fig. 2. Concentration-dependent cGMP accumulation induced by natriuretic peptides in BAE cells. Cells were incubated in the presence of different concentration of rANP (▨ in A), pBNP (□ in A), or pCNP (□ in B) for 5 min as described in Experimental Procedures. Data are the means ± standard deviations (error bars) for an experiment carried out in triplicate. Basal value seen in the absence of natriuretic peptides is also indicated.

(Fig. 2). Another important point is that rANP and pBNP increased cGMP levels in BAE cells by approximately 2 orders of magnitude more than seen in BASM cells, because a greater portion of ANP receptors are coupled to particulate guanylyl cyclase in BAE cells, compared with BASM cells (14). Again, HS-142-1 attenuated the increase in cGMP levels induced by 10^{-7} M rANP or pCNP in a dose-dependent manner at concentrations similar to those that inhibited the cGMP production in BASM cells (Fig. 3); IC_{50} values were calculated to be approximately 27 and 1.5 μg/ml, respectively. HS-142-1 also inhibited pBNP-induced cGMP production with an IC_{50} value almost equal to that for rANP-induced production (data not shown). HS-142-1 alone at concentrations up to 300 μg/ml had no effect on the intracellular accumulation of cGMP in BASM and BAE cells (data not shown).

Binding studies. Effects of unlabeled rANP, AP-1, and HS-142-1 on the specific binding of ^{125}I-rANP to the BASM and BAE cells were examined. Unlabeled rANP displaced ^{125}I-rANP binding to the BASM and BAE cells in a dose-dependent manner, with IC_{50} values of 5×10^{-10} M and 3×10^{-10} M, respectively; rANP at 10^{-7} M to 10^{-6} M displaced ^{125}I-rANP binding completely in both cell types (Figs. 4 and 5). In order to determine a possible binding specificity of HS-142-1 for the guanylyl cyclase-linked receptor or guanylyl cyclase-free receptor, we compared its effect with that of AP-1, which selectively binds to guanylyl cyclase-free receptors at rather lower concentrations (30, 48).

In BASM cells, AP-1 inhibited specific binding of ^{125}I-rANP up to 90% in a concentration-dependent manner, with an IC_{50} value of 4×10^{-9} M (Fig. 4); AP-1 at concentrations of 10^{-7} and 10^{-6} M almost completely inhibited the specific binding of ^{125}I-rANP, possibly due to its low specificity at these concentrations

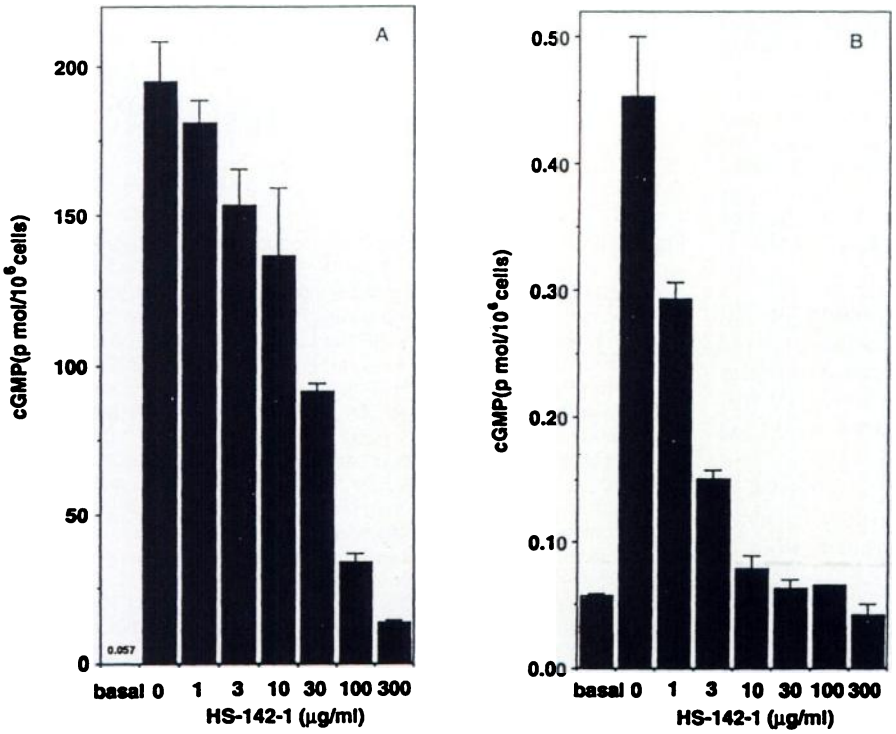

Fig. 3. Effects of HS-142-1 on the elevation of cGMP content induced by rANP, pBNP, or pCNP in BAE cells. cGMP production elicited by 10^{-7} M rANP (A) or pCNP (B) was measured in the presence of varying concentrations of HS-142-1. cGMP content was measured 5 min after the stimulation with natriuretic peptides, as described in Experimental Procedures. Data are the means ± standard deviations (error bars) for an experiment carried out in triplicate. Basal value seen in the absence of natriuretic peptides is also indicated.

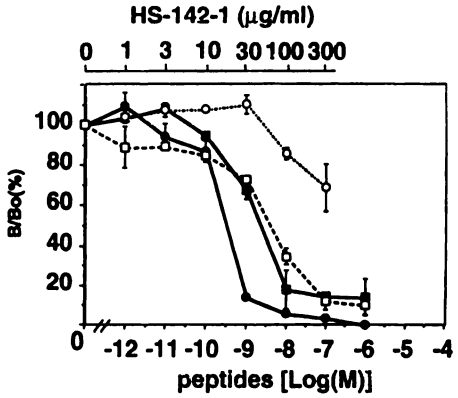

Fig. 4. Effects of rANP, AP-1, and HS-142–1 on the specific binding of ^{125}I-rANP to BASM cells. Competitive displacement of ^{125}I-rANP binding to BASM cells by rANP (●), AP-1 (■), HS-142–1 (○), or AP-1 in combination with 300 μg/ml HS-142–1 (□). Inhibition is expressed as the ratio of the specific binding of ^{125}I-rANP. *Bars*, standard deviation. Unlabeled rANP (1 μM) was used to determine the nonspecific binding, which was <10% of total ^{125}I-rANP binding at 3×10^{-11} M. Scatchard analysis of saturation binding data revealed that the equilibrium dissociation constant (K_d) of ^{125}I-rANP was 7.2×10^{-11} M and the maximal number of binding sites (B_{max}) was 129 fmol/10^6 cells. Binding assay was done as described in Experimental Procedures.

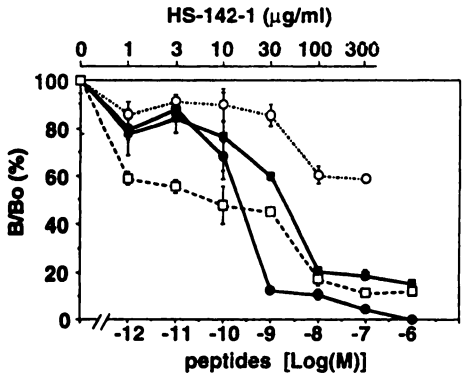

Fig. 5. Effects of rANP, AP-1, and HS-142–1 on the specific binding of ^{125}I-rANP to BAE cells. Competitive displacement of ^{125}I-rANP binding to BAE cells by rANP (●), AP-1 (■), HS-142–1 (○), or AP-1 in combination with 300 μg/ml HS-142–1 (□). Inhibition is expressed as the ratio of the specific binding of ^{125}I-rANP. *Bars*, standard deviation. Unlabeled rANP (1 μM) was used to determine the nonspecific binding, which was <10% of total ^{125}I-rANP binding at 3×10^{-11} M. Scatchard analysis of saturation binding data revealed that the equilibrium dissociation constant (K_d) of ^{125}I-rANP was 1.2×10^{-11} M and the maximal number of binding sites (B_{max}) was 13.5 fmol/10^6 cells. Binding assay was done as described in Experimental Procedures.

(30). HS-142–1 had no effect on the specific binding of ^{125}I-rANP to BASM cells at concentrations less than 30 μg/ml and weakly inhibited binding up to 20–30% at higher concentrations, (100–300 μg/ml). AP-1, in the presence of 300 μg/ml HS-142–1, displaced the specific binding of ^{125}I-rANP to BASM cells almost completely, as did AP-1 alone (Fig. 4).

In BAE cells, similar results were obtained as observed in BASM cells, except that HS-142–1 was more effective in inhibiting the specific binding of ^{125}I-rANP to BAE cells than in inhibiting the specific binding of ^{125}I-rANP to BASM cells; HS-142–1 inhibited binding up to 40% at higher concentrations (100–300 μg/ml) (Fig. 5). These results show that BASM and, to a lesser extent, BAE cells express guanylyl cyclase-free receptor (C-type receptor) predominantly and HS-142–1 selectively recognizes guanylyl cyclase-linked receptor.

Affinity cross-linking studies. To establish more conclusively that HS-142–1 selectively recognizes guanylyl cyclase-linked ANP receptor, ^{125}I-rANP affinity cross-linking studies were performed in the presence of varying concentrations of HS-142–1 and/or AP-1 with membranes prepared from BASM and BAE cells. Two major protein bands, with apparent molecular masses of 135 kDa and 60 kDa, were specifically labeled under reducing conditions in membranes from BASM cells (Fig. 6A, *lane 1*). The labeling of both bands was abolished by

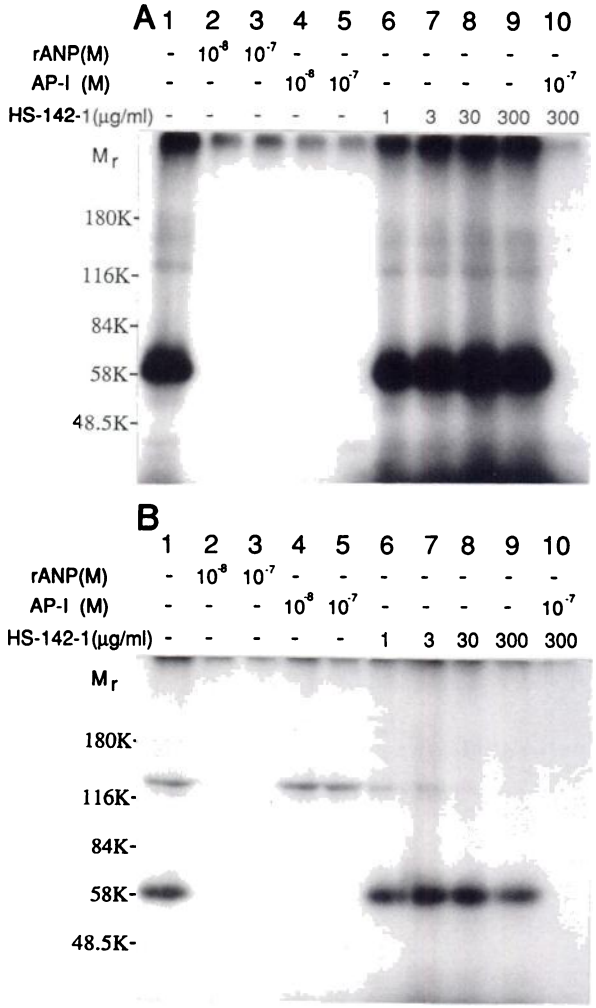

Fig. 6. Specific recognition by HS-142–1 of guanylyl cyclase-linked ANP receptor. A, Affinity cross-linking of ^{125}I-rANP to BASM cell membranes. Solubilized membranes (600 μg) were incubated with 5 nM ^{125}I-hANP in the absence of competing ligand (*lane 1*) or in the presence of different concentrations of nonlabeled hANP (*lane 2*, 1×10^{-8} M; *lane 3*, 1×10^{-7} M), different concentrations of AP-1 (*lane 4*, 1×10^{-8} M; *lane 5*, 1×10^{-7} M), different concentrations of HS-142–1 (*lane 6*, 1.0 μg/ml; *lane 7*, 3.0 μg/ml; *lane 8*, 30 μg/ml; *lane 9*, 300 μg/ml), or 300 μg/ml HS-142–1 in combination with 10^{-7} M AP-1 (*lane 10*) for 60 min at 0°. The cross-linking reaction was done by incubation of the membranes with DSS for 20 min at 5°, after which the reaction was quenched and the membranes were centrifuged. The washed membranes were solubilized in the sample buffer containing 5% 2-mercaptoethanol. The covalent incorporation of radioactivity into protein was monitored by SDS-gel electrophoresis and autoradiography, as described in Experimental Procedures. Standard molecular weight marker proteins are α_2-macroglobulin (180,000), β-galactosidase (116,000), fructose-6-phosphate kinase (84,000), pyruvate kinase (58,000), and fumarase (48,500). B, Affinity cross-linking of ^{125}I-rANP to BAE cell membranes. All procedures were the same as described for A except that BAE cell membranes were used in place of BASM cell membranes.

unlabeled rANP at 10^{-7} M and 10^{-8} M (Fig. 6A, *lanes 2* and *3*). The 135-kDa band should be derived from the labeling of the guanylyl cyclase-linked ANP receptor and the 60-kDa band should be derived from the C-type ANP receptor, as reported previously (13). AP-1 clearly inhibited the labeling of the 60-kDa band at concentrations of 10^{-7} and 10^{-8} M; at these doses, AP-1 also inhibited the labeling of the 135-kDa band, due to the lack of selectivity (Fig. 6A, *lanes 4* and *5*), as observed in the previous studies with adrenocortical membranes (30). AP-1 at 10^{-7} M in combination with 300 μg/ml HS-142-1 inhibited the labeling of the two bands, as did AP-1 alone (Fig. 6A, *lane 10*). Under identical conditions, unexpectedly, HS-142-1 only slightly inhibited the labeling of the 135-kDa band, sparing the labeling of the 60-kDa band at concentrations ranging from 1 to 300 μg/ml (Fig. 6A, *lanes 6–9*). This phenomenon was consistent and reproducible in four independent experiments with different batches of cultured cells.

In the case of membranes from BAE cells, the major protein bands with apparent molecular masses of 135 kDa and 60 kDa were also specifically labeled, as seen with membranes from BASM cells (Fig. 6B, *lane 1*). With BAE cell membranes, the 135-kDa band was more intensely labeled, with the 60-kDa band being labeled less intensely than with BASM cell membranes. The labeling of both bands was again completely abolished by unlabeled rANP at 10^{-7} M and 10^{-8} M (Fig. 6B, *lanes 2* and *3*). AP-1 selectively diminished the labeling of the 60-kDa band at concentrations of 10^{-7} and 10^{-8} M (Fig. 6B, *lanes 4* and *5*); AP-1 apparently inhibited the labeling of the 135-kDa band to a small degree in spite of its low specificity at these doses, because a greater portion of ANP receptors are coupled to particulate guanylyl cyclase in BAE cells, compared with BASM cells (14). In contrast to the studies with BASM cell membranes, HS-142-1 specifically abolished the labeling of the 135-kDa band in a concentration-dependent manner at concentrations ranging from 1 to 300 μg/ml but had no effect on the labeling of the 60-kDa band (Fig. 6B, *lanes 6–9*). The labeling of the two bands was abolished when 10^{-7} M AP-1 and 300 μg/ml HS-142-1 were added in combination (Fig. 6B, *lane 10*). This result in ^{125}I-rANP affinity cross-linking studies with membranes from BAE cells is consistent with our previous findings with adrenocortical and lung membranes (30). These results demonstrate that HS-142-1 specifically recognizes the 135-kDa band, but to different extents, in BASM and BAE cells; a major portion of the 135-kDa polypeptides are HS-142-1 insensitive and only a small portion of the 135-kDa ANP receptors are responsible for the blockade by HS-142-1 of cGMP production in BASM cells.

Specific antagonistic effects of HS-142-1 on isolated rabbit aortic rings. We tested the effects of HS-142-1 on hANP- or pBNP-induced relaxation of the isolated rabbit thoracic aorta induced to contract with phenylephrine (Figs. 7 and 8). hANP relaxed phenylephrine-contracted rabbit aortic rings in a concentration-dependent fashion. Compared with control, HS-142-1, in a concentration range of 30 to 300 μg/ml, caused a concentration-related parallel rightward shift in the concentration response of hANP but did not alter the maximal responses to hANP (Fig. 7). We next examined whether HS-142-1 also inhibits pBNP-induced vasorelaxation, because BNP has been shown to induce the same pharmacological responses as ANP (3). pBNP relaxed phenylephrine-contracted rings as did hANP, and HS-142-1 inhibited the

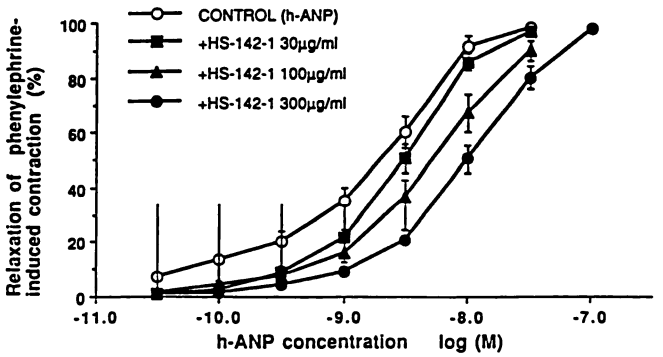

Fig. 7. Effect of HS-142-1 on vasorelaxation induced by hANP. hANP was cumulatively added to rabbit isolated thoracic aorta that had been previously induced to contract with phenylephrine (3×10^{-7} M), in the absence (○) or presence of 30 (■), 100 (▲), or 300 (●) μg/ml HS-142-1. Results are expressed as mean ± standard error (five to eight experiments).

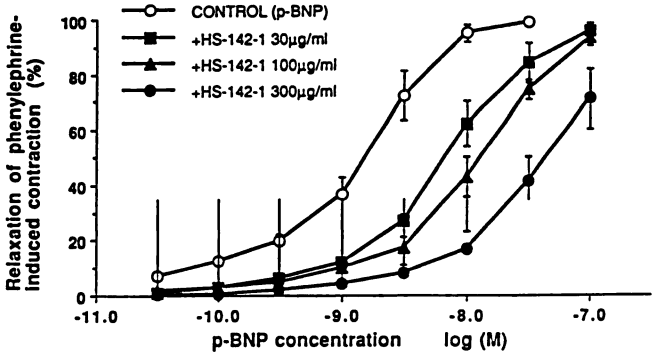

Fig. 8. Effect of HS-142-1 on vasorelaxation induced by pBNP. Experimental conditions were the same as described in the legend to Fig. 7 except that pBNP was used in place of hANP (four or five experiments).

relaxation in a concentration-dependent manner, as did hANP (Fig. 8). It is noteworthy that HS-142-1 is severalfold more potent in inhibiting the pBNP-induced relaxation than in blocking the hANP-induced relaxation.

That the inhibition by HS-142-1 (3–300 μg/ml) of hANP-induced relaxation of rabbit aortic rings was reversible and that the aortic rings were not damaged by HS-142-1 were shown by the ready reversibility of the effect of HS-142-1 at these concentrations. After the determination of contractile response to 3×10^{-7} M phenylephrine and subsequent relaxation of the ring by 3×10^{-8} M hANP, HS-142-1 was added stepwise to the organ chamber, followed by the readdition of hANP. HS-142-1 reversed the hANP-induced relaxation in a concentration-dependent manner, and hANP, even when added after the HS-142-1 treatment, led to an immediate relaxation of the ring. Residual contraction was relieved by washing of the ring (Fig. 9).

The action of HS-142-1 on the relaxation of aortic rings previously induced to contract with phenylephrine was specific for the relaxation elicited by treatment with natriuretic peptides. Other relaxants, such as SNP, ISO, and papaverine, induced concentration-dependent relaxation of precontracted rings. Pretreatment of aortic rings for 5 min with HS-142-1 at 100 μg/ml had no effect on relaxation induced by SNP, ISO, or papaverine at different concentrations (Table 1). HS-142-1 alone showed no contractile or vasorelaxant effects on rabbit aortic rings (data not shown). In addition, HS-142-1 had no effects on relaxation induced by either forskolin (3×10^{-6} M

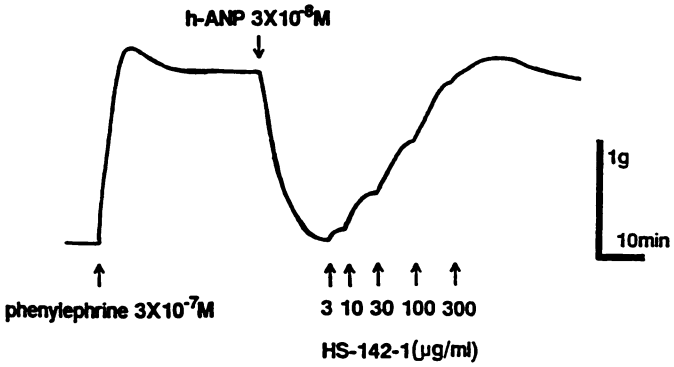

Fig. 9. Typical recording showing the reversibility of the inhibition by HS-142–1 of hANP-induced relaxation. After the determination of the contractile response to 3×10^{-7} M phenylephrine and subsequent relaxation of the rabbit aortic ring with 3×10^{-8} M hANP, HS-142–1 was added to the organ chamber at different concentrations (3–300 μg/ml). hANP was then added again to ensure the reversibility of the inhibition by HS-142–1.

TABLE 1

Effect of HS-142-1 on vasorelaxation induced by various relaxants

Relaxants were cumulatively added to rabbit isolated thoracic aorta that had been previously induced to contract with phenylephrine (2×10^{-7} M), in the presence or absence of HS-142-1 (100 μg/ml). Results are expressed as mean ± standard error (five experiments).

Agent	Relaxation	
	Absence of HS-142-1	Presence of HS-142-1
M	%	
SN		
1×10^{-8}	14.7 ± 1.3	14.2 ± 1.2
3×10^{-8}	33.8 ± 3.5	33.2 ± 2.8
1×10^{-7}	60.3 ± 4.7	63.1 ± 4.4
ISO		
1×10^{-7}	42.5 ± 11.9	44.4 ± 10.3
3×10^{-7}	53.1 ± 12.9	54.8 ± 11.4
Papaverine		
1×10^{-5}	25.3 ± 1.0	26.5 ± 3.3
3×10^{-5}	71.7 ± 5.7	71.9 ± 6.5

and 10^{-5} M) or adenylyl cyclase activator or on contraction induced by angiotensin II (10^{-8} M and 3×10^{-8} M), norepinephrine (10^{-8} M to 3×10^{-7} M), or KCl (20 mM and 40 mM) in aortic rings (data not shown).

In conclusion, HS-142–1 specifically recognizes the guanylyl cyclase-linked receptor and specifically and competitively antagonizes natriuretic peptide-induced vasorelaxation through the blockade of cGMP production.

Discussion

In the present study, we examined whether HS-142–1 inhibits the relaxation of isolated rabbit aorta and also inhibits cGMP accumulation by cultured vascular smooth muscle cells in response to natriuretic peptides. Although the functional consequences of ANP receptor activation in endothelial cells are a matter of speculation and study, we also used cultured vascular endothelial cells in the present study, because the endothelial cells have been shown to be rich in binding sites and to exhibit cGMP production in response to ANP (49–52). rANP, pBNP, and pCNP significantly elevate intracellular cGMP levels in cultured BASM and BAE cells; as expected, these stimulations were attenuated in a concentration-dependent fashion by HS-142–1 at concentrations ranging from 1 to 300 μg/ml. It was also confirmed that HS-142–1 recognizes only the guanylyl cyclase-linked 130-kDa receptor in binding and affinity cross-linking studies with the two types of intact cells and their membrane preparations, respectively. Further, we found that HS-142–1 does not inhibits the cGMP accumulation induced by SNP, which is known to be an activator of soluble guanylyl cyclase distinct from the ANP receptor-linked form of guanylyl cyclase (53). Taken together, these findings indicate that HS-142–1 specifically blocks natriuretic peptide-induced cGMP production through the specific blockade of the guanylyl cyclase-linked receptor.

HS-142–1 specifically inhibits vasorelaxation induced by hANP or pBNP. In rabbit aortic rings, pretreatment with HS-142–1 at 30–100 μg/ml, which is in the same concentration range as that required for the inhibition of cGMP production by BASM and BAE cells, significantly inhibited the vasorelaxation elicited by cumulative treatment with hANP or pBNP. On the other hand, HS-142–1 had no effect on vasorelaxation of the precontracted aortic rings induced by SNP, ISO, or papaverine, which cause relaxation through mechanisms different from that by which ANP acts. ISO binds to the β-adrenergic receptor. Papaverine causes vasorelaxation via rather nonspecific pathways, such as blockade of Ca^{2+} entry, inhibition of cAMP-dependent phosphodiesterase, or interference with the electron transport system. Accordingly, the actions of HS-142–1 on the relaxation of aortic rings are specific for relaxation elicited by treatment with natriuretic peptides. Thus, it is clear that HS-142–1 specifically antagonizes vasorelaxation through the specific blockade of guanylyl cyclase-linked receptor. The data presented here probably can also explain the attenuation by HS-142–1 of the hypotensive response induced by exogenously injected ANP *in vivo* (31). These results are consistent with our previous observations in which HS-142–1 was proven to recognize specifically the guanylyl cyclase-linked ANP receptor and to antagonize the actions of ANP both *in vitro* and *in vivo* (28–34).

HS-142–1 inhibits equally the rANP- and BNP-induced cGMP production by BASM cells. But, as shown in Figs. 8 and 9, HS-142–1 showed a more potent inhibitory activity against pBNP-induced relaxation than against relaxation induced by hANP, although pBNP and hANP induced vasorelaxation with almost equal potency in aortic rings. The reason for our failure to observe an equal inhibitory activity of HS-142–1 against the responses induced by the two peptides is not clear. To clarify this point further detailed experiments, including a homologous assay system with endogenous ligands and receptors of the same species, are needed.

Vascular smooth muscle and endothelial cells have been reported to have two types of receptors for natriuretic peptides. One type is coupled to particulate guanylyl cyclase, has a molecular mass of 120–140 kDa, and was designated the biologically active receptor. Thereafter, the presence of two subtypes of biologically active receptors, named the A-type (ANP-A or GC-A) and the B-type (ANP-B or GC-B), has been demonstrated (18, 19). The other type, named the C-type receptor, with a molecular mass of 60–70 kDa, is not coupled to guanylyl cyclase. The data presented in Figs. 4, 5, and 6 are compatible with the interpretation that a greater portion of ANP receptors are coupled to particulate guanylyl cyclase in BAE cells, compared with BASM cells. Indeed, rANP and pBNP increased cGMP level in BAE cells by approximately 2 orders of magni-

tude more than seen in BASM cells, as shown in Figs. 1 and 2. This is consistent with the previous observations reported by Leitman *et al.* (14). The rather weak inhibitory efficacy of HS-142-1 against rANP-induced cGMP production in BAE cells is possibly due to the presence of a large number of guanylyl cyclase-linked receptors in BAE cells, compared with BASM cells, as shown in Fig. 6.

In BASM cells, rANP, pBNP, and pCNP stimulated cGMP production to a similar degree; rANP and pBNP are 60–70-fold more potent than pCNP in BAE cells, as shown in Figs. 1 and 2. Recently, Koller *et al.* (22) and Suga *et al.* (21) revealed a ligand specificity of the two different subtypes of biologically active ANP receptors, ANP-A (or GC-A) and ANP-B (or GC-B) receptors. ANP and, to a lesser extent, BNP are efficient activators of ANP-A. ANP-B is activated most efficiently by CNP. BNP is also effective at stimulating this cyclase but is less potent. Thus, BASM and BAE cells have both ANP-A and ANP-B receptors, but the amount of ANP-A is much higher than that of ANP-B in BAE cells; BASM cells have almost equal amounts of ANP-A and ANP-B. Recently it has been reported that the majority of the functional receptors expressed in BASM cells are classified as ANP-B receptors, whereas almost all of the functional receptors expressed in BAE cells are classified as ANP-A receptors (21, 54). In order to determine the accurate proportions of these two receptor subtypes in BASM and BAE cells, further experiments, including studies with cells from different areas of the vasculature and with varying passage numbers of cultured cells, using the Northern blot technique, are needed.

In the present study, we used freshly prepared primary cells from vascular tissues instead of clonal cell lines, which have been used in our previous studies (32). The data obtained in the present studies with primary cells varied much more than those obtained in previous studies with the cell lines. The incompleteness of the inhibition by HS-142-1 or AP-1 observed in the binding and cGMP studies, as shown in Figs. 1, 3, 4, and 5, was not statistically significant, possibly because the experiments were performed with primary cells. Further, in the present study we chose concentrations of AP-1 based on the results obtained in the binding studies shown in Figs. 4 and 5; AP-1 clearly recognized guanylyl cyclase-free receptor at 10^{-7} M and 10^{-8} M and to a small degree at 10^{-9} M. However, whereas HS-142-1 did not lose its specificity at concentrations up to 300 μg/ml, AP-1 showed low specificity at 10^{-7} M and 10^{-8} M, as shown in Fig 6. We, therefore, presume that the reason for our failure to observe a correlation between the amount of ^{125}I-rANP binding resistant to displacement by HS-142-1 and that displaceable by AP-1 in the binding study is also due to the lack of specificity of AP-1. In any case, the results presented here are essentially consistent with our previous observations, in that HS-142-1 specifically recognized the guanylyl cyclase-linked ANP receptor *in vitro* (28, 30).

HS-142-1 is almost equally efficacious in inhibiting cGMP production by BASM and BAE cells stimulated by rANP, pBNP, or pCNP, suggesting that HS-142-1 recognizes ANP-A and ANP-B equally in the two different cell types. Similar results were obtained by using PC12 rat pheochromocytoma cells in combination with the three natriuretic peptides and HS-142-1 (32). In order to determine definitely the receptor subtype specificity of HS-142-1, more detailed experiments with cells that specifically express each subtype have to be undertaken. On the other hand, we showed, in the present affinity cross-linking studies with membranes prepared from BASM cells, that a major portion of the 135-kDa ANP receptors in BASM cells are HS-142-1 insensitive and a very small portion of the 135-kDa ANP receptors seem to be responsible for the blockade by HS-142-1 of cGMP production. Although the identity of the HS-142-1-insensitive ANP receptor is not yet known, it is noteworthy that this type of receptor is expressed only in BASM cells and not in BAE cells, bovine lung membranes, or bovine adrenal cortical membranes (30). Further, in our previous studies with NG108-15 mouse neuroblastoma-rat glioma hybridoma, HS-142-1 weakly inhibited the cGMP response induced by rANP or pBNP even at higher concentrations (32). The present results together with the previous observations suggest the possible existence of a novel subtype of ANP functional receptor, which is insensitive to HS-142-1, in BASM and NG108-15 cells. HS-142-1 does not have a general cytotoxic action in mice, as judged by the lack of acute toxicity of doses up to 10 mg/kg given intravenously in experiments not presented here, nor does it permanently damage the aortic rings, as shown by the ready reversibility of the inhibition by HS-142-1. These results show the usefulness of HS-142-1 as a natriuretic peptide antagonist both *in vivo* and *in vitro*.

In conclusion, HS-142-1 specifically recognizes the guanylyl cyclase-linked receptor in vascular cells and specifically and reversibly antagonizes natriuretic peptide-induced vasorelaxation through the blockade of cGMP production. HS-142-1 provides a powerful tool for elucidating the physiological and pathophysiological roles in vasculature of natriuretic peptides, which contribute to the homeostasis of blood pressure and intravascular volume. Further, HS-142-1 would be helpful for detecting a novel type of ANP receptor.

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