Role of guanylyl cyclase receptors for CNP in salt secretion by shark rectal gland

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Gunning, Mark, Richard J. Solomon, Franklin H. Epstein, and Patricio Silva. Role of guanylyl cyclase receptors for CNP in salt secretion by shark rectal gland. J. Physiol. 42: R1400-R1406, 1997.—The role of C-type natriuretic peptide (CNP) and its guanylyl cyclase-linked receptors in mediating salt secretion by the rectal gland of the spiny dogfish shark (Squalus acanthias) was investigated using HS-142–1, a competitive inhibitor of the binding of natriuretic peptides to their guanylyl cyclase receptors. CNP binds to receptors and activates guanylyl cyclase in rectal gland membranes in a way that is inhibited by HS-142–1. Guanylyl cyclase activation in rectal gland membranes is far more sensitive to CNP than to atrial natriuretic peptide, whereas the reverse is true for membranes derived from mammalian (rabbit) renal collecting duct cells. HS-142–1 inhibited the stimulatory effect of CNP on ouabain-inhibitable oxygen consumption by rectal gland tubules. In explanted rectal glands continuously perfused with blood from intact donor sharks, HS-142–1 inhibited the increase in salt secretion normally provoked by infusing isotonic saline solutions into the donor animal. These results strongly support the view that CNP released within the gland and initiated secretion by isolated perfused rectal glands (20), as well as cultured rectal gland cells, binds with high affinity to rectal gland plasma membranes, and activates membrane-bound guanylyl cyclase (3). Thus receptors for CNP on rectal gland cells have been presumed that may entrain cell transduction pathways (like guanylyl cyclase) other than the adenylyl cyclase cascade that is activated in response to VIP.

At the level of the intact shark, evidence for the role of CNP in the rectal gland’s homeostatic response to volume expansion has thus far been indirect, consisting chiefly of the facts that the mRNA for CNP is present in the heart of the shark (18), the peptide has been isolated from the heart (27), and that CNP stimulates isolated rectal gland preparations (20). In the present experiments we attempted to clarify the role of CNP by utilizing a newly discovered receptor antagonist of cardiac peptides (HS-142–1) on the coupled guanylyl cyclase receptors that are thought to mediate many of the cellular actions of this class of hormones.

METHODS

Dogfish of either sex were taken by gill nets from Frenchman Bay, Maine, and kept in marine live cars until used, usually within 3 days of capture. The dogfish were pithed, and their rectal glands were removed via an abdominal incision.

Explanted rectal gland. The rectal gland was excised from a dogfish, and the duct and vein were catheterized with PE-50 tubing. The artery of the gland was catheterized with the free end of a section of PE-50 tubing, which had previously been inserted (via percutaneous puncture through a thin-walled needle) into the dorsal aorta of a second heparinized perfusing fish that had been pithed and placed in a running-seawater (15°C) tank where the head and gills were kept submerged, dorsal side down, leaving the ventral surface of the abdomen and tail exposed above the water level. This blood-perfused rectal gland had no neural connection to the perfusing fish. The explanted gland was placed on a glass chamber cooled to 15°C with running seawater and positioned so as to maintain a hydrostatic perfusion pressure between 40 and 45 cmH2O (30–35 mmHg) at the level of the gland. In these experiments infusions were given to the fish through a second aortic catheter located downstream from the catheter supplying blood to the explanted gland. Infusions directly into the rectal gland artery of the explanted gland were performed with an infusion pump (Harvard Apparatus) connected to a T in the catheter between the explanted gland and the perfusing fish. All solutions were made up fresh before each experiment. The concentration of the infused solution was adjusted so that infusion rates of <10% of the simultaneously measured venous blood flow from the gland would deliver the desired concentration of drugs to the rectal gland. During control periods blood flow to the gland was maintained, but no additional substances were infused directly into the gland. Rectal gland duct flow and the secretion rate of chloride were measured for timed intervals during a control period before infusions and for experimental
periods during the infusions. Blood was sampled from the dorsal aorta at the midpoint of each collection.

Separated rectal gland tubules. Two rectal glands were used for each tubule preparation. The rectal glands were perfused in vitro as described previously with 100 ml of shark Ringer. The glands were then perfused with 10 ml of shark Ringer containing 0.2% collagenase and 10% fetal calf serum. The glands were sectioned longitudinally and minced into 1-mm cubes with a razor blade. The minced tissue was then incubated in shark Ringer containing collagenase and fetal calf serum, in the same proportions given previously, while constantly agitated for 45 min at room temperature. The tissue digest was then centrifuged at 50 g for 1 min in a refrigerated centrifuge to remove undigested tubules. The supernatant was then spun at 500 g for 3 min to harvest the tubules. The tubules were washed twice in shark Ringer and kept on ice until used.

Oxygen consumption measurement. Oxygen consumption was measured in a constant-temperature (25°C) chamber using a Clark type polarographic electrode connected to a recorder. The rate of oxygen consumption was calculated from the tangent of the recorded slope of the oxygen consumption, the solubility of oxygen in shark Ringer at 25°C, the barometric pressure, the volume of incubation solution in the measuring chamber, generally 2 ml, and the wet weight of the cells. The wet weight of the cells was determined at the end of the experiment by removing a measured aliquot of the cell suspension, spinning it down in a tared centrifuge tube, removing the supernatant, and reweighing the tube. Exogenous substrates used included (in mM) 5 glucose, 10 pyruvate, and 2.5 acetate. In the experiments reported here oxygen consumption was measured under basal conditions, and the rate of oxygen consumption was allowed to reach a steady state. A peptide was then added, and the rate of oxygen consumption was allowed to reach a new steady state. Once this new steady state was established, 10 mM ouabain was added and the rate of oxygen consumption was determined again. In the experiments in which HS-142-1 was used, it was added simultaneously with the peptide. Ouabain-sensitive oxygen consumption was calculated as the difference between the rate of oxygen consumption under basal or stimulated conditions and the rate after 10 mM ouabain was added.

Membrane preparation. Shark rectal glands were frozen in liquid nitrogen immediately on removal and subsequently stored at −80°C until use. Membrane preparations for binding studies and guanylyl cyclase assay were prepared in the same manner. Rectal glands were ground on ice and homogenized three times using a polytron at setting 6, for 30 s in a medium containing (in mM) 250 sucrose, 1 EDTA, and 1 g dithiothreitol, pH 7.4. The resulting homogenate was centrifuged at 100,000 × g for 60 min in a swinging bucket rotor, and the supernatant was used for measurement of guanosine 3′,5′-cyclic monophosphate (cGMP) content. cGMP concentration was measured by radioimmunoassay. In experiments using natriuretic peptides, membrane suspensions with and without added peptide were assayed simultaneously from the same preparation. Protein concentration was measured by a modification of the method of Bradford (1) (Bio-Rad).

Adenylyl cyclase assay. The rectal glands were removed, and all connective tissue was dissected out and discarded. The glands were diced into 2- to 3-mm cubes with a razor blade, suspended in ten times the volume of homogenizing solution in the presence of excess (10 mM) unlabeled hormone. Specific binding was defined as that amount of [125I]-CNP bound in the presence of excess (10−6 M) unlabeled hormone. Specific binding was always <3% of the total added radioactivity.

Guanylyl cyclase assay. Measurement of enzyme activity was performed by incubating 30–60 µg protein of the membrane preparation with the manganese salt of guanosine triphosphate (Mn3GTP, 5 mM) as substrate in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyloxanthine, 1 mM as described in detail previously (4). All incubations took place for 2 min in a water bath at 22°C in a final volume of 200 or 250 µl. The reaction was stopped with 12% trichloroacetic acid. Samples were centrifuged at 2,300 g for 15 min, and the supernatant was removed, extracted with 10 volumes of water-saturated ether four times, dried, and reconstituted in 4 ml of 50 mM sodium acetate buffer (pH 6.2) for measurement of guanosine 3′,5′-cyclic monophosphate (cGMP) content. cGMP concentration was measured by radioimmunoassay. In experiments using natriuretic peptides, membrane suspensions with and without added peptide were assayed simultaneously from the same preparation. Protein concentration was measured by a modification of the method of Bradford (1) (Bio-Rad).

Guanylyl cyclase receptors and shark rectal gland. shark Ringer with the following additions, 0.2% bovine serum albumin, 0.15% bacitracin, 1 mM phenylmethylsulfonyl fluoride, and 2 µM phosphoramidon and in the presence of radiolabeled human CNP (hCNP) and the indicated concentrations of the competing ligand. HS-142-1 was added to a final concentration of 100 µg/ml in the appropriate experiments. At the end of incubations bound radioactive hormone was separated from free by suction filtration through glass fiber filters (Whatman GF/C), pretreated with 1% polyethyleneimine, and the filters were washed with an additional 10 ml ice-cold 0.9% NaCl containing 0.2% bovine serum albumin. The filters on which the intact cells or membranes were retained were collected, and the associated radioactivity was counted in a gamma counter (LKB 1272 Clinigamma). Nonspecific binding was defined as that amount of [125I]-CNP bound in the presence of excess (10−6 M) unlabeled hormone. Specific binding was the difference between total binding and nonspecific binding. Nonspecific binding was always <3% of the total added radioactivity.
VIP and procaine were purchased from Sigma Chemical.

hCNP and human ANP (hANP) were purchased from Peninsu-

la Laboratories. Shark CNP (sCNP) was provided by

California Biotechnologies. HS-142–1 was a generous gift of

the Pharmaceutical Research Laboratories of Kyowa Hakko

Kogyo and Dr. Barry M. Brenner.

Chloride secretion is expressed as microequivalent per

hour per gram wet weight. Oxygen consumption is expressed

as micromole of oxygen consumed per hour per gram wet

weight. All values are means ± SE. Statistical significance

was determined using Student's t-test, paired t-test, and

analysis of variance wherever applicable.

RESULTS

Effect of HS-142–1 on binding of CNP to rectal gland

plasma membranes. CNP binds with high affinity to

abundant receptors in plasma membranes from shark

rectal gland (3). In mammalian tissues HS-142–1 is a

specific inhibitor of the natriuretic peptide receptors
coupled to guanylyl cyclase (9, 11, 16), but it does not

bind to the low-molecular-weight natriuretic peptide

receptor, the so-called clearance receptor (11). In iso-
lated shark rectal gland membranes, HS-142–1, at a

concentration of 100 µg/ml, inhibited ~40% of total

specific binding of hCNP without an apparent change

in the association constant (Fig. 1).

Effect of natriuretic peptides and HS-142–1 on gua-

nylyl cyclase and adenyl cyclase. The activation of
guanylyl cyclase by different classes of cardiac peptides

appears to be species specific. Figure 2A shows that

both shark and human forms of CNP stimulate the

production of cGMP by shark rectal gland membranes

in a similar and dose-dependent fashion. This effect

was several orders of magnitude greater than that of

hANP, which produced only a minor stimulation in

shark membranes even at the highest concentration

studied. Half-maximal stimulation with sCNP or hCNP

in shark rectal gland was 10 nM, similar to that

observed for hANP in rabbit kidney inner medullary

collecting duct cells (4). On the other hand, as shown in

Fig. 2B, hANP stimulates guanylyl cyclase in mem-

branes derived from the inner medulla of the rabbit

kidney, whereas sCNP does not.

As illustrated in Fig. 3, 100 µg/ml HS-142–1 reduced

the activation of guanylyl cyclase induced by CNP in

shark rectal gland membranes by ~60% when CNP

was present at 10 nM, close to the half-maximal

concentration seen in Fig. 2A. At higher concentrations

of CNP (500 nM) this concentration of HS-142–1 was

not sufficient to inhibit the effect of CNP.

CNP had no effect on adenylyl cyclase of the shark

rectal gland membranes. In the presence of 100 µM

theophylline, 1 nM VIP and 10 mM aluminum fluoride,

by contrast, briskly stimulated the production of cAMP

in the same preparation (Table 1).

Effect of CNP and HS-142–1 on ouabain-sensitive

oxygen consumption in rectal gland tubules. sCNP was

found to stimulate ouabain-inhibitable (transport-
dependent) oxygen consumption by separated rectal

gland tubules in a dose-dependent way (data not shown).
The lowest concentration of sCNP that had this effect was 10 nM, and maximal stimulation was reached between 50 and 100 nM. At 50 nM, sCNP increased ouabain-sensitive oxygen consumption approximately 10-fold, by 137.617 µM·h⁻¹·g wet wt⁻¹ (n = 16, P < 0.01). The stimulatory effect of sCNP was completely prevented by 500 µg/ml HS-142–1 (Fig. 4).

HS-142 blocks effect of volume expansion on chloride secretion by rectal gland. Having demonstrated that HS-142–1 inhibits the binding and action of C-type cardiac peptides in shark rectal gland cells, we next used HS-142–1 to determine whether endogenous natriuretic peptides mediate the effect of volume expansion on chloride secretion by the rectal gland in intact sharks (Fig. 5). After perfusion of explanted rectal glands was established, the donor fish was infused with 50 ml/kg weight of shark Ringer solution given over 30 min. Simultaneous with the volume load, a constant infusion of either shark Ringer or shark Ringer with HS-142–1 (5 mg/ml final concentration) was delivered into the arterial catheter connected to the explanted rectal gland. The infusion rate was maintained at 10% the rate of arterial blood flow to the gland for 150 min, so that the effective concentration of HS-142–1 perfusing the gland was ~500 µg/ml. The usual response to volume expansion of the donor fish is an increase in secretion of chloride by the explanted gland associated with an increase in blood flow that starts 30–60 min after the infusion is delivered and lasts for 2–3 h (22, 23). This pattern can be seen in the control experiments depicted in Fig. 5. Perfusion of the explaned rectal gland with 500

Table 1. CNP does not activate adenylyl cyclase

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Values are means ± SE; each number is average of quadruplicate measurements in 3 different membrane preparations. CNP, C-type natriuretic peptide; VIP, vasoactive intestinal peptide; AlF, aluminum fluoride.

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μg/ml of HS-142–1 completely inhibited the increase in chloride secretion observed after volume expansion of the donor fish. After cessation of the HS-142–1 infusion, chloride secretion by the explanted gland increased toward the levels found in control glands that had received vehicle only. There was no significant difference between the two groups in the rate of blood flow to the explanted rectal gland (37±4 vs. 39±3 ml·h⁻¹·g peak flow⁻¹ in HS-142–1 group vs. shark Ringer, respectively) or in perfusion pressure (46.7±2.7 vs. 43.5±0.9 cmH₂O peak pressure) in the HS-142–1 group vs. shark Ringer, respectively.

DISCUSSION

HS-142–1, a polysaccharide produced by Aureobasidiom pullulans var. melanigenum was shown by Mori­shita et al. (9) to inhibit the binding of ANP to its receptor in rabbit kidney cortex and bovine adrenocortical membranes. In mammalian tissues it reduces or suppresses the generation of cGMP evoked by ANP (5, 6, 9, 12, 15–17, 24, 28, 29), brain natriuretic peptide (BNP) (29), or CNP (6, 7, 12, 29, 30) in a variety of preparations from isolated membranes to live animals. It inhibits the binding of ANP, BNP, or CNP (9, 10, 12, 29) and prevents or reverses the effect of endogenous or exogenous natriuretic peptides (6, 10, 13, 17, 24, 29). It has been used to study the distribution of guanylyl cyclase-linked natriuretic peptide receptors (14, 30) and to explore the possible role of natriuretic peptides in a variety of pathophysiological conditions (8, 15, 28, 29). Because natriuretic peptides, including CNP, stimulate guanylyl cyclase, their effect has been thought to be mediated by cGMP.

The present experiments establish the validity of HS-142–1 as a competitive inhibitor of the action of natriuretic peptides in the shark rectal gland and provide direct evidence for the importance of CNPs in the native response of the rectal gland of intact sharks to salt loading. HS-142–1 inhibited the binding of human and shark CNP to plasma membranes derived from the rectal gland, inhibited its activation of membrane-associated guanylyl cyclase, and prevented the stimulation of transport-dependent oxygen consumption in separated rectal gland tubules by CNP. In experiments with explanted glands perfused with blood from intact donor sharks HS-142–1 prevented the effect of volume expansion of the donor to increase chloride secretion by the explanted gland. This inhibition was reversible, so that chloride secretion by the explant increased when glands were no longer exposed to HS-142–1. These results provide strong support for the notion that CNP released in response to a volume stimulus mediates the secretion of chloride by the shark rectal gland.

Marked species specificity was apparent when the action of various natriuretic peptides was tested on guanylyl cyclase from elasmobranch and mammalian tissues. Both sCNP and hCNP (which are 82% homologous, differing in 4 of 22 amino acids, 3 in the amino terminal end, those in positions 2, 4, 5, and 1 in the loop, that in position 16) stimulated guanylyl cyclase in plasma membranes of shark rectal gland and were equipotent. Half-maximal stimulation was observed at 10 nM, within the range of concentration at which chloride secretion is stimulated in intact glands (20) and comparable to the Michaelis constant found for ANP in guanylyl cyclase assays in membranes derived from rabbit kidney collecting duct (4). The potency of hANP to stimulate guanylyl cyclase in rectal gland membranes was almost negligible. On the other hand, sCNP did not stimulate guanylyl cyclase in rabbit collecting duct membranes, where hANP was highly active. These results support the view that in the shark, CNPs and their receptors predominate over ANP-type isoforms characteristic of mammalian species.

In a recently published report we showed that CNP binds to plasma membranes of shark rectal gland (3). In that report we demonstrated the presence of two receptors with similar affinity for CNP. Kinetic analysis of the data indicated that 50% of the receptors were highly specific for CNP and 50% exhibited characteristics for low-molecular-weight receptors. The experiments reported here support that conclusion. In the present experiments HS-142–1 displaced ~40% of the CNP binding to rectal gland plasma membranes. Because HS-142–1 prevents the binding of natriuretic peptide to guanylate cyclase-linked receptors and has no effect on the low-molecular-weight receptor, displacement by HS-142–1 of 40% of the CNP binding suggests that close to one-half of the receptors present in the rectal gland cell membrane are of the guanylate cyclase-linked type and the remainder must be of the low-molecular-weight type. This represents a second line of evidence indicating that there are two types of natriuretic peptide receptors in the rectal gland: a guanylyl cyclase-linked receptor that by binding and functional characteristics appears to be a GC-B receptor and a low-molecular weight receptor, the so-called clearance receptor.

Intravascular volume expansion of the spiny dogfish evokes an increase in blood flow to the rectal gland of ~300% as well as an increase in glandular secretion (22). In the explanted rectal gland preparation, the changes seen in the donor shark are mirrorrored in the explanted gland. The increased blood flow to the gland is not a result of an increase in aortic perfusion pressure and must therefore represent a decrease in local vascular resistance (23). A striking feature of the present experiments is that HS-142–1 inhibited chloride secretion without altering the increase in glandular blood flow that commonly accompanies volume expansion. Bumetanide similarly inhibits the secretory response of the explanted gland to volume expansion of the donor shark, without preventing the increase in blood flow (23). It seems reasonable to hypothesize that the vasodilation stimulated in explanted rectal glands by expansion of the donor blood stream is produced by a hormonal substance that may not be a cardiac peptide and is not an agonist of the guanylyl-cyclase-linked receptor inhibited by HS-142–1.
Perspectives

Natriuretic peptides, secreted by the heart in response to volume expansion, promote the excretion of salt by the mammalian kidney. This mechanism, which prevents the retention of salt, also operates in other vertebrates like the shark, where the target organ is the rectal gland and not the kidney. This represents an evolutionary convergence in vertebrates where a heart hormone has retained its specific action while the target organ has changed. The present report documents for the first time that CNP, the natriuretic peptide present in the shark heart, is the mediator of the effect of volume expansion to stimulate the secretion of salt by the rectal gland. Previous work in this area suggested that the effect of volume expansion might involve the sequential release of two hormones, a natriuretic peptide from the heart and VIP from nerves within the gland. The previous experiments were done using rat or hANP that stimulated the secretion of salt by the rectal gland. More importantly, in vivo, the effect of volume expansion is prevented by blockade of the natriuretic peptide receptor indicating that CNP mediates the effect of volume expansion.


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