Regulation of atrial natriuretic peptide secretion by cholinergic and PACAP neurons of the gastric antrum

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Am J Physiol Gastrointest Liver Physiol 284: G68–G74, 2003; 10.1152/ajpgi.00113.2002.—Atrial natriuretic peptide (ANP) released from enterochromaffin cells helps regulate antral somatostatin secretion, but the mechanisms regulating ANP secretion are not known. We superfused rat antral segments with selective neural agonists/antagonists to identify the neural pathways regulating ANP secretion. The nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) stimulated ANP secretion; the effect was abolished by hexamethonium but doubled by atropine. Atropine's effect implied that DMPP activated concomitantly cholinergic neurons that inhibit and noncholinergic neurons that stimulate ANP secretion, the latter effect predominating. Methacholine inhibited ANP secretion. Neither bombesin nor vasoactive intestinal polypeptide stimulated ANP secretion, whereas pituitary adenylate cyclase-activating polypeptide (PACAP) restored DMPP-stimulated ANP secretion. Although ANP secreted from atrial myocytes into the systemic circulation causes natriuresis and diuresis in the kidney, the fact that ANP and functional ANP receptors are coexpressed in the same tissues suggests that ANP may have local physiological roles that are related to the specific organ system within which it is produced (8, 18, 20, 26, 38).

In the stomach, ANP has been reported to relax smooth muscle cells (5, 34) and either inhibit or stimulate acid secretion (3, 32). Using molecular biological and immunohistochemical techniques, we have localized ANP to enterochromaffin (EC) cells of the gastric antrum (15), and we and others have demonstrated the presence of NPR-A and NPR-C receptors in antral mucosa (14, 15, 23, 26). These findings have led us to postulate that ANP may regulate gastric function, perhaps via a paracrine and/or autocrine pathway. In support of this notion, ANP stimulates cGMP production in rat pyloric glands (15, 26) and somatostatin secretion in superfused rat and human antral segments (14). The precise pathways that regulate ANP secretion from this region of the stomach, however, are not known.

In the present study, we have used the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP), alone and in combination with various selective antagonists, to identify the neural pathways that regulate gastrointestinal tract (22). ANP preferentially binds to two subtypes of natriuretic peptide receptors (NPR): type A (NPR-A) and type C (NPR-C). NPR-A, a transmembrane cell surface receptor with ligand-dependent guanylyl cyclase activity, mediates the biological effects of ANP in kidney, adrenal, and vascular tissues (9). NPR-C, a transmembrane cell surface receptor lacking guanylyl cyclase activity, originally thought to act primarily as a natriuretic peptide clearance receptor, may also inhibit adenylate cyclase activity (1, 9, 19, 34). Although ANP secreted from atrial myocytes into the systemic circulation causes natriuresis and diuresis in the kidney, the fact that ANP and functional ANP receptors are coexpressed in the same tissues suggests that ANP may have local physiological roles that are related to the specific organ system within which it is produced (8, 18, 20, 26, 38).

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ANP secretion in rat antrum. The results indicate that ANP secretion is regulated by intramural cholinergic and pituitary adenylate cyclase-activating polypeptide (PACAP) neurons. Activation of cholinergic neurons inhibits and activation of PACAP neurons stimulates ANP secretion.

MATERIALS AND METHODS

Materials. The nicotinic agonist DMPP, the muscarinic agonist methacholine, the nicotinic antagonist hexamethonium bromide, the muscarinic antagonist atropine sulfate, and the axonal blocker tetrodotoxin were purchased from Sigma (St. Louis, MO). PACAP-27, PACAP-38, and antiserum to rat ANP were purchased from Peninsula Labs (Belmont, CA). Bombesin and vasoactive intestinal polypeptide (VIP) were purchased from Bachem (Torrance, CA). Recombinant maxadilan [PACAP type I (PAC1) receptor agonist] and its deleted peptide M65 (PAC1 receptor antagonist) were produced in Escherichia coli and purified to homogeneity using reverse-phase high performance liquid chromatography by E. Lerner (25, 36). 125I-ANP and Amprep-mini C18 columns were purchased from Amersham (Arlington Heights, IL).

Superfusion of rat antral segments. Male Sprague-Dawley rats, weighing 250–350 g, were deprived of solid food overnight but allowed free access to water containing 5% dextrose. The animals were anesthetized with 20% urethane (5 ml/kg body wt ip). The serosal and muscle layers were partly removed from the antrum to improve drug diffusion, and a segment, ~1 cm², was cut into six to eight segments, washed with saline, and placed on a porous grid separating the two halves of a minichamber (Swinnex 25, 1.4 ml volume; Millipore, Bedford, MA) as previously described (31). Krebs-bicarbonate solution containing 0.2% bovine serum albumin, 4% dextran, and 4.5 mM glucose was perfused into the bottom of the chamber at a rate of 1 ml/min, and the effluent was collected via a catheter leading from a small aperture at the top of the chamber. The perfusate was gassed with 95% O₂ and 5% CO₂. Drugs were delivered at the rate of 0.1 ml/min via a side arm close to the inlet. The entire preparation was contained within a chamber maintained at 37°C. The protocol was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Experimental design. A 30-min equilibration period was followed by an 80- to 100-min sampling period. The sampling period consisted of a 30-min control basal period, a 20-min period during which DMPP (10 pM–10 μM), methacholine (10 μM–1 mM), PACAP-27 (10 pM–0.1 μM), PACAP-38 (10 pM–0.1 μM), maxadilan (10 pM–0.1 μM), M65 (10 nM), bombesin (10 pM–0.1 μM), or VIP (10 pM–0.1 μM) was superfused, and a final 30-min control period. In some experiments, atropine (0.1 μM or 0.1 mM), hexamethionum (1 mM), M65 (10 nM), or tetrodotoxin (5 μM) was superfused for 20 min before as well as during superfusion with DMPP, methacholine, PACAP-27, PACAP-38, or maxadilan. Five-milliliter samples of the effluent were obtained at 5-min intervals and stored at –20°C for subsequent measurement of ANP concentration by radioimmunoassay.

Radioimmunoassay. ANP was extracted from pooled aliquots of effluent collected for 5 min, and its concentration was measured in duplicate by radioimmunoassay as described in detail previously (6, 12). ANP-containing superfluids were applied to Amprep-mini C18 columns equilibrated with 0.1 N acetic acid and eluted with a mixture of acetoni trile and 0.1 M trifluoroacetic acid (60:40). The eluate was dried under nitrogen, resuspended in a minimal volume of radioimmunoassay buffer, and then assayed for immunoreactive ANP. The recovery of added rat ANP was 82 ± 3%. The limit of detection was 0.6 pg/tube, and the IC₅₀ was 12.2 pg/tube. Interassay and intra-assay coefficients of variability were 7.0% and 9.4%, respectively.

Data analysis. ANP secretion was expressed as the mean increase or decrease as picograms per minute or as percentage of basal level during the 10 min immediately preceding the experimental period. Data are presented as means ± SE of n experiments on different animals. Changes in secretion were tested for significance using Student’s t-test for unpaired values. Differences were considered significant at P < 0.05.

RESULTS

Effect of DMPP alone and in combination with various antagonists on ANP secretion from antrum. Basal secretion of ANP from rat antral segments was uniform from one experimental series to another and reverted to control basal levels at the end of the experimental period (start, 10 ± 3; end, 12 ± 4 pg/min).

Superfusion for 20 min with the nicotinic agonist DMPP, in the range of 10 pM–10 μM, caused a prompt, reversible, and concentration-dependent increase in ANP secretion (Figs. 1 and 2). The EC₅₀ was 3 nM, and maximal stimulation of ANP secretion, expressed as the integrated 20-min response, was 109 ± 29% at 1 μM (P < 0.01; n = 6).

Addition of the nicotinic antagonist hexamethionum (0.1 mM; n = 8), although having no significant effect on basal ANP secretion, abolished the increase in ANP secretion induced by DMPP (1 μM), indicating that DMPP acted specifically at nicotinic sites in this preparation (Fig. 2).

Fig. 1. Effect of the nicotinic agonist 1,1-dimethyl-4-phenylpipera
da trium (DMPP; 10 pM–10 μM) on basal atrial natriuretic peptide (ANP) secretion from rat antral segments. Results are expressed as integrated response during 20 min of superfusion with DMPP. Data are means ± SE of 5–8 experiments with each dose. *P < 0.05 vs. basal.
To determine whether cholinergic neurons are involved in the regulation of ANP secretion, experiments were performed in the presence of the muscarinic antagonist atropine. Addition of atropine (10 μM) to the superfusate, although having no significant effect on basal ANP secretion, augmented the stimulatory effect of DMPP (1 μM) on ANP secretion by twofold, from 109 ± 29% above basal level with DMPP alone to 210 ± 21% above basal level with DMPP plus atropine (P < 0.01 for the difference; Figs. 3 and 10). The results imply that DMPP activated cholinergic neurons that inhibit ANP secretion and noncholinergic neurons that stimulate ANP secretion, the effect of the latter predominating.

Consistent with this notion, superfusion of antral segments for 20 min with the muscarinic agonist methacholine, in the range of 10 pM–1 mM, caused a concentration-dependent decrease in ANP secretion (Fig. 4). The EC₅₀ was 5 nM, and maximal inhibition of ANP secretion, expressed as the integrated 20-min response, was 79 ± 4% below basal level (P < 0.001; n = 4). Addition of atropine (10 μM) to the superfusate, although having no significant effect on basal ANP secretion, abolished the decrease in ANP secretion induced by 0.1 μM methacholine (Fig. 5). Tetrodotoxin (5 μM) had no significant effect on the ANP response to 0.1 μM methacholine (62 ± 6% below above basal level with methacholine alone vs. 48 ± 6% below basal level with methacholine plus tetrodotoxin; no significant difference; Fig. 5).

Prime candidates for noncholinergic transmitter responsible for stimulation of ANP secretion include bombesin, VIP, and PACAP. All are present in gastric antral mucosal nerve fibers and capable of stimulating secretion from neuroendocrine cells (2, 7, 21, 33, 35, 40). Superfusion for 20 min with bombesin or VIP, in the range of 10 pM–0.1 μM, however, had no significant effect on ANP secretion (n = 4–6 each). In contrast, PACAP-27 (10 pM–0.1 μM), PACAP-38 (10 pM–0.1 μM), and the PAC1 receptor agonist maxadilan (10 pM–0.1 μM) each stimulated ANP secretion in a concentration-dependent manner, with threshold concentrations <10 pM (Fig. 6). Both the ANP response to 1
nM PACAP-27 and 1 nM PACAP-38 were abolished by the PAC1 receptor antagonist M65 (10 nM; Fig. 7 and 8). Tetrodotoxin (5 μM), on the other hand, inhibited the ANP response to 1 nM PACAP-27, 1 nM PACAP-38, and 1 nM maxadilan by 38–62% (PACAP-27, 165 ± 21% (P < 0.001) above basal level with PACAP-27 alone vs. 63 ± 6% (P < 0.001) above basal level with PACAP-27 plus tetrodotoxin (P < 0.01 for the difference); PACAP-38, 130 ± 20% (P < 0.01) above basal level with PACAP-38 alone vs. 80 ± 15% (P < 0.01) above basal level with PACAP-38 plus tetrodotoxin (P < 0.05 for the difference); and maxadilan, 116 ± 6% (P < 0.001) above basal level with maxadilan alone vs. 59 ± 8% (P < 0.001) above basal level with maxadilan plus tetrodotoxin (P < 0.01 for the difference), Figs. 7 and 8). The results suggest that PACAP stimulates ANP secretion directly as well as indirectly by activating noncholinergic neurons.

It should be noted that superfusion of antral segments for 20 min with M65 (10 nM) alone caused a prompt and reversible decrease in ANP secretion (mean integrated response, 28 ± 5% below basal level;
P < 0.001; n = 12), implying that endogenous PACAP, acting via the PAC1 receptor, stimulates ANP secretion (Fig. 9).

To determine the participation of PACAP neurons in DMPP-stimulated ANP secretion, experiments were performed in the presence of M65 (Figs. 3 and 10). Addition of M65 (10 nM) to the superfusate converted the increase in ANP secretion induced by DMPP (1 μM) from a 109 ± 29% increase above basal level to a 40 ± 5% (P < 0.001; n = 6) decrease below basal level, unmasking the cholinergic component and implying that DMPP activated PACAP neurons that stimulate ANP secretion. A combination of atropine (0.1 mM) and M65 (10 nM) had the same effect as hexamethonium, restoring DMPP-stimulated ANP secretion to control basal levels (Figs. 3 and 10).

DISCUSSION

Although quantitatively the most important source of ANP may be cardiac atrial myocytes, ANP is present in a variety of tissues, including the gastrointestinal tract (11). In the stomach, transcripts for ANP as well as its receptors, NPR-A and NPR-C, have been detected in antral mucosa (14, 15, 26). In this region, ANP has been localized to EC cells by in situ hybridization and immunohistochemistry (15). The presence of ANP and its receptor in antral mucosa suggests that ANP may regulate gastric function in a paracrine manner. In support of this notion, the NPR-A receptor antagonist, anantin, inhibits somatostatin secretion in rat antral segments (13). The results of the antagonist imply that endogenous ANP, acting via the NPR-A receptor, stimulates antral somatostatin secretion. The mechanisms, however, that regulate the secretion of ANP from this region of the stomach have not been examined.

Previous studies using superfused antral segments (28), antral sheets mounted in Ussing chambers (30), and vascularity perfused rat stomach (29), preparations that retain intact intramural neural pathways, have shown that peptide (i.e., gastrin and somatostatin) secretion from this region of the stomach is regulated by cholinergic and noncholinergic (i.e., bombesin and VIP) neurons. More recently, the release of gastric serotonin, which is colocalized with ANP in EC cells (15), has been shown to be regulated by cholinergic neurons in the vascularity perfused rat stomach preparation (39).

In the present study, we have demonstrated, for the first time, by using rat superfused antral segments, that ANP secretion from EC cells can also be regulated by intramural cholinergic and noncholinergic (i.e., PACAP) neurons. The evidence for this is based on the fact that pharmacological activation of intramural neurons by the ganglionic nicotinic agonist DMPP caused a concentration-dependent increase in ANP secretion. The ANP response to DMPP was completely inhibited by the ganglionic nicotinic antagonist hexamethonium but was augmented twofold by atropine. The effect of atropine implies that DMPP activated cholinergic neurons that inhibit ANP secretion and concomitantly noncholinergic neurons that stimulate ANP secretion; the effect of the latter appears to predominate, resulting in a net increase in ANP secretion. Consistent with this notion, the muscarinic agonist methacholine caused a concentration-dependent and atropine-sensitive decrease in ANP secretion.

The regulation of ANP secretion by cholinergic and noncholinergic intramural neurons parallels closely that of other gastric neuroendocrine cells as discussed above, in particular somatostatin-containing D cells. In both instances, activation of cholinergic neurons inhib-
its and activation of noncholinergic neurons (e.g., VIP) stimulates peptide secretion. We hypothesized that VIP and/or bombesin might be the noncholinergic transmitter responsible for stimulation of ANP secretion; both are present in antral mucosal nerve fibers and are capable of stimulating peptide secretion (VIP stimulates somatostatin and bombesin stimulates gastrin) in response to physiological stimuli such as mechanical distension and luminal protein (4, 7, 21, 27, 28, 30). Neither peptide, however, had any significant effect on ANP secretion in our preparation when superfused at concentrations ranging from 10 pM to 0.1 μM.

PACAP has recently been localized to efferent and afferent nerve fibers innervating gastric antral mucosa (10, 16, 35). PACAP, which shows 68% sequence homology with VIP, has two bioactive forms, with 38 (PACAP-38) and 27 (PACAP-27) amino acid residues. In rat, PACAP-38 and the COOH-terminally truncated PACAP-27 are derived from a common 175-amino acid precursor (24). PACAP exerts its actions through at least three distinct receptors: the PAC1 receptor, which binds PACAP with 1,000 times higher affinity than VIP, and the VPAC1 and VPAC2 receptors, which bind PACAP and VIP with equal affinities (17).

In the present study, PACAP-38 and PACAP-27 each stimulated ANP secretion in a concentration-dependent manner. The fact that VIP had no significant effect on ANP secretion led us to postulate that the effect of PACAP was mediated via the PAC1 receptor. Consistent with this notion, the PAC1 receptor agonist maxadilan caused a concentration-dependent increase in ANP secretion. The pattern of response led us to postulate that PACAP may be the noncholinergic transmitter responsible for ANP secretion.

To evaluate the role of endogenously released PACAP in neurally mediated ANP secretion, studies were performed in the presence of the PAC1 receptor antagonist M65. First, M65 abolished PACAP-38- and PACAP-27-stimulated ANP secretion, establishing its function as a specific PAC1 receptor antagonist in this preparation. Secondly, M65 inhibited basal ANP secretion, implying that endogenous PACAP tonically stimulates ANP secretion. Thirdly, M65 converted the ANP response to DMPP from an increase above to a significant decrease below basal levels, thus unmasking the cholinergic component and indicating that DMPP activated PACAP neurons that stimulate ANP secretion. The combination of atropine and M65, like hexamethonium, restored the ANP response to DMPP to control basal levels, implying that activation of cholinergic and PACAP neurons accounted for the entire response to DMPP.

The fact that the axonal blocker tetrodotoxin attenuated PACAP-38-, PACAP-27-, and maxadilan-stimulated ANP secretion by ~50% suggests that PACAP stimulates ANP secretion directly as well as indirectly by releasing a noncholinergic stimulatory neurotransmitter, the identity of which is not known. Although gastric EC cells have yet to be isolated, the fact that histamine-containing EC-like cells isolated from rat stomach express functional PAC1 receptors lends support to the notion that PAC1 receptors might also be present on EC cells (2, 40). In support of an indirect neurally mediated effect, PAC1 mRNA has been demonstrated in the muscle, but not mucosal, layers of the antrum (16, 35, 37). The fact that tetrodotoxin had no significant effect on the inhibition in ANP secretion induced by methacholine suggests that acetylcholine may directly inhibit ANP secretion, analogous to its effect on antral somatostatin secretion (28–31).

In summary, the present study demonstrates that ANP secretion from antral EC cells is regulated by at least two intramural neural pathways (i.e., postganglionic neurons): a cholinergic pathway that inhibits ANP secretion directly and a noncholinergic pathway involving the release of the neurotransmitter PACAP, which, acting via PAC1 receptors, stimulates ANP secretion directly as well as indirectly via activation of an additional noncholinergic neuron. The fact that ANP secretion can be regulated by intramural neurons suggests that ANP may participate physiologically in the regulation of gastric endocrine and/or exocrine secretion. In support of this notion, we have shown, in preliminary experiments, that endogenous ANP, acting via the NPR-A receptor, stimulates somatostatin and thus inhibits gastrin secretion (13). Because feeding stimulates and fasting inhibits ANP mRNA in rat antrum (15), we speculate that ANP may function, in this region of the stomach, in a paracrine feedback pathway that modulates somatostatin, and hence gastrin secretion; i.e., a decrease in somatostatin, as occurs during ingestion of a meal, stimulates ANP secretion, which, in turn, attenuates somatostatin secretion.

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