Different actions of secretin and Gly-extended secretin predict secretin receptor subtypes

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MANY REGULATORY PEPTIDES EXIST in multiple molecular forms generated by different degrees of posttranslational processing in their cell of synthesis. This differential processing can regulate the expression of biological activity for a peptide if receptor subtypes exist that select among the processed forms. One example is the family of PYY and NPY peptides and receptor subtypes: PYY-(1–36) and the more fully processed form PYY-(3–36) have different spectra of binding to their receptor subtypes (13). Preliminary evidence (38) suggests that two molecular forms of secretin exist in tissue and plasma: fully processed secretin [secretin-(1–27)-amide] and its immediate precursor, secretin-Gly. We recently reported (26) that COOH-terminally extended forms of secretin produced during posttranslational processing of preprosecretin were equipotent with fully processed secretin for stimulating pancreatic secretion. Only one secretin receptor has been cloned and characterized by molecular and pharmacological approaches (28), and this receptor is thought to mediate the stimulatory effect of secretin on pancreatic fluid and bicarbonate secretion. However, the relative potencies of COOH-terminally extended secretin forms for binding and activation of this receptor have not been determined.

Secretin has a wide range of actions, including stimulatory effects on pancreatic and biliary secretion, regulatory peptide release, cardiac and neural activity, and other functions; conversely, secretin has inhibitory effects on gastric acid secretion and motility (28, 33). In rats, secretin is a potent inhibitor of gastric acid secretion (25). Several observations indicate that the inhibitory action of secretin on gastric acid secretion is mediated by local release of somatostatin from oxyntic gland area D cells (25). Secretin has been reported to stimulate somatostatin secretion by cultured human antral D cells (3), suggesting that D cells may be one target that bears the inhibitory secretin receptor. The ability of COOH-terminally extended forms of secretin to inhibit gastric acid secretion has not been previously examined.

Although the stimulatory and inhibitory effects of secretin could be exerted through one receptor, there are precedents for receptor subtypes that differentially mediate stimulatory and inhibitory actions of other agonists (34). We examined the relative potencies of

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secretin and secretin-Gly on pancreatic secretion and gastric acid secretion to test the hypothesis that stimulatory and inhibitory actions of secretin are mediated through different secretin receptors. As a further test of this hypothesis, we compared the relative potencies of secretin and secretin-Gly for binding to the rat pancreatic secretin receptor subtype in stably transfected Chinese hamster ovary (CHO) cells and release of somatostatin from gastric mucosal D cells in vitro. These studies revealed distinct differences in the patterns of pancreatic and gastric secretory effects and in vitro actions of secretin and secretin-Gly, suggesting the existence of a second secretin receptor subtype.

MATERIALS AND METHODS

Peptides. Synthetic canine and rat secretins with COOH-terminal α-carboxyl Val-amide [secretin-(1–27)-amide] or Val-Gly [secretin-(1–27)-Gly] were synthesized by the UCLA Peptide Synthesis Core Facility under the direction of one of the authors (J. R. Reeve, Jr.). The peptides were purified by HPLC and evaluated by amino acid analysis, mass spectroscopy, and analytical reverse-phase HPLC. The details of these syntheses and purifications have been reported (26). Secretin peptides were weighed to the nearest microgram and dissolved in 2% (vol/vol) acetic acid. Aliquots of these solutions were stored frozen at −70°C; each aliquot was thawed only once, and any remainder was discarded after preparation of daily working solutions. Gastrin-17 was dissolved in 50 mM NH₄OH containing 1% (wt/vol) BSA (RIA grade, Sigma Chemical, St. Louis, MO), divided into aliquots, and stored in the same manner as for secretin stocks. The exact peptide concentrations in secretin stock solutions were determined by quantitative amino acid analysis, and the same stock solutions were used for all experiments reported. For administration, stocks were thawed and diluted to appropriate concentrations in 0.15 M NaCl containing 1% BSA. The diluted solutions were kept on ice until use within a few hours. Peptide solutions were administered using syringe pumps mounted with plastic disposable syringes and polyethylene tubing.

Animal models. Male Sprague-Dawley rats (250–320 g) were purchased from Sasco (Omaha, NE) and housed in an American Association for Accreditation of Laboratory Animal Care-approved facility. Rats were kept in the animal facility for at least 7 days before use and were maintained under controlled temperature and humidity on a standard rodent diet and an equal light-dark cycle.

For pancreatic studies, all animals were deprived of food for 24 h. Anesthesia was induced by administration of urethan (1.25 g/kg, given as divided im and ip injections). A jugular vein catheter (PE-50) was inserted, and 0.15 M NaCl containing 1% (wt/vol) BSA was given intravenously at 1.0 ml/h throughout the experiment. A midline celiotomy was performed, followed by ligation of the pylorus, ligation of the biliary duct proximal to its investment by pancreatic tissue, and insertion of a catheter (PE-50) into the distal bile-pancreatic duct for collection of pure pancreatic juice. Core body temperature was monitored using a rectal thermocouple and maintained at 35–37°C with a heating pad and heat lamp.

For gastric studies, rats were fasted for 24 h and then prepared with gastric cannulas and venous catheters using aseptic surgical techniques similar to those described previously (31). Anesthesia was induced with pentobarbital sodium (35–50 mg/kg ip). A polyethylene catheter (PE-50) was inserted into the jugular vein, tunneled subcutaneously to exit in the midscapular region, filled with saline containing 10 U/ml heparin, and capped. A stainless steel cannula was inserted into the forestomach and ligated in place with a purse-string suture. The cannula was brought through the abdominal wall to the left of the midline and capped, and the abdominal incision was closed in two layers. After awakening from anesthesia, the rats were allowed free access to food and water. Rats were allowed to recover for at least 5 days before experiments began, and at least 4 days elapsed between experiments. After each experiment, the rats were returned to individual cages and given free access to food and water. All studies reported were performed on animals that showed normal weight gain and appeared healthy.

Design of pancreatic and gastric secretory studies. After a 60-min stabilization period, pancreatic juice was collected during sequential 30-min periods. After two basal collections, canine secretin (n = 12 rats) or secretin-Gly (n = 16 rats) was administered in ascending doses of 1, 3, 10, 30, 100, 300, and 1,000 pmol·kg⁻¹·h⁻¹; each dose was given for 30 min. The volume of pancreatic juice was determined gravimetrically using preweighed collection tubes.

In studies of gastric secretion, rats were fasted 24 h before each study. To determine the effects of secretin and secretin-Gly on unstimulated (basal) acid secretion, an intravenous infusion of 0.15 M NaCl containing 1% BSA was given at 4 ml/h, and gastric juice was collected by gravity drainage in successive 30-min periods. After three control collections, canine secretin (n = 25 rats) or secretin-Gly (n = 23 rats) was added to the intravenous infusion to deliver 3, 10, 30, 100, 300, or 1,000 pmol·kg⁻¹·h⁻¹, each dose given sequentially for 30 min. As a control for time-dependent changes in gastric secretion, other rats (n = 22) received only saline plus BSA for the same total number of collections. The volume of each collection was recorded, and H⁺ was titrated electrometrically.

The effects of secretin forms on gastrin-stimulated secretion were studied in a group of 11 rats. After three control collections, gastrin-17 was administered intravenously at a dose of 1.25 nmol·kg⁻¹·h⁻¹ for 3.5 h. This dose was determined to cause maximal stimulation of gastric acid secretion in other groups of rats tested with a wide range of gastrin-17 doses. After 90 min of gastrin-17, canine secretin or secretin-Gly was added to deliver doses of 30, 100, 300, and 1,000 pmol·kg⁻¹·h⁻¹, each for 30 min. On a third experimental day, only gastrin-17 was given for the same total number of collections. These doses were selected because other studies in our laboratory indicated that inhibition of acid secretion was clear-cut at doses >10 pmol·kg⁻¹·h⁻¹ of secretin. Volume and H⁺ were measured as above.

Characterization of secretin receptor binding. Binding experiments were performed with a CHO cell line expressing the transfected rat secretin receptor (rSecR-1, a gift from Dr. Laurence J. Miller, Mayo Clinic, Rochester, MN). Cells were cultured in poly-L-lysine-coated 24-well plates with Dulbecco’s modified Eagle’s Medium (DME-F-12 medium, 20 mM HEPES, pH 7.4, 0.1% [wt/vol] bacitracin, 0.2% [wt/vol] BSA) and 1 ml of cell binding buffer [DMEM-F-12 medium, 20 mM HEPES, pH 7.4, 0.1% (wt/vol) bacitracin, 0.2% (wt/vol) BSA] was then added into each well. Binding assays were started by adding 125I-secretin-(1–27)-amide (rat peptide, 20 pM, -2,000 Ci/mmol) in the presence of increasing concentrations of unlabeled rat peptides as indicated. The radiolabeled tracer was prepared, purified, and stored as described previously (26). After 1-h incubation at 37°C, cells were washed twice with ice-cold PBS and solubilized in 1 ml of 1% (vol/vol) Triton X-100 in
PBS. Radioactivities of bound (cell lysate) and free (medium) fractions were counted, and these values were used to calculate specific binding, expressed as a percentage of maximal binding (with tracer alone). Total binding in these experiments was in the range of 4,000–5,000 cpm per well, whereas nonspecific binding in the presence of 10⁻⁶ M secretin was 200–300 cpm. Kinetic constants were calculated by nonlinear regression curve fitting using a one-site model (Prism 3.0, GraphPad, San Diego, CA).

Somatostatin release from gastric mucosal endocrine cells. Rat gastric endocrine cells were isolated by a combination of elutriation and density gradient centrifugation as described previously (37). The proportion of immunostained D cells in this preparation ranges from 12 to 30%. Freshly isolated endocrine cells were rinsed by gentle centrifugation in this preparation. Aliquots of the washed cell suspension were placed on glass coverslips precoated with Cell-Tak and incubated at 37°C for 45 min, allowing cell attachment to the coverslips. Then 0.8 ml of growth medium was added to the coverslips in six-well plates. Somatostatin release was determined after 48-h culture by incubating endocrine cells in Cell-Tak-precoated coverslips in six-well plates. Growth medium was replaced 3 h before the release experiments. After cells were incubated with graded concentrations of rat secretin and secretin-Gly for 2 h, the test medium was harvested, centrifuged, and stored at −20°C for somatostatin assay. Radioimmunoassay of somatostatin was performed with anti-somatostatin antiserum (AB 8401) as described previously (36). In each experiment, somatostatin secretion was calculated as the average of six wells exposed to each peptide concentration.

Calculations and statistical analysis. All data are presented as group means ± SE, with n as the number of rats in each group. Statistical significance of changes in secretory outputs was assessed both within and across groups. One-way repeated-measures analysis of variance was followed by appropriate repeated-measures individual comparisons within groups. One-way analysis of variance or Student’s t-test was used for comparisons across groups; appropriate corrections were made for multiple comparisons. Statistically significant changes were accepted at P < 0.05.

RESULTS

Stimulation of pancreatic secretion. Secretin and secretin-Gly caused indistinguishable patterns of pancreatic fluid secretion over the range of doses administered (Fig. 1). Bicarbonate concentration and output also showed similar increases in response to both secretin forms, and rat secretin and secretin-Gly produced identical results (data not shown).

Inhibition of acid secretion. The patterns of gastric acid secretion during basal and gastrin-stimulated conditions were stable and reproducible. This is illustrated in Fig. 2, which shows the time course of basal and gastrin-induced acid output in control studies. Unstimulated acid secretion did not vary over the 4-h period of observation. During intravenous infusion of 1.25 nmol·kg⁻¹·h⁻¹ gastrin-17, acid output increased about fourfold compared with basal output and was stable for at least 3 h. These data were used for comparison to patterns of acid secretion in rats treated with secretin and secretin-Gly during basal (Fig. 3) and gastrin-stimulated (Fig. 4) conditions.

Secretin strongly and significantly inhibited basal acid output at doses of 100 pmol·kg⁻¹·h⁻¹ and higher (Fig. 3); inhibition of acid output was the result of decreases in secretory volume and acid concentration (data not shown). In contrast, secretin-Gly did not inhibit basal acid output at any dose up to 1,000 pmol·kg⁻¹·h⁻¹. Acid output actually increased significantly (P < 0.05 and P < 0.01) at several doses of secretin-Gly (Fig. 3); this reflected significant increases in secretory volume with no change in concentrations of acid (data not shown).

Secretin caused marked, dose-related inhibition of gastrin-induced acid output (Fig. 4). Significant inhibi-
tion occurred at 30 pmol·kg⁻¹·h⁻¹ (P < 0.01), and acid output was reduced below basal levels at the highest infused dose of secretin. Secretin-Gly was markedly less potent for inhibition of gastrin-induced acid output, being significantly less potent than secretin at doses of 300 and 1,000 pmol·kg⁻¹·h⁻¹ (P < 0.01). In other experiments, a dose of 10 nmol·kg⁻¹·h⁻¹ of secretin-Gly was required for complete inhibition of submaximally stimulated (100 pmol·kg⁻¹·h⁻¹ of gastrin-17) acid secretion (data not shown). Identical results were obtained when rat secretin and secretin-Gly were compared under similar conditions.

Secretin receptor binding. As seen in Fig. 5, secretin and secretin-Gly were equipotent for displacing ¹²⁵I-secretin from CHO cells bearing the stably transfected rat secretin receptor. The dissociation constant values calculated for the two peptides were 5.3 ± 0.5 and 6.4 ± 0.6 nM, respectively. In other experiments, a dose of 10 nmol·kg⁻¹·h⁻¹ of secretin-Gly was required for complete inhibition of submaximally stimulated (100 pmol·kg⁻¹·h⁻¹ of gastrin-17) acid secretion (data not shown). Identical results were obtained when rat secretin and secretin-Gly were compared under similar conditions.

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**Somatostatin release from gastric mucosal D cells.** Figure 6 demonstrates the effects of secretin and secretin-Gly on somatostatin release from a partially purified rat gastric mucosal endocrine cell preparation. Secretin increased somatostatin secretion, whereas secretin-Gly was ineffective over the range of concentrations tested. Each point represents the mean ± SE, and the average of 6 wells for each condition was determined in each preparation.
purified preparation of rat gastric mucosal endocrine cells. In contrast to their similar patterns of displacement of radiolabeled secretin from the rat secretin receptor, there were striking differences in D cell somatostatin release by the two peptides. Secretin increased somatostatin release by nearly threefold at a concentration of 10^{-7} M, whereas secretin-Gly was ineffective at concentrations up to 10^{-6} M (Fig. 6).

DISCUSSION

The results of these studies can be summarized as follows: secretin and secretin-Gly were equipotent ligands for binding to the transfected rat pancreatic secretin receptor and for stimulating pancreatic fluid secretion in vivo, and secretin was significantly more potent than secretin-Gly for releasing somatostatin from gastric mucosal D cells in vitro and for inhibiting basal and gastrin-stimulated acid secretion in vivo. The most plausible explanation for these findings is the existence of secretin receptor subtypes. The subtype that stimulates pancreatic fluid secretion is likely to be the previously described pancreatic receptor (29), which we demonstrate here as recognizing secretin and secretin-Gly equally. A second receptor is preferentially activated by secretin and causes inhibition of gastric acid secretion. Other explanations for the different actions of secretin and secretin-Gly on gastric secretion, such as differences in metabolism of the two peptides or effects on non-secretin receptors, are unlikely.

We were not able to measure plasma levels of secretin-Gly to ensure that similar doses of secretin and secretin-Gly resulted in similar circulating concentrations. However, several points suggest that differences in metabolism of secretin and secretin-Gly probably do not account for the observed effects on pancreatic and gastric function. Fully processed and COOH-terminally extended secretins have equal or greater potency for stimulating pancreatic secretion in vivo (26). In contrast, secretin inhibited but secretin-Gly stimulated basal acid output, and secretin was distinctly more effective in inhibiting gastrin-induced acid secretion. These observations would require organ-specific differences in inactivation of the two forms of secretin if peptide degradation, rather than activation of different secretin receptor subtypes, was responsible for the two patterns of activity. A more plausible explanation for these disparate actions of fully processed and COOH-terminally extended secretins is that two receptors with different patterns of recognition are involved in stimulation of pancreatic fluid secretion on one hand and inhibition of gastric acid secretion on the other.

It is also highly unlikely that the actions of fully processed and COOH-terminally extended secretin were mediated by nonspecific actions on other receptors, such as those for vasoactive intestinal polypeptide (VIP) or pituitary adenylate cyclase-activating peptide (PACAP). A specific secretin receptor has been cloned from a rat pancreatic cDNA library (14, 29). This secretin receptor is localized to pancreatic duct and acinar cells (30) and almost certainly mediates the direct stimulatory effects of secretin on bicarbonate secretion by duct cells and enzyme secretion by acinar cells. Neither VIP nor PACAP is a potent stimulant of pancreatic secretion in the rat (1, 16). The crucial questions for the hypothetical existence of a different secretin receptor that mediates inhibition of gastric acid secretion involve the characteristics of secretin-induced inhibition of acid secretion and the localization (and thus the potential mechanism of action) of such a receptor. In rats, secretin is a potent inhibitor of gastric acid secretion under certain conditions (25); VIP does not inhibit acid secretion in rats (18), and PACAP is a weak inhibitor that acts indirectly (17, 18).

The mechanisms by which secretin inhibits gastric acid secretion appear to be indirect via release of somatostatin and prostaglandins. The effects of secretin on acid secretion are blocked by immunoneutralization of somatostatin and by inhibition of prostaglandin synthesis (25). Secretin increases gastric venous effluent levels of somatostatin (5–7, 24, 35) and prostaglandin E_{2} (7). These observations indicate that the inhibitory action of secretin on gastric acid secretion is mediated by local release of somatostatin by gastric mucosal D cells and prostaglandin E_{2} by unknown cells. Secretin has been reported to stimulate somatostatin secretion by cultured human antral D cells (3), suggesting that D cells may be one target that bears the inhibitory secretin receptor. Our finding that secretin—but not secretin-Gly—induced somatostatin release from partially purified rat mucosal D cells supports this hypothesis. However, we observed that secretin-Gly was weaker but not completely ineffective for inhibition of gastric acid secretion. This suggests that other inhibitory mechanisms such as prostaglandin generation may be activated by both molecular forms of secretin.

With Northern blot analysis of mRNA, the single rat secretin receptor that has been cloned to date (14, 29) has been localized to several tissues including the stomach (14). More precise definition of the cell types bearing this receptor is not currently available. The existence of secretin receptors on chief cells (23), fundic or antral D cells (3, 5–7, 24, 35), mucus-secreting cells (15), and forestomach smooth muscle cells (27) in rats is suggested by experimental data showing direct actions of secretin on the isolated stomach, mucosa, or cell populations in vitro. All of these experimental results were obtained using only fully processed secretin, because COOH-terminally extended secretin forms have not been available for characterization of their bioactivities.

Structure-activity relationships for binding and activation of the secretin receptor have been investigated using the cloned pancreatic receptor subtype. When this receptor or chimeric receptors composed of portions of the secretin, VIP, and glucagon receptors are studied, deletion of the NH_{2}-terminal histidyl residue of secretin reduces binding and activation by 1,000-fold (32), indicating a critically important role of the NH_{2}-terminal structure of secretin for binding and activation. For the pancreatic secretin receptor, the COOH-
terminal region of secretin also appears to be involved in receptor recognition, although to a lesser degree; peptides with deletions of the COOH-terminal Val-amide or Gly-Leu-Val-amide were only 10- and 50-fold less potent than intact secretin-(1–27)-amide (12). Recognition of the NH$_2$-terminal histidyl residue of secretin by the pancreatic secretin receptor subtype also appears to be sensitive to addition of amino acid residues, at least as judged by the distinctly lower in vivo bioactivity of an alternatively processed secretin form with an NH$_2$-terminal nonapeptide extension and COOH-terminal amidation (2). In contrast, COOH-terminal extensions of secretin do not reduce its pancreatic secretory bioactivity in vivo (present results and Refs. 4, 9, 10, 26) or binding (present results) and functional activation (19) of the pancreatic secretin receptor in vitro. The secretin receptor that mediates inhibition of gastric acid secretion appears to be very specific for the presence of an α-carboxyl amide group. We show here that addition of a COOH-terminal glycyl residue markedly reduces the acid inhibitory potency of secretin, and in unpublished studies we observed that the free acid form of secretin was also clearly less potent for this action. The acid-inhibitory effect of secretin is also affected by the NH$_2$-terminal structure of the peptide, because secretin-(5–27) is ineffective in blocking gastrin-induced acid secretion in rats (unpublished observation). Finally, it has been shown that residues 8–15 of secretin are important for the transfected pancreatic receptor to distinguish secretin from PACAP (11), suggesting that this region should also be evaluated for differences between the pancreatic and acid-inhibitory secretin receptor subtypes.

The data presented here indicate that there are at least two secretin receptor subtypes with different selectivities for fully processed vs. COOH-terminally extended secretins. In addition, the equipotency of secretin and secretin-Gly for binding to the cloned and transfected rat pancreatic secretin receptor suggests that previously described COOH-terminally extended molecular forms of secretin may be of physiological importance. There are several possible mechanisms that could result in the existence of a secretin receptor subtype with different selectivities for secretin molecular forms. First, there could be two structurally different receptors produced by two different genes. In general, most families of receptor subtypes appear to be produced by this mechanism, and members of these families show a substantial degree of structural homology at the nucleotide and amino acid levels (8). The presence of multiple bands on Northern blot analysis of various tissues with a secretin receptor probe is indirect evidence that structurally related receptors might indeed exist (21). Second, there may be only a single secretin receptor gene product that undergoes differential mRNA splicing, posttranslational processing, or G protein coupling to result in tissue-specific differences in agonist selectivity. Different patterns of mRNA splicing can produce distinct functional differences in receptors, as exemplified by splice variants of the PACAP receptor (22). The degree of posttranslational processing of the secretin receptor has in fact been shown to alter patterns of secretin binding and activation (20). It is at least theoretically possible that this could also result in different agonist selectivity of the known secretin receptor. It is also theoretically possible that tissue-specific differences in G protein coupling could alter receptor selectivity for different molecular forms of secretin.

Receptor subtypes provide the potential to select among structurally related agonists that are produced by different genes (i.e., the CCK-A receptor selects between CCK and gastrin) or by posttranslational processing of a single gene product (i.e., the Y$_1$ receptor selects between NPY/PYY-(1–36) and NPY/PYY-(3–36)). Our preliminary characterization of the relative amounts of secretin molecular forms in rat intestine indicates that secretin-Gly is present in at least 50% of the amount of secretin (38). The ratio of the two forms may be regulated by physiological conditions (postnatal development, feeding, acid secretion). These considerations suggest that the existence of secretin receptor subtypes could be functionally significant for selecting between secretin and secretin-Gly for actions on pancreatic and gastric secretion, as well as other potential targets of secretin. The use of secretin and secretin-Gly as pharmacological probes should facilitate the identification of putative secretin receptor subtypes and their participation in gastrointestinal and other regulatory events mediated by secretin.

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