Food-induced expression of orexin receptors in rat duodenal mucosa regulates the bicarbonate secretory response to orexin-A

Magnus W. Bengtsson,1* Kari Mäkelä,2* Markus Sjöblom,1 Sanna Uotila,2 Karl E. O. Åkerman,1,2 Karl-Heinz Herzig,2,3,4 and Gunnar Flemström1

1Department of Neuroscience, Division of Physiology, Uppsala University, Uppsala, Sweden; 2Departments of Biotechnology and Molecular Medicine and Neurobiology, A. I. Virtanen Institute for Molecular Sciences, University of Kuopio; 3Department of Internal Medicine, Kuopio University Hospital, Kuopio; and 4Department of Physiology, Oulu University, Oulu, Finland

Submitted 3 November 2006; accepted in final form 20 June 2007

Bengtsson MW, Mäkelä K, Sjöblom M, Uotila S, Åkerman KE, Herzig K-H, Flemström G. Food-induced expression of orexin receptors in rat duodenal mucosa regulates the bicarbonate secretory response to orexin-A. Am J Physiol Gastrointest Liver Physiol 293: G501–G509, 2007. First published June 21, 2007; doi:10.1152/ajpgi.00514.2006.—Presence of appetite-regulating peptides orexin-A and orexin-B in mucosal endocrine cells suggests a role in physiological control of the intestine. Our aim was to characterize orexin-induced stimulation of duodenal bicarbonate secretion and modulation of secretory responses and mucosal orexin receptors by overnight food deprivation. Lewis × Dark Agouti rats were anesthetized and proximal duodenum cannulated in situ. Mucosal bicarbonate secretion (pH stat) and mean arterial blood pressure were continuously recorded. Orexin-A was administered intraarterially close to the duodenum, intraluminally, or into the brain ventricles. Total RNA was extracted from mucosal specimens, reverse transcribed to cDNA and expression of orexin receptors 1 and 2 (OX1 and OX2) measured by quantitative real-time PCR. OX1 protein was measured by Western blot. Intra-arterial orexin-A (60–600 nmol·h−1·kg−1) increased (P < 0.01) the duodenal secretion in fed but not in fasted animals. The OX1 receptor antagonist SB-334867, which was also found to have a partial agonist action, abolished the orexin-induced secretory response but did not affect secretion induced by the muscarinic agonist bethanechol. Atropine, in contrast, inhibited bethanechol but not orexin-induced secretion. Orexin-A infused into the brain ventricles (2–20 nmol·kg−1·h−1) or added to luminal perfusate (1.0–100 nM) did not affect secretion, indicating that orexin-A acts peripherally and at basolateral receptors. Overnight fasting decreased mucosal OX1 and OX2 mRNA expression (P < 0.01) as well as OX1 protein expression (P < 0.05). We conclude that stimulation of secretion by orexin-A may involve both receptor types and is independent of cholinergic pathways. Intestinal OX receptors and secretory responses are markedly related to food intake.

bicarbonate secretion; enteroendocrine cells; fed and fasting state; perfused duodenum in situ; TRH

OREXIN-A AND OREXIN-B ARE orexigenic neuropeptides, initially thought to be located exclusively in hypothalamic neurons and involved in central nervous control of arousal and appetite (27, 40). More recently a role of orexins in the central nervous control of the gastrointestinal tract has also been indicated. Thus intracranial administration of orexin-A increases pancreatic secretion of protein and fluid (34) and gastric secretion of acid (37, 47). Moreover, there have been an increasing number of reports of orexin synthesis and presence of its receptors (OX1 and OX2) in peripheral tissues, including the gastrointestinal tract (26, 27, 35). In the small intestine, orexins are present in endocrine (enterochromaffin) cells in the mucosa as well as the enteric nervous system (26, 35). Furthermore, it has been proposed that peripherally produced orexins have a role in the physiological control of gastric and intestinal motility (11, 35).

The role of orexins in the central nervous control of appetite as well as in the peripheral control of the gastrointestinal tract make modulation of their expression and actions by food intake of particular interest. A short period of food deprivation was recently shown to profoundly modulate the small intestinal secretory response to orexin-A. Stimulation of duodenal alkaline secretion was thus abolished by overnight fasting (17, 18). Food deprivation for a longer period of time (2 days) did not affect preproorexin gene expression or hypothalamic orexin-A peptide levels in lactating rats, but there was a significant increase in hypothalamic orexin-B levels in these animals (6). Fasting for 3 days activated orexin-A-containing intestinal submucosal neurons as measured by immunoreactivity and phosphorylated Ca2+/cAMP response element binding protein (cCREB) expression (26).

The aim of the present study was to characterize the orexin-induced stimulation of duodenal HCO3− secretion. The alkaline secretion maintains pH at the duodenal surface at neutrality and is currently accepted as the primary mechanism of defense against acid discharge from the stomach. The duodenal secretion is furthermore a good model of small intestinal electrolyte transport in general (1, 15). Evidence is presented here that stimulation of duodenal bicarbonate secretion by orexin-A is mediated by peripheral OX1 receptors. Atropine does not affect this response, indicating its independence of cholinergic pathways. Furthermore, it is shown that overnight fasting significantly decreases OX1 and OX2 mRNA receptor expression as well as OX1 protein levels in the duodenal mucosa. This is compatible with the loss of orexin-induced stimulation of the intestinal secretion following overnight food deprivation.

MATERIALS AND METHODS

Animal preparation. Male F1-hybrids of Lewis × Dark Agouti rats (230–280 g, 7–9 wk old; Animal Department, Biomedical Center, Uppsala, Sweden) were housed in standard Macrolon cages (59 × 50 × 27 cm) and allowed ad libitum access to food and water. Food intake was recorded daily. Anesthetics and procedures were approved by the Animal Ethics Committee of Uppsala University.
38 × 20 cm) containing wood-chip bedding material, in groups containing two or more animals. Animals were kept under standardized temperature and humidity conditions (20 ± 1°C and 50 ± 10%, respectively) on a 12:12-h light-dark cycle. All animals had free access to water and, unless deprived of food overnight, free access to food pellets. In some experimental groups, animals were fasted for 16 h before experimentation. All experiments were approved by the Uppsala Ethics Committee for Experiments with Animals.

Animals were anesthetized at 9 am with Inactin, 120 mg/kg body wt ip. To minimize preoperative stress, the person who had previously handled the animals always performed the anesthesia within the Animal Department. Animals were tracheotomized, and body temperature was maintained at 37–38°C throughout the experiments by a heating pad controlled by a rectal thermistor probe. The surgical and experimental protocols have been described fully previously (13, 16, 44). A brief summary and some modifications are described here. A femoral artery and vein were catheterized with PE-50 polyethylene catheters (Becton-Dickinson, Franklin Lakes, NJ). For continuous recordings of the mean arterial blood pressure, the arterial catheter, containing 20 IU/ml heparin isotonic saline, was connected to a recordings of the mean arterial blood pressure, the arterial catheter, containing 20 IU/ml heparin isotonic saline, was connected to a recorder. A cannula into the duodenum (via the cranial pancreaticoduodenal artery) and the pancreas. The distribution of perfusate was checked visually at the start of each experiment by intra-arterial injection of a small amount (<0.1 ml) of Ringer solution.

Intra-arterial infusion to the duodenum. Orexin-A was administered to the duodenum by close intra-arterial infusion, described previously (44). Only small amounts of the compound were thus required, minimizing any central nervous actions. The hepatic artery was cannulated 3–4 mm proximal to its entrance into the liver and perfused in the retrograde direction at a rate of 17 μl/min. This perfusion resulted in distribution of the perfusate mainly to the duodenum (via the cranial pancreatocoduodenal artery) and the pancreas. The distribution of perfusate was checked visually at the start of each experiment by intra-arterial injection of a small amount (<0.1 ml) of Ringer solution.

Intracerebroventricular infusion. A stereotaxic instrument (model 900, Kopf Instruments, Tujunga, CA) was used to insert a metal cannula into the right cerebral ventricle. A skin incision was made over the right parietal bone and a 1-mm hole was drilled through the bone, 0.8 mm posterior to the bregma and 1.5 mm lateral to the midsagittal suture. The cannula was fixed in situ by cementing to the skull (Fuji type II, GC, Tokyo, Japan). Artificial cerebrospinal fluid (in mM: 151.5 Na+, 3.0 K+, 1.2 Ca2+, 0.8 Mg2+, 132.8 Cl−, 25 HCO3−, and 0.5 phosphate; pH 7.4) was infused through this cannula at a rate of 30 μl/h. All drugs infused intracerebroventricularly had been dissolved in the artificial cerebrospinal fluid. The presence of the cannula within the intracerebroventricular space was controlled after the end of experiments by adding Evans blue solution to the infusate and then dissecting the brain.

Quantitative real-time PCR. Continuously fed and overnight-fasted rats were killed by decapitation between 9 and 10 AM. A 10–15 mm segment of duodenum, starting ~5 mm distal to the pylorus was promptly excised via an abdominal midline incision and freed from mesentry. The segment was then opened along the antemesenterial axis, mounted as a sheet and cut into ~2-mm-thick slices. Slices were treated with RNA later (Qiagen, Hilden, Germany) and total RNA was extracted with RNeasy (Qiagen) according to manufacturer’s instructions. Genomic DNA was digested by treatment with DNase I (Qiagen). cDNA was synthesized from 1 μg RNA by using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA) with random hexamers as primers. Specific primers for rat OX1 and OX2 were used as reported by Beck et al. (3). All of the samples were normalized to β-actin mRNA. The primer sequences used were as follows: OX1, 5′-GCGCGATTATCCTCTATCCGAA-3′ (sense) and 5′-AAAGCTATGAGAAACACGGCC-3′ (antisense); OX2, 5′-GGAGTGCCATCTCTACTCTGTG-3′ (sense) and 5′-GATTTCCAGAGATTGCTCGGG-3′ (antisense); β-actin, 5′-CAACCCTGAAAAAT-GACCCAGA-3′ and 5′-AGCACAGAGGCATACAGGGAC-3′. Primers were synthesized either at the A. I. Virtanen Institute, Kuopio, Finland or at the TAG Copenhagen A/S, Denmark. PCR reactions were performed with ABI-PRISM 7700 sequence detection system (Applied Biosystems) in total volume of 30 μl. Reactions contained a 2-μl sample (400 ng). 1X SYBRgreen master mix (Applied Biosystems) and forward and reverse primers (15 pmol forward and 30 pmol reverse for OX1 primers, 15 pmol of both OX2 primers and 6 pmol of both rat β-actin primers). All samples were done as duplicates in following conditions: 2 min at 50°C and 10 min at 95°C followed by 42 cycles of 15 s at 95°C and 1 min at 62°C. Each assay included a relative standard curve of three serial dilutions of cDNA from fasted rat and no template controls. Results were calculated according to the manufacturer’s instructions (Applied Biosystems, ABI PRISM 7700 sequence detection system).

Western blotting. Samples of duodenal mucosa from four fed and four overnight-fasted rats were homogenized in 60 μl of solubilizing solution [25 mM Tris pH 7.4, 0.1 mM EDTA, 1 mM DTT, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)] by sonication at +4°C for 15 min. After centrifugation (12,500 rpm, 15 min, +4°C)
total protein concentrations were measured by the Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Munich, Germany). Equal amounts of protein (100 μg protein/lane) were electroforesed on a 12.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane [Bio-Rad, Trans-Blot transfer medium, Pure Nitrocellulose Membrane (0.2 μm), Bio-Rad Laboratories, CA]. Blocking was done with 5% nonfat milk powder in TBS containing 0.01% Tween 20 at room temperature for 1 h. Incubation with OX1 antibody (1:1,000, anti-rat/human orexin 1, cat no. OX1R11-A, Alpha Diagnostic International, San Antonio, TX) was performed overnight at +4°C. β-Actin antibody (1:2,000, no. 4967, Cell Signaling Technology, Danvers, MA) was used as a loading control. Membranes were then wasched and incubated with a secondary antibody [1:10,000, Zymax goat anti-rabbit IgG(H+L) horseradish peroxidase conjugate, Zymed, San Francisco, CA] for 1 h. Afterward, blots were washed and chemiluminescence detected with an ECL Plus (GE Healthcare, Amersham, UK) according to the manufacturer’s instructions. Detection was made with Typhoon 9400 Imager (GE Healthcare) and band densities were analyzed with ImageQuant TL program (GE Healthcare).

Chemicals and drugs. Atropine sulfate, bethanechol (carbamylmethylcholine chloride), orexin-A (human, bovine, rat), prostaglandin E2, thyrotropin-releasing hormone (TRH), and the anesthetic 5-ethyl-3-methylcholine chloride, orexin-A (human, bovine, rat), prostaglandin E2, thyrotropin-releasing hormone (TRH), and the anesthetic 5-ethyl-methylcholine chloride, orexin-A was stored at 20°C for a maximum of 6 days. Prostaglandin E2 was added as a small amount from an ethanol stock solution stored at −20°C.

Data analyses. Descriptive statistics are expressed as means ± SE. Rates of alkaline secretion by the duodenum are expressed as microequivalents of base (HCO3−) per centimeter of intestine per hour. The secretion and the mean arterial blood pressure were recorded continuously and registered at 10-min intervals. The statistical significance of data was tested by repeated-measures ANOVA. To test the difference within a group, a one-factor repeated-measure ANOVA was used followed by Tukey’s multiple comparison post hoc test. Between groups, comparison was made by two-factor repeated-measures ANOVA followed by a one-way ANOVA at each time point. If the ANOVA was significant at a given time point, a Tukey’s multiple comparison post hoc analysis was used. All statistical analyses were performed on an IBM-compatible computer using Prism 4.0 software (GraphPad Software, San Diego, CA). Orexin receptor expression data between groups were compared by the Student’s nonpaired t-test. P values of <0.05 were considered significant.

RESULTS

The rat duodenum in situ, in all cases, spontaneously secreted HCO3− at a steady basal rate, and neither this secretion nor the mean arterial blood pressure was influenced by intraarterial or luminal administration of vehicle (isotonic saline) alone. Intracerebroventricular infusion of vehicle (isotonic saline) alone did cause a slight and continuous rise in duodenal alkaline secretion but did not affect the arterial blood pressure. In some experimental groups, there was a slight decline in the mean arterial blood pressure during the (up to) 240 min-time period of the experiments. However, the blood pressure remained ≥90 mmHg in all animals studied. Animals were continuously infused intravenously with Ringer-bicarbonate solution, and blood acid-base balance was measured in all experimental groups. Mean blood pH was 7.36 ± 0.01 at the start of the first control period and 7.38 ± 0.01 at the end of experiments (n = 110). Blood HCO3− concentrations were 30.7 ± 0.3 and 28.7 ± 0.3 mM, respectively. The slight decrease in blood HCO3− concentration attained statistical significance (P < 0.05), but no significant differences between experimental groups were observed.

Intra-arterial administration of orexin-A and SB-334867. Close intra-arterial infusion of orexin-A (60–600 nmol·kg−1·h−1) caused a dose-dependent increase (P < 0.05 with 60 and P < 0.01 with 240 and 600 nmol·kg−1·h−1) in mucosal HCO3− secretion in fed animals (Fig. 2). Infusion of the lowest dose (60 nmol·kg−1·h−1) alone for extended period of time (150 min, n = 8) caused an initial (40 min) rise in secretion from 15.0 ± 2.4 to 17.8 ± 2.4 μeq·cm−1·h−1. The rate of HCO3− secretion then remained at the latter plateau throughout the

![Fig. 2](http://ajpgi.physiology.org/)

Fig. 2. Close intra-arterial infusion of orexin-A (60–600 nmol·kg−1·h−1) causes a dose-dependent increase in duodenal HCO3− secretion in continuously fed animals. All doses tested caused a significant rise in secretion. Pretreatment with the orexin receptor 1 (OX1) receptor antagonist SB-334867 (6 nmol/kg, intra-arterial bolus dose) inhibited orexin-induced stimulation of secretion. In contrast, the muscarinic antagonist atropine (0.75 μmol/kg iv followed by 0.15 μmol·kg−1·h−1 iv) had no effect on the stimulation induced by orexin-A. SB-334867 (SB) was injected and administration of atropine (Atr) started as indicated. Means ± SE of HCO3− secretion and mean arterial blood pressure (BP) in orexin-A-infused animals and in control animals receiving vehicle alone are shown (n ≥ 7 in all groups).

AJP-Gastrointest Liver Physiol • VOL 293 • AUGUST 2007 • www.ajpgi.org
Pretreatment with the OX1 receptor antagonist SB-334867 (6 nmol/kg intra-arterial bolus dose) inhibited the secretory response to orexin-A (Fig. 2). Not even the highest rate of orexin-A infusion (600 nmol·kg⁻¹·h⁻¹) caused a rise in secretion in animals pretreated with SB-334867 (P > 0.05). Intra-arterial bolus injection of SB-334867 alone (6 nmol/kg) tended to increase basal HCO₃⁻ secretion (Figs. 2 and 4), but the slight rise in secretion did not attain statistical significance (P > 0.05). In contrast, intra-arterial bolus injection of a tenfold higher dose of SB-334867 (60 nmol/kg) caused a significant (P < 0.05) rise in secretion (n = 5, not shown). The lower dose of 6 nmol/kg was thus selected for studies of the antagonist action of SB-334867 (Figs. 2 and 4).

A partial agonist action of SB-334867 was confirmed by continuous intra-arterial infusion of the compound at consecutively increasing rates (Fig. 3). In fed animals, there was a rise in HCO₃⁻ secretion that attained significance (P < 0.05) with the highest dose infused (6 nmol·kg⁻¹·h⁻¹). Effects of continuous infusion of SB-334867 were tested also in overnight food-deprived animals (Fig. 3). No significant increase in secretion was observed in the food-deprived group, indicating a partial agonist action of SB-334867, appearing only in fed animals and at infusion of higher doses of the compound.

Blood glucose concentration was 8.3 ± 0.8 mM at the start of the initial control period, preceding infusion of orexin-A alone, and 6.7 ± 0.7 mM at the end of these experiments. There was a decline in blood glucose of very similar magnitude in control animals infused with vehicle (isotonic NaCl) alone, from 9.5 ± 0.3 to 6.5 ± 0.5 mM (n = 6). A decline in blood glucose concentration of similar magnitude occurred also in animals pretreated with SB-334867.

Effects of muscarinic receptor ligands. The muscarinic antagonist atropine (0.75 μmol/kg bolus dose followed by 0.15 μmol·kg⁻¹·h⁻¹, both iv) did not affect the orexin-induced rise in mucosal HCO₃⁻ secretion (Fig. 2). Basal secretion as well as secretion stimulated by infusion of 60 and 240 nmol·kg⁻¹·h⁻¹ of orexin-A appeared higher in the atropine-treated group than in animals infused with orexin-A alone, but differences between groups did not attain statistical significance (P > 0.05). In contrast, atropine abolished stimulation induced by the muscarinic agonist bethanechol and also prevented the bethanechol-induced decline in mean arterial blood pressure (Fig. 4). Bethanechol alone significantly (P < 0.05) stimulated secretion at a dose of 5 μmol·kg⁻¹·h⁻¹. Higher doses of bethanechol caused a marked decline in the mean arterial blood pressure and were therefore not used.

Bethanechol-induced secretion was studied in some further experiments. The intra-arterial bolus dose of SB-334867 (6 nmol/kg) inhibiting stimulation by orexin-A (Fig. 2) did not prevent the rise (P > 0.05) in secretion induced by bethanechol (Fig. 4). Nor did this dose of SB-334867 affect the decrease (P < 0.05) in mean arterial blood pressure induced by the two larger doses of bethanechol. The absence of effects of atropine on orexin-induced secretion (Fig. 2) and of SB-334867 on bethanechol-induced secretion (Fig. 4) suggests independence between pathways for orexin-A and muscarinic-induced stimulation of the duodenal HCO₃⁻ secretion.

Luminal administration of orexin-A. Some agents, including the peptides glucagon, urogaquain, and heat-stable enterotoxin (14, 23) as well as the neurohormone melatonin (43) are potent stimuli of the duodenal secretion when added to the luminal perfusate. In addition, we have previously shown that orexin releases cholecystokinin (29), a duodenal secretagogue (1), from the neuroendocrine cell line STC-1. However, presence of orexin-A (1 to 100 nM) in the luminal perfusate did not affect the HCO₃⁻ secretion by the duodenal mucosa (Fig. 5). There were no significant differences in rates of secretion between orexin-A perfused and control animals. Nor did luminal orexin-A affect the mean arterial blood pressure or the decline in blood glucose concentration during the experimental period (not shown). Prostaglandin E₂ (20 μM), added to the luminal perfusate at the end of all experiments as a test of the viability of the preparation, caused a similar rise in HCO₃⁻ secretion in orexin-A-perfused and control animals (P < 0.01 in both groups).
Central nervous administration of orexin-A. It has been shown that central nervous (intracisternal) bolus injection of orexin-A increases pancreatic exocrine secretion of both protein and fluid (34) and also stimulates gastric secretion of acid (37, 47). Intracerebroventricular infusion of orexin-A (2 or 20 nmol·kg⁻¹·h⁻¹) caused a small continuous rise (P < 0.05) in duodenal HCO₃⁻ secretion, and this rise continued after cessation of orexin-A infusion (Fig. 6). However, there was a similar increase (P < 0.05) in secretion in control animals infused with vehicle alone. The mean arterial blood pressure in orexin-infused animals remained at a stable level (P > 0.05) and was very similar to that observed in animals infused with artificial cerebrospinal fluid alone (Fig. 6).

The absence of an effect of intracerebroventricular orexin-A on the duodenal alkaline secretion could in theory reflect operation-induced damage of the vagal supply to the cannulated duodenal segment. Central nervous administration of TRH has been shown before to elicit stimulation of the duodenal alkaline secretion mediated by the vagal nerves (16, 30). Intracerebroventricular TRH caused a rapid rise (P < 0.05) in duodenal secretion in the present study (Fig. 6), confirming that the cannulated segment had persistent efferent vagal supply, and also slightly increased (P < 0.05) the mean arterial blood pressure.

Blood glucose concentration decreased from 8.8 ± 0.5 to 8.2 ± 0.6 mM in animals infused intracerebroventricularly with 2 nmol·kg⁻¹·h⁻¹ of orexin-A, and from 10.3 ± 0.9 to 8.0 ± 0.9 mM in those infused with the higher dose of 20 nmol·kg⁻¹·h⁻¹. These decreases in glucose concentration were not different from that of 10.2 ± 0.5 to 8.0 ± 0.5 mM observed in the control animals infused with vehicle alone.

mRNA and protein expression of OX1 and OX2. Primers for quantitative real-time PCR were developed to detect OX1, OX2, and rat β-actin mRNA in the duodenal mucosa of fasted and fed rats. Expression of OX1 as well as OX2 were significantly downregulated in fasted animals compared with fed animals (P < 0.01). The mRNA levels of OX1 and OX2 in fasted animals were reduced by 44.5 and 70.1%, respectively.
Protein levels of OX1 in the rat duodenal mucosa samples were determined by Western blotting and compared between fed and fasted animals. OX1 was detected with an apparent molecular mass of 50 kDa whereas \( \beta \)-actin was detected as a 45-kDa peptide. Quantification analysis revealed a significantly \((P < 0.05)\) decreased amount of OX1 in fasted animals compared with fed animals. The protein level of OX1 in fasted animals was reduced by 49.3% compared with that in fed animals (Fig. 8).

**DISCUSSION**

Our results demonstrate that both OX1 and OX2 receptor mRNA are expressed in the duodenal mucosa. Orexin-induced stimulation of the \( \text{HCO}_3^- \) secretion by the duodenal mucosa was inhibited by the OX receptor antagonist SB-334867 which in vitro has up to 50-fold higher selectivity for OX1 over OX2 receptors and an excellent selectivity over range of other receptors (45). Furthermore, several groups have shown that SB-334867 antagonizes the elevation of food consumption induced by intracerebroventricular orexin-A and provided support for specific involvement of OX1 receptors in regulation of feeding patterns (5). Synthesis and actions of orexins in peripheral tissues is a recent area of research and selectivity of SB-334867 in gastrointestinal tissues in vivo has not yet been determined. The results from other preparations make it likely, however, that the orexin-A induced duodenal secretory response is mediated primarily by the OX1 receptor.

A role of OX2 receptors in mediation of small intestinal secretion cannot be excluded and would be an interesting subject of study. However, use of OX2-selective receptor antagonists would seem to be required. It should also be noted that OX1 and OX2 receptors are expressed in the same brain regions (27). Similarly, both receptors are expressed in some peripheral tissues (10, 24) and in cultured adrenocortical cells (46). Single-cell RT-PCR measurements from tuberomammillary neurons suggest that both receptors are expressed in the...
same cells (12, 41). Distinction between responses from the OX1 and OX2 receptors may presently prove difficult.

Higher doses of SB-334867 alone stimulated the duodenal secretion, suggesting a partial agonist action of the compound. As found with orexin-A, stimulation by SB-334867 occurred only in animals that had been continuously fed. It would seem of considerable interest to determine whether stimulation by endogenously produced orexins contributes to the basal HCO₃⁻ secretion by the duodenal mucosa. Inhibition of basal secretion by OX receptor antagonists would provide a clue. The likely partial-agonist action of SB-334867 made this compound unsuitable for such experiments.

Our use of close intra-arterial infusion enabled use of very small amounts of orexin-A and the antagonist SB-334867, minimizing any central nervous actions. It has been reported from studies in conscious rats that central nervous (intracerebroventricular) bolus injection of orexin-A (1.0 nmol, bolus dose), via activation of vagal efferents, stimulates pancreatic secretion of protein and fluid (34) and also that intracerebroventricular bolus injection of orexin-A (0.2–2.7 nmol) causes a dose-dependent increase in gastric acid secretion (37, 47). In contrast, peripheral administration of orexin-A affected neither the pancreatic nor the gastric secretion (34, 37). The duodenal alkaline secretion was stimulated by parenterol orexin-A but central nervous (intracerebroventricular) infusion of the peptide had no significant effect. The observed slight increase in HCO₃⁻ secretion was thus very similar to that in control animals infused with vehicle (artificial cerebrospinal fluid) alone. Experimental findings in the pancreas, stomach, and small intestine thus provide strong evidence that orexins have a role in peripheral as well as central neurohumoral control of the gastrointestinal tract. However, organ-dependent differences appear to be very considerable and effects on small intestinal secretion may in the main reflect peripheral actions of orexins.

The absence of a secretory response to intracerebroventricular orexin-A could, in theory, reflect operation-induced damage of vagal nerves mediating a centrally elicited response to the duodenum. After penetrating the diaphragm, the abdominal vagal trunks split into the paired gastric and celiac branches and the unpaired hepatic branch (4, 50). The duodenum receives fibers from all three branches and axons from the gastric branches travel through the pyloric sphincter to the duodenum. Our operative procedure might damage the latter pathway. However, the duodenum was never cut in the present experimental setup (Fig. 1). It should also be noted that the hepatic branch that enters the duodenum via the perivascular plexuses of the hepatic, gastroduodenal, and superior pancreaticoduodenal arteries innervates primarily the duodenum. Previous studies with the present type of in situ chamber preparation have shown that intracerebroventricular administration of the peptide hormone TRH (16, 30) or the catecholamine phenylephrine (28, 44) induces vagally mediated stimulation of duodenal alkaline secretion. Furthermore, it has been shown that electrical stimulation of the cut cervical vagal nerves in the distal direction does stimulate the secretion in rat and also cat duodenum in situ (22, 36). The combined evidence that the cannulated duodenum has functional vagal innervation must be considered very strong. In addition, we confirmed the stimulatory action of intracerebroventricular TRH in the present study (Fig. 6).

Most studies of the neurohumoral control of gastrointestinal functions in humans and in intact animals have used principles developed over a century ago in Pavlov’s laboratory (38), and by tradition such experiments are conducted after an overnight fast. It was early noted that longer periods of starvation (up to 4 days) resulted in organ-specific changes in peptide hormone concentrations in the gastrointestinal tract as well as in the central nervous system (42). Effects of short periods of food deprivation have been much less studied, and effects of overnight fasting would seem of particular physiological importance. Interestingly, our data demonstrate that overnight fasting results in downregulation of OX1 as well as OX2 receptor mRNA in the duodenal mucosa. The decline in expression of OX receptors is most likely the mechanism for the absence of a duodenal secretory response to exogenous orexin-A in fasted animals. It has been reported that fasting for 24 h regulates the orexin system in an organ-dependent manner. OX1 and OX2 mRNA as well as protein levels are upregulated in the hypothalamus in response to fasting (24, 32). In adrenal glands there is reduced expression of OX1 and OX2 in a similar manner to that shown in the present study (24). This could mean that the central and peripheral orexin systems are controlled in an opposite way by fasting.

The mechanisms by which feeding upregulates the intestinal responsiveness to orexin are not known. Influence from the central nervous system cannot be excluded but a likely explanation is that food contents have a stimulatory action on orexin receptor expression. The lining of the gastrointestinal tube contains a number of neuroendocrine cells and neurons that may respond to feeding. This allows the intestine to undergo an integrated response to changes in its luminal contents (19). Orexins are expressed both in enterochromaffin cells and in nerve endings of enteric neurons (26, 35). It would seem reasonable that such influences are responsible for upregulation of orexin receptors with a consequent paracrine/endocrine orexin-mediated response. Other neurohumoral mechanisms may also contribute to this difference. Leptin inhibits fasting-induced upregulation of preproorexin and OX1 receptor expression in rat hypothalamus (31). Furthermore, high-lipid diets in close association with elevated triglyceride levels increase orexin expression in the perifornical hypothalamus in rats and mice (48). The response to feeding or fasting might thus depend on the hormonal status at the time of fasting and the type of food.

We showed earlier that short fasting also altered the sensitivity (but not the magnitude) of cholinergic stimulation of secretion (18). A role for cholinergic mechanisms in orexin action on the contractility of guinea pig ileum has previously been shown (33) and many of the central actions of orexins are mediated by cholinergic mechanisms (40). Muscarinic stimulation causes potent stimulation of duodenal secretion in all species tested in vivo and in vitro (2, 8, 20, 22, 36) and atropine abolished the secretory response to the muscarinic subtype-independent agonist bethanechol in the present study (Fig. 4). However, atropine did not affect the secretory response to orexin-A. Furthermore, the OX1-antagonist SB-334867 had no effect on secretion induced by the muscarinic agonist bethanechol (Fig. 4). These findings provide evidence that orexin-induced stimulation of the duodenal secretion is independent of cholinergic pathways. In contrast to orexin-A and bethanechol, rises in HCO₃⁻ secretion in response to serotonin (39), mela-
tonin, or vasoactive intestinal polypeptide (18) are not altered by overnight food deprivation. This means that certain pathways for stimulation of intestinal secretion are selectively downregulated.

During the present experiments the acid-base balance remained unchanged and there was only a slight decline in blood $\mathrm{HCO}_3^-$ concentration in some groups, whereas the mean arterial blood pressure remained $\cong 90 \text{ mmHg}$ in all animals. These controls provide evidence that animals were maintained in condition proper for study of epithelial acid-base transport. In most animals, there was a decrease in blood glucose concentration during the course of the experiment. However, the declines in blood glucose occurring in animals treated parenterally with orexin-A or the antagonist SB-334867 were of magnitudes very similar to that observed in control animals infused with vehicle alone. Some groups have demonstrated dose-dependent increases in heart rate and mean arterial blood pressure after administration of orexin-A, intracerebroventricularly (49), or to specific brain areas such as the nucleus tractus solitarius (9) and the rostral medulla (7). Interestingly, the regulation of cardiovascular properties seems age dependent (21), and only 7- to 9-wk-old animals were used in the present study. This might contribute to the absence of blood pressure effects of central nervous orexin-A administration. It should also be emphasized that orexin-A is a highly lipophilic peptide that easily passes brain tissue (25). The mode of administration as well as the acid-base and general condition of the animals may influence the response.

Strong evidence is thus presented that the duodenal secretory response to orexin-A is mediated by peripheral OX receptors and independent of cholinergic pathways. Overnight food deprivation is the natural behavior in humans and several animal species and also a standard procedure in experiments on gastrointestinal function and pathophysiology. The results above show that studies made on intestinal secretion or effects of drug therapy may require particular evaluation with respect to feeding status. Furthermore, our studies demonstrate the involvement of a well-known appetite-increasing peptide in regulation of small intestinal secretion and duodenal bicarbonate-dependent protection against luminal acid. This provides a very interesting link between the central nervous system and the intestine.

ACKNOWLEDGMENTS

The authors thank Gunilla Jedstedt for excellent technical assistance and Dr. Adrian Allen for very helpful comments.

GRANTS

This research was supported by the Swedish Research Council (Grant 3515 to G. Flemström and M. Sjöblom), the Finnish Academy (Grants 108478 and 110525 to K.-H. Herzig), Novo Nordisk Foundation (to K.-H. Herzig), and the Jalmari and Rauha Ahokkaan and the Northern Savo Cultural Foundation (to K. Mäkelä).

REFERENCES


