Short food deprivation inhibits orexin receptor 1 expression and orexin-A induced intracellular calcium signaling in acutely isolated duodenal enterocytes

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Bengtsson MW, Mäkelä K, Herzig KH, Flemström G. Short food deprivation inhibits orexin receptor 1 expression and orexin-A induced intracellular calcium signaling in acutely isolated duodenal enterocytes. Am J Physiol Gastrointest Liver Physiol 296: G651–G658, 2009. First published December 31, 2008; doi:10.1152/ajpgi.90387.2008.—Close intra-arterial infusion of the appetite regulating peptide orexin-A stimulates bicarbonate secretion from the duodenal mucosa. The aim of the present study was to elucidate the ability of orexin-A to induce intracellular calcium signaling in acutely isolated duodenal enterocytes. Freshly isolated clusters of enterocytes, obtained from rat duodenal mucosa or human duodenal biopsies, were loaded with fura 2-AM and mounted in a perfusion chamber. Cryptlike enterocytes were selected (caged), and changes in intracellular calcium concentration ([Ca2+]i) were evaluated by fluorescence imaging. Total RNA was extracted from pellets of enterocytes and reverse transcribed to cDNA, and expression of orexin receptors 1 and 2 (OX1R and OX2R) was measured by quantitative real-time PCR. Orexin-A at all concentrations tested (1–100 nM) increased [Ca2+]i, in enterocytes isolated from continuously fed rats, and the OX1R-antagonist SB-334867 (10 nM) attenuated the response. The primary [Ca2+]i response was a slow increase to a sustained plateau persisting after orexin-A removal, and a similar response was observed in enterocytes from human biopsies. In contrast to orexin-A, the OX2R agonist (Ala11,D-Leu15)-orexin-B (1–10 nM) did not induce calcium signaling. There were no significant [Ca2+]i responses in enterocytes from animals food deprived overnight, and overnight fasting decreased (P < 0.01) enterocyte OX1R as well as OX2R mRNA. Induction of intracellular calcium signaling in isolated duodenal enterocytes is thus mediated primarily by OX1R receptors. Short (overnight) food deprivation markedly depresses receptor expression and inhibits orexin-A induced increases in [Ca2+]i. Studies of enterocyte signaling and intestinal secretion requires particular evaluation regarding feeding status.

(Ala11,D-Leu15)-orexin-B; bicarbonate secretion; fasting; feeding; SB-334867

OREXINS were DISCOVERED in 1998 and initially thought to be expressed exclusively in the hypothalamus and to be involved in central nervous control of arousal and appetite (9, 34). However, since the original discovery there have been an increasing number of studies demonstrating orexin synthesis and presence of orexin receptors in peripheral tissues, including the gastrointestinal tract (15). Expression and immunohistochemical location of orexins (orexin-A and orexin-B) and their receptors (OX1R and OX2R) have been established along the entire length of the gastrointestinal tract but seem to occur particularly in the duodenum (12, 15, 19, 29, 30).

It has been proposed that orexins have a number of different actions in the small and large intestine. Orexin-A is able to increase the excitability of ileal neurons in vitro (18, 19) and to induce both colonic (19) and ileal (27) motility. On the basis of findings that orexins and their receptors are expressed in mucosal enteroendocrine cells and enteric neurons, including secretory motor neurons (10, 12, 19, 30), we have recently investigated effects of intra-arterial infusion of low doses (60–600 pmol·kg−1·h−1) of orexin-A on the HCO3− secretion by the duodenal mucosa (4, 13). Interestingly, an increase in alkaline secretion occurred only in animals with continuous access to food. Short (overnight) fasting abolished the secretory response. It has also been suggested that orexins influence the gastrointestinal tract by actions elicited primarily within the central nervous system (7, 28, 40). Intracerebroventricular infusion of orexin-A is thus reported to induce gastric acid secretion (40) and pancreatic exocrine secretion (28). In contrast, intracerebroventricular administration of orexin-A did not affect the duodenal alkaline secretion (4).

The orexin family consists of two separate peptides, orexin-A and orexin-B, derived from the same precursor, preproorexin (PPO). Effects of these peptides are mediated by two specific G protein-coupled receptors, OX1R and OX2R (21). Signaling pathways downstream of orexin receptors are not fully clarified, but there are several plausible suggestions (21). A typical cellular response to orexin-A stimulation is an increase in intracellular calcium concentration ([Ca2+]i). It has been shown that orexins induce intracellular calcium transients in receptor-transfected cells (16, 34, 38), as well as in native-receptor-expressing cells (20, 24, 43), and these transients thus provide a useful indicator of orexin receptor activation. Generally, increases in [Ca2+]i, can depend on a variety of mechanisms (5, 6). Studies performed in Chinese hamster ovary (CHO) cells suggest that orexin-A-induced calcium transients originate from different calcium sources depending on the ligand concentration. At low concentrations of orexin-A, the primary calcium influx seems to be due to the opening of receptor-operated calcium channels. At higher concentrations of orexin-A, it is suggested that increases in [Ca2+]i, is the result of calcium release from the endoplasmic reticulum.

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as well as of influx via store-operated calcium channels (21, 22, 26).

The aim of the present study was to elucidate the ability of orexin-A to stimulate intracellular calcium mobilization in acutely isolated duodenal enterocytes. Freshly isolated clusters of enterocytes, devoid of enteric neurons, were obtained from rat duodenal mucosa or human duodenal biopsy samples. Cells were loaded with fura 2 acetoxymethyl ester (AM) and mounted in a perfusion chamber. Fluorescence imaging was then used to evaluate changes in [Ca\(^{2+}\)]. Our results demonstrate, for the first time, that orexin-A at all doses tested (1–100 nM) increases [Ca\(^{2+}\)] in isolated enterocytes. This occurred in enterocytes isolated from fed rats but, in contrast, not in enterocytes from animals deprived of food overnight. Responses to orexin-A were also observed in enterocytes from human biopsy samples.

Furthermore, pretreatment with the OX1R-selective antagonist SB-334867 attenuated the response to orexin-A, and the OX2R-selective agonist (Ala\(^{11}\),d-Leu\(^{15}\))-orexin-B did not induce intracellular calcium signaling. These results strongly suggest that orexin-A–induced [Ca\(^{2+}\)], responses are mediated by enterocyte membrane OX\(_{1R}\). Real-time PCR was used to study the expression of mRNA by the two orexin receptors in isolated enterocytes. The PCR data demonstrate marked down-regulation of orexinergic mRNA expression in enterocytes isolated from fasted animals. This is in line with the absence of orexin-induced calcium signaling in these cells.

**MATERIALS AND METHODS**

**Animals.** Male F1 hybrids of Lewis × Dark Agouti rats (230–280 g; Animal Department, Biomedical Center, Uppsala) were housed in standard Macrolon cages (59 × 38 × 20 cm) containing wood-chip bedding material, in groups of two or more animals. They were kept under standardized temperature and humidity conditions (20 ± 1°C and 50 ± 10%, respectively) on a 12-h light-dark cycle. All animals had free access to water and, unless deprived of food overnight, free access to pelleted food (Labfor, Kimstad, Sweden). Overnight deprivation of food implied 16 h of fasting before the experiments. All experiments were approved by the Uppsala Ethics Committee for Experiments with Animals.

**Isolation of duodenal enterocytes.** The procedures for isolation of clusters of duodenal enterocytes has been described in detail elsewhere (37) and tested in further studies (33). Experiments began at approximately 0930 and, after euthanasia of the animals, an abdominal midline incision was made by use of commercially available fura 2 calcium imaging kit (F-6774; Molecular Probes, Eugene, OR) and a diaphragm mirror (DM430; Nikon, Tokyo, Japan). Emission was performed at 37°C in a horizontal shaking water bath and was stopped by adding DTT to a final concentration of 0.3 mM. The solution was then centrifuged for 3 min at 1,000 g. The resulting pellet was washed three times by suspension in 10 ml DMEM/F-12 (15 mM HEPES and 2.5 mM glucose) followed by centrifugation (3 min at 1,000 g). HCO\(_3\)\(^{-}\) (1 mM), gentamicin (0.01 mg/ml), and fetal calf serum (2.0%) were always added to the DMEM/F-12, and the pH was adjusted to 7.40. The final pellet was again suspended in ~1 ml of DMEM/F-12 and immediately put on ice, a procedure found to substantially increase the viability of the enterocyte clusters compared with keeping them at 37°C (37). The preparation procedure yielded clusters of interconnected duodenal enterocytes as well as small numbers of single cells. During the experiments, clusters of 10–50 interconnected cells were chosen for imaging analysis and up to 50 enterocytes were selected (caged) prior to exposure to orexin receptor ligands or atropin. Calcium signaling in each caged enterocyte was stored by a computer and analyzed at the end of experiments. The clusters were composed predominantly of cells with morphological characteristics of crypt cells (8, 41). Submucosal tissue was not included and no enteric neurons were detectable by light microscopy or during fluorescence microscopy of fura 2-AM loaded preparations. Enteric neurons are markedly stained by fura 2 and would thus have been easy to identify. The viability of the enterocytes after preparation was tested by Trypan blue exclusion.

**Human biopsies.** Human duodenal biopsy samples was obtained from patients undergoing upper endoscopy at the Gastroenterology Unit, Uppsala University Hospital and found to have endoscopically normal duodenal and gastric mucosa. No food was taken in the morning before the endoscopy. The project was approved by the Ethics Committee of the Medical Faculty at Uppsala University, and all subjects provided written, informed consent. Biopsy samples were taken between 0900 and 1000 with a single-use biopsy forceps (Boston Scientific, Natick, MA) and were immediately placed into 20 ml of sterile DMEM/F-12 medium (plus 15 mM HEPES, l-glutamine, and 1 mM HCO\(_3\)\(^{-}\)) containing 2.0% fetal calf serum and 0.01 mg/ml gentamicin and transported to the laboratory. There, the samples were cut into fine pieces at room temperature within 20–40 min after removal. Clusters of duodenal enterocytes were then prepared and the viability was assessed as described above for rat duodenum.

**Calcium imaging.** For measurements of [Ca\(^{2+}\)], 70 μl of the cell cluster suspension was loaded at 37°C with fura 2-AM (2 μM) for 20–30 min in an electrolyte solution containing (in mM) 141.2 Na\(^{+}\); 3.0 K\(^{+}\); 1.0 Ca\(^{2+}\); 1.2 Mg\(^{2+}\); 121.8 Cl\(^{-}\); 1.2 SO\(_4\)\(^{2-}\); 6.0 phosphate, 15.0 HEPES, 1.0 pyruvate, and 10.0 glucose, plus 10 mg/ml phenol red, 0.1 mg/ml gentamicin, and 2.0% fetal calf serum. The pH was adjusted to 7.40 immediately before use, and the temperature was maintained at 37°C. The sheet of duodenal wall was then mounted on a precleaned glass slide (luminal side facing up) and the mucosa was gently scraped off. The scraping procedure used has been evaluated previously by morphological examination, and cells originating from Brunner’s glands were excluded from the preparations (37). The scraped-off mucosa was cut into pieces 0.3–0.8 mm in diameter that were dispersed and briefly shaken in NRM solution with addition of 0.5 mM diithiothreitol (DTT). After 2–3 min were allowed for sedimentation, the supernatant was removed and the tissue fragments remaining in the sediment were washed three times in DTT-free NRM solution. Following gassing with 100% O\(_2\), the tissue fragments (15–20 μl) were submitted to mild digestion for 2–3 min by inoculation in 10 ml NRM solution containing 0.1 mg/ml collagenase type H and 0.1 mg/ml dispase II. Digestion was performed at 37°C in a horizontal shaking water bath and was stopped by adding DTT to a final concentration of 0.3 mM. The solution was then centrifuged for 3 min at 1,000 g. The resulting pellet was washed three times by suspension in 10 ml DMEM/F-12 (15 mM HEPES and 2.5 mM glucose) followed by centrifugation (3 min at 1,000 g). HCO\(_3\)\(^{-}\) (1 mM), gentamicin (0.01 mg/ml), and fetal calf serum (2.0%) were always added to the DMEM/F-12, and the pH was adjusted to 7.40. The final pellet was again suspended in ~1 ml of DMEM/F-12 and immediately put on ice, a procedure found to substantially increase the viability of the enterocyte clusters compared with keeping them at 37°C (37). The preparation procedure yielded clusters of interconnected duodenal enterocytes as well as small numbers of single cells. During the experiments, clusters of 10–50 interconnected cells were chosen for imaging analysis and up to 50 enterocytes were selected (caged) prior to exposure to orexin receptor ligands or atropin. Calcium signaling in each caged enterocyte was stored by a computer and analyzed at the end of experiments. The clusters were composed predominantly of cells with morphological characteristics of crypt cells (8, 41). Submucosal tissue was not included and no enteric neurons were detectable by light microscopy or during fluorescence microscopy of fura 2-AM loaded preparations. Enteric neurons are markedly stained by fura 2 and would thus have been easy to identify. The viability of the enterocytes after preparation was tested by Trypan blue exclusion.

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coupled device camera (Cohu, San Diego, CA), and measurements were performed 30 times per minute. The relative increase in \([Ca^{2+}]_i\) was expressed as the ratio of the fluorescence intensity at 540 nm to that at 380 nm (340 nm/380 nm) and converted to \([Ca^{2+}]_i\).

**Quantitative real-time PCR.** After isolation, clusters of enterocytes devoid of duodenal submucosa were promptly frozen in liquid nitrogen and then kept at −78°C pending analyses. At the time of analysis total RNA was extracted with RNaseasy (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Genomic DNA was digested by treatment with Dnase I (Qiagen). cDNA was synthesized from 1 μg RNA by using the TagMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) with random hexamers as primers. Specific primers for rat OX1R and OX2R were used as reported by Beck et al. (3). The samples were normalized to 18s rRNA endogenous control (35). The primer sequences used were as follows: OX1R, 5′-GGCTATGAAACACGGCC-3′ (sense) and 5′-AA-GGCTATGAAACACGGCC-3′ (antisense); OX2R, 5′-GGAGTG-CGATCCTCTCACTCCTG-3′ (sense) and 5′-GATCCCCATAAGGAT-GCTGGG-3′ (antisense); 18s, 5′-GTAACCCGTTGAAACCCATT-3′ (sense) and 5′-CCATCTACCCGATGAGC-3′ (antisense). Primers were synthesized either at the A. I. Virtanen Institute, Kuopio, Finland or at TAG Copenhagen, Copenhagen, Denmark. PCR reactions were performed with ABI-PRISM 7700 sequence detection system (Applied Biosystems) in a total volume of 30 μL. Reactions contained 2 μL sample (400 ng), 1× SYBR Green master mix (Applied Biosystems) and forward and reverse primers (15 pmol forward and 30 pmol reverse OX1R primers, 15 pmol of both OX1R primers, and 5 pmol of both 18s primers). All samples were subjected in duplicate to the 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).

**Chemicals and drugs.** Membrane filters (polycarbonate, diameter 25 mm, thickness 25 μm, 3 × 10^6 pores/cm², pore diameter 3 μm) were obtained from Osmonics, Livermore, CA. Dispase II was purchased from Boehringer-Manheim (Mannheim, Germany) and orexin-A (human, bovine, rat), atropine sulfate, carbachol (carbamylcholine chloride), collagenase type H, Dulbecco’s modified Eagle’s medium with Ham’s nutrient mixture F-12 (DME-F12), and gentamicin (Sigma-Aldrich, St. Louis, MO). The OX1R antagonist SB-334867 [1-(2-methylbenzoxanzol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride] and the OX2R agonist (Ala11,D-Leu15)-orexin-B were purchased from Tocris Bioscience (Ellisville, MO), and Pluronic F-127 and the fura 2 calibration imaging kit (F-6774) were from Molecular Probes. Fura 2-AM was obtained from Calbiochem (La Jolla, CA), and fetal calf serum (Svanocolone, FBS Super) was from The National Veterinary Institute (Häタンholm, Sweden).

**Data analyses.** Descriptive statistics are expressed as ratio of proportions and 95% confidence interval for categorical data and otherwise as means ± SE. When appropriate the statistical significance of data was calculated and tested by Fisher’s exact test. Linear trend between doses was tested by the χ² test for trend. Orexin receptor expression data were compared between groups by Student’s nonpaired t-test. Some cells spontaneously increased \([Ca^{2+}]_i\) during the experimental period, probably reflecting presence of cell-to-cell signaling within the cluster preparations. Figures display the ratio of proportions between orexin-responding cells and spontaneously responding controls. A ratio larger than 1, indicated by a dashed line in figures, thus indicates orexin-A-induced stimulation of calcium signaling. All statistical analyses were performed on an IBM-compatible computer using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). P values of <0.05 were considered significant.

**RESULTS**

Experiments were run on preparations taken from the final perfusate up to 6 h after the isolation procedure, and selected (caged) enterocytes were studied for up to 25 min in the temperature-regulated perfusion chamber. Enterocytes were selected primarily from clusters of 10–50 interconnected enterocytes. As tested by Trypan blue exclusion, the enteroctye viability was >95% after preparation and remained at >80% after a 6-h period. Most caged cells displayed a stable \([Ca^{2+}]_i\) of ~100 nM during the initial prepeptide period of the experiments. Control experiments were performed by perfusion with ligand-free electrolyte solution.

**Orexin-A induced calcium signaling in rat enterocytes.** Fed animals had free access to their regular supply of food until the start of the experiments. Orexin-A was added to the perfusate at two concentrations in each experiment. Exposure to the peptide at a concentration of 1 nM was followed by exposure to 10 nM (271 cells in clusters from 8 rats), or exposure to a concentration of 10 nM was followed by 100 nM (220 cells in clusters from 5 rats). The duration of exposure to each concentration was 180 s. Orexin-A increased \([Ca^{2+}]_i\), at all concentrations tested \((P < 0.01)\) in enterocytes harvested from continuously fed animals (Fig. 1). The proportion of responding cells increased dose dependently from ~16% at the lowest concentration of 1 nM to ~20% at 10 nM and ~29% at 100 nM (trend, \(P < 0.05)\). In the majority of responding cells, \([Ca^{2+}]_i\), increased without a clear initial peak to reach a sustained plateau that remained stable within the experimental period. Cessation of exposure to orexin-A did not result in any immediate decline in \([Ca^{2+}]_i\). The magnitude of the rise in \([Ca^{2+}]_i\), was concentration dependent in cells that responded already to the lower of the two tested concentrations of orexin-A (Fig. 2). The cholinergic agonist carbachol (100 μM) was added to the perfusate in some experiments as a test of the viability of the enterocytes from fed animals. In contrast to orexin-A, carbachol induced intracellular calcium signaling \((P < 0.001,\) data not shown) in the majority of the caged enterocytes.

**Fig. 1.** At all concentrations tested (1–100 nM), 180 s of exposure to orexin-A induced intracellular calcium signaling in duodenal enterocytes acutely isolated from continuously fed animals. Despite a longer time of exposure (600 s), the same concentrations did not elicit a response in cells isolated from fasted animals. The proportion of caged cells that responded with increased intracellular calcium concentration (\([Ca^{2+}]_i\)) to orexin-A stimulation. Responding controls are cells showing spontaneous changes in \([Ca^{2+}]_i\). Ratio of proportions and 95% confidence interval (CI) are shown. Dashed line represents ratio of 1. **\(P < 0.01\); ***\(P < 0.001\).
Orexin-A (10 and 100 nM) increased \([\text{Ca}^{2+}]_i\) also in the absence of calcium in the perfusate (153 enterocytes in clusters from 5 rats). As in the presence of extracellular calcium, the peptide induced a \([\text{Ca}^{2+}]_i\) response in a significant \((P < 0.01)\) proportion of enterocytes. The signaling pattern was, however, distinctly different from that observed in cells with access to extracellular calcium; the rise in \([\text{Ca}^{2+}]_i\) displaying an initial peak response followed by a rapid decline (Fig. 3).

Fasted animals had been deprived of food overnight (16 h) before the isolation of enterocytes. This period of fasting inhibits the duodenal \(\text{HCO}_3^-\) secretory response to exogenous orexin-A in rats in vivo (4, 12). Initially, we tested the same concentrations of peptide and durations of exposure as were used in experiments with enterocytes from fed animals. The absence of significant effects (not shown) made it of interest to use a protocol with longer durations of exposure to orexin-A. Only one concentration of orexin-A was superfused in each experiment and the time of exposure to the peptide was extended to 600 s. The concentrations used were 1 nM (52 cells in clusters from 3 rats), 10 nM (139 cells in clusters from 4 rats), or 100 nM (132 cells in clusters from 4 rats). No significant responses to orexin-A were observed (Fig. 1). Furthermore, the cholinergic agonist carbachol (100 \(\mu\)M) was added to the perfusate at the end of experiments as a test of the viability of the enterocytes. Carbachol induced intracellular calcium signaling (Fig. 4) in a majority of enterocytes from fasted animals also.

**Controls with atropine.** The present findings strongly suggest that orexin-A exerts its action directly at enterocyte membrane receptor. Expression of orexin receptor mRNA in enteric neurons and endocrine cells has been demonstrated, however, in some studies (19, 29, 30). Thus the possibility could not be excluded that enterocyte responses to orexin-A reflect an action primarily at acetylcholine-releasing enteric neurons. However, only preparations microscopically devoid of enteric neurons were used in the present study. Enteric neurons are markedly stained by fura 2-AM and would thus have been easily detectable. Furthermore, pretreatment of cell preparations (fed animals) with the muscarinic antagonist atropine (1 \(\mu\)M) did not affect basal \([\text{Ca}^{2+}]_i\) nor the proportion of cells responding to 1 or 10 nM orexin-A (213 cells in clusters from 5 rats, not shown). Neither did atropine affect the

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**Fig. 2.** Rat duodenal enterocytes (clusters) were loaded with fura 2-AM, and changes in \([\text{Ca}^{2+}]_i\), were evaluated by fluorescence imaging and converted to actual levels of calcium (nM). Orexin-A at perfusate concentrations \(\geq 1\) nM, 180 s, caused a increase in \([\text{Ca}^{2+}]_i\) without a clear initial peak response. The magnitude of the \([\text{Ca}^{2+}]_i\), was concentration dependent in cells already responding to the lower concentration of orexin-A. A: perfusate concentration of orexin-A, 1 and 10 nM. B: perfusate concentrations of orexin-A, 10 and 100 nM.

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**Fig. 3.** Effects of orexin-A (100 nM, 600 s) in rat duodenal enterocytes perfused with calcium-free solutions. Orexin-A increased \([\text{Ca}^{2+}]_i\), also in the absence of extracellular calcium. The initial \([\text{Ca}^{2+}]_i\) response was followed by a rapid decline.

**Fig. 4.** Duodenal enterocytes (clusters) harvested from animals fasted overnight did not respond to any of the tested concentrations (1–100 nM, 600 s) of orexin-A. The viability of the cells was tested by addition of carbachol (100 \(\mu\)M, 180 s) to the perfusate at the end of experiments. Carbachol induced a robust increase in \([\text{Ca}^{2+}]_i\), in a majority (>50%) of the caged cells.
shape or magnitude of the orexin-induced $[\text{Ca}^{2+}]_i$ responses (not shown).

Enterocytes from human biopsies. The patients undergoing endoscopy took no food in the morning before the procedure. This is not directly comparable to overnight fasting, but we used the same protocol for enterocytes from human biopsies as for those from fasted rats. Orexin-A was added to clusters of human enterocytes (81 cells, 1 biopsy sample from each of 3 patients) for an extended period of time (600 s) and in separate experiments for each concentration. The results from experiments with a lower concentration of orexin-A (10 nM) were inconclusive, but at a higher concentration (100 nM) the peptide induced intracellular calcium signaling in $\sim 11\%$ of the human enterocytes ($P < 0.05$). The pattern of the increase in $[\text{Ca}^{2+}]_i$ was similar to that observed in rat enterocytes, but in general the magnitude of the response was smaller (Fig. 5).

Orexin receptor antagonist and agonist. A previous study in vivo provided evidence that the OX1R-selective antagonist SB-334867 at higher doses may act as a partial agonist (4). We therefore performed experiments in which SB-334867 alone (1, 10, and 100 nM) was added to the perfusate (503 cells in clusters from 6 rats), using the same protocol as for orexin-A. None of the tested concentrations of SB-334867 induced an increase in $[\text{Ca}^{2+}]_i$. In further experiments we used the high concentration of 1,000 nM of SB-334867 (128 cells in clusters from 5 animals). In contrast to the lower doses, this high concentration induced a robust increase in the intracellular calcium level in $\sim 24\%$ of the enterocytes ($P < 0.001$) (Fig. 6), with a calcium signaling pattern similar to that induced by orexin-A (not shown). These findings suggest that, at higher concentrations, SB-334867 acts in a partial agonistic fashion also in vitro.

The lower concentration of 10 nM of SB-334867 was therefore selected to test whether the antagonist inhibits the $[\text{Ca}^{2+}]_i$ response to orexin-A. The clusters were preperfused with SB-334867 for 180 s and the antagonist was then present in the perfusate throughout the experimental period. SB-334867 completely abolished ($P < 0.01$) the $[\text{Ca}^{2+}]_i$ response to orexin-A (1 and 10 nM, 180 s exposure, 530 cells in clusters from 6 rats) (Fig. 7).

In further experiments we examined effects of (Ala$^{11}$,d-Leu$^{15}$)-orexin-B, an agonist that displays a 400-fold selectivity for OX2R over OX1R as evaluated from $[\text{Ca}^{2+}]_i$ responses in the CHO-K1 cell line (2). The agonist was added to the perfusate according to the same protocol as was used for orexin-A (1, 10, and 100 nM, 714 cells from 7 rats). None of the tested concentrations induced intracellular calcium signaling (Fig. 8).

Quantitative real-time PCR. Primers for detection of OX1R and OX2R have been published previously (4). 18s primers were adapted from Schmittgen and Zakrajsek (35). Expression of both receptors was significantly downregulated in cells from fasted animals compared with those from fed animals ($P < 0.01$). The mRNA levels of OX1R and OX2R in fasted animals were reduced by 54.5 and 60.4%, respectively (Fig. 9). RNA was isolated from 2-mm thick slices of the duodenal wall in a previous study (4), and results with isolated enterocytes and slices seem quite similar.

**DISCUSSION**

Our results demonstrate that the appetite-regulating peptide orexin-A acts directly on intestinal enterocytes both from rats...
and humans and provide strong evidence that the actions are mediated by OX1R. All tested doses of orexin-A were capable of inducing intracellular calcium signaling. When the signaling pattern in the majority of responding cells was compared with that induced by carbachol and CCK-8 in enterocytes in primary culture (8), or with that induced by melatonin in cluster preparations (37), the rise in \([\text{Ca}^{2+}]\), in response to orexin-A appeared to be slower and no clear initial peak responses were observed. The increase in \([\text{Ca}^{2+}]\), reached a plateau that remained stable within the experimental time period, even after cessation of exposure to orexin-A (Fig. 2). The signaling pattern was similar, however, to that induced by serotonin in acutely isolated enterocytes (33) and that induced by orexin-A in subpopulations of neurons in the dorsal raphe and laterodorsal tegmentum (20). In contrast to the findings in the latter neurons, the initial part of the enterocyte \([\text{Ca}^{2+}]\), response persisted despite removal of extracellular calcium. It would seem likely that orexin-A increases enterocyte \([\text{Ca}^{2+}]\), by release of calcium from the endoplasmic reticulum as well as by influx of extracellular calcium via store-operated calcium channels (22, 23, 26).

Previous studies have indicated that food deprivation can influence the orexin system in an organ-specific manner. OX1R and OX2R mRNA as well as protein levels are upregulated in the hypothalamus in response to fasting (17, 25). We recently demonstrated that overnight fasting abolishes the duodenal \(\text{HCO}_3^-\) secretory response to orexin-A occurring in fed animals (4, 13). Furthermore, we provided evidence that fasting was associated with a decrease in OX1R and OX2R mRNA expression as well as OX1R protein expression in 2-mm-thick slices of the duodenal wall (4). Our present results show that orexin-A possesses the ability to induce intracellular calcium signaling in enterocytes acutely isolated from rat duodenal mucosa and that previous food intake is a prerequisite for the \([\text{Ca}^{2+}]\), response. The decline in orexin receptor expression in isolated enterocytes is quite similar to that observed in slices of the duodenal wall (4), suggesting that the fasting procedure in main affects enterocyte orexin receptor levels. Carbachol induced \([\text{Ca}^{2+}]\), responses also in enterocytes from starved animals that did not respond to orexin-A, excluding the possibility that short fasting induces a general decline in enterocyte receptor expression.

In contrast to our finding in enterocytes from fasted rats, orexin-A induced calcium signaling in human enterocytes, and no food had been taken in the morning before endoscopy in the patients undergoing that procedure. This restriction of food intake is not directly comparable to overnight fasting, and it should be noted that a high concentration of orexin-A and an extended duration of exposure were required for orexin-A induction of intracellular calcium signaling in the human enterocytes. Furthermore, the \([\text{Ca}^{2+}]\), response was substantially weaker than that seen in enterocytes from fed rats. The weaker \([\text{Ca}^{2+}]\), response in enterocytes from humans may reflect the shorter period of food deprivation. The comparatively lower metabolic rate and rates of intestinal fluid and water transport in humans should also be noted (32).

Two orexin receptors have been identified so far, OX1R and OX2R. When expressed in CHO cells, OX1R have a 10 times higher affinity for orexin-A than for orexin-B, whereas OX2R display the same affinity for both peptides (34). The same is true for measurements of potency (16, 31, 34, 38). In the present study, we tested effects of the OX1R specific antagonist SB-334867 and the OX2R specific agonist (Ala\(^{11},\text{D-Leu}^{15}\))-orexin-B. Only enterocytes from continuously fed rats were used in these experiments. SB-334867 inhibited the \([\text{Ca}^{2+}]\), response to orexin-A and (Ala\(^{11},\text{D-Leu}^{15}\))-orexin-B did not affect \([\text{Ca}^{2+}]\). These findings provide further evidence that the orexin-induced \([\text{Ca}^{2+}]\), response is mediated by OX1R.

Expression of PPO, OX1R, and OX2R mRNA in the small intestine has been established by RT-PCR (4, 19). Orexin-A and orexin receptors in nerve fibers have been detected by immunochemistry and are found in both submucosal and myenteric plexa and in interconnecting neurons (12, 19, 29, 30).
The muscarinic antagonist atropine did not inhibit orexin-A-induced duodenal HCO$_3^-$ secretion, indicating that stimulation in vivo is independent of enteric cholinergic pathways (4). In contrast, it has been suggested that orexin-A to induces ileal motility by release of acetylcholine from enteric cholinergic neurons (27). The present study demonstrates that orexin-A induces intracellular calcium signaling in clusters of enterocytes microscopically devoid of neurons and that atropine does not inhibit the response. Orexins and their receptors are also present, however, in enteroendocrine cells in the duodenal mucosa (19, 30). The proportion of enterocytes responding to orexin-A (up to 29%) is much higher than that of enteroendocrine cells in the intestinal mucosa (~1%), a difference that would seem to exclude that a certain kind of paracrine cell would be the primary target of orexin-A. Our combined findings provide strong evidence for a direct action of orexin-A at duodenal enterocyte receptors.

The proportion of enterocytes in clusters responding to orexin-A (~29%) was lower than the proportion (~40%) responding to melatonin or carbachol in cluster preparations (37). It would thus seem likely that enterocyte location, maturation, and development influence expression of orexin receptors and that feeding induces expression of orexin receptors in only a limited number of the enterocytes. However, it should be noted that [Ca$^{2+}$]$_i$ responses to melatonin and CCK-8 (37) spread throughout clusters of duodenal enterocytes, suggesting cell-to-cell communication and that the duodenal epithelium may act as a functional syncytium. The latter may be in line with an early study (39) using intracellular micropuncture techniques to study secretagogue-evoked electrical responses in enterocytes along the crypt-villus axis. It should also be noted that, most probably, villus as well as crypt cells export HCO$_3^-$ to the duodenal lumen (1, 42). A decline in orexin receptor expression or treatment with orexin receptor antagonist may, as found in vivo (4), markedly reduce the overall HCO$_3^-$ secretory response to orexin-A.

Effects of orexins on glucose absorption in rat jejunum were previously reported (11). Orexin-A as well as orexin-B inhibited the glucose-induced transepithelial short-circuit current and the inhibition by orexin-A, but not that by orexin-B, was reduced by tetrodotoxin and by a cholecystokinin antagonist. The authors proposed that orexin-B acts inhibitory by an action on enteroocyte OX$_2$R whereas inhibition by orexin-A involves neural and endocrine mediation. OX$_3$R in rat jejunal mucosa was expressed mainly in epithelial cells (enterocytes) and OX$_2$R in a nonepithelial fraction. Our results are in line with their finding of OX$_2$R mainly in enterocytes. Furthermore, the OX$_2$R specific agonist [Ala$^{11}$,d-Leu$^{15}$]-orexin-B did not affect [Ca$^{2+}$]$_i$ in isolated duodenal enterocytes (Fig. 8). It should be noted that the intermittently acid-exposed duodenum, in marked contrast to jejunum, exports HCO$_3^-$ at high rates (1) and that orexin-A does not inhibit but stimulates (4, 13) duodenal HCO$_3^-$ secretion.

In conclusion, the present study demonstrates that orexin-A induces intracellular calcium signaling in rat and human duodenal enterocytes. Signaling occurs in the absence of surrounding neural tissue and most probably reflects an action at enterocyte OX$_2$R. Overnight food deprivation significantly decreases receptor expression and prevents stimulation by orexin-A. Overnight food deprivation, is by century-old tradition, a standard experimental procedure in studies of gastrointestinal function and pathophysiology in humans and animals. Our results strongly suggest that studies of intestinal secretion and effects of drug therapy require particular evaluation with respect to feeding status.

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