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The STC-1 cells express functional orexin-A receptors coupled to CCK release

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Abstract

Orexins are newly discovered neuropeptides regulating feeding and vigilance and have been detected in neuroendocrine cells of the gut. Potential neuroendocrine functions of orexin are unknown. Therefore, the effects of orexin-A on the intestinal neuroendocrine cell line, STC-1, were investigated as a model system. RT-PCR demonstrated the presence of both OX_1 and OX_2 receptors. Stimulation with orexin-A produced a dose-dependent release of cholecystokinin (CCK), which was abolished by removal of extracellular Ca^{2+} or the presence of the voltage-gated L-type Ca^{2+}-channel blocker diltiazem (10 μM). Orexin-A (Ox-A) elevated intracellular Ca^{2+}, which was dependent on extracellular Ca^{2+}. Furthermore, orexin-A caused a membrane depolarization in the STC-1 cells. Ox-A neither elevated cAMP levels nor stimulated phosphoinositide turnover in these cells. These data demonstrate a functional orexin receptor in the STC-1 cell line. Ox-A produces CCK release in these cells, by a mechanism involving membrane depolarization and subsequently activation of L-type voltage-gated Ca^{2+}-channels.

Keywords: STC-1; Neuroendocrine; Gastrointestinal hormones; Orexin; Cholecystokinin; Secretion; Ca^{2+}-imaging; Fura-2; Patch-clamp

Orexin-A (Ox-A) and orexin-B (Ox-B) were originally isolated as hypothalamic peptides, which act on their targets via two G protein-coupled receptors called OX_1 and OX_2 receptors [1,2]. Orexin containing neurons have been shown to be involved in brain regions controlling appetite, wakefulness, thermoregulation, and autonomic control (reviewed in [3]). Recently, orexins and their receptors have also been described outside the CNS particularly in organs involved in feeding and energy metabolism, e.g., gastrointestinal tract, endocrine pancreas, and adrenal gland, suggesting that orexins may exert additional endocrine functions in the periphery (reviewed in [3,4]). In the enteric nervous system neurons, orexin immunoreactivity is seen both in the submucosa and myenteric plexi. Orexinergic neurons in these plexi were suggested to be sensory or secretomotor based on the coexpression of other markers, such as vasoactive intestinal peptide [5]. Moreover, neuroendocrine cells in the gut and stomach display orexin immunoreactivity [5,6]. OX_1 and OX_2 receptors are also widely distributed in populations of intestinal neurons and neuroendocrine cells [5,7]. The potential endocrine functions of orexin are unknown, but as speculated by Kirchgessner et al. [5] orexins could prime the gut for the gastric and intestinal phase of secretion. In the colon, orexins show stimulation of motility [5]. In the small intestine, their actions are complex and an overall effect is inhibition of the fasting motility [7]. Orexins, however, cause contraction of isolated ileum by a mechanism mediated through acetylcholine release [8,9]. A relaxing action of orexins in the small intestine has also been observed via nitric oxide-mediated mechanisms [9]. Orexins thus act at several levels and their actions most

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likely involve other neurohumoral systems in the intestine. At the cellular level, Ox-A stimulates electrical responses in guinea pig submucosal neurons [5]. On the other hand orexins have been shown to inhibit the CCK-stimulated discharge of afferent vagal fibers [10]. In the target neurons, orexins cause depolarization and increase in the action potential frequency (reviewed in [3]).

The mechanisms involved in the excitatory actions of orexins are not clear. Several mechanisms have been suggested based on experiments with central neurons. There is usually a considerable delay before excitation occurs suggesting a role of second messenger linked pathways (reviewed in [3]). Some studies have suggested that a reduction in potassium permeability is the primary mechanism behind orexin-mediated depolarization (reviewed in [3]). More recently, however, activation of nonselective cation channels by orexins has also been observed and suggested to be the primary target of orexin receptor-mediated signaling [11–14]. It has been reported that orexins increase the extracellular Ca^{2+} influx [15–17] both in neurons and recombinant systems [18,19].

Due to the complex actions of peptides in the intestine, cellular models would provide an important tool to study the effect and mechanism of action of orexins in vitro. Since no such cellular model currently exists, the aim of the present study was to investigate the effects of orexin on intestinal neuroendocrine cell lines. We demonstrate here responses to orexin in the STC-1 cell line. These cells have a high CCK content and serve as an established model for neuroendocrine peptide release [20–25].

Materials and methods

Materials

Amino acid solution (without L-glutamine), L-glutamine (50×), and penicillin/streptomycin were from Gibco-BRL (Karlsruhe, Germany), fetal calf serum, high-glucose Dulbecco's modified Eagle's medium (HG-DMEM), and horse serum were from Biochrom AG (Berlin, Germany). Carbamylcholine (CCh), diltiazem, tetrodotoxin, GTP (Na^+-salt), ATP (Mg^{2+}-salt), forskolin, and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma–Aldrich (Helsinki, Finland). Human orexin-A and B was from Peninsula Laboratories Europa (St. Helens, UK). The CatchPoint cyclic-AMP fluorescent assay kit was from Molecular Devices (Sunnyvale, CA). Cell culture plates were from Sarstedt (Nümbrecht, Germany). Cholecystokinin-8 (CCK) was from Saxon Biochemicals (Hannover, Germany) and collagenase was from Cell Systems (Remagen, Germany). *Myo*-[^{3}H]Inositol was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Fura-2 acetoxymethyl ester (fura-2 AM) and fura-2-penta-potassium-salt were from Molecular Probes (Eugene, OR). Ionomycin was from Calbiochem (Darmstadt, Germany).

Solutions

The Na^+-based medium consisted of (in mM): NaCl 137, KCl 5, $CaCl_2$ 1, glucose 10, $MgCl_2$ 1.2, KH_2PO_4 0.44, $NaHCO_3$ 4.2, and Hepes 20 adjusted to pH 7.4 with NaOH. The Ca^{2+}-free Na^+-based medium refers to nominally Ca^{2+} free medium, i.e., Na^+-based medium without added $CaCl_2$. This medium had a free Ca^{2+} concentration of ~1 μM as optically determined using fura-2-penta-potassium salt. The K^+-based medium consisted of (in mM): KCl 142, $CaCl_2$ 1, glucose 10, $MgCl_2$ 1.2, KH_2PO_4 0.44, $KHCO_3$ 4.2, and Hepes 20 adjusted to pH 7.4 with KOH (the final $[K^+] = 158$ mM). The extracellular bath perfusates used in patch-clamp recordings were Na^+-based medium (see above) or choline-based medium consisting of (in mM): Choline–Cl 137, KCl 5, $CaCl_2$ 10, glucose 10, $MgCl_2$ 1.2, KH_2PO_4 0.44, $NaHCO_3$ 4.2, and Hepes 20. Tetrodotoxin (200 nM) was finally added and pH was adjusted to 7.4 with TEA-OH. The intracellular pipette solution used in whole-cell voltage-clamp recordings consisted of either a Cs^+-based solution (in mM): Cs–Aspartate 136, Hepes 30, NaCl 10, ATP 2, and GTP 0.6. The $[Ca^{2+}]$ in the pipette solution was optically measured using fura-2 and calibrated to around 100 nM by addition of EGTA, pH was finally adjusted to 7.3 with CsOH. In whole-cell current-clamp experiments, the intracellular solution was identical to Cs^+-based medium, with the exception that 136 mM Cs–Aspartate was substituted with 136 mM K–Aspartate and pH was adjusted with KOH.

Cell culture

The STC-1 cell-line was kindly provided by Dr. Douglas Hanahan (University of California, San Francisco, CA) [26]. Cells were grown at 37 °C in HG-DMEM supplemented with 15% horse serum, 2.5% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin under 95% air/5% CO_2. Cells were plated onto cover glasses or well plates and used for experiments when they were grown in 70–80% confluence.

Expression of OX_1- and OX_2-receptors in STC-1 cells

RNA isolation and cDNA synthesis: total RNA was extracted from STC-1 cells using silica gel-based spin columns (RNeasy Kit, Qiagen, Hilden, Germany). Genomic DNA was digested by thorough treatment with deoxyribonuclease I (Qiagen). First-stranded cDNA was synthesized from 1.0 μg RNA using the TaqMan Reverse Transcription Reagents (Applied Biosystems, CA) and oligo(deoxythymidine)$_{15}$ primer. PCR: OX_1 and OX_2 receptor sense and antisense oligonucleotide primers were designed based on the published mRNA sequences using the Primer3 software [27,28]. Oligonucleotides were obtained from TAG Copenhagen (Copenhagen, Denmark). The oligonucleotide sequence and product size for each primer pair used are shown in Table 1. PCR was performed with 2 μl first-stranded cDNA using the Taq polymerase from Invitrogen (Karlsruhe, Germany).

Table 1
Mouse nucleotide sequences of PCR primers

Primer	Orientation	Sequence size	PCR product size	GenBank accession no.
OX_1	Sense	5′-TCGGAACAACTGGAGGCTCA-3′	287 bp	AF394596
	Antisense	5′-ATGATAGGGTTGGCGGCACT-3′		
OX_2	Sense	5′-TGACCATCACCTGCCTTCCA-3′	299 bp	AF394597
	Antisense	5′-ATTGGTTAGGCCAGGGAGCA-3′		

PCR in the presence of 1× PCR-buffer, 1.2 mM $MgCl_2$, 0.2 mM dNTPs (each), 2.5 U Taq polymerase, 0.5 μM sense primer, and 0.5 μM antisense primer. Thermocycling was performed in a Tpersonal (Biometra, Göttingen, Germany) for 37 cycles with the following profile: 94 °C for 45 s, 62 °C for 50 s, and 72 °C for 50 s and a final extension at 72 °C for 10 min. β-Actin mRNA levels were analyzed with β-actin primers from A.I. Virtanen-Institute (Kuopio, Finland) as a positive control for cDNA synthesis. Aliquots of the PCR products of cDNA were electrophoresed on 8% TBE–polyacrylamide gels, stained with ethidium bromide (0.5 μg/ml TBE), and photographed.

CCK measurement

Before the experiments, the cells were washed twice with Hepes-buffered Ringer-solution (in mM: Hepes 10, KCl 4.7, NaCl 128, $MgCl_2$ 0.56, $CaCl_2$ 1, NaH_2PO_4 1, glucose 6, 0.1% soybean trypsin inhibitor, 0.1% bovine serum albumin, and 2% Eagle's minimum amino acid supplement, adjusted to pH 7.4, and equilibrated with 100% oxygen) followed by the addition of the stimulus or control vehicle for 15 min as previously described in [25]. Samples from each well (125 μl/well) were taken for CCK analysis immediately following the secretion experiment. CCK secretion was then assayed using a pancreatic acini bioassay system as described previously [29]. Amylase release was measured using an autoanalyzer (Eppendorf ACP 5040, Hamburg, Germany) according to the method of Kruse-Jarres et al. [30].

Quantitative fluorescence imaging

The experiments were performed essentially as described in [31]. The coverslips with cells loaded with fura-2 AM (4 μM, 30 min, and 37 °C) were attached to the bottom of a thermostated (37 °C) perfusion chamber [24]. The cells were excited by alternating 340 and 380 nm UV-light with the use of filter changer under the control of an InCyt IM-2 system (Intracellular Imaging, Cincinnati, OH) and a dichroic mirror (DM430, Nikon), and the emission was measured through a 510-nm barrier filter with an integrating CCD camera. A new ratio (340/380) image was achieved every second. All the additions were made isotonic with the Na^+-based medium.

Patch-clamp recordings

Voltage-clamp and current-clamp modes were applied using the standard whole-cell patch-clamp technique [32]. Experiments were conducted at ~28 °C. Perfusates were applied with ~2.5 ml/min via a 40 μl fast perfusion chamber. Voltage-dependent Na^+- and Ca^{2+}-currents were evoked with step-pulses (+10 mV; 500 ms) from a resting potential of −80 mV and corrected for current-leak. Cells were patched in Na^+-based medium, which also served in the recordings of voltage-dependent Na^+-channels, whereas the intracellular pipette solution was Cs^+-based in order to abolish the voltage-dependent K^+-currents. In these experiments, we also recorded Ca^{2+}-currents following perfusion exchange with choline-based medium. Current-clamp recordings ($I = 0$) were performed in Na^+-based medium with the K^+-based pipette solution. Patch pipettes (model PG150T, Harvard Apparatus, UK) were flame polished to a resistance of 4.0–4.5 MΩ, measured in the Na^+-based medium. The patch-clamp amplifier Axopatch 200A was attached to a computer via a Digidata1320E AD/DA interface (Axon Instruments, CA). Voltage protocols and data acquisition were under the control of pClamp Clampex 8.1 (Axon Instruments, CA). In voltage-clamp experiments, the series resistance was compensated 60–70%, while in current-clamp experiments only the pipette capacitance was compensated. Voltage- and current-clamp data were digitally sampled at 4 and 10 kHz, respectively, and filtered at 2 and 4 kHz, respectively, using the analogue lowpass Bessel filter on the patch-clamp amplifier. Cells lacking large processes were selected for recording.

Inositol phosphate measurements

For inositol phosphate measurements, the cells were loaded with 3 μCi/ml *myo*-[^{3}H]inositol for 20 h in the culture medium. The cells were detached using phosphate-buffered saline containing 0.2 g/L EDTA, spun down, and resuspended in the Na^+-based medium supplemented with 10 mM LiCl. After 10 min preincubation in this medium, the cells were stimulated for 20–60 min and the reactions were terminated by spinning and replacement of the buffer with ice-cold perchloric acid. The cells were frozen down, thawed, and neutralized with $KOH/KHCO_3$. The cell debris and the precipitates were spun down and the total inositol phosphate fraction of the neutralized supernatants was isolated with anion exchange chromatography [33].

cAMP measurement

Cells in 24-well plates were washed twice with Na^+-based medium and subsequently preincubated for 10 min with 0.5 mM IBMX at 37 °C, after which drugs (orexin-A and/or forskolin) were added and incubation was continued for another 10 min. The reactions were stopped by addition of lysis buffer supplemented in the CatchPoint cyclic-AMP fluorescent assay kit. An aliquot of the lysed cells was taken and assayed with the cAMP assay kit according to the manufacturer's instructions. The fluorescence was recorded from 384-well plates with a FLUOstar plate reader (BMG LabTechnologies GmbH, Offenburg, Germany) and cAMP concentrations were calculated from the standard curve assayed simultaneously. Mean protein amount per well was determined with the Bradford dye reagent.

Data processing

CCK-release. CCK-release evoked with 1 nM orexin-A was normalized (%) to CCK-release in resting condition.

Quantitative fluorescence imaging. Data were imported into a prewritten worksheet in Microcal Origin 6.0 for visualization and analysis.

Voltage- and current-clamp recordings. Recordings were processed in Clampfit (version 8.1) and imported into Microcal Origin 6.0 for visualization and further analysis.

Data between two independent groups were compared using Student's non-paired *t* test. $P < 0.05$ (*) was considered significantly different. Data are expressed as means ± SEM. The number of experiments is indicated by *n*.

Results

Using RT-PCR, we demonstrated expression of both OX_1 and OX_2 receptor mRNA in STC-1 cells (Fig. 1). Since STC-1 cells have previously been used as model to study peptide secretion (i.e., cholecystokinin (CCK)) in response to neurotransmitters [21–23,25], we investigated whether orexin-A (Ox-A) could release CCK during a 15 min incubation period. As shown in Fig. 2A, orexin-A evoked a dose-dependent release of CCK. Release was detectable with Ox-A concentrations in the low pM range and was maximal with concentrations above 1 nM. Ox-A significantly stimulated CCK release (2.7-fold) compared to the non-stimulated control cells (Student's non-paired *t* test, Fig. 2B, a). For comparison, incubation of the cells with 70 mM K^+ produced a CCK release, which was 10-fold larger than the release observed with 1 nM Ox-A (Fig. 2C). The baseline CCK

secretion was found to be around 6 pM in the control situation and reduced by the absence of extracellular Ca^{2+} (Fig. 2B, b) or by the presence of the voltage-gated Ca^{2+}-channel blocker diltiazem (10 μM) (Fig. 2B, c). These treatments also abolished Ox-A-stimulated CCK-release (Fig. 2B, b and c).

As the Ox-A-stimulated CCK release was dependent on extracellular Ca^{2+}, we monitored the effect of Ox-A on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Recordings of $[Ca^{2+}]_i$ from individual STC-1 cells revealed spontaneous irregular oscillations under basal conditions. The frequency of the oscillations varied from cell to cell, while the amplitude of the basal Ca^{2+}-changes was fairly constant ranging from 100 to 200 nM. Due to irregular baselines, we were only able to obtain a clear response in 33% of the cells (40/120) to 1 nM Ox-A, similar to that seen in Fig. 2D.

The increase in $[Ca^{2+}]_i$ by Ox-A was fairly slow and mounted to 51 ± 23 nM ($n = 40$). When extracellular Ca^{2+} was removed the response to Ox-A was abolished (data not shown). Not surprisingly, a depolarization with 70 mM K^+ caused a substantially higher increase in the $[Ca^{2+}]_i$ as compared to Ox-A (Fig. 2E).

Ion substitution and electrophysiological experiments were then performed in order to evaluate the background of the irregular Ca^{2+} oscillations in STC-1 cells. Removal of extracellular Na^+ (by replacing with a choline-based medium) or change to Ca^{2+}-free medium abolished the spontaneous irregular Ca^{2+} oscillations. The oscillation returned upon restoration of the basal external medium (Figs. 3A and B). In line with this, step pulses in voltage-clamp mode (−80 to +10 mV; 500 ms) confirmed the presence of voltage-gated Ca^{2+}- and Na^+-channels in STC-1 cells (Figs. 3C and D). Because diltiazem inhibited the CCK release (see above), 10 μM of the drug was introduced to quantify the inhibitory effect on the Ca^{2+} currents. As shown in Fig. 3C, diltiazem inhibited the Ca^{2+} current. The current was restored

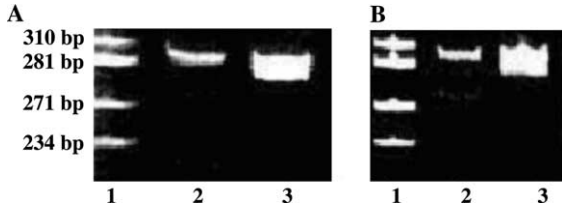

Fig. 1. (A) Amplicons of the PCR from STC-1 cells of OX_1 (2) and mouse brain (3). The product size is 287 bp. (B) Amplicons of the PCR of OX_2 from STC-1 cells (2) and mouse brain (3).

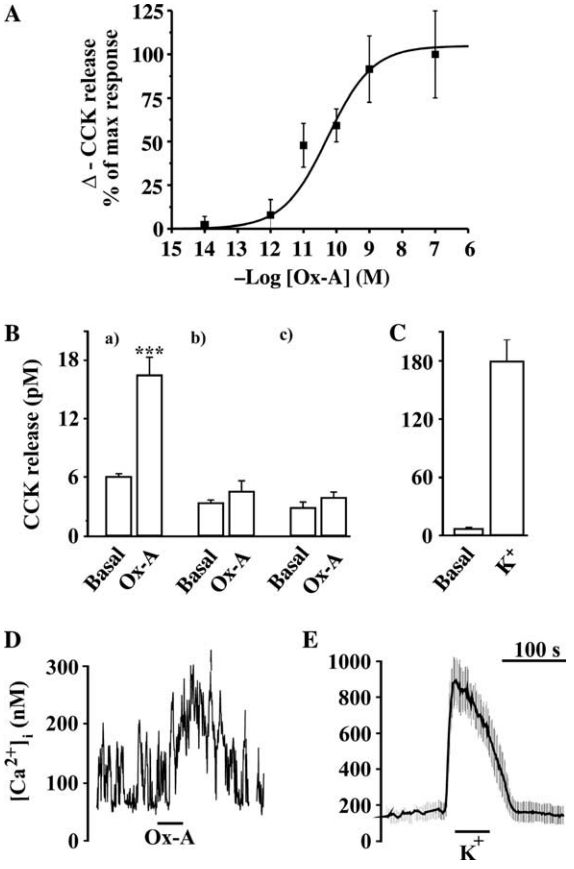

Fig. 2. Cholecystokinin (CCK) release in response to orexin-A (Ox-A) in STC-1 cells. (A) Dose-dependent CCK release measured after 15 min incubation. Points are data from 4–9 experiments. (B): (a) under normal conditions, (b) in the absence of extracellular Ca^{2+}, and (c) in the presence of 10 μM diltiazem. (C) Depolarization evoked CCK release by 70 mM K^+. Experiments were performed in duplicate and repeated 5 times. ***, $P < 0.001$. Data are means ± SEM. (D, E) The response in $[Ca^{2+}]_i$ to 1 nM orexin-A (Ox-A) and 70 mM K^+, respectively.

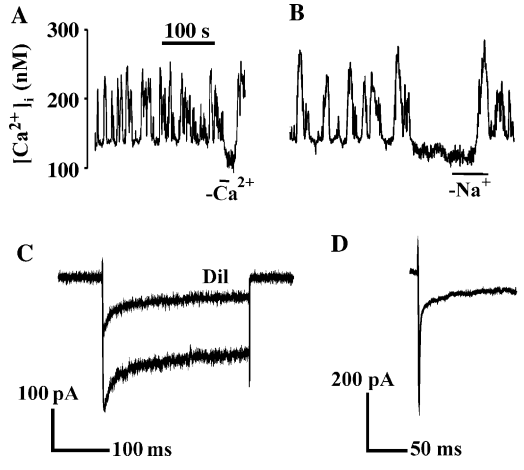

Fig. 3. Spontaneous Ca^{2+}-oscillation in STC-1 cells is dependent on extracellular Ca^{2+} and Na^+. Removal of extracellular Ca^{2+} ($-Ca^{2+}$) or Na^+ ($-Na^+$) abolishes the spontaneous Ca^{2+} oscillation, (A) respectively (B). Data were obtained with the Ca^{2+}-sensitive probe fura-2. (C) A voltage-clamp recording of a voltage-gated Ca^{2+}-current. The current was blocked with 10 μM diltiazem (Dil). The two current-traces were recorded with a 40 s interval. (D) A voltage-dependent Na^+-current in the same cell as used in (C). Currents were evoked with 500 ms pulses to +10 mV from a holding potential of −80 mV. A step-pulse was introduced every 20 s.

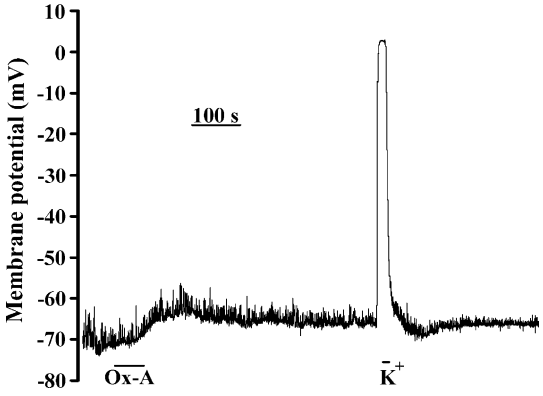

Fig. 4. Whole-cell current-clamp recording of orexin-A provoked membrane depolarization in STC-1 cells. The depolarization was evoked with 1 nM orexin-A (Ox-A). In comparison, depolarization by 70 mM K^+ is shown.

upon washout (data not shown). The average inhibition of the peak current was found to be $50 \pm 5\%$ ($n = 3$).

The whole-cell current-clamp configuration was applied in order to study the effect of Ox-A (10 nM) on the membrane potential. In the 12 cells tested, the average membrane potential was found to be -56 ± 4 mV. In 4/12 cells, with a resting membrane potential ranging between −35 and −45 mV spontaneous spiking activity was observed, while in the remaining 8/12 cells (membrane potential below −45 mV) no spiking was observed. Independently of the resting membrane potential, Ox-A provoked a depolarization of 8.3 ± 1.4 mV ($n = 8/12$) in response to a 30–40 s stimulation. The depolarization slowly returned towards baseline following washout. A representative recording is shown in Fig. 4. In comparison, 70 mM K^+ provoked a strong depolarization of 59 ± 3 mV ($n = 8$) which was fast and reversible upon washout as depicted in Fig. 4.

Orexin receptors have been shown to connect both to Ca^{2+} release via the phospholipase-C–inositol-1,4,5-trisphosphate system and to Ca^{2+} influx [19,34]. We therefore investigated whether orexins would cause any inositol phosphate (IP) release in STC-1 cells. Stimulation with Ox-A concentrations up to 1 μM for 20–60 min produced no change in the IP release (Fig. 5A). However, stimulation with 100 μM carbachol (CCh), a muscarinic receptor agonist which also causes Ca^{2+} elevation in STC-1 cells (Fig. 5B), produced a clear and significant IP elevation (Fig. 5A). Thus, orexin receptors may cause Ca^{2+} elevation in STC-1 cells independent of IP_3-mediated release. Fig. 5A also illustrates that mere Ca^{2+} elevation (1 μM ionomycin (IO)) is not sufficient to mobilize inositol phosphates in STC-1 cells.

Ox-A has also been shown to promote secretory events through the adenylate cyclase signaling pathway [35]. It was thus of interest to measure the influence of Ox-A on cAMP levels in STC-1 cells. Stimulation of STC-1 cells with 100 nM Ox-A had no effect on the basal

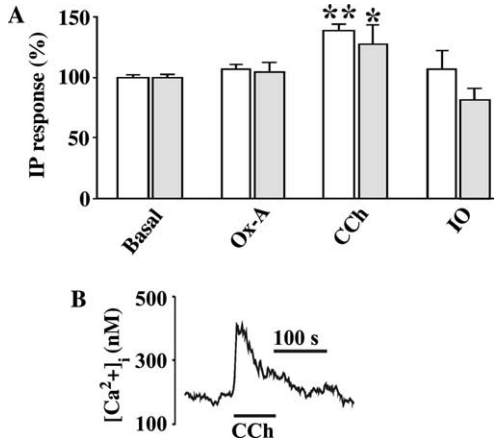

Fig. 5. Inositol phosphate responses to 300 nM orexin-A (Ox-A), 100 μM carbamylcholine (CCh), and 1 μM ionomycin (IO) in STC-1 cells. (A) The stimulants were tested with 1 mM (white stables) and 140 nM (grey stables) extracellular Ca^{2+}. Responses are normalized to the mean basal inositol phosphate level (100%). The incubation time with stimulants was 30 min. Experiments were performed in triplicate and repeated for three times. *, $P < 0.05$; **, $P < 0.01$. Data are means ± SEM. (B) A corresponding response in $[Ca^{2+}]_i$ to 100 μM CCh.

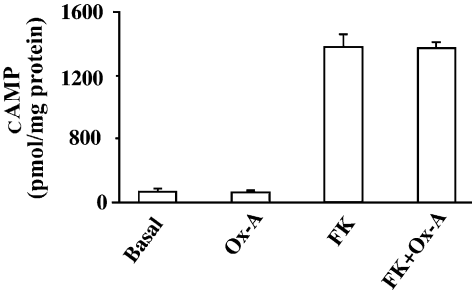

Fig. 6. Cyclic AMP levels measured in STC-1 cells grown in 24-well plates. Cells were preincubated (10 min) with the phosphodiesterase inhibitor IBMX prior to incubation (for another 10 min) with (100 nM) orexin-A (Ox-A), 10 μM forskolin (FK) or a combination of both (FK+Ox-A). The data are means ± SEM from two experiments performed in duplicate.

cAMP level, whereas 10 μM forskolin, a direct activator of adenylate cyclase, greatly stimulated the synthesis of cAMP (Fig. 6). The effect of forskolin was not affected by simultaneous stimulation with Ox-A (Fig. 6), suggesting that neither adenylate cyclase-stimulatory nor-inhibitory G proteins are involved in the response to orexins.

Discussion

We have in the present study demonstrated a functional orexinergic response in STC-1 cells. This is the first time an orexinergic response is characterized in an neuroendocrine cell-line. Ox-A stimulation resulted in

CCK release and also caused an elevation of $[Ca^{2+}]_i$ which was dependent on extracellular Ca^{2+}. Furthermore, Ox-A produced a membrane depolarization. The CCK release was comparable to those of other Ca^{2+} mobilizing peptides like bombesin, diazepam binding inhibitor fragment (DBI_{33-50}) or luminal CCK-releasing peptide (LCRF) [22,36,37]. The Ox-A-stimulated release of CCK was inhibited by Ca^{2+} removal or diltiazem, a blocker of voltage-gated Ca^{2+}-channels. However, also the basal release was sensitive to these treatments, which is explained by release stimulated by the irregular endogenous Ca^{2+}-oscillations observed under control conditions. The most likely pathway for CCK release in STC-1 cells is thus that Ox-A causes a depolarization followed by activation of voltage-gated Ca^{2+}-channels and a subsequent rise in intracellular Ca^{2+}. This is very similar to what has been observed in central neurons where activation of voltage-gated Ca^{2+}-channels appears to be the main mechanism for Ca^{2+} elevation in response to orexins [15,17]. In line with this, the electrophysiological data shown here suggest the presence of both voltage-gated Na^+- and Ca^{2+}-channels in the STC-1 cell-membrane. This together with a fairly low resting membrane potential explains the spontaneous activity of the STC-1 cells. It also explains why depolarization, as observed here, may cause the activation of voltage-gated Ca^{2+}-channels as the resting membrane potential of the cells is close to the threshold for initiation of action potentials. A direct activation of voltage-gated Ca^{2+}-channels has also been suggested in some studies [17,38].

The mechanism by which orexins mediate depolarization in central as well as peripheral neurons is presently unknown [11]. In some studies, a reduction in the potassium permeability has been suggested to be the main mechanism of depolarization (reviewed in [3]). However, Eriksson et al. [11] showed with current-clamp that the depolarization is abolished by removal of extracellular Na^+ and virtually unaffected by an increase in extracellular K^+ (to 18 mM) suggesting that the response is due to other mechanisms than modulation of K^+-channel permeability. In this study, we showed that removal of extracellular Ca^{2+} abolished the Ox-A response. Thus, it is also possible that Ox-A activates an inward current and a subsequent depolarization. Inward currents activated by orexins have also been observed in central neurons [12,13,39] and studies in recombinant systems also indicate that low concentrations of orexins stimulate the activation of Ca^{2+} permeable channels [19,34,40].

Orexins have previously been shown to stimulate secretion from endocrine cells but the mechanisms involved have been difficult to solve. Photoaffinity labeling experiments suggest that OX_2 receptor is able to couple to the G_s, G_q, and G_i [41]. However, there are no functional data suggesting an interaction of orexin receptors with these G-proteins except for activation of phosphoinositol hydrolysis by relatively high concentrations of orexins (>10 nM) in recombinant systems [19,34]. G_q-mediated activation of phosphoinositol hydrolysis does not appear to be the mechanism involved in orexin mediated CCK release as Ox-A failed to stimulate phosphoinositol hydrolysis. Cortisol secretion in response to orexin in human adrenocortical cells appears to occur by a cAMP-dependent mechanism [35] while the secretory response to orexins in pheochromocytoma cells appears to be mediated via activation of protein kinase C [42]. In agreement with data on central neurons [15], no Ox-A-stimulated cAMP accumulation could, however, be observed in STC-1 cells, suggesting that cAMP does not mediate the actions of orexins in these cells. It should also be noted that due to the complex control of adenylyl cyclases by a multitude of effectors it may be difficult to judge whether orexins stimulate cAMP-dependent mechanisms directly or indirectly via other second messenger pathways.

In conclusion, the results presented here demonstrate that low concentrations of orexins, similar to those causing robust Ca^{2+} elevation in recombinant system [19], stimulate CCK secretion in an intestinal neuroendocrine cell line STC-1. Orexin receptors are expressed on neuroendocrine cells of the intestine but so far no studies have been available on the action of orexin on these cells. In contrast to recombinant cell lines Ox-A induces only a slow and modest Ca^{2+} elevation in the STC-1 cells. The CCK secretion and Ca^{2+} elevations are mainly due to activation of voltage-gated channels. The slow modest depolarization seen in the STC-1 cells is reminiscent of that seen in central and peripheral neurons. The typical responses to orexins seen in recombinant systems, e.g., activation of phosphoinositide specific phospholipase C could not be observed in STC-1 cells. Neither could any changes in the cAMP content be detected. Our results thus suggest that in STC-1 cells orexins cause depolarization via a similar novel mechanism as that seen in neurons and that the Ca^{2+} elevation, which in these cells stimulates CCK release, is a consequence of this depolarization.

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