Orexins/Hypocretins Acting at G_i Protein-Coupled OX_2 Receptors Inhibit Cyclic AMP Synthesis in the Primary Neuronal Cultures

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Abstract Orexins A and B are newly discovered neuropeptides with pleiotropic activity. They signal through two G protein-coupled receptors: OX_1 and OX_2. In this study, we examined the expression of orexin receptors and effects of the receptors' activation on cyclic AMP formation in the primary neuronal cell cultures from rat cerebral cortex. Both types of orexin receptors were expressed in rat cortical neurons; the level of OX_2R was markedly higher compared to OX_1R. Orexin A (an agonist of OX_1R and OX_2R) and [Ala^{11}-D-Leu^{15}]orexin B (a selective agonist of OX_2R) did not affect basal cyclic AMP formation in the primary neuronal cell cultures. Both peptides (0.001–1 μM) inhibited, in a concentration-dependent manner and IC_{50} values in low nanomolar range, the increase in the nucleotide production evoked by forskolin (1 μM; a direct activator of adenylyl cyclase), pituitary adenylate cyclase-activating polypeptide (PACAP27; 0.1 μM), and vasoactive intestinal peptide (VIP; 3 μM). Effects of orexin A on forskolin-, PACAP27-, and VIP-stimulated cyclic AMP synthesis were blocked by TCS OX2 29 (a selective antagonist of OX_2R), and unaffected by SB 408124 (a selective antagonist of OX_1R). Pretreatment of neuronal cell cultures with pertussis toxin (PTX) abolished the inhibitory action of orexin A on forskolin- and PACAP-stimulated cyclic AMP accumulation. It is suggested that in cultured rat cortical neurons orexins, acting at OX_2 receptors coupled to PTX-sensitive G_i protein, inhibit cyclic AMP synthesis.

Abbreviations

FOR	Forskolin
GFAP	Glial fibrillary acidic protein
IBMX	3-Isobuthyl-1-methyxanthine
OX_1R	Type 1 orexin receptor
OX_2R	Type 2 orexin receptor
PACAP	Pituitary adenylate cyclase-activating polypeptide
PTX	Pertussis toxin
SB 408124	N-(6,8-difluoro-2-methyl-4-quinolinyl)-N'-[4-(dimethylamino)phenyl]urea
TCS OX2 29	($2S$)-1-(3,4-dihydro-6,7-dimethoxy-2(1H)-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride
VIP	Vasoactive intestinal peptide

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Introduction

Orexins (orexins A and B), also known as hypocretins (hypocretins 1 and 2), are multifunctional neuropeptides

discovered by two independent research groups in 1998 using orphan receptor technologies (Sakurai et al. 1998) and subtractive cDNA cloning (de Lecea et al. 1998). Both orexins are derived from a common precursor, preproorexin, by proteolytic cleavage (Sakurai et al. 1998, 1999), and share 46% aminoacid identity in humans (de Lecea and Sutcliffe 1999). Although production of the peptides is restricted to a discrete population of neurons in the lateral and posterior hypothalamus, the projection fields of these neurons have been identified in numerous brain regions, including the cortex, thalamus, hypothalamus, brain stem, and spinal cord (Matsuki and Sakurai 2008). This diffuse projection pattern suggests that the orexin system regulates multiple complex physiological functions. Indeed, accumulated experimental evidence indicate an involvement of orexins in the regulation of vigilance and sleep/wake cycle, feeding, appetite, reward seeking, and energy homeostasis (Kukkonen et al. 2002; Matsuki and Sakurai 2008; Carter et al. 2009; Kodatek and Cai 2010). The peptides also control hypothalamo–pituitary–adrenal axis and functions of miscellaneous peripheral organs, including heart, kidney, thyroid, lung, testis, ovaries, and adipose tissues (Voisin et al. 2003; Spinazzi et al. 2006; Heinonen et al. 2008; Okumura and Takakusaki 2008; Kagerer and Jöhren 2010). The loss or dysfunction of orexin neurons has been shown to cause human and animal narcolepsy [e.g., (Chemelli et al. 1999; Lin et al. 1999; Geraschenko et al. 2001; Thannickal et al. 2003; Mieda et al. 2004)].

Orexins orchestrate their diverse central and peripheral effects via two membrane-bound G protein-coupled receptors, OX_1R and OX_2R (Sakurai et al. 1998). These receptors share a 64% identity to each other in their amino acid sequences. Studies in heterologous expression systems have demonstrated that OX_2R has an equal affinity for both orexins A and B, whereas OX_1R has approximately tenfold greater affinity for orexin A than for orexin B (Sakurai et al. 1998; Ammoun et al. 2003). Signal coupling of orexin receptors has been rigorously investigated in few studies, the vast majority of them was performed on cell lines recombinantly expressing OX_1R or OX_2R. OX_1R primarily couples via the $G_q/_{11}$ subclass of G protein, while OX_2R couples to $G_q/_{11}$, G_s, and $G_i/_o$ subclasses (Sakurai et al. 1998; van den Pol et al. 1998; Randeva et al. 2001; Karteris and Randeva 2003; Kukkonen and Åkerman 2005; Ramanjaneya et al. 2009). One of the most marked responses to stimulation of orexin receptors is a robust increase in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, resulting from activation of two pathways: receptor-operated Ca^{2+} influx and phospholipase C—inositol-(1,4,5)-trisphosphate/diacylglycerol (Sakurai et al. 1998; Zhu et al. 2003; Holmqvist et al. 2005; Ekholm et al. 2007; Gorojankina et al. 2007; Tang et al. 2008; reviewed by Kukkonen and Åkerman 2005). A few reports suggest a link between orexin receptors and the cyclic AMP pathway

(Zhu et al. 2003; Holmqvist et al. 2005; Magga et al. 2006; Tang et al. 2008).

Although the expression pattern of the orexinergic system in the CNS has been well characterized, little is known about the presence of native orexin receptors and their signaling system(s) in the primary neuronal cultures. In this study, we investigated the expression of orexin receptors and the intracellular mechanism followed the receptors' activation that can affect cyclic AMP formation in neuronal cell cultures derived from rat cerebral cortex. We also determined which type of orexin receptors is involved in this action. Our results demonstrated that stimulation of OX_2R inhibits cyclic AMP production in a pertussin-toxin sensitive manner. This suggests an involvement of G_i protein in coupling of OX_2R to adenylyl cyclase.

Materials and Methods

Animals and Cell Culture

Experiments were performed on primary neuronal cell cultures prepared from Wistar rat embryos on day 16 of gestation. Animal procedures were in strict accordance with the Polish governmental regulations concerning experiments on animals (Dz.U.05.33.289), and the experimental protocol was approved by the Local Ethical Commission for Experimentation on Animals.

Primary neuronal cell cultures were prepared according to the method of Brewer (1995), described in detail by Nowak and coworkers (2007). Briefly, pregnant females were anesthetized with ether vapor, killed by decapitation, and subjected to cesarean section in order to remove fetuses. After brain's dissection, the cerebral cortex was isolated, incubated for 15 min in trypsin/EDTA (0.05%) at 37°C, triturated in a solution of DNase I (0.05 mg/ml) and fetal bovine serum (20%), and finally centrifuged at $210 \times g$ for 5 min at 21°C. The cells were suspended in neurobasal medium supplemented with B27, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and plated at a density of 300,000–350,000 cells/well onto poly-L-ornithine (0.01 mg/ml) coated multi-well plates. Cells were grown in humidified atmosphere of 95% air and 5% CO_2 at 37°C for 6–7 days prior to experimentation. Seventy-two hours after plating the solution of 1-β-D-arabinofuranosylcytosine (a final concentration—5 μM) was added to the neurobasal medium to stop the glial proliferation. The purity of neuronal cultures was verified by using antibodies against microtubule associating protein-2 for neurons, and against glial fibrillary acidic protein (GFAP), for astrocytes. The latter analysis revealed the presence of approximately 6–10% of GFAP-positive cells, which indicated that the primary neuronal cultures represented in fact neuron-enriched preparations.

Real-Time Quantitative RT-PCR

Total RNA was extracted from neuronal cells by using TRI Pure Isolation Reagent (Roche, Meylan, France) according to the manufacturer's instruction. For each sample, total RNA (1 μg) was subjected to reverse transcription (RevertAid H Minus First Strand cDNA Synthesis Kit, Fermentas, Burlington, Canada) according to the manufacturer's specifications.

Specific sense and antisense oligonucleotide primers for amplification of mRNAs of rat OX_1R and OX_2R were obtained from Invitrogen (Karlsruhe, Germany). The sequences of specific primers and the procedure of real-time quantitative PCR (qPCR) was published previously (Jöhren et al. 2001). Shortly, 2 μl of first strand cDNA reaction was incubated in the presence of 3 mM $MgCl_2$; 200 μM of dGTP, dATP, dCTP, and dUTP; Platinum Taq DNA Polymerase; the fluorescence dye SYBR green I; and the appropriate sense and antisense primers in a final volume of 25 μl (Platinum® SYBR® Green qPCR Super-Mix, Invitrogen) on the 7000 Sequence Detection System of Applied Biosystems (Darmstadt, Germany). Each sample was analyzed in duplicate along with standards and no template controls. Product purity was regularly confirmed for each sample by dissociation curve analysis.

Copy number calculations were based on the cycle threshold method (Higuchi et al. 1993). Serial dilutions of known amounts of specific cDNA fragments were used to generate standard curves. The threshold cycle number (C_T) for each sample was calculated using the 7000 Sequence Detection System software with an automatic baseline setting and a fluorescence threshold (R_n) of 0.2.

Assay of Cyclic AMP Formation

On the day of experiment, the culture medium was removed, fresh serum-free culture medium was added, and cells were incubated in the presence of [^{3}H]adenine for 30 min at 37°C. The formation of [^{3}H]cyclic AMP in [^{3}H] adenine prelabeled cells was assayed according to Shimizu et al. (1969), and the formed [^{3}H]cyclic AMP was isolated by a sequential Dowex-alumina chromatography according to Salomon et al. (1974). The results were individually corrected for percentage recovery with the aid of [^{14}C] cyclic AMP added to each column system prior to the nucleotide extraction. The mean recovery was in the range of 38–46%. The accumulation of cyclic AMP during a 15-min stimulation period was assessed as a percentage of the conversion of [^{3}H]adenine to [^{3}H]cyclic AMP. Antagonists of orexin receptors were added 10-min prior to addition of orexin A and [Ala^{11}-D-Leu^{15}]orexin B. All experiments were carried out in the presence of 0.1 mM 3-isobuthyl-1-methyxanthine (IBMX, an inhibitor of cyclic nucleotides' phosphodiesterase). Pertussis toxin (PTX; 100 ng/ml) was added 18 h prior to addition of orexin A. A day after addition of pertussis toxin, the culture medium was removed and fresh serum-free culture medium was added.

Chemicals

The peptides orexin A, [Ala^{11}-D-Leu^{15}]orexin B, PACAP27 (human, ovine, and rat), and vasoactive intestinal peptide (VIP; human, porcine, and rat) were from NeoMPS (Strasbourg, France). N-(6,8-difluoro-2-methyl-4-quinolinyl)-N′-[4-(dimethylamino)phenyl]urea (SB 408124) and (2S)-1-(3,4-dihydro-6,7-dimethoxy-2(1H)-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride (TCS OX2 29), selective antagonists of OX_1R and OX_2R, respectively, were purchased from Tocris Bioscience (Bristol, UK). Forskolin, IBMX, poly-L-ornithine, DNase I, trypsin, glutamine, penicillin, and streptomycin were from Sigma-Aldrich (Poznan, Poland). Neurobasal medium, B27, and fetal bovine serum were from Gibco (Paisley, Scotland, UK). dGTP, dATP, dCTP, and dUTP, and Platinum Taq DNA Polymerase were from Invitrogen (Karlsruhe, Germany). Anti-neuronal class III β-tubulin mouse monoclonal antibody, anti-glial fibrillary acidic protein rabbit polyclonal antibody, Texas Red® dye-conjugated goat anti-mouse antibody, and fluorescein (FITC)-conjugated goat anti-rabbit antibody were purchased from STEMCELL Technologies, Inc. (Vancouver, Canada). Petri dishes and multi-well plates for cell cultures were from Nunc (Wiesbaden, Germany). Radioactive compounds: [^{3}H] adenine (sp. activity 24 Ci/mmol) and [^{14}C]cyclic AMP (sp. activity 53 mCi/mmol) were from Hartmann-Analytic GmbH (Braunschweig, Germany). Other chemicals were of analytical purity and were obtained mainly from Sigma-Aldrich (Poznan, Poland).

Data Analysis

Data are expressed as mean ± standard error of the mean (SEM) values and were analyzed for statistical significance by one-way ANOVA followed by post hoc Student–Newman–Keul's test, using InStat version 3.05 for Windows 95 (GraphPad, San Diego, CA, USA).

Results

Expression of Orexin Receptors in the Primary Neuronal Cell Cultures

Quantitative RT-PCR analysis with the use of specific couples of primers demonstrated the expression of both types of orexin receptors, OX_1R and OX_2R, in the primary neuronal cell cultures derived from rat cerebral cortex.
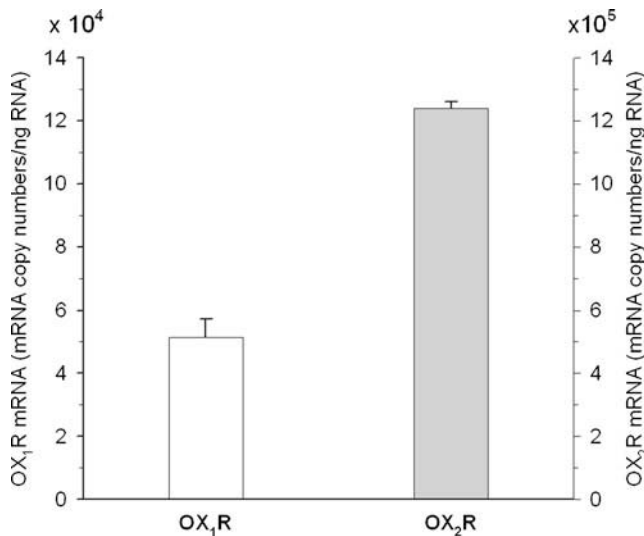
Fig. 1 Expression of orexin receptor subtypes OX_1R and OX_2R by qPCR in rat cultured neurons. Shown are the specific RNA copy numbers per nanograms of total RNA

tory action of forskolin, PACAP27, and VIP on cyclic AMP formation in cultured rat cortical neurons (Fig. 2). The calculated IC_{50} values for orexin A were: 24.5 ± 1.8 nM (experiments with forskolin), 26.3 ± 1.4 nM (experiments
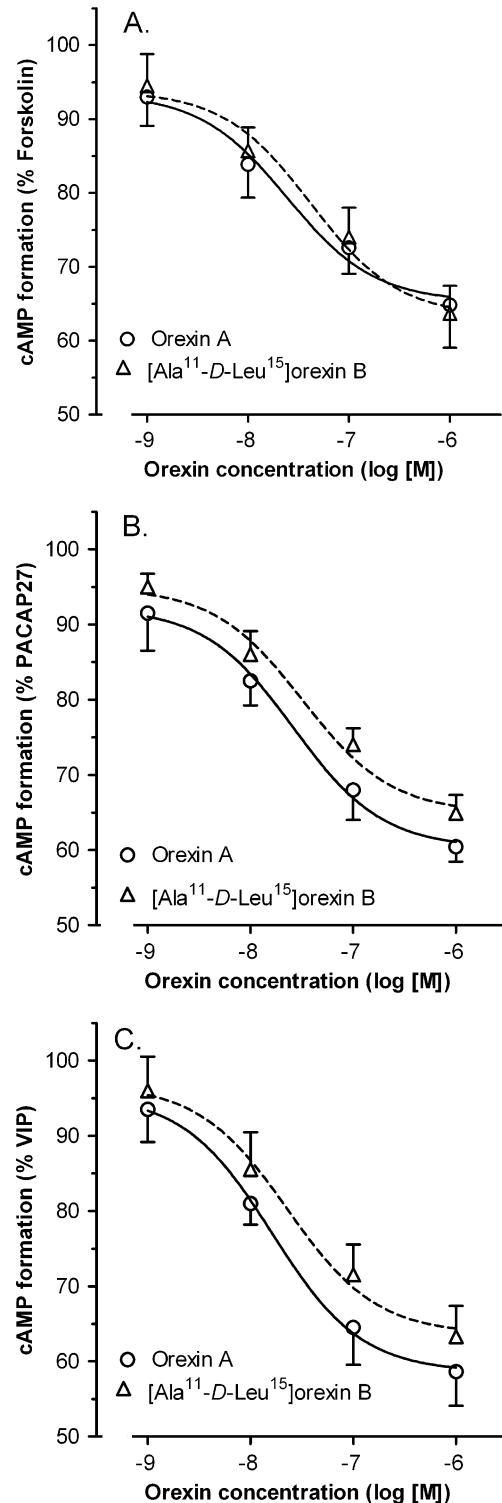
Interestingly, primary neuronal cells express about 24-fold more OX_2R than OX_1R at the mRNA level (Fig. 1).

Effects of Orexin A and [Ala^{11}-D-Leu^{15}]Orexin B on Cyclic AMP Formation in the Primary Neuronal Cells

No significant effects of orexin A (an agonist of OX_1R and OX_2R) and [Ala^{11}-D-Leu^{15}]orexin B (a selective agonist of OX_2R), both applied at 0.001–1 μM concentrations, on basal cyclic AMP accumulation in cultured rat cortical neurons were observed (data not shown). In line with previous demonstrations (Jozwiak-Bebenista et al. 2007), incubation of rat neuronal cell cultures with forskolin (a direct activator of adenylyl cyclase; 1 μM), PACAP27 (0.1 μM), or VIP (3 μM) resulted in a potent (three- to fivefold over the basal level) increase in cyclic AMP production (Figs. 2, 3 and 4). Orexin A and [Ala^{11}-D-Leu^{15}]orexin B (0.001–1 μM) inhibited, in a concentration-dependent manner, the stimula-

Fig. 2 Effects of orexin A (an agonist of OX_1R and OX_2R) and [Ala^{11}- ▶ D-Leu^{15}]orexin B (a selective agonist of OX_2R) on cyclic AMP formation in primary neuronal cell cultures from rat cerebral cortex stimulated by forskolin (**a**), PACAP27 (**b**), and VIP (**c**). Values are expressed as percent of the response to forskolin, PACAP27, and VIP, and are means ± SEM ($n=8$–16). Cyclic AMP accumulation: *experiments with forskolin*—control cultures, $0.86\pm0.12\%$ conversion ($n=18$); forskolin (1 μM) stimulated cultures, $3.39\pm0.22\%$ conversion ($n=20$); *experiments with PACAP27*—control cultures, $0.83\pm0.14\%$ conversion ($n=16$); PACAP27 (0.1 μM) stimulated cultures, $5.09\pm0.42\%$ conversion ($n=24$); *experiments with VIP*—control cultures, $0.88\pm0.15\%$ conversion ($n=16$); VIP (3 μM) stimulated cultures, $4.52\pm0.26\%$ conversion ($n=20$)

with PACAP27), and 16.2±1.2 nM (experiments with VIP); and for [Ala^{11}-D-Leu^{15}]orexin B: 42.0±2.0 nM (experiments with forskolin), 32.3±1.9 nM (experiments with PACAP27), and 22.9±1.6 nM (experiments with VIP).

Effects of OX$_1$R and OX$_2$R Antagonists on the Orexin A-Induced Decrease in Cyclic AMP Formation in the Primary Neuronal Cell Cultures

The observed by us similar potency of orexin A and [Ala^{11}-D-Leu^{15}]orexin B suggested an involvement of OX$_2$R in the regulation of cyclic AMP synthesis in cultured rat cortical neurons. In order to verify this hypothesis, in the next set of experiments selective antagonists of OX$_1$R (SB 408124) and OX$_2$R (TCS 0X2 29) were used. TCS 0X2 29 (10 μM), but not SB 408124 (10 μM), blocked the inhibitory actions of orexin A (1 μM) on increases of cyclic AMP formation produced by forskolin, PACAP27, and VIP (Fig. 3).

Effects of Pertussis Toxin on the Orexin A-Induced Decrease in Cyclic AMP Formation in the Primary Neuronal Cell Cultures

Pretreatment of rat neuronal cell cultures with pertussis toxin (18 h, 100 ng/ml) did not affect the basal as well as forskolin- and PACAP27-stimulated cyclic AMP accumulation. Pertussis toxin abolished the inhibitory action of orexin A on rises in the nucleotide formation produced by forskolin and PACAP27 (Fig. 4).

Discussion

A number of studies, performed predominantly on transfected cell lines, have demonstrated that orexin receptors could interact with three families of G proteins, i.e., G$_{q/11}$, G$_s$, and G$_i$/$_o$, with different efficacies, and activate several signaling pathways (e.g., Zhu et al. 2003; Holmqvist et al. 2005; Tang et al. 2008). The increase in [Ca^{2+}]$_i$ is the most typical response seen in many cell types (de Lecea et al. 1998; Zhu et al. 2003; Ammoun et al. 2006; Magga et al. 2006; Näsman et al 2006; Gorojankina et al. 2007). Regulation of adenylyl cyclase activity is also considered as an important component of orexin receptors signaling (Zhu et al. 2003; Holmqvist et al. 2005; Gorojankina et al. 2007; Tang et al. 2008), although this pathway has seldom been investigated.

The results obtained in the present study provide evidence that in the primary neuronal cell cultures from rat cerebral cortex expression of OX$_2$R is considerably higher than that of OX$_1$R. Our further studies on the primary astrocyte cultures from rat brain identified similar
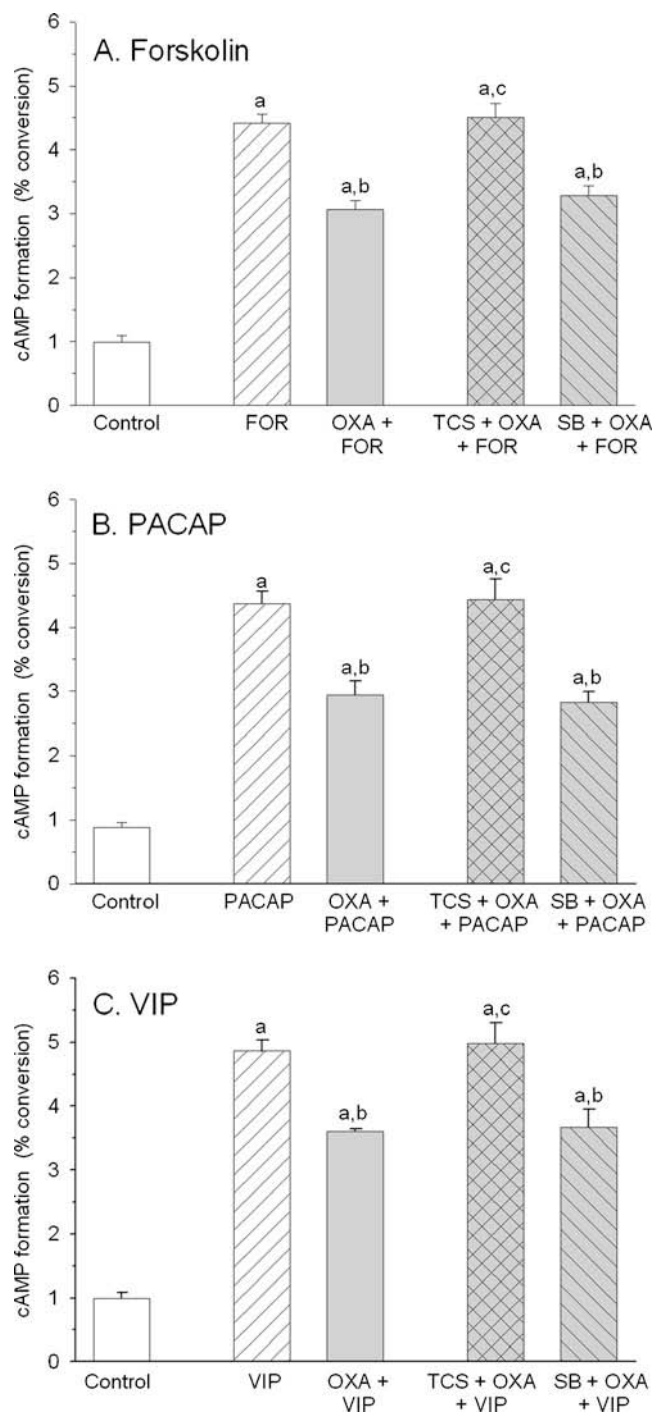

Fig. 3 a–c TCS OX2 29 (*TCS*, 10 μM), a selective antagonist of OX$_2$R, but not SB 408124 (*SB*, 10 μM), a selective antagonist of OX$_1$R, blocked the inhibitory action of orexin A (1 μM) on forskolin (1 μM), PACA27 (0.1 μM), and VIP (3 μM) induced increase in cyclic AMP formation in primary neuronal cell cultures from rat cerebral cortex. Values shown are means ± SEM (*n*=8–15). ^{a}P<0.05 versus control, ^{b}P<0.05 versus stimulatory agent (Forskolin, PACAP27, or VIP); ^{c}P<0.05 versus stimulatory agent and orexin

expressions of both types of orexin receptors at the mRNA level; however, they were markedly lower compared to neuronal cell cultures (unpublished data). This suggests that
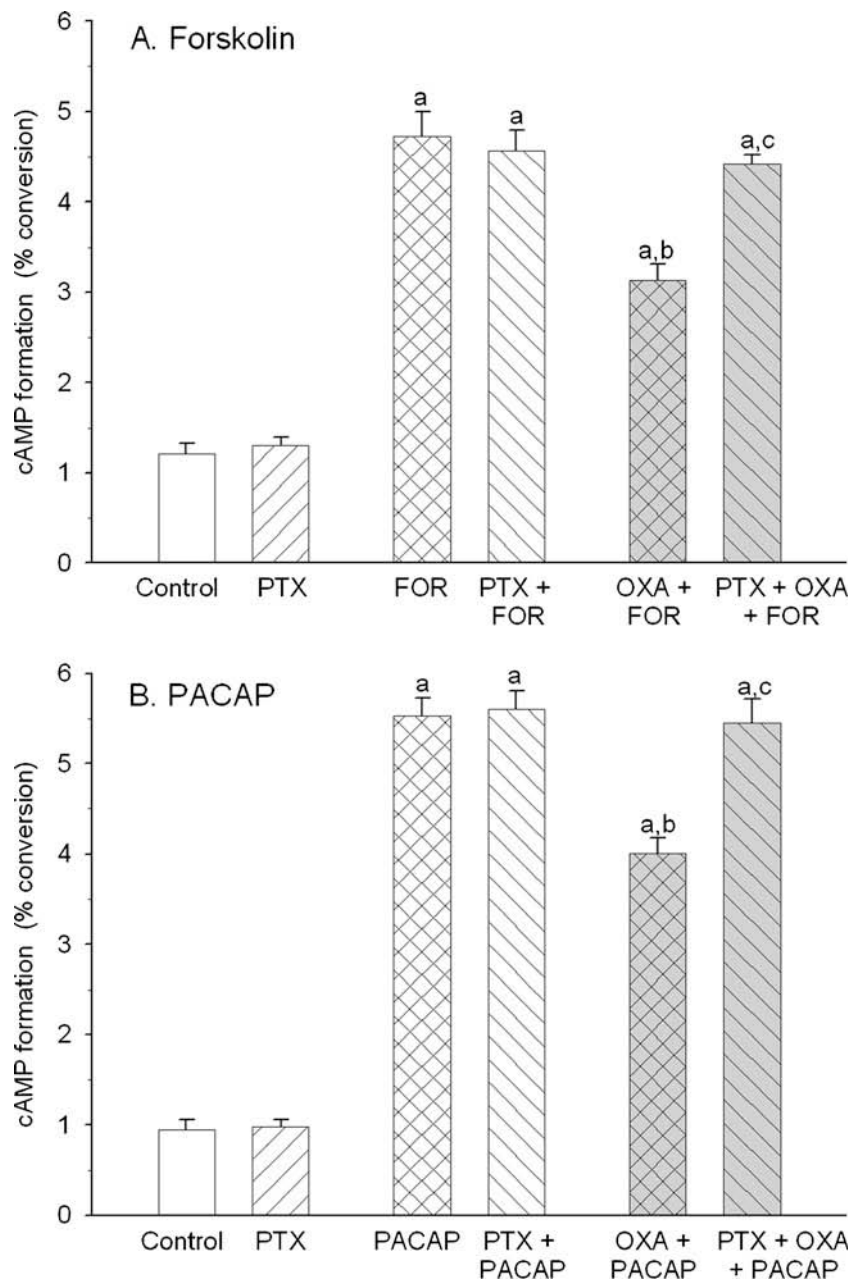
in the rat cerebral cortex the expression of orexin receptors is closely related to the cell type, and may trigger distinct cellular responses. To test this hypothesis we evaluated a potential role of the orexin receptors' activation in the regulation of cyclic AMP production in neurons. Treatment of cortical neuronal cultures with orexin A (the nonselective agonists of OX_1R and OX_2R) and [Ala[11]-*D*-Leu[15]]orexin B (the selective OX_2R agonist; Asahi et al. 2003) failed to significantly affect the basal cyclic AMP accumulation suggesting that orexin receptors expressed in this cell type do not couple to the G_s subclass of G protein. On the other hand, orexin A and [Ala[11]-*D*-Leu[15]]orexin B potently inhibited, in a concentration-dependent manner, cyclic

AMP production stimulated by the diterpene forskolin, the direct activator of adenylyl cyclase, and by two neuropeptides, i.e., PACAP and VIP, well-known activators of G_s proteins (Vaudry et al. 2009). The calculated IC_{50} values for both peptides were in low, nanomolar range, suggesting that this inhibition is of physiological relevance. Two lines of evidence point to the involvement of OX_2R in the studied phenomenon. Firstly, [Ala[11]-*D*-Leu[15]]orexin B was almost equipotent to orexin A. Secondly, the inhibitory action of orexin A was blocked by TCS 0X2 29, the selective OX_2R antagonist (Hirose et al. 2003), and not affected by SB 408124, the selective OX_1R antagonist (Porter et al. 2001). Pertussis toxin, which deactivates G_i/G_o through ADP

ribosylation, abolished the inhibitory effect of orexin A on stimulated cyclic AMP production. Hence, it would appear that this effect is mediated by OX_2R-coupling to G_i protein. A functional coupling between $G_{i/o}$ proteins and OX_2R has been previously demonstrated in HEK293 and BIM cell lines heterologously expressing human OX_2R (Hoang et al. 2003; Zhu et al. 2003; Tang et al. 2008).

In the context of the present study it is interesting to note that our previous results in astrocyte cultures from rat cerebral cortex demonstrated a stimulatory effect of orexin A, but not [Ala[11]-D-Leu[15]]orexin B, on cyclic AMP synthesis indicating a predominant OX_1R involvement in this action (Woldan-Tambor et al. 2011). Thus, it could be hypothesized that depending on a cell type in the rat cerebral cortex the biochemical responses to the tested peptides are mediated via different types of receptors interacting with distinct families of G proteins. It would be highly interesting to know whether the orexinergic system in astrocyte compartment in the rat brain can affect neuronal functions.

A physiological significance of the OX_2R-trigerred intracellular signaling, including cyclic AMP pathway, in rat cortical neurons is yet to be evaluated. Studies on HEK293-OX_2R cells suggested that adenylyl cyclase–cyclic AMP pathway is, in part, functionally linked to the activation by orexins of extracellular signal-regulated kinase (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK) (Tang et al. 2008). This hypothesis appears particularly interesting in light of the well-known involvement of MAPK signaling pathways in the regulation of cell growth, differentiation, and gene expression.

In conclusion, the results presented here demonstrate that in cultured rat cortical neurons orexins, acting at OX_2 receptors coupled to PTX-sensitive G_i protein, inhibit cyclic AMP synthesis.

Conflict of Interest The authors state no conflict of interest.

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