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Activation of orexin/hypocretin type 1 receptors stimulates cAMP synthesis in primary cultures of rat astrocytes

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Abstract:
The effects of orexins, which are also named hypocretins, on cAMP formation were examined in primary cultures of rat astrocytes. Orexin A, an agonist of OX_1 and OX_2 receptors, stimulated cAMP production with an EC_{50} value of 0.68 μM and potentiated the forskolin-induced increase in the nucleotide synthesis. [Ala[11]-D-Leu[15]]orexin B, an agonist of OX_2 receptors, was inactive. The effects of orexin A were antagonized by SB 408124, a selective blocker of OX_1 receptors, but were not affected by TCS OX2 29, a selective antagonist of OX_2 receptors. We hypothesized that the activation of OX_1 receptors stimulated cAMP synthesis in primary rat astrocyte cultures.

Key words:
orexin, orexin receptors, astrocytes, cerebral cortex, cAMP

Abbreviations: cAMP – cyclic 3',5'-adenosine monophosphate, GFAP – glial fibrillary acidic protein, IBMX – 3-isobutyl-1-methylxanthine, OX_1 – orexin type 1 receptor, OX_2 – orexin type 2 receptor, SB 408124 – *N*-(6,8-difluoro-2-methyl-4-quinolinyl)-*N'*-[4-dimethylamino)phenyl]urea, TCS OX2 29 – (2*S*)-1-(3,4-dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)-3,3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride

Introduction

Cyclic 3',5'-adenosine monophosphate (cAMP) is an ubiquitous second messenger in a vast array of physiological processes including, but not limited to, gene transcription, modulation of ion channels, regulation of hormone synthesis, glycogen metabolism, smooth muscle relaxation, cardiac contraction, olfaction, learning and memory [17]. The nucleotide is universally generated from ATP by adenylyl cyclase (AC; EC 4.6.1.1), a membrane-bound enzyme. There are nine known isoforms of mammalian AC (AC1 – AC9), and their activities are subject to many positive and negative regulatory inputs, such as those from G protein-coupled receptors (GPCRs). Previous studies have indicated that the cAMP responses differ from cell to cell based on the expression profile of AC isoforms and other proteins that participate in the regulation of enzymatic activity [10, 17]. Among numerous transmitters that act on membrane-bound GPCRs and exert their physiological actions *via* the AC–cAMP signaling pathway are the neuropeptides from the se-

cretin/vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating polypeptide (PACAP) family. VIP and PACAP are potent stimulators of cAMP production in two cultured cell types that had been isolated from the rat cerebral cortex, i.e., neurons and astrocytes [8].

Orexins (orexin A and orexin B), which are also known as hypocretins (hypocretin 1 and hypocretin 2), are recently discovered multifunctional neuropeptides that share some structure similarities with secretin. Orexin-containing neurons project from the lateral hypothalamus to numerous brain regions, and are involved in the regulation of vigilance and the sleep/wake cycle, feeding, appetite, and metabolic processes [11, 13]. Orexins also control the hypothalamo-pituitary-adrenal axis and the functions of miscellaneous peripheral organs, including the heart, the kidney, the thyroid, the lung, the testis, the ovaries, and the adipose tissues [4, 9, 13]. The loss or dysfunction of orexin neurons causes human and animal narcolepsy [11, 13].

Orexins orchestrate their diverse physiological effects *via* two GPCRs, OX_1 and OX_2 [11, 13]. These receptors share a 64% identity with each other in their amino acid sequences. Studies of orexin signaling pathways have suggested that orexin receptors interact with three families of G-proteins, i.e., $G_{q/11}$, $G_{s,}$ and $G_{i/o}$ with different efficacies [7, 12, 19, 21]. The most marked response to the stimulation of orexin receptors is a robust increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, which is partially caused by the activation of the phospholipase C (PLC) – inositol-1,4,5-trisphosphate (IP_3) pathway [3, 7, 19, 21]. Coupling between orexin receptors and the AC–cAMP signaling system has also been reported [3, 7, 21]. Several groups have postulated that the orexin-mediated activation of PLC or AC signaling may have tissue- or cell-specific characteristics [7, 11, 12, 15, 19, 20].

The aim of the current work was to investigate whether orexins affected cAMP formation in native astrocytes from the rat cerebral cortex and to identify the type of receptor that may mediate this action.

Materials and Methods

Chemicals

Orexin A and [Ala^{11}-D-Leu^{15}]orexin B were from NeoMPS (Strasbourg, France). *N*-(6,8-difluoro-2-methyl-4-quinolinyl)-*N'*-[4-dimethylamino)phenyl]urea (SB 408124) and (2*S*)-1-(3,4-dihydro-6,7-dimethoxy-2(1*H*)-isoquinolinyl)-3,3,-dimethyl-2-[(4-pyridinylmethyl) amino]-1-butanone hydrochloride (TCS OX2 29), which are selective antagonists of OX_1 and OX_2 receptors, respectively, were purchased from Tocris Bioscience (Bristol, UK). Forskolin, poly-L-ornithine, DNase I, trypsin, glutamine, penicillin, streptomycin and amphotericin B were purchased from Sigma-Aldrich (Poznań, Poland). The anti-neuronal class III β-tubulin mouse monoclonal antibody, the anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody, the Texas Red® dye-conjugated goat anti-mouse antibody and the fluorescein (FITC)-conjugated goat anti-rabbit antibody were purchased from STEMCELL Technologies, Inc. (Vancouver, Canada). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco Invitrogen Corp. (Carlsbad, CA, USA). Petri dishes and multi-well plates for cell cultures were purchased from Nunc (Wiesbaden, Germany). The radioactive compounds [^{3}H]adenine (specific activity of 24 Ci/mmol) and [^{14}C]cAMP (specific activity of 53 mCi/mmol) were purchased from Hartmann Analytic GmbH (Braunschweig, Germany). Other chemicals were of analytical purity and were obtained mainly from Sigma-Aldrich (Poznań, Poland).

Animals and cell culture

Experiments were performed on primary astrocyte cultures that had been prepared from newborn rats. Animal procedures were in strict accordance with the Polish governmental regulations (Dz.U.05.33.289), and the experimental protocol was approved by the Local Ethical Commission for Experimentation on Animals.

Primary astrocyte cultures were prepared from cerebral cortices that had been isolated from 1-day-old Wistar rat pups and were maintained according to Hertz et al. [5]. Briefly, a dissected cerebral cortex was cut into small fragments, incubated for 15 min in trypsin/EDTA (0.025%) at 37°C, triturated in a solution of DNase I (0.05 mg/ml) and fetal bovine serum (20%), and centrifuged at 210 × g for 5 min at 21°C. The cells were suspended in DMEM, which was supplemented with 10% newborn calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 mg/ml amphotericin B, and plated at a density of 10^6/ml onto poly-L-ornithine (0.01 mg/ml) coated 6-cm Petri dishes. Cells were grown in a humidified

atmosphere of 95% air and 5% CO_2 at 37°C. For subcultures, the astrocytes were harvested using trypsin-EDTA (0.25% trypsin, 1 mM EDTA) solution. The cells from the second and third passage were used for experiments. The homogeneity of the cell population was approximately 95%, which was verified by immunohistochemical staining for glial fibrillary acidic protein (GFAP) using the anti-GFAP antibody. No neurons, as confirmed using antibodies against neuronal class III β-tubulin, were detected.

Assay of cAMP formation

The primary cultures of rat astrocytes were seeded in 12-well plates at a density of 250,000–350,000 cells/well in 500 µl of culture medium and were cultured for 2 days. On the day of the experiment, the culture medium was replaced with fresh serum-free culture medium, and the cells were incubated in the presence of [^{3}H]adenine for 1.5 h at 37°C. The formation of cAMP in [^{3}H]adenine-prelabeled cells was assayed as described by Shimizu et al. [18]. The data were individually corrected for the percentage of cAMP recovery with the aid of [^{14}C]cAMP, which was added to each column system before the nucleotide extraction. The accumulation of cAMP during a 15-min stimulation period was measured as the percentage of the conversion of [^{3}H]adenine to [^{3}H]cAMP. Antagonists of orexin receptors were added 10 min before the application of peptides. All of the reactions were performed in the presence of 0.1 mM 3-isobutyl-1-methylxanthine (IBMX, a cyclic nucleotide phosphodiesterase inhibitor).

Data analysis

Data were expressed as the mean ± standard error of the mean (SEM) values and were analyzed for statistical significance using 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

To evaluate a putative role of the orexinergic peptides in the regulation of cAMP production, the primary cultures of rat astrocytes were treated with orexin A (an agonist of OX_1 and OX_2 receptors) and [Ala^{11}-D-Leu^{15}]orexin B (an agonist of OX_2 receptors). The incubation of cultures with 1 µM [Ala^{11}-D-Leu^{15}]orexin B had no effect on basal cAMP formation, whereas 1 µM forskolin (a direct activator of adenylyl cyclase) potently increased the nucleotide production. The effect of forskolin was not affected by the presence of [Ala^{11}-D-Leu^{15}]orexin B in the incubation medium (control: 0.69 ± 0.03 % conversion; 1 µM forskolin: 4.83 ± 0.17* % conversion; 1 µM [Ala^{11}-D-Leu^{15}]orexin B: 0.74 ± 0.02 % conversion; [Ala^{11}-D-Leu^{15}]orexin B + forskolin: 5.08 ± 0.14* % conversion; n = 6–9/group, *$p < 0.05$ *vs.* control). Orexin A (0.001–1 µM) increased the basal cAMP production in primary cultures of rat astrocytes in a concentration-dependent manner with a calculated EC_{50} value of 0.68 µM (Fig. 1). Orexin A (0.1–1 µM) potentiated the stimulatory action of forskolin (1 µM) on the nucleotide accumulation (Fig. 2). The simultaneous application of orexin A and forskolin induced a synergistic increase in cAMP accumulation, which was markedly higher than the sum of cAMP that was produced by these ligands separately (Fig. 2).

The pretreatment of primary cultures of rat astrocytes with SB 408124, which is a nonpeptide selective

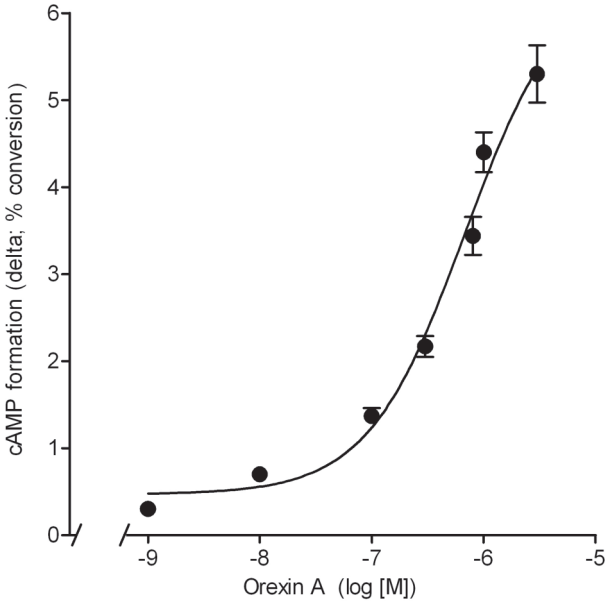

Fig. 1. Effects of orexin A on basal cAMP formation in primary astrocyte cultures from the rat cerebral cortex. The results are expressed as the percentage (%) of conversion, show the net increases (Δ) above the control values and represent the means ± SEM (n = 8–23). Control value: 0.92 ± 0.04% conversion (n = 16)

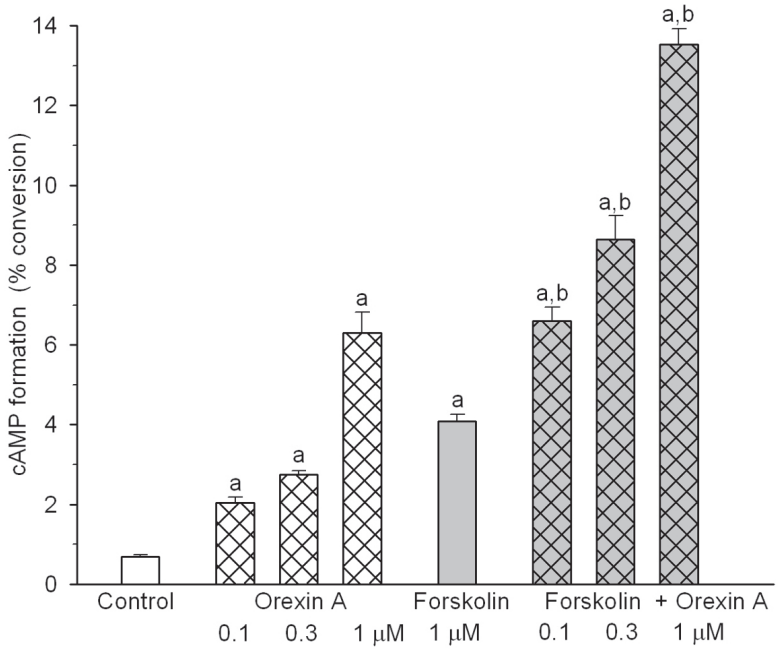

Fig. 2. Effects of orexin A on forskolin-stimulated cAMP formation in primary astrocyte cultures from the rat cerebral cortex. Values are shown as the means ± SEM (n = 5–9). [a] $p < 0.05$ *vs.* control, [b] $p < 0.05$ *vs.* forskolin

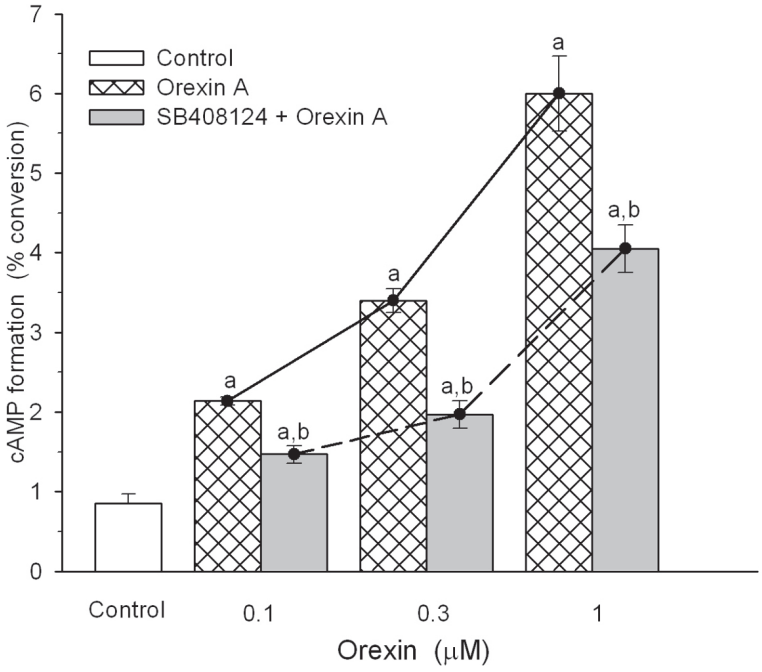

Fig. 3. The effect of SB 408124 (10 μM) on orexin-A-induced cAMP synthesis in primary astrocyte cultures from the rat cerebral cortex. Values are shown as the means ± SEM (n = 6–12). [a] $p < 0.05$ *vs.* control, [b] $p < 0.05$ *vs.* orexin A

antagonist of OX_1 receptors, before orexin A administration significantly reduced the stimulatory action of orexin A on both basal and forskolin-activated cAMP production (Fig. 3 and Fig. 4A). Conversely, TCS OX2 29, which is a selective antagonist of OX_2 receptors, had no effect on orexin A activity (Fig. 4B).

Discussion

Orexin A and orexin B (hypocretin 1 and hypocretin 2) are multifunctional neuropeptides that were discovered in 1998. Both orexins are derived from a common pre-

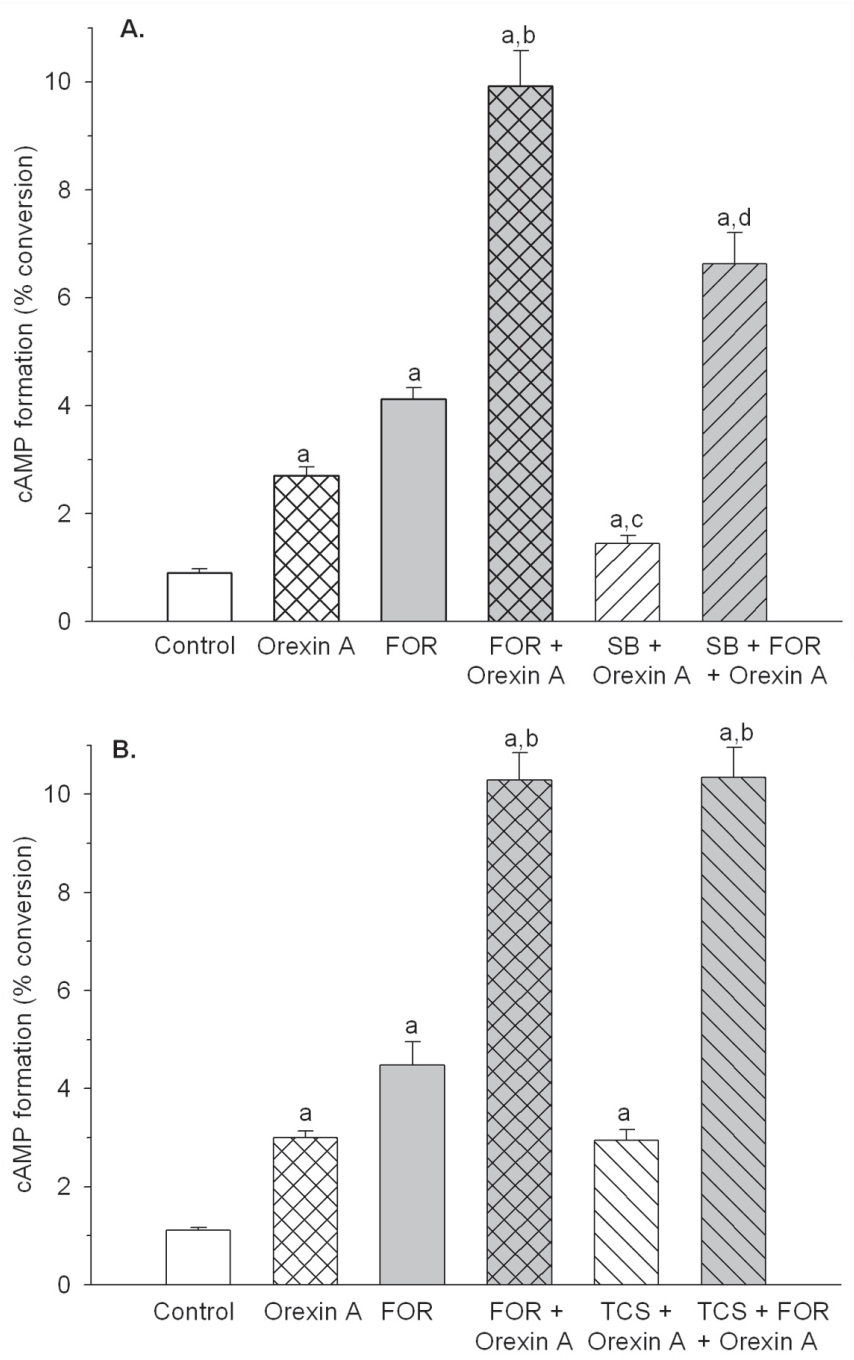

Fig. 4. Effects of SB 408124 (**A**) and TCS 0X2 29 (**B**) pretreatment on the stimulatory action of orexin A (0.3 μM) on basal and forskolin-activated cAMP formation in primary astrocyte cultures from the rat cerebral cortex. FOR – forskolin (1 μM), SB – SB 408124 (10 μM), TCS – TCS OX2 29 (10 μM). Values are shown as the means ± SEM (n = 6–12). $^{a}p < 0.05$ *vs.* control, $^{b}p < 0.05$ *vs.* forskolin, $^{c}p < 0.05$ *vs.* orexin A, $^{d}p < 0.05$ *vs.* forskolin + orexin A

cursor (prepro-orexin) by proteolytic cleavage, and share 46% amino acid identity in humans. The peptides exert their diverse effects *via* two membrane-bound G protein-coupled receptors, OX_1 and OX_2 [11, 13]. Studies in heterologous expression systems have demonstrated that the OX_2 receptor has an equal affinity for both orexin A and orexin B, whereas the OX_1 receptor has an approximately ten-fold higher affinity for orexin A than for orexin B [1, 13].

In the current work, the functional responses to orexinergic stimulation were analyzed in primary cultures of astrocytes that had been isolated from rat cerebral cortex. We demonstrated that orexin A (the nonselective agonist of OX_1 and OX_2 receptors) stimulated cAMP production in rat astrocytes. Conversely, [Ala^{11}-D-Leu^{15}]orexin B, which is the selective agonist of OX_2 receptors [2], failed to affect AMP formation even in the presence of forskolin. This ob-

servation suggests an involvement of OX_1-type receptors in mediating orexin A effects on cAMP synthesis in cortical astrocytes. Further support for the above hypothesis is derived from experiments that used the selective antagonists of orexin receptors. SB 408124, which is the potent nonpeptide antagonist of OX_1 receptors [14], strongly although only partially inhibited orexin A action, whereas TCS OX2 29, which is the OX_2 receptor antagonist [6], had no effect on orexin A activity. Previous studies have demonstrated that the action of orexin A on human OX_1 receptors that had been recombinantly expressed in CHO cells triggered two responses in cAMP production: a weak, high potency (EC_{50} at approximately 1 nM) inhibition, and a strong, low potency (EC_{50} at approximately 0.3 μM) stimulation [7]. Currently, we cannot exclude the hypothesis that a higher potency inhibitory component may contribute to the action of orexin A on cAMP synthesis in rat astrocytes. Further studies with the aid of pertussis toxin, which is an inhibitor of the G_i protein, are required to exclude the possibility of this hypothesis.

Cellular signals that are triggered in response to orexin receptors activation have been rigorously investigated in a few studies and are largely unknown. Ca^{2+} elevation is a prominent response that is seen in native neurons and all cell lines that heterologously expressed orexin receptors [1, 3, 7, 19, 21]. The activation of AC/cAMP is also considered as an important component of orexin receptors signaling, although this pathway has seldom been investigated [13]. In agreement with our results, other studies have shown that orexin A stimulated cAMP production in CHO and Odora (a cell line that is derived from olfactory sensory neurons) cells recombinantly expressing OX_1 receptors [3, 7]. OX_2 receptors are either positively or negatively coupled to the cAMP-generating system depending on the cell or tissue type that is examined and the experimental conditions [3, 7, 9, 20]. To our knowledge, the study has characterized an orexinergic response in astrocytes for the first time. The question remains concerning the functional relevance of orexin receptor-mediated signals in astrocytes. Recently, it has been demonstrated that orexins may markedly affect cell survival by exerting either proapoptotic [16] or proliferative [19] effects depending on the experimental model that is used. Because astrocytes constitute a very abundant cell type in the mammalian central nervous system and play critical roles in brain function, the concept that orexins modulate astrocyte survival is very attractive and warrants further investigation.

In conclusion, the results that are presented here demonstrate that OX_1 receptors stimulate cAMP production in primary rat astrocyte cultures. Further studies are required to evaluate the physiological role(s) of orexins in the regulation of glial cell functions.

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Note added in proof:
We have recently demonstrated that OX_1 and OX_2 receptors are expressed in rat cortical astrocytes. Biegańska K, Sokołowska P, Jöhren O, Zawilska JB: Pharmacol Rep, 2011, 63, 589.

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