Orexin signaling in recombinant neuron-like cells

Tomas Holmqvist[a,*], Karl E.O. Åkerman[a,b], Jyrki P. Kukkonen[a]

[a] *Department of Neuroscience, Physiology, Uppsala University, BMC, P.O. Box 572, SE-75123 Uppsala, Sweden*
[b] *A.I. Virtanen Institute for Molecular Sciences, BioTeknia, Kuopio, Finland*

Received 12 July 2002; accepted 16 July 2002

First published online 25 July 2002

Edited by Jesus Avila

Abstract To assess the role of orexin receptor signaling in neuron-like cells, Neuro-2a murine neuroblastoma and PC12 human pheochromocytoma cells were stably transfected with human OX_1 or OX_2 receptors. Activation of both receptors strongly elevated cellular inositol phosphates and Ca^{2+}. A difference in the potency between orexin-A and -B was seen for OX_1, but not OX_2 receptors. Dependence of the orexin-mediated Ca^{2+} response on extracellular Ca^{2+} and the observed Ba^{2+} influx indicate that in addition to phospholipase C, orexin receptors also may couple to similar non-voltage-gated Ca^{2+} channels in neuronal cells as previously characterized in non-neuronal cells.

Key words: Orexin; Hypocretin; Ca^{2+}; Inositol phosphate; Ca^{2+} influx

1. Introduction

The recently discovered neuropeptides orexin-A and orexin-B mediate their effects through two G-protein-coupled receptors named OX_1 and OX_2 receptors [1]. In the brain, orexins are thought to regulate sleep, feeding and autonomic functions, and similar functions may be mediated even in the periphery (reviewed in [2]). Little is known about the cellular effects of orexins. In neuronal slice preparations from the areas where orexinergic neurons terminate, the main response to application of orexins is an increase in synaptic activity (see e.g. [3]). Both presynaptic and postsynaptic effects have been shown (see e.g. [4]), but the mechanisms for these effects are rather unclear and the investigations are made difficult by methodological problems associated with the use of brain preparations. In neurons, orexins also cause Ca^{2+} elevations [5,6]. The pharmacological characteristics of orexin receptor responses have been determined so far only using recombinantly expressed receptors in Chinese hamster ovary (CHO) cells. In these cells, both orexin receptors have been shown to induce Ca^{2+} elevation [1], which to some degree is dependent on receptor-activated Ca^{2+} channels [7]. To avoid the problems associated with isolation of neuronal cells and the non-neuronal characteristics of CHO cells, we have in this study chosen to establish homogeneous model systems for orexinergic signaling in neuronal settings by transfection of two often used neuron-like model cell lines PC12 (human pheochromocytoma) and Neuro-2a (murine neuroblastoma).

*Corresponding author. Fax: (46)-18-506357.
E-mail address: tomas.holmqvist@fysiologi.uu.se (T. Holmqvist).

Abbreviations: CHO, Chinese hamster ovary; Ca_e^{2+}, extracellular Ca^{2+}; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; probenecid, *p*-(dipropylsulfamoyl)benzoic acid; PLC, phospholipase C; TBM, TES-buffered medium; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; VGCC, voltage-gated Ca^{2+} channel

2. Materials and methods

2.1. Chemicals

Dextrometorphane, EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), nifedipine and probenecid (*p*-(dipropylsulfamoyl)benzoic acid) were purchased from Sigma (St. Louis, MO, USA) and thapsigargin, ω-conotoxin GVIA and MVIIC and ω-agatoxin IVA from RBI (Natick, MA, USA). Fura-2 acetoxymethyl ester was purchased from Molecular Probes Inc. (Eugene, OR, USA) and human orexin-A and -B from Neosystem (Strasbourg, France). Digitonin was purchased from Merck AG (Darmstadt, Germany) and *myo*-[2-^{3}H]inositol (1 mCi/ml) from Amersham Pharmacia Biotech (Buckinghamshire, UK).

2.2. Cell culture and transfection

PC12 and Neuro-2a cells were grown continuously in Dulbecco's modified Eagle's medium (Life Technologies Inc., Paisley, UK) supplemented with 100 U/ml penicillin G (Sigma), 80 U/ml streptomycin (Sigma) and 10% (v/v) fetal calf serum (Life Technologies). The cDNA for human orexin receptor subtypes was cloned into the pCDNA 3.1 vector containing resistance for neomycin. These vectors were used together with the transfection reagents Fugene (Roche, Basel, Switzerland; PC12 cells) and Lipofectamine (Life Technologies; Neuro-2a cells). Forty-eight hours after transfection 800 μg/ml geneticin (Life Technologies) was added to the medium for selection and the cells then were cultured for 3 weeks to obtain stable pan-clones. Pan-clones of PC12 cells were diluted and plated on culture dishes and the expanded single-cell-based colonies were used for creation of clonal cell lines (see Section 3). The stable clonal cell lines were cultured in the presence of 500 μg/ml geneticin.

2.3. Intracellular Ca^{2+} measurements using Ca^{2+} imaging

The coverslips with Neuro-2a or PC12 cells were loaded with fura-2 acetoxymethyl ester (4 μM, 20 min, 37°C) in TES-buffered medium (TBM, consisting of 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose, and 20 mM TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) adjusted to pH 7.4 with NaOH). The measurements were performed as described in [8]. The additions in the chamber were made by perfusion.

2.4. Intracellular Ca^{2+} and inositol phosphate measurements in suspensions of cells

The experiments were performed as described previously [7]. For Ca^{2+} measurements, the cells were harvested and loaded with fura-2 acetoxymethyl ester. The measurements were performed in TBM containing 1 mM probenecid at 37°C using Hitachi F-2000 and F-4000 fluorescence spectrophotometers. Experiments were calibrated by adding 60 μg/ml digitonin (for the maximum fluorescence) and 10 mM

PII: S0014-5793(02)03101-0

EGTA (for the minimum fluorescence). 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 stimulated with the agonists for 20 min in the presence of 10 mM LiCl and the reactions terminated by spinning and replacement of the buffer with ice-cold perchloric acid. The total inositol phosphate fraction of the neutralized supernatants was isolated with anion exchange chromatography. Non-linear curve-fitting of the data was performed using SigmaPlot for Windows 4.01 (Jandel Scientific, Corte Madera, CA, USA).

3. Results

The stable pan-clones of OX_1 and OX_2 receptor-transfected Neuro-2a cells were examined using fura-2 Ca^{2+} imaging. This approach was chosen instead of receptor binding since the Ca^{2+} imaging allows examination of individual cells. In Neuro-2a pan-clones expressing any of the orexin receptors, all the cells responded to stimulation with orexin-A, and further experiments were thus performed with these. Orexin-A and -B stimulated inositol phosphate production in Neuro-2a cells expressing orexin receptors in a concentration-dependent manner (Fig. 1A,B). The EC_{50} values for orexin-A and -B acting at the OX_1 receptor were 2.9 ± 1.8 and 52 ± 20 nM, respectively, and at the OX_2 receptor 1.9 ± 1.1 and 2.2 ± 0.9 nM, respectively. The maximum response to stimulation with either orexin was approximately eight times the basal level in either cell line whereas endogenous P2Y purinoceptor [9] stimulation with ATP only elevated inositol phosphate levels 2–2.5-fold.

Concentration–response relationship for the orexin-elicited Ca^{2+} elevation was measured in cell suspension to obtain statistically more representative values. The concentration–response relationships were very similar to those obtained in inositol phosphate measurements (Fig. 1C,D). The EC_{50} values for orexin-A and -B acting at the OX_1 receptor were 1.9 ± 1.3 and 19 ± 2 nM, respectively, and at the OX_2 receptor 1.0 ± 0.3 and 1.0 ± 0.3 nM, respectively. Upon reduction of extracellular Ca^{2+} (Ca^{2+}_e) to 140 nM, the EC_{50} values were shifted one to three orders of magnitude to the right, which is in agreement with previous results from CHO cells [7].

Depolarization of Neuro-2a cells with 65 mM K^+ did not lead to any Ca^{2+} elevation, and the Ca^{2+} response to subsequent addition of orexins was not affected (data not shown). The Ca^{2+} responses were also insensitive to various inhibitors of voltage-gated Ca^{2+} channels (VGCC) such as nifedipine (10 μM), ω-conotoxin GVIA and MVIIC (2 μM), ω-agatoxin IVA (200 nM) and dextrometorphane (100 μM) (data not shown).

In the PC12 cell pan-clones, only 10% of cells responded to orexin-A with a Ca^{2+} elevation. Single-cell-based clones of transfected PC12 cells were therefore isolated. In the identified orexin receptor-positive clonal cell lines, all the cells responded to orexin-A (Fig. 2A). The same pattern of inositol phosphate response as with Neuro-2a cells was seen with PC12 cells. Thus orexin-A was 30-fold ($EC_{50} = 5.6 \pm 3.3$ nM) more potent than orexin-B ($EC_{50} = 100 \pm 13$ nM) at the OX_1 receptor whereas both orexins were equipotent at the OX_2 receptor ($EC_{50} = 39 \pm 5$ and 41 ± 12 nM, respectively) (Fig. 2B,C). The maximum response to orexins varied in different

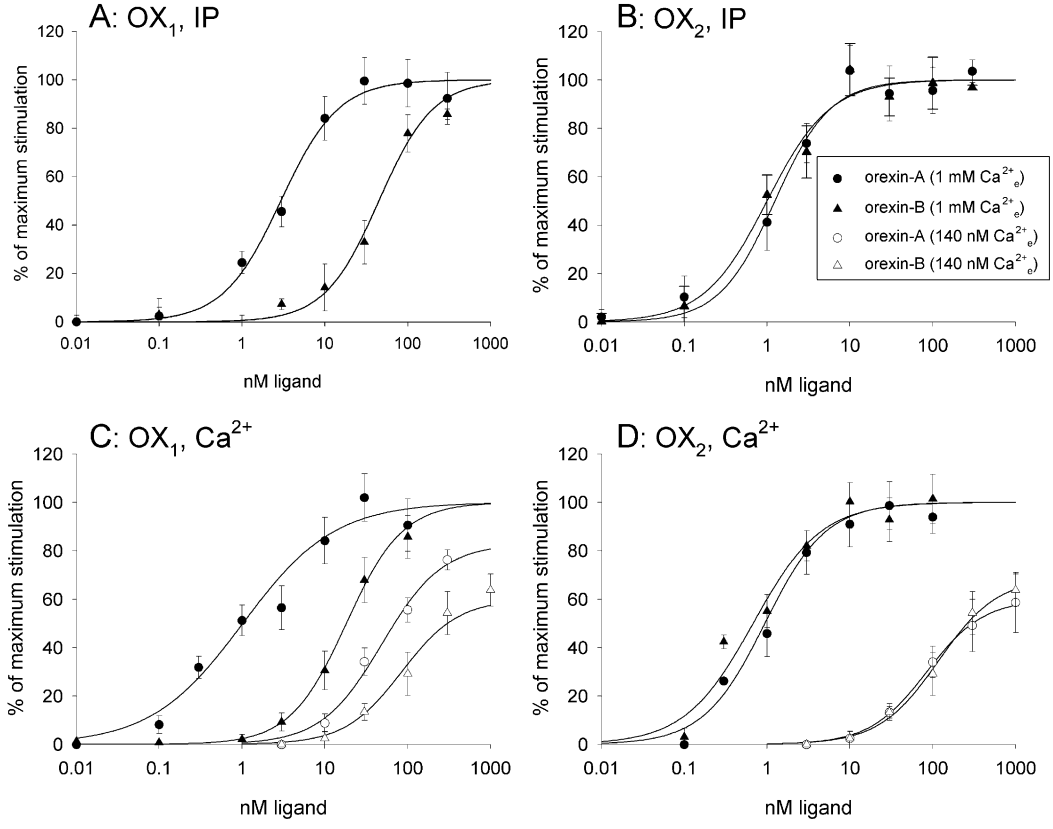

Fig. 1. Concentration–response relationships for orexin-A and -B at the OX_1 (A, C) and OX_2 receptors (B, D) in Neuro-2a cells with respect to the inositol phosphate mobilization (IP; A, B) and Ca^{2+} elevation (Ca^{2+}; C, D). Ca^{2+} responses are shown both in the presence of normal and low Ca^{2+}_e. All measurements were performed in triplicate with at least three different batches of cells; the error bars indicate standard error of the mean.

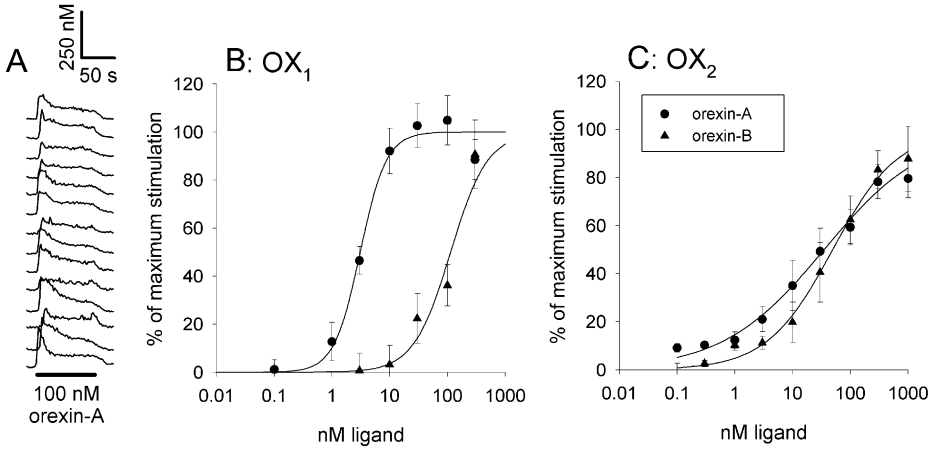

Fig. 2. In A, Ca^{2+} responses to stimulation with 100 nM orexin-A in a single-cell-based clonal cell line (PC12-hOX$_1$-H1). The data are from one coverslip. Each trace indicates an individual cell as measured using Ca^{2+} imaging. In B and C, concentration–response relationships for orexin-A and -B at the OX_1 (B) and OX_2 receptors (C) in PC12 cells with respect to the inositol phosphate mobilization. All measurements were performed in triplicate with at least three different batches of cells; the error bars indicate standard error of the mean.

cell lines, but the altogether highest responses (≈eight times the basal) were much higher than those elicited by endogenous P2Y purinoceptor stimulation with ATP (≈2–2.5 times the basal). Ca^{2+} imaging experiments indicated that the concentration–response curve for orexins was similarly shifted in PC12 cells as in Neuro-2a upon reduction of Ca_e^{2+} (data not shown). To judge whether this Ca_e^{2+} sensitivity was caused by Ca^{2+}-dependent binding of orexins to their receptors or by the dependence of the response on Ca^{2+} influx, we also performed experiments where Ca_e^{2+} was replaced with Ba^{2+}. Mere removal of Ca^{2+} abolished the response to low concentrations of orexins, but upon addition of Ba^{2+} a rapid and stable increase in the 340 nm fluorescence was seen. This increase, not present in the absence of orexins, is indicative of Ba^{2+} influx (data not shown).

As a control, non-transfected PC12 and Neuro-2a cells were exposed to orexins. No Ca^{2+} or inositol phosphate response was observed.

4. Discussion

Previous studies on coupling of orexin receptors to Ca^{2+} elevation are based on expression of these receptors in CHO cells [1,7,10] or recordings from isolated neurons [5,6]. In this study, we have stably expressed OX_1 and OX_2 orexin receptors in two neuron-like cell lines in order to investigate the function of these receptors in neuronal settings. In these cell lines, Neuro-2a and PC12 cells, we can confirm the result from rat neurons [5,6] and from non-neuronal CHO cells [7,11], which show that orexins produce Ca^{2+} elevations mainly in an Ca_e^{2+}-dependent manner. For the first time, we can also show that both orexin receptors, when activated by either of the endogenous orexin peptides, mobilize cellular inositol phosphates.

In rat neurons, orexin-stimulated Ca^{2+} influx has been suggested to relate to activation of VGCC [5,6]. In contrast, we have previously shown that OX_1 receptors connect to non-VGCC in CHO cells [7]. In this study we found no evidence for the involvement of VGCC, yet the Ca^{2+} and inositol phosphate responses were dependent on the Ca_e^{2+}. This is phenomenologically similar to CHO cells, where at low orexin-A concentrations Ca^{2+} influx is the primary response, but it can amplify phospholipase C (PLC) so that also Ca^{2+} release and IP_3 production are obtained. At 100-fold higher concentrations, orexin-A activates Ca^{2+} release even in the absence of Ca_e^{2+} [7]. It remains to be shown what lies behind the Ca_e^{2+}-dependence of orexin responses in Neuro-2a and PC12 cells. Our experiments with Ba^{2+} influx yet suggest – similarly to CHO cells – that this is due to Ca^{2+} influx and not to Ca^{2+}-dependence of orexin binding. Increased Ca^{2+} influx as an ubiquitous mechanism for orexinergic signaling could explain the increased transmitter release suggested as one of the mechanisms behind the orexin receptor-mediated increase in synaptic activity. Orexin-stimulated noradrenaline release from rat cerebral cortex shows a similar shift in the concentration–response curves upon removal of Ca_e^{2+} [12].

How orexin receptors regulate intracellular signaling pathways has not yet been clarified. OX_2 receptors have been shown to be able to activate members of the G_i, G_s and G_q G-protein families [13]. Though coupling to Ca^{2+} elevation has been shown in different systems, the relationship of this to PLC activation is unclear. The results of the present study show a strong coupling of orexin receptors to inositol phosphate mobilization, suggesting that PLC is one of the central effectors for orexin receptors even in neuronal cells. PLC-mediated protein kinase C activation might thus explain some of the depolarizing effects of orexins.

Pharmacological characterization of orexin receptors has been performed in CHO cells. There, orexin-A activates OX_1 receptors up to 10–100 times more potently than orexin-B but both peptides are equipotent at the OX_2 receptors [1,10]. It has been difficult to find unequivocal support for this selectivity in tissues since usually both receptor subtypes are expressed. Therefore, the results of the present study are important in showing that these pharmacological profiles hold true for the responses measured even in neuronal settings.

In conclusion, the results presented here indicate that activation of PLC and Ca^{2+} elevation are central for orexin signaling in neuron-like cells. Analysis of the pharmacological profile of orexins shows that orexin receptors behave similarly in neuron-like cells as in CHO cells. The results based on the Ca_e^{2+}-dependence of the Ca^{2+} responses suggest that Ca^{2+}-

permeable cation channels may be activated by orexin receptors, though further experiments are required to confirm this.

Acknowledgements: This study was supported by the European Union Contract no. QLG3-CT-2002-00826 and by grants from the Swedish Medical Research Council, the Swedish Society for Medical Research, the Cancer Research Fund of Sweden, the Lars Hierta Foundation, the Academy of Finland and the Sigrid Juselius Foundation. We also want to thank Dr. Michel Detheux (Euroscreen s.a., Bruxelles, Belgium) for the orexin receptor genes.

References

[1] Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., McNulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J. and Yanagisawa, M. (1998) Cell 92, 573–585.
[2] Willie, J.T., Chemelli, R.M., Sinton, C.M. and Yanagisawa, M. (2001) Annu. Rev. Neurosci. 24, 429–458.
[3] de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L., Gautvik, V.T., Bartlett, F.S., Frankel, W.N., van den Pol, A.N., Bloom, F.E., Gautvik, K.M. and Sutcliffe, J.G. (1998) Proc. Natl. Acad. Sci. USA 95, 322–327.
[4] Burlet, S., Tyler, C.J. and Leonard, C.S. (2002) J. Neurosci. 22, 2862–2872.
[5] van den Pol, A.N., Gao, X.B., Obrietan, K., Kilduff, T.S. and Belousov, A.B. (1998) J. Neurosci. 18, 7962–7971.
[6] Uramura, K., Funahashi, H., Muroya, S., Shioda, S., Takigawa, M. and Yada, T. (2001) NeuroReport 12, 1885–1889.
[7] Lund, P.E., Shariatmadari, R., Uustare, A., Detheux, M., Parmentier, M., Kukkonen, J.P. and Åkerman, K.E.O. (2000) J. Biol. Chem. 275, 30806–30812.
[8] Åkerman, K.E.O., Näsman, J., Lund, P.E., Shariatmadari, R. and Kukkonen, J.P. (1998) FEBS Lett. 430, 209–212.
[9] Kim, W.K. and Rabin, R.A. (1994) J. Biol. Chem. 269, 6471–6477.
[10] Smart, D., Jerman, J.C., Brough, S.J., Rushton, S.L., Murdock, P.R., Jewitt, F., Elshourbagy, N.A., Ellis, C.E., Middlemiss, D.N. and Brown, F. (1999) Br. J. Pharmacol. 128, 1–3.
[11] Kukkonen, J.P. and Åkerman, K.E.O. (2001) NeuroReport 12, 2017–2020.
[12] Hirota, K., Kushikata, T., Kudo, M., Kudo, T., Lambert, D.G. and Matsuki, A. (2001) Br. J. Pharmacol. 134, 1461–1466.
[13] Randeva, H.S., Karteris, E., Grammatopoulos, D. and Hillhouse, E.W. (2001) J. Clin. Endocrinol. Metab. 86, 4808–4813.