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SPECIAL REPORT

Characterization of recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR

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The cellular mechanisms underlying the physiological effects of the orexins are poorly understood. Therefore, the pharmacology of the recombinant human orexin receptors was studied using FLIPR. Intracellular calcium ($[Ca^{2+}]_i$) was monitored in Chinese hamster ovary (CHO) cells stably expressing orexin-1 (OX_1) or orexin-2 (OX_2) receptors using Fluo-3AM. Orexin-A and orexin-B increased $[Ca^{2+}]_i$ in a concentration dependent manner in CHO-OX_1 ($pEC_{50} = 8.03 \pm 0.08$ and 7.30 ± 0.08 respectively, $n = 5$) and CHO-OX_2 ($pEC_{50} = 8.18 \pm 0.10$ and 8.43 ± 0.09 respectively, $n = 5$) cells. This response was typified as a rapid peak in $[Ca^{2+}]_i$ (maximal at 6–8 s), followed by a gradually declining secondary phase. Thapsigargin (3 μM) or U73122 (3 μM) abolished the response. In calcium-free conditions the peak response was unaffected but the secondary phase was shortened, returning to basal values within 90 s. Calcium (1.5 mM) replacement restored the secondary phase. In conclusion, orexins cause a phospholipase C-mediated release of calcium from intracellular stores, with subsequent calcium influx.

Keywords: Orexin; hypocretin; calcium; protein kinase C; phospholipase C; phospholipase D; FLIPR

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; CHO, Chinese hamster ovary; FIU, fluorescence intensity units; FLIPR, flurometric imaging plate reader; OX_1, orexin-1 receptor; OX_2, orexin-2 receptor; PLC, phospholipase C; PLD, phospholipase D; PKA, protein kinase A; PKC, protein kinase C

Introduction Orexin-A and orexin-B are 33 and 28 residue peptides respectively, which were recently isolated from the rat hypothalamus (Sakurai *et al*., 1998). Both peptides are derived from a 130 amino acid precursor, prepro-orexin, which is encoded by a gene localized to chromosome 17q21 in humans (Sakurai *et al*., 1998). These peptides are located predominantly in the hypothalamus and locus coeruleus (Sakurai *et al*., 1998; Evans *et al*., 1999), but are also found elsewhere in the brain, and in the spinal cord (Smart, 1999; Van den Pol, 1999).

The orexins have a range of physiological functions, including the control of feeding and energy metabolism (Sakurai *et al*., 1998), modulation of neuroendocrine function (Van den Pol *et al*., 1998; Smart, 1999), and regulation of the sleep–wake cycle (Smart, 1999). However, the cellular mechanisms underlying these effects remain to be fully elucidated. Both peptides bind to two receptors, orexin-1 (OX_1) and orexin-2 (OX_2), although orexin-B apparently has a low affinity for OX_1 (Sakurai *et al*., 1998). The binding of these ligands is associated with an increase in intracellular calcium concentrations ($[Ca^{2+}]_i$), although the mechanism involved is controversial, with one group reporting mobilization from intracellular stores (Sakurai *et al*., 1998) and another reporting PKC-mediated calcium influx (Van den Pol *et al*., 1998). Therefore, the present study examined the pharmacology of the recombinant human orexin receptors using the calcium-sensitive dye, Fluo-3AM, in a fluometric imaging plate reader (FLIPR), and demonstrates that orexin-A is equipotent at OX_1 and OX_2, whilst orexin-B displays a moderate selectivity for OX_2. Moreover, we report here for the first time that activation of OX_1 with either orexin-A or orexin-B causes a biphasic calcium response consisting of a phospholipase C (PLC)-mediated mobilization of calcium from thapsigargin-sensitive intracellular stores, and a secondary calcium influx.

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Methods *Cloning and expression of OX_1 and OX_2 receptors in CHO cells* OX_1 and OX_2 were produced by PCR from in-house foetal and adult brain cDNA libraries respectively, using primers located across the start and stop codons. The receptors were sub-cloned into the pCDN vector (with neomycin resistance) and transfected into CHO cells using lipofectamine (Life Technologies). Clones were selected using 400 μg ml^{-1} G418 (Life Technologies) and single cell clones were produced by limiting dilution cloning.

Cell culture CHO-OX_1 and CHO-OX_2 cells were routinely grown as monolayers in MEM–Alpha medium supplemented with 10% foetal calf serum and 400 μg ml^{-1} G418, and maintained under 95%/5% O_2/CO_2 at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 16.

Measurement of $[Ca^{2+}]_i$ using the FLIPR CHO-OX_1 or CHO-OX_2 cells were seeded into black walled clear-base 96 well plates (Costar U.K.) at a density of 20,000 cells per well in MEM–Alpha medium, supplemented as above and cultured overnight. The cells were then incubated with MEM–Alpha medium containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, Texas, U.S.A.) and 2.5 mM probenecid at 37°C for 60 min. The cells were washed four

times with, and finally resuspended in, Tyrode's medium containing 2.5 mM probenecid and 1% gelatine, before being incubated for 30 min at 37°C with either buffer alone (control) or buffer containing various signal transduction modifying agents. In some studies calcium was omitted from the buffer. The plates were then placed into a FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence ($\lambda_{ex}=488$ nM, $\lambda_{EM}=540$ nM) (Sullivan *et al*., 1999) before and after the addition of orexin-A or orexin-B (10 pM – 10 μM).

Data analysis Responses were measured as peak fluorescence intensity (FI) minus basal FI, and where appropriate were expressed as a percentage of a maximum orexin-A-induced response. Data are expressed as mean ± s.e.mean unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 2.00 (Graphpad Software Inc., California, U.S.A.).

Materials Orexin-A and orexin-B were synthesized at SmithKline Beecham. All signal transduction modifying agents were purchased from Calbiochem, Nottingham, U.K. All cell culture media were obtained from Life Technologies, Paisley, U.K.

Results Orexin-A and -B caused a concentration-dependent increase in $[Ca^{2+}]_i$ in CHO cells expressing the human OX_1 receptor (Figure 1), with pEC_{50} values of 8.03 ± 0.08 and 7.30 ± 0.08 respectively ($n=5$). Similarly, both peptides increased $[Ca^{2+}]_i$ in CHO-OX_2 cells (Figure 1), with pEC_{50} values of 8.18 ± 0.10 and 8.43 ± 0.09 respectively ($n=5$). The response was typified at higher concentrations by a rapid initial peak (maximal 6 – 8 s after addition) followed by a gradual decline towards baseline values over a period of ~150 s (Figure 2).

The source of the calcium was also investigated. Depletion of the intracellular calcium stores with thapsigargin (3 μM for 30 min) or inhibition of PLC with U73122 (3 μM) abolished the orexin-A- or orexin-B-induced calcium response in CHO-OX_1 cells (data not shown). The inhibition by U73122 of the calcium response to 10 nM orexin-A was concentration-dependent, with a pIC_{50} of 5.28 ± 0.03 ($n=7$). In the absence of extracellular calcium, orexin-A still elicited an increase in $[Ca^{2+}]_i$ of comparable magnitude (12493 ± 1439 *vs* 13001 ± 733 FIU, $n=4$) to that obtained in the presence of calcium (Figure 2), but the potency of the orexin-A was reduced from 8.11 ± 0.04 to 7.22 ± 0.10 (pEC_{50} values, $n=3$). Similar results were also obtained for orexin-B (data not shown). Moreover, the response was more transient in calcium-free conditions, with the $[Ca^{2+}]_i$ returning to basal levels within 90 s (Figure 2). Calcium replacement (1.5 mM) 75 s after the addition of orexin-A in calcium-free conditions caused a secondary increase in $[Ca^{2+}]_i$ which was then maintained in a similar fashion to that seen in cells challenged with orexin-A in calcium-containing buffer (Figure 2).

Inhibition of PKC with chelerythrine chloride (10 μM) or Ro-31-8220 (10 μM) inhibited the calcium response to orexin-A by 79.0 ± 2.3% and 21.4 ± 3.0% respectively ($n=3-4$). Indeed, chelerythrine chloride inhibited the calcium response to 10 nM orexin-A in a concentration-dependent manner, with a pIC_{50} of 5.38 ± 0.01 ($n=8$). Inhibition of PKA with H-89 (10 μM) had no effect on the orexin-A induced calcium response. Wortmannin (10 μM), which inhibits PI-3 kinase and phospholipase D (PLD), inhibited the orexin-A (10 nM)-induced response by 32.9 ± 2.1% ($n=7$), but the PI-3 kinase specific inhibitor LY294002 (10 μM) had no effect ($n=4$). Similar results were also obtained for orexin-B (data not shown).

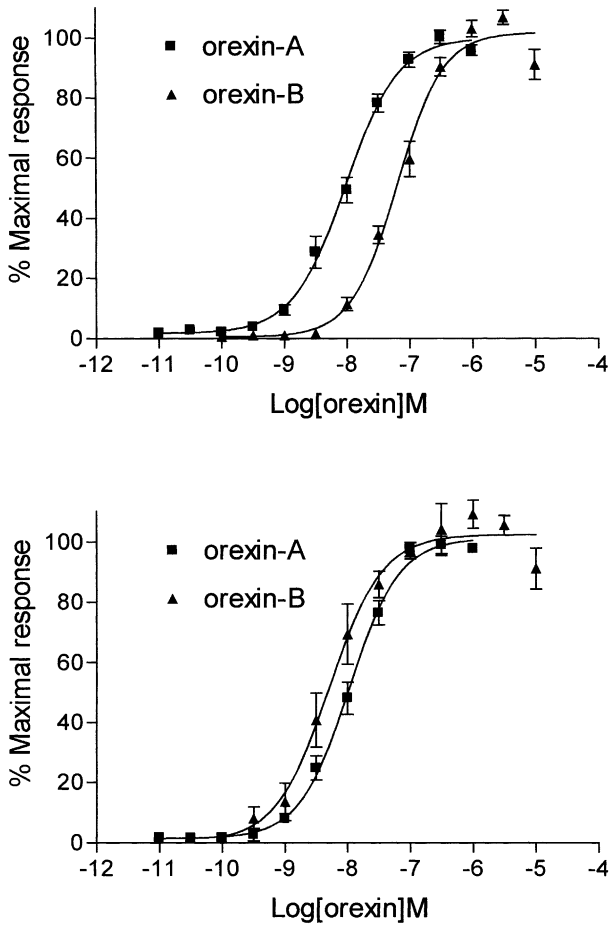

Figure 1 Orexin-A and orexin-B cause a concentration-dependent increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in CHO cells stably expressing OX_1 (upper panel) or OX_2 (lower panel) before and after addition of orexin-A (10 pM – 1 μM) or orexin-B (100 pM – 10 μM). Responses were measured as peak increase in fluorescence minus basal and are given as mean ± s.e.means, where $n=5$.

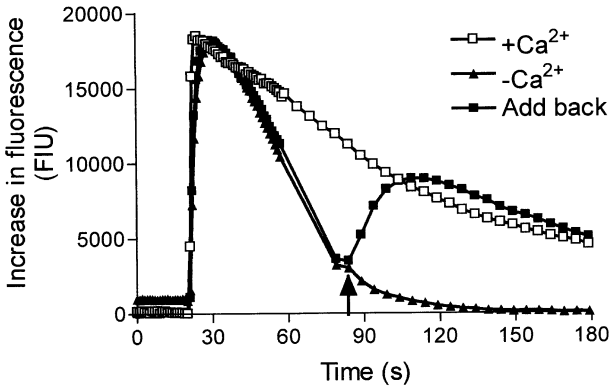

Figure 2 The role of intracellular and extracellular calcium in the orexin-A-induced response. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in CHO-OX_1 cells, incubated in calcium-containing or calcium-free buffer, before and after an orexin-A (1 μM) challenge at 20 s. In some calcium-free experiments the calcium (1.5 mM) was added back 75 s as indicated by the arrow. Data are representative traces, typical of at least $n=5$.

Discussion Orexins are a recently discovered family of neuropeptides (Sakurai *et al*., 1998) with a wide range of physiological functions (Smart 1999) but their cellular mechanism of action remains to be fully elucidated. We have demonstrated, in a recombinant CHO cell system, that orexin-A is equipotent at OX_1 and OX_2 receptors, whereas orexin-B displays a moderate selectivity for OX_2 over OX_1. Furthermore, we have shown clearly for the first time that activation of OX_1 with either orexin-A or -B causes a biphasic calcium response consisting of a phospholipase C mediated mobilization of calcium from intracellular stores, and a secondary influx of extracellular calcium.

In the present study orexin-A was shown to have a similar potency at OX_1 and OX_2 receptors whereas orexin-B displayed modest selectivity for the OX_2 receptor. Consistent with this, orexin-B has previously been reported to be selective for the OX_2 receptor in both radioligand binding (Sakurai *et al*., 1998) and functional (Ichinose *et al*., 1998; Sakurai *et al*., 1998) studies, whereas orexin-A was equipotent at the two receptors (Sakurai *et al*., 1998). However, the absolute EC_{50} values for orexin-B at OX_1 and OX_2 reported here are considerably higher than those reported previously (Sakurai *et al*., 1998), possibly reflecting methodological differences as the earlier study did not include gelatine in the buffer, a process that we have found necessary to accurately measure the potency of orexin-B (unpublished observations). The absolute EC_{50} values for orexin-A at the two receptors in the present study are consistent with the reported affinity from radioligand binding studies (Sakurai *et al*., 1998) and functional assays (Sakurai *et al*., 1998; Evans *et al*, 1999).

The orexin-induced calcium response was biphasic, with a rapid peak and a gradually declining plateau phase, suggesting both intracellular and extracellular components were involved, as reported for other receptors (Berridge, 1993). Similar biphasic orexin-induced calcium responses have recently been reported from studies using primary spinal cord cultures (Van den Pol, 1999). Moreover, we have shown that depletion of the intracellular calcium stores with thapsigargin abolished, whilst the PLC inhibitor, U73122, concentration-dependently inhibited, the orexin-induced response, indicating activation of OX_1 causes a PLC-mediated mobilization of calcium. Sakurai and colleagues originally hypothesized that orexins mobilized intracellular calcium, but offered no evidence to support this (Sakurai *et al*., 1998). However, others have reported that thapsigargin pretreatment had no effect on the orexin-induced calcium response in primary cultures of hypothalamic neurones (Van den Pol *et al*., 1998), although this study used an orexin-B-like peptide rather than orexin-B itself, and no attempt was made to characterize the receptor(s) involved in this heterogenous system (Van den Pol *et al*., 1998; Sakurai *et al*., 1998).

In the present study, the omission of extracellular calcium inhibited the plateau of the response to orexin, indicating that the maintained phase was dependent on calcium influx. Moreover, the reintroduction of calcium restored the maintained phase of the orexin-induced response. Orexin-induced calcium influx has also been reported for hypothalamic and spinal neuronal cultures (Van den Pol *et al*., 1998; Van den Pol, 1999). However, the orexin-induced calcium influx is secondary to the mobilization of intracellular calcium, as the peak response was not affected by the removal of extracellular calcium, and thapsigargin pretreatment abolished both phases of the response. This could reflect calcium-induced calcium influx or modulation of calcium channel activity by a second messenger (Berridge, 1993). Inhibition of PKC with chelerythrine chloride, or the structurally unrelated Ro 31-8220, partly inhibited the orexin-induced calcium response, whilst the inhibition of PKA with H-89, or PI-3 kinase with LY294002 was without effect. Taken collectively these data suggest that PKC-mediated, as well as calcium-induced calcium influx occurs in our cells. This is consistent with other reports that orexin causes PKC-mediated calcium influx in hypothalamic cultures (Van den Pol *et al*., 1998), and induces calcium spiking in spinal neurones (Van den Pol, 1999) similar to that associated with calcium-induced calcium influx in other systems (Berridge, 1993). Interestingly, inhibition of PLD with wortmannin also partly inhibited the orexin-induced response in the present study, suggesting that PLD-activated PKC could be involved in the secondary response.

In conclusion, we have shown that orexin-A is equipotent at OX_1 and OX_2 receptors expressed in CHO cells, whilst orexin-B displays modest selectivity for OX_2. Furthermore, activation of either receptor causes a biphasic calcium response that, at least in the case of OX_1, consists of a PLC-mediated mobilization of calcium from intracellular stores and a secondary influx of calcium, which is in part mediated by PKC.

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