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SPECIAL REPORT

The hypocretins are weak agonists at recombinant human orexin-1 and orexin-2 receptors

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The pharmacology of the orexin-like peptides, hypocretin-1 and hypocretin-2, was studied in Chinese hamster ovary (CHO) cells stably expressing orexin-1 (OX_1) or orexin-2 (OX_2) receptors by measuring intracellular calcium ($[Ca^{2+}]_i$) using Fluo-3AM. Orexin-A and orexin-B increased $[Ca^{2+}]_i$ in CHO-OX_1 ($pEC_{50} = 7.99 \pm 0.05$ and 7.00 ± 0.10 respectively, $n = 8$) and CHO-OX_2 ($pEC_{50} = 8.30 \pm 0.05$ and 8.21 ± 0.07 respectively, $n = 5$). However, hypocretin-1 and hypocretin-2 were markedly less potent, with pEC_{50} values of 5.31 ± 0.04 and 5.41 ± 0.04 respectively in CHO-OX_2 cells ($n = 5$). In CHO-OX_1 cells 10 μM hypocretin-1 only elicited a $37.5 \pm 3.4\%$ response whilst 10 μM hypocretin-2 elicited a $18.0 \pm 2.1\%$ response ($n = 8$). Desensitisation of OX_1 or OX_2 with orexin-A (100 nM) abolished the response to orexin-A (10 nM) and the hypocretins (10 μM), but not to UTP (3 μM). In conclusion, the hypocretins are only weak agonists at the orexin receptors.
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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

Introduction Orexin-A and orexin-B are 33 and 28 residue peptides respectively, which were recently isolated from the rat hypothalamus and are derived from a 130 amino acid precursor, prepro-orexin (Sakurai *et al*., 1998). 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 ($[Ca^{2+}]_i$) (Smart *et al*., 1999).

The orexins are located predominantly in the hypothalamus and locus coeruleus (Sakurai *et al*., 1998; Peyron *et al*., 1998), but are also found elsewhere in the CNS (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).

Independently, DeLecea and colleagues (1998) identified a hypothalamic-specific mRNA encoding a precursor protein which they called prepro-hypocretin, and predicted that processing of this prepro-peptide would yield two peptides, hypocretin-1 (residues 28–66) and hypocretin-2 (residues 69–97). Furthermore, they showed synthetic hypocretin-2 was excitatory when applied to hypothalamic neurons (DeLecea *et al*., 1998). Subsequent comparisons revealed that prepro-orexin and prepro-hypocretin were the same peptide (Flier & Maratos-Flier 1998; Sakurai *et al*., 1998), and that the sequences of the orexins and the hypocretins overlapped (Sakurai *et al*., 1998; DeLecea *et al*., 1998). This has led to some authors erroneously referring to the orexins as hypocretins in some published studies (Van den Pol, 1999; Samson *et al*., 1999). However, the pharmacology of the hypocretins at the orexin receptors has not been examined. Therefore, the present study examined the pharmacology of the hypocretins at the recombinant human receptors using the calcium-sensitive dye, Fluo-3AM, in a fluorometric imaging plate reader (FLIPR), and demonstrated that hypocretin-1 and hypocretin-2 are weak agonists at OX_1 and OX_2.

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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 18.

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) 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 orexin-A or orexin-B. 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, orexin-B, hypocretin-1 or hypocretin-2 (10 pM – 10 μM).

Reverse phase HPLC The purity of the commercial hypocretins was confirmed by reverse phase HPLC using a Waters Symmetry C18 (5 μM, 300A, 2.1 × 150 mm) column and a Hewlett-Packard HP1090 chromatograph at 40°C, with UV detection (210 nm). Eluent A was 0.1% TFA and Eluent B was 80 : 20 acetonitrile : water (+0.085% TFA), were run as a linear gradient (1%/min) from 5 – 95% Eluent B, with a flow rate of 200 μl min^{-1}. Synthetic orexin-A and orexin-B were run as standards.

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 3.00 (GraphPad Software Inc., CA, U.S.A.).

Materials Orexin-A and orexin-B were synthesized for SmithKline Beecham at California Peptides (CA, U.S.A.). Orexin-A, orexin-B, hypocretin-1 and hypocretin-2 were purchased from Phoenix Pharmaceuticals (CA, U.S.A.). 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-OX_1 cells (Figure 1), with pEC_{50} values of 7.99 ± 0.05 and 7.00 ± 0.10 respectively ($n=8$). Similarly, both peptides increased $[Ca^{2+}]_i$ in CHO-OX_2 cells (Figure 1), with pEC_{50} values of 8.30 ± 0.05 and 8.21 ± 0.07, respectively ($n=5$). Commercial orexin-A and orexin-B produced similar responses (data not shown).

Hypocretin-1 and hypocretin-2 also elicited concentration-related Ca^{2+} responses in CHO-OX_1 and CHO-OX_2 cells (Figure 1), but were markedly less potent than the orexins. Indeed, in CHO-OX_1 cells the concentration-response relationship could not be fully defined as the highest concentration tested (10 μM) only elicited a 37.5 ± 3.4% response for hypocretin-1 and a 18.0 ± 2.1% response for hypocretin-2 ($n=8$). However, in the CHO-OX_2 cells the concentration-response curves were better defined, allowing pEC_{50} values of 5.31 ± 0.04 and 5.41 ± 0.04 to be estimated for hypocretin-1 and hypocretin-2 respectively ($n=5$). The hypocretins had no effect on $[Ca^{2+}]_i$ in non-transfected CHO cells (data not shown).

The hypocretin-induced response had a similar kinetic profile to that of the orexin-induced response (Figure 2), with a rapid initial peak (maximal 6 – 10 s after addition) followed by a gradual decline towards baseline values over a period of ~150 s. Moreover, analysis by HPLC showed that there was no trace of contaminating orexins in either the hypocretin-1 or hypocretin-2 (data not shown).

In the absence of an available orexin receptor antagonist desensitization studies were carried out to confirm that the orexins and hypocretins were acting *via* the same receptors. Pre-exposure to orexin-A (100 nM) or orexin-B (100 nM) for 30 min abolished the Ca^{2+} response to a subsequent orexin-A (10 nM), orexin-B (10 nM), hypocretin-1 (10 μM) or hypocretin-2 (10 μM) challenge in CHO-OX_2 cells (Figure 2). Similarly, pretreatment with orexin-A (100 nM) or orexin-B (1 μM) abolished the Ca^{2+} response to all four ligands in CHO-OX_1 cells ($n=3$). However, the Ca^{2+} response to UTP (3 μM),

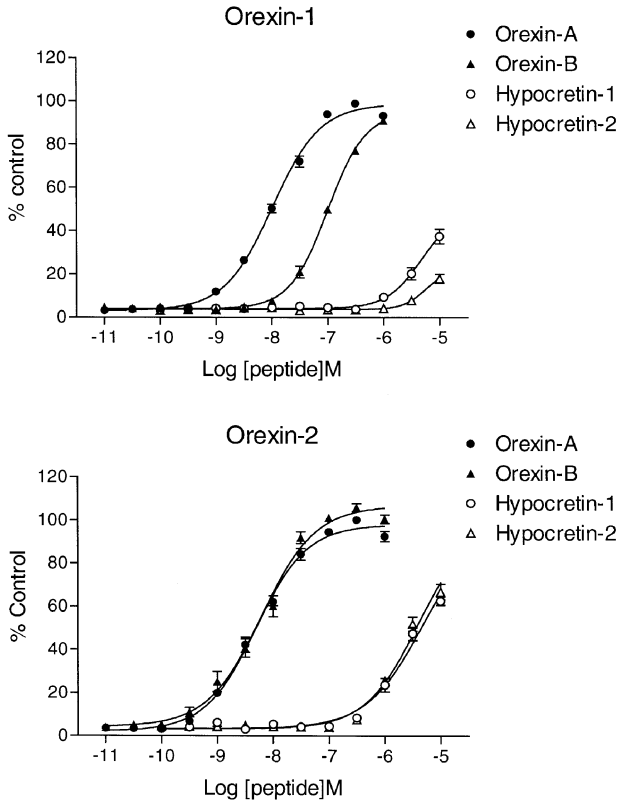

Figure 1 Orexins and hypocretins cause a concentration-dependent increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in CHO cells stably expressing OX_1 (upper panel) or OX_2 (lower panel) before and after addition of orexin-A (10 pM – 1 μM), orexin-B (10 pM – 1 μM), hypocretin-1 (100 pM – 10 μM) or hypocretin-2 (100 pM – 10 μM). Responses were measured as peak increase in fluorescence minus basal and are given as mean ± s.e.mean, where $n=5-8$.

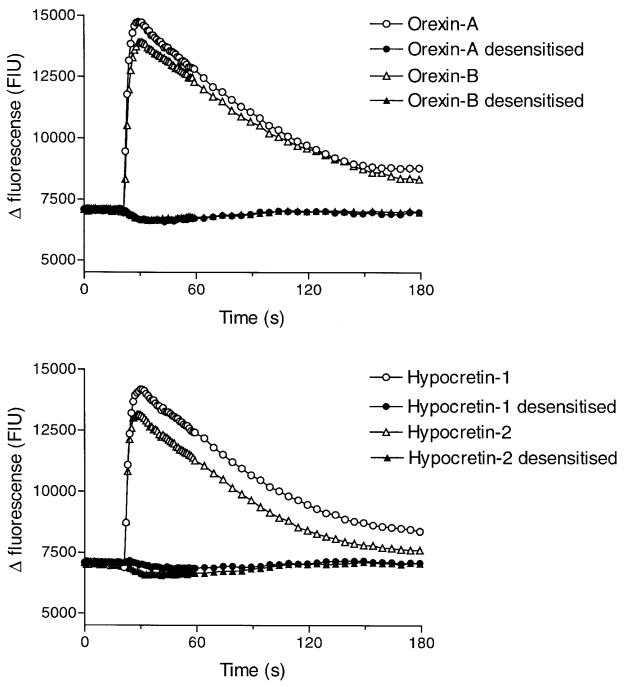

Figure 2 The hypocretin-induced Ca^{2+} responses are desensitized by orexin-A. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in CHO-OX_2 cells which had been incubated with buffer or orexin-A (100 nM) for 30 min prior to the addition of orexin-A (10 nM), orexin-B (10 nM), hypocretin-1 (10 μM) or hypocretin-2 (10 μM). Data are representative traces, typical of $n=3-8$.

which activates an endogenous purinergic receptor, was unaffected in these cells (data not shown).

Discussion The orexins are a recently discovered family of neuropeptides (Sakurai *et al*., 1998) with a wide range of physiological functions (Smart, 1999). These two peptides were originally isolated from rat hypothalamic extracts and shown to interact with two receptors, OX_1 and OX_2 (Sakurai *et al*., 1998). Another group independently identified a hypothalamic precursor protein, prepro-hypocretin, which sequence analysis indicated would yield two peptides, hypocretin-1 (residues 28–66) and hypocretin-2 (residues 69–97) although these were not isolated (DeLecea *et al*., 1998). Subsequent comparisons revealed considerable homology between the hypocretins and the orexins (Flier & Maratos-Flier, 1998; Sakurai *et al*., 1998). Hypocretin-1 has the same sequence as orexin-A, but with five additional N-terminal amino acids and a C-terminal glycine, whilst hypocretin-2 shares the same sequence as orexin-B, but with a C-terminal glycine (DeLecea *et al*., 1998; Sakurai *et al*., 1998). Thus, it was proposed that the hypocretins act at the orexin receptors, but no evidence has been offered to support this hypothesis (Van den Pol *et al*., 1998; 1999). Moreover, this has even resulted in some authors erroneously referring to having used hypocretins when they have actually used orexins (Samson *et al*., 1999). The present study has demonstrated that hypocretin-1 and hypocretin-2 act as weak agonists at OX_1 and OX_2, with $\sim$1000 fold lower potency than orexin-A and orexin-B respectively.

In the present study orexin-A and orexin-B caused a concentration-dependent increase in $[Ca^{2+}]_i$ in CHO cells expressing either OX_1 or OX_2, with potencies similar to those reported previously (Smart *et al*., 1999). The potency of orexin-A was also consistent with the published radioligand binding data (Sakurai *et al*., 1998). Furthermore, orexin-A was equipotent at OX_1 and OX_2, whilst orexin-B displayed moderate selectivity for OX_2, again consistent with the literature (Sakurai *et al.,* 1998; Smart *et al.,* 1999).

Hypocretin-1 and hypocretin-2 also elicited concentration-related Ca^{2+} responses in CHO-OX_1 and CHO-OX_2 cells, but were $\sim$1000 fold less potent than the orexins. Indeed, in CHO-OX_1 cells the concentration-response relationship could not be defined as the response to the highest concentration (10 μM) of either hypocretin tested was $<40\%$. However, the response to hypocretin-1 was greater than that to hypocretin-2, suggesting a similar rank order of potency to that displayed by the orexins at OX_1 (Sakurai *et al*., 1998; Smart *et al*., 1999). Neither hypocretin-1 nor hypocretin-2 affected $[Ca^{2+}]_i$ in parental CHO cells, indicating the hypocretin-induced Ca^{2+} response was mediated by the orexin receptor. Furthermore, the hypocretin-induced Ca^{2+} response displayed an identical kinetic profile to that of the orexin-induced Ca^{2+} response. This biphasic response is consistent with previous reports (Van den Pol, 1999; Smart *et al*., 1999) and indicative of an initial mobilization of intracellular Ca^{2+} and subsequent influx of extracellular Ca^{2+} (Smart *et al*., 1999).

Pre-exposure of the cells to EC_{80} concentrations of orexin-A or orexin-B for 30 min fully desensitized OX_1 and OX_2, as the Ca^{2+} response to a subsequent orexin-A or orexin-B challenge were abolished. Similarly, the responses to hypocretin-1 or hypocretin-2 were also abolished in orexin-desensitized CHO-OX_1 or CHO-OX_2 cells. However, the Ca^{2+} response elicited by UTP (3 μM), which activates an endogenous purinergic receptor, was not affected by orexin-pretreatment in these cells. Therefore, the desensitization of OX_1 and OX_2 was homologous, and thus receptor-specific (Lohse, 1993), confirming that the hypocretins and orexins were acting at the same receptors. Furthermore, the hypocretin-induced responses were not due to contaminating orexins as HPLC showed no trace of these peptides in either the hypocretin-1 or hypocretin-2.

In conclusion, the present study has shown that hypocretin-1 and hypocretin-2 act as weak agonists at OX_1 or OX_2, but are $\sim$1000 fold less potent than the orexins at these receptors.

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