Distinct recognition of OX₁ and OX₂ receptors by orexin peptides

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ABBREVIATIONS: $[Ca^{++}]_e$, extracellular free calcium concentration; $[Ca^{++}]_i$, intracellular free calcium concentration; CNS, central nervous system; $\Delta[Ca^{++}]_i$, change in $[Ca^{++}]_i$ ($[Ca^{++}]_{i/stimulated} - [Ca^{++}]_{i/basal}$); EC₅₀, concentration producing half-maximal response; EGTA, ethylene glycol-bis(β -aminoethyl ether)N,N,N',N'-tetraacetic acid; IP₃, inositol-1,4,5-trisphosphate; *N*, the number of batches of cells for the measurements; *n*_{Hill}, Hill-coefficient; pEC₅₀, $-\log(EC_{50})$; probenecid, *p*-(dipropylsulfamoyl)benzoic acid; TBM, TES Buffered Medium; TES, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino) ethane sulfonic acid

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ABSTRACT

In this study, we have compared the abilities of orexin-A and orexin-B and variants of orexin-A to activate different Ca^{++} responses (influx and release) in human OX_1 and OX_2 receptor expressing CHO cells. Responses mediated by activation of both receptor subtypes with either orexin-A or -B were primarily dependent on extracellular Ca^{++} , suggesting similar activation of Ca^{++} influx as we have previously shown for orexin-A and OX_1 receptor. Amino-acid-wise truncation of orexin-A reduced its ability to activate OX_1 and OX_2 receptors, but the response mediated by the OX_2 receptor was more resistant to truncation than the response mediated by the OX_1 receptor. We also performed a sequential replacement of amino acids 14-26 with alanine in the truncated orexin-A variant orexin-A₁₄₋₃₃. Replacement of the same amino acids produced a fall in the potency for each receptor subtype, but the reduction was less prominent for the OX_2 receptor. The most marked reduction was produced by the replacement of Leu20, Asp25 and His26 with alanine. Interestingly, extracellular Ca^{++} -dependence of responses to some of the mutated peptides was different to those of orexin-A and -B. The mutagenesis also suggests that although the determinants required from orexin-A for binding to and activation of the receptor are highly conserved between the orexin receptor subtypes, the OX_2 receptor requires fewer determinants. This might in part explain why orexin-B has the affinity and potency equal to orexin-A for this subtype, although it has 10-100 fold lower affinity and potency for the OX_1 receptor.

Recently, two novel hypothalamic peptides were isolated and subsequently named orexin-A and orexin-B (Sakurai *et al.*, 1998) or hypocretin-1 and hypocretin-2 (de Lecea *et al.*, 1998). Despite some initial confusion, orexin-A should now be considered identical to hypocretin-1 and orexin-B to hypocretin-2. Orexins act as agonists on two G-protein-coupled receptors called OX₁ and OX₂ receptors. Increased wakefulness and reduced sleep is a well-demonstrated response to central administration of orexin, and disruption of central orexinergic signaling leads to the sleep disorder narcolepsy in animal models and probably also in man (reviewed in Beuckmann and Yanagisawa, 2002; Kukkonen *et al.*, 2002; Sutcliffe and de Lecea, 2002). The other physiological roles for orexins may be regulation of energy homeostasis and stress response, probably both via central and peripheral mechanisms (reviewed in Willie *et al.*, 2001; Beuckmann and Yanagisawa, 2002; Kirchgesner, 2002; Kukkonen *et al.*, 2002; Smart and Jerman, 2002).

The two orexin peptides, orexin-A and -B, are both products of the same precursor peptide, preproorexin, cleavage of which results in equimolar amounts of orexin-A and orexin-B. Orexin-A is composed of 33 amino acids and it contains two disulfide bridges, whereas orexin-B is a linear peptide of 28 residues (Sakurai *et al.*, 1998). Though a product of a different part of the precursor peptide, orexin-B shows a 46% sequence identity with orexin-A, and these two peptide sequences seem to have arisen through duplication of a single sequence (Alvarez and Sutcliffe, 2002). Most striking is the homology in the more C-terminal parts of the peptide, which could make orexin-B an N-terminally truncated variant of orexin-A. The secondary structure of orexin-A is not known but orexin-B has been determined to consist of two α -helices in 60-80° angle to each other (Lee *et al.*, 1999). Orexin-A is much more lipophilic than orexin-B and it is also more stable in blood and cerebrospinal fluid (Kastin and Akerstrom, 1999). Yet,

the CNS (central nervous system) orexin-B levels are consistently 2-5 times higher than orexin-A levels (Mondal *et al.*, 1999a; Mondal *et al.*, 1999b; Date *et al.*, 2000a; Date *et al.*, 2000b).

Orexins act as agonists on two G-protein-coupled receptors called OX₁ and OX₂ receptors. Both of these subtypes show a high (91-98%) interspecies conservation between different mammals. Human variants of OX₁ and OX₂ receptors share a 64% sequence identity. Many studies suggest Ca⁺⁺ influx as the most immediate cellular response to orexin receptor activation in different systems (van den Pol *et al.*, 1998; van den Pol, 1999; Lund *et al.*, 2000; Hirota *et al.*, 2001; Kukkonen and Åkerman, 2001; Uramura *et al.*, 2001; Holmqvist *et al.*, 2002). This Ca⁺⁺ influx may in some systems occur via protein kinase C-dependent activation of voltage-gated Ca⁺⁺ channels (van den Pol *et al.*, 1998; Uramura *et al.*, 2001; Xu *et al.*, 2002) but a different type of Ca⁺⁺ channel has been implicated in other systems (Lund *et al.*, 2000; Kukkonen and Åkerman, 2001). In the CNS, the most prominent response to orexin application is an increase in synaptic activity (reviewed in Beuckmann and Yanagisawa, 2002; Kukkonen *et al.*, 2002). The putative role of increased Ca⁺⁺ influx for this process is largely unclear (reviewed in Kukkonen *et al.*, 2002). Orexin receptor subtypes are somewhat differentially distributed (reviewed in Willie *et al.*, 2001; Kukkonen *et al.*, 2002; Smart and Jerman, 2002), but since there is yet no evidence of subtype selective signaling, it is difficult to predict the significance of this difference.

When Ca⁺⁺ or inositol phosphate responses are measured in CHO (Chinese hamster ovary), PC12 or Neuro-2a cells recombinantly expressing orexin receptors, orexin-A is 10-100 times more potent than orexin-B on the OX₁ receptors, whereas both orexins are equipotent on the OX₂ receptors (Sakurai *et al.*, 1998; Smart *et al.*, 1999; Okumura *et al.*, 2001; Holmqvist *et al.*, 2002). These potencies appear to be direct reflections of the binding affinities (Sakurai *et*

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al., 1998), suggesting that in spite of the similarities between the orexin receptors there are interesting differences in the ligand binding domains. To further examine the parameters required for the orexin peptide–receptor interaction, we have in this study investigated the determinants of orexin-A required to activate OX₁ and OX₂ receptors. Truncated and alanine-substituted orexin-A peptides have been tested for their ability to induce Ca⁺⁺ elevations in CHO-K1 cells heterologously expressing OX₁ or OX₂ receptors. The results suggest that the structures responding to orexin-A are highly conserved between OX₁ and OX₂ receptors but also that there are some clear differences between these receptors.

Methods

Cell Culture. CHO-hOX₁-C1 cells were produced as described in (Lund *et al.*, 2000). CHO-hOX₂-C1 cells were produced in similar way; the clones used expressed receptors at approximately the same level, as determined with [¹²⁵I]orexin-A binding. CHO-hOX₁-C1 and CHO-hOX₂-C1 cells were grown in Ham's F12 medium (Gibco, Paisley, UK) supplemented with 100 U/ml penicillin G (Sigma Chemical Co., St. Louis, MO), 80 U/ml streptomycin (Sigma), 400 µg/ml geneticin (G418; Gibco) and 10% (v/v) fetal calf serum (Gibco) at 37°C in 5% CO₂ in an air ventilated humidified incubator in 260 ml plastic culture flasks (75 cm² bottom area; Nunc A/S, Roskilde, Denmark). For fluorometry, the cells were grown on circular plastic culture dishes (inner diameter, 94 mm; Nunc).

Drugs. EGTA (ethylene glycol-bis[β-aminoethyl ether]N,N,N',N'-tetraacetic acid) and probenecid (*p*-[dipropylsulfamoyl]benzoic acid) were purchased from Sigma (Natick, MA) and Fura-2 acetoxymethyl ester and fluo-3 acetoxymethyl ester from Molecular Probes Inc. (Eugene, OR). Human orexin-A and -B were from Peninsula Laboratories Europe Ltd. (St. Helens, UK) or Neosystem (Strasbourg, France) and digitonin was from Merck AG (Darmstadt, Germany).

Peptide synthesis. To avoid investigation of complex secondary structure changes caused by disulfide-bridge removals, we initially truncated orexin-A N-terminally to produce orexin-A₁₄₋₃₃, which is devoid of disulfide bonds, yet it has substantial activity on OX₁ and OX₂ receptors. A previous study has suggested that a significant part of the potency lost by truncation of orexin-A to orexin-A₁₅₋₃₃ is caused by the loss of the disulfide-bridges (Okumura *et al.*, 2001). All the further mutagenesis was done on orexin-A₁₄₋₃₃. We both performed a further stepwise N-terminal truncation of this peptide and one-by-one replacement of amino acids with alanine

("alanine scan") (table 1, fig. 1). The truncated and alanine-scanned orexin-A-peptides were synthesized using Fmoc (9-fluorenylmethoxycarbonyl) synthesis protocols with double or triple coupling reactions using TBTU (2-[H-benzotriazol-1-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate) as the activator on a Symphony synthesizer (Rainin Instrument Co., Woburn, MA). Purifications were performed by reverse phase-HPLC on a Delta-Pak C18 column (15 μ m - 100 Å - 25 $\times$ 100 mm) (Waters, Milford, MA) using a Waters liquid chromatography system consisting of Model 600 solvent delivery pump, a Rheodine injector and an automated gradient controller (solvent A: H₂O-0.125% TFA [trifluoroacetic acid]; solvent B: CH₃CN-0.1% TFA; gradient: 15% B to 60% B in 20 min). Detection was carried out using Model M2487 variable wavelength UV detector connected to the Waters Millennium software control unit. The Quality Control was performed by analytical reverse phase-HPLC on a Waters Delta-Pak C18 (5 μ m - 100 Å - 150 $\times$ 3.9 mm) column (same solvents as above; gradient: 0% B to 60% B in 20 min) using a Waters Alliance 2690 Separation Module equipped with a Waters 996 Photodiode Array Detector and by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry using a Voyager-DE instrument (PerSeptive Biosystems, Framingham, MA).

Media. The TES Buffered Medium (TBM) consisted of 137 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 10 mM glucose, 1 mM probenecid and 20 mM 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino) ethane sulfonic acid (TES) adjusted to pH 7.4 with NaOH.

Ca⁺⁺ measurements. Both the fluorescent Ca⁺⁺ indicators fura-2 and fluo-3 were used to monitor changes in intracellular [Ca⁺⁺] ([Ca⁺⁺]_i) since they offer detection of low and high Ca⁺⁺ elevations, respectively, with high accuracy due to different K_d-values for Ca⁺⁺ binding. The K_d of 224 nM was used for fura-2 whereas the K_d of 1000 nM was determined for fluo-3 in

simultaneous measurement with fura-2. The low responses of the truncated C-terminal orexin-A-peptides were thus determined using fura-2 whereas all the other experiments were performed using fluo-3. It should be noted that the K_d -value of the indicator does not affect the relative EC_{50} -values or the maximum responses determined. For the experiments, the cells were harvested using phosphate buffered saline containing 0.2 g/l EDTA, loaded with fura-2 acetoxymethyl ester or fluo-3 acetoxymethyl ester (4 μ M, 20 min, 37°C) in the culture medium (Ham-F12) supplemented with 10 μ g/ml bovine serum albumin and 1 mM probenecid and stored on ice as pellets (medium removed). For the measurement of intracellular free calcium, one pellet was resuspended in TBM at 37°C. The fluorescence was monitored in a stirred quartz microcuvette in a thermostated cell holder of either a Hitachi F-2000 or F-4000 fluorescence spectrophotometer at the wavelengths 340 or 340/380 (excitation), 505 (emission) for fura-2 or 480 nm (excitation), 540 nm (emission) for fluo-3. Experiments were calibrated by adding 60 μ g/ml digitonin, which gives the maximum value of fluorescence, and 10 mM EGTA, which gives the minimum value of fluorescence. The leaked fura-2 and fluo-3 were measured in separate experiments by adding 10 mM EGTA, which chelates Ca^{++} bound to extracellular indicator. The corrected fluorescence values were used to calculate $[Ca^{++}]_i$.

Calculations and data analysis. The extracellular free $[Ca^{++}]$ ($[Ca^{++}]_e$) was determined as described in (Lund *et al.*, 2000). Thus, addition of 1.5 mM EGTA in TBM gave a $[Ca^{++}]_e$ of approximately 140 nM. Values are given as mean $\pm$ S.E.M. unless otherwise indicated; N refers to the number of batches of cells on which the measurements were performed. Non-linear curve-fitting was performed using SigmaPlot for Windows 4.01 (Jandel Scientific, Corte Madera, CA). The difference in the potency (EC_{50}) and activity (max response) of orexin-A and orexin-B to elevate Ca^{++} in 1 mM and 140 nM $[Ca^{++}]_e$ (table 2) was evaluated using Student's paired two-

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tailed t test. The differences in the activity (maximum response) between different peptides was evaluated using Student's non-paired two-tailed t test.

Results

Orexin-A and -B differentially activate OX₁ and OX₂ receptors. As shown previously (Lund *et al.*, 2000; Holmqvist *et al.*, 2001; Kukkonen and Åkerman, 2001), orexin-A caused large elevations in intracellular [Ca⁺⁺] in OX₁ receptor-expressing CHO-K1 cells (table 2). Orexin-B was 7-fold less potent (table 2 and 3, fig. 2A). No difference in the potency between the ligands was seen for the OX₂ receptor (table 2 and 3, fig. 2B). We have previously shown that OX₁-mediated Ca⁺⁺-response to low concentrations of orexin-A requires extracellular Ca⁺⁺ in CHO cells (Lund *et al.*, 2000; Holmqvist *et al.*, 2001; Kukkonen and Åkerman, 2001). This is not specific for orexin-A as an external Ca⁺⁺-dependence was also seen with respect to both orexin-A and -B response with both OX₁ and OX₂ receptors (table 2; fig. 2). Reduction in extracellular Ca⁺⁺ caused a considerable increase in the n_{Hill} value for all combinations of ligand/receptor. This change was largest for the effect of orexin-B on the OX₂ receptor.

With each batch of cells, concentration-response relationships were determined with both orexin-A and orexin-B in normal (1 mM) and low (140 nM) extracellular Ca⁺⁺. This allows comparison within individual batches of cells and the slight variance between the batches of cells does not distort the results. From these data the significancies for any difference between high and low extracellular Ca⁺⁺ and orexin-A and orexin-B were calculated using the paired *t* test (table 3). These data confirm the fact that there is a difference in the potency between orexin-A and -B for the OX₁ receptor but not for the OX₂ receptor. Orexin-B appears to give a slightly (10-24%) lower maximum response than orexin-A in all the cases. A right-shift in the EC₅₀ values of both orexin-A and -B is seen upon reduction of extracellular Ca⁺⁺, but the

maximum responses appear unaffected. Notably, the shift appears lower for orexin-B than for orexin-A with both OX_1 and OX_2 receptors.

C-terminal Orexin-A peptides are agonistic for the OX_1 and OX_2 receptors

C-terminal truncation of orexin-A to orexin-A₁₄₋₃₃ (fig. 1) increased the EC_{50} to 27 nM from 4 nM (OX_1) or 7 nM (OX_2) (tables 4 and 5). The maximum response, however, was not affected, as calculated using non-paired *t* test. The effect of a further reduction in the peptide length is shown in fig. 3. The response to 100 nM peptide fell when the peptide length was reduced to 18 amino acids (orexin-A₁₆₋₃₃) (fig. 3A and B). Further truncation to 15 amino acids (orexin-A₁₉₋₃₃) completely abolished the Ca^{++} response to 100 nM peptide (fig. 3A and B). In a similar manner, both OX_1 - and OX_2 -expressing cells ceased responding to 1 μ M of the peptide when its length was reduced to 12 amino acids (orexin-A₂₂₋₃₃), though the response to 10 μ M peptide was retained (fig. 3A and B). With further truncation, the fall in activity was eminent in the OX_1 -expressing cells, and the C-terminal decapeptide (orexin-A₂₄₋₃₃) gave no response even at the concentration of 10 μ M. The decline in activity with reduced peptide length was markedly lower with OX_2 -receptors. At 10 μ M, the nonapeptide (orexin-A₂₅₋₃₃) gave a robust response, and a slight response was still observed with the heptapeptide (orexin-A₂₇₋₃₃) but not with the hexapeptide (orexin-A₂₈₋₃₃) (fig. 3A and B).

To evaluate the role of individual amino acid residues in the C-terminal orexin-A peptide for the binding and activation of the orexin receptors, we performed an alanine-scan of the residues 14-26 in the orexin-A₁₄₋₃₃ (table 1). Similar areas/residues of importance in orexin-A were observed both for OX_1 and OX_2 receptors. Replacements of Arg15, Leu16 or Tyr17 with alanine caused a 2-fold reduction in the potency of the peptide (tables 4 and 5, fig. 4). OX_1 receptor may be less sensitive to the mutation of Tyr17 than OX_2 receptor. More dramatic

reduction in the potency was seen with mutation of Leu20 to alanine: The potency for the OX₁ receptor was reduced 25-fold and the potency for the OX₂ receptor 11-fold (tables 4 and 5, figs. 4 and 5A). Remarkably, neither the mutation of the Leu19 nor His21 markedly affected the potency of the peptide (tables 4 and 5, figs. 4 and 5B). Mutation of Asn25 and His26 to alanine caused a progressive reduction in the potency, which was more pronounced for the OX₁ receptor (tables 4 and 5, figs. 4 and 5C). Thus, the results with OX₁ and OX₂ receptors are largely similar except for differences with some mutations (Tyr17Ala, Leu20Ala, Asn25Ala, His26Ala).

Some of the peptides, as also orexin-A, display high slope factors (n_{Hill}) (tables 4 and 5, fig. 5B). We have previously shown that Ca⁺⁺ influx can amplify the phospholipase C response at low concentrations of orexin-A in CHO-OX₁ cells (Lund *et al.*, 2000). Therefore, different slope factors could indicate different degree of synergism between the two signals to phospholipase C and thus a differential ability to activate Ca⁺⁺ influx and Ca⁺⁺ release responses. We therefore tested the ability of chosen peptides to elevate intracellular Ca⁺⁺ in low (140 nM) extracellular [Ca⁺⁺]. The potency of orexin-A was shifted 10-14-fold to the right and the potency of orexin-B somewhat less (6-fold) by the reduction of extracellular [Ca⁺⁺] (fig. 6). Orexin-A₁₄₋₃₃ was affected in a similar degree as orexin-A (11-fold shift; fig. 6). In contrast, orexin-A₁₄₋₃₃R15A was much less affected (4-5-fold shift), even less than orexin-B. On the other hand, the reduction in potency for orexin-A₁₄₋₃₃L16A, -Y17A and -H21A was much larger (18-67 fold), especially in the case of the OX₁ receptor. However, the difference in the shift of the EC₅₀ did not correlate with the slope factor. Note that the determination of the EC₅₀ for orexin-A₁₄₋₃₃L20A in the low extracellular Ca⁺⁺ was impossible due to its low potency.

We wanted to further confirm that the reduced potency of the mutated/truncated orexin peptides was due to reduced affinity for the orexin receptor. There is no pharmacologically

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satisfactory binding assay for orexin receptors; at the moment, orexin receptor binding has to be conducted using using radio- or fluorescently labeled orexin-A (an agonist) and intact cells (Kane *et al.*, 2000; Darker *et al.*, 2001; Holmqvist *et al.*, 2001; Smart *et al.*, 2001). Therefore, an alternative qualitative approach was used. For this the cells were exposed to a slightly lower concentration of the peptides than required to give a minimum detectable Ca^{++} response. After a 2-minute preincubation, orexin-A or orexin-A₁₄₋₃₃ at different concentrations was added. Neither orexin-A nor any of the peptides inhibited subsequent response to orexin-A or orexin-A₁₄₋₃₃ confirming that the reduced potency of the peptides was indeed due to reduced affinity. It should be noted that this approach cannot give an assumption of how much the affinity is reduced, only that the binding affinity is not higher than the EC_{50} .

Discussion

In the present study, we have investigated the differences between OX₁ and OX₂ receptors with respect to the specificity of activation by modified orexin peptides. The results obtained in this study indicate that the both OX₁ and OX₂ receptors require very similar determinants from orexin-A to allow binding to and activation of the receptor, which indicates that analogous domains of OX₁ and OX₂ receptors interact with orexin-A. However, some interesting differences are seen, the most obvious of which being the less strict requirements of the OX₂ receptor for ligand binding.

As also previously shown, orexin-B had potency equal to orexin-A for the OX₂ receptor, but 10-100-fold lower relative potency for the OX₁ receptor. Thus, the OX₂ receptor cannot distinguish between orexin-A and -B as the OX₁ receptor does. The OX₁ receptor was much more affected by the N-terminal truncation of orexin-A than the OX₂ receptor. The first truncation of orexin-A to orexin-A₁₄₋₃₃, which removes the sulfhydryl bridges, essentially abolished the difference between OX₁ and OX₂ receptors with respect to orexin-A. Since orexin-B is N-terminally more or less truncated as compared to orexin-A, these data from truncation of orexin-A may in part explain why orexin-B is as potent as orexin-A on the OX₂ receptor. However, domains interacting with the most N-terminal portion of orexin-A are not the only difference between the two receptors as the OX₂ receptor also resisted further truncation far better than the OX₁ receptor. Alanine-scan identified residues of particular importance for orexin receptor activation. These residues were the same with respect to the activation of both OX₁ and OX₂ receptors, but the potency for the OX₂ receptor was less markedly affected. This further supports the notion of less strict requirements for binding and activation of the OX₂ receptor.

Human OX₁ and OX₂ display 64% overall sequence identity (Sakurai *et al.*, 1998). An even higher degree of identity and similarity are found between the proximal N-termini and the extracellular loop 1 of the receptors. This might suggest that these parts of the receptors are important for the orexin binding, as is also suggested by the almost complete loss of orexin binding caused by the Glu54Lys (position homologous to Glu54 in human OX₂ receptor) mutation in the canine OX₂ receptor (Hungs *et al.*, 2001). No obvious differences, which could explain the different behavior of OX₁ and OX₂ receptor, are seen.

All the peptides displayed slope factors above one and in addition many of the truncated and alanine-scanned peptides, though not orexin-A itself, displayed slope factors (n_{Hill}) above 2.5. This may suggest cooperative binding, which in the case of G-protein-coupled receptors would indicate formation/presence of receptor di-/oligomers. However, this has not been seen in binding studies (Sakurai *et al.*, 1998)(Holmqvist *et al.*, unpublished), but the unsatisfactory conditions for the binding assay (see **Results**) may mask this. Apparent cooperativity could also originate from the functional level. We have previously reported that orexin-A activates Ca⁺⁺ influx as a primary response mechanism. Ca⁺⁺ influx and an unknown signal from the receptor act synergistically to activate phospholipase C, since no phospholipase C activation is seen with Ca⁺⁺ influx alone, and in the absence of Ca⁺⁺ influx phospholipase C is activated only at 100 times higher concentration of orexin-A (Lund *et al.*, 2000; Kukkonen and Åkerman, 2001). We hypothesized that the reduced ability of some of the peptides to activate one of the Ca⁺⁺ responses, influx or release, could explain the high slope factors. Our experiments indeed confirmed that there are clear differences in the relative potencies between different mutant peptides towards these responses. The Ca⁺⁺ responses mediated by the OX₂ receptor were for most of the peptides less affected by removal of extracellular Ca⁺⁺ than the responses via OX₁

receptor. However, these differences do not explain the apparent cooperativity, and other explanations may be required.

It has been shown before, that N-terminal truncation of orexin-A to orexin-A₁₅₋₃₃ (19 amino acids), which completely eliminates all cysteines, reduces the potency for the OX₁ receptor 60-170-fold (Darker *et al.*, 2001; Okumura *et al.*, 2001) but the reduction is only 20-fold for the OX₂ receptor (Okumura *et al.*, 2001). In the present study, the potency of orexin-A was decreased only 7- respectively 4-fold for the OX₁ and OX₂ receptors upon truncation to orexin-A₁₄₋₃₃. Darker *et al.* (2001) have characterized the effect of further truncation of orexin-A on the activation of the OX₁ receptor: truncation of orexin-A to 19, 17, 16, 15, 10 and 5 amino acids progressively reduced the response to 10 μ M peptide, and 5 and 10 amino acid-long peptides were inactive. In contrast to this, we did not observe any significant reduction in the maximum response until the chain length was reduced to 12 or 11 amino acids. In the present study, the shortened chain instead decreased the potency (increased the EC₅₀). This apparent discrepancy could be caused by somewhat higher expression level in our CHO cells than those used in (Darker *et al.*, 2001). In our CHO cells the EC₅₀-value for orexin-A is more than 10-fold lower than the binding affinity for OX₁ receptors (Holmqvist *et al.*, unpublished) as compared to only a 6-fold difference in (Darker *et al.*, 2001). Darker *et al.* (2001) have also shown that truncation of orexin-A to orexin-A₁₅₋₃₃ abolishes this difference in the EC₅₀ and binding affinity; thus this truncation may decrease both the binding affinity and the efficacy of the peptide. Our semiquantitative estimation of the binding affinity suggests that further truncation may only reduce the binding affinity and not the efficacy. In other words, once bound, both orexin-A₁₄₋₃₃ and the shorter peptides may have similar ability to activate the receptor. The most C-terminal amino acids may be the most important for the activation of the receptor whereas the additional

N-terminal amino acids may increase the binding affinity and efficacy by making contact with the receptor and stabilizing orexin-A structure. The importance of the C-terminus is evident since this area is highly conserved between orexin-A and orexin-B (fig. 1). The present results support the view that for this kind of studies, high receptor expression levels are useful.

Alanine-scan of residues 14-26 in orexin-A₁₄₋₃₃ identified three areas of interest, which were the same in both OX₁ and OX₂ receptors: amino acids 15-17, 20 and 25-26. Alanine-scan of orexin-A₁₅₋₃₃ was performed in (Darker *et al.*, 2001) with results similar to the results in the present study with respect to OX₁ receptor activation. This study also showed that the receptor response is extremely sensitive to mutations in the outermost C-terminus (amino acids 26-33), as can be expected from the highly similar C-termini of orexin-A and -B. In contrast to Darker *et al.* (2001), we failed to detect any decrease in the potency by mutation of residues 21-24. Neither we nor Darker *et al.* (2001) detected any mutation that would markedly increase the potency of the peptide. The most interesting mutation is the Leu20Ala, which causes an over 10-fold drop in the potency of the orexin-A₁₄₋₃₃. In contrast to the findings of Darker *et al.* (2001) we saw almost no effect of the mutation of the adjacent amino acids, Leu19 or His21. Mutation of a leucine to alanine does not cause any change in the charge of the peptide neither can the effect be explained by steric hindrance, which should instead be diminished by the less bulky alanine. Therefore, the effect of this mutation is likely to affect the secondary or tertiary structure of orexin-A₁₄₋₃₃ rather than the interaction with the orexin-binding site. If orexin-A, as orexin-B (Lee *et al.*, 1999), forms an α -helical structure in this region then replacement of leucine with alanine should also not have any remarkable effect on the secondary structure. Since the 3D-structure of orexin-A is unknown, no conclusion of its structure and the effects of mutations thereon can be made. The alanine-scanned peptides activated the OX₂ receptor with

potencies very similar to those for the OX₁ receptor. However, the OX₂ receptor once again proved less "fussy" in its requirements on the orexin-A₁₅₋₃₃ peptide than the OX₁ receptor.

Orexin receptor subtypes are somewhat differentially expressed in the CNS and in the periphery, but the significance of this is unknown since no separate cellular functions have been ascribed to the receptor subtypes and the relative amounts of orexin-A versus orexin-B seem rather constant in the different CNS areas (Kukkonen *et al.*, 2002). Therefore, some of the major issues are whether orexin receptor subtypes can couple to different signaling pathways and whether different orexin peptides can cause "signal-trafficking" (Kenakin, 1995) even via a single receptor subtype. The data from the experiments with reduced extracellular [Ca⁺⁺] suggest that orexin peptides may have an inherent ability to do this. Also some *in vivo* and *in vitro* data suggest that orexin-B is more efficacious than orexin-A (see Kukkonen *et al.*, 2002), which is unexplainable based on the recombinant pharmacology. We propose that the physiological roles of orexins can only be elucidated in the light of much further data on the receptor subtype specificity in ligand binding and signaling pathway activation.

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Footnotes

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TABLE 1

Alanine-scanned C-terminal Orexin-A-peptides

The replaced amino acid is shown in bold underlined.

Position of replacement	Sequence
Orexin-A ₁₄₋₃₃	CRLYELLHGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ C14A	<u>A</u> RLYELLHGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ R15A	C <u>A</u> LYELLHGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ L16A	CR <u>A</u> YELLHGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ Y17A	CRL <u>A</u> ELLHGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ F18A	CRLY <u>A</u> LLHGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ L19A	CRLYE <u>A</u> LHGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ L20A	CRLYEL <u>A</u> HGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ H21A	CRLYELL <u>A</u> AGAGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ G22A	CRLYELLH <u>A</u> AGNHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ G24A	CRLYELLHGA <u>A</u> NHAAGILTTL-amide
Orexin-A ₁₄₋₃₃ N25A	CRLYELLHGAG <u>A</u> HAAGILTTL-amide
Orexin-A ₁₄₋₃₃ H26A	CRLYELLHGAGN <u>A</u> AAGILTTL-amide

TABLE 2

Ca⁺⁺ responses of OX₁ and OX₂ receptors to orexin-A and orexin-B in normal and low extracellular Ca⁺⁺

N = 8.

		1 mM [Ca ⁺⁺] _e			140 nM [Ca ⁺⁺] _e		
		Max (nM)	pEC ₅₀	n _{Hill}	Max (nM)	pEC ₅₀ (M)	n _{Hill}
OX ₁	Orexin-A	1257 ± 99	8.43 ± 0.05	1.7	1346 ± 141	7.28 ± 0.08	2.5
	Orexin-B	1245 ± 132	7.59 ± 0.11	1.9	1212 ± 134	6.78 ± 0.09	2.2
OX ₂	Orexin-A	999 ± 193	8.17 ± 0.07	1.4	1080 ± 291	7.16 ± 0.07	2.1
	Orexin-B	767 ± 201	8.16 ± 0.10	1.8	835 ± 194	7.37 ± 0.06	3.6

TABLE 3

Normalized Ca^{++} responses of OX_1 and OX_2 receptors to orexin-A and orexin-B in normal and low extracellular Ca^{++}

The data from the same experiments as in table 3 were normalized to the response to orexin-A in 1 mM $[\text{Ca}^{++}]_e$ in each batch of cells.

		1 mM $[\text{Ca}^{++}]_e$		140 nM $[\text{Ca}^{++}]_e$	
		Max ($\times$ ctrl)	EC_{50} ($\times$ ctrl)	Max ($\times$ ctrl)	EC_{50} ($\times$ ctrl)
OX_1	Orexin-A	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	14 ± 3^a
	Orexin-B	0.90 ± 0.04	6.9 ± 1.8^a	0.87 ± 0.06^c	46 ± 9^{be}
OX_2	Orexin-A	1.0 ± 0.1	1.0 ± 0.2	1.1 ± 0.1^a	10 ± 2^a
	Orexin-B	0.76 ± 0.03^a	1.0 ± 0.2	0.80 ± 0.07^d	6.3 ± 0.9^{bc}

^a Significantly different ($p < 0.001$) as compared to orexin-A in 1 mM $[\text{Ca}^{++}]_e$

^b Significantly different ($p < 0.001$) as compared to orexin-B in 1 mM $[\text{Ca}^{++}]_e$

^c Significantly different ($p < 0.05$) as compared to orexin-A in 140 nM $[\text{Ca}^{++}]_e$

^d Significantly different ($p < 0.01$) as compared to orexin-A in 140 nM $[\text{Ca}^{++}]_e$

^e Significantly different ($p < 0.001$) as compared to orexin-A in 140 nM $[\text{Ca}^{++}]_e$

All the other reasonable comparisons yielded non-significant.

TABLE 4

Ca⁺⁺ responses of CHO-hOX₁ to Orexin-A and -B and alanine-scanned orexin-A C-terminal peptides in 1 mM [Ca⁺⁺]_e

N = 5-11.

	Max (% of Orexin-A response)	pEC ₅₀ (M)	n _{Hill}
Orexin-A	100 ± 1	8.43 ± 0.05	1.9
Orexin-A ₁₄₋₃₃	95 ± 4	7.57 ± 0.02	3.1
Orexin-A ₁₄₋₃₃ C14A	95 ± 4	7.42 ± 0.03	2.1
Orexin-A ₁₄₋₃₃ R15A	95 ± 3	7.15 ± 0.03	2.7
Orexin-A ₁₄₋₃₃ L16A	97 ± 6	7.29 ± 0.02	2.5
Orexin-A ₁₄₋₃₃ Y17A	94 ± 4	7.29 ± 0.03	2.7
Orexin-A ₁₄₋₃₃ E18A	97 ± 5	7.51 ± 0.03	2.8
Orexin-A ₁₄₋₃₃ L19A	98 ± 2	7.24 ± 0.03	1.9
Orexin-A ₁₄₋₃₃ L20A	93 ± 3	6.03 ± 0.04	1.2
Orexin-A ₁₄₋₃₃ H21A	97 ± 3	7.40 ± 0.03	2.7
Orexin-A ₁₄₋₃₃ G22A	94 ± 4	7.35 ± 0.03	2.6
Orexin-A ₁₄₋₃₃ G24A	97 ± 5	7.47 ± 0.02	2.6
Orexin-A ₁₄₋₃₃ N25A	100 ± 5	7.04 ± 0.05	1.8
Orexin-A ₁₄₋₃₃ H26A	95 ± 4	6.77 ± 0.05	1.3

TABLE 5

Ca⁺⁺ responses of CHO-hOX₂ to Orexin-A and -B and alanine-scanned orexin-A C-terminal peptides in 1 mM [Ca⁺⁺]_e

N =8-15.

	Max (% of Orexin-A response)	pEC ₅₀ (M)	n _{Hill}
Orexin-A	100 ± 1	8.17 ± 0.07	1.6
Orexin-A ₁₄₋₃₃	103 ± 5	7.57 ± 0.03	3.7
Orexin-A ₁₄₋₃₃ C14A	99 ± 4	7.43 ± 0.03	3.2
Orexin-A ₁₄₋₃₃ R15A	95 ± 4	7.07 ± 0.03	1.6
Orexin-A ₁₄₋₃₃ L16A	95 ± 4	7.32 ± 0.03	1.5
Orexin-A ₁₄₋₃₃ Y17A	106 ± 3	7.09 ± 0.04	2.1
Orexin-A ₁₄₋₃₃ E18A	98 ± 1	7.55 ± 0.06	3.1
Orexin-A ₁₄₋₃₃ L19A	98 ± 5	7.40 ± 0.03	3.0
Orexin-A ₁₄₋₃₃ L20A	99 ± 0.4	6.38 ± 0.03	1.6
Orexin-A ₁₄₋₃₃ H21A	97 ± 2	7.44 ± 0.02	2.8
Orexin-A ₁₄₋₃₃ G22A	100 ± 3	7.33 ± 0.02	2.9
Orexin-A ₁₄₋₃₃ G24A	95 ± 4	7.54 ± 0.02	3.5
Orexin-A ₁₄₋₃₃ N25A	96 ± 5	7.31 ± 0.02	3.4
Orexin-A ₁₄₋₃₃ H26A	104 ± 2	7.20 ± 0.02	2.1

Legends for figures

Fig. 1. Orexin-A and -B peptide sequences. The gray boxes mark the identical amino acids. The numbering of the amino acids is for orexin-A. Q* indicates the N-terminal pyroglutamoyl residue.

Fig. 2. Concentration-response curves for orexin-A and -B on OX₁ (A) and OX₂ (B) receptors in normal (1 mM) and low (140 nM) [Ca⁺⁺]_e. *N* = 8. The fitted maximum response of each curve is normalized to the maximum response to orexin-A in 1 mM [Ca⁺⁺]_e.

Fig. 3. Ca⁺⁺ responses to different concentrations of truncated orexin-A peptides in 1 mM [Ca⁺⁺]_e. Please observe the logarithmic ordinate. *N* = 3.

Fig. 4. Graphic representation of the pEC₅₀ values determined for alanine-scanned orexin-A₁₄₋₃₃ peptides in 1 mM [Ca⁺⁺]_e. The data are the same as presented in Tables 4 and 5.

Fig. 5. Concentration-response curves for three different mutated orexin-A₁₄₋₃₃ peptides in 1 mM [Ca⁺⁺]_e. In A, Leu20 to Ala mutation shifts the concentration response curve of orexin-A₁₄₋₃₃ 15-35-fold to the right. In, B His21Ala mutation results in peptides with apparent highly cooperative response (steep concentration-response curves). Leu20Ala (A) and His26Ala (C) mutations show that the OX₁ receptor is more sensitive to the orexin-A mutations than the OX₂ receptor. *N* = 9.

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Fig. 6. Comparison of the pEC₅₀ values of selected orexin-A₁₄₋₃₃ peptides in normal (1 mM) and low (140 nM) [Ca⁺⁺]_e. Please observe that the pEC₅₀ of Leu20Ala peptide could not be determined in 140 nM [Ca²⁺]_e because of its low potency.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6