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RESEARCH PAPER

Calcium affects OX_1 orexin (hypocretin) receptor responses by modifying both orexin binding and the signal transduction machinery

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BACKGROUND AND PURPOSE

One of the major responses upon orexin receptor activation is Ca^{2+} influx, and this influx seems to amplify the other responses mediated by orexin receptors. However, the reduction in Ca^{2+}, often used to assess the importance of Ca^{2+} influx, might affect other properties, like ligand–receptor interactions, as suggested for some GPCR systems. Hence, we investigated the role of the ligand–receptor interaction and Ca^{2+} signal cascades in the apparent Ca^{2+} requirement of orexin-A signalling.

EXPERIMENTAL APPROACH

Receptor binding was assessed in CHO cells expressing human OX_1 receptors with [^{125}I]-orexin-A by conventional ligand binding as well as scintillation proximity assays. PLC activity was determined by chromatography.

KEY RESULTS

Both orexin receptor binding and PLC activation were strongly dependent on the extracellular Ca^{2+} concentration. The relationship between Ca^{2+} concentration and receptor binding was the same as that for PLC activation. However, when Ca^{2+} entry was reduced by depolarizing the cells or by inhibiting the receptor-operated Ca^{2+} channels, orexin-A-stimulated PLC activity was much more strongly inhibited than orexin-A binding.

CONCLUSIONS AND IMPLICATIONS

Ca^{2+} plays a dual role in orexin signalling by being a prerequisite for both ligand–receptor interaction and amplifying orexin signals via Ca^{2+} influx. Some previous results obtained utilizing Ca^{2+} chelators have to be re-evaluated based on the results of the current study. From a drug discovery perspective, further experiments need to identify the target for Ca^{2+} in orexin-A–OX_1 receptor interaction and its mechanism of action.

Abbreviations

extracellular Ca^{2+}; $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration; CD (spectroscopy), circular dichroism (spectroscopy); Dyngo 4a, 3-hydroxy-*N*′-([2,4,5-trihydroxyphenyl]methylidene)naphthalene-2-carbohydrazide; IP_3, inositol-1,4,5-trisphosphate; KBM, K^+-based medium; NaBM, Na^+-based medium; SB-334867, 1-(2-methylbenzoxazol-6-yl)-3-(1,5)naphthyridin-4-yl-urea HCl; SKF-96365, (1-[β-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl]-1H-imidazole HCl); SPA, scintillation proximity assay; TCS 1102, *N*-biphenyl-2-yl-1-{[(1-methyl-1H-benzimidazol-2-yl)sulfanyl]acetyl}-l-prolinamide; TRPC channels, canonical transient receptor potential channels

Tables of Links

TARGETS		
GPCRs[b]	TRPC channels	PLA_2
Melanocortin receptor	Voltage-gated calcium channels	PLC
OX_1 receptor	**Enzymes**[d]	PLD
OX_2 receptor	Adenylyl cyclases	**Transporters**
Ion channels[c]	$cPLA_{2\alpha}$	Na^+/Ca^{2+} exchanger
IP_3 receptor	ERK	

LIGANDS
IP_3
Orexin-A
Orexin-B
SB-334867
SKF-96365

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([b,c,d]Alexander *et al.*, 2013b,c,d).

Introduction

Orexin (aka hypocretins; the orexin nomenclature used complies with that of the The Concise Guide to PHARMACOLOGY and IUPHAR guidelines; Alexander *et al.*, 2013a) receptor signalling is multifaceted (reviewed in Kukkonen, 2013; Kukkonen & Leonard, 2014; Leonard & Kukkonen, 2014). Nevertheless, some responses seem to be particularly prevalent; one such response is its ability to elevate Ca^{2+}. This has been seen in many recombinant systems and has also been measured in neurons (Sakurai *et al.*, 1998; Smart *et al.*, 1999; Lund *et al.*, 2000; Holmqvist *et al.*, 2005; Putula *et al.*, 2011; reviewed in Kukkonen & Leonard, 2014; Leonard & Kukkonen, 2014). The response was originally thought to rely on the canonical pathway of $G_q \rightarrow$ PLC $\rightarrow$ inositol-1,4,5-trisphosphate (IP_3) $\rightarrow$ Ca^{2+} release. However, an unexpected finding was that this response required extracellular Ca^{2+} (Ca^{2+}_e); under reduced extracellular [Ca^{2+}] ([Ca^{2+}]$_e$) (140 nM or 1–3 μM) conditions, the potency of orexin-A or -B to increase Ca^{2+} was 6–100-fold lower than under 'normal' [Ca^{2+}]$_e$ (1 mM), as seen in recombinant human OX_1 receptor-expressing CHO cells and other cell lines (Smart *et al.*, 1999; Lund *et al.*, 2000; Holmqvist *et al.*, 2002; Ammoun *et al.*, 2003). Similar to these results, Ca^{2+} responses to orexin in neurons are sometimes inhibited by a reduction in [Ca^{2+}]$_e$ (van den Pol *et al.*, 1998; Uramura *et al.*, 2001; Kohlmeier *et al.*, 2004; Ishibashi *et al.*, 2005; Nakamura *et al.*, 2010). Further studies in CHO cells, involving selective visualization or inhibition of Ca^{2+} influx, provided evidence that this dependence is the result of changes in orexin receptor-operated Ca^{2+} influx. This influx was deemed to be the primary response to orexin receptor stimulation (Lund *et al.*, 2000; Kukkonen and Åkerman, 2001), which is different from that seen in the canonical PLC–Ca^{2+} release pathway, where Ca^{2+} influx is involved in the secondary part of the response only, that is, following Ca^{2+} store release (store-operated Ca^{2+} influx; reviewed in Konieczny *et al.*, 2012). This concept was evaluated in further studies utilizing mainly CHO cells, but also in other recombinant cell models, which showed that this influx is also pharmacologically distinct from the store-operated Ca^{2+} influx, and that it does not require IP_3 (Kukkonen and Åkerman, 2001; Ekholm *et al.*, 2007; Johansson *et al.*, 2007; Turunen *et al.*, 2012; reviewed in Kukkonen, 2013; Kukkonen & Leonard, 2014; Leonard & Kukkonen, 2014). At the same time, non-selective cation channels were identified as one of the major mediators of orexin-induced depolarization in native CNS neurons (see, e.g. Brown *et al.*, 2002; Burlet *et al.*, 2002; Liu *et al.*, 2002; Yang and Ferguson, 2002; van den Pol *et al.*, 2002; reviewed in Kukkonen, 2013; Kukkonen & Leonard, 2014; Leonard & Kukkonen, 2014). A non-selective cation current was also isolated in OX_1-expressing CHO cells (Larsson *et al.*, 2005). Hence, it was hypothesized that the orexin receptor-induced Ca^{2+} influx is mainly mediated by non-selective cation channels. The identity of the channels as well as their activation mechanism is unclear, despite some suggestions of lipid signalling and the involvement of canonical transient receptor potential (TRPC) channels (Yang *et al.*, 2003; Larsson *et al.*, 2005; Näsman *et al.*, 2006; Kohlmeier *et al.*, 2008; Louhivuori *et al.*, 2010; Turunen *et al.*, 2012). However, it should be noted that the basis of the Ca^{2+} elevation in native neurons is usually not specified, and even in the case of Ca^{2+} influx, the mechanism may involve voltage-gated Ca^{2+} channels, a reverse-acting Na^+/Ca^{2+} exchanger, store-operated Ca^{2+} channels or non-selective cation channels (reviewed in Kukkonen & Leonard, 2014; Leonard & Kukkonen, 2014).

A reduction in Ca^{2+}_e, inhibitors of Ca^{2+} channels and a reduction in the driving force for Ca^{2+} entry have all been used to inhibit Ca^{2+} influx in response to orexin receptor activation. In more detailed studies, mainly done in CHO cells, it was found that many other OX_1 receptor-mediated responses are also inhibited by these treatments. Such responses include stimulation of adenylyl cyclases, PLA_2, PLC, PLD and ERK (Lund *et al.*, 2000; Ammoun *et al.*, 2006; Johansson *et al.*, 2007; Jäntti *et al.*, 2012; Turunen *et al.*, 2012). The inhibition is either observed as a significant shift in the orexin concentration–response curve or as an abolition of the response. In contrast, inhibition of Ca^{2+} release does not similarly affect the responses (as tested for ERK and PLD) (Ammoun *et al.*, 2006; Ekholm *et al.*, 2007; Jäntti *et al.*, 2012).

It has thus been suggested that an elevation in intracellular Ca^{2+} somehow enhances/facilitates orexin receptor signalling towards many targets by either a proximal (e.g. receptor–G-protein interaction) or distal (e.g. signal cascade components like PLC) effect (Ammoun *et al.*, 2006).

One major obstacle for obtaining conclusive evidence has been that no inhibitor of Ca^{2+} channels or signal pathways tested is able to completely inhibit the apparent orexin receptor-operated Ca^{2+} influx response, and therefore the studies have often resorted to investigating the effects induced by a reduction in $[Ca^{2}]_e$. However, the effect of reducing the $[Ca^{2}]_e$ on the orexin response is sometimes stronger than that induced by inhibition of Ca^{2+} influx by other means, as indicated, for example, in measurements of PLC activity (Johansson *et al.*, 2007). Although this may be explained by the fact that most of the means available for inhibiting this Ca^{2+} influx are less potent and thus do not produce full inhibition of the flux. A reduction in Ca^{2+} outside the cell may also affect the intracellular Ca^{2+} concentration; all PLCs and the PLA_2 involved here ($cPLA_2\alpha$) require some Ca^{2+} for activity (Kukkonen, 2014). Another explanation is that Ca^{2+}_e is also required for orexin binding, as shown for instance for melanocortin receptors (Salomon, 1990; Kopanchuk *et al.*, 2005). Therewith, a reduction in $[Ca^{2}]_e$ could produce multiple effects by affecting receptor binding and different levels of the signal cascade. The evidence currently available clearly cannot provide a definitive answer to this question. Hence, in the present study we assessed the effect of Ca^{2+} on orexin-A binding as well as on PLC activity. The results showed that Ca^{2+} indeed affects orexin-A binding from the extracellular side, but also that Ca^{2+} influx is required for a normal orexin response.

Methods

Cell culture

CHO-hOX_1 cells, expressing human OX_1 receptors (Lund *et al.*, 2000), were cultured in Ham's F12 medium (Gibco/Life Technologies, Paisley, UK) containing supplements on plastic cell culture dishes (56 cm^2 bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany) as described in Jäntti *et al.* (2012). For the binding and the PLC assay, the cells were plated on 48- or 96-well Cellstar cell culture plates (Greiner Bio-One), white, clear-bottom ViewPlate-96 cell culture plates (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) or, for the scintillation proximity assay (SPA), on Cytostar-T 96-well scintillation plates (PerkinElmer). All plates were coated with polyethyleneimine (25 $\mu g \cdot mL^{-1}$ for 1 h at 37°C; Sigma-Aldrich, St. Louis, MO, USA).

Media

Na^+-based medium (NaBM) was used as the basic experimental medium. It was composed of 137 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose and 20 mM HEPES and adjusted to pH 7.4 with NaOH. As determined utilizing Calcium Green-5N K^+ salt (Molecular Probes/Life Technologies, Paisley, UK), it had an 'ambient' $[Ca^{2+}]$ of 5–6 μM, originating mainly from the impurities in its constituent salts. The final $[Ca^{2+}]$ of 0.1, 1, 10, 100 μM and 1 mM were obtained by adding $CaCl_2$ and/or EGTA (Sigma-Aldrich) solution to the NaBM. The computer programme Bound and Determined (Brooks and Storey, 1992) was used for calculations, and all the buffers were tested with the help of Calcium Green-5N K^+-salt and Fura-2 K^+-salt (Molecular Probes) and adjusted with $CaCl_2$ and EGTA solutions; the probes were used at a concentration of 30 nM so as not to buffer $[Ca^{2+}]$. A Hitachi F-4000 fluorescence spectrophotometer (Hitachi, Ltd., Chiyoda, Tokyo, Japan) was used for fluorescence measurements. For some experiments, a mixture of the NaBM and K^+-based medium (KBM) was used. The KBM was identical to the NaBM except that all Na^+ salts were replaced with corresponding K^+ salts. For experimental use, a mixture of these two media, called High-K^+-NaBM, was prepared by mixing NaBM and KBM in the ratio 0.14:0.86, yielding a final $[Na^+] \approx 20$ mM and $[K^+] \approx 127$ mM, and 1 mM $CaCl_2$ was added; 20 mM Na^+ was retained in this medium so as not to affect any possible Na^+-dependent binding etc. In the TEA-NaBM, 68.5 mM of NaCl in the NaBM was replaced with TEA and 1 mM $CaCl_2$ was added. Ten micromolar 1-[β-(3-(4-methoxyphenyl) propoxy)-4-methoxyphenethyl]-1H-imidazole HCl (SKF-96365) was used in NaBM (including 1 mM $CaCl_2$).

When used in the experiments, all these media were additionally supplemented with 0.1% (w v^{-1}) stripped BSA (Turunen *et al.*, 2012). This was originally developed to reduce orexin binding to plastic surfaces for receptor binding experiments, but was included in all assays to allow direct comparison with receptor binding.

[^{125}I]-orexin-A binding

Binding experiments were performed using both the 'conventional' binding method as well as the scintillation proximity assay (SPA). For both methods, cells were cultured on multiwell plates. The conventional method was based on the counting of the cell-bound radioactivity in a γ-counter or scintillation counter after addition of scintillation cocktail, while for SPA, Cytostar-T SPA plates (PerkinElmer) already containing the scintillant were used. Wallac Wizard 1480 Gamma Counter and Wallac 1450 Microbeta TriLux Scintillation Counter (PerkinElmer) were used for sample counting. It should be noted that although ^{125}I is not highly efficiently counted with Cytostar-T plates (http://www.perkinelmer.com/fi/Resources/TechnicalResources/ApplicationSupportKnowledgebase/radiometric/cytostar.xhtml), the signals were high enough and the replicate variation low.

SPA was initially tested in two different ways: (1) the classical SPA way, where the scintillation is measured directly from the wells without any separation, and (2) in a manner similar to conventional binding assay, where all incubation medium is carefully removed but the wells are not washed. For both alternatives, the binding conditions were otherwise equal to the corresponding conventional method. Non-specific binding was higher with method 1, probably due to some radiation from the nearby, non-bound radiolabel from the medium phase picked up by the scintillant, and the read-out noise was also higher due to the shorter time available for reading of each well (due to experiment timing). These properties lead to a reduced signal-to-noise ratio. In addition, timing was more difficult and overlapping ligand incubations were needed due to the counting time. Finally,

the incubations could not be performed at 37°C, as they essentially took place within the scintillation counter. In method 2, the incubation of each well could be started essentially simultaneously, the incubations could be performed at 37°C and the incubations could be ended at the 'correct' time point. The plates could then be measured in the scintillation counter at any time, as the reactions with respect to the method were essentially stopped – for the SPA signal it does not matter, after the removal of the medium, whether the ligand is bound, the cells are alive, etc. The experimental results under the equilibrium (90 min, 21°C) and non-equilibrium (10 min, 37°C) conditions were thus produced using method 2, whereas the association and dissociation kinetics were measured using method 1. Equilibrium, binding experiments as well as kinetic studies are run for a long time, during which receptor internalization takes place (Jäntti *et al.*, 2013; P. M. Turunen and J. P. Kukkonen, unpubl. data). Therefore, 3-hydroxy-*N*′-([2,4,5-trihydroxyphenyl] methylidene)naphthalene-2-carbohydrazide (Dyngo 4a) was included in these experiments to inhibit dynamin-dependent internalization. Dyngo 4a was not used in the non-equilibrium studies, as there is very little internalization at 10 min (P. M. Turunen and J. P. Kukkonen, unpubl. data).

Homologous competition assay under apparent equilibrium conditions. The CHO-hOX_1 cells, 2×10^4 per well, were seeded on ViewPlate-96 (conventional assay) or Cytostar-T (SPA) 96-well plates and cultured overnight. The medium was changed to the experimental medium (NaBM with different Ca^{2+} concentrations including 30 μM Dyngo 4a), and 10 min later, the cells were exposed to 0.08 nM [^{125}I]-orexin-A mixed with non-labelled orexin-A to yield final orexin-A concentrations 0.08 nM –1 μM. The non-specific binding was determined separately under all conditions by including 10 μM of the orexin receptor antagonists SB-334867 or TCS 1102; both blocked orexin-A binding to the same extent. Binding of orexin-A to plastic- and glassware was also assessed under all conditions to make sure this did not contribute to the differences seen in binding under different conditions. After a 90 min incubation at 21°C, where the reaction was well in (apparent) equilibrium (reaction kinetics tested using the SPA method), the medium was removed by water suction and the plates allowed to dry. SPA plates were directly counted in Microbeta, whereas for the regular plates, 40 μL of Ultima Gold scintillation cocktail (PerkinElmer) was added, and the plates were counted after an overnight incubation. The detectors were normalized and well cross-talk compensated according to the instrument protocols. The results were converted to actual binding by compensating for the reduction in specific activity upon dilution of the radiolabel with the 'cold' ligand. Please observe that orexin-A and ^{125}I-orexin-A were considered equal here.

Non-equilibrium binding. Binding was also assessed under non-equilibrium conditions (10 min) at 37°C to allow direct comparison with the functional assay (PLC activity). For the conventional assay, 5×10^4 CHO-hOX_1 cells per well were seeded on 48-well plates and cultured overnight. The medium was changed to the experimental medium (NaBM with different Ca^{2+} concentrations, High-K^+-NaBM or TEA-NaBM), and the cells were exposed to [^{125}I]-orexin-A mixed with non-labelled orexin-A to yield final orexin-A concentrations of 1 and 10 nM (mixture of 'hot' and 'cold' of 1:50 and 1:400 respectively). The non-specific binding to cells and lab-ware was assessed as above. After a 10 min incubation at 37°C, the media were removed by water suction and the wells were rapidly washed twice with the same (but ice-cold) media. The cells were lysed in 200 μL NaOH, the wells washed with water, and NaOH + the wash pooled for each well and counted in the γ-counter. The results were converted to actual binding by compensating for the reduction in specific activity as above. For SPA, 2×10^4 cells per well were seeded on Cytostar-T 96-well SPA plates and cultured overnight. The experiments were essentially performed as described above except that the wells were not washed after removal of the experimental media. The plates were counted in Microbeta.

Binding kinetics. Binding kinetics were exclusively investigated using the SPA method. The cells (2×10^4 per well) were seeded on Cytostar-T 96-well SPA plates and cultured overnight. For association studies, the medium was changed to the experimental medium (NaBM with different Ca^{2+} concentrations including 30 μM Dyngo 4a), and 10 min later, the cells were exposed to 0.08 nM [^{125}I]-orexin-A. The non-specific binding was determined separately under all conditions using 10 μM TCS 1102. The association was monitored for 60–80 min in Microbeta. Twelve or 24 wells were run at each time, which gave a reading for each well approximately every 4 or 8 min, respectively (1 min reading time, three detectors). For dissociation studies, 0.08 nM [^{125}I]-orexin-A was allowed to reach equilibrium binding, 0.2 vol of the medium (NaBM with the same additions + 10 μM TCS 1102 was added), and the dissociation was monitored for 30–40 min in Microbeta. Twelve wells were run at a time, which gave a reading for each well approximately every 4 min. The incubation time difference between the wells caused by pipetting and reading in Microbeta was taken into account when analysing the results. Only 1 mM and 100 μM Ca^{2+}_e were used, as the results with 10 μM Ca^{2+}_e were too noisy due to very low binding. Because of the experimental procedure, the time points were not the same in different wells or experiments, and in order to present an average graph (Supporting Information Fig. S1), the data were averaged within time ranges (every 5 min for association, every 3 min for dissociation). This procedure increased the noise in the data; the original data and not the averaged data were used for calculation of the rate constants.

PLC activity

PLC activity was measured basically as described earlier (Johansson *et al.*, 2007). The cells, 5×10^4 per well, were plated on 48-well plates. After 24 h, they were labelled with 3 mCi·mL^{-1} *myo*-[2-^{3}H]-inositol for 16 h. The cells were washed once with PBS and then incubated in the experimental medium (NaBM with different Ca^{2+} concentrations, High-K^+-NaBM, TEA-NaBM or NaBM + 1 mM Ca^{2+} + 10 μM SKF-96365) + 10 mM LiCl for 10 min at 37°C. They were then stimulated with orexin-A for 10 min, after which the medium was rapidly removed and the reactions stopped by adding ice-cold 0.4 M perchloric acid and freezing. The samples were thawed and neutralized with 50 μL of 0.36 M KOH + 0.3 M

$KHCO_3$, and the insoluble fragments spun down (1100× *g*, +4°C). The total inositol phosphate fraction of the supernatants was isolated by anion-exchange chromatography, and the radioactivity determined by scintillation counting (HiSafe 3 scintillation cocktail and Wallac 1414 liquid scintillation counter; PerkinElmer).

Circular dichroism (CD) spectroscopy

CD spectra were measured using a Jasco J-715 instrument (Jasco Inc., Mary's Court, Easton, MD, USA) in a 1 mm light path cuvette. Wavelength scans were obtained in the range 190–250 nm, averaging five scans measured at the scan speed of 50 nm·min^{-1}, in 1 mM MOPS–5 mM Na_2SO_4. Because the pK_a value of MOPS was decreased on increasing the temperature, two different buffers were used in the temperature scans, with pH adjusted to 7.4 at 40 and at 70°C, respectively, with NaOH. Orexin-A was used at concentrations of 50–80 μM. $[Ca^{2+}]$ in the buffer solution was <1 μM, that is, negligible as compared with orexin-A. For the experiments in the presence of Ca^{2+}, 1 mM $CaSO_4$ was added to the buffer solution. The temperature sensitivity of the secondary structure was analysed in 5–10 °C steps from 25 to 90°C. The temperature was allowed to stabilize for 5 min before each scan. The buffer and cuvette background was subtracted from the data before the analysis.

Drugs

Human orexin-A was from NeoMPS (Strasbourg, France), and SB-334867 and TCS 1102 from Tocris Bioscience (Bristol, UK). *Myo*-[2-^{3}H]-inositol (PT6-271) and [^{125}I]-orexin-A were from PerkinElmer, SKF-96365 from Calbiochem (La Jolla, CA, USA) and Dyngo 4a from Abcam (Cambridge, UK).

Data analysis

All the data are presented as mean ± SEM. Each experiment was performed at least in quadruplicate at three independent occasions. All data are independent values. All group sizes (*N*) represent individual experiments, not replicates (triplicates, etc.). All statistics were undertaken on single individual independent values; in other words, if one sample were run in triplicate, only one numerical value was used for statistical analysis and the generation of group means, and the replication used only to validate the precision of the individual value. Student's paired or non-paired two-tailed *t*-test (with Bonferroni correction for multiple comparisons, when needed) was used for all statistical comparisons. Significances are as follows: ns (not significant), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Microsoft Excel (Microsoft Co., Redmond, WA, USA) was used for all data analyses including non-linear curve fitting. Eq. 1 was used for analysis of concentration–response curves, Eq. 2 for equilibrium-binding curves (Supporting Information Fig. S3), and Eqs. 3 and 4 for association and dissociation curve analysis respectively.

$$\text{Response} = \frac{[\text{orexin-A}]^n \times \text{response}_{\max}}{[\text{orexin-A}]^n + \text{EC}_{50}{}^n} \quad (1)$$

$$\text{Binding} = \frac{[\text{orexin-A}] \times \text{binding}_{\max-1}}{[\text{orexin-A}] + K_{d-1}} + \frac{[\text{orexin-A}] \times \text{binding}_{\max-2}}{[\text{orexin-A}] + K_{d-2}} \quad (2)$$

$$\text{Binding}_t = \text{binding}_{\max} \times (1 - e^{-kt}) \quad (3)$$

$$\text{Binding}_t = \text{binding}_0 \times e^{-kt} \quad (4)$$

Results

Ca^{2+}_e is required for orexin-A binding to OX_1 receptors

We first examined the effect of Ca^{2+}_e on orexin-A binding utilizing ^{125}I-orexin-A. No significant binding could be measured in cell homogenates or membrane preparations of CHO cells (not shown); the disruption of the cellular milieu may have caused this, and the rather high binding of orexin-A to plastic-ware easily masks more subtle specific binding (see, e.g. Jäntti *et al.*, 2013; Kukkonen, 2013). We thus conducted some binding utilizing intact CHO cells and the homologous competition assay during apparent equilibrium conditions (90 min at 21°C; Figure 1). Dyngo 4a was included to inhibit dynamin-dependent internalization, but it should be noted that, in intact cells, there may still be other processes affecting the binding over this long time, such as dynamin-independent internalization and receptor phosphorylation. Reducing the $[Ca^{2+}]_e$ from the physiological concentration of 1 mM to 100 μM and further to 10 μM reduced orexin-A binding over its entire concentration range (Figure 1). Please observe that at the upper end of the binding curve (300 nM orexin-A), the absolute binding level is uncertain, as the actual specific counts are rather low and thus the noise high.

Orexin causes rapid receptor responses, and we thus continued with the binding experiments under conditions that are most relevant for the response generation, that is, 10 min binding time, 37°C, orexin concentrations of 1 and 10 nM. Both orexin-A concentrations showed a robust binding in NaBM in the presence of 1 mM Ca^{2+}_e (Figure 2A). Increasing $[Ca^{2+}]_e$ to the supraphysiological concentrations 3 and 10 mM did not increase the orexin-A binding (Figure 2). Reducing the $[Ca^{2+}]_e$ caused a successive reduction in orexin binding (Figure 2). The half-maximal binding was obtained at 145 and 69 μM Ca^{2+}_e for 1 and 10 nM orexin-A (difference significant, $P < 0.05$) respectively (Figure 2B). These results indicated that Ca^{2+}_e is required for orexin binding to the OX_1 receptor. However, in this assay the cells were washed after the ligand had been removed in order to reduce non-specific counts, and the wash steps may induce ligand dissociation from the receptors (if its off-rate is high enough), despite precautions like rapid washes and cold wash buffer (like here). Thus, the results of the 10 min binding experiments may, alternatively, be explained by that Ca^{2+} renders the receptor ligand complex less readily dissociating. The latter alternative was scrutinized using SPA. On the SPA plates, the scintillant has been mixed with the bottom plastic where the cells are cultured. With SPA technology a scintillation signal is obtained only when the radiating isotope comes within the close vicinity of the scintillant (e.g. binding of the ligand to the receptors on the cell monolayer). Thus, the method does not, in principle, need separation of bound and free radioligand (Cooper, 2004). Similar to the conventional binding assay (Figure 2), SPA showed successive a reduction in

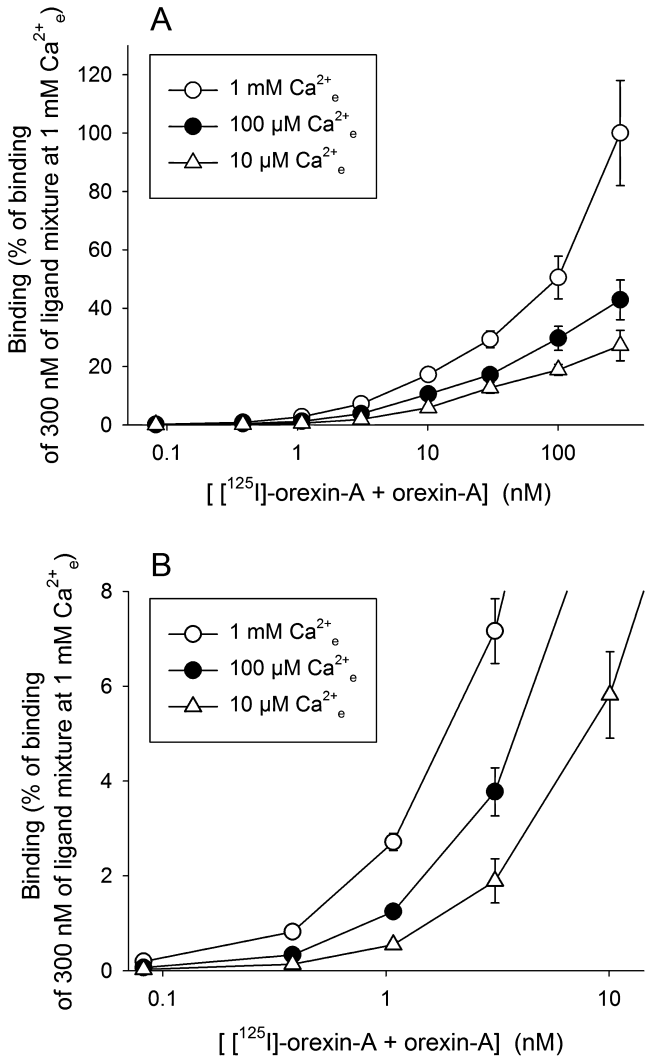

Figure 1

Ca^{2+} sensitivity of orexin-A binding to OX_1 receptors in the apparent equilibrium-binding assay. The results were obtained by use of the homologous competition assay (21°C, 90 min). The noise is high in the upper end of the concentration range of the competitor due to low actual counts, as happens in homologous competition assays, and thus the upper end of the scale (300 nM) is uncertain; results for 1000 nM were omitted due to this. (A) Full binding curves. (B) Magnification of the lower concentration range. N = 5–8. The difference between the results at 1 mM, 100 μM and 10 μM Ca^{2+}_e is significant, $P < 0.001$, for each orexin-A concentration except for 300 nM, where the difference between 100 and 10 μM Ca^{2+}_e is significant at $P < 0.01$.

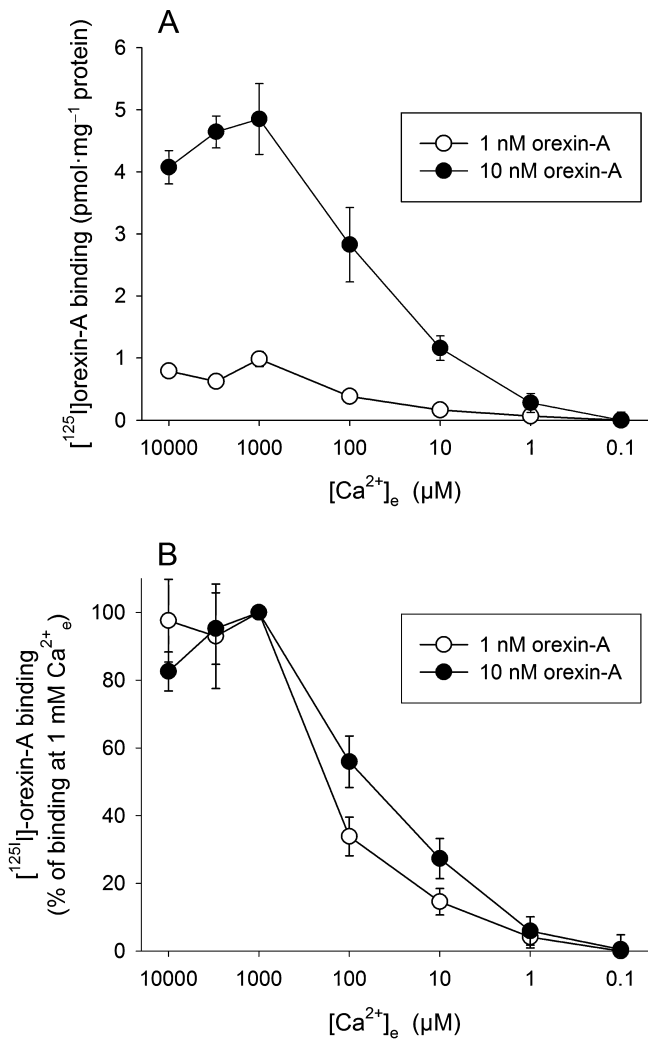

Figure 2

Ca^{2+} sensitivity of orexin-A binding to OX_1 receptors in the conventional binding assay under non-equilibrium conditions (37°C, 10 min). (A) Binding as pmol·mg^{-1} protein; (B) binding normalized to the binding at 1 mM Ca^{2+}_e. The normalized binding curves for 1 and 10 nM are significantly different ($P < 0.05$); N = 5.

orexin-A binding upon reduction of $[Ca^{2+}]_e$ (Figure 3). The same was also observed under the apparent equilibrium conditions (90 min, 21°C; not shown). We also directly monitored association and dissociation kinetic of ^{125}I-orexin-A. To keep the association slow enough for quantitation, a low concentration of ^{125}I-orexin-A, 0.08 nM, was used. The results indicate presence of initial association and dissociation phases, which are too fast to capture with this methodology, while the rest of the process could be fitted to a single exponential [(pseudo-) first-order reaction; Supporting Information Fig. S1]. By this analysis, the association rate constants (k_{+1}) were $4.19 \pm 0.56 \times 10^8$ and $4.80 \pm 1.03 \times 10^8$ M^{-1}·min^{-1} for 1 mM and 100 μM Ca^{2+}_e, respectively (N = 5), and the dissociation rate constants (k_{-1}) $2.57 \pm 0.002 \times 10^{-2}$ and $2.48 \pm 0.04 \times 10^{-2}$ min^{-1} for 1 mM and 100 μM Ca^{2+}_e respectively (N = 8). These correspond to differences of 13.6 ± 1.0 and 14.8 ± 1.4% (paired data; $P < 0.01$ for both). The dissociation could be more accurately modelled with dissociation from two populations, but there are too few data points to reliably suggest this, and therefore this is not shown.

Extracellular Ca^{2+} concentration affects OX_1 receptor responses through its effect on both orexin binding and Ca^{2+} influx

We previously investigated the importance of Ca^{2+}_e and Ca^{2+} influx on PLC activity induced by orexin-A in CHO-OX_1

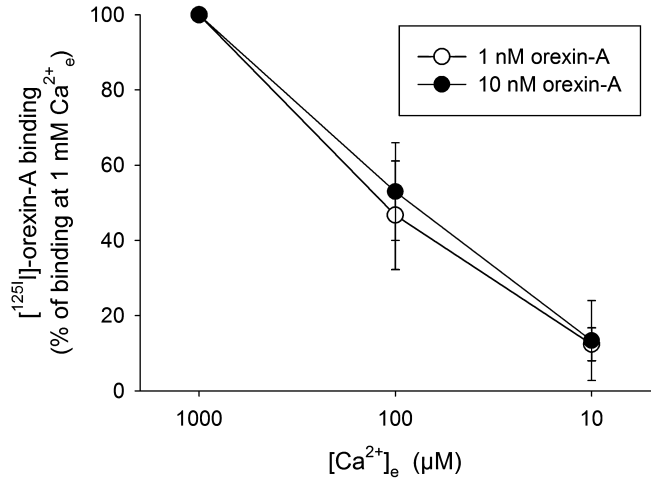

Figure 3

Ca^{2+} sensitivity of orexin-A binding to OX_1 receptors in SPA under non-equilibrium conditions (37°C, 10 min). The results are normalized, as in Figure 2B, to the binding at 1 mM Ca^{2+}_e; $N = 3$.

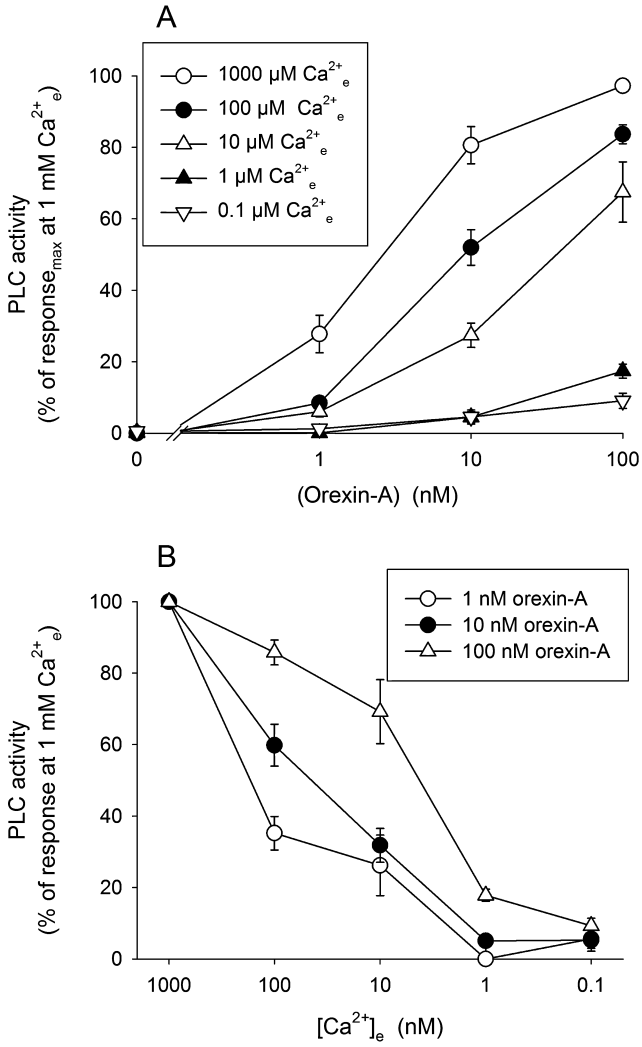

Figure 4

Ca^{2+} sensitivity of orexin-A stimulation of PLC. The experiments were performed at 37°C with 10 min stimulation. (A) Orexin-A concentration–response curves at different $[Ca^{2+}]_e$. (B) The same data as in (A) as Ca^{2+}_e concentration–response curves for different orexin-A concentrations. The responses in (A) were normalized to the basal and the maximum response to orexin-A in 1 mM Ca^{2+}_e (0 and 100%, respectively; obtained by curve fitting), and in (B) to the orexin-A-mediated response in 1 mM Ca^{2+}_e at each orexin concentration (100%), as in Figure 2B. $N = 5$.

cells (Johansson *et al.*, 2007). In the present study, we decided to re-examine this. However, we fixed the conditions for the PLC assay so that they were exactly the same as for the binding assay to make the results of these two assays directly comparable. Orexin-A robustly stimulated PLC in NaBM + 1 mM Ca^{2+}_e, to 10.1 ± 0.8-fold the basal ($N = 10$). PLC activity was seen to be highly sensitive to a reduction in $[Ca^{2+}]_e$; a reduction from 1 mM to 100 μM caused a 4.2-fold decrease in potency, and a reduction to 10 μM, a further 3.6-fold decrease. For even lower Ca^{2+} concentrations, it was difficult to determine whether the maximum response was also reduced (Figure 4).

The 'Ca^{2+} titration curves' from binding assays under the same conditions were essentially superimposable with corresponding curves for PLC activity with both 1 and 10 nM orexin-A (Figure 5). This suggests that the effect of Ca^{2+}_e on orexin receptor responses (like PLC here) is solely a result of its effect on orexin-A binding.

However, in previous studies it was suggested orexin-A-induced Ca^{2+} influx has a role in coupling orexin receptors to intracellular response pathways (see Discussion). Depolarization effectively attenuates Ca^{2+} influx by abolishing the driving force for Ca^{2+} entry (Lund *et al.*, 2000; Johansson *et al.*, 2007). We thus exposed cells to high-K^+-NaBM and assessed orexin binding and the PLC response. The PLC response was strongly attenuated, to an extent comparable with the reduction observed on decreasing $[Ca^{2+}]_e$ to 10 μM (Figure 6A and B), as also previously reported (Johansson *et al.*, 2007). TEA and SKF-96365 are selective (although not fully complete) inhibitors of the orexin receptor-operated Ca^{2+} influx and the store-operated Ca^{2+} influx pathways, respectively, in CHO cells (Johansson *et al.*, 2007). TEA strongly inhibited the PLC response, especially that to 1 nM orexin-A, while SKF-96365 was largely ineffective (Figure 6B). The effects of high-K^+-NaBM and TEA-NaBM were investigated, in parallel, on receptor binding. Both only weakly inhibited [^{125}I]-orexin-A binding, and were thus much more effective inhibitors of the PLC response, especially that to 1 nM orexin-A (Figure 6C). This suggests that Ca^{2+} entry also has a role in orexin receptor responses, and thus $[Ca^{2+}]_e$ affects the receptor behaviour at this level.

Ca^{2+} has no significant effect on the gross conformation of orexin-A

The fact that $[Ca^{2+}]_e$ affects orexin-A binding to OX_1 receptors suggests that Ca^{2+} probably binds to either orexin-A, the OX_1 receptor, or between the ligand and the receptors. Although the two latter options are difficult to investigate, the first-mentioned possibility can be more easily assessed

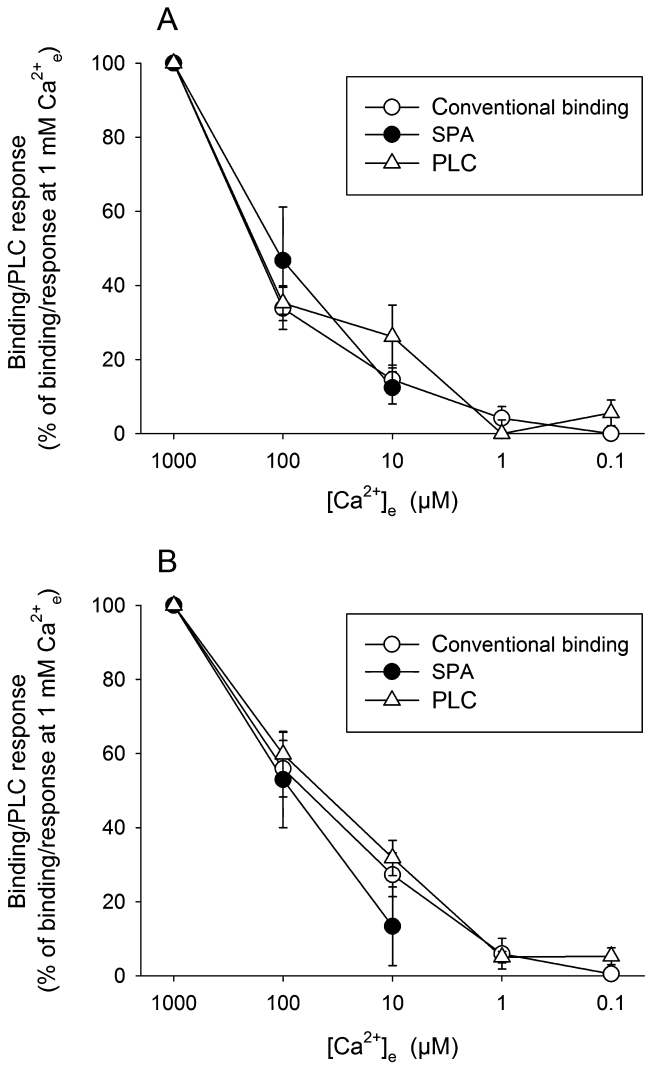

Figure 5

Comparison of orexin-A binding and PLC activation by orexin-A [(A) 1 nM, (B) 10 nM] under equal conditions (37°C, 10 min). The data are from Figures 2B, 3 and 4B.

using pure, synthetic orexin-A. CD spectroscopy was applied to measure the gross secondary structure of orexin-A in solutions containing different Ca^{2+} concentrations. The conditions (37°C, pH 7.4) were chosen to mimic physiological conditions, also used in the other assays of this study. Because we needed to assess also the effect of Ca^{2+}, we could not use the standard phosphate buffer. The CD spectrum of orexin-A showed a strong dominance of α-helix (Supporting Information Fig. S2A), as is logical in the light of previous NMR studies (Miskolzie and Kotovych, 2003; Kim *et al.*, 2004; Takai *et al.*, 2006). No significant difference in the CD spectrum of orexin-A was seen in the absence or presence of Ca^{2+} (Supporting Information Fig. S2A). Thermal denaturation was also monitored, in order to assess the possible effect of Ca^{2+} on the stability of orexin-A. The secondary structure of orexin-A was found to be very stable, with only minimal melting at the maximum temperature, 90°C, and no difference was seen in the absence and presence of Ca^{2+} (Supporting Information Fig. S2B). However, there was a minor difference in the conformation in the absence and presence of Ca^{2+}, as indicated by a slight (but significant) difference in the shape of the wavelength scan, as illustrated in the point where the scans cross the *x*-axis (Supporting Information Fig. S2C).

Discussion and conclusions

In previous studies in CHO cells, OX_1 receptor signalling to many cascades has been shown to be affected by $[Ca^{2+}]_e$. This has been explained by a strong Ca^{2+} influx induced by stimulation of the receptors and an effect of this Ca^{2+} on the signal cascades (see Introduction). Orexin-A activation of OX_1 receptors has been indicated under nominally Ca^{2+}-free conditions (no chelator or Ca^{2+} added; $[Ca^{2+}]_e \approx 1–5$ μM) or even under subnanomolar $[Ca^{2+}]_e$ (Lund *et al.*, 2000), and thus it has been assumed that Ca^{2+} has no direct effect on orexin binding. In addition to the receptor-operated Ca^{2+} influx, OX_1 receptor stimulation strongly activates PLC, Ca^{2+} release and store-operated Ca^{2+} influx in CHO cells (Lund *et al.*, 2000; Kukkonen and Åkerman, 2001). PLC enzymes are Ca^{2+}-sensitive and are directly amplified by intracellular Ca^{2+} (Ca^{2+}_i), most effectively via Ca^{2+} influx, to a degree that is dependent on the isoforms (Kukkonen, 2011). We previously showed that Ca^{2+} influx via store- and receptor-operated calcium channels has a significant effect on the PLC response in CHO cells, and that the receptor-operated Ca^{2+} influx appears to be more important for this effect (Johansson *et al.*, 2007). However, the finding that a stronger inhibition of the PLC response was obtained by a drastic Ca^{2+}_e reduction by chelation with EGTA than with any inhibitor, nominal Ca^{2+} free solution or reversal of the driving force for Ca^{2+} by depolarization, cast some doubt on this as the only explanation (Johansson *et al.*, 2007).

In the present study we directly assessed the Ca^{2+} sensitivity of the orexin-A binding. Orexin binding is not easily accomplished due to its high absorption to glass and plastic surfaces, making, for instance, filtration assays nearly impossible. To date, we have not managed to obtain consistent binding in membrane preparations of CHO cells, which indicates that some necessary conditions are lost upon cell homogenization. However, we previously found that some conditions, like inclusion of BSA and special plastic-ware, allow binding experiments with orexin-A (Jäntti *et al.*, 2013). Use of a short time window (10 min) mimics the time window for most of the responses, and also almost totally eliminates the internalization (Jäntti *et al.*, 2013), and may reduce other possible processes like receptor phosphorylation. Experiments under these conditions clearly showed that orexin-A binding is strongly influenced by $[Ca^{2+}]_e$, and even the studies under the apparent equilibrium conditions supported this conclusion. When compared with the PLC response (probably mediated by G_q) under the same conditions, the effect was equal, although the effects are difficult to quantify as the amplification of the PLC response and the mechanism by which Ca^{2+} affects binding have not been elucidated. Studies under apparent equilibrium conditions do not give a clear indication whether the affinity or the

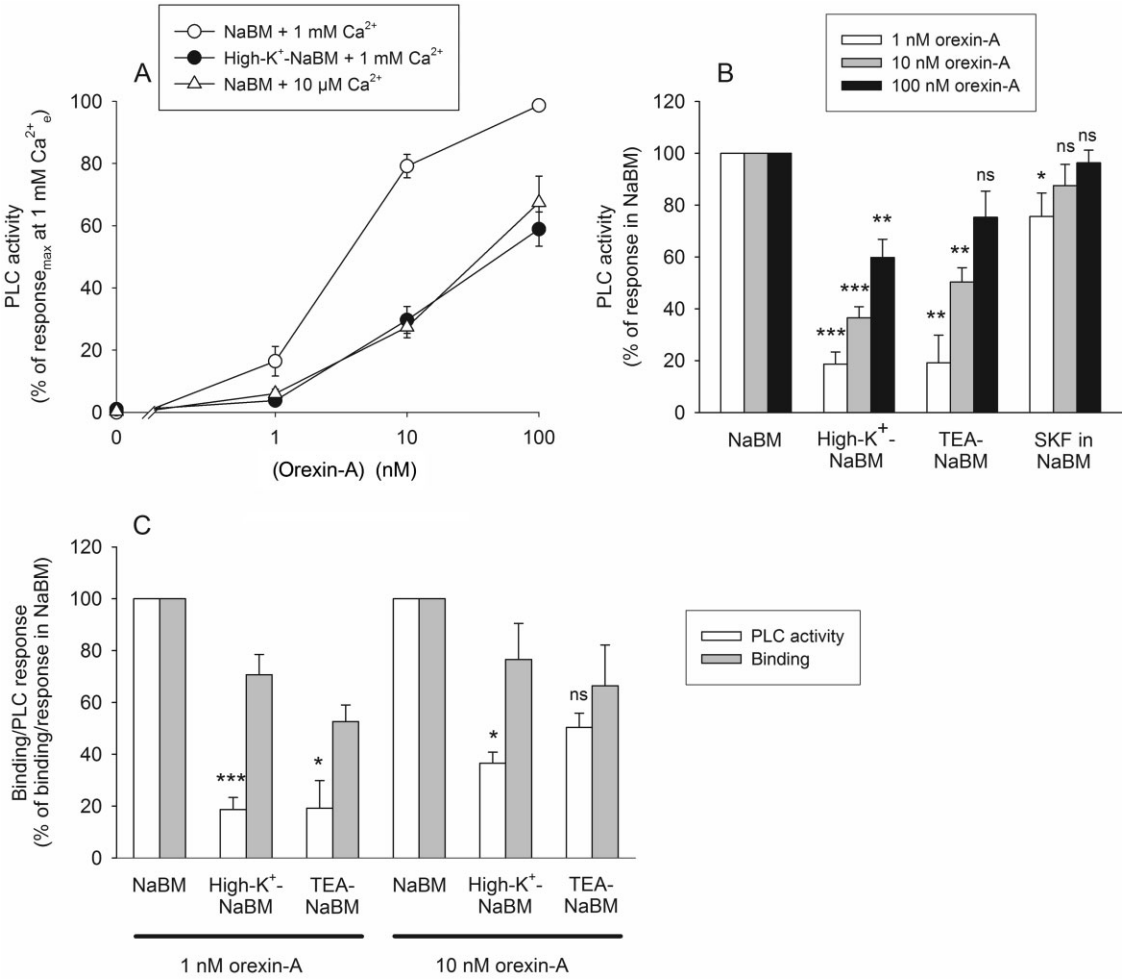

Figure 6

The effect of Ca^{2+} influx inhibitors on orexin-A binding and PLC activation by orexin-A. The experiments were performed under equal conditions (37°C, 10 min). (A) PLC response in high-K^+-NaBM as compared with responses in NaBM in the presence of 1 mM or 10 μM Ca^{2+}. The responses were normalized to the NaBM + 1 mM Ca^{2+}_e control (as in Figure 4A). (B) PLC response in high-K^+-NaBM, TEA-NaBM and SKF in NaBM (all containing 1 mM Ca^{2+}). The data were normalized to and the significances calculated from the corresponding controls in NaBM. N = 3–5. (C) Comparison of the PLC response and binding in high-K^+-NaBM and TEA-NaBM. The binding represents pooled results from regular binding and SPA, which gave similar results. The response/binding data were normalized to that observed in NaBM. The significance was calculated for the PLC response as compared with the binding for each condition. *P < 0.05, **P < 0.01, ***P < 0.001; N = 3–5.

maximum binding is affected; the data can be equally well fitted assuming either reduction in the binding affinity or maximum binding, if the most uncertain, upper end data are excluded (Supporting Information Fig. S3). The kinetic studies suggest that actually the binding affinity slightly (by 1.3-fold) increased at 100 μM Ca^{2+}_e as compared with 1 mM Ca^{2+}_e, and thus the effect might be on the number of binding sites available. Non-equilibrium binding of 1 and 10 nM orexin-A and PLC data suggest an effect on the binding affinity, but a possible receptor reserve may distort the latter. Ultimately, mechanistic studies on the binding would be conducted in membrane preparations, and the functional studies would benefit from procedures that effectively inhibit the Ca^{2+} influx pathways.

A comparison of the results of receptor binding and the PLC assay indicates that the entire inhibitory effect on orexin-A-mediated PLC activation seen upon a reduction in $[Ca^{2+}]a_e$ could be due to a decrease in binding. We thus determined the effect of strong depolarization (induced by the high-K^+-NaBM medium) on binding and PLC activation. The depolarization effectively inhibited orexin-A-mediated PLC activation but had only a weak effect on the binding. This suggests that Ca^{2+} may have a dual role in the PLC activation mediated by orexin receptors: Ca^{2+}_e is necessary for orexin binding but Ca^{2+}_e is also needed to flow in, stimulated by the receptor activity, to act on either PLC or another target required for the response. A similar effect was seen with the receptor-operated Ca^{2+} channel inhibitor, TEA, while the store-operated Ca^{2+} channel inhibitor SKF-96365 did not have a marked effect on the PLC response (not tested for effect on binding). This suggests, in agreement with the previous study (Johansson *et al.*, 2007), that Ca^{2+} influx, especially through the receptor-operated channels, is required for the PLC response.

The results of the current study unequivocally show that Ca^{2+}_e is required for orexin binding. However, there are several previous indirect observations of Ca^{2+}-independent binding of orexin based on the orexin responses observed in neurons (Burlet *et al.*, 2002; Wu *et al.*, 2002; 2004; Burdakov *et al.*, 2003; Kohlmeier *et al.*, 2008; Yamanaka *et al.*, 2010). Usually, relatively high concentrations of orexins (100 nM–1 μM) have been used when investigating the effect of decreasing $[Ca^{2+}]_e$ to 1–20 μM. This may not reduce the orexin binding enough to affect the response (Figure 1). Some studies have utilized orexin-B and may also target the OX_2 receptor; in the current study we did not assess the effect of Ca^{2+} on orexin-B responses or the OX_2 receptor. However, it is more difficult to comprehend similar observations with respect to orexin-A in the very same CHO cells as in the current study (Lund *et al.*, 2000); the apparent contradiction to earlier findings may be explained by some technical issue in the assay by Lund *et al.* (2000). In addition, the coupling of the binding and response is not well determined. It is possible that the actual response is generated from rapidly binding and dissociating ligands, which may not show up in the gross binding assays; instead the measured binding indicates an isomerized state of the receptor (as shown for inhibitors of some GPCRs and other targets) (see, e.g. Järv *et al.*, 1979; Hsu *et al.*, 1991), which is not relevant for (all) the responses. One such isomerization process could be receptor di-/oligomerization, which has also been suggested to be rather rapidly induced by exposure of OX_1 receptors to orexin-A (Xu *et al.*, 2011). However, it is difficult to understand how this would escape detection by SPA, and thus further studies are required to assess this.

Signalling of a number of GPCRs has previously been reported to be affected by the presence of Ca^{2+}. Some effects are attributed to the coupling of these receptors to Ca^{2+} influx responses that may be inhibited by the absence of Ca^{2+}_e. Signalling via, for instance, PLC, PKC, GPCR kinases and regulators of G-protein signalling proteins may be inhibited, leading to positive or negative effects on signalling, depending on the receptor (Levay *et al.*, 1998; Sallese *et al.*, 2000; Tosetti *et al.*, 2003; Kukkonen, 2011). A reduction in $[Ca^{2+}]_e$ may affect the cytosolic Ca^{2+} levels, without a requirement for Ca^{2+} influx, especially when Ca^{2+} chelators are used, which may even lead to effects that are not related to the signal cascades of the receptor being studied. Even the primordial stage of receptor activation, agonist binding, has been reported to be affected by Ca^{2+}, for instance for melanocortin peptide binding to melanocortin receptors (Salomon, 1990; Kopanchuk *et al.*, 2005). The studies are often performed using membrane preparations, and thus seldom identify whether the Ca^{2+} effect is on the extracellular or intracellular side of the cell. Why High-K^+-NaBM and TEA-NaBM have any effect at all on orexin binding in the current study might be explained by assuming that the receptor binding is also affected by intracellular Ca^{2+} concentration. Ca^{2+} has been shown to affect the structure of some peptide ligands (Peggion *et al.*, 1983; Siemion *et al.*, 1985; reviewed in Ananthanarayanan and Kerman, 2006), and thus the action of Ca^{2+} (or other metal ions) has been suggested to be on these peptides and not on the receptors (reviewed in Ananthanarayanan and Kerman, 2006). Our studies with CD spectroscopy – which as a method only assesses the net structure of the peptide – suggest only a slight conformational change in orexin-A in the presence of Ca^{2+}. More detailed studies are required to resolve the significance of the finding. Also many membrane proteins, like low-density lipoprotein receptors, some glutamate receptors and adhesion molecules, are positively or negatively regulated by Ca^{2+} (Hatta *et al.*, 1988; Fass *et al.*, 1997; Chaudhry *et al.*, 2009). Ca^{2+} could also be shared between the peptide and the receptor. Metal ions, like Ca^{2+} ion, are not only coordinated by carboxyl groups, which at least orexin-A would lack, but also histidines, cysteines and the peptide backbone (Lu *et al.*, 2009). Finally, it is possible that the Ca^{2+} sensitivity of the binding could come from other sites, like some receptor-interacting protein or intracellular site, where the calcium concentration may also be affected by extracellular manipulations.

The results of the current study show that Ca^{2+}_e affects orexin-A–OX_1 receptor interaction, and thus call for a reassessment of the procedure used to chelate Ca^{2+}_e to eliminate Ca^{2+} influx and the results hitherto obtained with such methods (Kukkonen and Leonard, 2014; Leonard and Kukkonen, 2014). However, the actual Ca^{2+} influx was also shown to be important for orexin responses, at least for the PLC response, and Ca^{2+}_e thus also acts at this level so complicating the situation. Further experiments need to identify the target for Ca^{2+} in orexin-A–OX_1 receptor interaction and its mechanism of action. This is also important from a drug discovery perspective. To fully distinguish the effect of Ca^{2+}_e on binding and responses, it is important to discover ways to more selectively and potently inhibit (or permanently activate) the orexin receptor-operated Ca^{2+} influx, which would, in the best possible case, reveal the molecular mechanisms for the activation of this pathway.

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Author contributions

J. P. K. planned the studies, T. P. supplied expertise on CD spectroscopy, J. P. and J. P. K. performed experiments and analysed data. All authors contributed to the manuscript by writing or commenting.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Binding kinetics of orexin-A. (A) Association; (B) dissociation. The data represent average of each independent

well. Before averaging, each well was curve fitted (using Eq. 3 and 4), normalized to its own curve fitting maximum and averaged. These averaged data were curve fitted again (just for the presentation), and the experimental data and curve fitting data normalized to the maximum of this. Please observe that the actual maximum binding at 100 μM Ca^{2+}_e is only 30–40% of the binding at 1 mM Ca^{2+}_e (see, e.g. Figure 1 or Figure 2), which also makes it more noisy. Due to the experimental procedure, the time points were not the same in different wells or experiments, and therefore the data were divided in time ranges (every 5 min for association, every 3 min for dissociation) and then averaged in both directions, to be able to produce this graph. This procedure increases the noise in the data.

Figure S2 CD spectroscopy of orexin-A. (A) Single scans of orexin-A in the absence and in the presence of 1 mM Ca^{2+} at 40°C. (B) Average circular ellipticity for 195 and 222 nm extracted from the wavelength scans at 5–10°C temperature steps. (C) The average *x*-axis crossing of the temperature scans. The curves in the absence and presence of Ca^{2+} are significantly different ($P < 0.001$).

Figure S3 The apparent equilibrium-binding data of orexin-A binding fitted to two different models. The data are the same as in Figure 1. The 300 nM point was excluded due to its rather high uncertainty and noise. The models used are two-site binding models (Eq. 2). For solid lines, the entire dataset was fitted to the model, which fixed the maximum binding for each site for all conditions but allowed variation of the binding affinity according to $[Ca^{2+}]_e$. For dotted lines, the affinities were fixed instead and the maximum binding was varied.