The Orexin OX₁ Receptor Activates a Novel Ca²⁺ Influx Pathway Necessary for Coupling to Phospholipase C*

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Ca²⁺ elevations in Chinese hamster ovary cells stably expressing OX₁ receptors were measured using fluorescent Ca²⁺ indicators fura-2 and fluo-3. Stimulation with orexin-A led to pronounced Ca²⁺ elevations with an EC₅₀ around 1 nm. When the extracellular [Ca²⁺] was reduced to a submicromolar concentration, the EC₅₀ was increased 100-fold. Similarly, the inositol 1,4,5-trisphosphate production in the presence of 1 mM extracellular Ca²⁺ was reduced to a submicromolar concentration, the EC₅₀ was increased 100-fold. The shift in the potency was not caused by depletion of intracellular Ca²⁺ but by a requirement of extracellular Ca²⁺ for production of inositol 1,4,5-trisphosphate. Fura-2 experiments with the "Mn²⁺-quench technique" indicated a direct activation of a cation influx pathway by OX₁ receptor independent of Ca²⁺ release or pool depletion. Furthermore, depolarization of the cells to +60 mV, which almost nullifies the driving force for Ca²⁺ entry, abolished the Ca²⁺ response to low concentrations of orexin-A. The results thus suggest that OX₁ receptor activation leads to two responses, (i) a Ca²⁺ influx and (ii) a direct stimulation of phospholipase C, and that these two responses converge at the level of phospholipase C where the former markedly enhances the potency of the latter.

The recently described hypothalamic peptides called orexins (1) or hypocretins (2) mediate their effects through G protein-coupled receptors called OX₁ and OX₂ receptors (1). The peptides and their receptors are widespread in the hypothalamus, cortex, and brainstem (2–5). The orexin/hypocretin peptides are encoded by a single mRNA giving rise to a 33-residue orexin-A peptide containing disulfide bridges and a linear 28-residue orexin-B (1). Orexin-A has a 10–100-fold higher affinity and potency for OX₁ receptor as compared with orexin-B, whereas no preference is displayed by the OX₂ receptor (1). The orexins cause robust increases in intracellular Ca²⁺ both in neurons cultured from rat medial and lateral hypothalamus (6) and spinal cord (7), and when studied using recombinant receptors (1). This has led to the suggestion that the receptors are coupled to the Gq family G proteins. Interestingly, the response in neurons is partially dependent on extracellular Ca²⁺, which may suggest that the receptors are connected to a Ca²⁺ influx pathway in neurons (6). Several different pathways for receptor-stimulated Ca²⁺ entry have been suggested based on functional studies with other G protein-coupled receptors. Suggested pathways include store-operated Ca²⁺ channels, second messenger-operated channels, as well as Ca²⁺-activated Ca²⁺ channels (reviewed in Refs. 8 and 9).

The aim of this study was to examine in detail the Ca²⁺ mobilizing actions of orexins on recombinant OX₁ receptors expressed in CHO-K1 cells. The results reveal the presence of a novel amplification mechanism at the level of phospholipase C that is dependent on activation of Ca²⁺ influx pathway upstream of phospholipase C.

EXPERIMENTAL PROCEDURES

Cell Cultures—To prepare the CHO-hOX₁-C1 cells used in this study CHO-K1 cells were transfected with a bidirectional vector containing the coding sequence of human OX₁ receptor as described previously for chemokine receptors (10). Neomycin resistant clones were then isolated by limited dilution. They were grown in nutrient mixture (Ham’s F-12) medium (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 100 units/ml penicillin G (Sigma), 80 units/ml streptomycin (Sigma), 400 μg/ml Geneticin (G418; Life Technologies, Inc.) and 10% (v/v) calf serum (Life Technologies, Inc.) at 32°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 Ca²⁺, inositol phosphate and IP₃ measurements in suspension, the cells were grown on circular plastic culture dishes (inner diameter, 94 mm; Nunc). For microfluorometry, the cells were grown on circular glass coverslips (inner diameter, 25 mm).

Materials—EGTA and probenecid (p-dipropylsulfamoylbenzoic acid) were purchased from Sigma and thapsigargin from RBI (Natick, MA). Fura-2, fura-2 acetoxyethyl ester, and fluo-3 acetoxyethyl ester were purchased from Molecular Probes Inc. (Eugene, OR) and thapsigargin from Merck AG (Darmstadt, Germany). myo-[2-²H]inositol (1 mCi/ml) was from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

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1 The abbreviations used are: CHO, Chinese hamster ovary; 2-APB, a 2-aminoethoxydiphenyl borate; [Ca²⁺][], extracellular free [Ca²⁺]; [Ca²⁺]i, intracellular free [Ca²⁺]; IP₃, inositol 1,4,5-trisphosphate; probenecid, p-dipropylsulfamoylbenzoic acid; TBM, TBS-buffered medium; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)aminoethanesulfonic acid; U-73122, 1H-[17β]-3-methoxyestr-1,3,5(10)-triene-17-y1 aminohexyl)-1H-pyrrrole-2,5-dione.
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**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 TES adjusted to pH 7.4 with NaOH.

**Ca²⁺ Measurements in Suspension**—The fluorescent Ca²⁺ indicator fluo-3 was used to monitor changes in intracellular Ca²⁺ instead of fura-2 since it has a higher Kᵣ (11), which allows more accurate estimation of the very high Ca²⁺ elevations induced by OX₁ receptor stimulation. The cells were harvested using phosphate-buffered saline containing 0.2 g/liter EDTA, loaded with fluo-3 acetoxymethyl ester (4 μM, 20 min, 37 °C) in TBM, 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 the thermostated cell holder of either a Hitachi F-2000, F-4000, or PTI QuantaMaster fluorescence spectrophotometer at the wavelengths 480 nm (excitation) and 540 nm (emission). Experiments were calibrated by adding 60 μg/ml digitonin, which gives the maximum value of fluorescence, and 10 mM EGTA, which gives the minimum value of fluorescence. The leaked fluo-3 was measured in separate experiments by adding 10 mM EGTA, which chelates Ca²⁺ bound to extracellular fluo-3. The corrected fluorescence values and the Kᵣ × 10⁴ (nm) were evaluated for each batch of cells, all the experiments were performed at least in duplicate.

**Photomultiplier-based Microfluorometry and Patch-Clamp**—For microfluorometric Ca²⁺ measurements, fura-2 was used instead of fluo-3 since it enables ratiometric Ca²⁺ measurement. The coverslips with cells were loaded with 1 μM fura-2 acetoxymethyl ester (30 min, 37 °C) in a buffer containing 140 mM NaCl, 2.8 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 3 mM glucose, 1 mM probenecid, and 10 mM Hepes (pH 7.4), rinsed with fura-2-free medium and used as the bottom of an open 0.4-ml chamber. The chamber was placed in a thermostat-controlled holder (32 °C) on the stage of an inverted microscope (Nikon). The additions in 6 were utilized in the patch-clamp experiments was 295 μl, containing 140 mM KCl, 2 mM MgCl₂, 3 mM glucose, 1 mM probenecid, and 10 mM Hepes (pH 7.4), held at a holding potential of −50 mV. Capacitative transient and series resistance were continuously monitored and compensated for. The extracellular solution was as above. The internal pipette solution containing 140 mM KCl, 2 mM MgCl₂, 3 mM Mg-ATP, 0.1 mM fura-2, and 10 mM Hepes adjusted to pH 7.2 with KOH. The osmolarity of all solutions utilized in the patch-clamp experiments (340/380) was achieved every 0.12 s by interpolation. Calibration constants for fura-2 were determined according to (12). Orexin-A was applied with a puff-pipette placed 30–50 μm away from the recording cell.

Patch-clamp experiments in the whole-cell configuration were performed using the same setup as above. Recorders were performed with 2–4-megohm pipettes (Kimax-51; Kimble, Vineland, NJ) and an EPC 9 patch-clamp amplifier together with Pulse software (HEKA). A new reading (340/380) was achieved every 0.12 s by interpolation. The experiments were performed with similar results with three to six batches of cells; for the statistics of A−C, see Fig. 2.

After 5 or 20 min of stimulation, the cells were rapidly spun down and the reactions were stopped by adding 200 μl of 0.1 M NaOH and vortexing. After a 10-min incubation at 37 °C, the solutions were neutralized with 80 μl of 0.2 M formic acid. The debris were removed, and the total inositol phosphate fraction was isolated with anion exchange chromatography (14). The radioactivity was determined by scintillation counting.

**IP₃ Measurements**—CHO-hOX₁-C1 cells were cultured and detached as for Ca²⁺ measurements in suspension. They were spun down, washed once with TBM, and resuspended in TBM, and the cell number was adjusted to 10⁶ cells/ml by counting in a Bürker chamber. The extraction of IP₃ was performed using the method of Palmer et al. (15) essentially as described (16). Briefly, the reactions were incubated for 10 min at 37 °C in a buffer containing 140 mM KCl, 2 mM MgCl₂, 3 mM glucose, 10 μM probenecid, and 10 mM Hepes (pH 7.4), containing 1 μM fura-2 (free acid form). Addition of 1.5 mM EGTA in TBM gave an approximate free concentration of approximately 1 μM.

**Measurements in Suspension**—Orexin-activated calcium elevations were measured with fluo-3 in the suspensions of CHO-hOX₁ cells. The responses to 1 nM orexin-A in Ca²⁺-free TBM, of 1 mM. Also indicated are the peak and stable Ca²⁺ elevations referred to in the text. B, the same response at [Ca²⁺]i of 140 nM and the response to restoration of [Ca²⁺], of 1 mM after orexin-A stimulation (control without orexin-A). C, the response to 100 nM orexin-A at [Ca²⁺], of 140 nM. D and E, the response to 1 μM tamapine and orexin-A [Ca²⁺]i of 140 nM, respectively. The experiments were performed with similar results with three to six batches of cells; for the statistics of A−C, see Fig. 2.

**Results**

**Orexin-induced Increase in Intracellular Free [Ca²⁺] in Suspension**—Addition of 1 nM orexin-A to CHO-hOX₁-C1 cells led to an immediate increase in intracellular free [Ca²⁺]i ([Ca²⁺]i) followed by a decay and a stable elevation of [Ca²⁺]i (Fig. 1A). When [Ca²⁺]i was reduced to 140 nM from 1 mM by addition of
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Fig. 2. Concentration-response curves for orexin-A stimulation of Ca\(^{2+}\) (A), inositol phosphate (B), and IP\(_3\) responses (C). Mean ± S.E. of maximum peak responses in the presence of 1 mM (○) and 140 nM Ca\(^{2+}\) (●) and the peak response after readdition 1 mM Ca\(^{2+}\) (as in Fig. 1B; □) from determinations with three or four (A) or two batches of cells (B and C) performed as in Fig. 1 are given. The results in B represent 20-min incubation with orexin-A; similar results, although with lower stimulation, were observed with 5-min incubation. In A, the response to addition of 2 μM thapsigargin in 140 nm extracellular Ca\(^{2+}\) is also shown (shaded bars). Different concentrations of orexin-A were added in 1 mM extracellular Ca\(^{2+}\) followed by addition of 1.5 mM EGTA 1 min later and 2 μM thapsigargin another 0.5 min later. Mean ± S.E. of five to eight determinations is given. **ns, not significant; p > 0.05; ***p < 0.001 (Student’s non-paired two-tailed t test).

1.5 mM EGTA immediately prior to addition of orexin-A, the [Ca\(^{2+}\)]\(_i\) response was abolished but a robust [Ca\(^{2+}\)]\(_i\) elevation was seen when 1.5 mM Ca\(^{2+}\) was added to saturate the EGTA and restore the [Ca\(^{2+}\)]\(_i\) of 1 mM (Fig. 1B). A higher concentration of orexin-A (100 nM) caused an increase in [Ca\(^{2+}\)]\(_i\), also when [Ca\(^{2+}\)]\(_i\), was reduced to 140 nM (Fig. 1C). To test whether the inhibitory effect of EGTA on the orexin-A response was caused by Ca\(^{2+}\) pool depletion by EGTA, 2 μM thapsigargin was added to the cells in the presence of 1 mM and 140 nM extracellular Ca\(^{2+}\) (Fig. 1, D and E). No depletion was observed since a response of similar magnitude was obtained under both conditions.

A concentration-response relation for the effect of orexin-A at normal and low [Ca\(^{2+}\)]\(_i\), is shown in Fig. 2. In 1 mM [Ca\(^{2+}\)]\(_i\), orexin-A dose-dependently increased [Ca\(^{2+}\)]\(_i\), with an EC\(_{50}\) value of 1 nM. In 140 nM [Ca\(^{2+}\)]\(_i\), the EC\(_{50}\) value was around 80 nM. The concentration dependence of the spike response seen upon Ca\(^{2+}\) readdition showed a bell-shaped curve with a half-maximal rising phase at 3 nM and a half-maximal falling phase at about 50 nM orexin-A. Fig. 2A also shows the response to 2 μM thapsigargin in the presence of 140 nM external Ca\(^{2+}\). When added after 1 mM orexin-A challenge in 1 mM Ca\(^{2+}\), the response to thapsigargin in 140 nM Ca\(^{2+}\) was the same as in control conditions, indicating that no change in the thapsigargin-releasable pool had occurred. When added after 100 nM orexin-A, the release by thapsigargin was almost completely abolished, indicating that the pool had been almost totally discharged. The concentration-response relation for orexin-A-induced accumulation (20 min) of total inositol phosphates in the presence of Li\(^+\) is shown in Fig. 2B. In 1 mM Ca\(^{2+}\), a concentration-dependent increase in inositol phosphates was seen with an EC\(_{50}\) around 1.5 mM. However, when the accumulation was measured in the presence of 140 nM Ca\(^{2+}\), no detectable accumulation could be seen. Ionomycin (1 μM) increased the total inositol phosphate accumulation in the presence of 1 mM Ca\(^{2+}\) by 72.4 ± 4.7%, whereas thapsigargin (2 μM) did not (5.3 ± 0.6%; mean ± S.E., n = 3). In the presence of 140 nM Ca\(^{2+}\), neither of these compounds affected the accumulation of inositol phosphates (3.5 ± 0.3% and −9.5 ± 0.9%, respectively).

The concentration-response relation for the rapid (7 s) orexin-A-induced IP\(_3\) accumulation in similar conditions is shown in Fig. 2C. Reduction of [Ca\(^{2+}\)]\(_i\) to 10 mM caused a considerable shift in the concentration-response relation. Whenever 1 mM [Ca\(^{2+}\)]\(_i\), was restored after orexin-A in conditions similar to those for the fluo-3 experiments in Figs. 1B and 2A, the concentration-response relation was almost identical to that in the presence of extracellular Ca\(^{2+}\) except that the maximum response might have been lower. Thus, the IP\(_3\) response has a similar extracellular Ca\(^{2+}\) dependence as the Ca\(^{2+}\) response except that the orexin-A has a 10-fold lower potency for the IP\(_3\) response as compared with the Ca\(^{2+}\) response. To investigate whether phospholipase C is involved in the external Ca\(^{2+}\)-dependent response to 1 mM orexin-A, the effect of the phospholipase C inhibitor U-73122 was tested. This compound, however, caused a considerable rise in [Ca\(^{2+}\)]\(_i\) on its own at a concentration as low as 1 μM (data not shown). This nonspecific effect is in line with other studies (18), and thus this inhibitor could not be used to probe a role of phospholipase C activation.

A bell-shaped relation was observed when the stable phase of Ca\(^{2+}\) elevation seen in Fig. 1A was plotted as a function of the orexin-A concentration (Fig. 3A). Thus, a more stable phase of the signal dominates at low concentrations of orexin-A as compared with high concentrations where this phase is virtually absent. A similar result was obtained with orexin-B although, as reported earlier for the OX\(_1\) receptor (1), the potency of this ligand was 10 times lower than that of orexin-A (data not shown).

**Extracellular Ca\(^{2+}\) Dependence of the Responses to Orexin-A**—In order to further characterize the Ca\(^{2+}\) dependence of the response to orexin-A, the stimulation was performed with a puff-pipette in a close contact with the cell being monitored. Cells stimulated with 10 nM orexin-A in Ca\(^{2+}\)-free conditions...
did, as expected (see Fig. 2A), not respond with an elevation of [Ca\(^{2+}\)]\(_i\) (Fig. 4A). If the bath perfusion immediately after the challenge was changed to a solution containing 2 mM Ca\(^{2+}\), there was an immediate robust increase in [Ca\(^{2+}\)]\(_i\), even though orexin-A was no longer present in the extracellular solution (Fig. 4A). If 2 mM Ca\(^{2+}\) was present together with orexin-A in the puff-pipette, the cells perfused either in 0 or 2 mM extracellular Ca\(^{2+}\) responded to the orexin-A-Ca\(^{2+}\)-challenge with an immediate robust increase in [Ca\(^{2+}\)]\(_i\) (Fig. 4B). Stimulation with the vehicle instead of orexin-A in any of these cases did not cause any response (data not shown). This indicates that the absence of orexin-A response under Ca\(^{2+}\)-free conditions is not caused by the depletion of intracellular Ca\(^{2+}\) stores, but by the absence of the Ca\(^{2+}\) influx.

Orexin-A response was also investigated in single cells under voltage-clamp conditions. When the normal holding potential of −50 mV was changed to +60 mV, which nearly nullifies the driving force for Ca\(^{2+}\) influx, the orexin-A response was abolished. A rapid change back to −50 mV in the presence of continued orexin-A application resulted in an immediate Ca\(^{2+}\) elevation. This indicates that, at this orexin-A concentration, the trigger for the Ca\(^{2+}\) response is a Ca\(^{2+}\) influx, most likely a channel.

**IP\(_3\) Receptor Dependence of the Responses to Orexin-A**—2-APB has been reported to block IP\(_3\)-mediated Ca\(^{2+}\) mobilization without affecting receptor-dependent IP\(_3\) production (19). Fig. 5A shows that 2-APB did not affect the orexin-A-induced Ca\(^{2+}\) elevation in the presence of 2 mM Ca\(^{2+}\). However, in the presence of 140 nM Ca\(^{2+}\), the Ca\(^{2+}\) response was almost completely abolished (Fig. 5B). Xestospongin C has been shown to block some subtypes of the IP\(_3\) receptor. For blocking in intact cells, this compound requires preincubation for about 20 min (20, 21). Therefore, these experiments were performed in suspension. Cell suspensions were preincubated for 20 min with 20 μM xestospongin C before challenge with 3 or 100 nM orexin-A. In the presence of 1 mM extracellular Ca\(^{2+}\), xestospongin C did not affect Ca\(^{2+}\) at either concentration of orexin-A. However, with 140 nM extracellular Ca\(^{2+}\), the response to 100 nM orexin-A was almost totally blocked. Thus, both 2-APB and xestospongin C give the same result, e.g., have no effect in the presence of 1 mM extracellular Ca\(^{2+}\) but block the orexin-A response in the presence of 140 nM extracellular Ca\(^{2+}\).

**Mn\(^{2+}\) Influx in Responses to Orexin-A as Determined with Fura-2**—Many of the Ca\(^{2+}\) influx pathways activated by G protein-coupled receptors are also permeable to Mn\(^{2+}\), and quenching of the fluorescence of fura-2 by this cation can be seen at the 3 nM orexin-A in the presence of 140 nM Ca\(^{2+}\) since no response is observed at this concentration (see Fig. 2). ***, ***, \(p<0.001\) (Student’s non-paired two-tailed \(t\) test).

Fura-2—Many of the Ca\(^{2+}\) influx pathways activated by G protein-coupled receptors are also permeable to Mn\(^{2+}\), and quenching of the fluorescence of fura-2 by this cation can be used for monitoring the activation of such pathways (22). Cells were challenged with different concentrations of orexin-A in nominally Ca\(^{2+}\)-free solutions to see whether orexin-A activates an influx pathway at concentrations that do not cause intracellular Ca\(^{2+}\) mobilization. The fura-2 response in individual cells was viewed by monitoring the fluorescence at 340 nm (to detect the fura-2-Ca\(^{2+}\) complex) and 360 nm (insensitive to changes in Ca\(^{2+}\) but sensitive to Mn\(^{2+}\) binding). 100 nM orexin-A caused a Ca\(^{2+}\) release as indicated by the fluorescence increase at 340 nm, whereas no response was seen at 360 nm (Fig. 6A). A further addition of 100 μM Mn\(^{2+}\) quenched the fura-2 fluorescence at both wavelengths, indicating an influx through, e.g., store-operated Ca\(^{2+}\) channels. The addition of 100 μM Mn\(^{2+}\) before stimulation with 100 nM orexin-A did not result in any fluorescence response (Fig. 6B). After the addition of orexin-A, however, a similar Ca\(^{2+}\) release and Mn\(^{2+}\) influx, as in Fig. 6A, was seen. Simultaneous addition of 100 nM orexin-A and 100 μM Mn\(^{2+}\) resulted in rapid fluorescence increase at 340 nm and a decrease at 360 nm (Fig. 6C). Simultaneous addition of 100 μM Mn\(^{2+}\) and 1 nM orexin-A resulted in a quenching of the fluorescence at both wavelengths (Fig. 6D). A further addition of 10 nM orexin-A lead to a further quenching at both wavelengths. This indicates that 1 and 10 nM orexin-A, which do not activate Ca\(^{2+}\) release in these nominally Ca\(^{2+}\)-free conditions, still activate a cation influx pathway seen as Mn\(^{2+}\) influx.

**Effect of Thapsigargin-induced Store Depletion on Orexin-induced Ca\(^{2+}\) Elevation and Mn\(^{2+}\) Influx**—Thapsigargin caused a transient and sustained Ca\(^{2+}\) elevation in the presence of 1 mM extracellular Ca\(^{2+}\) (Figs. 1D and 7A), whereas the
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The results of the present study strongly support the idea that the primary response upon activation of the OX1 receptor is the activation of a Ca²⁺ influx pathway, which triggers the phospholipase C activation. Thus, no changes in [Ca²⁺], total inositol phosphates, or IP₃, could be detected upon activation of the receptor with low concentration of orexin-A when the [Ca²⁺] was reduced immediately before the challenge. Additionally, reduction of the driving force for the Ca²⁺ influx had similar effect. Furthermore, low concentration of orexin-A in nominally Ca²⁺-free conditions caused a quenching of fura-2 fluorescence by Mn²⁺ in conditions where no change in [Ca²⁺], could be detected. The fact that purified phospholipase C-β is activated both by Ca²⁺ and by G protein subunits α₁ and βγ, would allow such an amplification mechanism (23).

Phospholipase C-β1 has been shown to be synergistically activated by both Ca²⁺ and Gq (24). As shown here, neither thapsigargin nor ionomycin alone is able to activate accumulation of inositol phosphates to the same extent as orexin-A. This suggests that Ca²⁺ alone is not sufficient for phospholipase C-β activation but an additional receptor-associated mechanism is also required. An enhancement of the maximum IP₃ production by elevated [Ca²⁺], has been previously shown for muscarinic receptors (25, 26). However, there has not been any indication of any receptor-operated pathway that would enhance the primary IP₃ response; on the contrary, the potentiating effect of Ca²⁺ on IP₃ production has been seen after activation of voltage-sensitive Ca²⁺ channels (25) or artificial regulation of [Ca²⁺], in permeabilized cells (26). The results of the present study suggest that the primary response of OX1 receptors almost completely relies on a receptor-operated, extracellular Ca²⁺-dependent amplification mechanism, since the concentration-response curve is shifted 100-fold to the right when this mechanism is disabled. Such a mechanism has not been described for any other receptor.

The lack of Ca²⁺ elevation due to depletion of intracellular stores in 140 nm extracellular Ca²⁺ can be excluded as high concentrations of orexin-A and thapsigargin were able to cause Ca²⁺ mobilization in these conditions. The same was seen in the fluorometric experiments, where even low perfusion with even lower Ca²⁺ concentrations did not result in reduction of orexin-A response, which was immediately fully restored when orexin-A was added together with Ca²⁺. Therefore, it is evident that the response at low concentration of orexin-A is dependent on extracellular Ca²⁺. Although xestospongin C and 2-APB block the response to high orexin-A, Fig. 6. Representative fluorescence traces of Ca²⁺ elevations and Mn²⁺ influx measured with fura-2 in the Ca²⁺ imager. All the experiments were performed in nominally Ca²⁺-free solutions. In A the addition of 100 nm orexin-A causes a Ca²⁺ release as indicated by the fluorescence increase at the 340 nm, whereas no response is seen at 360 nm. Addition of 100 μM Mn²⁺ quenches the fura-2 fluorescence at both wavelengths, indicating an influx through, e.g., store-operated Ca²⁺ channels. In B, the addition of 100 μM Mn²⁺ before stimulation with 100 nm orexin-A does not result in any fluorescence response. After the addition of orexin-A, however, a similar Ca²⁺ release and Mn²⁺ influx, as in A, are seen. Simultaneous addition of 100 nm orexin-A and 100 μM Mn²⁺ in C results in rapid fluorescence increase at 340 nm and a decrease at 360 nm. In D, simultaneous addition of 100 μM Mn²⁺ and 1 nm orexin-A results in a quenching of the fluorescence at both wavelengths. Addition of 10 nm orexin-A leads to a further quenching at both wavelengths.

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The lack of Ca²⁺ elevation due to depletion of intracellular stores in 140 nm extracellular Ca²⁺ can be excluded as high concentrations of orexin-A and thapsigargin were able to cause Ca²⁺ mobilization in these conditions. The same was seen in the fluorometric experiments, where even low perfusion with even lower Ca²⁺ concentrations did not result in reduction of orexin-A response, which was immediately fully restored when orexin-A was added together with Ca²⁺. Therefore, it is evident that the response at low concentration of orexin-A is dependent on extracellular Ca²⁺. Although xestospongin C and 2-APB block the response to high orexin-A.
concentrations in 140 mM extracellular Ca\(^{2+}\), the response to low and high orexin-A concentrations in 1 mM extracellular Ca\(^{2+}\) is unaffected, suggesting that influx of Ca\(^{2+}\) into the cells is the primary pathway. Ca\(^{2+}\) elevation via the OX1 receptor in these cells may thus reflect the function of a receptor-operated influx pathway.

Previously, a variety of phosphatidylinositol hydrolysis-associated Ca\(^{2+}\) influx pathways have been described using electrophysiological techniques (27–30) and measurements of [Ca\(^{2+}\)]\(_i\) (30–33). Most of the described pathways for Ca\(^{2+}\) entry appear to be activated secondary to IP\(_3\) production. Inositol phosphates have been shown to activate Ca\(^{2+}\) entry pathways in some cells (34–36). There are, however, also reports on Ca\(^{2+}\) entry pathways (involving G protein-coupled receptors) which are not secondary to phospholipase C activation (37–40). Potential activators include, e.g., lipid metabolites (41, 42). Most receptors coupled to mobilization of Ca\(^{2+}\) activate so-called store-operated Ca\(^{2+}\) channels. An often found Ca\(^{2+}\) current in this category is I\(_{\text{CRAC}}\), which is activated in many cells by Ca\(^{2+}\) store depletion (43). A family of non-voltage-activated Ca\(^{2+}\) channels called Trp has been identified. Although some members of this family show some properties similar to store-operated channels (44–48), others appear to be fairly distinct (49–51). At least some members have been reported to be activated by lipid metabolites (52, 53). Evidence has been presented that one channel belonging to this family, called TRPL, is directly activated by the G\(_{\text{O11}}\) G protein subunits (54).

The results shown here suggest that the orexin-A mediated Ca\(^{2+}\) elevation is not primarily due to an inositol phosphate-activated Ca\(^{2+}\) influx pathway. At low orexin-A concentrations, both the Ca\(^{2+}\) and IP\(_3\) responses required extracellular Ca\(^{2+}\). The same was reflected in the assay of inositol phosphates. In addition, the IP\(_3\) production in the presence of Ca\(^{2+}\) required 10 times higher concentrations of orexin-A than the Ca\(^{2+}\) response. Accumulation of inositol phosphates was seen at lower concentrations of orexin-A, so it cannot be completely excluded the Ca\(^{2+}\) influx would be amplified by an inositol phosphate-activated mechanism.

The store-operated mechanism for Ca\(^{2+}\) entry also appears unlikely. Store-operated Ca\(^{2+}\) channels are activated by pool depletion (43), whereas the extracellular Ca\(^{2+}\)-dependent response here is independent of IP\(_3\) production and Ca\(^{2+}\) pool depletion as well as insensitive to blockers of IP\(_3\)-receptor mediated Ca\(^{2+}\) release. Also, influx of Mn\(^{2+}\) was initiated immediately by the addition of low concentrations of orexin-A, whereas activation of I\(_{\text{CRAC}}\) by pool depletion is usually delayed (7 s latency; Ref. 43). The stable level of Ca\(^{2+}\) elevation as well as part of the Ca\(^{2+}\) response seen upon Ca\(^{2+}\) readdition could well be due to a store-operated Ca\(^{2+}\) channel. Secondary Ca\(^{2+}\) influx pathways like I\(_{\text{CRAC}}\) show very fast inactivation (\(\tau\) around 100 ms) by intracellular Ca\(^{2+}\) (55, 56). This could explain the bell-shaped relation of the response to Ca\(^{2+}\) readdition and the steady state Ca\(^{2+}\) level upon the orexin-A concentration. Thus, the elevated Ca\(^{2+}\) could reduce the influx. However, the bell shape could also result from, e.g., (i) a faster inactivation of the receptor response or (ii) a stronger activation of Ca\(^{2+}\) extrusion or both (see also below).

Whether the Ca\(^{2+}\) response at low orexin-A concentrations is exclusively due to influx or whether it is part of a Ca\(^{2+}\)-dependent amplification mechanism is worth some consideration. Mn\(^{2+}\) influx seen at low orexin-A concentrations supports the concept of inositol phosphate- and Ca\(^{2+}\)-independent activation of an influx pathway, and a possible mechanism involving IP\(_3\)-sensitive release is unlikely in light of the discussion above. The lack of an effect of orexin-A at steady state after the thapsigargin elevation could be explained by a markedly enhanced Ca\(^{2+}\) extrusion. Evidence for this was the considerable Mn\(^{2+}\) influx, which was apparent in conditions where [Ca\(^{2+}\)]\(_i\) was close to the basal level. Thus, low Ca\(^{2+}\) levels are maintained despite an active Ca\(^{2+}\) influx pathway. This would mean that strong activation of Ca\(^{2+}\) extrusion by Ca\(^{2+}\) (and calmodulin) limits the Ca\(^{2+}\) elevation.

The results of this study thus suggest that activation of a Ca\(^{2+}\) influx pathway is the primary response upon activation of OX1 receptors. Moreover, activation of the inositol phosphate pathway appears to be secondary to the activation of this pathway; only very high concentrations (1 \(\mu\)M) of orexin-A were able to activate IP\(_3\) production in the absence of Ca\(^{2+}\) influx. The reason for the requirement for the amplification of the Ca\(^{2+}\) release is not clear at the moment. Even a small overall Ca\(^{2+}\) influx can result in high submembrane Ca\(^{2+}\) levels, which are enough to result in full Ca\(^{2+}\) stimulation of phospholipase C. Since the inositol phosphate and IP\(_3\) production at moderate orexin-A concentrations (1–100 nM) required external Ca\(^{2+}\), the trigger of the influx has to be upstream of phospholipase C. The mechanism would thus be a direct or G protein-mediated activation of an influx pathways in close apposition to phospholipase C. It may be, that the signaling mechanisms, e.g., G proteins, for the Ca\(^{2+}\) influx and direct phospholipase C activation are different, i.e. that the latter is activated only at higher concentrations of orexin-A. This is different from the usually described sequence of activation of G\(_i\)-coupled receptors. This raises the possibility that activation of a parallel Ca\(^{2+}\) influx pathway may be involved in the signal transduction of other receptors as well. The parallel activation of phospholipase C and Ca\(^{2+}\) influx by the OX1 receptor may represent compartmentalization of the signal transductions, i.e. signal steering to the interior of the cells or to the submembrane areas. It is thus also possible that the Ca\(^{2+}\) influx is involved in activation of other cellular effector pathways like ion channels etc.

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