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RESEARCH PAPER

Arachidonic acid release mediated by OX_1 orexin receptors

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Background and purpose: We have previously shown that lipid mediators, produced by phospholipase D and C, are generated in OX_1 orexin receptor signalling with high potency, and presumably mediate some of the physiological responses to orexin. In this study, we investigated whether the ubiquitous phospholipase A_2 (PLA_2) signalling system is also involved in orexin receptor signalling.
Experimental approach: Recombinant Chinese hamster ovary-K1 cells, expressing human OX_1 receptors, were used as a model system. Arachidonic acid (AA) release was measured from ^{3}H-AA-labelled cells. Ca^{2+} signalling was assessed using single-cell imaging.
Key results: Orexins strongly stimulated [^{3}H]-AA release (maximally 4.4-fold). Orexin-A was somewhat more potent than orexin-B (pEC_{50} = 8.90 and 8.38 respectively). The concentration–response curves appeared biphasic. The release was fully inhibited by the potent $cPLA_2$ and $iPLA_2$ inhibitor, methyl arachidonyl fluorophosphonate, whereas the $iPLA_2$ inhibitors, R- and S-bromoenol lactone, caused only a partial inhibition. The response was also fully dependent on Ca^{2+} influx, and the inhibitor studies suggested involvement of the receptor-operated influx pathway. The receptor-operated pathway, on the other hand, was partially dependent on PLA_2 activity. The extracellular signal-regulated kinase, but not protein kinase C, were involved in the PLA_2 activation at low orexin concentrations.
Conclusions and implications: Activation of OX_1 orexin receptors induced a strong, high-potency AA release, possibly via multiple PLA_2 species, and this response may be important for the receptor-operated Ca^{2+} influx. The response coincided with other high-potency lipid messenger responses, and may interact with these signals.
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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; $cPLA_2$, cytosolic phospholipase A_2; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; GF109203X (bisindolylmaleimide I, Gö6850), 2-(1-[3-dimethylaminopropyl]-1H-indol-3-yl)-3-(1H-indol-3-yl)-maleimide; IP_3, inositol-1,4,5-trisphosphate; IP_3-5P1, type I IP_3 5-phosphatase; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; Lp-PLA_2, lipoprotein-associated PLA_2; MAFP, methyl arachidonyl fluorophosphonate; NaBM, Na^+-based medium; PA, phosphatidic acid; PD 98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; probenecid, *p*-(dipropylsulphamoyl) benzoic acid; *R*-BEL, (*R*)-bromoenol lactone; *S*-BEL, (*S*)-bromoenol lactone; 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; $sPLA_2$, secreted phospholipase A_2; TEA, tetraethylammonium chloride; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; TRP, transient receptor potential; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene

Introduction

The orexin receptors, OX_1 and OX_2 (nomenclature follows Alexander *et al.*, 2008), constitute a family of G-protein-coupled receptors with a wide spectrum of engaged intracellular signalling pathways (see Kukkonen and Åkerman, 2005).

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These receptors, or specifically OX_1 receptors, which have been a target for most investigations, have been shown to connect to multiple Ca^{2+} signalling mechanisms in all the native and recombinant cell types where this has been investigated (see Kukkonen and Åkerman, 2005). Based on detailed analyses, orexin receptors, at low orexin concentrations, have been suggested to activate primarily a receptor-operated Ca^{2+} influx pathway (Lund *et al.*, 2000; Kukkonen and Åkerman, 2001), and at higher concentrations, orexins also activate a phospholipase C (PLC)-dependent Ca^{2+} release and a secondary (store-operated) Ca^{2+} influx (Kukkonen and Åkerman, 2001; Johansson *et al.*, 2007). Transient receptor potential (TRP) family non-selective cation channels have in some cases been suggested to mediate the primary influx (Larsson *et al.*, 2005; Näsman *et al.*, 2006), but these channels may even in these cases be responsible for only a part of the response. In the native cells, the identity of the channels involved is not known, nor are the mechanisms utilized for channel activation in orexin receptor signalling. Ca^{2+} influx seems to be important for orexin receptor signalling at different levels. The receptor-operated influx couples orexin receptors to several signal pathways, including the extracellular signal-regulated kinase (ERK), adenylyl cyclase and part of the PLC response (Ammoun *et al.*, 2006a; Johansson *et al.*, 2007). The store-operated pathway amplifies the PLC response and may sometimes substitute for the receptor-operated pathway (Ammoun *et al.*, 2006a; Johansson *et al.*, 2007).

Furthermore, a more recently discovered signalling system significantly targeted by orexin receptors involves lipid messengers. We have previously shown that orexin receptor activation regulates both PLC and phospholipase D (PLD) species (Lund *et al.*, 2000; Holmqvist *et al.*, 2005; Johansson *et al.*, 2008), which generate at least the messengers phosphatidic acid (PA), diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3), and subsequently most likely other derivatives of these as well. Lipid messengers represent likely candidates to regulate many types of Ca^{2+} channels (reviewed in Delmas *et al.*, 2005; Montell, 2005), and are thus also candidates for the orexin receptor-operated Ca^{2+} channel activation, albeit they might also affect other orexin receptor responses, such as cell plasticity and death.

Phospholipase A_2 (PLA_2) enzymes cleave the sn2-linked acyl chain of glycerophospholipids, although several PLA_2 species are not strictly specific for the sn2 linkage (reviewed in Six and Dennis, 2000; Leslie, 2004). PLA_2 enzymes (more than 20 are known) are commonly divided into three or four major types of secreted PLA_2s (sPLA_2; GI-III, -V, -IX–XIV PLA_2 families), cytosolic PLA_2s (cPLA_2; GIV PLA_2 family), Ca^{2+}-independent PLA_2s (iPLA_2; GVI PLA_2 family) and lipoprotein-associated PLA_2 (Lp-PLA_2; also known as platelet-activating factor acetyl hydrolase and GVIIA PLA_2), of which cPLA_2 and iPLA_2 have been suggested to be receptor-regulated (see Akiba and Sato, 2004; Leslie, 2004; Balsinde and Balboa, 2005; Ghosh *et al.*, 2006; Schaloske and Dennis, 2006; Zalewski *et al.*, 2006). cPLA_2, but not iPLA_2, enzymes are brought to their lipid substrates by Ca^{2+} elevation, which interacts with the C2-domain of cPLA_2. Some members of the intracellular cPLA_2 and iPLA_2 have been shown to be regulated by phosphorylation, for instance by protein kinase C (PKC), Ca^{2+}-calmodulin kinase II and mitogen-activated kinase (MAPK) pathways (see Six and Dennis, 2000; Akiba and Sato, 2004; Leslie, 2004; Balsinde and Balboa, 2005; Ghosh *et al.*, 2006). Arachidonic acid (AA) is classically thought to be a major product of PLA_2 activity, although very few PLA_2 isoforms show high specificity for AA. AA is, most importantly, a precursor for prostaglandin and leukotriene synthesis, but it also acts directly as an intracellular, autocrine or paracrine messenger. One of the elusive AA targets is the AA-activated ARC channel (see Shuttleworth *et al.*, 2004). ARC channels have been reported in a number of cell types using Ca^{2+} measurements or electrophysiology, and a significant amount of data have accumulated on their regulation, yet their molecular identity remains unclear. In addition, AA may modulate the activity of several other types of ion channels, such as TRP channels, Na^+ channels, Cl^- channels and voltage-gated Ca^{2+} channel (see Delmas *et al.*, 2005; Meves, 2008).

In this study, we set out to investigate the involvement of PLA_2 in OX_1 receptor signalling. We found a robust release of AA upon orexin receptor activation, with a pharmacology that indicates that PLA_2 enzymes are indeed involved. The results also suggest involvement of PLA_2 activity in orexin receptor-mediated Ca^{2+} influx.

Methods

Test systems used

Chinese hamster ovary cells, expressing human OX_1 receptors (CHO-hOX_1), have been described previously (Lund *et al.*, 2000). CHO cells were propagated in Ham's F-12 medium (Gibco, Paisley, UK) supplemented with 100 U·mL^{-1} penicillin G (Sigma Chemical Co., St Louis, MO, USA), 80 U·mL^{-1} streptomycin (Sigma), 400 mg·mL^{-1} geneticin (G418; Gibco) and 10% (v/v) fetal calf serum (Gibco) at 37°C in 5% CO_2 in an air-ventilated humidified incubator on plastic culture dishes (56 cm^2 bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany). Wild-type (wt) CHO cells (not expressing OX_1 receptors) were propagated under same conditions except that geneticin was omitted. For the ^{3}H-AA release experiments, the cells were cultivated on Primaria 24-well plates (1.77 cm^2 well bottom area; BD Biosciences, Erembodegem, Belgium), and for Ca^{2+} imaging, on uncoated circular glass coverslips (diameter 25 mm; Menzel-Gläser, Braunschweig, Germany).

Transfection

Cells were transfected to introduce pcDNA3-InsP3-5-phosphatase-I (type I inositol-1,4,5-P_3 5-phosphatase; here referred as IP_3-5P1) (De Smedt *et al.*, 1997); pEGFP-C1 (Clontech, Palo Alto, CA, USA; 10% of the total DNA) was used as a marker for transfected cells (both for IP_3-5P1- and mock-transfected cells). Briefly, CHO-OX_1-cells were grown to 40–50% confluence, washed with PBS and transfected in OPTI-MEM (Gibco) using Lipofectamine (Invitrogen, Carlsbad, CA, USA) (Ammoun *et al.*, 2006a; Johansson *et al.*, 2007). After 5 h, this medium was replaced with fresh Ham's F-12 medium with the standard supplements (see above), and the cells were studied 48 h after the initiation of the transfection.

The total amount of DNA was kept constant in all transfections using empty plasmids.

AA and oleic acid release

The experiments were performed essentially as described by Mounier *et al*. (2004). Cells were plated on 24-well plates (2×10^4 cells per well), and left to grow for 24 h. Then, 0.1 μCi [^{3}H]-AA (or [^{3}H]-oleic acid) was added to each well, and the cells were cultured for another 20 h. The incubation medium was removed and the cells were washed twice with the Na^+-based medium (NaBM; composition in mM: NaCl, 137; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1.2; KH_2PO_4, 0.44; $NaHCO_3$, 4.2; glucose, 10; and HEPES, 20 adjusted to pH 7.4 with NaOH) supplemented with 2.4 mg·mL^{-1} bovine serum albumin (BSA), and finally left in NaBM without BSA at 37°C. The cells were then immediately stimulated with orexins, thapsigargin, ionomycin or 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) for 7 min, after which 200 μL of the total volume of 250 μL in each well was transferred to an Eppendorf tube on ice. These samples were centrifuged for 1.5 min at 4°C, and 100 μL of the medium was transferred to a scintillation tube. The cells on the 24-well plates were dissolved in 0.1 M NaOH, and transferred to separate scintillation vials. Scintillation cocktail (HiSafe 3, Wallac-PerkinElmer, Turku, Finland) was added, and the radioactivity was measured in a Wallac 1414 liquid scintillation counter.

In some cases, orexin stimulation was preceded by some inhibitor preincubation. In such cases, the inhibitor used was added to the cells after one wash with NaBM and the cells preincubated for 30 min in the absence of BSA. Then, the cells were washed once more with NaBM + BSA, changed to fresh NaBM without BSA still containing the inhibitor and immediately stimulated with orexin-A for 7 min. Controls (vehicle only) were treated in the same manner. This procedure was designed to remove the radioactivity leaking out of the cells during the preincubation period. For other inhibitors {TEA-BM [70 mM of the NaCl in NaBM replaced with tetraethylammonium chloride (TEA)], KBM [all the Na^+ salts replaced with corresponding K^+ salts], SKF-96365 [1-(β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole HCl]} not requiring such long preincubation periods, the inhibitors were added in NaBM without BSA after the two washes with NaBM + BSA, and the cells stimulated directly in this medium after a 5 min preincubation. Controls (vehicle only) were treated in the same manner.

Ca^{2+} measurements

Cellular Ca^{2+} measurements were performed as microfluorometric Ca^{2+} imaging of individual CHO cells attached on glass coverslips as described previously (Ammoun *et al*., 2006a). Briefly, the cells were loaded with 4 mM fura-2 acetoxymethyl ester for 20 min at 37°C in NaBM + 0.5 mM probenecid, rinsed once and used immediately. TILLvisION version 4.01 imaging system (TILL Photonics GmbH, Gräfelding, Germany) on Nikon TE200 fluorescence microscope (×20/0.5 air objective) was used for measurements. The cells were excited with 340 and 380 nm light from a xenon lamp through a monochromator, and the emitted light was collected through a 400 nm dichroic mirror and a 470 nm barrier filter with a high-resolution (1280 × 1024) cooled CCD camera. One 340 and one 380 reading were obtained each second, and the ratio was calculated after background subtraction.

The concentration of free Ca^{2+} ions in nominally Ca^{2+}-free NaBM (no added $CaCl_2$) was measured in FlexStation (Molecular Devices, Sunnyvale, CA, USA). Ten nM CalciumGreen 5N free acid was included in this medium, and the Ca^{2+} concentration was calculated from the fluorescence utilizing the maximum and minimum defined by saturating Ca^{2+} concentration (1 mM) and 0 Ca^{2+} (10 mM EGTA), respectively.

Data analysis and statistical procedures

All the data are presented as mean ± SE; *n* refers to the number of batches of cells. Each experiment was performed at least three times. The AA release experiments were performed with six parallel data points, and in imaging regularly about 30 or more data points (cells per coverslip) were obtained in parallel. Student's two-tailed *t*-test with Bonferroni correction was used in all pairwise comparisons except for the data in Figure 4A and B, where, because of the non-parametric nature of cell counting, the χ^2-test was used.

Microsoft Excel was used for the non-linear curve fitting used for the determination of the EC_{50} values; fits obtained with different models were compared using the *F*-test individually for each batch of cells (Figure 2). The effect of inhibitors on the orexin-stimulated AA release was calculated from the cpm values using a formula that compensates for the possible effect of the inhibitors also on the basal release: release [in % of the ctrl (non-inhibited)] = $(orexin_{inhibitor} - basal_{inhibitor})/(orexin_{ctrl} - basal_{ctrl}) \times 100\%$ (Figures 3–5). In this manner, the non-treated controls (basal, 1 nM orexin-A, 100 nM orexin-A) are set to 100%, and the full inhibition to 0%.

Materials

Human orexin-A and -B were from NeoMPS (Strasbourg, France), while the methyl arachidonyl fluorophosphonate (MAFP), (*R*)-bromoenol lactone (*R*-BEL) and (*S*)-bromoenol lactone (*S*-BEL) were from Cayman Europe (Tallinn, Estonia). Ionomycin, SB-334867 (1-[2-methylbenzoxazol-6-yl]-3-[1,5]naphthyridin-4-yl-urea HCl), thapsigargin and U0126 (1,4-diamino-2,3-dicyano-1,4-bis[*o*-aminophenylmercapto]butadiene) were from Tocris Cookson Ltd. (Avonmouth, UK). GF109203X (= bisindolylmaleimide I = Gö6850 = 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide), PD 98059 (2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one) and SKF-96365 were from Calbiochem (La Jolla, CA, USA); fura-2 acetoxymethyl ester and CalciumGreen 5N free acid were from Molecular Probes (Eugene, OR, USA); and AA, EGTA, probenecid, TEA and TPA were from Sigma. [^{3}H]-AA ([5,6,8,9,11,12,14,15-^{3}H]-AA) and [^{3}H]-oleic acid ([9,10-^{3}H]-oleic acid) were from New England Nuclear Corp. GesmbH (Wien, Austria).

Results

Orexin receptor stimulation strongly stimulates ^{3}H-AA release

In order to measure PLA_2 activity, we loaded CHO cells with ^{3}H-AA and measured the released ^{3}H-radioactivity. In the classical methodology, the released ^{3}H-AA is sequestered by adding BSA to the medium (see Mounier *et al.*, 2004). However, as the usual BSA contains fatty acids, and even the commercial 'fatty acid-free' BSA is seldom devoid of fatty acids (our unpublished observations), the addition of BSA may induce cell responses by itself. We thus first evaluated the necessity of including BSA in the assay. As seen in Figure 1A and B, both the basal and the orexin-A-stimulated releases were higher in the presence of BSA. As a very robust release was seen with orexin-A even in the absence of BSA, we decided to perform the rest of the experiments without BSA. Independent of the presence of the BSA, a much weaker orexin-stimulated release of radioactivity was observed from ^{3}H-oleic acid-loaded cells than from ^{3}H-AA-loaded cells (Figure 1A and B), indicating that this orexin receptor-stimulated enzyme activity prefers AA over oleic acid (for instance, $cPLA_2\alpha$ [GIVA] or $iPLA_2\zeta$ [GVIE] (Jenkins *et al.*, 2004; Schaloske and Dennis, 2006).

The basal level of release of ^{3}H-AA was 0.44 ± 0.04% of the total incorporated radioactivity, and orexin-A stimulated the release by 4.4 ± 0.5-fold ($n = 21$, $P < 0.001$). The response was concentration-dependently inhibited by the OX_1 receptor antagonist, SB-334867 (Smart *et al.*, 2001) with full inhibition at 10 μM; also, no response was seen in CHO cells not expressing OX_1 receptors (Figure 1C). This confirms that the response was specific for the OX_1 receptor. While the maximum response to orexin-B was equal to the response to orexin-A [101 ± 3% of the response to orexin-A; $n = 3$, $P > 0.05$ (ns)], orexin-A was more potent than orexin-B (apparent $pEC_{50} = 8.9 \pm 0.1$ and 8.4 ± 0.1, respectively; $n = 3$, $P < 0.05$). We also observed clearly biphasic agonist concentration–response curves in each individual batch of cells (Figure 2). Altogether, ^{3}H-AA release occurred much in the same concentration range as the high-potency DAG production by PLD and the high-potency PLC (Johansson *et al.*, 2008).

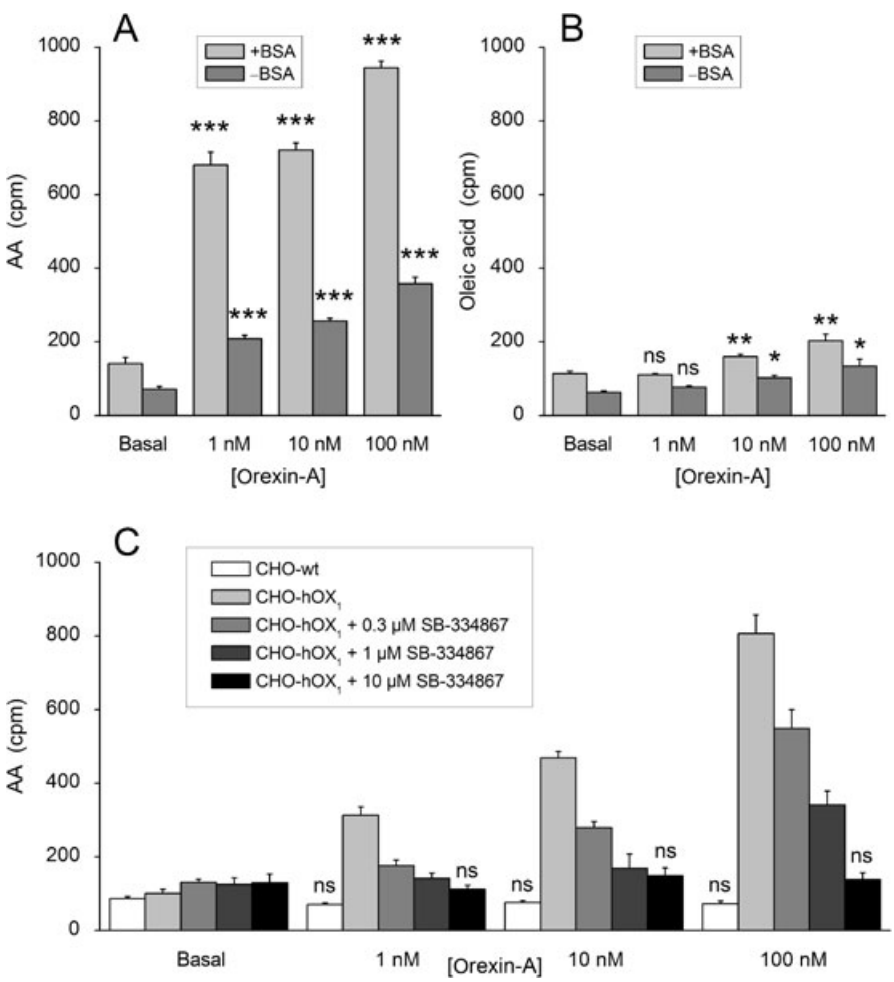

Figure 1 Orexin-A-stimulated ^{3}H-AA (A, C) and ^{3}H-oleic acid (B) release. (A and B) OX_1 receptor-expressing CHO cells (CHO-hOX_1) were used. The release was assessed in NaBM in the presence of 2.4 mg/mL BSA (+BSA) or in the absence of BSA (–BSA). (C) Both wild-type CHO cells (CHO-wt) and CHO-hOX_1 cells were used, both in the absence of BSA. CHO-hOX_1 cells were preincubated for 30 min with SB-334867, changed to fresh NaBM containing the SB-334867 and immediately stimulated with orexin-A. ns (not significant), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; comparisons in all subfigures are to the corresponding basal in C, significances are only calculated for wild-type CHO cells and CHO-hOX_1 with 10 μM SB-334867.

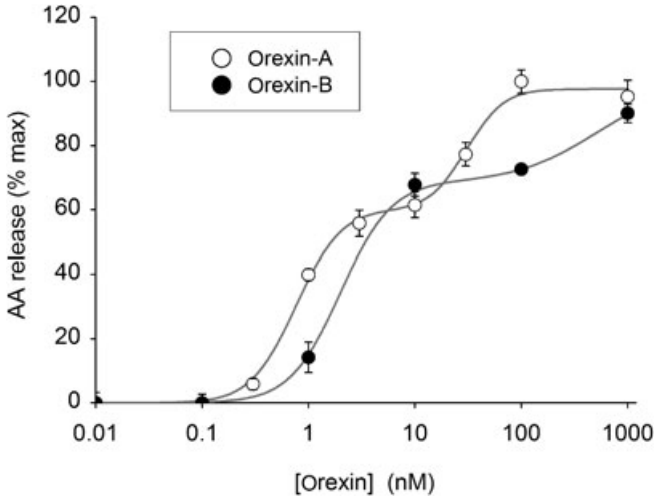

Figure 2 Concentration–response relationships for orexin-A and orexin-B for AA release in CHO-hOX$_1$ cells in NaBM. A two-site fit was significantly better ($P < 0.001$) than a one-site fit for both orexin-A and orexin-B.

Different PLA$_2$ species are involved in the AA release

Traditionally, PLA$_2$, and in particular cPLA$_2\alpha$, has been thought to be the key enzyme in AA release (Leslie, 2004). However, because orexin receptors also activate PLD and PLC, di- and monoacylglycerol lipases might also be involved. We found, here, that MAFP, a potent iPLA$_2$ and cPLA$_2$ inhibitor, fully inhibited the AA release at the concentrations of 3 and 10 μM (Figure 3A). In contrast, the selective iPLA$_2$ inhibitors *R*- and *S*-BEL were significantly less active, and could only inhibit a specific component of the response even at an apparently saturating concentration (Figure 3B). Ten μM was considered to be a saturating concentration, as 25 μM did not produce any stronger inhibition, and as 25 μM of each BEL enantiomer produced a strong decrease in the basal AA release, we decided to use 10 μM. These data together thus indicate that both iPLA$_2$ and cPLA$_2$ are involved in the response. We could not observe any significant difference in the inhibitory potency of either BEL enantiomer (Figure 3B), which could be used as an indication of involvement of either iPLA$_2\beta$ (GVIA-1 or -2) or iPLA$_2\gamma$ (GVIB) (see Discussion and Conclusions). In contrast to MAFP, the effect of *R*- and *S*-BEL varied between different batches of cells (see, for instance, the size of the error bars for 3 μM *R*- and *S*-BEL in Figure 3B), indicating that the expression of the BEL-inhibited component may thus vary. In conclusion, several PLA$_2$ species seem to be involved in the orexin-induced AA release, which could offer one explanation to the apparent biphasic concentration–response curves.

Ca^{2+} dependence of the AA release

The influx of Ca^{2+} is the most characteristic consequence of orexin receptor activation (Lund *et al.*, 2000; Kukkonen and Åkerman, 2005), and it may mediate/amplify orexin receptor-mediated signals at different levels (Ammoun *et al.*, 2006a; Johansson *et al.*, 2007). In addition, cPLA$_2$, based on the inhibitor activity of MAFP, may be the most likely candidate to mediate a part of the AA release response. We thus evaluated the role of Ca^{2+} influx in this response. Reduction of extracellular Ca^{2+} to 30 μM, from the normal 1 mM, abolished the AA release (Figure 4A). Ca^{2+} influx can be greatly decreased by depolarizing the cells with high-K$^+$ medium (reduction of the driving force for Ca^{2+} entry; Larsson *et al.*, 2005; Johansson *et al.*, 2007). Upon exposure of the cells to such medium (KBM), AA release was eliminated at 1 nM orexin-A and markedly inhibited at 100 nM orexin-A (Figure 4B). TEA has been suggested to strongly, although not fully, inhibit the receptor, but not the store-operated Ca^{2+} influx (Larsson *et al.*, 2005; Johansson *et al.*, 2007). In our experiments, TEA (TEA-BM; 70 mM TEA), strongly inhibited AA release at 1 nM orexin-A, but less at 100 nM orexin-A (Figure 4B). In contrast, the store-operated Ca^{2+} influx blocker SKF-96365 (10 μM) (Johansson *et al.*, 2007) only weakly inhibited AA release both at 1 and 100 nM orexin-A (Figure 4B).

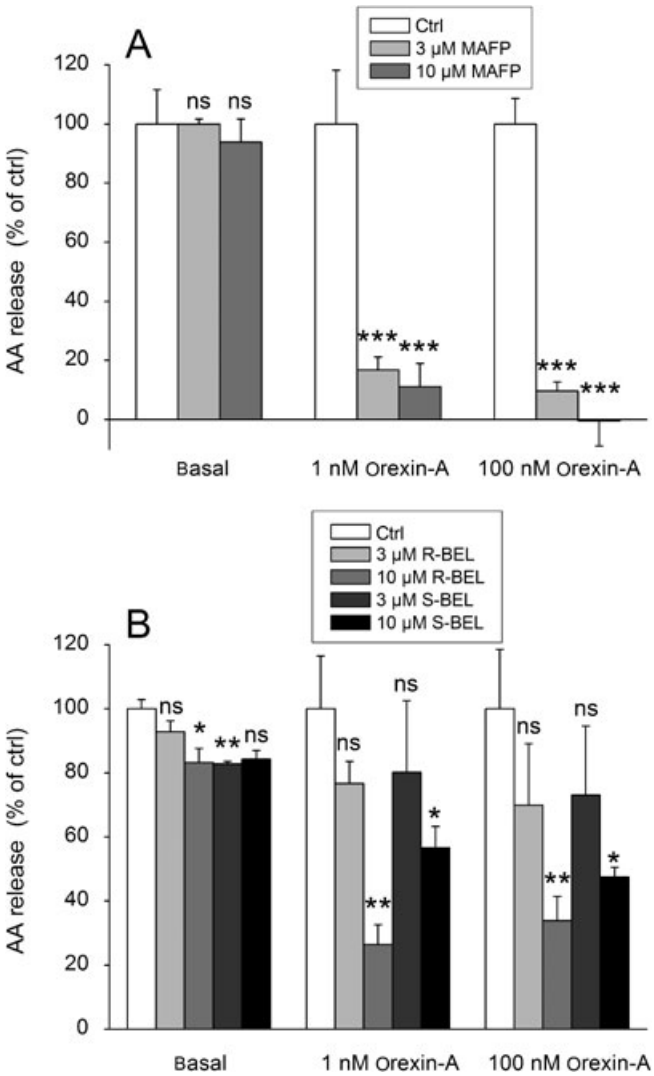

Figure 3 The effect of the PLA$_2$ inhibitors MAFP (A) and *R*- and *S*-BEL (B) on the orexin-A-stimulated AA release in CHO-hOX$_1$ cells. The cells were preincubated for 30 min with the inhibitor, changed to fresh NaBM containing the inhibitor and immediately stimulated with orexin-A. The responses are normalized so that each control response (basal, 1 nM orexin-A, 100 nM orexin-A) amounts to 100% (see Data analysis); ns (not significant), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

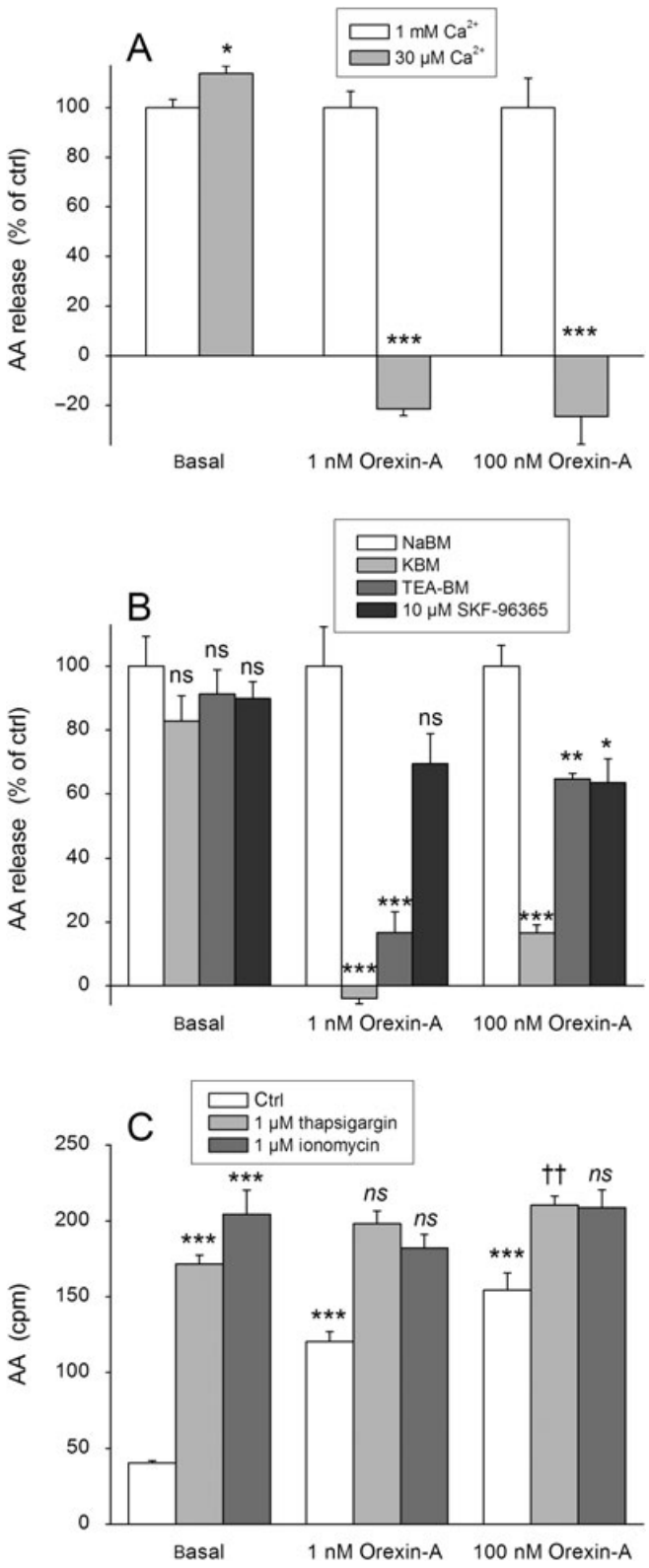

Figure 4 The effect of Ca^{2+} on AA release in CHO-hOX_1 cells. (A) The cells were stimulated with orexin-A in the normal extracellular concentration of Ca^{2+} (1 mM) or in the reduced concentration (30 μM) in NaBM. Data are normalized as explained in Data analysis and Figure 3. (B) The cells were, before stimulation with orexin-A, exposed to the treatments that reduce the driving force for Ca^{2+} entry (KBM) or inhibit particular Ca^{2+} channel types [TEA-BM (70 mM TEA), SKF-96395 (10 μM)]. The cells were preincubated for 5 min in the presence of the specific medium/inhibitor before stimulation with orexin-A in the same medium. Data are normalized as explained in Data analysis and Figure 3. (C) The cells were stimulated with orexin-A in the absence (ctrl) or presence of the Ca^{2+}-elevating compounds thapsigargin or ionomycin in NaBM. The first comparison (***) is to the basal (i.e. Do thapsigargin, ionomycin and orexin-A stimulate AA release over the basal?) and the second comparison (†† and ns) to thapsigargin or ionomycin (i.e. Does orexin-A stimulate AA release over thapsigargin or ionomycin?); ns (not significant), $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. ††$P < 0.01$.

Based on these results, intracellular Ca^{2+} elevation, more specifically Ca^{2+} influx, would be a strong candidate to mediate orexin receptor-induced AA release. We therefore exposed the cells to the Ca^{2+}-elevating agents thapsigargin and ionomycin. Thapsigargin, by inhibiting the endoplasmic/sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), causes a modest Ca^{2+} release and a sustained Ca^{2+} influx via the store-operated Ca^{2+} channels (Ammoun *et al.*, 2006a). The Ca^{2+} ionophore ionomycin, at high concentration, makes all the cellular membranes Ca^{2+} permeable, thus elevating the Ca^{2+} concentration in all cellular compartments to the level of the extracellular medium. Thus, thapsigargin may induce a more modest and localized Ca^{2+} elevation than ionomycin. Both thapsigargin and ionomycin (both at 1 μM) strongly activated AA release in CHO cells (Figure 4C). Orexin-A only very weakly stimulated Ca^{2+} release in the presence of thapsigargin, but not ionomycin.

ERK pathway, but not PKC, is involved in AA release

Phosphorylation has been identified as one major mechanism for $cPLA_2$ and $iPLA_2$ activation, and PKC and ERK have often been implicated in this process. Here, we have used an inhibitor of both conventional and novel PKC, GF109203X (1 μM) (see e.g. Holmqvist *et al.*, 2005; Ammoun *et al.*, 2006a), and found it did not inhibit the orexin receptor-induced AA release (Figure 5A). TPA (1 μM), an activator of these same PKC subfamilies, did not stimulate AA release (Figure 5B). It can thus be concluded that members of the conventional or novel subfamilies of PKC are unlikely to be involved in the orexin-induced AA release. In contrast, the MEK1/2 (MAPK/ERK kinase 1/2) inhibitors U0126 (10 μM) and PD98059 (100 μM) strongly inhibited AA release (Figure 5A), suggesting that the ERK pathway is partly involved in this response. The inhibition was clearly stronger at 1 nM orexin-A than at 100 nM.

Modulation of Ca^{2+} signalling by AA

Ca^{2+} influx and release are central orexin receptor responses, and we thus wanted to investigate the putative interaction between AA and Ca^{2+} responses. To optimally target different components of the Ca^{2+} signal (see Ekholm *et al.*, 2007), orexin-A concentrations of 0.3, 3 and 30 nM were used. The receptor-operated Ca^{2+} influx can be specifically investigated when the inositol-1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} release is inhibited by over-expression of the IP_3-hydrolyzing

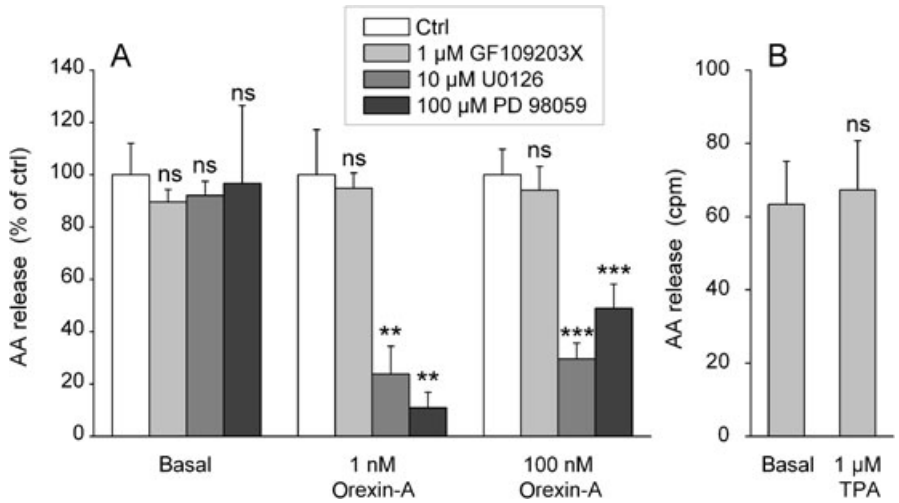

Figure 5 The effect of PKC and ERK activation on AA release in CHO-hOX_1 cells. (A) Cells were preincubated for 30 min with the MEK inhibitors U0126 and 98059 as indicated, changed to fresh NaBM containing the inhibitor and immediately stimulated with orexin-A. Data are normalized as explained in Data analysis and Figure 3. (B) Cells were stimulated with 1 mM TPA for 7 min in NaBM. ns (not significant), $P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

enzyme IP_3-5-phosphatase type 1 (IP_3-5P1) (Ekholm *et al.*, 2007). Under these conditions, MAFP (3 μM) significantly reduced the number of cells responding with Ca^{2+} elevation (Figure 6A), and this effect was also clearly seen in the marked reduction of the average cell response (Figure 6C). Pure Ca^{2+} release-dependent Ca^{2+} responses can be investigated in isolation when Ca^{2+} is excluded from the bathing medium (no influx possible). Under these conditions, MAFP did not affect the cell responses, that is, the number of cells responding (Figure 6B) or the response magnitude (Figure 6D). These data thus strongly suggest that AA or its metabolites contribute to the orexin receptor-mediated Ca^{2+} influx, but not Ca^{2+} release.

Discussion and conclusions

We have shown here that activation of OX_1 orexin receptors results in a marked release of AA, and the inhibitor profile suggests that PLA_2 enzymes are responsible for this response. The orexin concentration–response curves and the inhibitor data suggest that more than one pathway regulates AA release. AA release observed links strongly to the Ca^{2+} signalling of the orexin receptors, much in the line of evidence from other responses such as ERK, adenylyl cyclase and PLC (Ammoun *et al.*, 2006b; Johansson *et al.*, 2007). On the other hand, AA released (or its metabolites) apparently itself modulates the Ca^{2+} influx.

Generally, AA is thought to be released from glycerophospholipids by PLA_2 enzymes. That PLA_2 would be responsible for the AA release upon orexin receptor activation is supported by the inhibitor experiments. MAFP, an inhibitor of both $cPLA_2$ and $iPLA_2$, efficiently inhibited AA release. The $iPLA_2$-selective inhibitors *R*- and *S*-BEL induced only partial inhibition of the response. This suggests that both $cPLA_2$ and $iPLA_2$ may contribute to the AA release. *R*- and *S*-BEL show concentration-dependent selectivity for $iPLA_2\gamma$ and -β, respectively (Jenkins *et al.*, 2002). We could not observe any significant selectivity, which may indicate that both γ- and β-isoforms are involved. However, it should be noted that the potencies or activities of the BEL enantiomers for other $iPLA_2\beta$ splice variants or other $iPLA_2$ isoforms are not known. BEL enantiomers may not only show different affinities for different $iPLA_2$ isoforms, but they may also display partial inhibition (Jenkins *et al.*, 2002), which can complicate the analysis in a native cell putatively expressing several $iPLA_2$ isoforms, like the cells in the current study. Finally, it should be noted that no PLA_2 inhibitor shows absolute selectivity for PLA_2 only, but may inhibit other lipid-metabolizing enzymes as well (see Akiba and Sato, 2004; Burke and Dennis, 2009). Therefore, although it seems rather likely that PLA_2 species are primarily responsible for the AA release in response to orexin receptor stimulation, further studies are needed to exclude other possible enzymes. Under any circumstance, the biphasic orexin concentration–response curves, and the incomplete inhibition of the orexin response by the inhibitors of $iPLA_2$ (BEL) and ERK suggest that several enzyme species are involved in the AA release response.

Ca^{2+} influx seems to hold an essential role in the AA release: when either the extracellular Ca^{2+} concentration or the driving force for Ca^{2+} entry was reduced, AA release was abolished. We utilized the previously devised approach to target the Ca^{2+} influx pathways (receptor- and store-operated influx) by the inhibitors TEA and SKF-96365, respectively (Johansson *et al.*, 2007). These results suggest that the receptor-operated pathway is most important at 1 nM orexin-A and less than at 100 nM, while the store-operated pathway plays an altogether less significant part. This is remarkably similar to the regulation of PLC (Lund *et al.*, 2000; Johansson *et al.*, 2007) A key question, though, is whether Ca^{2+} influx mediates the AA release response or if it rather plays a more permissive role in orexin receptor signalling, as has been suggested for the ERK, adenylyl cyclase and PLC responses (Ammoun *et al.*, 2006b; Johansson *et al.*, 2007). There is, however, one important difference between the AA release observed in the current

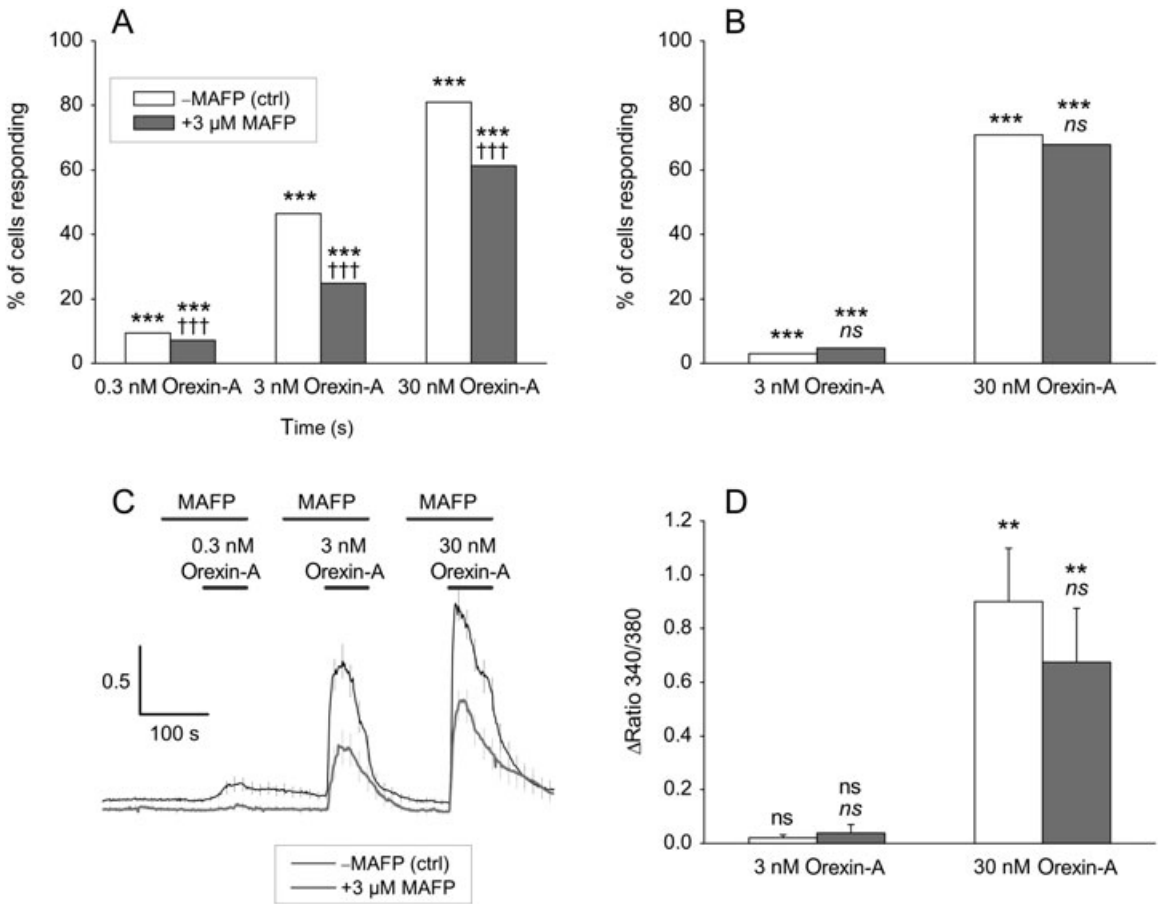

Figure 6 The effect of AA release inhibition on orexin-induced receptor-operated Ca^{2+} influx (A, C) and Ca^{2+} release (B, D) in CHO-hOX_1 cells in NaBM. (A, C**)** Cells are over-expressing IP_3-5P1, which precludes Ca^{2+} release. In (A) data from counting of responsive and non-responsive cells (n = 458–496 cells/treatment) and in (C) a representative experiment (n = 29–31 cells/trace). Some error bars (C) are omitted for purpose of clarity. (B, D) Cells were stimulated in nominally Ca^{2+}-free conditions (no $CaCl_2$ added to the NaBM), which gives an extracellular [Ca^{2+}] ≈ 2.5–3.3 μM as measured using CalciumGreen 5N, which is low enough concentration to abolish the Ca^{2+} influx. In (B) data from counting of responsive and non-responsive cells (n = 551–577 cells/treatment) and in (D) the average Ca^{2+} responses from these cells. The first comparisons (*/**/***) in (A, B and D) are to the basal (i.e. Does orexin cause a Ca^{2+} response?) and the second comparison (††† and ns) to the corresponding orexin response (i.e. Does MAFP inhibit the response to orexin-A?); ns (not significant), $P > 0.05$; **$P < 0.01$; ***$P < 0.001$, †††$P < 0.001$.

study and the activation of ERK, adenylyl cyclase and PLC: while the latter are only weakly stimulated by cytoplasmic Ca^{2+} elevation alone, that is, by thapsigargin or ionomycin (Ammoun *et al*., 2006b; Johansson *et al*., 2007), AA release was strongly stimulated by these Ca^{2+}-elevating compounds. This supports the possibility that orexin receptor-induced Ca^{2+} influx could also directly activate AA release via $cPLA_2$. This, however, would be contradictory to the BEL data, which suggest involvement of $iPLA_2$ in this response as well, because Ca^{2+} may rather regulate $iPLA_2$ in a negative manner (Jenkins *et al*., 2001; reviewed in Akiba and Sato, 2004). It would, though, be possible that Ca^{2+} influx activates $iPLA_2$ indirectly. With the help of pharmacological inhibitors and activators, we could show that ERK, but not PKC, is likely to be involved in AA release. ERK is very strongly activated by OX_1 receptors in CHO cells possibly with both cytosolic and nuclear targeting (Milasta *et al*., 2005; Ammoun *et al*., 2006a), and thus with different molecular targets (Shenoy and Lefkowitz, 2005). The ERK pathway constitutes one possible candidate target for Ca^{2+} influx-stimulated AA release. As pointed out earlier, ERK is only modestly activated by Ca^{2+} influx alone, as compared to the strong response triggered by orexin receptor activation (Ammoun *et al*., 2006a). However, this finding does not exclude the possibility of ERK being involved in AA release, and the levels of active ERK required for this may be much lower than the maximal. In summary, we conclude that the target(s) of Ca^{2+} influx and its *modus operandi* in AA release remain unresolved to date, and further experiments are thus needed.

What then could be the role of AA release in orexin receptor signalling? In this study, we observed a very marked inhibition of the receptor-operated Ca^{2+} influx when AA release was blocked by MAFP. This effect was clearly stronger than the inhibition obtained previously with the specific dominant-negative TRPC channels in CHO cells (Larsson *et al*., 2005). As exogenous AA did not appear to cause a Ca^{2+} influx response (our unpublished experiments) and there is, to our knowledge, no previous report of AA-induced Ca^{2+} influx in CHO cells, it may be that AA (or a metabolite of it) is a supporting factor in orexin receptor signalling to the receptor-operated

Ca^{2+} influx. Interestingly, as Ca^{2+} influx, on the other hand, seems to be a key requirement for AA release, Ca^{2+} influx and AA release could constitute a positive feedback loop. However, although the release of AA was the parameter measured in this study, it is possible that another product of the phospholipase reaction, such as another fatty acid or a lysophospholipid, is more relevant for orexin receptor signalling to receptor-operated Ca^{2+} channels.

The concentration–response curves for orexin-A and -B showed untypical and unexpected features. First, the shape was significantly biphasic. In a recombinant cell system, as we have used here, it seems likely that the high and low potency components are generated by different signal cascades. Our previous studies suggest that orexin receptor responses in CHO cells may be put in a potency order of PLD > high potency PLC ≈ Ca^{2+} influx > low potency PLC > G_s (Lund *et al*., 2000; Holmqvist *et al*., 2005; Johansson *et al*., 2007). Thus, the ability to activate different signal cascades at different agonist concentrations seems characteristic for orexin receptors. The reason for this is unknown, but processes such as affinity differences of activated receptors for particular G-proteins and preferential localization in signal complexes may be involved. We hope to resolve the factors governing AA release and also other determinants of the wide concentration–response range of orexin receptors in our future studies.

Another feature attracting attention was the weak preference for orexin-A over orexin-B, that is, twofold, that we have found in the present experiments, compared to the 10- to 100-fold preference expected (see Sakurai *et al*., 1998; Holmqvist *et al*., 2002; Ammoun *et al*., 2003). As recently shown, rank order of agonist efficacies may not be fixed for different responses due to ligand-selective receptor conformations (Kenakin, 1995; 2003; Kukkonen *et al*., 2001; Kukkonen, 2004). In the present context, orexin-A and -B potencies have mainly been determined with respect to Ca^{2+} elevation and PLC activation in recombinant cells. Nevertheless, orexin-A and orexin-B have been used in native tissues to determine the involvement of OX_1 or OX_2 receptors, a practice we have tried to discourage (Kukkonen *et al*., 2002). The results of the present study would strongly support caution in using orexin-A and -B for such 'diagnostic' purposes. Our data may also lead to re-evaluation of the physiological role of orexin-B. The OX_1 receptor has been proposed to mediate signals only from orexin-A under physiological conditions, but the current results suggest that – at least selected – signals from orexin-B are also transduced. This calls for investigations of orexin-B with respect to many other OX_1 receptor responses and of the molecular players involved. For instance, do orexin-B-activated OX_1 receptors show a G-protein activation profile different from that of orexin-A-activated OX_1 receptors?

In conclusion, the results presented here show that OX_1 orexin receptor activation results in robust AA release. The inhibitor profile strongly suggests that this response is mediated by PLA_2, and PLA_2 thus seems to join the lipid signalling systems activated by orexin receptors, of which PLD and PLC and (indirectly) phosphatidylinositol-3-kinase (PI3K) have previously been identified (Lund *et al*., 2000; Randeva *et al*., 2001; Ammoun *et al*., 2006a; Johansson *et al*., 2008). The response occurs with a rather high potency, although not quite equal to the recently described PLD response (Johansson *et al*., 2008). Accordingly, the PLA_2 pathway could be of high relevance for the orexin receptor signalling in many physiological settings, where the orexin peptide levels are low. Receptor-operated Ca^{2+} influx is required for the AA release, and AA release seems to enhance Ca^{2+} influx, constituting a positive feedback loop. Further experiments are required to confirm the identity of the AA-releasing enzymes and the relationship of this signalling to other lipid signalling systems engaged in orexin receptor signalling.

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