Research Article

Multiple phospholipase activation by OX_1 orexin/hypocretin receptors

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Abstract. We investigated coupling of OX_1 receptors to phospholipase activation and diacylglycerol generation in Chinese hamster ovary (CHO) cells using both biochemical and fluorescence "real-time" methods. The results indicate that at lowest orexin-A concentrations (highest potency), diacylglycerol generated results from phospholipase D activity. At 10–100-fold higher orexin-A concentrations, phospholipase C is activated, likely hydrolyzing phosphatidylinositol (PI) or phosphatidylinositol monophosphate (PIP) but not phosphatidylinositol bisphosphate (PIP_2). At further 7-fold higher orexin-A concentrations, PIP_2 is hydrolyzed, releasing both diacylglycerol and inositol-1,4,5-trisphosphate. Thus, OX_1 orexin receptors connect to multiple phospholipase activities, apparently composed of at least one phospholipase D and two different phospholipase C activities. At low agonist concentrations, diacylglycerol and phosphatidic acid are the preferred products, and interestingly, it seems that even the primarily activated phospholipase C mainly works to increase diacylglycerol and not inositol-1,4,5-trisphosphate.

Keywords. Orexin, hypocretin, phospholipase C, phospholipase D, phosphoinositides, phosphatidylcholine, diacylglycerol, live cell imaging.

Introduction

Phospholipid-derived messengers are central biological signal substances. Phospholipid hydrolyzing enzymes, phospholipase A, C and D (PLA, PLC and PLD, respectively) release fatty acids, diacylglycerol (DAG), inositol phosphates and phosphatidic and lysophosphatidic acid, which either act directly as messengers or can be converted to such *via* action of, for example, kinases/phosphatases. Also normal membrane constituents, most prominently phospha-tidylinositol-4,5-bisphosphate (PIP_2), act as messengers, in this case as a regulator of ion channel function [1].

Phospholipase activity is regulated by many intracellular signals such as Ca^{2+}, heterotrimeric and monomeric G-proteins and tyrosine phosphorylation, and therewith by many plasma membrane receptor signaling systems. One of the most classic G protein-coupled receptor (GPCR) responses is PLC activation, which results in the hydrolysis of phosphoinositides and subsequent generation of intracellular signal molecules, such as DAG and water-soluble inositol (poly) phosphates [especially the Ca^{2+}-releasing second messenger inositol-1,4,5-

trisphosphate (Ins-1,4,5-P$_3$)]. The PLC family includes subfamilies PLCβ, -γ, -δ, -ε, -ζ and -η, each with divergent regulatory properties, in addition to the common requirement for Ca^{2+} for activity (reviewed in [2–4]). Although GPCRs most obviously signal to PLCβ, all PLC families can, at least theoretically, respond to GPCR signaling. Consequently, GPCR signaling also to PLCγ [5, 6], -δ [7–9] and -ε [10, 11] has been shown. DAG is a central lipid messenger activating ion channels and membrane targeting/activating signaling enzymes such as protein kinase C (PKC) species (reviewed in [12, 13]). Beside PLC, other cellular signaling systems, e.g., sphingosine synthase and PLD, are well capable of producing DAG. While the role of sphingosine synthase in GPCR or other receptor signaling remains elusive, PLD may be a significant effector for receptors (reviewed in [4, 14]). PLD breaks down phosphatidylcholine (PC) to choline and phosphatidic acid, the latter of which is a substrate for phosphatidic acid phosphohydrolase producing DAG (reviewed in [15, 16]).

Orexin receptors, OX$_1$ and OX$_2$ receptors, are recently discovered GPCRs (reviewed in [17]). The physiological agonists for these receptors are orexin-A and orexin-B. Orexins and orexin receptors are found both in the central and peripheral nervous systems and in peripheral organs, and orexins are thought to act both as synaptic, extrasynaptic, paracrine and endocrine mediators. At the cellular level, orexin receptor signaling is very versatile (reviewed in [17, 18]). Most characteristic for orexin receptors is Ca^{2+} signaling, which is seen in all cells expressing orexin receptors endo- or exogenously where this has been investigated. In essentially all cases, connection to a Ca^{2+} release-independent Ca^{2+} influx pathway is also seen (recombinant cells, primary neurons). Ca^{2+} signaling *via* the classic PLC-mediated Ca^{2+} release (PIP$_2$ → Ins-1,4,5-P$_3$ → Ca^{2+} release) is also potently stimulated by orexin receptors. Indirect evidence from the extracellular signal-regulated kinase (ERK) pathway also suggests activation of protein and lipid kinases [PKC, Src, phosphoinositide-3-kinase (PI3K)] and monomeric G-proteins (Ras) in OX$_1$ receptor signaling [19]. In OX$_1$ receptor-expressing Chinese hamster ovary (CHO) cells, where the Ca^{2+} signaling has been investigated in detail, we have recently isolated the receptor-operated Ca^{2+} influx pathway using electrophysiology [20]. In these cells, low concentrations of orexin-A activate this Ca^{2+}-permeable channel and apparently amplify the PLC activity [21]. At higher orexin-A concentrations, PLC is activated in a manner independent of the Ca^{2+} influx. This could be interpreted to mean that different PLC isoforms could be activated at low and high orexin-A

concentrations. Interestingly, PLC-regulated lipids PIP$_2$ and DAG are potent regulators of many receptor-operated transient receptor potential (TRP) channels. In this study, we wanted to focus on the PLC activities seen upon OX$_1$ receptor activation in CHO cells, and at the same time evaluate different methods of measurement. The results suggest that low concentrations of orexin-A indeed trigger a PLC activity different from that triggered at high orexin concentrations, as distinguished based on different substrate specificity. At very low orexin concentrations, DAG is produced by orexin receptor-activated PLD activity.

Materials and methods

Cell culture. CHO-OX$_1$ cells [22] were grown in Ham's F-12 medium (Gibco, Paisley, UK) supplemented with 100 U/ml penicillin G (Sigma Chemical Co., St Louis, MO), 80 U/ml streptomycin (Sigma), 400 μg/ml geneticin (G418; Gibco) and 10 % (v/v) fetal calf serum (Gibco) at 37 °C in 5 % CO$_2$ in an air-ventilated humidified incubator in 260-ml plastic culture flasks (75 cm^2 bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany). For the total inositol phosphate generation experiments, the cells were cultivated on 24-well plates (1.77 cm^2 well bottom area; Greiner Bio-One). For imaging, the cells were grown on uncoated circular glass coverslips (diameter 13 or 25 mm; Menzel-Gläser, Braunschweig, Germany) and for all other experiments on circular plastic culture dishes (8.3 or 56 cm^2 bottom area; Greiner Bio-One).

Chemicals. Human orexin-A was from Neosystem (Strasbourg, France) and [^{3}H]*myo*-inositol (PT6–271 TRK911) and D-*myo*-inositol-1,4,5-trisphosphate – [^{3}H]Biotrak Assay (TRK1000) from Amersham Biosciences (Buckinghamshire, UK). D609 [tricyclodecan-9-yl-xanthate or *O*-(octahydro-4,7-methano-1H-inden-5-yl) carbonopotassium dithioate] was from Tocris Cookson Ltd (Avonmouth, UK)

Media. 2-{[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]amino} ethane sulfonic acid (TES)-buffered medium (TBM) consisted of 137 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1.2 mM MgCl$_2$, 0.44 mM KH$_2$PO$_4$, 4.2 mM NaHCO$_3$, 10 mM glucose, and 20 mM TES adjusted to pH 7.4 with NaOH.

Expression vectors. pEGFP-C1-PLCdelta-PH (here referred as GFP-PH-PLCδ1) [23] was from Prof. Tobias Meyer (Stanford University School of Medicine, Stanford, CA), pEGFP-N1-PKCα-C1 (here referred as GFP-C1-PKCα) [24] from Dr. Christer

Larsson (Lund University, Lund, Sweden) and the PLD plasmids pCGN-hPLD1-K898R [dominant-negative (catalytically inactive) human PLD1] and pCGN-mPLD2-K758R [dominant-negative (catalytically inactive) mouse PLD2] [25] from Prof. Mark Frohman (University Medical Center at Stony Brook, Stony Brook, NY).

Transfection. Transfection of the cells was performed to introduce "green fluorescent protein (GFP) probes" (GFP-PH-PLCδ1, GFP-C1-PKCα) and dominant-negative PLD constructs to the cells. CHO-OX_1 cells were grown to 40–50% confluence, washed with PBS and transfected in OPTI-MEM (Gibco) using Lipofectamine reagent (Invitrogen Corp., Carlsbad, CA) [19, 21]. After 5 h, this medium was replaced with fresh Ham's F-12 medium with all the usual supplements (see above), and the cells were used 48 h after the initiation of the transfection. The total amount of DNA was kept the same in all transfections using empty plasmids.

Ion exchange separation of inositol phosphates. The cells were cultured on plastic culture dishes (8.3 cm^2) and prelabeled with 3 μCi/ml [^{3}H]inositol for 20 h. The medium was replaced with TBM and the cells incubated for 10 min, after which orexin-A was added. The experiments were terminated 10 s later by replacement of the medium with ice-cold 0.4 M perchloric acid and immediate freezing on dry ice. The thawed supernatants were neutralized with 0.36 M KOH + 0.3 M KHCO$_3$ and the inositol phosphate fractions with different charges were isolated with AG 1-X8-anion-exchange chromatography as in [26]. The elutions were as follows: 20 ml H$_2$O (inositol; discarded), 8 ml 5 mM sodium tetraborate + 60 mM ammonium formate (glycerophosphoinositol; discarded), 20 ml 5 mM sodium tetraborate + 0.15 M ammonium formate [inositol monophosphates (IP$_1$); collected], 18 ml 0.1 M formic acid + 0.4 M ammonium formate [inositol bisphosphates (IP$_2$); collected], 12 ml 0.1 M formic acid + 0.7 M ammonium formate [inositol trisphosphates (IP$_3$); collected], 24 ml 0.1 M formic acid + 1.5 M ammonium formate [inositol tetrakisphosphates (IP$_4$); discarded]. Radioactivity was determined using scintillation counting.

Cumulative inositol phosphate liberation. Membrane phosphoinositides were prelabeled by incubating the cells with 3 μCi/ml [^{3}H]inositol for 20 h in culture medium, after which the cells were washed and incubated in TBM at 37 °C containing 10 mM LiCl (to inhibit inositol monophosphatase) for 10 min. The cells were then stimulated with orexin-A for 2 or 20 min and the reactions were stopped by rapid

removal of TBM, addition of ice-cold perchloric acid and freezing. After thawing, the samples were neutralized, the insoluble fragments were spun down, and the supernatant were subjected to anion-exchange chromatography [19, 21, 27]. The radioactivity of the inositol phosphate fraction was determined by scintillation counting.

Binding-protein measurement of Ins-1,4,5-P$_3$. Cells cultured on plastic culture dishes (56 cm^2) were detached using PBS containing 0.2 g/l EDTA. They were spun down, washed once with TBM and resuspended in TBM, and the cell number was adjusted to 10^7 cells/ml by counting in a Bürker chamber. The determination of Ins-1,4,5-P$_3$ concentration was performed essentially as described in [22]. The cells were kept at 37 °C. For stimulation, orexin-A was added, the cell suspension was rapidly mixed and the reactions were terminated by adding ice-cold perchloric acid to a final concentration of 4% (v/v), vortexing and submersion in ice. The precipitate was sedimented by centrifugation at 2000 g for 15 min at 4 °C. The supernatants were neutralized with 1.5 M KOH + 60 mM HEPES, and 0.5 M Tris-HCl (pH 8.6) was added to a final concentration of 0.1 M (final pH 8.6). The resulting KClO$_4$ sediment was removed by centrifugation at 2000 g for 15 min at 4 °C. The Ins-1,4,5-P$_3$ concentration of the samples was determined using D-*myo*-inositol-1,4,5-trisphosphate – [^{3}H] Biotrak Assay essentially as described for another similar assay kit in [22].

Microfluorometric "real-time" imaging. Microfluorometric imaging was performed to measure translocation of GFP-PH-PLCδ1 and GFP-C1-PKCα. The transfected cells on coverslips were excited at 490 nm and the emission light was collected at 520 nm with TILLvisION v. 4.01 imaging system (TILL Photonics GmbH, Gräfelding, Germany) [28]. The coverslips were constantly perfused with TBM at a rate of 1 ml/min in an ~160-μl perfusion chamber at 37 °C. The additions into the chamber were made by perfusion. The data were analyzed using the TILL software and Microsoft Excel.

Data analysis. Student's two-tailed t-test was used in all pairwise comparisons. Significances are as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Significances are indicated only for the data where the results are not self evident. In the figures, mean $\pm$ SEM is given. Each experiment was performed at least three times, unless specifically indicated. Microsoft Excel was used for non-linear curve-fitting.

Results

Optical and biochemical detection of PLC activity against PIP$_2$ give same results. The PH domain of PLCδ1 (PH-PLCδ1) displays high affinity for only two phosphoinositide derivatives, PIP$_2$ and Ins-1,4,5-P$_3$, and has accordingly been used – fused to green fluorescent protein (GFP-PH-PLCδ1) – to resolve the dynamics of these derivatives [23, 29].

GFP-PH-PLCδ1 fluorescence was mainly located in the membrane of the resting cells, which is expected as there should not be any significant basal PIP$_2$ breakdown. When measured in "real-time", OX$_1$ receptor stimulation with orexin-A caused a rapid and reversible translocation of the GFP-PH-PLCδ1 to the cytosol (Fig. 1A). Translocation was rather stable as long as the cells were stimulated with orexin-A (not shown). The concentration-response relationship of the response corresponded to that measured using the binding-protein assay for Ins-1,4,5-P$_3$ (Fig. 1B, compare empty triangles to filled circles), suggesting that GFP-PH-PLCδ1 offers a rather equal sensitivity and dynamic range of detection. While GFP-PH-PLCδ1 does not separate elevated Ins-1,4,5-P$_3$ concentration from the lowered membrane PIP$_2$ level, in contrast to the binding protein assay, it has the advantage of covering the temporal aspect of PLC activity towards PIP$_2$.

PIP$_2$ is not the only substrate for PLC. Total inositol phosphate measurements similarly indicated strong OX$_1$ receptor-stimulated PLC activity (Fig. 1C). Interestingly, total inositol phosphate production appeared to occur at 15-fold higher potency than the production of Ins-1,4,5-P$_3$. To make the assays more comparable, we performed total inositol phosphate release experiments using a 2-min reaction time instead of the usual 20 min. The shorter assay time shifted the EC$_{50}$ only 1.8-fold to the right as compared to 20-min incubation (see below). Thus, the difference in reaction time does not explain the large difference in the concentration-response curves (7-fold).

To investigate this further, we performed experiments with short stimulation time (10 s) and separated the liberated inositol mono-, bis- and trisphosphates. Short stimulation time should minimize the generation of inositol mono- and bisphosphates as a result of Ins-1,4,5-P$_3$ breakdown. At 1 nM orexin-A, essentially only inositol monophosphates (IP$_1$) were generated, whereas 300 nM orexin-A stimulated generation of both inositol monophosphates (IP$_1$), bisphosphates (IP$_2$) and trisphosphates (IP$_3$) (Fig. 2). Thus, it seems that at low concentrations of orexin-A, primarily PI or phosphatidylinositol-4-phosphate (PIP) instead of PIP$_2$ is used as a substrate by PLC. This would be a
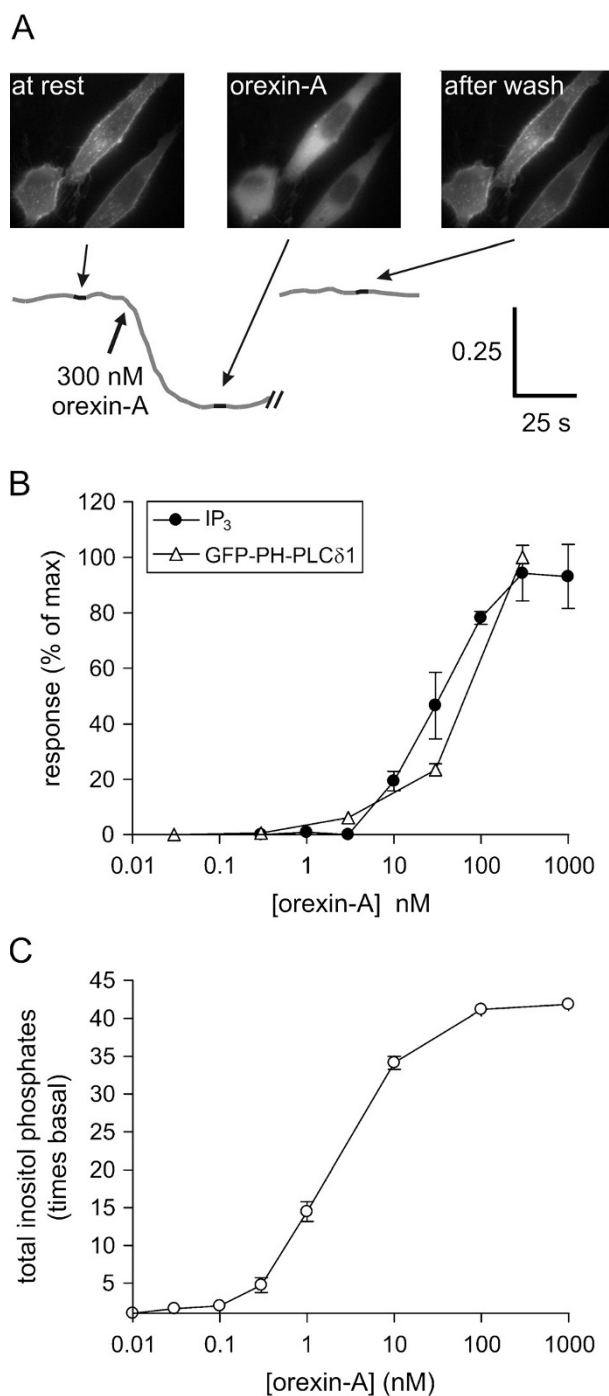
Figure 1. Measurement of orexin-A-stimulated phospholipase C (PLC) activity towards phosphatidylinositol-4,5-bisphosphate (PIP$_2$) (*A*, *B*) and the total phosphoinositide pool (*C*). (*A*) Transiently expressed GFP-PH-PLCδ1 translocates upon OX$_1$ receptor stimulation with orexin-A indicating hydrolysis of PIP$_2$/generation of inositol trisphosphates (IP$_3$). The trace gives the plasma membrane fluorescence divided by the cytosolic fluorescence. Note that the apparently saturated pixels were actually not saturated in the measurements, but this is a result of the contrast enhancement for better visualization. (*B*) IP$_3$ generation was assessed with the binding-protein assay (filled circles; EC$_{50}$ = 35 nM) and by the GFP-PH-PLCδ1 translocation (empty triangles; EC$_{50}$ = 59 nM). (*C*) Total inositol phosphate release was assessed by ion exchange chromatography (EC$_{50}$ = 2.2 nM).
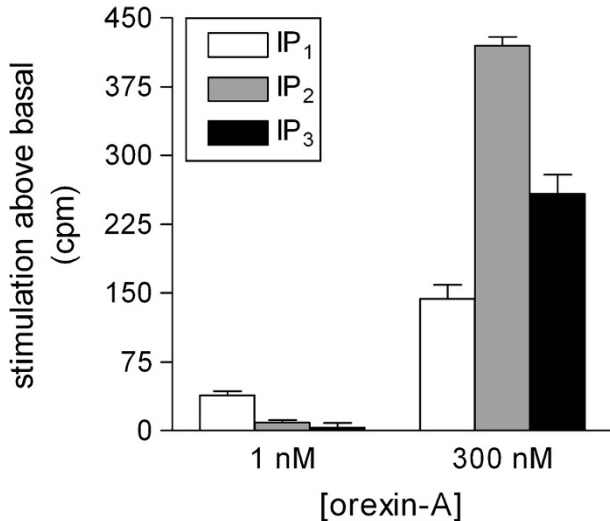
Figure 2. Rapid release of inositol monophosphates (IP$_1$) is seen upon OX$_1$ receptor stimulation with a low concentration of orexin-A. CHO cells were stimulated with 1 nM or 300 nM orexin-A for 10 s followed by stop with ice-cold perchloric acid. Inositol phosphates were separated with ion exchange chromatography.

likely explanation for the fact that at low orexin-A concentrations (0.3–3 nM), no Ins-1,4,5-P$_3$ is produced despite phosphoinositide hydrolysis (Figs 1A, B and see below).

DAG generation. Upon PLC activation, equimolar amounts of DAG and inositol phosphates should be generated. The C1 domains of conventional and novel protein kinase C (PKC) isoforms bind DAG with high affinity and they centrally contribute to the membrane translocation of these PKC family isoforms (see e.g. [30]). Thus, isolated C1 domains fused to GFP have been used to detect DAG [24, 31, 32]; here we used the C1 domain of PKCα (GFP-C1-PKCα; Fig. 3). GFP-C1-PKCα fluorescence was mainly located in the cytosol of the resting cells, which is expected as there should not be any significant basal DAG production. Orexin-A caused a rapid and reversible translocation of the GFP-C1-PKCα to the membrane (Fig. 3A). GFP-C1-PKCα apparently detected two different components in DAG generation, a high-potency minor component (~10 %) and a low-potency major component (~90 %) (Fig. 3B). When the concentration-response relationship of DAG generation (Fig. 4A, empty inverted triangles) is compared to Ins-1,4,5-P$_3$ production (filled circles) and total inositol phosphate release (at 2 min; empty circles), it can be seen that DAG production largely follows total inositol phosphate release at higher orexin concentrations (>3 nM), while at low orexin concentrations DAG production is clearly more efficient than inositol phosphate release (Fig. 4A),
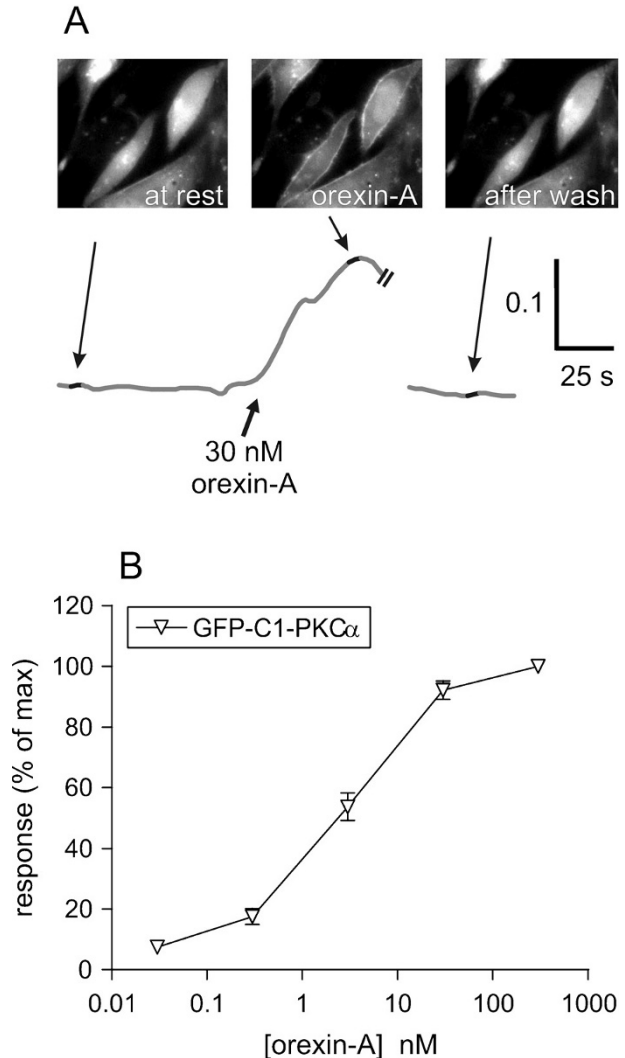
Figure 3. Measurement of orexin-A-stimulated diacylglycerol (DAG) generation. (*A, B*) Transiently expressed GFP-C1-PKCα translocates upon OX$_1$ receptor stimulation with orexin-A indicating generation of DAG. The trace (*A*) gives the plasma membrane fluorescence divided by the cytosolic fluorescence. Note that the apparently saturated pixels were actually not saturated in the measurements, but this is a result of the contrast enhancement for better visualization. (*B*) DAG generation was assessed by quantitation of the GFP-C1-PKCα translocation (EC$_{50-1}$ < 0.1 nM, 10 % of the magnitude; EC$_{50-2}$ = 3.3 nM, 90 % of the magnitude).

owing to the apparent high-potency component of DAG production. It therefore appears likely that the low-potency component of DAG generation originates from the phosphoinositide pool, whereas high-potency component would depend on some other source and likely some other enzyme than PLC.

We have previously shown that PKCδ is responsible for the high-potency cAMP production upon OX$_1$ receptor activation in CHO cells [28]. The PKCδ response corresponds to two apparent components with a higher potency–lower magnitude and lower potency–higher magnitude (Fig. 4B). Interestingly,

A

B
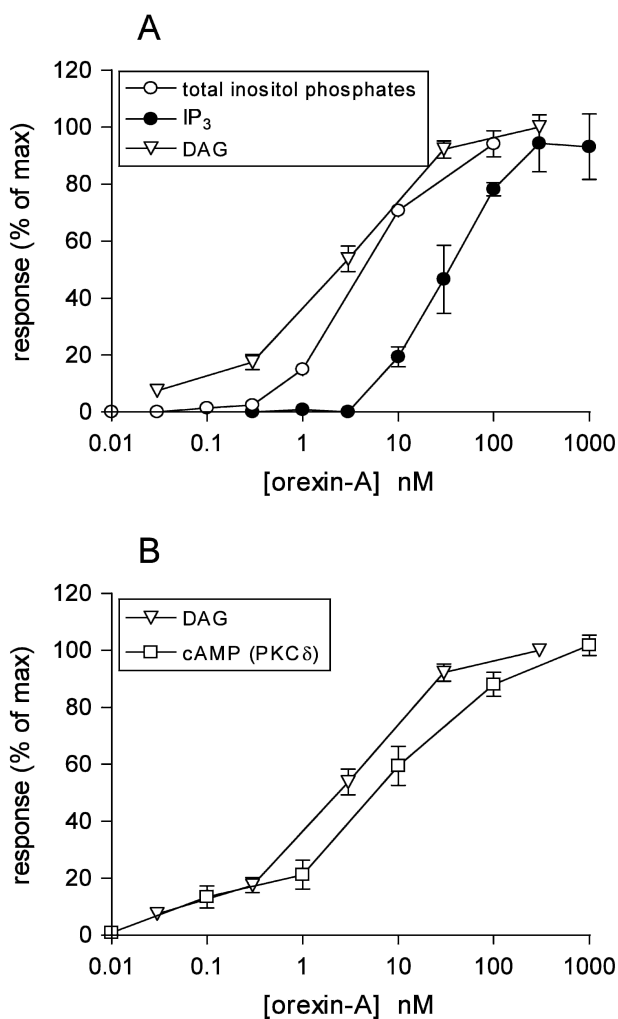
Figure 4. Comparison of orexin-A-stimulated responses. (*A*) Comparison of total inositol phosphate release (2-min stimulation; ion exchange chromatography; empty circles), DAG generation (GFP-C1-PKCα translocation; empty inverted triangles) and IP$_3$ generation (binding-protein assay; filled circles). (*B*) Comparison of DAG generation (GFP-C1-PKCα translocation; empty inverted triangles) and cAMP elevation in the presence of cholera toxin (a fully PKCδ-dependent response; empty squares; EC$_{50-1}$ = 0.045 nM, 16% of the magnitude; EC$_{50-2}$ = 9.9 nM, 84% of the magnitude). The cAMP data are from [28].

the higher potency component of cAMP generation also perfectly overlaps the high-potency component of DAG production (Fig. 4B). It therefore appears likely that the DAG produced at highest potency upon OX$_1$ receptor activation is effective in activating PKCδ. Most obvious candidates for the high-potency DAG production are PLD and PC-specific-PLC/sphingomyelin synthase acting on PC. No inhibition of DAG generation was seen with the PC-specific-PLC/sphingomyelin synthase inhibitor D609 [33] (Fig. 5A). Thus, it seems unlikely that PC-specific-PLC/sphingomyelin synthase would be responsible for DAG generation. In contrast, inhibition of PLD activity with dominant-negative PLD constructs fully reversed the high-potency component of DAG generation (Fig. 5BC), after which DAG generation followed the release of total inositol phosphates (Fig. 5C).

Discussion

The aim of this study was to primarily investigate the PLC signaling of OX$_1$ orexin receptors. When the different methods of measurement of DAG, inositol phosphates and PIP$_2$/Ins-1,4,5-P$_3$ were applied on the pharmacological analysis the OX$_1$ receptor signaling, an interesting picture emerged: total inositol phosphates and DAG were produced by significantly higher potency (lower orexin concentration) than Ins-1,4,5-P$_3$. This is in sharp contrast to the expected equipotent production of Ins-1,4,5-P$_3$, total inositol phosphates and DAG seen in another recent study with other GPCRs [34]. Based on this, it appears likely that a part of the inositol phosphates come from phosphoinositides other than PIP$_2$ (i.e., PI, PIP). This was further supported by direct measurements of release of IP$_1$ at 1 nM orexin without subsequent release of Ins-1,4,5-P$_3$. While inositol phosphate analysis is tricky to perform as the metabolites are readily converted to each other, these two different pieces of information, the difference in orexin-A concentration-response and the detection of IP$_1$ in the absence of IP$_3$ makes it likely that (at least) two different PLC activities follow activation of OX$_1$ receptors, one with high potency and specificity for PI and/or PIP and another with low receptor-coupling potency and specificity for PIP$_2$ (or low specificity for different phosphoinositides). The method used and the high rate of metabolism of inositol phosphates makes the separation of PI and PIP as substrates impossible in the current setting. The identity of each PLC isoform is at present unknown. Previous studies have shown interaction of OX$_1$ receptors with G$_{q/11}$ proteins [35, 36], making PLCβ a likely candidate for one of the PLCs, but GPCRs are likely to be able to activate members of all the other PLC families as well (see Introduction).

PIP$_2$ is generally believed to be the preferred PLC substrate, but this may rather be based on its known role in the generation of the second messenger Ins-1,4,5-P$_3$ than on firm experimental evidence. In some tissues the experimental data can be interpreted to support this, whereas in many other cases the situation is unclear and some studies indicate that even other phosphoinositides (PI, PIP) are hydrolyzed to a significant degree [37–43]. The specificity of individual PLC isoforms is unclear. PI and PIP are abundantly present in membranes to act as potential
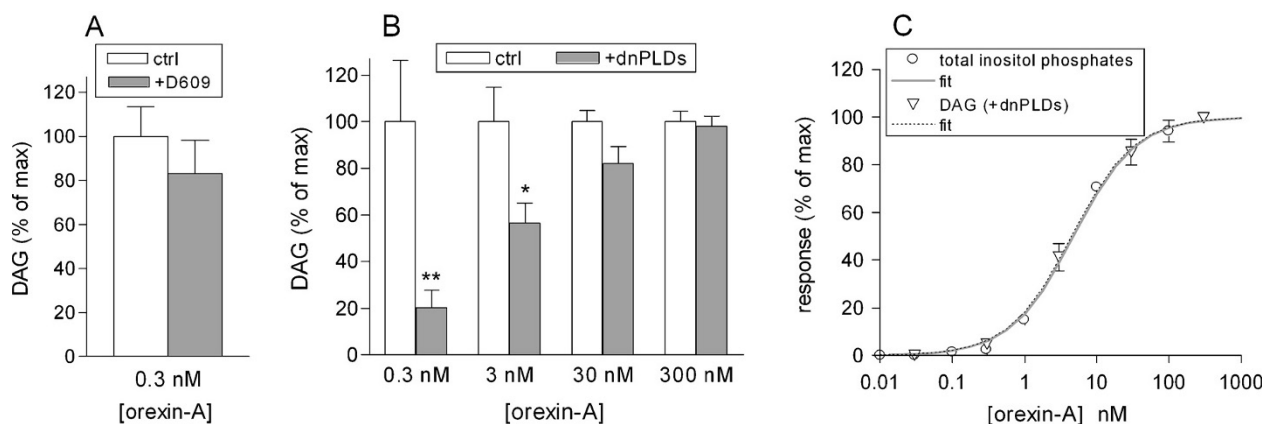
Figure 5. PLD but not phosphatidylcholine (PC)-specific-PLC/sphingomyelin synthase is responsible for the high-potency DAG generation. (*A*) CHO cells transiently transfected with GFP-C1-PKCα were stimulated with 0.3 nM orexin-A in the absence or presence of the PC-specific-PLC/sphingomyelin synthase inhibitor D609 (10 μM, 30-min preincubation). (*B*) CHO cells transiently transfected with GFP-C1-PKCα with (+dnPLDs) or without (ctrl) dominant-negative PLD1 and -2 were stimulated with different concentrations of orexin-A. (*C*) Comparison of the total inositol phosphate release (2 min; empty circles and gray solid line; $EC_{50} = 4.7$ nM) and DAG generation (GFP-C1-PKCα translocation) in the absence of PLD activity (empty inverted triangles and black dotted line; $EC_{50} = 4.5$ nM).

substrates. PI seems to be the dominant species, making up to 80–98% of the total phosphoinositides, while the relative amounts of PIP and PIP$_2$ may vary between different cell types; on average these are approximately equal, making up ~1–10% of the total phosphoinositide pool each [44–49].

While most of the DAG production is likely to result from PI/PIP and PIP$_2$ hydrolysis, the high-potency component of DAG production occurred without a concomitant phosphoinositide hydrolysis. Therefore, this component reflects activity of enzymes other than PLC, i.e., PLD or PC-specific-PLC/sphingomyelin synthase. PC-specific-PLC/sphingomyelin synthase has previously been suggested to be involved in orexin receptor signaling in neurons based on the inhibitory potency of D609 ([50, 51] and reviewed in [18]). D609 is chemically unstable in solution and was therefore always freshly prepared, as in the previous studies. Yet no inhibition of the DAG generation was seen and we thus consider that PC-specific-PLC/sphingomyelin synthase is not involved in DAG generation. PLD has been associated with intracellular signaling of different types of receptors (reviewed in [14]). Beside the known signaling role of phosphatidic acid produced upon PLD activity, a major metabolic pathway leads from phosphatidic acid to DAG (reviewed in [16]). Inhibition of PLD activity with dominant-negative variants produced a full inhibition of the high-potency component of DAG generation. Consequently, DAG production perfectly followed the phosphoinositide hydrolysis when PLD was inhibited. Interestingly, the high-potency component of cAMP production (i.e., activation of PKCδ) [28] perfectly superimposes on the PLD-mediated DAG production, suggesting that the DAG released from PC

efficiently activates PKCδ (Fig. 4B). In contrast, it appears that the DAG released from phosphoinositides is less potent in activating PKCδ (the low-potency components in Fig. 4B). If this was the case, it might depend on the different fatty acid composition between the PC and phosphoinositides (preferably saturated/monounsaturated and polyunsaturated, respectively) [15]. However, the few previous studies suggest that the more saturated DAG species derived from phosphatidic acid are less potent in activating PKC [52–54]. As yet, the role of the fatty acid composition of DAG in OX$_1$ receptor signaling is highly speculative in the current setting, and will be pursued in future studies.

We applied dominant-negative variants of both PLD1 and PLD2 together, and thus the results of this study cannot point out the isoform responsible or the activation pathway. Previously, monomeric G proteins of Rho, Arf and Ras families and ERK, among others, have been involved in GPCR signaling to PLD (reviewed in [14]). In comparison to previous studies, cAMP elevation and ERK activation appear unlikely since these are not substantially activated at the very low orexin-A concentrations where PLD apparently is active [19, 28]. One possible pathway from the receptor could be offered by pertussis toxin-sensitive G$_{i/o}$ proteins, which appear to be activated with very high potency by OX$_1$ receptors [$EC_{50} < 1$ nM (not exactly determined)] [28].

In conclusion, the data suggest that OX$_1$ receptors involve, at least, one PLD and two different types of PLC activities in their signaling (Fig. 6), but the molecular identity or regulatory mechanism of none of the enzymes is currently known. The results have implications both on orexin receptor signaling and on
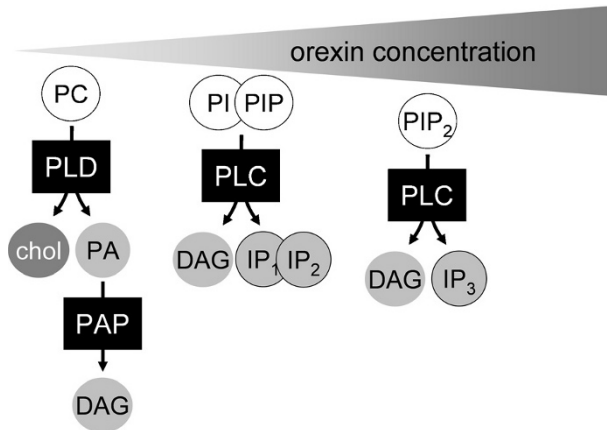
Figure 6. The scheme of phospholipase regulation by OX_1 orexin receptors based on the results of the current study. chol, choline; IP_3, Ins-1,4,5-P_3; PAP, phosphatidic acid phosphohydrolase.

PLC behavior in general. The fact that DAG production by OX_1 receptors is stimulated by PLD at 100–1000-fold lower orexin concentrations than Ins-1,4,5-P_3 production, and also by PLC at 7-fold lower orexin concentrations, suggests that DAG is an important messenger in orexin receptor signaling. We can show that PKCδ is very potently activated by this DAG. Another interesting target candidate are the receptor-operated Ca^{2+} channels that seem to be ubiquitous signal transducers of orexin responses (see Introduction). The data also indicate that some receptor-activated PLC isoforms preferentially hydrolyze PI or PIP (summarized in Fig. 6), and thus DAG may the signal of preference for some PLC isoforms, stipulating further studies on the substrate specificity of PLC isoforms under physiological conditions.

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