ADP-ribosylation Factor-dependent Phospholipase D2 Activation Is Required for Agonist-induced µ-Opioid Receptor Endocytosis*

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Agonist exposure of many G protein-coupled receptors induces a rapid receptor phosphorylation and uncoupling from G proteins. Resensitization of these desensitized receptors requires endocytosis and subsequent dephosphorylation. Using a yeast two-hybrid screen, the rat µ-opioid receptor (MOR1, also termed MOP) was found to be associated with phospholipase D2 (PLD2), a phospholipid-specific phosphodiesterase located in the plasma membrane, which has been implicated in the formation of endocytic vesicles. Coinmunoprecipitation experiments in HEK293 cells coexpressing MOR1 and PLD2 confirmed that MOR1 constitutively interacts with PLD2. Treatment with the µ receptor agonist DAMGO ([d-Ala2, Me Phe4, Glyol5]-enkephalin) led to an increase in PLD2 activity, whereas morphine, which does not induce MOR1 receptor internalization, failed to induce PLD2 activation. The DAMGO-mediated PLD2 activation was inhibited by brefeldin A, an inhibitor of ADP-ribosylation factor (ARF) but not by the protein kinase C (PKC) inhibitor calphostin C indicating that opioid receptor-mediated activation of PLD2 is ARF- but not PKC-dependent. Furthermore, heterologous stimulation of PLD2 by phorbol ester led to an accelerated internalization of the µ-opioid receptor after both DAMGO and morphine exposure. Conversely the inhibition of PLD2-mediated phosphatidic acid formation by 1-butanol or overexpression of a negative mutant of PLD2 prevented agonist-mediated endocytosis of MOR1. Together, these data suggest that PLD2 play a key role in the regulation of agonist-induced endocytosis of the µ-opioid receptor.

Phospholipase D (PLD) is a widely distributed phospholipid-specific diesterase that hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA) and choline and is assumed to play an important function in cell regulation (1, 2). Signal-dependent activation of PLD was demonstrated in numerous cell types stimulated by various hormones, growth factors, cytokines, neurotransmitters, adhesion molecules, drugs, and physical stimuli (reviewed in Ref. 3). Pathways leading to PLD activation include protein serine/threonine kinases, e.g. protein kinase C, small GTPases, e.g. ADP-ribosylation factor (ARF), RhoA and Ral, phosphatidylinositol 4,5-bisphosphate (PIP2), and tyrosine kinases (4–6). Recently two mammalian PLDs (PLD1 and PLD2) have been identified (7–10). Subcellular fractionation studies have demonstrated the presence of PLD1 in intracellular membranes, e.g. ER, Golgi, and vesicular compartment (9, 11), whereas PLD2 was largely associated with the plasma membrane (9). After stimulation with serum, redistribution of PLD2 from the plasma membrane into submembraneous endocytic vesicles (early endosomes) was observed (9). Another study revealed that PLD2 is associated with the EGF receptor (12). Interestingly, EGF receptor endocytosis is impaired when PLD activity is inhibited (13) suggesting a role for PLD2 in receptor trafficking.

Using the yeast two-hybrid system to identify proteins that interact with the µ-opioid receptor, we isolated a rat cDNA encoding for the NH2 terminus of PLD2. We therefore investigated the potential role of PLD2 in the process of agonist-mediated endocytosis of the µ-opioid receptor. We show that PLD2 is constitutively associated with the µ-opioid receptor. Furthermore, we provide evidence that the opioid receptor-mediated activation is ARF-dependent and essential for receptor endocytosis.

MATERIALS AND METHODS

Yeast Two-hybrid Studies—The µ-opioid receptor was subcloned into pGBK7 vector (Clontech, Heidelberg, Germany) and used as bait to screen a rat brain MATCHMAKER cDNA library (Clontech). The yeast two-hybrid screen was carried out according to the protocol of the manufacturer. 280 clones were positive on plates lacking leucine, tryptophan, histidine, and adenine, 6 of which were further confirmed by yeast mating and filterlift assays for β-galactosidase: one encoding for PLD2, two clones encoding for known proteins, and three clones encoding for novel proteins.

Epitope Tagging and Cloning of cDNA—The rat µ-opioid receptor (MOR1) was tagged at the NH2 terminus with the HA epitope tag sequence MYPYNVPYNA using polymerase chain reaction and then subcloned into the pEAK10 expression vector (Edge Bio Systems, Gaithersburg, MD). Dr. S. Ryu (Pohang, South Korea) kindly provided the human PLD1b and PLD2 cDNAs subcloned into pcDNA3.1 expression vector. The PLD2 truncation mutant (nPLD2) expressing only the NH2-terminal amino acids 1–235 of PLD2 lacking the active site motif of the enzyme was constructed by PCR mutagenesis. To introduce a HindIII restriction site, the forward primer 5'-GCC GCC GCC GCG CGC CTG ATG ACC GCG ACC CCT GAG-3' was synthesized. The sequence for the reverse nPLD2-mutagenesis primer introducing a Xhel restriction site and an amber stop codon in amino acid position 235 of the PLD2 gene was 5'-CCA GCC ACC TCT AGA ACC GAC GAT AAC AAA

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CTT-3. The amplified 695-bp NH2-terminal fragment of PLD2 (pNL2) was subcloned in the HindIII and XhoI sites of pcDNA3.1.

Generation of Cell Lines Coexpressing µ-Opioid Receptor and PLD1b, PLD2, or the NH2-terminal Fragment of PLD2 (pNL2)—Cells were first transfected with peak10: MOR1 plasmid containing puromycin resistance using the calcium phosphate precipitation method (14). Stable transfectants were selected in the presence of 1 µg/ml puromycin (Sigma). To generate cell lines coexpressing HAMOR1 and PLD1b, PLD2, or pNL2D, cells were subjected to a second round of transfection using Effectene (Qiagen, Hilden, Germany) and selected in the presence of 1 µg/ml puromycin and 500 µg/ml G418 (Invitrogen). The whole pool of resistant cells was used without selection of individual clones. Receptor expression and PLD1b or PLD2 expression was monitored using receptor ligand binding assays, PLD activity assays, Western blot analysis, and confocal microscopy as described below.

Results

Identification of PLD2 as a µ-Opioid Receptor-interacting Protein by Yeast Two-hybrid Screen—To identify tail-interacting proteins that could modulate endocytosis of rat µ-opioid receptor we used the yeast two-hybrid system (MATCHMAKER GAL4 Two-Hybrid-System 3); (Clontech). A yeast two-hybrid screen of a rat cDNA library using the rat MOR1-tail domain (COOH-terminal amino acids 340–398) as bait led to the finding of a cDNA encoding only the NH2-terminal amino acids 116–226 (representing exons 4–8) of PLD2. This NH2-terminal fragment of the PLD2 harbors a major part of the phosphoinositide-binding Phox homologous (PX) domain (amino acids 63–192) of the enzyme. The interaction between the COOH terminus of MOR1 (fused with Gal4 binding domain as bait protein) and the NH2-terminal domain of PLD2 (fused with the Gal4-activating domain) was verified in a yeast mating and β-galactosidase assay according to the protocol of the manufacturer. As negative control empty Gal4-BD with Gal4-AD-PLD2 and empty Gal4-AD vector with Gal4-BD-MOR1, as well as a fusion of Gal4-BD with human lamin C, which neither forms complexes nor interacts with most other proteins, were used.

MOR1 Stimulates PLD2 Activity in HEK293 Cells—Since PLD2 activation has been previously described for various G protein-coupled receptors, the association of MOR1 and PLD2 indicated that agonist stimulation of the MOR1 might activate the PLD2. Therefore, we stably expressed MOR1 and full-length human PLD2 in HEK293 cells. MOR1 and PLD2 expression was monitored by ligand binding experiments, Western blot, and immunocytochemical analyses. Saturation binding experiments (n = 3–5) revealed no substantial differences between MOR1 and MOR1-PLD2 expressing cells with respect to their affinities (KD) to [3H]DAMGO (1.4 ± 0.3 nM and 1.4 ± 0.4 nM for MOR1 and MOR1-PLD2, respectively) and their numbers of binding sites (Bmax) (1329 ± 399 fmol/mg protein and 938 ± 107 fmol/mg protein for MOR1 and MOR1-PLD2, respectively). We then incubated MOR1-PLD2 expressing HEK293 cells for 2, 5, 10, 20, and 30 min with µ-opioid DAMGO and determined the relative PLD activity. As shown in Fig. 1A, treatment with the µ-receptor selective agonist DAMGO led to a time-dependent increase in the PLD2 activity with a maximum (3-fold increase in activity) after 30 min, whereas an incubation for 30 min with morphine failed to induce activation of PLD2. The observed DAMGO-induced PLD2 activation was opioid receptor-mediated, because it could be completely blocked by the opioid antagonist naloxone. Activation of PKC by PMA also promoted a 4.5-fold increase in PLD2 activity, which was not blocked by naloxone (data not shown). Furthermore, DAMGO-mediated activation of PLD2 could not be blocked by the PKC inhibitor calphostin C (Fig. 1B), indicating that activation of PKC seems not to play a major role in the opioid receptor-induced PLD2 activation in these cells. We therefore examined whether activation of PLD2 by the opioid receptor involves ARF-GTP proteins. Fig. 1B shows that DAMGO-mediated activation of PLD2 was completely blocked by the ARF inhibitor brefeldin A indicating that PLD2 activation by the µ-opioid receptor is ARF-dependent. BFA had no effect on the PMA-induced PLD2 activation (data not shown).

In MOR1 and human PLD1b coexpressing HEK293 cells, PLD1b activity was stimulated by PMA treatment, whereas DAMGO incubation did not lead to an increase in the PLD1b activity (Fig. 1C) indicating that the opioid receptor specifically activates PLD2.

MOR1 Interacts with PLD2 and ARF in HEK293 Cells—To analyze an interaction between MOR1, PLD1b, PLD2, and ARF1 in HEK293 cells, we carried out coimmunoprecipitation studies. Expression of HAMOR1, PLD1b, PLD2, and ARF was examined by directly immunoblotting lysates from these cells with specific antibodies against HA tag, PLD1b, PLD2, or

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In MOR1 and human PLD1b coexpressing HEK293 cells, PLD1b activity was stimulated by PMA treatment, whereas DAMGO incubation did not lead to an increase in the PLD1b activity (Fig. 1C) indicating that the opioid receptor specifically activates PLD2.

MOR1 Interacts with PLD2 and ARF in HEK293 Cells—To analyze an interaction between MOR1, PLD1b, PLD2, and ARF1 in HEK293 cells, we carried out coimmunoprecipitation studies. Expression of HAMOR1, PLD1b, PLD2, and ARF was examined by directly immunoblotting lysates from these cells with specific antibodies against HA tag, PLD1b, PLD2, or
ARF1 (Fig. 2, lysate). For communoprecipitation, HAMOR1 receptors were precipitated from lysates of HAMOR1-expressing cells and cells coexpressing HAMOR1 and PLD1b or PLD2 using anti-HA antibodies. The resulting precipitates were immunoblotted with antibodies directed against PLD1b, PLD2, or ARF1. As shown in Fig. 2A (lanes 2 and 3), PLD2 was detected in immunoprecipitates from cells coexpressing HAMOR1 and PLD2, suggesting that MOR1 is physically associated with PLD2 in vivo. Surprisingly, after agonist treatment we observed a decrease in the amount of coimmunoprecipitated PLD2 in HAMOR1-PLD2-expressing cells (Fig. 2A, lane 3). In immunoprecipitates from HAMOR1-expressing control cells no

PLD2 was detected, which might be because of the low basal PLD2 expression levels (Fig. 2A, lane 4).

In addition, ARF was coimmunoprecipitated from HAMOR1-PLD2-coexpressing cells but not from cells stably expressing HAMOR1 alone (Fig. 2A, lanes 2–4), indicating that ARF binds to the HAMOR1-PLD2 signaling complex and not directly to the MOR1 receptor. After DAMGO treatment an increase in the amount of coimmunoprecipitated ARF protein was detected in HAMOR1-PLD2 cells (Fig. 2A, lane 3). PLD2 and ARF were not nonspecifically immunoprecipitated with anti-HA antibodies, because in PLD2-expressing control cells no PLD2 and ARF were detected after precipitation with anti-HA antibodies (Fig. 2A, lane 6). Furthermore, from HAMOR1-PLD1b-coexpressing control cells no PLD1b or ARF was coimmunoprecipitated with MOR1 (Fig. 2B, lane 2) indicating that MOR1 is not associated with PLD1b in vivo.

Heterologous Activation of PLD2 by Phorbol Ester Influences the Agonist Selectivity of μ-Opioid Receptor Endocytosis—First, we examined the subcellular distribution of HAMOR1 and PLD2 using double immunofluorescence and confocal microscopy. As shown in Fig. 3, A and A’, both MOR1 and PLD2 were confined to the plasma membrane in HEK293 cells. To test the possibility that physical association would promote cointernalization of PLD2 and MOR1 after agonist-exposure, MOR1-PLD2-expressing cells were incubated in the presence of 1 μM DAMGO for 30 min. The results in Fig. 3, B and B’ reveal
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Fig. 3. Subcellular distribution of MOR1 and PLD2 in HEK293 cells. HEK293 cells stable coexpressing HAMOR1 and PLD2 were either not treated (A and A') or treated with 1 μM DAMGO for 30 min (B and B'). Cells were subsequently fixed, subjected to double immunofluorescent staining using a mixture of rat anti-HA and rabbit anti-PLD2 antibodies, and examined by confocal microscopy. Note that in untreated cells both HAMOR1 and PLD2 were confined to the plasma membrane. In contrast, after treatment with DAMGO only the μ-opioid receptor but not PLD2 was internalized into the vesicular endocytotic compartment. Shown are representative results from one of three independent experiments performed in duplicate. Scale bar, 20 μm.

that the MOR1 receptor is rapidly internalized while PLD2 remained at the plasma membrane.

We next examined whether the activation of PLD2 by the μ-opioid receptor influences the agonist-induced receptor endocytosis. Therefore, HEK293 cells stable expressing HAMOR1 or HAMOR1 and PLD2 were incubated with anti-HA antibodies at 4 °C to label cell surface receptors. The cells were then treated with DAMGO, morphine in the presence or absence of the PKC activator PMA for 30 min at 37 °C. During the experiment, the culture medium was supplemented with 5 μM monensin to prevent internalized receptors from recycling back to the plasma membrane. Cells were subsequently fixed, permeabilized, and bound anti-HA antibodies were immunofluorescently detected. The subcellular distribution of the receptor proteins was then analyzed by confocal microscopy. Fig. 4 shows that without agonist incubation (control), μ-opioid receptors were almost exclusively confined to the plasma membrane. After 30 min of DAMGO incubation at 37 °C, the μ-opioid receptor exhibited robust receptor endocytosis. In contrast, after incubation with morphine the MOR1 receptor was highly resistant to agonist-mediated endocytosis. Preincubation with PKC activator PMA alone did not increase the endocytosis of MOR1. In the presence of overexpressed PLD2, however, the combination of morphine and PMA resulted in a robust receptor internalization suggesting that both the agonist-induced conformational change and stimulation of PLD2 activity were required for receptor endocytosis under these conditions.

Quantitative analysis of receptor endocytosis by ELISA confirmed that in HAMOR1-PLD2 coexpressing HEK293 cells, PMA treatment enhances the rate of receptor endocytosis after both DAMGO and morphine in HAMOR1-PLD2 coexpressing HEK293 cells (Fig. 5). In contrast, in cells expressing the MOR1 receptor alone, PMA treatment did not significantly affect the rate of agonist-mediated receptor endocytosis indicating that the PMA-induced facilitation of receptor endocytosis depends on PLD2 and is not simply caused by increased PKC activity in these cells.

Agonist-induced MOR1 Endocytosis Is Inhibited by Primary but Not Secondary Alcohol—Next, we examined whether the production of PA by PLD2 is required for the induction of opioid receptor endocytosis in HEK293 cells. For inhibition of PA production the primary alcohol 1-butanol was used, which is known to be preferentially used over water by PLD2 in the transphosphatidylation reaction to generate phosphatidylalcohol instead of phosphatidic acid. This reaction is highly specific for primary alcohols, whereas secondary alcohols (such as isobutyl alcohol) are not utilized by PLD2 (1). Since 1.5% of butanol was found to inhibit the PLD2 activity but also to induce toxic effects (19), we used 1% of butanol for inhibition of PA production. Fig. 6A shows that DAMGO-mediated endocytosis of HAMOR1 was strongly inhibited by the primary alcohol (1-butanol) but not the secondary alcohol (isobutyl alcohol). This inhibitory effect of primary alcohol (1-butanol) on the agonist-induced endocytosis of the μ-opioid receptor was confirmed by quantitative analysis (Fig. 6B). These data strongly suggest that PA production by PLD2 is required for the induction of opioid receptor endocytosis.

Agonist-induced MOR1 Endocytosis Is Decreased by Coexpression of an NH2-terminal Fragment of PLD2—In a yeast two-hybrid screen we identified the NH2 terminus of the PLD2 as the important MOR1 interacting site of the enzyme. This region harbors the phosphoinositide binding domain of the PLD2 but lacks the PLD2 active site motif. After overexpression of the NH2 terminus (amino acids 1–235) of the PLD2 in HAMOR1 cells (HAMOR1-nPLD2), the DAMGO-induced MOR1-internalization was reduced by nearly 50% compared with control cells expressing HAMOR1 alone (Fig. 7), suggesting that nPLD2 competes for binding of full-length endogenous PLD2 to MOR1 and impairs opioid receptor-mediated PLD2 activation and subsequent receptor internalization. Furthermore, costimulation of HAMOR1-nPLD2-expressing cells with DAMGO and PMA resulted in a restoration of receptor endocytosis to the same extent as in HAMOR1-expressing cells (Fig. 7), indicating that PMA stimulation of PLD2 bypasses the nPLD2-mediated impairment of opioid receptor induced PLD2 activation. This effect is not simply due to increased PKC activity in these cells, because PMA treatment alone did not induce the endocytosis of MOR1 in both HAMOR1- and HAMOR1-nPLD2-expressing cells.

DISCUSSION

Stimulation of PLD activity has been observed for numerous G protein-coupled receptors including the VPAC 1 and 2 (for vasoactive intestinal polypeptide) receptors, PAC1 (for pituitary adenylate cyclase-activating peptide) receptor (20), metabotropic glutamate receptors (21, 22), m1-m4 muscarinic receptors (23), the endothelin receptor (24), the α2-adrenergic receptor (25), and the D2 dopamine receptor (26). However, the mechanisms of PLD activation by G protein-coupled receptors as well as the cellular functions of activated PLD are still incompletely understood. For the first time, the present study provides evidence for an essential role of PLD2 in agonist-induced endocytosis of a G protein-coupled receptor namely the μ-opioid receptor.

The present study demonstrates that the μ-opioid receptor interacts specifically with PLD2 and not with PLD1b. Using a yeast two-hybrid technique we identified the Phox homologous (PX) domain in the NH2 terminus of the PLD2 to be the important site for the interaction with the COOH terminus of the μ-opioid receptor. PX domains are known to be phosphoinositide-binding motifs found in a number of signaling and adapter proteins (Ref. 27 for review), but their precise function is still poorly defined. Recently, PX domains in proteins mediating the protein trafficking were shown to be required not only for the association with cellular membranes but also for the association with various receptors (e.g. epidermal growth factor, platelet-derived growth factor) (28, 29). The fact that PX domains
ANOV A followed by Bonferroni test. 0.001) between HAMOR1- and HAMOR1-PLD2-expressing cells.

The specific interaction between MOR1 and PLD2 was confirmed by coimmunoprecipitation experiments in HEK293 cells stable expressing MOR1 and PLD2. The interaction between MOR1 and PLD2 was shown to be constitutive and not inducible by agonist-treatment. In HAMOR1-PLD2 cells ~68% of the expressed PLD2 was communoprecipitated with the MOR1, whereas agonist-stimulation resulted in only ~35% communoprecipitated PLD2. This reduction might be due to the separation of MOR1 and membranal localized PLD2 after agonist-induced receptor internalization. It was further shown that stimulating the μ-opioid receptor with DAMGO activates PLD2 and that this DAMGO-mediated elevation of PLD2 activity was completely blocked by the opioid antagonist naloxone. The DAMGO-mediated PLD2 activation was ARF-dependent because it was completely blocked by the ARF inhibitor brefeldin A, which inhibits ARF-specific guanine nucleotide exchange proteins by locking them in an abortive complex with ARF-GDP. In addition, after DAMGO stimulation a 50% increase in the amount of communoprecipitated ARF1 with MOR1 was detected. Thus, it is possible that the opioid-mediated PLD2 stimulation may involve a direct interaction of the μ-opioid receptor with small G proteins, e.g. ARF. The NPXXY motif, which is highly conserved within the seventh transmembrane domain of many G protein-coupled receptors including the μ-opioid receptor, was previously demonstrated to represent a specific ARF binding site implicated in receptor-mediated PLD activation (30). However, for communoprecipitation of MOR1 and ARF the presence of PLD2 seemed to be required, because ARF was only detected in communoprecipitates from MOR1-PLD2- but not from MOR1-expressing cells. Therefore, it is reasonable to assume that ARF binds directly to PLD2 rather than to MOR1, but it cannot be excluded that a conformational change of MOR1 in the MOR1-PLD2 signaling complex is necessary to facilitate ARF binding to MOR1. However, after agonist-stimulation we observed an increase in the communoprecipitation of ARF (from ~4 to ~8% of total ARF) from MOR1-PLD2-expressing cells supporting the hypothesis of an ARF-dependent activation pathway of PLD2 by MOR1.

We further demonstrate that PLD2 can be activated by heterologous stimulation of PKC with PMA. However, the finding that DAMGO-induced PLD2 stimulation was not blocked by calphostin C suggests that PKC is not required for opioid receptor-mediated activation of PLD2. Similar to phosphoinositide-specific phospholipase C, phospholipase A2, and sphingomyelinase, PLD2 is a signal- and receptor-activated phospholipase; however, little is known about the cellular effects of PLD2 and its primary metabolite PA. It has been demonstrated that the second messenger PA can activate phosphatidylinositol 4-phosphate-5-kinase leading to the production of PIP2 (31, 32), which can further activate PLD in a positive feedback. In addition, an increase in the level of PA after PLD activation results in a change of the physical properties, e.g. charge and pH, of cellular membranes, thereby facilitating vesicle formation. Whereas PLD1 seems to play a role for the vesicle formation from the Golgi apparatus (33, 34), PLD2 has been suggested to be involved in vesicle formation from the plasma membrane (9).
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Performed in duplicate. The sis rates. Data are presented as means ± S.E. of five independent experiments performed in duplicate. The double asterisks indicate significant difference (p < 0.001) between cells treated only with DAMGO and cells treated with DAMGO plus 1-butanol or isobutyl alcohol (ANOVA followed by Bonferroni test).

Thus, it is reasonable to speculate that local activation of PLD2 by the μ-opioid receptor might play a key role in the stimulation of vesicle formation during μ-opioid receptor endocytosis. Because PLD2 levels in wild-type HEK293 cells were below the detection limit in Western blot and immunocytochemical assays, we stably coexpressed PLD2 and MOR1. Immunocytochemical studies revealed that both proteins were localized at the plasma membrane and that coexpression of PLD2 led to a significant increase in the agonist-induced endocytosis of the μ-opioid receptor. However, coininternalization of PLD2 and MOR1 was not observed in agonist-treated HAMOR1-PLD2-expressing cells. On the other hand, preventing the PLD2-mediated production of PA by treatment with the primary alcohol (1-butanol) led to an ~80% reduction of agonist-induced receptor endocytosis. Furthermore, overexpression of an inactive nPLD2 mutant which competes for binding of full-length PLD2 to the MOR1 resulted in a ~50% reduction from agonist-induced receptor endocytosis. Heterologous activation of endogenous PLD2 by PMA together with DAMGO-treatment bypasses the nPLD2-mediated block of receptor-induced activation of PLD2 and led to a robust μ-receptor internalization in HAMOR1-PLD2-expressing cells. In addition, it should be noted that morphine, which failed to induce μ-opioid receptor internalization, did not activate PLD2. However, heterologous PLD2 activation by PMA permitted morphine to induce robust μ-receptor internalization suggesting that both the agonist-induced conformational change and stimulation of PLD2 activity were required for receptor endocytosis. Desensitization of the μ-opioid receptor after agonist-treatment is induced by a rapid receptor phosphorylation and β-arrestin binding resulting in an uncoupling of the receptor from G proteins (15, 35–37). Since morphine failed to induce μ-opioid receptor internalization, it was speculated that morphine represses the MOR1 receptor in a conformation that is recalcitrant to GRK-mediated phosphorylation and subsequent β-arrestin binding (15, 38). This is supported by our previous finding that morphine failed to induce MOR1 phosphorylation, whereas splice variants (MOR1D and MOR1E) of the mouse μ-opioid receptor, which are internalized after morphine treatment, revealed a marked morphine-induced receptor phosphorylation (15). Moreover, β-arrestin was shown to interact with β2-adaptin (AP-2) leading to the initiation of clathrin-mediated receptor endocytosis (39). Furthermore, it was demonstrated that the recruitment of AP-2 is facilitated by the acidic phospholipid-enriched membrane resulting from PA production by ARP-activated PLD2 (40, 41). This indicates that receptor endocytosis can be modulated not only by receptor phosphorylation and β-arrestin binding but also by the PLD2-mediated PA production which, in turn, facilitates recruitment of AP-2 and clathrin to the plasma membrane. Consistent with this hypothesis we observed that inhibition of PLD2 activity led to a marked decrease in receptor endocytosis, whereas activation of PLD2 by PKC resulted in an enhanced agonist-induced endocytosis of the μ-opioid receptor even after morphine treatment. Thus, it can be suggested that activation of PLD2 may be a key step during the induction of agonist-mediated endocytosis of the μ-opioid receptor.
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REFERENCES

1. Morris, A. J., Frohman, M. A., and Engebrecht, J. (1997) Anal. Biochem. 252, 1–9
2. Liscovitch, M., and Cantley, L. C. (1994) Cell 77, 329–334
3. Liscovitch, M., Castryn, M., Fuciuci, G., and Tang, X. (2000) Biochem. J. 345, 401–415
4. Kiss, Z. (1996) Chem. Phys. Lipids 80, 81–102
5. Natarajan, V., Scribner, W. M., and Vepa, S. (1996) Chem. Phys. Lipids 80, 103–116
6. Exton, J. H. (1998) Biochim. Biophys. Acta 1436, 105–115
7. Hammond, S. M., Altschuller, Y. M., Sung, T. C., Rudge, S. A., Ross, K., Engebrecht, J., Morris, A. J., and Frohman, M. A. (1995) J. Biol. Chem. 270, 29640–29643
8. Park, S. K., Provost, J. J., Bae, C. D., Ho, W. T., and Exton, J. H. (1997) J. Biol. Chem. 272, 29261–29271
9. Colley, W. C., Sung, T. C., Roll, R., Jenco, J., Hammond, S. M., Altschuller, Y., Bar-Sagi, D., Morris, A. J., and Frohman, M. A. (1997) Curr. Biol. 7, 191–201
10. Kodaka, T., and Yamashita, S. (1997) J. Biol. Chem. 272, 11408–11413
11. Sung, T. C., Zhang, Y., Morris, A. J., and Frohman, M. A. (1999) J. Biol. Chem. 274, 3659–3666
12. Slabry, R., Jensen, T., Hansen, H. S., Frohman, M. A., and Seedorf, K. (1998) J. Biol. Chem. 273, 33722–33727
13. Shen, Y., Xu, L., and Foster, D. A. (2001) Mol. Cell. Biol. 21, 595–602
14. Chen, C. A., and Okayama, H. (1988) BioTechniques 6, 632–638
15. Koch, T., Schulz, S., Pfeffer, M., Kutzny, M., Schroeder, H., Kahl, E., and Haltt, V. (2001) J. Biol. Chem. 276, 31408–31414
16. Pfeffer, M., Koch, T., Schroeder, H., Kutzny, M., Kirsch, S., Kreienkamp, H. J., Haltt, V., and Schulz, S. (2001) J. Biol. Chem. 276, 14027–14036
17. Pfeffer, M., Koch, T., Schroeder, H., Laugusch, M., Haltt, V., and Schulz, S. (2002) J. Biol. Chem. 277, 19762–19772
18. Ketter, K., and Klein, J. (1999) J. Neurochem. 73, 2517–2523
19. Skippen, A., Jones, D. H., Morgan, C. P., Li, M., and Cockcroft, S. (2002) J. Biol. Chem. 277, 5825–5831
20. McCulloch, D. A., Lutz, E. M., Johnson, M. S., Robertson, D. N., MacKenzie, C. J., Holland, P. J., and Mitchell, R. (2001) Mol. Pharmacol. 59, 1523–1532
21. Shimomura, T., del Rio, E., Breen, K. C., Downes, C. P., and McLaughlin, M. (2000) Br. J. Pharmacol. 131, 1011–1018
22. Kanumilli, S., Toms, N. J., Venkateswarlu, K., Mellor, H., and Roberts, P. J. (2002) Neuropharmacology 43, 1–8
23. Sandmann, J., Peralta, E. G., and Wurtman, R. J. (1991) J. Biol. Chem. 266, 6031–6034
24. Ambar, I., and Sokolovsky, M. (1985) Eur. J. Pharmacol. 245, 31–41
25. MacNaught, E. E., McClue, S. J., Carr, I. C., Jess, T., Waleman, M. J., and Milligan, G. (1992) J. Biol. Chem. 267, 2149–2156
26. Sengesle, S. E. (2000) Mol. Pharmacol. 58, 455–462
27. Xu, Y., Seet, L. F., Hanson, B., and Hong, W. (2001) Biochem. J. 360, 13–30
28. Phillips, S. A., Barry, V. A., Haft, D. H., Taylor, S. L., and Haft, C. R. (2001) J. Biol. Chem. 276, 5574–5584
29. Haft, C. R., de la Luz Sierra, M., Barr, V. A., Haft, D. H., and Taylor, S. I. (1998) Mol. Cell. Biol. 18, 7278–7287
30. Mitchell, R., McCulloch, D., Lutz, R., Johnson, M., McMenina, C., Fennell, M., Fink, G., Zhou, W., and Sealfon, S. C. (1998) Nature 392, 411–414
31. Moritz, A., De Graan, P. N., Gispen, W. H., and Wirtz, K. W. (1992) J. Biol. Chem. 267, 7207–7210
32. Honda, A., Nogami, M., Yokosuki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanako, Y. (1999) Cell 99, 521–532
33. Kistakas, N. T., Brown, H. A., Sternweis, P. C., and Roth, M. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4952–4956
34. Kistakas, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) J. Cell Biol. 134, 295–306
35. Zhang, J., Ferguson, S. S., Barak, I. S., Bodduluri, S. R., Laporte, S. A., Law, P. Y., and Caron, M. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7157–7162
36. Kusvoor, A., Nappey, V., Kieffer, B. L., and Chavkin, C. (1997) J. Biol. Chem. 272, 27605–27611
37. Bohn, L. M., Gnaidetinov, R. R., Lin, F. T., Lefkowitz, R. J., and Caron, M. G. (2000) Nature 408, 720–723
38. Whistler, J. L., Chuang, H. H., Chu, P., Jan, L. Y., and von Zastrow, M. (1999) Neuron 23, 737–746
39. Laporte, S. A., Miller, W. E., Kim, K. M., and Caron, M. G. (2002) J. Biol. Chem. 277, 9247–9254
40. Liscovitch, M., and Cantley, L. C. (1995) Cell 81, 659–662
41. De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996) Science 271, 1533–1539
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