μ-Opioid Receptor Activates Signaling Pathways Implicated in Cell Survival and Translational Control*

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The μ-opioid receptor mediates the analgesic and ad- dictive properties of morphine. Despite the clinical importance of this G-protein-coupled receptor and many years of pharmacological research, few intracellular signaling mechanisms triggered by morphine and other μ-opioid agonists have been described. We report that μ-opioid agonists stimulate three different effectors of a phosphoinositide 3-kinase (PI3K)-dependent signaling cascade. By using a cell line stably transfection with the μ-opioid receptor cDNA, we show that the specific agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO) stimulates the activity of Akt, a serine/threonine protein kinase implicated in protecting neurons from apoptosis. Activation of Akt by DAMGO correlates with its phosphorylation at serine 473. The selective PI3K inhibitors wortmannin and LY294002 blocked phosphorylation of this site, previously shown to be necessary for Akt enzymatic activity. DAMGO also stimulates the phosphorylation of two other downstream effectors of PI3K, the p70 S6 kinase and the repressors of mRNA translation, 4E-BP1 and 4E-BP2. Upon μ-opioid receptor stimulation, p70 S6 kinase is activated and phosphorylated at threonine 389 and at threonine 421/serine 424. Phosphorylation of p70 S6 kinase and 4E-BP1 is also repressed by PI3K inhibitors as well as by rapamycin, the selective inhibitor of FRAP/mTOR. Consistent with these findings, DAMGO-stimulated phosphorylation of 4E-BP1 impairs its ability to bind the translation initiation factor eIF-4E. These results demonstrate that the μ-opioid receptor activates signaling pathways associated with neuronal survival and translational control, two processes implicated in neuronal development and synaptic plasticity.

The μ-opioid receptor mediates the effects of morphine as well as the actions of endogenous opioid ligands involved in diverse functions in the central and peripheral nervous system (1, 2). Activation of the μ-opioid receptor modulates neuronal excitability as it inhibits neuronal firing and neurotransmitter release in different regions of the nervous system (1). In addition to antinociception, the μ-opioid receptor has been also implicated in many other functions such as hippocampal plasticity, gastrointestinal motility, and modulation of the immune response (1, 3). Recent evidence using mice lacking the μ-opioid receptor underscored its role in hematopoiesis and in reproductive physiology (4). Although the behavioral and pharmacological properties of this receptor have been extensively studied, analysis of intracellular signaling has focused almost entirely on inhibition of the cAMP system. Opioid agonists inhibit adenylyl cyclase through coupling of the receptor to a Gαi pertussis-sensitive protein (1, 5). This activity is mediated by the Gα subunit of the G-protein. Studies on other G-protein-coupled receptors have shown that, upon agonist binding, the GTP-bound Gα subunit dissociates from the Gβγ subunit. The Gβγ subunit then serves as an independent activator of different effector pathways that require a phosphoinositide 3-kinase (PI3K) activity as an early step in the signaling cascade (6-8). PI3K was initially described as a phosphatidylinositol kinase associated with tyrosine phosphorylation and receptor tyrosine kinase signaling (9, 10). However, a PI3K isoform specifically activated by Gβγ subunits was later identified and demonstrated to be necessary for Gβγ-mediated mitogen-activated protein kinase (MAPK) signaling (7, 8, 11).

Three PI3K downstream effectors, the serine/threonine kinase Akt (also called protein kinase B or RAC), p70 S6 kinase, and the repressors of protein synthesis initiation factor 4E (eIF-4E), 4E-BP1 and 2, are stimulated by growth factors and thus possibly by G-protein-coupled receptors (12, 13). Activation of Akt in different cells leads to cell survival, glucose uptake, and regulation of glycogen metabolism (14). p70 S6 kinase phosphorylates the 40S subunit ribosomal protein S6 and is involved in the translational control of mRNA transcripts that contain a polypyrimidine tract at their 5’ ends (15, 16). 4E-BP1 and -2 (4E-binding protein 1 and 2), also known as PHAS-1/2, are pivotal to translational control by negatively regulating initiation factor eIF-4E and thereby protein synthesis (17). In this study, we report that stimulation of the μ-opioid receptor by specific agonists induces the phosphorylation and activation of Akt and p70S6k, as well as phosphorylation and inactivation of 4E-BP1 and 4E-BP2.

EXPERIMENTAL PROCEDURES

Reagents—[D-Ala²,N-Me-Phe⁴,Gly⁵-ol]Enkephalin (DAMGO), naloxone, and pertussis toxin (PTX) were from Sigma. Rapamycin, wortmannin, GSK3-β, glycogen synthase kinase-3 β, PTX, pertussis toxin, HEK, human embryonic kidney; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; GSK3-β, glycogen synthase kinase 3 β; eIF-4E, eukaryotic initiation factor 4E; MEK, MAPK/extracellular signal-regulated kinase 4E.

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nn, and LY294002 were from Calbiochem. PD98059 was from New England Biolabs. Rabbit polyclonal antibodies (New England Biolabs) specific for the following proteins were used: Akt phosphorylated at serine 473; control Akt (phosphorylation-independent); p70S6k phosphorylated at threonine 389; p70S6k phosphorylated at both threonine 421 and 424 and antibodies recognizing human 4E-BP1 and 4E-BP2 respectively, both expressed as glutathione S-transferase fusion proteins (Ponce Rabbit Farm, Canadensis, PA) (18).

Production of Antiphosphopeptide Antibodies—Phospho-specific antibodies directed against the various sites on Akt, p70S6k, and GSK-3β were produced as described (19). Briefly, immunization of New Zealand White rabbits was carried out with the following synthetic phosphopeptides coupled to keyhole limpet hemocyanin: Akt (Ser-473), RPPHPQPS*YSASGTC; p70S6k (Thr-389), NQVFLGFT*YVPKKC; p70S6k (Thr-421/Ser-424), SPTP*PVS*PVKFS; GSK-3β (crosstide, corresponding to Ser-9 in GSK-3β, and Ser-21 in GSK-3α), RRRRRRTSS*FAEGC. A phosphorylation-independent Akt antibody (control Akt) was raised against the same peptide sequence containing nonphosphorylated Ser-473 (RPPHPQPSYSASGTC); enzyme-linked immunosorbent assay (ELISA) using phosphopeptides, was used to identify positive rabbits, and IgG was purified using protein A-Sepharose. Further purification steps were performed using adsorption of nonspecific material to nonphosphopeptide affinity column, followed by elution of reactive material from a phosphopeptide affinity column at low pH. Antibodies were then dia-lyzed and characterized by enzyme-linked immunosorbent assay against phospho- and nonphosphopeptides to determine the extent of phosphospecificity and by Western blotting to examine specificity against whole cell extracts.

Cell Culture and Transient Transfections—A Chinese hamster ovary cell line stably transfected with the murine µ-opioid receptor was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and G418 (0.5 mg/ml) (20). In a typical experiment, cells were grown in 6-well plates for 24 h prior to treatment, washed three times with phosphate-buffered saline, and incubated in serum-free medium for 2 h prior to agonist stimulation in the presence or absence of the different inhibitors. This treatment is sufficient for detecting phosphorylation in vivo over background levels, although it does not block the complete quiescence. Incubation with the different inhibitors was initiated either 16 h (pertussis toxin), 1 h (PD98059), 30 min (wortmannin, LY294002, and rapamycin), or 5 min (naloxone) prior to agonist stimulation. HEK293 cells were transfected with DNA constructs expressing the murine µ-opioid receptor and HA-tagged Akt constructs (generous gifts from T. Franke and J. Woodgett) using the FuGENE 6 transfection reagent and following the manufacturer’s instruc-tions (Boehringer Mannheim).

Extract Preparation and Immunoblotting—For most Western blot experiments, cell extracts were prepared by lysing the cells immediately after treatments in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% w/v bromphenol blue). Extracts were subjected to SDS-10% PAGE and chemiluminescent detection (New England Biolabs), was performed as described using the antibodies indicated above (21-23).

Immunoprecipitations and In Vitro Kinase Assays—For kinase activity assays, cells were grown in 10-cm plates for 24 h, serum-starved for 2 h, and treated as indicated in the figures. After treatment, cells were scraped into 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, protease, and phosphata-se inhibitors). Extracts were then sonicated and centrifuged for 5 min at 14,000 × g. Immunoprecipitations were performed by adding the appropriate antibodies to cell extracts and incubating with protein A-coupled sepharose for 1 h. Immunoprecipitated complexes were washed twice and subjected to SDS-15% PAGE (18). Western blotting, using chemiluminescent detection (New England Biolabs), was performed as described using the antibodies indicated above (21-23).

RESULTS

Activation and Phosphorylation of Akt by the µ-Opioid Receptor—The mechanism of Akt regulation involves the interaction between the phospholipids produced by PI3K and the amino-terminal pleckstrin homology domain of Akt (26, 27). This interaction is critical for translocating Akt to the plasma membrane and for the ability of a newly identified kinase termed phosphatidylinositol (3,4,5)-trisphosphate-dependent kinase-1 (PKD1) to phosphorylate Akt at Thr-308 in its activation loop (28, 29). PI3K-dependent phosphorylation of Ser-473 is also essential for Akt activation (30), although the kinase responsible for phosphorylation of this site has yet to be identified (28).

Due to the importance of Ser-473 for Akt activation and regulation (30), we have developed an antibody that specifically recognizes Akt only when that residue becomes phosphorylated. We used this antibody to study the activation and phosphorylation of Akt by µ-opioid receptor agonists. To prepare the antibody, rabbits were immunized with a synthetic phosphopeptide containing phosphorylated Ser-473 (RPHPPQPS*YSASGTC) coupled to keyhole limpet hemocyanin and IgG purified as described under “Experimental Procedures” and elsewhere (19). Both insulin and insulin-like growth factor-1 activate Akt, and this activation results from the phosphorylation of Ser-473 and Thr-308 (28-30). By using this phosphopeptide antibody, we first examined whether phosphorylation at
Ser-473 could also be detected after µ-opioid receptor stimulation with the specific agonist DAMGO. Fig. 1a shows that the specific agonist DAMGO induced a strong immunoreactivity signal, as detected with the phosphopeptide antibody, with a molecular weight corresponding to that of Akt. This signal was inhibited by the opioid receptor-specific antagonist naloxone and by pertussis toxin. Equal levels of immunoreactivity were observed in all samples with a control antibody raised against the same non-phosphorylated peptide sequence. These results suggest that the phosphopeptide antibody recognized phosphorylated Akt and confirmed the loading of equal amounts of protein in the gel. They also indicate that the DAMGO-stimulated increase in Akt phosphorylation is mediated by the Gαi1/Gβ2-coupled µ-opioid receptor. Identical results were obtained with other µ-receptor-specific agonists (not shown). To test further the specificity of this antibody for Akt phosphorylated at Ser-473, HEK293 cells were cotransfected with a mammalian expression vector encoding the µ-opioid receptor cDNA and either of three HA-tagged Akt constructs expressing wild-type Akt protein, a “kinase dead” version of Akt (with lysine 179 changed to alanine, K179A), or a K179A Akt with Ser-473 mutated to the nonphosphorylatable residue alanine (K179A/S473A). Following a 5-min stimulus with 1 μM DAMGO, cell extracts were prepared, and HA-Akt material was immunoprecipitated using the anti-HA monoclonal antibody 12CA5. Immunoprecipitated complexes were then analyzed by Western blotting using the phospho-Akt (Ser-473), control Akt, and anti-HA antibodies. Fig. 1a shows that the antibody raised against the phosphopeptide can detect wild-type and K179A but not K179A/S473A Akt protein. The control Akt and HA antibodies detected similar levels of all three versions of Akt proteins present in the precipitated material. This demonstrates that the binding of phosphopeptide antibody to Akt requires serine at position 473. We next verified whether the µ-receptor agonist could induce not only phosphorylation but also Akt enzymatic activity. For this purpose we took advantage of the fact that Akt phosphorylates glycogen synthase kinase 3-β (GSK3-β) at Ser-9 in vivo and in vitro (31). Cells stably expressing the µ-opioid receptor were stimulated with DAMGO for 5 min in the presence or absence of the antagonist naloxone and then lysed under non-denaturing conditions. Akt polypeptides were immunoprecipitated using the phosphorylation-independent Akt antibody, and the immunocomplexes were incubated together with a bacterially expressed 46-kDa GSK3-β protein in the presence of ATP. To detect phosphorylated GSK3-β, aliquots of the kinase reactions were analyzed by Western blotting using an antibody that recognizes GSK3-β when phosphorylated at Ser-9. As shown in Fig. 1c, a 46-kDa band corresponding to phosphorylated GSK3-β was detected in kinase reaction samples containing immunoprecipitated Akt from cells stimulated with DAMGO. GSK3-β phosphorylation at Ser-9 was not observed using Akt immunoprecipitated from unstimulated cells or cells treated with the antagonist naloxone. Analysis of the same samples with a phosphorylation-independent GSK3-β antibody showed that equal amounts of GSK3-β protein were present in all reactions and loaded onto the Western gels. The same blots were probed with the phospho- and control Akt antibodies showing that similar amounts of Akt kinase were present in the reactions. We observed a precise correlation between the content of phosphorylated Akt in the immunocomplexes and Akt kinase activity (Fig. 1c). These results demonstrate that, similar to the effects of insulin and insulin-like growth factor-1, DAMGO strongly induced Akt enzymatic activity, and this activation correlates with phosphorylation of Akt at Ser-473.

Cells expressing the µ-opioid receptor were next incubated for increasing times with the specific agonist DAMGO (100 nM) and cell extracts analyzed by Western blotting using the phospho-Ser-473 Akt antibody. Phosphorylation of Akt was transient, peaking at 5 min after exposure to the agonist, followed by a rapid decline to reach undetectable levels after 1 h of agonist exposure (Fig. 2a). Akt phosphorylation was detectable after exposure to low concentrations of DAMGO (1–10 nM) increasing in a dose-dependent manner (Fig. 2b). To test
whether μ-receptor stimulation of Akt phosphorylation requires an active PI3K, we exposed the cells to two different chemical inhibitors of PI3K, wortmannin and LY294002. Both drugs completely blocked the activation of Akt at concentrations considered to be specific for PI3K isoforms (8). At 50 nM wortmannin, phosphorylation of Akt was reduced to approximately 50% and was completely abolished at 200 nM (Fig. 3a); LY294002 totally blocked the induction of Akt phosphorylation by DAMGO at 5 μM (Fig. 3b). In addition, DAMGO-induced phosphorylation at Ser-473 was not affected by rapamycin at concentrations as high as 50 nM (Fig. 7, d and e). These results suggest that the μ-opioid induction of Akt phosphorylation is a PI3K-dependent and FRAP/mTOR-independent process.

Phosphorylation of p70 S6 Kinase at Thr-389 and Thr-421/Ser-424—Activation of p70 S6 kinase (p70S6k) requires a complex sequence of Ser/Thr phosphorylation events at as many as 10 residues (16). At least two sites are critical for kinase function: Thr-229 in the catalytic domain and Thr-389 in the linker domain (16). Phosphorylation at Thr-389 was shown to be essential for p70S6k activity and stimulation of 5’-oligopyrimidine tract mRNA translation (32); Thr-389 is also the major wortmannin and rapamycin-sensitive phosphorylation site (15), and prior phosphorylation at this site is required for the action of 3-phosphoinositide-dependent protein kinase-1 (PDK-1) on Thr-229 (32, 33). A cluster of Ser-Pro and Thr-Pro phosphorylation sites in the non-catalytic carboxy tail is also critical for p70S6k activation in vivo (16, 34). It was postulated that, unless phosphorylated at sites including Thr-421 and Ser-424, a detailed analysis of these antibodies using constructs expressing versions of p70S6k in which these sites were mutated to nonphosphorylatable alanines or to glutamic acid was recently reported (19). This study demonstrated the specificity of the antibodies for their phosphorylated target residues in the p70S6k protein. Moreover, a comparison between insulin-stimulated phosphorylation of these and other sites in p70S6k and its enzymatic activity revealed that the activity of this kinase in vivo most closely correlates with the phosphorylation state of Thr-389 (19).

We first tested whether agonist μ-opioid receptor stimulation leads to the induction in p70S6k activity and phosphorylation of the kinase at Thr-389 and Ser-421/Thr-424. Cells expressing the μ-opioid receptor were incubated with DAMGO for 10 min, or DAMGO in the presence of the antagonist naloxone, prior to extract preparation under non-denaturing conditions. p70S6k polypeptides were subsequently immunoprecipitated using a p70S6k phosphorylation-independent antibody. Aliquots of the immunocomplexes were used in a kinase reaction utilizing the peptide KRKRRASLAA as substrate (24) and analyzed in parallel for the presence or absence of phosphorylated p70S6k by immunoblotting. As shown in Fig. 4a, the activity of p70S6k was enhanced 6-fold in DAMGO-treated cells as compared with basal levels in unstimulated cells. The antagonist naloxone inhibited this effect almost completely. The immunocomplexes displaying active p70S6k were also those containing phosphorylated p70S6k at Thr-389 and Thr-421/Ser-424 (Fig. 4b, upper and middle panels, respectively). The same phosphorylation-independent antibody used to immunoprecipitate the enzyme detected similar amounts of total p70S6k in the three treatment samples. This indicates that the signals revealed by the Thr-389 and Thr-421/Ser-424 antibodies truly reflect the presence of phosphorylated polypeptides rather than differences in immunoprecipitated protein amounts. This antibody also revealed the presence of an 85-kDa protein, presumably the p70S6k isoform derived from alternative mRNA splicing and an alternative translational start site containing a putative nuclear localization signal (35). The 85-kDa band could also be detected with the phosphopeptide antibodies at very long exposures (not shown). The clear “band shift” due to the slower migrating phosphorylated p70 and p85 isoforms further indicates a high level of p70/85 protein phosphorylation upon DAMGO stimulation (Fig. 4b, bottom panel). This suggests that the different intensities in the 70- and 85-kDa bands obtained with the control antibody versus the phosphopeptide antibodies are probably due to different antibody sensitivities rather than the actual ratio between phosphorylated and unphosphorylated material upon stimulation. Thus, phosphorylation at Thr-389 and Thr-421/Ser-424 induced by DAMGO correlates accurately with a dramatic increase in p70S6k kinase activity, as previously reported for serum or insulin (16). The kinetics of this induction is shown in Fig. 5a. Phosphorylation of both

FIG. 3. DAMGO-induced phosphorylation of Akt at Ser-473 is blocked by PI3K inhibitors. Dose-dependent inhibition of Akt phosphorylation by wortmannin (a) and LY294002 (b). Cells were incubated for 30 min in the presence of the indicated concentrations of wortmannin and LY294002 before stimulation by 100 nM DAMGO for 5 min. Extracts were prepared, immunoblotted, and probed with an Akt phosphoserine 473-specific antibody (upper panels) and control Akt (phosphorylation-independent) antibody (lower panels) as described under “Experimental Procedures.”
Thr-389 and Thr-421/Ser-424 is first detected at 5 min, peaking after 10 min of agonist exposure. Although there is a clear decay in the signal intensity, the stimulation persists for at least 1 h. This time course differs from that of Akt, whose strong signal pulse decays almost immediately (Figs. 2a–c). This suggests a differential regulation of these two kinases, despite their common upstream activating pathway, and/or the action of different phosphatases. Both Thr-389 and Thr-421/Ser-424 sites are phosphorylated in a dose-dependent manner upon stimulation with increasing agonist concentrations (Fig. 5a). Phosphorylation of Thr-389, and more surprisingly of Thr-421/Ser-424 sites in the autoinhibitory domain. Consistent with previous studies, rapamycin abolished phosphorylation of p70S6k at Thr-389 at concentrations as low as 1 nM, without affecting phosphorylation of Akt at Ser-473 (compare Fig. 7, a and d). However, the same concentrations of rapamycin also blocked the μ-receptor-induced phosphorylation at Thr-421/Ser-424 (Fig. 7b), previously reported not to be affected by this drug upon serum stimulation (36). Thus, based on the use of well described pharmacological inhibitors, it appears that both PI3K and FRAP/mTOR, but not MAPK, are required for μ-opioid-dependent phosphorylation of p70S6k.

Phosphorylation of 4E-BP1 and 4E-BP2 and Release of 4E-BP1 from Its Complex with eIF4E by Activation of the μ-Opioid Receptor—Initiation factor 4E binds to the cap structure (m^7GpppX, where X is any nucleotide) of eukaryotic mRNA promoting translation initiation, the rate-limiting step in protein synthesis (17). A major level of eIF4E regulation is mediated by the G_{o,i}-coupled μ-opioid receptor (Fig. 6a). Phosphorylation of both sites was also repressed by the PI3K-selective inhibitors in a dose-dependent manner. Wortmannin and LY294002 blocked both Thr-389 and Thr-421/Ser-424 phosphorylation signals at 100 nM and 1 μM, respectively (Fig. 6, b and c, respectively). Phosphorylation of Thr-389, and more surprisingly of Thr-421/Ser-424 sites, which are embedded in a canonical MAPK phosphorylation site (PX(S/T)P), was not sensitive to PD98059 (Fig. 6a). Western analysis of the same samples with a phospho-specific MAPK (Erk1/2) antibody showed total inhibition of MAPK phosphorylation by the same PD98059 concentration indicating that the inhibitor used in this experiment was effective (data not shown). This result suggests that, as reported before (16), MAPK is not responsible for the μ-opioid-induced in vivo phosphorylation of Thr-421/Ser-424 sites in the autoinhibitory domain.

FIG. 4. Activation of p70 S6 kinase by μ-opioid receptor stimulation correlates with phosphorylation at Thr-389 and Thr-421/Ser-424. a, cells were incubated with DAMGO (1 μM) for 10 min in the presence or absence of naloxone (10 μM) activity before extracts were prepared, and p70S6k polypeptides were immunoprecipitated using a control (phosphorylation-independent) antibody. Kinase reactions were performed using as substrate the peptide KRRRLASLAA as described under “Experimental Procedures.” The data are mean ± S.D. for n = 5 from three experiments (where error bars are not shown, they are within the boundaries of the histograms). b, aliquots of the same immunoprecipitates were analyzed by Western blotting using the phosphopeptides antibodies directed against Thr-389 (upper panel), Thr-421/Ser-424 (middle panel), and the control p70S6k antibody (bottom panel).

FIG. 5. Time- and dose-dependent stimulation of p70 S6 kinase phosphorylation at Thr-389, and Thr-421/Ser-424 sites by the μ-agonist DAMGO. a, time course of p70S6k phosphorylation. Cells were incubated for the indicated times with 100 nM DAMGO before extract preparation. b, dose-dependent induction of p70S6k phosphorylation. Cells were incubated with increasing concentrations of DAMGO for 10 min before extract preparation. Extracts were processed for Western blotting as described before and probed with antibodies that specifically recognize p70S6k when phosphorylated at Thr-389 (upper panels) and Thr-421/Ser-424 (middle panels). The same blots were stripped and re-probed with a control p70S6k antibody showing equal loading and confirming the phospho-specificity of the signal detected with the phosphopeptide antibodies (bottom panels).
preventing its interaction with the eIF-4G to form a functional eIF-4F complex, which is required for translation initiation (17). Upon mitogenic stimulation, 4E-BP1 is phosphorylated and inactivated, thereby releasing a functional eIF-4E (17). We analyzed whether the activation of \( \mu \)-opioid receptor signaling would lead to 4E-BP1 phosphorylation and its dissociation from eIF-4E. Cells stably expressing the \( \mu \)-opioid receptor were exposed to increasing concentrations of DAMGO. Cell extracts were prepared after 10 min of exposure, immunoblotted and analyzed with 4E-BP1-specific antibodies (Fig. 8a).

As previously reported, phosphorylation of 4E-BP1 was monitored by an increase of the ratio between the slower (\( \gamma \)) over the faster (\( \alpha \) and \( \beta \)) migrating electrophoretic isoforms of 4E-BP1 (23). In unstimulated cells, we could predominantly detect the \( \beta \) form of 4E-BP1 and lower levels of the \( \alpha \) and \( \gamma \) forms. An agonist concentration as low as 1 nM increased the amount of the highly phosphorylated, slower migrating \( \gamma \) form (Fig. 8a). At 1 \( \mu \)M DAMGO the \( \gamma \) form predominated, whereas the unphosphorylated \( \alpha \) isoform disappeared, indicating a high level of phosphorylated 4E-BP1 (Fig. 8a). The phosphorylation of 4E-BP1 followed rapid induction kinetics (Fig. 8b). The highly phosphorylated \( \gamma \) form was induced after 5 min. Maximum
Phosphorylation of Akt, p70S6k, and 4E-BP1/2 by w-opioid agonists is inhibited by the antagonist naloxone, pertussis toxin, and by wortmannin and LY294002, indicating that the receptor specifically activates a G-linked, PI3K-dependent signaling cascade. Upon w-opioid receptor activation, these three PI3K-dependent effectors appear to be interrelated in a similar fashion to signaling cascades stimulated by growth factors.

For instance, Akt is apparently required for insulin-mediated 4E-BP1 phosphorylation and inactivation (22). We are currently investigating whether Akt kinase activation might also represent a necessary step in the cascade leading to the w-opioid-stimulated phosphorylation of 4E-BP1. Both p70S6k and 4E-BP1 are regulated by the target of rapamycin, FRAP/mTOR (15, 37–39). Recent studies suggest that FRAP/mTOR may directly phosphorylate 4E-BP-1, inhibiting 4E-BP1 association with eIF-4E in vivo (40, 41). In agreement with these reports, FRAP/mTOR is likely to regulate the w-opioid-induced phosphorylation of p70S6k as well as 4E-BP1 and 4E-BP2, since both are blocked by rapamycin (Figs. 7 and 9). Moreover, w-opioid-induced phosphorylation of p70S6k at both Thr-389 and Thr-421/Ser-424 sites is blocked by 1 nM rapamycin (Fig. 7). The inhibitory effect of rapamycin on Thr-421/Ser-424 phosphorylation stands in contrast to previous studies indicating that serum-stimulated phosphorylation of that site is not sensitive to rapamycin (36). To explain this discrepancy, it could be postulated that different stimuli may have different effects on the rapamycin sensitivity of this site. However, Weng et al. (19) have also observed that rapamycin (200 nM) caused a marked reduction of insulin-stimulated phosphorylation of Thr-421/Ser-424. We found no effects of rapamycin on phosphorylation of Akt at Ser-473 induced by the w-opioid receptor (Fig. 7), despite the sequence homology between protein segments of Akt and p70S6k containing Ser-473 and Thr-389, respectively (Figs. 1a and 3a). With a consensus FXXFxT/S/Y, also found in many PKCs and p90RSK, these sites were postulated to be the substrates of a putative PDK2 kinase (42). It is also possible that FRAP/mTOR regulates both p70S6k and Akt by direct phosphorylation at Thr-389 (41) and Ser-473, respectively, whereas rapamycin could act to stimulate a phosphatase selective for p70S6k (16). The precise nature of the signaling steps involved in this receptor cascade leading to activation of p70S6k and inactivation of 4E-BP1/2 remains to be elucidated. However, it is likely that, similar to growth factor stimulation, an array of complex interactions among kinases and effectors downstream of PI3K also takes place upon w-opioid receptor stimulation.

A variety of G coupled receptors have been implicated in transmitting mitogenic signals (43). Opioid receptors appear to follow that paradigm as well since they induce MAPK activity in cultured cells (44–46), presumably via a G /PI3K-mediated mechanism. In agreement with this concept and with our results, the d-opioid receptor was also shown to induce p70S6k activity (47). Therefore, based on our results and these other reports, we postulate that PI3K-dependent effectors, such as Akt, p70S6k, and 4E-BP1/2, as well as the MAPK pathway, represent important intracellular mechanisms mediating the diverse physiological functions of the w-opioid receptor. Although the functional significance of these signaling events is not yet known, results from the analysis of w-opioid receptor-deficient mice suggest that this receptor does not play a major role in normal animal development or growth (2). w-Opioid receptor activation of signaling pathways connected to growth could still have a role in neuronal development, although w-opioids have apparently inhibitory effects on proliferation of neuronal and glial progenitors (48). Consistent with this possibil-

**DISCUSSION**

We have shown that agonist stimulation of the w-opioid receptor leads to activation of Akt (Fig. 1) and p70 S6 (Fig. 4) protein kinases as well as inactivation of 4E-BP1 (Fig. 8). These effects correlate precisely to a marked increase in the phosphorylation of these proteins. In the case of Akt and p70S6k, the sites phosphorylated by w-opioid receptor stimulation were previously documented to be critical for the activation of both protein kinases by serum or growth factors (16, 30). We studied phosphorylation of those sites (Akt at Ser-473, and p70S6k at Thr-389 and Thr-421/Ser-424) using polyclonal antibodies raised against the corresponding phosphopeptides. The specificity of these antibodies was demonstrated using constructs with alanine substitutions of the target-phosphorylated residues in Akt and p70S6k (Fig. 1 and Ref. 19, respectively). Phosphorylation of Akt, p70S6k, and 4E-BP1/2 by w-opioid agonists is inhibited by the antagonist naloxone, pertussis toxin, and by wortmannin and LY294002, indicating that the receptor specifically activates a G-linked, PI3K-dependent signaling cascade. Upon w-opioid receptor activation, these three PI3K-dependent effectors appear to be interrelated in a similar fashion to signaling cascades stimulated by growth factors.

For instance, Akt is apparently required for insulin-mediated 4E-BP1 phosphorylation and inactivation (22). We are currently investigating whether Akt kinase activation might also represent a necessary step in the cascade leading to the w-opioid-stimulated phosphorylation of 4E-BP1. Both p70S6k and 4E-BP1 are regulated by the target of rapamycin, FRAP/mTOR (15, 37–39). Recent studies suggest that FRAP/mTOR may directly phosphorylate 4E-BP-1, inhibiting 4E-BP1 association with eIF-4E in vivo (40, 41). In agreement with these reports, FRAP/mTOR is likely to regulate the w-opioid-induced phosphorylation of p70S6k as well as 4E-BP1 and 4E-BP2, since both are blocked by rapamycin (Figs. 7 and 9). Moreover, w-opioid-induced phosphorylation of p70S6k at both Thr-389 and Thr-421/Ser-424 sites is blocked by 1 nM rapamycin (Fig. 7). The inhibitory effect of rapamycin on Thr-421/Ser-424 phosphorylation stands in contrast to previous studies indicating that serum-stimulated phosphorylation of that site is not sensitive to rapamycin (36). To explain this discrepancy, it could be postulated that different stimuli may have different effects on the rapamycin sensitivity of this site. However, Weng et al. (19) have also observed that rapamycin (200 nM) caused a marked reduction of insulin-stimulated phosphorylation of Thr-421/Ser-424. We found no effects of rapamycin on phosphorylation of Akt at Ser-473 induced by the w-opioid receptor (Fig. 7), despite the sequence homology between protein segments of Akt and p70S6k containing Ser-473 and Thr-389, respectively (Figs. 1a and 3a). With a consensus FXXFxT/S/Y, also found in many PKCs and p90RSK, these sites were postulated to be the substrates of a putative PDK2 kinase (42). It is also possible that FRAP/mTOR regulates both p70S6k and Akt by direct phosphorylation at Thr-389 (41) and Ser-473, respectively, whereas rapamycin could act to stimulate a phosphatase selective for p70S6k (16). The precise nature of the signaling steps involved in this w-receptor cascade leading to activation of p70S6k and inactivation of 4E-BP1/2 remains to be elucidated. However, it is likely that, similar to growth factor stimulation, an array of complex interactions among kinases and effectors downstream of PI3K also takes place upon w-opioid receptor stimulation.

A variety of G coupled receptors have been implicated in transmitting mitogenic signals (43). Opioid receptors appear to follow that paradigm as well since they induce MAPK activity in cultured cells (44–46), presumably via a G /PI3K-mediated mechanism. In agreement with this concept and with our results, the d-opioid receptor was also shown to induce p70S6k activity (47). Therefore, based on our results and these other reports, we postulate that PI3K-dependent effectors, such as Akt, p70S6k, and 4E-BP1/2, as well as the MAPK pathway, represent important intracellular mechanisms mediating the diverse physiological functions of the w-opioid receptor. Although the functional significance of these signaling events is not yet known, results from the analysis of w-opioid receptor-deficient mice suggest that this receptor does not play a major role in normal animal development or growth (2). w-Opioid receptor activation of signaling pathways connected to growth could still have a role in neuronal development, although w-opioids have apparently inhibitory effects on proliferation of neuronal and glial progenitors (48). Consistent with this possibil-

![Fig. 9. Effect of different inhibitors on DAMGO-induced 4E-BP1 and 4E-BP2 phosphorylation.](Image 74x628 to 273x729)
ity, an increase in proliferation of several hematopoietic cell lineages was also observed in mice lacking the \( \mu \)-opioid receptor (4).

Considering the involvement of Akt in neuronal survival (49), it is tempting to speculate that \( \mu \)-opioid receptor signaling could also prevent apoptosis of specific developing neurons. This hypothesis is supported by two early studies indicating that morphine and endogenous opioids inhibit neuronal cell death in the avian ciliary ganglion (50, 51). In addition, signaling cascades typically associated with cell proliferation should have non-mitogenic functions in postmitotic neurons. For example, we have recently shown that the MAPK pathway is required for \( \mu \)-receptor desensitization (52). This signaling pathway also participates in hippocampal long term potentiation (53). Moreover, opioids have been postulated as mediators of hippocampal plasticity (3), a process that requires local protein synthesis (54). A role for hippocampal \( \mu \)-opioids in neuronal plasticity through p70S6k and 4E-BP-1/2-mediated regulation of protein synthesis is an intriguing possibility.

In summary, this study describes, for the first time, the ability of the \( \mu \)-opioid receptor to activate signaling pathways associated with neuronal survival (Akt) and translational control (p70S6k and 4E-BP-1 and 4E-BP-2), two processes implicated in neuronal development, long term memory, and synaptic plasticity. Future efforts should be directed to explore the physiological significance of these signaling cascades in vivo.

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