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A Mitogen-activated Protein Kinase Pathway Is Required for μ-Opioid Receptor Desensitization*

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The μ-opioid receptor mediates not only the beneficial painkilling effects of opiates like morphine but also the detrimental effects of chronic exposure such as tolerance and dependence. Different studies have linked tolerance to opioid receptor desensitization. Agonist activation of the μ-opioid receptor stimulates a mitogen-activated protein kinase (MAPK) activity, but the functional significance of this pathway remains unclear. We have focused on the MAPK signaling cascade to study μ-opioid receptor desensitization. We report that inhibition of the MAPK pathway blocks desensitization of μ-opioid receptor signaling as well as the loss of receptor density due to internalization. Our results suggest that a feedback signal emanating from the MAPK cascade is required for μ-opioid receptor desensitization.

Agonists for the μ-opioid receptor are the therapeutic choice in the management of severe acute and chronic pain despite the potential development of tolerance, dependence, and addiction. The mechanisms of tolerance, defined as the diminishing effect of the drug in response to chronic exposure, are not fully understood. It has been postulated that receptor desensitization and down-regulation could be associated with aspects of tolerance *in vivo* (1). Opioid receptors undergo homologous desensitization upon continuous or repeated agonist exposure in a fashion similar to other G protein-coupled receptors (GPCR)[1] (2–4). Desensitization of the μ-opioid receptor involves agonist-dependent phosphorylation of the receptor, most likely by a member of the family of G-coupled receptor protein kinases (GRKs) (4, 5). According to this model, upon phosphorylation and uncoupling from the G protein, the receptor is bound to β-arrestins and internalized into endosomes, reducing the number of receptors available at the cell surface for further agonist binding (6, 7).

Desensitization of the μ-opioid receptor has been mainly described as the attenuated reduction of forskolin-stimulated cAMP levels in response to the agonist (2–4). However, other signaling pathways might also be desensitized upon prolonged agonist exposure. GPCRs can trigger a Gβγ-mediated activation of a phosphoinositide 3-kinase (PI3K)/Ras-dependent MAPK signaling pathway (8, 9). Opioid receptors stimulate MAPK activity as well, although the components of this signaling cascade have not been fully described (10–12). In this report we have examined the role of the MAPK (Erk1/2) signaling pathway on μ-opioid receptor desensitization. This pathway, which involves the activation of a phosphatidylinositol 3-kinase activity as well as Raf and MEK1/2, is essential for μ-opioid receptor desensitization.

EXPERIMENTAL PROCEDURES

Reagents—[D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), dermorphin, morphine, Met-enkephalin, naloxone, D-Pen^2,D-Pen^5-enkephalin (DPDPE), lysophosphatidic acid (LPA), pertussis toxin, cycloheximide, forskolin, and isobutylmethylxanthine (IBMX) were from Sigma. Wortmannin and LY294002 were from Calbiochem. Antibodies that recognize only the phosphorylated forms of p42 and p44 MAPK (Thr-202/Tyr-204), Elk-1 (Ser-383), MEK1/2 (Ser-217/221), as well as a control (phosphorylation-independent) MAPK antibody, and the MEK inhibitor PD98059 were from New England Biolabs.

Cell Culture—A stable Chinese hamster ovary cell line expressing the murine μ-opioid receptor cDNA (13) was maintained in DMEM supplemented with 10% fetal bovine serum and G418 (0.5 mg/ml). In a typical MAPK induction experiment cells were grown in 6-well plates for 24 h prior to treatment, washed, and incubated in serum-free medium for 1–2 h prior to agonist stimulation as indicated in the figures. Incubation with vehicle (0.04% dimethyl sulfoxide) or the inhibitors was initiated 1 h (PD98059), 15 min (wortmannin and LY2940092), 5 min (naloxone), or 16 h (pertussis toxin) prior to agonist stimulation. For desensitization experiments, cells were washed and incubated for 1 h in serum-free medium before 100 nM–1 μM of DAMGO or morphine, and different inhibitors were added to the medium for 2 additional hours. The cells were then washed three times in phosphate-buffered saline and subjected to a second agonist stimulus, typically 100 nM DAMGO for 5 min. C6 glioma cells were transfected with DNA constructs expressing the murine μ-opioid receptor and dominant negative forms of MEK (S221A) (14) and Ras (Asn-17) (15), using the FuGENE 6 transfection reagent (Boehringer Mannheim). Forty-eight hours after transfection, the cells were stimulated with 100 nM DAMGO for 5 min prior to extract preparation.

Immunoblotting and MAPK Assays—For Western blot experiments, cell extracts were prepared using Laemmli sample buffer and subjected to SDS-10% polyacrylamide gel electrophoresis and immunoblotted as described (16). For MAPK activity assays, cells were grown in 10-cm plates for 24 h, treated as indicated, and extracted using a non-denaturing lysis buffer (10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl), protease, and phosphatase inhibitors. Active MAPK was immunoprecipitated using a phospho-MAPK specific antibody and used in kinase reactions to phosphorylate 1 μg of GST-Elk-1 (307–428) fusion protein as substrate. Elk-1 phosphorylation was then analyzed by immunoblotting using a phospho-Elk-1 specific antibody (16).

cAMP Assays—Cells were grown in 6-well plates for 24 h prior to experimental treatment, washed, and incubated in serum-free medium for 1 h, followed by 2 h of incubation in the presence of agonists and different inhibitors. The cells were then washed three times with phosphate-buffered saline and 5 μM forskolin, and 0.5 mM IBMX was added with or without 1 μM DAMGO for 10 min. Cells were then extracted using 0.1 N HCl and assayed using the Biotrak enzyme immunoassay system (Amersham Pharmacia Biotech).

[^{3}H]Naloxone Binding Assay—Before membrane preparation, we fol-

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; GRK, G protein-coupled receptor kinase; PI3K, phosphoinositide 3-kinase; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin; DPDPE, D-Pen^2,D-Pen^5-enkephalin; LPA, lysophosphatidic acid; IBMX, isobutylmethylxanthine; MEK, MAP kinase/ERK kinase.

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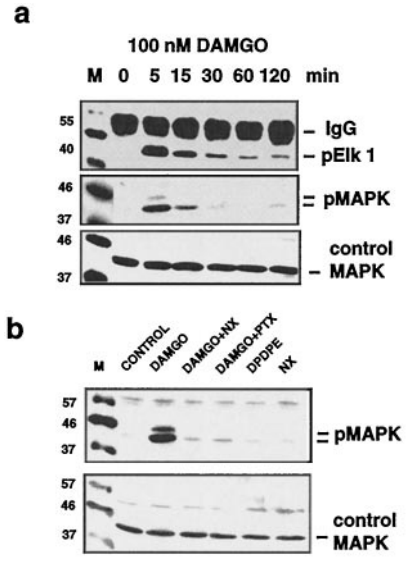

FIG. 1. **Induction of MAPK by μ-opioid agonist DAMGO.** *a*, time course DAMGO induction of MAPK activity (*top*) and phosphorylation (*center, bottom*). *b*, DAMGO-induced MAPK phosphorylation is blocked by naloxone (*NX*, 10 μM) and pertussis toxin (*PTX*, 100 ng/ml) but is not induced by a δ-receptor-specific agonist DPDPE (1 μM) or by naloxone (10 μM) alone. *Control lane* contains extract from non-induced cells. Active MAPK (*a*, *upper panel*) was extracted and assayed as described under "Experimental Procedures." Phosphorylated MAPK was detected by immunoblotting using phospho-MAPK (*pMAPK*) and control MAPK antibodies. In this cell line, the 42 kDa is the predominant MAPK isoform, whereas the 44 kDa is only seen in longer exposures when phosphorylated. *M*, molecular mass marker in kilodaltons.

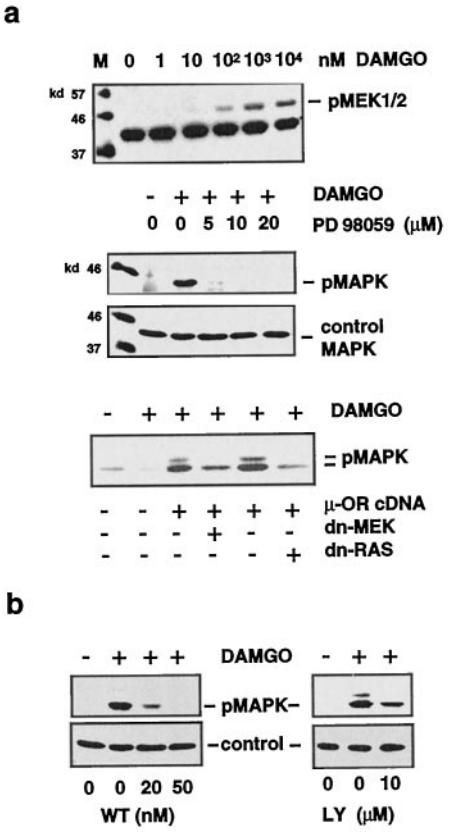

FIG. 2. **Induction of MAPK cascade by μ-opioid receptor involves activation of PI3K, Ras and Raf, MEK1/2.** *a*, *upper panel*: dose-dependent DAMGO induction of MEK1/2 phosphorylation; *center panel*: dose-dependent inhibition of MAPK phosphorylation by the MEK inhibitor, PD98059; *bottom panel*: inhibition of DAMGO-induced MAPK phosphorylation by dominant negative forms of MEK and Ras (*dn-MEK, dn-Ras*). C6 glioma cells were transfected with plasmids expressing the μ-opioid receptor alone (*μ-OR cDNA*) or together with plasmids expressing dominant negative MEK (S221A) or dominant negative Ras (Ha-Ras (Asn-17)). *b*, inhibition of DAMGO-induced MAPK phosphorylation by the PI3K inhibitors wortmannin (*WT*) and LY294002 (*LY*). In all *panels*, extracts were prepared for immunoblotting using phospho-MAPK (*pMAPK*), control MAPK, and phospho-MEK (*pMEK*) specific antibodies. *M*, molecular mass markers in kilodaltons (*kd*).

lowed the same desensitization procedures described above and in Fig. 4. Plasma membranes were prepared essentially as described (3). Binding of [^{3}H]naloxone (specific activity, 53.7 Ci/mmol, NEN Life Science Products) using 25 μg of membrane protein was performed according to the previously described method (3, 17). To generate saturation isotherm the ligand concentration was varied from 0.2 nM to 16 nM. The reaction (90 min at 25 °C) was terminated by a rapid filtration followed by three washes with ice-cold buffer using a Brandel harvester. Cold naloxone, 100 nM, was used to define nonspecific binding. Binding data from saturation experiments were analyzed using the GraphPad Prism 2.01 program (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

We first confirmed that selective μ-opioid agonists were capable of inducing MAPK activity in a Chinese hamster ovary cell line stably transfected with the murine μ-opioid receptor (13). MAPK activity and phosphorylation peaked at 5 min after agonist addition, rapidly decreasing to reach background levels between 30 and 60 min after stimulation (Fig. 1*a*). Increased phosphorylation and enzymatic activity were already detectable at low levels (10 nM) of the μ-receptor agonist DAMGO (data not shown). MAPK phosphorylation was not induced by DPDPE, a selective agonist for the δ-opioid receptor, and was blocked by naloxone and pertussis toxin, confirming that MAPK activation in these cells is specifically mediated by a G_o/G_i-coupled μ-opioid receptor (Fig. 1*b*). In all experiments, MAPK phosphorylation correlated with enzymatic activity as measured in Fig. 1*a*. Identical results were obtained using morphine or peptide μ-agonists such as dermorphin and Met-enkephalin (data not shown). Consistently, DAMGO also induced the phosphorylation of the MAPK-activating kinase MEK1/2 in a dose-dependent manner (Fig. 2*a*, *upper panel*), which is indicative of Raf kinase activation, and following a similar time course as shown for MAPK (not shown). DAMGO-induced MAPK phosphorylation was blocked by the specific inhibitor of MEK activation, PD98059 (18), at concentrations as low as 5 μM (Fig. 2*a*, *center panel*), and by expression of dominant negative forms of MEK1/2 and Ras (Fig. 2*a*, *bottom panel*). MAPK phosphorylation was also reduced by the selective inhibitors of PI3K, wortmannin, and LY294002 at concentrations considered to be specific for PI3K inhibition (8) (Fig. 2*b*). These results suggest that μ-agonist activation of the MAPK pathway requires the involvement of a wortmannin/LY294002-sensitive PI3K, Ras, Raf, and MEK, as has been shown for other G_i-coupled receptors (8, 9).

The rapid decline of MAPK activity despite continuous agonist presence (Fig. 1*a*) is reminiscent of short-term receptor desensitization (6, 7). Receptor desensitization has been extensively studied in the β-adrenergic receptor system (6) and has also been described for opioid receptors mostly in terms of the diminishing agonist inhibition of cAMP levels induced by forskolin (2–4). We investigated whether desensitization of the μ-opioid receptor could be analyzed by monitoring phosphorylation of MAPK. We first measured the time necessary for cells exposed continuously to 100 nM DAMGO to lose their capability to respond to a second agonist stimulus. Fig. 3*a* shows the progressive desensitization of the μ-receptor-stimulated MAPK phosphorylation. A significant decrease (approximately 50–60% of maximum levels) in phosphorylated MAPK was already detected after 5 min of agonist exposure corresponding to the time frame of short-term desensitization (6). No receptor re-

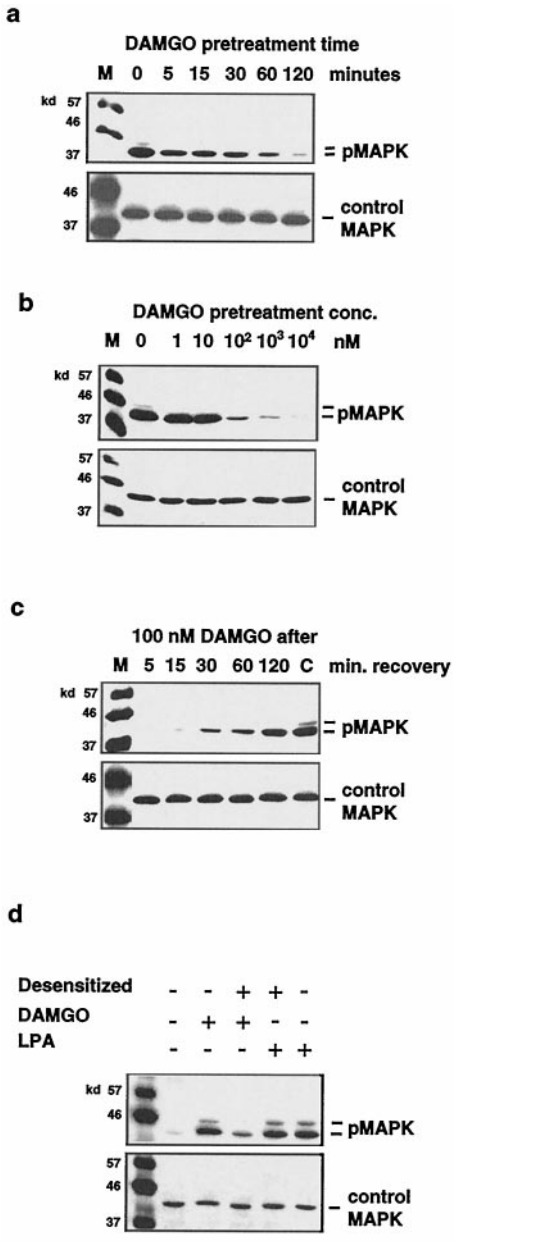

FIG. 3. **Short-term homologous desensitization of MAPK signaling induced by μ-opioid receptor agonists.** *a*, time course of MAPK signal desensitization. Cells were incubated for the indicated periods of time with 100 nM DAMGO, washed, and subjected to a second 100 nM DAMGO stimulus for 5 min. *b*, dose-dependent desensitization of MAPK phosphorylation. Cells were exposed for 1 h to increasing concentrations of DAMGO, washed, and exposed to a second 100 nM DAMGO stimulus. *c*, resensitization time course of μ-receptor-induced MAPK phosphorylation. Cells were exposed for 2 h to 100 nM DAMGO and washed, and fresh serum-free medium added to the wells. After the indicated times, the cells were exposed to a second agonist stimulus for 5 min. Control (*C*) is non-desensitized cells stimulated with the same DAMGO concentration. *d*, homologous desensitization of μ-receptor-induced MAPK signaling. Cells were incubated in serum-free medium with (desensitized, +) or without (−) 100 nM DAMGO for 2 h, washed, and incubated for 5 min in serum-free medium alone (−) or with 100 nM DAMGO (+) or 10 μM LPA (+). After the indicated treatments (*a, b, c, d*) extracts were prepared for immunoblotting with phospho-MAPK (*pMAPK*) and control MAPK-specific antibodies. *M*, molecular mass marker in kilodaltons (*kd*).

sponse, in terms of MAPK phosphorylation stimulated by a second agonist stimulus, could be detected after 2 h of agonist exposure (Fig. 3*a*). Identical results were obtained using morphine instead of DAMGO for the 2-h desensitizing period. Desensitization of MAPK phosphorylation was also dependent upon agonist concentration used in the first stimulus (Fig. 3*b*). The attenuated receptor response is reversed upon agonist removal (19). We next measured the time required for the MAPK signal to become resensitized. The cells recovered their responsiveness to a second DAMGO stimulus only after 15–30 min of incubation in agonist-free medium (Fig. 3*c*). This parallels the resensitization timing reported for the β-adrenergic receptor (20). Similar levels of MAPK phosphorylation as those induced in non-desensitized cells are nearly reached after a 2-h relapse in agonist-free medium (Fig. 3*c*). The μ-agonist-induced desensitization of the MAPK response could be homologous (the loss of response to a specific stimulus) or heterologous (the loss of response to diverse stimuli). To distinguish between these two possibilities we used LPA, which stimulates MAPK phosphorylation via an endogenous G_i-coupled receptor (8). Cells preincubated for 2 h with DAMGO were washed and then exposed to LPA or DAMGO for 5 min (Fig. 3*d*). Whereas DAMGO pretreatment greatly reduced the response to a second DAMGO stimulus, it did not affect the levels of MAPK phosphorylation induced by LPA as compared with those of cells stimulated with LPA but not pretreated with the μ-agonist (Fig. 3*d*). This indicates that the MAPK signal desensitization provoked by a 2-h exposure to a μ-specific agonist had no effect upon any component of the LPA-induced MAPK pathway. Therefore, the MAPK pathway induced by μ-opioid receptor agonists is subject to homologous desensitization in a dose- and time-dependent manner (Fig. 3).

The desensitization process is critical for timing the duration of the cell response to a particular stimulus. Upon signaling through GPCRs the regulation of this process can theoretically occur at any level of the interaction between receptor, G-protein, and effector pathway. The role of GRKs and β-arrestins in receptor phosphorylation, uncoupling, and internalization has been described (6, 21), as well as the importance in these processes of GRK-specific interactions with the Gβγ subunit (22, 23). However, it is possible that other signals contribute to the desensitization process as well. We hypothesized that a feedback signal originating at the effector pathway itself, in this case the MAPK pathway, may help initiate the desensitization mechanism. This signal could induce the molecules that actively uncouple the receptor from the downstream effector pathways. We tested whether blocking the MAPK signal transduction cascade would affect the desensitization of a μ-opioid receptor stimulus. Cells expressing the μ-receptor were exposed to 100 nM DAMGO alone or together with 10 μM naloxone or 20 μM PD98059 for 2 h, the time required for complete desensitization as shown in Fig. 3*a*. The cells were then extensively washed to eliminate agonist and inhibitors and subjected to a second stimulation with 100 nM DAMGO for 5 min before extracts were harvested to assay MAPK phosphorylation (Fig. 4*a, upper* and *middle panels*) and MAPK activity (Fig. 4*a*, *bottom panel*). Cells exposed to DAMGO alone for 2 h did not respond to a second agonist stimulus. In contrast, the presence of PD98059 during preincubation with DAMGO prevented the desensitization of μ-receptor signaling, similar to the expected effect of the antagonist naloxone. We also obtained identical results in these experiments using morphine as the first desensitizing stimulus. At the concentrations used in this study, PD98059 was shown to be highly specific in preventing the activation of MEK, without affecting the activity of 18 other tested Ser/Thr kinases (18). Thus the inhibition of MEK activation and consequently of MAPK caused the receptor to remain sensitive despite 2 h of agonist exposure. If MAPK induced a desensitizing feedback signal that acts at the level of the receptor or G-protein, then inhibition of this signal should also affect the desensitization of other downstream effectors,

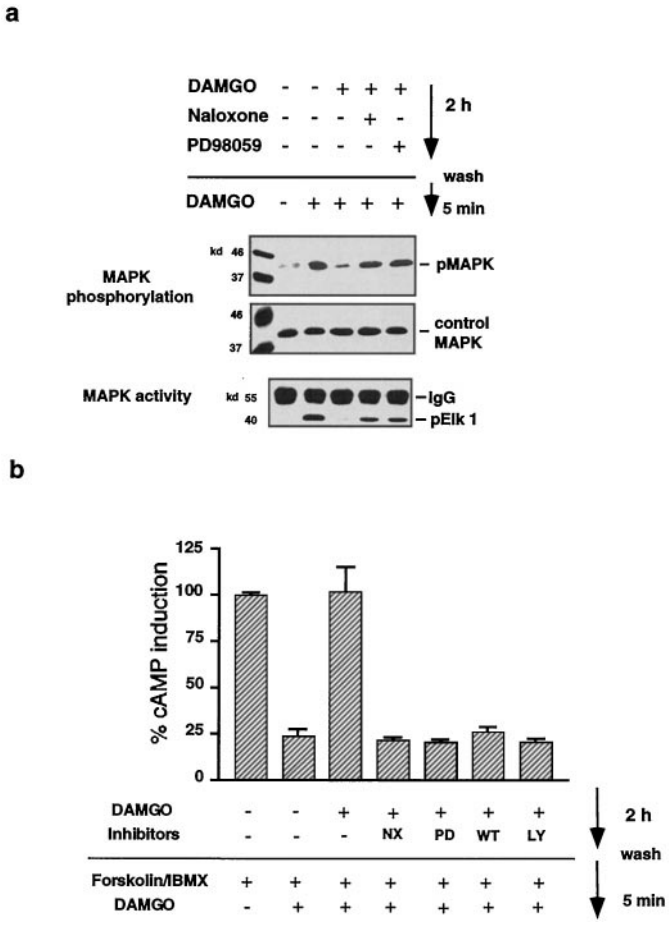

FIG. 4. **Inhibition of the MAPK pathway blocks desensitization of μ-opioid receptor signaling.** *a*, cells were washed and cultured in serum-free medium for 2 h in the absence or presence of DAMGO (100 nM) alone or together with naloxone (10 μM) or PD98059 (20 μM). After 2 h, the cells were washed and re-exposed to DAMGO (100 nM) for 5 min, and extracts were harvested for immunoblotting (*upper* and *middle panels*) and MAPK kinase assay as in Fig. 1*a* (*bottom panel*). *b*, inhibition of MAPK pathway blocks desensitization of cAMP signaling induced by a μ-agonist. Cells were incubated in serum-free medium for 2 h without or with 1 μM DAMGO alone or together with naloxone (*NX*, 10 μM), PD98059 (*PD*, 20 μM), wortmannin (*WT*, 100 nM), and LY294002 (*LY*, 10 μM). After 2 h, cells were washed and incubated with forskolin (5 μM), IBMX (0.5 mM), and with or without 1 μM DAMGO for 10 min. Extracts were prepared and assayed for cAMP concentrations as described under "Experimental Procedures." The results are plotted as a percentage of the cAMP concentration induced by stimulation with forskolin and IBMX alone and expressed as mean ± S.D. of at least three experiments, each data point measured in triplicate.

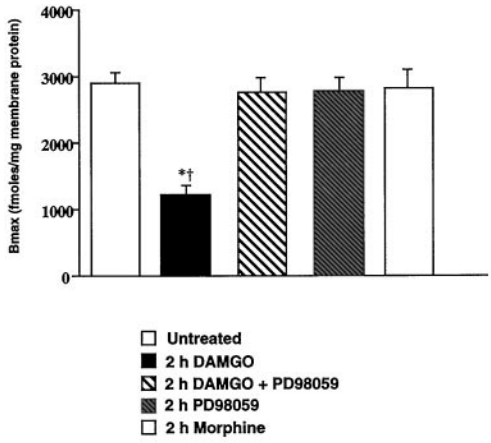

FIG. 5. **The MEK inhibitor, PD98059, blocks the loss of receptor density caused by 2 h agonist exposure.** [^{3}H]Naloxone-specific binding to membranes of the μ-opioid receptor-expressing cell line. Membranes were prepared from untreated cells or cells exposed for 2 h with: 1 μM DAMGO, 1 μM DAMGO + 20 μM PD98059, 20 μM PD98059 alone, and 1 μM morphine as indicated. Saturation curves and Scatchard analysis were performed to obtain B_{max} data (not shown). The bar graph shows the B_{max} data expressed in fmol/mg protein of the five treatment groups. The values represent mean ± S.E. of three experiments determined in triplicates. *, indicates significantly different when compared with membranes from untreated cells; †, indicates significantly different when compared with membranes from treatment groups desensitized in the presence of PD98059 and PD98059 alone (one-way analysis of variance, $p < 0.05$).

such as the cAMP pathway. We then examined whether desensitization of the μ-opioid receptor adenylate cyclase signaling could be blocked by PD98059, as well as by wortmannin and LY294002. These MEK and PI3K inhibitors block the MAPK pathway (Fig. 2) but do not affect the cellular cAMP levels induced by forskolin (not shown). As described previously (13), DAMGO reduced cAMP levels induced by forskolin and the phosphodiesterase inhibitor IBMX (Fig. 4*b*). Pre-exposure to DAMGO for 2 h desensitized the receptor, thereby abolishing this effect. However, when the cells were preincubated with DAMGO in the presence of naloxone, PD98059, wortmannin, or LY294002, a second agonist stimulus reduced the forskolin-stimulated levels of cAMP as in non-desensitized cells. Thus, multiple inhibitors acting at different points along the MAPK cascade are capable of blocking the μ-agonist-induced desensitization of cAMP signaling (Fig. 4*b*).

It has been shown that, except for morphine (24), agonist-induced μ-opioid receptor desensitization is accompanied by the loss of membrane receptor (3, 24). Total receptor binding using the opioid antagonist [^{3}H]naloxone was determined to test whether inhibition of the MAPK pathway would block not only desensitization but also the agonist-induced receptor internalization. [^{3}H]Naloxone binding to membranes of desensitized cells was performed by following the same experimental protocol described in Fig. 4; we expose the cells to no agonist (vehicle, 0.04% dimethyl sulfoxide) or to 1 μM DAMGO, 1 μM morphine, 1 μM DAMGO in the presence of 20 μM PD98059, or to 20 μM PD98059 alone. After 2 h of treatment the cells were extensively washed, and plasma membranes were prepared for radioligand binding assays. [^{3}H]Naloxone bound in a saturable manner to all cell membrane preparations (not shown). Scatchard analysis of the binding results was performed to calculate B_{max} data for each treatment, and these values are compared in the histogram shown in Fig. 5. The results revealed a decrease of approximately 60% in receptor number in cells pretreated with DAMGO (B_{max}, 1217 ± 136 fmol/mg protein, $n = 4$) as compared with untreated cells (B_{max}, 2894 ± 144 fmol/mg protein, $n = 4$). A similar degree of internalization caused by agonist exposure was reported for the μ- and δ-opioid receptors (24–26). Total receptor number in cells pretreated with morphine shows no significant difference to that of untreated cells (B_{max}, 2813 ± 123 fmol/mg protein, $n = 3$). Pretreatment with the agonist in the presence of PD98059 was able to prevent the loss of μ-opioid receptor density (B_{max}, 2753 ± 311 fmol/mg protein, $n = 3$), compared with untreated cells, whereas pretreatment with PD98059 alone had no effect upon μ-opioid receptor density (B_{max}, 2761 ± 98 fmol/mg protein, $n = 3$). The K_d measured for the four treatment groups are: untreated cells (0.9 ± 0.2 nM, $n = 3$); desensitized with DAMGO, (0.9 ± 0.2 nM, $n = 3$); desensitized with morphine (3.7 ± 0.4); desensitized with DAMGO in the presence of PD98059 (1.6 ± 0.6 nM, $n = 3$), and PD98059 treatment alone (0.5 ± 0.08 nM, $n = 3$). Thus, the inhibition of the MAPK pathway at the level of MEK1/2 also impaired the agonist-induced receptor internalization yielding a number of coupled receptors available sufficient to maintain an intact signaling

response (Fig. 4).

Our results suggest that upon agonist activation a feedback signal emanates from the MAPK cascade to promote the desensitization of receptor signaling, since blocking this pathway at different points with different inhibitors prevented the desensitization of two independent effector cascades (Fig. 4). Moreover, the results of the binding studies indicate that an active MAPK pathway also contributes to receptor internalization (Fig. 5). Morphine does not cause receptor internalization even at concentrations that strongly inhibit adenylate cyclase and stimulate MAPK (24). Thus, morphine activation of the MAPK pathway (Fig. 1 and Ref. 12) may not require immediate receptor internalization as recently reported for the β-adrenergic and LPA receptors (27). Our results also indicate that morphine is capable of desensitizing the μ-opioid receptor-stimulated MAPK pathway, and this effect is blocked by PD98059, without affecting internalization (Figs. 4 and 5). This is consistent with reports suggesting that sequestration is not required for desensitization (20). Our study also supports the notion that different μ-agonists can induce two distinct receptor conformational changes, one necessary for signaling and desensitization and a second required for internalization (7, 24). Whereas morphine would induce only the former, other opioid agonists would always induce both.

What then are the potential targets for this desensitizing signal emanating from the MAPK cascade? According to our results (Figs. 4 and 5), particularly the effects of morphine on signaling, desensitization, and internalization, we speculate that this signal should act before internalization is initiated. One possibility is the direct phosphorylation of the receptor by MAPK as was reported for the AT_1 receptor (28). Another possibility is the direct or indirect interaction between the MAPK cascade and GRK2, as GRK2 has been recently implicated in agonist-induced phosphorylation and desensitization of opioid receptors (4, 5, 29). We are currently investigating this hypothesis. A feedback signal promoting uncoupling at the level of the G-protein itself is also possible. Such signal could act on either subunit of the heterotrimeric G-protein or affect the function of regulators of G-protein signaling (RGS proteins) (30). Further investigation on how the MAPK pathway affects μ-opioid receptor desensitization will contribute to elucidate the molecular mechanisms of tolerance to opiate drugs.

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