The mu opioid receptor (MOR) has been shown to desensitize after 1 h of exposure to the opioid peptide, [d-Ala², N-MePhe⁴, Gly-ol⁵]enkephalin (DAMGO), largely by the loss of receptors from the cell surface and receptor down-regulation. We have previously shown that the Thr³⁹⁴ in the carboxyl tail is essential for agonist-induced early desensitization, presumably by serving as a primary phosphorylation site for G protein-coupled receptor kinase. Using a T³⁹⁴A mutant receptor, we determined that Thr³⁹⁴ was also responsible for mu opioid receptor down-regulation. The T³⁹⁴A mutant receptor displayed 50% reduction of receptor down-regulation (14.8%) compared with wild type receptor (34%) upon 1 h of exposure to DAMGO. Agonist-induced T³⁹⁴A receptor down-regulation was unaffected by pertussis toxin treatment, indicating involvement of a mechanism independent of G protein function. Interestingly, pertussis toxin-insensitive T³⁹⁴A receptor down-regulation was completely inhibited by a tyrosine kinase inhibitor, genistein. Tyrosine kinase inhibition blocked wild type MOR down-regulation by 50%, and the genistein-resistant wild type MOR down-regulation was completely pertussis toxin-sensitive. Following DAMGO stimulation, MOR was shown to be phosphorylated at tyrosine residue(s), indicating that the receptor was a direct substrate for tyrosine kinase action. Mutagenesis of the four intracellular tyrosine residues resulted in complete inhibition of the G protein-insensitive MOR internalization. Therefore, agonist-induced MOR down-regulation appears to be mediated by two distinct cellular signal transduction pathways. One is G protein-dependent and GRK-dependent, which can be abolished by pertussis toxin treatment of wild type MOR or by mutation of Thr³⁹⁴. The other novel pathway is G protein-independent but tyrosine kinase-dependent, blocked by genistein treatment, and one in which Thr³⁹⁴ has no regulatory role but phosphorylation of tyrosine residues appears essential.

Although the acute actions of opioids can induce a number of beneficial effects including analgesia and euphoria, chronic use of opioids produces tolerance and dependence (1, 2), which are among the major factors limiting the clinical use of these compounds. The molecular mechanisms underlying these phenomena are poorly understood, but receptor desensitization has been implicated as having a major role.

It has been shown that opioid receptor desensitization is directly related to receptor phosphorylation (3–5), possibly mediating receptor down-regulation (6, 7). A role for several kinases has been postulated in opioid receptor desensitization, including cAMP-dependent protein kinase (PKA) (4), calcium-dependent protein kinase (PKC) (4, 8), G protein-coupled receptor kinase (GRK) (5, 9), and mitogen-activated protein kinase (10). A large body of evidence for many G protein-coupled receptors supports the contention that phosphorylated receptors are translocated into the cytosol through binding of arrestin to the phosphorylated receptor. Thus, phosphorylation of the receptors appears to be the prerequisite event for receptor down-regulation (11, 12). In addition to the general involvement of kinases in receptor function, specific phosphorylation sites have been mapped in the third intracellular loop and the carboxyl tail of the muscarinic cholinergic receptor and β₂ adrenergic receptor (β₂AR) (12, 13). However, few studies have directly addressed the involvement of receptor phosphorylation in the down-regulation of opioid receptors. In the case of the δ opioid receptor, deletion of the carboxyl tail of receptor expressed in CHO cells completely abolished receptor down-regulation, suggesting that the carboxyl tail is the necessary structural determinant for δ opioid receptor down-regulation (15, 16). In contrast to this study, the same δ opioid receptor deletion mutant expressed in HEK 293 cells showed the identical degree of receptor internalization compared with the wild type δ opioid receptor in the absence of any detectable phosphorylation, suggesting that phosphorylation of G protein-coupled receptors may not be an absolute requirement for receptor internalization (17).

In the case of the μ opioid receptor (MOR), the reduction of total membrane receptors appears to be the major mechanism for functional desensitization of the MOR (7, 9). Furthermore, we and others have reported that the structural determinants necessary for agonist-induced MOR desensitization are Thr³⁹⁴ and its preceding acidic amino acid stretch (6, 9). Thus, in present study, we examined the further functional roles of these amino acid residues in the carboxyl tail to establish the structural determinants mediating MOR down-regulation.

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¹The abbreviations used are: PKA, cAMP-dependent protein kinase; PKC, calcium-dependent protein kinase; GRK, G-protein-coupled receptor kinase; MOR, μ opioid receptor; CHO, Chinese hamster ovary; DAMGO, [D-Ala², N-Me-Phe⁴, Gly-ol⁵]enkephalin; β₂AR, β₂-adrenergic receptor; PBS, phosphate-buffered saline.
EXPERIMENTAL PROCEDURES

Generation of Mutants and Stable Cell Lines Expressing Wild Type or Mutant Receptors—The full-length cDNA for the rat MOR was cloned into the mammalian expression vector pRC/CMV (InVitrogen, San Diego, CA). This construct was used as a template for mutagenesis and for subsequent stable transfection of the wild type and mutant receptor cDNAs into CHO cell lines. Mutant MORs such as T394A, T394S (AT) (see Fig. 3), Y326F, Y372F, Y165F, and Y386F (see Fig. 5B) were constructed by substituting single or multiple amino acid residues. Site-directed mutagenesis was performed using a polymerase chain reaction-based technique as described (18). Briefly, oligonucleotide primers corresponding to unique EcoRI/Apal restriction sites (located at amino acids 304–305 and at 3′-region of vector, respectively) or NolI (located at the 5′-region of vector/EcoRI) were designed in combination with two mutagenic primers. Polymerase chain reaction fragments were digested with unique restriction enzymes and subcloned into the corresponding restriction sites of the pRC/CMV encoding a MOR construct. All mutations were verified by dideoxy sequencing. For stable expression, the cell line CHO-K-1 (number CCL61; American Type Culture Collection) was grown to 60% confluence in a 100-mm dish and subsequently transfected with wild type or mutant constructs in pRC/CMV vector using a calcium phosphate transfection kit (Life Technologies, Inc.) according to the manufacturer’s recommendations. Stable transfectants were selected in 1 mg/ml genetin (Life Technologies, Inc.), and clones with the appropriate expression level were screened by radioligand binding assay. 30–90 clones expressing varying numbers of receptors were screened to select those with comparable expression levels.

Determinations and Assay conditions—CHO cells expressing wild type or mutant MORs at confluent monolayer were treated with either medium alone or 1 μM DAMGO (final concentration) and incubated for 1 h at 37 °C in 5% CO2. Incubation was terminated by washing plates three times with 12 ml of ice-cold PBS. Membranes were prepared as described above. Adenylyl cyclase assays were conducted essentially as described previously (9). The assay mix contained 20 mM Tris/HCl, pH 7.4, 15–20 μM GDP, 0.1 μM [3H]cAMP, 0.12 μM ATP, and 0.05 μM GTP, 53 μM GTP, 2.7 mM phosphoenopyruvate, 0.2 μM of pyruvate kinase, 1 unit of myokinase, and 0.13 μCi of [3H]cAMP. The amounts of [3H]cAMP were determined in a parallel assay. Samples were assayed in quadruplicate for each condition. Results were expressed as the means ± S.E. of triplicate samples. Data from multiple experiments were analyzed by nonlinear least squares regression using the data analysis program LIGAND (20). Data from multiple experiments were averaged and expressed as the means ± S.E. The results were considered significantly different when the probability of randomly obtaining a mean difference was <0.05 using the paired Student’s t test.

RESULTS

We have previously reported that the MOR is desensitized after 1 h of treatment with an agonist, 1 μM DAMGO (7). The underlying mechanism of this agonist-induced form of desensitization is likely phosphorylation of MOR, because mutation of a potential GRK phosphorylation site, Thr394 in the carboxyl tail of MOR, completely abolished agonist-induced desensitization (9). During 1 h of DAMGO exposure, the absolute number of MOR in the high affinity state was reduced, resulting from a reduction in total membrane receptors, and this seemed to be the major mechanism causing functional desensitization (7). Therefore, we have investigated the possible role of specific carboxyl tail residues including GRK-dependent phosphorylation sites in MOR down-regulation (Fig. 1).

With wild type MOR, [3H]naloxone competition by DAMGO following exposure to agonist for 1 h showed that the total number of receptors was decreased by 34 ± 1.6% (n = 6) compared with that of untreated membranes (Fig. 2A). The proportion of receptors in the agonist-detected high affinity state and in low affinity state (Table I) remained unchanged following treatment with DAMGO, suggesting that prolonged exposure of agonist did not affect the coupling of wild type MOR to G protein (Fig. 2B). We tested the involvement of Thr394 and the proximal 7 serine/threonine amino acid residues in agonist-
induced MOR down-regulation. Interestingly, none of these mutations resulted in complete abolishment of receptor down-regulation, even though only the T394A mutant receptor showed complete inhibition of DAMGO-induced desensitization. However, compared with wild type receptor, T394A mutant receptors were down-regulated to a lesser extent following agonist exposure; 34 ± 1.6% (n = 6) for wild type, 14.8 ± 3.19% (n = 8) for the T394A receptor (Fig. 2A). The AT mutant receptor showed desensitization of DAMGO inhibition of forskolin-stimulated cAMP accumulation after 1 h of agonist treatment, with maximal inhibition that was only 70% of that observed with wild type receptor (9). This mutant receptor also showed down-regulation of receptor by 21.7 ± 1.95% (n = 4), which was 64% of wild type MOR down-regulation (p < 0.05) (Fig. 2A). The ATT mutant (Fig. 1) showed an identical degree of desensitization compared with wild type receptor (9). This mutant receptor also showed an identical degree of receptor down-regulation (29.9 ± 0.2%, n = 3, p > 0.05) compared with the wild type receptor (Fig. 2A). Down-regulation of the AT and ATT mutant receptors were significantly different from each other (p < 0.05), suggesting that these receptors differing by a single amino acid demonstrated differential capabilities of agonist-induced down-regulation. However, these mutations did not affect agonist affinity or proportion of receptors in the high affinity state (Table I) compared with wild type receptor, just as shown for the T394A mutant (Fig. 2C), indicating that the properties of G protein coupling were identical to that of wild type MOR. These data imply that the mutated residues in the carboxyl tail of MOR are not involved in G protein coupling but may serve as site(s) regulating down-regulation and desensitization. Furthermore, all mutant receptors displayed the identical degree of agonist-mediated maximal inhibition of adenyl cyclase activity and also similar IC_{50} values (Table II), indicating that the mutant receptors retained

**TABLE I**

| [^3]H|Naloxone | [^3]H|Naloxone/DAMGO |
|---|---|---|
| | K_H | K_L | R_H | R_L |
| WT | B_{max} | 2.9 ± 0.5 pmol/mg | 3.9 ± 0.7 | 305 ± 47 | 68 ± 2 | 32 ± 2 |
| | K_d | 1.1 ± 0.1 nM | | | | |
| T394A | B_{max} | 1.5 ± 0.1 pmol/mg | 3.2 ± 0.5 | 326 ± 83 | 76 ± 1 | 24 ± 1 |
| | K_d | 1.0 ± 0.3 nM | | | | |
| AT | B_{max} | 4.3 ± 0.3 pmol/mg | 4.9 ± 1.3 | 379 ± 102 | 67 ± 1 | 33 ± 1 |
| | K_d | 1.0 ± 0.1 nM | | | | |
| ATT | B_{max} | 4.0 ± 0.1 pmol/mg | 4.9 ± 1.2 | 354 ± 32 | 70 ± 1 | 30 ± 1 |
| | K_d | 1.0 ± 0.1 nM | | | | |
| 4YF | B_{max} | 0.17 ± 0.02 pmol/mg | 6.0 ± 5.7 | 100 ± 68 | 36 ± 4 | 63 ± 4 |
| | K_d | 1.9 ± 0.2 nM | | | | |
The ability to stimulate the adenyl cyclase effectors system through normal G protein activation, even though they differed with respect to receptor down-regulation (Fig. 2A). This strongly suggests that MOR down-regulation is independent of G protein activation.

To determine the involvement of G protein activation in MOR down-regulation, CHO cells expressing wild type MOR were treated with 1 μg/ml of pertussis toxin for 24 h to uncouple the receptor from G proteins. Membranes treated with pertussis toxin were then exposed to DAMGO for 1 h. Pertussis toxin treatment resulted in a complete loss of the agonist detected high affinity state, with receptor existing in a single low affinity, indicating complete uncoupling from G protein (Fig. 3A). However, DAMGO-induced down-regulation of MOR still occurred after pertussis toxin treatment. The down-regulation of MOR by agonist following pertussis toxin treatment was confirmed by Scatchard analysis of [3H]naloxone saturation isotherms (Fig. 3A, inset). The reduction of cell surface receptors by DAMGO in pertussis toxin treated membranes was 21.65 ± 3.75% (n = 3) of that observed with pertussis toxin treatment alone (Fig. 3B). This degree of down-regulation was significantly different from wild type MOR (34 ± 1.6% n = 6) but not different from the T394A mutant receptor down-regulation (14.8 ± 3.2, n = 8) (Fig. 3B). Therefore, agonist-induced MOR down-regulation was reduced by 40% with pertussis toxin treatment, indicating partial dependence on G protein activation. However, the residual MOR down-regulation unaffected by pertussis toxin treatment was therefore clearly independent of G protein activation. Thus, it is tempting to speculate that MOR down-regulation, which is partially blocked by the T394A mutation, may be dependent on G protein activation and GRK-dependent phosphorylation, whereas the residual down-regulation seen in the T394A mutant or after pertussis toxin treatment may be independent from G protein activation and, further, independent from GRK-dependent phosphorylation. This led us to test the possibility that the agonist may induce MOR down-regulation through activation of protein kinases other than GRKs.

To determine the identity of the kinases involved in G protein-independent receptor down-regulation, we used a panel of kinase activators and inhibitors. Because there are several reports of the possible role of PKA or PKC on receptor phosphorylation (4, 8) and the target motif is present in the third intracellular loop of MOR, we first examined for involvement of these kinases. To test the possibility of PKA involvement in MOR down-regulation, H89-dihydrochloride was used to inhibit PKA activation. CHO cells expressing wild type MOR were treated with H89-dihydrochloride 5 μM for 15 min prior to and during 1 μM DAMGO treatment for 1 h. The PKA activator, 8-bromo cAMP 100 μM, was also tested without pretreatment with DAMGO to determine whether PKA involvement was an agonist-specific phenomenon. In the same way, the PKC inhibitor, bis-indolylmaleimide I (1 μM) and activator, phorbol 12-myristate 13-acetate (1 μM) were also tested. Surprisingly, neither PKA nor PKC inhibitors blocked agonist-induced MOR down-regulation (Fig. 4A). The PKA inhibitor resulted in increased DAMGO-induced MOR down-regulation (45.5 ± 3.2%, n = 4), whereas the PKC activator alone did not affect MOR down-regulation (Fig. 4A). MOR down-regulation by agonist in the presence of the PKC inhibitor was unaffected (37.63 ± 2.5%, n = 4), and the PKC activator alone did not cause any changes in receptor down-regulation (Fig. 4A). However, the tyrosine kinase inhibitor, genistein (final concentration, 100 μM) partially blocked DAMGO-induced MOR down-regulation (17.3 ± 3.3%, n = 4) by 50% compared with wild type MOR (Fig. 4A). But genistein in the absence of DAMGO without activation of MOR did not affect the receptor density on the cell surface (data not shown), indicating that MOR down-regulation because of activation of tyrosine kinase was agonist-specific. Furthermore, the blockade of MOR down-regulation using genistein was saturable, suggesting that genistein could not block MOR down-regulation further with higher concentrations (data not shown).

Because the study of wild type MOR suggested that receptor down-regulation was partially G protein-independent and genistein sensitive, we examined whether this was dependent on tyrosine kinase activation. Because DAMGO-induced receptor down-regulation was present in the T394A mutant receptor (14.8 ± 3.2%) (Fig. 3B), we first tested whether this residual T394A mutant receptor down-regulation was also G protein-independent. CHO cells expressing the T394A mutant receptor were treated with 1 μg/ml pertussis toxin for 24 h prior to DAMGO treatment, and receptor down-regulation was compared with cells treated with pertussis toxin alone. Indeed, DAMGO-induced T394A mutant receptor down-regulation occurred even following pertussis toxin treatment, and its extent (13.15 ± 0.8%, n = 3) was identical to that of pertussis toxin untreated membranes (14.8 ± 3.2%) (Fig. 4B), suggesting that DAMGO-induced T394A mutant receptor down-regulation was entirely G protein-independent.

We further tested whether the G protein-independent T394A mutant receptor down-regulation could be blocked by the tyrosine kinase inhibitor. When CHO cells expressing the T394A mutant receptor were treated with 100 μM genistein, down-regulation of receptors because of DAMGO exposure was completely blocked (Fig. 4B). We therefore explored the possibility that MOR might be a direct substrate for tyrosine kinase. When wild type MOR expressed in CHO cells was precipitated using the anti-MOR antibody and probed with an anti-phosphotyrosine antibody, a tyrosine-phosphorylated protein of ~70 kDa appeared only in the transfected cells exposed to DAMGO (Fig. 5A, lane 3 in upper panel). Similar amounts of MOR protein were detected in transfected but agonist-unstimulated cells when the anti-MOR immunoprecipitates were Western blotted (Fig. 5A, lanes 5 and 6 in lower panel). Because there are four tyrosine residues in intracellular loops and the carboxyl tail that may serve as potential phosphorylation sites (Fig. 5B), we mutated all four tyrosines to phenylalanine (4YF) to test whether these residues were functionally involved in MOR down-regulation. The 4YF mutant receptor stably expressed in CHO cells consistently demonstrated a low expression level in 45 cell lines screened, suggesting that some or all of these tyrosine residues may have a role in cell surface expression (Table II). We have previously reported that the receptor expression level did not affect the degree of DAMGO-induced receptor down-regulation (7). Interestingly, the 4YF mutant receptor showed comparable receptor down-regulation with the T394A receptor: 14.53 ± 3.8% (n = 3) for 4YF (Fig. 5C) and 14.8 ± 3.19% for T394A receptor (Fig. 2A), suggesting that

| Maximum inhibition of adenyl cyclase activity | IC_{50} |
|---------------------------------------------|---------|
| Wild type                                   | 54.0 ± 1.7 | n. n. |
| T394A                                       | 60.3 ± 2.1 | 5.6 ± 1.2 |
| AT                                          | 52.1 ± 1.9 | 8.5 ± 4.1 |
| ATT                                         | 52.4 ± 2.8 | 25.1 ± 9.0 |

TABLE II

Functional properties of wild type and mutant MORs

IC_{50} is the concentration yielding half-maximal functional response. Values shown are the means ± S.E. from at least three independent experiments.
the G protein-dependent pathway was still active in the 4YF mutant receptor.

To examine the involvement of G protein-dependent receptor down-regulation in the 4YF mutant, cells expressing this receptor were treated with pertussis toxin. DAMGO-induced 4YF mutant receptor down-regulation was completely abolished by pertussis toxin treatment (1.6 ± 1.6%, n = 3) in Fig. 5C, indicating that the residual receptor down-regulation observed in 4YF was entirely due to the G protein-dependent pathway mediated by Thr^{394} and, further, that the tyrosine residues mutated were functionally involved in receptor down-regulation. Thus, MOR down-regulation seems to be governed by two distinct and mutually exclusive cellular signal transduction pathways.

DISCUSSION

Our results demonstrate that MOR down-regulation is dually regulated by two distinct equally important cellular signal transduction pathways; one is a G protein-dependent, GRK-dependent pathway, and the other is a G protein-independent,
tyrosine kinase-dependent pathway. Agonist-induced down-regulation of G protein-coupled receptors has been proposed to be due to phosphorylation of the agonist-bound form of the receptors and subsequent internalization, because of binding of arrestin to the phosphorylated form of receptors (reviewed in Refs. 11 and 12). The recognition sites for the G protein-coupled receptor kinases have been identified as repeated serines and threonines mostly in the carboxyl tail, by phosphopeptide sequencing of high pressure liquid chromatography purified peptides derived from proteolysis of phosphorylated receptors (21, 22). However, the involvement of these amino acid residues in receptor down-regulation has been unclear. For instance, Strader et al. (23) reported that a mutant hamster β2AR lacking both the GRK-dependent phosphorylation sites and the putative PKA sites in the carboxyl terminus was seques tered normally in response to the agonist, isoproterenol. In contrast to this study, using a chimeric β2/β3AR in which single or multiple intracellular domains and carboxyl tail of the β2AR were exchanged with the corresponding regions of the β2-adrenergic receptor, Jockers et al. (14) have shown the involvement not only of the carboxyl tail of β2AR but also the second intracellular loop in receptor sequestration. Because a chimeric receptor, in which the carboxyl tail of β2AR containing GRK-dependent phosphorylation sites was exchanged into β2-adrenergic receptor, did not show β2AR like sequestration, this result suggested that factors other than GRK-dependent ones may be involved in receptor sequestration. However, in certain other receptor systems, there is evidence that receptor down-regulation may be solely due to GRK-dependent phosphorylation. For instance, in the case of the human muscarinic cholinergic receptor types 1 and 3, serine/threonine rich domains in the third intracellular loop are involved in receptor internalization; amino acid residues ESLTSSE for type 1 and EN- SASSD for type 3, which are GRK-dependent phosphorylation sites, were the only determinants for receptor internalization (24). Additionally, in the case of the gastrin-releasing peptide receptor, a mutant in which all serines and threonines in the carboxyl tail were deleted showed complete abolishment of receptor internalization (25). However, this deletion mutant receptor showed normal activation of the effector system in increasing total inositol phosphate similar to wild type receptor, indicating that in this case, receptor internalization was regulated independently from receptor-G protein coupling. Furthermore, β2AR expressed in the cyc-mutant of S49 cells that are functionally uncoupled from adenyl cyclase because of an inherent mutant GCα present, still were able to internalize normally (26, 27). These results strongly support distinct and independent mechanisms for G protein activation in signal transduction and for receptor internalization. In our study, the MOR was partly down-regulated by DAMGO even after uncoupling the receptor from G protein by pertussis toxin treatment, indicating that MOR down-regulation was mediated by G protein-dependent phosphorylation of receptors and subsequent internalization, because of binding of arrestin to the phosphorylated form of receptors. The percentages of DAMGO-induced receptor down-regulation are presented as the means ± S.E. of at least three independent experiments. Significant difference from wild type is denoted by an asterisk (p < 0.05), and the double asterisks indicate significant difference from 4YF (p < 0.05).

What other kinases are likely to be involved? Although PKA- dependent phosphorylation of β2AR was known to play an important role in agonist-specific desensitization together with GRK, the direct involvement of this kinase in agonist-induced receptor internalization has not been well characterized (32–35). However, the tyrosine residue (NPXXY), which is highly conserved among G protein-coupled receptors has been re-
ported to be responsible for β2AR sequestration (28, 36, 37). Indeed, agonist-induced receptor sequestration was essentially abolished with the Y326A mutant β2AR (NPXXA) but was only slightly reduced in the conservatively substituted Y326F receptor (38). This result indicates that this tyrosine residue may not be involved in receptor phosphorylation because of tyrosine kinase activation, but this sequence motif seems to provide the distinct structure for the binding of some other cellular proteins involved in receptor sequestration. However, there has been a substantial body of evidence that tyrosine kinases could be activated through G protein-coupled receptor stimulation. Activation of Gα2-coupled m1 muscarinic cholinergic receptor has been shown to activate a Src-related tyrosine kinase, Lyn, and Gα1-coupled m2 muscarinic cholinergic receptor has been shown to activate another nonreceptor tyrosine kinase, Syk (39). Furthermore, phosphoamino acid analysis establishes that β2AR residues Tyr132, Tyr141, Tyr350/353, and Tyr364 are phosphorylated by insulin in vitro, suggesting that β2AR is a substrate for the insulin receptor tyrosine kinase (40). More directly, upon agonist stimulation, the YIPP motif in the carboxyl tail of angiotensin AT1 receptor was reported to be phosphorylated by c-Src tyrosine kinase (41). However, the role of the activation of these tyrosine kinases on G protein-coupled receptor function has been unclear.

In the case of MOR, PKA and PKC have been reported to be differentially related to MOR function (4). Activation of PKC with phorbol ester potentiated the desensitization of MOR-induced G protein-activated K+ channel activity, but injection of the catalytic subunit of PKA completely abolished the desensitization (4). Interestingly, another group reported contradictory results with the same system, where the desensitization of MOR-induced G protein-activated K+ channel activity was not affected by PKA or PKC activation (42). These controversial effects of PKA and PKC on MOR desensitization may be due to effects of these two kinases on other components of the signaling pathways. Furthermore, the PKC inhibitor staurosporine failed to block morphine-induced receptor phosphorylation and subsequent receptor desensitization, although the PKC activator phorbol 12-myristate 13-acetate enhanced receptor phosphorylation (8). Our data showed that PKA or PKC did not affect MOR down-regulation and very likely MOR desensitization following agonist exposure for 1 h. Recently, mitogen-activated protein kinase affecting MOR function has been reported, whereby agonist activation of MOR stimulated mitogen-activated protein kinase activity and, further, promoted receptor desensitization and internalization (10). It has been suggested that activation of MOR increases c-Src tyrosine kinase activity (43), but the consequence of this activation on MOR function has not been elucidated. We have shown for the first time that MOR is directly phosphorylated by a protein-tyrosine kinase upon receptor activation and that this has a significant role in receptor down-regulation. This result confirms our conclusion that GRK-independent protein kinases are also stimulated by MOR activation.

In summary, we have demonstrated that there are two distinct but equally important cellular mechanisms mediating agonist-induced MOR down-regulation; one is a G protein-dependent, GRK-dependent pathway, and the other is a G protein-independent, tyrosine kinase-dependent pathway. One pathway appears unaffected when the other pathway is blocked, and we have identified discrete structural elements that separately participate in each pathway. This is the first demonstration of a role for tyrosine kinase in agonist-induced MOR down-regulation. Further investigation of the specific tyrosine residues that may be substrates for tyrosine kinase action and the other cellular proteins that may participate following tyrosine phosphorylation will elucidate the detailed molecular mechanism of tyrosine kinase activation leading to MOR internalization.

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