Identification of Serine 356 and Serine 363 as the Amino Acids Involved in Etorphine-induced Down-regulation of the \( \mu \)-Opioid Receptor

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Agonist-induced internalization of G protein-coupled receptors is influenced by many structural determinants including the carboxyl tail. To investigate the role of serine and threonine residues within the carboxyl tail, several mutants were constructed by truncating the carboxyl tail of the hemagglutinin-tagged \( \mu \)-opioid receptor, thereby removing serines and threonines systematically. Neuro2A cells stably expressing the truncated receptors did not exhibit a significant alteration in the affinity of \([^{3}H]diprenorphine\) or etorphine for the receptor or the potency of etorphine to inhibit forskolin-stimulated adenyl cyclase activity. Chronic etorphine treatment resulted in a time-dependent down-regulation of all the truncated receptors, except MORITAG355D, thus revealing the importance of the carboxyl tail, several mutants were constructed by truncating the carboxyl tail of the hemagglutinin-tagged \( \mu \)-opioid receptor, thereby removing serines and threonines systematically. Neuro2A cells stably expressing the truncated receptors did not exhibit a significant alteration in the affinity of \([^{3}H]diprenorphine\) or etorphine for the receptor or the potency of etorphine to inhibit forskolin-stimulated adenyl cyclase activity. Chronic etorphine treatment resulted in a time-dependent down-regulation of all the truncated receptors, except MORITAG355D, thus revealing the importance of the four amino acids between Ser\(^{355}\) and Glu\(^{359}\) (STIE). Surprisingly, deletion of the STIE sequence resulted in a receptor that down-regulated the same as the wild-type receptor. The involvement of multiple amino acids within the carboxyl tail was demonstrated by combining alanine substitutions of several putative G-protein-coupled receptor kinase phosphorylation sites. Systematic analysis of these receptors indicated that mutation of Ser\(^{356}\) and Ser\(^{363}\) to alanine attenuated agonist-mediated down-regulation. The magnitude of etorphine-induced phosphorylation of this mutant receptor, however, was similar to that of the wild-type \( \mu \)-opioid receptor. Thus, phosphorylation of the carboxyl tail of the \( \mu \)-opioid receptor is not an obligatory event for etorphine-induced down-regulation of the receptor.

Prolonged exposure to opioid agonists results in a loss of effector response termed desensitization. It is thought that the overall desensitization of opioid receptors follows that of other G-protein-coupled receptors (GPCR), where cellular adaptations result in conformational changes that uncouple the receptors from their respective G-proteins. Such molecular changes also lead to an ultimate loss of receptors from the cell surface via internalization and degradation pathways. The mechanisms and specific amino acids which facilitate these processes, however, have not been delineated.

While nearly all GPCRs follow this overall process of desensitization, the \( \beta \)-adrenergic receptor has served as a model for characterizing such mechanisms. In the \( \beta \)-adrenergic receptor, agonist-activation initiates receptor phosphorylation by multiple kinases including protein kinase C (PKC), protein kinase A (PKA), and G-protein-coupled receptor kinases (GRK). Phosphorylation of the receptor, in turn, enhances the affinity of \( \beta \)-arrestin, an associative protein, which advances receptor internalization leading to receptor degradation (1). This paradigm of receptor phosphorylation has been shown to account for the agonist-induced receptor internalization of numerous other members of the GPCR family (2–6). There is, however, no consensus among the GPCR family as to a common motif for down-regulation, but phosphorylation of the receptor appears to be a prerequisite.

Studies have indicated that opioid receptors are rapidly phosphorylated after agonist activation as well. Zhang and colleagues (7) demonstrated that both morphine and phorbol 12-myristate 13-acetate, a PKC activator, stimulated phosphorylation of the \( \mu \)-opioid receptor expressed in Chinese hamster ovary cells within 5 min of exposure. They then suggested a correlation between the time course of this phosphorylation event with that of the desensitization of the inwardly rectifying potassium channel (GIRK) to opioid agonists in \textit{Xenopus} oocytes expressing both the \( \mu \)-opioid receptor and channel (7). These studies in the oocytes were supported by a study with Chinese hamster ovary cells stably expressing the wild-type and mutant receptors when Pak and colleagues (8) reported that point mutation of Thr\(^{394}\) to alanine completely blocked desensitization of the receptor to DAMGO after just 1 h of exposure. They proposed that phosphorylation of this specific threonine individually gated the desensitization of the \( \mu \)-opioid receptor. However, studies by Kovoor and co-workers (9) directly contradicted the notion that a phosphorylation event is involved in the desensitization of the \( \mu \)-opioid receptor. They demonstrated that the rate of desensitization was not altered by staurosporine, a PKC inhibitor, and concluded that the desensitization of the receptor does not involve a phosphorylation dependent mechanism (9). Interestingly, this study was supported by El-Kouhen and colleagues who recently demonstrated that the loss of adenylyl cyclase to DAMGO had a \( t_{1/2} \) of 7 h in HEK 293 cells stably expressing the \( \mu \)-opioid receptor. This loss of response, however, did not correspond with DAMGO-induced phosphorylation which was maximal after just 20 min, suggesting that the phosphorylation of the \( \mu \)-opioid receptor.

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† The abbreviations used are: PKA, cAMP-dependent protein kinase; PKC, calcium-dependent protein kinase; GPCR, G-protein coupled receptor; GRK, G-protein coupled receptor kinase; PCR, polymerase chain reaction; Taps, 3-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)-1-propanesulfonic acid.

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Opioid receptor does not directly induce desensitization of the receptor. 2

Despite these discrepancies, phosphorylation has been demonstrated to play a critical role in the down-regulation of the receptor after long-term agonist exposure. The addition of phorbol 12-myristate 13-acetate potentiated agonist-induced down-regulation of the δ-opioid receptor, and staurosporine, a PKC inhibitor, blocked down-regulation of the phorbol 12-myristate 13-acetate stimulated receptors, but not those receiving agonist alone (10), favoring PKC as the mediating kinase. Additionally, dominant negative mutants of β-adrenergic receptor kinase 1 and GRK 5 did not block agonist-induced phosphorylation of the δ-receptor, again arguing that phosphorylation occurs through multiple kinases (11). Another study that examined down-regulation of the δ-opioid receptor demonstrated that Thr353 alone mediated agonist-induced down-regulation and argued that phosphorylation was responsible for this event (12). Thr353, however, is not a conserved residue for the μ- or κ-receptors. Furthermore, through the use of fluorescently labeled deltorphin I and [Lys3]dermorphine analogs, Gaudriault et al. (13) demonstrated that the μ- and δ-opioid receptors internalize via different pathways utilizing distinct, yet separate, proteins. This would support the hypothesis that there is a difference in the down-regulation motif of the δ- and μ-opioid receptors. In addition, Capeyrou and colleagues (14) showed that agonist-induced down-regulation was blocked when all the serine and threonine residues within the third intracellular loop and carboxyl tail of the μ-opioid receptor were exchanged with alanine. The study unfortunately did not evaluate the carboxyl tail and third intracellular loop separately, but the findings suggest a role for phosphorylation in agonist-induced down-regulation of the μ-opioid receptor.

The studies presented here investigate whether phosphorylation plays a pivotal role in agonist-induced down-regulation of the μ-opioid receptor. The carboxyl tail of the μ-opioid receptor contains 12 serine and threonine residues which constitute numerous GRK and PKC consensus sites. The importance of this domain in agonist-induced down-regulation was evaluated through the use of (i) truncations of the carboxyl tail; (ii) deletions of specific motifs within the carboxyl tail; and (iii) point mutations of serine and threonine residues within the carboxyl tail.

**EXPERIMENTAL PROCEDURES**

**Materials—Oligonucleotides were synthesized by an automated DNA synthesizer (Millipore model 8905). Taq polymerase and restriction enzymes were obtained from Boehringer-Mannheim (Indianapolis, IN). Expression vector pcDNA, and cloning vector pCR II were from Invitrogen (San Diego, CA). Cell culture reagents, Dulbecco’s modified Eagle’s medium, minimal essential medium, fetal calf serum, and G418 were purchased from Life Technologies, Inc. (Grand Island, NY). [3H]Adenine and [3H]diprenorphine were purchased from Amersham and 125I-labeled acetylated cAMP was purchased from Linco Research (St. Charles, MO). All opioid peptides and alkaloids, with the exception of naloxone (supplied by Endo Lab) were obtained from the National Institute on Drug Abuse. Polyclonal antibodies for the cAMP radiomunnoassay were a generous gift from Dr. T. Gettys (Medical University of South Carolina, Charleston, SC).

**Construction of Mutants of the μ-Opioid Receptor—**The human influenza virus hemagglutinin epitope-tagged μ-opioid receptor as described previously (15) was utilized in the construction of the truncated μ-opioid receptors. Polymerase chain reactions (PCR) were used to truncate the receptor by adding a stop codon after amino acids Ser356, Ser359, Glu359 or Ser355. The PCR reactions contained (final concentration) 25 mM Taps-HCl (pH 9.3), 50 mM KC1, 2 mM MgCl2, 1 μM dithiothreitol, 0.2 mM upstream and downstream primers, 0.2 mM dNTP, and 2.5 units of Taq polymerase (Boehringer-Mannheim) with the following conditions: 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min for 25 cycles. All the downstream primers contained the XbaI site used in later subcloning. The resulting products were subcloned into a pCRII plasmid (Invitrogen) and were then sequenced with Sequenase using theideoxy nucleotide termination method to verify the distinct mutations. Fragments isolated from the EcoRI/HindIII (nucleotide 1120 in the wild-type receptor/ XbaI restriction digest of the mutants were subcloned into MORITAG previously subcloned into pcDNA (Invitrogen) at the EcoRI/XbaI mult cloning sites with the fragment between EcoRI/GIII and XbaI sites retained, SC).

The deletion of the amino acids between Ser356 and Glu359 (MORITAG355/359) was also constructed by PCR. The resulting PCR products were subcloned into pCRII, sequenced, and subsequently subcloned into pcDNA as described above. Additional point mutations alone or in combination with other point mutations were constructed in multiple steps using 5′ and 3′ site-directed mutagenesis method as outlined by Stratagene (La Jolla, CA). Forward and reverse oligonucleotides with the desired mutation and a designed endonuclease site were synthesized. First using cDNA from the wild-type μ-opioid receptor and then subsequently using cDNA with a desired serine and/or threonine mutation as a template, PCR was performed. The conditions for PCR were as follows: 10 mM KCl, 6 mM (NH4)2SO4, 20 mM Tris-HCl, pH 8.0, 2 mM MgCl2, 0.1% Triton X-100, 10 μg/ml bovine serum albumin, 44 mM oligonucleotide, 0.1 mM dNTP, 40 ng of template, and 2.5 units of Pfu DNA polymerase in a total volume of 50 μl; denatured at 95 °C for 30 s, annealed at 56–60 °C for 30 s, and extended at 68 °C for 14 min for 18 cycles. After PCR, 10 units of DpnI endonuclease was added to each reaction and the reactions were incubated at 37 °C for 1 h so as to remove the original templates. Finally, 5 μl of the resulting incubation mixtures were used to transform competent XL-1B1 cells. The DNA sequences of the mutants were confirmed by dideoxynucleotide termination reactions using Sequenase II.

**Transfection of Neuroa and HEK 293 Cells with Wild-type and Mutant μ-Opioid Receptors—**Stable transfections of wild-type and truncated MORITAG into Neuroa cells were carried out by the calcium phosphate precipitation method as described by Chen and Okayama (16). After 10–14 days of selection with 1 mg/ml genicin (G-418), colonies were isolated. Clones were initially selected to have similar counts/min/mg of protein and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 250 μg/ml genicin under humidified atmosphere with 10% carbon dioxide. For transient transfections, HEK 293 cells were also transfected by the calcium phosphate precipitation method and assays were performed 48–60 h after transfection.

**Intracellular cAMP Levels—**The ability of the opioid agonist, etorphine, to inhibit 10 μM forskolin-stimulated intracellular [3H]cAMP production in these Neuroa cells was determined using [3H]diprenorphine as a competitive antagonist (15). Intracellular ATP levels were measured after 1 h of stimulation (17). [3H]diprenorphine (as indicated in the legends). The IC50 values of the agonists and maximal inhibitory levels were obtained by curve-fitting the dose-response curves using Sigma Plot (Jandel Scientific). For HEK 293 cells transiently expressing mutant opioid receptors, inhibition of 5 μM forskolin-stimulated intracellular cAMP was determined using 125I-labeled cAMP receptor binding and rabbit polyclonal antibodies which recognize the acetylated cAMP (19). For these experiments, the cells were incubated for 15 min in the same reagents described above. Each reaction was terminated with 50 μl of 3 M perchloric acid, neutralized with 2 μl KOH, 1 μl Tris, and 60 μl EDTA, and subsequently assayed for the amount of cAMP without further purification. The IC50 values of etorphine were obtained by curve-fitting the dose-response curves using GraphPad Prism.

**Opioid Receptor Binding—**Characterization of the μ-opioid receptor binding sites in Neuroa cells expressing wild-type and truncated receptors was carried out with membrane (100,000 × g × 60 min membrane preparations minus nucleus) in 25 μM HEPES buffer, pH 7.5, containing 5 mM MgCl2, at 24 °C for 90 min as described (17). The Kd and Bmax values were determined for each clone by computer analysis using the Ligand program (20).

Competition binding studies measured the ability of increasing concentrations of opioids to compete for the binding of either 0.5 or 1 nM [3H]diprenorphine (as indicated in the legends). The IC50 values of the competition binding studies were then obtained by curve-fitting the dose-response curves using Sigma Plot (Jandel Scientific) or the Ligand program. The Kd values were calculated from the IC50 values using the Cheng-Prusoff relationship (21).

As for the measurement of opioid receptor down-regulation, cells were pretreated with 100 nM etorphine for the times indicated in the figure legends. The same amount of opioid ligands was added to control cells 5 min prior to harvesting so as to equalize the ligand concentration in all the cells. The cells were then washed by incubating at 37 °C for 15
Ser355, Glu359, Ser363, and Ser375 are indicated in the receptor sequence. The mutations indicate that there was no change in the receptor sequence. The mutations or truncations are marked accordingly.

Our study clearly demonstrates through \[^{[^3]}H\]diprenorphine binding that MOR1TAG355D down-regulates after chronic treatment with etorphine, whereas MOR1TAG355D does not.

\[\text{RESULTS}\]

Within the carboxyl tail of the \(\mu\)-opioid receptor there are 12 serine and threonine residues which pose as potential kinase-mediated phosphorylation sites. However, as defined by the motif of acidic amino acids directly upstream (N terminus) of each phosphorylated amino acid, there are only nine putative GRK sites, Thr354, Ser355, Ser356, Thr357, Ser358, Thr359, Ser375, Ser376, and Thr376 (22). To explore the region of the carboxyl tail necessary for agonist-induced down-regulation, truncations of the \(\mu\)-opioid receptor were performed systematically to remove the 12 serine and threonine residues in groups of four. These truncations were made by the addition of stop codons after amino acids positioned after 375, 363, and 355 (Fig. 1) and will be referred to as MORITAG375D, MORITAG363D, and MORITAG355D, respectively. MOR1B, a splice variant of the \(\mu\)-opioid receptor, has sequence homology with the wild-type receptor up to Glu366 where the sequence then differs by only five amino acids and is ultimately seven amino acids shorter than the wild-type receptor (23).

Receptor down-regulation, a decrease in the total cellular receptor concentration, occurs after prolonged exposure to agonists. This loss of surface receptors is dependent on both time and agonist concentration. Initially to investigate etorphine-induced down-regulation, Neuro2A, and clones expressing receptors with similar \[^{[^3]}H\]diprenorphine binding (\(K_d\) and \(B_{max}\); Table I) were selected to negate the possibility of receptor density influencing the action of opioid agonists. As summarized in Table I, the potency of etorphine, an opioid agonist, to inhibit forskolin-stimulated intracellular \[^{[^3]}H\]cAMP production and its affinity for the receptor are similar for all the truncated and splice variant receptors (Table I).

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Receptor Phosphorylation—Phosphorylation of HEK 293 cells transiently expressing the \(\mu\)-opioid receptor was determined as described previously. Briefly, cells were washed with phosphate-free Dulbecco’s modified Eagle’s medium incubated with 100 \(\mu\)M/\(\mu\)l \[^3\]Porthophosphate (ICN) for 2 h at 37°C in 5% CO\(_2\). Etorphine was added as indicated in the figure legends. Each reaction was terminated on ice, the cells were washed with ice-cold phosphate-buffered saline, and subsequently lysed (lysis buffer: 25 mM HEPES pH 7.4, 1% (v/v) Triton X-100, 5 mM EDTA, with 100 \(\mu\)g/ml bacitracin, 10 \(\mu\)g/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 100 \(\mu\)g/ml soybean trypsin inhibitor, 10 \(\mu\)g/ml pepstatin A, and 20 \(\mu\)g/ml benzamidine as protease inhibitors and with 50 mM sodium fluoride, 10 mM sodium phosphate, and 0.1 mM sodium vanadate). After solubilization, insoluble debris was removed by centrifugation and the receptor was partially purified by wheat germ lectin affinity columns. These purified samples were incubated in the presence of hemagglutinin-monoclonal antibody 12CA5 (Boehringer-Mannheim) and prewashed immunopure protein G-agarose beads (Pierce Chemical Co.). The final samples were eluted from the agarose beads and separated on a 10% SDS-polyacrylamide gel electrophoresis gel. After electrophoresis, the gels were dried and the phosphorylated proteins were visualized and quantified by using the PhosphorImager Storm 840 system (Molecular Dynamics).

Statistical Analysis—

\(t\) tests were performed by use of the statistical program Statview.
The key to the difference between these two responses may be related to the eight amino acids which lie between the two truncation sites. The original truncations were made to remove serines and threonines in a systematic manner, thereby removing potential phosphorylation sites within the carboxyl tail of the opioid receptor. To pursue the possibility that some or all of the specific amino acids responsible for the down-regulation of the \( \mu \)-opioid receptor lie within this eight amino acid stretch, another truncation after Glu 359, referred to as MOR1TAG359D, was constructed (Fig. 1). MOR1TAG359D was stably expressed in Neuro2A cells and a clone having a similar \( K_D \) and \( B_{\text{max}} \) to the other truncated receptors was selected. As shown in Fig. 3, MOR1TAG359D down-regulated 51 ± 6.9% after etorphine treatment. These results are consistent with those of the other truncated receptors and suggest that only four amino acid residues, STIE, between MOR1TAG355D and MOR1TAG359D might be necessary for agonist-induced down-regulation.

To further investigate whether this STIE motif mediates agonist-induced down-regulation of the \( \mu \)-opioid receptor, these four amino acids were deleted while leaving the remaining carboxyl tail intact. The receptor construct referred to as MOR1TAG355/359 (Fig. 1) was transiently expressed in human embryonic kidney 293 cells, HEK 293. \([3H]\)Diprenorphine binding revealed a loss of surface receptors after treatment with 1 \( \mu \)M etorphine for 12 h (Fig. 4). Thus, these data suggested that the STIE motif is not sufficient in determining the ability of the receptor to be down-regulated by agonist. To ensure that the behavior or regulation of the expressed opioid receptors was comparable between HEK 293 and Neuro2A cells, transient transfection of HEK 293 cells with MOR1TAG and MOR1TAG355D were performed in parallel as controls (Fig. 4). Similar to the data previously obtained with Neuro2A cells, etorphine could down-regulate the wild-type receptor, but not agonist-induced down-regulation of the \( \mu \)-opioid receptor, these four amino acids were deleted while leaving the remaining carboxyl tail intact. The receptor construct referred to as MOR1TAG355/359 (Fig. 1) was transiently expressed in human embryonic kidney 293 cells, HEK 293. \([3H]\)Diprenorphine binding revealed a loss of surface receptors after treatment with 1 \( \mu \)M etorphine for 12 h (Fig. 4). Thus, these data suggested that the STIE motif is not sufficient in determining the ability of the receptor to be down-regulated by agonist. To ensure that the behavior or regulation of the expressed opioid receptors was comparable between HEK 293 and Neuro2A cells, transient transfection of HEK 293 cells with MOR1TAG and MOR1TAG355D were performed in parallel as controls (Fig. 4). Similar to the data previously obtained with Neuro2A cells, etorphine could down-regulate the wild-type receptor, but not

### Table I

| \( \mu \)-Opioid receptor | \( B_{\text{max}} \) (pmol/mg protein) | \( K_D \) (nM) | \( IC_{50} \) (nM) | Maximal inhibition |
|--------------------------|--------------------------------------|----------------|----------------|-----------------|
| MOR1TAG                  | 1.9 ± 0.14                           | 0.30 ± 0.04    | 2.0 ± 0.65     | 0.53 ± 0.50     | 61 ± 8.7        |
| MOR1BTAG                 | 2.4 ± 0.06                           | 0.4 ± 0.02     | 0.65 ± 0.01    | 0.46 ± 0.11     | 80 ± 2.2        |
| MOR1TAG355D              | 0.7 ± 0.07                           | 0.22 ± 0.03    | 1.1 ± 0.38     | 4.1 ± 0.16      | 59 ± 0.96       |
| MOR1TAG355D              | 2.6 ± 0.05                           | 0.32 ± 0.16    | 0.80 ± 0.06    | 1.03 ± 0.16     | 86 ± 2.2        |
| MOR1TAG356D              | 2.3 ± 0.14                           | 0.43 ± 0.16    | 3.7 ± 1.5      | 3.2 ± 1.5       | 74 ± 8.8        |
| MOR1TAG357D              | 1.1 ± 0.43                           | 0.32 ± 0.05    | 1.5 ± 0.29     | 6.4 ± 3.5       | 75 ± 4.6        |
| MOR1TAG359D              | 2.6 ± 0.05                           | 0.32 ± 0.16    | 0.80 ± 0.06    | 1.03 ± 0.16     | 86 ± 2.2        |
| MOR1TAG363D              | 2.3 ± 0.14                           | 0.43 ± 0.16    | 3.7 ± 1.5      | 3.2 ± 1.5       | 74 ± 8.8        |
| MOR1TAG375D              | 1.1 ± 0.43                           | 0.32 ± 0.05    | 1.5 ± 0.29     | 6.4 ± 3.5       | 75 ± 4.6        |
| MOR1TAG375D              | 2.3 ± 0.14                           | 0.43 ± 0.16    | 3.7 ± 1.5      | 3.2 ± 1.5       | 74 ± 8.8        |
| MOR1TAG375D              | 1.1 ± 0.43                           | 0.32 ± 0.05    | 1.5 ± 0.29     | 6.4 ± 3.5       | 75 ± 4.6        |

a S. Chakrabarti, unpublished result.
compared with treated.

Fig. 4. Etorphine-induced down-regulation in HEK 293 cells transiently expressing wild-type and deletion mutant \(\mu\)-opioid receptors. HEK 293 cells were cultured in six separate 100-mm plates and transiently transfected with MOR1TAG, MOR1TAG355D, and MOR1TAG355/359 receptors. These transiently expressing HEK 293 cells were treated with (three plates) or without (three plates) \(1 \mu M\) etorphine for 12 h. The loss of surface receptors as an index of down-regulation was measured by \(^{3}H\)diprenorphine whole cell binding (see "Experimental Procedures"). Each bar represents the mean (% control) \(\pm\) S.E. from three separate transfection experiments. *, \(p < 0.05\) as compared with treated.

MOR1TAG355D. Likewise, HEK 293 cells stably expressing MOR1TAG verified that the rate of down-regulation in response to etorphine was comparable to that in Neuro2A cells (data not shown, Fig. 2).

While the deletion of the STIE motif may indicate that this region is not critical in the etorphine-induced down-regulation of the receptor, it may also reveal the complexity of the regulation, suggesting that multiple motifs or amino acids are needed to attenuate down-regulation. The carboxyl tail of the \(\mu\)-opioid receptor is the primary site of phosphorylation and plays an integral role in GRK-mediated internalization (24). When reviewing the amino acid sequence of the carboxyl tail of the \(\mu\)-opioid receptor, GRK consensus sites involve around Thr\(^{354}\), Ser\(^{355}\), Ser\(^{356}\), Thr\(^{357}\), Ser\(^{363}\) and Thr\(^{364}\), Ser\(^{375}\), Thr\(^{376}\), as well as Thr\(^{394}\). Therefore, on the premise that phosphorylation is a prerequisite for etorphine-induced down-regulation, multiple alanine substitutions of the nine putative GRK consensus sites were constructed by site-directed mutagenesis (Fig. 1). Fig. 1 contains a key of the combinations of amino acid residues which have been mutated to alanine. Thr\(^{394}\) was immediately excluded from the potential GRK phosphorylation sites that are involved in down-regulation of the receptor because MOR1BTAG, the splice variant of the \(\mu\)-opioid receptor, which does not contain Thr\(^{394}\), down-regulates the same as the wild-type receptor (Fig. 3). Since we have previously shown the possible importance of Ser\(^{356}\) and Thr\(^{357}\), the first cluster of amino acids examined included these amino acids in Mutant I, a combination of Thr\(^{354}\), Ser\(^{355}\), Ser\(^{355}\), and Thr\(^{357}\). This cluster (TSST) of amino acids was also combined with the remaining potential GRK consensus sites in specific combinations. Mutant II included Ser\(^{356}\) and Thr\(^{354}\), Mutant III included Ser\(^{375}\) and Thr\(^{357}\) in addition to the TSST cluster (Fig. 1). Mutants I–III as well as the wild-type receptor were transiently transfected into HEK 293 cells. After 12 h of \(1 \mu M\) etorphine treatment, \(^{3}H\)diprenorphine whole cell binding indicated that Mutant I and III down-regulated 32 \(\pm\) 2.6% and 36 \(\pm\) 4.4%, respectively (Fig. 5). However, Mutant II significantly attenuated etorphine-induced down-regulation of the \(\mu\)-opioid receptor when compared with the wild-type receptor (Fig. 5). Taken together a combination of potential GRK consensus sites Thr\(^{354}\), Ser\(^{355}\), Ser\(^{356}\), Thr\(^{356}\), Ser\(^{363}\) and Thr\(^{364}\) mediates etorphine-induced down-regulation of the \(\mu\)-opioid receptor.

It was then questioned whether a combination of all six amino acids (Mutant II) or rather a subset of these amino acids was necessary to attenuate etorphine-induced down-regulation of the receptor. Since the potential importance of Ser\(^{356}\) and Thr\(^{357}\) has been demonstrated by the difference of the truncation after Ser\(^{355}\) versus Glu\(^{359}\), additional combination mutations of only the four amino acids, Ser\(^{356}\), Thr\(^{357}\), Ser\(^{363}\), and Ser\(^{364}\), were constructed by site-directed mutagenesis (Fig. 1). Again a key for each alanine combination mutation is found in Fig. 1. Mutants IV and V combined Ser\(^{356}\) with either Ser\(^{363}\) or Thr\(^{364}\), respectively. Likewise Thr\(^{357}\) was combined with Ser\(^{363}\) and Thr\(^{364}\) individually for Mutants VI and VII, respectively. Mutants IV–VII, as well as the wild-type receptor were transiently expressed in HEK 293 cells. After 12 h of \(1 \mu M\) etorphine treatment, all the combination mutations had a loss of binding sites except combinations containing alanine substitutions at Ser\(^{356}\) and Ser\(^{363}\) (Fig. 6). To verify that both Ser\(^{356}\) and Ser\(^{363}\) were needed in combination to attenuate agonist-induced down-regulation, both individual substitutions were evaluated for a loss of binding sites in response to etorphine treatment. In both cases there was a loss of binding sites after 12 h of etorphine treatment (data not shown).

Down-regulation of the \(\mu\)-opioid receptor requires a high affinity complex between the receptor and its G-protein. To demonstrate that the attenuation of down-regulation by several mutants (Mutants II and IV) was not due to either an inability of the receptor to bind etorphine or to activate the respective G-proteins, the ability of each mutant to inhibit forskolin-stimulated adenylyl cyclase was determined (Table II). In each case the mutant maintained an IC\(_{50}\) to etorphine which was comparable to the wild-type receptor. The binding affinity to diprenorphine and etorphine were further determined for the mutants which attenuated etorphine-induced down-regulation. Again, the binding affinities were comparable to the wild-type receptor indicating that mutation of the serine and threonine residues within the carboxyl tail of the receptor had no effect on the functionality of the receptor.

Since receptor phosphorylation has been suggested to be involved in the regulation of the \(\mu\)-opioid receptor (7, 8, 10, 11, 14, 25), several combination mutations that attenuated etorphine-induced down-regulation were evaluated for a loss of phosphorylation. As shown in Fig. 7, removal of the putative GRK sites, Thr\(^{354}\), Ser\(^{355}\), Ser\(^{356}\), Thr\(^{357}\), Ser\(^{363}\), and Thr\(^{364}\) (i.e. Mutant II) resulted in a pronounced decrease in etorphine-induced phosphorylation. This reduction in phosphorylation could be the cause for the attenuation in etorphine-induced down-regulation observed (Fig. 5). However, the mutation of Ser\(^{356}\) and Ser\(^{363}\) to alanine (i.e. Mutant IV), which also resulted in a statistically significant attenuation of etorphine-induced down-regulation (Fig. 6), did not alter receptor phosphorylation (Fig. 7). Hence, the ability of Mutant IV to block...
etorphine-induced down-regulation was not due to the removal of putative GRK sites.

DISCUSSION

The present study examines the role of the carboxyl tail of the \( \mu \)-opioid receptor in agonist-induced down-regulation of the receptor. The \( \mu \)-opioid receptor was evaluated by truncation analysis or replacement of specific threonine and serine residues within the carboxyl tail with alanine. Initially, the wild-type and truncated receptors were stably expressed in Neuro2A cells, while the alanine replacement receptors were transiently expressed in HEK 293 cells. In either case, all the mutated receptors had similar efficacies for etorphine and exhibited high affinity binding comparable to that of the wild-type receptor (Table I), indicating that the receptors are efficiently coupled to G-proteins and, thus, should be able to down-regulate.

Of all the truncated receptor mutants examined, only one, MOR1TAG355D, failed to down-regulate in response to agonist treatment (Fig. 3). Interestingly, truncation after Glu359 resulted in a receptor that could be down-regulated by etorphine.
Opioid Receptor Deletion Mutation and Down-regulation

The IC50 for etorphine to inhibit 5 μM forskolin-stimulated intracellular cAMP production was determined by RIA 48 h after transfection (see “Materials and Methods”). Mutant II, alanine replacement at Thr354, Ser355, Ser356, Thr357, Ser363, and Thr364; Mutant IV, alanine substitution at Ser356 and Ser363. The binding affinities are representative of two separate transfections and the IC50 values were determined from at least three separate transfections.

| μ-Opioid receptor | Diprenorphine Kd (nM) | Etorphine Kd (nM) | IC50 for Etorphine (nM) |
|------------------|------------------------|-----------------|------------------------|
| MORITAG          | 0.27 ± 0.07            | 1.4 ± 0.9       | 8.1 ± 1.4              |
| Mutant II        | 0.46 ± 0.08            | 6.3 ± 1.7       | 2.9 ± 0.31             |
| Mutant IV        | 0.38 ± 0.14            | 3.5 ± 2.1       | 1.9 ± 0.39             |

These data suggested that the residues between Ser355 and Glu359, STIE, could be the key for etorphine-induced down-regulation of the μ-opioid receptor. Deletion of STIE, while leaving the remaining carboxyl tail intact, however, still allowed down-regulation in response to agonist exposure (Fig. 4). Such a result suggests that either more than one motif is necessary to mediate agonist-induced receptor down-regulation or this region does not play a critical role in receptor down-regulation. In the rat-neurotensin receptor Thr422 and Tyr424 are critical for agonist-induced internalization of the receptor. If, however, these amino acids were individually modified there was little or no effect on internalization (6). Chabry and colleagues (6) maintained that removal of one amino acid at the amino-terminal side of the phosphorylated residues, suggesting that a specific motif is necessary for GRK-mediated phosphorylation. This motif of acidic amino acids was also shown for the rhodopsin receptor and perfectly aligns with phosphorylation by GRK. When evaluating the amino acid sequence of the μ-opioid receptor carboxyl tail (Fig. 1), there are two possible motifs, Glu349 and Glu359 which could be the acidic amino acids necessary for GRK-mediated phosphorylation of either Thr354, Ser355, Ser363, and Ser364, or Ser356 and Ser363 and Ser364. Alamine replacement of Thr354, Ser355, Ser356, and Ser364 in combination with Ser363 and Thr364 (Mutant II), a combination which attenuates down-regulation, had a pronounced decrease in phosphorylation (Fig. 7). Such results suggest that the removal of these GRK phosphorylation sites was the direct cause of the attenuation observed. Interestingly, substitution of only Ser356 and Ser363 with alanine (Mutant IV), yielding a mutant which also blocks etorphine-induced down-regulation, had no obvious effect on phosphorylation when compared with the wild-type receptor (Fig. 7). This result was not too surprising since the STIE deletion that removed Glu359 rendering Ser363 and Ser364 nonsubstrates for GRK phosphorylation had no effect on etorphine-induced down-regulation (Fig. 4). Altogether, it is evident that phosphorylation of the receptor is not an obligatory event for etorphine-induced down-regulation.

The pronounced decrease in phosphorylation of Mutant II (Thr354, Ser355, Ser356, Thr357, Ser363, and Thr364) (Fig. 7), however, suggests that there is a correlation between phosphorylation and down-regulation, but that only a subset of the phosphorylation sites actually mediates the process. For GPCRs, after agonist activation the receptors are rapidly internalized. This rapid endocytosis leads to either the resensitization of the receptor through a recycling pathway or a total loss of receptors via degradation. Our study does not preclude the possibility that phosphorylation of the receptor is involved in the rapid internalization of the receptor. In fact, the role of phosphorylation and the rapid internalization of the μ-opioid receptor was recently supported by Zhang and co-workers (24). They demonstrated that the overexpression of GRK 2 allowed morphine, which normally does not induce internalization, to promote internalization. Zhang maintained that phosphorylation by GRKs is critical in initiating agonist-mediated internalization of GPCRs. In addition, deletions of the third intracellular loop of the muscarinic receptor, hindered GRK 2 induced internalization without affecting the long-term down-regulation of the receptor (26). This study suggested that there are
different receptor motifs for the rapid internalization and long-term down-regulation and that these processes are mediated via separate pathways. In our own laboratory, overexpression of GRKs with Mutant II and Mutant IV reversed the observed attenuation of down-regulation suggesting that not only is phosphorylation pivotal in the down-regulation and possibly the rapid endocytosis of the μ-opioid receptor, but also that Ser356 and Ser363, the sites which mediate etorphine-induced down-regulation, are probably not phosphorylated. This data also confers that additional factors are pertinent for etorphine-induced down-regulation of the receptor.

In the β2-adrenergic receptor, β-adrenergic receptor kinase 1 recruits associative protein, β arrestin, and cytoskeletal protein, dynamin, to assist in the internalization of the receptor (1,27). It has been demonstrated that arrestin preferentially binds to a phosphorylated receptor with greater affinity than an unphosphorylated receptor. It is interesting to speculate that the role of phosphorylation in the down-regulation of the μ-opioid receptor is similar to the β-adrenergic receptor in promoting arrestin-receptor interactions. Zhang and co-workers (24) have demonstrated the involvement of arrestin and dynamin with the μ-opioid receptor after etorphine activation. However, the magnitude of their involvement has yet to be identified. The involvement of other proteins or internalization pathways cannot be ruled out. In addition, it has been demonstrated that the muscarinic receptor subtypes internalize via different pathways. The m2 muscarinic receptor internalizes by an arrestin-dependent pathway while m1, m3, and m4 internalize by a dynamin-dependent and arrestin-independent pathway (28). From these studies of the muscarinic receptor (26,28) it is interesting to speculate that there are multiple internalization pathways which may lead to receptor degradation. Therefore, the observed attenuation of down-regulation by Mutant IV may be due to blocking a specific internalization pathway. In addition, the six amino acids which attenuate etorphine-induced down-regulation may also be a sequence/motif either at the phosphorylated or dephosphorylated state that interacts with proteins or with other amino acids within the carboxyl tail or intracellular loops of the μ-opioid receptor. Interestingly, Smith and colleagues (31) isolated a protein which interacts with the phosphorylated μ-opioid receptor carboxyl tail. This protein, named μRAM-1, has homology with a region of the human ubiquitin specific protease3 and indicates that the μ-opioid receptor may be a substrate for ubiquitin-dependent proteolysis. This would not be surprising since the degradation of the rhodopsin receptor outer rod segments and G-protein, Gt, are via a ubiquitin-dependent proteolytic pathway (29,30). Therefore, while Ser356 and Ser363 are not the key phosphorylated residues, they may be directly involved in protein interactions or at least in the proximity of amino acid residues within the carboxyl tail that are protein-binding sites. The identification of these additional motifs, novel proteins, and the overall mechanism of down-regulation of the μ-opioid receptor are currently under investigation in our laboratory.

In summary, truncation of the carboxyl tail of the μ-opioid receptor, thereby removing nearly all the serine and threonine residues in the carboxyl tail, abolished agonist-induced down-regulation of the receptor. The region responsible was narrowed to the amino acids between Ser356 and Glu359 by truncation analysis. Deletions and point mutations of serines and threonines have demonstrated the importance of this region and specifically Ser356 and Ser363 in the attenuation of etorphine-induced down-regulation. However, neither serine has an obvious effect on the etorphine-mediated phosphorylation suggesting that other factors are involved in the down-regulation of the μ-opioid receptor.

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Identification of Serine 356 and Serine 363 as the Amino Acids Involved in Etorphine-induced Down-regulation of the \( \mu \)-Opioid Receptor

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