Agonist-induced Desensitization of the μ Opioid Receptor Is Determined by Threonine 394 Preceded by Acidic Amino Acids in the COOH-terminal Tail*

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To identify the structural determinants necessary for μ opioid receptor desensitization, we serially ablated potential phosphorylation sites in the carboxyl tail of the receptor and examined their effects on [d-Ala²,N-Me-Phe³,Gly-ol⁵]enkephalin (DAMGO)-induced desensitization. First, we replaced Thr³⁹⁴ with alanine (T394A) and stably expressed this mutant receptor in Chinese hamster ovary cells. The T394A receptor did not desensitize after 1 h of treatment with DAMGO, indicating that Thr³⁹⁴ is required for agonist-induced early desensitization. To test whether Thr³⁹⁴ was the only residue necessary, we investigated the importance of 7 potential phosphorylation sites between residues 383 and 388, which were all replaced by alanines with the Thr³⁹⁴ maintained. This mutant (AT) showed partial loss of desensitization (30%), which was attributable to the Ala mutation at Thr³⁹⁴, since complete desensitization was achieved by restoring Thr³⁹⁴ (ATT). These results suggest that Thr³⁹⁴ is the primary recognition site for G protein-coupled receptor kinases, but Thr³⁹⁴ is also required for complete agonist-induced desensitization. The specificity of Thr³⁹⁴ as the primary initiation site appears to be dependent on the preceding acidic amino acid stretch, because in a mutant in which glutamic acid residues at 388, 391, and 393 were replaced by glutamines (EQ), agonist-induced desensitization was completely abolished, identical to the T394A mutant.

Desensitization is defined as loss of a biological response to a ligand despite its continuous presence. This phenomenon of desensitization is observed with almost every receptor, and some of the mechanisms involved in desensitization of G protein-coupled receptors have been well characterized using the β-adrenergic receptor (βAR) and rhodopsin. For these receptors, the primary mechanism of desensitization appears to be receptor phosphorylation mediated by G protein-coupled receptor kinases (GRKs). Since these kinases have the unique feature of phosphorylating receptors only when they are in an active or a ligand-bound conformation, GRK-mediated phosphorylation is considered to be the main mechanism for agonist-induced desensitization. These kinases have been shown to phosphorylate a number of G protein-coupled receptors including the βAR (1, 2), rhodopsin (3, 4), α₂-adrenergic receptor (α₂AR) (5, 6), platelet-activating factor receptor (7), thrombin receptor (8), and C5a anaphylatoxin receptor (9).

Endogenous opioids play an important role in a variety of physiological processes through pharmacologically distinct, three major receptor subtypes that are coupled to inhibitory G proteins (10). Although the acute action of opioids can induce a number of beneficial effects, chronic use of opioids produces tolerance and dependence (11, 12), which are among the major factors limiting the clinical use of opioids. The molecular mechanisms underlying these phenomena are poorly understood, and receptor desensitization has been implicated as a possible mechanism.

It has been shown recently that opioid receptor desensitization is directly related to receptor phosphorylation. Protein kinase C (PKC) is involved in the functional uncoupling of the delta opioid receptor (DOR) from G protein in the striatum of guinea pigs (13). However, cellular depletion of PKC failed to alter DOR phosphorylation in HEK 293 cells (14), indicating a role for other kinases as well. In the case of the μ opioid receptor (MOR), it was reported that activation of PKC enhanced desensitization of the receptor, leading to a decrease in the receptor-activated potassium channel current (GIRK1) in Xenopus oocytes (15, 16). However, the PKC inhibitor staurosporine showed differential effects on the PKC-mediated response and the agonist-induced response. Staurosporine blocked PKC-activated MOR phosphorylation and eliminated PKC influence on desensitization, but failed to block desensitization induced by repeated administration of agonist (16). This result suggests participation of other kinases in agonist-mediated desensitization. Indeed, a recent study of agonist-induced phosphorylation of DOR suggests involvement of one or more GRKs in receptor desensitization (14). It has also been shown that overexpression of a functionally impaired mutant form of GRK2 blocks μ receptor desensitization in COS-7 cells (17). In contrast to PKC manipulation, agonist-dependent phosphorylation of DOR in HEK 293 cells was altered by overexpression of GRK2, GRK 5, or a functionally impaired mutant form of GRK2 (14). These data suggest that GRKs are more directly involved in receptor desensitization and function together with PKC-mediated mechanisms.

In a previous study, we reported that 1-h pretreatment of MOR with the agonist DAMGO caused agonist-specific desensitization (18). The mechanisms underlying agonist-induced...
desensitization could include receptor phosphorylation, similar to that described for other G protein-coupled receptors. Recently, a splice variant of the MOR cDNA has been cloned from rat that differs at the 3’-end (19). This variant (MOR1B) shares 100% amino acid sequence identity with MOR1 up to amino acid 386 but differs from residue 387 to the carboxyl tail (rMOR1, 387LENLEATAPIL398, rMOR1B, 387LIKDIPL391).

Within that region of the carboxyl tail, one potential site for phosphorylation by GRKs is found in MOR that is absent in MOR1B. Interestingly, this shorter variant, rMOR1B, has been shown to be resistant to agonist-induced desensitization, unlike the longer form, rMOR1 (19). The cytoplasmic tail of the MOR contains potential phosphorylation sites for GRKs which may explain the differences between the variants and has led us to map the amino acid residues important in the functional desensitization of MOR.

**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 Chinese hamster ovary (CHO) cell lines. Mutant MORs such as T394A, T/S363–383A (AT), T/S363–379A (ATT), E388–393Q (EQ) (Fig. 1) were constructed by substituting single or multiple amino acid residues with alanine(s). In case of the mutant EQ, glutamic acids between residues 388 and 393 were substituted with glutamine. Site-directed mutagenesis was performed using a polymerase chain reaction-based technique as described previously (20). Briefly, oligonucleotide primers corresponding to unique EcoRIIII and Apal restriction sites (located at amino acids 304–305 and at vector, respectively) were utilized in combination with two mutagenic primers. Polymerase chain reaction fragments were digested with unique restriction enzymes and subcloned into the corresponding restriction sites of pRC/CMV encoding a MOR. All mutations were verified by dideoxy sequencing. For stable expression, cell line CHO K-1 (no. CCL61; American Type Culture Collection) was grown to 60% confluency 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, Toronto, Ontario, Canada) and clones with the appropriate expression level were screened by radioligand binding assay. 90–90 clones expressing varying numbers of receptors were screened to select those with comparable expression levels.

**Membrane Preparation and Radioligand Binding Assays**—Cells were grown until apparent confluency and then washed twice with 12 ml of ice-cold phosphate-buffered saline, harvested and centrifuged at 100 × g for 10 min. Cells were then lysed in hypotonic buffer (5 mM Tris-HCl, pH 7.8, 2 mM EDTA, containing a protease inhibitor mixture (10 mg/ml leupeptin, 5 mg/ml soybean trypsin inhibitor and 5 mg/ml benzamidine)) with a Polytron homogenizer (Brinkman Instruments, Westbury, NY) for two 30-s bursts at the 5.5 setting. The lysate was then centrifuged at 100 × g for 10 min to pellet unbroken cells and nuclei. The supernatant was collected and centrifuged at 30,000 × g for 30 min, and the resulting pellet was resuspended in buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 1 mM EGTA with the protease inhibitor mixture and used immediately for radioligand binding studies.

Protein content was determined according to the method of Bradford (21). For saturation experiments, cell membranes (20–30 μg of protein/tube) were incubated with increasing concentrations of [3H]naloxone to the wild type or T394A mutant receptor was prepared (Fig. 1). The binding of antagonist [3H]naloxone to the wild type or T394A mutant receptor was saturable and of high affinity. The mu agonist, DAMGO (1 μM) (18). During agonist exposure, basal adenylyl cyclase activity and forskolin-stimulated adenylyl cyclase activity did not change, suggesting this desensitization was specifically agonist-induced, with no evidence of heterologous desensitization. The underlying mechanism of this agonist-induced form of desensitization is likely due to the involvement of GRKs. Since phosphorylation of receptors plays an important role in receptor desensitization, and the carboxyl tail region of MOR contains a potential phosphorylation site for GRKs (Thr394), Thr394 was substituted by alanine in the longer variant of the receptor to test the involvement of this residue in agonist-induced desensitization of MOR.

Initially, stable CHO cell lines expressing wild type or mutated receptor in which the Thr394 was changed to alanine (T394A) were prepared (Fig. 1). The binding of antagonist [3H]naloxone to the wild type or T394A mutant receptor was saturable and of high affinity. The mu agonist, DAMGO detected two affinity states of the wild type and mutant receptors. With each of the receptors, 70% of the total receptor population was in a high affinity state for agonist with a dissociation constant of 3 nM, and 30% of the receptor population was in a low affinity state with a dissociation constant of 330 nM. To determine the functional coupling of the wild type and mutant receptors (T394A) to adenylyl cyclase, dose-response curves for the stimulation of forskolin-stimulated cAMP accumulation with increasing concentrations of DAMGO were tested. The IC50 values for adenylyl cyclase inhibition for both receptors in CHO cells were identical; 8.5 ± 2.6 nm (n = 7) for wild type receptors and 5.6 ± 1.2 nm (n = 4) for mutant (T394A) receptors (Fig. 2, A and B). However, the maximum percentage inhibition of adenylyl cyclase activities was slightly different, but statisti-
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FIG. 2. Effects of substitution of serines and threonines in the COOH terminus of the MOR on agonist-induced desensitization. CHO cells expressing wild type and mutant receptors were incubated in the absence (○), and presence (□) of 1 μM DAMGO for 1 h, and subsequently the ability of increasing concentrations of DAMGO (10^{-11} to 10^{-3} M), to inhibit forskolin-stimulated cAMP accumulation was tested. Data are presented as a percentage of forskolin-stimulated activity, and are shown as follows. A, wild type MOR; B, a mutant MOR in which the last threonine (T394A) was substituted to alanine; C, a mutant MOR in which 2 serines and 5 threonines between residues 363 and 383 (AT) were substituted by alanines except for the Thr^{394} which was left intact (AT mutant) (Fig. 1). This mutant receptor was created in which 6 potential phosphorylation sites in the carboxyl tail, resulted from the stretch of acidic amino acids at the carboxyl tail of the MOR are the key residues required for agonist-induced desensitization occurring within 1 h.

The effect on wild type MOR (●), wild type MOR without pretreatment of DAMGO; ○, wild type MOR with DAMGO pretreatment; □, AT mutant receptor with DAMGO pretreatment). Data shown are the means ± S.E. of independent experiments (n = 4–8).

Analysis of phosphorylation of a synthetic peptide based on β_{2AR} or α_{2AR} sequence showed that negatively charged residues are required for a peptide to serve as a substrate for GRK2 (23). Support for the idea that the Thr^{394} serves as the primary recognition site for GRKs, compared with the other potential sites in the carboxyl tail, resulted from the stretch of acidic amino acids in front of it, a mutant receptor was created in which glutamic acid residues at 388, 391, and 393 were replaced by glutamines and Thr^{394} was left intact (EQ). This mutant (EQ) was expressed at low densities and naloxone binding affinity was marginally higher (Table I). However, K_{i} values for DAMGO at high and low receptor affinity states and their proportions were comparable to those of wild type receptors, indicating no changes in G-protein coupling ability. As shown in Fig. 3C, agonist-induced desensitization was completely abolished in the EQ mutant. Thus, the acidic amino acid residues preceding the Thr^{394} are absolutely necessary for Thr^{394} to play the critical role in desensitization, possibly by facilitating phosphorylation of Thr^{394} by a GRK.

DISCUSSION

Our results demonstrate that the Thr^{394} and preceding acidic amino acids at the carboxyl tail of the MOR are the key residues required for agonist-induced receptor desensitization.
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**TABLE I**

| Binding parameters for the stably transfected wild type or mutant μ opioid receptors | [<sup>3</sup>H]Naloxone | [<sup>3</sup>H]Naloxone/DAMGO |
|---|---|---|
| | | \(K_m\) | \(n_m\) | \(R_H\) | \(R_L\) |
| Wild type | \(B_{max}\) | 3.8 ± 0.9 pmol/mg | 2.9 ± 0.7 | 326 ± 25 | 69 ± 2 | 31 ± 2 |
| T394A | \(B_{max}\) | 3.5 ± 0.5 pmol/mg | 2.7 ± 1.4 | 329 ± 14 | 74 ± 2 | 26 ± 2 |
| T5S63–383A (AT) | \(B_{max}\) | 4.3 ± 0.3 pmol/mg | 2.2 ± 1.7 | 370 ± 23 | 70 ± 1 | 30 ± 1 |
| T5S63–379A (ATT) | \(B_{max}\) | 4.0 ± 0.1 pmol/mg | 3.2 ± 0.2 | 383 ± 97 | 71 ± 2 | 29 ± 2 |
| E388–393Q (EQ) | \(B_{max}\) | 2.0 ± 0.1 pmol/mg | 2.9 ± 1.8 | 431 ± 215 | 73 ± 2 | 27 ± 2 |

**FIG. 3. Effects of Thr<sup>394</sup>, Thr<sup>383</sup>, and acidic amino acids on the NH<sub>2</sub>-terminal side of Thr<sup>394</sup> on agonist-induced desensitization of MOR.** A, the effect of a mutant MOR in which 2 serines and 4 threonines between residue 363 and 379 (ATT) of MOR were changed to alanines but Thr<sup>394</sup> and Thr<sup>383</sup> were unaltered. This mutant receptor was incubated in the absence (●) and presence (○) of 1 μM DAMGO and subsequently DAMGO inhibition of forskolin-stimulated adenyl cyclase activity was tested. Data shown are the mean ± S.E. of eight independent experiments. B, the effect of the mutant receptor ATT on agonist-induced desensitization (●) is presented by overlapping with the effect seen with the wild type receptor, in the absence (●) and presence (○) of 1 μM DAMGO pretreatment. C, the effect of a mutant MOR (EQ) in which three glutamic acids on the NH<sub>2</sub>-terminal side of Thr<sup>394</sup> were changed to glutamines and the effect on agonist-induced desensitization was tested (●, EQ mutant MOR without pretreatment by DAMGO; ○, EQ mutant MOR with DAMGO pretreatment). Data shown are representative of four independent experiments.

Agonist-induced receptor desensitization has been proposed to be due to phosphorylation of the agonist-bound form of receptors by GRKs (reviewed in Inglese et al. (24) and Lefkowitz (25)). Furthermore, the recognition sites for these kinases have been identified as repeated serines and threonines at the carboxyl tail by phosphopeptide sequencing of high pressure liquid chromatography-purified peptides derived from proteolysis of phosphorylated receptors (1, 26). Since there are usually several repeated serine/threonine residues in the carboxyl tails of G protein-coupled receptors, it is of interest to determine whether all of these residues are required to be phosphorylated to attenuate prolonged agonist-induced stimulation.

Numerous studies have attempted to answer this question by using truncation mutations of the carboxyl tail of various lengths, to interfere with both receptor phosphorylation and agonist-induced desensitization (1–8). These studies demonstrated that phosphorylation of particular residues is necessary for desensitization. These results raise the question of how GRKs distinguish certain serine or threonine residues from others. Analysis of the phosphorylation of several synthetic peptides based on β<sub>2</sub>AR sequence identified two peptides that were phosphorylated by GRK2. Interestingly, the feature common to both substrate peptides was the presence of acidic amino acids, mostly glutamic acids, in close proximity to serine and threonine residues (1, 6, 23, 27–31). However, peptides with glutamic acid residues on the carboxyl-terminal side of the serine or threonine were not substrates for GRK2 (23). A peptide with glutamic acid residues on both sides of the serine residue was also a poor substrate. Furthermore, a peptide that was a good GRK2 substrate became poorly phosphorylated when acidic amino acids at the amino-terminal side of the phosphorylation sites were changed to their neutral counterparts (6, 23). This strongly suggests that amino-terminal acidic amino acids relative to the phosphate acceptor group is a requirement for GRK2 recognition. In contrast to GRK2, rhodopsin kinase (GRK1) recognizes a serine or threonine residue located on the carboxyl-terminal side of basic amino acids (1, 23). This seems to indicate that even though there are several GRKs in this family of kinases, they each have different substrate specificity together with different tissue distributions (24, 25).

Sequential phosphorylation of different residues has been demonstrated in the case of rhodopsin, in which light-dependent phosphorylation occurs first at the serine in position 338, and subsequently at serine 343 and then threonine 336 (4, 32–34). In the case of the human C5α anaphylatoxin receptor, phosphoamino acid analysis of C5α-dependent phosphorylation showed the maximal stoichiometry to be 6 moles of phosphate/mole of receptor. However, mutation of 2 particular serines at positions 332 and 338 completely abolished agonist-dependent phosphorylation, further suggesting multiple phosphorylations occurring in a sequential manner (9). However, in the case of α<sub>2</sub>AR, there are 4 consecutive serines in the third intracellular loop and substitution of each serine decreased overall phospho-
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Phosphorylation by 25% when compared with that of wild type \(\alpha_{2AR}\) (6). Since both C5a anaphylatoxin receptor and \(\alpha_{2AR}\) are substrates for the same kinase, GRK2, it is unlikely that these two different patterns of phosphorylation are due to different substrate and kinase interactions. It remains to be determined if the nonsequential nature of \(\alpha_{2AR}\) phosphorylation reflected the close proximity of each serine residue or its third loop location. However, in our study, substitution of the Thr\(^{394}\) completely abolished agonist-induced desensitization, whereas for achieving the full agonist-induced desensitization, both Thr\(^{383}\) and Thr\(^{394}\) appear necessary in the carboxyl terminus. This would be best explained by a sequential phosphorylation model, in which Thr\(^{383}\) can be phosphorylated only after Thr\(^{394}\). This speculation is very interesting to speculate that the slower time course of desensitization, suggesting that the structure of the carboxyl terminus. Subsequent sequential phosphorylation of Thr\(^{383}\) after Thr\(^{394}\) phosphorylation, would effect the complete desensitization of the MOR in response to agonist.

REFERENCES

1. Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 13796–13803
2. Preumont, R. T., Koch, W. J., Inglese, J., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 6832–6841
3. Palczewski, K., and Benovic, J. L. (1991) Trends. Biochem. Sci. 16, 387–391
4. Ohguro, H., Van Hooser, J. P., Milan, A. H., and Palczewski, K. (1995) J. Biol. Chem. 270, 14259–14263
5. Jewell-Motz, E. A., and Liggitt, S. B. (1996) J. Biol. Chem. 271, 18682–18687
6. Eason, M. G., Moreira, S. P., and Liggitt, S. B. (1995) J. Biol. Chem. 270, 4681–4688
7. Takano, T., Honda, Z., Sakanaka, C., Izumi, T., Kameyama, K., Haga, K., Haga, T., Kurokawa, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 22453–22458
8. Vuore-Craviani, V., Auberger, P., Pouyssegur, J., and Van Obberghen-Schilling, E. (1995) J. Biol. Chem. 270, 4813–4821
9. Giannini, E., Brouchon, L., and Boulay, F. (1995) J. Biol. Chem. 270, 19166–19172
10. Simon, E. (1991) Med. Res. Rev. 11, 357–374
11. Koob, G., Maldonado, R., and Stinus, L. (1992) Trends. Neurosci. 15, 186–191
12. Koob, G., and Bloom, F. (1992) Science 256, 715–719
13. Fukushima, N., Ueda, H., Hayashi, C., Katayama, T., Miyamae, T., and Misu, Y. (1994) Neurosci. Lett. 176, 55–58
14. Pei, G., Kieffer, B. L., Lefkowitz, R. J., and Freedman, N. J. (1995) Mol. Pharmacol. 48, 173–177
15. Chen, Y., and Yu, L. (1994) J. Biol. Chem. 269, 7839–7842
16. Zhang, L., Yu, Y., Mackin, S., Weight, F. F., Ulj, G. R., and Wang, J. B. (1996) J. Biol. Chem. 271, 11449–11454
17. Raynor, K., Hong, J., Hines, J., Kong, B., Benovic, J., Yasuda, K., Bell, G. I., and Residence, T. (1994) J. Pharmacol. Exp. Ther. 270, 1381–1386
18. Pak, Y., Kouvelis, A., Scheideler, M. A., Rasmussen, J., O’Dowd, B. F., and George, S. R. (1996) Mol. Pharmacol. 50, 1214–1222
19. Zergrich, A., Simon, T., and Holt, V. (1995) FEBS Lett. 359, 142–146
20. Liggett, S. B., Ostrowski, J., Chesnut, L. C., Kurokawa, K., Haga, T., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 4740–4746
21. Bradford, M. M. (1976) Anal. Biochem. 110, 248–254
22. Salomon, Y., Loudias, C., and Rodbell, M. (1974) Anal. Biochem. 58, 541–548
23. Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., and Benovic, J. L. (1991) Biochemistry 30, 5118–5125
24. Inglese, J., Freedman, N. J., Koch, W. J., and Lefkowitz, R. J. (1993) J. Biol. Chem. 268, 23735–23738
25. Lefkowitz, R. J. (1995) Cell 74, 499–412
26. Palczewski, K., Arendt, A., McDowell, J. H., and Hargrave, P. A. (1989) Biochemistry 28, 8764–8770
27. Richardson, R. M., Kim, C., Benovic, J. L., and Hosey, M. M. (1993) J. Biol. Chem. 268, 13630–13656
28. Blaxall, B. B., Cerutis, K., Hass, N. A., Iversen, L. L., and Bylund, D. B. (1994) Mol. Pharmacol. 45, 176–181
29. Barton, A. C., and Sibley, D. R. (1991) Mol. Pharmacol. 38, 531–541
30. Barton, A. C., Black, L. E., and Sibley, D. R. (1991) Mol. Pharmacol. 39, 650–658
31. Bates, M. D., Senogles, S. E., Bunzow, J. R., Liggett, S. B., Civelli, O., and Caron, M. G. (1994) Biochemistry 33, 45136–45142
32. Haga, T., Kurokawa, K., and Shimizu, T. (1994) J. Biol. Chem. 269, 22453–22458
33. Palczewski, K., Ohguro, H., Premont, R. T., and Inglese, J. (1995) J. Biol. Chem. 270, 15294–15298
34. Pullen, N., and Akhtar, M. (1994) Biochemistry 33, 45136–45142
35. Haga, T., and Lefkowitz, R. J. (1994) J. Biol. Chem. 269, 10993–10999
36. Chen, J., Makino, C. L., Peachey, N. S., Baylor, D. A., and Simon, M. I. (1995) Science 267, 314–377