Role for the C-Terminus in Agonist-Induced μ Opioid Receptor Phosphorylation and Desensitization†

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ABSTRACT: Determining which domains and amino acid residues of the μ opioid receptor are phosphorylated is critical for understanding the mechanism of μ opioid receptor phosphorylation. The role of the C-terminus of the receptor was investigated by examining the C-terminally truncated or point-mutated μ opioid receptors in receptor phosphorylation and desensitization. Both wild-type and mutated receptors were stably expressed in Chinese hamster ovary (CHO) cells. The receptor expression was confirmed by receptor radioligand binding and immunoblottting. After exposure to 5 μM of DAMGO, phosphorylation of the C-terminally truncated receptor and the mutant receptor T394A was reduced to 40 and 10% of that of the wild-type receptor, respectively. Mutation effects on agonist-induced desensitization were studied using adenylyl cyclase inhibition assays. The C-terminally truncated receptor and mutant receptor T394A both showed complete loss of DAMGO-induced desensitization, while the mutant T/S-7A receptor only lost part of its ability to desensitize. Taken together, these results suggest that the C-terminus of the μ opioid receptor participates in receptor phosphorylation and desensitization with threonine 394, a crucial residue for both features. DAMGO-induced μ opioid receptor phosphorylation and desensitization are associated and appear to involve both the μ opioid receptor C-terminus and other domains of the receptor.

The μ opioid receptor is a seven transmembrane domain, G-protein coupled receptor. Prolonged or repeated exposure to an opioid agonist results in attenuation of agonist-activated opioid responses, a phenomenon termed desensitization (*1*−*3*). Desensitization occurs in several G-protein-coupled receptors, and mechanisms have been extensively studied in the β-adrenergic receptors. In the β_2-adrenergic receptor, the agonist-activated receptor is first phosphorylated by protein kinase A (PKA) and G-protein coupled receptor kinase 2 (GRK2).[1] A β-arrestin isoform then binds the GRK-phosphorylated β_2-adrenergic receptor and leads to uncoupling of the receptor from G-proteins (*4*, *5*). Phosphorylation of specific amino acid residues located in the intracellular domains is thus believed to be one crucial step for the agonist-induced regulation of many G-protein coupled receptors.

μ opioid receptor can undergo rapid phosphorylation upon stimulation by μ-selective agonists (*6*−*8*), perhaps through activation of GRKs related phosphorylation (*9*−*12*). Several lines of evidence have suggested that this phosphorylation plays important roles in receptor desensitization. Agonist-induced desensitization of the μ opioid receptor can be reduced by protein kinase inhibitors (*2*). Treatments that decreased the μ opioid receptor phosphorylation can reduce tolerance to morphine analgesia effects in mice (*13*). Parallels of the time courses and dose−response curves between μ opioid receptor phosphorylation and acute desensitization can also be demonstrated (*6*). These results suggest that phosphorylation of the μ opioid receptors may be one prerequisite for desensitization.

Much less is known about the domains and specific amino acid residues involved in μ opioid receptor phosphorylation and desensitization. Mutagenesis analysis for several other G-protein-coupled receptors indicated that third cytoplasmic loops and C-termini are most likely involved in both the coupling of receptor−G-protein complexes and in functional regulation of the receptors (*14*−*16*). Phosphorylation of serine or threonine residues located in the G-protein coupled receptor C-terminus is often implicated in receptor desensitization (*17*−*22*). Several serine and threonine residues

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[1] Abbreviations: DAMGO, [D-Ala^2, MePhe^4, Glyol^5]-enkephalin; CHO, Chinese hamster ovary cells; E-HμCHO, Chinese hamster ovary cells stable expressing epitope-tagged human μ receptor; E-HμCHO3$^-$, Chinese hamster ovary cells stably expressing epitope-tagged C-terminally truncated human μ receptor; GRKs, G-protein-coupled receptor kinases.

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located in the C-terminus in G-protein coupled receptors have been identified as the recognition sites for GRKs (*18*, *23*). Evidence for phosphorylation of the μ receptor can be further studied at a minor splice variant of the rat μ opioid receptor (MOR1B), which lacks amino acids from residue 387 to the 3′-end and thus omits one potential phosphorylation site, compared to the more aboundant μ opioid receptor (*24*). This receptor exhibits more resistance to agonist-induced desensitization, implying the importance of the C-terminus in μ opioid receptor phosphorylation and desensitization (*24*, *25*). Each of these results adds interest to the determination of which residues in the C-terminus of the μ opioid receptor are required for receptor phosphorylation and, possibly desensitization.

In this study, we used two types of mutant μ opioid receptors: (1) a C-terminally truncated mutant; (2) point- and cluster-mutants with alanine substituted for one or multiple serine or threonine residues located in the C-terminus. These mutant receptors were expressed individually in the CHO cell line and used to investigate the role of the μ opioid receptor C-terminus in agonist-induced phosphorylation and desensitization events.

EXPERIMENTAL PROCEDURES

Construction of Mutant μ Opioid Receptors and Establishment of Stable Cell Lines. An epitope-tagged wild-type μ opioid receptor was PCR-constructed in which the N-terminus has been fused to nine amino acid residues of the HA epitope (YPYDVPDYA). The C-terminal 45 amino acids were deleted from the epitope-tagged receptor by incorporating a stop codon at the end of the cDNA sequence of the desired amino acid via PCR. The epitope and deletion sequences were confirmed by DNA sequencing. The epitope-tagged wild-type and C-terminally truncated μ opioid receptors stably expressed in CHO cell lines, named E−HμCHO and E−HμCHO3$^-$ respectively, were established by subcloning the receptor cDNAs into the pcDNAI/Neo vector, transfecting the constructs into CHO cells, and growing the transfected cells in media containing the selective agent, Geneticin (G418 sulfate; GIBCO BRL). After limiting dilution and Geneticin selection for 4−6 weeks, cell lines stably expressing the epitope-tagged wild-type or epitope-tagged C-terminally truncated μ opioid receptor cDNA were identified by ligand-binding assays and preserved for further study. The point mutant receptor T/S-3A was generated following the procedure described by Surratt and colleagues (*26*) and stably expressed in CHO cell using the procedure described above. The point- and cluster-mutant receptors T394A, T/S-6A, and T/S-7A were generated and stably expressed in CHO cell lines by the procedure reported previously (*21*). The mutants were named by the number of threonine or serine residues substituted: T/S-nA represented the mutant where *n* serines or threonines were substituted by alanines.

Radioligand Binding Assay. Cells expressing the wild-type or mutant μ opioid receptors on the plate (150 mm) were rinsed briefly with 50 mM Tris-HCl buffer, pH 7.4, and harvested by scraping. Whole cells from one plate were suspended in 4.8 mL of Tris-HCl (about 4×10^8 cells). [^{3}H]-DAMGO ([D-Ala^2,MePhe^4,Gly-ol^5] enkephalin, 37 Ci/mmol) binding of suspended cells was assayed as reported previously (*26*). Borosilicate glass tubes containing 400 μL cell suspension were incubated with different concentrations of [^{3}H]DAMGO in a final volume of 500 μL. For saturation binding, six or seven concentrations of [^{3}H]DAMGO ranging from 0.1 to 20 nM were evaluated in duplicate. The mixture was incubated at room temperature for 90 min and then filtered through Whatman GF/B filter paper (FP-100, Brandel) washed three times with 4 mL of ice-cold 50 mM Tris-HCl, pH 7.4 at room temperature using a Brandel filtration device. Radioactivity was assayed by liquid scintillation counting. Data were analyzed by nonlinear analysis using the Prism computer program (GraphPad Software, Inc.). Specific binding was determined as total binding minus the nonspecific binding measured in the presence of 1 μM naloxone. The B_{max} and K_d values for each mutant receptor were compared with those of the wild-type receptor using the Student *t*-test.

Preparation of the Cell Lysate and Immunoblotting. CHO cells stably expressing wild-type or mutant μ opioid receptors were grown on 150 mm plates to a density of 10^9 cells. Cells were rinsed with PBS buffer three times, and 1 mL of sample buffer (4% SDS, 25 mM Tris-HCl, pH 6.8, 5% glycerol, 0.5% 2-mercaptoethanol, 0.005% bromophenol blue) was added to the plates. After incubation on ice for 10 min, the cells were scraped and sheared with a sonicator for 20 s at 50% force, and then boiled at 95 °C for 10 min. Samples were centrifuged at 10 000*g* for 10 min, and the supernatants were saved. Protein concentrations for cell lysates was determined using a Bio-Rad protein assay kit. A total of 20 μg of CHO cell lysates was resolved using 8% SDS−PAGE gels. Proteins were transferred to a nitrocellulose membrane and incubated for 2 h in 5% nonfat dry milk dissolved in the TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) to block nonspecific binding. The nitrocellulose filter was treated with anti-epitope monoclonal antibody 12CA5-A (from Boehringer Mannhem, 1:10000 dilution) or anti-μ opioid receptor antibody, μOR-A, a polyclonal antibody directed to against the C-terminal 18 amino acids of rat and human μ opioid receptor (1:5000 dilution) in 20 mL of NP-40 solution [0.15 M NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.25% (v/w) gelatin, 0.05% (v/v) NP-40] for 2 h at room temperature. The nitrocellulose filter was washed three times in TBST buffer and then incubated with anti-mouse IgG (Boehringer Mannheim Corp.) for 12CA5-A, or anti-rabbit IgG (H+L)-alkaline phosphatase (Boehringer Mannheim Corp.) for μOR-A, at 1:5000 dilution in TBST for 1 h at room temperature. The nitrocellulose membrane was again washed three times in TBST and visualized with the Supersignal Chemiluminescent substrates (Pierce).

Phosphorylation of the μ Opioid Receptor. Cells stably expressing wild-type or mutant μ opioid receptors were plated at 80% confluence in six-well plates, grown for 24 h in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 100 μg of penicillin and 100 mg/L streptomycin, washed twice with phosphate-free DMEM, and incubated at 37 °C for 2 h with 300 μCi/mL of [^{32}P]-orthophosphate (8500 Ci/mmol; Dupont/NEN) in phosphate-free DMEM. Metabolically labeled cells were then exposed to 5 μM DAMGO for 20 min. Cells were cooled to 4 °C by washing with ice-cold phosphate-buffered saline (PBS). Proteins were extracted for 60 min at 4 °C with 0.8 mL of

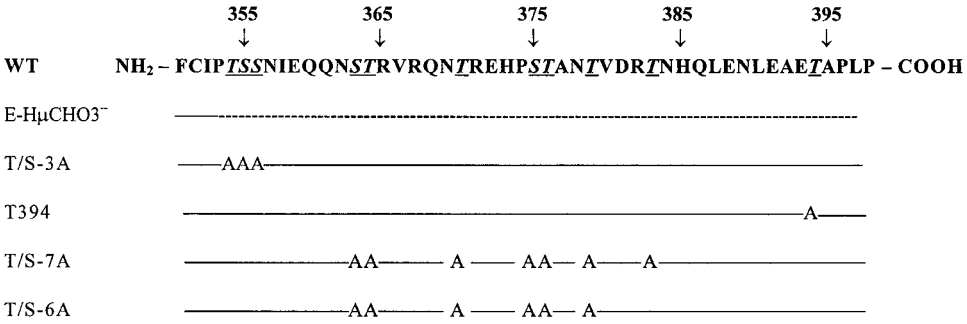

FIGURE 1: Schematic representation showing the positions of mutations in the C-terminus of the μ opioid receptor. In the wild-type receptor (WT), the C-terminal residues 350−398 are shown by single-letter amino acid codes. The 11 serine/threonine sites are indicated by underlined italics. The numbers above indicate the amino acid positions. In the truncated receptor, the deleted segment of the C-terminus is indicated by a dashed line; in the receptors containing point mutations, the letters indicate the sites of substitution of serine (S) or threonine (T) by alanine (A). The amino acid residues of the mutants which are identical to the wild-type are represented by a solid line.

RIPA$^+$ buffer (1% NP-40, 0.5% Na_2deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM NaF, 10 mM Na_2pyrophosphate, 1 μM okadaic acid, 0.1 mM PMSF, 10 ug/mL leupeptin, and 1 ug/mL pepstatin A in PBS buffer). After a 15 min centrifugation at 150000*g*, the supernatant was removed, assayed for protein concentration using a Bio-Rad protein assay kit and preadsorbed by incubation with 100 μL of presoaked protein A-sepharose beads (Pharmacia), followed by centrifugation. For immunoprecipitation, 700 μL of the supernatant from the preadsorption step was incubated for 2 h with 100 μL of the protein A-sepharose bead slurry and a 1:250 final dilution of the 12CA5-A or μOR-A antibody. Beads were washed three times by resuspension with 1 mL of RIPA$^+$ followed by microcentrifugation, and immunoprecipitated proteins were then dissociated from the beads by extraction with 60 μL of SDS−PAGE gel loading buffer (4% SDS, 25 mM Tris-HCl, pH 6.8, 5% glycerol, 0.5% 2-mercaptoethanol and 0.005% bromophenol blue). Then 20 μL of the immunoprecipitated proteins was separated on 8% SDS−PAGE gels, and radiolabeled proteins were identified by autoradiography using Hyperfilm-MP (Amersham) with intensifying screens. Densities for the bands of interest were quantified using a phosphoimage and IMAGEQUANT software (Molecular Dynamies) and were normalized to the amounts of extracted cell protein or the number of receptors subjected to immunoprecipitation. Phosphorylation differences between μ receptors subjected to different treatments were analyzed by the Student *t*-test.

Determination of Adenylyl Cyclase Activity. Wild-type or mutant μ opioid receptors were cultured and harvested as described in the phosphorylation studies. Cell suspensions were treated with medium only or 5 μM DAMGO in medium at 37 °C in 5% CO_2 for 20 min. Cells were collected by centrifugation at 2500*g* for 10 min. Cell membranes were prepared by homogenizing the cells with buffer containing 2.0 mM Tris-HCl, pH 7.4, and 2.0 mM EDTA, and centrifuged twice at 10000*g* for 10 min. Cell membranes corresponding to 30 μg of protein were added to the assay tubes. Then they are coincubated with 10 μM forskolin and varying concentrations of DAMGO in the assay buffer: Tris-HCl, 80 mM (pH 7.4); theophylline, 10 mM; $MgSO_4$, 1 mM; EGTA, 0.8 mM; NaCl, 30 mM; ATP, 0.25 mM; and GTP, 0.01 mM for 5 min at 30 °C. Reaction was terminated by boiling for 2 min. [^{3}H]cAMP (at a final concentration of 4 nM) in a citrate-phosphate buffer (pH 5.0), and binding protein prepared from bovine adrenal glands, were added to each sample. The samples were incubated on ice for 90 min, and the reaction was terminated by addition of charcoal and then centrifugation. Supernatant radioactivity containing bound cAMP was measured by liquid scintillation counting and was converted to picomoles of cAMP by comparison to a standard curve. Dose−response curves were obtained by nonlinear regression analyses using Prism (GraphPad Software, Inc.). The protein concentration was determined using the Bio-Rad protein assay kit. Comparisons of the dose−response curves were analyzed by two-way ANOVA. If a significant treatment difference was observed, a Tukey's HSD test was used to determine the significance.

Table 1: [^{3}H]DAMGO-Binding Properties and Inhibition of the Forskolin-Stimulated Adenylyl Cyclase Activity in the Wild-Type or Mutant μ Opioid Receptors[a]

	binding to [^{3}H]DAMGO		inhibition of adenylyl cyclase activity	
	B_{max} (pmol/mg)	K_d (nM)	EC50 (nM)	maximum inhibition (%)
E−HμCHO	2.9 ± 0.4	1.8 ± 0.21	13.9 ± 4.2	76.6 ± 3.8
E−HμCHO3$^-$	2.7 ± 0.1	1.2 ± 0.2	17.3 ± 4.8	48.5 ± 4.9**
WT	2.3 ± 0.8	1.6 ± 0.6	11.2 ± 3.9	72.6 ± 3.1
T/S-3A	1.6 ± 0.5	1.7 ± 0.5	19.7 ± 2.6	68.6 ± 4.6
T394A	2.9 ± 0.2	2.1 ± 0.3	16.4 ± 2.7	71.9 ± 8.5
T/S-7A	3.7 ± 0.9	2.2 ± 0.3	12.7 ± 4.6	72.9 ± 3.2
T/S-6A	3.3 ± 0.6	2.0 ± 0.2	14.0 ± 5.1	71.5 ± 4.4

[a] The binding of [^{3}H]DAMGO to the wild-type or mutant μ opioid receptors was performed as described in the Experimental Procedures, and two cloned cell lines of E−HμCHO3$^-$ were examined. Each experiment was duplicated. In the adenylyl cyclase assay, membranes from the cells stably expressing the wild-type or mutant μ receptors were treated with 10 μM forskolin and varying concentrations of DAMGO. Then the adenylyl cyclase activity was measured following the procedures described in the Experimental Procedures. Each assay was performed in triplicate. Data shown are mean ± SE from at least three independent experiments. **P < 0.01 compared with that of E−HμCHO (Student *t*-test).

RESULTS

To address the role of the C-terminus in μ opioid receptor phosphorylation and desensitization, we produced cell lines that stably express E−HμCHO and E−HμCHO3$^-$ as well as several alanine substituted μ opioid receptors (Figure 1). [^{3}H]DAMGO-binding assays revealed that mutant receptors showed K_d and B_{max} values similar to those of the wild-type receptor (Table. 1). When we examined the ability of the mutant receptors to inhibit forskolin-stimulated adenylyl

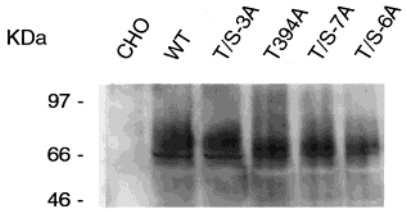

FIGURE 2: Immunoblotting analysis of the wild-type and mutant μ opioid receptors stably expressed in CHO cell lines using μOR-A antibodies. Approximately 20 ug of cell membrane protein was resolved by SDS−PAGE. Size standards, which were derived from prestained markers, were electrophoresed in adjacent lanes. The resolved proteins were transferred to a nitrocellular membrane, blotted with diluted antibody, and visualized with the Supersignal Chemiluminescent substrates.

cyclase activity, each mutant receptor except E−HμCHO3$^-$ exhibited normal DAMGO-induced inhibition of forskolin-stimulated adenylyl cyclase activity. Although the E−Hμ-CHO3$^-$ displayed an EC_{50} value for inhibition of adenylyl cyclase similar to that of the wild-type E−HμCHO, the maximal effect was significantly reduced to 63% of that of E−HμCHO.

Because the N-termini of E−HμCHO and E−HμCHO3$^-$ have been fused to nine amino acid residues of HA epitope, these two epitope-tagged μ opioid receptors were recognized specifically by a commercially available anti-12CA5 monoclonal antibody, 12CA5-A. Another antibody used in our experiments is μOR-A, which was raised against the rat μ opioid receptor C-terminal 18 amino acids. The ability of this antiserum to recognize the μ opioid receptor has been characterized either in a transfected cell line or rat brain tissue using immunoprecipitation, immunohistochemical, immunoblotting assays (*6*, *26*, *27*). We also tested the ability of μOR-A to recognize the point- and cluster-mutant μ opioid receptors, which several amino acids were changed in the C-terminus. These mutant μ opioid receptors were recognized by μOR-A specifically (Figure 2), although it cannot be excluded that the antibody may recognize mutant receptors quantitatively different than wild-type receptor.

The impact of C-terminal truncation or serine/threonine substitutions on agonist-induced μ opioid receptor phosphorylation was determined by metabolically labeling CHO cells that stably expressed mutant receptors with [^{32}P]orthophosphoric acid and then immunoprecipitating the receptor. Both E−HμCHO and E−HμCHO3$^-$ were phosphorylated under basal condition, without drug treatment. Phosphorylation was enhanced by DAMGO agonist treatment (Figure 3). However, the E−HμCHO3$^-$ exhibited less than half of the DAMGO-stimulated enhancement of phosphorylation displayed by E−HμCHO (40%, $P < 0.05$, *t*-test). These data suggest involvement of C-terminus in μ opioid receptor phosphorylation. To identify the specific residues required for receptor phosphorylation, we examined the phosphorylation event in four point- and cluster-mutant μ opioid receptors. Mutant T394A lost more than 90% of DAMGO-induced phosphorylation. All other point- and cluster-mutant receptors were phosphorylated at a density similar to that of wild-type, after data were normalized to the number of receptors subjected to immunoprecipitation (Figure 4). Therefore, threonine 394 is a crucial residue for agonist-induced μ opioid receptor phosphorylation.

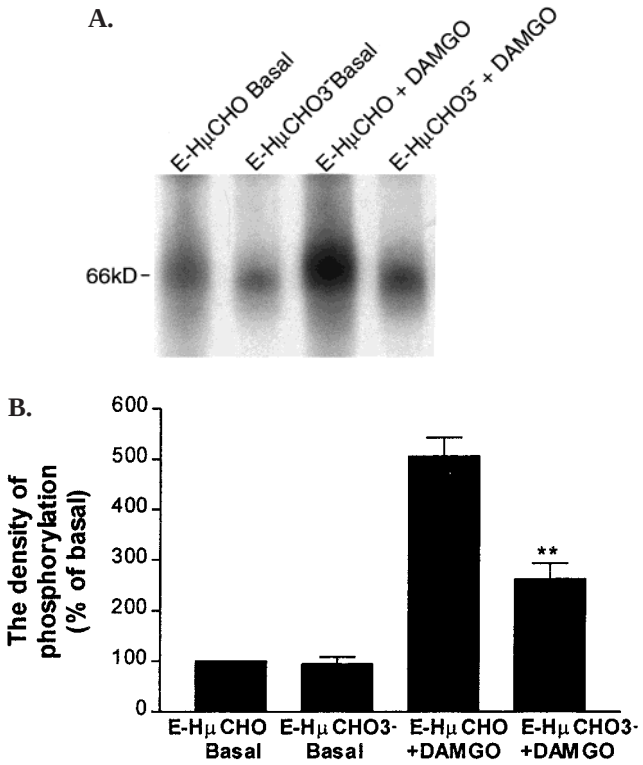

FIGURE 3: (A) Agonist-induced receptor phosphorylation of E−HμCHO and E−HμCHO3$^-$. Cell extracts from E−HμCHO or HμCHO3$^-$ were immunoprecipitated with 12CA5-A, and separated on an SDS−PAGE gel. Cells were pretreated for 20 min at 30 °C in the presence or absence of 5 μM DAMGO. The radiolabeled proteins were identified by autoradiography using Hyperfilm-MP (Amersham) with intensifying screens. Molecular size standards were prestained markers, electrophoresed in adjacent lanes. (B) Phosphorylation density presented as the percentage of basal phosphorylation (without drug treatment) in E−HμCHO. The density of the receptor phosphorylation was quantitatively analyzed by phosphoimaging and normalized to the amount of cell protein subjected to immunoprecipitation. (**) $P < 0.01$ compared to that of E−HμCHO (Student *t*-test).

In E−HμCHO cells, DAMGO-mediated inhibition of adenylyl cyclase activity was also attenuated after 20 min of DAMGO pretreatment, displaying 2-fold increase in EC_{50} and a reduced maximal response (43%) compared with those observed in no DAMGO pretreatment (Figure 5A). Two-way ANOVA analysis revealed significant differences between the two dose−response curves obtained before and after pretreatment ($P < 0.01$). In contrast, pretreated mutant E−HμCHO3$^-$ cells with DAMGO for 20 min failed to induce significant desensitization. These results again imply an important role of the C-terminus in acute μ opioid receptor desensitization (Figure 5B). When we examined the effect of the alanine-substituted point- and cluster-mutation on acute receptor desensitization by assessing their K_d and E_{max} values (Table 2) and their desensitization pattern. We found that mutant T/S-3A still underwent acute DAMGO-mediated desensitization with a pattern similar to that of the wild-type (Figure 6A, Figure 6B). Strikingly, mutation of T394 led to complete abolition of DAMGO-mediated acute desensitization. This mutant displayed no change of the dose−response curve and no reduction in the maximal response with DAMGO pretreatment (Figure 6C). However, the mutant T/S-7A, which possesses seven substituted serine or threonine residues, exhibited only 28% of wild-type

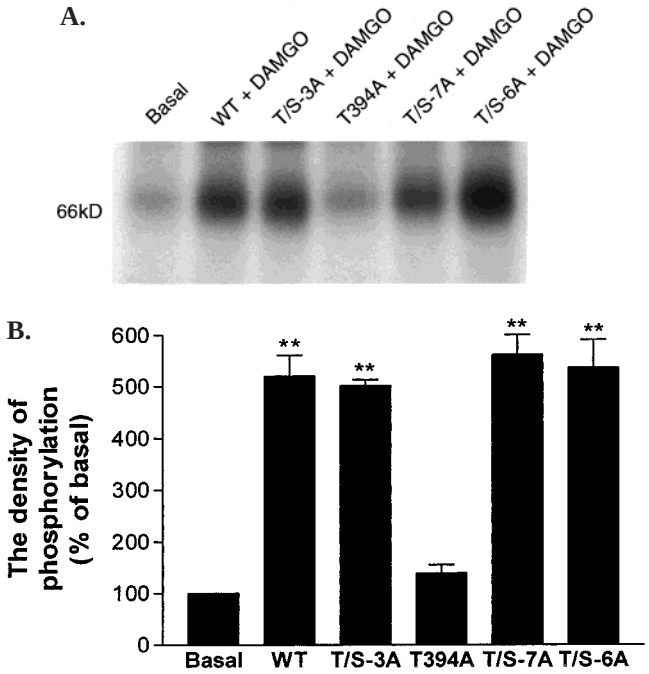

FIGURE 4: (A) Agonist-induced receptor phosphorylation of point- and cluster-mutant μ opioid receptors. Cell extracts from CHO cells stably expressing mutant μ opioid receptor as indicated were immunoprecipitated with μOR-A antiserum, and separated on an SDS−PAGE gel. Cells were pretreated for 20 min at 30 °C in the presence or absence of 5 μM DAMGO. The radiolabeled proteins were identified by autoradiography using Hyperfilm-MP (Amersham) with intensifying screens. Molecular size standards were prestained markers electrophoresed in adjacent lanes. (B) Phosphorylation density presented as the percentage of basal phosphorylation (without drug treatment). The density of receptor phosphorylation was quantitatively analyzed by phosphoimaging and normalized to the number of receptors subjected to immunoprecipitation. (**) $P < 0.01$ compared to basal phosphorylation (Student *t*-test).

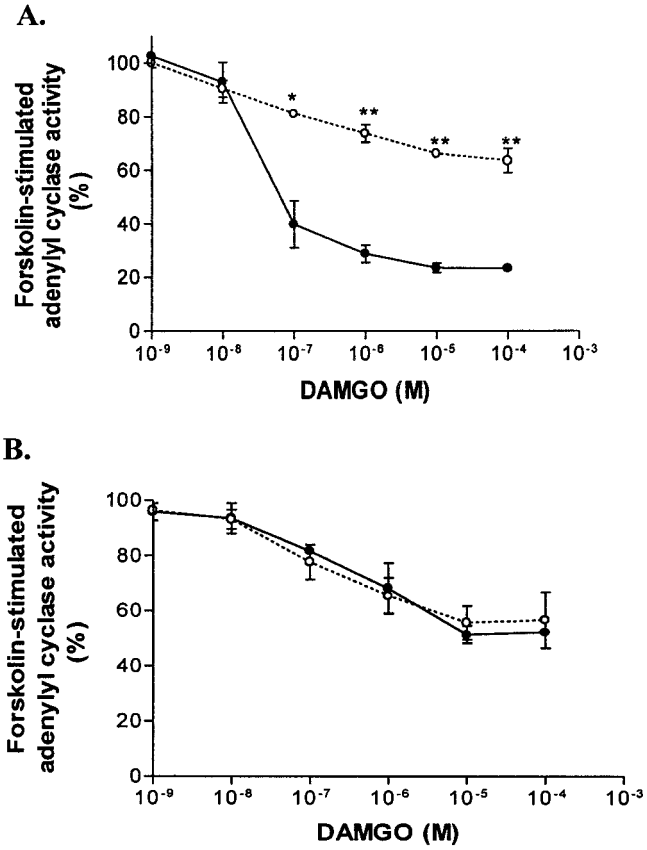

FIGURE 5: Effect of DAMGO pretreatment on E−HμCHO (panel A) and E−HμCHO3$^-$ (panel B) adenylyl cyclase activity. E−HμCHO or E−HμCHO3$^-$ were preincubated for 20 min with (○) or without (●) 5 μM DAMGO in the culture medium. The cells were then tested for μ receptor mediated adenylyl cyclase inhibition as described in the Experimental Procedures, with application of varying concentrations of DAMGO ranging from 10^{-9} to 10^{-4} M. Data are represented as the percentage of the forskolin-stimulated activity. Data points represent mean ± SE of three independent experiments performed in triplicate. (*) $P < 0.05$; (**) $P < 0.01$ compared to the response without pretreatment (Tukey's HSD test).

desensitization following 20 min DAMGO pretreatment. Two-way ANOVA analysis revealed significant differences between the dose−effect curves generated by DAMGO in this and wild-type receptors ($P < 0.05$). These data contrast with data from mutant T/S-6A, which has the same mutations as T/S-7A except that T383 was left intact. This multiple-substituted receptor displayed desensitization similar to that of the wild-type receptor ($P < 0.01$, two-way ANOVA). These results also implicate threonine 383 in acute desensitization.

DISCUSSION

Understanding the mechanisms of μ opioid receptor phosphorylation and desensitization requires determining which domains and amino acid residues are phosphorylated in response to an agonist and their relationship to desensitization. In the present study, we investigated these issues by studying a C-terminally truncated μ opioid receptor, E−HμCHO3$^-$. The truncated receptor showed normal binding properties, but reductions in both phosphorylation and desensitization extent compared to E−HμCHO. The C-terminus of the μ opioid receptor is thus involved in receptor phosphorylation and desensitization. This truncation of mutant receptor may render a normal μ receptor into a desensitized form of the receptor. However, fewer phosphorylation sites may not in themselves account for the reductions in receptor phosphorylation and desensitization; impaired G-protein coupling due to the C-terminal truncation could also cause these reductions. Indeed, E−HμCHO3$^-$ did not function as well as E−HμCHO in inhibiting adenylyl cyclase activity. Our findings that this receptor displays much lower maximal inhibition than E−HμCHO suggest that the C-terminal truncation may impair the receptor G-protein

Table 2: Inhibition of the Forskolin-Stimulated Adenylyl Cyclase Activity in the Wild-Type or Mutant μ Opioid Receptors after 5 μM DAMGO Pretreatment[a]

	EC_{50} (nM)	maximal inhibition (%)
WT	31.5 ± 4.2	33.2 ± 3.8
T/S-3A	44.0 ± 2.8	35.8 ± 7.2
T394A	19.4 ± 4.9	70.5 ± 3.6
T/S-7A	27.8 ± 6.4	61.8 ± 4.2
T/S-6A	48.3 ± 2.7	41.2 ± 6.0

[a] After pretreatment, membranes from the cells stably expressing the wild-type or mutant μ receptors were prepared and treated with 10 μM forskolin and varying concentrations of DAMGO. Then, the adenylyl cyclase activity was measured following the procedures described in the Methods. Each assay was performed in triplicate. Data shown are mean ± SE from at least three independent experiments.

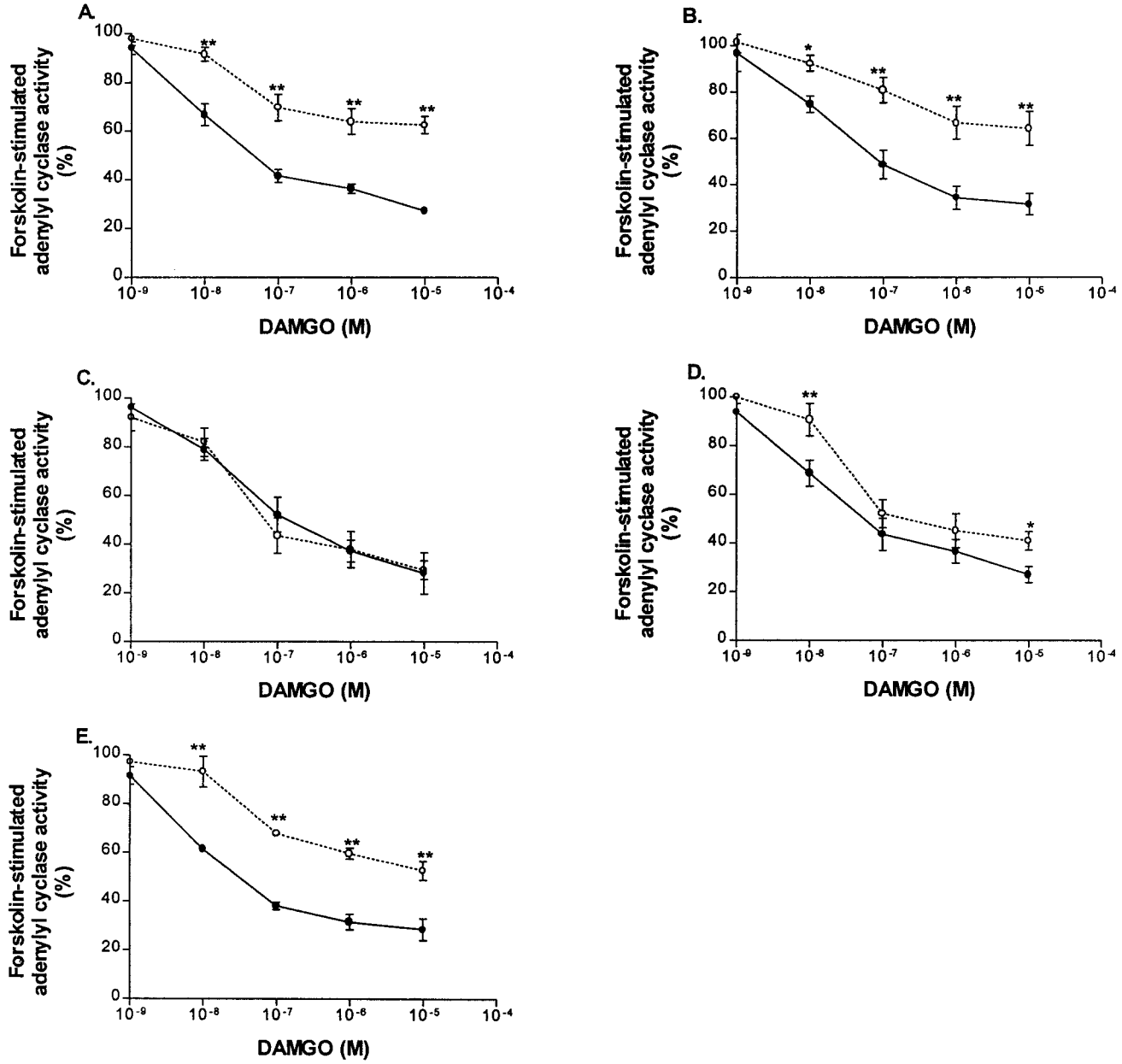

FIGURE 6: Effects of point and cluster mutations on agonist-induced desensitization of μ opioid receptor mediated adenylyl cyclcase inhibitoin. CHO cells stably expressing mutant μ receptors were preincubated for 20 min with (○) or without (●) 5 μM DAMGO and tested for adenylyl cyclase inhibition with application of varying concentrations of DAMGO ranging from 10^{-9} to 10^{-5} M as described in the Experimental Procedures. Data are represented as the percentage of the forskolin-stimulated activity. (A) Wild-type μ opioid receptor. (B) T/S-3A. (C) T394A. (D) T/S-7A. (E) T/S-6A. Data points represent mean ± SE from three to five independent experiments performed in triplicate. (*) $P < 0.05$; (**) $P < 0.01$ compared to the response without pretreatment (Tukey's HSD test).

interaction upon agonist stimulation and lead to decreased agonist efficacy. Mutagenesis studies of other G-protein coupled receptors have also implicated third cytoplasmic loop and proximal C-terminus regions in the coupling of the receptors with intracellular G-proteins (*14*−*16*).

To narrow the investigations to specific serine and threonine residues, we used μ opioid receptors with alanine substitutions for all C-terminal domain serines and threonines. Each of these mutant receptors behaved like wild-type receptors in binding, inhibiting adenylyl cyclase and expression patterns. Substitution of threonine 394 by alanine resulted in an μ opioid receptor mutant with greatly reduced agonist-induced phosphorylation. Threonine 394 is thus a crucial residue for agonist-induced μ opioid receptor phosphorylation. The current results are in accord with previous studies of the μ opioid receptor splice variant MOR1B and the role of the C-terminus in μ opioid receptor desensitization (*24*, *25*). In addition to that, T394A mutation of μ opioid receptor severely attenuates DAMGO-induced shortOLINIT-term desensitization, this mutant seems also be able to facilitate internalization and resensitization of the μ opioid receptor after a longer time exposure of the receptor to DAMGO (*28*)

G-protein coupled receptors are frequently regulated by protein kinases of two classes: relatively receptor-specific protein kinases and second messenger-activated kinases. The second messenger-activated kinases include cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and calcium/calmodulin kinases. The receptor-specific protein kinases, such as the β-adrenergic receptor kinase (GRK2),

are GRKs. They have the unique feature of phosphorylating G-protein-coupled receptors when the receptors are in their active or stimulated conformations. Studies from our laboratory and other laboratories suggested that μ opioid receptor phosphorylation could involve both types of protein kinases under different circumstances. μ opioid receptor desensitization is potentiated by calcium/calmodulin dependent protein kinase (*29*), and agonist-induced μ opioid receptor phosphorylation can be calcium dependent (*8*). Chronic morphine administration increased PKA activity in the locus coeruleus (*30*, *31*). Although activation of PKC could phosphorylate the μ opioid receptor, PKC may not be directly linked with agonist-induced phosphorylation, but could relate to the second messenger interaction (*6*). Recently, evidence points to participation of GRKs in μ opioid receptor phosphorylation (*10*, *11*). Interestingly, it is proposed that GRK2 has a preference for phosphorylating serine or threonine residues which have acidic amino acids juxtaposed to their N-terminal side (*14*, *18*). T394 meets these conditions with several acidic glutamic acid residues in close proximity to its N-terminal side. Thus, GRK2 may be the kinase that is responsible for phosphorylating T394. GRKs may also play roles in desensitization of μ and other opioid receptors. Chronic morphine treatment increased levels of GRK2 in the locus coeruleus (*12*). A dominant negative mutant of GRK2 blocked κ opioid receptor desensitization (*32*). Desensitization of δ opioid receptor following acute agonist stimulation in transfected cell lines was also changed by the overexpression of GRK2 or an impaired mutant GRK2 (*9*). Whether β-arrestins and GRKs are involved in agonist-specific regulation of μ receptor is still unclear. Two studies (*39*, *49*) failed to show interference of β-arrestin with μ receptor, while other studies (*10*, *11*) reported that GRKs and arrestins play a fundamental role in mediating agonist-selective regulation of the μ receptor. It is also noted that there is a marked difference in the ability of different agonists in promoting μ receptor phosphorylation and internalization. The apparent discrepancy may reflect the need of different types of GRKs and arrestins for their optimal interaction with μ receptor.

Studies of G-protein-coupled receptor phosphorylation generally postulate sequential and nonsequential phosphorylation events. In the sequential phosphorylation, the first phosphorylation step greatly enhances subsequent phosphorylation events. Replacement of upstream serine or threonine residues produces a substantial reduction or even complete abolition of the whole phosphorylation event. Sequential phosphorylation occurs in the phosphorylation of rhodopsin by rhodopsin kinase (*33*−*36*) and for the human C_{5a} anaphylatoxin receptor (*37*). In nonsequential phosphorylation, serine or threonine residues are phosphorylated randomly and independently. Substitution of any serine or threonine residue leads to only a reduction of the substituted residue phosphorylation level from the whole phosphorylation event, as was shown for the α_2-adrenergic receptor (*14*). In our study, we found that replacement of T394 substantially reduced agonist-induced phosphorylation by up to 90%. Agonist-induced μ opioid receptor phosphorylation could thus be explained by the sequential phosphorylation model, in which T394 served as a primary phosphorylation site. Its phosphorylation may initiate the following phosphorylation events. However, the residues phosphorylated following T394 appeared to be located somewhere other than in the C-terminus because the other C-terminal cluster-mutated receptors in our study exhibited similar phosphorylation to that of the wild-type. Serine or threonine residues located in other cytoplasmic domains, most likely the third intracellular domain of the μ opioid receptor, may also contribute to the entire agonist-induced μ receptor phosphorylation events. Future phosphopeptide mapping and phosphoamino acid analysis will reveal how many phosphorylation sites are involved in the entire agonist-induced μ opioid receptor phosphorylation event and further delineate the receptor phosphorylation processes.

If T394 were considered an essential residue for initiation of μ receptor phosphorylation, we would expect that the truncation of C-terminal of μ receptor could eliminate the receptor phosphorylation. However, in E−HμCHO3$^-$, the 40% reduction in phosphorylation that we observed was less than that found in the T394A mutant receptor. A simple explanation would be that the truncation of the receptor may cause normally nonaccessible phosphorylation residues to become readily available substrates for protein kinase. This also may be due to a compensatory effect of the C-terminus deletion. Because the C-terminus is involved in G-protein coupling, deletion of the C-terminus could cause a conformational change of the intracellular part of the receptor. Subsequent phosphorylation of serine or threonine residues lying in other intracellular loops could thus be increased due to activation of compensatory mechanisms.

Deletion or mutation of putative phosphorylation sites in G-protein-coupled receptors has revealed the importance of receptor phosphorylation in agonist-mediated desensitization (*17*, *21*, *38*). For the μ opioid receptor, C-terminal threonine 394 has been implicated in receptor desensitization (*21*, *24*, *25*). However, the desensitization observed in these studies occurred slowly with 1 h agonist exposures. The phosphorylation change in our experiment was detected within 20 min of agonist application, and therefore, we wondered whether T394 was also an essential residue for acute desensitization corresponding to rapid phosphorylation. Indeed, after exposure to DAMGO for 20 min, the T394A mutant receptor failed to show agonist-mediated desensitization of its inhibitory effect on adenylyl cyclase activity, while the wild-type receptor showed significant desensitization. This receptor apparently becomes a poor substrate for kinase and, hence, is resistant to acute desensitization.

Receptor phosphorylation is not the only mechanism involved in receptor desensitization. We found that mutant T/S-7A showed a partial loss of its desensitization (21% of the wild-type) while T/S-6A desensitized to the same extent as wild-type receptor. This result indicated the involvement of T383 in agonist-mediated acute desensitization, although T383 does no directly participate in agonist-induced phosphorylation. The role that T383 plays in agonist-mediated desensitization is thus not clear, but does not appear to be due to phosphorylation. It cannot be excluded that a lesser extent of phosphorylation may occur which cannot be detected by our method. Recent studies demonstrated the involvement of β-arrestin in acute μ receptor desensitization and internalization (*10*, *11*). Conceivably, T383 may be required for binding β-arrestin or a β-arrestin like cytoplasmic component. This residue may also be involved in other cellular processes, such as rapid internalization or resensitization (*28*).

In conclusion, our results demonstrate that the C-terminus is an important domain that influences μ opioid receptor G-protein coupling and receptor desensitization. Threonine 394 is a crucial residue required for receptor phosphorylation and desensitization. Threonine 383 was required for complete agonist-induced μ opioid receptor desensitization, but may be not required for agonist-induced receptor phosphorylation. Agonist-induced receptor phosphorylation and desensitization are thus closely associated, but other cellular components or processes are likely to be involved as well.

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