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Sensitization of the Adenylyl Cyclase System in Cloned κ-Opioid Receptor-Transfected Cells Following Sustained Agonist Treatment: A Chimeric Study Using G Protein α_{i2}/α_q Subunits

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ABSTRACT—Chronic and/or sustained opioid treatment has been shown to result in development of sensitization of the adenylyl cyclase (AC) system or cAMP overshoot. In this study, we investigated the molecular mechanism responsible for sensitization of the AC system using CHO cells co-expressing cloned κ-opioid receptor and some chimeric G protein α_{i2}/α_q subunits. In CHO cells co-expressing the κ-opioid receptor and pertussis toxin-insensitive chimeric α_{i2}/α_q subunits with α_{i2} residues $Met^{244}-Asn^{331}$, despite pretreatment with pertussis toxin, acute treatment with the κ-opioid-receptor-selective agonist U69,593 suppressed forskolin-stimulated cAMP accumulation, while sustained treatment with U69,593 (4 h) induced cAMP overshoot over the naive level by the κ-opioid-receptor-selective antagonist norbinaltorphimine (sensitization of the AC system). On the other hand, in CHO cells co-expressing the κ-opioid receptor and pertussis toxin-insensitive chimeric α_{i2}/α_q subunits without α_{i2} residues $Met^{244}-Asn^{331}$, pretreatment with pertussis toxin completely blocked these acute and sustained effects of U69,593 on cAMP accumulation. These results suggested that the presence of the specific region of α_{i2} ($Met^{244}-Asn^{331}$), which was reported to be responsible for the inhibition of AC, and continuous inhibition of AC by α_{i2} is necessary for the development of sensitization.

Keywords: κ-Opioid receptor, Adenylyl cyclase, Sensitization, Chimeric G protein α subunit, CHO cell

Although opiates are widely used clinically for their potent analgesic effects, chronic treatment with these agents results in the development of physical and psychological dependence (1). Investigations of the cellular and molecular mechanisms underlying these phenomena have focused on the adaptive changes of signal transduction systems via opioid receptors. Opioid receptors have been pharmacologically classified into at least three types, designated as μ, δ and κ, each with distinct binding properties for various opioid ligands and with distinct distributions in the nervous system (2). All types of opioid receptors belong to the superfamily of seven-transmembrane domain GTP-binding protein (G protein)-coupled receptors. Stimulation of opioid receptors leads to the activation of heterotrimeric pertussis toxin-sensitive $G_{i/o}$ proteins, leading to inhibition of the AC system (2).

On the other hand, it has been reported that sustained activation of opioid receptors followed by withdrawal of the opioid agonist leads to an increase of adenylyl cyclase (AC) activity over the naive level in several cell lines such as NG108-15 neuroblastoma×glioma hybrid cells, and human neuroblastoma cells SH-SY5Y (3-7) and in several neuronal tissues such as the locus coeruleus, nucleus accumbens, and amygdala (8, 9). Sensitization of the AC system, as characterized by the production of cAMP overshoot, has been suggested to be the cellular and molecular mechanisms of opioid dependence and/or withdrawal syndrome (3-11), although the molecular mechanisms underlying this phenomenon remain unclear.

Chinese hamster ovary (CHO) cells stably expressing one of the cloned μ-, δ- and κ-opioid receptors have been reported to be a suitable system for molecular studies of their binding and signal transduction properties, including alterations in the signal transduction system by sustained opioid treatment (2, 12). Indeed, we and others have characterized sensitization of the AC system induced by sustained agonist treatment in CHO cells expressing

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κ- and μ-opioid receptors (13–15).

Although it is clear that chronic activation of μ-opioid receptors induces the development of opioid dependence and/or expression of withdrawal syndrome, there is evidence that chronic activation of κ-opioid receptors also contributes significantly to the response (16, 17). In this study, to elucidate the molecular mechanism responsible for sensitization of the AC system by sustained activation of κ-opioid receptors, we used CHO cells co-expressing cloned κ-opioid receptor and some chimeric G protein α subunits between α_{i2}, which inhibits AC, and α_q as a representative α subunit with no effects on AC. The results obtained in the present study indicated that the presence of a specific region of α_{i2} (Met^{244}–Asn^{331}), which is responsible for the interaction with AC, and continuous inhibition of AC by α_{i2} is necessary for the development of sensitization.

MATERIALS AND METHODS

Materials

Rat κ-opioid receptor cDNA was cloned as described (18). The κ-opioid selective agonist (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro-(4,5) dec-8-yl]benzeneacetamide (U69,593) was a gift from the Upjohn Company (Kalamazoo, MI, USA). The κ-opioid selective antagonist norbinaltorphimine (norBNI) was a gift from Dr. H. Nagase (Toray Industries, Kamakura). Forskolin and pertussis toxin were purchased from Wako Pure Chemical Industries (Osaka). Anti-α_{i2} rabbit polyclonal antibody, raised against the peptide EEQGMLPEDLS corresponding to the amino acid sequence from rat α_{i2} residues 115 to 125, was from Gramsch Laboratories (Schwabhausen, Germany). Anti-α_q rabbit polyclonal antibody, raised against the peptide EVDVEKSAFENP YVDAIK corresponding to the amino acid sequence from mouse α_q residues 115 to 133, was from Chemicon (Temecula, CA, USA). [γ-^{32}P]GTP (6000 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA, USA). All other reagents were of the highest quality available from commercial sources.

Construction of mutated or chimeric G protein α subunits

The rat α_{i2} cDNA was cloned from the cortex of Sprague-Dawley rats by an RT-PCR-based method. Similarly, mouse α_q was cloned from the whole brain of ddY mice. For site-directed mutagenesis, the coding regions of α_{i2} and α_q were subcloned into pBluescript II (Stratagene, San Diego, CA, USA). In vitro site-directed mutagenesis was carried out using a Transformer™ Site-Directed Mutagenesis Kit (2nd version) (Clontech Laboratories, Palo Alto, CA, USA) as previously described (19). The chimeric G protein α subunits between α_{i2} and α_q were constructed using intrinsic (*Nsi* I site in α_{i2} cDNA) and introduced (*Nsi* I site in α_q and *Sca* I site in α_{i2} and α_q cDNAs) restriction enzyme sites at corresponding locations in both α subunit cDNAs (Fig. 2). The appropriate restriction enzyme fragments of α_{i2} and α_q cDNAs were ligated. Each fragment containing the full-length coding region of the wild-type, mutated or chimeric α subunits was subcloned into the *Eco*RV-*Not* I site of the pTracer-CMV eukaryotic expression vector (Invitrogen, Carlsbad, CA, USA). The sequence of each construct was confirmed by sequencing analysis using an ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA).

Cell culture

CHO cells stably expressing the rat κ-opioid receptor were established as described (12) and cultured in F-12 medium containing 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 200 μg/ml G418 (Gibco BRL, Gaithersburg, MD, USA) under a 5% CO_2 atmosphere at 37℃. The cells were seeded into 75-cm^2 flasks at a density of 10^4–10^6 cells/cm^2 and grown to subconfluence for 5–7 days. The growth medium was changed every 2 or 3 days.

Isolation of CHO cells co-expressing κ-opioid receptors and G protein α subunits

Isolation of CHO cells co-expressing κ-opioid receptors and G protein α subunits was performed as described previously (13). Briefly, CHO cells stably expressing the κ-opioid receptor were seeded in 60-mm culture dishes and grown to 60–80% confluency before transfection. For transfection, aliquots of 2.0 μg of the plasmids encoding G protein α subunits were mixed with 8 μl of lipofectAMINE reagent (Gibco BRL) in 2.0 ml of serum-free F-12 medium according to the manufacturer's instructions. CHO cells were exposed to the DNA/lipofectAMINE mixture for 6 h, and then the medium was replaced with fresh growth medium. After 72 h, cells expressing each G protein α subunit were selected by culture in the presence of 200 μg/ml Zeocin (Invitrogen), and the expression of α subunits was analyzed by immunoblotting with anti-α_{i2} or anti-α_q antibody to isolate CHO cells co-expressing the κ-opioid receptor and G protein α subunits. The transfectants were cultured in F-12 medium containing 10% fetal calf serum, 200 μg/ml G418 and 100 μg/ml Zeocin under a 5% CO_2 atmosphere at 37℃.

Immunoblotting

For immunoblotting, aliquots of 50 μg of membrane protein from CHO cells were resolved by SDS-polyacrylamide gel electrophoresis (12% acrylamide), trans-

ferred onto nitrocellulose membranes, and probed with antibodies specific for either α_{i2} or α_q. The antigen-antibody complexes were detected using biotin-conjugated goat anti-rabbit immunoglobulin G and visualized using 3,3′-diaminobenzidine tetrahydrochloride as a substrate with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA).

GTPase assay

GTPase activity was measured based on ^{32}Pi liberation from [γ-^{32}P]GTP by the procedure of Cassel and Selinger (20) with slight modifications. CHO cells were seeded at a density of 1×10^6 cells / 60-mm dish. After 24 h, the cells were washed and harvested in phosphate-buffered saline containing 1 mM EDTA. The cells were homogenized with a Polytron homogenizer and centrifuged at $100{,}000 \times g$ for 5 min at 4℃. The membranes (4–8 μg of proteins) were incubated for 5 min in 20 mM HEPES (pH 7.5) containing 2 mM $MgCl_2$, 0.1 M NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM App(NH)p, 0.2 mM ATP, 2 mM phosphocreatine, 10 U/ml creatine phosphokinase and 0.5 μM [γ-^{32}P]GTP in the presence or absence of U69,593 at 37℃. The reaction was terminated by addition of a 5% suspension of charcoal (Norit SX plus) containing 0.1% bovine serum albumin in 20 mM phosphate buffer (pH 7.0), and chilled for 10 min. Nonspecific GTPase was assessed by parallel assays containing 100 μM GTP. The assay tubes were centrifuged to sediment the charcoal, and ^{32}P in the supernatant was counted by liquid scintillation counting. GTPase assays were performed in duplicate and the results are presented as means±S.E.M. of 3–5 separate experiments.

cAMP assay

cAMP assay was performed in duplicate as described previously (12) with slight modifications. Briefly, CHO cells were seeded into 24-well plates at a density of 1×10^5 cells/well. After 20 h, cells were incubated in the presence or absence of U69,593 (1 μM) for 4 h. At the end of this treatment, the cells were rapidly washed once with HEPES-buffered saline (140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM HEPES, pH 7.4) and then incubated for 10 min at 37℃ with HEPES-buffered saline containing 1 mM 3-isobutyl-1-methylxanthine and 10 μM forskolin in the presence or absence of opioid ligands. The reaction was stopped by adding an equal volume of ice-cold 10% trichloroacetic acid to each well. The concentration of cAMP was measured by a radioimmunoassay kit (Amersham, Buckinghamshire, UK). In each experiment, forskolin-stimulated cAMP accumulation in naive cells was assigned a value of 100% (61.8±3.72 and 50.0±3.18 pmol/10 min per well in the absence and presence of pertussis toxin, respectively). The results are presented as means±S.E.M. of 3–6 separate experiments.

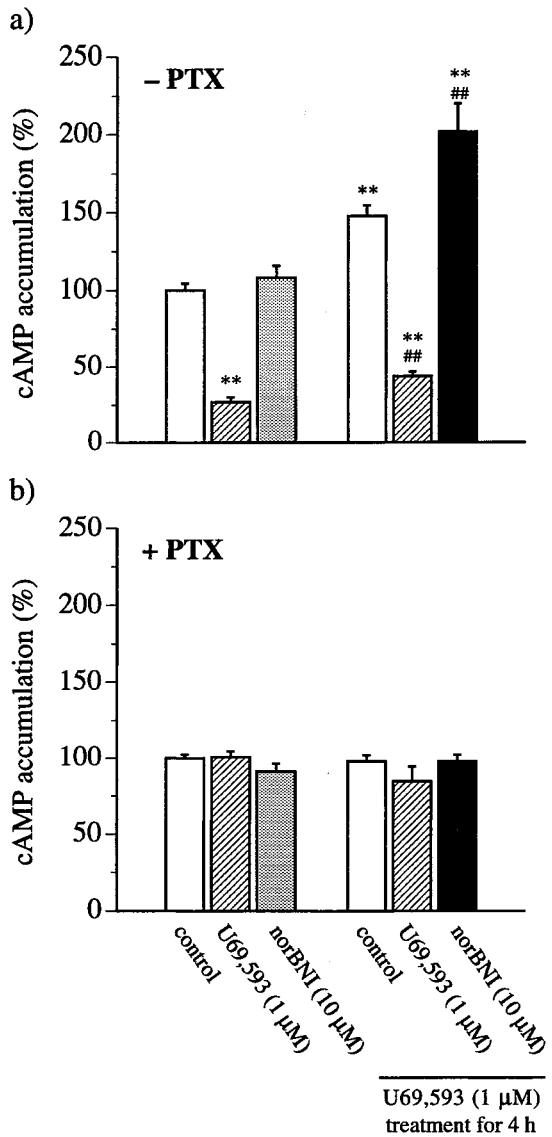

Fig. 1. Effects of acute and sustained treatment with U69,593 on the AC system in CHO cells expressing the κ-opioid receptor. a: For acute treatment, CHO cells were incubated with forskolin (10 μM) in the presence or absence of U69,593 (1 μM) or norBNI (10 μM) for 10 min. For sustained treatment, U69,593 (1 μM) was added to the growth medium 4 h before the cAMP assay. After a rapid wash, the cells were challenged with forskolin in the presence or absence of U69,593 (1 μM) or norBNI (10 μM) for 10 min. b: To block the activation of endogenous G_i-like G proteins, pertussis toxin (20 ng/ml) was added to the growth medium 12 h before and was present during 4 h of sustained treatment with U69,593. In each experiment, forskolin-stimulated cAMP accumulation in naive cells with (+PTX) or without (−PTX) pertussis toxin pretreatment was assigned a value of 100%. Data are presented as means±S.E.M. of 6 separate experiments. **$P < 0.01$, compared with forskolin-stimulated cAMP accumulation in naive cells. ##$P < 0.01$, compared with forskolin-stimulated cAMP accumulation in the cells treated with U69,593 for 4 h (Mann-Whitney *U*-test).

RESULTS

Sensitization of the AC system in CHO cells expressing the κ-opioid receptor

In naive CHO cells expressing the κ-opioid receptor, acute treatment with U69,593 (1 μM) significantly suppressed forskolin-stimulated cAMP accumulation to 26.9±3.1% of the control level, although norBNI (10 μM) had no effect (107.9±7.8%). In the cells treated with U69,593 (1 μM) for 4 h, challenge with forskolin in the absence of opioid ligand significantly increased forskolin-stimulated cAMP accumulation to 147.4±7.04% of the naive control level. Readdition of U69,593 (1 μM) with forskolin after sustained agonist treatment significantly suppressed cAMP accumulation to 43.7±2.97% of the naive control level. When forskolin-stimulated cAMP accumulation in the cells was assigned a value of 100%, the inhibitory effect by readdition of U69,593 was similar (29.6±2.02%) to that in the naive cells. In the cells, challenge with forskolin in the presence of norBNI (10 μM) induced significant cAMP overshoot to 201.4±17.9% of the naive control level (sensitization of the AC system) (Fig. 1a). Pretreatment of the cells with pertussis toxin (20 ng/ml for 16 h) completely blocked both acute and sustained effects of U69,593 on cAMP accumulation (100.6±3.80% and 97.9±4.21%, respectively) (Fig. 1b).

Isolation of CHO cells co-expressing κ-opioid receptors and wild-type, mutated or chimeric G protein α subunits

First, we generated pertussis toxin-insensitive α_{i2} in which Cys^{352} was replaced with glycine (α_{i2} C352G). This pertussis toxin-insensitive α_{i2} was used to generate chimeric α subunits between α_{i2} and α_q. To generate the first chimeric α subunit, the segment of α_{i2} C352G from residues 1–243 was replaced by the corresponding 248-residue segment of α_q (residues 1–248: GQI *Nsi* I). Similarly, the segment of α_{i2} C352G from residues 1–331 was replaced by the corresponding 336-residue segment of α_q (residues 1–336) to generate the second chimera, GQI *Sca* I. For the third chimeric α subunit, the 88-residue segment of α_{i2} C352G from residues 244–331 was replaced by the corresponding 88-residue segment of α_q (residues 249–336: GIQI) (Fig. 2).

After subcloning of these α subunit fragments (wild-type α_{i2}, α_{i2} C352G, GQI *Nsi* I, GQI *Sca* I, GIQI) into the pTracer-CMV expression vector, CHO cells stably expressing the κ-opioid receptor were transfected with these plasmids. CHO cells were selected by culture in the presence of Zeocin and analyzed by immunoblotting. Finally, the expression of each of the α subunits in the cloned CHO cells was confirmed by immunoblotting with

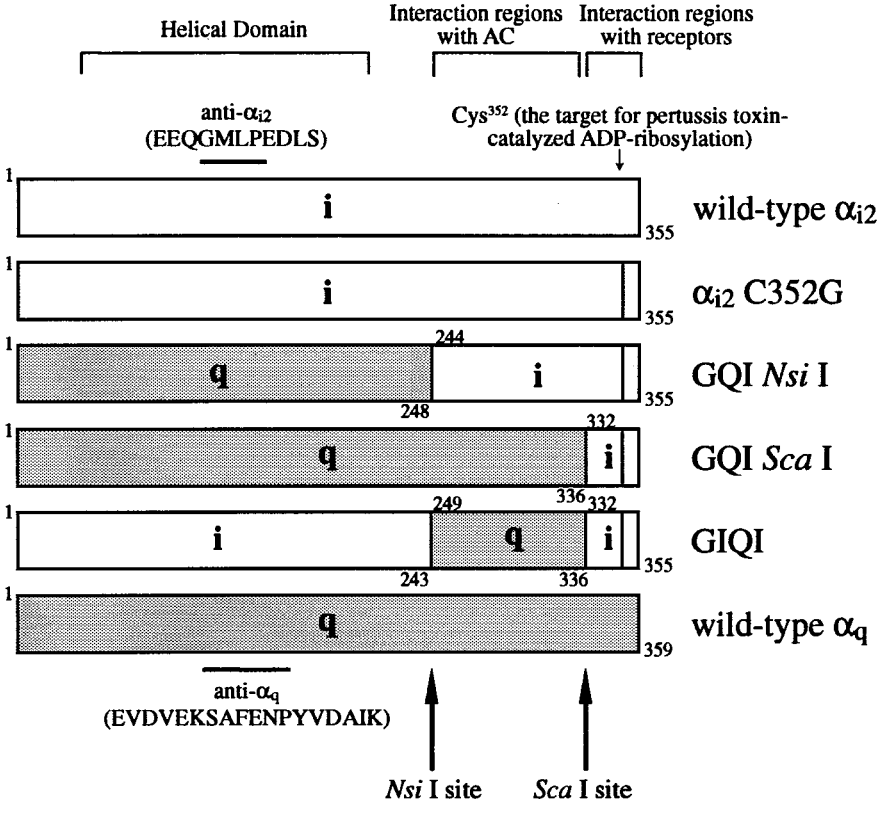

Fig. 2. Construction of wild-type, mutated and chimeric G protein α subunits. Chimeric G protein α subunits are depicted as segments with numbers that represent α_{i2} (open) or α_q (shaded) residues at the beginning and end of segments derived from these proteins.

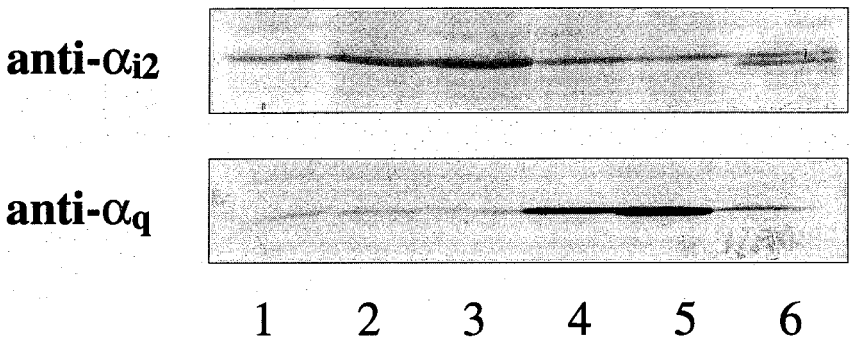

Fig. 3. Expression of recombinant G protein α subunits in CHO cells expressing the κ-opioid receptor. The CHO cells were transfected without (lane 1) or with wild-type α_{i2} (lane 2), α_{i2} C352G (lane 3), GQI *Nsi* I (lane 4), GQI *Sca* I (lane 5) and GIQI (lane 6) cDNAs. Membrane proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis (12% gels), transferred onto nitrocellulose membranes and probed with antibodies specific for either α_{i2} (recognizing the α_{i2} unique sequence Glu^{115}–Ser^{125}) or α_q (recognizing the α_q sequence Glu^{115}–Lys^{133}).

anti-α_{i2} and anti-α_q antibodies (Fig. 3). As these antibodies recognize the N-termini of the α subunits, wild-type α_{i2}, α_{i2} C352G and GIQI could be detected by the anti-α_{i2} antibody, and GQI *Nsi* I and GQI *Sca* I were detected by the anti-α_q antibody. Our results confirmed that all of the recombinant G protein α subunits were expressed in CHO cells expressing the κ-opioid receptor.

Functional coupling of κ-opioid receptors to recombinant G protein α subunits

In the membranes from CHO cells expressing the κ-opioid receptor alone, acute treatment with U69,593 (10 μM) significantly stimulated GTPase activity by activation of the endogenous G protein. Pretreatment of the cells with pertussis toxin (20 ng/ml for 16 h) completely blocked this effect. Similarly, in the membranes from CHO cells co-expressing κ-opioid receptor and wild-type α_{i2}, pretreatment with pertussis toxin completely blocked the stimulatory effect of U69,593 on GTPase activity. On the other hand, in the membranes from CHO cells co-expressing κ-opioid receptor and each of the mutated or chimeric α subunits, α_{i2} C352G, GQI *Nsi* I, GQI *Sca* I or GIQI, pretreatment with pertussis toxin did not block the stimulatory effect of U69,593 on GTPase activity (Table 1).

Sensitization of the AC system in CHO cells co-expressing the κ-opioid receptor and wild-type, mutated or chimeric G protein α subunits

In naive CHO cells co-expressing the κ-opioid receptor and wild-type α_{i2}, acute treatment with U69,593 (1 μM) significantly suppressed forskolin-stimulated cAMP accumulation to 11.9±6.99% of the control level, although norBNI (10 μM) had no such effect (111.2±23.3%). In the cells treated with U69,593 (1 μM) for 4 h, challenge with forskolin in the presence of norBNI (10 μM) induced significant sensitization of the AC system to 177.7±23.1% of the control level. Pretreatment of the cells with pertussis toxin completely blocked both acute and sustained effects of U69,593 on cAMP accumulation (88.7±8.46% and 89.4±5.27%, respectively) (Fig. 4a). On the other hand, although CHO cells co-expressing the κ-opioid receptor and pertussis toxin-insensitive α_{i2} C352G were pretreated with pertussis toxin, acute treatment with U69,593 significantly suppressed forskolin-stimulated cAMP accumulation (68.5±5.58%) and sustained treatment led to significant sensitization of the AC system (137.4±0.30%) (Fig. 4b). In the CHO cells co-expressing the κ-opioid receptor and GQI *Nsi* I, acute treatment with U69,593 significantly suppressed forskolin-stimulated cAMP accumulation (78.2±6.06%) and sus-

Table 1. Effects of agonist treatment on GTPase activity in the membrane from CHO cells co-expressing κ-opioid receptor (κ-OPR) and wild-type, mutated or chimeric G protein α subunits

	U69,593 (10 μM)-stimulated GTPase activity (%)	
	−PTX	+PTX
κ-OPR / CHO	121.1±3.95*	98.8±2.88
κ-OPR + wild-type α_{i2} / CHO	122.7±5.80**	94.9±3.94
κ-OPR + α_{i2}C352G / CHO	133.8±5.29**	132.5±6.41*
κ-OPR + GQI *Nsi* I / CHO	122.9±6.35*	128.8±6.26*
κ-OPR + GQI *Sca* I / CHO	127.8±8.96*	125.4±6.29*
κ-OPR + GIQI / CHO	135.9±6.12**	133.2±6.43*

CHO cells were cultured in either the absence (−PTX) or presence (+PTX) of pertussis toxin (20 ng/ml for 16 h). Cell membranes were prepared and treated with U69,593 (10 μM), and GTPase activity was measured as described in Materials and Methods. The values are expressed as % of the control values obtained from membranes not treated with U69,593. n=3–5. *$P<0.05$, **$P<0.01$, compared with the untreated control values (Mann-Whitney *U*-test).

tained treatment led to significant sensitization of the AC system (122.0±3.27%) despite pretreatment with pertussis toxin (Fig. 4c). However, in the CHO cells co-expressing the κ-opioid receptor and GQI *Sca* I, both acute and

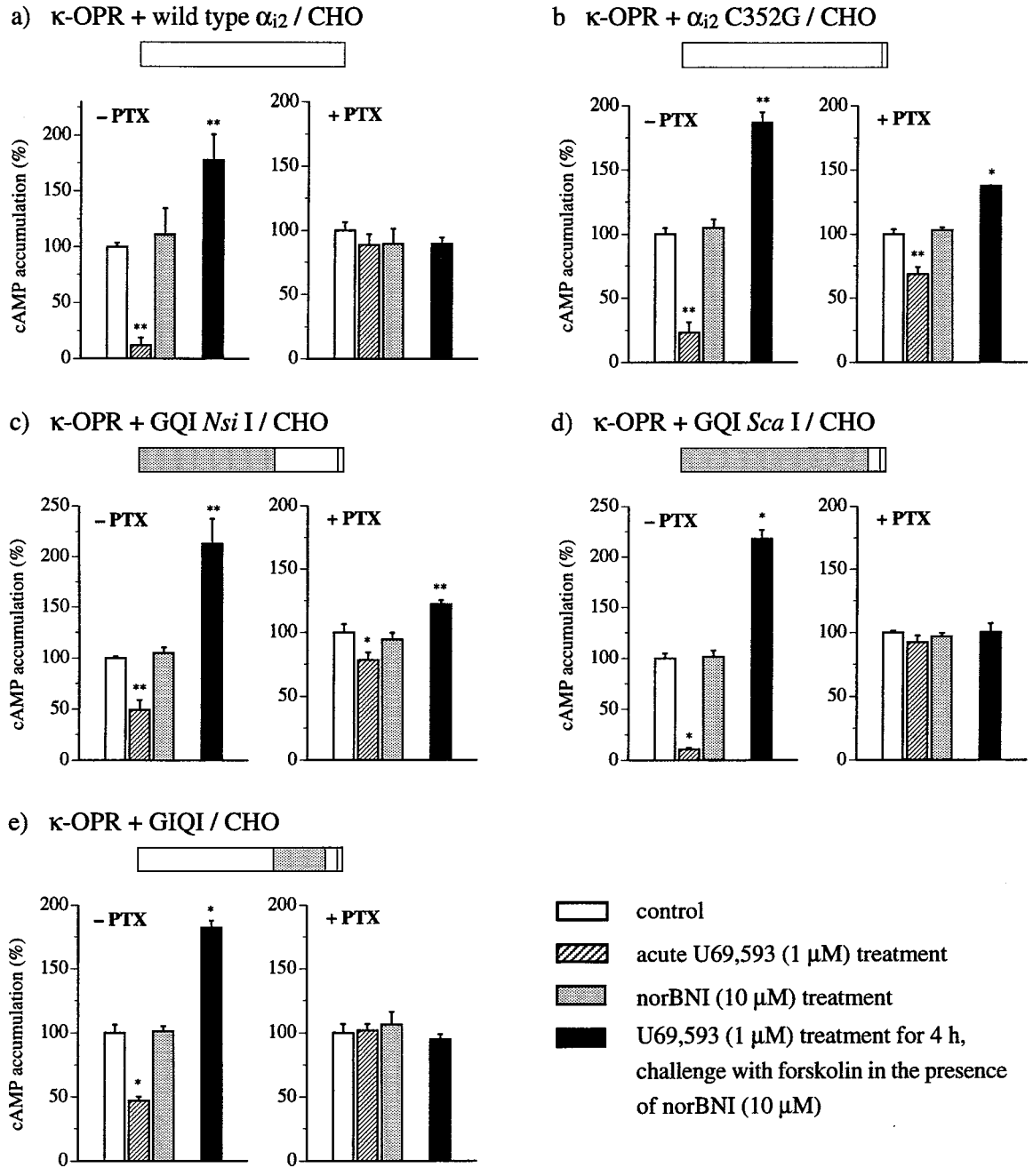

Fig. 4. Effects of acute and sustained U69,593 treatment on the AC system in CHO cells co-expressing the κ-opioid receptor and wild-type, mutated or chimeric G protein α subunits. In naive CHO cells, acute treatment with 1 μM U69,593 (10 min), but not 10 μM norBNI, suppressed forskolin (10 μM)-stimulated cAMP accumulation. In CHO cells treated for 4 h with U69,593 (1 μM), challenge with forskolin (10 μM) in the presence of norBNI (10 μM) induced cAMP overshoot to above the control level. To block the activation of endogenous G_i-like G proteins, pertussis toxin (20 ng/ml) was added to the growth medium 12 h before and was present during 4 h of sustained treatment with U69,593 in the CHO cells co-expressing κ-opioid receptor and wild-type α_{i2} (a), α_{i2} C352G (b), GQI *Nsi* I (c), GQI *Sca* I (d) or GIQI (e). In each experiment, forskolin-stimulated cAMP accumulation in naive cells with (+PTX) or without (−PTX) pertussis toxin pretreatment was assigned a value of 100%. Data are presented as means±S.E.M. of 3–6 separate experiments. *$P<0.05$, **$P<0.01$, compared with the control (Mann-Whitney *U*-test).

sustained effects of U69,593 on cAMP accumulation were completely blocked by pretreatment with pertussis toxin (92.2±5.45% and 100.2±6.95%, respectively) (Fig. 4d). Similarly, in the CHO cells co-expressing κ-opioid receptors and GIQI, pretreatment with pertussis toxin completely blocked both effects (101.8±4.88% and 95.1 ±3.79%, respectively) (Fig. 4e).

DISCUSSION

Sensitization of the AC system following sustained opioid agonist treatment has been demonstrated in several cell lines such as NG108-15 neuroblastoma × glioma hybrid cells (3, 4) and human neuroblastoma cells SH-SY5Y (5–7) and in several neuronal tissues such as the locus coeruleus, striatum, nucleus accumbens and amygdala (9, 21, 22). The recent cloning of cDNAs for opioid receptors has allowed us to study cells transfected with a single type of opioid receptor. In the present study, we used CHO cells expressing cloned κ-opioid receptor to study the molecular mechanism responsible for sensitization of the AC system (13). Acute treatment with κ-opioid agonist suppressed forskolin-stimulated cAMP accumulation, while sustained treatment with κ-opioid agonist led to development of sensitization of the AC system in CHO cells expressing the κ-opioid receptor, consistent with previous findings (13, 14). These results suggested that CHO cells are a useful model system in which to study the molecular mechanism of the development of sensitization.

In CHO cells expressing the κ-opioid receptor treated with U69,593 for 4 h, withdrawal of the agonist by a rapid washing induced significant sensitization of the AC system, indicating that the induction of this phenomenon is due to removal of the agonist. However, the sensitization by a rapid washing was significantly lower than that in the presence of norBNI. Previous studies indicated that vigorous washing for complete removal of the agonist could induce full sensitization, as well as withdrawal of the agonist by addition of an antagonist (14). In our preparation, the agonist may not have been removed completely by one wash. Furthermore, we observed that readdition of U69,593 suppressed forskolin-stimulated cAMP accumulation to a similar extent as that in naive cells, despite sustained treatment with the agonist. This result suggested that the κ-opioid receptors stably expressed in CHO cells have the capacity to continuously inhibit AC during sustained treatment with the agonist. In our preparations, we observed no desensitization of κ-opioid receptors by sustained agonist treatment, consistent with recent findings using opioid receptor-transfected cells (14, 15, 23). On the other hand, these findings are different from previous observations using cells endogenously expressing opioid receptors (24–26). Whether desensitization of opioid receptors can be observed may be due to the cell lines examined, the amount of opioid receptors expressed in the cells or the ligands used (23, 27). Moreover, our present findings and other recent ones suggested that the mechanisms of sensitization of the AC system and desensitization of opioid receptors can be dissociated from each other. Indeed, the development of sensitization was not affected by pretreatment with several protein kinase inhibitors (our unpublished data), although phosphorylation of the receptors is known to contribute to the mechanisms of desensitization.

To examine the involvement of the interaction between α_i and AC in sensitization of the AC system, we used CHO cells co-expressing the κ-opioid receptor and some chimeric G protein α subunits between α_{i2} and α_q, which are approximately 50% identical in amino acid sequence and specifically interact with their respective effectors, adenylyl cyclase and phosphoinositide phospholipase C. In the absence of pertussis toxin, the expression of recombinant α subunits did not affect the effects of acute or sustained treatment with U69,593. We postulated that the effects of U69,593 on the recombinant α subunits were masked by those on endogenously expressed α subunits of G_i-like G protein in CHO cells. To investigate the effects of recombinant α subunits on the development of sensitization, it was necessary to exclude the effects of endogenously expressed α_i. Therefore, we performed site-directed mutagenesis of the cysteine residue that acts as the acceptor for pertussis toxin-catalyzed ADP-ribosylation (28), and produced a pertussis toxin-insensitive α_i subunit (α_{i2} C352G) (29). On the other hand, previous studies have clarified the regions of G protein α subunits responsible for interactions with receptors and effectors (30). The C-terminus of the α subunit plays a central role in determining the selectivity of receptor / G protein interactions, and the proper recognition of the C-terminus of the α subunits by receptors is sufficient to trigger G protein activation (30, 31). The effector-specifying region of α_{i2} has been shown to be localized within a 78-residue segment from His^{245} to Thr^{322} to inhibit AC by the study using chimeric α subunits between α_{i2} and α_q (32). Based on these findings, we constructed some pertussis toxin-insensitive chimeric G protein α subunits and isolated several CHO cell lines co-expressing the κ-opioid receptor and wild-type, mutated or chimeric α subunits.

To investigate whether these α subunits could be functionally coupled with opioid receptors, we measured GTPase activity stimulated by U69,593 in CHO cell membranes pretreated with pertussis toxin. Pertussis toxin pretreatment of CHO cells expressing either κ-opioid receptor alone or the receptor and wild-type α_{i2} com-

pletely blocked the stimulation of GTPase activity by U69,593. In contrast, despite pertussis toxin pretreatment of CHO cells co-expressing the κ-opioid receptor and either mutated or chimeric α subunits, α_{i2} C352G, GQI *Nsi* I, GQI *Sca* I or GIQI, treatment with U69,593 significantly stimulated GTPase activity. These results suggested that all mutated or chimeric α subunits have the capacity to interact functionally with κ-opioid receptors, and treatment with agonists results in activation of these α subunits, consistent with previous findings concerning α_{2A}-adrenoceptor-G protein interactions (29). Pretreatment of the CHO cells expressing either κ-opioid receptor alone or the receptor and wild-type α_{i2} with pertussis toxin completely blocked both acute and sustained effects of U69,593. These observations suggested that sustained activation of pertussis toxin-sensitive G_i-like G proteins during sustained agonist treatment is necessary for the development of sensitization. In the CHO cells co-expressing the κ-opioid receptor and pertussis toxin-insensitive α_{i2} C352G, both acute and sustained effects were observed despite pretreatment with pertussis toxin, indicating that α_{i2} C352G can functionally interact with and inhibit AC and that the development of sensitization was mediated by α_{i2} C352G. Similar results were seen in CHO cells co-expressing the κ-opioid receptor and GQI *Nsi* I, which has the regions necessary for interaction with opioid receptors and AC. However, in the presence of pertussis toxin there is only a partial suppression of forskolin-stimulated cAMP accumulation by U69,593 and a partial sensitization of the AC system. The full effects in the absence of pertussis toxin were considered to be mediated by both the recombinant chimeric α subunits and endogenously expressed G_i-like G proteins. Since pretreatment of the cells with pertussis toxin blocked the activation of endogenous G_i-like G proteins, these partial effects were thought to be due to the activation of recombinant chimeric α subunits. On the other hand, in CHO cells co-expressing the κ-opioid receptor and either GQI *Sca* I or GIQI, which has the region required for interaction with opioid receptors but not with AC, pretreatment with pertussis toxin completely blocked both effects. These results indicated that a specific region of α_{i2}, namely Met^{244}–Asn^{331}, which was reported to be responsible for the interaction with AC (32), is necessary for the development of sensitization via the κ-opioid receptor. In other words, regardless of whether other regions of α_{i2}, namely Met^{1}–Arg^{243} and Val^{332}–Phe^{355}, are activated in a sustained manner, α_{i2} lacking the ability to inhibit AC is not able to lead to the development of sensitization. It has been shown that sensitization of the AC system is AC isozyme-specific. Acute activation of the μ-opioid receptor suppressed the activity of AC I, V, VI and VIII, stimulated that of AC II, IV and VII and did not affect that of AC III, while sustained activation of the μ-opioid receptor induced sensitization of AC I, V, VI and VIII, but not AC II, III, IV and VII, indicating that a class of AC isozymes, which are suppressed by activation of the μ-opioid receptor via α_i, are able to induce sensitization (33–36). Taken together, these findings suggested that the continuous inhibition of AC by the α_i subunit, but not continuous activation of G protein itself, plays an important role in the development of sensitization.

It has been shown that sensitization of the AC system developed by sustained treatment with agonists for G_i-coupled receptors is not due to a decrease in cAMP concentration in NG108-15 cells or S49 mouse lymphoma cells (37). In addition, pretreatment with forskolin, 3-isobutyl-1-methylxanthine or dibutyryl cAMP did not affect the development of sensitization (our unpublished data). Taken together, these findings suggested that the molecular mechanism for sensitization involves the interaction between the α_i subunit and AC, but not the downstream second messenger cAMP.

In conclusion, the present study using several CHO cell lines co-expressing the κ-opioid receptor and some chimeric G protein α subunits between α_{i2} and α_q revealed that the presence of a specific region of α_{i2} (Met^{244}–Asn^{331}), which has been reported to be responsible for the inhibition of AC, and continuous inhibition of AC by α_{i2} are necessary for the development of sensitization. Additional investigations are needed to determine the site and to identify the functional alterations essential for the development of sensitization via the κ-opioid receptor.

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