Conformation State-sensitive Antibodies to G-protein-coupled Receptors

Received for publication, September 29, 2006, and in revised form, November 24, 2006 Published, JBC Papers in Press, December 4, 2006 Published, JBC Papers in Press, December 4, 2006

A growing body of evidence indicates that G-protein-coupled receptors undergo complex conformational changes upon agonist activation. It is likely that the extracellular region, including the N terminus, undergoes activation-dependent conformational changes. We examined this by generating antibodies to regions within the N terminus of μ-opioid receptors. We find that antibodies to the midportion of the N-terminal tail exhibit enhanced recognition of activated receptors, whereas those to the distal regions do not. The enhanced recognition is abolished upon treatment with agents that block G-protein coupling or deglycosylate the receptor. This suggests that the N-terminal region of μ receptors undergoes conformational changes following receptor activation that can be selectively detected by these region-specific antibodies. We used these antibodies to characterize μ receptor type-specific ligands and find that the antibodies accurately differentiate ligands with varying efficacies. Next, we examined if these antibodies can be used to investigate the extent and duration of activation of endogenous receptors. We find that peripheral morphine administration leads to a time-dependent increase in antibody binding in the striatum and prefrontal cortex with a peak at about 30 min, indicating that these antibodies can be used to probe the spatio-temporal dynamics of native μ receptors. Finally, we show that this strategy of targeting the N-terminal region to generate receptor conformation-specific antisera can be applied to other Gαs-coupled (δ-opioid, CB1 cannabinoid, α{sub 2}{alpha}-adrenergic) as well as Gα{gamma}-coupled (AT1 angiotensin) receptors. Taken together, these studies describe antisera as tools that allow, for the first time, studies probing differential conformation states of G-protein-coupled receptors, which could be used to identify molecules of therapeutic interest.

Family A GPCRs{sup 2} play a critical role in normal cell function and are the focus of intense studies and targets for drug development. A tremendous effort has been put toward understanding the mechanism of activation of family A GPCRs at a molecular level. Spin label studies with rhodopsin have shown that exposure to light leads to a movement of helices relative to one another that is important for activation of transducin (1–4). Studies using a variety of techniques suggest that small agonists bind to a pocket formed by the surrounding transmembrane helices, and in addition peptide ligands contact additional determinants in extracellular loops and possibly the N-terminal tail (5–10). Binding of agonists, but not antagonists, leads to the stabilization of the helical bundle into a conformation, which, in turn, leads to the uncovering of molecular determinants at the bottom of the core required for G-protein binding and activation (11). Although a comprehensive mechanism for receptor activation, including the N- and C-terminal regions, is not yet available, accumulating evidence suggests that these regions undergo strong structural perturbations upon receptor activation. Studies with receptors with a large N-terminal tail, such as glycoprotein hormone receptors (12), as well as those with a smaller N-terminal tail, such as opioid and other family A receptors, have provided support for the participation of the N-terminal region in receptor activation (13–15). However, relatively little is known about agonist-mediated change in conformation of the N terminus of native receptors.

Opioid receptors belong to family A GPCRs, and their activation induces systemic responses, such as analgesia, euphoria, and decreased intestinal motility (16–18). These receptors are activated by opioid peptides as well as opiate alkaloids. The alkaloid, morphine, is widely used as an analgesic in the treatment of chronic pain; however, its long term use leads to the development of tolerance and addiction. Therefore, a major research focus has been toward the understanding of the spatio-temporal events that are critical to the development of these side effects. Understanding the mechanisms of receptor activation would allow the design of new drugs that are as effective as morphine in the treatment of chronic pain but with fewer side effects.

{sup 2} The abbreviations used are: GPCR, G-protein-coupled receptor; Ab, antibody; BSA, bovine serum albumin; DAMGO, [beta-endorphin]; Enk, enkephalin; ELISA, enzyme-linked immunosorbent assay; MAP, multiple antigenic peptide; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; GTPγS, guanosine 5’-3-O-(thio)triphosphate; GPP(NH)p, guanosine 5’-(beta,y-imido)triphosphate.
Antibodies have been useful in exploring the mechanisms of activation by delineating the domains involved in activity-mediated conformational changes in the case of a variety of signaling proteins and ligand-receptor interactions (19, 20). In the case of GPCRs, although antibodies have been generated to different regions of the receptor, the involvement of the N-terminal region in activity-dependent conformational changes has not been well explored. In a few instances, antibodies to the N terminus appear to discriminate between naive and activated receptors (21, 22). These results suggested that we should be able to target a region of the N terminus for the generation of antibodies that could detect conformational changes in this region following receptor activation. We tested this possibility by generating antibodies against a region in the N terminus of μ-opioid receptors. We find that these antibodies differentially recognize inactive and agonist-activated receptors. Next, we examined if a similar strategy could be used to generate antibodies that detect conformational changes in the N-terminal region of a variety of family A GPCRs (differing in the nature of endogenous ligand and G-protein coupling). We find that these antisera can distinguish between inactive and agonist-activated receptors.

Next, we focused on the characterization of μ antibodies because of their high clinical relevance. We show that these antibodies can be used to characterize and screen ligands by whole cell ELISA or flow cytometry. Finally, we show that these antibodies recognize native receptors and that we can quantitate the spatio-temporal dynamics of receptor activation in the brain following peripheral drug administration.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—CHO cells stably expressing FLAG-tagged mouse μ receptors were grown in F-12 medium (23). COS and SKNSH cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. COS cells were transfected with FLAG-tagged wild type μ, δ, α2A, CB1, β2, or AT1 receptors using Lipofectamine as per the manufacturer’s protocol (Invitrogen).

Generation of Antibodies—The following peptides (Fig. 1A and supplemental Fig. 1) were used to generate antisera: μ, 1SDPOLAPSCSPA25 for SA25 and 5G8 and 32GSHLCPQTGSPS64 for NT1 and 3D6; δ, 3LVPSARAELOSSPLV17 for LV17; α2A, 15GETAPGGGTRATPS29 for GS29; CB1, 27DIQYEDIKGLK12 for LK12 antibodies. These regions were chosen with the help of ExPaSy software so as to exclude regions containing predicted N-glycosylation and phosphorylation sites. The peptides were subjected to a Blast search of the mouse NCBI database to ensure that they represent unique sequences (<10% overlap). These peptides were synthesized, on a polylysine backbone, as multiple antigenic peptides (MAPs) by Research Genetics (Huntsville, AL). Antisera to μ (SA25 and NT1), δ (LV17), α2A (GS29), or CB1 (DL41) MAPs were generated in rats and to β2 (SE27) or AT1 (LK12) MAPs in rabbits using a standard protocol (24). Monoclonal Abs to μ (5G8 and 3D6) MAPs were generated in mice as described previously (25). These antibodies are highly receptor-specific, since they exhibit low cross-reactivity against other closely related receptors, as examined using a whole cell ELISA (described below) with COS cells expressing the various receptors indicated above. Specificity of the antisera was also examined using an antigen depletion assay, where a 1 mM concentration of the specific MAP or an unrelated MAP (CB1 receptor MAP was used as a nonspecific peptide for SA25, LV17, GS29, SE27, and LK12 antibodies, whereas α2A receptor MAP was used as a nonspecific peptide for DL41 antibody) was incubated with 10 μg of Abs for 24 h (4 °C) prior to incubation with cells.

Western Blot Analysis—Membranes were prepared from CHO cells or from those stably expressing μ- or δ-opioid receptors (26). Membranes (15 μg) were subjected to Western blot analysis using a 1:1000 dilution of SA25 antiserum or SA25 antiserum preabsorbed (10 mg/ml) with the specific peptide used to generate this antiserum and a 1:15,000 dilution of IR Dye 800CW anti-rat IgG. Blots were visualized using the Odyssey Imaging system (Li-Cor Biosciences, Lincoln, NE).

Effect of Ligand Treatment on Receptor Recognition by Abs—CHO cells stably expressing FLAG-tagged μ receptors, COS cells transiently expressing FLAG-tagged μ- or δ-opioid, α2A or β2-adrenergic, CB1 cannabinoid, or AT1 angiotensin receptors, or SKNSH cells (endogenously expressing these receptors) were plated on poly-l-lysine-treated 24-well plates (2 × 105 cells/well). The next day, cells were washed with phosphate-buffered saline (PBS) and incubated without or with 8–10 doses of ligands in 50 mM Tris-Cl, pH 7.5, or in isotonic HEPES buffer (10 mM HEPES containing 300 mM sucrose and 0.2 mM EDTA, pH 7.4) for 30 min at 37 °C (in the presence of a protease inhibitor mixture; Sigma). There is no significant receptor internalization under these conditions3 due to a lack of K+ and Ca2+ ions, which are required for receptor internalization. Cells were quickly rinsed three times (within 5 min) with cold PBS (washing with 20 μM antagonist in PBS produced similar results) and fixed with ice-cold methanol for 10 min at −20 °C. This treatment was included to help reduce cell loss during multiple washings as determined by protein estimation or recognition by FLAG Ab. We do not observe any significant differences in receptor recognition by SA25 antiserum or FLAG monoclonal Ab in CHO-μ cells that were subjected or not to methanol fixation (0.29 ± 0.01 without and 0.35 ± 0.04 with methanol fixation for SA25 Ab and 0.22 ± 0.01 without and 0.23 ± 0.01 with methanol fixation for FLAG Ab). ELISA was carried out by incubating cells with 3% BSA in PBS for 1 h at 37 °C, followed by overnight incubation at 4 °C with a 1:500 dilution of primary antiserum in 1% BSA in PBS. The wells were then washed three times with 1% BSA in PBS (5 min each wash) followed by a 1-h incubation at 37 °C with 1:500 dilution (in 1% BSA in PBS) of secondary antibody coupled to horseradish peroxidase. The wells were washed three times with 1% BSA in PBS (5 min each wash) and color was developed by addition of the substrate, o-phenylenediamine (5 mg/ml in 0.15 M citrate buffer, pH 5, containing 15 μM of H2O2). Absorbance at 490 nm was measured with a Bio-Rad ELISA reader. We do not see a difference in the levels of protein at the end of the assay (197 ± 8 and 197 ± 7 μg/well, respectively, for SA25 and FLAG Ab).

3 A. Gupta, I. Gomes, and L. A. Devi, unpublished observations.
Activation State-sensitive Antibodies

For screening compounds (Table 2), CHO-μ cells (1 × 10^6) were plated on 96-well Nunc-Immuno™ plates (Nalge Nunc International, Rochester, NY), air-dried at room temperature. The wells were washed with PBS, incubated without or with ligands for 30 min at 37 °C. The extent of receptor recognition by the SA25 Ab was assayed by ELISA as described above.

Flow Cytometry—Cells (3 × 10^5/well) were plated onto a 24-well plate. After 48 h, the wells were treated with or without 1 μM [D-Ala2,NMe-Phe4,Gly-ol5]enkephalin (DAMGO) in 50 mM Tris-Cl buffer, pH 7.5, or 1 μM morphine in Dulbecco’s modified Eagle’s medium for 30 min at 37 °C. The extent of cell surface receptor recognition was determined using receptor-specific antiserum and Alexa 488-conjugated goat anti-mouse/anti-rat IgG (1:400 in 50% fetal bovine serum in PBS for 2 h at 4 °C) as described previously (27) using a FACScan flow cytometer (BD Biosciences). Cells remaining in the tubes following fluorescence-activated cell sorting analysis were routinely examined under the microscope to check for cell lysis.

Effect of Peptide:N-glycanase on Antibody Recognition—CHO cells stably expressing μ-opioid receptors (2 × 10^5) plated on 24-well plates were incubated with or without 1 μM DAMGO in the absence or presence of peptide:N-glycanase F (40 units) for 3 h at 37 °C in 50 mM Tris-Cl buffer, pH 7.5. Cells were quickly washed with ice-cold PBS and fixed with ice-cold methanol for 10 min at −20 °C. The extent of antibody recognition was monitored by ELISA using either SA25, 5G8, NT1, or 3D6 antibodies as described earlier.

Studies with Endogenous Receptors—Membranes (1 μg/well) from C57BL/6 mice brain cortex were coated overnight on a high binding 96-well ELISA plate (Fisher). The next day, cells were washed with PBS and incubated without or with 8–10 doses of ligands in 50 mM Tris-Cl, pH 7.5. Membranes were quickly rinsed three times (within 5 min) with cold PBS; washing with 20 μM antagonist in PBS produced similar results. Wells were treated with ice-cold methanol for 10 min at −20 °C, and the extent of receptor recognition by Abs was assayed by ELISA as described above. Specificity of the antisera in endogenous tissue was determined using the antigen-depleted antisera; these gave a signal that was 10–20% that of sera undepleted or treated with unrelated peptide (supplemental Table 4). To examine the effect of modulators of G-protein activity, cortical membranes (10 μg) were pretreated with a 100 μM concentration of either GTPγS, GPP(NH)p, AlF3, or NaF for 30 min at 37 °C in the presence of a protease inhibitor mixture (Sigma). This was followed by a 30-min treatment with 1 μM DAMGO, and the extent of Ab recognition was assayed by ELISA as described above. The pertussis toxin (50 ng/ml) pretreatment was carried out overnight at 37 °C; the protease inhibitor mixture was used to protect membrane proteins, including receptors, from degradation. The effect of membrane incubation at 37 °C on receptor integrity was measured by ligand binding in the presence of protease inhibitor mixture using [3H]diprenorphine as described (23). The receptor binding following overnight incubation was 97.2 ± 4.8% of that observed following 30 min of incubation at 37 °C, indicating that under these conditions, no significant loss of receptor binding was observed.

In Vivo Receptor Activation Studies on Brain Sections—Four-month-old male C57BL/6 mice (3–5/group) or μ−/− animals and age-matched sex-matched littermate controls (3–5/group) were injected intraperitoneally with either 10 mg/kg morphine, 10 mg/kg morphine plus 10 mg/kg naloxone, or saline and sacrificed 30 min later or at the indicated times. Brains were dissected and frozen at −80 °C until use. The brains were embedded in M-1 embedding matrix (Thermo Electron Corp., Waltham, MA), and 10-μm serial sections were cut using a MICROM HM 560 CryoStar (Richard-Allan Scientific, Kalamazoo, MI). Areas representing the prefrontal cortex (3.0-2.58 mm; Bregma), striatum (1.5-1 mm; Bregma), or cerebellum (−5.6 to −6.5 mm; Bregma) were collected; at least 10 sections from each animal were probed. Two sections from each region were placed on Fisher Superfrost Plus slides and circled immediately with an ImmEdge PAP pen (Vector Laboratories, Burlingame, CA) to form a waterproof barrier; the resulting wells held ~200 μl. ELISA was performed in these wells using a 1:500 dilution of primary antiserum and 1:500 dilution of secondary antibodies. The reaction product was transferred to a 96-well plate, and absorbance at 490 nm was measured with a Bio-Rad ELISA reader.

RESULTS

Detection of Agonist-induced Conformational Changes in the N Terminus of μ-Opioid Receptors—Studies with rhodopsin indicate that the N-terminal region undergoes conformational changes following receptor activation. This suggests that antibodies directed against this region of family A GPCRs should be able to distinguish between inactive and activated receptors. In order to see if a similar strategy could be applied to μ-opioid receptors, we targeted the N-terminal domain and generated antibodies to a region proximal (SA25 antibodies) and a region distal to the N terminus (NT1 antibodies) (Fig. 1). These antibodies were characterized in cell lines stably (CHO-μ) or transiently (COS-μ) expressing recombinant μ receptors or expressing native receptors (SKNSH). In all cell lines, these antibodies specifically recognize μ receptors. There was no significant recognition in cells expressing either δ-opioid, CB1 cannabinoid, δ2/δ3, or β2-adrenergic, or AT1 angiotensin receptors (Table 1). The specificity of SA25 antiserum was confirmed by Western blotting; a specific signal was observed with membranes from CHO cells expressing μ receptors and not from CHO cells alone or from CHO cells expressing δ receptors. Furthermore, when Western blotting was carried out with SA25 antiserum preabsorbed with the specific peptide used to generate the antiserum there was no signal (Fig. 1B), supporting the specificity of the antiserum. This was further supported by studies with membranes from animals lacking μ receptors (supplemental Tables 1 and 2). We examined if these antibodies were able to differentially recognize activated receptors by treating cells with the μ receptor-specific agonist, DAMGO. We find that this leads to a dose-dependent increase in receptor recognition by SA25 but not NT1 antibodies (Fig. 1C). Similar results were obtained upon treatment with morphine (not shown). The increase in recognition by SA25 antibodies does not occur upon treatment with the antagonist, naloxone, or unrelated peptide (Fig. 2). This increase in recognition follow-
Activation State-sensitive Antibodies

FIGURE 1. Agonist-induced conformational changes in the N terminus of \( \mu \)-opioid receptors. A, schematic representation of \( \mu \)-opioid receptors. The FLAG epitope (black ellipse), putative N-linked glycosylation site (branch), and amino acid sequences used for the generation of multiple antigenic peptides (black lines) are indicated. B, Western blot analysis of membranes prepared from CHO cells alone or CHO cells stably expressing \( \mu \)-opioid receptors (CHO-\( \mu \) receptors) with SA25 antiserum preabsorbed without or with the peptide used to generate the antiserum (blocking peptide, 10 mg/ml). C, CHO cells stably expressing \( \mu \)-opioid receptors were incubated without (shaded) or with (open) DAMGO or morphine, stained with SA25 or NT1 antibodies followed by Alexa-488-labeled secondary antibodies, and analyzed by flow cytometry. Data are shown from a representative experiment of three.

TABLE 1

Specificity of \( \mu \)-opioid receptor antibodies

COS-7 cells were untransfected or transfected with \( \mu \)- or \( \delta \)-opioid, CB1 cannabinoid, \( \alpha_{2A} \)- or \( \beta_{2} \)-adrenergic, or AT1 angiotensin receptor cDNA and probed with polyclonal antibodies generated to peptides against a region proximal (SA25) or distal to the N terminus (NT1) of \( \mu \)-opioid receptors (Fig. 1) by ELISA as described under “Experimental Procedures.” Data from vehicle-treated cells were taken as 100%. Treatment of cells with indicated doses of agonists or antagonists and probed with SA25 or NT1 antibodies by ELISA as described under “Experimental Procedures.” Data from vehicle-treated cells were taken as 100%. Percentage of Ab binding was calculated as 100% (OD value obtained with cells expressing SA25 or NT1 antibodies followed by Alexa-488-labeled secondary antibodies, and analyzed by flow cytometry. Data are shown from a representative experiment of three.

| Cells expressing receptors | \( \mu \)-Opiao | \( \delta \)-Opiao | CB1 cannabinoid | \( \alpha_{2A} \)-Adrenergic | \( \beta_{2} \)-Adrenergic | AT1 angiotensin |
|----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| SA25                      | 100 ± 1.0       | 5.1 ± 4.1       | 7.1 ± 1.0       | 4.1 ± 1.0       | 5.1 ± 4.2       | 3.1 ± 2.0       |
| NT1                       | 100 ± 2.0       | 2.0 ± 2.0       | 0.0 ± 2.0       | 4.0 ± 2.0       | 6.0 ± 2.0       | 6.0 ± 2.0       |
In order to further explore this, we carried out dose-response curves and compared the Ab binding properties with the signaling properties. We find a positive correlation between the $E_{\text{max}}$ for Ab binding and that for GTP$\gamma$S binding (Table 2). These results suggest that our conformation-sensitive Abs can be used to develop rapid and highly sensitive screening assays for the identification of novel small molecule ligands of therapeutic interest.

Detection of Native $\mu$ Receptors in the Brain—Next, we examined the extent of recognition of the native receptors using membranes from different brain regions and probing them with SA25 antibodies. The relative levels of Ab binding to various brain regions were measured using a standard curve (generated with cells expressing known levels of $\mu$ receptors). Results in Fig. 4 show that there is a good match between the level of antibody binding and the reported level of these receptors; binding is highest in the cortex and lowest in the cerebellum. These results suggest that these antibodies can be used to probe changes in conformation in the N-terminal region following activation of endogenous receptors.

$Ab$ Recognition of Native Receptors in the Brain following Drug Administration—Next we used these antibodies to study the spatio-temporal dynamics of native $\mu$ receptors following drug administration. For this, drugs were peripherally administered, and the change in antibody recognition was monitored after 30 min. We find a significant increase in recognition by SA25 antibodies in the brain (striatum) 30 min after peripheral morphine administration (Fig. 5A). This increase could be blocked by co-administration of naloxone with morphine (Fig. 5A). The effect of morphine is selective for $\mu$ receptors, since morphine treatment does not lead to significant changes in the recognition of $\delta$ receptors by LV17 antibody (Fig. 5A). In order to ensure the specificity of receptor recognition, we used mice lacking $\mu$ receptors ($\mu^{-/-}$) and found that there is a significantly decreased recognition that is not affected by morphine administration (Fig. 5B). Under these conditions, the recognition of $\delta$ receptors by $\delta$ receptor-specific antibodies is not altered (Fig. 5B). Thus, our data show that changes in conformation of native receptors following drug administration can be monitored by N-terminal antibodies.

In order to study the time course of drug-induced change in receptor conformation, we collected brains at various times following peripheral administration of morphine and evaluated receptor-specific antibodies as described under “Experimental Procedures.” Angiotensin II was used as a nonselective peptide ligand. Data from untreated “control” were taken as 100%. Statistically significant differences from control are indicated. **, $p < 0.001$ ($n = 3$). The OD values with CHO cells not expressing the receptor were <0.005 with or without ligands. The values with CHO-$\mu$ cells were 0.4 ± 0.02 for SGB, 0.38 ± 0.03 for 3D6, 0.42 ± 0.01 for SA25, and 0.28 ± 0.01 for NT1. The values with SKNSH cells were 0.62 ± 0.02 for SGB, 0.19 ± 0.01 for 3D6, 0.3 ± 0.02 for NT1, and 0.40 ± 0.03 for SA25 antibodies.

Next, we characterized the increased antibody binding by examining the effect of deglycosylation on antibody recognition. We found that treatment with peptide-N-glycanase F abolished the increased recognition following receptor activation by both the SA25 polyclonal and SGB monoclonal antibodies (Fig. 3B). This, taken with the fact that peptide-N-glycanase treatment does not affect receptor recognition in the basal state, suggests that receptor glycosylation plays a role in the conformational switch that occurs at the N terminus upon receptor activation. Taken together, these results support the idea that these antibodies recognize changes in conformation at the N terminus following receptor activation.

Screening of Ligands Using Conformation-sensitive Antibodies—Next, we examined if these antibodies can be used as a tool to screen and characterize specific ligands. For this, we set up a simple and rapid screening assay in a 96-well format and probed a series of $\mu$ ligands differing in their chemical and pharmacological properties; these included clinically relevant drugs, endogenous peptides, synthetic peptidic and nonpeptidic agonists, and antagonists (Table 2). We found a correlation between the extent of Ab receptor recognition and the reported efficacies of these ligands; morphine, a partial agonist with lower efficacy, exhibits a lower $E_{\text{max}}$ compared with DAMGO, a highly potent full agonist that exhibits a higher $E_{\text{max}}$ (Table 2).
maximal at 30 min and returned to basal levels by 60 min (Fig. 5C). In contrast, recognition by the NT1 antibody or the DL41 antibody (that recognizes CB1 receptors) was not altered at any time point following morphine administration. Cerebellar slices did not show significant recognition by the SA25 antiserum, and morphine administration had no effect at any of the times tested (not shown). These results are consistent with the notion that morphine administered intraperitoneally reaches the brain and activates the receptor within 30 min; the receptor returns to the basal state of activity by 60 min. This correlates well with the reported time course of behavioral effects of peripherally administered morphine.

**Detection of Agonist-induced Conformational Changes in the N Terminus of Family A GPCRs—**We next examined if the strategy of targeting the N terminus can be used to generate similar conformation-sensitive antibodies to other GPCR family members. We chose receptors that couple to different G-proteins and are activated by different types of ligands (i.e. 

**Activation State-sensitive Antibodies**

GPCRs undergo conformational changes following receptor activation. Although the involvement of the transmembrane regions in conformational changes following the binding of the agonist to the receptor has been extensively documented (1–10), not much is known about the involvement of the N-terminal region. Antibodies have been useful tools in exploring the domains involved in activity-mediated conformational changes of signaling proteins, including receptors (19, 20, 29–36). For example, a monoclonal antibody to the N-terminal region of rhodopsin exhibited a higher degree of recognition for bleached (activated) receptors than for unbleached (inactive) receptors even after detergent treatment. This indicates that photoactivation of rhodopsin induces a conformational change at the N terminus that exposes an epitope that is recognized by the monoclonal antibody (22). In this study, we generated antibodies to distinct regions of the N terminus of µ-opioid receptors to show that a region within the N terminus undergoes significant local movement following receptor activation. We find that this could be a general mechanism shared by other members of family A, since we observe that irrespective of the nature of the ligand or G-protein selectivity, receptor activation leads to the exposure of the epitope recognized by antibodies targeted to the midportion of the N terminus.

The role of the N-terminal region in ligand binding and receptor activation has been mainly explored in receptors with long N-terminal regions. For example, for glycoprotein hormone receptors, the long N-terminal tail constitutes the primary high affinity and selective binding site for receptor agonists (12). In the case of receptors of family C, the very large extracellular N terminus is organized into a domain called the Venus flytrap module that contains the ligand binding pocket
Activation State-sensitive Antibodies

TABLE 2
Screening of μ ligands using anti-μ (SA25) antibody
CHO cells expressing μ receptors were treated with 8–10 doses of ligands and probed with μ receptor antibody (SA25) as described under “Experimental Procedures.” Data from untreated cells were taken as 100%. The agonist-mediated increase in [35S]GTPγS binding was measured under the same conditions of antibody binding as described under “Experimental Procedures.” Basal values obtained in the absence of agonist were taken as 100%. Results represent mean ± S.E. (n = 3–5). Statistically significant differences are indicated. ND, not determined. DALDA, H-Tyr-D-Arg-Phe-Lys-NH2; CTAP, D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH2; CTOP, D-Phe-c[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH2.

| μ ligands      | EC50 (nM) | Emax (% control) | GTPγS binding12 | Emax (% control) |
|---------------|-----------|------------------|------------------|------------------|
|               | m         |                  |                  |                  |
| Endo-1        | 2.35 × 10^-8 | 230 ± 11*       | ND               | ND               |
| Endo-2        | 8.00 × 10^-10| 196 ± 25*       | ND               | ND               |
| DAMGO         | 1.49 × 10^-9 | 161 ± 8.2*      | 13 × 10^-9       | 176 ± 2.1*       |
| Methadone     | 2.00 × 10^-9 | 167 ± 2.1*      | 42 × 10^-9       | 169 ± 2.2*       |
| Binalphine    | 6.85 × 10^-8 | 165 ± 5*        | ND               | ND               |
| Fentanyl      | 1.64 × 10^-8 | 158 ± 2*        | 19 × 10^-9       | 160 ± 6.8*       |
| Super DALDA   | 3.06 × 10^-9 | 153 ± 1*        | ND               | ND               |
| Met-enk       | 1.72 × 10^-9 | 151 ± 1*        | ND               | ND               |
| Meperidine    | 9.50 × 10^-9 | 149 ± 3*        | ND               | ND               |
| β-Endorphin   | 7.93 × 10^-9 | 145 ± 1*        | ND               | ND               |
| Morphine      | 3.45 × 10^-8 | 141 ± 3*        | 52 × 10^-9       | 149 ± 4.6*       |
| Naltrexone    | >10^-6      | 120 ± 2         | ND               | ND               |
| CTOP          | >10^-6      | 111 ± 6         | ND               | ND               |
| CTAP          | >10^-6      | 108 ± 4         | ND               | ND               |
| Naloxone      | >10^-6      | 107 ± 9         | ND               | ND               |

* p < 0.0001 (n = 3–5).
* p < 0.01 (n = 3–5).
* p < 0.001 (n = 3–5).

(37, 38). Interestingly, the smaller N-terminal tail of family A GPCRs, such as opioid receptors as well as other peptide or amine receptors, has also been proposed to participate in receptor activation (39). In the case of δ-opioid receptors, a random mutagenesis study identified 5 amino acids in the N-terminal region that enhanced the spontaneous activity of the receptor (15). In this study, each mutation substantially modified the chemical nature of the amino acid side chain, suggesting that the N terminus of δ receptors is folded as a domain whose structure and spatial orientation affects receptor function (15). This is also suggested by the structure of rhodopsin, in which the N terminus is folded as a β-sheet over the helical bundle covering it like a lid (40). Although a comprehensive molecular mechanism for receptor activation that includes the extracellular loops and the N- and C-terminal tails is not yet available, the accumulating evidence to date is consistent with the notion that these regions of the receptor undergo substantial structural perturbations upon activation.

An observation in this study has been that increased recognition of the receptor by antibodies targeted to the midportion of the N-terminal region of μ-opioid receptors remains for a
prolonged period (ever after the removal of the agonist). In
addition, the formation of the ternary complex does not appear
to be sufficient, since the recognition persists for a prolonged
period of time (well beyond the formation of the ternary com-
plex). It can be postulated that the antibody recognizes the acti-
vation-mediated post-translationally modified receptor. It
should be pointed out that our assays (in whole cells and mem-
branes) were carried out under conditions that do not allow
receptor internalization (and dephosphorylation/resensitiza-
tion). Hence, the changes in receptor conformation would per-
sist for a long time, as seen in our studies with cells, membranes,
and animals. Taken together, these results are consistent with
the notion that the SA25 antibody recognizes changes (in the N
terminus) that are induced by the activation-mediated long
lasting changes to the receptor. We are currently exploring
these possibilities.

Although a number of studies have investigated the activa-
tion state of heterogeneously expressed opioid receptors, a
major research focus has been to identify brain regions and
molecular partners playing critical roles in the development of
side effects, such as tolerance to and physical dependence on
opiates. However, no definite model has emerged that could be
used to design a new category of drugs as powerful as morphine
but with less abuse potential. This is partly due to difficulties in
distinguishing the brain regions targeted by a drug, which
depend on the route of administration, dose of the drug, and its
bioavailability. For example, morphine has been shown to
induce the activation of mitogen-activated protein kinase in a
set of cells quite distinct from those that express μ receptors
(41). Studies have also shown that the pharmacological proper-
ties of a ligand in vitro can be different from those seen in vivo.
For example, [d-Pen₂,d-Pen⁵]enkephalin and deltorphin II, two
highly μ-selective peptide agonists, are thought to induce anal-
gesia through μ receptors (42). The lack of suitable reagents,
thus far, has not allowed the direct evaluation of the extent
of activation of receptors of interest. Attempts have been made
to probe this using indirect markers (mitogen-activated protein
kinase and GTPγS) or receptor knock-out mice. Apart from the
radiolabeled GTPγS binding performed on slices (43), few tech-
niques are available that allow the investigation of the spatio-
temporal dynamics of receptor activation. This emphasizes the
dire need for reliable tools for identification of brain regions
where GPCRs are activated at the cellular level. Our antibodies
represent a useful and direct approach compared with other
time-consuming and labor-intensive techniques. This approach
seems to be applicable to many family A GPCRs and opens the
way to examine the localization of active receptors as well as the
extent of modulation of receptor activity by cross-talk between
receptors.

The results of this study not only demonstrate the structural
mobility exhibited by the small N-terminal region of distinct
types of family A GPCRs but also provide a new and powerful
technique to examine the duration and extent of activation of
endogenous receptors as well as to screen for drugs that are
allosteric modulators of family A GPCRs, which would be of
potential therapeutic value.

Acknowledgments—We thank Drs. Raphael Rozenfeld for critical
reading of the manuscript and Toni Shippenberg for providing the
brains from μ −/− mice and littermate controls, Phil Portoghese for
biphalin, and Peter Schiller for Super DALDA (H-Tyr-d-Arg-Phe-Lys-NH₂).

FIGURE 6. Dose-response curves for recognition of native μ, δ, CB1, α₂A,
β₂, and AT1 receptors. SKNSH cells (A) or membranes (1 μg) from mouse
cortices (B) were treated with the indicated doses of each ligand and probed
with antisera raised against μ- or δ-opioid, CB1 cannabinoid, α₂A- or β₂-adre-
nergic, and AT1 angiotensin receptors by ELISA as described under “Experi-
mental Procedures.” Data from untreated cells were taken as 100%. Results are
the mean ± S.E. (n = 4–5). CTOP, d-Phe-c[Cys-Tyr-d-Trp-Orn-Thr-Pen]-Thr-NH₂.
REFERENCES

1. Altenbach, C., Yang, K., Farrens, D. L., Farahbakhsh, Z. T., Khorana, H. G., and Hubbell, W. L. (1996) Biochemistry 35, 12470–12478
2. Altenbach, C., Klein-Seetharaman, J., Hwa, J., Khorana, H. G., and Hubbell, W. L. (1999) Biochemistry 38, 7945–7949
3. Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770
4. Yang, K., Farrens, D. L., Altenbach, C., Farahbakhsh, Z. T., Hubbell, W. L., and Khorana, H. G. (1996) Biochemistry 35, 14040–14046
5. Fadhil, I., Schmidt, R., Walpole, C., and Carpenter, K. A. (2004) J. Biol. Chem. 279, 21069–21077
6. Lu, Z. L., Saldanha, J. W., and Hulme, E. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12622–12627
7. Xu, W., Li, J., Chen, C., Huang, P., Weinstein, H., Javitch, J. A., Shi, L., de Zoolog. Sci. 19, 971–977
8. Decaillot, F. M., Befort, K., Filliol, D., Yue, S., Walker, P., and Kieffer, B. L. (2003) J. Biol. Chem. 277, 30429–30435
9. Dhawan, B. N., Cesselin, F., Raghubir, R., Reisine, T., Bradley, P. B., Portoghese, P. S., and Hamon, M. (1996) J. Biol. Chem. 271, 545–552
10. Archer, E., Maigret, B., Escrieut, C., Pradayrol, L., and Fourmy, D. (2003) Trends Biochem. Sci. 28, 192–198
11. Gether, U. (2000) Trends Pharmacol. Sci. 21, 119–126
12. Vignal, E., Blangy, A., Martin, M., Gauthier-Rouviere, C., and Fort, P. (2001) Mol. Cell Biol. 21, 8022–8034
13. Cha, K., Reeves P. J., and Khorana, H. G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3016–3021
14. Vignal, E., Blangy, A., Martin, M., Gauthier-Rouviere, C., and Fort, P. (2001) Mol. Cell Biol. 21, 8022–8034
15. Gomes, I., Gupta, A., Filipovska, J., Szeto, H. H., Pintar, J. E., and Devi, L. A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 5135–5139
16. Fujisawa, Y., Furukawa, Y., Ohta, S., Ellis, T. A., Dembrow, N. C., Li, L., Floyd, P. D., Sweedler, J. V., Minakata, H., Nakamaru, K., Mori, F., Matsushima, O., Weiss, K. R., and Vilim, F. S. (1999) J. Neurosci. 19, 9618–9634
17. Takao, M., Iwasa, T., Yamamoto, H., Takeuchi, T., and Tokunaga, F. (2002) Zoolog. Sci. 19, 651–659
18. Takao, M., Iwasa, T., Yamamoto, H., Takeuchi, T., and Tokunaga, F. (2002) Zoolog. Sci. 19, 651–659
19. Schmidt, R., Walpole, C., and Carpenter, K. A. (2004) J. Biol. Chem. 279, 21069–21077
20. Schmidt, R., Walpole, C., and Carpenter, K. A. (2004) J. Biol. Chem. 279, 21069–21077
21. Khorana, H. G. (1996) Science 274, 768–770
22. Vignal, E., Blangy, A., Martin, M., Gauthier-Rouviere, C., and Fort, P. (2001) Mol. Cell Biol. 21, 8022–8034
23. Takao, M., Iwasa, T., Yamamoto, H., Takeuchi, T., and Tokunaga, F. (2002) Zoolog. Sci. 19, 651–659
24. Fujisawa, Y., Furukawa, Y., Ohta, S., Ellis, T. A., Dembrow, N. C., Li, L., Floyd, P. D., Sweedler, J. V., Minakata, H., Nakamaru, K., Mori, F., Matsushima, O., Weiss, K. R., and Vilim, F. S. (1999) J. Neurosci. 19, 9618–9634
25. Gomes, I., Gupta, A., Singh, S. P., and Sharma, S. K. (1999) FEBS Lett. 456, 126–130
26. Gomes, I., Filipovska, J., Jordan, B. A., and Devi L. A. (2003) Methods 27, 358–365
27. Trapaidze, N., Gomes, I., Bansinath, M., and Devi, L. A. (2000) DNA Cell Biol. 19, 195–204
28. Gouarderes, C., Sutak, M., Zajac, J. M., and Jhamandas, K. (2000) Eur. J. Pharmacol. 406, 391–401
29. Lu, J., Chunjieh, A., Sanderson, S. D., Scholz, W., Buchner, Y., Ye, R. D., and Hugli, T. E. (1999) J. Immunol. 163, 1782–1788
30. Morgan, E. L., Ember, J. A., Sanderson, S. D., Scholz, W., Buchner, Y., Ye, R. D., and Hugli, T. E. (1999) J. Immunol. 163, 377–388
31. Garzon, J., Castro, M. A., Juarros, J. L., and Sanchez-Blazquez, P. (1994) Life Sci. 54, PL191–PL196
32. Bozon, V., Di Scala, E., Eftekhar, P., Hoebeke, J., Lezoualc’h, F., Fischmeister, R., and Argibay, J. (2002) Receptors Channels 8, 113–121
33. Peter, J. C., Eftekhar, P., Billiard, P., Wallukat, G., and Hoebeke, J. (2003) J. Biol. Chem. 278, 36740–36747
34. Kunishima, N., Shimada, Y., Tsuji, Y., Sato, T., Yamamoto, M., Kumasaka, T., Nakanishi, S., Jingami, H., and Morikawa, K. (2000) Nature 407, 971–977
35. Silve, C., Petrel, C., Leroy, C., Bruel, H., Mallet, E., Rognans, D., and Ruat, M. (2005) J. Biol. Chem. 280, 37917–37923
36. Meng, F., Hoversten, M. T., Thompson, R. C., Taylor, L., Watson, S. D., and Aki, H. (1995) J. Biol. Chem. 270, 12730–12736
37. Palczewski, K., Kumasaka, T., Hori, T., Behne, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
38. Eitan, S., Bryant, C. D., Saliminejad, N., Yang, Y. C., Vojdani, E., Keith, D., Akil, H. (1995) J. Biol. Chem. 270, 12730–12736
39. Palczewski, K., Kumasaka, T., Hori, T., Behne, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
40. Eitan, S., Bryant, C. D., Saliminejad, N., Yang, Y. C., Vojdani, E., Keith, D., Akil, H. (1995) J. Biol. Chem. 270, 12730–12736
41. Palczewski, K., Kumasaka, T., Hori, T., Behne, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Science 289, 739–745
42. Eitan, S., Bryant, C. D., Saliminejad, N., Yang, Y. C., Vojdani, E., Keith, D., Jr., Polakiewicz, R., and Evans, C. J. (2003) J. Neurosci. 23, 8360–8369
43. Scherrer, G., Befort, K., Contet, C., Becker, J., Matias, A., and Kieffer, B. L. (2004) Eur. J. Neurosci. 19, 2239–2248
44. Wang, D., Raehal, K. M., Lin, E. T., Lowery, J.-J., Kieffer, B. L., Bulsky, E. J., and Sadee, W. (2004) J. Pharmacol. Exp. Ther. 308, 512–520