The Third Intracellular Loop Stabilizes the Inactive State of the Neuropeptide Y1 Receptor*

Melissa J. S. Chee‡§,* Karin Mörl‡, Diana Lindner‡, Nicole Merten‡, Gerald W. Zamponi¶, Peter E. Light§, Annette G. Buck-Schickinger‡, and William F. Colmers‡§†

From the ‡Department of Pharmacology and §Centre for Neuroscience, University of Alberta, Edmonton, Alberta T6G 2H7, Canada, the ¶Institute of Biochemistry, Leipzig University, Brüderstr. 34, 04103 Leipzig, Germany, and the †Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Constitutively active G-protein-coupled receptors (GPCRs) can signal even in the absence of ligand binding. Most Class I GPCRs are stabilized in the resting conformation by intramolecular interactions involving transmembrane domain (TM) 3 and TM6, particularly at loci 6.30 and 6.34 of TM6. Signaling by G\textsubscript{i}/G\textsubscript{o}-coupled receptors such as the Neuropeptide Y1 receptor decreases already low basal metabolite levels. Thus, we examined constitutive activity using a biochemical assay mediated by a G\textsubscript{i}/G\textsubscript{o} chimeric protein and a more direct electrophysiological assay. Wild-type (WT-Y1) receptors express no measurable, agonist-independent activation, while \(\mu\)-opioid receptors (MOR) and P2Y\textsubscript{12} purinoceptors showed clear evidence of constitutive activation, especially in the electrophysiological assay. Neither point mutations at TM6 (T\textsubscript{6.30}A or N\textsubscript{6.34}A) nor substitution of the entire TM3 and TM6 regions from the MOR into the Y1 receptor increased basal WT-Y1 activation. By contrast, chimeric substitution of the third intracellular loop (ICL3) generated a constitutively active, Y1-ICL3-MOR chimera. Furthermore, the loss of stabilizing interactions from the native ICL3 enhanced the role of surrounding residues to permit basal receptor activation; because constitutive activity of the Y1-ICL3-MOR chimera was further increased by point mutation at locus 6.34, which did not alter WT-Y1 receptor activity. Our results indicate that the ICL3 stabilizes the Y1 receptor in the inactive state and confers structural properties critical for regulating Y receptor activation and signal transduction. These studies reveal the active participation of the ICL3 in the stabilization and activation of Class I GPCRs.

Neuropeptide Y (NPY)\textsuperscript{2} is one of the most abundant peptides in the nervous system. NPY and its receptors have been impli-

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* The work was supported by the Canadian Institutes of Health Research (CIHR) Grant MT10520, the German Research Foundation (DFG SFB610, TP A1), and the Saxonian Ministry for Science and Culture (SMWK, Saxonia/Alberta-collaboration 4-7531.50-02-0361-05/1). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: 9-36 Medical Sciences Bldg., Dept. of Pharmacology, University of Alberta, Edmonton, AB T6G 2H7, Canada. Fax: 780-492-4325; E-mail: william.colmers@ualberta.ca.

2 The abbreviations used are: NPY, neuropeptide Y; Y, NPY receptor; GPCR, G-protein-coupled receptor; TM, transmembrane; IP, inositol phosphate; P, pre-pulse facilitation; BPF, basal PF; ICL, intracellular loop; MOR, \(\mu\)-opioid receptor; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; h, human; P2Y, purinergic receptor; DMEM, Dulbecco’s modified Eagle medium; p, porcine; Fmoc/Bu, 9-fluorenylmethoxycarbonyl-tert-butyloxycarbonyl-lysine; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; WT, wild-type; TRITC, tetramethylrhodamine isothiocyanate; VDCC, voltage-dependent calcium channel; ECFP, enhanced cyan fluorescent protein; \(\Delta\)barium current; P, test pulse; \(\Delta\)V\text{rest}, holding potential; PP, pre-pulse; \(\Delta\)barium current produced by test pulse; DAMGO, [d-Ala\textsubscript{2},N-MePhe\textsubscript{4},Gly-ol\textsubscript{2}]enkephalin; GDP-\(\beta\)-S, guanosine 5’-(\(\beta\)-thio)di phosphate; \(\Delta\)V\text{rest}/P, ratio of current produced by P2 versus P1.
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was it susceptible to constitutive activation by single point mutations at position 6.30 (T6.30A) or 6.34 (N6.34A). By chimeric substitution of functional regions, such as a complete TM or intracellular loop (ICL), into the Y1 receptor from analogous regions in the constitutively active μ-opioid receptor (MOR), we identified the structural region that stabilizes the Y1 receptor. The loss of the region that imparts the most relevant stabilizing interactions would result in the generation of a constitutively active chimeric Y1 receptor. Our findings indicate that the Y1 receptor is stabilized in the inactive state by the ICL3 region and suggest the relative importance of this atypical region for regulating the activation of other Y receptors and class I GPCRs.

EXPERIMENTAL PROCEDURES

Generation of Y1 Receptors

The subcloning of the human Y1 receptor cDNA into the eukaryotic expression plasmid pEGFP-N1 (Clontech, Heidelberg, Germany) has been described previously (17). The C-terminal fusion to the enhanced green fluorescent protein (EGFP) or yellow fluorescent protein (EYFP) allowed for fluorescence detection of the heterologously expressed receptor and does not alter the function of Y receptors (17). The human (h) Y1 receptor EYFP fusion gene was additionally subcloned into the eukaryotic expression plasmid pVitro2-mcs(hygro) (Invivogen, Toulouse, France) via a BamHI and an AvrII site. Mutations were introduced with the QuikChange™ site-directed mutagenesis method (Stratagene). The entire coding region was sequenced to ensure the accuracy of all constructs. In the Y1-ICL3-MOR construct, the nucleotides coding for amino acids 232–264 of the hY1 receptor were replaced by nucleotides coding for amino acids 145–168 and 274–305 of the hY1 receptor.

Y1-TM3-TM6-MOR chimera, nucleotides coding for amino acids 116–139 and 255–286 of the hY1 receptor were replaced by nucleotides coding for amino acids 6.34K

TABLE 1

Agonist binding and signal transduction for the WT-Y1 receptor and Y1 chimera

| Specific binding % relative to WT-Y1 | Ligand potency EC50 nm |
|--------------------------------------|------------------------|
| WT-Y1 100                          | 1.97 ± 0.18            |
| T6.30A-Y1 102 ± 5                  | 1.49 ± 0.12            |
| N6.34A-Y1 78 ± 5                   | 1.69 ± 0.21            |
| Y1-ICL3-MOR 75 ± 9                 | 5.4 ± 0.34             |
| Y1-TM3-TM6-MOR 20 ± 3              | N/A*                  |
| Y1-ICL3(T6.34K)-MOR 50 ± 5         | N/A*                  |

* Capacity for agonist binding was retained, but receptor could not be activated.

Inositol Phosphate Accumulation Assay

COS-7 Cell Transfection—African green monkey kidney COS-7 cells were maintained in standard Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and 0.01% penicillin-streptomycin. Cells were grown as monolayers at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. All cell culture agents were obtained from Invitrogen.

The phospholipase Cβ pathway signal, IP accumulation, was used to determine the activation of Gαi5/Go13-GPCRs by co-transfection of the chimeric G-protein Gα5δq4myr (kindly provided by Dr. E. Kostenis, 7TM Pharma A/S, Hoersholm, Denmark). A Gαi5/Go13-GPCR can couple to the Gαi5 pathway via this chimeric protein, which has a Gα5 backbone with an altered C terminus comprised of residues from Gαi3 and an N-terminal myristoylation consensus site (12, 13). COS-7 cells were seeded into 24-well plates (1.0 × 105 cells/well) and transiently transfected with cDNA plasmids to be tested (0.20 μg), plus the Gα5δq4myr (0.05 μg) using 0.75 μl of metofectene (Biontex, München, Germany). As a positive control, cells were transiently co-transfected with the Gi5i3i6m-co-protein-coupled P2Y12 receptor (0.20 μg) and Gα5δq4myr (0.05 μg). As a negative control, cells were transiently co-transfected with the cDNA plasmid encoding EGFP (0.20 μg) and Gα5δq4myr (0.05 μg).

Determination of IP Accumulation—IP formation was measured 2 days after transfection. Cells were incubated with 2 μCi/ml of myo-[3H]-inositol (25.0 Ci/mmol; Amersham Biosciences, Freiburg, Germany) 16 h prior to measurement. The cells were washed once and stimulated in DMEM containing 10 mM LiCl. Cells were incubated for 1, 3, or 6 h at 37 °C in the absence, or in differing concentrations (1 nM to 10 μM) of an agonist, either pNPY (synthesized by automated solid phase peptide synthesis with the 9-fluorenlymethoxycarbonyl-tert-buty (Fmoc/tBu) strategy (19); or 10 μM ADP (Sigma-Aldrich), as appropriate. The reactions were stopped by the aspiration of DMEM and cell lysis with 300 μl of 0.1 M NaOH. After adding 100 μl of 0.2 M formic acid, intracellular IP levels were determined by anion-exchange chromatography as described (20, 21). Ligand potency of generated chimeric GPCRs was determined (Table 1).

Radioligand Binding Studies—Competitive radioligand binding studies were performed with N-[propionyl-[3H]pNPY (GE Healthcare Europe GmbH, Braunschweig, Germany) in the absence (total binding) and presence (nonspecific binding) of 1 μM unlabeled NPY as described previously (22; Table 1).

Receptor Localization and Quantification

To estimate plasma membrane localization, a cell surface, enzyme-linked immunosorbent assay (ELISA; 23) was performed with the WT-Y1 receptor and its mutants carrying an N-terminal hemagglutinin (HA) tag. The N-terminal nine amino acid residue epitope (YPYDVPDYA) was derived from
the influenza virus HA protein and inserted downstream of the start codon. The entire coding region was sequenced to ensure the accuracy of all constructs. COS-7 cells were seeded into 48-well plates (35,000 cells/well) and transfected (0.20 μg/H9262 plasmid DNA and 0.60 μl of metafectene/well). Three days after transfection, the cells were fixed with formaldehyde without disrupting the cell membrane and probed with a biotin-labeled anti-HA antibody (1 μg of 12CA5 with biotin/ml; Sigma-Aldrich). Bound anti-HA antibody was detected by a peroxidase-labeled streptavidin conjugate (0.29 μg/ml; Sigma-Aldrich). Following removal of excess unbound conjugate, H2O2 and o-phenylenediamine (2.5 mM each in 0.1 M phosphate-citrate buffer; pH 5) were added to serve as substrate and chromogen, respectively. After 10–30 min, the enzyme reaction was stopped by addition of 1 M HCl containing 50 mM Na2SO3. Color development was measured bichromatically at 492 nm and 620 nm using an ELISA reader (Sunrise, Tecan).

Immunofluorescence studies were carried out to determine the subcellular distribution of P2Y12, MOR, WT-Y1, T6.30A-Y1 (data not shown), N6.34A-Y1 (data not shown), Y1-ICL3-MOR, Y1-TM3-TM6-MOR, and Y1-ICL3(T6.34K)-MOR receptors (Fig. 1). COS-7 cells were transferred into 24-well plates that contained sterilized glass cover slips and then transfected. After 48 h, the cells were fixed with formaldehyde. They were treated with an anti-HA monoclonal antibody (10 μg 12CA5/ml; Sigma-Aldrich) and then incubated with a tetramethylrhodamine isothiocyanate (TRITC)-linked anti-mouse IgG secondary antibody (Sigma-Aldrich). The nuclei were visualized with Hoechst 33342 (1 μg/ml; Sigma-Aldrich). Fluorescence images were obtained with a confocal laser-scanning microscope (Leica TCS SP2; Heidelberg, Germany).

**Electrophysiological Assay: Prepulse Facilitation**

**tsA-201 Cell Transfection—Human embryonic kidney-derived tsA-201 cells were maintained in standard DMEM supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin-streptomycin. Cells were grown and maintained as monolayers at 37 °C in a humidified atmosphere of 5% CO2 and 95% O2. All cell culture agents were obtained from Invitrogen.**

When the tsA-201 cells reached 90% confluence, they were detached with trypsin-EDTA (0.25% trypsin with EDTA 4 mM Na) and plated onto glass coverslips at 50% confluence 7 h prior to transfection. Using a standard calcium phosphate precipita-
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...tion protocol (24), the plated cells were always transiently transfected with cDNA constructs encoding for the N-type voltage-dependent calcium channel (VDCC) subunits (α1A, α2δ, and β1δ; 3:2:2 ratio with a total of 7.0 μg/ml of DMEM). In most cases, proteins of interest were intrinsically tagged with a fluorescent protein that does not affect their functions; otherwise, a plasmid encoding EGFP (0.05 μg/ml) was additionally co-expressed. Where indicated, N-type VDCC subunits were co-expressed with the (1.0 μg/ml each) WT-Y1-EGFP, T6.34K-A-Y1-EGFP, N634A-Y1-EGFP, WT-MOR, T6.34K-MOR-EYFP, Y1-ICL3-MOR-EYFP, Y1-ICL3(T6.34K)-MOR-EYFP, or Y1-TM3-TM6-MOR-EYFP receptor construct. Where indicated, the chimeric Gα protein (Gαqα7-ECFP; 0.6 μg/ml; kindly provided by Dr. N. Gautam, Washington University, St. Louis, MO) was also transiently co-expressed in the tsa-201 cells as scavengers of Gβγ. The cDNA constructs were first added to a transfection mixture containing (in mM): 640 NaCl, 25 HEPES, 20 CaCl2, 120 NaCl, 0.75 Na2HPO4, H2O before being added to the cells. Following a 16-h incubation at 37 °C in the calcium phosphate/cDNA mixture, the cells were washed with fresh DMEM and allowed to recover at 37 °C for 6 h. The cells were then moved to a 30 °C incubator to prevent further cell division.

Patch Clamp Recordings—A coverslip with the transfected cells attached was placed in a recording chamber, attached to a fixed stage upright microscope (Axioskop FS-2, Carl Zeiss) that was continuously perfused with external recording solution composed of (in mM): 108 Cs-methanesulfonate, 4 MgCl2, 9 EGTA, 100 HEPES, 108 CsCl (adjusted to pH 7.20 with TEA-OH; 256–260 mOsm/liter). The microscope was equipped with epifluorescence optics (excitation 450–490 nm; emission 515 nm; Chroma, Burlington, VT), and only those cells exhibiting fluorescence were considered for patching. Patch pipettes (borosilicate glass Sutter Instruments, Novato, CA) were pulled using a Sutter P-97 microelectrode puller, then fire-polished with a Micro-Forge 200 (World Precision Instruments, Sarasota, FL) to yield tip resistances of 5–7 MΩ when back-filled with internal solution composed of (in mM): 108 Cs-methanesulfonate, 4 MgCl2, 9 EGTA, 9 HEPES, 1 Na-GTP (adjusted to pH 7.20 with CsOH; 238–243 mOsm/liter). All electrophysiological recordings were conducted at room temperature (22 °C). Patch pipettes were connected to the headstage of either an Axopatch 200B or Axopatch 1D amplifier (Axon Instruments, Foster City, CA) used in voltage-clamp mode and linked via a Digidata 1322 interface (Axon) to a computer equipped with Clampex 8.2 software (Axon). The whole cell currents were filtered at 1 kHz (-3 dB) and the cell membrane capacitance and series resistance were manually compensated. Current traces were digitized at 10 kHz and analyzed offline. An online leak-subtraction protocol was used, where 8 pulses of opposite polarity and 1/8th amplitude were acquired immediately prior to the test protocol.

The barium current (Ib) elicited by each test pulse (P1 or P2) consisted of a 105 mV, 14 ms depolarizing voltage step from a holding potential (Vh) of −90 mV. In the prepulse (PP) protocol, a 15-ms depolarizing voltage step from Vh to +105 mV (a 195-mV depolarization) was applied immediately following the first test pulse (P1); the second test pulse (P2) was then preceded by a brief (4 ms) interpulse interval at the holding potent-

tial (24). In the absence of the prepulse, the two test pulses were separated from each other by 19 ms. The parameters of the voltage protocols used here: Vh; amplitude and duration of P1, P2, and PP; and duration of the interpulse interval, were optimized to maximize the effect of prepulse facilitation. Only cells showing peak inward Iba amplitudes between 150 pA and 2000 pA in response to P1 (Ib1) were included in the data set.

Drug Applications—NPY (Peptide Technologies, Pierrefonds, QC, Canada) and [d-Ala2,N-MePhe4,Gly-ol5]enkephalin (DAMGO; Bachem, Torrance, CA) were diluted into the external recording solution from concentrated stock solutions to their final concentrations immediately prior to use. Stock solutions of 10 mM ADP, 100 μM NPY, and 100 μM DAMGO was prepared in HPLC grade water (Fisher Scientific) and stored at −20 °C. During drug application, 1 mM ADP, 50 nM NPY, or 1 μM DAMGO was perfused into the recording chamber for 3–5 min. Following drug application, the cells were washed out with bath perfusion solution for at least 8 min. In experiments involving guanosine 5’-β-thio)diphosphate (GDP-β-S; Sigma-Aldrich), it was added directly to the internal pipette solution to a final concentration of 1 mM immediately prior to use.

Data Analysis

Current traces were analyzed using Clampfit 9.2 (Axon Instruments). Sample traces were reproduced using Axum 5.0 (MathSoft, Miami Beach, FL). Statistics and graphs were produced using GraphPad Prism v 4.03 (GraphPad Software, San Diego, CA). Statistical significance for mean comparisons involving only 2 groups and 3 or more groups was determined using the unpaired Student’s t test and Tukey’s Multiple Comparison Test, respectively, unless indicated otherwise. Statistical significance for single means was compared with 0 with a one-sample Student’s t test. Statistical significance for slope comparisons between lines of best-fit was determined using a comparison of Linear Regression. The differences were considered to be significant at p < 0.05. Results were presented as means ± S.E. where appropriate.

RESULTS

WT-Y1 Receptor Is Not Constitutively Active—Basal activation of the WT-Y1 receptor was determined in the absence of agonist, with a biochemical assay measuring IP accumulation and an electrophysiological assay measuring basal prepulse facilitation (BPF). Activation of the Gαq-subunit (12) coupled the activation of all Gαq-protein-coupled receptors tested to the normally Gαq-mediated stimulation of the phospholipase Cβ pathway and subsequent accumulation of IP.

In this functional biochemical assay, basal activity of the highly constitutively active P2Y12 receptor (25, 26) was readily detected, but not that of the native MOR (27). There was a significant increase in intracellular IP accumulation following the co-expression of the constitutively active P2Y12 receptor but not of the demonstrably constitutively active MOR relative to the EGFP control (Fig. 2). The IP assay is therefore not sensitive to subtle levels of GPCR constitutive activity. Further-
more, this assay did not reveal significant basal receptor activation by the WT-Y1 receptor (Fig. 2).

The electrophysiological assay measuring basal prepulse facilitation (BPF) can detect constitutive activity of G\text{q}/G\text{o}-coupled receptors expressed in individual cells (14–16). Whole cell I_{Ba} was elicited using a double-pulse protocol, with or without an intervening depolarizing prepulse (PP) and results were reported as the ratio (I_{P2/P1}) of the peak current elicited by the second test pulse (P2) relative to that of the first (P1; Fig. 3A). BPF refers to the percent increase in I_{P2/P1} elicited by the PP in the absence of an agonist (Fig. 3A).

Constitutive activity of both the P2Y_{12} receptor and MOR were revealed by the electrophysiological BPF assay. Co-expression of either the P2Y_{12} receptor or the MOR resulted in a significant increase in BPF relative to control cells in which no GPCRs were co-expressed with the N-type VDCCs. In contrast, co-expression of the WT-Y1 receptor did not cause a significant increase in BPF over control, indicating that it is quiescent in the absence of an agonist (Fig. 3A).

Even in the absence of any co-expressed GPCRs, a significant level of BPF was observed (Fig. 4A). We tested the hypothesis that the BPF produced in the absence of any co-expressed GPCRs was due to the actions of endogenous G\beta\gamma, expressed in excess of the G\text{o}-subunit within the cell. A scavenger of G\beta\gamma, the chimeric Go subunit G\alpha_q\alpha_o, that is activated by a G\alpha_q-protein-coupled GPCR but transduces via the G\alpha_o pathway (28), was introduced to the cell. Co-expression of G\alpha_q\alpha_o completely abolished the BPF observed in the absence of any co-expressed GPCRs, as well as that observed in the presence of the WT-Y1 or even the known constitutively active MOR (Fig. 4A).

To test the possibility that the endogenous expression of excess G\beta\gamma could impede the detection of possible constitutive WT-Y1 receptor activation, we examined the effects of GDP-\beta-S, an inhibitor of G-protein activation, on BPF. We hypothesized that GDP-\beta-S will determine whether or not the BPF observed by the WT-Y1 receptor was caused by receptor-mediated turnover of G\beta\gamma. In all our experiments, we observed a gradual decline of BPF with time that was independent of GPCR expression (Fig. 4B). When 1 mM GDP-\beta-S was applied intracellularly via the pipette to inhibit basal GPCR-mediated activation of BPF, it did not affect the rate of BPF decline in cells expressing the WT-Y1 receptor, or in cells that do not co-express any GPCRs (Fig. 4B). However, GDP-\beta-S accelerated the rate of BPF decline in MOR-expressing cells (Fig. 4B).
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**Table 2**

Inhibition of barium currents at the first test pulse ($I_{\text{pf}}$) and increase in PF following agonist-mediated receptor activation

| Agonist        | Receptor                | $n$ | $I_{\text{pf}}$ inhibition | $p$ value | PF increase | $p$ value |
|----------------|-------------------------|-----|----------------------------|-----------|-------------|-----------|
| 50 nM NPY      | WT-Y1                   | 21  | 73.8 ± 3.6                 | ***       | 94.7 ± 11.6 | *        |
|                | T6.30A-Y1               | 7   | 84.5 ± 4.1                 | ***       | 188.4 ± 65.0 | *b       |
|                | N6.34A-Y1               | 12  | 82.1 ± 2.5                 | ***       | 197.5 ± 56.0 | ***      |
|                | Y1-TM3-TM6-MOR          | 4   | 7.8 ± 9.0                  | ns             | N/A       | N/A       |
|                | Y1-ICL3-MOR             | 17  | 60.6 ± 2.8                 | ***       | 26.7 ± 9.5 | ns        |
|                | Y1-ICL3(T6.34K)-MOR     | 9   | 62.4 ± 1.3                 | ***       | 11.6 ± 6.9 | ns        |
| 1 μM DAMGO     | WT-MOR                  | 4   | 61.2 ± 4.2                 | ***       | 34.4 ± 5.7  | ***      |
|                | T6.34K-MOR              | 4   | 36.0 ± 6.2                 | **        | −13.3 ± 9.8 | ns        |

$a=p<0.0001$.

$^b$ $p<0.05$.

$^c$ not significant; one-sample t test.

$^d$ $p<0.001$.

produced by MOR-expressing cells was significantly reduced after a 6 min GDP-β-S application (Fig. 4C). The sensitivity of BPF to inhibition by GDP-β-S was observed in cells expressing the highly constitutively active P2Y12 receptor as well (Fig. 4C). This is consistent with the hypothesis that the basal activity of the MOR and P2Y12 receptors resulted in an accelerated rate of GDP-β-S binding to Goα-subunits, thus reducing the Gβγ subunits available for binding to the N-type VDCCs. In contrast, intracellular GDP-β-S did not decrease BPF produced by cells expressing the WT-Y1 receptor (Fig. 4C). This suggested that the BPF observed in WT-Y1 receptor-expressing cells was not attributed to basal WT-Y1 receptor activation.

**WT-Y1 Receptor Is Resistant to Constitutive Activation by Single Point Mutation at Loci 6.30 and 6.34**—Constitutive GPCR activation is governed by a loss of intramolecular interactions that constrain the receptor in an inactivated state (6, 7, 29). The involvement of the cytoplasmic end of TM6, specifically at locus 6.30 and 6.34, has been implicated in such restraining intramolecular interactions (30). A single point mutation at locus 6.30 or 6.34 was introduced to the WT-Y1 receptor to test the hypothesis that the quiescence of the WT-Y1 receptor is regulated at these specific loci. The mutant T6.30A-Y1 and N6.34A-Y1 receptors were normally trafficked and expressed at the plasma membrane, as revealed by the ELISA cell surface imaging assay (data not shown), and these mutant receptors retained agonist binding capacity similar to that of the WT-Y1 receptor (Table 1).

Agonist-mediated activation of both mutant Y1 receptors resulted in the robust inhibition of $I_{\text{pf}}$ and produced a significant increase in BPF over the WT-Y1 receptor (Fig. 5B). Furthermore, the Y1-TM3-TM6-MOR chimerae did not display agonist sensitivity (Table 2) though it was able to bind NPY (Table 1). This indicated that the Y1-TM3-TM6-MOR chimeric receptor does not express constitutive activity and suggested that exchange of the TM3 and TM6 regions from the WT-Y1 receptor interfered with agonist-mediated receptor activation and signal transduction despite retaining the capacity for agonist binding.

**ICL3 Domain Governs WT-Y1 Receptor Activation**—As seen above, the WT-Y1 receptor was not susceptible to constitutive activation by single point mutation at specific loci on TM6. The loci 6.30 and 6.34 on the WT-Y1 receptor lie on the interface between TM6 and the ICL3 regions of the receptor. To test the hypothesis that the ICL3 region contributes residues that stabilize the inactive state of the WT-Y1 receptor (31, 32), the ICL3 region of the WT-Y1 receptor was replaced with that from the MOR. The resulting Y1-ICL3-MOR chimera is a functional receptor that responded to 50 nm NPY agonist application. In the absence of the agonist, the Y1-ICL3-MOR chimera also demonstrated a robust BPF response. The BPF observed with expression of the Y1-ICL3-MOR chimera was significantly greater than that of the WT-Y1 receptor (Fig. 6). Furthermore, following a 5-min intracellular application of 1 mM GDP-β-S, the BPF produced in cells expressing the Y1-ICL3-MOR chimeric receptor was significantly reduced by 87.0 ± 14.9% ($n = 6$; $p < 0.005$, paired $t$ test). This suggested that the BPF observed in cells expressing the chimeric Y1-ICL3-MOR receptor was in part attributable to basal activation of the receptor. Thus, exchange of the ICL3 region of the WT-Y1 receptor with that of the MOR resulted in the generation of a mainly Y1 receptor that is constitutively active.

We then tested the hypothesis that following the ICL3 substitution, the constitutive activity of the WT-Y1 receptor is susceptible to further modulation. In the MOR, basal activity was increased following a single Thr (T) to Lys (K) point mutation at locus 6.34 of the WT to result in the T6.34K-MOR receptor mutant (33). We examined the constitutive activity of the Y1-ICL3-MOR chimera following an additional T6.34K point mutation at the junction between the TM6 and ICL3 domain. If the hypothesis is correct, then a comparison of the increase in constitutive activity between the Y1-ICL3-MOR and Y1-ICL3(T6.34K)-MOR chimerae should be similar to that
between the WT-MOR and T6.34K-MOR. Both the T6.34K-MOR and Y1-ICL3(T6.34K)-MOR responded to agonist application (Table 2). The Y1-ICL3(T6.34K)-MOR chimeric mutant produced a similar inhibition of IP₁ as the Y1-ICL3-MOR, but the T6.34K-MOR mutant was less responsive to the agonist than the WT-MOR.

The T6.34K point mutation produced a similar increase in the BPF produced by the WT-MOR (74.9 ± 26.0%; n = 15; p < 0.01) and the Y1-ICL3-MOR (77.7 ± 24.3%; n = 17; p < 0.01; Fig. 7). Despite the robust elevation in constitutive activity observed in the BPF assay for both mutant and chimeric receptor containing the T6.34K point mutation, the IP assay revealed evidence of constitutive activity for the T6.34K-MOR (IP accumulation: 3.27 ± 0.29-fold over WT-MOR; n = 30; p < 0.05) only. Our results showed that following the exchange of the native Y1 ICL3 region with that from the WT-MOR, the Y1 receptor demonstrated a similar susceptibility to structural changes that also increased basal receptor activation in the WT-MOR.

**DISCUSSION**

Detection of constitutive activity in G_i/G_o-protein-coupled GPCRs is more complex than with GPCRs coupling to G-proteins such as Gα or Gq that naturally stimulate the production of readily examined second messengers. In the biochemical assay here, the coexpression of a chimeric Gqα₆δα₄q₅γ₅γ₅ protein converted the inhibitory G_i/G_o-signaling pathway into a stimulatory Gq signal that was identified by the accumulation of IP (12, 25, 34, 35). The IP assay was insensitive to low levels of GPCR activation but responded with a wider dynamic range; thus may be particularly useful to study GPCRs that express strong constitutive activity. While a potentially sensitive biochemical method of measuring activity in potassium channels activated by G_i/G_o-coupled GPCRs has been described recently (36), its suitability and sensitivity to constitutive activity is unknown. In contrast, the electrophysiological detection of BPF can track even subtle changes in constitutive activity over a short period of time, including the gradual loss of GPCR-mediated Gβγ turnover in the presence of GDP-β-S in an individual cell. The sensitivity of the BPF assay can be largely attributed to the presence of an internal control, because the expression of BPF produced by the activated and inactivated GPCR state was evaluated during one continuous current recording.

The WT-Y1 receptor differs from many class I GPCRs in that it expresses neither any detectable constitutive activity nor sensitivity to simple structural changes that enhance constitutive

46.0 ± 8.0%; Presence of Gα₆δα₄qγ₅γ₅EGFP: −4.9 ± 1.7%; WT-Y1: −2.3 ± 6.6%; MOR: −10.0 ± 3.6%). *** p < 0.0001. B, comparison of GPCR-mediated BPF decline following an intracellular application of 1 mM GDP-β-S (dashed line, open circle). In the absence of GDP-β-S (solid line, solid circle), a gradual decline in BPF (slope) was observed in all conditions of GPCR expression (EGFP: 2.4 ± 0.7% min⁻¹; thin black; MOR: 3.4 ± 1.7% min⁻¹, bold black; WT-Y1: 3.2 ± 1.4% min⁻¹, bold gray). GDP-β-S accelerated the gradual decline of BPF over time only in MOR-expressing cells (10.5 ± 2.2% min⁻¹; p < 0.05; EGFP: 2.8 ± 0.5% min⁻¹; WT-Y1: 1.6 ± 1.1% min⁻¹). Slopes of best-fit lines were generated using linear regression. C, comparison of BPF produced at 1 min (black; EGFP: 18.3 ± 6.8%; MOR: 49.5 ± 16.8%; P2Y₁₂: 32.6 ± 11.0%; WT-Y1: 28.2 ± 6.0%) and 6 min (gray; EGFP: 17.0 ± 6.9%; MOR: 17.6 ± 14.6%; P2Y₁₂: 5.5 ± 2.5%; WT-Y1: 18.5 ± 10.8%) following the application of intracellular GDP-β-S.+ ** p < 0.05; * p < 0.001 (paired t test).
activity in other similar receptors. By substituting a complete TM or ICL3 region from the MOR into the Y1 receptor, we identified which functional region is most important for the activation of the Y1 receptor. Though the activation of most class I GPCRs involves the TM3 and TM6 regions (37), they do not appear to be critical for stabilizing the inactive state of the Y1 receptor. Replacement of the ICL3 but not the TM3 and TM6 region of the WT-Y1 receptor generated a constitutively active, chimeric Y1 receptor. This suggests that the ICL3 region of the Y1 receptor contributes significant intramolecular interactions that stabilize the Y1 receptor in the inactive state. Interestingly, following the loss of constraining interactions from the WT-

**FIGURE 5.** 
T<sup>6.30A</sup>-Y1 and N<sup>6.34A</sup>-Y1 receptors do not express constitutive activity. A, neither the T<sup>6.30A</sup>-Y1 (27.6 ± 7.2%) nor N<sup>6.34A</sup>-Y1 (27.8 ± 6.4%) receptor demonstrated a significant increase in BPF relative to the WT-Y1 receptor (25.4 ± 5.4%). B, substitution of TM3 and TM6 from the MOR into the WT-Y1 receptor resulted in the generation of the Y1-TM3-TM6-MOR chimera, which did not produce a significant increase in BPF (EGFP: 19.2 ± 2.6%; MOR: 47.1 ± 3.6%; WT-Y1: 23.4 ± 4.8%; Y1-TM3-TM6-MOR: 41.8 ± 4.5%). *, p < 0.05; **, p < 0.01.

**FIGURE 6.** Increased basal Y1 receptor activation following mutagenesis at ICL3. Substitution of ICL3 from the Y1 receptor with that from the MOR produced a constitutively active Y1-ICL3-MOR chimeric receptor. BPF produced by the Y1 chimera (41.8 ± 4.5%) was greater than that produced by the positive EGFP control (19.2 ± 2.6%) or WT-Y1 receptor (23.6 ± 3.1%) and was similar to that produced by the MOR (41.1 ± 4.0%). *, p < 0.05.

**FIGURE 7.** T<sup>6.34K</sup> point mutation increased the basal activity of constitutively active MOR and Y1 receptor chimera. The T<sup>6.34K</sup> point mutation within the ICL3 region (gray; T<sup>6.34K</sup>-MOR: 95.0 ± 14.2%; Y1-ICL3(T<sup>6.34K</sup>)-MOR: 78.8 ± 9.2%) further increased the BPF produced by both the WT-MOR (52.4 ± 8.2%) and Y1-ICL3-MOR receptor (41.8 ± 4.5%). *, p < 0.05.

ICL3, the residues at the cytoplasmic end of TM6 appear to become more important for stabilizing the inactive conformation of the Y1 receptor.

**Contributions of TM6 to WT-Y1 Receptor Activation**—Others have indicated that the Y receptor family displays little or no basal activation (38). The class I GPCRs, which include the Y
receptors, is stabilized in the inactive state by an ionic lock (5, 8, 39, 40). The ionic lock comprises a network of hydrogen bonds and charge interactions between the conserved (E/D)RY motif on TM3 and specific residues at locus 6.30 and 6.34 along TM6 (5). Constitutively active mutants can be generated in several receptors by point mutation(s) at locus 6.30 and/or 6.34. However, the applicability of this mutagenesis depends on the presence of a conserved D/E\textsuperscript{6.30} residue. Although D/E\textsuperscript{6.30} is nearly 100% conserved among rhodopsin and monoamine GPCRs (10), it is not universally conserved among all class I GPCRs (i.e. MOR: 33, 41; gonadotrophin releasing hormone receptor (GnRHR): 42; angiotensin I receptor (AT\textsubscript{1}R): 43, 44), and it is not present in the Y1 receptor (45, 46).

Two different phenotypes of GPCR activation can be identified based on the presence or absence of D/E\textsuperscript{6.30} in the native GPCR. When D/E\textsuperscript{6.30} is present in the wild-type GPCR, a constitutively active mutant receptor could be generated by point mutation at either position 6.30 or 6.34 (α\textsubscript{1h}-AR: 6, 9; B\textsubscript{2}-AR: 10, 11; 5-HT\textsubscript{2A} receptor 47, 48; luteinizing-stimulating hormone receptor (LSHR): 49, 50; thyrotropin-stimulating hormone receptor (TSHR): 51–53). This corresponded with the rhodopsin model, which suggested that both residues at loci 6.30 and 6.34 are necessary to regulate GPCR activation (10, 47). The second phenotype arose in the absence of D/E\textsuperscript{6.30} in the wild-type receptor. Constitutive activity was observed only following site-directed mutagenesis at locus 6.34 (33 but not 6.30 (41). This suggested that only one constraining intramolecular interaction existed at TM6 for GPCRs lacking a D/E\textsuperscript{6.30} residue (41).

Our studies on the Y1 receptor provide evidence of a third phenotype for GPCR activation where neither point mutation at locus 6.30 nor 6.34 can elicit constitutive, agonist-independent GPCR activation in the absence of a native D/E\textsuperscript{6.30}. A similar insensitivity to constitutive activity was also observed in GnRHR (42) and AT\textsubscript{1}R (43, 44). In contrast to the distinctive role of TM3 and TM6 for receptor stabilization in many Class I GPCRs, our studies revealed that neither of these TM regions contains residues that play a significant role in constitutive activation of the WT-Y1 receptor.

Role of ICL3 in Stabilizing the WT-Y1 Receptor—As with the α\textsubscript{1}-adrenergic receptor (α\textsubscript{1}-AR; 31, 32), the ICL3 of the Y1 receptor confers properties important for activation and signal transduction. GPCR activation becomes a physiologically relevant event when it affects an intracellular signal. Several residues within the ICL3 (54–58) and at the TM6/ICL3 junction (59, 60) are essential for the formation of a G-protein-binding pocket (61–63). Because the ICL3 mediates G-protein recognition and activation (61), structural changes involving the ICL3 can mimic conformational changes of agonist-mediated GPCR activation even in the agonist-unbound state. It is interesting to note that, despite the change in basal activity conferred upon the Y1 receptor with a chimeric ICL3, the ability to activate G\textsubscript{i0}/G\textsubscript{s}-proteins appeared unimpaired. This implies that the functional aspects of the region are at least partly independent of the effect of basal activity. Although identification of those specific ICL3 residues that have the most significant impact on Y1 receptor activation can be addressed by single point mutations along the ICL3, it is beyond the scope of the present study.

The ICL3 substitution also caused structural changes to nearby regions of the Y1 receptor. Point mutation at locus 6.34 of the quiescent WT-Y1 receptor had no effect on its basal activity. By contrast, the basal activity of the Y1-ICL3-MOR chimera was substantially increased by the T\textsuperscript{6.34}K point mutation at this previously disengaged locus. This indicates that the structural change in the Y1-ICL3-MOR chimera unmasked the previously unseen participation of residues along TM6 in stabilizing the inactive conformation of the Y1 receptor.

The NPY system appears to be relatively quiescent during basal physiological conditions in vivo. Thus, there is little or no effect of germine knock-out of NPY or its receptors on basal physiological responses (64). Similarly, there are only modest effects in vivo following the administration of NPY antagonists (e.g. 65, 66), except under circumstances where the NPY system is strongly activated (e.g. 67). Activation of G\textsubscript{i0}/G\textsubscript{s}-coupled GPCRs tends to inhibit neuronal activity via a number of mechanisms (68), and signaling via G\textsubscript{i0}/G\textsubscript{s}-coupled GPCRs appears to interact with that of G\textsubscript{i} or G\textsubscript{q11}-coupled receptors (69–71). Given this, there may be significant adaptive advantages to agonist/receptor systems where there is no signaling in the absence of any agonist.

CONCLUSION

Mutant GPCRs that demonstrate the activation of their G-proteins even in the absence of agonist-binding have advanced the understanding of the molecular mechanisms involved in GPCR activation. Constitutively active mutant GPCRs are useful tools for identifying specific regions or residues that comprise the structural machinery conferring GPCR activation. Our experiments identified the relative quiescence of the WT-Y1 receptor, which is resistant to constitutive activation by disturbing specific intramolecular interactions along TM6. Instead, we observed that the ICL3 region plays an important role for its constitutive activation, which is highly unusual relative to most class I GPCRs.

Acknowledgments—We thank Dr. W. F. Dryden for his comments in revising the manuscript. We thank Dr. T. P. Snutch for providing calcium channel cDNAs, Dr. E. Kostenis for the gift of the G\textsubscript{a}\textsubscript{Gqi1}myr, and Dr. M. Statnick for the gift of the P2Y\textsubscript{12} construct, and Dr. B. El Bahh and C. Dammann for providing technical assistance.

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