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Ligand Specific Up-Regulation of a *Renilla reniformis* Luciferase-Tagged, Structurally Unstable Muscarinic M_3 Chimeric G Protein-Coupled Receptor

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ABSTRACT

The rat muscarinic acetylcholine receptor subtype 3 was modified by swapping the third intracellular loop with the corresponding region of a constitutively active mutant human β_2-adrenergic receptor and attaching *Renilla reniformis* luciferase to its C terminus. The chimeric fusion receptor displayed constitutive G_q- and G_s-coupled activity as demonstrated in nuclear factor of activated T cell and cAMP response element reporter gene assays. The chimeric receptor displayed a pharmacological binding profile comparable with that of the wild-type receptor for agonists, antagonists, and inverse agonists but showed a large decrease in expression in both human embryonic kidney 293 and COS-7 cells. Long-term treatment of cells expressing the chimeric receptor with agonists, antagonists, and inverse agonists resulted in a concentration-dependent up-regulation in the steady-state levels that was not observed for the wild-type receptor. The EC_{50} of neutral antagonists and inverse agonists was significantly correlated to their binding affinities at the wild-type receptor, whereas agonists demonstrated greater EC_{50} values for the chimeric receptor. To validate the approach as a means of discovering novel receptor modulators, a cell-based, high-throughput screening assay was developed and used to screen a small molecule compound collection against the chimeric fusion receptor. Several novel hits were identified and confirmed by ligand binding assay and functional assays using the wild-type rat muscarinic acetylcholine receptor subtype 3.

The rat muscarinic acetylcholine receptor subtype 3 (M_3) belongs to the seven transmembrane-containing superfamily of G protein-coupled receptors (GPCRs) and stimulates intracellular inositol trisphosphate production by a $G_{q/11}$-mediated mechanism (Wess, 1993). Much research has been focused on the mechanisms of agonist-induced regulation of muscarinic receptors (Koenig and Edwardson, 1996; Edwardson and Szekeres, 1999), and it is generally accepted that short-term treatment with agonists causes receptor internalization and long-term treatment leads to receptor down-regulation of wild-type receptors (Koenig and Edwardson, 1996; Edwardson and Szekeres, 1999). Conversely, recent studies have shown that prolonged treatment of a constitutively active mutant β_2-adrenergic receptor (CAM–β_2-AR) with antagonists/inverse agonists can result in a significant up-regulation of receptor levels (Samama et al., 1993; MacEwan and Milligan, 1996a,b; McLean et al., 1999). Interestingly, studies using purified receptors have shown that all ligands, including agonists, can stabilize the conformation of the CAM–β_2-AR (Gether et al., 1997a,b). These and a range of other studies suggest that constitutively active mutant GPCRs exhibit structural instability and that both inverse agonists and agonists can stabilize the inherently unstable receptor conformation, resulting in receptor up-regulation (Milligan et al., 2002). Attachment of easy assay reporters, such as green fluorescent protein (McLean et al., 1999) or *Renilla reniformis* luciferase (Ramsay et al., 2001) to the C-terminal tail of CAM–β_2-AR has allowed direct detection of ligand-induced up-regulation without the need to perform ligand binding assays.

Because the CAM–β_2-AR but not the wild-type receptor exhibits structural instability, we proposed that the mutated

ABBREVIATIONS: M_3, rat muscarinic acetylcholine receptor 3; GPCR, G protein-coupled receptor; CAM, constitutively active mutant; AR, adrenergic receptor; CAM-i3, third intracellular loop of CAM β-adrenergic receptor; IC3, third intracellular loop; Rlu, *Renilla reniformis* luciferase; HEK, human embryonic kidney; NMS, *N*-methyl scopolamine; LOPAC, library of pharmacologically active compounds; MG132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; CMV, cytomegalovirus; CRE, cAMP response element; NFAT, nuclear factor of activated T cell; PCR, polymerase chain reaction; TM, transmembrane; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; DMSO dimethyl sulfoxide; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; QNB, *R*(−)-quinuclidinyl benzilate; ICI-118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; As-1397, 10-(α-diethylaminopropionyl)-phenothiazine.

third intracellular loop (CAM-i3) of the CAM–β_2-AR may play a key role in conferring the structural instability. To test this hypothesis, M_3 was modified by swapping its third intracellular loop (IC3) with the corresponding IC3 of the CAM–β_2-AR (CAM-i3). The difference between the wild-type and CAM–β_2-AR is that the C terminus of the third intracellular loop in the CAM–β_2-AR was replaced with the equivalent segment of the hamster α_{1B}-AR (Samama et al., 1993). The *R. reniformis* luciferase gene was linked in-frame to the C terminus of M_3(CAM-i3) to facilitate quantitative determination of receptor expression. The resulting chimeric fusion receptor [M_3(CAM-i3)-Rlu] displayed an increase in constitutive activity and could activate both G_q- and G_s-mediated second messenger responses, but retained the same binding specificity and affinity as the wild-type receptor for both agonists and antagonists. Sustained treatment (37°C, 20–24 h) of cells expressing this chimeric fusion receptor with either agonists or antagonists resulted in a concentration-dependent up-regulation of receptor expression in transiently transfected cells and stable cell lines. Based on these observations, we developed a high-throughput cell-based assay. This assay was validated by screening a HEK293 cell line stably expressing the M_3(CAM-i3)-Rlu fusion against a compound library containing known reference compounds. The assay provides a novel strategy to identify ligands for GPCRs by a ligand and second messenger-independent process.

Materials and Methods

Materials. All materials for cell culture and general molecular biology experiments were supplied by Invitrogen (Carlsbad, CA). [^{3}H]*N*-methyl scopolamine chloride (NMS; 80 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). All chemicals and the LOPAC compound library were obtained from Sigma (St. Louis, MO). Oligonucleotides were obtained from Genset (San Diego, CA) and MG132 was purchased from Calbiochem (La Jolla, CA). Native coelenterazine was obtained from Biotum (Hayward, CA). The *R. reniformis* luciferase-containing vector plasmid pRLCMV was purchased from Promega (Madison, WI) and reporter gene plasmids containing a firefly luciferase gene (pCRE-luc and pNFAT-luc) were purchased from Stratagene (La Jolla, CA). The luciferase reporter gene assay kit (LucLite) was bought from PerkinElmer Life and Analytical Sciences.

Construction of Fusion Receptor Genes. Both rat wild-type M_3-Rlu and M_3(CAM-i3)-Rlu were generated as described in Fig. 1. Briefly, M_3-Rlu was constructed in two steps. First, a full-length cDNA encoding *R. reniformis* luciferase (Rlu; 312 amino acids), generated by PCR amplification of an *R. reniformis* luciferase-containing vector plasmid (pRLCMV), was digested with EcoRI and XbaI, and the resulting 1-kilobase EcoRI-XbaI fragment was subcloned into a pCDNA3.1(+) vector, resulting in Rlu-pCDNA3.1. In the second step, M_3 was generated by PCR amplification of M_3-pCD plasmid (kindly provided by Dr. J. Wess, National Institutes of Health, Bethesda, MD). The PCR product was then inserted, after digestion with NheI and EcoRI, into Rlu-pCDNA3.1, resulting in the final construct, M_3-Rlu. The introduction of an EcoRI restriction site between the C terminus of M_3 and the N terminus of Rlu resulted in the insertion of three additional amino acids (Glu-Asn-Ser) (Fig. 1).

M_3(CAM-i3)-Rlu was constructed as follows: first, a PCR fragment encoding the entire IC3 loop (55 amino acids) of the constitutively active mutant human β_2-adrenergic receptor (CAM-i3) was inserted into the Rlu-pCDNA3.1 plasmid after digestion and ligation with KpnI and EcoRI, resulting in the CAM-i3-Rlu construct. Second, a PCR product encoding amino acids 1 to 249 of M_3 (from the N-terminal Met to the junction of TMV and IC3) was subcloned into the upstream region of CAM-i3-Rlu using NheI and KpnI restriction sites. Finally, a PCR product encoding amino acids 495 to 589 (spanning from the junction of TMVI and the IC3 loop to the C terminus) was inserted by nondirectional cloning with EcoRI. Constructs in each step were then verified by DNA sequence analysis. Our cloning strategy resulted in the introduction of mutations at two positions. First, the introduction of a KpnI site resulted in mutation of two amino acids within the end of TMV (Tyr-Trp to Gly-Thr); second, the addition of an EcoRI site to the end of TMVI changed Ser-Ala in M_3 to Glu-Phe.

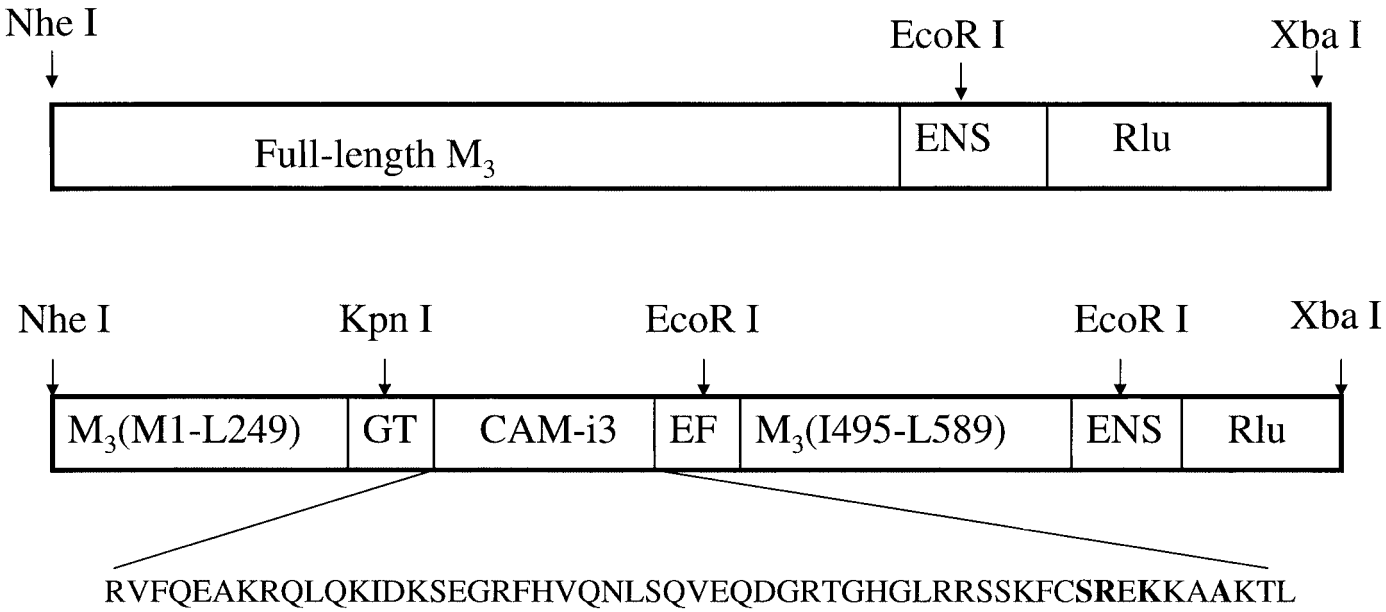

Fig. 1. Schematic representations of the wild-type and chimera M_3 fusion receptors. A, the C terminus of full-length M_3 was linked in-frame to the N-terminal Met of Rlu with insertion of amino acids Glu-Asn-Ser. B, the entire IC3 loop of the wild-type M_3 (amino acids 250–494) was replaced with the corresponding IC3 loop of CAM–β_2-AR (55 amino acids, sequence as indicated) with the introduction of KpnI and EcoRI sites, resulting in mutations of two amino acids at the membrane junctions of TMV (Tyr-Trp to Gly-Thr) and TMVI (Ser-Ala to Glu-Phe). The chimeric M_3(CAM-i3) was then linked in-frame to the N-terminal Met of the *R. reniformis* luciferase gene, resulting in the insertion of the three amino acids Glu-Asn-Ser.

Cell Culture and Transfection. HEK293 or COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Cells (1×10^6) were seeded into 100-mm dishes; after incubation for 24 h, the cells were transfected with 4 μg of plasmid DNA/dish using LipofectAMINE reagent according to the manufacturer's instructions (Invitrogen). To generate stable cell lines, cells were seeded 2 days after transfection and maintained in DMEM supplemented with 500 μg/ml G-418 (Geneticin sulfate). The medium was replaced every 3 days with DMEM supplemented with 500 μg/ml G-418 sulfate. To determine the effect of compounds on the steady-state receptor expression, cells were split 20 h after transfection into the poly-D-lysine–pretreated 96-well plates (Greiner America Inc.) and treated with test compounds for 20 to 24 h before the luciferase activity assay was performed.

Luciferase Activity Assay. Untreated or drug-treated cells were washed once with phosphate-buffered saline (PBS) solution. Cells were incubated with 50 μl of lysis buffer (0.25% Nonidet P-40 in assay buffer, 100 mM sodium phosphate, pH 7.4, and 500 mM NaCl) at room temperature for 30 min. The *R. reniformis* luciferase activity was immediately measured using a Wallac 1450 Microbeta counter (PerkinElmer Wallac, Gaithersburg, MD) after adding 100 μl of 2 μM coelenterazine diluted in assay buffer.

Ligand Binding Assays. COS-7 cells were transiently transfected with various constructs. After 2 days of transfection, cells were washed once with ice-cold PBS, then detached with PBS/0.5 mM EDTA and resuspended in ice-cold binding buffer (25 mM sodium phosphate, pH 7.4, 2 mM EDTA, and 10 mM $MgCl_2$). Cells were then homogenized using a Polytron homogenizer (PT-MR3100; Kinematica AG, Littau, Switzerland), and the crude cell homogenate was used for the [^{3}H]NMS binding assay. To study the effect of ligands on the expression of Rlu-tagged fusion receptors, cells were incubated for 24 h after transfection and subsequently treated overnight (20 h) with 1 μM of atropine or other ligands. The nontreated or drug-treated cells were extensively washed with ice-cold PBS to remove the bound compound. Binding assays were carried out with cell homogenates using [^{3}H]NMS as a radioligand. Samples (150 μl) were incubated in binding buffer for 2 h at room temperature in a 96-well plate. To measure maximal saturation binding sites, 2 nM [^{3}H]NMS was used. For competitive binding experiments, the membrane homogenates were preincubated with various concentrations of ligands at room temperature for 30 min before addition of 0.5 nM [^{3}H]NMS. Nonspecific binding was determined in the presence of 10 μM atropine. Reactions were terminated after incubation for 90 min at room temperature, and the bound [^{3}H]NMS was separated by rapid filtration through GF/C 96-well filters (Whatman, Clifton, NJ) followed by three washes with ice-cold wash buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4). After soaking in scintillation fluid, filters were counted in a liquid scintillation counter (Wallac 1409 DSA; PerkinElmer Wallac, Gaithersburg, MD).

Reporter Gene Assays. A CRE-firefly luciferase reporter gene assay was used to measure G_s-mediated cAMP production and an NFAT-firefly luciferase reporter gene assay was used to measure G_q-mediated intracellular calcium release in HEK293 cells (Hill et al., 2001). HEK293 cells were seeded in a 96-well white plate (Greiner Bio-One, Longwood, FL) (10,000 cells/well). After incubation for 24 h, the cells were cotransfected with receptor plasmids and reporter gene plasmids (pCRE-luc or pNFAT-luc) using LipofectAMINE 2000 reagent. Twenty-four hours after transfection, cells were exposed to serum-free medium (phenol red-free) and incubated in the absence or presence of drugs for another 12 to 16 h. Luciferase activity was then measured using the luciferase reporter gene assay kit LucLite according to manufacturer's instructions (PerkinElmer Life and Analytical Sciences).

Screening of LOPAC Compound Collection. All compounds were dissolved in DMSO at 400 μM and plated in columns 2 to 11 on 96-well plates. Atropine (400 μM in DMSO) was plated in column 1 and DMSO in column 12. One microliter of the stock solution was transferred to assay plates containing 100 μl of normal cell culture medium using a MiniTrak liquid handling device (PerkinElmer Life and Analytical Sciences). HEK293 cells, stably expressing M_3(CAM-i3)-Rlu (100 μl containing about 50,000 cells), were added into the assay plate using a Multidrop liquid handling device (Thermo Electron, Beverly, MA) resulting in a final medium volume of 200 μl and a final DMSO concentration of 0.5%. Cells were incubated for another 20 h and the medium was removed by aspiration. Luciferase activity was determined as described above.

Miscellaneous Methods. Protein was determined according to the method of Bradford (1976), using bovine serum albumin as the standard. All data were analyzed by a nonlinear least-squares curve fitting procedure, using the computer program Prism (GraphPad Software, San Diego, CA).

Results

M_3(CAM-i3)-Rlu Fusion Protein Displays Lower Protein Expression Levels Than the Wild-Type Receptor. To facilitate quantitative determination of receptor expression, the luciferase gene from the sea pansy *R. reniformis* was fused in-frame to the C terminus of the wild-type M_3. The resulting fusion protein displayed a similar binding affinity for [^{3}H]NMS compared with the nontagged wild-type receptor (data not shown). However, saturation binding studies demonstrated that the Rlu-tagged receptor was expressed in much lower quantities than the nontagged wild-type receptor, as evidenced by a dramatic decrease in [^{3}H]NMS binding sites (Fig. 2). Replacement of the IC3 loop with the IC3 loop of CAM–β_2-AR further decreased [^{3}H]NMS binding sites by approximately 2-fold compared with the M_3-Rlu (Fig. 2). Overall, M_3(CAM-i3)-Rlu exhibited a ~6-fold decrease in steady-state protein expression levels compared with the nontagged M_3 receptor. These results demonstrate that replacement of the IC3 loop with the IC3 loop of CAM–β_2-AR and addition of Rlu to the C terminus generated a chimeric mutant receptor that displayed much lower steady-state pro-

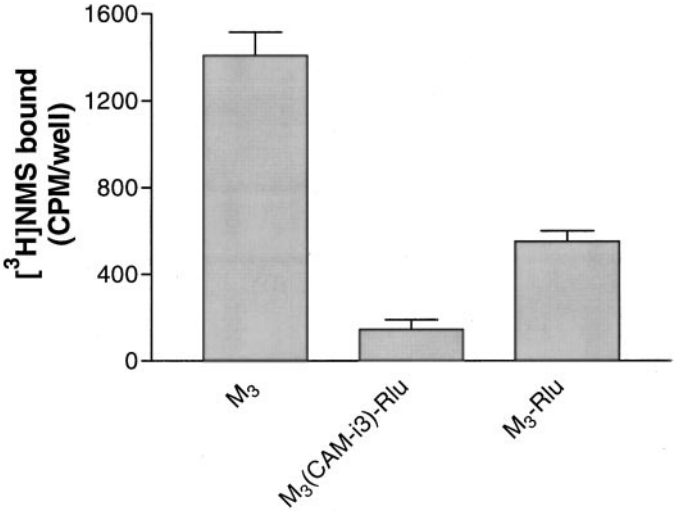

Fig. 2. Expression comparison of the wild-type M_3, M_3-Rlu, and M_3(CAM-i3)-Rlu in COS-7 cells. COS-7 cells were transiently transfected with the wild-type M_3, M_3-Rlu, and M_3(CAM-i3)-Rlu plasmids and cells were harvested after incubation for 48 h. Cell homogenates (20 μg/well) were incubated with 2 nM [^{3}H]NMS at room temperature for 2 h in the absence (total binding) or in the presence of 10 μM atropine (nonspecific binding). Data, expressed as specific counts per minute per well, are from one of three typical experiments performed in triplicate.

tein expression levels, suggesting that the chimeric fusion receptor M_3(CAM-i3)-Rlu was conformationally unstable.

M_3(CAM-i3)-Rlu Fusion Receptor Retains Binding Affinity Similar to That of the Wild-Type Receptor for Agonists and Antagonists. To examine the possibility that swapping the IC3 loop may change overall receptor conformation, we performed competitive ligand binding experiments with both agonists and antagonists/inverse agonists. M_3(CAM-i3)-Rlu showed a affinity for [^{3}H]NMS similar to that of the wild-type receptor [K_D = 0.63 ± 0.16 nM for wild-type M_3 and K_D = 0.37 ± 0.08 nM for M_3(CAM-i3)-Rlu]. As shown in Fig. 3, A and B, the antagonists atropine and NMS showed a slight increase (1- to 2-fold) in binding affinity to M_3(CAM-i3)-Rlu compared with the wild-type receptor (left shift of competitive binding curves). However, the antagonist 4-DAMP, had a slight decrease in binding affinity for the M_3(CAM-i3)-Rlu compared with the wild-type receptor (Fig. 3C), and the agonist carbachol showed a similar affinity for the wild-type and chimeric fusion receptor (Fig. 3D). Thus, the M_3(CAM-i3)-Rlu fusion receptor showed ligand binding properties analogous to those of the wild-type receptor.

M_3(CAM-i3)-Rlu Displays a Large Decrease in Agonist Potency and Efficacy to Activate G_q Protein. To examine whether the C-terminally tagged Rlu affects the interaction of the receptor with its cognate G proteins, the NFAT reporter gene assay was used to measure G_q-mediated intracellular calcium responses (Boss et al., 1996, 1998). Carbachol stimulated the NFAT gene expression of both wild-type and M_3-Rlu receptors with a similar potency and efficacy, indicating that the Rlu-tagging did not affect G protein-coupled coupling in this system (Fig. 4B). Replacement of the IC3 loop with the IC3 loop of CAM–β_2-AR resulted in an increase of at least 2-fold in basal activity in the NFAT-reporter gene assay, and this activity could be completely inhibited by the inverse agonist atropine (data not shown), suggesting that M_3(CAM-i3)-Rlu constitutively couples to the G_q protein (Fig. 4A). Of note is that the C-terminal Rlu luciferase tag does not contribute to this signal, because Rlu luciferase does not recognize the same substrate as firefly luciferase, which is driven by the NFAT reporter system. Moreover, addition of Rlu to M_3 and M_3(CAM-i3) did not significantly affect the constitutive or carbachol-stimulated activity in the NFAT reporter gene assay, but carbachol did display a dramatic decrease in the ability to stimulate the M_3(CAM-i3)-Rlu receptor (Fig. 4B). These results support the notion that the IC3 loop of M_3 plays an important role in the efficiency and specificity of G protein coupling. We also used CRE-reporter gene assay and whole-cell cAMP assay to determine whether the replacement of the IC3

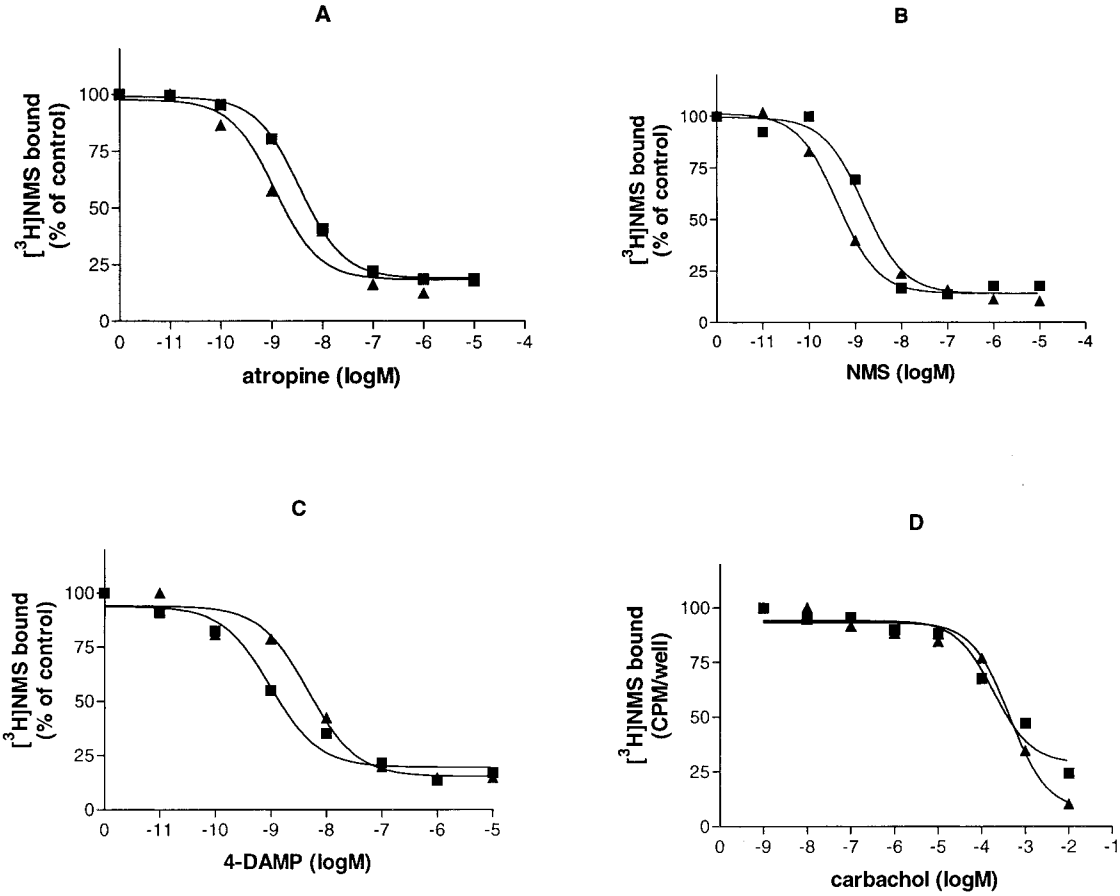

Fig. 3. Displacement of specific [^{3}H]NMS binding to the wild-type M_3 and M_3(CAM-i3)-Rlu by agonists and antagonists. COS-7 cell homogenates (20 μg/well), transiently expressing the wild-type M_3 (■) and M_3(CAM-i3)-Rlu fusion receptor (▲) were incubated in binding buffer with different concentrations of the indicated ligands at room temperature for 30 min. [^{3}H]NMS was added to a final concentration of 500 pM, and the mixture was incubated at room temperature for a further 1 h. M_3(CAM-i3)-Rlu showed an affinity for [^{3}H]NMS similar to that of wild-type receptor [K_D = 0.63 ± 0.16 nM for wild-type M_3 and K_D = 0.37 ± 0.08 nM for M_3(CAM-i3)-Rlu]. Data are presented as a percentage of control (in the absence of unlabeled ligand, as 100%) from one of three representative experiments performed in triplicate.

loop with the IC3 loop of CAM–β_2-AR can make the chimeric receptor couple to G_s protein. M_3(CAM-i3)-Rlu displayed an increase of ~2-fold in basal CRE-reporter gene expression and whole-cell cAMP assay (data not shown), suggesting that this chimeric receptor exhibited significant constitutive G_s-coupling activity compared with the wild-type receptor.

Antagonists Specifically Induce Up-Regulation of M_3(CAM-i3)-Rlu but Not The Wild-Type M_3-Rlu Receptor. The addition of Rlu to the receptors facilitates the determination of the fusion receptor protein expression by directly measuring the luciferase activity. We did not observe a significant up-regulation of the wild-type M_3-Rlu by sustained treatment of cells with a range of muscarinic antagonists (Fig. 5A). By contrast (Fig. 5B), several antagonists (10 μM) specific for muscarinic acetylcholine receptors (NMS, atropine, 4-DAMP, and QNB) led to a 4- to 5-fold increase in the luciferase activity of M_3(CAM-i3)-Rlu compared with untreated cells, demonstrating that the effect was unique to an inherently unstable form of the receptor. Importantly, under the same conditions, ligands specific for the adrenergic receptors (isoproterenol, yohimbine, ICI-118,551) and other compounds that have no activity for the muscarinic receptors had no significant effect on the luciferase activity of this fusion receptor (Fig. 5B). Moreover, other compounds known not to interact with the muscarinic receptors, such as ones specific for serotonin (serotonin, metergoline) and histamine (imetit, ranitidine) receptors, did not alter the luciferase activity (data not shown). These results clearly indicate that the increase in enzyme activity of this fusion receptor was driven by a ligand-specific mechanism. In addition, to exclude the possibility that these ligands directly affected the luciferase enzyme activity, cells were transfected with the wild-type Rlu gene alone before exposure to test compounds and, in a second experiment, the ligands were introduced directly into a luciferase activity assay. In both cases, all antagonists had no effect on luciferase enzyme activity (data not shown).

Agonists Induce Up-Regulation of Both the Wild-Type M_3-Rlu and M_3(CAM-i3)-Rlu Fusion Receptors. In contrast to antagonists, carbachol (10 μM) caused an increase of ~2-fold in luciferase activity for both the wild-type M_3-Rlu and M_3(CAM-i3)-Rlu receptors (Fig. 5, A and B). Other agonists such as oxotremorine M and acetylcholine could also increase luciferase activity in a specific manner (data not shown). Gallamine, an allosteric ligand for muscarinic receptors (Krejci and Tucek, 2001), had no effect on the luciferase activities of the wild-type (data not shown) and M_3(CAM-i3)-Rlu receptors, respectively (Fig. 5B).

Verification of Ligand-Induced Receptor Up-Regulation by Radioligand Binding Assay. To verify that the ligand induced increase in luciferase activity was responsible for the increase in receptor number, we determined the receptor expression levels of untreated and ligand treated cells by measuring [^{3}H]NMS binding sites in a ligand binding assay. Treatment of cells expressing M_3(CAM-i3)-Rlu for 20 h with 1 μM atropine or 100 μM carbachol led to an increase of approximately 3- to 4-fold in [^{3}H]NMS binding sites (Fig. 5C). Similar treatment of the wild-type receptor M_3-Rlu with carbachol gave about a 3-fold increase in [^{3}H]NMS binding sites, but atropine had no significant effect on [^{3}H]NMS binding sites of M_3-Rlu (Fig. 5C). These results are consistent with those obtained in luciferase assays (Fig. 5, A and B), confirming that the increased luciferase activity was caused by an actual increase in receptor number.

Inhibition of Receptor Internalization and Down-Regulation by Addition of Rlu to the C Terminus of Wild-Type M_3. To further understand why agonists cause receptor up-regulation of M_3-Rlu, we examined carbachol-induced receptor internalization of both nontagged and Rlu-tagged receptors. As shown in Fig. 6A, carbachol (100 μM, 37°C, 1 h) resulted in about 60% loss of surface receptors as determined by a membrane-impermeable [^{3}H]NMS binding assay, whereas the same treatment reduced surface receptor numbers of M_3-Rlu by only <10%. Furthermore, long-term treatment with carbachol led to a significant decrease in total [3H]NMS binding sites for wild-type M_3 but resulted in a 3-fold increase in total [3H]NMS binding sites for the M_3-Rlu receptor (Fig. 6B). These results suggest that the C-terminally tagged Rlu may block agonist-induced receptor internalization and down-regulation.

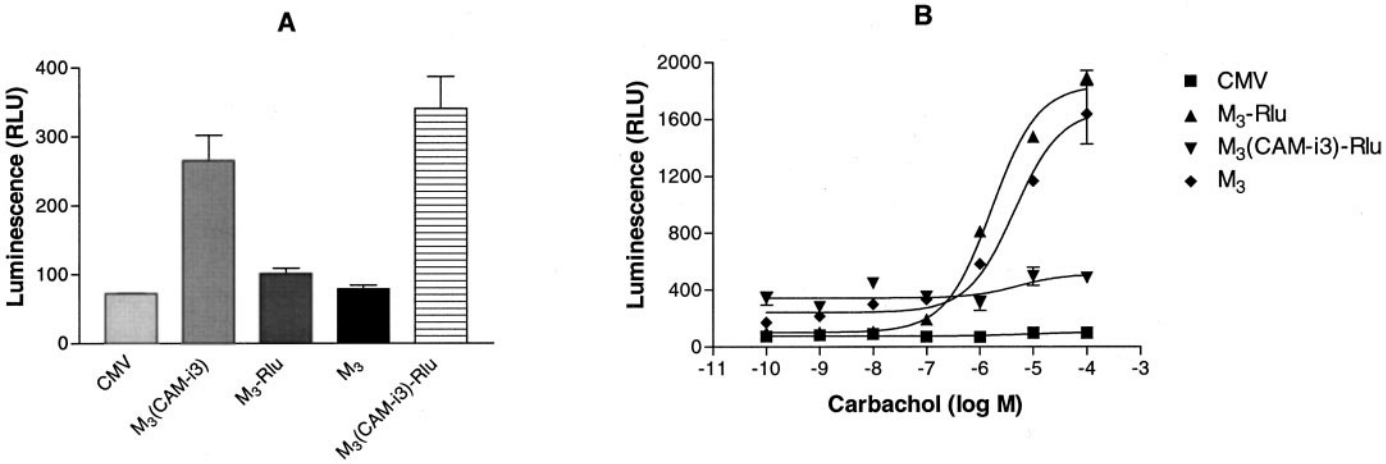

Fig. 4. NFAT reporter gene assays. HEK293 cells, plated in white 96-well plates, were cotransfected with the pNFAT-Luc plasmid, vector alone (CMV), or receptor plasmids using LipofectAMINE 2000. After transfection and incubation for 20 h, cells were washed once with serum-free medium (phenol red-free) and incubated with various concentrations of carbachol for another 12 to 16 h. Luciferase activity, driven from the pNFAT-Luc plasmid, was measured using a firefly luciferase reporter gene assay kit according to the manufacturer's instructions. Note that the C-terminal Rlu luciferase tag does not contribute to the signal because Rlu luciferase does not recognize the same substrate as firefly luciferase. Activity was measured in the absence (A) or presence (B) of varying concentrations of carbachol. Data are presented as the mean ± S.E. of triplicate.

Time- and Concentration-Dependence of Ligand-Induced M_3(CAM-i3)-Rlu Up-Regulation. Ligand induced up-regulation of M_3(CAM-i3)-Rlu was both time- and concentration-dependent. Significant up-regulation could be detected after incubation for 6 h at 37°C. After treatment of cells with 10 μM atropine, the luciferase activity increased with incubation time from 6 to 96 h (data not shown). Generally, cells were incubated for 20 to 24 h, which gave sufficient time for compound interaction without compromising compound stability at 37°C. As shown in Fig. 7, atropine and 4-DAMP increased the luciferase activity of the M_3(CAM-i3)-Rlu receptor in a concentration dependent manner. All other antagonists tested also showed a dose-dependent up-regulation of the chimeric fusion receptor (data not shown), but potency (EC_{50} values) for these ligands did not always correlate to the K_i values obtained from competitive binding assays (Fig. 3). For example, the EC_{50} value for atropine (~6 nM) in the luciferase up-regulation assay (Fig. 7) was only ~5-fold higher than that obtained in the competitive binding assay (1.3 nM). A small right-shift in the EC_{50} values was also observed for several other antagonists/inverse agonists, such as NMS, QNB, and himbacine, and several identified antagonists (Table 1). In contrast, the EC_{50} value for 4-DAMP (~900 nM) in the luciferase up-regulation assay was ~1000-fold higher than that obtained in the competitive binding assay (~1 nM) (Fig. 3). The agonist carbachol also induced receptor up-regulation in a dose-dependent manner (Fig. 7), but the EC_{50} (> 100 μM) had a rightward shift of at least 10-fold compared with that seen in the competitive binding assay (Fig. 2). In addition, oxotremorine M exhibited

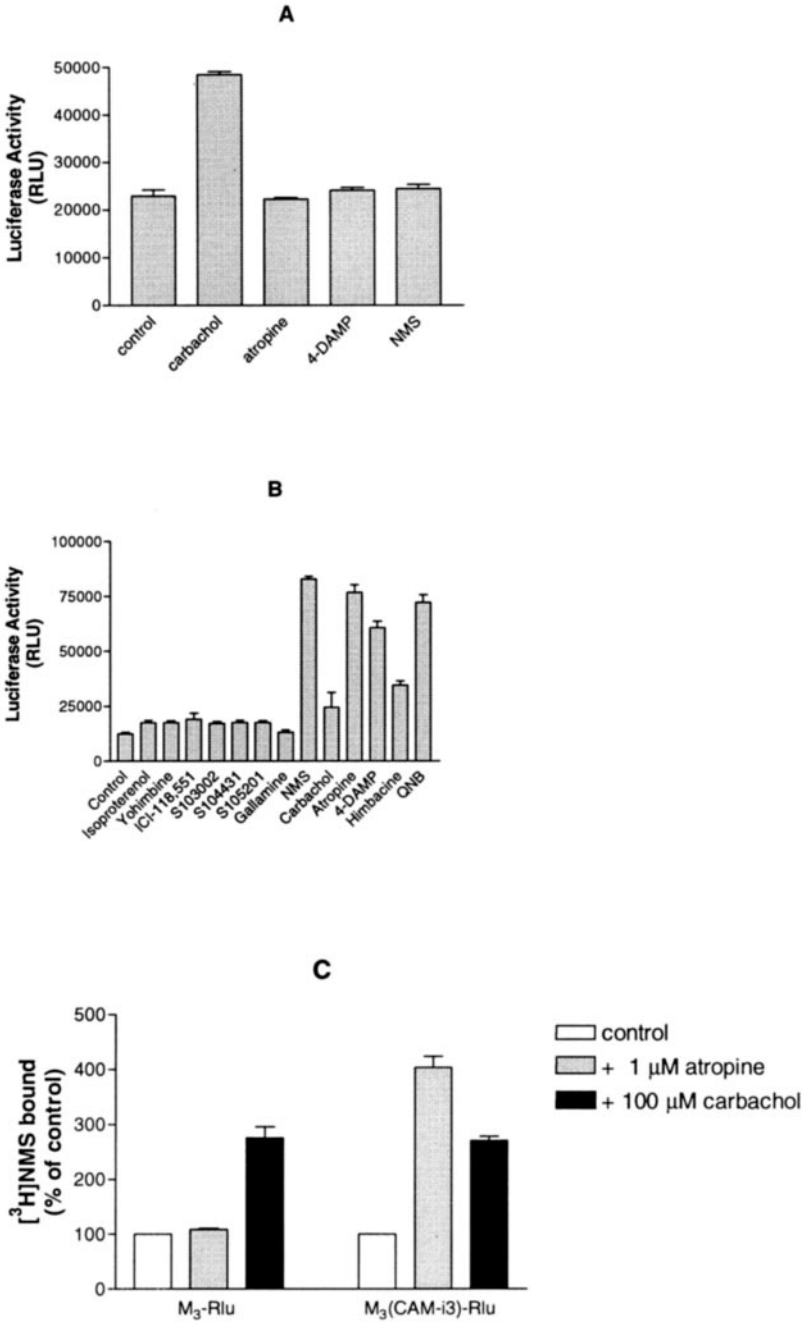

Fig. 5. Ligand specific up-regulation of the wild-type M_3-Rlu and M_3(CAM-i3)-Rlu receptor transiently expressed in HEK293 cells. HEK293 cells were transfected with the wild-type M_3-Rlu (A) and M_3(CAM-i3)-Rlu receptor fusion constructs (B). After a 24-h transfection, cells (10-cm dish) were seeded into 96-well plates (50,000 cells per well) and compound was added to the culture medium to a final concentration of 10 μM. Cells were cultured for a further 20 to 24 h before carrying out the luciferase activity assays as described under *Materials and Methods*. All data are expressed as mean ± S.E. of luciferase activity [relative light units (RLU)] from one of six independent experiments, each carried out in duplicate. Compounds S103002, S104431, and S105201 are compounds from the Arena library that are known not to interact with the muscarinic M_3 receptor. C, ligand-induced receptor up-regulation was verified by [^{3}H]NMS binding. HEK293 cells stably expressing M_3-Rlu or M_3(CAM-i3)-Rlu were incubated in culture medium for 20 h in the absence (□) or in the presence of 1 μM atropine (▨) or 100 μM carbachol (■). Cells were harvested and extensively washed with ice-cold PBS to remove bound ligand. Binding was carried out with cell homogenates using [^{3}H]NMS (2 nM) as described under *Materials and Methods*. Data are presented as percentage of untreated cells (100%) and represent one of three independent experiments, each carried in duplicate.

an EC_{50} that was 1000-fold higher than the K_i value (Fig. 7). The rightward shift of EC_{50} values in the luciferase up-regulation assay may have been caused by ligand instability and agonist-induced receptor down-regulation under the assay conditions used (37°C and >20-h treatment) (also see *Discussion*). Indeed, we found that 4-DAMP was less stable than atropine under these conditions, as evidenced by a decrease in binding affinity (data not shown).

Ligands Act on Receptors by Inhibiting Receptor Degradation. Mutated or improperly processed receptors are often specifically targeted for degradation. In this regard, we examined effects of the proteasome inhibitor, MG132, on both basal and ligand-induced receptor up-regulation to determine whether proteasomes are involved in the degradation of the chimeric fusion receptor. Treatment with this agent in the absence of ligand resulted in up-regulation of luciferase activity and thus receptor number (Fig. 8, A and C) consistent with receptor degradation at least partially via proteasome pathways. In the presence of MG132, atropine and carbachol still caused concentration-dependent increases in luciferase activity but as a percentage, this was lower than the effect of atropine or carbachol in the absence of inhibitor (Fig. 8, B and D). These results show that at least a component of the mechanism of ligand-induced up-regulation is a result of blockade of receptor degradation by proteasome pathways.

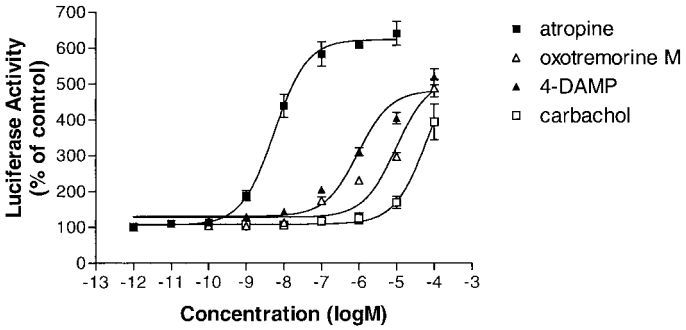

Fig. 7. Dose-dependent up-regulation of the M_3(CAM-i3)-Rlu fusion protein by ligands. HEK293 cells stably expressing M_3(CAM-i3)-Rlu were seeded into 96-well plates and incubated with different concentrations of atropine (■), 4-DAMP (▲), carbachol (□), and oxotremorine M (△) for a further 20 h before measuring receptor expression using the luciferase activity assay. Data are expressed as a percentage of the control luciferase activity in the absence of ligand (defined as 100%) and represent observations from three independent experiments, each carried out in triplicate.

Development of a Screening Platform Based on Ligand Specific Up-Regulation of M_3(CAM-i3)-Rlu. To explore the possibility that the ligand-specific receptor up-regulation assay could be adapted as a high-throughput screening assay, we screened a LOPAC compound library of about 700 compounds (Sigma) that included many ligands for

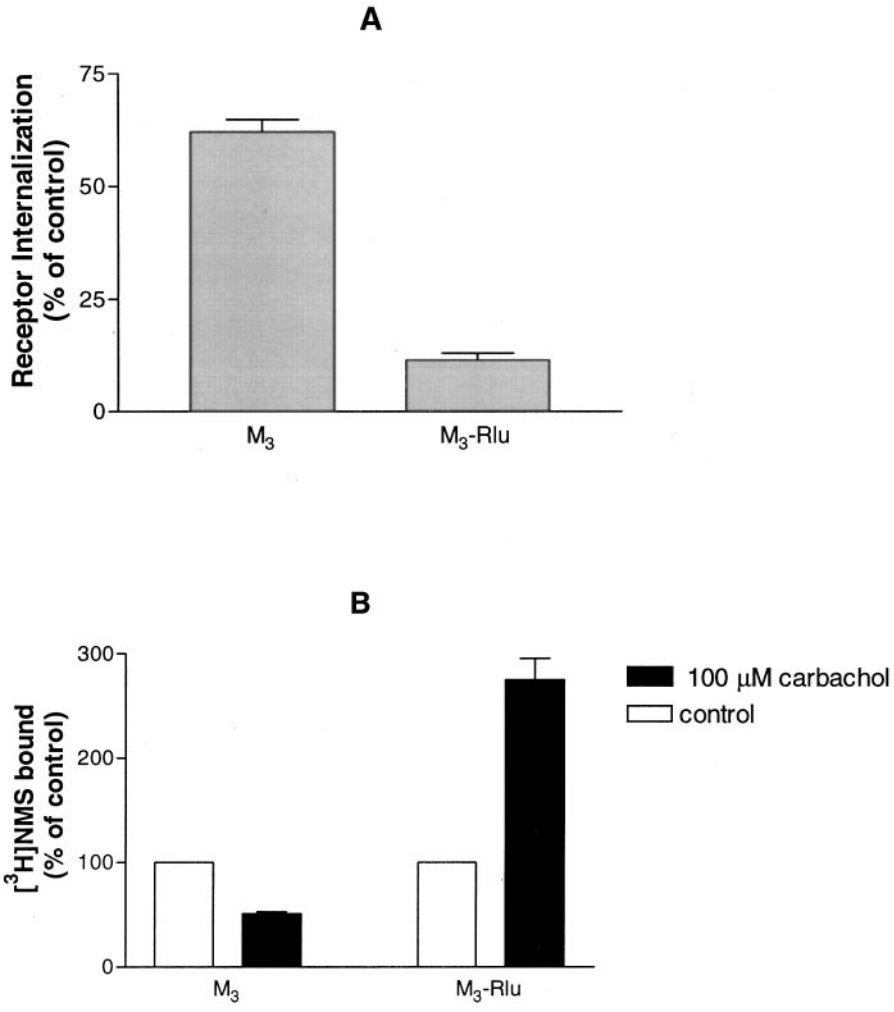

Fig. 6. Agonist-induced receptor internalization and down-regulation. A, receptor internalization. HEK293 cells were transiently transfected in six-well dishes with wild-type M_3 or M_3-Rlu; 40 h after transfection, cells were treated with or without 100 μM carbachol in normal culture medium at 37°C for 1 h. Cells were washed once with ice-cold PBS, then incubated with 2 nM [^{3}H]NMS in binding buffer in the absence (total binding) of or presence of 10 μM atropine (nonspecific binding) at 4°C for 2 h. B, receptor down- or up-regulation. HEK293 cells were transiently transfected with wild-type M_3 or M_3-Rlu; 20 h after transfection, cells were treated with or without 100 μM carbachol in normal culture medium at 37°C for 20 h. Cells were extensively washed with PBS and homogenized. Cell membrane (20 μg/well) was subjected to [^{3}H]NMS binding assay. Data are presented as percentage of untreated cells (100%) and represent one of three independent experiments, each carried in duplicate.

TABLE 1

Verification of hits identified in the receptor up-regulation assay by competitive ligand binding and functional NFAT reporter gene assays.

The receptor up-regulation assay was performed in HEK293 cells stably expressing M_3(CAM-i3)-Rlu as described in Fig. 7. The competitive ligand binding assay was carried out in membrane homogenates of COS-7 cells transiently transfected with wild-type M_3 using 500 pM [^{3}H]NMS (K_D = 0.63 ± 0.16 nM). The NFAT reporter gene assay was performed on HEK293 cells transiently transfected with wild-type M_3 in the presence of 5 μM carbachol. Data are presented as the mean ± S.E. of three experiments performed in triplicate.

Compounds	EC_{50}		IC_{50}
	Up-Regulation Assay	NFAT Reporter Gene Assay	Competition Binding Assay
	M	*M*	*M*
As-1397	$3.01 \pm 0.25\times10^{-7}$	$1.45 \pm 0.16\times10^{-8}$	$1.37 \pm 0.14\times 10^{-7}$
Octoclothepin	$1.24 \pm 0.36\times10^{-6}$	$1.63 \pm 0.30\times10^{-6}$	$2.88 \pm 0.81\times 10^{-7}$
Thioperamide	$6.87 \pm 1.48\times10^{-5}$	$1.87 \pm 0.21\times10^{-5}$	$8.30 \pm 1.41\times 10^{-5}$
Thioridazine	$1.55 \pm 0.18\times10^{-6}$	$2.82 \pm 0.35\times10^{-7}$	$6.75 \pm 1.25\times 10^{-7}$
Quinidine sulfate	$2.17 \pm 0.34\times10^{-6}$	$1.07 \pm 0.13\times10^{-7}$	$1.41 \pm 0.40\times 10^{-6}$
Nicardipine	$5.60 \pm 1.27\times10^{-6}$	$4.90 \pm 0.24\times10^{-7}$	$1.00 \pm 0.21\times 10^{-6}$
Atropine	$6.73 \pm 0.16\times10^{-9}$	$2.56 \pm 0.14\times10^{-9}$	$1.41 \pm 0.16\times 10^{-9}$
NMS	$7.15 \pm 0.50\times10^{-9}$	$1.09 \pm 0.16\times10^{-9}$	$7.00 \pm 1.40\times10^{-10}$
QNB	$3.33 \pm 0.60\times10^{-9}$	$1.60 \pm 0.57\times10^{-9}$	$7.50 \pm 1.12\times10^{-10}$
Himbacine	$1.05 \pm 0.21\times10^{-6}$	$4.25 \pm 0.61\times10^{-7}$	$5.00 \pm 0.57\times 10^{-8}$

muscarinic receptors. The assay tolerated DMSO concentrations up to 1%. All compounds were screened at a single concentration of 2 μM with a final concentration of 0.5% DMSO. Atropine (2 μM) was used as positive control and normalized to 200% of the response, and DMSO was used as a negative control and normalized to 100% of the response. Both 96- and 384-well formats generated similar standard deviations (<10%) and Z′ values (0.5–0.7) (data not shown). An example of a screening plate (96 wells) is shown in Fig. 9. All compounds that gave >130% response were selected for reconfirmation by retesting the compound in triplicate. The reconfirmation rate was generally >75% and known M_3 ligands could be identified with great accuracy (Fig. 9). Ligands with low affinity (K_i > 2 μM), such as carbachol, and/or instability, such as acetylcholine, could not be identified with the 130% cut-off criteria. However, several other compounds displayed very strong activity (>150% response) (Table 1). These compounds included octoclothepin maleate (D_2 dopamine receptor antagonist), thioperamide (H_3 histamine receptor antagonist), As-1397 (selective butyrylcholinesterase inhibitor), thioridazine (dopamine receptor antagonist), quinidine sulfate (Na^+ channel blocker), and nicardipine (Ca^{2+}

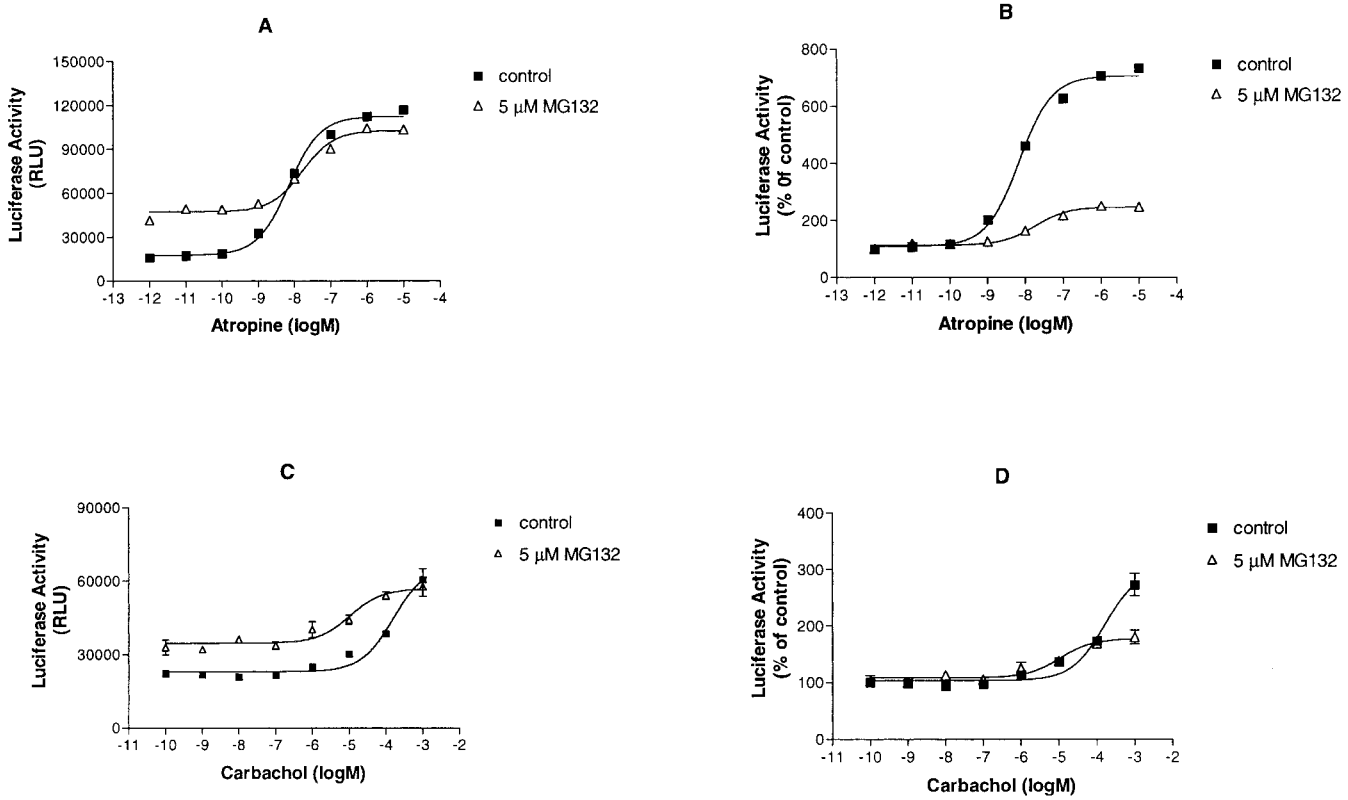

Fig. 8. Effect of proteosome inhibitor on ligand induced up-regulation of M_3(CAM-i3)-Rlu. HEK293 cells stably expressing M_3(CAM-i3)-Rlu were seeded into 96-well plates and incubated with different concentrations of atropine or carbachol in the absence (■) or in the presence of 5 μM MG132 (△) for a further 20-h period before measuring receptor expression levels by luciferase assay. Data are presented as raw data (A and C) or as percentage of control luciferase activity in the absence of ligand (100%) (B and D) and represent one experiment of three independent experiments, each carried out in triplicate.

channel antagonist). Of these identified compounds, octoclothepin maleate, thioperamide, and As-1397 have not previously been reported as M_3 ligands, and thioridazine and quinidine have been previously shown to have antimuscarinic effects (Nakajima et al., 1989; Niedzwiecki et al., 1989). In addition, quinidine and nicardipine have been reported to be able to inhibit [^{3}H]QNB binding to rat brain membranes, indicating that they may interact with muscarinic receptors (Cohen-Armon et al., 1985; Katayama et al., 1987). These compounds were further subjected to the competitive ligand binding assay and functional NFAT reporter gene assay using the wild-type M_3 to ascertain whether they also interacted with the wild-type receptor. Indeed, all these compounds could inhibit [^{3}H]NMS binding and carbachol-stimulated NFAT gene expression in a concentration-dependent manner (TABLE 1), suggesting that they interacted with the wild-type receptor at the ligand binding site. EC_{50} values of these compounds in the up-regulation assay were generally consistent with those obtained in the competition binding assay or NFAT reporter gene assay (Fig. 10). These results demonstrate that this assay can identify novel compounds with accuracy and reproducibility in both 96- and 384-well screening formats.

Discussion

In this study, we have demonstrated that replacement of the IC3 loop of the rat M_3 with the corresponding IC3 loop of the CAM–β_2-AR results in constitutive activation and destabilization of the chimeric receptor.

The M_3(CAM-i3)-Rlu demonstrated a dramatic decrease in the potency and efficacy to activate G_q protein and a clear increase in the ability to activate G_s protein. Even with low expression levels, M_3(CAM-i3)-Rlu was constitutively active. In this regard, previous studies have indicated that the constitutively active mutant β_2-AR was consistently expressed at lower levels than the wild-type receptor in either stable or transient expression systems (MacEwan and Milligan, 1996a; Milligan and Bond, 1997; Gether et al., 1997b; Lee et al., 1997; Rasmussen et al., 1999), suggesting that the receptor conformation responsible for constitutive activation was less stable than the wild-type form. Furthermore, the steady-state protein expression levels were significantly correlated to the constitutive activity of these receptor proteins, suggesting that ligand-independent receptor activation may lead to the structural instability of these fusion receptors. The decreased expression levels found for this chimeric fusion receptor may be explained by an increase in basal receptor down-regulation as a consequence of constitutive activation, as previously reported to be the case for many other receptors (Heinflink et al., 1995; MacEwan and Milligan, 1996b; Smit et al., 1996; Lee et al., 1997; Milligan and Bond, 1997; Alewijnse et al., 1998; Miserey-Lenkei et al., 2002). Another possibility is that the low protein expression levels may be a result of structural instability of the mutant receptors as observed for the constitutive active mutant rat μ-opioid receptor (Li et al., 2001), H_2 histamine receptor (Alewijnse et al., 2000), and β_2-adrenergic receptor (Gether et al., 1997a; Rasmussen et al., 1999). Indeed, upon incubation of membranes at 37°C, M_3(CAM-i3)-Rlu lost [^{3}H]NMS binding activity at a greater rate and to a greater extent than the wild-type receptor (data not shown), indicating that the M_3(CAM-

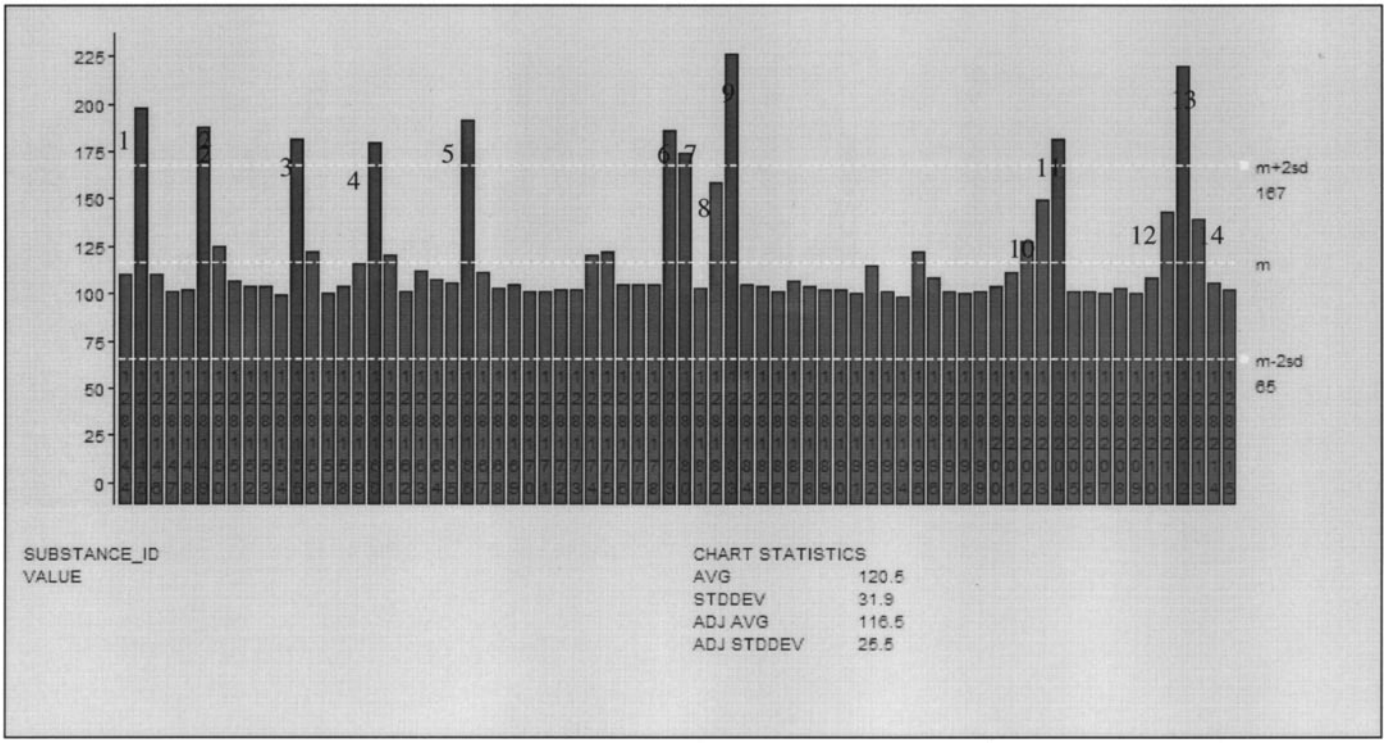

Fig. 9. One example of LOPAC compound screening in 96-well format. Nine plates of the LOPAC compound collection (Sigma) were screened at a concentration of 2 μM. Results for one of these plates are shown here. The positive control (2 μM atropine) is set as 200% and the negative control (0.5% DMSO) is set as 100%. Compounds that gave >140% activity are: 1, atropine sulfate; 2, aminobenztropine; 3, benztropine; 4, As-1397; 5, 4-DAMP; 6, hexahydro-sila-difenidol; 7, hexahydro-sila-difenidol, *p*-fluoro analog; 8, himbacine; 9, ipratropium bromide; 10, pilocarpine; 11, pirenzepine; 12, quinidine sulfate; 13, (−)-scopolamine; 14, (−)-*N*-butyl scopolamine.

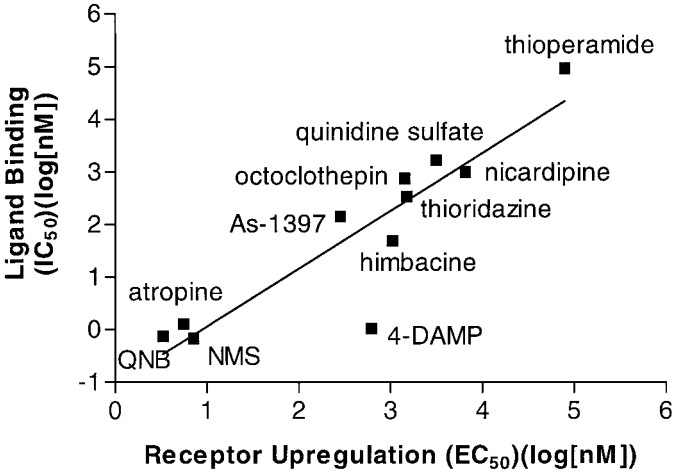

Fig. 10. Correlation of EC_{50} values in receptor up-regulation assay and ligand binding assay. EC_{50} values of compounds obtained from receptor up-regulation assay using the chimeric M_3(CAM-i3)-Rlu are plotted against IC_{50} values obtained from competitive ligand binding assay using the wild-type M_3. R^2 is 0.804.

i3)-Rlu was more structurally unstable. The ability of the proteasomal inhibitor MG132 to increase levels of Rlu activity in cells expressing M_3(CAM-i3)-Rlu in the absence of receptor ligands indicates an important role for proteasomal destruction of the chimeric receptor. Indeed, recent studies suggest that these ligands up-regulate CAM receptor expression by blocking constitutive receptor internalization (down-regulation) and inhibiting receptor degradation in lysosomes and proteasomes (Milligan and Bond, 1997; Li et al., 2001; Ramsay et al., 2001). In this regard, we showed that proteasome enzyme inhibitors effectively reduced M_3(CAM-i3)-Rlu up-regulation by atropine and carbachol (Fig. 8). These results indicate that inhibition of degradation of the mutant receptors in proteasomes is likely to be one mechanism involved. The structural instability of mutant GPCRs may also enhance interaction with some plasma membrane proteases, resulting in an increase in receptor down-regulation (Jockers et al., 1999). The structural instability can be induced by mutations that enhance G protein-coupling activity and constitutive activation (Ramsay et al., 2001; McLean et al., 2002; Milligan et al., 2002, Li et al., 2001) or that impair G protein-coupling activity (Wilson and Limbird, 2000; Wilson et al., 2001). Our studies with many GPCRs suggest that the CAM-i3 loop (IC3 loop of CAM–β_2-AR) swapping approach may represent a general strategy to generate structurally instability of chimeric receptors.

The ligand up-regulated receptors displayed the same properties as the wild-type receptor as demonstrated in the ligand binding assay and NFAT reporter gene assay (Fig. 5C and data not shown). However, EC_{50} values for some ligands in the receptor up-regulation assay were higher than their K_i values obtained from the ligand binding assay (Figs. 3 and 7). A similar rightward shift of the EC_{50} values has also been observed for other receptors. The inverse agonist naloxone up-regulated a constitutively active mutant μ-opioid receptor with an EC_{50} value approximately 1000-fold above its K_i value, as measured by a competitive binding assay (Li et al., 2001). In addition, inverse agonists/antagonists up-regulated the constitutively active mutant α_{1B}-AR with EC_{50} values in the micromolar range despite having low nanomolar affinities to the receptor in a binding assay (Stevens et al., 2000). In contrast to these receptors, agonists and inverse agonists could up-regulate the C-terminally Rlu-tagged CAM–β_2-AR with EC_{50} values similar to their K_i values in the binding assay (Ramsay et al., 2001). Figure 10 shows the correlation of EC_{50} values obtained from the receptor up-regulation assay and the competitive ligand binding assay. All antagonists other than 4-DAMP had a good correlation between these two assays (correlation coefficient is 0.9094; $R^2 = 0.8040$). The rightward shift in EC_{50} values in the receptor up-regulation assay seems to be dependent on the properties of the mutant receptors and stability of the compounds. The increase in EC_{50} values may be caused by a decrease in compound stability and ligand (agonist)-mediated receptor down-regulation under the assay conditions (37°C, >20 h), but the exact mechanisms are still not clear.

Many studies have demonstrated that inverse agonists can up-regulate the expression of constitutively active mutant GPCRs, including α_{1B}-AR (Stevens et al., 2000), α_{2A}-AR (Wilson and Limbird, 2000; Wilson et al., 2001), β_1-AR (McLean et al., 2002), histamine H_2 receptor (Alewijnse et al., 2000), μ-opioid receptor (Li et al., 2001), M_1 muscarinic acethylcholine receptor (Lu and Hulme, 1999), and β_2-AR (Milligan and Bond, 1997; Leurs et al., 1998). These studies suggest that inverse agonists can stabilize the inherently unstable structures of constitutively active mutant GPCRs. However, our results show that a variety of ligands, including agonists, neutral antagonists, and inverse agonists can up-regulate the M_3(CAM-i3)-Rlu fusion receptor. We also found that agonists such as isoproterenol and albuterol could up-regulate the expression of the CAM–β_2-AR-Rlu, as shown by an increase in the luciferase activity (Ramsay et al., 2001), and some studies have shown that agonists can stabilize the purified CAM–β_2-AR in detergent solutions (Gether et al., 1997a,b). Recent studies using the constitutively active histamine H_2 receptor (Alewijnse et al., 2000), α_{1B}-AR receptor (Stevens et al., 2000), α_{2A}-AR (Betuing et al., 1997), and μ–opioid receptor (Li et al., 2001) demonstrated that agonists and neutral antagonists could also stabilize the conformationally unstable receptor structure, leading to receptor up-regulation. Treatment with the full agonist carbachol also resulted in up-regulation of the wild-type M_3-Rlu (Fig. 5A). Discrepancy of our results with those previously reported is most probably caused by the type of protein that is tagged on the C terminus and how these different proteins can effect the overall receptor conformation (MacEwan and Milligan, 1996b; Milligan and Bond, 1997; Samama et al., 1997; Ramsay et al., 2001). Indeed, C-terminal Rlu tagging could inhibit agonist-induced receptor internalization and down-regulation (Fig. 6). Agonist mediated receptor internalization and down-regulation is known to be initiated by specific interaction of the receptor intracellular surface with downstream proteins such as G protein-coupled receptor kinases, arrestins, and dynamins (Gagnon et al., 1998; Lefkowitz, 1998). It is possible that the attachment of a large motif such as Rlu to the C-terminal tail may influence the interaction of the receptor, with these proteins preventing down-regulation for the Rlu-tagged receptor, but the exact molecular mechanisms involved in agonist-induced CAM receptor up-regulation remain to be determined.

The assay has been successfully adapted as a high-throughput screening assay in 96- and 384-well formats (Fig.

9). Compounds identified by the up-regulation assay were subsequently shown to interact with the wild-type receptor by ligand binding assays and other functional assays (Table 1). Moreover, because this assay is ligand-independent and does not rely on a second messenger response, it provides a novel strategy to identify ligands for orphan GPCRs for which the natural ligands and signal pathways are currently unknown. However, because this assay measures changes in total receptor expression, any compound that increases protein expression levels in a ligand-independent manner, such as protease inhibitors and compounds that enhance transcription and translation, will be identified as false positives. These nonspecific compounds can easily be separated from receptor-specific compounds by assessing their selectivity against a range of other receptors in the same assay.

In summary, we have demonstrated that swapping the IC3 loop of a GPCR with the IC3 loop of the CAM–β_2-AR leads to constitutive activation and structural instability of the receptor. Furthermore, we have demonstrated that a variety of receptor ligands, including agonists, neutral antagonists, and inverse agonists, can up-regulate the steady-state receptor protein expression by blocking receptor degradation. In some cases, antagonists and inverse agonists displayed EC_{50} values that correlate to their K_i values if the compounds were stable under the assay conditions. Last, this approach offers promise to constitutively activate orphan G protein-coupled receptors in the absence of their endogenous natural ligand(s) and provides a novel approach to screen chemical libraries in a high-throughput manner to identify novel small molecule modulators of GPCRs.

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