Protean Effects of a Natural Peptide Agonist of the G Protein-Coupled Secretin Receptor Demonstrated by Receptor Mutagenesis

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ABSTRACT

G protein-coupled receptors initiate signaling cascades after associating with heterotrimeric G proteins. This is typically initiated by agonist binding, but can also occur spontaneously, particularly in receptors bearing distinct missense mutations. Two such mutations in the parathyroid hormone receptor are associated with constitutive activity, manifesting clinically as Jansen’s metaphyseal chondrodysplasia. We introduce analogous mutations separately and together into the secretin receptor to explore their impact on another family member. Constructs were expressed transiently in COS cells, and had binding and signaling (cAMP generation) studied. Each construct was processed appropriately to lead to cell surface expression and signaling. Secretin bound to the wild-type receptor with two affinity states recognized, 1% of sites in the high affinity state ($K_i = 0.5 \pm 0.1 \text{ nM}$) and 99% in the low affinity state ($K_i = 23 \pm 3 \text{ nM}$). Mutant receptor binding best fit a single affinity state, having values for $K_i$ of 5 $\pm$ 1 nM (H156R), 8 $\pm$ 1 nM (T322P) and 6 $\pm$ 1 nM (H156R/T322P), with each of these demonstrating a shift to higher affinity than the predominant low affinity state of the wild-type receptor. Each mutant receptor expressed small to moderate constitutive activity, with basal levels of cAMP activity greater than control ($P < .01$): H156R, 1.4-fold; T322P, 4.5-fold and H156R/T322P, 6.8-fold. The level of basal activity of even the most active construct was only 15% of the maximal response of wild-type receptor. Although each of the single site mutants responded to secretin by increasing their cAMP levels in a concentration-dependent manner, the dual mutant decreased its cAMP in response to hormone (EC50 = 13 nM). Thus, a natural agonist had become an inverse agonist at this unique construct. Because this could reflect reduced normal coupling with $G_s$ or increased aberrant coupling with $G_i$, the mechanism was further explored using pertussis toxin and a stable analogue of GTP. Although ligand-binding determinants were retained in the dual receptor mutant, the conformation of this receptor upon secretin binding effected a reduction in its basal coupling with $G_i$, thereby resulting in inverse agonism.

Ternary complex models provide the best current understanding of the molecular events responsible for initiation of cellular signaling processes at guanine nucleotide-binding protein- (G protein) coupled receptors (Kenakin, 1996). The critical focus for this process is the complementarity of conformations of the cytosolic face of the receptor and its associated G protein. Initially, it was thought that an agonist ligand was key to select or induce an active conformation of the receptor (DeLean et al., 1980). As such models have evolved, they have accommodated concepts of receptor-G protein coupling that may exist transiently in the absence of agonist ligand and of receptor mutations that can result in various levels of constitutive signaling activity (Samama et al., 1993; Weiss et al., 1996a; Weiss et al., 1996b). The latter has now been recognized to exist in several disease states (Lefkowitz et al., 1993). This has also changed how we think about antagonists. With constitutively active receptor expression systems now widely available, it has become common to find that molecules previously believed to represent antagonists actually possess inverse agonist activity (Costa and Herz, 1989; Lefkowitz et al., 1993). Most agonists at wild-type receptors continue to have agonist activity at constitutively active receptors (Lefkowitz et al., 1993), although there is one reported example of such a receptor (the D578L mutant of the lutropin/choriogonadotropin receptor) having constitutive activity, but having no response to its native agonist ligand (Kosugi et al., 1996).

As medicinal chemists have become experienced in understanding the structural determinants for agonist activity of ABBREVIATIONS: G protein, guanine nucleotide-binding protein; PCR, polymerase chain reaction; KRH, Krebs-Ringers-HEPES; DMEM, Dulbecco’s modified Eagle’s medium.
receptor ligands, it has become possible to make small structural modifications in a ligand to change its effect on a receptor from that of an agonist or partial agonist to an antagonist (Aquino et al., 1996). In this report, we have achieved the same type of reversal of biological action by modifying a receptor rather than a ligand.

For this project, we constructed a series of mutants of the rat secretin receptor, incorporating separately and together each of two point mutations that have been reported to produce constitutive activity in the structurally related parathyroid hormone receptor (Schipani et al., 1985; Schipani et al., 1996). The single point mutations behaved as predicted, producing different degrees of constitutive activity of the secretin receptor, and binding and signaling in response to secretin. However, the dual mutant provided an extremely novel effect. This construct did express mild constitutive activity, in the range of 15% of the normal maximal cAMP response in these cells. It bound the natural agonist ligand with almost normal affinity, but rather than having this event result in a receptor conformation that promoted coupling with its G protein, it resulted in a receptor conformation that became and remained uncoupled. At this receptor construct, the natural agonist had become an inverse agonist. Presumably the determinants for ligand binding were still intact in the mutant receptor, but the net effect of ligand binding was to shift the conformation of the cytosolic surface of the receptor to one that did not effectively couple with G protein. This may provide important clues to the molecular basis for transmitting the conformational changes in receptors from the external sites of ligand binding to the cytosolic domain of G protein-coupling. It may also have interesting and important clinical implications.

Methods

Materials. Rat secretin-27 and its analogue, (Tyr<sup>10</sup>)-secretin-27, were synthesized in our laboratory, as we previously described (Ulrich et al., 1993). Enzymes for mutagenesis were purchased from Boehringer Mannheim (Indianapolis, IN). All other reagents were analytical grade.

Secretin receptor mutagenesis. Secretin receptor mutagenesis strategies included utilization of ligation at naturally occurring restriction sites, PCR mutagenesis, and applications of the method of Kunkel (1985). Constructs were built in the pBK-CMV eukaryotic expression vector (Stratagene, La Jolla, CA), with the wild-type rat secretin receptor cDNA positioned between the BamHI and HindIII sites. Constructs included the single site mutants of the secretin receptor, replacing His<sub>156</sub> with Arg (H156R) and Thr322 with Pro (T322P), and the dual mutant with both changes (H156R/T322P). H156R was generated using the Kunkel (1985) method, and T322P was prepared using PCR mutagenesis. Restriction digestion of the two constructs with BssHII and religation of relevant fragments was used to prepare the dual mutant.

All PCR reactions were performed in a thermocycler with Taq DNA polymerase utilizing 35 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. PCR and restriction digestion products were separated on 1% agarose gels and purified on QIAEX resin or on 1% low melt agarose gels before subcloning. The complete sequences of expected products of mutagenesis were confirmed by direct DNA sequencing, using the method of Sanger et al. (1977).

Secretin receptor expression. Receptor constructs were expressed transiently in COS-1 cells (American Type Culture Collection, Rockville, MD). Cells were maintained in culture on tissue culture plastic in DMEM with 5% Fetal Clone II (HyClone Laboratories, Logan, UT) in a humidified incubator at 37°C in an atmosphere of 5% CO<sub>2</sub>. They were transfected with amounts of DNA ranging from 11 ng to 2.7 μg per dish using the DEAE-dextran method, with dimethyl sulfoxide shock and 0.1 mM chloroquine diphosphate treatment (Lopata et al., 1984). The day after transfection, cells were lifted using trypsin, and were transferred to 24-well plates at a density of 1.0 × 10<sup>5</sup> cells/well. Cells were studied 72 hr posttransfection.

Secretin receptor signaling. The ability of receptor constructs to transduce a signal was assessed by quantitation of intracellular cAMP stimulated in response to secretin. COS cells that had been transfected 72 hr previously were washed in phosphate-buffered saline, and placed in KRH medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor, which was supplemented with 1 mM 3-isobutyl-1-methylxanthine. Cells were stimulated with secretin for 30 min at 37°C, and lysed by shaking for 15 min after the addition of ice-cold 6% perchloric acid. The pH was adjusted to 6.0 with KHCO<sub>3</sub>, and lysates were cleared by centrifugation at 2000 rpm for 10 min. Supernatants were used for determination of cAMP levels with a [3H] cAMP kit from Diagnostic Products Corp. (Los Angeles, CA) following the manufacturer’s instructions. Assays were performed in duplicate and repeated in at least three independent experiments. Radioactivity was quantified in a Beckman LS6000 scintillation counter.

Effect of pertussis toxin. In select experiments, cells were incubated for 18 hr at 37°C in the presence of 100 ng pertussis toxin/ml of medium before their being stimulated with secretin or acetylcholine (positive control). The cAMP assays were then performed as described above.

Secretin receptor binding. The radioligand was prepared by oxidative radioiodination of the secretin analogue, [Tyr<sup>10</sup>Phe<sup>27</sup>]secretin-27, with purification by reversed-phase high performance liquid chromatography to specific radioactivity of 2000 Ci/mmol, as we have described (Ulrich et al., 1993). Intact transfected COS-1 cells were mechanically lifted and suspended in KRH medium. After trituration, cells were incubated for 1 hr at room temperature with a constant amount of radioligand (3–5 pM) and concentrations of nonradioabeled secretin up to 1 μM. A Skatron cell harvester with glass fiber filtermat that had been soaked in 0.3% polybrense was used to separate bound from free radioligand. Bound radioactivity was measured with a gamma spectrometer. Nonspecific binding was assessed in the presence of excess unlabeled secretin (1 μM).

Effect of GppNHP. The stable analogue of GTP, GppNHP, was used in select experiments to determine the sensitivity of radioligand binding to this reagent. Experiments were performed analogous to the competition-binding experiments described above, except using enriched plasma membranes from the cells.

Data analysis. All observations were performed in duplicate and repeated at least three times in independent experiments and are expressed as means ± S.E.M. Binding data were analyzed using both the Prism software nonlinear regression analysis routine for radioligand binding (GraphPad Software, San Diego, CA) and the LIGAND program of Munson and Rodbard (Munson and Rodbard, 1980). Differences were determined by using Student’s t test, with P < .05 considered to be significant.

Results

Each of the secretin receptor constructs were synthesized and delivered to the COS cell surface in a form that was able to bind secretin radioligand with high affinity. Competition-binding curves are shown in figure 1. Binding to cells expressing the wild-type secretin receptor best fit a model having two affinity states with K<sub>s</sub> of 0.5 ± 0.1 and 23 ± 3 nM. More than 99% of the saturable binding sites were in the low affinity state. Binding data with the mutant constructs, how-
ever, did not fit a two-site model statistically better than a single site model. Affinities for that site were $5 \pm 1$ nM for the H156R receptor, $8 \pm 1$ nM for the T322P receptor and $6 \pm 1$ nM for the H156R/T322P receptor. These affinities were higher than that of the predominant low affinity site of the wild-type secretin receptor. Attempting to fit these curves to a two-site model resulted in the presence of too few high affinity sites in an affinity too close to that of the low affinity site to recognize.

The efficiency of biosynthesis and delivery to the cell surface was reduced for each of the receptor mutants relative to the wild-type secretin receptor. Using the same amount of DNA for each, the number of sites per cell were $7.6 \pm 1.2 \times 10^6$, $2.4 \pm 1.5 \times 10^6$, $2.5 \pm 0.4 \times 10^6$ and $3.4 \pm 1.1 \times 10^6$ for the WT, H156R, T322P and H156R/T322P mutants, respectively. The reduced level of surface expression for the mutants (single mutant receptor density reduced to approximately one-third the wild-type receptor density and double mutant receptor density reduced to approximately one-twentieth the wild-type receptor density) could not be overcome by transfecting cells with larger amounts of DNA (up to 2.7 μg/dish). A series of studies was, therefore, performed using reduced amounts of wild-type receptor construct. Over a range from 3 μg/dish to 100 ng/dish, direct radioligand binding analysis confirmed reduced receptor expression parallel with reduced DNA. The lowest dose resulted in expression of $3.8 \times 10^5$ receptors per cell. This was similar to the receptor density of the most sparsely expressed mutant receptor. Of note, despite this range in receptor expression, basal and maximal secretin-stimulated cAMP was not different from that observed with the higher receptor density used in all other studies in this series. This supports the presence of spare receptors under these transfection conditions, and the absence of constitutive basal activity of the wild-type secretin receptor.

Figure 2 illustrates cAMP responses to secretin for each of the receptor constructs. The top panel shows the absolute levels of cAMP per cell in response to secretin for each of the constructs, relevant to responses in cells transfected with wild-type secretin receptor (basal less than 1 pmol cAMP per tube, maximal 25 pmol cAMP per tube). The bottom panel expresses the same data normalized to the maximal responses observed in cells transfected with that receptor construct. Values represent means ± S.E.M. for data from a minimum of three independent experiments.
cept-receptor-expressing cells (P < .05). This was also true using matched receptor densities per cell for wild-type and mutant receptor constructs.

The bottom panel shows these responses normalized to the maximal responses of each receptor construct. This clearly demonstrates that secretin stimulated cAMP accumulation in cells expressing wild-type secretin receptor and those expressing the single mutant receptors, although it inhibited cAMP accumulation in cells expressing the dual mutant receptor construct. The EC_{50} values for H156R (0.9 ± 0.1 nM) and T322P (1.6 ± 0.8 nM) were not significantly different from that of the WT receptor (0.7 ± 0.1 nM). The dual mutant, however, responded paradoxically with a concentration-dependent inhibition of cAMP production (EC_{50} = 12.8 nM). cAMP production in response to 1 μM secretin fell to 42.3% of the unstimulated level of cAMP in cells expressing this construct (P < .01).

The data in figure 2 also demonstrate constitutive activity for each of the secretin receptor mutants. The unstimulated cAMP data for each receptor construct is again shown in figure 3a, with the addition of data from mock-transfected cells. Comparison of basal unstimulated cAMP levels for each of the constructs with that observed in control transfections demonstrated that the mutation in the sixth transmembrane domain (T322P) conferred significant constitutive activity (4.5 times that of control; P < .01), although the mutation in the second transmembrane domain (H156R) conferred statistically significant, but biologically modest constitutive activity (1.4 times that of control; P < .01). The receptor construct with both mutations (H156R/T322P) had basal cAMP production of 6.8 times that of control (P < .01). Another measure of constitutive activity is basal activity as a proportion of maximal response (fig. 3b). For the WT receptor, basal cAMP production was 3.6% of maximal, although for the mutant receptor constructs it was 32% (H156R; P < .01), 67% (T322P; P < .01) and 100% (H156R/T322P; P < .01) of the maximal responses observed.

Due to the unusual response to secretin observed in the dual receptor mutant, with the natural agonist acting as an inverse agonist, G protein coupling to this construct was investigated. The stable analogue of GTP, GppNHp, had no effect on the binding of the secretin radioligand to this receptor, although having an inhibitory effect on the binding of the same radioligand to the wild-type receptor (fig. 4). Pertussis toxin treatment to inactivate G, had no effect on the cAMP responses to secretin observed for wild-type receptor or the dual receptor mutant (fig. 5). This treatment, however, was effective in reducing the ability of acetylcholine to inhibit cellular cAMP in these cells.

**Discussion**

Circulating hydrophilic agonist ligands are dependent on the critical position of receptor molecules that span the plasma membrane lipid bilayer to transduce their signal and initiate intracellular effector machinery. Although conformational change in the receptor has been assumed to represent the mechanism for this transduction event, the precise nature of this change and the molecular basis to effect it are unclear. This is not surprising, given the current low resolution insight into the structure of these molecules (Unger and Schertler, 1995; Grigorieff et al., 1996). Most insights have been indirect.

The association of a heterotrimeric G protein with an active state of the receptor seems to be key for the initiation of intracellular signal cascades. As noted in the introduction, this can be achieved in the classical ternary complex with agonist ligand or in the few naturally occurring examples of constitutively active receptor mutations (Latronico et al., 1995; Parma et al., 1993; Robinson et al., 1992) or the more
numerous examples of artificially constructed receptor mutations having the same effect (Kjelsberg et al., 1992; Ren et al., 1993). One particularly interesting series of such mutations involved the replacement of a single residue near the carboxyl-terminal end of the third cytosolic loop of the alpha-1B adrenergic receptor (Ala293) with each of the 19 remaining amino acids (Kjelsberg et al., 1992). In every case, the mutant receptor was more active (higher basal activity) than the wild-type receptor, leading to the suggestion that this residue was key to hold the wild-type receptor in the least favorable conformation for G protein coupling, and that this constraint could be overcome by agonist binding or mutagenesis, with the combination of the two even more effective (Lefkowitz et al., 1993; Kenakin, 1996).

Most examples of constitutively active G protein-coupled receptors are in the rhodopsin-beta-adrenergic receptor family (Lefkowitz et al., 1993). This includes syndromes of hyperthyroidism (TSH receptor), male precocious puberty (luteinizing receptor) and visual disturbances (rhodopsin) (Latronico et al., 1995; Parma et al., 1993; Robinson et al., 1992). Only recently have similar examples of constitutively active receptors been described for the structurally distinct secretin receptor mutants, the same domain has likely been conserved Cys residues and presumed to have a conserved critical disulfide bonding pattern, appears to be a critical domain for ligand binding and activation (Jüppner et al., 1994; Holtmann et al., 1995; Cao et al., 1995; Vilaradaga et al., 1995). This insight comes from receptor mutagenesis and truncation studies. Other extracellular loop regions also contribute important complementary determinants (Holtmann et al., 1995, 1996). Thus, it is likely that such ligands normally exert tension across distinct extracellular receptor domains to effect movement of some transmembrane domain relative to another such domain or relative to the lipid bilayer, and to thereby expose a receptor domain that is key for G protein association. In the case of constitutively active secretin receptor mutants, the same domain has likely been exposed as a function of the missense mutations in the second or sixth transmembrane domains. With the single site mutants, the determinants for agonist binding are still accessible and further facilitate the exposure of this critical site of G protein association. In the unique setting of the dual mutant, although ligand binding determinants are still accessible, that binding interaction reduces the level of cellular cAMP rather than increasing it.

Secretin normally stimulates cellular cAMP accumulation as a result of coupling with Gs and activating adenylate cyclase (Trimble et al., 1987). The opposite effect of secretin at the dual mutant receptor could reflect a reduced level of normal coupling with Gs, or increased aberrant coupling with Gi that is known to inhibit adenylate cyclase. This family of receptors commonly exhibits promiscuous G protein coupling, but the G proteins normally involved are in the Gs and Gq families, with increased cAMP responses most sensitive and increased intracellular calcium response observed in response to high concentration of some agonists (Chabre et al., 1992; Abou Samra et al., 1992). Secretin also follows this pattern (Trimble et al., 1987).

Two series of experiments in this report support the interpretation of secretin binding to the dual mutant receptor resulting in reduced coupling with Gs, rather than increased aberrant coupling with Gi. This includes the persistence of the biological response in the presence of pertussis toxin receptors are consistent with predictions from other constitutively active G protein-coupled receptors, although the observation that natural secretin acts like an inverse agonist at the secretin receptor construct incorporating two mutations that individually produce constitutive activity is particularly novel. As with most of such previously described receptors, the constitutively active secretin receptor mutants had apparent shifts to higher affinities than wild-type receptor. The binding data for the wild-type secretin receptor best fit a two-state model, while the binding data for the mutants did not fit a two-state model significantly better than a single state model. Two states may actually exist for the mutants, but are less apparent, due to the low frequency of the high affinity state and to the shift in affinity of the low affinity state towards that of the high affinity state. This increase in affinity is consistent with our current models of agonist binding to constitutively active receptors (Lefkowitz et al., 1993; Kenakin, 1996).

The secretin family receptors all normally bind moderately large peptide ligands having diffuse pharmacophoric domains (Laburthe et al., 1996). The relatively long and complex amino-terminal tail of these receptors, having six highly conserved Cys residues and presumed to have a conserved critical disulfide bonding pattern, appears to be a critical domain for ligand binding and activation (Jüppner et al., 1994; Holtmann et al., 1995; Cao et al., 1995; Vilaradaga et al., 1995). This insight comes from receptor mutagenesis and truncation studies. Other extracellular loop regions also contribute important complementary determinants (Holtmann et al., 1995, 1996). Thus, it is likely that such ligands normally exert tension across distinct extracellular receptor domains to effect movement of some transmembrane domain relative to another such domain or relative to the lipid bilayer, and to thereby expose a receptor domain that is key for G protein association. In the case of constitutively active secretin receptor mutants, the same domain has likely been exposed as a function of the missense mutations in the second or sixth transmembrane domains. With the single site mutants, the determinants for agonist binding are still accessible and further facilitate the exposure of this critical site of G protein association. In the unique setting of the dual mutant, although ligand binding determinants are still accessible, that binding interaction reduces the level of cellular cAMP rather than increasing it.

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treatment to inactivate $G_{o}$ and the absence of effect of stable analogues of GTP (GppNHp) on secretin binding to this unique receptor construct, although having an inhibitory effect on secretin binding to wild-type receptor. This is most consistent with this natural agonist ligand having a protean effect, acting as a full agonist at the wild-type receptor and as an inverse agonist at the dual mutant receptor construct.

In addition to providing important insights into the molecular basis for G protein-coupled receptor action, this may also have clinical relevance. Although to date no such occurrence has been recognized, this suggests that a specific missense mutation or combination of such changes in a receptor could not only lead to constitutive activity, but also to reversal of normal responses to its natural endogenous agonist ligand. Since all physiologic mechanisms for feedback inhibition of biological responses are designed to limit normal effects, such mechanisms would even be expected to worsen the biological implications of this type of receptor mutation. If such a signaling system normally resulted in cell growth or trophic action, the mutant receptor could contribute to atrophy, although a normal allele might protect the tissue from this. However, if such a signaling system normally resulted in slowed growth or cellular activity, this type of mutant receptor could lead to uncontrolled growth or activity, with normal feedback inhibitory pathways potentially worsening the biological response.

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References


