

Role of Receptor Phosphorylation in Desensitization and Internalization of the Secretin Receptor*

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The secretin receptor is prototypic of a recently described family of G protein-coupled receptors. We recently demonstrated its phosphorylation in response to agonist stimulation and elimination of this covalent modification by C-terminal truncation (F. Ozcelebi *et al.* (1995) *Mol. Pharmacol.* 48, 818–824). Here, we explore the functional impact of receptor phosphorylation and structural determinants for desensitization by comparing receptor behavior after agonist exposure in cell lines expressing wild-type and truncated receptor. To characterize receptor internalization, a novel fluorescent full agonist, [rat secretin-27]-Gly-rhodamine, was developed, which bound specifically and with high affinity. Both receptor constructs bound secretin normally, leading to normal G protein coupling and cAMP accumulation and prompt receptor internalization. Exposure to 10 nM secretin for 5 min or 12 h prior to washing and restimulation with a full range of concentrations demonstrated absent cAMP responses in wild-type receptor-bearing cells and responses 25 to 30% of control and shifted 1 order of magnitude to the right in the truncated receptor-bearing cells. Thus, the major mechanism of desensitization was phosphorylation-independent receptor internalization. Phosphorylation was associated with a distinct process that likely represents interference with G protein coupling, manifest as a reduced rate of cAMP stimulation. Thus, dual distinct mechanisms of desensitization exist in the secretin receptor family that should help protect receptor-bearing cells from overstimulation.

Desensitization in response to agonist stimulation is a ubiquitous property of biological systems, which helps to prevent overstimulation and potential damage. Although mechanisms for this exist at many levels within the cell, a number of processes have been reported to affect the initial step in stimulus-activity coupling, agonist binding to cell surface receptors. These have been best characterized for the β -adrenergic receptor family of guanine nucleotide-binding protein (G protein)-coupled receptors (2), where receptor phosphorylation has been identified as playing a key role (3). It has been implicated in mediating the binding of an arrestin-like protein that interferes with coupling of the receptor to its G protein, as well as affecting the cycle of receptor sequestration and resensitization (4).

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However, it is not clear if the same mechanisms play a similar role in a recently recognized and distinct family of receptors within this superfamily, the secretin receptor family (5). This family of G protein-coupled receptors shares little structural homology with receptors in the β -adrenergic receptor family of this superfamily (6–8). The types of natural ligands differ, with the secretin receptor family binding moderately large peptide ligands having a diffuse pharmacophore (5, 9, 10). Binding motifs also differ, with the secretin receptor family having a large and complex extracellular domain that appears to contribute key binding epitopes (11–13). Even the large number of signature sequences that are conserved throughout the β -adrenergic receptor family, and presumably play some structural role there, are absent in the secretin receptor family (6, 8, 14).

Although there is clear physiological evidence that receptors in the secretin receptor family exhibit agonist-stimulated desensitization (15–17), the molecular mechanisms for their desensitization are not clearly delineated. Like some members of the β -adrenergic receptor family, the secretin receptor is phosphorylated in response to agonist occupation (1). We have shown that this occurs rapidly in an agonist concentration-dependent manner and involves serine and threonine residues within the C-terminal tail of that receptor (1). The functional impact of this modification of the receptor, however, is not known. Another potentially important process for desensitization, receptor internalization, has been quite difficult to study in this family due to the difficulties in producing appropriately tagged ligands or receptor antibodies.

In this work, we have used a unique CHO cell line that we established to express a truncated form of the secretin receptor missing 27 residues at its C terminus, which binds secretin and signals normally, yet is not phosphorylated (1). By comparing receptor function in this line to that in an analogous CHO cell line which expresses the wild-type receptor, it has been possible to explore the functional roles of this region of the receptor and its regulated phosphorylation. Furthermore, we developed and characterized a novel fluorescently tagged, biologically active analogue of secretin that has allowed us to directly follow agonist-stimulated internalization of this receptor.

With these reagents and techniques, we have demonstrated that this receptor is completely desensitized in response to acute and chronic exposure to high concentrations of secretin, and that it undergoes agonist-stimulated internalization. The presence of the C-terminal domain of the secretin receptor, which is the site of its agonist-stimulated phosphorylation, contributes to a more prolonged yet lower amplitude signaling (cAMP) response, which is consistent with dynamic modulation of G protein coupling. However, elimination of this domain had no effect on receptor internalization, supporting the distinct determinants for that process. This provides clear and important new insights for a prototypic member of the secretin receptor family.

MATERIALS AND METHODS

Reagents—Secretin and its radioiodinatable, biologically active analogue, [Tyr¹⁰,pNO₂-Phe²²]rat secretin-27], were synthesized as we described previously (18). We also synthesized a new, biologically active, fluorescent analogue of secretin for morphologic analysis of cellular handling of the agonist-occupied receptor. [Rat secretin-27]-Gly-rhodamine (Rho-sec)¹ was designed based on existing structure-activity relationships (19) to position the fluorophore at the C terminus of the secretin analogue. This 29-residue peptide had to be synthesized completely *de novo*, since derivatization of an existing peptide would place the fluorophore in an N-terminal position, which interferes with biological activity. Synthesis was accomplished using manual, solid-phase techniques analogous to those used previously (18, 20).

Restriction enzymes were purchased from Boehringer Mannheim. All other reagents were analytical grade.

Receptor Constructs and Cell Lines—As we reported recently, the wild-type and truncated rat secretin receptor constructs, WTSecR and SecR,1–398, were cloned from a pancreatic cDNA library and produced by polymerase chain reaction mutagenesis, respectively (1, 18). Final constructs were inserted into the *Bam*HI and *Hind*III sites of the eukaryotic expression vector, pBK-CMV (Stratagene, La Jolla, CA). Sequences were confirmed by direct sequence analysis using the dideoxynucleotide chain termination method of Sanger *et al.* (21). These were then used to transfect CHO-K1 cells (American Type Culture Collection), and lines of receptor-bearing cells were established by G418 resistance and cell sorting (22). Cell lines were cultured in Hams F-12 medium with 5% Fetal Clone 2 (HyClone Laboratories, Logan, UT) on Falcon plasticware.

Biological Activity Studies—Intracellular cAMP levels were determined by radioimmunoassay with a [³H]cAMP tracer, using reagents from Diagnostic Products Corp. (Los Angeles, CA). This assay is sensitive to 0.1 pmol cAMP/tube, with the standard curve ranging from 0.1 to 27 pmol/tube, and the EC₅₀ of 1 pmol/tube. Cross-reactivity with cGMP is less than 0.1%. Wild-type and mutant secretin receptor-bearing CHO cells were studied in suspension, after being rinsed with PBS, pH 7.4, and harvested mechanically. Cells were then resuspended in Krebs-Ringers-Hepes (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 2.5 mM D-glucose, 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, essential and nonessential amino acids, and glutamine) and triturated by gentle pipetting. For generation and subsequent assay of cAMP responses, 1 mM 3-isobutyl-1-methylxanthine was incorporated into the medium.

For dose-response studies, cells were incubated with varied concentrations of secretin for 10 min at 37 °C. Time course experiments used 100 nM secretin at 37 °C for the noted intervals. For desensitization studies, aliquots of each cell line were incubated with 10 nM secretin for 12 h and for 5 min and were then washed thoroughly three times with PBS, prior to stimulation with the full range of concentrations of secretin for 10 min at 37 °C.

At the end of each incubation, cAMP generation was stopped by the addition of ice-cold 6% perchloric acid, the pH was adjusted to 6.0 with KHCO₃, and lysates were cleared by centrifugation at 2000 rpm for 10 min at 4 °C. Cyclic AMP content was then determined in supernatants. All conditions were assayed in duplicate in a minimum of three independent experiments.

Radioligand Binding—The secretin analogue, [Tyr¹⁰,pNO₂-Phe²²]rat secretin-27], was radioiodinated and purified by high performance liquid chromatography to yield a specific radioactivity of 2000 Ci/mmol, as we described previously (18). Intact CHO-WTSecR cells and CHO-SecR,1–398 cells were suspended in KRH medium and incubated with a constant amount of radioiodinated secretin (3–5 pM) and increasing concentrations of non-radiolabeled peptide (0–1 μM) for 1 h at room temperature. For separation of bound from free radioligand, a Skatron cell harvester with glassfiber filtermats that had been soaked in 0.3% Polybrene were used. Bound radioactivity was quantified in a gamma spectrometer. Nonspecific binding was assessed in the presence of excess unlabeled secretin (1 μM). Data were analyzed and plotted using the Prism software package (GraphPad, San Diego, CA).

Receptor Internalization and Recycling—Internalization of agonist-occupied secretin receptor was assayed morphologically, using the fluorescent Rho-sec analogue described above, and following experimental protocols we described previously (23). Receptor-bearing CHO cells were grown on glass coverslips in 24-well tissue culture plates. Immediately before labeling, they were washed three times with PBS at 37 °C

and once at 4 °C. Labeling occurred for 1 h at 4 °C with 100 nM Rho-sec. For the 0 time point, the cells were washed with iced PBS and immediately fixed with 2% paraformaldehyde in PBS. The other coverslips were washed with 37 °C PBS and incubated at 37 °C for 1, 5, and 30 min, then fixed and subsequently mounted on slides. The specificity of secretin receptor labeling was determined in experiments incorporating competition with 100-fold excess unlabeled peptide and by attempting to label untransfected CHO cells. Cells were examined with a Zeiss inverted microscope equipped for epifluorescence. Pictures were taken with a 35-mm camera using TMAX 3200 film.

To determine whether secretin receptors returned to the plasma membrane following internalization, we conducted internalization experiments as described above but initially used nonfluorescent native secretin and cycloheximide-treated receptor-bearing cells. After internalization at 37 °C, the cells were washed three times with iced PBS and then incubated with the fluorescent Rho-sec analogue at 4 °C for determination of surface receptors. Specimens were fixed and analyzed as described above.

Statistical Analysis—Results are expressed as means ± S.E. of a minimum of three independent experiments. When appropriate, differences were assessed using the Mann-Whitney nonparametric test of unpaired values, with *p* < 0.05 considered to be statistically significant.

RESULTS

Characterization of Secretin Receptor-bearing CHO Cell Lines—We previously established the abilities of the C-terminally truncated secretin receptor construct to bind secretin normally and to elicit normal cAMP responses to secretin when expressed transiently in COS cells (1). Consistent with that, secretin stimulated similar biological responses in a concentration-dependent manner in both of the receptor-bearing CHO cell lines. The wild-type receptor-bearing cells (CHO-WTSecR) responded to secretin with an EC₅₀ of 2.7 ± 1.4 nM, whereas the truncated secretin receptor-bearing cells (CHO-SecR,1–398) responded with an EC₅₀ of 2.9 ± 0.3 nM. The magnitude of the responses was also not statistically different (CHO-WTSecR, 7.8 ± 2.9-fold increase over basal values; CHO-SecR,1–398, 5.2 ± 1.1-fold increase over basal values).

Characterization of the Fluorescent Secretin Analogue—Rho-sec bound to the secretin receptor with high affinity, as demonstrated in competition binding for a well-characterized radiolabeled secretin analogue (Fig. 1). Rho-sec competed for radioligand binding with the same potency as the native peptide (IC₅₀, 2.3 ± 1.1 nM; *p* = 0.5). It was also a full agonist, stimulating cAMP responses in CHO-WTSecR cells with an EC₅₀ of 1.0 ± 0.2 nM, which was not different from that of the native peptide (*p* = 1.1) (Fig. 1).

Desensitization—Secretin responsiveness of the cells expressing the wild-type receptor (CHO-WTSecR cells) was completely desensitized after exposures to 10 nM secretin of 5 min and 12 h. After such exposures and extensive washing, stimulation with increasing concentrations of secretin did not elicit any cAMP responses (Fig. 2). However, when the truncated receptor-bearing CHO-SecR,1–398 cells were pretreated with 10 nM secretin, they were still capable of responding to stimulation with high concentrations of agonist, although the responses were considerably blunted and shifted to the right by 1 order of magnitude (Fig. 2). This was true both after 5-min and 12-h preexposures (generating 25 and 30% of the maximal responses observed in the control incubation with cells which had not been pretreated with hormone). These responses were significantly different from those for the wild-type receptor (*p* < 0.02).

To further explore potential desensitization that might occur at very early time points after agonist exposure, detailed time course experiments were performed. Rates of cAMP generation were determined from data representing cAMP that was accumulated within the cell in the presence of a phosphodiesterase inhibitor. These data were fit to a curve using the Prism software package (GraphPad). The best fit was a single-phase

¹ The abbreviations used are: Rho-sec, [rat secretin-27]-Gly-rhodamine; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.

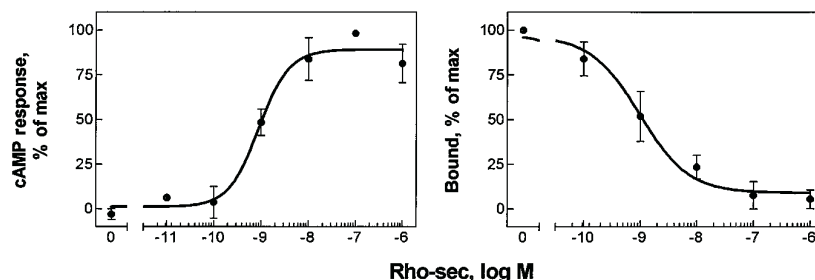


FIG. 1. **Characterization of fluorescent secretin analogue.** Shown are cAMP responses to various concentrations of Rho-sec (*left*) and the ability of this hormone analogue to compete for secretin radioligand binding to its receptor on membranes from WTSecR cells (*right*). Data are expressed as the means (bars, S.E.) of three independent experiments. cAMP responses are normalized relative to maximal responses, whereas binding data represent saturable binding relative to the control condition in the absence of competitor.

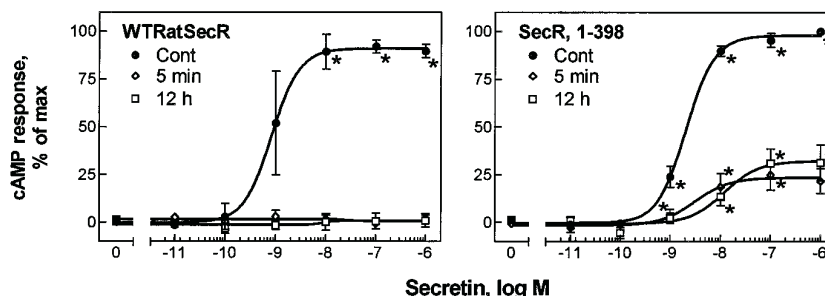


FIG. 2. **Desensitization of secretin receptor-bearing cell lines, CHO-WTSecR (*left*) and CHO-SecR,1-398 (*right*).** Shown are cAMP responses to increasing concentrations of secretin after pretreatment with 10 nM secretin for either 5 min or 12 h. The control curves for cAMP responses to secretin in cells not pretreated with hormone are shown as reference. Values are expressed as means (bars, S.E.) of four experiments in CHO-WTSecR cells and seven experiments in CHO-SecR,1-398 cells, with values expressed relative to maximal responses in controls. Statistically significant increases in cAMP responses over basal levels are indicated with and asterisk (*).

exponential of the form, $Y = Y_{\max}(1 - e^{-KX})$. As can be seen in Fig. 3, the raw data fit these curves extremely well. Data analysis using a dual exponential curve fit was not statistically better than this curve (data not shown). Also shown in Fig. 3 are curves representing the first derivatives of these equations to reflect rates of cAMP generation. The rate of cAMP generation was much more rapid in the cells expressing the truncated SecR,1-398 receptor than those expressing the wild-type receptor.

Internalization and Recycling—To study internalization of the agonist-occupied secretin receptor constructs, the fluorescent secretin analogue Rho-sec was used in a direct morphologic assay. This fluorophore labeled the secretin receptor specifically and with high affinity, with no labeling observed in untransfected CHO cells and with the labeling of the receptor-bearing cells eliminated in the presence of competing nonfluorescent secretin (Fig. 4).

Fig. 5 shows representative fluorescent images of the two cell lines over time. At the end of the 1-h incubation period at 4 °C, both CHO-WTSecR cells and CHO-SecR,1-398 cells were labeled throughout their plasmalemmal surface. Upon warming to 37 °C, the fluorescent labeling patterns changed rapidly. After 1 min, the pattern of fluorescence became more punctate at or near the plasma membrane in both cell lines. By 5 min, most of the fluorescence had moved to the perinuclear region of these cells, where it remained over a period of 30 min. There was no consistent or quantifiable difference in the internalization of the fluorescent agonist-receptor complex between the two cell lines.

Receptor recycling studies were conducted after exposure of the cells incubated with nonfluorescent native secretin by probing for surface secretin receptor with fluorescent ligand. A decrease in the fluorescent signal on the plasma membrane was observed at 30 min, representing receptor internalization. Cell surface fluorescence increased after 60 min, indicating that secretin receptors returned to the plasma membrane fol-

lowing internalization. These results are similar to those we observed previously for the analogous CCK receptor-bearing CHO cell line (23).

DISCUSSION

Agonist-stimulated phosphorylation of the secretin receptor, limited to threonine and serine residues within the C-terminal tail, can be eliminated by truncation of this domain of the receptor, resulting in a molecule that binds agonist ligands normally, couples to its G protein normally, and elicits a cAMP response with normal agonist concentration dependence. This covalent modification of the secretin receptor, which has been functionally linked to mechanisms for desensitization of receptors in the β -adrenergic receptor family, likely plays a similar role for the secretin receptor, since its absence in the truncated receptor construct was associated with reduced desensitization. Of particular interest, however, was the observation that substantial agonist-induced desensitization still occurred in the absence of receptor phosphorylation. These observations led us to postulate the existence of at least two separate mechanisms for desensitization of receptors in the secretin receptor family, with each having distinct structural determinants.

The most prominent mechanism for desensitization observed was secretin receptor internalization. This process moves the receptor to an intracellular location in which the very hydrophilic natural peptide ligand cannot bind and activate it. This process was totally independent of receptor phosphorylation, since it proceeded similarly in cell lines expressing both the wild-type receptor and the C-terminally truncated receptor construct. The kinetics of internalization were indistinguishable in these cell lines. It is important that we were able to observe this process directly in a morphological assay. Although internalization of secretin family receptors has been suggested based on acid washing data (24), we now understand that such observations are not specific for internalization (25). In a recent report, receptor movement into a compartment of

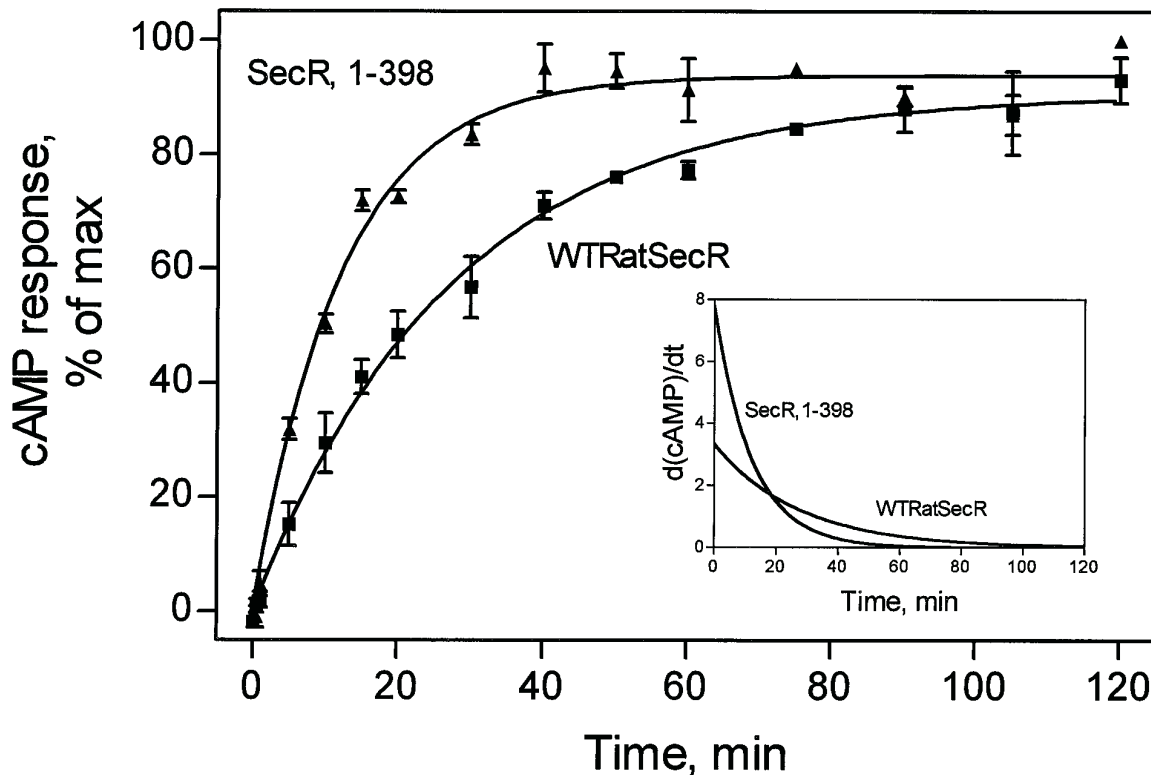


FIG. 3. **Time courses of cAMP generation** CHO cell lines expressing secretin receptor constructs. Data points represent cAMP responses to 100 nM secretin over time. Values represent means (bars, S.E.) of three independent experiments, with data normalized relative to maximal responses that were not significantly different from each other. The curves represent the single-phase exponentials best fitting each data set, with $Y = 93.86 (1 - e^{-0.082t})$ for SecR,1-398 and $Y = 90.79 (1 - e^{-0.037t})$ for WTSecR. The time point of half-maximal increase in cAMP was 8.4 min for the truncated receptor (95% confidence interval, 7.3 to 10 min) and 18.9 min for the wild-type receptor (95% confidence interval, 17.3 to 20.9 min). The rate of cAMP generation of the receptor constructs, reflected in the first derivative of these equations ($Y = 7.73 e^{-0.082t}$ for SecR,1-398 and $Y = 3.33 e^{-0.037t}$ for WTSecR shown in the inset graph) suggests that the truncated receptor elicited cAMP generation with a rate two times higher than the wild-type receptor.

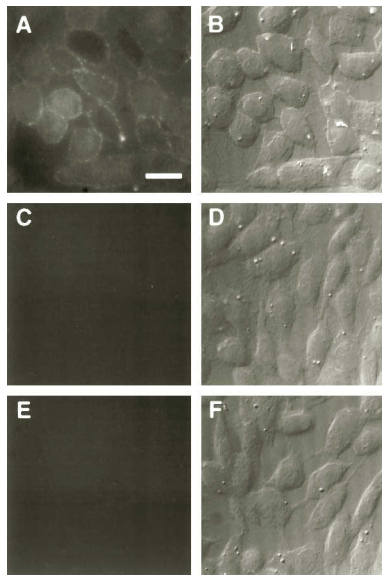


FIG. 4. **Fluorescent labeling of the secretin receptor with Rho-sec.** Shown are fluorescent (a and c) and differential interference contrast (b and d) images of CHO-WTSecR cells labeled for 1 h at 4 °C with 100 nM Rho-sec (a and b) and those labeled under identical conditions in the presence of 100-fold excess secretin (c and d). Fluorescent (e) and differential interference contrast (f) images of untransfected CHO cells labeled under identical conditions are also shown. Images are representative of three independent experiments. Bar, 20 μ m.

“insulation” at the level of the plasmalemma, which is relatively depleted in G proteins, also resulted in resistance to acid elution (25). It is difficult to directly demonstrate internaliza-

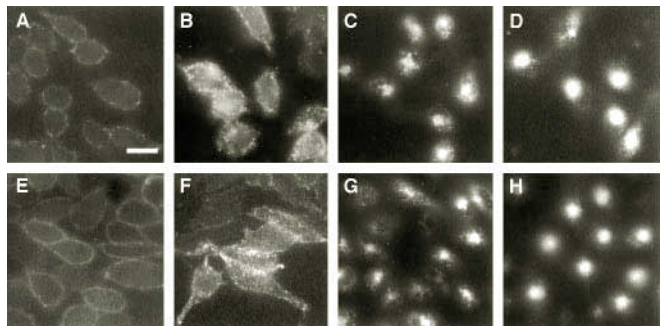


FIG. 5. **Time course of internalization of the Rho-sec-occupied secretin receptor constructs in CHO-SecR,1-398 (a-d) and CHO-WTSecR (e-h) cells.** Cells were preincubated at 4 °C in 100 nM Rho-sec, washed, and warmed to 37 °C for 1, 5, and 30 min. Aliquots were fixed with paraformaldehyde at time 0 or after warming for the noted times. Both cell lines were labeled diffusely over their plasmalemmal surfaces at the end of the preincubation period (a and e). After 1 min of warming, the fluorescence displayed a more punctate pattern at or near the plasmalemma (b and f). By 5 min, most fluorescence was present in vesicular structures in the perinuclear region (c and g), with this pattern persisting through the 30-min time point (d and h). Images are representative of three independent experiments. Bar, 20 μ m.

tion of receptors in this family, due to structure-activity considerations in producing fluorescent ligands. Unlike peptide hormone receptors in the β -adrenergic receptor family with ligands that can often be easily derivatized by chemical methods at their N terminus since their critical binding determinants reside at their C terminus, ligands for receptors in the secretin receptor family have been suggested to have critical binding determinants present more diffusely (5, 8, 9). Of interest, the N-terminal histidine residue of secretin has been

shown to be a critical binding determinant that cannot be derivatized without interfering with its activity at this receptor (26, 27). Therefore, when it came to designing a fluorescent analogue of secretin for the morphologic studies, there was no clear opportunity to acylate the α amino group or to derivatize the native peptide. Instead, we had to synthesize a fluorescent derivative *de novo*, to position the rhodamine at its C terminus. Precedent existed for us to modify secretin at its C terminus without interfering with its binding and biological activity (19). Indeed, in this work we demonstrated that the derivative is a full agonist and binds with high affinity to the secretin receptor.

Although receptor phosphorylation can be ruled out as a positive signal for internalization, the structural basis for this process is unclear. Many receptors in the G protein-coupled superfamily have an "NPX₂₋₃Y" motif in their seventh transmembrane domain, and this has been implicated in internalization of the β -adrenergic receptor (28). Of interest, this motif is not required for internalization of the gastrin-releasing peptide and angiotensin II receptors (29, 30). Such a motif is absent in the secretin receptor family. In addition to phosphorylation, our observations also allow us to rule out any sequence within the C-terminal 27 residues that were eliminated by truncation in the receptor construct which internalized normally. Additional mutagenesis will be necessary to further localize a potential internalization signal that might be exposed by the conformational change induced by agonist occupation of this receptor.

The potential phosphorylation-dependent desensitization mechanism was more difficult to clearly define. The existence of such a process is supported by the desensitization assays performed in cells that had been exposed to secretin for 5 min or 12 h prior to washing and restimulation with secretin. After these treatments, the wild-type receptor-bearing cells were completely unresponsive, whereas those expressing the truncated receptor had a significant, although reduced, response. This response was of lower amplitude and required a higher concentration of secretin to elicit than the control response in the same cells that had not been preincubated with hormone. We believe that this is an effect of receptor phosphorylation, rather than representing an effect of some other determinant that might have been eliminated by the truncation, since this region of the tail of the wild-type receptor was associated with normal signaling before that receptor was modified by this agonist-stimulated posttranslational modification. The phosphorylation likely interfered with G protein coupling, analogous to a process reported for the β -adrenergic receptor (3). Like that receptor, this could be an indirect effect that is mediated by binding of an arrestin-like protein (4), or it could be a direct effect of the phosphorylation.

Although we do not have direct evidence for receptor phosphorylation interfering with G protein coupling, the differences in the kinetics of generation of cAMP observed in the cell lines expressing wild-type and truncated secretin receptors may well reflect this. Despite similar secretin-binding affinities and similar concentration dependences of secretin-stimulated cAMP accumulation, the receptor construct that lacked its C-terminal 27 residues exhibited more rapid stimulation of cAMP accumulation in response to secretin than did the wild-type receptor. This analysis is most reflective of the time points after 1 min of agonist stimulation, when we know that the wild-type receptor has already achieved its maximal level of phosphorylation, which remains at that plateau through at least 30 min (1). Due to the extremely small amounts of cAMP generated in the first seconds of agonist exposure, before phosphorylation of the receptor is apparent, we cannot be certain that the initial kinetics

of cAMP generation in the wild-type receptor were not actually similar to those of the truncated receptor.

The observed data for the kinetics of cAMP generation best fit a single exponential event for both of the receptor constructs, even though the rate constant was different for each. We know that both internalize the receptors similarly; however, that process is much slower than the kinetic event described. Since a dual exponential process did not fit the data better than the single exponential, it is likely that internalization was not limiting for the observed kinetics of stimulation of cAMP. The only events that we now understand which occur in the time frame necessary to affect the kinetic event of interest are the changes in receptor conformation that occur upon agonist occupation and the phosphorylation of that receptor. Presumably, the conformational changes are not the primary explanation since the concentration dependence for secretin to stimulate cAMP production was similar for both cell lines. Conversely, there is a clear and well-documented difference in phosphorylation of the two receptor constructs.

Because it was not possible to directly measure the rate of cAMP generation in the first few seconds of agonist stimulation, before the wild-type receptor was phosphorylated, it is theoretically possible that the slower rate of cAMP generation in the wild-type receptor construct reflected stable steric inhibition provided by the C-terminal domain, rather than slowing of this dynamic event by the phosphorylation. To definitively distinguish between these possibilities will require selective interference with the phosphorylation itself, either by site-directed mutagenesis or by kinase inhibition. The former would require the identification of the actual sites of phosphorylation among the 10 potential candidates within this domain. However, even if such mutations were established and reproduced the effects of the truncated construct, it would still be possible that this resulted from secondary effects on conformation rather than by a direct effect of a phosphorylated residue. The second approach of kinase inhibition would likely interfere nonspecifically with a large number of other cellular events that are regulated by phosphorylation.

This work provides evidence for two independent mechanisms for secretin receptor desensitization that possess different structural determinants. This duplication of mechanisms helps assure protection of the cell from agonist overstimulation. Similar dual distinct mechanisms for desensitization have been described for receptors in the β -adrenergic receptor family (2, 3). The quantitative importance of the different mechanisms for receptor desensitization will likely vary with different receptors and different cell types. Each cell may possess differences in sensitivity to damage by overstimulation and have differences in the need to maintain sensitivity to agonist stimulation. In our CHO cell model system, internalization appears to be the predominant mechanism for desensitization of the secretin receptor, unlike what has been described for the β -adrenergic receptor in an analogous heterologous expression system (31, 32). However, since the CHO cell is not a natural site of expression of the secretin receptor, the relative importance of internalization *versus* phosphorylation-induced interference with G protein coupling may be different in such a cell. This will ultimately have to be established in each different native cellular environment. The techniques developed in this work and the insights provided here should be valuable tools to facilitate this.

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