CCK receptor phosphorylation exposes regulatory domains affecting phosphorylation and receptor trafficking

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Rao, Ram Mohan V., Eileen L. Holicky, Susan M. Kuntz, and Laurence J. Miller. CCK receptor phosphorylation exposes regulatory domains affecting phosphorylation and receptor trafficking. Am J Physiol Cell Physiol 279: C1986–C1992, 2000.—Agonist-stimulated phosphorylation of guanine nucleotide-binding protein (G protein)-coupled receptors has been recognized as an important mechanism for desensitization by interfering with coupling of the activated receptor with its G protein. We recently described a mutant of the CCK receptor that modified two of five key sites of phosphorylation (S260,264A) and eliminated agonist-stimulated receptor phosphorylation, despite normal ligand binding and signaling (20). As expected, this nonphosphorylated mutant had impaired rapid desensitization but was ultimately able to be desensitized by normal receptor internalization. Here we demonstrate that this mutant receptor is also defective in resensitization, with abnormal recycling to the cell surface. To explore this, another receptor mutant was prepared, replacing the same serines with aspartates to mimic the charge of serine-phosphate (S260,264D). This mutant was expressed in a Chinese hamster ovary cell line and shown to bind CCK normally. It had accelerated kinetics of signaling and desensitization and was phosphorylated in response to agonist occupation, with all other normal sites of phosphorylation modified. It was internalized like wild-type receptors and was resensitized and trafficked normally. This provides evidence for an additional important function for phosphorylation of G protein-coupled receptors. Phosphorylation may induce a conformational change in the receptor to expose other potential sites of phosphorylation and to expose domains involved in the targeting and trafficking of endosomes. The hierarchical phosphorylation of these sites may play a key role in receptor regulation.

G protein-coupled receptor; receptor phosphorylation; endocytosis

GUANINE NUCLEOTIDE-BINDING protein (G protein)-coupled receptors represent the largest superfamily of plasma membrane receptors, likely expressed on every excitable cell in the body. The regulation of these receptors is particularly important to prevent overstimulation that may be damaging to the cell and to maintain an appropriate level of hormonal responsiveness. Receptor phosphorylation has been proposed as an important mechanism for a very proximal step in this regulation by rapidly interfering with the coupling of G proteins to the activated receptor (7, 10, 13, 26). The details of receptor phosphorylation, including the enzymes involved, the sites modified, and the general functional impact of this modification, have been the focus of many studies on multiple receptors.

Many receptors in the G protein-coupled receptor superfamily are phosphorylated on multiple sites. Two major classes of kinases acting on these receptors have been identified: the signaling kinases with action initiated by events within the activated signaling cascades emanating from the receptor and the G protein-coupled receptor kinases whose action is dependent on the active conformation of the receptor (1). Multiplicity of kinases and potential sites of phosphorylation suggest the opportunity for differential receptor regulation. In the β2-adrenergic receptor, protein kinase A and β-adrenergic receptor kinase have been shown to act on distinct receptor domains (3), and recent work suggests that differential phosphorylation of the receptor by each kinase may modify the selectivity of G protein coupling with shift from calcium to cAMP signaling (6). In this work, we postulate that agonist-stimulated receptor phosphorylation also plays additional distinct roles that add flexibility and cell specificity to the themes of receptor action and regulation.

We have accumulated substantial experience exploring various aspects of the agonist-stimulated phosphorylation of the CCK receptor (4, 11, 14, 17–20). This includes examination of the kinases involved, the stoichiometry and specific sites of phosphorylation affected, and even the involvement of a regulated receptor protein phosphatase. Both staurosporine-sensitive and -insensitive kinases act on the CCK receptor to phosphorylate five serine and threonine residues within the third intracellular loop and carboxy-terminal tail domains. Protein kinase C is an important enzyme that is activated in the signaling cascade initiated by receptor occupation with CCK and has been shown to be responsible for most of the agonist-stimulated phosphorylation of this receptor when it is expressed in the model cell system, the Chinese hamster...
ovary CCK receptor (CHO-CCKR) cell (19). By serendipity, we recently constructed a mutant CCK receptor (S260,264A) that eliminated two of the prominent sites of protein kinase C action within the third intracellular loop, resulting in total elimination of agonist-stimulated phosphorylation of this receptor (20). This construct bound ligand and signaled normally but interfered with the access of relevant kinases (including protein kinase C) to their other normal sites of action.

In the current work, we found that the nonphosphorylated S260,264A mutant receptor did not undergo the expected normal desensitization, with the occupied receptor delayed in the endosomal compartment and not recycling to the plasma membrane. To explore the mechanisms of this, we constructed another CCK receptor mutant in which the same serine residues were changed to aspartates to mimic the charge of serine-phosphates in these positions and expressed this in a CHO cell line. This strategy was previously used successfully (8, 9), with the acidic aspartate residue mimicking the charge of the phosphoserine, even though it is slightly smaller than that residue. This construct, S260,264D, had a number of extremely interesting differences from the wild-type receptor and from the S260,264A mutant receptor expressed in the same type of cell. We believe that these differences provide important insights into previously unrecognized events. Site-directed mutagenesis of the rat CCK Type A receptor delayed in the endosomal compartment (20). It also supports a regulatory role for the endocytic cargo in targeting and trafficking of the internalized receptor.

**METHODS**

**Reagents.** CCK-8 was synthesized or purchased from Peninsula Laboratories (Belmont, CA). The fluorescent CCK analog rhodamine-Gly-[(Nle28,31)CCK-(26–33)] (Rho-CCK) was prepared as we have described (23). [3H]inositol 1,4,5-triphosphate (IP3; 20 Ci/mmol) was purchased from DuPont-NEN (Boston, MA). All other chemicals were analytical grade.

**Cell preparations.** We previously established and characterized CCK receptor-expressing CHO-CCKR cells (5). These express ~125,000 wild-type rat CCK-A receptors per cell and bind and signal normally in response to hormone. Cells were cultured in Ham's F-12 medium containing 5% Fetal Clone 2 supplement (HyClone Laboratories, Logan, UT) in tissue culture plastic ware in a 37°C humidified environment containing 5% CO2. Analogous cell lines were also prepared to express the S260,264A mutant CCK receptor, which was previously characterized (20), and a new S260,264D mutant CCK receptor construct. The DNA sequence of the resulting construct was determined using the dideoxynucleotide chain termination method (24). The S260,264D CCK receptors were expressed in similar density to the wild-type receptors expressed on the CHO-CCKR cells. The S260,264A mutant has been shown to bind, signal, and be internalized normally (20). The S260,264D mutant will be characterized as part of this work. These cells were maintained in culture similarly to the CHO-CCKR cells.

**CCK receptor binding.** CCK radioligand binding was performed using conditions previously established (5). Binding was performed using intact cells grown in 24-well culture dishes (approximately one million cells per well), incubated at room temperature for 1 h in Krebs-Ringer-HEPES medium (KRH) containing (in mM) 25 HEPES, pH 7.4, 104 NaCl, 5 KCl, 1.2 MgSO4, 2 CaCl2, 1 KH2PO4, 1 phenylmethylsulfonyl fluoride, and 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor. Incubations included 3–5 pM radioligand ([125I]-labeled b-Tyr-Gly-[(Nle28,31)CCK-(26–33)]) and various concentrations of unlabeled competing peptides. Cells were washed with iced medium and then lysed with 0.5 M NaOH to release bound radioligand that was quantified in a gamma spectrometer. Binding data were analyzed using the LIGAND program of Munson and Rodbard (15) and were graphed with the nonlinear regression analysis for radioligand binding in the Prism software (GraphPad Software, San Diego, CA).

**CCK receptor phosphorylation.** CCK receptor constructs were phosphorylated in situ in intact cells in response to agonist stimulation after radiolabeling the ATP pool by loading the cells with 10 mCi H32P04 as we described previously (19). For each condition, the number of receptors per tube was established at a fixed and equal number based on direct analysis of radioligand binding (11). After the labeling of the ATP pool and stimulation of the cells with specific agonists, the CCK receptor was solubilized from a plasma membrane preparation and purified by affinity adsorption to a CCK-like ligand affinity column (19). Following this protocol, all the radioactivity in the relative molecular mass = 85,000–95,000 region of an SDS-polyacrylamide gel represented CCK phosphoreceptor. This was then quantified by densitometric analysis with subtraction of the local background using the National Institutes of Health Image program.

**Phosphopeptide fingerprinting.** Phosphoreceptor fingerprinting of the CCK phosphoreceptor was performed as we previously described (18, 19). This involved the proteolytic digestion with subtilisin of the radiochemically pure phosphoreceptor that had been eluted from an SDS-polyacrylamide gel. This mixture was then separated in two dimensions on a microcrystalline cellulose thin layer plate using electrophoresis in basic buffer followed by ascending chromatography. Radioactive spots were identified by autoradiography.

**Biological activity and desensitization and resensitization assays.** For determination of biological activity, IP3 was measured in intact cells after stimulation with CCK. We used a rapid, sensitive, and specific radioligand competition-binding assay that used [3H]IP3 and a specific IP3-binding protein prepared from rat cerebellum (2, 16, 20). We previously established and validated this assay in CCK receptor-bearing CHO-CCKR cells (20).

For determination of desensitization, paired aliquots of cells were treated with vehicle alone or with 10 nM CCK for 5 s at 37°C to provide control basal and control stimulated responses, while analogous aliquots were preincubated with 1 μM CCK at 37°C for 10 min and then washed extensively with KRH medium. For experiments exploring the time course of desensitization, cells were incubated with 1 μM CCK for various durations before being extensively washed. In all cases, paired samples demonstrated that these washes were adequate to return cellular IP3 content to basal unstimulated levels. The second aliquot of each pair was stimulated with 10 nM CCK for 5 s at 37°C. A desensitized response was less than the stimulated response in the control cells that had not been preexposed to CCK. CCK-stimulated IP3 responses were expressed as percentages of the range.
from basal to the maximal stimulated response of the control aliquots.

For determination of resensitization, cells were desensitized for 10 min, as described above. After this preexposure to CCK and washing, paired aliquots of cells were incubated at 37°C in the absence of additional CCK for various periods of time. At each test point in time, the pairs were stimulated with vehicle or 10 nM CCK for 5 s at 37°C. The cells were again assayed for IP₃ content as described above. Resensitization was expressed as a percentage of the control response of a similar aliquot of cells that had not been desensitized.

Receptor internalization and recycling. CCK receptor internalization and recycling were determined using morphological assays with a fluorescent ligand that were previously fully validated (22, 23). Direct analysis of CCK receptor location using receptor antibody demonstrated the validity of these assays in these cells (27). The fluorescent ligand used was a full agonist analog of CCK, Rho-CCK, that has been demonstrated to bind with specificity and with high affinity (Kᵢ = 8.4 ± 2 nM) (23).

For determination of internalization, cells were grown on coverslips and washed with iced phosphate-buffered saline at 4°C. While this temperature was maintained, 50 nM Rho-CCK was incubated for 1 h to saturate surface receptors. Cells were then warmed to 37°C for various periods of time before fixation and morphological analysis. For determination of recycling of the internalized receptors back to the cell surface, nonfluorescent native CCK was used in place of the Rho-CCK, and cells were incubated in the presence of 40 μg cycloheximide/ml to inhibit new receptor synthesis. At each time point, cells were cooled to 4°C and surface receptors were satura\n
**RESULTS**

Similar to the wild-type CCK receptor and to the S260,264A mutant receptor construct, the S260,264D mutant receptor was expressed normally on the surface of a CHO cell line. It had similar density to the other two receptor-bearing cell lines (wild-type, 1.0 ± 0.2 × 10⁵; S260,264A, 1.5 ± 0.3 × 10⁵; S260,264D, 0.6 ± 0.1 × 10⁵ sites per cell). It also had similar binding parameters, with the wild-type receptor having a Kᵢ for CCK of 2.2 ± 0.3 nM and the S260,264D mutant having a Kᵢ of 1.9 ± 0.9 nM. Competition-binding curves are shown in Fig. 1.

We previously characterized the phosphorylation of the CCK receptor expressed on the CHO-CCKR cells and the absence of agonist-stimulated phosphorylation of the S260,264A mutant receptor in the same cellular environment (20). The latter was surprising, given the stoichiometry of 5 mol phosphate/mol receptor, with only two sites represented by serine residues in positions 260 and 264. Nevertheless, phosphopeptide fingerprints demonstrated the absence of agonist-stimulated phosphorylation of the other sites in the S260,264A mutant receptor construct, despite normal CCK binding and signaling (20). This was true for both CCK- and phorbol ester-stimulated receptor phosphorylation.

Agonist-stimulated phosphorylation of the S260,264D mutant receptor construct returned toward normal (Fig. 2), reaching 70% of the control phosphorylation observed in wild-type receptor-bearing cells. This is in the range expected for phosphorylation of all the sites normally modified, except for the serines in positions 260 and 264. Indeed, the pattern of the phosphopeptide fingerprint of this receptor supported this interpretation (Fig. 3).

Similar to our previous observations for the wild-type receptor expressed on the CHO-CCKR cells, agonist-stimulated phosphorylation of the S260,264D mutant receptor was rapid, reaching a maximal level in 15
CCK stimulated \( \text{IP}_3 \) responses in each of the cellular systems, the recombinant wild-type receptor-bearing CHO-CCKR cell and the mutant receptor-bearing CHO cell lines (20). The magnitude of \( \text{IP}_3 \) responses per million cells differed for each cell type. For the wild-type receptor-bearing CHO-CCKR cells, there was a 15- to 18-fold increase in \( \text{IP}_3 \) compared with the basal unstimulated levels. There was a fivefold increase observed in the S260,264A receptor-bearing cells and an eight- to tenfold increase observed in the S260,264D receptor-bearing cells.

All the cellular systems were completely desensitized in response to CCK, although the rates of desensitization varied (Fig. 5). The rate of desensitization was most rapid for the wild-type receptor and for the S260,264D mutant receptor, while being significantly slowed \((P < 0.05)\) for the S260,264A mutant receptor. The half time to achieve complete desensitization was 0.7 ± 0.1 min for the wild-type receptor in the CHO-CCKR cells, 0.5 ± 0.1 min for the S260,264D receptor, and 1.7 ± 0.4 min for the S260,264A receptor. Previous analysis has established that the later stages of desensitization of the CCK receptor on the CHO-CCKR cells is due to receptor internalization (20). This has been observed to occur normally for both wild-type CCK receptor-bearing CHO-CCKR cells and the nonphosphorylated S260,264A mutant receptor-bearing CHO cell line (20). We now see that normal and complete receptor internalization also occurs for the S260,264D mutant receptor in an analogous cell line (Fig. 6).

The time courses of resensitization of these cellular systems are illustrated in Fig. 7. Wild-type receptor-bearing cells CHO-CCKR cells completely resensitized within 90 min. Of note, resensitization remained in-

Fig. 3. Shown are typical autoradiographs of 2-dimensional phosphopeptide maps of the CCK receptor after subtilisin cleavage and a numbered map for the maximal number of radioactive spots observed after CCK stimulation of the WT receptor. These represent WT and mutant CCK receptors expressed on CHO cell lines that had been stimulated with 0.1 \( \mu \text{M} \) CCK (left) or 1 mM \( \text{O-tetradecanoylphorbol 13-acetate} \) (TPA) (right). As previously reported (20), the S260,264A mutant receptor was not phosphorylated in any sites. The S260,264D mutant receptor was phosphorylated to an intermediate degree, representing the normal sites except for the serine residues in the 260 and 264 positions. Phosphopeptides absent in the mutant that were present in the control setting (WT receptor treated similarly) are highlighted.

Fig. 4. Time course of phosphorylation of the S260,264D CCK receptor construct in response to CCK stimulation. Shown is a typical autoradiograph of the CCK phosphoreceptor region of an SDS-polyacrylamide gel and quantitation of data from 3 independent experiments. Values represent means ± SE.

Fig. 5. Time courses of desensitization of \( \text{IP}_3 \) responses to CCK. Shown are signaling responses to 10 nM CCK after WT CCK receptor-expressing CHO-CCKR cells and CHO cell lines expressing similar numbers of S260,264A and S260,264D mutant CCK receptors were exposed to hormone for various periods of time. Each of the 3 types of cells had clear signaling responses to CCK and could be fully desensitized by previous exposure to hormone, although the rates of desensitization varied. Values represent means ± SE for a minimum of 3 independent experiments.
complete throughout this entire time period in the S260,264A mutant receptor-bearing CHO cell line. Nearly complete resensitization was, however, achieved in the S260,264D mutant receptor-bearing cell line, similar to that observed in the control wild-type receptor-bearing cells. Figure 8 explores the recycling of these receptors to the cell surface. As is apparent in the illustration and the quantitation of these data, recycling to the cell surface was substantial in the wild-type receptor-bearing cells and in the S260,264D mutant receptor-bearing cells, but was below detectability throughout this time period in the S260,264A mutant receptor-bearing cells.

DISCUSSION

Phosphorylation of G protein-coupled receptors has been recognized as being an important rapid and reversible molecular mechanism for desensitization to protect target cells from overstimulation. This occurs both directly and indirectly, with receptor phosphorylation interfering with the coupling of the activated receptor to its heterotrimeric G protein and with this modification mediating the binding of arrestin molecules to the phosphoreceptor to achieve the same outcome (7, 10, 13, 26). The current work has provided new insights into additional regulatory roles of agonist-stimulated receptor phosphorylation. It is now clear that some sites of receptor phosphorylation act to functionally expose previously hidden receptor domains. Such domains can be further acted on by kinases to extend the agonist-stimulated phosphorylation of the receptor and to thereby interfere with G protein coupling. Receptor domains can also be made available to affect other regulatory processes, unrelated to G protein coupling. The process affected in the present work is the targeting and trafficking of the receptor-bearing endosome. This is also remarkable for providing important new evidence that the cargo of such a cellular compartment can determine its destination.

In our previous report of the characterization of the S260,264A mutant CCK receptor, the only clear functional impact of absent receptor phosphorylation that was identified was defective early desensitization due to the absence of the effect of phosphorylation to interfere with G protein coupling (20). That receptor construct was shown to bind ligand, to signal, and to be internalized normally. In the current report, we demonstrated another more dramatic effect of this mutation: the defective receptor resensitization due to abnormal recycling from the endocytic compartment back to the plasma membrane. In exploring the molecular mechanism for this, we also found that the charge modification affected by the phosphorylation of serines in positions 260 and 264 had the additional effect of exposing other portions of the receptor to kinases for their phosphorylation and resulted in the normalization of the defect in recycling and resensitization.

We previously established that there were at least five distinct sites of agonist-stimulated phosphorylation of serines and threonines within the CCK receptor (18) and that mutation of two key sites of protein kinase C action on serines within the third intracellular loop to nonphosphorylatable alanines eliminated all receptor phosphorylation (20). This occurred despite the intact nature of the other sites of phosphorylation, some of which were known to be substrates of protein kinase C, an enzyme known to be activated by agonist occupation of this mutant receptor. The only likely explanation for the absence of phosphorylation of those sites is related to inaccessibility to the enzyme. Even the potent and prolonged activation of cellular protein kinase C with phorbol ester failed to phosphorylate this receptor construct. The current report confirms that by mutating these serine residues to aspartic acids to mimic the charge of the serine-phosphates in these key positions, the other phosphorylation events were allowed to proceed.

Of particular interest, despite mimicking the already phosphorylated state of this key region of the third intracellular loop, the S260,264D mutant was still able to signal normally, suggesting normal coupling to Gq. In fact, there was even a trend toward a more rapid than normal IP3 response to CCK occupation of this mutant receptor. The opposite was also true, with absence of phosphorylation occurring at those sites in

![Fig. 6. Internalization of the S260,264D CCK receptor construct.](http://ajpcell.physiology.org/)

![Fig. 7. Resensitization of CCK receptor constructs.](http://ajpcell.physiology.org/)

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**CHO-CCKR**

**S260,264D**

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**C1990 CHOLECYSTOKININ RECEPTOR REGULATION**
the S260,264A mutant, the IP₃ response was significantly slowed from normal. There was no evidence for the S260,264D mutant simulating the charge effect of this early phosphorylation event that facilitates other events to lead to any constitutive activation of the signaling pathway in the unliganded state or to stimulate receptor internalization that might occur independent of ligand occupation.

The nature of the conformational change in the receptor that initiates receptor internalization remains a mystery. We now know that this event is independent of any component of the signaling pathway, including G protein association, because it can occur even with the occupation of the CCK receptor by an antagonist (21). It is also completely independent of receptor phosphorylation, because the nonphosphorylated mutant is internalized normally (20). It also must be independent of receptor epitopes exposed by mimicking the charge of the serine-phosphates in positions 260 and 264 of the third loop.

The majority of the work on G protein-coupled receptor regulation has focused on the desensitization mechanisms, with the regulated events of receptor phosphorylation leading to G protein uncoupling and of receptor internalization. Once a receptor entered the endocytic compartment, it was felt to likely represent passive cargo, with targeting and trafficking occurring as a result of the endogenous machinery of that cellular compartment. Resensitization and recycling events have been much less well studied.

Of interest, receptor dephosphorylation has been implicated in resensitization of some G protein-coupled receptors (12). However, if receptor phosphorylation is not necessary for receptor entry into the endocytic compartment, it would make little sense to be dependent on dephosphorylation to direct the receptor out of the compartment. We were, therefore, somewhat surprised by the inability of the S260,264A mutant CCK receptor to recycle and resensitize. Given the correction of these events by the charge mimic mutant, S260,264D, a more likely explanation is the effect of this mutation to expose key receptor domains involved in regulating the targeting and trafficking of the internalized receptor. This will

Fig. 8. Recycling of CCK receptor constructs. Shown are typical morphological findings for the presence, clearance, and reappearance of CCK receptor constructs on the surface of CHO cell lines after occupation with nonfluorescent CCK. A: surface receptor was visualized with saturable concentrations of CCK-like fluorescent ligand. B: quantitative data for recycling experiments with these cells, representing means ± SE from a minimum of 3 independent experiments.

A
CHO-CCKR
Control
0'
30'
60'
90'
S260,264D
Control
0'
30'
60'
90'
S260,264A
Control
0'
30'
60'
90'
B

Receptor on cell surface, % of control

Time, min

S260,264D
○ WT
× S260,264A

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also provide important and powerful tools to dissect the molecular basis for regulating recycling.

This series of studies adds new and important insights into our thinking about receptor phosphorylation. This regulated and reversible event can play key roles in uncoupling the receptor from its proximal effector G protein, can lead to conformational changes making additional sites of phosphorylation of the receptor possible, and can expose hidden receptor domains that can affect targeting and trafficking of the internalized receptor. These molecular mechanisms likely provide additional flexibility for the cell-specific handling of receptors in a way that might be optimal for that target. This should change the paradigm in the way we think about and explore this regulatory mechanism.

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