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Roles of Cholecystokinin Receptor Phosphorylation in Agonist-Stimulated Desensitization of Pancreatic Acinar Cells and Receptor-Bearing Chinese Hamster Ovary Cholecystokinin Receptor Cells

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SUMMARY

Receptor phosphorylation has been implicated in desensitization responses to some agonist ligands, in which receptors may become uncoupled from G proteins and move into cellular compartments inaccessible to hydrophilic ligands. Understanding of the linkage between these processes, however, has come largely from recombinant receptor-bearing cell systems with consensus sites of kinase action mutagenized. We recently established methodology permitting direct assessment of sites of phosphorylation of the cholecystokinin receptor (CCKR) in its native milieu in the pancreatic acinar cell and in a Chinese hamster ovary (CHO)–CCKR cell line (1, 2). Although CCK binding leads to phosphorylation of serine residues within the third intracellular loop of the receptor in both cell types, there are clear differences in the time course of phosphorylation, in the balance of action of kinases and a receptor phosphatase, and in a few of the distinct sites phosphorylated. In this work, we have directly assessed the inositol 1,4,5-triphosphate responses to CCK and desensitization of these responses in both cells. CHO cell lines expressing receptor mutants with protein kinase C consensus sites modified were also studied. CCK-stimulated inositol 1,4,5-triphosphate responses in both cells expressing wild-type receptors were rapidly and completely desensitized, associated with the onset of receptor phosphorylation. However, despite maintenance of the phosphorylated state of the receptor in the CHO-CCKR cell and its dephosphorylation returning the receptor to its basal state in the acinar cell, desensitization continued to be present in both. Mutagenesis of Ser260 and Ser264 to alanines individually reduced receptor phosphorylation by approximately 50%, whereas the dual mutant completely eliminated agonist-stimulated phosphorylation. Because other sites of phosphorylation were still intact in this construct, this raises the possibility of hierarchical phosphorylation with these two sites key in making other sites accessible to kinases. Constructs modifying Ser264 delayed the onset of desensitization, whereas all constructs proceeded to achieve complete desensitization by 10 min. Receptor internalization occurred independent of its phosphorylation state in the CHO cell lines, explaining the desensitization observed. In the acinar cell in which the receptor remains on the cell surface after agonist occupation, we postulate that receptor insulation achieves similar uncoupling from G protein association as is achieved by receptor phosphorylation early after agonist occupation.

Eukaryotic cells use a variety of mechanisms to dampen their responses to sustained hormonal agonist stimulation. Among these mechanisms for desensitization are processes involving the receptor itself, including uncoupling from G protein signal transducers and movement into cellular compartments inaccessible to hydrophilic ligands (3–7). A key regulatable and reversible biochemical modification of the receptor that has been implicated in these events in selected systems is phosphorylation (8). However, it has been difficult to correlate specific receptor phosphorylation events with specific desensitization or resensitization events because a detailed understanding of this covalent modification is only available for a few receptors in this superfamily. Also, most of our insights come from recombinant receptor-bearing cells in which consensus sites of action of kinases have been mutagenized.

We have extensive information about the phosphorylation of the CCKR as it resides in its natural setting in pancreatic acinar cells, as well as in recombinant receptor-bearing cells

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ABBREVIATIONS: CCK, cholecystokinin; CHO, Chinese hamster ovary; CCKR, cholecystokinin receptor; IP_3, inositol 1,4,5-triphosphate; TPA, 12–0-tetradecanoylphorbol-13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; PKC, protein kinase C.

(1, 2, 9–12). This is a physiologically important G protein-coupled receptor linked to the phospholipase C pathway, which has diverse roles in mediating pancreatic exocrine secretion, gallbladder contraction, enteric motility, and satiation after a meal. We demonstrated that this receptor is phosphorylated in response to agonist stimulation of native receptor-bearing pancreatic acinar cells (9) and recombinant receptor-bearing CHO-CCKR cells (1). Furthermore, we have characterized some of the kinases and a protein phosphatase contributing to the phosphorylation status of this protein (10–12) and have mapped the sites of receptor phosphorylation in both of these cellular systems (1, 2). Although the CCKR in both of these cells is phosphorylated predominantly on serine residues within the third intracellular loop, there are cellular differences in the time course of phosphorylation, the balance between the action of kinases and the receptor phosphatase, and the use of a few distinct sites within the receptor for phosphorylation (1, 2, 9).

In the current work, we have directly assessed IP_3 responses to CCK and desensitization of these responses in the wild-type CCKR in both of these cellular systems, as well as in a series of CHO cells bearing receptor phosphorylation site mutants. In both cells bearing wild-type receptors, phosphorylation occurred rapidly (1, 9), as did the onset of receptor desensitization. There was divergence in the phosphorylation state of the receptor in the two cells over time, with the CHO-CCKR cell maintaining its receptor phosphorylation through the time in which it is internalized (13), whereas the acinar cell receptor is dephosphorylated promptly to its basal state (9, 11) while remaining on the cell surface (14). Of interest, both cellular systems maintain their desensitized status throughout this time. The likely explanation for maintenance of desensitization in the acinar cell receptor after dephosphorylation is its entry into the "insulation compartment" we described recently (14). The receptor phosphorylation mutants provide additional insights. Some of these that interfered with receptor phosphorylation also interfered with the rate of desensitization, consistent with the early desensitization being mediated by specific sites of this covalent modification. Agonist occupation of this receptor stimulated receptor internalization in the CHO-CCKR cell independent of the phosphorylation of that molecule, with a dual mutant (S260/264A) not phosphorylated, but internalized normally upon agonist occupation.

Experimental Procedures

Materials. Synthetic CCK-8 was purchased from Peninsula Laboratories (Belmont, Ca). The CCK analog (CCK-OPE)-agarose affinity resin was synthesized as we described (15). Subtilisin was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and TPA, 1,1,2-trichloro-trifluoroethane, and tri-*n*-octylamine were from Sigma Chemical (St. Louis, MO). [^{3}H]IP_3 (20.0 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA).

CCKR expression systems. Male Sprague-Dawley rats weighing between 80 and 100 g were used for preparation of the dispersed pancreatic acini, which naturally express the CCK-A receptor. We

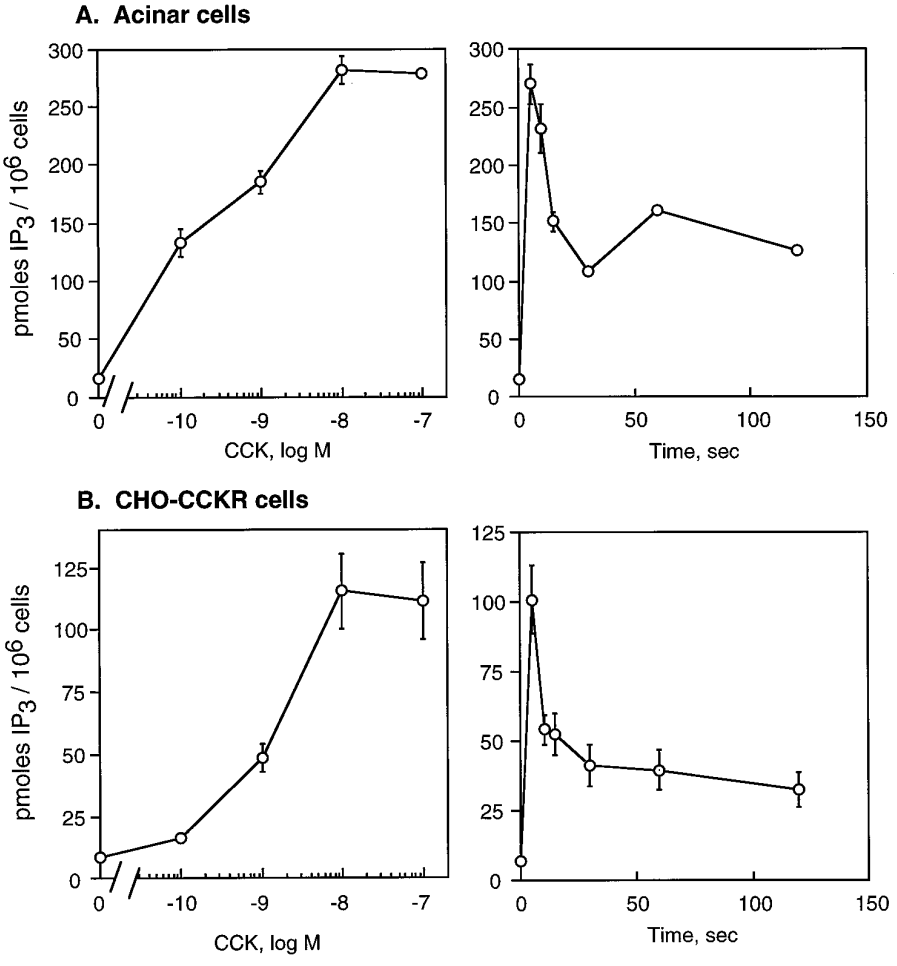

Fig. 1. Concentration dependency and time course of IP_3 production in response to CCK-8 in pancreatic acinar cells (A) and CCKR-bearing CHO-CCKR cells (B). For the concentration-dependency data, cells were stimulated with increasing concentrations of CCK-8 for 5 sec. For time-course studies, cells were stimulated with 10 nM CCK-8 for noted periods of time. IP_3 mass was measured by radioreceptor binding assay and is expressed as pmol/10^6 cells. Values represent mean ± standard error of three independent experiments.

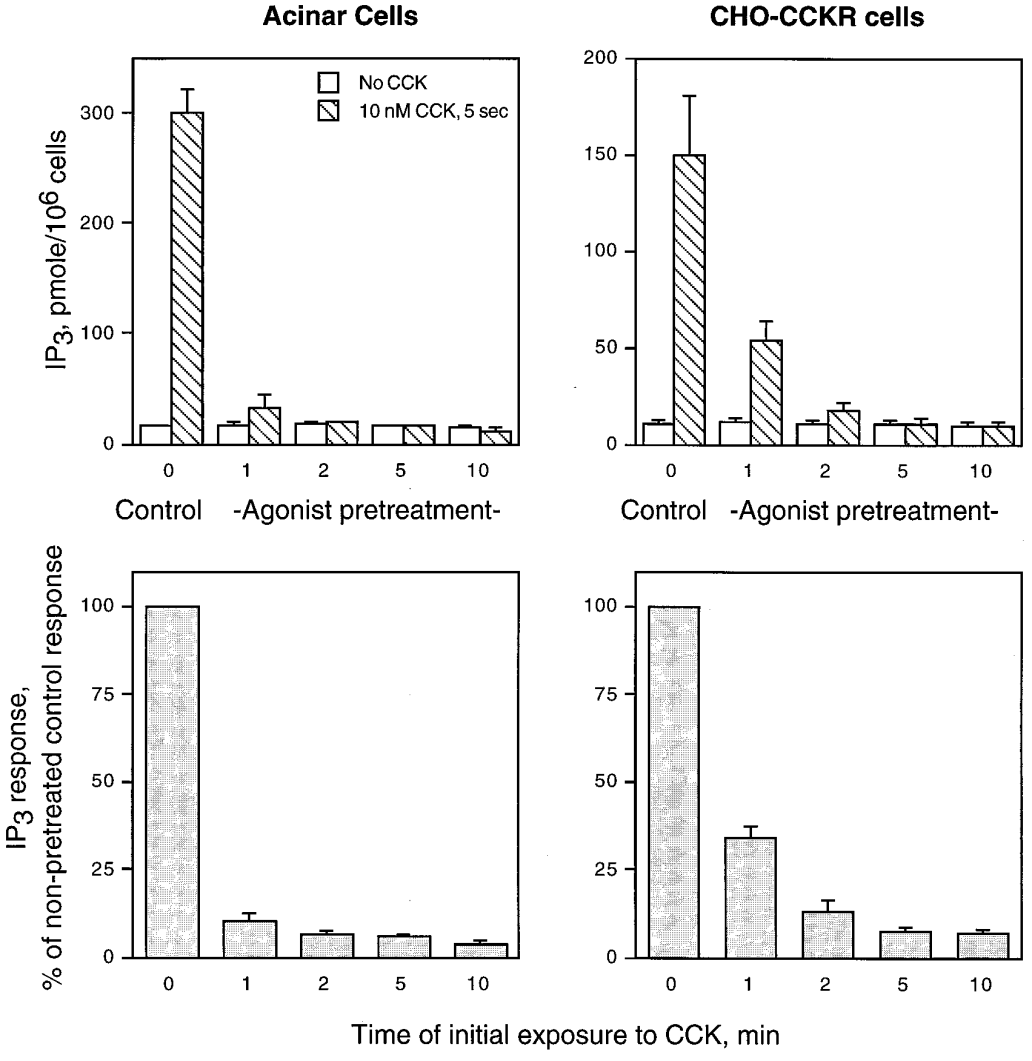

Fig. 2. Desensitization of IP_3 responses to CCK in pancreatic acinar cells (*left*) and CHO-CCKR cells (*right*) expressing wild-type CCKRs. Shown are the time courses of desensitization, with the absolute values of IP_3 at each assayed condition (*top*), as well as the responses to 10 nM CCK (in excess of a time-specific unstimulated control) expressed as percentages of the control response in cells that had not been preincubated with CCK (*bottom*). As described in Experimental Procedures, *time points* represent the amount of time that the cells were incubated with 1 μM CCK before extensive washing. Values represent mean ± standard error of three independent experiments.

followed the dissociation protocol described previously (11). These experimental protocols were reviewed and approved by the Mayo Clinic Institutional Animal Care and Use Committee.

The recombinant rat CCK-A receptor-bearing CHO-CCKR cell line was previously established and characterized (16). This cell line was maintained in culture in Hams-F12 medium containing 5% fetal clone 2 supplement (Hyclone Laboratories, Logan, UT) in a 37° humidified incubator containing 5% CO_2.

For mutagenesis, the rat CCK-A receptor cDNA we cloned previously (16) was subcloned into the pBK-CMV expression vector (Stratagene, LaJolla, CA), and site-directed mutagenesis was performed using the method of Sayers *et al.* (17). The sequences of all constructs were confirmed by DNA sequencing using the dideoxynucleotide chain termination method (18). CHO-K1 cells were acquired from American Type Culture Collection (Rockville, MD) and were cultured similarly to the CHO-CCKR cells described above. Cells were transfected with 2–4 μg of DNA using DEAE-dextran or lipofectin methods (19) and selecting stable receptor-bearing cell lines as we described (16).

Receptor constructs, as expressed on the surface of the CHO cell lines, were characterized directly for CCK binding properties as we have performed previously (16). For this, we used the fully validated radioligand ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] (20) under standard conditions in which steady state had been achieved. Binding data were analyzed and graphed using the nonlinear regression analysis routines for radioligand binding in the Prism software package (GraphPAD Software, San Diego, CA).

Measurement of IP_3 by radioreceptor assay. IP_3 was measured in intact pancreatic acinar cells or CCKR-bearing CHO cell lines in the basal state and in response to CCK-8 using a previously characterized radioreceptor assay incorporating [^{3}H]IP_3 and a specific binding protein for the 1,4,5-isomer of IP_3 that is present in rat cerebellar membranes (21, 22). This assay is rapid and sensitive and specifically determines the mass of the biologically active isomer of IP_3. Reagents were prepared in our laboratory. Freshly harvested cerebella from adult rats were homogenized in a polytron homogenizer in 10 ml of ice cold buffer per animal (50 mM Tris·HCl, pH 7.7, 1 mM EDTA, 1 mM 2-mercaptoethanol, 4 mg/ml BSA). This was pelleted by centrifugation (25,000 rpm, 15 min), resuspended in buffer three times, with the final pellet resuspended at 1.5 mg protein/ml, and stored at −20°.

In the typical assay, cells were incubated with agonist in Krebs-Ringer-HEPES medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 2.5 mM D-glucose, 0.2% BSA, 0.01% soybean trypsin inhibitor, essential and nonessential amino acids, and glutamine. Reactions were terminated by addition of equal volumes of ice-cold 10% perchloric acid. The tubes were kept on ice for 10 min and centrifuged at 3000 rpm for 10 min. The supernatant was added to 0.1 ml of 10 mM EDTA, pH 7.0, and a one-half volume of a 3:1 (v/v) mixture of 1,1,2-trichloro-trifluoroethane:tri-*n*-octylamine was added. After vortexing, the samples were centrifuged at 3000 rpm for 10 min, yielding three phases. The upper IP_3-containing phase was collected and used for subsequent binding assays.

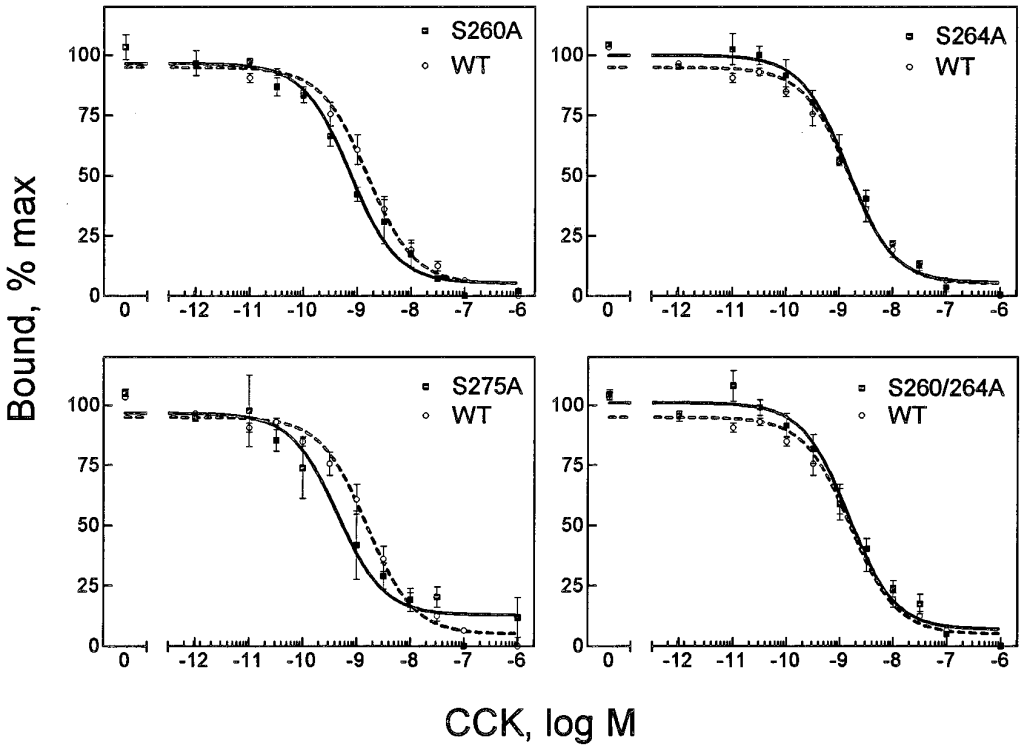

Fig. 3. Shown are competition-binding curves for CHO cells expressing the CCKR constructs. For each, CCK displaced the binding of the CCK radioligand similarly to its ability to achieve this with cells expressing the wild-type receptor. Values represent mean ± standard error of three independent experiments. EC_{50} values for wild-type (*WT*), S260A, S264A, S275A, and S260/264A, respectively, are the following: 1.7 ± 0.6, 1.0 ± 0.4, 1.5 ± 0.3, 1.1 ± 0.3, and 1.5 ± 0.4 nM CCK. None is significantly different from any other.

Of this phase, 150 μl was added to the IP_3 assay buffer (50 mM Tris·HCl, pH 8.4, 1 mM EDTA, 1 mM 2-mercaptoethanol, 4 mg/ml BSA) along with 5 nCi of [^{3}H]IP_3. The incubation was initiated with the addition of 150–200 μg of the receptor binding protein and this was continued on ice for 30 min, followed by centrifugation at 5500 rpm for 15 min. The supernatant was aspirated off, and the pellet was reconstituted in 75 μl of 0.15 N NaOH. Scintillation fluid (10 ml) was added to all tubes and radioactivity was quantified in a scintillation counter. Nonspecific binding was defined as binding not displaced by 300 pmol of standard IP_3. Each condition was assayed in duplicate and repeated in a minimum of three independent experiments.

Desensitization was defined as a reduced IP_3 response to CCK after the cells had previously been exposed to stimulation with this hormone. In the experimental protocol, paired incubations of identical aliquots of cells were performed in the absence or presence of 10 nM CCK for 5 sec at 37° before IP_3 determination. Control cells had no preincubation with CCK, whereas experimental conditions included preincubation with 1 μM CCK at 37° for indicated periods of time. Three large-volume washes with Krebs-Ringer-HEPES medium were performed after the preincubation period. In validation studies with both acinar cells and CHO-CCKR cells, these washes were adequate to return IP_3 levels to the levels present in the basal state (absolute values for each of these conditions illustrated in the top panels of Fig. 2).

To be certain that washes were adequate to remove the CCK that had been bound during the preincubation period, providing the opportunity for fresh hormone to bind during re-exposure, an additional control was performed. In this, analogous incubations were performed with CHO-CCKR cells and with the CHO cell line, which expresses a nonphosphorylatable CCKR mutant (S260/264A). In these assays, 1 μM unlabeled CCK was used in the initial exposure and 10 pM CCK radioligand was used in the absence or presence of 100-fold excess unlabeled CCK in the second incubation. The binding assay was performed for 5 min at 37°. Bound radioligand was quantified after rapid centrifugation, washing with iced buffer, and repetition of the wash procedure. At every time point tested, substantial saturable radioligand binding was found to be present on these cells (CHO-CCKR cells: 1 min pre-exposure, 33 ± 4% of binding observed in control cells that had not been pre-exposed to CCK; 5 min, 33 ± 5%; 10 min, 28 ± 2%. S260/264A mutant-bearing cells: 1 min, 74 ± 13%; 5 min, 47 ± 16%; 10 min, 36 ± 8%). Therefore, for both cell lines, these data reflect substantial access for CCK to bind to its receptors during re-exposure in the desensitization assay. The apparent lower access for the wild-type receptor probably reflects its lower affinity state, as a function of agonist-stimulated receptor phosphorylation interfering with G protein coupling. Data quantifying the nonphosphorylated CCKR construct accessible for binding parallels the amount of receptor suggested to be present on the cell surface in independent studies quantifying receptor internalization by acid-washing.[1]

***In situ* CCKR phosphorylation.** CCKR expressed on rat pancreatic acinar cells and receptor-bearing CHO cell lines were phosphorylated in response to agonist stimulation of the intact cells, after radiolabeling the endogenous ATP pool by incubation with 10 mCi of $H_3{}^{32}PO_4$, as we have carefully described and validated (1, 9). For each condition, the number of receptors per tube was established at a fixed and equal amount based on direct analysis of radioligand binding, as described previously (9). Cells were then fractionated to yield plasmalemma, which was solubilized, and the CCK phosphoreceptor was purified by affinity chromatography on (CCK-OPE)-agarose and separation on a sodium dodecyl sulfate-polyacrylamide gel as we described (1). Following this protocol, all of the radioactivity in the M_r = 85,000–95,000 region of the gel represented CCK phosphoreceptor (1, 9). Phosphoreceptor was then quantified by analysis on a phosphorimager or by densitometric analysis using Image Software (National Institutes of Health, Bethesda, MD).

Two-dimensional phosphopeptide mapping. For high resolution and fine mapping of the CCKR phosphorylation sites, the radiochemically pure phosphoreceptor was cleaved with subtilisin and the resulting fragments were separated in two dimensions on a cellulose

[1] W. Go, B. Roettger, E. Holicky, E. Hadac, and L.J. Miller. Quantitative dynamic multicompartmental analysis of cholecystokinin receptor movement in a living cell using dual Fluorophores and reconstruction of confocal images. Manuscript in preparation.

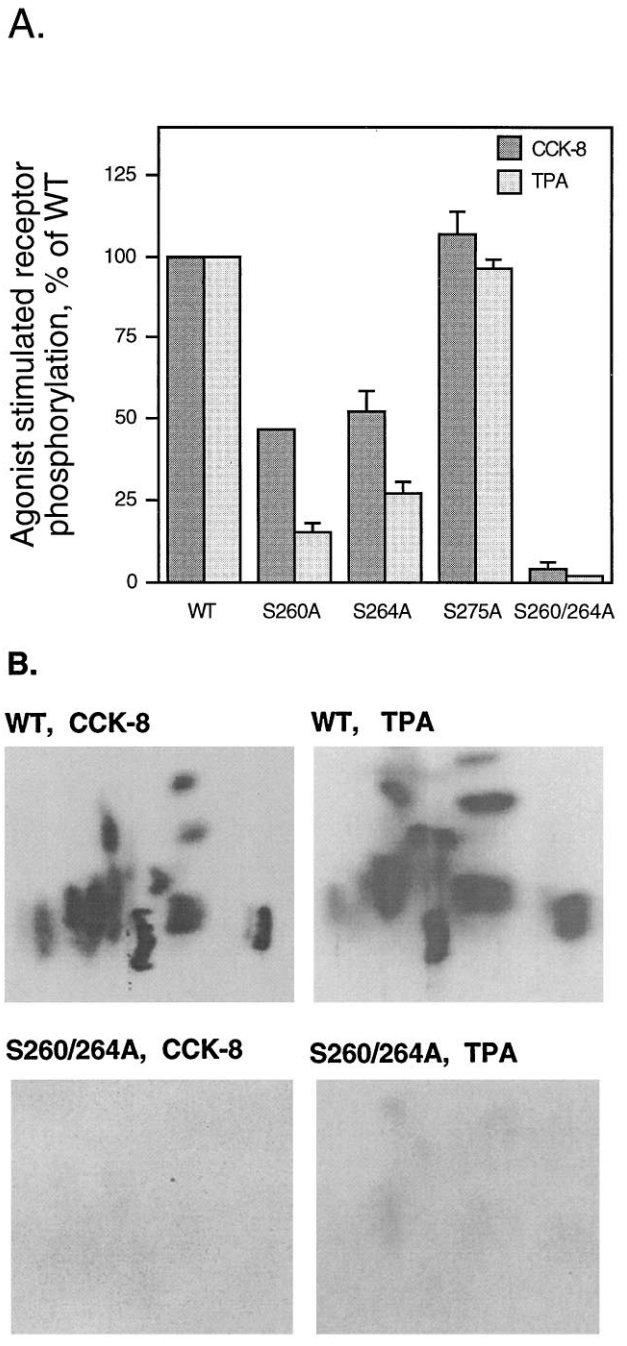

Fig. 4. Phosphorylation of CCKR constructs expressed in CHO cell lines. Bar graphs in A represent quantitation of CCK phosphoreceptor migrating in the M_r = 85,000–95,000 region of sodium dodecyl sulfate gels in which cells expressing similar amounts of CCKR (established by radioligand binding assay) were treated with either 1 μM CCK or 0.1 mM TPA for 2 min at 37°. Data are presented relative to the responses of the wild-type (*WT*) receptor and reflect three independent experiments (mean ± standard error). Images in B show representative two-dimensional phosphopeptide maps of similar amounts of purified WT and dual mutant receptors after subtilisin treatment. Images of the maps represent similar length exposures in the phosphorimager; however, even long exposures with enhancement failed to demonstrate significant amounts of phosphopeptide in the mutant receptor.

plate using the method of Nairn and Greengard (23), which was applied previously to this receptor (1, 2).

Internalization of the CCKR. CCKR internalization in response to agonist occupation in the CHO cell lines expressing the mutant receptors was determined morphologically using the methodology we previously established and validated (13). In brief, cells were incubated for 1 hr at 4° with the biologically active fluorescent analog of CCK, rhodamine-[Gly-(Nle^{28,31})CCK-26-33], and then warmed to 37° for variable periods of time. The temperature-dependent internalization process was followed using epifluorescence microscopy. Time courses were observed in at least three independent experiments for each construct.

Statistical analysis

Values are presented as mean ± standard error. Differences were determined using the Mann-Whitney test for unpaired values, with $p < 0.05$ considered to be significant.

Results

CCK stimulated prompt and concentration-dependent increases in cellular IP_3 in both native rat pancreatic acinar cells and wild-type receptor-bearing CHO-CCKR cells (Fig. 1). Responses reached a peak in 5 sec in both cells, followed by rapid reduction to a plateau level at 30–40% of the maximal responses.

Complete desensitization of the CCK-stimulated IP_3 responses were achieved rapidly in both the pancreatic acinar cells and the wild-type receptor-bearing CHO-CCKR cells. Fig. 2 illustrates the absolute values for each of the conditions assayed, as well as expression of the subsequent responses to CCK as percentages of the control response in cells that had not been previously exposed to hormone. In both cells, complete desensitization was achieved within 2 min. To be certain that apparent desensitization did not reflect depletion of components of the postreceptor signaling machinery, acinar cells that had been exposed to 1 μM CCK for 5 min were washed and exposed to 100 nM bombesin. This resulted in an IP_3 response representing 79 ± 8% of its control response.

We have previously characterized the CCKR phosphorylation observed in the acinar cells (2, 9) and the CHO-CCKR cells (1). We also established that the third intracellular loop was the location of >95% of agonist-stimulated phosphorylation in both (1, 2). There are three strong consensus sites [using the PROSITE definition (24)] for the action of PKC within that domain, Ser260, Ser264, and Ser275. We established that both Ser260 and Ser264 are used by these cells, by direct sequence analysis of purified phosphopeptides, by demonstrating that these were present on two-dimensional phosphopeptide maps of the receptor from both cell systems, and by demonstrating that these were absent in similar maps of the mutant receptors (1, 2). In this work, we have extended the previous observations by quantifying the net effect on CCKR phosphorylation of changing each of these PKC consensus sites to an alanine. Stable CHO cell lines expressing each of these constructs (as well as a dual mutant, S260/264A) were characterized directly. Each receptor was synthesized and expressed on the cell surface and exhibited binding affinity for CCK that was not different from that of the wild-type receptor (Fig. 3). As expected from our sequencing of phosphopeptides on the two-dimensional map (1), the S275A mutant, representing a consensus site not actually used by the cell, had no effect on agonist-stimulated receptor phosphorylation (Fig. 4). Both the S260A mutant and the S264A mutant reduced CCK-stimulated phosphorylation by approximately 50% (Fig. 4). TPA-stimulated phosphorylation

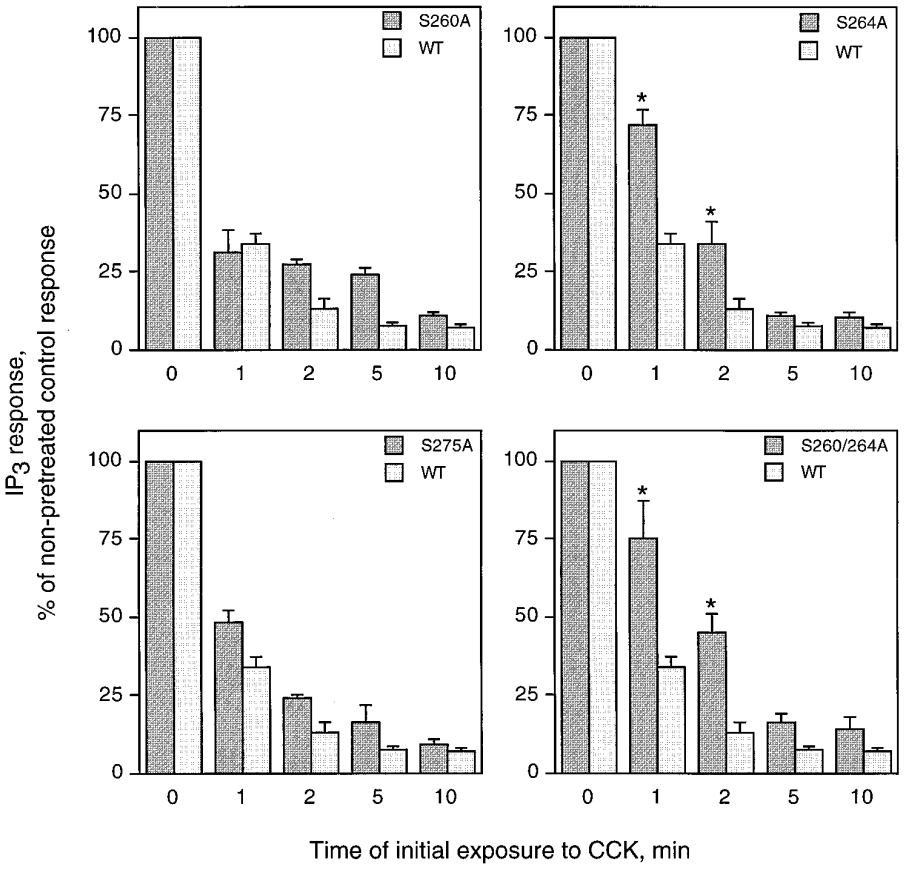

Fig. 5. Desensitization of IP_3 responses to CCK in CHO cells expressing CCKRs with mutations in potential sites of PKC action on the third intracellular loop. Shown are the time courses of desensitization, with the specific responses to 10 nM CCK (in excess of a time-specific unstimulated control) expressed as percentages of the control response in cells that had not been preincubated with CCK. As described in Experimental Procedures, each time point represents the amount of time that the cells were incubated with 1 μM CCK before extensive washing. Values represent mean $\pm$ standard error. Control data from CHO-CCKR cells expressing wild-type (*WT*) receptor are shown as well, with asterisks marking those points for the receptor mutants that were significantly different from the control.

of each of these constructs paralleled the effects on CCK-stimulated receptor phosphorylation (Fig. 4).

Although Ser260 and Ser264 are prominent sites of CCKR phosphorylation (1), they are clearly not the only sites (1). Other distinct sites have previously been directly demonstrated by sequence analysis as well, and this is consistent with the stoichiometry determined as approximately 5 mol of phosphate/mol of receptor after CCK stimulation (2). We were, therefore, quite surprised by the effect of creating the dual mutant, S260/264A, on receptor phosphorylation (Fig. 4). This essentially eliminated any CCKR phosphorylation in response to agonist occupation. Two-dimensional phosphopeptide maps established that the other sites of phosphorylation of the CCKR, which are distinct from Ser260 and Ser264, also were not present after stimulation of this construct (Fig. 4).

Each of the consensus phosphorylation site mutants had biological responses to occupation with CCK (Fig. 5). All such constructs eventually completely desensitized in response to CCK, with their IP_3 responses, to repeat exposure to hormone not different from basal levels (Fig. 5). Of interest, desensitization was significantly delayed in the receptor mutants involving Ser260. In these, reduced desensitization was observed at the earliest time points tested (1 and 2 min), before the internalization of the agonist-occupied receptor. The S275A mutant that affects a computer-predicted site of action of PKC known not to be used by the cell (1, 24) did not have any significant effect on the desensitization observed in cells bearing wild-type receptor. At the 10-min time point, when the wild-type receptor is known to be substantially internalized in CHO-CCKR cells (13), all of the mutant constructs had achieved near-complete desensitization as well.

To study whether the mutations of the phosphorylation sites affected internalization mechanisms, we studied CCK-induced internalization of the mutant receptors and compared this to that of the wild-type receptors in the same type of cell (13). As shown in Fig. 6, all of these constructs internalized promptly upon agonist occupation and warming, analogous to the wild-type receptors (13). This predominantly involved clathrin-dependent endocytosis, with ultimate movement deep within the cell into perinuclear compartments, including lysosomes (13). The time courses for internalization of the mutant receptors could not be distinguished from that of the wild-type receptor.

Discussion

Using both native and recombinant receptor-expressing cell models, we have been able to gain insights into the regulatory roles played by agonist-stimulated phosphorylation of the CCKR. The earliest desensitization that occurs in response to agonist occupation of this receptor seems to be highly dependent on this covalent modification and probably represents "uncoupling" from its G protein. Signal transduction, however, occurs totally independent of receptor phosphorylation, and longer-term desensitization ultimately occurs by independent mechanisms as well. These correlate best with the removal of the receptor from its natural environment, in which it is fully mobile within the lipid bilayer of the plasmalemma (14), to enter a compartment of "insula-

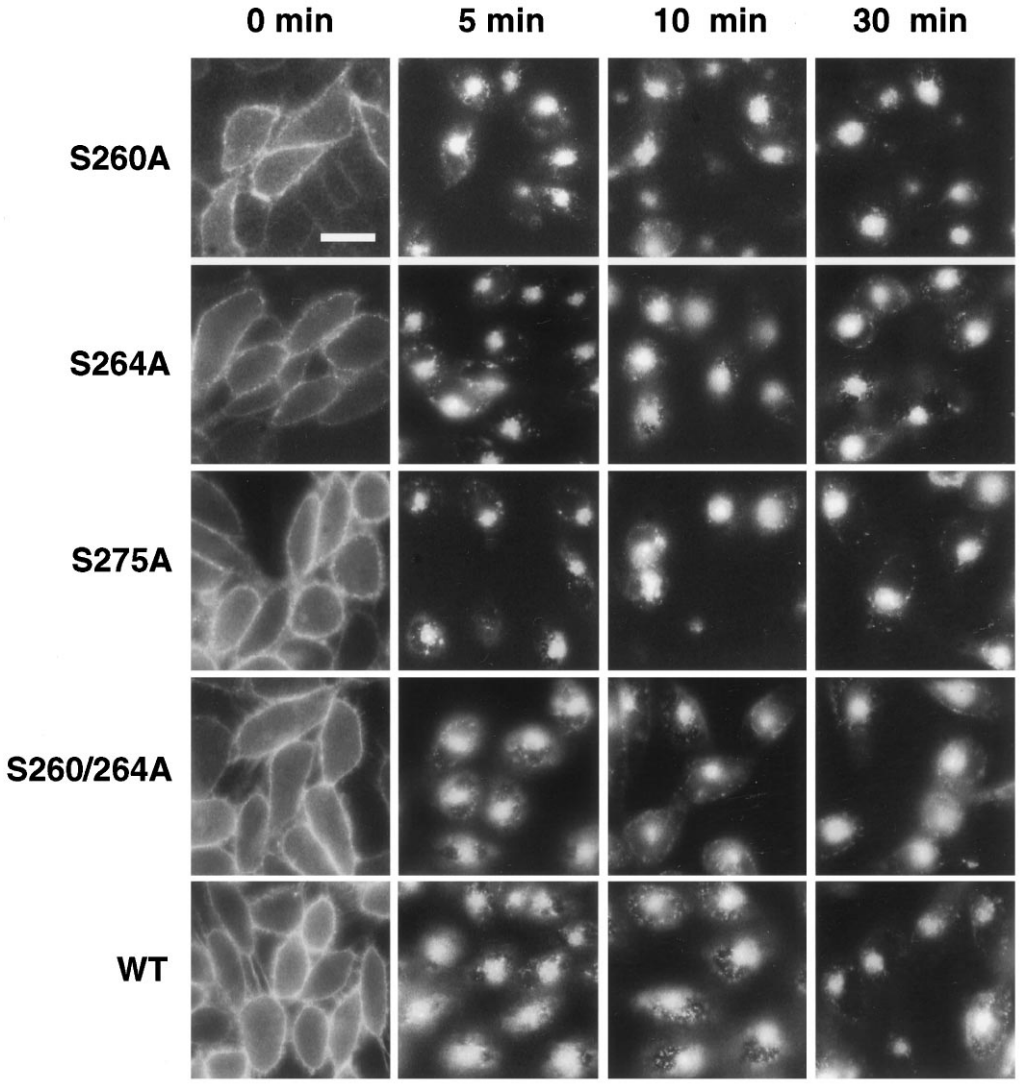

Fig. 6. Comparison of CCKR internalization in CHO cell lines expressing wild-type (*WT*) and mutant receptors. Cells were labeled with 100 nM rhodamine-[Gly-(Nle^{28,31})CCK-26-33] at 4° for 1 hr and then warmed to 37° for the times indicated. Images are representative of at least three independent experiments. For these determinations, each of the mutant receptors behaved similarly to the WT receptor. *Bar*, 20 μM.

tion" in pancreatic acinar cells (14) or to be internalized into the receptor-bearing CHO cell line (13).

Protein phosphorylation currently is recognized as a prominent biochemical mechanism for the rapid and reversible regulation of cellular proteins (8, 25). This has resulted in a proliferation in the identification of cellular protein kinases and phosphatases and in rapid expansion in our understanding of the regulation of these enzymes. Receptor molecules have been a very attractive target for these enzymes, because receptor occupation with agonists initiates activation cascades, which include modification of relevant enzyme activities. Until recently, such focus was on the many protein kinases known to be in signaling cascades. Currently, it also is clear that receptor phosphatase activity can be regulated as well (11). To correlate with these enzyme activities, there is direct evidence for the rapid and reversible phosphorylation of receptor molecules in response to agonist occupation (9, 11).

We have collected a large and unique series of detailed observations relevant to the phosphorylation of the CCKR (1, 2, 9–12). Of particular interest are the differences in receptor phosphorylation that occur dependent on the cell in which this receptor resides (1). Furthermore, we have direct evidence for differences in the cellular handling of this receptor by different cells (13, 14). In the current work, we have directly determined CCK-stimulated responses in a proximal effector, IP_3 production, in these wild-type receptor-expressing cells and have determined desensitization of this response. This provides an opportunity to correlate these functional effects with the details of receptor phosphorylation.

Additionally, we also have studied other receptor constructs expressed in CHO cell lines that represent site mutants with consensus sites of PKC action in the third intracellular loop changed to alanine residues. The latter were particularly interesting, because one site (Ser275) seems not to be phosphorylated by the cell, whereas the two other sites (Ser260, Ser264) are phosphorylated in both CHO cells and acinar cells (1). A dual mutant in which both of these sites were modified resulted in a receptor that was synthesized and transported to the cell surface normally, where it bound hormone with appropriate affinity, and initiated signaling normally, yet which was not phosphorylated on these or any other sites. This suggests a key role played by these sites, and because this eliminated other established sites of phosphorylation that were not mutagenized, this raises the interesting possibility of sequential phosphorylation of distinct sites oc-

curring in a hierarchical manner in the intact cell. It is possible that early phosphorylation of these two sites of PKC action result in a conformational change that exposes other sites of kinase action within the third intracellular loop. Precedent for hierarchical phosphorylation exists in rhodopsin and the *N*-formyl peptide receptor (26, 27). A clear advantage of this construct is the ability to dissociate agonist-stimulated phosphorylation from all of the other functions and handling of the receptor that we have observed to date with the wild-type receptor.

When the wild-type CCKR is expressed on pancreatic acinar cells or CHO-CCKR cells and occupied by CCK, it becomes phosphorylated very rapidly (1, 9), before the receptor moves into distinct cellular compartments of desensitization that are cell specific (13, 14). In the CHO-CCKR cell, these represent endocytic compartments, as well as caveolae (13). In the pancreatic acinar cell, this represents the recently described plasmalemmal compartment of "insulation" in which the receptor becomes immobilized in a domain that probably is depleted in G proteins (14). In both cases, the compartment provides the mechanism for desensitization that does not depend on the phosphorylation state of the receptor.

The cell seems to have multiple mechanisms for protecting itself from overstimulation. It seems wise that these are not affected by phosphorylation, and are, therefore, not dependent on the same biochemical mechanism as the early "uncoupling" event. The CCKR does have a "NP*xx*Y" motif in the predicted seventh transmembrane domain that has been suggested as playing a key role for internalization in other G protein-coupled receptors (28).

It is only before the receptor leaves its highly mobile state on the plasmalemma, where it is capable of interacting with G proteins, that phosphorylation plays a key role in desensitization. This may be a direct effect or, like for the β-adrenergic receptor, this may be mediated by binding to an arrestin-like molecule (4, 5). It is critical to remember how rapidly signaling processes are initiated and how much opportunity there may be for amplification along such pathways. Although the early desensitization that we now attribute to receptor phosphorylation may seem to be relatively minor in the desensitization time course, it may well be critically important in protecting the cell from overstimulation, having biological significance far in excess to what is apparent.

Acknowledgments

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