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Impaired Resensitization and Recycling of the Cholecystokinin Receptor by Co-expression of its Second Intracellular Loop

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ABSTRACT

Intermolecular interaction represents an important theme in regulation of intracellular trafficking of organelles that can be interrupted by competitive overexpression of a relevant molecular domain. We attempted to identify the functional importance of intracellular domains of the cholecystokinin (CCK) receptor by their over-expression in receptor-bearing Chinese hamster ovary (CHO-CCKR) cell lines. Although clathrin-dependent endocytosis and recycling of this receptor are well-established (*J Cell Biol* **128:**1029–1042, 1995), any influence of distinct receptor domains is not understood. In this work, constructs representing each of the intracellular domains of the CCK receptor were coexpressed with wild-type receptor, and stable clonal cell lines were selected. Each was characterized for ligand binding and agonist-stimulated biological activity (inositol 1,4,5-trisphosphate generation), desensitization, resensitization, receptor internalization, and recycling. Each cell line expressed normal CCK radioligand binding, signaling, internalization, and desensitization. Three independent cell lines that coexpressed the 25-residue second intracellular loop domain exhibited deficient resensitization. In morphological assessment of receptor trafficking, this construct was also shown to interfere with receptor recycling to the plasma membrane. As a control, recycling of an unrelated G protein-coupled receptor was demonstrated to occur normally in this cell line. These observations suggest that rather than representing passive cargo within an endosome, a receptor can influence its own trafficking within the cell.

Receptor trafficking represents an extremely important mechanism for regulation of cellular responsiveness to hormone, on a time-scale shorter than can be addressed by de novo receptor synthesis. Receptor recycling to the cell surface after its internalization reestablishes hormonal responsiveness, a process that needs to be regulated for optimal function. Although we know much about the molecular basis of regulation of receptor desensitization, very little is understood about reversing this process.

The most rapid desensitization of G protein-coupled receptors occurs by agonist-induced receptor phosphorylation uncoupling association that for many G protein-coupled receptors is followed by arrestin binding. This leads to uncoupling of receptor association with G proteins (Ferguson et al., 1998; Lefkowitz, 1998). The receptor phosphorylation may occur in response to a kinase in the signaling cascade, or by the action of a member of the G protein-coupled receptor kinase (GRK) family on an active conformation of the receptor (Benovic et al., 1989). On a slightly slower time scale and using mechanisms dependent on receptor phosphorylation or on a phosphorylation-independent conformational change, receptor internalization moves the binding domain out of the reach of hydrophilic ligands.

The major cellular pathway for the internalization of G protein-coupled receptors is clathrin-dependent endocytosis (Roettger et al., 1995b). Here, too, much is known of molecular mechanisms, with interactions with adapter proteins playing a role (Goodman et al., 1996). After entry into this pathway, receptors and their ligands can become passive cargo. As the endosome becomes acidified, some ligands are known to become dissociated and to move into the lysosome for degradation, whereas some receptors can remain with the portion of the vesicular membrane that recycles back to the cell surface (Koenig and Edwardson, 1997). Protein phosphatase action has been implicated in this process for selected G protein-coupled receptors (Krueger et al., 1997; Lin et al., 1997); however, the molecular mechanisms for this and its regulation are unclear.

In this work, we use the cholecystokinin (CCK) receptor as a model system. The type A CCK receptor belongs to the superfamily of G protein-coupled receptors and is structurally related to the β-adrenergic receptor and rhodopsin. These receptors, located on pancreatic acinar cells, smooth muscle cells of the gallbladder and selected regions of the gastrointestinal tract, and neurons in regions of the periph-

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ABBREVIATIONS:GRK, G protein-coupled receptor kinase; CCK, cholecystokinin; IP_3, inositol 1,4,5-trisphosphate; Rho-CCK, rhodamine-Gly-[(Nle^{28,31})CCK-26–33]; KRH, Krebs-Ringers-HEPES.

eral and central nervous systems, are physiologically important in mediating postcibal pancreatic exocrine secretion, gallbladder contraction, enteric motility, and satiety (Mutt, 1980). Agonist occupation activates CCK receptors, leading to hydrolysis of inositol phospholipid with generation of diacyl glycerol, increases in levels of intracellular calcium, and activation of protein kinase C (Pandol et al., 1985). The CCK receptor, as is typical of most G protein-coupled receptors, is desensitized by undergoing phosphorylation and being internalized (Roettger et al., 1995b; Rao et al., 1997) and is resensitized by processes that include recycling to the cell surface (Roettger et al., 1995b). Although phosphorylation of this receptor is responsible for the earliest desensitization, by uncoupling receptor and G protein, it can be fully dissociated from receptor internalization. This is based on observations with a nonphosphorylated receptor mutant that binds its ligand, signals, and is internalized entirely normally (Rao et al., 1997; Roettger et al., 1997).

Because phosphorylation of the CCK receptor is not necessary for its internalization, a mechanism requiring receptor dephosphorylation for regulation of its recycling seems unlikely. A more exact mechanism might involve the receptor directly. It is possible that dephosphorylation can expose such a receptor domain and that the conformational change induced by the pH change in the endosome could achieve the same thing. Here, we explore the relevance of each of the intracellular domains of the CCK receptor to receptor function using a strategy of competitive coexpression of these domains with intact receptor. We studied ligand binding, signaling, and receptor internalization and recycling.

When expressed individually, no single domain had adequate influence to modify the normal binding of CCK, its initiation of signaling, or its stimulation of desensitization and receptor internalization. However, competitive coexpression of the second intracellular loop peptide resulted in impaired resensitization and in the abnormal intracellular trafficking of the internalized CCK receptor. Three independently derived cell lines expressing this construct exhibited similar behavior. As control, a structurally unrelated G protein-coupled receptor was shown to traffic normally in these cells. We postulate that the second intracellular loop of the CCK receptor contributes important determinants for molecular interactions key to receptor recycling and resensitization. The most important implication of this is that the internalized receptor may directly influence its own trafficking, rather than representing passive cargo with its destination determined by an acidification-induced dissociation of ligand and receptor (Koenig and Edwardson, 1997). This also suggests that receptor internalization and recycling have different dynamic mechanisms that provide substantial opportunity for diversity in cellular handling of receptors.

Experimental Procedures

Materials. All reagents were analytical grade. Particular sources included soybean trypsin inhibitor from Worthington Biochemicals (Freehold, NJ) and [^{3}H]inositol 1,4,5-trisphosphate (IP_3) (20 Ci/mmol) from DuPont-New England Nuclear (Boston, MA).

Peptides. Synthetic CCK was purchased from Peninsula Laboratories (Belmont, CA). Radioiodinated and fluorescent analogs of CCK, which we have established and characterized previously (Pearson et al., 1987; Roettger et al., 1995b) were freshly prepared in our laboratory. Both have been shown to be fully biologically active and to bind with high affinity, with no difference from natural CCK. The CCK-like radioligand ^{125}I-D-Tyr-Gly[(Nle^{28,31})CCK-26–33] was radioiodinated oxidatively and purified by reversed-phase, high-performance liquid chromatography to a specific radioactivity of 2000 Ci/mmol (Pearson et al., 1987). The fluorescent analog rhodamine-Gly-[(Nle^{28,31})CCK-26–33] (Rho-CCK) was prepared as described previously (Roettger et al., 1995b).

Cell Lines. The CCK receptor-bearing CHO cell line (CHO-CCKR), which we established and characterized previously (Hadac et al., 1996), was used as the wild-type receptor-bearing control. Cells were grown on tissue culture plasticware in Ham's F12 medium supplemented with Fetal Clone 2 (Hyclone Laboratories, Logan, UT). Cells were passaged twice weekly. For experiments, cells were lifted mechanically, triturated, and washed with appropriate medium before use.

Unique clonal cell lines were prepared for this work, representing cells that simultaneously expressed wild-type CCK receptor in fixed density identical with the control cells, along with a peptide fragment representing an intracellular domain of the receptor. Each cell line was prepared by transfection of the CHO-CCKR cells with an appropriate expression vector. The CHO-CCKR cell line was originally prepared using a G-418-resistant vector encoding the wild-type CCK receptor (Hadac et al., 1996). The strategy for preparing the new cell lines made use of the pCEP-4 eukaryotic expression vector with hygromycin resistance (Invitrogen, San Diego, CA). Each derivative cell line was selected clonally using limiting dilution techniques and was characterized to be certain of coexpression of the region of interest.

Relevant regions of the CCK receptor cDNA, shown in Fig. 1, were amplified by polymerase chain reaction using the wild-type CCK receptor construct as template. The codon numbering used was started at residue 16 of the originally published rat CCK receptor sequence to be consistent with cloned CCK receptor sequences from other species, such as the human sequence. Regions included the first intracellular loop (1i), the second intracellular loop (2i), the amino-terminal portion of the third intracellular loop (3iN), the carboxyl-terminal portion of the third intracellular loop (3iC), the entire carboxyl-terminal tail (4i), and the region of the carboxyl-terminal tail including receptor residues 409 through 423 ($4i_{409–423}$). Each amplification product was designed to contain a restriction site (*Hin*dIII) at the 5′ end for subcloning, followed by a consensus sequence for the initiation of translation (GCCGCCACC), as well as codons for methionine and glycine upstream of the receptor-specific sequences. At the 3′ end of each receptor-derived sequence was a stop codon and a restriction site (*Bam*HI) for subcloning. Constructs were cloned into the multiple cloning site of the pCEP4 vector, and identities were proven by direct dideoxynucleotide DNA sequence analysis (Sanger et al., 1977). CHO-CCKR cells were transfected using lipofectin (Life Technologies, Rockville, MD). Stable transfectants were initially enriched by addition of 0.5 mg/ml hygromycin and clonal lines were then selected by limiting dilution. The presence of the individual receptor domains was confirmed in each cell line using polymerase chain reaction with primers complementary to sequences spanning both vector and insert.

A series of experiments was performed on CHO cell lines transiently expressing the secretin receptor. These cells were prepared by the transfection of an expression vector incorporating the secretin receptor cDNA (Ulrich et al., 1993). The cells were studied 48 h after transfection, using methods and reagents that have been fully characterized and validated (Ulrich et al., 1993; Holtmann et al., 1996).

Quantification of Receptor and Loop Peptide by Radioimmunoassay. The peptide corresponding to the amino acid residues 160 through 172 within the second intracellular loop domain of the CCK receptor (RPLQSRVWQTKSH) was synthesized by solid-phase techniques, both on an octameric-lysine support (Tam, 1988) and as a linear peptide having an amino-terminal extension of Tyr-Gly to provide a site for oxidative radioiodination. Peptides were purified to

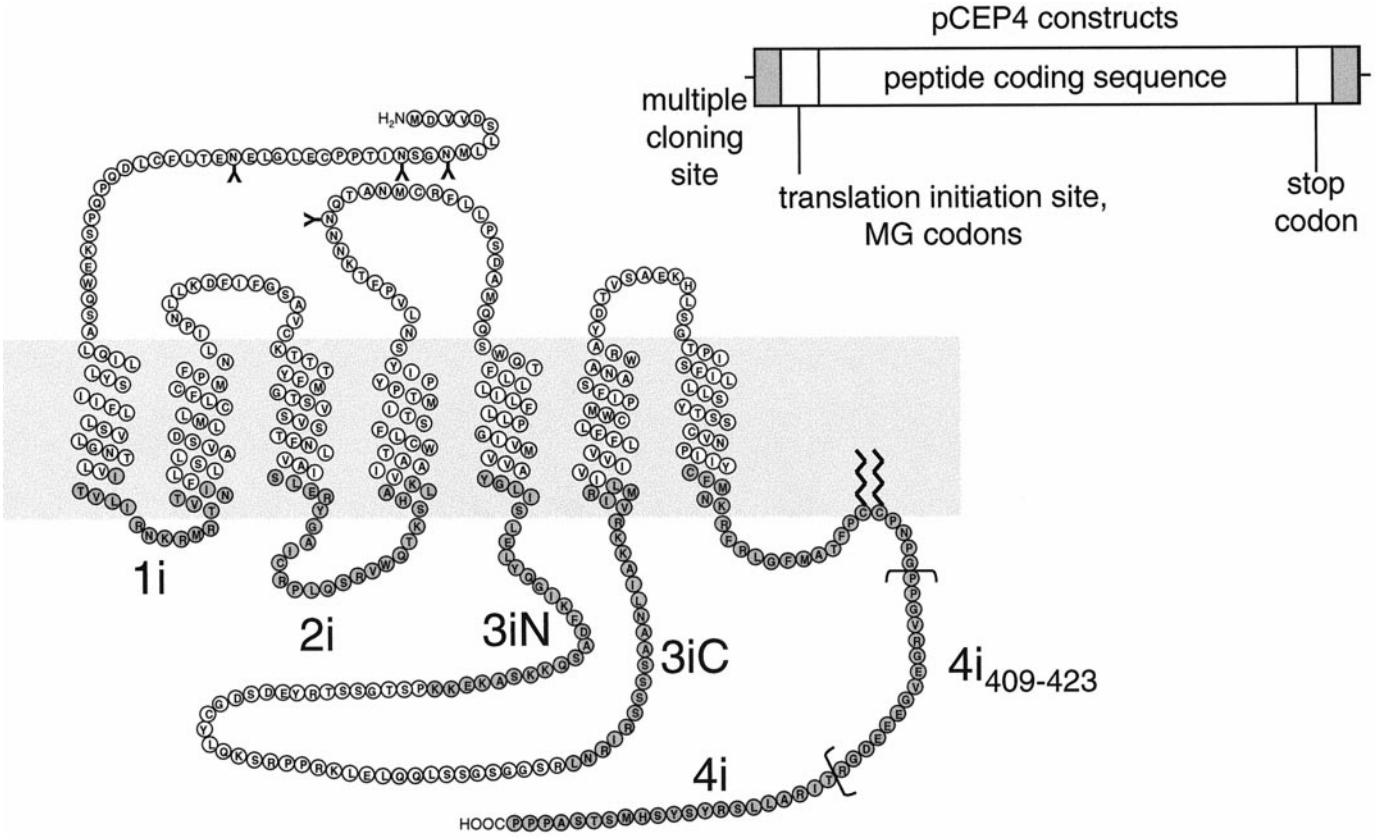

Fig. 1. CCK receptor intracellular domain peptides. Shown is the putative topology of the G protein-coupled CCK receptor, with filled residues representing the peptide sequences employed in the construction of the coexpression vectors. The design of these constructs is also depicted: 1i, first intracellular loop, residues 78 to 93; 2i, second intracellular loop, residues 151 to 175; 3iN, N-terminal portion of the third intracellular loop, residues 244 to 269; 3iC, C-terminal portion of the third intracellular loop, residues 309 to 331; 4i, C-terminal tail, residues 387 to 444; $4i_{409-423}$, residues 409 through 423 of the C-terminal tail.

homogeneity by reversed-phase, high-performance liquid chromatography. The polymeric peptide was used as an immunogen in two New Zealand White rabbits, whereas the linear peptide was used as tracer after oxidative radioiodination and as competing unlabeled peptide in a radioimmunoassay.

For peptide quantification, cytosolic and membrane fractions of CHO cell lines were prepared by differential centrifugation techniques. For this, cells were harvested mechanically, suspended in 15 volumes of iced PBS, and sonicated. Unbroken cells and the nuclei were removed by centrifugation at 1,000*g* for 10 min at 4°C. The resulting supernatant was centrifuged at 225,000*g* for 1 h at 4°C, to separate the cytosolic (supernatant) and membrane (pellet) fractions. The pellet was then washed with iced 1 M NaCl and was solubilized with 1% Nonidet P-40 in PBS overnight at 4°C.

The radioimmunoassay was performed using a double-antibody method (Camarillo et al., 1998). For this assay, serial dilutions of unlabeled peptide (standard) or samples were incubated with the antiserum at a dilution of 1:250 and 20,000 cpm of radioiodinated peptide in buffer (50 mM Na_2HPO_4, pH 7.5, 10 mM EDTA, 1.2 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.2% BSA, and 0.05% NaN_3) at 4°C for 20 to 24 h. Bound and free tracer were separated by addition of goat-antirabbit serum, normal rabbit serum and polyethylene glycol (M_r 8000), followed by centrifugation at 3000*g* at 4°C for 30 min. A representative standard curve for this assay is shown in Fig. 2.

Radioligand Binding and Biological Activity Studies. Cells were plated at a density of 20,000 cells/well in 24-well tissue culture plates 2 days before the competition-binding assay. Cells were incubated with 3 to 5 pM radioligand in the absence or presence of increasing concentrations of unlabeled CCK in 0.5 ml of Krebs-Ringers-HEPES (KRH) medium containing 25 mM HEPES, pH 7.4, 1 mM KH_2PO_4, 104 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.01% soybean trypsin inhibitor, and 0.2% BSA at room temperature for 60 min. Nonspecific binding was defined as radioactivity bound in the presence of 1 μM unlabeled CCK, and represented less than 10% of total binding. After termination of the binding reaction by washing the cells twice with iced medium, cells were lysed with 0.5 ml of 0.5 M NaOH and radioactivity in the lysate was quantified using a gamma counter. Binding parameters, K_i and B_{max}, were determined using the nonlinear least-squares curve fitting computer program LIGAND (Munson and Rodbard, 1980).

Biological activity was assessed by quantification of IP_3 production in response to CCK stimulation, as we established and validated previously for pancreatic acinar cells and CHO-CCKR cells (Rao et al., 1997). This was performed by a rapid, sensitive, and specific competition binding assay that used [^{3}H]IP_3 and a specific IP_3-binding protein that we prepared from rat cerebellum (Bredt et al., 1989). This assay has a sensitivity of 0.1 pmol of IP_3 per tube. Cells were incubated with 10 nM CCK in KRH medium at 37°C for 5 sec. Reactions was terminated by the addition of an equal volume of iced 10% perchloric acid. After centrifugation, the supernatant was diluted with 0.1 ml of 10 mM EDTA and 0.5 volumes of a 3:1 (v/v) mixture of 1,1,2-trichloro-trifluroethane and tri-*n*-octylamine. The samples were centrifuged at 2000*g* for 10 min, yielding three phases. The upper IP_3-containing phase was collected and used for the subsequent binding assay. Of this phase, 150 μl was incubated with 150 to 200 μg of the binding protein in IP_3 assay buffer for 30 min at 4°C, followed by centrifugation at 3000*g* for 15 min. The pellets were reconstituted in 75 μl of 0.15 M NaOH and radioactivity was quantified in a scintillation counter. Each assay condition was performed

in duplicate and each set of experiments was repeated a minimum of three independent times.

Receptor Regulation Studies. Desensitization and resensitization experiments were performed using protocols established previously (Rao et al., 1997). In the desensitization experiments, paired aliquots of cells were treated with or without 10 nM CCK for 5 s at 37°C to provide control basal and control stimulated responses. Analogous aliquots of cells were preincubated with 1 μM CCK at 37°C for 10 min and were then washed extensively with KRH medium. Under all conditions, these washes were adequate to return cellular IP_3 content to basal unstimulated levels. The second aliquot of the pair was stimulated with 10 nM CCK for 5 sec at 37°C. A desensitized response was less than the stimulated response in the control cells that had not previously been exposed to CCK. This was expressed as a percentage of the range from basal to the maximal stimulated response of the control aliquots.

For the resensitization experiments, cells were desensitized as described above (Rao et al., 1997). After the desensitizing exposure to CCK and washing, paired aliquots of cells were incubated at 37°C in KRH medium in the absence of additional CCK for 60 min. One of each pair was then stimulated with 10 nM CCK for 5 sec at 37°C. Cells were again assayed for IP_3 content as described above. Resensitization was expressed as a percentage of the control response of an aliquot of cells that had not previously been exposed to CCK.

CCK Receptor Internalization and Recycling. CCK-stimulated CCK receptor internalization and recycling were assayed morphologically using a fluorescent CCK ligand that has been previously characterized and fully validated (Roettger et al., 1995a,b). The fluorescent ligand Rho-CCK is a full agonist and binds to the CCK receptor with high affinity (Roettger et al., 1995a,b). Direct analysis with CCK receptor antibody demonstrated the validity of this assay in these cells, with the receptor trafficking along with the fluorescent ligand (Roettger et al., 1995b; Toledo et al., 1997). In brief, in the internalization assay, cells grown on glass coverslips were incubated with 50 nM Rho-CCK for 1 h at 4°C to saturate surface receptors. Cells were then warmed to 37°C for various periods of time before washing, fixation with 2% paraformaldehyde, and morphologic analysis. Cells were examined using a Nikon Microphot FXA microscope (Nikon, Tokyo, Japan) equipped for epifluorescence or using a Zeiss 510 confocal microscope.

For determination of recycling of the internalized receptor back to the cell surface, nonfluorescent CCK was used in place of Rho-CCK to initially saturate surface receptors, and cells were incubated in the presence of 5 mg of cycloheximide per milliliter in all steps. The assay was initiated by warming the cells to 37°C. At each relevant time point, cells were again cooled to 4°C and incubated with 50 nM Rho-CCK for 1 h at 4°C to occupy and saturate surface receptors. With receptor synthesis inhibited, receptors reappearing on the cell surface represented recycled receptor. This assay has been validated previously (Roettger et al., 1995b).

Data Analysis. Each experimental condition was studied in a minimum of three independent experiments. Data were expressed as means ± S.E.M., with differences examined using Student's *t* test for unpaired values. Significant differences were considered at *P* values less than .05.

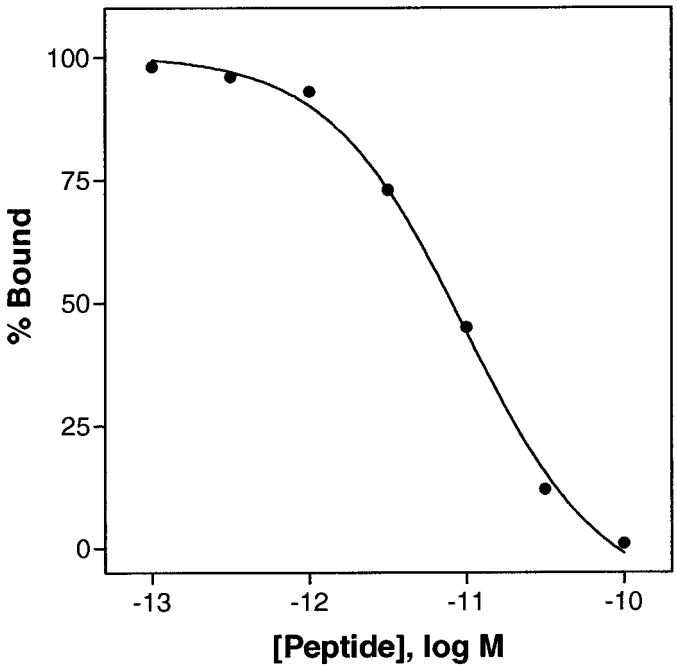

Fig. 2. Quantification of intracellular second loop peptide of the CCK receptor. Shown is a typical standard curve for the radioimmunoassay developed to quantify CCK receptor and its competing second loop peptide (residues 160–172). Serial dilutions of unlabeled peptide standard were incubated with the antiserum at a dilution of 1:250 and 20,000 cpm of radiolabeled peptide in phosphate buffer at 4°C for 20 to 24 h. Bound and free tracer were separated by the double-antibody technique, as detailed under Experimental Procedures.

Results

Competitive Coexpression of the Intact Receptor and its Intracellular Domain Peptides. Each of the cell lines described under *Experimental Procedures* was established by transfection of the parental CHO-CCKR cell line, leading to clonal lines that expressed similar density of wild-type CCK receptors (Hadac et al., 1996). Presence of the coexpressed receptor fragment was established for each cell line by PCR analysis of the integrated construct using primers that spanned the vector and insert sequences. Any positive effect observed was evaluated in a minimum of three independently derived and characterized cell lines.

The concentrations of CCK receptor in the membrane fraction and of the receptor intracellular second loop peptide in the cytosolic fraction of relevant cell lines were quantified by radioimmunoassay (Fig. 2). Immunoreactive peptide that diluted in parallel to the standard curve was detected in the membrane fractions from CHO-CCKR cells and CHO-CCKR cells coexpressing the receptor second intracellular loop domain and in the cytosolic fraction of the latter cells. Cytosolic and membrane preparations of non-receptor-bearing CHO cells, having no such dilutable immunoreactivity, were included as negative controls in each assay. The concentration of the second loop peptide in the cytosol of the relevant cell lines was in the range of 27 to 50 pmol/mg of protein. This represented a concentration of approximately 10- to 20-fold that of the membrane fraction containing the intact CCK receptor (1 to 2 pmol/mg of protein). The amount of receptor on the cell surface determined this way was similar to that determined by LIGAND analysis of competition-binding data (Munson and Rodbard, 1980; Hadac et al., 1996).

Receptor Binding and Signaling. CCK bound specifically and with high affinity to each of the cell lines. Competition by CCK for binding the CCK-like radioligand was always similar to that observed in the parental CHO-CCKR cell line (Fig. 3). Data for binding constants and receptor density quantification are shown in Table 1. Agonist-stimulated IP_3 signaling was also similar for each of the cell lines (Fig. 4). We previously characterized the time- and concentration-dependence for CCK to stimulate IP_3 responses in the CHO-CCKR cells (Rao et al., 1997). The response reached a

peak in 5 s and was followed by a rapid reduction to a plateau at 30 to 40% of the maximal response. Complete desensitization occurred within 2 min of high dose stimulation. Each of the cell lines exhibited basal and maximally stimulated IP_3 responses to CCK similar to that of the parental cell line (Fig. 4A). Figure 4B shows the concentration-response curves for CCK to stimulate IP_3 in cell lines coexpressing the second intracellular loop peptide and the wild-type receptor and the parental cell line expressing only the wild-type receptor. Each of these cell lines displayed the same potency and efficacy of CCK-stimulated IP_3 responses, with EC_{50} values of 1.0 ± 0.1 nM (CHO-CCKR + 2i) and 0.9 ± 0.1 nM (CHO-CCKR).

Receptor Desensitization and Resensitization. We have previously characterized the patterns of CCK receptor desensitization and resensitization after agonist stimulation of the CHO-CCKR cells (Rao et al., 1997). This receptor undergoes rapid and complete desensitization after CCK stimulation (Rao et al., 1997). In the present study, the influence of coexpression of each of the intracellular domain peptides on agonist-stimulated receptor desensitization was examined. Each of the cell lines exhibited desensitization of CCK-stimulated IP_3 responses after 10 min of exposure to CCK that were not different from control parental cells treated the same way (Fig. 5A). The responses after this treatment were in the range of 5 to 15% of the values of the control cells that had not been preincubated with CCK.

The CCK receptor-bearing CHO-CCKR cells have also had their ability to resensitize after exposure to CCK was quantified (Rao et al., 1997). In the present work, the influence of coexpression of each of the intracellular receptor domain peptides on resensitization was also examined. Each of the cell lines, except the line expressing the second intracellular loop peptide, resensitized in manner similar to the control parental cells (Fig. 5B). Three independently derived cell lines that expressed the second loop domain peptide behaved similarly to each other and quite distinctly from all of the other cell lines. These constructs exhibited markedly defective resensitization, with their IP_3 response to CCK stimulation remaining at essentially the same level achieved after the desensitization protocol. This was in contrast to the return to approximately 70% of the maximal control IP_3 response after 60 min at 37°C in the other cell lines.

Receptor Internalization and Recycling. Each of the cell lines internalized agonist-occupied CCK receptors promptly upon warming, in manner and quantity analogous to that in the control cells. Figure 6 illustrates representative time-courses of receptor internalization after fluorescent ligand occupation in control cells and in cell lines coexpressing the second intracellular loop of the receptor. There were no differences in the kinetics of internalization between these cell lines.

CCK receptor recycling after agonist-induced internalization of the wild-type receptor expressed on CHO-CCKR cells has been described previously (Rao et al., 1997). In the present work, quantitative morphometry was performed on the control parental cell line and on the cell lines coexpressing the receptor intracellular domain peptides. Consistent with the impaired resensitization observed in the cell lines coexpressing the second intracellular loop peptide (Fig. 5B), receptor recycling back to the cell surface was significantly inhibited in the cell lines expressing this construct (Fig. 7A). Receptor on the cell surface reached only 10% of control levels after 30 min in these cells compared with almost 50% of control levels in the parental cell line (Fig. 7B).

To assess the specificity of this defect in recycling, we transiently expressed the secretin receptor in the CHO-CCKR cells and in the cell line coexpressing the second intracellular loop domain peptide. As shown in Fig. 8A, this receptor was fully internalized and recycled similarly in both

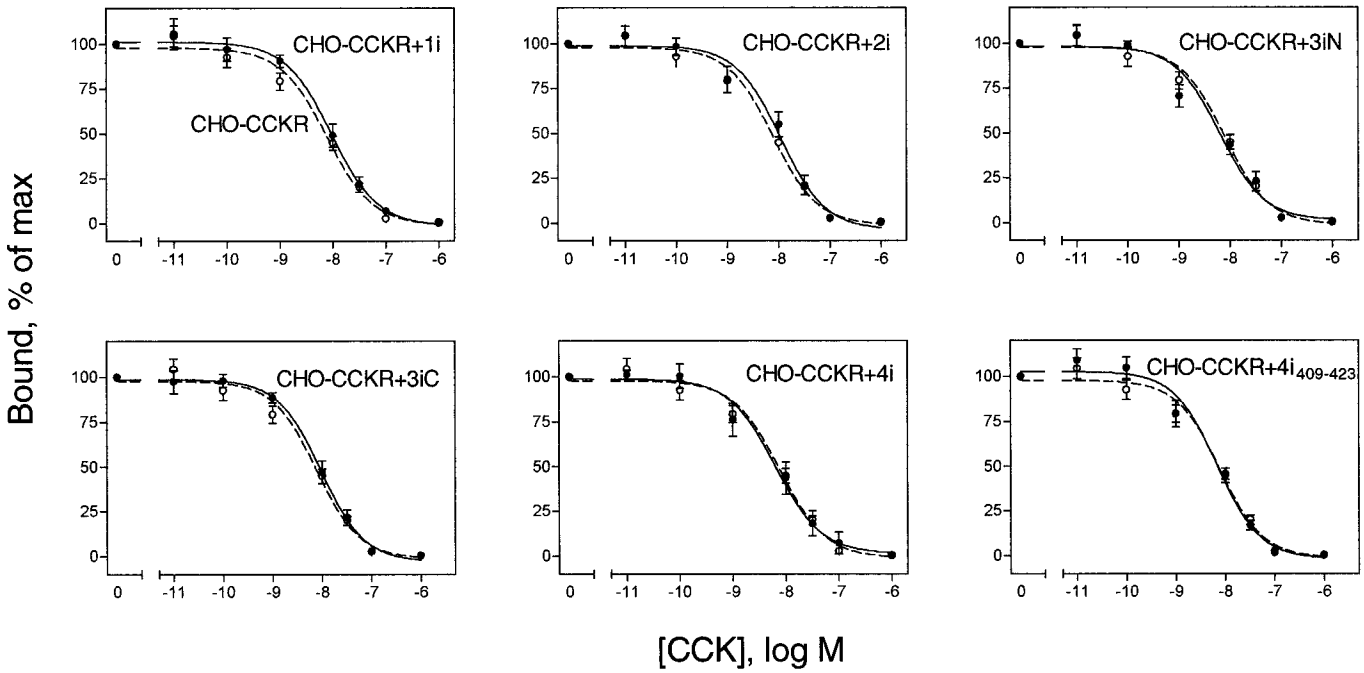

Fig. 3. Effect on CCK receptor binding. Shown is the ability of CCK to compete for binding of the CCK-like radioligand to CHO-CCKR cells and cells coexpressing the receptor intracellular domain peptides. Competition binding was similar for each of the cell lines studied. Data represent means ± S.E.M. for a minimum of three independent experiments.

the experimental and control cell lines. Quantitative morphometry demonstrated completely normal secretin receptor recycling in these cells, despite the abnormal recycling of the CCK receptor in the same cells (Fig. 8B).

Discussion

Cellular trafficking of plasma membrane receptor molecules is an important mechanism for the regulation of the sensitivity of hormone-stimulated signaling. We previously explored CCK receptor movement through the cell using receptor antisera and fluorescently tagged and electron-dense receptor ligands (Roettger et al., 1995b; Toledo et al., 1997). These parameters were also explored with cell lines expressing mutant and truncated receptor constructs that interfered with agonist-stimulated receptor phosphorylation (Rao et al., 1997). This work has demonstrated that agonist occupation of the CCK receptor on the CHO-CCKR cell line results in its internalization via clathrin-dependent endocytosis, with a minor population moving into caveolae (Roettger et al., 1995b). Agonist-stimulated phosphorylation of these receptors contributes to the earliest phase of desensitization, by uncoupling receptor and G protein; however, this seems to play no role in directing receptor internalization (Rao et al., 1997; Roettger et al., 1997). After internalization, substantial recycling of the CCK receptor back to the plasma membrane of these cells was also shown to occur (Rao et al., 1997; Roettger et al., 1995b).

In the present study, cell lines were established that coexpressed peptides corresponding to each of the intracellular domains of the CCK receptor along with the intact receptor. The rationale for this was to explore the ability of individual structural domains to compete for functionally important processes. Precedent for this experimental design came from the studies of Hawes et al. (1994), in which coexpression of the third intracellular loop domains of the β-adrenergic and muscarinic M_3 receptors with their respective wild-type receptors was shown to interfere with ligand-stimulated signaling in intact cells. Similar observations have also been made for receptors in this superfamily in membrane preparations (Dalman and Neubig, 1991). The ability of the coexpressed peptides derived from receptor sequences to interact with the same molecular partners as the intact receptor has been well established. Third intracellular loop domain peptides derived from sequences of the β-adrenergic receptor, the M_4 muscarinic receptor, and the α-adrenergic receptor have all been shown to mimic their activated receptors by coupling with and activating the relevant G proteins in vitro (Dalman and Neubig, 1991; Okamoto et al., 1991).

In the present series of experiments, only the second intracellular loop domain of the CCK receptor had recognizable impact on function of the intact receptor. Unlike the previous studies in which a proximal event occurring at the level of the plasma membrane was affected, here the impact was substantially later in time and space. The cell line expressing the second intracellular loop peptide along with the wild-type CCK receptor exhibited normal ligand binding, signaling, and internalization, but abnormally slow resensitization compared with the control cell line expressing a similar density of wild-type receptors. Receptor recycling was also impaired in this cell line, correlating with the impairment in resensitization. These results were observed with the competing peptide present in the cytosolic fraction in concentration 10- to 20-fold that of the intact receptor within the plasma membrane, a ratio that is consistent with biological specificity.

The absence of demonstrable effects of the other intracellular domains of the CCK receptor in this series of competitive coexpression experiments should not be overinterpreted. Although this could mean that a specific domain is not important for a function, it is also consistent with representing

TABLE 1
Characterization of CCK binding to CHO-CCKR cells and cell lines coexpressing the receptor intracellular domain peptides

Cell lines	CCK Binding K_i	CCK Receptors B_{max}
	nM	*× 10^5 sites/cell*
CHO-CCKR	6.6 ± 0.7	1.2 ± 0.3
CHO-CCKR + 1i	8.8 ± 1.0	0.5 ± 0.1
CHO-CCKR + 2i	10.3 ± 1.4	1.0 ± 0.3
CHO-CCKR + 3iN	3.1 ± 0.4	1.3 ± 0.4
CHO-CCKR + 3iC	6.7 ± 0.3	1.6 ± 0.5
CHO-CCKR + 4i	5.3 ± 1.6	0.9 ± 0.3
CHO-CCKR + $4i_{409–423}$	6.2 ± 0.7	1.5 ± 0.5

Data represent means ± S.E.M. for a minimum of three independent experiments.

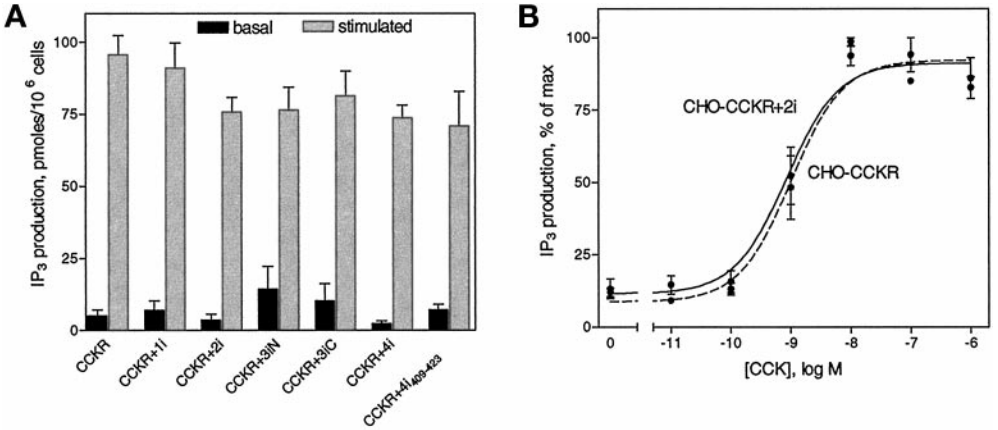

Fig. 4. Effect on CCK-stimulated cell signaling. A, IP_3 levels in the basal state and in response to 10 nM CCK stimulation for 5 s were quantified in CHO-CCKR cells and in cell lines coexpressing the receptor intracellular domain peptides. No significant differences were observed between the control and experimental cell lines. B, full concentration-response curves are shown for CCK-stimulated IP_3 levels for the wild-type CCK receptor-bearing CHO-CCKR cell line and for the cell lines coexpressing the receptor second intracellular loop domain. Data represent means ± S.E.M. for a minimum of three independent experiments.

a functionally important domain with a molecular interaction that is particularly difficult to displace. This could reflect a very high affinity interaction that is established early during biosynthesis, an interaction with a huge stoichiometric excess of soluble partner, or an interaction that is broad and involves multiple distinct domains. The CCK receptor-G protein interaction probably falls into the latter category, as seems to be true of dopamine and muscarinic M_1 receptors (Luttrell et al., 1993). We also cannot exclude the possibility of subtle effects of the other peptides, such as effects on rate of desensitization, rather than on the extent of desensitization that was monitored.

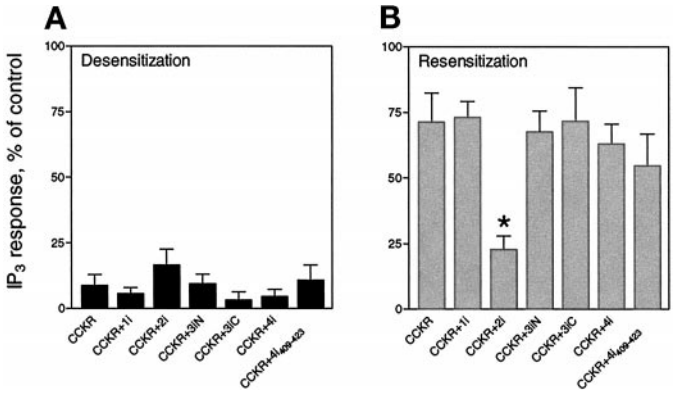

Fig. 5. Effect on desensitization and resensitization of IP_3 responses to CCK. A, each of the cell lines coexpressing the receptor intracellular domain peptides were desensitized to the CHO-CCKR cell line to a similar degree after being incubated with 1 μM CCK for 10 min at 37°C, washed, and stimulated with 10 nM CCK for 5 sec at 37°C. B, in the resensitization experiments, cells were desensitized as described above, washed, and incubated in KRH buffer at 37°C for 1 h before CCK stimulation. After 60 min, the CHO-CCKR cells recovered approximately 70% of their control maximal response to CCK. Each of the cell lines coexpressing intracellular domain peptides behaved similarly to this, except for the cell line expressing the second loop domain. Resensitization in three independently derived cell lines coexpressing that construct along with the wild-type CCK receptor was essentially absent. Data represent means ± S.E.M. for a minimum of three independent experiments. **, significant difference from the control CHO-CCKR cells ($P < .01$).

Still another possibility is that the relevant domain is modified in the natural receptor, such as by phosphorylation, and that the nonmodified peptide does not reflect the relevant structure, and therefore does not effectively compete for the binding of its partner. This, too, would be important for the third intracellular loop peptides that include the residues of this receptor that are known to be phosphorylated in response to agonist occupation (Ozcelebi and Miller, 1995; Ozcelebi et al., 1996). We know from our previous studies (Ozcelebi and Miller, 1995; Ozcelebi et al., 1996), however, that the second intracellular loop does not contain any such residues.

In this experimental design that incorporates peptide competition, one cannot be certain that a positive effect reflects the competition with an external partner (soluble cytosolic) molecule. It is also theoretically possible that the domain interacts intramolecularly with another domain of the receptor itself. It is, however, unlikely that such an interaction could be effectively competed off by a soluble coexpressed peptide because of the effective local concentrations of each. The effective concentration of two domains held near each other because they are part of the same molecule are likely to be much higher than that of a soluble coexpressed peptide.

In our experimental design, we chose to express the intracellular domain peptides without any epitope tags, because of concern that the latter might alter the conformation and accessibility of a potentially critical region. We were certain that the appropriate construct was incorporated into the cell line by using a polymerase chain strategy that involved primers spanning vector and insert sequences that would have been absent or of distinct size in the intact receptor construct. We also controlled for positional effects of incorporation of the experimental constructs by preparing and studying multiple independent clonal cell lines for any positive effect. For the single construct that had this clear effect, we developed specific new methods to quantify expression of peptide and receptor. This involved raising a polyclonal rabbit antiserum against a peptide epitope within the second intracellular loop

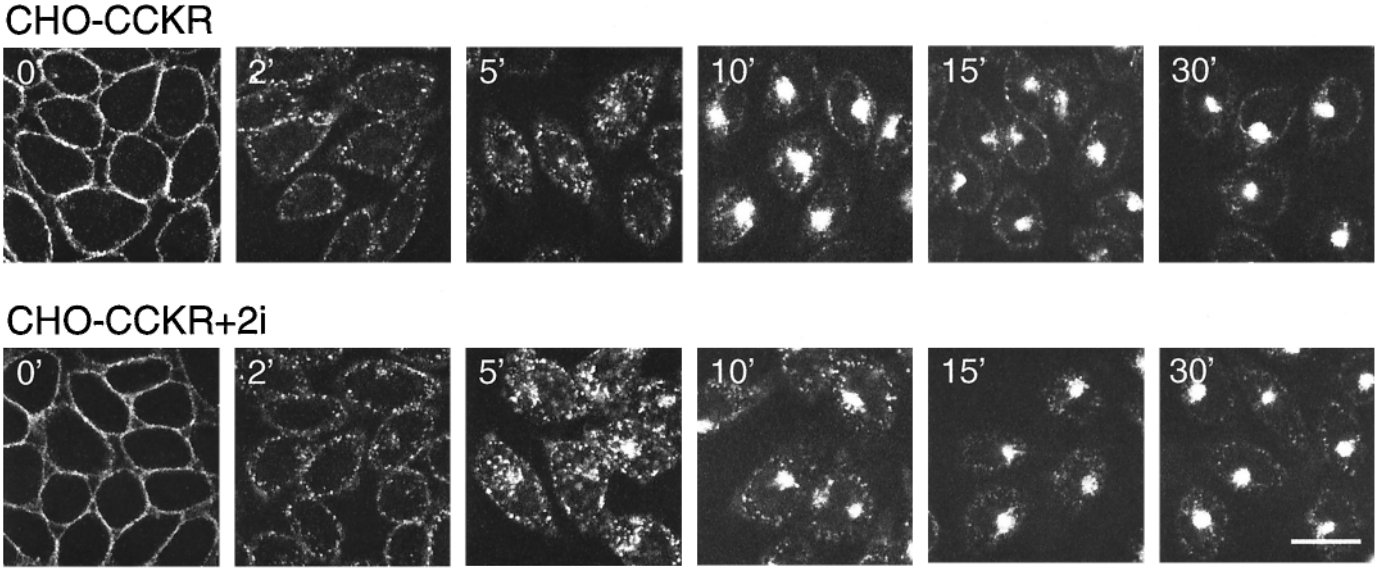

Fig. 6. Effect on CCK receptor internalization. Shown are representative fluorescent images of Rho-CCK movement over time into the CHO-CCKR cells and into cells coexpressing the receptor second intracellular loop domain peptide. Cell lines coexpressing each of the receptor intracellular domains internalized agonist ligand-occupied CCK receptor in manner similar to that of the control CHO-CCKR cell line. Images are representative of at least three independent experiments. Bar, 20 μM.

domain and developing a sensitive and specific radioimmunoassay that was able to quantify both receptor in the membrane and peptide in the cytosolic fraction. This assay gave a value for the membrane CCK receptor similar to that of the calculation based on equilibrium radioligand binding using the LIGAND program (Munson and Rodbard, 1980). The concentration of the competing peptide in the cytosol was 10- to 20-fold that of the intact receptor. Thus, the expression was in an excellent range to be biologically specific and not at such a high level of expression of the competitor to have nonspecific effects.

We recently demonstrated that mutation of two key sites of

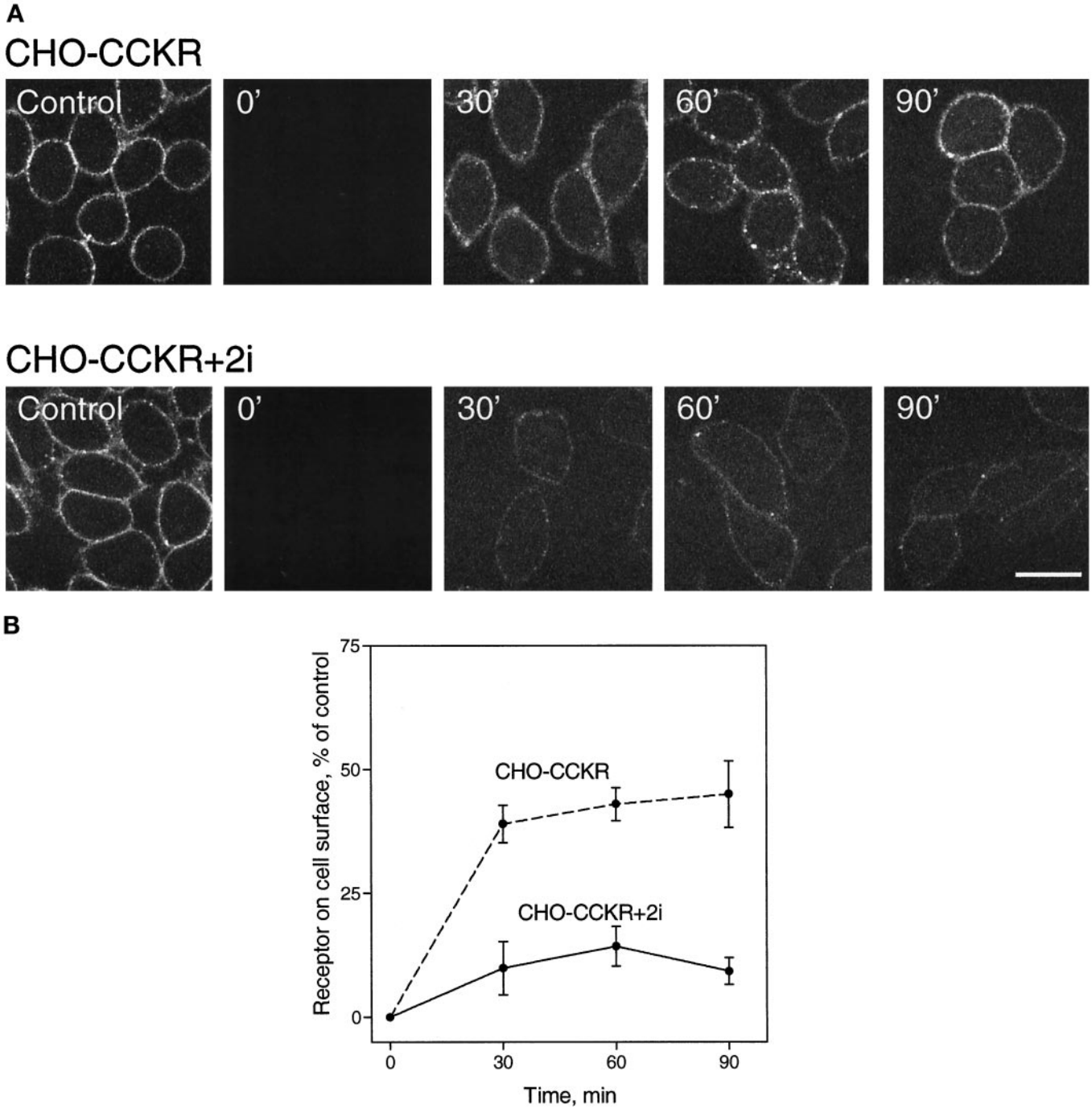

Fig. 7. Effect on CCK receptor recycling. Shown are representative images for the control CHO-CCKR cells and for the cell line coexpressing the receptor second intracellular loop domain peptide. In the recycling assay, surface receptors were first saturated by incubation with 50 nM nonfluorescent CCK at 4°C for 1 h, followed by washing with buffer at 4°C. The assay was then initiated by warming the cells to 37°C. After this, at each time point, aliquots of cells were cooled to 4°C and receptor on the cell surface at that time point was morphologically probed with 50 nM Rho-CCK for 1 h at 4°C. In control cells that were not preincubated with CCK, this reflected the normal starting density of surface receptor. Recycling was expressed as the percentage of the control receptor density that returned to the cell surface over time. A, both cell lines promptly and effectively cleared the CCK-occupied surface receptors, but the cell line coexpressing the second loop peptide exhibited a markedly reduced rate and extent of receptor recycling back to the cell surface relative to the control cells. Images are representative of at least three independent experiments. Bar, 20 μM. B, the graph shows quantification of data (means ± S.E.M.) from a minimum of three independent experiments.

action of protein kinase C within the third intracellular loop of the CCK receptor, changing serines 260 and 264 into alanines (S260,264A), resulted in the absence of agonist-induced receptor phosphorylation, despite the presence of at least three other normal sites of phosphorylation within other intracellular domains (Ozcelebi and Miller, 1995; Ozcelebi et al., 1996; Rao et al., 1997). Interestingly, even the direct activation of protein kinase C using phorbol ester in cells expressing this receptor mutant exhibited no receptor phosphorylation (Rao et al., 1997). This led us to postulate the hierarchical phosphorylation of the CCK receptor, with these most prominent sites of protein kinase C action being phosphorylated first and thereby opening up the third loop and exposing the other sites of receptor phosphorylation (Rao et al., 1997). Consistent with this hypothesis, another CCK receptor mutant in which these serines were replaced with aspartate residues (S260,264D) to mimic the charge of the phosphoserines has been shown to exhibit agonist-stimulated phosphorylation of the other normal sites (Rao et al., 2000). A most interesting observation with the S260,264A mutant was its deficient resensitization and abnormal recycling to the cell surface, with both of these ameliorated by the S260,264D mutation (Rao et al., 2000). We postulate that the phosphorylation of the third loop exposes the second loop domain that we now find key for this receptor trafficking event.

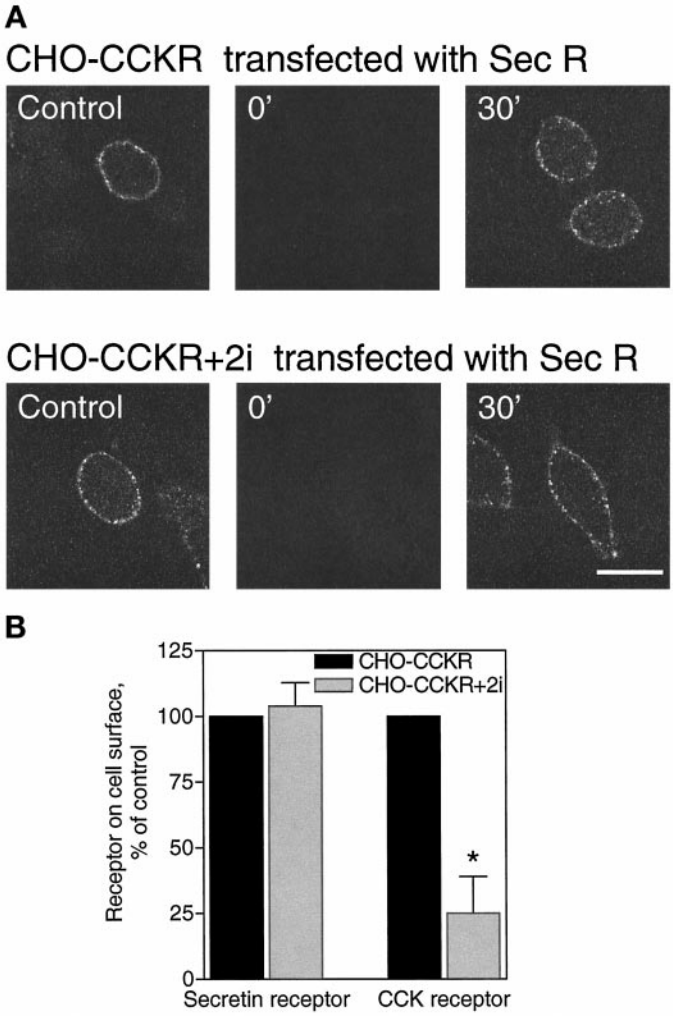

Fig. 8. Control receptor recycling. The CHO-CCKR cells and the cell line coexpressing the receptor second intracellular loop domain were transfected with an expression vector containing the secretin receptor cDNA. Internalization and recycling of secretin receptors expressed on these cells were then studied morphologically, using a well-characterized fluorescent secretin analog. Both cells expressed functional receptor that promptly and completely internalized upon agonist occupation. A, after 30 min, receptor recycling to the cell surface was clearly apparent, and similar in both cell lines. This is in marked contrast to the deficient recycling of the CCK receptors on the cell line coexpressing the second intracellular loop domain peptide. Bar, 20 μM. B, the graph shows quantification of data (means ± S.E.M.) for a minimum of three independent experiments. **, significant difference from CHO-CCKR cells ($P < .01$).

Structural motifs that are important for mediating intermolecular interactions are being widely recognized and found to be prominent in signaling cascades (Bairoch, 1993). The second intracellular loop peptide was 25 residues long, containing five basic and one acidic residues, but no currently recognized functional sequence motif. This primary sequence pattern is not conserved in the G protein-coupled receptor superfamily and not even in the members of the rhodopsin-β-adrenergic receptor family that are most structurally similar to this receptor. It is possible that this will ultimately constitute a conformational structural motif that will be more broadly represented in this receptor family. However, no structural data are now available to confirm or refute this hypothesis.

Molecular partners for such motifs appearing in modular form can be identified by affinity purification approaches, such as yeast two-hybrid screening and affinity chromatography. Our observation in the current work that the 25-residue peptide representing the second intracellular loop of the CCK receptor specifically influences the intracellular trafficking of the intact receptor supports the possibility that an important motif could be present within this region. Because this peptide is relatively short and is not a recognized site for post-translational modification, it should be an ideal tool to explore potential molecular partners that might mediate the observed effect.

The theme of hiding such a structural motif under a receptor loop that moves upon its phosphorylation and thereby exposes the module of interest satisfies the need for regulation of key intermolecular interactions. As we learn more about the molecular details of this proposed event, it should become clearer whether other events in the signaling cascade (i.e., phosphorylation of partner molecules) or in the changing milieu within the endosome (i.e., acidification) are important as well. The evolving theme of a hidden structural motif within an internalized receptor affecting the trafficking of the receptor adds a new level of complexity and ability to regulate key events in management of the sensitivity of a specific cell to hormone-induced signaling.

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