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ACCELERATED COMMUNICATION

Antagonist-Stimulated Internalization of the G Protein-Coupled Cholecystokinin Receptor

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SUMMARY

Receptor-mediated endocytosis has been observed after agonist occupation of several G protein-coupled receptors, which contributes to the desensitization response to agonist stimulation; however, the cellular signals required to initiate this process are unclear. In this study, we developed and characterized a new antagonist analogue of cholecystokinin (D-Tyr-Gly-[(Nle^{28,31},D-Trp^{30})cholecystokinin-26–32]-phenethyl ester) that can be tagged with a fluorescent rhodamine and radioiodinated. This has permitted us to demonstrate that antagonist occupation of the cholecystokinin receptor also results in receptor internalization, which dissociates this response from second messenger signaling activities and receptor phosphorylation. Immunolocalization of this receptor after occupation with an established nonpeptidyl antagonist confirmed this phenomenon. Antagonist-induced receptor internalization probably results from stabilization of the receptor in a conformation that exposes a domain critical to directing it into the clathrin-dependent endocytic pathway. This work provides evidence for a new and independent mechanism for receptor internalization, provides a mechanism for the rarely observed phenomenon of antagonist-induced desensitization, and raises important issues regarding the approach to establish optimal treatment regimens for antagonist drugs.

Treatment with agonist drugs often results in desensitization of target responses and tachyphylaxis. This response has been correlated with the intrinsic efficacy and potency of the agonists, and molecular mechanisms have often been attributed to biochemical effects of cellular signaling cascades (1, 2). Antagonist therapy, however, is typically initiated without consideration of such phenomena, titrating dose to the acute blockage of an active process. This reflects the recognized absence of traditional signaling events after antagonist administration. However, it is possible that the conformational change in the receptor induced by or stabilized by an antagonist results in the unveiling of a molecular domain that could result in a bimolecular interaction leading to receptor internalization. Indeed, there are reports of reduced agonist responses effected by administration of apparent antagonists (3, 4), but the mechanisms of such phenomena are not clear. Inverse agonism has been raised as a possible mechanism for desensitization, but the intrinsic inverse efficacies of antagonists have not correlated with this phenomenon (4). It has also been suggested that agents leading to such a response might have actually been agonists with low amplitude, transient responses, which stimulated desensitization.

In this work, we explore the possibility that true antagonists could lead to the internalization of their receptors, and we provide insights into the mechanism of that response. We focus on the possibility of antagonist-induced internalization of the receptor into a cellular compartment which would clearly be inaccessible to natural, hydrophilic agonist ligands. For this, we have developed a new fluorescent peptidyl analogue of CCK that allows morphologic tracking of the ligand-receptor complex and have rigorously demonstrated its antagonist status. Receptor kinesis was also tracked after occupation with an established nonpeptidyl antagonist (5) using immunolocalization with a receptor antibody (6). Both lines of evidence demonstrated the clear and substantial

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ABBREVIATIONS: CCK, cholecystokinin; D-Trp-OPE, D-Tyr-Gly-[(Nle^{28,31},D-Trp^{30})CCK-26–32]-phenethyl ester; Rho-D-Trp-OPE, rhodamine-Gly-[(Nle^{28,31},D-Trp^{30})CCK-26–32]-phenethyl ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; TPA, phorbol-12-myristate-13-acetate; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline.

internalization of the CCK receptor in response to antagonist occupation, disproving the hypothesis that receptor internalization is correlated with the efficacy and potency of agonist occupation. Although this may be an unusual response, recognition that this phenomenon can occur should change the way we establish optimal dosages and treatment schedules for antagonist drugs and point toward a distinct mechanism for the induction and regulation of receptor endocytosis, which can be independent of signaling cascades.

Materials and Methods

Reagents

Synthetic CCK-8 (CCK-26–33) was purchased from Peninsula Laboratories (Belmont, CA). The putative antagonist analogue of CCK, D-Trp-OPE, and its fluorescent analogue, Rho-D-Trp-OPE, were synthesized in our laboratory using established methods (7) and purified to homogeneity by reversed-phase, high pressure liquid chromatography (8). All products were analyzed by amino acid analysis and mass spectrometry. Peptides were radioiodinated oxidatively using the solid-phase oxidant iodobeads (Pierce Chemical, Rockford, IL), and products were purified by high pressure liquid chromatography, as we have described (9). The nonpeptidyl CCK receptor antagonist L-364,718 was generously provided by Dr. R. Freidinger of Merck, Sharp, and Dohme Laboratories (West Point, PA).

The rat type A CCK receptor-bearing CHO cell line, CHO-CCKR, which we previously established and characterized (10), was used as cellular source of this receptor. These cells express 125,000 CCK receptors per cell, with binding and signaling fully intact. The parent cell line, CHO-K1 (American Type Culture Collection, Rockville, MD), was used as a non-receptor-bearing negative control. Both cell lines were maintained in culture in Ham's F12 medium supplemented with 5% Fetal Clone 2 (Hyclone Laboratories, Logan, UT) on Falcon (Cowley, United Kingdom) plasticware. Cells were passaged twice weekly.

CCK receptor binding studies

Receptor binding studies were performed with enriched plasma membranes prepared from the CHO-CCKR cells using the technique previously described (10). The radioligand ^{125}I-D-Tyr-Gly[(Nle^{28,31})-CCK-26–33] has been previously fully characterized and validated as binding identically to native CCK-8 (9). The antagonist radioligand ^{125}I-D-Trp-OPE was used similarly. Membranes representing 5–25 μg of protein were incubated with 10 pM radioligand in 0.5 ml of Krebs-Ringer-HEPES medium containing 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 0.2% bovine serum albumin, 0.01% soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride for 60 min at 25°. This provided steady state conditions before separation of bound from free radioligands using the Skatron cell harvester (Sterling, VA) with receptor-binding filtermats. Filters were counted in a γ-spectrometer, and data were analyzed using the LIGAND program, using a weighted least-squares nonlinear regression method (11). All conditions were assayed a minimum of three independent times, in duplicate. Data were graphed using the Prism program (GraphPad, San Diego, CA).

Biological activity studies

Intracellular calcium. For the determination of intracellular calcium responses, cells plated on glass coverslips were loaded with 5 μM Fura-2/AM (Molecular Probes, Eugene, OR) in Ham's F-12 medium for 20 min at 25°. Coverslips were then washed twice and brought to 37° in the same medium. They were then rinsed with a physiological saline buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 1.28 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 2 mM L-glutamine, 5.5 mM D-glucose, and 0.2% bovine serum albumin) and mounted onto a temperature-controlled stage of a Zeiss (Oberkochen, Germany) Axiovert microscope at 37°. The cells were stimulated with varied concentrations of CCK and the putative antagonist analogue, D-Trp-OPE (10). Relative calcium levels were determined using ATTOFLUOR RatioVision software (Zeiss, Thornwood, NY) and standard ratiometric techniques, with an excitation wavelength of 340 nm and emission of 380 nm (12).

cAMP

Intracellular cAMP levels in ligand-stimulated cells were determined by radioimmunoassay with a [^{3}H]cAMP tracer, using reagents from Diagnostic Products (Los Angeles, CA), as we reported previously (13). This assay is sensitive to 0.1 pmol per tube and has less than 0.1% cross-reactivity with cGMP. For maximal sensitivity, 1 mM 3-isobutyl-1-methylxanthine was incorporated into the medium during the cell stimulation, which was performed for 10 min at 37°. All conditions were assayed a minimum of three times in duplicate.

Microphysiometry

Microphysiometry was performed to determine proton flux across the plasma membrane of the CHO-CCKR cells using a Cytosensor apparatus from Molecular Devices (Menlo Park, CA) (14). This technique is sensitive to all types of cell signaling events now recognized, making it a highly sensitive screen for agonist activity. All observations were repeated in three independent experiments.

CCK receptor internalization studies

Fluorescent ligand studies. Internalization of ligand-bound CCK receptor was assayed morphologically, using rhodamine-conjugated ligands and following the experimental protocols we previously reported (15). CHO-CCKR cells grown on glass coverslips were washed three times with 37° PBS (1.5 mM NaH_2PO_4, 8 mM Na_2HPO_4, 0.145 M NaCl, 0.1 mM $MgCl_2$, and 0.08 mM $CaCl_2$, adjusted to pH 7.4), transferred to iced buffer for 10 min, and incubated for 1 hr at 4° with either 2 nM Rho-D-Trp-OPE or 50 nM fluorescent CCK-like analogue, rhodamine-Gly[(Nle^{28,31})CCK-26–33]. In select experiments, cells were exposed to 1 μM TPA for 10 min before incubations with the fluorescent ligand. Fixation was performed with 2% paraformaldehyde in PBS.

Immunolocalization of CCK receptor

Receptor internalization was also studied more directly by immunolocalization with a CCK receptor antiserum using methods established previously (6).

CCK receptor phosphorylation

CCK receptor phosphorylation was studied in living CHO-CCKR cells in response to ligand binding, following protocols we previously established (16, 17). This involved the radiolabeling of the cellular ATP pool by incubation with ^{32}P, followed by stimulation of cells with receptor ligands and the rapid and efficient cell fractionation and purification of the CCK phosphoreceptor to radiochemical purity. The amount of phosphoreceptor was quantified by densitometry using a PhosphorImager (Molecular Dynamics).

Results and Discussion

For this work, we synthesized a fluorescent analogue of the previously reported peptidyl antagonist of the CCK receptor, JMV-179 (18), using manual, solid-phase techniques, and purifying the product by reversed-phase high pressure liquid chromatography. This molecule, Rho-D-Trp-OPE, had its identity confirmed by amino acid analysis and mass spectrometry. It bound to the CCK receptor saturably, specifically, and with high affinity [K_i = 22 ± 1.6 nM, based on analysis with the LIGAND program (11)] (Fig. 1a). CCK is

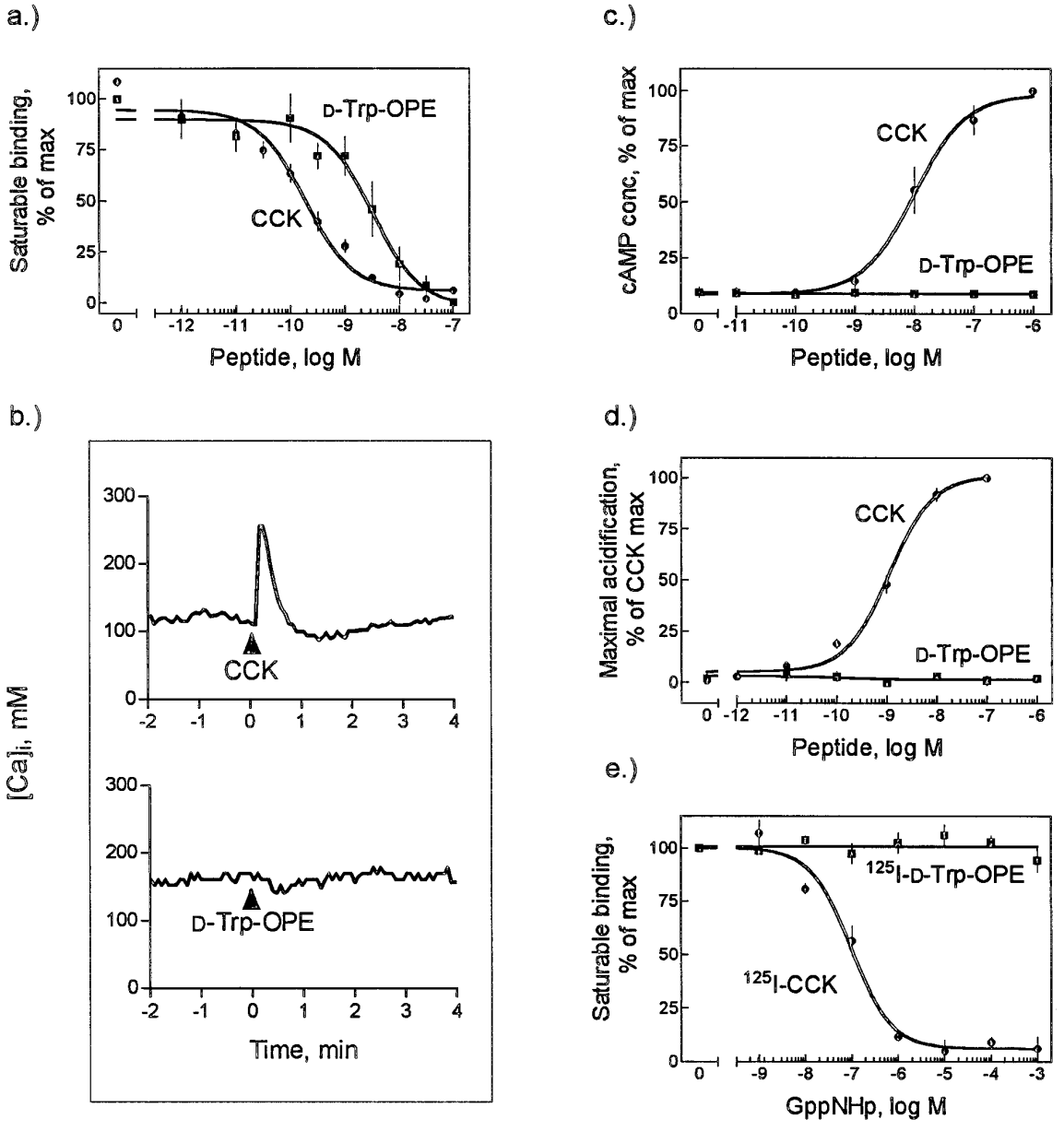

Fig. 1. Binding and biological activity of D-Trp-OPE analogue. a, Competition for the binding of radioiodinated D-Trp-OPE analogue of CCK to CHO-CCKR cell membranes by Rho-D-Trp-OPE and CCK. Shown is saturable binding, with the values representing the mean ± standard error for three separate experiments performed in duplicate. b, Intracellular calcium responses to CCK and its D-Trp-OPE analogue. Single-cell calcium levels were determined as previously validated, using fluorescence ratio imaging (10, 12). Values shown are representative of 26 CHO-CCKR cells exposed to 10 nM CCK in three separate experiments and 23 cells exposed to 10 μM D-Trp-OPE in two experiments. Similar patterns of responsiveness to agonist and unresponsiveness to antagonist were observed in three separate experiments each with 1 nM CCK and 100 nM and 1 μM D-Trp-OPE. c, Cellular cAMP responses to CCK and its D-Trp-OPE analogue. Values represent mean ± standard error of cAMP responses of the CHO-CCKR cells observed in three separate experiments performed in duplicate. d, Proton efflux across the plasmalemma of CHO-CCKR cells determined by microphysiometry (14). Values shown represent mean ± standard error of the maximal responses to peptides observed in three separate experiments performed in duplicate. e, Effects of a nonhydrolyzable GTP analogue on binding of radioiodinated analogues of CCK and D-Trp-OPE. CHO-CCKR cell membranes were incubated with radioligands in the presence of varied concentrations of 5′-guanylyl-imidodiphosphate. Values represent mean ± standard error for three separate experiments performed in duplicate.

known to be a potent stimulant of the phospholipase C cascade of events leading to an increase in intracellular calcium and also to stimulate adenylate cyclase and accumulation of intracellular cAMP after higher agonist concentrations (19). Both of these signaling events were prominently observed after stimulation of the CCK receptor-bearing CHO-CCKR cells with CCK (10) (Fig. 1, b and c), whereas concentrations of Rho-D-Trp-OPE that fully saturate the surface receptors had no effect on these parameters (Fig. 1, b and c). Microphysiometry is an extremely sensitive measure of cellular metabolic activity as reflected in the rate of proton efflux across the plasma membrane, which is known to be affected by all recognized cell signaling cascades (14). This makes it a highly sensitive screen for agonist activity. Another advantage of this technique is the continuous monitoring of this potential activity after ligand administration, to assure that

potential transient effects are not missed. CCK stimulated a concentration-dependent increase in this parameter, whereas Rho-D-Trp-OPE had no effect (Fig. 1d). Also consistent with the absence of agonist activity of this compound was the lack of effect of the nonhydrolyzable GTP analogue, 5′-guanylyl-imidodiphosphate, on binding of a radioiodinated derivative of D-Trp-OPE, whereas this GTP analogue markedly inhibited CCK radioligand binding (Fig. 1e).

Despite the absence of measurable signaling responses to CCK receptor occupation by Rho-D-Trp-OPE, this process resulted in substantial internalization of the ligand-receptor complex (Fig. 2). The cellular compartments traversed by the receptor seemed to be similar for both agonist- and antagonist-occupied receptors (Fig. 2a). Initiation of internalization was observed for both at the 5-min time point. The extent of internalization, however, was different, with a less complete response to antagonist occupation than to agonist occupation. For the agonist-occupied receptors, 90 ± 2% were internalized to the perinuclear compartment after 30 min at 37°, whereas only 37 ± 4% of antagonist-occupied receptors moved similarly (quantified by reconstruction of serial confocal images, with summation of signal above background in each). A representative series of confocal sections through the agonist-stimulated CHO-CCKR cells at this time point is shown in Fig. 2b. We previously used manipulations such as hypertonic sucrose treatment and ultrastructural evaluation to establish that CCK receptor internalization after agonist occupation in these cells was predominantly via the clathrin-dependent pathway (15). As is typical for a clathrin-mediated endocytic process, hypertonic sucrose treatment also inhibited the internalization of the antagonist-occupied CCK receptor (Fig. 2c).

To be certain that the observed internalization was not a constitutive process in these cells and to confirm that the receptor movement correlated with that of the fluorescent ligand, receptor immunolocalization was also performed. No internalization of CCK receptors was observed in unstimulated cells, whereas intracellular compartments were prominently labeled by the receptor antibody in cells exposed to the nonfluorescent native hormone ligand, CCK (6) and the well established nonpeptidyl CCK receptor antagonist, L-364,718 (5) (Fig. 2d). Therefore, two chemically distinct receptor antagonists, a peptide analogue of CCK (Rho-D-Trp-OPE) and the benzodiazepine derivative L-364,718 both stimulated CCK receptor internalization.

Although receptor phosphorylation has been suggested as a trigger for internalization (1, 20) and arrestin-like molecules, which bind to phosphorylated domains of some receptors in this family, have been implicated in receptor internalization (21), it is unlikely that receptor occupation by an antagonist would stimulate this covalent modification. We therefore used methodology previously established and validated in our laboratory (16) to directly assess the phosphorylation state of the CCK receptor after antagonist stimula-

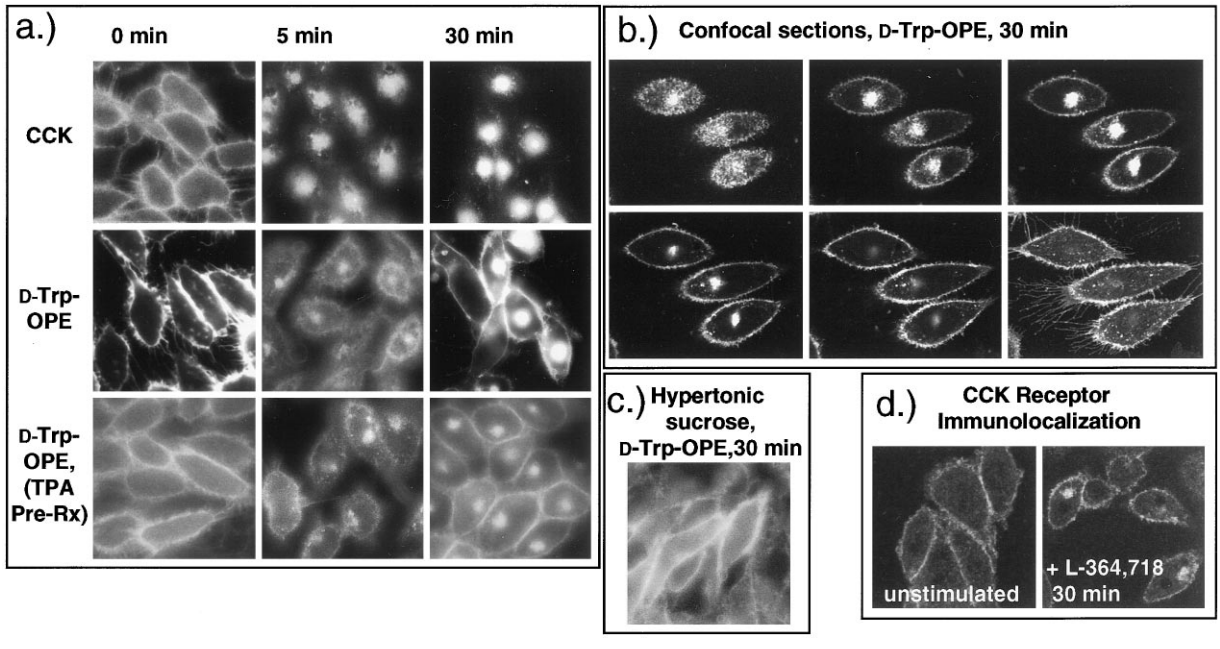

Fig. 2. Morphological assessment of cellular handling of D-Trp-OPE analogue. a, Shown are time courses of CHO-CCKR cell handling of fluorescent analogues of CCK and D-Trp-OPE. *Top row*, typical internalization of the agonist ligand (50 nM, Rho-D-Trp-OPE).*Middle row*, typical internalization of the antagonist ligand (2 nM Rho-D-Trp-OPE) under control conditions. *Bottom row,* after treatment with 1 μM TPA for 10 min. Images are representative of three separate experiments. b, Serial optical sections through the 2-nM Rho-D-Trp-OPE-labeled CHO-CCKR cells 30 min after binding at 37° using confocal microscopy. The internalized receptor represented 37 ± 4% (n = 21) of the antagonist-occupied receptor population and 90 ± 2% (n = 9) of the agonist-occupied receptors, determined by summation of the fluorescent signal above background in serial 1-μm confocal sections of each cell on a Zeiss LSM 310 microscope, using the IBAS Image Analysis System (Kontron Elektronik, Munich, Germany). Analyses were conducted on data from three separate experiments. c, Disruption of clathrin-dependent endocytosis by hypertonic sucrose treatment. CHO-CCKR cells were treated with 0.45 M sucrose in PBS before and during 5-, 10-, and 30-min incubations with 2 nM Rho-D-Trp-OPE at 37°, as previously described (22). The image shown is for a 30-min incubation and is representative of four separate experiments. d, Immunolocalization of internalized CCK receptors after antagonist occupation of receptors on CHO-CCKR cells. CCK receptors were localized using a polyclonal antiserum directed against a carboxyl-terminal domain of the CCK receptor, which was previously validated (6). Cells were incubated at 37° for 30 min in the presence or absence of 10 nM L-364,718 following the procedure outlined (6). Images are representative of three separate experiments.

tion. Unlike the native agonist, the receptor antagonists stimulated no significant increase in receptor phosphorylation (Fig. 3). We also explored the influence of CCK receptor phosphorylation, which was stimulated by exposure of the cells to the phorbol ester, TPA. This treatment did not modify the extent or time course of antagonist-stimulated receptor internalization (Fig. 2a).

Thus, the process of internalization of antagonist-occupied CCK receptors seems to be independent of G protein-coupling, intracellular calcium or cAMP signaling, proton efflux across the plasma membrane, or even phosphorylation of the receptor. We postulate that the internalization occurs because of a signal that is intrinsic to the receptor itself, whereby occupation of the CCK receptor by the antagonists leads to a conformational change or stabilizes a conformation that exposes a receptor domain which mediates internalization. This likely occurs via a bimolecular interaction that directs the receptor into the endocytic pathway.

This work demonstrates that internalization of the G protein-coupled CCK receptor can occur in response to occupation with peptidyl and nonpeptidyl antagonists, independent of activation of second messenger pathways and receptor phosphorylation. This does not rule out roles for signaling events in desensitization of the agonist-occupied receptor but provides evidence for an independent and previously unrecognized mechanism for receptor internalization. The ability of antagonist occupation to reduce the surface complement of receptors and lead to their kinesis into the clathrin-dependent endocytic pathway raises the possibility that it leads to down-regulation of the receptor. Clearly, for native peptide hormone to have access to its binding domain on the receptor, that molecule must be on the cell surface. Thus, antagonist exposure can reduce subsequent responsiveness to agonist stimulation. Awareness of this possibility should affect the establishment of dosage schedules for antagonists of the CCK receptor and possibly for other receptors in this family.

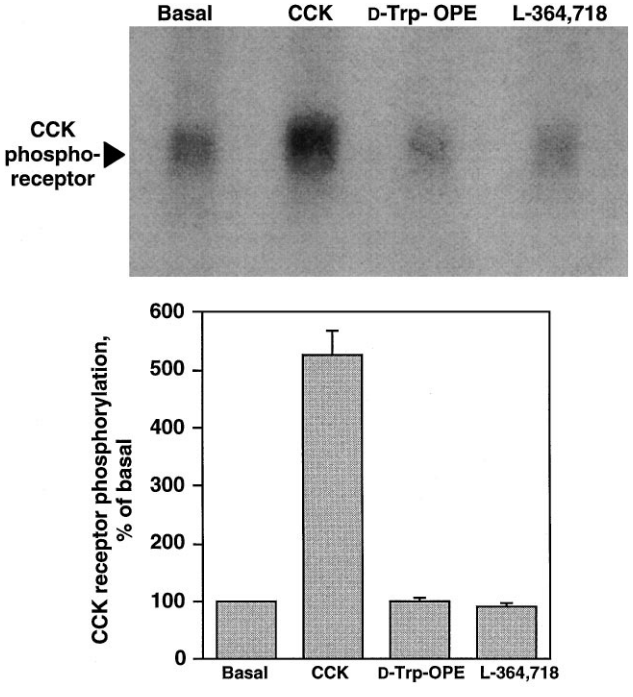

Fig. 3. Effect of CCK receptor antagonists on receptor phosphorylation. a, Using the techniques well established previously (16), we explored the ability of CCK receptor ligands to affect the state of phosphorylation of the CCK receptor expressed on CHO-CCKR cells. Shown is a representative autoradiograph of the CCK phosphoreceptor in the basal state and after stimulation with the full agonist, CCK, and the peptidyl and nonpeptidyl antagonists, Rho-D-Trp-OPE and L-364,718. Quantitation of data from a minimum of three such experiments is also shown, with data expressed as mean ± standard error.

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