Src Regulates Constitutive Internalization and Rapid Resensitization of a Cholecystokinin 2 Receptor Splice Variant*

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The third intracellular loop domain of G protein-coupled receptors regulates their desensitization, internalization, and resensitization. Colorectal and pancreatic cancers, but not the nonmalignant tissue, express a splice variant of the cholecystokinin 2 receptor (CCK2R) called CCK2i4svR that, because of intron 4 retention, contains an additional 69 amino acids within its third intracellular loop domain. This structural alteration is associated with agonist-independent activation of Src kinase (Olszewska-Pazdrak, B., Townsend, C. M., Jr., and Hellmich, M. R. (2004) J. Biol. Chem. 279, 40400–40404). The purpose of the study was to determine the roles of intron 4 retention and Src on CCK2i4svR desensitization, internalization, and resensitization. Gastrin1–17 (G17) binds to both CCK2R and CCK2i4svR and induces intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) increases. Agonist-induced increases in [Ca\(^{2+}\)]\(_i\) were used to assess receptor activity. Src kinase activity was inhibited by transducing cells with a retrovirus containing a dominant-negative mutant Src (A430V). The subcellular location of enhanced green fluorescent protein-tagged receptors was monitored using laser scanning confocal microscopy. Both receptor variants desensitized at the same rate; however, CCK2i4svR desensitized five times faster than CCK2R. Without agonist, 80% of CCK2i4svR is located in an intracellular compartment. In contrast, 80% of CCK2R was located on the plasma membrane. Treatment with inverse agonist (YM022) or expression of dominant-negative Src blocked the constitutive internalization of CCK2i4svR, resulting in its accumulation on the plasma membrane. Expression of dominant-negative Src slowed the rate of CCK2i4svR desensitization. Inhibition of Src did not affect G17-induced internalization of either receptor variant. Constitutive internalization of CCK2i4svR increases its rate of resensitization by creating an intracellular pool of receptors that can rapidly recycle back to the plasma membrane.

Receptor desensitization and resensitization are essential mechanisms for maintaining physiologically appropriate cellular responses to extracellular stimuli. For G protein-coupled receptors (GPCR\(^2\)(s)), these processes are regulated, in part, by the intracellular domains of the receptors. Within seconds to minutes of agonist binding, the activated receptor is desensitized when specific amino acid residues within the 3il and/or C-terminal tail domain of the receptor are phosphorylated, creating binding sites for proteins, such as β-arrestins and Src kinase, which block further receptor interactions with heterotrimeric G proteins and initiate receptor internalization (1–3). Resensitization involves the dephosphorylation of the intracellular domains and recycling of the intact receptor back to the plasma membrane where it can once again bind ligand (4, 5). Dysregulation of these processes can lead to disease (6, 7).

CCK2R is a GPCR that exists as various splice variant isofoms (8). Previously, we reported the identification and cloning of a variant, called CCK2i4svR, which, as a result of intron 4 retention, contains an additional 69 amino acid residues in its 3il domain (9). In contrast to CCK2R, which is widely expressed in normal tissues of the gastrointestinal tract and nervous system, CCK2i4svR appears to be the predominant variant expressed by some hyperplastic polyps, colorectal and pancreatic cancer cells, and pancreatic cancer-derived cell lines (9–12). Several lines of evidence support a role for CCK2i4svR in the progression of these cancers. First, CCK2i4svR, but not CCK2R, coimmunoprecipitates with activated Src kinase in an agonist-independent manner (13). Increased Src activity is associated with the development and progression of breast, brain, pancreas, and colon cancers (14, 15). Second, expression of CCK2i4svR stimulates cell growth in vitro (9) and HEK293 tumor growth in vivo in a Src-dependent fashion (16). Third, inhibition of CCK2i4svR expression, using antisense oligonucleotides, slowed agonist (gastrin1–17 (G17))-stimulated growth of BxPC3 pancreatic cancer cells in vitro (17). Finally, treatment with G17 promotes proliferation, motility, and invasion in various in vitro and in vivo cancer models in pancreatic and colorectal cancer cells (18–21). Together, these studies suggest that CCK2i4svR is involved in the pathophysiology of pancreatic and colorectal cancers through both agonist-independent and -dependent mechanisms.

Because the 3il domain of GPCRs plays a critical role in regulating agonist-dependent receptor activities, including desensitization, internalization, and resensitization, the purpose of this study was to determine the effects of intron 4 retention on these processes. We report that both CCK2R splice variants desensitize at a similar rate. However, CCK2i4svR resensitizes significantly faster than CCK2R. The rapid resensitization rate of CCK2i4svR is a consequence of its constitutive, Src-dependent internalization, which creates an intracellular pool of receptors that rapidly recycle back to the plasma membrane. These studies suggest an additional mechanism by which Src can contribute to the cancer phenotype: the rapid resensitization of CCK2i4svR to the growth-promoting agonist, G17.
EXPERIMENTAL PROCEDURES

Plasmid Expression Constructs and Transfected Cell Lines—Receptor constructs containing a C-terminal enhanced green fluorescent protein (EGFP), tag, designated CCKR-EGFP and CCK2i4svR-EGFP were constructed and transfected into HEK293 cells as described previously (13).

Retroviral Expression Construct—The dominant-negative (dn)Src cDNA was cloned into a bicistronic packaging murine oncoretroviral vector based on pFB (Stratagene) using Sall and EcoRI sites. The vector contains the murine leukemia retrovirus packaging sequence and a multiple cloning site, flanked by the murine leukemia retrovirus long terminal repeat regions. The 5’-long terminal repeat functions as a strong promoter upon chromosomal integration of proviral DNA. The pFB plasmid was modified to contain a caspase comprising an ECMV internal ribosome entry site followed by a gene encoding β-galactosidase (modified with a nuclear localization signal), which enabled retrovirus titer and transcript expression levels to be determined by staining for β-galactosidase (22). Retroviruses were made by simultaneous transfection of HEK293FT cells (Invitrogen) with the dnSrc expression plasmid and plasmids encoding murine leukemia retrovirus gag-pol and vesicular stomatitis virus envelope protein. Cell supernatants were collected and filtered through a 0.45-μm filter to remove cell debris prior to infection.

Quantification of Receptor Expression—The level of receptor expression for each clonal line was quantified using 125I-labeled agonist binding to isolated cell membranes (23). Receptor expression levels by the various clones ranged from ~0.43 to 1.8 pmol receptor/mg of membrane protein. The smallest difference in level of receptor expression between EGFP-tagged clones was ~2-fold. In all cases, cells transfected with CCK-R-EGFP expressed higher levels of receptor than cells transfected with CCK2i4svR-EGFP. For the studies presented, we used CCK2i4svR-EGFP clonal lines that expressed between 2 and 3.5-fold fewer receptors than the cell line expressing CCKR-EGFP.

Receptor Desensitization and Resensitization—The rates of receptor desensitization and resensitization were compared using agonist-induced increases in [Ca2+]i as a measure of receptor activity. The change in the concentration to [Ca2+]i, was determined using the Ca2+ binding dye, Fura 2/AM (Molecular Probes, Eugene, OR) as described previously (24). Briefly, cells were cultured on 25-mm glass coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, washed with a physiological medium (KRH) containing NaCl (125 mM), KCl (5 mM), KH2PO4 (1.2 mM), MgSO4 (1.2 mM), CaCl2 (2 mM), and incubated with 2 μM Fura 2/AM for 50 min at room temperature. Single cell changes in [Ca2+]i were recorded using a Nikon Diaphot inverted microscope (Garden City, NY) and a CCD camera (Dage-MTI, Inc., Michigan City, IN). At least 30 cells were analyzed per time point in each experiment and each experiment was repeated three times.

Light Scanning Confocal Microscopy (LSCM) and Image Analysis—LSCM was performed on both live and 4% paraformaldehyde-fixed cells. For live cells, the HEK293 cells expressing either CCKR-EGFP or CCK2i4svR-EGFP were grown on laminin-coated (15 μg/ml) (Trevigen Inc., Gaithersburg, MD) coverslips for 24 h. The cells were then rinsed and placed into a heated stage incubator containing KRH solution (pH 7.4) at 37 °C. The EGFP fluorescence distribution was observed using a Zeiss LSM510 META laser scanning confocal microscope (63× objective with oil immersion; excitation 488 nm, emission 505-530 nm). Sequential confocal images were collected at 0.8 μm sections throughout the cells. 17–22 optical sections were collected for each cell scanned. After acquiring the initial Z-series scan, the cells were treated with agonist (100 nM), and additional Z-series images were acquired at various time points.

For fixed cells, the cells were plated on laminin-coated glass coverslips, treated as described in the figure legends and fixed in 4% paraformaldehyde for 30 min. The plasma membrane of cells was stained with 2.5 μg/ml of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) for 8 min at 37 °C. The coverslips were mounted with Vectashield mounting medium on microscope slides, and confocal images were collected as described above. Image processing and colocalization analysis were performed using Metamorph 5.05 software (Universal Imaging, West Chester, PA). The percentage of plasma membrane associated receptor was defined as the percentage of EGFP (green) pixels that colocalized with plasma membrane dye DiI (red) pixels. At least 20 individual cells/condition were analyzed for each experiment, and each experiment was repeated three times.

RESULTS AND DISCUSSION

Both CCKR Variants Exhibit Similar Rates of Desensitization; However, CCK2i4svR Resensitizes Faster—Because the 3il domain of GPCRs plays a critical role in receptor desensitization, endocytosis, recycling, and resensitization, it is reasonable to hypothesize that these processes are differentially regulated by the two CCKR receptor splice variants. To test this hypothesis, we compared the rates of receptor desensitization and resensitization using agonist-induced increases in [Ca2+]i as a measure of receptor activity.

To determine the rate of receptor desensitization, HEK293 cells expressing either recombiant CCK2i4svR or CCKR were repeatedly stimulated with G17 (10 nM) over a time course. The initial change in [Ca2+]i, induced by a 15-s exposure to 10 nM agonist at 22 °C was defined as the maximum response. Following this first stimulation, cells were washed with KRH and rechallenged with 10 nM G17 for another 15-s period. This process was repeated 10 times at 2.5-min intervals over a 25-min time course. Both receptors rapidly desensitized with first-order decay kinetics. Analyses of the desensitization curves revealed t1/2 maximum Ca2+ response values of 2.9 min (95% confidence interval, 2.6–3.3 min, R2 = 0.99) and 2.6 min (95% confidence interval, 2.2–3.1 min, R2 = 0.99) for CCK2i4svR and CCKR, respectively (Fig. 1A). At the end of the time course, treatment with thapsigargin confirmed that the decrease in the G17-induced Ca2+ responses were not because of depletion of the intracellular Ca2+ stores (data not shown).

In contrast to their similar rates of desensitization, the two receptor variants differ significantly in their rates of resensitization. Cells expressing either CCK2i4svR or CCKR were first treated with 100 nM G17 and 10 μM cycloheximide for 1 h to completely desensitize the receptors and block new protein synthesis, respectively. Treatment with 10 μM cycloheximide for up to 3 h did not cause any measurable cell damage. After the cells were washed with KRH supplemented with cycloheximide, they were restimulated at various time points with 10 nM G17 at 22 °C, and the changes in [Ca2+]i, were recorded. A plot of percent maximum Ca2+ response versus time showed that the G17-induced Ca2+ response recovered up to 6.5 times faster in cells expressing CCK2i4svR compared with cells expressing CCKR (Fig. 1B). The averaged data from three experiments indicated that CCK2i4svR resensitized ~5 times faster at 22 °C.

The Two Receptor Variants Exhibit Different Subcellular Distributions at Steady State—GPCR desensitization typically involves a rapid and reversible agonist-dependent phosphorylation of specific serine and/or threonine residues within the 3il and C-terminal domains of the receptor by one or more kinases, including second messenger-dependent kinases, such protein kinases A and C, G protein-coupled receptor kinases, and/or casein kinase 1α (1, 2). Subsequently, resensitization of
the receptor-mediated response involves the internalization of the phosphorylated receptor, dephosphorylation of the intracellular domains, and recycling of the intact receptor back to the plasma membrane (4). To assess the role of receptor trafficking in the rapid resensitization of CCK2svR, we fused the full-length receptor cDNA to the N-terminus of EGFP and examined its subcellular distribution before and after agonist-stimulation using LSCM.

At steady state (before agonist stimulation) the subcellular distributions of CCK2svR-EGFP and CCK2R-EGFP were distinctly different. Cells expressing CCK2R-EGFP exhibited predominantly plasma mem-

FIGURE 1. Comparison of the rates of CCK2svR (closed circles) and CCK2R (open circles) desensitization (A) and resensitization (B). The average peak increase in [Ca^{2+}]_i, at each time point was divided by the average peak height of the initial stimulation and plotted as a percentage of the initial (maximum) response. Thirty cells were analyzed for each data point. Error bars represent the S.E.

FIGURE 2. Effects of intron 4 retention and YM022 on CCK2svR subcellular distribution. A, LSCM images of HEK293 cells expressing either EGFP-tagged CCK2R or CCK2svR. Green color indicates the location of EGFP-tagged receptors. The cells were treated with either vehicle (0.001% Me_2SO) or YM022 (100 μM) for 2 h at 37°C. Scale bar indicates 10 μm. B, summary data quantifying the percentage of total membrane-associated EGFP fluorescence from 30 cells for each condition is shown (*, p < 0.001 CCK2svR for vehicle- versus YM022-treated cells).

FIGURE 3. Effects of dnSrc expression on CCK2svR and CCK2R subcellular distribution. A, LSCM images of receptor expressing cell transduced with either a control retroviral vector or a vector containing a dnSrc (kinase-dead) expression construct. Scale bar indicates 10 μm. B, summary data quantifying the percentage of total membrane-associated EGFP fluorescence from 30 cells for each condition is shown (*, p < 0.001 CCK2svR for vector- versus dnSrc-expressing cells).
brane-associated fluorescence (Fig. 2A), whereas the EGFP fluorescence in cells expressing CCK2i4svR-EGFP was located primarily in an intracellular membranous compartment concentrated around, but not in, the nucleus (Fig. 2A). To quantify the amount of plasma membrane-associated receptor for each variant, we determined the percentage of total cellular EGFP fluorescence that colocalized with plasma membrane dye, DiI, using LSCM. At steady state, only \( \frac{20}{1000} \% \) of the total cellular EGFP was associated with the plasma membrane in CCK2i4svR-EGFP-expressing cells, whereas \( \frac{80}{1000} \% \) of the fluorescence was plasma membrane-associated in cells expressing CCK2R-EGFP (Fig. 2B).

**Intracellular Localization of CCK2i4svR Is due to Constitutive Receptor Activity**—Previously, we and others have shown that CCK2i4svR exhibits ligand-independent (constitutive) activation of intracellular signaling pathways (9, 13, 25). To determine whether the intracellular distribution of CCK2i4svR-EGFP was because of its constitutive activity, we treated the cells with the inverse agonist, \((R)-1-[2,3	ext{-dihydro-1-(2'\text{-methylphenacyl})-2\text{-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl}]\text{-3-(3-methylphenyl)urea (YM022)}}. Although initially classified as a CCK2R-selective antagonist (26), Beinborn et al. (27) demonstrated the inverse agonist activity of YM022 using an engineered mutant of CCK2R that induced increased basal levels of inositol 1,4,5-triphosphate production. YM022 treatment of COS cells expressing the mutated receptor resulted in a decrease in the basal levels of inositol 1,4,5-triphosphate when compared with cells expressing the wild-type receptor (27). Furthermore, Harris et al. (25) showed that YM022 decreased the elevated basal level of phospho-Akt associated with CCK2i4svR expression in the esophageal cancer cell line, OE-18. When we treated HEK293 cells expressing CCK2i4svR-EGFP with YM022 (100 nM for 2 h), we observed an accumulation of EGFP fluorescence on the plasma membrane (Fig. 2A). Compared with either untreated or vehicle (0.001% DMSO) treated cells in which only 20% of the total EGFP was associated with the plasma membrane, YM022-treated cells exhibited \( \frac{77}{1000} \% \) of their total cellular EGFP fluorescence on the plasma membrane (Fig. 2B), suggesting that the intracellular location of CCK2i4svR is because of constitutive internalization.

**Inhibition of Src Kinase Causes the Redistribution of CCK2i4svR from the Intracellular Compartment to the Plasma Membrane**—CCK2i4svR coimmunoprecipitates with activated Src kinase in an agonist-independent manner (13). Because Src regulates GPCR endocytosis (28–32), we
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assessed its role in regulating the subcellular distribution of CCK$_{2/4svR}$. Receptor-expressing cells were infected with a retroviral expression construct containing either empty vector or a kinase-dead Src mutant (A430V) that functions as a dominant-negative (33). In the presence of dnSrc, CCK$_{2/4svR}$ was located primarily on the plasma membrane (Fig. 3A). Approximately 74 ± 1.5% of the total cellular EGFP fluorescence was associated with the plasma membrane in cells expressing dnSrc compared with just 19 ± 1.7% in cells expressing a control retroviral vector (Fig. 3A). Coexpression of dnSrc did not affect the distribution pattern of CCK$_2$R-EGFP (Fig. 3A). In the presence of dnSrc, 82 ± 1.3% of the EGFP fluorescence was associated with the plasma membrane compared with 80 ± 1.3% in cells transduced with the control retrovirus (Fig. 3B). Together, these data suggest that the constitutive activation of Src kinase by CCK$_{2/4svR}$ leads to its agonist-independent internalization.

Expression of dnSrc Does Not Inhibit Agonist-induced Receptor Internalization—A simple model for the constitutive internalization of CCK$_{2/4svR}$ is that the additional 69 amino acid residues in the 3il domain cause the receptor to transition into an activated conformation that mimics the conformation of the agonist-bound receptor, activating a common internalization mechanism. Because Src has been shown to regulate agonist-induced receptor internalization through both clathrin- and caveolin-dependent mechanisms (28, 29, 32, 34), we questioned whether dnSrc could also block agonist-induced receptor internalization. To address this question, cells expressing either dnSrc or a control retroviral vector were stimulated with G17 (10 nM), and the subcellular distribution of the EGFP-tagged receptors was monitored in live cells over a 40-min time course. Treatment with G17 induced a time-dependent redistribution of the EGFP-tagged receptors from the plasma membrane to intracellular membrane vesicles, which eventually concentrated in a perinuclear compartment by 40 min (Fig. 4). Thus, unlike constitutive CCK$_{2/4svR}$ internalization, expression of dnSrc did not inhibit the agonist-induced internalization of either CCK$_{2/4svR}$ or CCK$_2$R (Fig. 4, dnSrc), suggesting that different mechanisms mediate the agonist-dependent and -independent receptor internalization.

Expression of dnSrc Does Not Affect the Rates of Receptor Desensitization but Significantly Slows the Rate of CCK$_{2/4svR}$ Resensitization—Consistent with the hypothesis that agonist-induced and constitutive receptor internalization involves distinct mechanisms, expression of dnSrc had no effect on the rates of G17-induced desensitization of either receptor (Fig. 5A). Similar to the data shown in Fig. 1A, in the presence of dnSrc, both receptors rapidly desensitize in accordance with first-order decay kinetics. Analyses of the desensitization curves showed $t_{1/2}$ maximum Ca$^{2+}$ response values of 2.7 min (95% confidence interval, 2.3–3.2 min, $R^2$ = 0.99) and 2.9 min (95% confidence interval, 2.6–3.2 min, $R^2$ = 0.99) for CCK$_{2/4svR}$ and CCK$_2$R, respectively (Fig. 5A). In contrast to desensitization, expression of dnSrc significantly slowed the rate of CCK$_{2/4svR}$ resensitization (Fig. 5B). A plot of percent maximum Ca$^{2+}$ response versus time showed that the G17-induced Ca$^{2+}$ response in cells expressing CCK$_{2/4svR}$ and dnSrc recovered significantly slower than cells expressing CCK$_{2/4svR}$ and control retroviral vector (Fig. 5B). In fact, the rate of resensitization of CCK$_{2/4svR}$ in the presence of dnSrc was the same as rates of resensitization of CCK$_2$R-expressing cells with or without dnSrc expression (Fig. 5B).

FIGURE 6. Determination of the rate of CCK$_{2/4svR}$ recycling. Plot of percent change in membrane EGFP fluorescence over time. Cells were treated with cycloheximide (10 μM) alone (open diamonds) or in combination with either YM022 (10 μM) (open circles) or JB93182 (10 μM) (closed triangles). Cells treated with 0.001% Me$_2$SO are labeled vehicle (closed circles). Membrane fluorescence was monitored by LSCM and quantified as % Plasma Membrane EGFP.$^\text{a}$

FIGURE 7. Model of CCK$_{2/4svR}$ internalization and rapid resensitization. CCK$_{2/4svR}$ is internalized via two distinct pathways: 1) agonist-independent (constitutive), which requires Src, and 2) agonist-dependent, which does not require Src. The agonist-independent (constitutive) Src-dependent internalization creates an intracellular pool of ready receptors that recycles back to the plasma membrane at a rate similar to the rate of CCK$_{2/4svR}$ resensitization. The agonist-dependent pathway is shared by both CCK$_2$R splice variants.

\* Yu, W. C., and T. L. Snider. 2005. Rapid Resensitization of CCK$_{2/4svR}$ is a Slow Process. J. Biol. Chem. 280:33372–33380.
That recycling of receptor from the intracellular pool is responsible for desensitization, internalization, and resensitization of the CCK2 receptor similar to the rate of receptor resensitization (Figs. 1A and 5B), suggesting that recycling of receptor from the intracellular pool is responsible for the rapid recovery of the agonist-induced Ca$^{2+}$ response. Interestingly, the CCK2 receptor antagonist, J93182, did not affect CCK2i4svR subcellular distribution, confirming that YM022 acts an inverse agonist and suggesting that the CCK2i4svR conformation and mechanism involved in its constitutive internalization are distinct from those involved in agonist-induced internalization.

Model for the Rapid Resensitization of CCK2i4svR—In comparing the desensitization, internalization, and resensitization of the CCK2 receptor splice variants, we have shown that intron 4 retention and the resulting addition of 69 amino acid residues to the 3il domain of CCK2i4svR affects both its subcellular distribution and rate of agonist-dependent resensitization. Based on the data presented, we developed the model shown in Fig. 7. CCK2i4svR is internalized through two mechanisms. The first involves constitutive receptor internalization through a Src kinase-dependent pathway (Fig. 7). Internalization of CCK2i4svR through the agonist-independent pathway is blocked both by the inverse agonist, YM022, and expression of dnSrc. The second mechanism of receptor internalization, induced by agonist binding, is common to both receptor variants and does not require Src kinase activity. The rapid resensitization of CCK2i4svR, in this two-compartment model, results from the recycling of constitutively internalized receptor back to the plasma membrane in a process that is driven largely by the amount of receptor in the intracellular pool (4). As predicted by this model, decreasing the amount of receptor in the intracellular recycling compartment by inhibiting Src-mediated receptor internalization slowed the rate of CCK2i4svR resensitization.

Biological Significance—The rapid resensitization of CCK2i4svR may contribute to the potential role of the receptor in regulating the progression and/or spread of colorectal and pancreatic cancers, where it is ectopically expressed. It is well established that gastrin promotes tumor cell proliferation, motility, and invasion of these cancers (18–21). Because many colorectal cancer cells also express gastrin, and experimental evidence suggests that the peptide hormone acts in an autocrine and/or paracrine manner to stimulate tumor growth, the hypothesis that rapid resensitization of CCK2i4svR contributes to the agonist-mediated tumor promoting properties will be the focus of future studies.

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