G protein-coupled receptor kinases (GRKs) initiate pathways leading to agonist-dependent phosphorylation and desensitization of G protein-coupled receptors. However, the role of GRKs in modulation of signaling properties of native receptors has not been clearly defined. Here we addressed this question by generating Chinese hamster ovary (CHO) cells stably expressing a dominant-negative mutant of GRK2 (DN-GRK2), K220R, using retrovirally mediated gene transfer, and we assessed function of the endogenously expressed calcitonin (CT) receptors. We found that CT-mediated responses were prominently enhanced in CHO cells expressing DN-GRK2 compared with mock-infected control CHO cells with ~3-fold increases in CT-promoted cAMP production in whole cells and adenylyl cyclase activity in membrane fractions. CT-promoted phosphoinositide hydrolysis was also enhanced in DN-GRK2 cells. The number of CT receptors was increased ~3-fold in DN-GRK2 cells, as assessed by 125I-salmon CT-specific binding, and this was associated with increased CT receptor mRNA levels. These results indicate that DN-GRK2 has multiple consequences for CT receptor signaling, but a primary effect is an increase in CT receptor mRNA and receptor number and, in turn, enhanced CT receptor signaling. As such, our findings provide a mechanistic basis for previous observations regarding agonist-promoted down-regulation of CT receptors and for resistance and escape from response to CT in vitro and in vivo. Moreover, the data suggest that blunting of receptor desensitization by DN-GRK2 blocks a GRK-mediated tonic inhibition of CT receptor expression and response. We speculate that GRKs play a similar role for other G protein-coupled receptors as well.

Stimulation of G protein-coupled receptors (GPCRs) in the plasma membrane triggers two events, activation of G protein-mediated signal transduction pathways and, in parallel, a deactivation or desensitization of signaling. One component of this desensitization, in particular homologous, receptor-specific desensitization, is agonist-dependent phosphorylation of the receptor by specific G protein-coupled receptor kinases (GRKs). This phosphorylation event leads to the recruitment of cytosolic proteins, β-arrestins, to the receptor-signaling complex, the uncoupling of receptor from heterotrimeric G proteins, and loss of receptor responsiveness. Recent evidence indicates that GRKs and β-arrestins not only promote receptor uncoupling but also may directly participate in GPCR sequestration and the initiation of events leading to clathrin-coated pit-mediated internalization of receptors (for recent reviews see Refs. 1–5). In addition, GRK activation may be required to initiate certain events unrelated to receptor desensitization (3, 4).

At least six different isoforms of the GRK family have been isolated. GRK2, formerly termed ßARK, is a widely expressed member of this family and has been shown to phosphorylate various GPCRs (1, 2). GRK2 K220R, a dominant-negative GRK2 mutant (DN-GRK2) in which lysine at position 220 has been mutated to arginine to disrupt kinase activity (6), has been used to attenuate desensitization of several GPCR systems such as the ß2-adrenergic receptor, a1B-adrenergic receptor, adenosine A2 receptor, thyrotropin receptor, follitropin receptor, and CCR2B receptor (6–11).

To date, however, relatively little is known about the role of GRKs in desensitization to peptide hormones, in particular, in the regulation and expression of endogenously expressed GPCRs. Thus, most studies of GRKs have involved the use of transfected cells expressing relatively nonphysiological levels of GPCRs. In the current studies, we utilized a model cell, Chinese hamster ovary (CHO) cells, which endogenously expresses calcitonin (CT) receptors, and we evaluated the role of endogenously expressed GRKs on signaling. We stably expressed DN-GRK2 in CHO cells using retroviral mediated gene transfer and found that CT receptor expression and signaling were markedly enhanced by the DN-GRK2. The results suggest a key role for GRKs in establishing the steady-state level of GPCR expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Salmon calcitonin, human calcitonin, forskolin, and monoclonal M5 anti-FLAG antibody were purchased from Sigma. Antibodies against GRK2 (C-15), GRK-3 (C-14), GRK5 (C-20), and GRK6 (polyclonal, rabbit; C-20) were generous gifts from Dr. Howard Wiley (Washington University). Recombinant DN-GRK2 was produced by subcloning the K220R mutation into pSV-GRK2, a plasmid expressing wild-type human GRK2 in a baculovirus vector (CGS Laboratories). VSV-G and VSV-N expression plasmids were generous gifts from Dr. Richard Lerner (Cleveland Clinic Foundation). Affinity-purified polyclonal antibodies against GRK2 (C-15), GRK-3 (C-14), GRK5 (C-20), and GRK6 (polyclonal, rabbit; C-20) were generous gifts from Dr. Howard Wiley (Washington University). Recombinant DN-GRK2 was produced by subcloning the K220R mutation into pSV-GRK2, a plasmid expressing wild-type human GRK2 in a baculovirus vector (CGS Laboratories). VSV-G and VSV-N expression plasmids were generously provided by Dr. Richard Lerner (Cleveland Clinic Foundation).

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G418 (400 µg/ml) was purchased from NEN Life Science Products. GTP-γ-S was from Roche Molecular Biochemicals. Cell culture media and fetal bovine serum were from Life Technologies, Inc. 125I-H2O was purchased from Amersham Pharmacia Biotech. Recombinant rhodopsin was kindly provided by Ryan Adams from Dr. Alexandra Newton’s laboratory (University of California, San Diego).

Cells and Cell Culture—CHO 1001 cells were seeded from a CHO Pro-5 line of cells were kindly provided by Dr. Michael Gottesman, National Institutes of Health (13). Cells were maintained in Ham’s F12 medium with 10% fetal bovine serum and antibiotics (50 units/ml penicillin and 50 µg/ml streptomycin (Life Technologies, Inc.) in gelatin-coated 75-cm² flasks until 80% confluent. COS7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics.

Construction of Expression Vectors—A pcDNA3/FLAG-tagged DN-GRK2 construct was generated by excising the EcoRI fragment from pcDNA3/FLAG-tagged DN-GRK2 cDNA and subsequent ligation of the blunt EcoRI fragment by Klenow enzyme (Life Technologies, Inc.) into amino-terminally FLAG-tagged pcDNA3 (Invitrogen) at a blunted BamHI site. Confirmation of the plasmid was accomplished by sequencing the coding region of murine GRK5 cDNA. The FLAG-DN-GRK2 construct (LFDRNL) was generated by insertion of the blunt BamHI/XhoI fragment of pcDNA3/FLAG-tagged DN-GRK2 into a blunt SalI site of pLRNL vector. All constructs were verified by sequencing. The coding region of murine GRK5 cDNA was isolated from murine S49 lymphoma cell cDNA using PCR primers specific to the murine GRK5 sequence (sense, 5′-CAA TGG AGC TG GAA AAA ACA TCG TGG CC-3′; antisense, 5′-GAG CAG AAA CTA GCT GGT CTC TGG CCT CGT G-3′), and sequencing of the clone was revealed to be identical to the published murine GRK5 cDNA (GenBank™ accession number AF040746). Murine GRK5 cDNA and rat GRK6a cDNA were cloned into amino-terminally FLAG-tagged pcDNA3 at EcoRI/XhoI sites, and the expression of those constructs were confirmed by immunoblotting with monoclonal M5 anti-FLAG antibody.

Generation of FLAG-DN-GRK2 Retrovirus—FLAG-DN-GRK2 pseudotyped retrovirus was generated by Dr. Atsushi Miyahara, Vector Development Laboratory, University of California, San Diego. The method for generation of pseudotyped retrovirus with G-glycoprotein in the envelope of vesicular stomatitis virus (VSV-G) envelope was developed by immunoblotting with an anti-FLAG M2 monoclonal antibody (Eastman Kodak Co.). The clone that produced the highest amount of the FLAG-DN-GRK2 gene was then expanded and used for subsequent production of the pseudotyped virus by removal of tetracycline. The culture medium was replaced with fresh medium, and the pseudotyped virus was collected between 24 and 96 h after removal of tetracycline. The collected culture medium was condensed by centrifugation and then filtered through a 0.45-µm filter. This amphotropic retrovirus was used to infect another packaging cell line derived from canine thymus cells and kindly provided by Dr. Robert J. Lefkowitz (Duke University) and rat calcitonin receptor (CTR) C1a (sense, 5′-CAA TGC ATG ATG CAG TCC TT-3′; antisense, 5′-GAG GCC TTC TTT TTA GAC CAC CAG-3′), murine GRK4 and rat GRK5a (sense, 5′-GAA CCG CCA AAG AAA GGG CTG-3′; antisense, 5′-CTA GCT GCT TCT AGT GGA G-3′), murine and rat calcitonin receptor (CTR) C1a (sense, 5′-GAG CAG CTG CAT CAG CTG-3′; antisense, 5′-AGG AAA GAA GTT GAC CAG AAC-3′), and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense, 5′-CCA AGA AGC CCG GTG G-3′; antisense, 5′-GAA CTT GCA CAT GAG-3′). The PCR conditions for each GRK isoform consisted of 35 thermal cycles at 60 °C annealing temperature. The condition for CTR C1a consisted of 40 thermal cycles at 55 °C annealing temperature and that for GAPDH was 30 thermal cycles at 65 °C annealing temperature. To visualize the PCR products, the samples were subjected to electrophoresis.
G Protein-Coupled Receptor Kinase DN-GRK Enhances Endogenous Calcitonin Receptor

RESULTS

Expression of GRK Isoforms in CHO Cells—Before studying the role of GRKs in regulating endogenous calcitonin (CT) receptors in CHO cells, we examined the profile of GRK isoforms expressed in the cells. We investigated the expression of GRK isoforms mRNAs by RT-PCR analysis using CHO cell cDNA as a template. PCR primers for GRK2 and GRK3 were designed at the catalytic region of the kinase, whereas those for GRK4, GRK5, and GRK6 were designed at the carboxyl-terminal part of each GRK isoform. Each pair of PCR primers was highly specific for a single corresponding GRK isoform. Murine GRK2 cDNA, rat GRK3 cDNA, rat GRK4a cDNA, murine GRK5 cDNA, and rat GRK6a cDNA were used as controls. Fig. 1A shows the result of RT-PCR for each GRK isoform. PCR products specific for GRK2, GRK5, and GRK6 (572-, 144-, and 117-bp length, respectively) were obtained from CHO cell cDNA, whereas there were no amplified products for GRK3 and GRK4.

Expression of GRK proteins was studied by Western blot analysis (Fig. 1B). Relatively abundant expression of GRK2 and GRK6 proteins was demonstrated in CHO cells; limited
Substrate phosphorylation by GRK2 activity obtained from CHO Cells—To investigate GRK2-dependent phosphorylation activities in CHO cell lines, an in vitro phosphorylation study was performed using either purified tubulin or rhodopsin as a substrate. The highly purified tubulin preparation that we used was free of endogenous tubulin kinase activity (data not shown). Post-nuclear fractions from mock-infected cells (Mock) or DN-GRK2 expressing (DN) cells were immunoprecipitated with anti-GRK2 antibody, and the immune complexes were used as sources for kinase activity. As shown in Fig. 3A, we detected time-dependent phosphorylation of tubulin in both Mock cells and DN cells. When the cell lysates were immunoprecipitated with nonimmune rabbit IgG, very little phosphorylation was detected in the two cell lines (data not shown). Fig. 3B shows the radioactivities of phosphorylated tubulin from four independent assays. The result shows that GRK2-depend-ent phosphorylation was inhibited by approximately 40% in DN cells compared with Mock cells. Further evidence for inhibition of GRK2-dependent phosphorylation by DN expression was obtained in studies assessing light-dependent phosphorylation of rhodopsin, in which the intensity of phosphorylation was inhibited about 70% (Fig. 3C). Thus the DN-GRK2 acts as a functional inhibitor of GRK2 activity.

Stimulation of cAMP Production in CHO Cells by Calcitonin (CT) Agonists—Calcitonin is known to activate the Gs/adenylyl cyclase and Gq/Pi hydrolysis–Ca2+ signaling systems (20). Previous studies have documented that various CHO isolates express CT receptor C1a mRNA and CT receptor C1a-like receptors (21, 22). We investigated whether functional CT receptor was expressed in CHO 10001 cells and, if so, whether DN-GRK2 expression affected CT-stimulated cAMP responses. Both Mock cells and DN cells were incubated with salmon CT (sCT) or human CT (hCT) in the presence of IBMX (0.2 mM) for 10 min, and whole cell cAMP production was assayed. As shown in Fig. 4A, we found that sCT increased cAMP formation at ≥0.1 nM in both cell lines and that maximal response occurred at ≥0.1 μM. Although EC50 values were similar for Mock cells and DN cells (for sCT, Mock cells: 0.57 nM, 95% confidence intervals (CI) 0.17–1.88 nM; DN cells: 1.10 nM, 95% CI 0.81–1.51 nM, for hCT, Mock cells: 3.09 nM, 95% CI 1.01–9.44 nM, DN cells: 9.39 nM, 95% CI 5.30–16.6 nM), maximal response in DN cells was approximately 3-fold higher for both CT ligands compared with that in Mock cells (Fig. 4, A and B). Thus the DN-GRK2 increased maximal response without substantially altering EC50.

To determine if stable expression of DN-GRK2 altered either the rate of cAMP production or the time to peak response after CT stimulation, Mock cells and DN cells were assayed either in the presence or in the absence of IBMX (0.2 mM) at several times after sCT (1 nM) stimulation. In Mock cells, cAMP levels in the presence of IBMX increased linearly up to 10 min after sCT stimulation, reached a maximal level by 20 min, and then gradually decreased to a low level by 60 min (Fig. 5, A and B). Without IBMX, the level of cAMP in Mock cells was maximal by 10 min but was only one-third of that in the presence of IBMX. In DN cells, the cAMP level in the presence of IBMX increased linearly up to 10 min after sCT stimulation and reached a maximal level at 30 min. The cAMP level in DN cells without IBMX reached a maximal level at 20 min and gradually decreased by 30 min. The peak cAMP level in DN cells without IBMX was 80% of that in the cells with IBMX. As shown in Fig. 5C, expression of the DN-GRK2 appeared to increase the rate of cAMP formation relative to that of Mock cells. In addition, retroviral DN-GRK2 expression did not alter basal cAMP levels or forskolin-stimulated cAMP levels.

Impairment of Desensitization of sCT-induced cAMP Production by DN-GRK2 Expression—To investigate whether DN-GRK2 expression affected desensitization of CT receptor in CHO cells, sCT (1 nM)-induced cAMP production was assayed after treatment of cells with sCT (1 nM) or vehicle for 30 min

![Image](image-url)
and 2.2 pmol/mg protein/min for Mock cells and DN cells, respectively; forskolin-stimulated adenylyl cyclase activities, 28.3 pmol/mg protein/min for Mock cells and DN cells, respectively. A, cells were incubated with sCT at various concentrations for 10 min at 37 °C in the presence of 0.2 mM IBMX. Data are normalized to cAMP levels stimulated by 1 µM sCT with 0.2 mM IBMX in DN cells (226.8 ± 4.7 pmol cAMP/mg protein). Open circles, Mock cells; closed circles, DN cells. B, cells were incubated with hCT for 10 min at 37 °C in the presence of 0.2 mM IBMX. Data are normalized to cAMP levels stimulated by 1 µM sCT with 0.2 mM IBMX in DN cells (161.1 ± 5.6 pmol cAMP/mg protein). The data shown are mean ± S.E. from three independent experiments.

In Mock cells, sCT-induced cAMP response following sCT treatment was approximately one-half of the response following incubation with vehicle; thus, sCT treatment homologously desensitized sCT-induced cAMP response in the control cells as well as in three separate clonal isolates of cells expressing construct lacking DN-GRK. In DN cells, however, sCT treatment did not alter the sCT-stimulated cAMP response; thus, desensitization of sCT-induced cAMP production was impaired in DN cells. In contrast to sCT-induced cAMP response, forskolin (10 µM)-induced cAMP responses was not altered by sCT treatment in either Mock cells or DN cells (16.5 ± 0.3-fold versus 15.4 ± 0.3-fold over basal for Mock cells with vehicle and sCT treatment, respectively; 16.1 ± 0.4-fold versus 16.8 ± 0.5-fold over basal for DN cells with vehicle and sCT pretreatment, respectively).

Potentiation of CT-induced Adenylyl Cyclase Activity by DN-GRK2 Expression—To investigate further whether CT-promoted cAMP responses were elicited through G<sub>S</sub>/adenylyl cyclase activation, we measured adenyl cyclase activities in membrane preparations from CHO cells. Basal adenylyl cyclase and GTPγS- and forskolin-stimulated adenylyl cyclase activities were not altered by DN-GRK2 expression (basal adenylyl cyclase activities, 1.3 ± 0.2 versus 1.6 ± 0.2 pmol/mg protein/min for Mock cells and DN cells, respectively; GTPγS-stimulated adenylyl cyclase activities, 22.3 ± 0.9 versus 24.7 ± 1.4 pmol/mg protein/min for Mock cells and DN cells, respectively; forskolin-stimulated adenylyl cyclase activities, 28.3 ± 0.7 versus 30.1 ± 1.0 pmol/mg protein/min for Mock cells and DN cells, respectively). As shown in Fig. 7, both sCT and hCT stimulated adenylyl cyclase activity in Mock cells (4.5 ± 1.0- and 2.2 ± 0.5-fold over basal for 1 µM sCT and 1 µM hCT, respectively). In DN cells, the stimulation of adenylyl cyclase by sCT and hCT was significantly increased compared with Mock cells (11.5 ± 1.3- and 6.7 ± 0.7-fold over basal for 1 µM sCT and 1 µM hCT, respectively). The result strongly suggest that potentiation of CT-induced adenylyl cyclase activity in DN cells is not secondary to changes in either G proteins or adenylyl cyclase as the response to GTPγS or forskolin was unchanged by DN-GRK2 expression.

Enhancement of CT-induced IP Formation by DN-GRK2 Expression—Previous reports have shown that CT couples to G<sub>S</sub>-dependent signaling pathways as well as to the G<sub>S</sub>-dependent signaling pathway (23). To investigate whether DN-GRK2 expression also potentiated G<sub>S</sub>-dependent signaling, we assayed CT-induced IP formation. Although in Mock cells, we could not reproducively detect an sCT (1 µM)-mediated increase in IP formation, in DN cells, sCT (1 µM) stimulated...
approximately a 2-fold increase over basal (Fig. 8). ATP (10 μM) also stimulated a 2-fold increase over basal in DN cells. Therefore, the data suggest that DN-GRK2 expression enhances Gq-dependent CT signaling and that of another Gq-dependent receptor (P2Y receptor).

Up-regulation of sCT-specific Binding Sites by DN-GRK2 Expression—To determine whether the enhanced CT responses in DN cells were dependent on the number of CT receptors, we performed a saturation binding study of the radioligand 125I-sCT using intact Mock cells and DN cells (Fig. 9). Whole cells were incubated with CT ligands for 3 h in chilled medium containing 0.1% bovine serum albumin and protease inhibitors to prevent agonist-induced internalization and peptide degradation. Binding affinity to 125I-sCT was not significantly different between both cell lines (Kd values: for Mock cells, 24.1 nM, 95% CI 8.0–40.2 nM, for DN cells, 22.3 nM, 95% CI 7.3–37.4 nM). The maximal number of CT specific binding sites in DN cells was, however, 2.5-fold larger than that found in Mock cells (610 ± 55 sites/cell versus 240 ± 40 sites/cell, p < 0.005). The level of specific binding sites for sCT in DN cells was comparable to the enhancement of sCT-promoted cAMP production in the cell line relative to that in Mock cells. The results of the whole cell binding studies demonstrate that stable expression of DN-GRK2 up-regulates CT receptor expression in CHO cells.

CT Receptor C1a mRNA Expression—To investigate further whether up-regulation of CT receptors was dependent on CT receptor mRNA expression, the relative abundance of CT receptor mRNA expression was examined by RT-PCR. Since other CHO cell strain lines have been reported to express CT receptor C1a (21, 22), expression of CT receptor C1a mRNA in our CHO cell lines was examined using PCR primers that were designed based on rodent CT receptor C1a cDNAs. As shown in Fig. 10, a 253-bp PCR product obtained with primers specific to C1a was detectable in parental and Mock CHO cells using 40 thermal cycles (not detectable using 35 thermal cycles), whereas higher expression of the amplified product was shown in DN cells, and a signal could be detected using 35 thermal cycles (data not shown). Sequencing of the 253-bp PCR product obtained from rat brain cDNA was identical to the published rat CT receptor C1a sequence (GenBankTM accession number L13041); sequencing of the cloned PCR products obtained from CHO cell lines revealed 90.5% identity with the rat CT receptor C1a mRNA. A housekeeping gene GAPDH mRNA was used as an internal standard for RT-PCR amplification, and the expression levels of GAPDH in Mock cells and DN cells were similar. Although the RT-PCR assay was only semi-quantitative, the data strongly suggest that DN cells express more CT receptor C1a mRNA than do Mock cells.

DISCUSSION

Calcitonin is an important hormone in the regulation of serum calcium levels and bone mineral density through its effects on bone resorption and renal calcium excretion. Previous studies have not clearly defined the mechanisms involved in the regulation of CT receptor expression, in particular, agonist-mediated desensitization and down-regulation of these receptors (24–26). In the current studies, we used CHO 10001 cells as a model to examine CT receptor expression by GRKs. We found that retrovirally mediated gene transfer is a useful means to establish stable expression of DN-GRK2. Stable expression of DN-GRK2 inhibited GRK2-mediated substrate phosphorylation by approximately 40% and impaired CT-mediated desensitization of cAMP formation. Potentiation of cAMP generation in DN-GRK2-expressing cells, however, appeared to be much greater than what would have been expected.
from the loss in GRK2 phosphorylating activity and appeared to relate to the increase in CT receptor number. Enhancement of CT receptor signaling by DN-GRK2 expression was also observed in the phosphoinositide pathway. The up-regulation of CT receptor number in DN-GRK2-expressing cells was associated with an increase in mRNA for CT receptor C1a. These data suggest that the CT receptor, one of the Class II family of GPCRs, can be regulated by GRKs and that CT receptor mRNA and protein expression are negatively influenced by GRK activity.

The GRK isoforms that regulate CT receptor signaling were not precisely defined in our studies, but the data suggest that GRK2 may play an important role for desensitization of the receptor. Recent studies reveal that various GPCRs can be regulated by multiple GRKs in vitro (e.g. Refs. 9, 10, and 27). The secretin receptor, another of the class II GPCRs, can be desensitized by expression of GRK5 as well as that of GRK2 or GRK3 (27). In the CHO cells that we used, in addition to GRK2, GRK6 and GRK5 are also expressed. Although we found that the K220R DN-GRK2 construct inhibited GRK2-mediated activity (Fig. 3), we cannot rule out an effect of the DN construct on activity of GRK5 or GRK6 as well. Indeed, based on structural similarities, to be described below, we believe this is quite likely.

Previous workers have employed several different techniques to modulate GRK expression (e.g. Refs. 1–5). Many previous studies have involved overexpression of GRK isoforms. The more limited efforts designed to inhibit GRK expression have included use of a carboxyl-terminal fragment from GRK2 (termed βARKct), antisense oligonucleotides, and studies in cells or tissues from mice with a knockout of a GRK isoform. We believe that use of a retrovirally expressed DN construct offers advantages relative to those other methods. Thus, for example, βARKct is an inhibitor of Gβγ and some of its actions in cells may be attributable to Gβγ-dependent, but GRK-independent, events (3, 4). Moreover, because Gβγ is not involved in the action of all GRK isoforms, studies with βARKct will not assess the role of Gβγ-independent isoforms. Antisense oligonucleotides, which are generally isoform-selective, are primarily useful in acute experiments and not for generation of stably inhibited cells. Material from knockout animals is limited thus far to murine tissues and generally only from heterozygotic animals that have loss of expression of a single type of GRK. We believe that the retrovirally engineered DN-GRK2 construct provides a useful complementary approach to those other methods because of its theoretical ability to block function of multiple GRK isoforms (the K220R in GRK2 represents a conserved region in the catalytic domain ATP-binding site of all GRK isoforms), its potential utility to generate stably expressing cells, such as those we have used here, and the rather widespread tropism of the retroviral vector. Since CHO cells are often used for the heterologous expression of GPCRs, the stable cell line that we have developed may prove useful to examine GRK-mediated regulation of transfected GPCRs.

Our findings in the DN-GRK CHO cells strongly suggest that GRKs not only regulate receptor desensitization but also play a role in the steady-state level of receptor expression. Activity of GRK to regulate CT receptor desensitization presumably reflects phosphorylation of one or more of the 8 serine/threonine residues in the carboxyl-terminal portion of C1a receptors. Mutagenesis and related approaches will be necessary to define the precise sites that are regulated by the GRKs. Although such sites likely are involved in GRK-mediated desensitization of the receptors, it is not clear how receptor phosphorylation by GRK would regulate receptor expression. Presumably, GRKs impact on the CT receptor life cycle through events subsequent to receptor phosphorylation and internalization. The evidence that DN-GRK2 cells have an increase in CT receptor mRNA suggests that GRKs regulate the transcription and/or turnover of CT receptor mRNA. In this regard, it is of further interest that although the focus of the current studies is on CT receptor, ATP-mediated phosphoinositide hydrolysis, presumably via one or more P2Y receptors, was also enhanced in the DN cells (Fig. 8). Perhaps GRKs are able to influence expression of multiple types of GPCRs.

In summary, the current studies with CHO cells show that these cells express CT receptor C1a and GRK2, GRK5 and GRK6, but not GRK3 and GRK4. We found that stable expression of DN-GRK2 by retrovirally mediated gene transfer inhibited GRK2-promoted substrate phosphorylation, potentiated CT receptor signaling (both cAMP generation and phosphoinositide hydrolysis) in CHO cells, and blunted desensitization of CT receptors. Moreover, DN-GRK2-expressing CHO cells had an up-regulation in expression of CT receptors and receptor mRNA. The findings thus provide a mechanistic explanation for previous observations regarding agonist-mediated down-regulation of CT receptors, a phenomenon that has been implicated in resistance and escape from response to CT (25, 26). In addition, the data indicate that GRKs are involved not only in desensitization of the CT receptor but also in the regulation of CT receptor expression. We speculate that through their effects on receptor phosphorylation, GRKs are able to inhibit expression of CT receptor mRNA and protein in CHO cells and perhaps more generally of GPCRs in other cell types as well.

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