Phosphorylation and Desensitization of Human Endothelin A and B Receptors

EVIDENCE FOR G PROTEIN-COUPLED RECEPTOR KINASE SPECIFICITY

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Neil J. Freedman‡, Alan S. Ament, Martin Oppermann§, Robert H. Stoffel∥, Sabrina T. Exum, and Robert J. Lefkowitz†

From the Howard Hughes Medical Institute, Departments of Medicine (Cardiology) and Biochemistry, Duke University Medical Center, Box 3821, Durham, North Carolina 27710

Although endothelin-1 can elicit prolonged physiologic responses, accumulating evidence suggests that rapid desensitization affects the primary G protein-coupled receptors mediating these responses, the endothelin A and B receptors (ET\textsubscript{A}-R and ET\textsubscript{B}-R). The mechanisms by which this desensitization proceeds remain obscure, however. Because some intracellular domain sequences of the ET\textsubscript{A}-R and ET\textsubscript{B}-R differ substantially, we tested the possibility that these receptor subtypes might be differentially regulated by G protein-coupled receptor kinases (GRKs). Homologous, or receptor-specific, desensitization occurred within 4 min both in the ET\textsubscript{A}-R-expressing A10 cells and in 293 cells transfected with either the human ET\textsubscript{A}-R or ET\textsubscript{B}-R. In 293 cells, this desensitization corresponded temporally with agonist-induced phosphorylation of each receptor, assessed by receptor immunoprecipitation from \textsuperscript{32}P\textsubscript{i}-labeled cells. Agonist-induced receptor phosphorylation was not substantially affected by PKC inhibition but was reduced 40% (p < 0.03) by GRK inhibition, effected by a dominant negative GRK2 mutant. Inhibition of agonist-induced phosphorylation abrogated agonist-induced ET\textsubscript{A}-R desensitization. Overexpression of GRK2, -5, or -6 in 293 cells augmented agonist-induced ET-R phosphorylation ~2-fold (p < 0.02), but each kinase reduced receptor-promoted phosphoinositide hydrolysis differently. While GRK5 inhibited ET-R signaling by only ~25%, GRK2 inhibited ET-R signaling by 80% (p < 0.01). Concomitant with its superior efficacy in suppressing ET-R signaling, GRK2, but not GRK5, co-immunoprecipitated with the ET-Rs in an agonist-dependent manner. We conclude that both the ET\textsubscript{A}-R and ET\textsubscript{B}-R can be regulated indistinguishably by GRK-initiated desensitization. We propose that because of its affinity for ET-Rs demonstrated by co-immunoprecipitation, GRK2 is the most likely of the GRKs to initiate ET-R desensitization.

A variety of vital cardiovascular and developmental func-

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‡ To whom correspondence should be addressed. Tel.: 919-684-2974; Fax: 919-684-8975.
§ Present address: Dept. of Immunology, Universitätskliniken Göttingen, Kreuzbergring 57, D-37075 Göttingen, Germany.
∥ Present address: Bristol Myers Squibb Pharmaceutical Research, 5 Research Pkwy., Wallingford, CT 06492-7660.

1 The abbreviations used are: G protein, guanine nucleotide-binding regulatory protein; ET-R, endothelin receptor; ET\textsubscript{A}-R, endothelin receptor subtype A; ET\textsubscript{B}-R, endothelin receptor subtype B; AT\textsubscript{1A}-R, rat type 1A angiotensin II receptor; ET-1, endothelin-1; GRK, G protein-coupled receptor kinase; PKC, protein kinase C; SFLLRN, single-letter amino acid code for the human thrombin receptor agonist peptide; t-ET\textsubscript{A}-R and t-ET\textsubscript{B}-R, epitope-tagged ET\textsubscript{A}-R and ET\textsubscript{B}-R, respectively; TPA, 12-O-tetradecanoylphorbol-13-acetate; 293 cells, human embryonic kidney cells; PCR, polymerase chain reaction.
regulated differently by GRKs, or regulated by different GRKs. To date, no data directly address this issue. However, experiments with cytoplasmic tail truncation mutants of the ET₄-R (24) and the ET₅-R (25) suggest that large portions of each receptor’s cytoplasmic tail may be removed without impairing agonist-induced desensitization of endothelin-1-stimulated chloride currents (24) or calcium transients (25). To test directly whether or not homologous desensitization of the human ET₄-R and ET₅-R proceeds via a GRK-initiated mechanism, we used a transfected, intact cell model for assessing both receptor phosphorylation and signaling through phospholipase C.

In this model system, we also overexpressed individual GRKs with the ET₄-R and ET₅-R to test the hypothesis that different GRKs might phosphorylate and initiate desensitization of the ET₄-R and ET₅-R.

**EXPERIMENTAL PROCEDURES**

**Materials—**Embryonic rat thoracic aortic smooth muscle (A10) cells and human embryonic kidney 293 cells were obtained from the American Type Culture Collection, and all cell culture supplies were obtained from Life Technologies, Inc. 

**Embryonic rat thoracic aortic smooth muscle (A10) cells** were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum as well as penicillin (100 units/ml) and streptomycin (100 µg/ml) and incubated in 95% air, 5% CO₂ at 37 °C in a humidified incubator.

**Human embryonic kidney 293 cells** were obtained from the American Type Culture Collection, and all cell culture supplies were obtained from Life Technologies, Inc. 

**Embryonic rat thoracic aortic smooth muscle (A10) cells** were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum as well as penicillin (100 units/ml) and streptomycin (100 µg/ml) and incubated in 95% air, 5% CO₂ at 37 °C in a humidified incubator. Human 293 cells were propagated and transfected by the calcium phosphate method as described (21).

For most ET₄-R transfections, 10 µg of receptor plasmid was used, along with 5 µg of GRK, GRK2 polypeptide, or empty vector plasmid per 100-mm dish. For homologous desensitization experiments, 293 cells were transfected with 4 µg of t-ET₄-R and 10 µg of AT₅A-R per 100-mm dish, to ensure that phosphoinositide hydrolysis mediated by the AT₅A-R exceeded that mediated by the t-ET₄-R. For t-ET₄-R transfections with the receptor plasmid utilized, along with GRK, 2–6 µg of empty vector, or 6 µg of AT₅A-R plasmid (for a total of 10 µg) per 100-mm dish. For AT₅A-R experiments, cells were transfected as described (18). The day after transfection, cells from all identically transfected plates were pooled and aliquoted, at the same density, for functional or receptor expression assay (21). With transfection on day 1, cells were assayed on day 3 or 4 (with equivalent results).

**Antibodies for Receptor Expression Assays—**ET₄-R and ET₅-R expression levels were assessed in pilot experiments by [32P]ET-1 binding assays, modified from Sakamoto et al. (23). Assays were performed in triplicate, and data were analyzed by least squares nonlinear regression with Prism™ software (Graphpad). These assays demonstrated receptor expression levels of 1–3 pmol/mg of total cell protein for both receptor subtypes.

The native receptor expression among cell lines co-transfected with various plasmids was routinely assessed by immunofluorescence and flow cytometry, performed as described previously (21). Typical transfection efficiencies ranged from 35 to 50% and from 45 to 70% for the t-ET₄-R and t-ET₅-R, respectively. To obtain a surrogate for receptor expression in each transfected cell line, the mean fluorescence/cell was multiplied by the percentage of cells staining positive for receptor. Cells co-transfected with various plasmids typically expressed receptor expression levels within 30% of that measured in empty vector-co-transfected control cells. Cells with receptor expression levels outside this range were not used.

**Cellular Phosphoinositide Hydrolysis and Desensitization Assays—**A10 and 293 cells were metabolically labeled for 18–24 h with 2 µCi of [3H]inositol/ml of labeling medium, which differed from the cell-specific growth medium only in that it contained 5% fetal bovine serum. A10 cells were plated in labeling medium at 5.3 × 10⁵/cm² in 12-well plates. After labeling, cells were washed with Dulbecco’s phosphate-buffered saline and then exposed at 37 °C to 0.2% bovine serum albumin and 20 µM LiCl in Dulbecco’s phosphate-buffered saline (“PI medium”), without (basal) or with (stimulated) 100 nM ET-1 for the indicated times. Each time point comprised a single 12-well plate containing triplicate wells for both basal and stimulated cells. Reactions were stopped by the addition of an equal volume of 0.8 M perchloric acid to the wells, and total inositol phosphates were assayed by anion exchange chromatography as described (18). To facilitate comparisons between independent experiments, cellular inositol phosphates obtained from each well were normalized to the amount of intracellular [3H] counts in that well (determined by counting a 50-µl aliquot of neutralized cell extract before chromatography). The resulting value, multiplied by 100, is referred to as “percent conversion of 3H into inositol phosphates.”

Signaling assays with 293 cells (see Figs. 7–9) were performed in an analogous manner with the following modifications: cells were plated at 1.6 × 10⁵/cm² in labeling medium; each 12-well plate comprised triplicate wells of each transfected cell type; and agonist stimulation transpired for 8 min. Homologous desensitization assays with 293 cells were performed with three 12-well plates manipulated in concert. During the “first period,” cells were stimulated with the indicated stimulus for 3 min (ET-1) or 10 min (TPA, forskolin) in “PI medium with or without (control plate) or with (‘desensitized plates’) the indicated concentrations of ET-1. PI medium was then removed from all wells. Next, during the “signaling period,” cells from both control and desensitized plates were exposed for 5 min at 37 °C to PI medium containing vehicle (“basal”), 100 nM ET-1, or 100 nM angiotensin II. Reactions were then terminated, as described previously (21). For desensitized cells, basal inositol phosphate values for the signaling period were obtained by terminating reactions on one of the desensitized plates at the outset of the signaling period.

**Intact Cell Phosphorylation—**These assays were performed as described previously (21). Briefly, cells metabolically labeled with [32P]P, were stimulated with the indicated stimulus for 3 min (ET-1) or 10 min (TPA), washed, and solubilized in detergent buffer. Cells expressing the t-ET₄-R were solubilized, and receptors were immunoprecipitated with 12C5, as described (21). Receptor desensitized in “M2 detergent buffer” (1% (w/v) Triton X-100™, 0.05% SDS, 5 mM EDTA, 50 mM Tris-Cl, pH 8.0 (± 0.5), 200 mM NaCl, with phosphatase and protease inhibitors as described (21). The t-ET₄-Rs were immunoprecipitated as described (21), but with the M2 monoclonal IgG, and protein G-Sepharose. Immune complexes were dissociated and resolved on SDS-10% polyacrylamide gels, which were loaded with equivalent amounts of receptor protein per lane, as described (21). Autoradiograms...
Stoichiometry of Receptor Phosphorylation—Equilibrium biosynthetic labeling and immunoprecipitation were employed to determine the stoichiometry of ET$_{m}$-R phosphorylation in intact 293 cells, as described previously (21).

Membrane GTPase Assays—293 cells plated at 1 × 10$^6$/cm$^2$ in 150-mm dishes were subsequently exposed to 100 nM ET-1 ("desensitized") or vehicle ("naive") at 37 °C, in minimal essential medium containing 20 mM HEPES, pH 7.4, and 1 μM okadaic acid (Calbiochem) to inhibit possible phosphatase activity during the subsequent acid wash (30). After 3 min, the cells were washed three times at 4 °C in 50 mM glucose, pH 2.5, in protocol shown by Chun et al. (31) to remove receptor-bound ET-1. After a further wash in Dulbecco’s phosphate-buffered saline, the cells were scraped at 0 °C into 10 ml Tris-Cl, 2 mM EDTA, pH 7.4 (25 °C), supplemented with protease inhibitors (21). Cells were lysed with a motorized Teflon pestle, and crude membranes were pelleted at 30,000 × g at 4 °C for 15 min. Membranes were washed once at 4 °C in 10 ml triethanolamine, pH 7.4 (30 °C), supplemented with protease inhibitors (21), and finally resuspended at a protein concentration of 0.3 mg/ml in the same buffer. ET-1-stimulated hydrolysis of [γ-32P]GTP was determined with triplicate 15-μg aliquots of these membranes, as described (18). Reactions were stopped at 10 min, a time at which reaction rates were still linear.

Co-immunoprecipitation and Immunoblotting—293 cells were transfected with either an endothelin receptor or the AT$_{1}$-R and the indicated plasmids (see Fig. 10); they were plated at 7–9 × 10$^5$/cm$^2$ in 90-mm dishes and assayed the next day. As in the inositol phosphate signaling assays, cells were washed and then exposed to PI medium for 8 min (37 °C) in the absence (unstimulated) or presence (stimulated) of 100 nM ET-1 or 100 nM angiotensin II. (Albumin was omitted from the PI medium for these experiments.) PI medium was then replaced with 1 ml of cross-linking buffer (Dulbecco’s phosphate-buffered saline) containing 10 mM HEPES, pH 7.4 (to increase buffering capacity), 10% (v/v) Me$_2$SO, and 2.5 mM of the cell-permeant homobifunctional cross-linking agent dithiobis(succinimidylpropionate) (Pierce). Cells were incubated at 25 °C for 30 min, with continuous rocking. (In AT-R experiments, the cross-linking buffer on stimulated plates contained 100 nM angiotensin II.) Cross-linking buffer was then replaced with 1 ml plate of the detergent buffer appropriate for the monol IgG to be used for immunoprecipitating the receptor (see above) and receptor immunoprecipitation proceeded as described previously (18, 21), but with 17 μg of IgG/sample. Before the addition of IgG to the detergent-solubilized cells, aliquots were removed for protein assay and for subsequent immunoblotting to determine the approximate fraction of cellular GRK co-immunoprecipitated with the receptor. Dithiobis(succinimidylpropionate)-mediated cross-links were reduced, and immune complexes were dissociated by incubation in Laemmli buffer (32) at 37 °C for 90 min. As described above for phosphoinositide assays, equivalent amounts of receptor protein were resolved on SDS-10% polyacrylamide gels and then transferred to nitrocellulose by semidry blotting (32). Immunoblots were probed with biotinylated monoclonal IgGs, either the GRK2/3-specific C97 or the GRK4/5/8-specific A19/17 (33), and horse-radish peroxidase-conjugated streptavidin. Enhanced chemiluminescence (Amersham Corp.) was used to develop the immunoblots, which were exposed to Biomax MR™ film (Eastman Kodak Co.). Quantitation of band density was performed with a laser densitometer. To confirm that equivalent amounts of receptor protein had been loaded in each gel lane, immunoblots processed for detecting GRK2 or -5 were stripped (50 °C for 3–4 h in 2% (w/v) SDS, 100 mM 2-mercaptoethanol, 62.5 mM Tris-Cl, pH 6.7 at 25 °C) and reprobed with either biotinylated M2 IgG (for the t-ET$_{m}$-R) or biotinylated 12CA5 (for the t-ET$_{m}$-R and AT$_{m}$-R).

Statistical Analysis—The t test for independent means was used to compare results for different cell types, and two-sided p values were calculated using Excel™ software (Microsoft), assuming equal sample variances (34). Because of considerable variability in membrane GTPase activity among experiments, paired t testing was used to compare naive with desensitized cells and to compare GRK2 K220R cells with control cells in the membrane GTPase experiments. Concentration/response curves were fitted by least squares nonlinear regression with variable slope, using Prism™ software (Graphpad). In the text, numerical values are given as means ± S.D., whereas means ± S.E. are plotted in all figures.

RESULTS

To demonstrate rapid desensitization of the ET$_{m}$-R, we investigated the time course of ET-1-stimulated phosphoinositide hydrolysis in intact A10 cells, which endogenously express the ET$_{m}$-R (31). Within 10 min, the A10 cell phospholipase C response to ET-1 ceased, consistent with ET$_{m}$-R desensitization (Fig. 1). When these ET-1-desensitized cells were challenged with thrombin agonist peptide, however, their phospholipase C response resumed. Thus, the ET$_{m}$-R desensitization observed in A10 cells was homologous, or receptor-specific. Moreover, because the ET$_{m}$-R-dependent phosphoinositide hydrolysis in A10 cells waned substantially by 6 min, this desensitization would seem to involve mechanisms other than receptor sequestration, which has been shown to follow a somewhat longer time course in these cells (31).

To examine the molecular mechanisms responsible for endothelin receptor desensitization and to facilitate direct comparison between the cloned human ET$_{m}$-R and ET$_{1}$-R, we employed transfected human 293 cells as a model system. To evaluate homologous desensitization in this system, we assayed ET-1-stimulated phosphoinositide hydrolysis in 293 cells co-transfected with the rat AT$_{1}$-R and either the human t-ET$_{m}$-R or t-ET$_{1}$-R. A 3-min challenge with ET-1 reduced the subsequent inositol phosphate response to ET-1 in a dose-dependent manner for both the t-ET$_{m}$-R and ET$_{1}$-R (Fig. 2). However, the initial challenge with ET-1 had no effect on the subsequent inositol phosphate response to angiotensin II. Thus, rapid homologous desensitization characterizes the signaling behavior of both the t-ET$_{m}$-R and the t-ET$_{1}$-R in this model 293 cell system.

Receptor sequestration attendant to the first challenge with ET-1 could not explain the desensitization findings in transfected 293 cells. Separate aliquots of cells challenged with 100 nM ET-1 and then processed for flow cytometry demonstrated that only 12 ± 5% of the cell surface t-ET$_{m}$-Rs and 9 ± 1% of the t-ET$_{1}$-Rs were internalized within 3 min. These small decrements in cell surface receptor number were modeled by transfected cells with varying amounts of receptor plasmid, and had
no effect on ET-1-stimulated phosphoinositide hydrolysis (data not shown).

To determine whether or not receptor phosphorylation was involved in the observed agonist-promoted desensitization of the t-ET\(_A\)-R and t-ET\(_B\)-R, we immunoprecipitated these receptors from 293 cells metabolically labeled with \(^3\)Hinositol and assayed for agonist-stimulated phosphoinositide hydrolysis as described under "Experimental Procedures." Cells received vehicle without (control) or with (desensitized) the indicated concentrations of ET-1 for 3 min. After vehicle removal, cells received vehicle without (basal) or with (stimulated) 100 nM ET-1 or 100 nM angiotensin II (Ang II). Reactions were stopped with HClO\(_4\) either immediately after the second addition of vehicle (basal for desensitized cells), or after an additional 5 min. Total cellular inositol phosphates were determined as described under "Experimental Procedures." Phosphoinositide hydrolysis (stimulated – basal) is plotted, as a percentage of that measured in control cells. Depicted are the means ± S.E. from three experiments performed in triplicate. The percent incorporation of \(^3\)H into inositol phosphates in the basal, ET-1-stimulated, and angiotensin II-stimulated states, respectively, was as follows: (i) control: 0.6 ± 0.3, 3.0 ± 1.7, and 2.9 ± 1.4 (ET\(_A\)-R); 1.2 ± 0.5, 3.9 ± 1.5, and 4.3 ± 1.8 (ET\(_B\)-R); (ii) desensitized with 100 nM ET-1: 1.6 ± 0.8, 3.3 ± 2, and 3.9 ± 2 (ET\(_A\)-R); 3 ± 1, 4.4 ± 1.9, and 6 ± 3 (ET\(_B\)-R). *, p < 0.05 for the comparison with control cells (paired t test).

Because endothelin receptor stimulation results in activation of PKC isoforms, we sought to determine the extent to which PKC might participate in the rapid agonist-dependent phosphorylation of the endothelin receptors. In experiments presented in Fig. 3, 293 cells were incubated with a PKC-selective concentration of staurosporine briefly and then challenged with either ET-1, to evoke agonist-induced receptor phosphorylation, or TPA, to activate PKC. Whereas staurosporine abolished the TPA-stimulated t-ET\(_B\)-R phosphorylation by PKC, it had no significant effect on agonist-promoted t-ET\(_B\)-R phosphorylation. Similar results were obtained with the ET\(_A\)-R (data not shown). Thus, for both the t-ET\(_A\)-R and the t-ET\(_B\)-R, agonist-induced phosphorylation must be mediated by kinases other than PKC.

To test the possibility that agonist-promoted t-ET\(_A\)-R phosphorylation is mediated by one or more of the GRKs endogenously expressed by 293 cells (21), we performed phosphorylation experiments in cells transfected with a dominant-negative K220R mutant of GRK2 (21, 35). This K220R mutant...
of GRK2 cannot perform a phosphotransferase reaction (35); therefore, for any receptor substrate of GRK2, the K220R mutant can act as a competitive inhibitor of any GRK that could bind to and subsequently phosphorylate the receptor (18, 21, 35–37). Characteristically, however, the K220R mutant inhibits GRK action incompletely; even when present in 15-fold molar excess over GRK2, the K220R mutant inhibits only 60% of agonist-stimulated β₂-adrenergic receptor phosphorylation in vitro (35). Co-expression of the K220R with the t-ETₐ-R in 293 cells resulted in a 40% reduction in agonist-induced ETₐ-R phosphorylation (Fig. 4). In contrast, GRK2 K220R expression had no effect on TPA-stimulated ETₐ-R phosphorylation (Fig. 4). Thus, a substantial fraction of the agonist-induced ETₐ-R phosphorylation appears to be mediated by one or more GRKs expressed endogenously in 293 cells.

The foregoing experiments demonstrate a temporal correlation between agonist-induced phosphorylation and desensitization of the t-ETₐ-R and t-ETₐ-R. To correlate the extent of GRK-mediated receptor phosphorylation with the extent of desensitization, we inhibited agonist-promoted ETₐ-R phosphorylation in 293 cells with dominant-negative GRK2 and assessed the effect of such inhibition on ET-1-stimulated membrane GTPase activity in membranes derived from these cells (Fig. 5A). A 3-min exposure of cells to ET-1 reduced the subsequent ET-1-stimulated membrane GTPase activity by 40%, compared with that observed in membranes derived from control cells. With cells expressing dominant-negative GRK2, however, exposure to ET-1 did not affect the subsequent ET-1-stimulated membrane GTPase activity. Thus, abrogation of desensitization appears to attend the inhibition of GRK-mediated ETₐ-R phosphorylation by GRK2 K220R. In contrast to dominant-negative GRK2-transfected cells, native GRK2-transfected cells demonstrated desensitization indistinguishable from control cells (Fig. 5B), although GRK2 overexpression resulted in augmented agonist-induced ETₐ-R phosphorylation (see below; Fig. 6). In 293 cells, agonist-induced ETₐ-R phosphorylation mediated by endogenous GRKs therefore appears both necessary and sufficient for ETₐ-R desensitization.

Which of the anatomically disseminated (12) mammalian GRKs can effect agonist-induced phosphorylation of the endothelin receptors? To address this question, we overexpressed GRK2, -5, and -6 along with each endothelin receptor in 293 cells and studied the effects of GRK overexpression on agonist-induced receptor phosphorylation. Overexpression of any of the three GRKs tested significantly increased the level of agonist-induced receptor phosphorylation (Fig. 6). For the t-ETₐ-R, co-overexpression of either GRK2, GRK5, or GRK6 augmented agonist-induced phosphorylation 1.6 ± 0.1, 1.7 ± 0.3, or 2.0 ± 0.6-fold, respectively, over that observed in control cells (p < 0.05) (Fig. 6A). For the t-ETₐ-R, co-overexpression of GRK2, -5, or -6 augmented agonist-induced phosphorylation 2.6 ± 0.4,
2.1 ± 0.8-, or 2.4 ± 0.8-fold, respectively, over that observed in control cells (p, 0.02) (Fig. 6B). Under the conditions prevailing in these cellular assays, then, the t-ET A-R and t-ET B-R each appear susceptible to agonist-promoted phosphorylation mediated by each of the three widely expressed mammalian GRKs.

To correlate GRK-mediated receptor phosphorylation with effects on receptor signaling in the intact cell, we assayed ET-1-stimulated phosphoinositide hydrolysis in cells co-expressing the ET-Rs with GRK2 and GRK5 (Fig. 7, A and B). Cells overexpressing GRK2 with the t-ET A-R or t-ET B-R produce only 16 ± 4% or 22 ± 5%, respectively, of the maximal inositol phosphate response observed in control cells after an 8-min challenge with ET-1 (p, 0.002). By contrast, cells overexpressing GRK5 with the t-ET A-R or t-ET B-R produce 75 ± 8% or 73 ± 13%, respectively, of the maximal inositol phosphate response observed in control cells (p, 0.03) (Fig. 7, A and B). Despite these consistent and distinct effects on ET-R-promoted phosphoinositide hydrolysis, overexpression of GRK2 or GRK5 failed to affect phosphoinositide hydrolysis engendered by fluoromalicate, which stimulates G proteins directly (38) (data not shown). Thus, overexpression of either GRK2 or GRK5 appears to diminish agonist-promoted phosphoinositide hydrolysis at the level of the endothelin receptor and not at the level of even the proximate signal-transducing protein.

To ascertain that the overexpressed GRKs were indeed inhibiting ET-1-promoted phosphoinositide hydrolysis at the level of the endothelin receptors, we tested the 293 cell GRK overexpression system with two additional Gq-coupled receptors. First, we transfected 293 cells with plasmids encoding the...
GrK overexpression. This apparent paradox prompted us to test further the mechanism by which GrK overexpression suppresses Gα-coupled receptor phosphoinositide signaling in 293 cells.

To determine whether or not receptor phosphorylation is required for receptor-specific signal dampening by overexpressed GrKs, we examined the effect of the dominant-negative GrK2 on receptor-mediated phosphoinositide hydrolysis in intact 293 cells. Surprisingly, the dominant-negative (K220R) GrK2 mutant suppressed receptor signaling as effectively as the wild type GrK2 for both ET-Rs (Fig. 9, and data not shown). Moreover, by transfecting graded amounts of GrK2 and GrK2 K220R plasmid DNA in parallel cell groups, we could find no significant disparity between the wild type and mutant GrK effect on signaling at any level of GrK protein expression (data not shown). Thus, GrK-mediated receptor phosphorylation appears unimportant to the suppression of receptor signaling observed in intact 293 cells overexpressing certain GrKs.

Because GrK2 had the most profound dampening effect on endothelin receptor signaling, we investigated the ability of the noncatalytic GrK2 polypeptide domains (14) to accomplish such signal dampening. Cellular expression of the 180-amino acid GrK2 N-terminal domain polypeptide diminished t-ETAR-dependent phosphoinositide hydrolysis, by 27 ± 6% (p < 0.02). Expression of the 195-amino acid C-terminal domain polypeptide (28), however, had no effect on signaling (Fig. 9).

Both the phosphorylation-incompetent GrK2 mutant (GrK2 K220R) and the GrK2 N-terminal domain polypeptide suppressed endothelin receptor signaling. Could a simple physical association of GrK with the activated receptor, by itself, engender the observed suppression of signaling? To test this hypothesis, we immunoprecipitated the t-ETα-R, t-ETβ-R, or AT1AR from 293 cells co-transfected with either GrK2 or GrK5 and immunoblotted these immunoprecipitates to detect the presence of GrK co-immunoprecipitated with each receptor. Data obtained with the t-ETβ-R and AT1AR are depicted
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DISCUSSION

Because of their exceptionally high affinity for their agonists, the ET_A-R and ET_B-R may remain stably associated with their agonists long after initial agonist binding (2, 39). The resulting persistence of the receptors’ activated states makes intracellular mechanisms of receptor/effector desensitization exceedingly important for cellular homeostasis. In this investigation, we have shown for the first time that rapid, agonist-promoted desensitization of both the ETA_R and ETB_R involves phosphorylation of the receptors, and that this phosphorylation results from GRK activity in an intact, transfected cell model. Surprisingly, despite the dissimilarity of their cytoplasmic C-terminal tail domains, the ETA_R and ETB_R appear to interact with GRKs indistinguishably.

That the ET_A-R and ET_B-R are regulated by one or more GRKs in vivo is anatomically plausible, since the ET-Rs (40) as well as GRKs 2, 5, and 6 (12) are all expressed together, for example, in human myocardium. Moreover, the paradigm for GRK-initiated desensitization of only agonist-occupied receptors (12) is consonant with the homologous ETA-R and ETB-R desensitization observed in a multitude of experimental systems (5–11). The rapid rate of receptor/effector desensitization observed in some of these systems (4, 5, 7–9, 11) is also consistent with a GRK-initiated mechanism (15, 16). In this study, the use of dominant-negative GRK2 to inhibit both agonist-induced phosphorylation and desensitization of the ETA-R demonstrates that these events are indeed mediated by a GRK mechanism (18, 21, 35, 36).

This study describes three distinct aspects of the relationship between GRKs and the ET-Rs: (i) the dependence of ET-R desensitization upon GRK-mediated ET-R phosphorylation, demonstrated in experiments that use dominant-negative GRK2 to inhibit both processes; (ii) the ability of individual GRKs to phosphorylate the ET-Rs, demonstrated in experiments that show augmented agonist-induced ET-R phosphorylation in cells transfected with specific GRK expression plasmids; and (iii) the affinity of individual GRKs for the ET-Rs, suggested by GRK-specific inhibition of ET-R-dependent signaling in intact cells and demonstrated by agonist-dependent association of GRK with receptor. As we discuss below, this last point seems most informative regarding GRK substrate specificity.

The incremental ET-R phosphorylation seen in cells overexpressing a particular GRK demonstrates the ability of that GRK to phosphorylate the receptor under the conditions studied. However, this incremental phosphorylation is probably unimportant with regard to desensitization of the receptor/G protein signaling system. This latter inference is supported by our membrane GTPase data with the ETA-R. Desensitization of ET-1-stimulated GTPase was equivalent in cells that overexpressed GRK2 and in cognate control cells that expressed only endogenous levels of GRK2. We obtained similar results with the AT1A-R previously (18). In 293 cells expressing endogenous GRKs, both the ET-R and the AT1A-R undergo GRK-mediated phosphorylation to a stoichiometry of \(-1\) mol of phosphate/mol of receptor. For these receptors in 293 cells, then, it appears that this stoichiometry of \(-1\) suffices for maximal binding of arrestins to the receptor and hence for maximal desensitization assessed by receptor signaling in membrane preparations.

The most unexpected finding of this study emerged from examining the ability of overexpressed GRKs to inhibit ET-R-mediated phosphoinositide hydrolysis in intact 293 cells. We anticipated that cellular overexpression of GRKs would diminish ET-R-dependent phosphoinositide hydrolysis in intact cells by augmenting both agonist-promoted ET-R phosphorylation and desensitization. Surprisingly, however, GRK-mediated suppression of ET-R signaling in intact cells failed to correlate with the incremental, GRK-mediated receptor phosphorylation observed. Rather, the ability of an overexpressed GRK to suppress receptor-mediated signaling correlated with the GRKs’ ability to bind to the receptor in an agonist-dependent manner, as revealed by co-immunoprecipitation experiments. Bound to the GRK, the agonist-occupied receptor appears sterically hindered from stimulating its G protein and thus from signaling. As assessed by the parameters of agonist-dependent receptor binding and signal dampening, both the ETA-R and ETB-R appear to interact selectively with GRK2, as opposed to GRK5 or GRK6. Contrastingly, the AT1A-R appears relatively nonselective with regard to GRK2 and GRK5.

Although probably attributable to our level of GRK overex-
pression. GRK-dependent dampening of receptor-mediated signaling nonetheless reveals a novel perspective on GRK substrate specificity. We showed that GRK-dependent suppression of signaling was receptor-specific by controlling for GRK expression in distinct cell lines expressing different receptors and by stimulating different receptors within the same GRK-overexpressing cell. Since this GRK-dependent signal dampening correlates with agonist-promoted binding of the GRK to the receptor, it must reflect the affinity of a given GRK for the activated form of a particular receptor. This receptor affinity is likely to be very important for GRK-mediated receptor phosphorylation in untransfected cells, which express physiologic (i.e. very low (41)) levels of both receptor and GRK. Applying this reasoning to our results with the ET-Rs, we would expect GRK2 to be the most active of the GRKs tested in desensitizing the ET-Rs in vivo.

Why does a GRK’s affinity for the ET-Rs, manifested by co-immunoprecipitation, fail to correlate with the observed degree of agonist-induced phosphorylation in our 293 cell overexpression system? Two explanations seem relevant. First, ET-R phosphorylation is facilitated by the high expression levels of both receptor and GRK in the transfected 293 cells (20–50-fold above those seen physiologically). By mass action, even GRKs with relatively low affinity for the agonist-occupied receptor may phosphorylate it in this system, although they may not do so when enzyme and substrate are present at physiological concentrations. Second, ET-R phosphorylation may be bridled by other intracellular proteins (particularly the arrestins), which may compete with even the high-affinity GRK(a)s for receptor binding. In this regard, it is notable that phosphorylation of the receptor has been shown both to decrease the affinity of GRK binding (42) and to increase the affinity of arrestin isofrom binding to the receptor (43).

GRK-mediated phosphorylation of the ET-Rs does not, of course, preclude a role for other kinases in the phenomenon of homologous ET-R desensitization. Since they are activated by the ET-Rs (2), PKC isoforms would seem likely candidates for regulating the ET-Rs. In support of this idea, both the ET\(_A\)-R (five sites) (22) and the ET\(_B\)-R (seven sites) (23) possess numerous serines and threonines that occur within consensus sequences for PKC-mediated phosphorylation (44), and we have shown that PKC can clearly phosphorylate the ET-Rs in our experimental system. Despite these data, PKC appears to play no role in the rapid, agonist-induced ET-R phosphorylation we have studied here. Indeed, second messenger-dependent kinases, probably because of the multitude of their potential substrates, have been shown to effect receptor phosphorylation of/and desensitization at rates considerably slower than that evinced for GRKs (16, 18). In other cell systems or at later time points after agonist stimulation, however, it would not be surprising to observe a more prominent role for PKC isoforms in ET-R desensitization.

The site or sites of GRK phosphorylation on the ET-Rs remain speculative. Suggesting as yet unidentified diversity in GRK phosphorylation sequences, neither ET-R (22, 23) possesses serine or threonine residues residing in sequences conforming to those defined for the best characterized GRK substrates (20). By analogy with other receptors shown to be GRK substrates, the molecular architecture of the ET-Rs suggests that the ET-R cytoplasmic tail domains would most likely serve as sites for GRK phosphorylation (20, 22, 23, 45). Previous mutational studies have contended, though, that the C-terminal cytoplasmic tail of neither the ET\(_A\)-R (24) nor the ET\(_B\)-R (25) is important for agonist-promoted receptor desensitization.

Inferences drawn from these studies regarding ET-R desensitization and its potential mechanisms should be cautious, however. “ET-R desensitization” in both of these studies was monitored by signals considerably downstream from the ET-Rs themselves (calcium-activated chloride currents in Xenopus oocytes and cystolic calcium transients in Ltk\(^{-}\) cells, respectively). Results from these downstream signaling mechanisms may be confounded by rapid desensitization of the inositol trisphosphate receptor (46) and perhaps the chloride channels, too. Furthermore, complete ET\(_B\)-R desensitization appeared to be achieved with very low fractional receptor occupancy and in the setting of considerable heterologous desensitization (25). Such an observation conflicts strongly with the behavior of a GRK-initiated mechanism (47). Even if these mutational studies are correct, however, GRK phosphorylation sites on the ET-Rs may reside in the C tail remnants proximal to the truncation sites or in other cytoplasmic receptor domains (22, 23).

In light of the rapid desensitization of the ET-R/G\(_{i}\)/phospholipase C signaling system by GRK-initiated (and perhaps other) mechanisms, how can we explain the prolonged physiologic action of ET-1 administered pharmacologically (48)? Whereas ET-R-promoted phospholipase C activity desensitizes rapidly, ET-R-promoted phospholipase D activity (2) may desensitize much more slowly, as has been observed for the AT\(_1\)-A-R (18, 49). Furthermore, the attenuating effect of nitric oxide on ET-1-stimulated signaling (39), which may facilitate ET-1 signal termination in vivo, may be unphysiologically deficient in experimental systems demonstrating sustained ET-1-promoted activity (1, 48).

In investigating the role of GRKs in the homologous desensitization of the ET\(_A\)-R and ET\(_B\)-R, we have employed a co-transfected cell technique used by our laboratory (21) and subsequently others (37, 50–52) to show the effect of overexpressed GRKs on agonist-induced receptor phosphorylation and signaling. Our current study reveals how overexpressed GRKs, by binding to agonist-occupied receptors, can inhibit receptor signaling in a manner that may manifest GRK substrate specificity previously unappreciated. Using the approach employed with the ET-Rs and the AT\(_1\)-A-R, future studies should substantially expand our understanding of GRK substrate specificity.

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