THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 45, pp. 32138–32148, November 8, 2013

Desensitization and Internalization of Endothelin Receptor A

*IMPACT OF G PROTEIN-COUPLED RECEPTOR KINASE 2 (GRK2)-MEDIATED PHOSPHORYLATION**

Received for publication, February 13, 2013, and in revised form, August 22, 2013 Published, JBC Papers in Press, September 24, 2013, DOI 10.1074/jbc.M113.461566

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Background: The involvement of phosphorylation of endothelin receptor A (ET_A) in its desensitization and internalization is unclear.
Results: Phosphorylation-deficient ET_A mutants display defective regulation, whereas receptor internalization is not affected.
Conclusion: GRK2-mediated phosphorylation contributes to ET_A desensitization, but not to ET_A internalization.
Significance: This work contributes to the understanding of the complex GRK2-mediated ET_A regulation involving multiple mechanisms.

Endothelin receptor A (ET_A), a G protein-coupled receptor, mediates endothelin signaling, which is regulated by GRK2. Three Ser and seven Thr residues recently proven to be phosphoacceptor sites are located in the C-terminal extremity (CTE) of the receptor following its palmitoylation site. We created various phosphorylation-deficient ET_A mutants. The phospholipase C activity of mutant receptors in HEK-293 cells was analyzed during continuous endothelin stimulation to investigate the impact of phosphorylation sites on ET_A desensitization. Total deletion of phosphoacceptor sites in the CTE affected proper receptor regulation. However, proximal and distal phosphoacceptor sites both turned out to be sufficient to induce WT-like desensitization. Overexpression of the $G\alpha_q$ coupling-deficient mutant GRK2-D110A suppressed ET_A-WT signaling but failed to decrease phospholipase C activity mediated by the phosphorylation-deficient mutant ET_A-6PD. In contrast, GRK2-WT acted on both receptors, whereas the kinase-inactive mutant GRK2-D110A/K220R failed to inhibit signaling of ET_A-WT and ET_A-6PD. This demonstrates that ET_A desensitization involves at least two autonomous GRK2-mediated components: 1) a phosphorylation-independent signal decrease mediated by blocking of $G\alpha_q$ and 2) a mechanism involving phosphorylation of Ser and Thr residues in the CTE of the receptor in a redundant fashion, able to incorporate either proximal or distal phosphoacceptor sites. High level transfection of GRK2 variants influenced signaling of ET_A-WT and ET_A-6PD and hints at an additional phosphorylation-independent regulatory mechanism. Furthermore, internalization of mRuby-tagged receptors was observed with ET_A-WT and the phosphorylation-deficient mutant ET_A-14PD (lacking 14 phosphoacceptor sites) and turned out to be based on a phosphorylation-independent mechanism.

The potent vasoconstrictive peptides endothelin-1–3 (1) mediate their multiple physiological effects in various tissues and cell types (2) via activation of the G protein-coupled receptors (GPCRs)[2] endothelin receptor A (ET_A) and receptor B (3), which couple to $G\alpha_q$ proteins to induce phospholipase C (PLC) activity.

A general property of GPCR signaling is that with continuous or repeated agonist stimulation, GPCR activity wanes. This regulatory process is called desensitization (4). Phosphorylation of activated receptors by GPCR kinases (GRKs) in the third intracellular loop and the C-terminal domain plays a major role as an early regulatory event in GPCR desensitization. This is mediated by increasing the affinity of the phosphorylated receptor for arrestins, which uncouple the receptor from its G protein, initiate receptor internalization, and redirect the signaling to alternative pathways (5, 6). In addition, phosphorylation-independent mechanisms are known to be involved in the regulation of GPCR signaling (7). Phosphorylation-independent GRK-mediated GPCR desensitization may occur by direct binding of the kinase to the receptor (8, 9) or to the activated form of $G\alpha_{q/11}$ (10–12) via the RH (regulator of G protein signaling homology) domain of GRK (13).

Stannard *et al.* (14) identified 15 phosphorylation sites essentially in the C-terminal tail of ET_A in human lung fibroblasts by mass spectrometry (Fig. 1). In the same study, palmitoylation of cysteines 383, 385, 386, 387, and 388 in the C-terminal domain was demonstrated. The anchoring of this palmitoylated cysteine cluster to the plasma membrane leads to the development of a fourth intracellular loop (15). Among the identified phosphorylation sites in this study, three Thr residues and seven Ser residues are located in the region distal to the palmitoylation site (amino acids 389–427, subsequently referred to as the C-terminal extremity (CTE)), indicating that this part of the C-terminal tail is a major site of phosphorylation. Within

* This work was supported by Actelion Pharmaceuticals (Allschwil, Switzerland) and the Erich and Hanna Klessmann Foundation (Gütersloh, Germany) (to F. G. and H. M.).
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[2] The abbreviations used are: GPCR, G protein-coupled receptor; ET_A, endothelin receptor A; PLC, phospholipase C; GRK, GPCR kinase; CTE, C-terminal extremity; IP_1, *myo*-inositol 1-phosphate; ANOVA, analysis of variance.

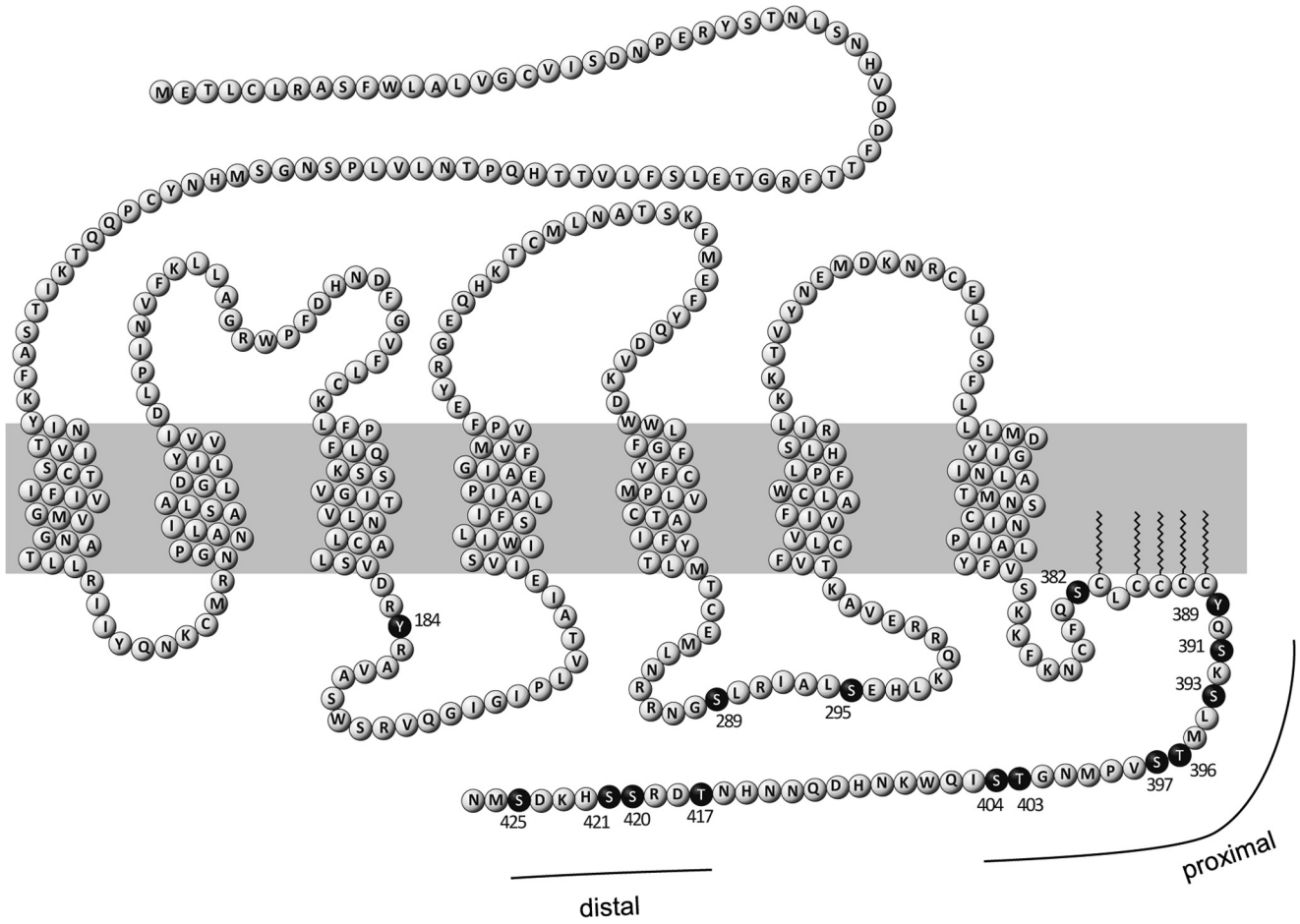

FIGURE 1. **Post-translational modifications of human ET_A.** The primary sequence of human ET_A is shown as a snake plot. The plasma membrane is represented by the *gray box*. Transmembrane amino acids are depicted according to NCBI entry P25101. In this study, mutations of ET_A were introduced at known phosphorylation sites, which are represented by *black spheres*. Palmitoylation sites are marked by *zigzag lines*. Post-translational modifications are depicted according to Stannard *et al.* (14). Clustering of phosphoacceptor sites appears in the C-terminal domain of the receptor in the region following the palmitoylation site. This region is referred to as the CTE. Clustered phosphorylation occurs in a distal and a proximal region of the CTE.

the CTE, two clusters of phospho-Ser and phospho-Thr residues can be distinguished: in the proximal region (amino acids 391–404), six phosphoacceptor sites were identified, whereas in the distal region (amino acids 417–425), four amino acids were shown to be phosphorylated.

As shown by overexpression of dominant-negative GRK mutants, GRK2 is involved in the desensitization of ET_A (8, 16). However, the importance of GRK2-mediated receptor phosphorylation is still unclear in this context. Furthermore, because GRK2 does not recognize a well defined consensus sequence but rather shows high specificity toward agonist-occupied GPCRs (17), the location of potential GRK2 phosphorylation sites in ET_A is yet unknown.

Therefore, we analyzed the desensitization of a set of different ET_A mutants lacking several phosphoacceptor sites essentially in the CTE of the receptor. This was done 1) to gain information about the significance of GRK2 phosphorylation in triggering ET_A regulation and 2) to investigate which phosphoacceptor sites, if any, are herein involved.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—The coding sequence of ET_A in pCMV-XL5 (SC118901, Origene, Rockville, MD) was amplified by PCR using oligonucleotides 5′-CTCGAGTATTTCCTCAAATTTGCCTCAAGATGGA-3′ and 5′-GCGGCCGCCATAAAAGCTAGCCATGTACTTGAAAGC-3′. The PCR fragment was subcloned into the XhoI and NotI sites of the pLPCX vector (Clontech). The mutant receptor constructs ET_A-4PD, ET_A-6PD, ET_A-8PD, ET_A-10PD, ET_A-PDZPD, ET_A-6E, and ET_A-14PD were generated by the exchange of a C-terminal sequence of ET_A with a corresponding synthetically generated DNA sequence (ATG:biosynthetics, Merzhausen, Germany) containing the respective point mutations via EcoRI (in the coding sequence of ET_A) and NotI. All other constructs were derived from these plasmids by site-directed mutagenesis using the QuikChange Lightning kit (Agilent Technologies, Santa Clara, CA). mRuby (18)-tagged receptor constructs were created by amplification of the respective receptor DNA lacking the stop codon with the use of oligonucleotides 5′-CTCGAGTATTTCCTCAAATTTGCCTCAAGATGGA-3′ and 5′-AAGACCGGTCCGTTCATGCTGTCCTTATGGCTGCTC-3′ (ET_A-WT and ET_A-PDZPD) or 5′-AAGACCGGTCCGTTCATGGCGTCCTTATGGGCG-3′ (receptor constructs containing S421A and S425A mutations), thereby flanking the PCR product with XhoI and AgeI restriction sites. The amplified products were introduced into a construct similar to the pECFP-N1

vector (Clontech) containing mRuby instead of the enhanced cyan fluorescent protein sequence to obtain ET_A constructs C-terminally fused to mRuby with Gly-Pro-Val-Ala-Thr as a linker sequence. Human GRK2 in the pcDNA3 vector was kindly provided by Prof. Martin Lohse (University of Würzburg). Site-directed mutagenesis was performed to obtain GRK2-D110A and GRK2-D110A/K220R. The coding sequence of every construct was verified.

Cell Culture and Transfection—HEK-293 cells were cultured in DMEM supplemented with 10% FBS (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) and incubated at 37 °C with 5% CO_2 in a humidified incubator. Transfection of trypsinized cells was performed 24 h before the beginning of the experiments using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Unless stated otherwise, we used 400 ng of receptor plasmid DNA/cm^2 of culture surface. Cotransfection of GRK2 was performed using 5 ng of GRK2 plasmid DNA/cm^2 of culture surface to obtain a molar ratio of 1:75 GRK2 to ET_A plasmid DNA. For high level cotransfection experiments, the amount of GRK2 plasmid DNA was increased by 5-fold. For knockdown experiments, 75 nM validated Silencer® Select siRNA (Ambion AM51331) or negative control siRNA (Ambion AM4611) was transfected along with 200 ng of receptor plasmid DNA/cm^2 of culture surface 48 h before the beginning of the experiment.

Western Blotting—HEK-293 cells were lysed in PBS containing 0.2% Triton X-100 and protease inhibitor mixture (Sigma-Aldrich P2714). After shaking for 30 min at 4 °C, the lysate was cleared by centrifugation at 20,000 × *g* for 15 min. Protein content was determined using bicinchoninic acid with BSA as a standard. 7 μg of protein was separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. GRK2 was detected using an anti-GRK2/3 antibody (1:10,000 dilution; Upstate 05-465) and an HRP-coupled anti-mouse IgG antibody (1:2000 dilution; BD Pharmingen 554002). Densitometric analysis of chemiluminescence was performed using a MultiImage light cabinet (Alpha Innotech Corp., San Leandro, CA).

Membrane Preparation—2.85 × 10^6 HEK-293 cells were transiently transfected with receptor plasmid DNA in a 6-well plate. After 24 h, cells were washed with PBS containing protease inhibitor mixture and transferred to 1.5-ml reaction tubes. The additional procedure was performed according to Elshourbagy *et al.* (19).

Radioligand Binding—4 μg of membrane preparation was mixed with 10–1000 pM ^{125}I-ET-1 and incubated in a total volume of 120 μl of binding buffer (10 mM Tris (pH 7.4) and 154 mM NaCl). After binding at 37 °C for 4.5 h, 10 ml of binding buffer was added, and the samples were filtered through glass-fiber filters presoaked in 0.1% (w/v) BSA. After three washes with 10 ml of binding buffer, the activity of the filters was quantified in a γ-counter.

Determination of Cell Surface Receptors with ^{125}I-ET-1—1.14 × 10^6 HEK-293 cells in 12-well plates were transiently transfected with plasmids encoding receptor variants. After 24 h, cells were washed with binding buffer (Hanks' balanced salt solution with 20 mM HEPES (pH 7.4), 0.2% BSA, and 0.1% glucose) and incubated with 470 μl of 100 pM ^{125}I-ET-1 (23 μCi; PerkinElmer Life Sciences) in binding buffer for 3 h at 10 °C. Cells were transferred to glass-fiber filters and washed three times with PBS. The activity of ^{125}I was quantified in a γ-counter.

Determination of PLC Activity—Accumulation of *myo*-inositol 1-phosphate (IP_1) was determined using the IP-One kit (Cisbio Bioassays, Codolet, France) according to the manufacturer's protocol. 30,000 HEK-293 cells were transfected with plasmids encoding receptor and, in the case of cotransfection, GRK2 variants in a white 384-well plate (Greiner Bio-One, Kremsmünster, Austria). Stimulation was performed with 100 nM ET-1 at 37 °C in the presence of 50 mM LiCl to inhibit *myo*-inositol 1-phosphatase. Time-resolved fluorescence signals were measured using an Infinite M1000 plate reader (Tecan, Männedorf, Switzerland).

Confocal Microscopy—Confocal microscopy experiments were performed with transiently transfected HEK-293 cells in a Nunc Lab-Tek Chamber Slide™ system on a Zeiss LSM 780 microscope using a 63× water immersion objective. For analysis of receptor internalization, HiLyte Fluor™ 488-labeled ET-1 (ET-1-HiLyte; Eurogentec, Seraing, Belgium) was added to the cells expressing the mRuby-tagged receptor to a final concentration of 100 nM. Images were captured immediately after agonist administration (~1 min) and after 15, 30, 45, and 60 min. HiLyte Fluor™ 488 was excited with the 488-nm line of an argon laser, and emission was detected at a range of 493–551 nm. For excitation of mRuby, a DPSS laser (561 nm) was used. Fluorescence intensities were recorded from 569 to 652 nm. In both cases, a 488/561 nm main beam splitter was used. Signal amplification was constant for all measurements.

Data Analysis—Data were analyzed using GraphPad Prism Version 6 software (GraphPad Software, La Jolla, CA). Mean values from individual treatment groups were statistically analyzed by one-way analysis of variance (ANOVA) with subsequent Bonferroni correction. Direct comparison of two treatment groups was performed using Student's *t* test.

RESULTS

Human ET_A was analyzed with respect to the involvement of phosphorylation sites in receptor desensitization and internalization. Therefore, various constructs of ET_A lacking phosphoacceptor sites were generated (Fig. 2). Binding of ^{125}I-ET-1 to HEK-293 cells transiently transfected with the corresponding constructs demonstrated comparable surface expression of ET_A-WT and all mutant receptors used in this study (Fig. 3). Furthermore, the binding characteristics of the maximally mutated variant ET_A-14PD did not differ significantly from those of ET_A-WT (Table 1). Because of the nearly irreversible binding of ET-1 to ET_A (20, 21), pre-stimulation with ET-1 to induce receptor desensitization was not possible. Ca^{2+} flux experiments revealed desensitization of ET_A-mediated Ca^{2+} release (data not shown), but they are not suited for monitoring specific ET_A desensitization because the inositol trisphosphate receptor itself is rapidly desensitized (22). Therefore, in this study, impairment of ET_A desensitization was analyzed by measuring inositol production. An augmented inositol accu-

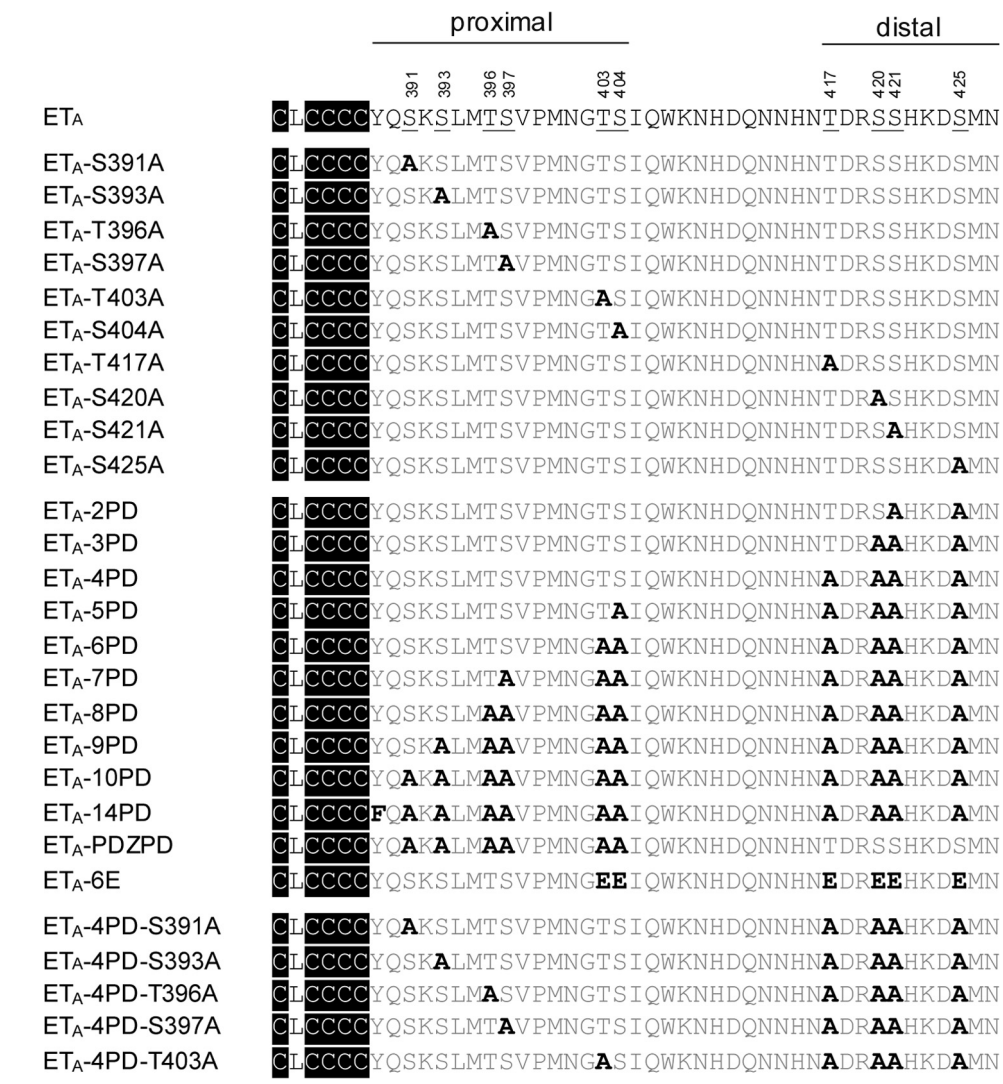

FIGURE 2. **ET_A variants used in this study.** Shown are the amino acid sequences of the WT and mutant ET_A CTEs. The *numbers* above indicate the amino acid positions in the receptor protein. Palmitoylation occurs at cysteine residues indicated in *black* (14). Phosphorylated amino acids in ET_A-WT (14) are *underlined*; mutant amino acids are highlighted in *boldface*. Additional substitutions of ET_A-14PD (S289A, S295A, and S382A) located upstream of the palmitoylation site are not shown.

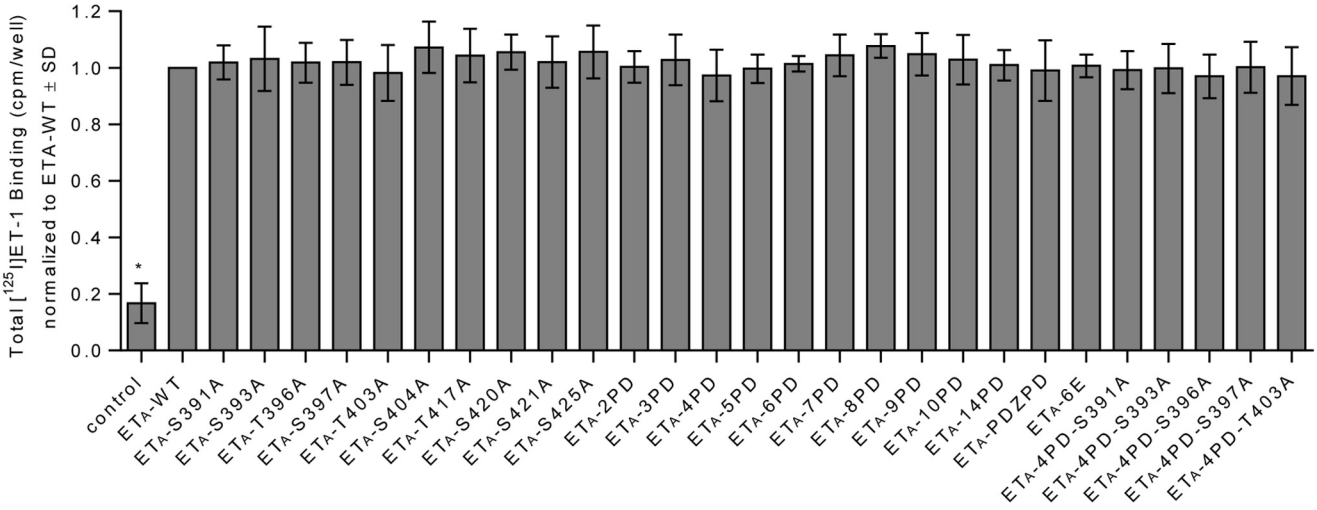

FIGURE 3. **Surface expression of ET_A-WT and mutants.** HEK-293 cells were transiently transfected with constructs of ET_A-WT or mutants. Transfection of empty vector was performed as a control. Total binding of ^{125}I-ET-1 was determined as described under "Experimental Procedures." The binding of mutant receptors was normalized to the binding of ET_A-WT. The means ± S.D. of four independent experiments were compared with ET_A-WT using one-way ANOVA with Bonferroni correction. *, $p < 0.001$.

TABLE 1

Radioligand binding characteristics of ET_A-WT and ET_A-14PD

^{125}I-ET-1 binding to membrane preparations of HEK-293 cells expressing the WT or mutant receptor was performed to obtain the equilibrium dissociation constants (K_d) of the two receptor variants and the maximal binding (B_{max}) of membranes from cells expressing receptors. The data represent the mean ± S.E. of six experiments. There were no significant differences in ^{125}I-ET-1 binding of ET_A-WT and ET_A-14PD.

	K_d	B_{max}
	pM	*pmol/mg protein*
ET_A-WT	287.2 ± 80.5	2.33 ± 1.08
ET_A-14PD	435.0 ± 106.3	2.00 ± 1.06

mulation during continuous ET-1 stimulus was interpreted as a desensitization defect.

15 phosphorylation sites, including Tyr-184, were previously identified in human ET_A (Fig. 1) (14). Tyr-184 is part of the (E/D)RY motif, one of the most highly conserved sequences in the class A family of GPCRs. This motif is involved in the adjustment of conformational states of receptors (23). Because the exchange of Tyr-184 resulted in decreased PLC activity (Fig. 4), we excluded this amino acid from further investigations. The phosphorylation-deficient mutant ET_A-14PD is depleted of all other phosphorylation sites described by Stannard *et al.* (14). Their involvement in the desensitization of ET_A was analyzed by investigating the PLC activity in HEK-293 cells overexpressing ET_A-14PD under continuous ET-1 stimulation (Fig. 4). This mutant showed a significant increase in IP_1 accumulation, reflecting a decreased desensitization compared with ET_A-WT. We concluded that proper regulation of ET_A correlates with the ability of the receptor to become phosphorylated. Next, we tried to identify which of the phosphorylation sites are involved in receptor regulation. We first analyzed ET_A-10PD, depleted of all 10 phosphoacceptor sites within the CTE. With this mutant receptor, an impaired desensitization was observed (Fig. 4), showing similar augmented IP_1 accumulation as with ET_A-14PD. Hence, phosphorylation sites located somewhere in the CTE of ET_A play a pivotal role in its desensitization. To address the impact of each single Ser and Thr residue located in the CTE, 10 mutants were generated in which these amino acids were individually exchanged with Ala. No influence on agonist-stimulated receptor desensitization was detected (Fig. 4). Thus, none of the Ser or Thr residues located in the CTE of the receptor is indispensable for ET_A regulation. Accordingly, only the combined deletion of several phosphoacceptor sites at once influences the desensitization of ET_A-10PD. Therefore, we created a series of ET_A multiple mutants including two to nine Ala substitutions. We started with the deletion of the two most distally located phosphorylation sites and removed additional Ser and Thr residues successively from the C terminus heading toward the palmitoylation site (Fig. 4). Desensitization was not affected by deletion of two to four phosphorylation sites in the distal region but was attenuated by the additional replacement of Ser-404 (ET_A-5PD) and was further impaired by removing Thr-403 (ET_A-6PD) to a maximum extent. Desensitization of ET_A-6PD was indistinguishable from that obtained with ET_A-14PD or ET_A-10PD. In addition, no further impact on receptor regulation was detected with ET_A-7PD, ET_A-8PD, or ET_A-9PD.

From these experiments, it appears that phosphorylation of the distal region of the CTE is not involved in ET_A desensitization (or is involved only if Thr-403 and Ser-404 are present). To investigate the involvement of distal phosphoacceptor sites in ET_A desensitization, we analyzed the second messenger accumulation of the mutant ET_A-PDZPD, lacking all phosphoacceptor sites in the proximal region. Remarkably, phosphorylation sites in the distal part of the C terminus were sufficient to trigger the desensitization process as indicated by the WT-like signal of this mutant receptor.

We conclude that phosphorylation of ET_A initiating its desensitization is located at the CTE and is not restricted to one cluster of Ser/Thr residues. Rather, phosphorylation may occur redundantly at either proximal (ET_A-4PD) or distal (ET_A-PDZPD) phosphoacceptor sites.

As presented above, desensitization of ET_A takes place without phosphorylation of distal Ser and Thr residues (ET_A-4PD) but is impaired by deleting further phosphorylation sites in the proximal part (ET_A-5PD and ET_A-6PD). Subsequently, we investigated whether Thr-403 and Ser-404 play a particular role in ET_A-4PD desensitization or if instead the amount of phosphoacceptor sites at arbitrary positions in the proximal region is sufficient for ET_A-4PD regulation, regardless of their precise location. This was done by the insertion of extra Ala substitutions into the proximal region of ET_A-4PD. It turned out that each phosphorylation site deleted in this experiment is involved in the desensitization of ET_A-4PD because an increased IP_1 accumulation was found for all investigated mutants (Fig. 5), but not for ET_A-4PD (Fig. 4). However, the complete presence of the three phosphorylation sites Ser-397, Thr-403, and Ser-404 appears to play a more important role compared with the complete presence of Ser-391, Ser-393, and Thr-396. The absence of one of the latter phosphorylation sites reduced the ability for desensitization to a lesser extent. The simultaneous deletion of two phosphoacceptor sites belonging to the highlighted triplet (Ser-397, Thr-403, and Ser-404) even reduced the desensitization competence to a maximum degree as shown with the mutant ET_A-6PD containing the mutations T403A and S404A in the proximal region (Fig. 5).

In summary, every Ser and Thr phosphorylation site located in the proximal part of the CTE is obligatory for proper receptor regulation in the absence of distal phosphoacceptor sites. However, the lack of Ser-397, Thr-403, and Ser-404 influences desensitization to a greater degree compared with the lack of Ser-391, Ser-393, and Thr-396.

To verify that the desensitization incompetence of ET_A-6PD results from its lack of phosphorylation, we generated a mutant containing six substitutions with Glu instead of Ala to mimic a phosphorylated receptor. Indeed, the IP_1 accumulation of this mutant (ET_A-6E) was significantly reduced compared with the ET_A-6PD signal (Fig. 4), indicating that the increased signal of ET_A-6PD does not result from the absence of Ser and Thr residues but rather from the phosphorylation deficiency of this mutant. However, the signal of ET_A-6E was not reduced to an extent comparable to that of the WT receptor.

To assess the influence of GRK2 on the desensitization of ET_A-WT and ET_A-6PD, we investigated the IP_1 accumulation in HEK-293 cells coexpressing receptor and kinase (Fig. 6). For

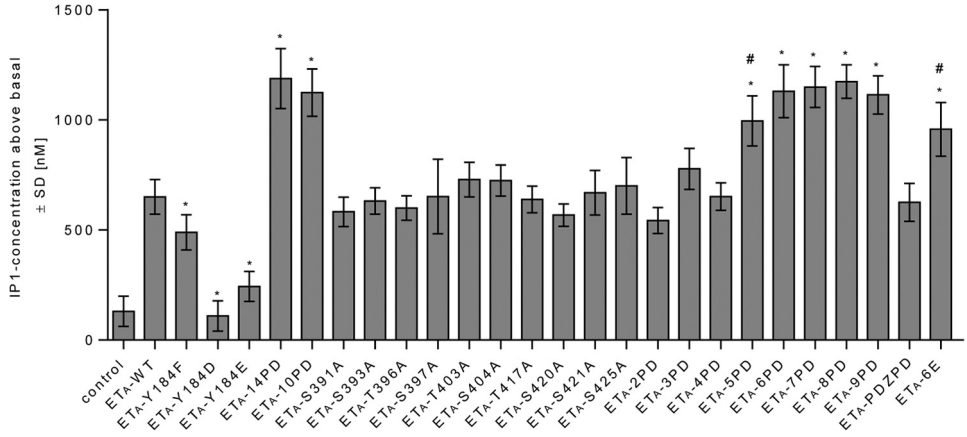

FIGURE 4. **Inositol production of ET_A-WT and mutants.** HEK-293 cells were transiently transfected with constructs of ET_A-WT or mutants as indicated. Cells were incubated for 2 h in the absence (basal) or presence of 100 nM ET-1. IP_1 accumulation was quantified as described under "Experimental Procedures." Values are presented as signal above the basal level. An impairment of receptor desensitization was observed upon enhanced IP_1 accumulation compared with the WT-induced signal. Data are expressed as the means ± S.D. of three independent experiments, each performed in at least quadruplicate, and were compared with ET_A-WT using one-way ANOVA with Bonferroni correction (*, $p < 0.001$). Furthermore, *t* test analysis (#, $p < 0.01$) was performed to compare the signals of ET_A-5PD and ET_A-6E with the signal of ET_A-6PD.

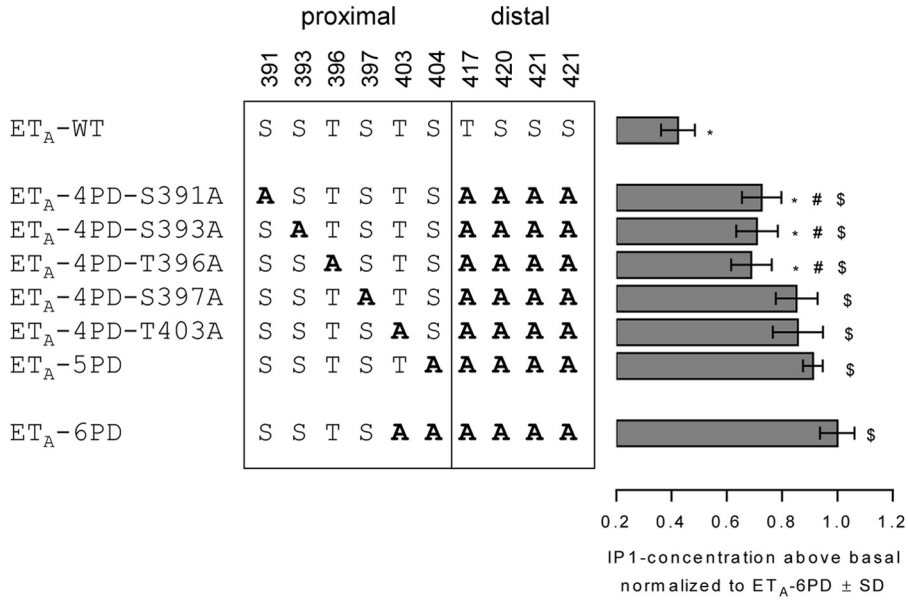

FIGURE 5. **Desensitization of mutants derived from ET_A-4PD.** HEK-293 cells were transiently transfected with constructs of ET_A-WT or mutants as indicated. Cells were incubated for 2 h in the absence (basal) or presence of 100 nM ET-1. IP_1 accumulation was quantified as described under "Experimental Procedures," and values above the basal level were normalized to the value above the basal level of ET_A-6PD. *Bars* represent the mean ± S.D. of two independent experiments, each performed at least in triplicate. Data were compared with the values of ET_A-6PD (*, $p < 0.001$), ET_A-5PD (#, $p < 0.01$), or ET_A-WT ($, $p < 0.001$) using one-way ANOVA with Bonferroni correction.

the cotransfection we used GRK2-WT and mutants GRK2-D110A and GRK2-D110A/K220R. The point mutation D110A (11, 24) in the RH domain of GRK2 influences binding of the kinase to $G\alpha_{q/11}$ (12), whereas the mutation K220R in the catalytic domain results in kinase-deficient GRK2 (25). As verified by Western blot experiments, protein expression of GRK2 was not different between the WT and mutants in HEK-293 cells (Fig. 6*A*).

Coexpression of GRK2-WT along with ET_A or ET_A-6PD decreased the second messenger response of both receptors. However, the PLC activity of ET_A-WT was inhibited by ~80%, whereas the second messenger response of ET_A-6PD was influenced to a lesser degree (~35%), suggesting that receptor phosphorylation is involved in signal inhibition. Accordingly, GRK2-D110A (which does not bind $G\alpha_{q/11}$) inhibited activity of ET_A-WT by 45% but had no significant impact on ET_A-6PD signaling. Finally, the mutant GRK2-D110A/K220R (lacking kinase activity in addition) did not influence ET_A-WT or ET_A-6PD receptor activity. Thus, desensitization of ET_A-WT is mediated by two GRK2-dependent mechanisms: binding to $G\alpha_{q/11}$ and receptor phosphorylation. However, in the case of ET_A-6PD, kinase-dependent desensitization is disabled, which proves the impact of phosphorylation sites in the ET_A CTE for receptor regulation.

The PLC activity of ET_A-WT and ET_A-6PD along with the high level cotransfection of GRK2 constructs (Fig. 6*C*) resembles the results described above, albeit all signals are decreased. However, enhanced desensitization cannot be explained only

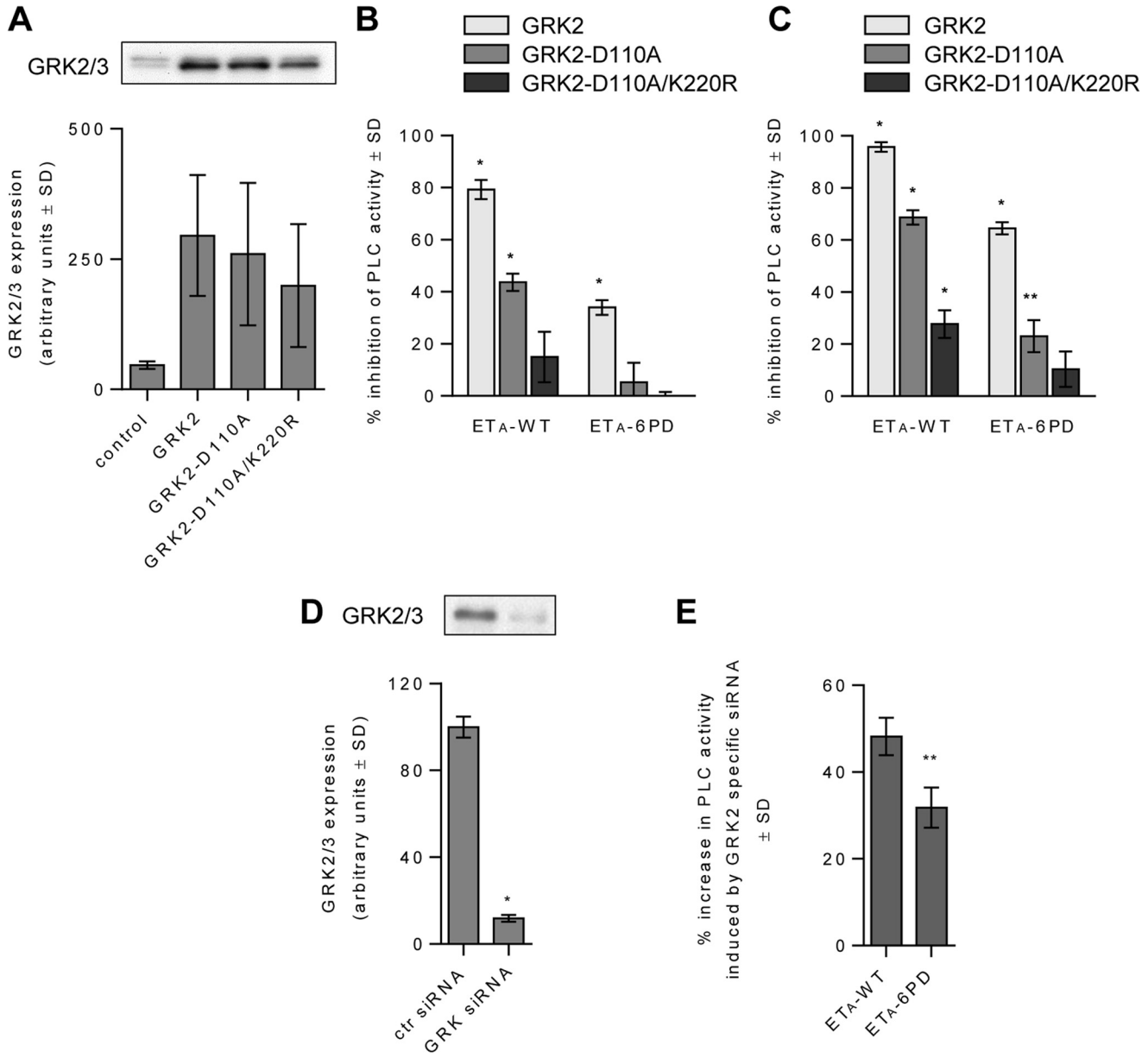

FIGURE 6. **GRK2 involvement in ET_A desensitization.** *A*, for expression control, HEK-293 cells were transiently transfected with ET_A-WT and with the indicated GRK2 construct. Control cells were transfected exclusively with ET_A-WT. After 24 h, cells were lysed, and the expression of GRK2/3 was investigated in a Western blot experiment using a GRK2/3-specific antibody (*upper*) and by densitometric analysis (*lower*). *Bars* represent the mean ± S.D. of three independent experiments. No significant differences in GRK2 expression could be observed. *B*, HEK-293 cells were transiently transfected with constructs of ET_A-WT or ET_A-6PD. Cotransfection was performed with 5 ng of plasmid DNA/cm^2 of culture surface coding for WT or mutant GRK2 as indicated. No GRK2 cotransfection was performed with control cells. The cells were stimulated with 100 nM ET-1 for 30 min. IP_1 accumulation was quantified as described under "Experimental Procedures." Data were collected in triplicate. From each data point, the basal value obtained with non-stimulated control cells was subtracted to obtain PLC activity above the basal level (PLC_{net}). Inhibition of PLC activity is given in percent (means ± S.D.) and calculated as follows: 100 × (PLC_{net} of control cells − PLC_{net} of GRK-transfected cells)/(PLC_{net} of control cells). Results were compared with the values obtained with control cells using one-way ANOVA and Bonferroni correction. *C*, High level cotransfection using 25 ng of plasmid DNA/cm^2 of culture surface encoding GRK2 variants. The experimental conditions and data processing are analogous to those described for *B*. *D*, knockdown of GRK2 using siRNA. HEK-293 cells were transfected with ET_A-WT and control siRNA or GRK2-specific siRNA. After cell lysis, the expression of GRK2/3 was investigated in a Western blot experiment (*upper*) and by densitometric analysis (*lower*). *Bars* represent the mean ± S.D. of three independent experiments. Statistical analysis was performed using Student's *t* test. *E*, HEK-293 cells were transfected with ET_A-WT or ET_A-6PD and 75 nM control siRNA (control cells) or GRK2-specific siRNA. After 48 h, the cells were stimulated with 100 nM ET-1 for 30 min. IP_1 accumulation was quantified as described under "Experimental Procedures." From each data point, the basal value obtained with non-stimulated control cells was subtracted to obtain PLC_{net}. *Bars* represent the additional signal intensity obtained by treatment with GRK2-specific siRNA compared with control cells given in percent (means ± S.D.) and calculated as follows: 100 × ((PLC_{net}/PLC_{net} of control cells) − 1). Data were collected in triplicate. Statistical analysis was performed using Student's *t* test. *, $p < 0.001$; **, $p < 0.01$.

by an augmentation of both aforementioned GRK2 effects because expression of GRK2-D110A and GRK2-D110A/K220R affected ET_A-6PD- and ET_A-WT-mediated signaling, respectively, to a significant degree.

Congruent with the data obtained by GRK2 overexpression, siRNA-mediated down-regulation of endogenously expressed GRK2 resulted in augmented PLC activity of both receptors (Fig. 6, *D* and *E*), verifying a role of GRK2 in ET_A desensitiza-

tion. However, the impact of GRK2 silencing on the signal mediated by ET_A-6PD (with disabled kinase-dependent desensitization) was less pronounced.

To investigate the role of phosphorylation sites in the internalization of ET_A, we fused the red fluorescent protein mRuby to the C termini of the receptors (18). HEK-293 cells transfected with ET_A-WT-mRuby or the phosphorylation-deficient mutant ET_A-14PD-mRuby were incubated with fluorescent ET-1-HiLyte. Binding of ET-1-HiLyte to cells expressing receptor constructs could be observed immediately after the addition of the ligand (Fig. 7). However, non-transfected cells showed no detectable binding of the fluorescent agonist (data not shown). After 15, 30, 45, and 60 min, cells expressing ET_A-WT-mRuby and ET_A-14PD-mRuby showed an increasing number of vesicles containing ET-1-HiLyte, indicating internalization of agonist-bound receptors (Fig. 7). No impact of mutations on the internalization was detected. We conclude that the mechanism of ET_A internalization is independent of receptor phosphorylation in HEK-293 cells.

DISCUSSION

For endothelin receptors, the mechanism of desensitization, especially the role of receptor phosphorylation, is not unambiguously resolved. In a study performed by Freedman *et al.* (8), GRK2 overexpression in HEK-293 cells led to an increase of ET_A phosphorylation. Furthermore, agonist-induced receptor phosphorylation, as well as agonist-induced reduction of ET_A-mediated GTPase activity, was abolished by inhibition of GRK2 activity caused by coexpression of the dominant-negative mutant GRK2-K220R. Congruent with these observations, overexpression of GRK2-D110A/K220R and introduction of siRNA targeting endogenous GRK2 both attenuated desensitization of endogenous ET_A in mesenteric arterial smooth muscle cells of adult rats (16). These reports propose that ET_A regulation is mediated by GRK2 phosphorylation of the receptor.

However, there are data suggesting that this canonical model for GPCR desensitization does not suffice to explain ET_A desensitization. Because GRK2-K220R, although lacking phosphotransferase activity, retains the ability to suppress phosphoinositide hydrolysis (8), it was suggested that GRK-mediated ET_A phosphorylation is not relevant for decreasing of receptor signaling.

In this study, we have demonstrated a defective regulation of phosphorylation-deficient ET_A mutants (Fig. 4). This suggests that receptor phosphorylation contributes to ET_A desensitization. Furthermore, the involvement of phosphorylation-independent desensitization as proposed by Freedman *et al.* (8) was verified in our study by transfection of GRK2 constructs lacking kinase activity and the ability to bind $G\alpha_{q/11}$ proteins (Fig. 6*B*). Both phosphorylation-dependent and -independent regulation of PLC activity were shown to be GRK2-mediated.

Physical association of GRK2 with the receptor may be a complementary regulatory action of the kinase (8). In our results obtained with high level co-transfection of GRK2 variants, inhibition of ET_A-WT- and ET_A-6PD-mediated signaling was induced even with the use of GRK2-D110A/K220R or GRK2-D110A, respectively (Fig. 6*C*), most likely attributable to the latter mechanism.

The increase in PLC activity mediated by silencing of endogenously expressed GRK2 underlines the existence of both 1) a phosphorylation-independent desensitization mechanism by the enhancement of ET_A-6PD signaling and 2) a phosphorylation-dependent regulatory component by the additional signal increase in the case of ET_A-WT.

Little is known about the localization of phosphoacceptor sites involved in GRK2-mediated desensitization of endothelin receptors. Clustering of Ser and Thr residues within the extreme C terminus is a common feature of many GPCRs. For rhodopsin (26), PAR2 (protease-activated receptor-2) (27), and the β_2-adrenergic receptor (28) among other GPCRs, GRK-mediated phosphorylation of Ser and Thr residues is located within the C-terminal tail subsequent to the palmitoylation site. These phosphoacceptor sites are an important determinant for arrestin interaction, receptor desensitization, or both. The CTE of ET_A contains a high density of phosphorylatable residues, and all 10 of them are indeed phosphorylated in human lung fibroblasts (14). In this study, we have shown that the combined deletion of these 10 phosphoacceptor sites in ET_A results in an impaired desensitization most likely due to the lack of GRK2-mediated phosphorylation.

However, individual phosphorylation of each site is dispensable for receptor desensitization as shown with single mutant receptors. Thus, ET_A regulation is not based on a key phosphorylation of a specific priming site initiating a hierarchical receptor phosphorylation as seen for other GPCRs, such as rhodopsin (29), the complement 5a receptor (30), and the δ-opioid receptor (31). Our data instead suggest a comparable unspecific phosphorylation of Ser and Thr residues located within the CTE of ET_A. This fits the fact that GRKs do not recognize a defined consensus sequence of specific receptors but bind specifically to activated receptors. Upon activation of GPCRs, a pocket in the cytoplasmic receptor surface is shaped (32), supporting GRK binding. Once docked, the kinase is activated and is able to phosphorylate any phosphoacceptor site located nearby in an unspecific fashion (17). The redundancy of phosphorylation of either proximal or distal phosphoacceptor sites in the regulation of ET_A leads to the conclusion that the desensitization process is initiated by a certain number of phosphorylated amino acids, regardless of their precise location. Similarly, in the m2 muscarinic acetylcholine receptor, two redundant clusters of phosphoacceptor sites were reported to be involved in receptor internalization (33). However, arrestin binding (34) and desensitization (33) were shown to be dependent on the phosphorylation of one particular cluster.

For desensitization, however, the phosphorylation of ET_A is not entirely interchangeable. Phosphorylation of Ser-397, Thr-403, and Ser-404 (despite not being indispensable) seems to have a more important role in receptor desensitization than phosphorylation of Ser-391, Ser-393, and Thr-396. This may be attributable to the different locations of phospho groups within the tertiary structure of the cytoplasmic domain, which are unequally accessible for arrestin.

For internalization studies, we used ET_A constructs C-terminally fused to a fluorescent protein. This tag does not alter the internalization characteristics of ET_A (35). We showed that internalization of ET_A is independent of phosphoaccep-

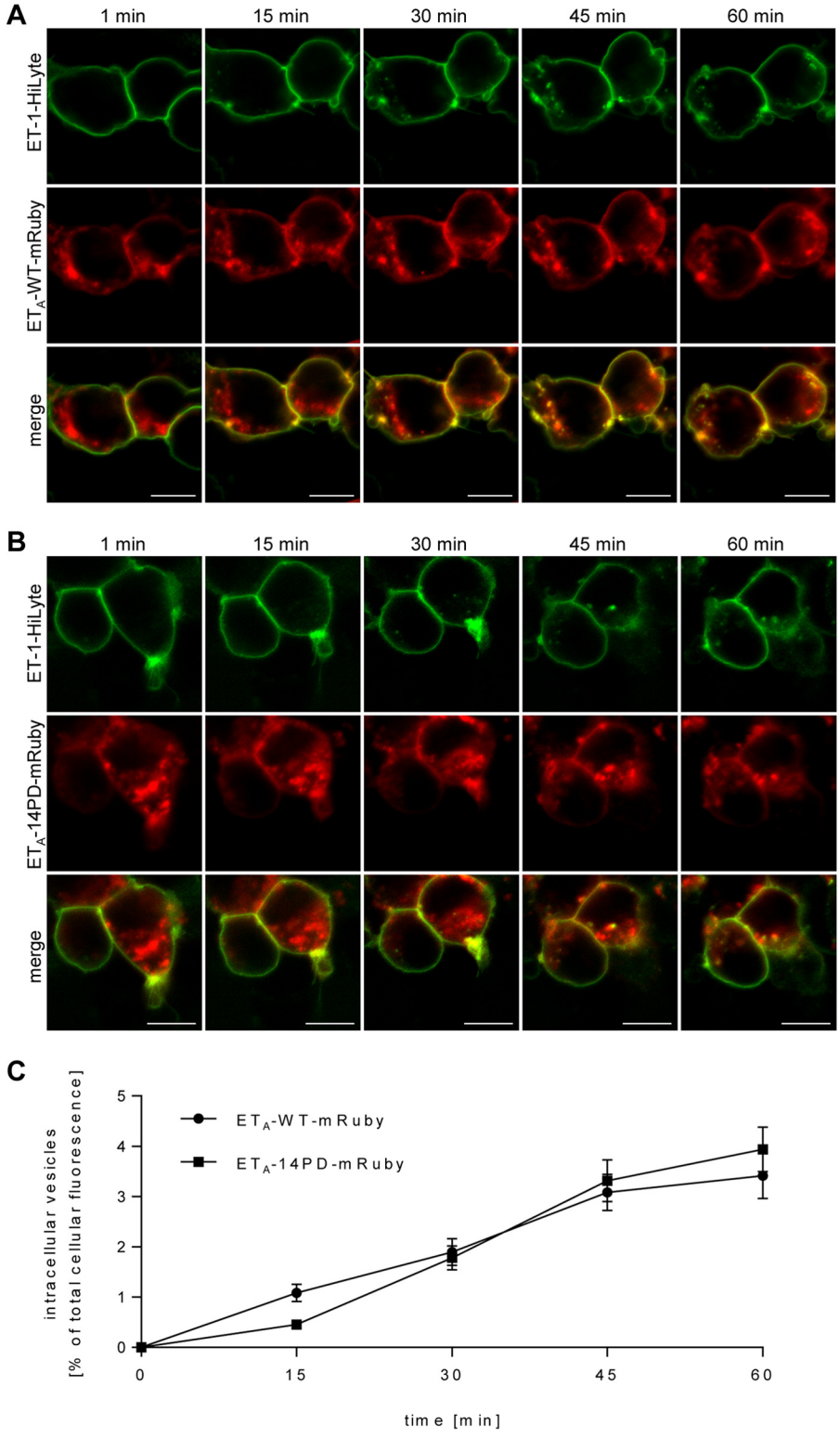

FIGURE 7. **Internalization of ET_A-WT and ET_A-14PD.** HEK-293 cells expressing ET_A-WT-mRuby (*A*) or ET_A-14PD-mRuby (*B*) were loaded with 100 nM ET-1-HiLyte and studied for internalization of the membrane-bound ligand by confocal laser scanning microscopy. The cells were observed over a period of 60 min. *Scale bars* = 10 nm. *C*, quantification of receptor internalization at the indicated time points after the addition of the fluorescent agonist was performed using ImageJ software. The summarized ET-1-HiLyte signal intensity of intracellular vesicles was background-subtracted and normalized to the total cellular fluorescence intensity to account for possible photobleaching. The value was then multiplied by 100 to obtain the percent of total cellular ET-1-HiLyte fluorescence intensity. The mRuby signal served as a control and was not incorporated into the calculation. Data points represent the mean ± S.E. of at least 25 cells for each construct. No significant differences were observed between ET_A-WT and ET_A-14PD.

tor sites (at least of phosphoacceptor sites proven in Ref. 14). Similar results were obtained using a truncated ET_A mutant with deletion of the CTE, including 10 phosphoacceptor sites (36), in CHO cells. In concert with these findings, GRK2 overexpression failed to affect ET_A internalization in CHO cells (35).

Arrestin binding is essential for internalization of GPCRs (37, 38). Especially for ET_A expressed in CHO (35) and HEK-293T (39) cells, arrestin-dependent receptor internalization was demonstrated. It was claimed for a long time that receptor phosphorylation is essential for arrestin binding (40). In this context, a mutant of the human P2Y1 receptor lacking Ser-352 and Thr-358 in the distal portion of the C-terminal tail failed to induce the translocation of arrestin and consequently receptor internalization in HEK-293 cells (41). This contrasts with our data suggesting a phosphorylation-independent internalization mechanism of ET_A.

However, the arrestin-GPCR interaction seems to reflect a more complex process than just binding or not. Binding to a non-phosphorylated receptor may induce an arrestin conformation different from that resulting from the binding to a phosphorylated receptor. This can result in different downstream effects. Such differential arrestin binding was shown, for example, for PAR2. Here, a stable arrestin interaction was lost upon a truncation in the C-terminal tail impairing prolonged ERK2 activation, whereas receptor internalization mediated by a more unstable arrestin association was not influenced (27).

GPCR desensitization and internalization likewise may have different requirements for arrestin conformations resulting from different arrestin receptor associations. Consequently, both events are not necessarily correlated in phosphorylation-deficient GPCR mutants. This is the case, for example, for the somatostatin subtype 2A receptor. A Thr-deficient mutant of this receptor inhibited internalization but did not affect desensitization (42). Recently, results analogous to ours were obtained with BLT1 (B4 leukotriene receptor-1) in rat basophilic leukemia RBL-2H3 cells (43). In this study, arrestin-mediated internalization of the receptor was not influenced by conversion of phosphoacceptor sites in the C-terminal domain to Ala residues, whereas desensitization of the same construct was reduced as shown by enhanced G protein-mediated activities. These and other (*e.g.* Refs. 27, 30, 44, and 45) results agree with our finding that phosphorylation of GPCRs is not an absolute requirement for arrestin binding or internalization. The phosphorylation-independent interaction of a ligand-occupied receptor with arrestin may be mediated through the activation sensor of arrestin (5).

The flexibility of arrestins is a prerequisite for our current view on these multifunctional adaptor proteins. For instance, binding of different arrestin-biased agonists to GPCRs leads to the establishment of distinct phosphorylation patterns of the same receptor by different GRKs leading to the adoption of different arrestin conformations and functional outcomes (46).

Our investigations using the phospho-mimic mutant ET_A-6E revealed a partly reduced desensitization. This demonstrates that negative charges are not sufficient to promote proper arrestin binding required for ET_A desensitization. One would anticipate that mutant ET_A-6E would show low signal intensity and behave as a constitutive desensitized receptor, as is the case for BLT1. Here, a mutant containing Asp/Glu insertions mimicking the phosphorylation of substituted Ser/Thr behaved as a constitutive desensitized receptor (43). On the other hand, for the β_2-adrenergic receptor, a reduction of GRK-mediated desensitization was observed upon the substitution of Asp for putative GRK sites (47). This shows that signaling of different GPCRs can be individually influenced by the introduction of phospho-mimic mutations. In the case of ET_A-6E, the phospho-mimic does not equal real receptor phosphorylation with respect to the ability to induce receptor internalization.

In this study, we have shown that ET_A desensitization is a complex process involving phosphorylation-dependent and -independent regulatory mechanisms. The participation of diverse desensitization mechanisms presumably allows cell-specific fine-tuning of endothelin signaling. Understanding the flexible nature of endothelin receptor regulation may help in the development of drugs targeting tissue-specific receptor desensitization, *e.g.* for the treatment of pulmonary hypertension.

Acknowledgments—We thank Prof. Gerd Ulrich Nienhaus (Karlsruhe Institute of Technology) for providing the mRuby construct; Prof. Hanns Hatt (Ruhr University Bochum) for providing the opportunity to measure Ca^{2+} fluxes; Dr. Andreas Brodehl, Prof. Martin Lohse, and Prof. Karl-Josef Dietz (University of Bielefeld) for helpful discussions; and Désirée Gerdes, Birte Bohms, and Ramona Cebulla for excellent technical assistance.

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