EFR3s are palmitoylated plasma membrane proteins that control responsiveness to G protein-coupled receptors

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ABSTRACT

The yeast Efr3p protein is a major regulator of the Stt4p phosphatidylinositol 4-kinase at ER-PM contact sites. Its mutant fly homologue, Rbo displays diminishing light responses attributed to progressively impaired PLC signaling. Here we find that Efr3s play a role in maintaining responsiveness to angiotensin II (AngII) receptors. RNAi-mediated depletion of EFR3A and EFR3B impaired the sustained phase of cytosolic Ca\(^{2+}\) response to high concentration of AngII in HEK293 cells expressing the wild type but not a truncated AT1a receptor, missing the phosphorylation sites. Efr3 depletion had minimal effect on the recovery of plasma membrane phosphoinositides during stimulation, and AT1 receptors still underwent ligand-induced internalization. A higher level of basal receptor phosphorylation and a larger response was observed after stimulation. Moreover, Gq activation more rapidly desensitized after AngII stimulation in Efr3 downregulated cells. Similar but smaller effect of EFR3 depletion was observed on the desensitization of the cAMP response after isoproterenol stimulation. These data suggest that mammalian Efr3s contribute to the control of the phosphorylation state and hence desensitization of AT1a receptors and could affect GPCR responsiveness in higher eukaryotes.

INTRODUCTION

A selected group of G protein-coupled receptors (GPCRs) activate phospholipase C (PLC) enzymes via coupling to heterotrimeric Gq/11 proteins to induce a cytosolic Ca\(^{2+}\) increase and trigger a variety of downstream responses depending on the cell type. PLC enzymes hydrolyze the plasma membrane (PM) lipid, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)\(P_2\)] to generate the two messenger molecules, Ins(1,4,5)\(P_3\) and diacylglycerol (DG) (Berridge, 1984). Due to the limited size of the PM PtdIns(4,5)\(P_2\) pools, continuous signal generation requires a steady resynthesis of this lipid by PI 4-kinase and PIP 5-kinase enzymes. The PI4K implicated in this process is PI4KA (Balla et al., 2007; Balla et al., 2005) whose yeast orthologue, Stt4 is also responsible for the maintenance of the PM PtdIns(4,5)\(P_2\) pools (Audhya and Emr, 2002).

Cells possess several protective mechanisms to prevent excessive stimulation of their GPCRs. High concentrations of GPCR ligands often lead to rapid receptor desensitization that diminishes the receptor’s ability to couple to G proteins. The most common cause of desensitization is due to the rapid phosphorylation of receptors by one of several GPCR kinases (GRKs) that lead to uncoupling from the G proteins and association with arrestins (Moore et al., 2007). Arrestin binding triggers receptor endocytosis thereby limiting the number of the receptors on the cell surface. However, arrestin-
association also lends new signaling profile to endocytosed receptors triggering specific cellular responses from endocytic compartments (Luttrell and Lefkowitz, 2002; Reiter et al., 2012; Wei et al., 2003). While these molecular events have been studied and characterized in great detail, little is known about the processes that restore the sensitivity of receptors at the PM (Vasudevan et al., 2011). Early studies on β-adrenergic receptors suggested that dephosphorylation requires the receptors to be endocytosed (Yu et al., 1993) but this question has never been resolved.

The present studies were intended to investigate the possible role of the two mammalian EFR3 proteins, EFR3A and EFR3B in the regulation of the PI4KA enzyme in mammalian cells. The yeast orthologue of these proteins, Efr3p was found to be essential for the localization of the yeast PI4K, Stt4 into discrete signaling domains and together with another protein, Ypp1, to control Stt4 function (Baird et al., 2008). A similar role has been described in the mammalian system where EFR3 and the TTC7 proteins were shown to keep an active pool of PI4KA at the plasma membrane (Nakatsu et al., 2012). While studying the role of EFR3 in the maintenance of the signaling phosphoinositide pools, we discovered that EFR3 proteins were important for AT1 receptors to maintain their signaling competence, an effect that was not caused directly by rundown of the phosphoinositide pool, but rather the regulation of the phosphorylation-state of the receptor. Therefore, our studies identified EFR3 as a component of the angiotensin II receptor signaling cascade that controls receptor re-sensitization and thereby could serve as a hitherto unrecognized player in determining angiotensin II and possibly other GPCR responsiveness.

RESULTS

EFR3s are widely expressed but show distinct enrichment in various tissues
Mammalian cells contain two EFR3 proteins, EFR3A and EFR3B with several putative splice variants annotated in GenBank. To determine the expression pattern of the two EFR3 isoforms, we performed quantitative PCR using various mouse tissues. Fig. 1 shows that the two EFR3 isoforms are widely expressed although they do show notable differences: while EFR3A showed highest expression in the testis, EFR3B had highest expression in the brain followed by small intestine and the eye but showed very low expression in the testis and the kidney. These results did not suggest a narrow tissue-specific function for the two EFR3 proteins.

EFR3s are localized to the plasma membrane by palmitoylation
To determine the subcellular distribution of the two EFR3 proteins, we obtained cDNAs of both of them and epitope-tagged them at their C-termini either with a HA-tag, GFP or mRFP. When these proteins were expressed in HEK293 cells or COS-7 cells, the proteins were localized to the PM with some of the signal being in the cytosol (Fig. 2A). Similar PM localization was found with the HA-tagged versions in fixed and immunostained cells (not shown). A shorter form of EFR3B truncated at the N-terminus (missing 148 residues) was found completely cytosolic (Fig. 2A). The presence of cysteines (four in EFR3A and three in EFR3B) within this N-terminal segment that are conserved in the EFRs of higher eukaryotes (but missing in yeast) raised the possibility that the full-length proteins are kept in the membrane by palmitoylation. Therefore, we mutated the four cysteines to serine residues in EFR3A and this mutant protein was found in the cytosol (Fig. 2B). Using metabolic labeling of cells overexpressing GFP-tagged EFR3A or 3B with [3H]-palmitate, we were able to confirm that indeed, EFR3A and EFR3B are palmitoylated proteins (Fig. 2C). Lastly, we determined whether the isolated N-terminus (residues 1-37 of EFR3A) was sufficient to target GFP to the PM. We found that this construct was mostly localized to the Golgi and it required the cysteines for Golgi localization (Fig. 2B). This suggested that other determinants of the full-length protein were needed to localize the protein to the PM. These results collectively suggested that contrary to previous suggestions, EFR3 proteins are not transmembrane proteins but peripheral membrane proteins associated with the plasma membrane via palmitoylation. The same conclusion about EFR3 localization and palmitoylation was reached in a recently published study (Nakatsu et al., 2012).

**EFR3s are important for sustained Ca\(^{2+}\) signaling**

If EFR3 proteins are important for the proper function of PI4KA, one expects that the PM phosphoinositide pools are run-down upon strong stimulation of Gq/11-coupled receptors, since this enzyme was found to be primarily responsible for the synthesis of PtdIns4P in the PM to serve as precursor for PtdIns(4,5)P\(_2\) (Balla et al., 2007; Balla et al., 2005). This happens when the enzyme is pharmacologically inhibited as shown initially by using high concentrations of wortmannin (Wm) or a concentration of PAO that primarily inhibits the PI4KA enzyme (Balla et al., 2007) or recently by specific PI4KA inhibitors (Bojjireddy et al., 2014). The easiest way to test for such an effect was to follow the cytosolic Ca\(^{2+}\) increase after stimulation, since the sustained phase of this response requires continuous PtdIns(4,5)P\(_2\) resynthesis (Nakanishi et al., 1995). For this experiment, we used HEK293 cells stably expressing the rat AT1a angiotensin receptors (HEK-AT1) and stimulated with AngII, since the AngII responses of these cells have been thoroughly characterized in our laboratory. We used siRNA-mediated silencing (for 3 days) to knockdown EFR3 isoforms (either alone or in combination) and studied the Ca\(^{2+}\)
responses of Fura-2-loaded cells to AngII (100 nM). The efficiency of the knock down was tested using western blot analysis following either the endogenous EFR3A or transfected EFR3A and EFR3B (Fig. 3A and B). We found that EFR3A/B depleted cells showed a Ca$^{2+}$ response with a diminished plateau phase, while cells treated with control siRNA maintained the sustained Ca$^{2+}$ plateau, characteristic of these cells (Fig. 3C). Notably, the Ca$^{2+}$ response to a lower concentration of AngII was not affected by EFR3 depletion (Fig. 3C). Knock down of the individual EFR3s have indicated that EFR3B knockdown had a stronger effect than that of EFR3A (not shown). Therefore, some of the later experiments were performed only with EFR3B knockdown. These data were consistent with a run-down of the PM PtdIns(4,5)P$_2$ pools in the EFR3A/B-depleted cells which would be more severe at a higher level of stimulation.

**EFR3-depleted cells do not show prominent defects in PtdIns4P and PtdIns(4,5)P$_2$ resynthesis during stimulation**

Next we tested the effects of EFR3 down-regulation by RNAi on the PtdIns4P and PtdIns(4,5)P$_2$ responses to AngII stimulation using metabolic labeling of these lipids. Cells were labeled with $^{32}$P-phosphate or myo-$[^3]$H]inositol for 3 hrs or 24 hrs, respectively before stimulation with AngII (100 nM) for the indicated times. Lipids were extracted and separated by TLC and quantified with PhosphorImaging (for $^{32}$P) or densitometry after exposure of Enhance-sprayed plates to X-ray film ($[^3]$H]inositol). As shown in Fig. 3D for $^{32}$P-labeled PtdIns4P and PtdIns(4,5)P$_2$ changes, we did not observe the run-down of these inositide pools in EFR3-depleted cells even after stimulation with the high concentration of AngII. The PtdIns4P and PtdIns(4,5)P$_2$ kinetics were very similar between the control and siRNA-treated groups although the drop in PtdIns(4,5)P$_2$ at early time points was slightly deeper (Figure 3C). These results suggested that the partial depletion of EFR3 (to the extent it was achieved in these experiments) had little if any impact on PtdIns4P synthesis. It is worth pointing out, however, that even the siRNA-mediated knockdown of PI4KA exerted only subtle effects on PtdIns(4,5)P$_2$ resynthesis (Balla et al., 2007) or Ca$^{2+}$ signaling (Korzeniowski et al., 2009) reported in our previous studies. Therefore these results did not argue against a role of EFR3 proteins in PI4KA-mediated PtdIns4P synthesis as it was convincingly shown in recent studies (Nakatsu et al., 2012). However, they did raise the question of whether the impaired Ca$^{2+}$ response was caused by a mechanism other than the depletion of PtdIns(4,5)P$_2$ pools under these experimental conditions.

**EFR3 depletion has no major effect on the Ca$^{2+}$ signal in cells expressing a truncated AT1a receptor**

There are a number of possibilities that could cause an alteration of the Ca$^{2+}$ signal including modification of Ca$^{2+}$ pumps and influx pathways. However, one possible cause of a diminishing Ca$^{2+}$ signal that can
be easily tested is the desensitization of the receptor due to phosphorylation and internalization. Rapid receptor desensitization could also terminate PLC activation before the PtdIns(4,5)P$_2$ pools can run down. Therefore, we generated stable HEK293 cells expressing a truncated AT1a receptor (Y319stop) lacking the phosphorylation sites (HEK-AT1Δ) and hence unable to interact with β-arrestins and unable to internalize and desensitize (Hunyady et al., 1994). We knocked down EFR3s in these cells to study their Ca$^{2+}$ responses. As expected, the cells displayed a more pronounced plateau in their cytoplasmic Ca$^{2+}$ responses because of the lack of desensitization. Remarkably, this more sustained Ca$^{2+}$ elevation was only minimally affected by EFR3 down-regulation (Fig. 4A). This finding was important as it suggested that EFR3 knockdown did not have a major impact on the Ca$^{2+}$ handling machinery or a massive effect on the phosphoinositide pools of the cell and hence, the impaired Ca$^{2+}$ signal observed with the wild-type AT1a receptor must have been due to a process involving the receptor tail containing the phosphorylation sites.

**AT1a receptors are hyperphosphorylated in EFR3 depleted cells**

These findings turned our attention to receptor phosphorylation. Since our HEK-AT1 cells express receptors tagged with a Flag-tag at the N-terminus, we used these cells and labeled them with $^{32}$P-phosphate for 4 h followed by stimulation with AngII (100 nM) for the indicated times. Receptors were then immunoprecipitated and analyzed by western blotting and phosphorimaging. As shown in Fig. 4B, AT1a receptors isolated from EFR3 depleted cells, showed increased basal phosphorylation and a substantially larger phosphorylation response following AngII stimulation. However, at later times, the receptor phosphorylation subsided and was indistinguishable from the controls at 20 minutes after stimulation. This finding was consistent with an impaired dephosphorylation of the AT1a receptor and could explain the faster receptor desensitization during stimulation.

**AT1a receptor internalization is not impaired in EFR3-depleted cells**

Previous studies suggested that EFR3 may be important for bulk endocytosis of membranes (Vijayakrishnan et al., 2009) and a genetic linkage was found in flies between dynamin and the *Rbo* mutation, suggesting that EFR3 could play a role in endocytosis (Vijayakrishnan et al., 2010). Without internalization, the receptor may not be dephosphorylated and regain its G protein signaling competence. Therefore, next we examined whether EFR3 knock down affects the endocytosis of the AT1a receptor. For this, we used several different approaches. First, we examined the translocation of β-arrestin2 tagged with GFP in the HEK293-AT1 cell line after AngII stimulation. In these experiments we used siRNA
against EFR3B only. These experiments showed that EFR3 knockdown had no appreciable effect on the ability of the AT1 receptor to recruit β-arrestin2 GFP to the membrane or on subsequent intracellular trafficking of the receptor-β-arrestin2 complex (Fig. 5A). Similar results were found with GFP-tagged β-arrestin1, although the association of this construct with the receptor was less pronounced both in the control and EFR3-depleted cells (not shown).

Second, we followed the distribution of the AT1 receptors in a stable cell line expressing the AT1a receptor tagged with GFP at the C-terminus (Hunyady et al., 2002). In previous studies we have thoroughly characterized the trafficking of these GFP-tagged AT1a receptors (Hunyady et al., 2002). There was a notable decrease in the GFP signal at the PM of EFR3B-depleted cells before AngII stimulation relative to the internal signal (Fig. 5B). However, the GFP-tagged AT1a receptors showed clustering and endocytosis similar to those found in cells treated with either control or EFR3 siRNAs (Fig. 5B).

We also performed a more quantitative assessment of receptor distribution using flow cytometry. We determined the total and cell-surface receptors using the HEK293 cells expressing an AT1a receptor HA-tagged at the N-terminus and GFP-tagged at the C-terminus. Cells were treated with RNAi for EFR3 A/B or control knockdown prior to trypsinization, pelleting and fixation in 4% PFA. Cells were next immunostained using a mouse antibody against the extracellular HA epitope under non-permeabilizing conditions. Secondary antibody staining was performed using the PerCP-conjugated goat anti-mouse antibody and both the total (GFP) and surface (Ab signal) were analyzed by a Flow Cytometer. These studies showed no decrease in the amount of cell surface receptors in EFR3-depleted cells and a slight but statistically not significant increase in the amount of total receptors (Fig. 6AB). To examine the extent of receptor internalization, we used HEK293-AT1 cells. Cells were treated with EFR3 A/B or control siRNA duplexes. Two days after the second knockdown treatment, cells were incubated with 1μM Alexa Fluor 488-conjugated AngII for 5 min at 37 °C to allow receptor internalization. At that point the cell-surface-bound ligand was washed away with an acid wash and the cells were subjected to live-cell flow cytometry. These experiments showed no significant difference in the amount of acid-resistant signal between control and EFR3-depleted cells (Fig. 6CD).

**AT1a receptors uncouple from Gq proteins more rapidly in cells depleted in EFR3.**

These results did not indicate a major problem with AT1 receptor endocytosis. In fact, if anything, the receptor internalized more rapidly. However, hyperphosphorylation of the receptor could mean more internalization as well as a more rapid uncoupling of the receptor from its partner G protein(s). To test this latter possibility, we next examined the kinetics of Gq activation in HEK293 cells transfected with the
AT1a receptor, together with a plasmid that drives the stoichiometric expression of the three Gq subunits: an mTorquoise-tagged Gαq, a YFP-tagged Gγ2, and an untagged Gβ subunit (Goedhart et al., 2011). This reporter system is capable of monitoring Gq activation directly based on a decreasing FRET between the two FP-tagged subunits once they dissociate (or change orientation) following activation by the liganded receptor (Fig. 7A). As shown in Fig. 7B, stimulation of AT1 receptors induced a rapid activation of Gq reflected in the rapid decrease in the FRET ratios. This activation was followed by a rapid dose-dependent deactivation, which became more prominent at high AngII concentrations (> 30 nM). Depletion of EFR3B (or EFR3A and -B) caused no change in the activation kinetics, but the receptors uncoupled from the Gq protein more quickly than in control siRNA-treated cells (Fig. 7C). In cells expressing the truncated AT1 receptors, the activation of Gq was more robust and without deactivation and it was not affected by depletion of the EFR3 proteins (Fig.7D). These results were consistent with the phosphorylation data and suggested that higher phosphorylation of the receptor results in a more rapid uncoupling from the Gq proteins.

**Gₛ-coupled β-adrenergic cAMP responses are also affected by EFR3 depletion.**

To determine whether the rapid desensitization in EFR3-depleted cells similarly affects Gₛ-coupled receptors, we investigated the effects of EFR3 depletion on the cAMP responses of HEK293 cells to β-adrenergic stimulation by isoproterenol. For these measurements cells were transfected with a FRET-based cAMP sensor based on EPAC (Klarenbeek and Jalink, 2014). As shown in Fig. 7D, isoproterenol (10⁻⁵ M) evoked a rapid elevation of cAMP (reflected as a decreased FRET signal) that slowly started to decline after 5 min of stimulation. In cells treated with EFR3A/B siRNA, the amplitude of the cAMP response was similar but there was an enhanced decay in the response (Fig. 7D). This difference was statistically significant, but it was not as pronounced as those observed with the AT1 receptor.

**DISCUSSION**

These studies were designed to explore the importance of the EFR3 proteins in the regulation of signaling from PLC-activating GPCRs. Studies in yeast have clearly demonstrated that Efr3 is critical for the organization of the PI4KA orthologue, Stt4 into active signaling complexes (Baird et al., 2008). Since our previous studies suggested that PI4KA is the kinase that supplies the plasma membrane with PtdIns4P for PtdIns(4,5)P₂ synthesis (Balla et al., 2005; Bojjireddy et al., 2014), we set out to investigate whether mammalian EFR3 proteins (A and B) play any role(s) in maintaining the PtdIns4P and PtdIns(4,5)P₂ supply during strong agonist activation by affecting the function of PI4KA. An Efr3 homologue has
already been identified in *Drosophila* and its mutation was responsible for the Rolling blackout (Rbo) phenotype (Huang et al., 2004). Rbo inactivation is lethal in flies, but Rbo mutants show a rapidly diminishing light response in the retina and a phenotype somewhat reminiscent of the dynamin mutant Shibire (Vijayakrishnan et al., 2010). Rbo was believed to be an integral membrane protein with features of a lipase (Huang et al., 2004). When we expressed the human EFR3A and EFR3B proteins either tagged with a HA-epitope or GFP at the C-terminus, we found the protein exclusively PM localized. However, expression of an N-terminally truncated form was found in the cytosol that drew our attention to a cysteine-rich region characteristic of these proteins. We suspected that these proteins were palmitoylated, which we were able to confirm with \(^3\)H-palmitate labeling of the immunoprecipitated proteins. A mutant protein lacking the cysteines was found in the cytosol indicating that the protein was not an integral membrane protein. While our experiments were in progress, a similar conclusion was reached in a study from the DeCamilli laboratory (Nakatsu et al., 2012).

In order to determine whether the supply of PtdIns4\(P\) and PtdIns(4,5)\(P_2\) might be limited in cells depleted in EFR3A and B, we used HEK293-AT1 cells stimulated with AngII and measured cytoplasmic Ca\(^{2+}\) responses and the kinetics of PtdIns4\(P\) and PtdIns(4,5)\(P_2\) in *myo-*\(^3\)H*inositol-or \(^32\)P*phosphate-labeled cells during stimulation. These experiments showed a highly consistent decrease in the sustained phase of the Ca\(^{2+}\) increase after AngII stimulation in EFR3-depleted cells, but no indication of a run-down of either the PtdIns4\(P\) and PtdIns(4,5)\(P_2\) pools. We reasoned that our failure to show such lipid depletion might be related to the inability of the AT1 receptors to evoke a robust sustained PLC activation due to the known desensitization of the receptor. Therefore, we turned to cells that express a truncated AT1 receptor that shows no internalization and desensitization with the idea that stimulation of such receptors would pose a larger challenge for the PtdIns(4,5)\(P_2\) synthesizing machinery (Olivares-Reyes et al., 2001). However, contrary to our expectation, EFR3 knock down failed to affect the Ca\(^{2+}\) signal in these cells, suggesting that the effects of EFR3 depletion on the Ca\(^{2+}\) responses observed with the wild-type AT1 receptor were not caused by either a short supply of inositol lipid precursors or an alteration of Ca\(^{2+}\) handling of the cells. Instead, these results indicated that EFR3s might control the receptor itself via a process that is linked to the receptor tail lacking in the deletion mutant. Since this region contains the phosphorylation sites, next we examined the phosphorylation status of the receptor and found that EFR3 depletion caused an increased basal phosphorylation and an enhanced phosphorylation response following stimulation. In parallel experiments we also determined that AT1 receptor internalization and the subsequent trafficking were not inhibited in EFR3 depleted cells. These findings together pointed to a possible defect in the receptor’s ability to maintain Gq activation in EFR3-depleted cells caused by hyper-phosphorylation and uncoupling from the G-proteins, in other words, a more rapid desensitization. This
was studied directly by using a FRET-based Gq activation sensor (Adjobo-Hermans et al., 2011) and the results showed that EFR3-depletion, indeed, yielded a faster inactivation of the wild-type receptor but not the tail-deleted mutant.

Taken all these findings together, we assume that there is a defect in the resensitization of the receptors from their desensitized state in EFR3-depleted cells. This would also explain the rapidly diminishing light responses of the Rbo fly and their increased PtdIns(4,5)P$_2$ pool due to the photoreceptor’s inability to couple to transducin and activate PLC (Huang et al., 2004). There is limited knowledge on the processes how phosphorylated GPCRs are dephosphorylated and return to their active state. In Drosophila, a Ca$^{2+}$-activated protein phosphatase, a product of the rdgC gene was found to be responsible for the dephosphorylation of the photoreceptor (Steele et al., 1992; Vinos et al., 1997). Mammalian homologues of this protein have been identified as PFEF-1 and PFEF-2 (Montini et al., 1997; Sherman et al., 1997), but mice deficient in these proteins showed no signs of problems with GPCR desensitization or reactivation either in the retina or other tissues (Ramulu et al., 2001). In mammalian cells several phosphatases can dephosphorylate GPCRs, but it is generally agreed that PP2A dephosphorylates β2-receptors and perhaps other GPCRs as well (Vasudevan et al., 2011). A special “latent” pool of PP2A residing in internal membranes was postulated as the relevant activity that would act on β2-receptors (Yang et al., 1988) and it was suggested that β-adrenergic receptors have to be internalized and recycled to regain their coupling competence (Yu et al., 1993). However, other studies showed that GRK-mediated GPCR phosphorylations could be reversed at the PM (Iyer et al., 2006). Since EFR3s themselves do not show internalization we assume that their function is linked to the PM. It should also be noted that the higher level of receptor phosphorylation still subsided after a 20 minutes stimulation. This suggests either a delayed dephosphorylation response or the existence of more than one mechanism responsible for receptor dephosphorylation at different stages of receptor trafficking. No signs of a defect in AT1 receptor internalization or subsequent trafficking were observed in the present study. The notable decrease in the AT1aR-GFP signal at the PM relative to the cytoplasm in EFR3-depleted cells may suggest a problem with the return of the phosphorylated and internalized receptors to the PM. However, flow cytometry did not indicate a decreased receptor level on the cell surface after EFR3A/B knockdown, and we found no signs of accumulated receptor-containing vesicles beneath the PM at the light microscopy level.

Recent reports have shown that EFR3 and TTC7 proteins are responsible for the recruitment of PI4KA to the PM in yeast (Baird et al., 2008) and also in mammalian cells (Nakatsu et al., 2012), a finding we were able to confirm (Bojjireddy and Balla, unpublished observation). The question then naturally arises whether the rapid desensitization of the receptor in EFR3-depleted cells is related to a
defective function of PI4KA. We found no indication of a problem with PtdIns(4,5)P₂ pool maintenance in EFR3-depleted cells even when stimulated through a non-desensitizing AT1R. However, it is important to emphasize that these results do not argue against the role of EFR3s in controlling PI4KA function. They merely suggest that at the level of EFR3 knockdown that we achieved, PI4KA can still supply the necessary phosphoinositides. It should be also noted that it has proven to be extremely difficult to achieve PI4KA knockdowns that would limit PtdIns4P production at the PM (Balla et al., 2007; Balla et al., 2005). This, however, also suggests that the process that controls the resensitization of the AT1R is more sensitive to EFR3 depletion than the PI4KA-mediated PtdIns(4,5)P₂ pool maintenance. Furthermore, there were no signs of rapid desensitization of the AT1a receptor in our previous studies in PI4KA-depleted cells.

We also examined whether the same phenomenon is observed when the cells are stimulated via a Gₛ-coupled receptor, and found that the cAMP response to isoproterenol in HEK293 cells expressing endogenous β-receptors is also affected by EFR3A/B knockdown, although the effect was not as pronounced as those observed with the AT1a receptor. This may be caused by the fact that we did not measure directly Gₛ coupling but only cAMP levels, which may not reflect so directly the extent of desensitization. More studies will be needed to extend the generality of this phenomenon to other GPCRs and to address the exact mechanism by which receptor resensitization occurs. Nevertheless, the cAMP studies also suggest that that observed phenomenon might not be related to phosphoinositide changes as evoked by Gq-coupled receptors.

In summary, the present results identified a hitherto unrecognized and unexpected function of the EFR3 proteins in mammalian cells, namely their importance in controlling the responsiveness of GPCRs via affecting receptor phosphorylation. AT1Rs in EFR3-depleted cells are hyperphosphorylated and uncouple from Gq proteins more rapidly after stimulation but maintain their ability to internalize. These findings highlight the caveats in our knowledge of the mechanism(s) that control GPCR dephosphorylation and resensitization and should facilitate further studies to uncover the molecular details of these processes as well as the role of the EFR3 proteins in them.
MATERIALS AND METHODS

Materials: Angiotensin II (human octapeptide) was from Bachem (Torrance, CA). All other chemicals were of the highest analytical grade. \([\gamma-^{32}P]\)ATP (6000 Ci/mmol) was purchased from Perkin-Elmer. \(\text{Myo-}[^{3}H]\text{inositol}\) (30-80 Ci/mmol) was from Amersham and American Radiolabeled Chemicals (St Louis, MO). The monoclonal anti-HA Ab (HA.11) was from Covance, the anti-Flag and the EFR3A antibodies were from Sigma (St Louis, MO). Antibodies for Tubulin and Actin were from Sigma Aldrich and Cell Signaling, respectively.

Plasmids and cells: The GFP-fused \(\beta\)-arrestin-2 (Barak et al., 1997) was kindly provided by Marc Caron (Duke University). The Gq FRET construct (Goedhart et al., 2011) was a kind gift from Dr. Joachim Goedhart and Theodorus W. Gadella Jr. (University of Amsterdam). The HEK293-AT\(_{1}\) cells stably express the rat AT\(_{1a}\) angiotensin receptor (Balla et al., 2005). HEK293-AT1-GFP cells stably express the rat AT\(_{1a}\) angiotensin receptor fused to GFP (Hunyady et al., 2002), and the HEK-AT1Δ cell line stably expresses the rat AT\(_{1a}\) angiotensin receptor with a stop at Y319 (Hunyady et al., 1994). EST clones for EFR3A (EHS1001-213247615) and EFR3B (MHS6278-202802246) (IHS1382-8680872, containing the N-terminal EFR3B piece missing from clone MHS6278-202802246) were from Open Biosystems. The coding sequence of EFR3A, EFR3B short form and long form were subcloned into the pCDNA3.1(+) vector with an HA-tag at C-termini. For localization studies EFR3A and 3B were also subcloned into pEGFP-N1 and mRFP-N1 plasmids. Palmitoylation sites EFR3A-GFP (amino acids 5-CCCC-11) and EFR3B-GFP (amino acids 4-CGCC-9) were mutated to EFR3A (5-SSSS-11) and EFR3B (4-SGSS-9) by site directed mutagenesis using the Quikchange mutagenesis kit from Promega.

Transfection of cells: Cells (50,000 cells/ well) were plated onto 25-mm-diameter circular glass cover slips treated with Poly-lysine in 6 well plates and plasmid DNAs (0.5-1 \(\mu\)g/well) were transfected with the indicated constructs (Szentpetery et al., 2009) using the Lipofectamine2000 reagent (Invitrogen) and OPTI-MEM (Invitrogen) following the manufacturer’s instructions. For siRNA treatment, cells were either cultured on cover slips in 6 well dishes for imaging or 12 well dishes for metabolic labeling. Cells were treated with 100 nM siRNA using oligofectamine. Experiments were carried out with the knockdown cells after 3 days post siRNA treatment.

Live-cell imaging: After 20-24 hrs of transfection, cells were washed on the glass cover slips with a modified Krebs-Ringer solution, containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl\(_{2}\), 0.7 mM MgSO\(_{4}\),
10 mM glucose, 10 mM Na-Hepes, pH 7.4 and the coverslip was placed into a metal chamber (Atto, Invitrogen) that was mounted on a heated stage (35°C). Cells were incubated in 1 ml of the Krebs-Ringer buffer and the stimuli were dissolved and added in 200 μl warm buffer removed from the cells. Cells were examined in inverted microscopes. Confocal images were obtained with a Zeiss LSM 510-META laser confocal microscope (Carl Zeiss MicroImaging, Inc.) using a 63x oil-immersion objective equipped with an objective heater (Bioptech).

**Cytoplasmic Ca^{2+} measurements:** HEK-AT1 cells (4x10^5 cells) cultured on cover slips were treated with siRNA as described above. Cells were loaded with 3 μM Fura 2/AM at room temperature for 45 min in Hepes-buffered M199-Hank’s salt solution containing 200 μM sulfinpyrazone and 0.06% pluronic acid. Calcium measurements were performed in the modified Krebs-Ringer solution (see above) containing 200 μM sulfinpyrazone. Single cell calcium measurements were carried out at room temperature using an Olympus IX70 inverted microscope equipped with Lamda-DG4 illuminator and a MicroMAX-1024BFT digital camera with appropriate filters. Data was acquired using MetaFluor software (Molecular Devices).

**Analysis of myo-[^3H]Inositol- or[^32P]Phosphate-labeled lipids:** HEK293-AT1 cells plated on 12-well plates (at a density of 30,000 cells/ml) were labeled with myo[^3H]inositol (20 μCi/ml) in 1 ml of inositol-free DMEM supplemented with 2% dialyzed FBS for 24 h or with 2 μCi/ml o-[^32P]phosphate for 3 h in phosphate-free DMEM supplemented with 2% dialyzed FBS. Cells were stimulated with angiotensin II (100 nM) for the indicated times or left unstimulated. Reactions were terminated by the addition of ice-cold perchloric acid (5% final concentration), and cells were kept on ice for 30 min. After scraping and freezing/thawing, the cells were centrifuged and the cell pellet was processed to extract the phosphoinositides by an acidic chloroform/methanol extraction followed by thin layer chromatography (TLC) essentially as described previously (Balla et al., 2007; Nakanishi et al., 1995). TLC plates were sprayed with Enhance solution (Perkin-Elmer) and were subjected to autoradiography (in the case of ^3H-labeling) or were analyzed by a PhosphorImager (for the ^32P-labeled samples). Autographic films were exposed several times to find the proper exposure for densitometric analysis.

**Analysis of incorporation of[^3H]-palmitate:** The procedure described by Linder et al. has been followed (Linder et al., 1995). Briefly, COS-7 cells were transiently transfected with pGFP-N1-EFR3A or pEGFP-N1-EFR3B. After 24 hrs, cells were metabolically labeled with[^3H]-palmitate (0.3 mCi/ml) for 4 hrs. Cells were washed once with ice-cold PBS pH 7.4, prior to lysis. Lysates were immunoprecipitated
with GFP-trap (ChromoTek GmbH, Germany) overnight and washed three times with lysis buffer. Proteins were resolved on SDS-PAGE gel and the gel was incubated with Enhance solution for 2 hrs, dried and subjected to autoradiography. pEGFP-N1-PI4KIIα was used as a positive control in this experiment.

**Analysis of receptor phosphorylation:** HEK293-AT1 cells (ctrl siRNA or EFR3AB siRNA treated) in 6 well plates were metabolically labeled with 100 μCi/mL ortho-32PO4 for 4 hrs at 37 °C in phosphate-free DMEM containing 2% dialyzed FBS. Cells were stimulated with 100 nM AngII for various time points and cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, 1 mM DTT, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin). Cell lysates were incubated on ice for 15 min and centrifuged at 16,000 x g. Supernatants were further incubated with a mixture of anti-HA and anti-Flag antibodies and Protein G agarose beads overnight at 4°C. Immunoprecipitates were further washed three times with lysis buffer and incubated in sample buffer for 1 h at 48°C followed by SDS-PAGE. The SDS-PAGE gel was dried and subjected to analysis by a PhosphorImager.

**FRET analysis of Gq activation and cAMP changes:** To explore the kinetics of the Gαq or cAMP FRET reporter, the appropriate HEK293 cells were cultured on glass coverslips and transfected with the pGβ-2A-YFP-Gγ2-IRESGαq-mTq construct (a kind gift from Drs Joachim Goedhart and Theodorus Gadella, University of Amsterdam (Goedhart et al., 2011)) or the EPAC-based cAMP FRET sensor (Klarenbeek and Jalink, 2014) (kindly provided by Dr. Kees Jalink, The Netherlands Cancer Institute, Amsterdam) for 24 h. Coverslips were placed into a metal Attofluor cell chamber (Invitrogen) and the cells were incubated in 1 ml modified Krebs-Ringer buffer (see above) at room temperature. Cells were stimulated with Angiotensin II (0.1-100 nM) (for Gq activation) or isoproterenol (10^{-5} M) (for cAMP) (stimuli, dissolved in 200 µl buffer removed from the cells, were added with proper mixing). FRET measurements were performed on an Olympus IX70 inverted microscope equipped with a Lambda-DG4 illuminator (Sutter Instruments, Novato, CA) and a MicroMAX-1024BFT digital camera (Roper Scientific, Trenton, NJ). A beam-splitter (DualView, Photometrics) with the appropriate filter sets was used to obtain a time-lapse of CFP and YFP images. Data acquisition and processing was performed by the MetaFluor software (Molecular Devices), where YFP/CFP ratios were formed after background-subtraction and normalized to the initial pre-stimulatory ratio values. For the EFR3A and EFR3B siRNA
experiments, HEK293-AT1 cells were treated with the respective siRNAs (100 nM) for 3 days before transfection with the pGβ-2A-YFP-Gγ2-IRES-GαqmTq or the EPAC-FRET construct.

**Flow cytometry:** Measurement of AngII internalization rates by flow cytometry was performed on HEK293-AT1 cells at sub-confluent density previously treated with EFR3 A/B or control siRNA duplexes. Two days following the second knockdown treatment cells were incubated with 1μM Alexa Fluor 488-conjugated AngII for 5 min, washed with the acid wash solution (150 mM NaCl and 50 mM acetic acid, pH 4.0) in order to remove the surface-bound ligand, and assayed by live-cell flow cytometry.

Analysis of total and surface AT1aR levels was performed in HEK19 cells stably expressing HA-AT1aR-GFP that were treated for EFR3 A/B or control knockdown prior to trypsinization, pelleting and fixation in 4% PFA. Cells were next immunostained with a mouse antibody against the extracellular HA epitope under the non-permeabilizing conditions. Secondary antibody staining was performed using the PerCP-conjugated goat anti-mouse secondary antibody. Data acquisition was performed on Becton Dickinson FACScan cytometer (Franklin Lakes, NJ) using CELLQuest software, processing 10,000 cells per sample.

**Quantitative PCR:** Total RNA from various mouse tissues (replicate tissues from 3 mice) were isolated using RNeasy Kit (Qiagen), and cDNA was synthesized using Omniscript reverse transcriptase and random hexamers according manufacturers instructions (Qiagen). Real Time PCR was performed in duplicates using SYBER green (Bio-rad). Primers used for real time PCR were EFR3A-F: 5’-CAGCTGACAAGAAGAGAACA-3’, EFR3B-R: 5’-ACTGAGCCTGGATAGAATAC-3’ and EFR3B-F: 5’-GAGCATGAGGAGTGCATGTTCCAG-3’, EFR3B-R: 5’-GAAACCTGTGGATACCTGAAGGAGGGA-3’. The Real Time PCR results for the mRNA levels of each gene were normalized to 18S rRNA levels.
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REFERENCES


LEGENDS TO FIGURES

Figure 1. Relative expression of EFR3A and EFR3B mRNAs in various mouse tissues. Total RNA from various mouse tissues was isolated and cDNA synthesized as described in the Materials and Methods. Real Time PCR was performed using SYBER green using primers specific for the respective mRNAs (see Methods). The signal was then normalized to that of 18S ribosomal RNA. Means ± SEM (n=6) are shown from duplicate determinations from 3 independent mouse and tissue isolations. See the predominant expression of EFR3B in the brain and that of EFR3A in the testis.

Figure 2. Cellular localization and palmitoylation of the EFR3 proteins. (A) EFR3A and –B proteins were tagged with GFP at their C-termini and expressed either in COS-7 cells or HEK293-AT1 cells. Cells were imaged live by confocal microscopy. Both proteins were found to primarily localize to the plasma membrane (PM) and this localization requires the N-terminus of the proteins containing several cysteine residues. (B) Mutation of the four cysteine residues to serines eliminates the PM localization of the full-length EFR3A (and EFR3B, not shown) proteins. The short N-terminal fragment of EFR3A, however, shows Golgi localization suggesting that other determinants are also important for the PM localization of the protein. Without the cysteines, this short construct also loses its Golgi localization. (C) Palmitoylation of the EFR3 proteins: GFP-tagged EFR3A or EFR3B were expressed in COS-7 cells (PI4K2A-GFP was used in a separate dish as control) and the cells were labeled with [3H]-palmitate for 4 hrs. Cells were lysed and the proteins immunoprecipitated using GFP-trap beads. Immunoprecipitated proteins were resolved on SDS-PAGE gel and the gel was incubated with Enhance solution before drying and autoradiography (left). Parallel samples were run for western blotting using an anti-GFP antibody (right).

Figure 3. Depletion of EFR3s results in an impaired cytoplasmic Ca\(^{2+}\) response without a notable depletion of PtdIns(4,5)P\(_2\) levels. (A) The efficiency of EFR3A knockdown using two different siRNA duplexes. Here the levels of the endogenous protein were measured. (B) EFR3B knockdown efficiency was tested using an expressed GFP-tagged protein because of the lack of suitable antibody to determine
endogenous levels. (C) HEK293-AT1 cells cultured on 25 mm coverslips were treated with the indicated siRNAs for 3 days. Cells were loaded with Fura-2/AM and their Ca^{2+} responses to 100 nM (left) or 1 nM (right) AngII monitored in ratiometric imaging as described in Materials and Methods. The difference between the Ca^{2+} responses to 100 nM AngII were highly significant (for analyzing all time points > 60 sec, p<0.0001) using either unpaired t-test or the non-parametric Mann-Whitney test. (D) HEK293-AT1 cells treated with EFR3A and EFR3B siRNAs or with a control siRNA were labeled with [32P]phosphate for 3 h before stimulation with angiotensin II (100 nM) for the indicated times. Reactions were terminated and lipids extracted and separated by TLC as detailed in Materials and Methods. Radioactivity was quantified by Phosphorimaging and expressed as percent of the prestimulatory value. Means ± SEM from 3 independent experiments are shown performed in duplicates.

Figure 4. Depletion of EFR3s failed to affect the cytoplasmic Ca^{2+} response of cells expressing a C-terminally truncated AT1a receptor and augmented phosphorylation of the wild-type AT1a receptor. (A) HEK293 cells stably transfected with an AT1a receptor truncated at its C-terminus (to remove all phosphorylation sites) were treated with EFR3A and EFR3B siRNAs or with a control siRNA before measurements of cytoplasmic Ca^{2+} responses to AngII as described in the legend to Figure 3 (B) Phosphorylation of the wild-type AT1a receptor immunoprecipitated from HEK293-AT1 cells labeled with [32P]phosphate and stimulated with AngII for the indicated times. Note the already increased phosphorylation of the receptor before and the larger response after AngII stimulation. Representative phosphorimage is shown from one of three similar observations. (C) Quantification and summary from three independent experiments. All radioactivity values in each experiment were expressed as a percent of the value measured at the 5 minutes time-point in the EFR3AB-depleted cells. Means ± S.E.M (n=3).

Figure 5. Internalization of the AT1a receptor is not inhibited by EFR3B depletion. (A) HEK293-AT1 cells treated with the indicated siRNAs were transfected with GFP-β-arrestin2 for 24 h and studied live with confocal microscopy at 30 °C. Cells were treated with 100 nM AngII and images were acquired at the times indicated. (B) HEK293 cells stably expressing the AT1a receptor fused to GFP were treated with the indicated siRNAs and studied live with confocal microscopy at 30 °C. Cells were stimulated with 100 nM AngII and images were recorded at various time points. Note the higher signal in the plasma membrane of control cells at 0 min compared to the cells treated with EFR3B siRNA. These results were reproduced two times in multiple dishes from independent knockdown experiments.
Figure 6. Effects of EFR3 depletion on AT1aR levels and AngII uptake. (A,B) HEK19 cells stably expressing HA-AT1aR-GFP were treated with the control- or EFR3 A/B targeting siRNA oligos. Cells were then fixed and immunostained against the HA epitope under non-permeabilizing conditions and analyzed by flow cytometry, recording the GFP signal of the total AT1aR population (left panel) and the cell-surface HA epitope signal (right panel). Control knockdown levels (black) were compared to those in EFR3 A/B knockdown cells (gray) (n=10,000 cells). Shown are grand averages ± range from two experiments. (C,D) HEK293-AT1 cells treated with control siRNA or siRNA directed against EFR3 A/B, were incubated with Alexa Fluor 488-conjugated AngII for 5 min prior to acid wash and detection of internalized AngII by flow cytometry. EFR3 A/B depletion (right panels) did not alter the rate of AngII internalization when compared to the control knockdown (left panels) (n=10,000 cells).

Figure 7. Effect of EFR3B knockdown on Gq activation kinetics in HEK293 cells expressing the wild-type or tail-deleted AT1a receptor. (A) Schematic cartoon of the principles of monitoring Gq activation using Förster’s resonance energy transfer (FRET). Upon excitation, the mTq fluorescent protein engineered into the Gq α-subunit will transfer its energy to Venus, which is attached to the γ-subunit of the βγ complex in the tightly associated heterotrimer. After AT1a receptor activation, the dissociation of the heterotrimer will result in a decrease in direct energy transfer, which can be detected as an increased emission from mTq and a decreased emission from Venus (see Goedhart et al., 2011 for details). (B) FRET measurements in HEK293-AT1 cells expressing the wild-type AT1a receptors and stimulated with increasing concentrations of AngII. (C) HEK293 cells expressing the wild-type AT1a receptors and treated for 3 days either with control siRNA (grey trace) or with EFR3B siRNA (red trace). Means ± S.E.M. are shown for 250 and 216 cells in control and EFR3B-depleted cells, respectively, acquired in three independent knockdown experiments (note that the error bars are too small to be visible in the red trace). (D) Same FRET experiments described in (B) except for using HEK293 cells expressing the truncated AT1a receptors. Means ± S.E.M. are shown for 53 and 91 cells in control and EFR3B-depleted cells, respectively, acquired in two independent knockdown experiments. (E) cAMP changes in HEK293-AT1 cells stimulated by isoproterenol. Cells were treated with siRNA against EFR3A/B or control and transfected with a FRET-based cAMP sensor (Klarenbeek and Jalink, 2014) to monitor changes in cAMP levels. A decreased FRET means and increase in cAMP. Means ± S.E.M from 50-52 cells from 6 independent dishes obtained in two different knockdown experiments. The difference between the two curves for time points larger than 240 sec is statistically significant (p < 0.0001) using either non-paired t-test or the non-parametric Mann-Whitney test.
Figure 5

A

GFP-β-arrestin2 0 min 1 min 5 min 10 min

EFR3B/TTC7B siRNA

0 min 1 min 5 min 10 min

B

Control siRNA

AT1aR-GFP

0 min 7.5 min

EFR3B/TTC7B siRNA

0 min 7.5 min
Figure 6
Figure 7

A

AT1aR

Gqα

Gβγ

mTq

Venus

AngII

AT1aR

Gqα

Gβγ

mTq

Venus

B

AngII

FRET ratio (F525/F475)

Time (sec)

1 nM

3 nM

10 nM

30 nM

100 nM

1 μM

C

AngII 100 nM

EFR3B-si

Control-si

D

AngII 100 nM

EFR3B-si

Control-si

E

Isoproterenol

cAMP-sensor FRET (% of initial)

Time (sec)