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G protein-coupled receptor kinase 4 γ interacts with inactive $G\alpha_s$ and $G\alpha_{13}$

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Abstract

G protein-coupled receptors (GPCRs) are regulated by multiple families of kinases including GPCR kinases (GRKs). GRK4 is constitutively active towards GPCRs, and polymorphisms of GRK4 γ are linked to hypertension. We examined, through co-immunoprecipitation, the interactions between GRK4 γ and the $G\alpha$ and $G\beta$ subunits of heterotrimeric G proteins. Because GRK4 has been shown to inhibit $G\alpha_s$ -coupled GPCR signaling and lacks a PH domain, we hypothesized that GRK4 γ would interact with active $G\alpha_s$, but not $G\beta$. Surprisingly, GRK4 γ preferentially interacts with inactive $G\alpha_s$ and $G\beta$ to a greater extent than active $G\alpha_s$. GRK4 γ also interacts with inactive $G\alpha_{13}$ and $G\beta$. Functional studies demonstrate that wild-type GRK4 γ , but not kinase-dead GRK4 γ , ablates isoproterenol-mediated cAMP production indicating that the kinase domain is responsible for GPCR regulation. This evidence suggests that binding to inactive $G\alpha_s$ and $G\beta$ may explain the constitutive activity of GRK4 γ towards $G\alpha_s$ coupled receptors.

G protein-coupled receptor kinases (GRKs) comprise a family of seven proteins that possess an amino terminal regulator of G protein signaling homology (RH) domain and a serine/threonine kinase domain similar to protein kinase C [1]. GRKs initiate homologous desensitization through phosphorylation of the third cytoplasmic loop or carboxy terminus of ligand bound/activated target receptors [1]. This desensitization of the activated receptor is crucial in regulating signal transduction as well as ligand-mediated receptor endocytosis. The GRK1-like family, GRK1 and GRK7, are found in rod and cone cells and are associated with opsins [2]; the GRK2-like family, GRK2 and GRK3 (also known as β -adrenergic receptor kinase 1 and 2, respectively), are ubiquitously expressed and associate with $G\alpha_{q/11}$ -coupled receptors [1]; and finally, the GRK4-like family comprises GRK4, GRK5, and GRK6, which compared to the other GRKs little is known about their biology [3]. GRK5 and GRK6 are expressed in multiple tissues [1], whereas GRK4 is expressed in the testis [4], kidney tubular cells [5], brain [6], and myometrium [7]. In this study we focused on GRK4.

GRK4 was first identified during positional cloning of the Huntington's disease locus [8]. Early *in vitro* studies demonstrated that GRK4 phosphorylates the β_2 -adrenergic receptor (β_2 AR) in a PIP₂-dependent manner [4]. Additionally, both GRK4 and GRK2, significantly decrease luteinizing hormone/chorionic gonadotropin receptor-mediated cAMP production [4]. These

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data suggest that GRK4 is similar to GRK2 in its action; however, further studies demonstrate that in a comparable system GRK4, but not GRK2, phosphorylates the β_2 AR in an agonist-independent manner resulting in increased recycling of the β_2 AR [9]. Additionally, GRK4, and particularly GRK4 γ , is the only GRK that appears to be constitutively active [9]. In support of this, recent studies demonstrate that GRK4 α phosphorylates the D1 dopamine receptor (D1R) independently of D1R agonists [10].

There are four published splice variants of GRK4 [4]. GRK4 α is the longest transcript with 16 exons encoding a protein of 578 amino acids. GRK4 β is missing exon 2 and produces a protein of 546 amino acids; the 32 missing amino acids occur after Gln¹⁷ of GRK4 α , which, based upon the GRK6 crystal structure [11], correspond to the first two alpha helices $\alpha 0$ and $\alpha 1$ of the RH domain. GRK4 γ , the first GRK4 reported [8], lacks exon 15 and is 532 amino acids in length; the 46 missing amino acids occur after Glu⁵¹⁵ and correspond to less than half of alpha helix $\alpha 10$ and all of $\alpha 11$ of the RH domain as well as a portion of the unstructured region following the RH domain [11]. GRK4 δ , a 500 amino acid protein, is missing both exons 2 and 15. None of the alternative splicing events eliminate GRK4 activity [4]; however, most studies of GRK4 are conducted with GRK4 α . Polymorphisms in GRK4 (numbering based on GRK4 α : R65L, A142V, and A486V) are associated with hypertension [5;12;13]. Two of the polymorphisms, R65L and A142V, lie within the RH domain and transgenic mice carrying the polymorphisms of GRK4 γ are hypertensive compared to mice carrying human wild-type GRK4 γ [5] (personal communication with P.A. Jose). The mechanism of action of GRK4 γ , with regard to hypertension, is proposed to be hyper-phosphorylation/desensitization of the dopamine D1R [5]. This desensitization prevents excretion of salt and relieves the renal dopaminergic-mediated inhibition of the renin-angiotensin system [14].

We examined which of the G α subunits interact with GRK4 γ in an effort to understand the biochemistry of GRK4 γ and eventually examine the polymorphisms in the RH domain associated with hypertension. We hypothesized that GRK4 γ would interact with active/GTP-bound G α_s but not inactive/GDP-bound G α_s , and also possibly interact with other G proteins in a GTP-dependent manner. Counter-intuitively, the data indicate that GRK4 γ interacts preferentially with inactive/GDP-bound G α_s .

Materials and Methods

Materials

All agonists and drugs were obtained from Sigma-Aldrich (St. Louis, MO). The AU1 antibody was obtained from Covance (Berkley, CA). All other antibodies and materials for immunoprecipitation were obtained from Santa Cruz (Santa Cruz, CA). The following lysis buffers were used: TX-100 based buffer (1% Triton X-100, 100mM NaCl, 20mM Tris pH 7.5, 2mM EDTA, 10mM MgCl₂, 10mM NaF, 40mM β -glycerol phosphate, and 1mM PMSF), RIPA buffer (TX-100 based buffer plus 0.1% SDS and 0.5% deoxycholate), and detergent-free buffer (TX-100 buffer without Triton X-100) with cells passed through a 27 gauge needle 10 times. The cAMP EIA Kit was obtained from Cayman Chemical (Ann Arbor, MI) and used per the manufactures specifications.

cDNA Constructs

GRK2 cDNA was purchased from ATCC (Manassas, VA). Human wild-type GRK4 γ and GRK2 were subcloned into the expression vector pcDNA4/TO (Invitrogen). Kinase dead (K216M, K217M) GRK4 γ [15] was created with the Stratagene (La Jolla, CA) quick change mutagenesis kit. The wild-type and constitutively active QL mutant G proteins were gifts of Dr. J.S. Gutkind. Wild-type G proteins were subcloned into pEF-AU1, a derivative of pCEFL, obtained from Dr. J.S. Gutkind.

Cell Culture and Transfection

HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS and 100 units/ml of penicillin/streptomycin (Invitrogen, Carlsbad, CA) in 60mm culture dishes coated with poly-D-Lysine (Sigma). HEK293T cells were transiently transfected using Qiagen's PolyFect Transfection Reagent (Germantown, MD). Functional assays were conducted using immortalized renal proximal tubule cells (RPTCs) isolated from Wistar-Kyoto (WKY) rats (a gift from Dr. U. Hopfer). PTCs were cultured in DMEM/F12 containing 5% (v/v) FBS, 10 ng/ml epithelial growth factor (EGF), 4 μ g/ml dexamethasone, ITS+ diluted at 4 ml per bottle of media, and 100 units/ml of penicillin/streptomycin. EGF and ITS+ premix were obtained from BD Biosciences (Bedford, MA). PTCs were grown in 60mm culture dishes for the cAMP assay and transfected utilizing the FuGene 6 protocol (Roche, Indianapolis, IN).

Preparation of Cellular Extracts and Co-IP

Transfected HEK293T cells were serum starved overnight (at least 8 hours) prior to all experiments. For experiments with the AU1-tagged G proteins, all samples and buffers were supplemented with 10 μ M GDP. To activate G α proteins, samples were treated with 100 μ M AlCl₃ and 10mM NaF for 15 minutes at 37°C to generate AlF₄⁻. All buffers used with the AlF₄⁻ treated samples contained 100 μ M AlCl₃ and 10mM NaF. For experiments with the wild-type and QL mutant G α subunits, GDP and AlF₄⁻ were not used. Medium was removed and cells were lysed in one of the three lysis buffers, and clarified by centrifugation for 5 minutes at 4°C. An aliquot of the supernatant was reserved for analysis of the total cell lysate and the remaining sample was used for co-immunoprecipitation with an antibody directed against the AU1 tag, G α subunit, or G β common for one hour at 4°C. Protein A/G PLUS-Agarose beads (Santa Cruz) were added for 30 additional minutes. Immune complexes were washed with PBS containing 1% NP-40 and 1mM PMSF, followed by SDS-PAGE on a 9% gel and western blot analysis.

Results and Discussion

Examination of common G α subunits

Before conducting any co-immunoprecipitation (co-IP) studies, the levels of endogenous and transfected GRK4 γ were examined in 293T cells. No endogenous GRK4 γ was detected by western blot in 293T cells. Initial co-IP studies were conducted with a panel of the commonly used heterotrimeric G protein alpha subunits: s, i1-3, q, 11, 12, and 13 (Fig. 1). The G α subunits were subcloned into pEF-AU1 in order to conduct the analyses in parallel using a single antibody for detection. Each G α subunit was examined in the active, AlF₄⁻ treated, conformation (Fig. 1a and 1b) and inactive conformation (Fig. 1c and 1d). After stimulation with AlF₄⁻, GRK4 γ co-immunoprecipitates with AU1-G α_s and to a lesser extent AU1-G α_{13} (Fig. 1a). Surprisingly, when the experiment is conducted without AlF₄⁻, representing the inactive G α subunits, GRK4 γ also co-immunoprecipitates with AU1-G α_s and to a lesser extent AU1-G α_{13} (Fig. 1c). These results were opposite to our initial hypothesis.

To confirm that the conditions represent active and inactive G α subunits, we conducted identical studies with GRK2 (Fig. 1b and d). As shown in Figure 1b GRK2 interacts with AlF₄⁻ treated (active) AU1-G α_q . Moreover, in samples lacking AlF₄⁻ (inactive) GRK2 is not co-immunoprecipitated with any of the AU1-G α subunits (Fig 1d). These data partially fit what is known about GRK2 and G protein interactions[16; 17]; however, GRK2 should also interact with G α_{11} . Most likely this interaction is not observed due to low expression of AU1-G α_{11} . The expression levels of the various G proteins are significantly different, and G α_q and G α_{11} are expressed the least (Figure 1). Therefore, we utilized wild-type G α_q and G α_{11} and constitutively active Q209L G α_q and Q209L G α_{11} in co-IP experiments with GRK2. As

previously reported [16; 17], GRK2 interacted with the active form of $G\alpha_q$ and $G\alpha_{11}$ (data not shown). Therefore, IP is an accurate measure of GRK4 γ interactions with active and inactive G proteins.

Interaction of active and inactive $G\alpha_s$ with GRK4 γ

Due to the experimental setup of the $G\alpha$ subunit panel, direct comparison of the inactive and AlF_4^- activated blots is not appropriate. To directly compare each $G\alpha$ subunit in the active and inactive conformation, two series of experiments were conducted. First, experiments similar to those shown in figure 1 were conducted; each $G\alpha$ subunit in the active and inactive conformation was run on the same gel for direct comparison of their interaction with GRK4 γ . As shown in figure 2a, GRK4 γ co-immunoprecipitates with inactive AU1- $G\alpha_s$ to a greater extent than with AlF_4^- activated AU1- $G\alpha_s$. Second, in order to avoid artifacts that may arise from using tagged constructs and AlF_4^- , untagged wild-type and constitutively active Q213L $G\alpha_s$ subunits were expressed to obtain the inactive and active state of the G protein, respectively. GRK4 γ robustly co-immunoprecipitated with inactive $G\alpha_s$, but little interaction was found with Q213L $G\alpha_s$ (Fig. 2b). These data confirm the AU1-tagged/ AlF_4^- treatment results. Thus indicating that the observed interaction between GRK4 γ and inactive $G\alpha_s$ is not an artifact of the tagged $G\alpha_s$, AlF_4^- , or the antibodies used. However, in all experiments presented thus far, the co-IP was conducted in Triton X-100 based lysis buffer. To determine if the interaction of inactive $G\alpha_s$ with GRK4 γ is an artifact of the lysis buffer, we repeated the studies shown previously with three different lysis buffers (Fig. 2c). GRK4 γ interacts with wild-type $G\alpha_s$ to a much greater extent than Q213L $G\alpha_s$ in all detergents examined, including detergent-free lysis. Overexposure of the IP blot indicates that native $G\alpha_s$ will also pull down the overexpressed GRK4 γ . Similar experiments were conducted with AU1- $G\alpha_s$ with and without AlF_4^- , and showed GRK4 γ interaction with inactive $G\alpha_s$ (data not shown). Therefore, the data are not an artifact of the lysis buffer. GRK4 γ preferentially interacts with inactive $G\alpha_s$.

Interaction of an RH-domain containing protein with an inactive $G\alpha$ subunit has never been described. As stated, these results run counter to our hypothesis. Given that this is an overexpression system, and 293T cells are known to generate copious amounts of protein, we investigated the interaction of GRK4 γ , $G\alpha_s$, and $G\beta$ subunits. $G\beta$ subunits are bound to inactive $G\alpha$ subunits. If GRK4 γ displaces $G\beta$ from $G\alpha_s$, then it is likely that the binding is an artifact of overexpression. Using endogenous $G\beta$, overexpressed GRK4 γ , and overexpressed wild-type $G\alpha_s$, we conducted co-IP studies with antibodies to $G\alpha_{s/olf}$ (Fig. 2d) and $G\beta$ common (Fig. 2e). In both cases GRK4 γ was pulled down only in the presence of overexpressed $G\alpha_s$. $G\beta$ was pulled down with the antibody to $G\alpha_{s/olf}$ (Fig. 2d). Similarly, $G\alpha_s$ was pulled down with the antibody to $G\beta$ common, and GRK4 γ appears to increase the pull down of $G\alpha_s$ (Fig. 2e). Because there is no apparent displacement of $G\beta$ by GRK4 γ , the interaction is most likely specific. The $G\beta$ data are difficult to interpret because the antibody is interacting with four of the five $G\beta$ variants, which will pull down all the $G\alpha$ subunits. Alternatively, it is unknown if $G\beta$ binds to GRK4 γ . $G\beta$ binds to the plextrin homology (PH) domain of GRK2 [17], however the GRK4 family members do not contain a PH domain [11]. Although it is unlikely that $G\beta$ directly binds to GRK4 γ , the experiments conducted here cannot discriminate between direct binding and indirect binding through $G\alpha_s$ or $G\alpha_{13}$. However, it is clear that GRK4 γ , $G\alpha_s$, and $G\beta$ are bound together in one complex.

Interaction of active and inactive $G\alpha_{13}$ with GRK4 γ

Experiments similar to those presented in figures 2a and 2b were conducted with all $G\alpha$ subunits examined in figure 1. Initially weak interactions between GRK4 γ and many of the $G\alpha$ subunits were observed, but only the AU1- $G\alpha_{13}$ interaction with GRK4 γ proved to be specific when using the wild-type and QL mutants. This supports previous findings that GRK4 does not

interact with $G\alpha_q$ or $G\alpha_{11}$ [18]. In accordance with figure 1, GRK4 γ is co-immunoprecipitated with inactive AU1- $G\alpha_{13}$ and to a lesser extent when treated with AlF_4^- (Fig. 3a). Repetition with wild-type and constitutively active Q226L $G\alpha_{13}$ indicated that inactive $G\alpha_{13}$ binds to GRK4 γ significantly more than active $G\alpha_{13}$ (Fig. 3b). To confirm these results, the wild-type and Q226L $G\alpha_{13}$ co-IP was repeated under detergent-free conditions. As shown in figure 3c, inactive $G\alpha_{13}$ co-immunoprecipitates with GRK4 γ , whereas constitutively active $G\alpha_{13}$ does not interact with GRK4 γ . Furthermore, GRK4 γ is co-immunoprecipitated with $G\beta$ only in the presence of overexpressed $G\alpha_{13}$ (Fig. 3d) similar to what is seen with $G\alpha_s$ (Fig. 2e). However, $G\beta$ pulled down an equal amount of $G\alpha_{13}$ in all conditions suggesting that the system was saturated. As noted previously, the binding order cannot be determined in such an assay; however, the data indicate that GRK4 γ , $G\alpha_{13}$, and $G\beta$ are in a complex. However, the interaction of GRK4 γ with $G\alpha_{13}$ is weaker than that of GRK4 γ with $G\alpha_s$ (Fig. 1a and 1c).

Function of GRK4 γ

Recent structural studies indicate that the interaction of $G\alpha_q$ with the RH domain of GRK2 occurs in a manner distinct from $G\alpha_q$ /RGS interactions in that the interaction is more like an interaction with an effector not a RGS [17]. GRK2 does not increase the intrinsic catalytic rate of $G\alpha_q$, which is proposed to be a general method of action of GRKs. The RH domain of GRK6, a member of the GRK4 subfamily, structurally resembles GRK2 suggesting that the mechanisms of action and areas of $G\alpha$ subunit binding are similar [11]. However, the area of GRK2 that interacts with $G\alpha_q$, $\alpha 5$ - $\alpha 6$ helices of the bundle sub-domain of the RH domain, is highly diverged in GRK6 (3.1% identity and 34.4% similarity). Furthermore, the predicted $\alpha 5$ - $\alpha 6$ helices of GRK4 has a 15.5% identity and 28.1% similarity with GRK2, and GRK4 and GRK6 only share 25% identity and 59.4% similarity over the same sequence. Although the $\alpha 5$ - $\alpha 6$ region of GRK4 and GRK6 may not be the region that interacts with $G\alpha$ subunits, the absence of significant similarity suggests that the interaction of the $G\alpha$ subunit with its respective GRK does not necessarily have one unified mechanism.

In order to examine the physiological consequence of the interactions with inactive $G\alpha$ subunits, functional assays were conducted to test the effects of GRK4 γ and kinase dead K216M, K217M GRK4 γ (KD GRK4 γ) on $G\alpha_s$ - and $G\alpha_{13}$ -mediated cellular signaling. We generated KD GRK4 γ as previously reported [15] (Fig. 4a). As a control, we examined the interaction of KD GRK4 γ with wild-type $G\alpha_s$ (Fig. 4b). KD GRK4 γ interacts identically to wild-type GRK4 γ (Fig. 2d). Similar studies were conducted with Q213L $G\alpha_s$, wild-type $G\alpha_{13}$, and Q226L $G\alpha_{13}$ that demonstrated KD GRK4 γ interacts with the inactive $G\alpha$ subunits (data not shown). Thus, the dual lysine to methionine mutation can be used to discriminate between kinase activity driven effects of GRK4 γ and interactions with $G\alpha$ subunit driven events.

To examine $G\alpha_s$ -mediated signaling, we utilized WKY RPTCs; one of the few cells to express endogenous GRK4 [4–7]. WKY RPTCs were transfected with empty vector, GRK4 γ , KD GRK4 γ , or GRK2 and then stimulated with vehicle, or 30 nM or 10 μ M Isoproterenol for 30 min in the presence of 500 μ M IBMX, a phosphodiesterase inhibitor. Intracellular cAMP measurements indicated that 30 nM Isoproterenol is saturating, thus, only this data is shown (Fig. 4c). All data were normalized to the control empty vector transfection. GRK2 served as a positive control. No effects were observed in unstimulated RPTCs. Stimulation doubled cAMP levels in KD GRK4 γ and vector transfected RPTCs, but had no significant effect in GRK4 γ and GRK2 transfected RPTCs. An independently conducted experiment utilizing 1 μ M fenoldopam, a partial D1-like receptor agonist [19], and Isoproterenol confirmed GRK4 γ 's inhibitory action, and revealed that KD GRK4 γ can enhance fenoldopam-mediated cAMP accumulation (data not shown). Fenoldopam had a marginal effect on the RPTCs except when KD GRK4 γ was present, suggesting that KD GRK4 γ acts in a classic dominant negative

fashion. Experiments were conducted to examine $G\alpha_{13}$ -mediated events; however, these studies were inconclusive.

Summary and Conclusion

A caveat of these studies is that they are conducted with GRK4 γ . The gamma isoform is missing a portion of the carboxy terminus of full-length GRK4. Based on the co-crystallization of GRK2, $G\alpha_q$, and $G\beta$, the missing portion of GRK4 γ is most likely not directly involved in the interaction with the $G\alpha$ subunit [17]. However, this does not preclude a variation in protein folding that alters GRK4 γ - $G\alpha$ interactions. Therefore, these studies should not be extended to all GRK4 variants or the other GRK4 family members.

GRK4 is the most active GRK under basal conditions, and GRK4 γ appears to have the lowest level of receptor-mediated activity [9]. This may, in part, be explained by our results indicating that GRK4 γ binds preferentially to inactive $G\alpha_s$. Binding of GRK4 γ to the inactive receptor- $G\alpha$ complex would allow GRK4 γ greater access to the G protein-coupled receptor, and thus could result in increased phosphorylation of the inactive receptor. Moreover, the apparent lower affinity for active $G\alpha_s$ may also explain the aforementioned low level of receptor-mediated activity. GRK4 γ and GRK2 functionally inhibit $G\alpha_s$ -mediated accumulation of cAMP (Fig. 4). Thus, in RPTCs, GRK4 γ functions as hypothesized, but through a novel mechanism. This may be a cell type specific response since similar studies show little effect of wild-type GRK4 γ in regulating dopamine-mediated cAMP production in CHO cells [5].

In conclusion, GRK4 γ binds to inactive $G\alpha_s > \text{inactive } G\alpha_{13} \gg \text{active } G\alpha_s \gg \text{active } G\alpha_{13}$, yet still appears to function normally by inhibiting receptor-mediated signaling in a physiologically relevant cell type. These phenomena may account for the previously reported increased basal activity of GRK4 γ . The molecular mechanism mediating this interaction requires further elucidation. Once the mechanism of wild-type GRK4 γ binding to $G\alpha_s$ is determined, the effects of the polymorphisms in the RH domain associated with hypertension can then be clearly examined.

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Figure 1.

GRK4 γ interacts with G-proteins in the active and inactive state. 293T cells were transfected with an AU1-tagged G α subunit, and GRK4 γ (a & c) or GRK2 (b & d). The G-proteins were stimulated as indicated and immunoprecipitated with AU1 antibody. The first of the three blots in each set is the co-immunoprecipitation, and the lower two blots are the controls to indicate that the GRK and G α subunits were expressed. Note, G α_q and G α_{11} are not well expressed, which is why the IP blot was used instead of total cell lysate (TCL) in panels a, b & d. Each experiment was conducted a minimum of two times.

Figure 2.

GRK γ interacts preferentially with inactive G α_s and G β . 293T cells were transfected with GRK4 γ and either AU1-tagged G α_s (a), or wild-type and constitutively active G α_s (b & c). AU1 tagged G α_s was stimulated with AIF $_4^-$. 293T cells were lysed with either a triton X-100 (TX-100) based lysis buffer, a RIPA buffer, or a detergent-free buffer to examine the effects of the lysis buffer (c). Endogenous G β is co-immunoprecipitated with GRK4 γ by G α_s (d), conversely endogenous G β co-immunoprecipitates GRK4 γ (e). In all panels the upper blots are the co-immunoprecipitation and the lower blots are the control total cell lysates (TCL). Each experiment was conducted a minimum of two times

Figure 3.

GRK4 γ interacts with inactive G α ₁₃. 293T cells were transfected with GRK4 γ and either AU1 tagged G α ₁₃ (a), or wild-type and constitutively active G α ₁₃ (b & c). The AU1 tagged G α ₁₃ was stimulated with AIF₄⁻. To test if the lysis buffer had any effects, 293T cells were lysed in detergent-free buffer (c). Endogenous G β co-immunoprecipitates GRK4 γ and G α ₁₃ (d). In all panels the upper blots are the co-immunoprecipitation and the lower blots are the control total cell lysates. Each experiment was conducted twice.

Figure 4.

Functional properties of GRK4 γ . Kinase dead (KD) GRK4 γ was generated through oligonucleotide-directed mutagenesis (a). This mutation does not abrogate binding to inactive G α_s (b). cAMP production was measured in WKY RPTCs transfected with cDNA expression vectors, indicated at the base of the histogram, and stimulated for 30 min with Isoproterenol (n = 4). Bars with different letters are significantly different (p < 0.05) via ANOVA with Tukey-Kramer multiple-comparison post hoc test.