Antisense oligonucleotides against receptor kinase GRK2 disrupt target selectivity of β-adrenergic receptors in atrial myocytes

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Abstract K^+ channels composed of GIRK subunits are predominantly expressed in the heart and various regions of the brain. They are activated by βγ-subunits released from pertussis toxin-sensitive G-proteins coupled to different seven-helix receptors. In rat atrial myocytes, activation of $K_{(ACh)}$ channels is strictly limited to receptors coupled to pertussis toxin-sensitive G-proteins. Upon treatment of myocytes with antisense oligodesoxynucleotides against GRK2, a receptor kinase with $G_{\beta\gamma}$ binding sites, in a fraction of cells, $K_{(ACh)}$ channels can be activated by β-adrenergic receptors. Sensitivity to β-agonist is insensitive to pertussis toxin treatment. These findings demonstrate a potential role of $G_{\beta\gamma}$ binding proteins for target selectivity of G-protein-coupled receptors

Key words: Potassium channel; GIRK; G-protein; β-receptor; GRK2; Antisense

1. Introduction

An important pathway controlling the membrane excitability in cardiac and neuronal cells is the regulation of K^+ ion channel activity by receptors for neurotransmitters or hormones via heterotrimeric G-proteins in a membrane-delimited fashion (for reviews see [1,2]). The prototype of this ion channel family is the muscarinic K^+ ($K_{(ACh)}$) channel predominantly expressed in supraventricular myocytes. It is composed of two related subunits (GIRK1/GIRK4, also termed Kir3.1/Kir3.4) [3], which form a heterotetramer. It is now commonly accepted that channel gating is directly controlled by physical interaction of the GIRK subunits with $G_{\beta\gamma}$ [4–7]. In the heart, $K_{(ACh)}$ channels can be activated by different receptors that couple to pertussis toxin (Ptx)-sensitive G-proteins, such as M_2 muscarinic, A_1 purinergic [8] and a sphingolipid receptor with a high affinity to sphingosine-1-phosphate [9,10]. In excised inside-out membrane patches, $K_{(ACh)}$ channels can be activated by a number of different $G_{\beta\gamma}$ combinations [4,11], as would be expected given that the specificity of βγ-heterodimers to target proteins is weak [12]. In atrial myocytes, activation of $K_{(ACh)}$ channels is usually limited to receptors coupled to Ptx-sensitive G-proteins [13]. In this regard, GIRK channels expressed in oocytes behave differently. They can be activated by co-expressed β_2-adrenergic receptors [14]. This finding suggests that in the oocyte expression system, unlike in a post-mitotic differentiated cardiac myocyte, G-protein βγ-subunits are promiscuously coupled to the GIRK channels.

Recently, [15] described a low incidence of Ptx-insensitive activation of $I_{K(ACh)}$ by isoprenaline in native canine atrial myocytes which was enhanced by adenoviral infection. The latter had been described previously to result in a non-specific increase in G_s. We have found recently a Ptx-insensitive activation of $I_{K(ACh)}$ by isoprenaline in rat atrial myocytes overexpressing a G-protein β_1-subunit [13]. This finding was interpreted by assuming that the overexpressed (inactive) β-subunits might saturate proteins with $G_{\beta\gamma}$ binding motifs which might act as 'βγ-scavengers', thereby limiting the spatial spreading of βγ released upon receptor activation.

In the present study, the expression level of GRK2, a receptor kinase with a βγ binding sequence was inhibited in post-mitotic ('adult') atrial myocytes, using an antisense approach. In the majority of cells treated with GRK2 antisense ODN's, $K_{(ACh)}$ channels were activated by the β-adrenergic agonist isoprenaline in a Ptx-insensitive fashion. The requirement for a normal expression level of GRK2 to maintain the target specificity in a receptor-coupled signaling pathway suggests a novel role of this protein in cellular signaling.

2. Materials and methods

2.1. Isolation and culture of atrial myocytes

WKY rats of either sex weighing around 200 g were anesthesized by intravenous injection of urethan (1 g/kg). The chest was opened and the heart was removed and mounted on the cannula of a Langendorff apparatus for coronary perfusion at a constant flow. The method of enzymatic isolation of atrial myocytes and culture conditions have been described in detail previously [16]. Cells were plated at a low density (several hundred cells per dish) on 36 mm culture dishes. The medium was changed 24 h after plating and then every second day. Cells were used experimentally from about 4 h after isolation up to 6 days in culture.

2.2. Solutions and chemicals

For the patch-clamp measurements, an extracellular solution of the following composition was used (mM): NaCl 120, KCl 20, $CaCl_2$ 2.0, $MgCl_2$ 1.0, HEPES/NaOH 10.0, pH 7.4. The solution for filling the patch-clamp pipettes for whole-cell voltage clamp experiments contained (mM) K-aspartate 110, KCl 20, NaCl 10, $MgCl_2$ 1.0, MgATP 2.0, EGTA 2.0, GTP 0.01, HEPES/KOH 10.0, pH 7.4. The K^+ equilibrium potential under this condition is −50 mV. This experimental condition was chosen because of the strongly inward rectifying properties of $I_{K(ACh)}$, which yields very small currents in the outward direction, i.e. at a membrane potential positive to E_K. In the majority of experiments, this solution was supplemented with cAMP (100 μM) to fully activate the cAMP-dependent signaling pathway. The rationale for this was to exclude that the β-adrenergic effects described here were cAMP-dependent. Standard chemicals were from Merck (Darmstadt, Germany). EGTA, HEPES, MgATP, adenosine GTP and ACh-chloride were from Sigma (Deisenhofen, Germany).

2.3. Current measurement

Membrane currents were measured by means of whole-cell voltage patch-clamp. Pipettes were fabricated from borosilicate glass with filament (Clark, Pangbourne, UK) and were filled with the solution listed above. The DC resistance of the filled pipettes ranged from 2 to

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6 MΩ. Current measurements were performed by means of a patch-clamp amplifier (List LM/EPC 7, Darmstadt, Germany) as described in detail recently [13]. Experiments were performed at an ambient temperature (22–24°C). Cells were voltage-clamped at a holding potential of −90 mV, i.e. negative to E_K. K^+ channel currents under this condition are in the inward direction. This experimental condition was chosen because of the strongly inward rectifying properties of $I_{K(ACh)}$. Current-voltage relations were determined by means of a ramp protocol. From −90 mV, the holding potential was stepped to −120 mV, followed by a voltage ramp at 360 mV/s to +60 mV and a step back to −90 mV. Rapid application and withdrawal of different solutions was performed by means of a solenoid-operated flow system that permitted switching between up to six different solutions. The half time of exchange of solution seen by the superfused cell was less than 100 ms and was not limiting the activation kinetics of $I_{K(ACh)}$.

2.4. Treatment with AsODNs

AsODNs (5′-CTTTTGGAAGATGTCG-3′) against nucleotides 480–495 of the rat GRK2 cDNA sequence were synthesized with the phosphorothioate backbone by Biognostik (Göttingen, Germany). As control ODN, a randomized sequence with the above DN composition was used. Antisense and control ODNs were diluted in M199 culture medium to a final concentration of 6 μM. Cells were incubated on day 2 after plating for 3–5 days in 1 ml ODN-supplemented medium. Optical monitoring the uptake of ODNs using a fluorescein tag yielded a half time for loading in the order of 2 h. After 48 h, ODN containing medium was replaced by standard medium. Measurements were performed after another 24 or 48 days, i.e. on days 5 and 6 in vitro. The rationale for using this time window was that on day 4, no effects were seen ($n=6$), whereas after periods of time in vitro >7 days, an increasing number of cells developed vacuole-like structures. This did not affect the viability, adult myocytes could be kept in vitro for >3 months, but impeded formation of stable Gigaohm seals.

2.5. Western blots

For Western blots, 10^4 cells per dish were plated and incubated with either control or AsODNs. On day 5, cells were harvested, pelleted and washed once in PBS. Each pellet was diluted and boiled for 10 min in 2×SDS-Laemmli buffer followed by PAGE using a 12% acrylamide (29:1) gel with a 6% stacking gel. Following electrophoresis, proteins were blotted to a nylon membrane using the Electro-Trans-Blot (Bio-Rad, Germany). The membrane was washed and blocked according to the manufacturer's protocol. Rabbit polyclonal anti-GRK2 antibody (Santa Cruz Biotechnology, USA) was diluted in blocking solution (1 μg/ml), incubated with the membrane for 30 min at room temperature and washed 3×5 min in H_2O and once with TBS. Alkaline phosphatase conjugated anti-rabbit IgG antibody was diluted in blocking solution (1:6000) and incubated with the membrane at room temperature for 30 min. GRK2-antibody complex was detected using chemiluminescent substrate sheets (Schleicher and Schuell, Germany).

3. Results and discussion

Fig. 1A shows changes in the membrane current induced by rapid superfusion with ACh at a saturating concentration (2 μM) of a myocyte, cultured for 6 days. The cell was treated with control ODNs by incubation on day 2 after isolation. In this and the subsequent experiments for about 5 min before the trace was recorded, cAMP was allowed to diffuse into the cell from the recording pipette. At the standard holding potential (−90 mV) and E_K (−50 mV), K^+ channel currents are in the inward direction. Repetitive brief exposures to ACh resulted in inward currents of a reproducible amplitude. From superimposed voltage ramps (−130–+60 mV), the current-voltage relation of the ACh-activated current was obtained by subtraction of the background I/V curve (Fig. 1B, b−a). As in previous work, the I/V relation of this current is characterized by strong inward rectification, which is assumed to result from a block by intracellular polyamines [17].

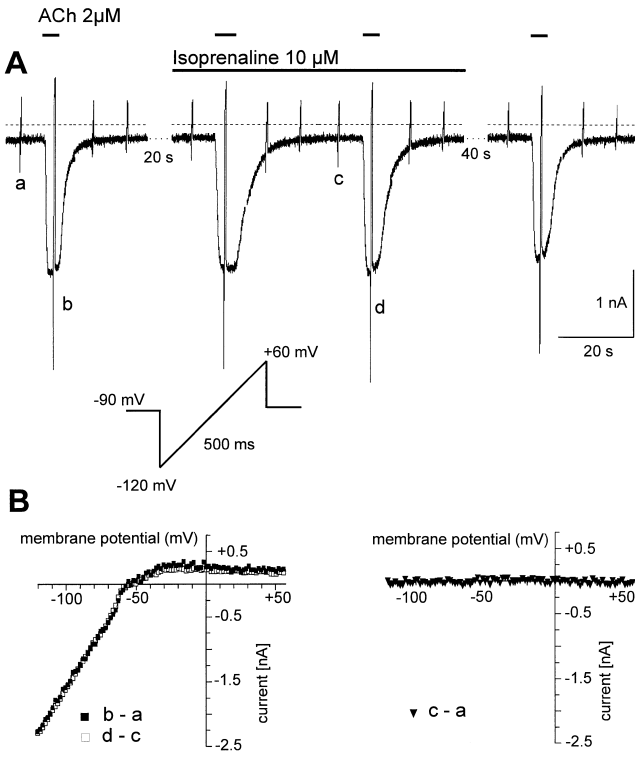

Fig. 1. β-adrenergic stimulation does not interfere with the muscarinic signaling pathway in myocytes expressing normal levels of GRK2. (A) Representative recording of the membrane current at a −90 mV holding potential from a myocyte treated with a randomized ODN sequence as described in Section 2. Rapid deflections in this and subsequent figures represent changes in the membrane current caused by a voltage ramp protocol shown in the inset. ACh and Iso were superfused as indicated by the horizontal bars. (B) (left panel) Current-voltage relations of ACh-activated current in the absence (b-a) and in the presence (d-c) of isoprenaline. I/V curves represent difference currents obtained by background subtraction as indicated by the labelling. (B) (right panel) Subtraction of the control background current (a) from the background current in the presence of iso (c) yields zero over the whole range of membrane potentials.

In line with a recent report [13], simultaneous superfusion with the β-receptor agonist Iso at a highly saturating concentration (10 μM) had no effect on the holding current or on the size of the ACh-induced current. Such a large concentration was used to maximally activate β-adrenergic receptors. The absence of an effect of Iso is confirmed by the current-voltage relations obtained by subtraction as indicated by the labelling. Fig. 1B shows I/V curves of ACh-activated current in the absence and presence of Iso. The two curves are perfectly superimposable, demonstrating that there is no effect of β-adrenergic stimulation on M_2 receptor-activated $I_{K(ACh)}$ under these experimental conditions. Fig. 1C shows the background current at a −90 mV holding potential in the presence of Iso (c) minus control (a). The difference is zero over the entire range of membrane potentials. This behavior, i.e. a complete lack of an effect of Iso on the holding current and receptor-activated $I_{K(ACh)}$, was found in 28/28 untreated cells and 15/15 cells incubated with the control ODN. If cAMP was omitted from the pipette filling solution, identical results were obtained with regard to the current under study ($n=6$). Furthermore, superfusion with forskolin (10 μM) had no effect on the holding current or the voltage ramp-induced current (not

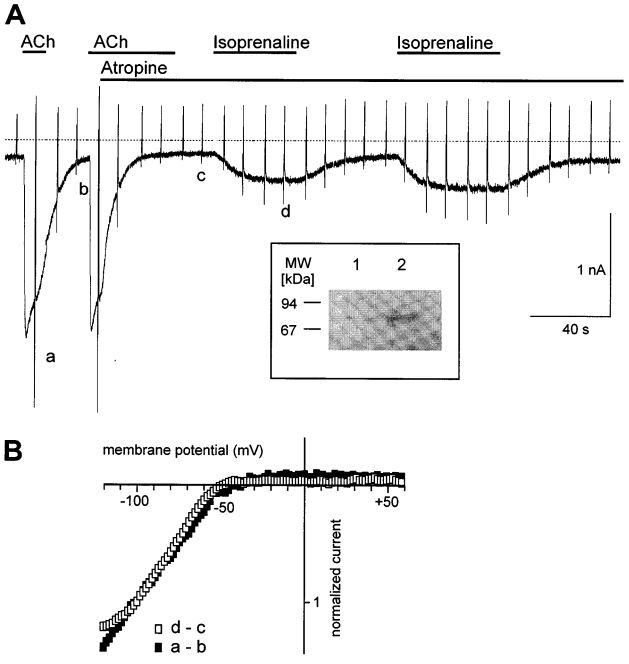

Fig. 2. Activation of $I_{K(ACh)}$ by isoprenaline in a myocyte treated with GRK2 AsODN. (A) Recording of the membrane current at a −90 mV holding potential. ACh, atropine and isoprenaline were superfused as indicated. The inset shows a representative immunoblot for rat GRK2 (molecular weight 76 kDa). Line 1 represents GRK2-AsODN-treated culture; line 2, a sister culture treated with control ODN. (B) Current-voltage relationship of the ACh-induced current (a-b in A) and isoprenaline-induced current (d-c), multiplied by a factor of 6.21 to match the size of the ACh-induced current at −100 mV.

shown). Consistent with previous studies of $I_{K(ACh)}$, there is no [18] or little [15] direct cross-talk of the muscarinic and the β-adrenergic signaling pathways with regard to this target in native myocytes. This is in contrast to findings in the oocyte system, where expressed GIRK subunits form functional channels that can be substantially activated by co-expressed β-adrenergic receptors [14]. We hypothesized that in differentiated cells, other proteins with βγ binding sites might affect the spatio-temporal distribution of free βγ-subunits upon receptor stimulation. Such a mechanism could contribute to preventing an unphysiological interaction of $G_{s\beta\gamma}$ with the GIRK1/GIRK4 subunits. One candidate for a protein that could play this role is GRK2 (βark1), a receptor kinase which is activated and targeted to the membrane by binding to the β-subunit of heterotrimeric G-proteins [19]. In order to test this hypothesis, we used an AsODN directed against a part of the encoding sequence of rat GRK2. Inhibition of expression of GRK2 was verified by Western blot analysis, comparing sister cultures 4 days after incubation with either the GRK2-specific or the scrambled sequence (see Section 2). An example representative of three pairs of culture dishes is illustrated in Fig. 2 (inset). Although the signals are weak due to the small amount of material and do not permit a quantitative evaluation of the difference of GRK2 expression, the blots clearly indicate a reduced amount of GRK2 in the antisense AsODN-treated myocytes. A representative example of a recording of the membrane current from a myocyte treated with the GRK2 AsODN is illustrated in Fig. 2A. The cell responded normally to a saturating concentration of ACh (2 μM). During the second challenge by ACh, superfusion was switched to a solution additionally containing the muscarinic antagonist atropine (1 μM). This resulted in a complete inhibition of $I_{K(ACh)}$. Iso (10 μM) in the continuous presence of atropine caused a reversible and reproducible inward current of 350 pA. An analysis of the current-voltage relation of the Iso-induced current (Fig. 2C) yielded a curve which, after scaling up, was perfectly superimposable on the I/V curve of the ACh-activated current. As activation of the current by Iso was insensitive to a saturating concentration of atropine, a participation of M_2 receptors in the Iso-activated current and so also artefacts, e.g. leak of trace amounts of ACh from the superfusion device, can be safely excluded. Activation of the inward rectifying K^+ current ranging from ≥5%–22% of peak $I_{K(ACh)}$ induced by ACh was found in 70% (23 of 32) of GRK AsODN-treated myocytes. The large variability is not surprising, given that neither cellular uptake of ODNs nor the reduction in expression of the protein are likely to be homogenous in all cells in a dish. The concentration of Iso used in these experiments (10 μM) is highly saturating and might even cause unspecific effects. It should be noted, however, that the same concentration of the agonist was completely ineffective in 100% of the control cells.

The strongly inward rectifying I/V relation identifies the K^+ current induced by Iso as $I_{K(ACh)}$. This is further supported by cross-desensitisation between the ACh- and Iso-activated cur-

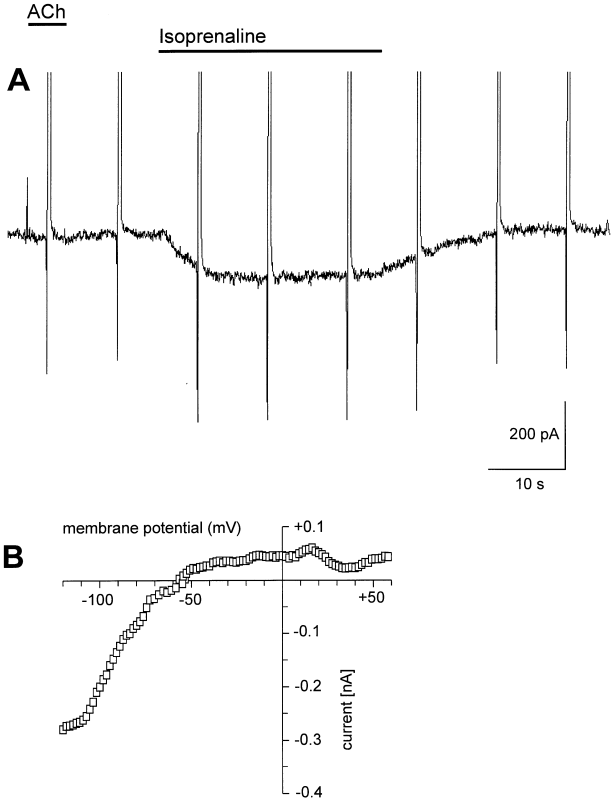

Fig. 3. Activation of $I_{K(ACh)}$ by Iso in a myocyte pre-incubated with pertussis toxin. (A) Membrane current at a −90 mV holding potential. ACh was used at a highly saturating concentration (10 μM). (B) Difference I/V relationship of the Iso-induced current. The upward deflections representing the ramp-induced outward current have been cut off. The non-specifc leak current was significantly larger after Ptx treatment, resulting in a more pronounced contamination of background currents.

rent using similar protocols as shown recently [13] (data not shown). Coupling of receptors to $K_{(ACh)}$ channels normally proceeds via βγ-subunits of (a) Ptx-sensitive G-protein(s). If activation of $I_{K(ACh)}$ by Iso proceeds via G_s, it should be insensitive to treatment with Ptx. As shown previously, receptor-mediated activation of whole-cell $I_{K(ACh)}$ in the system under study can be completely abolished by incubation of myocyte cultures with Ptx at 2 μM for ~6 h. A representative recording from a GRK2 antisense-treated Ptx-incubated cell is illustrated in Fig. 3. ACh at the standard concentration of 2 μM failed to induce a significant current. Iso (10 μM) caused activation of an inward current at a 90 mV holding potential of about 140 pA, which by its voltage-dependence (Fig. 3B) was identified as $I_{K(ACh)}$. This was found in three of five Ptx-treated myocytes which were completely insensitive to ACh. In 5/5 myocytes from time-matched control cultures treated with Ptx, no activation of $I_{K(ACh)}$ by Iso was detected.

In excised inside-out patches, activation of $K_{(ACh)}$ channels by different βγ-subunit combinations has been demonstrated [20], in line with the notion that specificity of different βγ-heterodimers for target proteins is not particularly pronounced. Together with the finding that $K_{(ACh)}$ channels expressed in *Xenopus* oocytes can be activated by co-expressed β_2-adrenergic receptors [14], this raises the question what prevents activation of this channel by free $G_{s\beta\gamma}$ produced by activation of β-adrenergic receptors in a differentiated atrial myocyte. As a matter of fact, Sorota et al. [15] recently described that a fraction of acutely isolated or cultured canine atrial myocytes at 37°C showed a slight activation of $I_{K(ACh)}$ by Iso. This was insensitive to Ptx and thus most likely proceeded via βγ released from G_s. Increasing the concentration of G_s by adenoviral infection caused a substantial increase in sensitivity of $I_{K(ACh)}$ to β-adrenergic stimulation. If the low basal sensitivity of $I_{K(ACh)}$ to Iso observed in a fraction of cells in that study reflects a species property or a combination of several factors (species, temperature, etc.) at present is not known. The finding as such lends further support to the notion that signaling via βγ-subunits in principle is promiscuous in nature.

Although most of the actions of β-adrenergic receptors are mediated through G_s-proteins and the cAMP system, few examples of G_i coupling of β_2-R have been described. In the heart, evidence based on pharmacological experiments for a coupling of β_2-R to Ptx-sensitive G-proteins has been provided [21]. More recently, [22], using a heterologous expression system, found that phosphorylation of β_2 receptors by PKA causes a switch in their coupling from G_s (Ptx-insensitive) to G_i (Ptx-sensitive), resulting in activation of the MAP kinase pathway in that model.

We can exclude an analogous mechanism as the basis of the β-receptor-induced activation of $I_{K(ACh)}$ in the present study for two reasons: (i) β-receptor-induced $I_{K(ACh)}$ is not sensitive to Ptx and (ii) in control and AsODN-treated myocytes, intracellular loading with cAMP and superfused with forskolin had no effect on the signaling pathway under study. Furthermore, activation of $I_{K(ACh)}$ by β-adrenergic stimulation was never seen in a total of 43 control myocytes, either untreated or incubated with the scrambled AsODN sequence.

Although βγ binding to GRK2 is a key step in mediating homologous desensitization of various G-protein-coupled receptors (see [23,24] for reviews), this kinase is not a priori selective for a distinct receptor. Specificity is assumed to result from its targeting to an agonist-activated receptor by free βγ [19]. This concept implies that the kinase is retained in the micro-environment of an activated receptor by binding to βγ. The binding site on GRK2 for the (β) subunit overlaps with a pleckstrin homology (PH) domain at the C-terminus of GRK2 [19,25,26]. PH domains are modules of around 100 amino acids that are found in a large number of proteins (see [27] for reviews). Apart from an interaction motif for β-subunits, they share a phosphoinositide binding site. It has been demonstrated that GRK2 translocation to the membrane and kinase activity require both binding to βγ and a phosphoinositide, presumably PIP_2 [28]. It is conceivable that the coordinated binding of GRK2 to two membrane-associated ligands (PIP_2 and $G_{\beta\gamma}$) contributes to limiting its lateral mobility. Formation of such a complex, in turn, would also affect the lateral mobility of the ligand, i.e βγ. In this way, GRK2 could contribute to preventing interaction of βγ with a wrong target protein. This interpretation is in line with our recent observation that overexpressing a β-subunit in atrial myocytes by transient transfection, apart from reducing the $I_{K(ACh)}$ amplitude, renders this current sensitive to β-adrenergic stimulation to about the same extent as in the present study [13].

It has been demonstrated that peptides derived from the C-terminus of GRK2 (βARK1), containing the βγ binding sequence, inhibit agonist-dependent activity of heterologously expressed as well as native cardiac $K_{(ACh)}$ channels when applied to the internal face of the membrane in excised inside-out membrane patches [29] and whole-cell recordings [15]. These findings not only underscore the role of βγ as activator of this target but also demonstrate the βγ sequestering potency of GRK2 and its putative physiological role in tuning the availability of βγ subunits released from different G-proteins to target proteins.

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