The role of members of the pertussis toxin-sensitive family of G proteins in coupling receptors to the activation of the G protein-gated inwardly rectifying potassium channel

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Edited by Lily Y. Jan, University of California, San Francisco, CA, and approved February 18, 2000 (received for review December 28, 1999)

Inwardly rectifying potassium (K^+) channels gated by G proteins (Kir3.x family) are widely distributed in neuronal, atrial, and endocrine tissues and play key roles in generating late inhibitory postsynaptic potentials, slowing the heart rate and modulating hormone release. They are directly activated by $G_{\beta\gamma}$ subunits released from G protein heterotrimers of the $G_{i/o}$ family upon appropriate receptor stimulation. Here we examine the role of isoforms of pertussis toxin (PTx)-sensitive G protein α subunits ($G_{i\alpha1-3}$ and $G_{o\alpha A}$) in mediating coupling between various receptor systems (A_1, α_{2A}, D_{2S}, M_4, $GABA_B$1a+2, and $GABA_B$1b+2) and the cloned counterpart of the neuronal channel (Kir3.1+3.2A). The expression of mutant PTx-resistant $G_{i/o\alpha}$ subunits in PTx-treated HEK293 cells stably expressing Kir3.1+3.2A allows us to selectively investigate that coupling. We find that, for those receptors (A_1, α_{2A}) known to interact with all isoforms, $G_{i\alpha1-3}$ and $G_{o\alpha A}$ can all support a significant degree of coupling to Kir3.1+3.2A. The M_4 receptor appears to preferentially couple to $G_{i\alpha2}$ while another group of receptors (D_{2S}, $GABA_B$1a+2, $GABA_B$1b+2) activates the channel predominantly through $G_{\beta\gamma}$ liberated from G_{oA} heterotrimers. Interestingly, we have also found a distinct difference in G protein coupling between the two splice variants of $GABA_B$1. Our data reveal selective pathways of receptor activation through different $G_{i/o\alpha}$ isoforms for stimulation of the G protein-gated inwardly rectifying K^+ channel.

Inwardly rectifying K^+ channels gated by the direct action of G proteins are present in neurones, atrial myocytes, and endocrine cells and are responsible for mediating postsynaptic inhibitory effects, in slowing the heart rate in response to vagal nerve stimulation and in modulating hormone release. Their molecular counterparts have been identified and the channel has been shown to be a heteromultimeric structure comprised of members of the Kir3.x family of K^+ channels (1–5). Co-expression of Kir3.1 with Kir3.2, Kir3.3, or Kir3.4 results in currents that show many of the basic characteristics of the native channels in neurones and atria (6–8). Channel activation is abolished by pertussis toxin (PTx) treatment, implicating the $G_{i/o}$ family of G proteins (9–11). Although initially controversial, it is now well established that activation of these channels in native tissues and of the cloned counterparts in heterologous expression systems is via a membrane-delimited mechanism involving a direct interaction with the $G_{\beta\gamma}$ dimer (12–14). Indeed the studies on this channel have become a paradigm of how $G_{\beta\gamma}$ can be important in signaling to downstream effectors. Current studies have focused on domains on the channel important for binding $G_{\beta\gamma}$ (15–20), trafficking of the channel complex (21–24), and the role of anionic phospholipids in regulating channel activity (25–29).

We have recently shown that the G_α subunit is the key determinant of specificity of channel activation for receptors coupling predominantly to $G_{i/o}$ as against those that couple to G_s (30). In this study, we investigate the role of different $G_{i/o\alpha}$ variants in determining selective receptor coupling between receptors and the cloned G protein-gated inwardly rectifying K^+ channel, Kir3.1+3.2A. The PTx-sensitive G protein family is made up of G_i, encoded by three separate gene products ($G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$), and G_o, made up of two splice variants ($G_{o\alpha A}$ and $G_{o\alpha B}$) (31). It is apparent that some heptahelical receptors exhibit a preference for the type of G_α subunit they couple to within a G protein family—for example, evidence exists that suggests that the D_2 dopamine receptor splice variants preferentially couple to some $G_{i/o\alpha}$ subunits rather than to others (32–35). In addition, it has been proposed that $G_{i/o\alpha}$ subunit variants, in particular $G_{i\alpha1}$, may have direct inhibitory actions on the G protein-gated K^+ channel (36). However, the significance of this for receptor-mediated activation has not been addressed. In this study, we demonstrate that all $G_{i/o\alpha}$ variants are able to liberate $G_{\beta\gamma}$ to mediate coupling between receptor and Kir3.1+3.2A channels, but that some receptors have a preference for the $G_{i/o\alpha}$ subunit variant with which they interact to activate the channel.

Methods

Molecular Biology, Cell Culture, and Transfection. Standard molecular cloning and mutagenesis techniques were used throughout. Cell culture, generation of stable cell lines, construction of the bicistronic vector, and point mutations of G_α subunits were as described (30, 37). For this study we used a similar PCR-based strategy to introduce a C→G mutation at analogous positions in $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha A}$. We transfected 400 ng of each receptor cDNA and 500 ng of each $G_{i/o\alpha}$ cDNA. We examined the effects of varying cDNA ratios for the α_{2A} adrenergic receptor and the $G_{i\alpha2}$C352G and $G_{i\alpha3}$C351G mutants in the HKIR3.1/3.2 line treated with PTx. We found that reducing the cDNA concentration for the mutant G protein (from 500 ng to 125 ng) alters the magnitude but not the selectivity difference whereas decreasing the receptor concentration (from 400 ng to 100 ng) loses any response (data not shown). Increasing the amount of $G_{i/o\alpha}$ cDNA beyond 1 μg was toxic to cells. It should be noted that the IRES-containing vector we constructed does not ensure that translation starts in the optimal position from the end of the IRES element (38). It is thus likely that protein translation from the second cistron will be reduced. However, in our particular case, the expression of Kir3.1 and Kir3.2A from the IRES plasmid and from separate plasmids [pcDNA3 and pcDNA3.1(+)/Zeo (Invitrogen)] gave similar basal current levels (separate plas-

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This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PTx, pertussis toxin; NECA, 5′-*N*-ethylcarboxyamidoadenasine.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.080572297. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.080572297

mids: 147 ± 22 pA/pF, n = 17; IRES vector: 112 ± 17 pA/pF, n = 41, P = 0.26).

Electrophysiology. Whole-cell membrane currents were recorded by using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Patch pipettes were pulled from filamented borosilicate glass (Clark Electromedical Instruments, Pangbourne, U.K.) and had a resistance of 1.5–2.5 MΩ when filled with pipette solution (see below). Before filling, tips of patch pipettes were coated with a Parafilm/mineral oil suspension. Records were filtered at 1 kHz and were digitized at 5 kHz, and data were acquired and analyzed by using a Digidata 1200B interface (Axon Instruments) and PCLAMP 6.0 software (Axon Instruments). Cell capacitance was approximately 15 pF, and series resistance (<10 MΩ) was at least 75% compensated. Recordings of membrane current were commenced after an equilibration period of approximately 5 min. Currents were measured at the end of each voltage step. Current densities were measured at −100 mV (unless otherwise stated), and all data are presented as mean ± SEM. Student's t tests were performed to examine statistical significance, and an asterisk in Figs. 2–4 indicates that $P \leq 0.05$.

Dose-response curves were constructed in the HKIR3.1/3.2/A1 cell line. It was not feasible to apply more than four concentrations of 5′-*N*-ethylcarboxyamidoadenasine (NECA) to individual cells because of receptor "desensitization" and a subsequent decline in response. Therefore, for each experimental condition (i.e., receptor coupling to either endogenous G_α or exogenously expressed G_α mutants), data were pooled from at least 12 cells, and the responses obtained by using different concentrations of NECA were normalized (I/I_{max}) to those obtained by using a maximal concentration (1 μM) that was applied to every cell recorded from. Concentrations of NECA were applied randomly, but 1 μM NECA was always applied twice to each cell at the start and end of the experiment. Curves were fitted by using nonlinear regression with PRISM 3.0 software (GraphPad, San Diego). Data were obtained from at least two independent transfections of each G_α/receptor combination.

Materials and Drugs. Solutions were as follows (concentrations in mM): pipette solution, 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 Hepes, 2 MgATP, 0.3 Na_2GTP (KOH to pH 7.2, ≈140 mM total K^+); bath solution, 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 Hepes (pH 7.4). Cell culture materials were from GIBCO/BRL and Invitrogen. Molecular biology reagents were obtained from New England Biolabs or Roche Molecular Biochemicals, and oligonucleotides were from Genosys (Cambridge, U.K.). All chemicals were from Sigma or Calbiochem. Drugs were made up as concentrated stock solutions and were kept at −20°C or −80°C.

Results

The studies detailed here were performed on stably transfected HEK293 cells expressing Kir3.1 and Kir3.2A alone (HKIR3.1/3.2) or on another line (HKIR3.1/3.2/A1) that additionally stably expressed the A_1 adenosine receptor together with Kir3.1 and Kir3.2A (30).

Characterization of PTx-Insensitive Mutants of $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha1}$. PTx catalyzes the ADP ribosylation of the $G_{i/o\alpha}$ subunit at a cysteine residue four amino acids from the C-terminal end of the protein. The PTx-treated subunit is thus unable to participate in signaling. However, mutation of this residue to glycine or isoleucine renders the mutant subunit insensitive to the effects of PTx (34, 39). Such mutants have been shown to still functionally interact with receptors as determined by agonist-stimulated $^{35}S[GTP\gamma S]$ binding (37, 40, 41). We have previously shown that $G_{i\alpha1}$C351G is able to rescue coupling between Kir3.1+3.2A and the transiently transfected A_1 and α_{2A} receptors in the HKIR3.1/3.2 cell line after PTx treatment (30).

We now characterize the behavior of $G_{i\alpha1}$C351G, $G_{i\alpha1}$C351I,

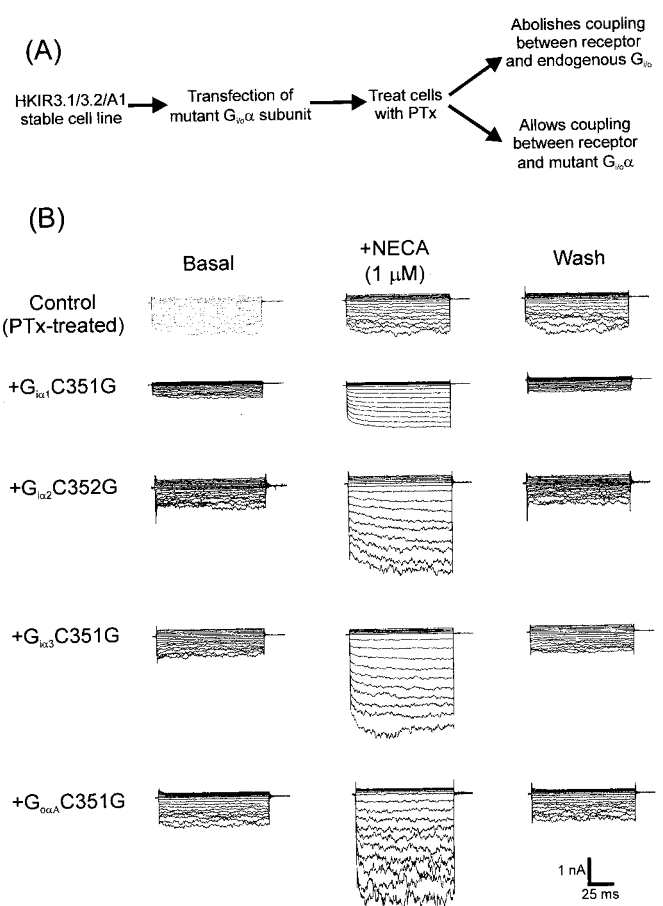

Fig. 1. The A_1 adenosine receptor couples to Kir3.1+3.2A channels via $G_{i\alpha1-3}$ and $G_{o\alpha A}$. (*A*) This illustrates the experimental protocol used. The HKIR3.1/3.2/A1 monoclonal cell line stably expressing Kir3.1+3.2A channels together with the A_1 receptor was transiently transfected with mutant PTx-insensitive $G_{i/o\alpha}$ subunits, and then cells were treated with PTx 1 day before electrophysiological recording. (*B*) These are examples of traces showing the effects of stimulating A_1 receptors in the HKIR3.1/3.2/A1 cell line in PTx-treated cells (top traces) and when each of the mutated $G_{i/o\alpha}$ variants was co-expressed. Currents were elicited by holding cells at 0 mV and stepping to potentials between −100 and +50 mV in 10-mV increments for 100 ms. Traces indicate current responses before (Basal), during (+ NECA), and after (Wash) receptor stimulation.

$G_{i\alpha2}$C352G, $G_{i\alpha3}$C351G, and $G_{o\alpha A}$C351G after transient expression and PTx treatment in the stable cell line HKIR3.1/3.2/A1 in which there are constant levels of expression of the A_1 receptor and Kir3.1+3.2A channel current. The experimental protocol we used is shown in Fig. 1*A*. Expression of the mutants alone did not enhance membrane currents and in fact significantly reduced basal current density similarly to wild-type G_α (e.g., $G_{i\alpha1}$C351G: 34 ± 8 pA/pF, n = 8, P = 0.05; $G_{i\alpha2}$C352G: 18 ± 4 pA/pF, n = 10, P < 0.01). In PTx-treated cells, A_1 receptor stimulation was unable to enhance Kir3.1+3.2A currents (Fig. 1*B*, top traces). However, when any of the mutant $G_{i/o\alpha}$ subunits were co-expressed, NECA stimulation of HKIR3.1/3.2/A1 cells led to a large enhancement of currents (Fig. 1*B*). Thus, the PTx-insensitive G_α subunits were able to rescue signaling between the A_1 receptor and Kir3.1+3.2A in PTx-treated cells.

We have quantitatively investigated the behavior of these G_α mutants by constructing dose-response curves for NECA in the HKIR3.1/3.2/A1 line. The dose-response curve obtained from control, non-PTx-treated cells expressing endogenous G protein is illustrated in Fig. 2*A*. From this curve, it can be seen that the $\log EC_{50}$ for NECA is −7.48 ± 0.20, equivalent to a concentration

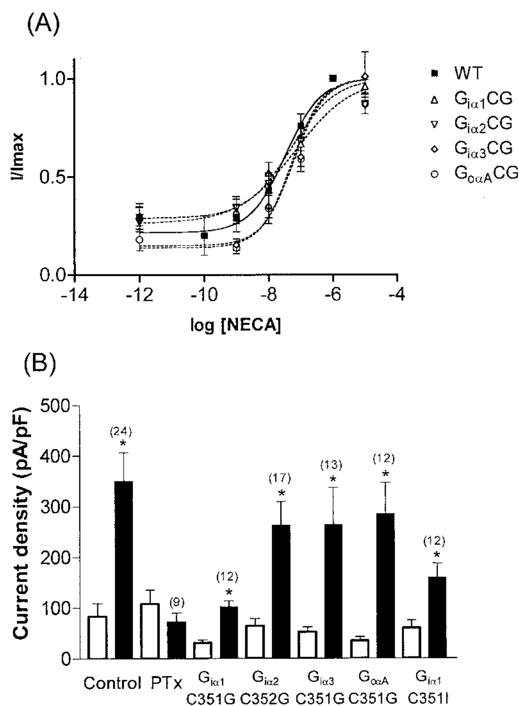

Fig. 2. The $G_{i/o\alpha}$ subunits have similar affinities in mediating signaling between A_1 receptors and Kir3.1+3.2A. (*A*) Superimposed dose-response curves for NECA-induced activation of Kir3.1+3.2A channels in control, non-PTx treated cells (solid line) and in PTx-treated cells in which the mutant $G_{i/o\alpha}$ subunits (dashed lines), $G_{i\alpha1}$C351G, $G_{i\alpha2}$C352G, $G_{i\alpha3}$C351G, and $G_{o\alpha A}$C351G, have been co-expressed. (*B*) Bar chart summarizing the data obtained with the HKIR3.1/3.2/A1 cell line and expression of each of the $G_{i/o}$ variants. Open bars represent basal currents, and solid bars represent current in response to receptor stimulation. Numbers in parentheses refer to the number of cells recorded from for each experiment. Current density was measured at −100 mV.

of 33.1 nM, and the Hill coefficient is 0.79 ± 0.27 (data pooled from 13 cells). We constructed dose-response curves to NECA for each of the G_α mutants. These are also illustrated in Fig. 2*A*, and the results are summarized in Table 1. To examine whether the EC_{50} values varied with the different G_α subunits, we compared the logEC_{50}s measured with exogenous expression of each of the mutant G_α with endogenous G_α. No significant differences were observed when compared with endogenous $G_{i/o}$ (P = 0.31–0.51). We also found no significant differences in the Hill coefficients when the G_α point mutants were compared with endogenous G protein (P = 0.3–0.95). These findings suggest that the mutant $G_{i/o\alpha}$ subunits ($G_{i\alpha1}$C351G, $G_{i\alpha1}$C351I, $G_{i\alpha2}$C352G, $G_{i\alpha3}$C351G, and $G_{o\alpha A}$C351G) are still able to couple the A_1 receptor to the channel complex with approximately equal affinity and that this affinity is similar to that displayed by the endogenous $G_{i/o}$ proteins present in the HEK293 cells.

The efficacy of the response was next investigated. We compared the responses obtained with a maximal concentration of NECA (1 μM) to see whether the efficacy of coupling had been altered by the mutant G_α subunits (Fig. 2*B*). In cells in which $G_{i\alpha1}$C351G had been expressed, the NECA-induced increase in current density was significantly smaller than in control non-PTx-treated cells (P = 0.01). Similarly, we found that, when $G_{i\alpha1}$C351I was expressed, the induced currents were also significantly smaller than in control cells (P = 0.01) and moreover were not significantly different from those obtained with $G_{i\alpha1}$C351G (P = 0.25). None of the other G protein mutants tested had any significant effects on NECA-induced currents (P = 0.28–0.83), suggesting that $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha A}$ all have similar efficacies.

It is likely that the mutant G proteins are all expressed to similar high levels as they are expressed in essentially the same plasmid under the control of the same cytomegalovirus promoter. This has been demonstrated for different mutations of Cys351in $G_{i\alpha1}$ (37) and also for C→G mutations in $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ (40). The data presented above suggest that the levels of expression achieved are sufficient to functionally complement the response.

Delineating Different Patterns of Receptor Stimulation of Channel Activation. Our studies with the A_1 receptor establish that the mutant G protein subunits can substitute both qualitatively and quantitatively for the endogenous G proteins expressed in HEK293 cells. Given the broad ability of the A_1 receptor to activate the isoforms of $G_{i/o}$, we next examined other $G_{i/o}$-coupled receptors, including the α_{2A}, D_{2S}, M_4, and $GABA_B$. We compared the ability of a concentration of standard, full agonist that would lead to maximal receptor occupancy (3 μM noradrenaline, 10 μM quinpirole, 10 μM carbachol, and 100 μM baclofen, respectively) to activate currents in the HKIR3.1/3.2 cell line transiently transfected with receptor and mutant G protein and treated with PTx.

When the mutant G_α subunits were expressed in PTx-treated cells, the channel was still able to be activated via noradrenaline-mediated stimulation of the α_{2A} receptor coupling to all of the G_α subunits tested. However, as observed with the A_1 receptor, some G_α subunits appeared to be more efficient than others, although a different pattern was observed (Fig. 3*A*). Although there did not appear to be much difference between $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{o\alpha A}$, the responses obtained with expression of $G_{i\alpha3}$ were smaller. We also looked at the $G_{i\alpha1}$C351I mutant: the noradrenaline-induced currents obtained when this α subunit was expressed were not significantly different to those obtained with $G_{i\alpha1}$C351G (100.2 ± 33.6 pA/pF, n = 10, P = 0.33). We next investigated the coupling of D_{2S} to $G_{i\alpha1-3}$ and $G_{o\alpha A}$. Interestingly, the expression of neither $G_{i\alpha1}$C351G, $G_{i\alpha2}$C352G, nor $G_{i\alpha3}$C351G was able to efficiently rescue signaling between the receptor and Kir3.1+3.2A. However, the D_{2S} receptor was able to activate channels in PTx-treated cells to a similar level observed in control cells when $G_{o\alpha A}$C351G was co-expressed (Fig. 3*B*). Finally, we examined the coupling of the M_4 muscarinic receptor to the channel whereupon we observed that co-expression of $G_{i\alpha2}$C352G was able to support channel activation. $G_{i\alpha3}$C351G could also support channel activation, but to a lesser extent, whereas $G_{i\alpha1}$C351G and $G_{o\alpha A}$C351G were not nearly as effective (Fig. 3*C*).

Studies on Cloned $GABA_B$ Receptors. The stimulation of postsynaptic $GABA_B$ receptors in neurones and the subsequent activation of G protein-gated inwardly rectifying K^+ channels is a major mechanism for generating late inhibitory postsynaptic potentials. It has recently been established that native $GABA_B$ receptors are a heterodimeric complex comprised of a combination of the two subunits $GABA_B$1 and $GABA_B$2 (42–45). There are now three

PHYSIOLOGY

Table 1. Summary of data from dose-response curves constructed to NECA in the HKIR3.1/3.2/A1 cell lines

	Endogenous	$G_{i\alpha1}$ C351G	$G_{i\alpha2}$ C352G	$G_{i\alpha3}$ C351G	$G_{o\alpha A}$ C351G
EC_{50}	33.1 nM	50.7 nM (P = 0.51)	60.3 nM (P = 0.31)	59.4 nM (P = 0.51)	69.2 nM (P = 0.35)
Hill coefficient, n_H	0.79 ± 0.27	0.64 ± 0.15 (P = 0.64)	0.5 ± 0.12 (P = 0.3)	0.9 ± 0.51 (P = 0.85)	0.82 ± 0.34 (P = 0.95)

Numbers in brackets refer to level of significance in comparing each exogenously expressed $G_{i/o\alpha}$ with endogenous G protein.

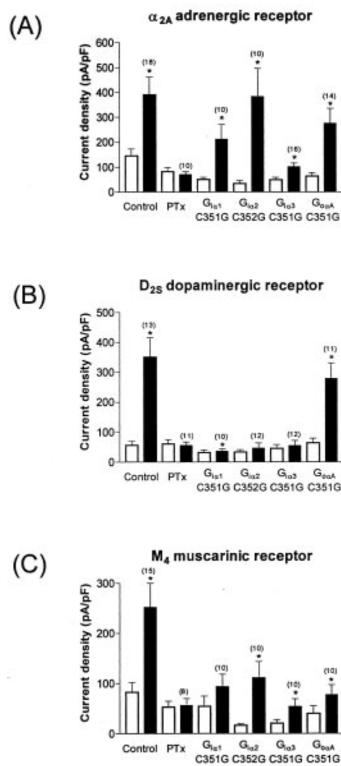

Fig. 3. The α_{2A} adrenergic receptor, D_{2S} dopaminergic receptor, and M_4 muscarinic receptor exhibit different coupling profiles to $G_{i/o\alpha}$ subunits. Summary of data obtained from studying coupling between the α_{2A} adrenergic receptor (*A*), the D_{2S} dopaminergic receptor (*B*) and the M_4 muscarinic receptor (*C*), and Kir3.1+3.2A channels via the $G_{i\alpha}1$, $G_{i\alpha}2$, $G_{i\alpha}3$, and $G_{o\alpha A}$ CII G mutants. Open bars represent basal currents, and solid bars represent current in response to receptor stimulation. Numbers in parentheses refer to the number of cells recorded from for each experiment. Current density was measured at −100 mV.

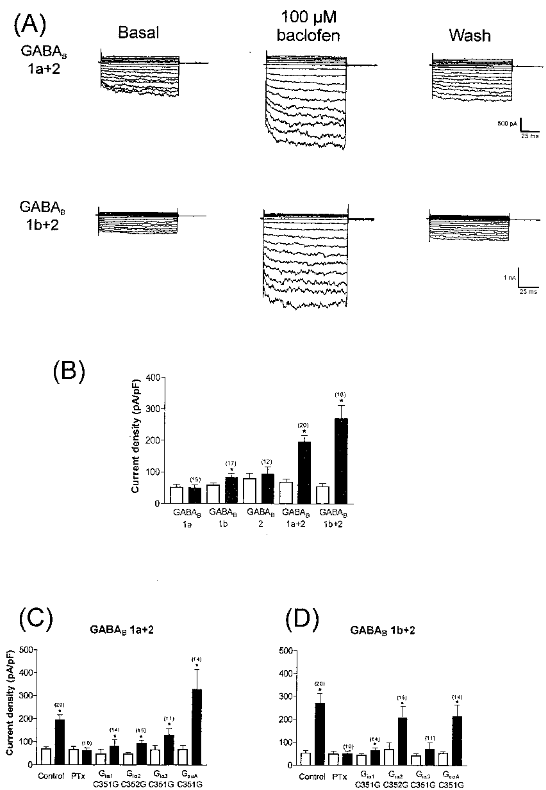

Fig. 4. The two forms of the $GABA_B$ receptor activate Kir3.1+3.2A channels through different $G_{i/o\alpha}$ subunits. (*A*) Stimulation of both the $GABA_B1a+2$ and the $GABA_B1b+2$ receptor (100 μM baclofen) led to robust activation of the Kir3.1+3.2A channels. Currents were elicited as described in Fig. 1*B*. (*B*) Summary of data obtained from expression of the $GABA_B1a$, -1b, and -2 subunits alone, and when expressed as the dimeric receptors $GABA_B1a+2$ and $GABA_B1b+2$. (*B* and *D*) Bar charts summarizing the data obtained with the $GABA_B1a+2$ receptor (*C*) and the $GABA_B1b+2$ receptor (*D*) when the mutant $G_{i/o\alpha}$ mutants were co-expressed in PTx-treated cells. Open bars represent basal currents, and solid bars represent current in response to receptor stimulation. Numbers in parentheses refer to the number of cells recorded from for each experiment. Current density was measured at −100 mV.

splice variants of $GABA_B1$, -1a, -1b, and -1c (46, 47). It has been reported that neither $GABA_B1a$, $GABA_B1b$, nor $GABA_B1c$ can express efficiently alone: the $GABA_B2$ subunit is required to form a functional heterodimeric receptor.

We first established the behavior of the cloned receptors in our system. We transiently expressed the $GABA_B$ splice variants $GABA_B1a$, -1b, or -2 alone and investigated whether these could activate the Kir3.1+3.2A channels. When either $GABA_B1a$ or $GABA_B2$ were expressed, we observed no stimulation of Kir3.1+3.2A currents in response to 100 μM baclofen. However, expression and stimulation of the $GABA_B1b$ splice variant did lead to an enhancement of Kir3.1+3.2A currents in approximately one-third of cells recorded from, revealing a small but significant enhancement of currents (Fig. 4*B*). We then expressed receptors comprised of $GABA_B1a+2$ and $GABA_B1b+2$ (1:1 cDNA ratio) and investigated their coupling to Kir3.1+3.2A. Stimulation of $GABA_B1a+2$ receptors significantly increased current density, and this was sensitive to PTx. Likewise, stimulation of receptors composed of $GABA_B1b+2$ subunits also potentiated currents in a PTx-sensitive fashion (Fig. 4 *A* and *B*).

The $GABA_B1a+2$ receptor was able to signal most prominently via $G_{o\alpha A}$C351G to Kir3.1+3.2A whereas signaling to the other G_α subunits was not so pronounced (Fig. 4*C*). Interestingly, $GABA_B1b+2$ was able to signal to an equal extent through both $G_{i\alpha 2}$C351G and $G_{o\alpha A}$C351G to a similar extent to that observed in control cells with coupling to endogenous G_α (Fig. 4*D*).

Discussion

The aims of the present study were to investigate the role of $G_{i/o\alpha}$ isoforms in coupling receptors to the G protein-gated inwardly rectifying K^+ channel. Specificity of this phenomenon could lie at two levels: in the ability of receptors to couple to various G_α subunits or in the ability of liberated $G_{\beta\gamma}$ from a particular heterotrimer to activate the channel. To address this, we have used a series of PTx-insensitive $G_{i/o}$ point mutants in which a cysteine residue four amino acids from the C terminus of the α subunit is replaced by a glycine or isoleucine residue. In all cases, we could always rescue signaling between any of the receptors tested and the Kir3.1+3.2A channels in PTx-treated cells, and we observed different patterns of preferences between the different receptors and G_α subunits.

The first question is, are these mutants good reporters of the coupling between receptor and channel? We investigated this quantitatively by constructing dose-response curves in a stable line expressing both the A_1 receptor and Kir3.1+3.2A channel complex. The data indicate that the EC_{50} for all G_α point mutants is similar and comparable to that displayed when the A_1 receptor couples to endogenous G proteins. The lack of a statistically significant change in the EC_{50} and the Hill coefficient for channel stimulation via the A_1 receptor is a strong result suggesting that the mutations, at these levels of expression, do

not affect the ability of the receptor to interact with the G protein or the innate ability of these different G protein heterotrimers to liberate $G_{\beta\gamma}$ for Kir3.1+3.2A channel activation.

Are all $G_{i/o\alpha}$ subunits, through the liberation of $G_{\beta\gamma}$, able to activate the channel to a similar extent? Schreibmayer *et al.* (36) showed inhibition of $G_{\beta1\gamma2}$-induced currents by activated $G_{i\alpha1}$ (but not $G_{i\alpha2}$ or $G_{i\alpha3}$) added as purified proteins to inside-out patches containing cloned or native Kir3.x channels. The significance of this for receptor-mediated activation was not addressed, the implication being that stimulation would not occur through liberation of $G_{\beta\gamma}$ from $G_{i\alpha1}$ heterotrimers due to simultaneous inhibition by the G_{α} subunit. Our studies suggest that it is not only possible to activate the channel via $\beta\gamma$ released from G_{i1} heterotrimers but that there are only moderate quantitative differences between the variants of $G_{i/o}$ in the ability to mediate activation of the channel. The most profound response observed with $G_{i\alpha1}$C351G was in its coupling to the α_{2A} receptor (mean current density: 211.51 ± 59.53 pA/pF, n = 10), a response not dissimilar to those observed with other $G_{i/o\alpha}$/receptor combinations (α_{2A}/$G_{i\alpha2}$C352G: 384.07 ± 113.31 pA/pF, n = 10, P = 0.19; A_1/$G_{i\alpha3}$C351G: 263.85 ± 73.56 pA/pF, n = 13, P = 0.61; $GABA_B$1a+2/$G_{o\alpha A}$C351G: 329.17 ± 86.69 pA/pF, n = 14, P = 0.32), suggesting that all $G_{i/o\alpha}$ variants can mediate channel activation to a similar extent.

To summarize, the $G_{i/o\alpha}$ point mutants are good reporters of receptor/channel coupling, and there are only moderate differences in the efficacy of their ability to activate currents from liberation of $G_{\beta\gamma}$. Thus, any major quantitative differences in the coupling pattern between $G_{i/o\alpha}$ variants and a particular $G_{i/o}$-coupled receptor is likely to be attributable to differences in the ability of the receptor to "talk" to a particular variant. Indeed, this experimental approach may be a useful model system to assay the specificity of such interactions.

It is apparent that the receptors we have studied exhibit different patterns of coupling to $G_{i/o\alpha}$ subunits to activate Kir3.1+3.2A channels. The receptors we investigated were the A_1 adenosine receptor, the α_{2A} adrenergic receptor, the D_{2S} dopaminergic receptor, the M_4 muscarinic receptor, and the two subtypes of the $GABA_B$ receptor, $GABA_B$1a+2 and $GABA_B$1b+2. The A_1 receptor seemed to couple almost equally well to all $G_{i/o\alpha}$ subunits, although $G_{i\alpha1}$ appeared to be not quite as efficient. This is consistent with previous studies showing that recombinant A_1 receptors have been shown to interact equally well with $G_{i\alpha1-3}$ (41). Differences do occur across species however, notably between human and bovine A_1 receptors (48).

A completely different pattern was observed with the α_{2A} adrenergic receptor. In other studies examining the coupling of α_{2A} to $G_{i/o}$ proteins, it was found to equally activate $G_{i\alpha1-3}$ (40). However, we found that $G_{i\alpha3}$ was much less effective than the other G_{α} subunits. Our observation that the D_{2S} dopamine receptor couples exclusively to $G_{o\alpha A}$ but not any of the G_{α} subunits is an interesting one and one that is in contrast to some studies by other investigators. A point to note is the disagreement between different studies concerning D_{2S} and G_{α} coupling. Some investigators report that D_{2S} couples to both $G_{i\alpha2}$ and $G_{i\alpha3}$ (33), others report selective coupling to $G_{i\alpha2}$ (34), and others report preferred coupling to $G_{i\alpha1}$ rather than $G_{i\alpha2}$ (35). We found that the M_4 muscarinic receptor, in agreement with previous findings (49), preferentially coupled to the $G_{i\alpha2}$ subunit. Some coupling to $G_{i\alpha3}$ was also observed, but this was less effective. A number of issues should be considered when comparing these differences and similarities. Studies done by other groups have been concerned with effector mechanisms linked to the G_{α} subunit: e.g., the inhibition of forskolin- or receptor-stimulated cAMP accumulation or agonist-induced [^{35}S]GTPγS binding. In contrast, the reporter system we are using (i.e., Kir3.1+3.2A channel activation) is one that is mediated directly by the $G_{\beta\gamma}$ subunit. In addition, there is less amplification in this system than, for example, looking at cAMP accumulation. Furthermore, we have made a comparative study of a much larger number of $G_{i/o\alpha}$ family members ($G_{i\alpha1-3}$ and $G_{o\alpha A}$) whereas, in some of the studies above, only a select number were examined.

A further finding is the different G protein coupling profiles observed with two different heterodimers of $GABA_B$ receptors. The $GABA_B$ receptors belong to the class 3 family of seven-pass receptors and as such share most homology with the metabotropic glutamate receptors. Members of this family have extended N termini that bear some sequence similarity to periplasmic amino acid binding proteins found in bacteria (50) that are thought to be involved in ligand binding (51, 52) but not G protein coupling (53). The splice variants $GABA_B$1a and $GABA_B$1b differ by a 117-aa stretch that is missing at the N terminus of $GABA_B$1b: this region contains amino acid motifs, termed "Sushi repeats," thought to be involved in protein-protein interactions (54). The regions involved in coupling the metabotropic glutamate receptors to G proteins are thought to be the second and third intracellular loops (55–57), so it might be likely that the same applies to $GABA_B$ receptors (58). However, we have clearly shown that $GABA_B$1a+2 receptors have a different G_{α} coupling profile to the $GABA_B$1b+2 receptors. This is unlikely to be attributable to differences in binding affinity of the splice variants for baclofen because the two variants have identical pharmacological agonist/antagonist profiles (58). The most likely mechanism we envisage is that the agonist-occupied active state is different between the two splice variants and that this is reflected by different conformation and G protein preferences in the receptor/G_{α} coupling domain, despite the only differences between the two receptor splice variants being at the proximal N terminus. However, we cannot exclude differences in trafficking or the possibility that one, but not the other, splice variant may interact with a protein that influences the above functional property.

In conclusion, we have examined the role of different $G_{i/o}$ isoforms in coupling receptors to the activation of G protein-gated inwardly rectifying K^+ channels. Our data indicate that PTx-insensitive point mutants of $G_{i/o}$ are able to report these interactions meaningfully and that there are only minor differences in the ability of these variants to activate the channel. Different receptors appear to prefer different G_{α} subunits to couple to the channels: indeed, the N-terminal splice variants of the $GABA_B$ heterodimeric receptor show different patterns of selectivity. Thus, we have revealed a mechanism for selective receptor activation of the channel that lies at the interface between the receptor and $G_{i/o}$ variant. Differential or localized expression of $G_{i/o}$ variants (59) could lead to selective pathways of channel activation.

We thank the following people for providing us with cDNAs: B. Conklin ($G_{i\alpha2}$, $G_{i\alpha3}$, $G_{o\alpha A}$), L. Y. Jan (α_{2A} receptor and Kir3.1), M. Lazdunski (Kir3.2A), F. Marshall ($GABA_B$ clones), G. Milligan, ($G_{i\alpha1}$C351G and $G_{i\alpha1}$C351I), T. Palmer (A_1 receptor), E. G. Peralta (M_4), and W. Xu (D_{2S} receptor) and the members of the Human Frontiers Science Program collaboration for fruitful discussions (L. Y. Jan, Y. Kurachi, and E. Reuveny). We also thank G. Milligan for extensive discussions concerning the PTX-insensitive variants, Z. Hafeez and other members of the lab for technical help and discussion, and L. Clapp for helpful comments. This work was supported by the Human Frontiers Science Program and the Wellcome Trust.

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