G\(\beta\gamma\)-activated inwardly rectifying K\(^+\) (GIRK) channels have distinct gating properties when activated by receptors coupled specifically to \(G_\alpha_i\) versus \(G_\alpha_o\) subunit isoforms, with \(G_\alpha_o\)-coupled currents having a 3-fold faster agonist-evoked activation kinetics. To identify the molecular determinants in \(G_\alpha\) subunits mediating these kinetic differences, chimeras were constructed using pertussis toxin (PTX)-insensitive \(G_\alpha_o\) and \(G_\alpha_i\) mutant subunits (\(G_\alpha_o\text{AC351G}\) and \(G_\alpha_i\text{AC352G}\)) and examined in PTX-treated Xenopus oocytes expressing m\(2\) receptors and Kir3.1/3.2a channels. These experiments revealed that the \(\alpha\)-helical N-terminal region (amino acids 1–161) and the switch regions of \(G_\alpha\) (amino acids 162–262) both partially contribute to slowing the GIRK activation time course when compared with the \(G_\alpha_o\text{AC351G}\)-coupled response. When present together, they fully reproduce \(G_\alpha_o\text{AC352G}\)-coupled GIRK kinetics. The \(G_\alpha_o\) C-terminal region (amino acids 263–355) had no significant effect on GIRK kinetics. Complementary responses were observed with chimeras substituting the \(G_\alpha_o\) switch regions into the \(G_\alpha_i\text{AC352G}\) subunit, which partially accelerated the GIRK activation rate. The \(G_\alpha_o\)/\(G_\alpha_i\) chimera results led us to examine an interaction between the \(\alpha\)-helical domain and the Ras-like domain previously implicated in mediating a 4-fold slower in vitro basal GDP release rate in \(G_\alpha_i\) compared with \(G_\alpha_o\). Mutations disrupting the interdomain contact in \(G_\alpha_i\text{AC352G}\) at either the \(\alpha\)-b-\(\alpha\)E loop (R145A) or the switch III loop (L233Q/E240T/M241T), significantly accelerated the GIRK activation kinetics consistent with the \(G_\alpha_i\) interdomain interface regulating receptor-catalyzed GDP release rates in vivo. We propose that differences in \(G_\alpha_i\) versus \(G_\alpha_o\)-coupled GIRK activation kinetics are due to intrinsic differences in receptor-catalyzed GDP release that rate-limit \(G\beta\gamma\) production and is attributed to heterogeneity in \(G_\alpha\) and \(G_\alpha\) interdomain contacts.

Cardiac and neuronal \(G\beta\gamma\)-gated inwardly rectifying K\(^+\) channels (GIRKs) are activated by G protein-coupled receptors (GPCRs) selectively coupled to pertussis toxin (PTX)-sensitive \(G_\alpha_o\)/\(G_\beta\gamma\) proteins (1, 2). The time course for GIRK channel activation elicited by application of receptor agonist can be influenced by the multiple intervening steps of the G protein cycle that begin with agonist binding to the GPCR, and end with \(G\beta\gamma\) binding to the GIRK channel subunits that promote gating transitions to the open state. In addition to the G protein activation steps, signal termination with GTP hydrolysis by the \(G_\alpha\) subunit and \(G\beta\gamma\) reassociation also impacts the kinetics and amplitude of agonist-activated GIRK currents. The ternary complex consisting of agonist, GPCR, and G protein influences the time course of agonist-elicted GIRK channel currents (3), supporting the notion that isoform composition of different GPCR-\(G_\alpha_i\)/\(G_\beta\gamma\) protein-RGS protein-GIRK channel signaling complexes have different kinetic properties that affect their functional output (4).

We recently reported notable differences in the gating properties of GIRK channels activated by muscarinic m\(2\) receptors coupled specifically to PTX-insensitive \(G_\alpha_o\) isoforms (\(G_\alpha_o\text{i1}, G_\alpha_o\text{o}o\), or \(G_\alpha_o\text{r}A\)) versus \(G_\alpha_i\) isoforms (\(G_\alpha_i\text{A}o\) or \(G_\alpha_i\text{A}o\)) in the Xenopus oocyte system (4). The ACh-elicted activation time course for \(G_\alpha_i\)-coupled GIRK currents was a 3-fold faster than for \(G_\alpha_o\)-coupled GIRK currents, and \(G_\alpha_o\) expression was significantly more effective than the \(G_\alpha_i\) isoforms at reducing receptor-independent basal GIRK channel activity. To identify the molecular determinants responsible for these differences, we constructed several PTX-insensitive \(G_\alpha_o\)/\(G_\alpha_i\) chimeras and examined their functional properties in the Xenopus oocyte system via coupling to muscarinic m\(2\) receptors and GIRK channels. The \(G_\alpha_o\)/\(G_\alpha_i\) chimera experiments indicate the kinetic differences between \(G_\alpha_o\) and \(G_\alpha_i\) involve an interdomain interaction that led us to examine a previously identified domain-domain contact in \(G_\alpha_i\) that slows the basal (receptor-independent) GDP release rate compared with \(G_\alpha_o\) in vitro (5). \(G_\alpha_o\) mutations designed to disrupt the domain-interdomain interaction accelerated GIRK activation rates consistent with the biochemical studies of mutant \(G_\alpha_i\) subunits that increased basal GDP release rates (5). Thus our findings establish experimental conditions whereby the GPCR-catalyzed \(G_\alpha\) GDP release rate is the rate-limiting step in receptor-dependent GIRK activation as originally proposed by Breitwieser and Szabo (6) for \(I_{K_ACh}\) in atrial myocytes, and importantly identifies molecular determinants that mediate functional differences in GIRK channels activated by GPCRs coupled to \(G_\alpha_i\) and \(G_\alpha_o\) proteins that may impact the kinetics and magnitude of inhibitory GIRK channel-mediated postsynaptic currents (7).

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\(^{1}\) The abbreviations used are: GIRK, \(G\beta\gamma\)-activated inwardly rectifying K\(^+\) channel; GPCR, G protein-coupled receptor; PTX, pertussis toxin; ACh, acetylcholine.
FIG. 1. Construction of PTX-insensitive G_{i/o}A chimera subunits. 

A. Amino acid sequence alignment of G_{i1}, G_{i2}, G_{i3}, G_{oA}, and G_{oB} subunits. The asterisk (*) at the conserved cysteine residue in the C-terminal tail (−4 position) denotes the site of PTX-mediated ADP-ribosylation. All G_{i/o} subunits and chimeras used in this study were rendered PTX insensitive by mutating the −4 cysteine to a glycine residue (Cys → Gly mutation). Black arrows (↓) indicate G_{i2}/G_{i3} junction sites in the chimeras. The red residues in the G_{o} sequences denote non-conserved amino acid differences with the three G_{i} subunits. Secondary structures (α-helices, bars; β-sheets, arrows) are aligned with the amino acid sequences and are based on the G_{i1} crystal structure (8). The color coding highlights the three major structural domains of the G_{i} subunit; the αN domain.
**Determinants Mediating Go-specific GIRK Activation Kinetics**

| Targeted Go coding region | Primer | Primer Sequence (5′→3′) | T<sub>a</sub> | Features |
|---------------------------|--------|-------------------------|-------------|----------|
| amino acids               |        |                         |             |          |
| Go<sub>G</sub>(1–161)     | 1      | F-CTTTAATAGGCGCTGACCTATAAG |             |          |
| Go<sub>G</sub>(162–282)   | 2      | R-GGAGACCTGCCGGAATGCTTTC | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(161–355)   | 3      | F-GGACAGCCTCGAGCGTGTACGCGGCACT |             |          |
| Go<sub>G</sub>(161–272)   | 4      | R-CTTTAATAGGCGCTGACCTATAAG | 56          | targets native Go<sub>G</sub> XhoI site |
| Go<sub>G</sub>(172–355)   | 5      | F-GGACAGCCTCGAGCGTGTACGCGGCACT |             |          |
| Go<sub>G</sub>(136–355)   | 6      | R-CTTTAATAGGCGCTGACCTATAAG | 56          | targets native Go<sub>G</sub> XhoI site |
| Go<sub>G</sub>(1–160)     | 7      | F-GGACAGCCTCGAGCGTGTACGCGGCACT |             |          |
| Go<sub>G</sub>(162–272)   | 8      | R-CTTTAATAGGCGCTGACCTATAAG | 56          | targets native Go<sub>G</sub> XhoI site |
| Go<sub>G</sub>(161–355)   | 9      | F-GGACAGCCTCGAGCGTGTACGCGGCACT |             |          |
| Go<sub>G</sub>(172–355)   | 10     | R-CTTTAATAGGCGCTGACCTATAAG | 56          | targets native Go<sub>G</sub> XhoI site |
| Go<sub>G</sub>(136–355)   | 11     | F-GGACAGCCTCGAGCGTGTACGCGGCACT |             |          |
| N-terminal fragment       |        |                         |             |          |
| Go<sub>G</sub>(C<sub>B</sub>) | 12     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| C-terminal fragment       |        |                         |             |          |
| Go<sub>G</sub>(C<sub>B</sub>) | 13     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(1–145)     | 14     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(145–355)   | 15     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(1–160)     | 16     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(162–272)   | 17     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(161–355)   | 18     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(172–355)   | 19     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |
| Go<sub>G</sub>(136–355)   | 20     | F-CTTTAATAGGCGCTGACCTATAAG | 56          | targets T7 promoter of pCI vector |

*F*, forward; *R*, reverse; underlined sequences denote restriction enzyme recognition site.

**EXPERIMENTAL PROCEDURES**

**Design and Construction of PTX-insensitive Go<sub>A</sub>/Go<sub>A</sub> Chimeras**—Fig. 1A shows the amino acid sequence alignment of all five Go<sub>A</sub> isoforms with corresponding secondary structures determined from crystallographic studies of Go<sub>A</sub> (8). PTX-insensitive Go<sub>A</sub>/Go<sub>A</sub> chimeras. Go<sub>A</sub> subunits are comprised of three major domains: 1) an N-terminal α-helix (αN) that contains lipophilic residues for membrane attachment and mediates in part GPCR and G<sub>βγ</sub> interactions, 2) a closely packed α-helical domain (αHD) that encloses the guanine nucleotide binding cleftued and is thought to regulate GDP/GTP exchange rates, and 3) a Ras-like domain that binds guanine nucleotides at highly conserved “switch regions” to regulate conformational changes effecting Gβγ association and dissociation (9). Six different PTX-insensitive Go<sub>A</sub>/Go<sub>A</sub> chimeras were constructed exchanging three major regions: 1) the combined αN-αHD region (with the exception of the eA-eR linker and eP), 2) the three switch regions (SWI-III) located within the Ras-like domain, and 3) the carboxyl remaining elements of the Ras-like domain (Fig. 1, A and C). The Go<sub>A</sub>C<sub>B</sub>, or Go<sub>A</sub>C<sub>B</sub>, sequence corresponding to these three swapped regions is indicated in the naming of each constructed chimera; e.g., Go<sub>A</sub>C<sub>B</sub>, Go<sub>A</sub>C<sub>B</sub>, Go<sub>A</sub>C<sub>B</sub>, Go<sub>A</sub>C<sub>B</sub>, (Fig. 1C). The two junction sites within the Go<sub>A</sub>C<sub>B</sub> region correspond to Glu<sup>161</sup>/Arg<sup>162</sup> and Arg<sup>162</sup>/Thr<sup>163</sup> (Fig. 1A). Non-conserved amino acid differences between Go<sub>A</sub> and Go<sub>A</sub> isoforms are heavily biased in the αHD region although differences exist in all major structural domains (see Fig. 1A).

The PTX-insensitive Go<sub>A</sub>/Go<sub>A</sub> chimeras were constructed by PCR amplification (Vent DNA polymerase, New England Biolabs) of selected regions of the rat Go<sub>A</sub>C<sub>B</sub> and mouse Go<sub>A</sub>C<sub>B</sub> cDNAs cloned in the pCI vector (kindly provided by Stephen Ikeda, NIAAA, National Institutes of Health). Existing or new restriction sites flanking targeted regions were introduced with oligonucleotide primers that preserved the desired coding sequence (Fig. 1B). The various PCR products were gel-purified, digested with appropriate restriction enzymes, and ligated (T4 ligase, Promega) into the pcDNA3.1(+) cloning vector (Invitrogen).

All primers used in the construction of the Go<sub>A</sub>/Go<sub>A</sub> chimeras are provided in Table 1. The full-length Go<sub>A</sub>/Go<sub>A</sub> chimeras were confirmed by automated DNA sequencing (Molecular Biology Core Facility, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL).  

**Isolation and cRNA Injection of Xenopus Oocytes—**All procedures for the use and handling of Xenopus laevis (Xenopus Express, Plant City, FL) were approved by the University of South Florida Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Methods to enzymatically isolate and culture oocytes were as described elsewhere (4). Stage V-VI oocytes were maintained at 19°C in oocyte culture medium (OCM) consisting of 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 1.0 mM NaHPO<sub>4</sub>, 5.0 mM Hepes, 2.5 mM Na pyruvate, and 2% heat-inactivated horse serum, at pH 7.5 (NaOH).

Mixtures of cRNAs were injected the day after oocyte isolation at a final injection volume of 50 nl (Nanoliter2000, World Precision Instruments). Each 50-nl mixture consisted of cRNA encoding the rat Kir3.1 (Kir<sub>3.1</sub>) and mouse G<sub>A</sub>C<sub>B</sub> subunits. Rat and mouse Kir<sub>3.1</sub> cDNA templates. Kir<sub>3.1</sub> subunits were initially superfused with a minimal salt solution composed of 98 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 1.0 mM NaHPO<sub>4</sub>, 5.0 mM Hepes, 2.5 mM Na pyruvate, and 2% heat-inactivated horse serum, at pH 7.5 (NaOH). The switch to high K<sup>+</sup>-specific GIRK Activation Kinetics (29789)
FIG. 2. Receptor-independent and receptor-dependent GIRK channel activity associated with expression of PTX-insensitive \( \text{Go}_{\alpha}/\text{Go}_{\alpha} \) chimeras. Receptor-independent (\( I_{K,basal} \) open bars) and receptor-dependent (\( I_{K,ACH} \), filled bars) GIRK current amplitudes with expression of PTX-insensitive \( \text{Go}_{\alpha}/\text{Go}_{\alpha} \) chimeras in comparison to the parental PTX-insensitive \( \text{Go}_{\alpha/A352G} \) (HD) and \( \text{Go}_{\alpha/AC351G} \) (GoA) subunits. \( I_{K,basal} \) was evoked by application of 20 mM K\(^+\) solution and \( I_{K,ACH} \) evoked by a saturating concentration of ACh (10 \( \mu \)M) in 20 mM K\(^+\) solution. Values are the mean \( \pm \) S.E. (\( n \geq 8 \)).

consisting mostly of basal receptor-independent GIRK current (\( I_{K,basal} \)). To evoke receptor-dependent GIRK currents (\( I_{K,ACH} \)), a computer-controlled superfusion system (SF-77B, Warner Instruments) was used to rapidly apply and washout various concentrations of acetylcholine (ACh, Sigma-Aldrich) in high K\(^+\) solution (11). All recordings were performed at room temperature (21–23 °C).

Data Analysis—GIRK current kinetics were analyzed using non-linear curve fitting software that fit single exponential functions to derive the activation time constant (\( \tau_{a} \)) and the deactivation time constant (\( \tau_{d} \)) (pCLAMP software, Axon Instruments). ACh dose-response relations were analyzed by fitting peak \( I_{K,ACH} \) amplitudes with the Hill function shown in Equation 1,

\[
\frac{I_{K,ACH}}{I_{basal}} = \frac{1}{1 + \left(\frac{[ACh]}{EC_{50}}\right)^{n_{H}}} (\text{Eq. 1})
\]

where the ACh concentration producing a 50% response (\( EC_{50} \)) and the Hill coefficient (\( n_{H} \)) were derived from a non-linear least-squares best fit (Origin 6.0 software, OriginLab Corp., Northampton, MA). Statistical comparisons between the various experimental groups were performed by one-way analysis of variance where \( p < 0.05 \) was considered significant. Each experiment was replicated in oocytes derived from at least two separate oocyte batches (dissections).

RESULTS

Functional Expression of \( \text{Go}_{\alpha}/\text{Go}_{\alpha} \) Chimeras—Each of the six PTX-insensitive \( \text{Go}_{\alpha}/\text{Go}_{\alpha} \) chimeras effectively rescued m2 receptor-activated GIRK currents in PTX-S1-expressing oocytes (Fig. 2). PTX-mediated uncoupling of endogenous Go\(_{\alpha/o}\) subunits (>95%) was confirmed in parallel groups of oocytes that did not receive RNA encoding a PTX-insensitive Go subunit (data not shown), indicating the ACh-activated GIRK currents were evoked via coupling to the PTX-insensitive Go subunit. As reported previously (4), expression of the parental \( \text{Go}_{\alpha/A351G} \) subunit produced smaller \( I_{K,basal} \) amplitudes and larger receptor-activated currents (\( I_{K,ACH} \)) compared with \( \text{Go}_{\alpha/C352G} \) and \( \text{Go}_{\alpha/AC351G} \) subunits. Expression of each of the PTX-insensitive Go\(_{\alpha/o}\)/Go\(_{\alpha} \) chimeras yielded \( I_{K,basal} \) and \( I_{K,ACH} \) amplitudes that ranged between the properties of the \( \text{Go}_{\alpha/A351G} \) and \( \text{Go}_{\alpha/C352G} \) subunits (Fig. 2). Since each chimera was expressed under identical conditions (see “Experimental Procedures”), and expression of the parental \( \text{Go}_{\alpha/A351G} \) and \( \text{Go}_{\alpha/C352G} \) subunits produce equivalent protein levels (4), the differences in receptor-independent and receptor-dependent GIRK channel activity with the different \( \text{Go}_{\alpha/o}/\text{Go}_{\alpha} \) chimeras is not readily attributable to differences in Go subunit protein levels but instead to differences in intrinsic Go function. Moreover, the results demonstrate that each \( \text{Go}_{\alpha/o}/\text{Go}_{\alpha} \) chimera expresses a functional PTX-insensitive Go subunit capable of m2 receptor coupling, receptor-catalyzed GDP/GTP exchange, endogenous G\( \beta \gamma \) association/dissociation, and intrinsic GTPase activity.

Regions of \( \text{Go}_{\alpha} \) That Accelerate ACh-evoked GIRK Activation Kinetics—The time course for ACh-elicited GIRK current activation and the ACh dose-dependence varied among the six \( \text{Go}_{\alpha/o}/\text{Go}_{\alpha} \) chimeras tested, yet ranged between the properties of the parental \( \text{Go}_{\alpha/A351G} \) and \( \text{Go}_{\alpha/C352G} \) subunits as anticipated. Replacing either the \( \alpha \)N/o/HD region of \( \text{Go}_{\alpha/C352G} \) with the corresponding \( \alpha \)N/o (\( \text{Go}_{\alpha} \) chimera), or replacing the Go\(_{\alpha} \) switch regions with Go\(_{\alpha} \) switch regions (\( \text{Go}_{\alpha} \) chimera), both accelerated the GIRK activation time course compared with \( \text{Go}_{\alpha/A351G} \)-coupled responses (Fig. 3A). The effects of each \( \text{Go}_{\alpha} \) region substitution, however, was only partial and did not fully reconstitute the activation kinetics of the \( \text{Go}_{\alpha/A351G} \)-coupled response. The effects on GIRK activation kinetics were also reflected in the steady-state ACh dose-response curves, where \( \text{Go}_{\alpha} \)-coupled GIRK responses were leftward shifted and more characteristic of \( \text{Go}_{\alpha/A351G} \)-coupled GIRK currents than \( \text{Go}_{\alpha/C352G} \)-coupled GIRK currents (Fig. 3A). Differences in \( EC_{50} \) values with \( \text{Go}_{\alpha/C352G} \) coupling (0.43 \( \pm \) 0.07 \( M \), \( n = 9 \)) versus \( \text{Go}_{\alpha/A351G} \) coupling (0.21 \( \pm \) 0.05 \( M \), \( n = 10 \)) are small yet significantly different (Fig. 4B) and have comparable Hill coefficients (\( Go_{\alpha/C352G} \) 1.11 \( \pm \) 0.04; \( Go_{\alpha/A351G} \) 1.44 \( \pm \) 0.07). Replacing the HD region with the parental Go\(_{\alpha} \) carboxyl region with the \( \text{Go}_{\alpha/A351G} \) carboxyl region (\( \text{Go}_{\alpha} \) chimera) had no significant effect on GIRK activation kinetics or the ACh dose-response curve, and was indistinguishable from the \( \text{Go}_{\alpha/C352G} \)-coupled GIRK channel (Fig. 3A). Thus isoform differences in the last 92 residues do not mediate the kinetic differences between \( \text{Go}_{\alpha/C352G} \)-coupled and \( \text{Go}_{\alpha/A351G} \)-coupled GIRK currents (12).

Regions of \( \text{Go}_{\alpha} \) That Slow GIRK Activation Kinetics—For the reciprocal set of experiments where \( \text{Go}_{\alpha/C352G} \) regions replaced the equivalent regions of \( \text{Go}_{\alpha/A351G} \), only substituting the Go\(_{\alpha} \) switch regions (\( \text{Go}_{\alpha} \) chimera) caused a significant yet partial slowing in the GIRK activation kinetics (Fig. 3B). Neither the Go\(_{\alpha} \) \( \alpha \)N/o/HD region (\( \text{Go}_{\alpha} \) chimera) nor the carboxyl region (\( \text{Go}_{\alpha} \) chimera) significantly slowed the GIRK activation characteristics of \( \text{Go}_{\alpha/A351G} \)-coupled responses. Yet the combined presence of both the Go\(_{\alpha} \) \( \alpha \)N/o/HD region plus the Go\(_{\alpha} \) switch regions (\( \text{Go}_{\alpha} \) chimera), was sufficient to fully reproduce the slower \( \text{Go}_{\alpha/C352G} \)-coupled GIRK activation kinetics (Fig. 3A). Similarly, the combined presence of the Go\(_{\alpha} \) \( \alpha \)N/o/HD region plus the switch regions (\( \text{Go}_{\alpha} \) chimera) was sufficient to fully reproduce the faster \( \text{Go}_{\alpha/A351G} \)-coupled GIRK activation kinetics (Fig. 3B). Thus in both cases the \( \alpha \)N/o/HD region plus the switch regions are necessary and sufficient to fully reproduce the parental Go subunit effects on GIRK activation kinetics.

Effects of \( \text{Go}_{\alpha}/\text{Go}_{\alpha} \) Chimeras on Basal GIRK Channel Activity—In addition to receptor-dependent GIRK activation kinetics, the receptor-independent basal GIRK channel activity \( I_{K,basal} \) associated with expression of each \( \text{Go}_{\alpha}/\text{Go}_{\alpha} \) chimera was also examined. A reduction in \( I_{K,basal} \) caused by Go subunit expression in parallel with an increased \( I_{K,ACH} \) amplitude, is largely a consequence of Ga sequestration of free endogenous G\( \beta \gamma \) dimers and increased heterotrimeric Ga\(_{\alpha}/\text{GDP}\gamma \) formation (4, 13). For comparisons among the different \( \text{Go}_{\alpha}/\text{Go}_{\alpha} \) chimeras, \( I_{K,basal} \) was expressed as the fraction of the total GIRK current elicited from each oocyte.
Determinants Mediating Ga-specific GIRK Activation Kinetics

\( I_{\text{K, total}} = I_{\text{K, basal}} + \text{maximal } I_{\text{K, CAMP}} \). As reported previously (4), \( G_a(G_{\alpha A(C351G)}) \) reduced \( I_{\text{K, basal}} \) amplitudes significantly greater than \( G_a(G_{\alpha A(C352G)}) \) under equivalent expression conditions, with \( I_{\text{K, basal}} \) representing \(-10\% \) of the total GIRK current with \( G_a(G_{\alpha A(C351G)}) \) expression compared with \(-45\% \) with \( G_a(G_{\alpha A(C352G)}) \) expression (Fig. 4A). The fractional \( I_{\text{K, basal}} \) amplitudes for each of the \( G_{\alpha A/(G_{\alpha A})} \) chimeras indicated a significant effect of the switch region substitutions (Fig. 4A). Expression of the \( G_{\alpha A} \) chimera produced a significantly lower fractional \( I_{\text{K, basal}} \) amplitude \((22 \pm 4\% \), \( n = 6 \) \) compared with the parental \( G_a(G_{\alpha A(C352G)}) \) expression \((45 \pm 6\% \), \( n = 9 \) \). Conversely, expression of \( G_{\alpha o} \) significantly increased the fractional \( I_{\text{K, basal}} \) amplitude to \( 28 \pm 6\% \) \( (n = 9) \) compared with \( 11 \pm 1\% \) \( (n = 10) \) for parental \( G_a(G_{\alpha A(C351G)}) \) expression (Fig. 4A). Again, the effects of the individual domain substitutions were partial, where substitutions of both the \( \alpha N/oHD \) region plus the switch regions \( (G_{\alpha iu} \) and \( G_{\alpha ou} \)) constituted the parental \( I_{\text{K, basal}} \) amplitude (Fig. 4A). Interestingly, the effects of the switch regions on \( I_{\text{K, basal}} \) correlate well with the effects on receptor-evoked GIRK activation kinetics (Fig. 4C), with the exception of the \( G_{\alpha oA(oN/oHD} \) region chimera \( (G_{\alpha ou}) \), which displayed a slightly accelerated receptor-evoked GIRK activation time course without significantly affecting the fractional \( I_{\text{K, basal}} \) amplitude.

The deactivation time course of GIRK currents after rapid ACh washout, a process dependent on Ga GTPase activity, was previously found to be similar among all the PTX-insensitive \( G_a \) isoforms (4). However, in the current set of experiments, \( G_a(G_{\alpha A(C351G)}) \) expression \((\tau_{\text{deact}} = 14.7 \pm 0.4 \text{ s}, n = 10) \) was different \((p < 0.05) \) from the deactivation time constant derived with \( G_a(G_{\alpha A(C352G)}) \) expression \((\tau_{\text{deact}} = 23.2 \pm 1.6 \text{ s}, n = 9) \). Introducing either the \( G_{\alpha i2} \) \( \alpha N/oHD \) region \( (G_{\alpha ou}) \) or the switch regions \( (G_{\alpha ou}) \) into \( G_a(G_{\alpha A(C351G)}) \) was sufficient to convert the deactivation time constant to a \( G_a(G_{\alpha A(C352G)}) \)-like value (Fig. 4D).

**DISCUSSION**

The goal of this study was to identify molecular determinants in \( G_{\alpha o} \) and/or \( G_{\alpha a} \) subunits that mediate differences in GIRK channel gating kinetics associated with selective Ga-GPCR coupling (4). Our \( G_{\alpha d}/G_{\alpha o} \) chimera results implicated an interdomain interface previously linked to \( G_{\alpha o}/G_{\alpha a} \) isoform differences in basal GDP release rates (5, 14). They also ruled out the possible role of the carboxyl region known to be involved in GPCR coupling and previously implicated in \( G_{\alpha d}/G_{\alpha o} \) differences in nucleotide exchange (12). Isomorph differences in basal GDP release rate among \( G_{\alpha a} \) and \( G_{\alpha o} \) subunits (14) correlate well with the kinetic profile we observed with receptor-activated GIRK currents coupled to individual PTX-insensitive \( G_{\alpha o} \) and \( G_{\alpha a} \) subunits (4). That is, the three \( G_{\alpha} \) isoforms that have a significantly slower basal GDP release rate compared with \( G_{\alpha o} \) subunits in vitro (14), also produce slower receptor-activated GIRK currents compared with \( G_{\alpha a} \) subunits in vivo (4). Thus, these findings are consistent with the rate-limiting step in GPCR activation of GIRK channels being receptor-catalyzed GDP release from associated \( G_{\alpha o} \) subunits (6).

The αd-Helix Switch III Interaction in Ga Subunits and Receptor-catalyzed GDP Release—Domain-domain interactions involving the αd-αe loop and the switch III loop have previously been implicated in regulating basal and receptor-activated nucleotide exchange for different Ga subunits (5, 15–18). Indeed, inherited mutations in the human \( G_{\alpha i} \) switch III domain enhance GDP release and cause Albright hereditary osteodystrophy characterized by skeletal and developmental abnormalities (19). Mutational analysis of \( G_{\alpha o} \) subunits indicates that substitution of \( G_{\alpha o} \) switch III residues (conserved in all three \( G_{\alpha} \) isoforms) into \( G_{\alpha a} \) impairs β-adrenergic receptor-mediated G protein activation (15), and substitution of corresponding \( G_{\alpha a} \) switch III residues into \( G_{\alpha o} \) (L232Q/E235H/E239T/M240T) speed up the basal GDP release rate similar to wild-type \( G_{\alpha o} \) (5). Together these findings indicate the conserved \( G_{\alpha o} \) switch III loop slows the rate of GDP release in \( G_{\alpha o} \) subunits through interactions with the α-helical domain, principally through hydrophobic interactions between Leu232 in switch III and Arg144 in the αd-αe loop (5). In our experiments, m2 receptor-activated GIRK currents coupled to \( G_{\alpha a}(G_{\alpha A(C351G)}) \) containing the \( G_{\alpha a} \) switch III loop (i.e. the \( G_{\alpha d}(G_{\alpha SW3}) \) chimera) displayed accelerated activation kinetics consistent with a faster Ga GDP release rate. Introducing the R145A mutation in the αd-αe loop of \( G_{\alpha a}(G_{\alpha A(C352G)}) \), to disrupt the domain-domain interaction, more dramatically accelerated the GIRK activation kinetics which were indistinguishable from \( G_{\alpha a}(G_{\alpha A(C351G)}) \)-coupled GIRK kinetics. Since the equivalent mutation in \( G_{\alpha a} \) (R144A) increases basal GDP release rates equivalent to the kinetics of wild-type \( G_{\alpha o} \) (5), the PTX-insensitive \( G_{\alpha d}(G_{\alpha A(R145A/C352G)}) \) subunit appears to similarly have faster receptor-stimulated GDP release rates compared with the parental \( G_{\alpha d}(G_{\alpha A(C352G)}) \) subunit as reflected in the GIRK activation time course. Since GIRK channels are activated by Gaβγ subunits, the different ACh-evoked GIRK activation time courses reflect differences in receptor-catalyzed production of Gaβγ subunits that are rate-limited by GDP release from the heterotrimeric Ga(GDP)βγ complex. The
**Fig. 3.** Receptor-dependent GIRK activation kinetics via coupling to PTX-insensitive $G_\alpha_i/G_\alpha_o$ chimeras. A. effects of introducing $G_\alpha_{oo}$ regions into $G_\alpha_{oo}$. Left panels, representative ACh-evoked GIRK currents from oocytes expressing PTX-insensitive $G_\alpha_{oo}$, $G_\alpha_{oi}$, and $G_\alpha_{io}$ subunits (red traces). GIRK current activated by m2 receptors coupled to $G_\alpha_{oo}G_\alpha_{oi}$ is superimposed (gray traces) on each $G_\alpha$ chimera-coupled response for comparison. The peak currents were normalized for kinetic comparisons. The horizontal bar above the traces indicate the 15 s period of ACh (1 $\mu$M) application. Middle panels, GIRK activation time constants ($\tau_{act}$) derived from exponential fits of the ACh-evoked GIRK current activation time course at four different ACh concentrations. Values are the mean ± S.E. ($n \geq 8$). Red symbols are values for the corresponding PTX-insensitive $G_\alpha_{oo}/G_\alpha_{oi}$ chimera indicated in the adjacent left panel. Activation time constants from $G_\alpha_{oo}G_\alpha_{oi}$-coupled GIRK currents (gray symbols) and $G_\alpha_{oo}G_\alpha_{oi}$-coupled GIRK currents (black symbols) are included for comparisons with the chimera values. Right panels, ACh dose-response relations with m2 receptor coupling to $G_\alpha_{oo}$, $G_\alpha_{oi}$, and $G_\alpha_{io}$ chimeras. GIRK current ($I_{K,ACH}$) amplitudes from the application of five different ACh concentrations were normalized to the maximal current ($I_{max}$) elicited within each oocyte (10 $\mu$M ACh). The mean values were fit with
much faster GTP binding step, Ga(GTP)-Gβγ dissociation step, and GIRK channel gating steps apparently do not affect the receptor-dependent GIRK activation time course under our expression conditions. Interestingly, native GIRK currents in hippocampal neurons activated by either GABA_B or adenosine A1 receptors in Go_0-knockout mice have significantly slower activation kinetics (7), analogous to the slower Go_i-coupled GIRK currents reconstituted in Xenopus oocytes. Thus Go_o versus Go_i-coupled GIRK currents clearly have distinct kinetics that could impact the kinetics of GIRK-mediated postsynaptic currents in neurons and atrial myocytes. Our findings reported here indicate this is attributable to the Go isoform differences in GDP release kinetics. Further swapping of isoform-specific residues in the switch III loop and HD will better refine and ultimately resolve all the key residues that contribute to the slower Go_i-coupled GIRK channel kinetics, including the switch III leucine residue (5).

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**FIG. 4.** Effects of PTX-insensitive Ga_o/Ga_i2 chimera expression on receptor-independent basal GIRK channel activity and receptor-evoked GIRK deactivation kinetics. **A**, receptor-independent basal GIRK channel activity associated with the expression of each PTX-insensitive Ga_o/Ga_i2 chimera. Left panel, effects of introducing Ga_o regions into Ga_i(C352G) (open bars) compared with the parental Ga_i(C352G) subunit (gray bar). I_{K,basal} amplitudes are expressed as a fraction of the total GIRK current (I_{K,basal} + I_{K,ACh}) elicited from each oocyte expressing the indicated Ga subunit. B, derived EC_{50} values from GIRK currents elicited by a range of ACh concentrations as shown in Fig. 3, via muscarinic m2 receptor coupling to each PTX-insensitive Ga_o/Ga_i2 chimera. C, receptor-dependent GIRK activation time constants (τ_{act}) associated with expression of each PTX-insensitive Ga_o/Ga_i2 chimera. Time constant values are derived from exponential fits of ACh-elicited GIRK current responses with maximal receptor stimulation (100 μM ACh). Data are also presented in Fig. 3 and included here for easier comparison with the basal GIRK activity and GIRK deactivation data. D, I_{K,ACh} deactivation time constants (τ_{deact}) with expression of each PTX-insensitive Ga_o/Ga_i2 chimera. I_{K,ACh} deactivation was assessed after washout of a 15 s application of 1 μM ACh. Values in all panels are the mean ± S.E. (n ≥ 8). Statistical comparisons were made between the chimera values and the corresponding parental subunit in each panel, with p ≤ 0.05 considered significant (*).
subunits in *Xenopus* oocytes, important for regulated oocyte maturation (20, 21). Exogenous expression of Go subunits effectively reduce I$_{K,basal}$ by sequestering the free G$\beta$y subunits, and increasing the pool of GoGDP$\beta$y complexes available for receptor-dependent GIRK channel activation (13). Expression of PTX-insensitive Go$_i$ versus Go$_o$ subunits differentially reduce I$_{K,basal}$ with Go$_i$ isoforms being more effective than Go$_o$, Go$_{i1}$, or Go$_{i2}$ subunits (4). In light of our findings reported here implicating Go$_i$/Go$_o$ isoform differences in GDP release kinetics, we propose the I$_{K,basal}$ differences are due to Go$_i$/Go$_o$ isoform differences in GDP binding affinity (5). Although the basal rate of GDP dissociation from Go$_o$ is ~4 times faster than Go$_i$ isoforms (14), the measured equilibrium binding affinity for GDP to Go$_i$ is ~2 times greater than Go$_o$, indicating a faster GDP association rate as well (5). Thus under equilibrium conditions in our oocyte expression experiments, the PTX-insensitive Go$_i$ isoforms are expected to be more effective than the Go$_o$ isoforms at reducing basal GIRK channel activity by more readily occupying the GDP-bound state and sequestering free G$\beta$y dimers. This notion is contrary to that originally proposed by Remmers et al. (5), who suggested the faster Go$_i$ GDP release rate would promote a higher level of basal G protein activity versus Go$_o$. Our findings correlating GIRK channel basal activity, receptor-dependent GIRK activation rates, with the transient and steady-state biochemical GDP binding kinetics suggest that, alternatively, the basal G protein activity is determined instead by the steady-state GDP binding affinity.

Implications for the RGS-accelerated G Protein Cycle and GIRK Channel Gating Kinetics—RGS proteins accelerate both the activation and deactivation phase of receptor-activated GIRK currents by accelerating the GTPase activity of Go subunits (22, 23). Yet since RGS proteins do not reduce steady-state GIRK current amplitudes during enhanced GTPase activity (signal termination), other processes must promote G protein activation to maintain steady-state GIRK current amplitudes. RGS proteins do not affect the basal (receptor-independent) GDP/GTP exchange rates for Go subunits in *vitro* (24), but do increase receptor-dependent G protein cycling (e.g., GTPase activity) in reconstituted cell membranes containing GPCRs, G proteins, and RGS proteins (25–28). These findings have led to the hypothesis that the RGS-accelerated GTPase activity allows for rapid heterotrimeric GoGDP$\beta$y re-formation and receptor re-activation, thus enabling multiple G protein cycles from an agonist-bound receptor (25, 28). This process requires 1) receptor-catalyzed GDP release to be sufficiently fast to sustain the multiple cycling with agonist-bound receptor, and 2) proximity or association of the agonist-GPCR-G protein ternary complex with the effector molecule (GIRK channel) for efficient signal transduction (G$\beta$y gating of the GIRK channel) over multiple G protein cycles. These events may also be facilitated by the ability of RGS proteins to increase the pool of Go subunits to the plasma membrane (29, 30) through a process not very well understood but requiring the RGS N-terminal domain (31).

There is now compelling evidence that GIRK channels form stable signaling complexes with GPCRs, given GIRK channel proteins bind both Go and G$\beta$y subunits in *vitro* (32, 33) and are immunoprecipitated with GPCRs and G proteins assembled in *vivo* (34). Recent fluorescence resonance energy transfer (FRET)-based assays suggest that heterotrimeric G proteins within GPCR-G protein-GIRK channel complexes do not actu-
Statistical comparisons were made with values from Gα panels A and B. Characterizing the kinetic properties of m2 receptor-activated GIRK currents primarily by slowing the caten endogenous oocyte RGS proteins do indeed impact receptor activation time constants (τact) derived from the GIRK deactivation time course following washout of 1 μM ACh with PTX-insensitive Gα activation as in panels A and B. All values are the mean ± S.E. (n ≥ 8). Statistical comparisons were made with values from Gα(A1C352G) expression, with p ≤ 0.05 considered significant (*).

Fig. 6. Summary of receptor-independent and receptor-dependent GIRK channel gating properties with expression of Gα2(G145A) and Gα12/13(G145A) mutant subunits. A, receptor-independent basal GIRK channel activity (I(basal)) associated with expression of Gα2(G145A) and Gα12/13(G145A) mutant subunits. For comparison, I(basal) values from the Gα1 chimera, Gα2(C352G) (Gα2 gray bar), and Gα12(C352G) (Gα12 black bar) are included. I(basal) amplitudes are expressed as a fraction of the total GIRK current (I(basal) + I(ACh)) elicited from each oocyte expressing the indicated Gα subunit. B, EC50 values derived from GIRK currents elicited by ACh dose-response experiments via m2 receptor coupling to Gα2(G145A) and Gα12/13(G145A). C, activation time constants (τact) derived from exponential fits of the GIRK activation time course in response to 100 μM ACh (15 s application) with expression of the indicated PTX-insensitive Gα mutant subunits. D, deactivation time constants (τdeact) derived from the GIRK deactivation time course following washout of 1 μM ACh with PTX-insensitive Gα activation as in panels A and B. All values are the mean ± S.E. (n ≥ 8). Statistical comparisons were made with values from Gα12(C352G) expression, with p ≤ 0.05 considered significant (*).

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Gβγ-activated Inwardly Rectifying K⁺ (GIRK) Channel Activation Kinetics via Gαi and G αo-coupled Receptors Are Determined by Gα-specific Interdomain Interactions That Affect GDP Release Rates
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