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Coupling of the Muscarinic m2 Receptor to G Protein-activated K^+ Channels via $G\alpha_z$ and a Receptor-$G\alpha_z$ Fusion Protein

FUSION BETWEEN THE RECEPTOR AND $G\alpha_z$ ELIMINATES CATALYTIC (COLLISION) COUPLING*

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G protein-activated K^+ channel (GIRK), which is activated by the $G_{\beta\gamma}$ subunit of heterotrimeric G proteins, and muscarinic m2 receptor (m2R) were coexpressed in *Xenopus* oocytes. Acetylcholine evoked a K^+ current, I_{ACh}, via the endogenous pertussis toxin (PTX)-sensitive $G_{i/o}$ proteins. Activation of I_{ACh} was accelerated by increasing the expression of m2R, suggesting a collision coupling mechanism in which one receptor catalytically activates several G proteins. Coexpression of the α subunit of the PTX-insensitive G protein G_z, $G\alpha_z$, induced a slowly activating PTX-insensitive I_{ACh}, whose activation kinetics were also compatible with the collision coupling mechanism. When GIRK was coexpressed with an m2R·$G\alpha_z$ fusion protein (tandem), in which the C terminus of m2R was tethered to the N terminus of $G\alpha_z$, part of I_{ACh} was still eliminated by PTX. Thus, the m2R of the tandem activates the tethered $G\alpha_z$ but also the nontethered $G_{i/o}$ proteins. After PTX treatment, the speed of activation of the m2R·$G\alpha_z$-mediated response did not depend on the expression level of m2R·$G\alpha_z$ and was faster than when m2R and $G\alpha_z$ were coexpressed as separate proteins. These results demonstrate that fusing the receptor and the $G\alpha$ strengthens their coupling, support the collision-coupling mechanism between m2R and the G proteins, and suggest a noncatalytic (stoichiometric) coupling between the G protein and GIRK in this model system.

Members of the $G_{i/o}$ family of heterotrimeric G proteins (G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_{o2}, and G_z) regulate numerous effectors such as adenylyl cyclase, ion channels, protein kinases, etc. (1–5). A great number of heptahelical G protein-coupled receptors (GPCRs)[1] activate $G_{i/o}$ proteins while usually being rather ineffective in interacting with the other three large families (G_s, G_q, and G_{12}) (2, 6). The diversity of $G_{i/o}$-coupled GPCRs and of the $G\alpha_{i/o}$ subunits suggests that the various GPCRs should specifically activate different cellular responses via different $G_{i/o}$ proteins. Many outstanding examples of specific regulation of effectors by certain $G_{i/o}$-coupled GPCRs have been described *in vivo*; however, only limited specificity is observed on the level of receptor-$G\alpha_{i/o}$ interaction in intact cells and especially in model expression systems (for review see Ref. 2). It is widely accepted that the overall GPCR-effector coupling specificity is defined by factors such as colocalization or scaffolding of the signaling components, the presence of additional regulatory proteins such as regulators of G proteins signaling, effector/G protein specificity, etc. (6–9). However, in the $G_{i/o}$-related pathways, these factors still remain poorly understood. One proposed mechanism of ensuring a specific activation of a certain $G\alpha$ by a GPCR is the existence of a stable complex between the receptor and the G protein in the absence of agonist. The existence of such complexes is supported by several lines of data, among them coimmunoprecipitation of several GPCRs with the corresponding $G\alpha$ proteins in many cell types (6–9). However, in other systems, a collision coupling-type mechanism (10, 11) between GPCRs and the G proteins has been demonstrated. In these systems, the receptor is not *a priori* coupled to $G\alpha$, and the coupling takes place only after the binding of an agonist to the receptor. A receptor activated in this way shuttles between and catalytically activates several G proteins in succession.

To study activation and effector coupling of individual $G\alpha_{i/o}$ proteins in separation from the other members of the family, it is necessary to overcome the problem that each of the $G_{i/o}$ proteins can be activated by almost any $G_{i/o}$-interacting GPCR. Attempts to achieve specific coupling in GPCR-$G\alpha$ pairs were made by creating GPCR·$G\alpha$ fusion proteins (12–14). However, in $G_{i/o}$-containing GPCR·$G\alpha$ tandems, the tethered receptor still activates "nearby" nontethered $G\alpha_{i/o}$ proteins (15). This problem has been partly overcome (14) by utilizing the pertussis toxin (PTX) sensitivity, which is a characteristic of all $G\alpha_{i/o}$ proteins except $G\alpha_z$ (16, 17). PTX catalyzes ADP-ribosylation of a cysteine near the end of the C terminus, preventing the coupling of the agonist-bound receptor to $G\alpha$, the dissociation of $G\alpha$ and $G_{\beta\gamma}$ subunits, and the activation of their effectors (1). Changing the C-terminal cysteine to glycine or serine renders $G\alpha$ insensitive to PTX, but it can still be activated by the receptors (18, 19). Thus, after PTX treatment, the GPCR of the tandem containing such a mutant $G\alpha_{i/o}$ interacts only with the tethered $G\alpha$. However, another problem arose. In the best studied case, a tandem of the α2A adrenoreceptor with a PTX-resistant mutant $G\alpha_{i1(C351G)}$, both the receptor-activated GTPase activity and the coupling to the effector (adenylyl cyclase) were substantially impaired as compared with the wild-type $G\alpha_i$ (20, 21). This is not surprising, given the importance of the C-terminal end of $G\alpha$ in receptor recognition and coupling (18, 19) and the devastating effects of some (although not all) mutations of the C-terminal cysteine on receptor-$G\alpha$ coupling (22, 23).

To avoid the use of mutant $G\alpha$ and to still be able to compare effector activation by free *versus* receptor-fused $G\alpha_{i/o}$ protein,

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; GIRK, G protein activated K^+ channel (Kir3); m2R, muscarinic m2 receptor; PTX, pertussis toxin; ACh, acetylcholine.

we utilized the naturally PTX-resistant Gα$_z$ (24, 25) and the *Xenopus* oocyte expression system. Gα$_z$ is activated by a variety of Gα$_{i/o}$-coupled GPCRs and inhibits adenylyl cyclase like other Gα$_{i/o}$ proteins (reviewed in Refs. 16 and 17), but Gα$_z$ shows a slower GDP-GTP exchange rate and a very low GTPase activity (17). Recently, endogenous α-adrenergic receptors have been shown to inhibit N-type Ca^{2+} channels and to activate the G protein-activated, inwardly rectifying K^+ channels (GIRK) in a PTX-resistant manner in sympathetic neurons after overexpression of Gα$_z$ (26). Both Ca^{2+} channel inhibition and GIRK activation are mediated by a direct interaction of these channels with G$_{βγ}$ normally released from Gα$_{i/o}$ proteins (4, 27–29). The use of GIRK channels expressed in *Xenopus* oocytes as an assay to study the receptor-G protein-effector coupling has been widely utilized (28, 30). It allows a controlled expression of different amounts of proteins under study, simply by injecting different amounts of the encoding RNAs, enabling a quantitative study of various aspects of the coupling mechanism. Furthermore, the binding and unbinding of G$_{βγ}$ to and from the channel are fast. The rise and decay times of the GIRK current upon agonist application and washout are believed to be limited primarily by the rate of G protein activation (normally, the GDP-GTP exchange at the Gα) and by the rate of GTP hydrolysis by Gα, respectively (28, 31, 32). Therefore, the kinetics of GIRK currents reflect the kinetics of G protein activation and deactivation. Here, using this system, we demonstrate a collision coupling-type (10) mechanism in activation of the GIRK by m2R via PTX-sensitive G$_{i/o}$ proteins and via G$_z$ and a substantial improvement of the efficiency of coupling by tethering m2R and the Gα in tandem.

EXPERIMENTAL PROCEDURES

cDNA Constructs and mRNA—Materials and enzymes for molecular biology were from Roche Molecular Biochemicals, Promega, or MBI Fermentas. The cDNA of GIRK2 (33) was a gift from H. A. Lester. cDNA of cβARK (34) was a gift from E. Reuveni. The coding sequences of m2R (35) were a gift from E. Peralta. Gα$_z$ (24) and Gα$_{i3}$ (36) (gifts from M. I. Simon) were subcloned into the pGEMHJ vector (34) using a standard polymerase chain reaction procedure in which an *Eco*RI restriction site was created immediately before the ATG initiation codon, and another *Eco*RI (m2R), *Hin*dIII (Gα$_z$), or *Bst*EII (Gα$_{i3}$) site was created immediately after the stop codon. The pGEMHJ vector provides 5′- and 3′-untranslated regions of the *Xenopus* β-globin RNA (37), ensuring a high level of protein expression in the oocytes. The m2R·Gα$_z$ and m2R·Gα$_{i3}$ tandem cDNAs were created by ligating the m2R cDNA sequence into the *Eco*RI site of the corresponding Gα cDNA. Thus, in each tandem protein, the full primary sequences of m2R and the Gα are connected by a short, 2-amino acid linker (Glu-Phe) encoded by the *Eco*RI restriction site sequence GAATTC. High quality capped RNA was prepared as described (38).

Oocytes and Electrophysiology—*Xenopus laevis* oocytes were prepared and injected with RNAs as described (39) and incubated for 3–5 days before the experiment in the ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes/NaOH) supplemented with 50 μg/ml gentamycin and 2.5 mM sodium pyruvate. cβARK RNA (5 ng/oocyte) was injected 2 days after the other RNAs, 2 days before the experiment. Two-electrode voltage clamp experiments were performed as described (39). Data acquisition and analysis were done using pCLAMP software (Axon Instruments). The membrane potential was set at −80 or −40 mV in the ND96 solution, and GIRK currents were induced and measured in the high K^+ solution (KCl, 96; NaCl, 2; $CaCl_2$, 1; $MgCl_2$, 1; Hepes/NaOH, 5). Exchange of solutions was performed using the BSP-4 fast perfusion system (ALA Instruments, New York). Full exchange of solution in the experimental bath (50 μl volume) occurred within less than 0.5 s. The shift from ND96 to the high K^+ solution evokes a basal current, I_{basal} (see Fig. 1*A*), which flows mainly via the GIRK channels, with a minor (<0.1 μA) contribution of a G protein-independent endogenous current, I_{native} (39, 40). Therefore, I_{basal} was corrected by subtracting the average I_{native} measured in native (not injected with RNA) oocytes. PTX treatment was done by injecting the oocytes with the activated A protomer of PTX (Alomone Labs, Jerusalem, Israel) 4–24 h before the experiment (39).

Immunochemistry of the Expressed m2R and m2R·Gα Tandems in Xenopus Oocytes—was performed essentially as described (41). Oocytes were injected with mRNAs and incubated in NDE solution containing 0.5 mCi/ml [^{35}S]methionine/cysteine (Amersham Pharmacia Biotech) for 3–4 days at 22 °C. 5 oocytes were homogenized, and proteins were solubilized, immunoprecipitated with 5 μl of a polyclonal antibody against m2R (Alomone Labs, Jerusalem, Israel), and electrophoresed on 7.5% polyacrylamide-SDS gel.

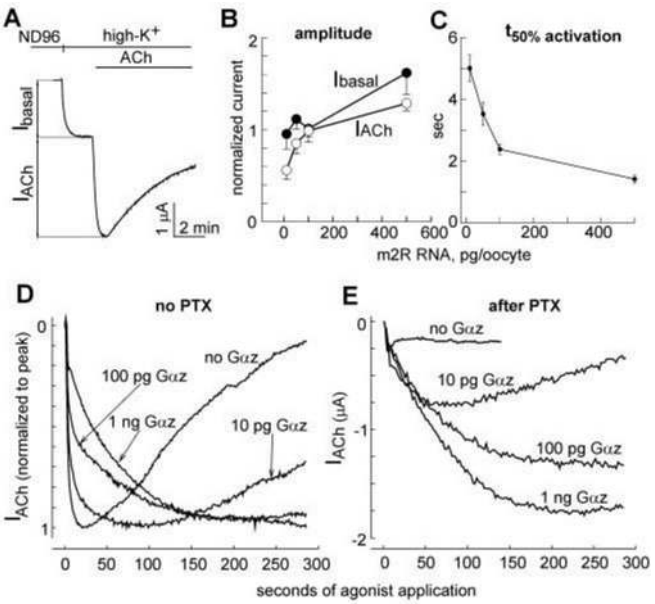

FIG. 1. **Activation of GIRK by m2R via the endogenous G$_{i/o}$ proteins and via coexpressed G$_z$.** *A*, a standard experimental protocol and the definition of I_{basal} and I_{ACh}. *B* and *C*, the dependence of the amplitude of I_{basal} and I_{ACh} (*B*) and the speed of activation of I_{ACh} (*C*) on the level of expression of m2R. Summary of three experiments is shown. Each point is mean ± S.E. of 9–11 oocytes. In *B*, current amplitude was normalized to the average of the group of oocytes of the same donor injected with 100 pg/oocyte of m2R RNA. *D* and *E*, ACh-evoked currents in oocytes from one donor expressing the channel, m2R (100 pg of RNA/oocyte) and various doses of Gα$_z$. The amounts of Gα$_z$ RNA, in pg/oocyte, are shown near each trace. In *E*, the oocytes were injected with 100 ng/oocyte of the A promoter of PTX.

RESULTS AND DISCUSSION

Collision Coupling of m2 Receptor to GIRK via the Endogenous PTX-sensitive G Proteins—We used the GIRK1/GIRK2 subunit composition of the G protein-gated channels, which is abundant in the brain (see Refs. 28 and 30 for review). Expression of m2R with GIRK1 and GIRK2 in *Xenopus* oocytes gave rise to inwardly rectifying K^+ currents (42, 43) that were measured using the two-electrode voltage clamp method (Fig. 1*A*). Exchanging the high Na^+ solution (ND96) to a high K^+ solution elicited a basal current (I_{basal}), and the addition of 10 μM agonist (acetylcholine (ACh)) caused an additional current (I_{ACh}), which gradually inactivated over a few minutes (39, 44, 45). 10 μM of ACh was a saturating concentration, because a maximal response was observed already at 1 μM (data not shown). In each experiment, all oocytes were injected with the same amount of channel RNA. We used relatively low doses of RNA of the channel subunits (50, 80, or 100 pg/oocyte each) to ensure that the amount of the endogenous G$_{βγ}$ subunits was sufficient to activate all the channels. Indeed, larger currents were evoked by ACh at 200–500 pg/oocyte of GIRK1 and GIRK2 RNAs (data not shown). I_{ACh} was inhibited by ~90% by the injection of the A promoter of PTX (Ref. 39; Figs. 1*E* and 2*B*), suggesting that the response is mediated by the endogenous PTX-sensitive G$_{i/o}$ proteins.

Increasing the dose of m2R RNA caused an increase in I_{ACh}; the dose-response relationship almost saturated at 500 pg/oocyte (Fig. 1*B*, ○). I_{basal} was unchanged by coexpression of

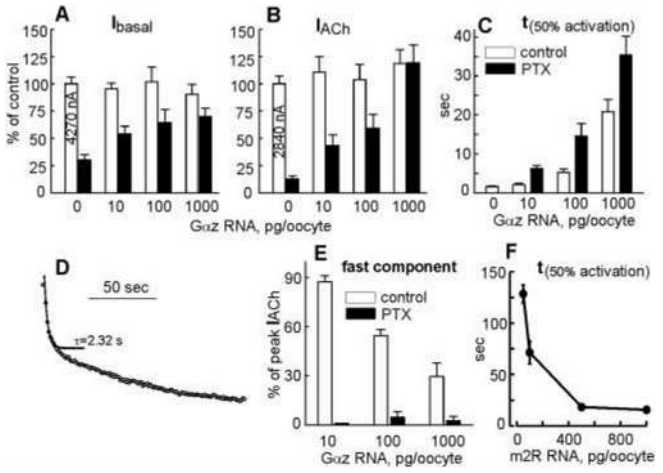

FIG. 2. **Effect of varying the level of expression of Gα_z and of PTX treatment on GIRK currents.** *A–C*, dependence of amplitude of I_{basal} (*A*), amplitude of I_{ACh} (*B*), and speed of activation of I_{ACh} (*C*) on the level of expression of Gα_z and on PTX treatment. Each point represents mean ± S.E. from 12–33 oocytes from at least three donors. All oocytes were injected with 100 pg/oocyte of m2R RNA and the indicated amounts of Gα_z RNA. *Empty bars*, no PTX treatment; *black bars*, cells treated with PTX. In each oocyte, the currents were normalized to the average value recorded in the control group (Gα_z = 0). *D*, an example of the fitting procedure used to estimate the contribution of the fast component to the total I_{ACh}. The oocyte was injected with RNAs of m2R, Gα_z, GIRK1, and GIRK2 (100 pg of each). *E*, summary of the analysis of the kind shown in *D*, 4–6 oocytes from two batches. *F*, the dose dependence of $t_{50\%\ act}$ on the dose of m2R RNA in oocytes of one batch, treated with PTX (Gα_z RNA was 1 ng/oocyte). The points are mean ± S.E. of 4–6 oocytes.

m2R at RNA doses between 10 and 100 pg/oocyte (40) but increased at 500 pg/oocyte (Fig. 1*B*, ●), suggesting that at high m2R densities, the agonist-free receptor activates the G proteins. The speed of activation of I_{ACh} (expressed as the time by which I_{ACh} reaches 50% of peak amplitude, $t_{50\%\ act}$) became progressively faster with increasing expression of m2R, reaching 1.4 s at 500 pg of m2R RNA/oocyte (Fig. 1*C*). The simplest explanation for this phenomenon is a collision coupling-type mechanism (11, 46), in which one receptor shuttles between and catalytically activates several G proteins in succession. The active state of a Gα persists after unbinding of the receptor until GTP is hydrolyzed; only then it can bind G$_{\beta\gamma}$. Until then, G$_{\beta\gamma}$ remains free ("active") too. This model predicts that the response will develop faster when more receptors are present, because each receptor has to activate less molecules of Gα, saving the shuttling and binding/unbinding time. There is an alternative theoretical possibility that each m2R is always found in a tight complex with a G$_{i/o}$ and each complex releases one G$_{\beta\gamma}$. In such a case, to explain the acceleration of activation at increased levels of m2R, one must assume that each G$_{\beta\gamma}$ activates several channels in succession, and a channel previously activated by G$_{\beta\gamma}$ remains open at least until and during the activation of the next one. This, however, is highly unlikely in view of the fast channel deactivation upon unbinding of G$_{\beta\gamma}$ (31, 32).

GIRK Channels Are Activated by G$_{\beta\gamma}$ Released from G$\alpha_z\beta\gamma$ Heterotrimers—Expression of increasing amounts of Gα_z while keeping the levels of m2R and GIRK constant slowed down the speed of activation of I_{ACh} (Fig. 1*D*). The activation appeared composed of a fast and a slow component, with the slow one becoming more prominent as the concentration of Gα_z RNA was increased. At the same time, the inactivation of I_{ACh} became less apparent. The deactivation of the current upon washout of ACh became extremely slow, taking many minutes for completion (data not shown), in line with the slow GTPase activity of Gα_z. These features are similar to those described by Jeong and Ikeda (26) for G$_z$-mediated activation of GIRK in sympathetic neurons, suggesting that the slow component is contributed by G$_{\beta\gamma}$ released from G$\alpha_z\beta\gamma$ heterotrimers. Indeed, PTX treatment eliminated the fast component of activation, leaving the slow one intact (Figs. 1*E* and 2*E*). On the other hand, the ACh-evoked response was still mediated by G$_{\beta\gamma}$, because coexpression of a myristoylated C-terminal part of β-adrenergic receptor kinase (cβARK), which is a strong G$_{\beta\gamma}$ chelator and blocks agonist-evoked GIRK currents in the oocytes (47), reduced I_{ACh} by 85 ± 6% (mean ± S.E., n = 3) in oocytes injected with 1 ng of Gα_z RNA.

Fig. 2 summarizes the results of Gα_z coexpression experiments. Without PTX, the amplitudes of either I_{basal} or I_{ACh} (Fig. 2, *A* and *B*) were not changed by coexpression of Gα_z, supporting the notion that the overall amplitude of the response was limited by the number of expressed channels. Without Gα_z, PTX strongly reduced I_{basal} and almost fully suppressed the I_{ACh} (39), and both I_{basal} and I_{ACh} were dose-dependently rescued by coexpression of Gα_z (Fig. 2, *A* and *B*). At 1 ng of Gα_z RNA/oocyte, I_{basal} and I_{ACh} were already almost fully restored. The speed of activation of I_{ACh} became progressively slower with increasing dose of Gα_z, with $t_{50\%\ act}$ reaching 35 ± 5 s at 1 ng of Gα_z RNA in the presence of PTX (Fig. 2*C*). At all concentrations of Gα_z, $t_{50\%\ act}$ was lower in cells untreated with PTX, although this difference was reduced from 2.9-fold at 10 pg to 1.7-fold at 1 ng of Gα_z RNA (Fig. 2*C*). The fast component of activation could be reasonably well fitted by a single exponent with time constant of activation (τ) of 1–3 s in a majority of cells (Fig. 2*D*). This allowed to estimate its contribution to the peak amplitude of I_{ACh} (Fig. 2*E*). This analysis showed that in PTX the fast component of activation was practically absent. As expected, in the absence of PTX the contribution of the fast component decreased as the expression of Gα_z increased; about 30% of total agonist-evoked current was still contributed by the endogenous G$\alpha_{i/o}$ even at 1 ng of Gα_z RNA.

The results of Fig. 2 (*A*—*E*) demonstrate that the endogenous G$\alpha_{i/o}$ and the expressed Gα_z compete for the available m2R, to donate G$_{\beta\gamma}$ for channel activation. In the absence of PTX, a fast component of activation is contributed by the endogenous G$\alpha_{i/o}$ at all doses of Gα_z RNA, even 1 ng/oocyte, and only after PTX treatment all channels can be activated solely via G$_{\beta\gamma}$ coming from G$_z$. PTX reveals the actual kinetics of the response evoked via G$_z$.

These experiments also have an important general implication: PTX is often used to suppress agonist-evoked responses mediated by G$_{i/o}$ proteins, to evaluate in this way what part of the cellular response evoked by this agonist is mediated by PTX-insensitive G proteins. Our results call for caution in such interpretations by showing that after PTX treatment, PTX-insensitive G proteins may "take over" the function of PTX-sensitive G$_{i/o}$ proteins and couple to a larger proportion of effector molecules than under normal conditions.

The smaller amplitude of I_{ACh} at low levels of Gα_z expression suggests that G$_z$ protein expression is limiting; each G$_z$ may donate G$_{\beta\gamma}$ for the activation of a limited number of channels (maybe only one). The progressive increase in $t_{50\%\ act}$ with increasing Gα_z expression in the presence of PTX (Fig. 2*C*) is compatible with the collision coupling mechanism: when more Gα_z are available, each receptor can activate more G proteins (hence a greater I_{ACh}; compare with Fig. 2*B*); but activation of more G proteins by each receptor simply takes more time. An alternative explanation is the presence of a diffusional barrier between G$_z$ and GIRKs (because of channel scaffolding with other G proteins such as G$_i$, etc.), when some of the channels are poorly accessible to G$_{\beta\gamma}$ derived from G$_z$. Expression of

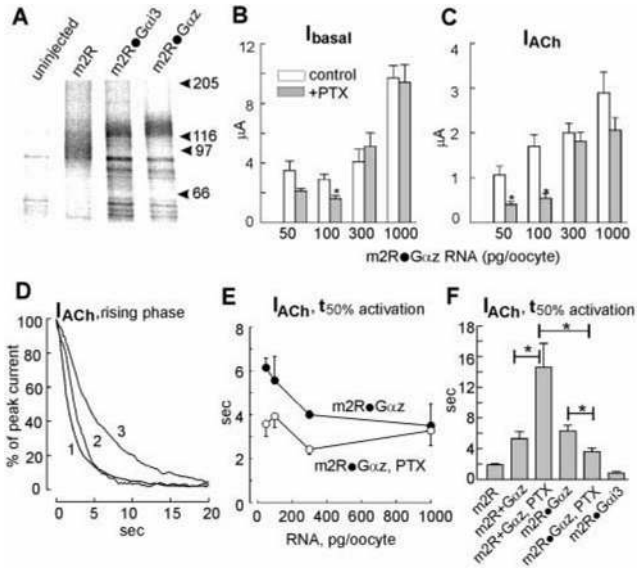

FIG. 3. **Expression of the m2R·Gα$_z$ tandem and its coupling to GIRK.** *A*, a polyclonal antibody against m2R immunoprecipitates metabolically labeled m2R, m2R·Gα$_{i3}$, and m2R·Gα$_z$ tandems from oocytes injected with 100 pg of each RNA but not from uninjected oocytes. *B* and *C*, effect of the level of expression of the m2R·Gα$_z$ tandem and of PTX on I_{basal} (*B*) and I_{ACh} (*C*). Results from a representative batch of oocyte are shown; similar results were obtained in two more batches. The *bars* show mean ± S.E. of 3–7 oocytes. *Asterisks* indicate $p < 0.05$ or better (*t* test). *D*, representative traces of the responses to ACh in oocytes expressing the GIRK channels and either m2R (*trace 1*, 100 pg/oocyte) or m2R·Gα$_z$ tandem (100 pg/oocyte) with (*trace 3*) or without (*trace 2*) PTX treatment. *E*, dependence of $t_{50\%\ act}$ of I_{ACh} on the level of expression of m2R·Gα$_z$. Same experiment as in *B* and *C*. Similar results were obtained in two additional batches. *F*, comparison of $t_{50\%\ act}$ from all experiments in which 100 pg RNA/oocyte of each of the indicated constructs were injected. Each *bar* shows mean ± S.E. of 15–53 oocytes from 5–12 batches, except for the m2R·Gα$_{i3}$ tandem (7 oocytes, two batches). *Horizontal bars* with *asterisks* indicate statistically significant differences ($p < 0.05$ or better) obtained in comparisons between selected groups (two-tailed *t* test).

more G$_z$ might eventually force the activation of the previously inaccessible population of channels, but with a slower time course, reflecting the slower diffusion of G$_{βγ}$ to these channels. We view the collision coupling mechanism between m2R and G$_z$ as a more plausible explanation, because it is supported by an independent experiment showing acceleration of activation of I_{ACh} by increasing the level of expression of m2R, in the presence of PTX (Fig. 2*F*). Importantly, the activation of GIRK by G$_z$ is much slower than by PTX-sensitive G$_{i/o}$. Comparison of the group in which all the channels were activated by the endogenous G$_{i/o}$ (0 pg of Gα$_z$, no PTX) and the group in which the same amount of channels was activated by G$_z$ (1 ng of Gα$_z$ RNA, with PTX) reveals a 22-fold difference in $t_{50\%\ act}$ (Fig. 2*C*). To understand the reasons for this poor speed of activation, we have created an m2R·Gα$_z$ tandem and studied its interaction with GIRK. For comparison, an m2R·Gα$_{i3}$ tandem was also made and tested.

Stoichiometric Coupling of the m2R·Gα$_z$ Tandem to GIRK—Metabolically labeled m2R, m2R·Gα$_z$, and m2R·Gα$_{i3}$ were immunoprecipitated from oocytes injected with 100 pg of each RNA with a polyclonal antibody against m2R. The amounts of all three proteins were approximately equal (Fig. 3*A*). m2R (calculated molecular mass, 51.7 kDa) is a glycosylated protein (48) with an apparent molecular mass of 65–80 kDa in mammalian cells (48, 49). In the oocytes, it ran as a diffuse band of ~90 kDa (Fig. 3*A*), suggesting that it was glycosylated stronger than in mammalian cells. The apparent sizes of the tandem proteins were 116–120 kDa, above the calculated ~92 kDa, suggesting that these proteins were also glycosylated. Uninjected oocytes were devoid of label.

In oocytes coexpressing the m2R·Gα$_z$ tandem with GIRK, I_{basal} was stable in the range 50–300 pg of m2R·Gα$_z$ RNA but grew at higher doses (Fig. 3*B*), suggesting a substantial basal activity of the receptor at high expression levels. I_{ACh} grew dose-dependently with increasing amounts of tandem RNA (Fig. 3*C*). PTX reduced both I_{basal} and I_{ACh} at lower doses of m2R·Gα$_z$, confirming that a GPCR, even when tethered to a Gα, can still activate other nontethered G proteins (15). Above 300 pg/oocyte of tandem RNA, PTX had little effect on either I_{basal} or I_{ACh} (Fig. 3, *B* and *C*), suggesting that there were enough tandems to donate G$_{βγ}$ for activation of most channels. The m2R·Gα$_z$-mediated I_{ACh} was fully G$_{βγ}$-dependent, because coexpression of cβARK completely abolished it (data not shown). Examination of the rising phase of I_{ACh} (Fig. 3*D*) revealed that activation by m2R·Gα$_z$ was *accelerated* by the PTX treatment (Fig. 3*D*, *trace 2*, and Fig. 3*F*). This was true at most doses of m2R·Gα$_z$ (Fig. 3*E*). These results reveal that the coupling of the tethered receptor to Gα$_z$ within the tandem is better (faster) than its coupling to the nontethered Gα$_{i/o}$. Again, PTX treatment reveals the real kinetics of GIRK activation by the tandem. Fig. 3*F* summarizes the differences in the kinetics of GIRK activation, obtained in several experiments at the same doses of RNAs of m2R, Gα$_z$, or m2R·Gα: 100 pg/oocyte. The activation of GIRK by the m2R·Gα$_z$ (in PTX) is 4-fold faster than by separately expressed m2R and Gα$_z$, suggesting that the slowness of coupling of m2R to GIRK via G$_z$ is primarily the result of poor receptor-Gα$_z$ coupling. Yet, activation by the m2R·Gα$_z$ tandem is still slower ($p < 0.001$) than by m2R via the endogenous G$_{i/o}$ ($t_{50\%\ act}$, 3.6 ± 0.4 s, $n = 16$, *versus* 1.9 ± 0.1 s, $n = 53$). This may reflect the slow intrinsic kinetics of Gα$_z$ activation, because a structurally similar tandem, m2R·Gα$_{i3}$, activated the channel even faster than m2R via the endogenous G$_{i/o}$ ($t_{50\%\ act}$, 0.8 ± 0.2 s, $n = 7$).

The most striking consequence of the physical link between m2R and Gα$_z$ was revealed after PTX treatment, which eliminated of the contribution of the endogenous G$_{i/o}$ (Fig. 3*E*): the speed of activation of the m2R·Gα$_z$-mediated response did not depend on the expression level of the tandem. This result clearly demonstrates a stoichiometric interaction between m2R and Gα$_z$ in the tandem protein, confirming that tethering produces an active GPCR-Gα complex and eliminates the collision coupling. Furthermore, it confirms that collision coupling (if any) between free G$_{βγ}$ and GIRK does not contribute to the activation kinetics. This, in turn, strengthens the conclusion reached above, that the collision coupling under normal conditions (free expressed m2R; Figs. 1*C* and 2*F*) occurs on the receptor-G protein level. The stoichiometric (noncatalytic) coupling between the released G$_{βγ}$ and the channel may simply reflect the fast dissociation of G$_{βγ}$ (see above). A more tantalizing possibility is that there is a high affinity preformed complex between a G protein heterotrimer and the GIRK (50, 51), so that dissociation between Gα and G$_{βγ}$ is immediately followed by the activation of the effector, saving the time lag introduced by the diffusion. Such a scheme explains well the fast kinetics of GIRK activation in atrial myocytes and in neurons (32, 52).

Conclusions—Using the *Xenopus* oocyte expression system, we have performed a quantitative comparison of activation of an effector (GIRK) by a GPCR (m2R) via endogenous PTX-sensitive G proteins, via a coexpressed Gα$_z$, and via an m2R·Gα$_z$ tandem (fusion) protein. The efficiency (in terms of kinetics) of coupling of the components of the signaling pathway was monitored by measuring the activation kinetics of the GIRK current. Our results demonstrate a collision coupling between m2R and the G$_{i/o}$ proteins (either endogenous G$_{i/o}$ or

coexpressed G_z). In contrast, the relationship between the amount of activated G proteins and the activated effector is stoichiometric. The latter is in line with the existence of a preformed complex between a G protein heterotrimer and the effector. Although m2R is able to elicit a full effector response via G_z, the slow kinetics of the response reveals an inherent inefficiency of this signaling pathway, compared with m2R-GIRK signaling via PTX-sensitive $G_{i/o}$ proteins. The use of m2R·Gα reveals that the poor coupling between m2R and $G\alpha_z$ and the intrinsic slow activation of G_z are important rate-limiting steps in the G_z pathway.

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