The Dynamics of Formation and Action of the Ternary Complex Revealed in Living Cells Using a G-protein-gated K+ Channel as a Biosensor*

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Traditionally the consequences of activation of G-protein-coupled receptors (GPCRs) by an agonist are studied using biochemical assays. In this study we use live cells and take advantage of a G-protein-gated inwardly rectifying potassium channel (Kir3.1+3.2A) that is activated by the direct binding of Gβγ subunit to the channel complex to report, in real-time, using the patch clamp technique the activity of the “ternary complex” of agonist/receptor/G-protein. This analysis is further facilitated by the use of pertussis toxin-resistant fluorescent and non-fluorescent Ga/o subunits and a series of HEK293 cell lines stably expressing both channel and receptors (including the adenosine A1 receptor, the adrenergic α2A receptor, the dopamine D2S receptor, the M4 muscarinic receptor, and the dimeric GABA-B1b/2 receptor). We systematically analyzed the contribution of the various inputs to the observed kinetic response of channel activation. Our studies indicate that the combination of agonist, GPCR, and G-protein isoform uniquely specify the behavior of these channels and thus support the importance of the whole ternary complex at a kinetic level.

The activation of G-protein-coupled receptors (GPCRs)† by extracellular ligands is an important mechanism involved in a multitude of physiological responses and is of central importance in drug development and therapeutics (1). The activated receptor couples to G-proteins of various subtypes that then activates effector pathways either directly or indirectly. This combination of agonist, receptor, and G-protein is referred to as the “ternary complex” and is thought to be the key essential determinant of the magnitude of the downstream response (2, 3). The most recent formulations propose a cubic ternary complex model with a large number of equilibrium constants between various states governing efficacy (2, 4, 5). The important species is the activated receptor/agonist/G-protein complex. It is proposed that for any combination of these three elements the particular active conformation (or conformational space) is unique and can thus have distinctive signaling consequences (2, 4–6). An agonist binds more favorably to the active receptor species and thus at equilibrium favors its formation. Recently this model has been extended to also incorporate the kinetics of G-protein activation and deactivation and indicate that a kinetic model, as opposed to an equilibrium model, may potentially have quite different properties (7).

Generally these phenomena have been studied by the use of biochemical assays, using cell homogenates or fractions, or by measuring the behavior of a physiological response many steps downstream from the G-protein cycle. It is apparent that there is a gap in our understanding about how these signaling pathways behave dynamically in intact cells. This is important, because, in reality, the release of hormones and neurotransmitters varies over the second time scale. Agonist binding to receptor is generally agreed to be diffusion-limited and much faster than the activation of downstream signaling events. However, there are a number of more controversial issues regarding models of receptor activation of G-proteins. Is there kinetic evidence for the unique conformation of the ternary complex? What is the role of both the isoform and concentration of G-protein in dictating the dynamic behavior? Is the encounter of receptor with the G-protein rate-limiting, and do receptor and G-protein exist in a pre-coupled complex? To address these questions we have used members of the Kir3.0 family of inwardly rectifying K+ channels that are gated by G-proteins as a reporter. G-protein-gated inwardly rectifying K+ channels were first identified in atrial myocytes where they are activated by acetylcholine at muscarinic M2 receptors (8–10). It was subsequently shown that this activation was membrane-delimited (11), mimicked by non-hydrolysable GTP analogues (12), and sensitive to pertussis toxin (PTx), implicating the inhibitory family of G-proteins (Gi/o) (13). It is now apparent that G-protein-gated inwardly rectifying K+ currents are also present in many neuronal cell types (14–16). Cloning efforts have revealed the molecular counterparts of these currents, and the channel is a heteromultimer of members of the Kir3.0 family of K+ channels (16–23). It is now accepted that activation of native and cloned Kir3.0 channels involves a direct interaction with the Gβγ dimer not the Ga subunit (16, 24, 25).

In our previous studies we have developed a series of molecular tools to study these issues including stable cell lines expressing the channel complex along with GPCRs and both fluorescent and non-fluorescent Ga/o subunits engineered to be resistant to the action of pertussis toxin (26–28). In this study we combine the use of these tools with whole-cell patch clamping to record Kir3.1+3.2A currents in response to GPCR stim-

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† The abbreviations used are: GPCR, G-protein-coupled receptor; PTx, pertussis toxin; CFP, cyan fluorescent protein; GABA, γ-aminobutyric acid; GFP, green fluorescence protein; Tet, tetracycline; ttp, time to peak; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, 5′-[(N-Ethylcarboxamido)adenosine. This paper is available on line at http://www.jbc.org

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Receptor mediated kinetics. A, an example of a NECA-induced current recorded in the HKIR3.1/3.2/A1 cell line at ~60 mV in response to a 20-s application of NECA (in this and subsequent figures, agonist application is indicated by the solid horizontal bar). The expanded current trace shows the parameters measured. Channel activation kinetics is represented by "lag" (time between onset of agonist application and channel activation) and "time-to-peak (ttp)" (time between onset of channel activation and peak current amplitude). B, representative examples from the three channel-plus-receptor expressing stable cell lines: HKIR3.1/3.2/A1 (upper panel), HKIR3.1/3.2/M4 (middle panel), and HKIR3.1/3.2/D2 (lower panel) in response to a 20-s application of relevant agonist (A1, 1 μM NECA; α2A, 3 μM noradrenaline; and M4, 10 μM quinpirole). C, radioligand binding using tritiated receptor antagonists was used to assess levels of receptor expression in the HKIR3.1/3.2/A1, HKIR3.1/3.2/M4, and HKIR3.1/3.2/D2 stable cell lines. All three receptor types were expressed at equivalent levels (p > 0.05), and these data are summarized in the bar chart. D, we measured channel activation (lag+ttpt) in the three cell lines shown in A and additionally in a cell line expressing the channel plus the GABA-B1b variant (HKIR3.1/3.2/GGB) and a cell line expressing the channel complex and the M4 muscarinic receptor (HKIR3.1/3.2/M4). One-way analysis of variances with Bonferroni’s multiple comparisons test were used to compare data from the HKIR3.1/3.2/A1, HKIR3.1/3.2/M4, and HKIR3.1/3.2/D2 cell lines in which receptors were expressed to similar levels. Channel activation via stimulation of the D2S receptor was significantly shorter than that via stimulation of either the A1 (p < 0.001) or the α2A receptors (p < 0.001).

Experimental Procedures

Molecular Biology, Cell Culture, and Transfection—PTx-resistant Gα subunits and CFP-tagged PTx-resistant Gα were generated and used as previously described (27, 28). GABA-B2 and GABA-B3 were expressed in the dual promoter vector pBudCE4.1 (Invitrogen). Standard molecular cloning techniques were used to excise the relevant clones from the previous vector (GABA-B2 was excised from pcDNA3.1 neo with PaeII and GABA-B3 from pcDNA3.1 neo (+) with KpnI/XhoI) and they were introduced into the two polylinkers in Scul/Sall sites for GABA-B2 and KpnI/XhoI sites for GABA-B3. Inducible expression of Gα-CPF was achieved using the TReX system (Invitrogen). Gα-CPF was removed from the previously described construct (28) and subcloned into pcDNA5/TO and pCDNA5/TO using a KpnI/XhoI digest. The A1-Gi1 fusion construct was as previously described (29), and cDNA was excised from pcDNA3 and subcloned in to pcDNA3.1 neo (+) (28).

The methods for cell culture and the generation of stable cell lines were as previously described (26, 30). In addition to our established cell lines (Kir.1.1-2.2A channel plus either the A1 adenosine receptor (HKIR3.1/3.2/A1) or the D2 dopamine receptor (HKIR3.1/3.2/D2)), we made a further four dual receptor plus channel stable lines that were designated as follows: α2A adrenergic receptor, HKIR3.1/3.2/α2; GABA-B1b receptor, HKIR3.1/3.2/GGB; M4 muscarinic receptor, HKIR3.1/3.2/M4; and A1-Gi1 fusion, HKIR3.1/3.2/A1-Gi1. Monoclonal cell lines were established by picking single colonies of cells following transfection and growth under selective pressure. For all the dual receptor and channel expressing lines we used a dual selection strategy with 727 μg/ml G418 and 364 μg/ml Zeocin (Invitrogen). Stable cell lines, expressing a fluorescent G-protein α subunit (Gα-CPF) in an inducible system, were made after transfection of Gα-CPF in pcDNA5/TO and pcDNA5/TO (both Invitrogen) into the HKIR3.1/3.2/A1 and subsequent selection with 727 μg/ml G418, 364 μg/ml Zeocin, 400 μg/ml hygromycin, and 5 μg/ml blasticidin (Invitrogen). This stable line was designated as HKIR3.1/3.2/A1(Gα,CPF-T).

Transiently transfected cells suitable for patch clamping were identified by epifluorescence from the co-transfection of 100 ng of the enhanced variant of the green fluorescent protein (pEGFP-N1, Clontech) Data were obtained from at least two independent transfections.

Radioligand Binding—Radioligand binding was performed on crude membrane preparations isolated from the relevant stable lines (HKIR3.1/3.2/A1, HKIR3.1/3.2/M4, and HKIR3.1/3.2/D2). Cells were harvested into binding buffer (50 mM Tris-HCl, pH 7.4) and stored at -80 °C. Cells were hypotonically shocked (10 mM Tris-HCl and 10 mM EDTA) on ice and then homogenized using a glass-on-glass Dounce homogenizer. The homogenate was spun at 600 × g for 15 min to sediment nuclei and large cell debris. The membrane fraction was then obtained by spinning the supernatant at 100,000 × g at an ultracentrifuge (Beckman, Optima LE-80K). The pellet was resuspended in binding buffer and incubated with radioligand at room temperature for 1 h. Specific binding was assessed using saturating concentrations of radiolabeled receptor antagonists: 8 nM [3H]DPCPX for adenosine A1 receptors, 30 nM [3H]SR225002 for adrenergic α2A receptors, and 4 nM [3H]spiperone for dopamine D2 receptors. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled antagonist: 8 μM DPCPX (A1), 30 μM rauwolscine (α2A), and 4 μM spiperone.
Binding was performed in triplicate and repeated at least four times. Data were corrected for total protein content in each sample and are expressed as fmol/µg of protein (mean ± S.E.).

Electrophysiology—Whole-cell membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments). Patch pipettes were pulled from filamented borosilicate glass (Clark Electromedical) and had a resistance of 1.5–2.5 MΩ when filled with pipette solution (see below). Prior to filling, tips of patch pipettes were coated with a Parafilm/mineral oil suspension. Data were acquired and analyzed using a Digidata 1200B interface (Axon Instruments) and pClamp software (version 6.0, Axon Instruments). Cell capacitance was ~15 pF, and series resistance (<10 MΩ) was at least 75% compensated using the amplifier circuitry. Recordings of membrane current were carried out after an equilibration period of ~5 min. Immediately following patch rupture, a current-voltage relationship was performed to establish that currents were inwardly rectifying. Theretofe cells were voltage-clamped at ~60 mV, and agonist-induced currents were measured at this potential. For current-voltage relationships, records were filtered at 1 kHz and digitized at 5 kHz. For continued data acquisition where cells were voltage-clamped at ~60 mV, records were digitized at 100 Hz.

Rapid Drug Application and Barium Calibration—Drugs were applied using a “sewer pipe” system (Rapid Solution Changer RSC-160, Bio-Logic) whereby an array of perfusion capillaries was placed in the bath ~40 μm from the recorded cell. This system allowed rapid solution switching between capillary tubes and localized application of drugs due to the laminar flow over the studied cells from the tubes as previously described (31). A number of parameters were determined using this system (Fig. 1A, part ii). Upon agonist application current activation with an initial delay (lag) followed by a rapid rise to peak amplitude (time-to-peak (ttpeak)). Current subsequently became desensitized during continued agonist application. In this study agonist was applied for 20 s. Upon agonist removal currents deactivated back to baseline levels.

For each cell we assessed whether there were any flow artifacts resulting from the pressure of drug application. We did this by applying bath solution from one of the sewer pipes and recording any flow artifacts that would lead to full receptor occupancy. We observed an initial lag followed by a rapid rise to a peak amplitude (lag plus ttp) and a plateau phase. Block of the current occurred with an initial delay before reaching equilibrium. It was assumed that this lag reflected the intracellular delivery time to the cell. A barium calibration was performed prior to the start of experiments to ascertain correct positioning of the sewer pipe and was repeated on several cells during each recording session. In general the results were highly reproducible (the lag time for barium block was 237.3 ± 11.7 ms (n = 73)).

Confocal Microscopy and Western Blotting—Confocal microscopy and acquisition of images were as previously described (28). In the current study we used a 40× oil objective, 40× laser power, gain was set to 50%, and iris aperture was opened to 1.5 nm (optimum aperture, 1.1 nm). Western blotting of CFP-tagged Gαs, subunits was performed using a polyclonal rabbit GFP antibody as previously described (28).

Materials and Drugs—Materials and procedures were as follows (concentrations in millimolar): pipette solution, 107 KCl, 1.2 MgCl2, 1 CaCl2, 10 EGTA, 5 HEPES, 2 MgATP, 0.3 Na3GTP (KOH to pH 7.2, ~140 ms total K+); bath solution, 140 KCl, 2.6 CaCl2, 1.2 MgCl2, 5 HEPES (pH 7.4). Cell culture materials were from Life Technologies, Inc. and Invitrogen. All chemicals were from Sigma or Calbiochem. Drugs were made up as concentrated stock solutions and kept at ~20 °C.

RESULTS

In our previous studies (26, 27), we generated an HEK293 stable cell line expressing the Kir3.1+3.2A channel complex. FIG. 2. Changes in channel kinetic properties with agonist concentration.

A, two traces recorded from a HKIR3.1/3.2/GGB cell voltage-clamped at ~60 mV and exposed to 1 μM (left-hand panel) and 100 μM (right-hand panel) baclofen for 20 s. Channel activation was greatly and significantly slowed when using a lower agonist concentration (p < 0.001). C, this bar chart summarizes data obtained from similar experiments using the HKIR3.1/3.2/2A1 cell line and two concentrations of NECA, 30 nm and 1 μM. Similarly to the findings with the HKIR3.1/3.2/GGB5 line, channel activation was significantly slowed using the lower agonist concentration (p < 0.001).

On this background of channel expression we subsequently generated dual receptor-plus-channel stable lines in which we have investigated the kinetic properties of receptor-mediated currents (see “Experimental Procedures”). The HKIR3.1/3.2/GGB cell line was used in Fig. 1B, whereas the dual receptor-plus-channel stable lines in Fig. 1C were used in a representative current recordings from three stable lines (A1; HKIR3.1/3.2/2A1, α2δ1; HKIR3.1/3.2/2α2, D2s; HKIR3.1/3.2/D2) in response to 20-s applications of maximal concentrations of agonist. To determine absolute levels of receptor expression we used radioligand binding with tritiated antagonists (see “Experimental Procedures”) and found similar levels in the HKIR3.1/3.2/2A1, HKIR3.1/3.2/2α2, and HKIR3.1/3.2/D2 clonal isolates used in Fig. 1B. These data are shown in Fig. 1C. We found that, although activation kinetics were quite similar through these three receptors, D2s-mediated currents did exhibit slower time courses of activation than α2δ1- or A1-mediated currents. We also investigated the kinetics of activation in two other cell lines (M4: HKIR3.1/3.2/M4 and GABA-B1b/2: HKIR3.1/3.2/GGB), and the mean data are summarized.
in Fig. 1D. Representative recordings from HKIR3.1/3.2/M4 and HKIR3.1/3.2/GGB are shown in subsequent figures.

The experiments just described were performed using maximal concentrations of agonist likely to result in full receptor occupancy. We next examined the effects of agonist concentration, and thus receptor occupancy, on channel activation. We used the HKIR3.1/3.2/GGB (Fig. 2A) and the HKIR3.1/3.2/A1 cell lines (Fig. 2B) and used the agonists at a high, saturating concentration and at a lower concentration, which was approximately the EC50 value. We found that, with the lower concentration of agonist, channel activation was significantly slowed (Fig. 2, A and B).

We have shown previously that by using engineered PTX-resistant Gα subunits, it is possible to look exclusively at coupling between a receptor and the channel via specific Gαi/o isoforms (27). Furthermore, we have recently made a series of cyan fluorescent protein (CFP)-tagged PTX-resistant Gαi/o isoforms and have shown that they are both membrane-targeted and functional (coupling to both βγ subunits and the adenylate cyclase pathway), and we have established conditions where these constructs are expressed at equivalent levels (28). In our previous work we have shown that the A1 receptor appears to couple to the channel with equal efficacy and potency via all the Gαi/o isoforms tested (27). We also now demonstrate that all CFP-tagged Gαi/o subunits are able to participate in A1-mediated channel activation with similar magnitudes of response and similar kinetic profiles except via GαoAC351G, where we observed that channel activation via this G-protein exhibited slower activation kinetics (Fig. 3) (28). However, we know that other receptors, for example the M4 muscarinic and the GABA-B1b/2 receptors (using the HKIR3.1/3.2/M4 and HKIR3.1/3.2/GGB lines, respectively). With the HKIR3.1/3.2/M4 cell line we used the CFP-tagged Gα subunits (Gα12-CFP and Gα25-CFP, at equivalent concentrations) and observed that, although channel activation via this receptor was intrinsically slower than through the A1 receptor, there was no significant difference in channel activation via Gαi2-CFP and Gαi2-CFP (Fig. 4). However with the HKIR3.1/3.2/GGB line we were unable to rescue coupling between the GABA-B1b/2 receptor and the channel in PTX-treated cells using the CFP-tagged G-proteins. This is the only receptor to date where we have observed this, and such lack of coupling may be related to their unique heterodimeric receptor formation. Instead we used the non-CFP-tagged GαoAC352G and GαoAC351G to study channel activation through the GABA-B1b/2 receptor. In the HKIR3.1/3.2/A1 cell line, expression of these constructs yielded comparable activation kinetics to the CFP-tagged variants (not shown). In contrast to both the A1 and M4 receptors, activation via this receptor was much faster through Gαi2 than GαoA (Fig. 5). For comparison the magnitude of current potentiation and kinetics of activation via native G-proteins are included (see Figs. 3–5). Importantly, we see different kinetic profiles of channel activation through different receptor and G-protein combinations despite robust coupling apparent from the magnitude of the response.

G-protein α isoforms are present at varying levels in different cells, and thus it is important to know what role the G-protein concentration has in determining these kinetic responses. To do this we established an inducible system whereby we could regulate the levels of expression of GαoA-CFP in a cell line stably expressing the channel complex and the A1 receptor (referred to as HKIR3.1/3.2/A1/GαoA-T). This was done using a commercially available system (see “Experimental Procedures”) whereby gene expression is conditional on the addition
of the antibiotic tetracycline (Tet). We titrated the concentration of Tet (0.01–100 μg/ml) to determine a high, medium, and low level of Gαi3-CFP expression. Fig. 6A shows the induction of Gαi3-CFP expression at the membrane in the HKIR3.1/3.2/A1/Gi0-T cell line with increasing concentration of Tet. Using Western blotting we showed the induction of graded expression of Gαi3-CFP (Fig. 6B). In addition, in PTx-treated cells, increasing concentrations of Tet progressively enhanced the amplitude of NECA-induced currents (Fig. 6C). However, importantly the kinetics of activation of the channel response was not altered (Fig. 6D).

Finally, we examined the potential role that diffusion of the receptor to G-protein might have on activation kinetics. We took advantage of an approach in which the receptor is physically tethered to an engineered PTx-resistant G-protein, specifically the A1 receptor and the Gαi3-C351G subunit. We both transiently and stably expressed this construct in the HKIR3.1/3.2 cell line. In addition, we characterized receptor expression density using radioligand binding with [3H]DPCPX (8 nM) in a clonal isolate and found that the fused A1 receptor was expressed at more than 2-fold higher levels (72.7 ± 13 fmol/μg of protein, n = 9) than the non-fused A1 receptor (28.6 ± 7.2 fmol/μg of protein, n = 6) (Fig. 1C). We compared the activation kinetics of A1 via endogenous G-proteins to that of the fused A1-Gαi3 construct after both transient and stable expression, with and without PTx treatment (Fig. 7). Under both sets of conditions activation was significantly slower via the fused construct both before and after PTx treatment. However, if Gαi3-C351G was transfected into the HKIR3.1/3.2/A1 line, signaling was also slowed in an analogous manner. Thus, it is the nature of the mutant G-protein subunit rather than the tethering per se that determines the change in activation kinetics. We also observed that the deactivation rate was increased and this is discussed below.

DISCUSSION

In this report we have taken advantage of the fact that Kir3.0 channels are gated directly by Gβγ subunits, and thus they act as biosensors for Gβγ concentration at the plasma membrane. One of the major advantages of this approach is that the release of Gβγ is directly measured and the system output is not dependent upon downstream events. In combination with electrophysiological recordings (and rapid agonist application) this results in high temporal resolution. We have analyzed the kinetic contribution of the ternary complex to channel activation. We have a number of major findings, namely that increased occupancy of the receptor accelerates activation kinetics, that the activation kinetics via a Gαo isoform are determined by the particular receptor/G-protein combination, and that receptor diffusion to the G-protein and the concentration of the G-protein have little influence on the activation kinetics. The G-protein amount simply determines the amplitude of the response. Our data all support the hypothesis that the assembly of the ternary complex of agonist, receptor, and G-protein is not rate-limiting. It is, however, the unique conformation of the active ternary complex that specifies the kinetic behavior of the channel response.

Channel activation kinetics through a number of different receptors with saturating agonist concentration occurs rapidly via the mixed pool of G-proteins endogenously expressed in HEK293 cells. However, there are significant differences with activation, with that through M4 being the slowest and α2A being the most rapid. Indeed, the nature and numerical details of channel kinetics are very similar to those occurring with the channel expressed in neurons (15, 32). Receptor-mediated currents elicited using high agonist concentration have a typical profile comprising an initial lag followed by a subsequent sharp rise to a peak amplitude after drug application. This pattern
likely reflects the occurrence of a number of sequential steps. In a classic “collision” coupling view these steps might consist of agonist binding to receptor followed by diffusion of the agonist/receptor complex to the G-protein, activation and dissociation of the G-protein heterotrimer, diffusion of Gβγ to the channel, and finally, activation and opening of the channel. Binding of Gβγ and channel activation are assumed to be fast and not rate-limiting. Indeed there is also evidence that channel activation is intrinsically cooperative, because the Hill coefficient for Gβγ-mediated channel stimulation is between 1.5 and 3 and more than one Gβγ subunit needs to occupy one of the four equivalent binding sites to initiate channel opening (33, 34). Such considerations would account for why there is a small discrepancy between the point at which induced G-protein is detected and the point at which significant coupling begins to occur in Fig. 6.

As might be expected from the principles of mass action, agonist concentration clearly influences the onset kinetics. Agonist binding to receptors is diffusion-limited, agonists bind preferentially to active receptor conformations, and thus within the timescale of signaling events the establishment of this equilibrium between active and inactive receptor conformations will not be rate-limiting. It is the proportion of active species that will subsequently determine the kinetics of the downstream response. But, what about other steps in the G-protein signaling pathway? To address the role of receptor diffusion to the G-protein we fused the A1 receptor directly to Gαi1. It is possible that a receptor might activate sequentially that deactivation rates are increased and this too is consistent with data of Waldhoer et al. (36). They find that the A1-Gα1i fusion releases bound radioligand agonist more readily than the A1 receptor and argue that the ternary complex is less stable (36). It is difficult to be quite so categorical with our approach, but our data are potentially consistent with this hypothesis.

Receptor occupancy is not the only factor that has important consequences for channel kinetics. We demonstrate that act-

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**Fig. 5. Effects of the Go isoform on channel kinetics via GABA<sub>1</sub>**. A, representative current traces obtained from HKIR3.1/3.2/GGB cells voltage-clamped at −60 mV. Recordings are made from control cells (in the absence of PTx) or transiently transfected cells (PTx, 100 ng/ml, >16 h) as indicated. Baclofen (100 μM) was applied as indicated by the horizontal bar. B, the bar chart shows the magnitude of current responses before, on, and after agonist application in conditions as indicated. C, bar chart shows a summary of channel activation data obtained from the HKIR3.1/3.2/GGB5 cell line transiently transfected with the non-tagged Go<sub>1i</sub> or Go<sub>1a</sub> subunits (1 μg of cDNA). Channel activation was significantly faster with Go<sub>1a</sub> than with Go<sub>1i</sub> (p < 0.01).
vation occurs faster via $G_{i1}$, $G_{i2}$, and $G_{i3}$ than $G_{oA}$ for the $A_1$ receptor. This pattern was reversed when channels were activated via the GABA-B$_{1b/2}$ receptor, and a similar non-significant trend was observed with the M4 receptor, thus illustrating the particular receptor/G-protein combination dictates the response. These observations can be accounted for by greater “kinetic efficacy,” i.e. some agonist-receptor-G-protein ternary complexes promote the faster release of GDP from the G-protein $\alpha$ subunit. This proposal is not unreasonable given the accumulating data supporting the idea that different GPCRs have differing affinities for the various $G\alpha$ isoforms and that even different agonists at the same receptor may couple with varying degrees to different G-protein isoforms (2, 6, 27, 38, 39). It has been argued on theoretical grounds that there may be significant differences in the predicted behavior of signaling cascades when considered in kinetic models compared with that in equilibrium models (3, 7). Our data argue that the ternary complex uniquely determines the kinetic as well as the steady-state properties.

Intriguingly, the G-protein amount simply modifies the amplitude of the response and does not influence the activation kinetics. This is consistent with the potential existence of a complex between the G-protein $\alpha$ subunit and channel (40) or a high degree of precoupling between receptor and G-protein being important for channel activation (28, 41), and it is cons-

![Diagram A](image1.png)

**Fig. 6.** Inducible $G_{oA}$-CFP expression system in the HKIR3.1/3.2/A1/$G_{oA}$-T stable cell line. **A,** upper panel shows laser scanning confocal images of live cells treated with tetracycline for 24 h to induce the expression of $G_{oA}$-CFP. Concentrations of tetracycline (Tet) are indicated ($\mu$g/ml). The lower panels show a brightfield image of the same field of cells. The scale bar represents 10 $\mu$m. Cells were imaged under identical conditions in each instance (see “Experimental Procedures”). **B,** we performed a Western blot using an antibody directed against GFP as previously described (28) to show graded expression of $G_{oA}$-CFP (band indicated by the asterisk) in response to various concentrations of Tet as indicated. Markers are for 50- and 80-kDa proteins. WT indicates lysate of HEK293 cells, and C indicates zero Tet in the HKIR3.1/3.2/A1/$G_{oA}$-T line. The symbol “*” indicates a background band that we observe using this antibody (as previously reported (28)) but that has a different mobility to GFP. **C,** summary of electrophysiological data showing mean current densities measured at $-60$ mV from HKIR3.1/3.2/A1/$G_{oA}$-T cells treated with combinations of Tet (concentrations as indicated) and PTx ($100$ ng/ml) for 24–30 h prior to recording. A representative sample of cells from each condition was recorded from. Not all Tet-induced cells produced agonist-stimulated currents: 25% of patched cells, which had been treated with Tet and PTx, were unresponsive to agonist and so were not included in the analysis. NECA-induced currents were significantly larger for the 10 $\mu$g/ml Tet-treated group than the 0.5 $\mu$g/ml group ($p < 0.05$). **D,** measured activation kinetic parameters show no significant differences between treatment groups.

![Diagram B](image2.png)

**Fig. 7.** The consequences of physically linking the receptor and G-protein. **A,** representative example of a current trace obtained from a HKIR3.1/3.2/A1-$G_{oA}$ cell voltage-clamped at $-60$ mV and exposed to NECA (1 $\mu$m, 20 s). **B,** bar chart showing a comparison of channel activation kinetics between the fusion protein $A_1-G_{oA}$ (“Fused”) and the $A_1$ receptor (“Unfused”) transiently transfected into the HKIR3.1/3.2/cell line. Channels were significantly slower ($p < 0.001$) to activate when the $A_1$ receptor was fused to $G_{oA}$, C351G. **C,** we compared channel activation kinetics via the $A_1$ receptor either alone (“Unfused”), when co-expressed with $G_{oA}$, C351G (+$G_{oA}$), or when fused to $G_{oA}$, C351G (“Fused/PTx”) and treated with PTx (“Fused/PTx”). Here channel activation kinetics were not significantly different.
sistent with theoretical studies (3). Our studies here have mostly focused on the A1 receptor, and it is possible that variations in precomplexing, for example promoted by GOS proteins, might influence activation kinetics via other receptors. It is intriguing that a recent report has shown dopamine receptors and Kir3.0 channels potentially existing in complexes suggesting that all three components may be associated in a macromolecular complex (42). It is also interesting that the kinetics of K+ and Ca2+ channel modulation in Goi, knockout mice are slowed (43); our results suggest that this may be related to differences in the efficacy of GPCR coupling to the remaining G-proteins rather than changes in amount of the total G-protein pool.

Our results and conclusions depend on the use of a number of tools, in particular that of PTX-resistant G-proteins with a slowed (43): our results suggest that this may be related to the GABA-B receptor clones. Annu. Rev. Biochem. 2. Kenakin, T. (2002) J. Pharmacol. Exp. Ther. 302, 1010–1018. 3. Shea, L., and Linderman, J. J. (1997) Biochem. Pharmacol. 53, 519–530. 4. Christopoulos, A., and Kenakin, T. (2002) Pharmacol. Rev. 54, 323–374.

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In summary our data support the concept that the formation of the ternary complex is not rate-limiting for signaling. It is the release of GDP from the G-protein heterotrimer that is important, and the rate at which that happens is determined by the conformation of the ternary complex.

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