Rapid desensitization of G protein-gated inwardly rectifying K⁺ currents is determined by G protein cycle

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Leaney, Joanne L., Amy Benians, Sean Brown, Muriel Nobles, David Kelly, and Andrew Tinker. Rapid desensitization of G protein-gated inwardly rectifying K⁺ currents is determined by G protein cycle. Am J Physiol Cell Physiol 287: C182–C191, 2004—Activation of G protein-gated inwardly rectifying K⁺ (GIRK) channels, found in the brain, heart, and endocrine tissue, leads to membrane hyperpolarization that generates neuronal inhibitory postsynaptic potentials, slows the heart rate, and inhibits hormone release. During stimulation of G protein-coupled receptors and subsequent channel activation, it has been observed that the current desensitizes. In this study we examined mechanisms underlying fast desensitization of cloned heteromeric neuronal Kir3.1+3.2A and atrial Kir3.1+3.4 channels and also homomeric Kir3.0 currents in response to stimulation of several Gᵦₒ G protein-coupled receptors (GPCRs) expressed in HEK-293 cells (adenosine A₁, adrenergic β₂A, dopamine D₂S, M₄ muscarinic, and GABA_B1B2 receptors). We found that all agonist-induced currents displayed a similar degree of desensitization except the adenosine A₁ receptor, which exhibits an additional desensitizing component. Using the nonhydrolyzable GTP analog guanosine 5’-O-(3-thiotriphosphate) (GTPγS), we found that this is due to a receptor-dependent, G protein-independent process. Using Ca²⁺ imaging we showed that desensitization is unlikely to be accounted for solely by phospholipase C activation and phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis. We examined the contribution of the G protein cycle and found the following. First, agonist concentration is strongly correlated with degree of desensitization. Second, competitive inhibition of GDP/GTP exchange by using nonhydrolyzable guanosine 5’-O-(2-thiodiphosphate) (GDPSβS) has two effects, a slowing of channel activation and an attenuation of the fast desensitization phenomenon. Finally, using specific Gα subunits we showed that ternary complexes with fast activation rates display more prominent desensitization than those with slower activation kinetics. Together our data suggest that fast desensitization of GIRK currents is accounted for by the fundamental properties of the G protein cycle.

G protein-coupled receptor; potassium channel; inward rectifier; kinetics

INWARDLY RECTIFYING K⁺ CHANNELS gated by G proteins (GIRK channels) were first characterized in atrial myocytes and are activated by acetylcholine binding to muscarinic M₂ receptors (33). Stimulation of this current is responsible in part for slowing of the heart rate in response to vagal nerve stimulation (14, 44). It is now clear that analogous currents are present in central neurons and neuroendocrine cells. In central neurons GIRK currents are activated by a large variety of Gαₒ-coupled receptors (34), including GABA_B and adenosine A₁, generating late inhibitory postsynaptic potentials (31, 41, 45). Activation of the current is membrane delimited (42), mimicked by nonhydrolyzable GTP analogs (6), and sensitive to pertussis toxin (PTX), implicating the inhibitory family of G proteins (Gᵦₒ) (36). Activation of native and cloned G protein-gated K⁺ channels has been shown to involve a direct interaction with the Gβγ dimer that is released from the activated G protein heterotrimer (30, 39, 45). The cloning of a subfamily of inwardly rectifying K⁺ channels (Kir3.0) revealed that the native channel is a heterotetrameric complex composed of Kir3.1 with Kir3.2, Kir3.3, or Kir3.4 subunits (8, 12, 17, 19, 21, 22, 29, 45). Recently it has become apparent that functional homotetrameric complexes of Kir3.0 may also occur in native cells (1, 11, 17) and that Kir3.2 and Kir3.3 may coassemble (18).

The receptor-mediated response displays characteristic activation, desensitization, and subsequent deactivation phases in response to agonist application and removal. It was noted in some of the earliest studies on the atrial channel that the current desensitizes with a two-component time course: the fast component has a time constant of a few seconds, whereas the slower process occurs over tens of seconds (7, 24). Fast current desensitization also occurs in neurons (7, 16, 35), occurring within seconds of the peak current response. The slow component of desensitization is likely to occur at the level of the receptor and involve phosphorylation by G protein-coupled receptor (GPCR) kinases, uncoupling from G proteins, and receptor internalization (24, 37, 40, 45). However, the molecular events that underlie the fast component of desensitization remain elusive and controversial. A number of theories have been proposed—receptor-dependent effects that are independent of the G protein (5), effects at the level of the channel (1), a mechanism that is accounted for by the intrinsic hydrolysis cycle of the G protein (24), and, more recently, depletion of phosphatidylinositol 4,5-bisphosphate (PIP₂) by concurrent activation of a G₁₁₁-coupled muscarinic receptor (10, 20).

We previously investigated processes accounting for activation and deactivation kinetics (2, 3), and we now focus on desensitization. In this study we systematically tested the various hypotheses suggested to account for this phenomenon. Our data show that desensitization is a fundamental property of all GPCRs and indicate that fast desensitization is a property of the G protein cycle.

MATERIALS AND METHODS

Molecular biology, cell culture, and transfection. The methods for cell culture and transient transfection and the techniques for estab-
lishing stable cell lines have been described previously (15, 27). In addition to the stable lines described previously (2, 3), a line expressing Kir3.1 and Kir3.4 channel subunits was established. Kir3.1 and Kir3.4 were subcloned into the dual-promoter vector pBudCE4.1. Monoclonal cell lines were generated after transfection and growth under selective pressure (364 μg/ml Zeocin). Single colonies were picked, expanded, and screened by patch clamping. The mutants (Kir3.1F137S and Kir3.4S143T) that generate currents as homomeric channels (43) were constructed with a QuickChange kit (Stratagene). Mutations were confirmed by automated sequencing.

Electrophysiology. Whole cell membrane currents were recorded with 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 Materials and drugs). Before filling, the tips of patch pipettes were coated with a Parafilm-mineral oil suspension. Data were acquired and analyzed with a Digidata interface (1200B or 1322; Axon Instruments) and pCLAMP software (version 6.0 or 8.0; Axon Instruments). Cell capacitance was ~15 pF, and series resistance (~10 MΩ) was at least 75% compensated with the amplifier circuitry. Recordings of membrane current were commenced after an equilibration period of ~5 min. Immediately after patch rupture a current-voltage relationship was determined to establish that currents were inwardly rectifying. Thereafter 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 continual data acquisition where cells were voltage clamped at ~60 mV, records were digitized at 100 Hz. Rapid drug application was performed as previously described (2, 3, 26) with a “sewer pipe” system (Rapid Solution Changer RSC-160; Bio-Logic). Agonist was applied for at least 20 s, and current responses followed a typical profile: after rapid activation of currents to a peak amplitude, current subsequently waned during the presence of agonist—we termed this “desensitization.” After removal of agonist, currents returned to baseline—we termed this “deactivation.” In this article we focus on desensitization, the magnitude of which was quantified by measuring the relative reduction from peak current at a series of different time points. This is illustrated in Fig. 1A. Channel activation characteristically exhibited an initial “lag” between drug application and onset of current, which was then followed by a subsequent rise to peak amplitude (‘time to peak,” TTP). Activation kinetics were therefore quantified as “lag + TTP,” which is the time period between initial drug application and the peak current amplitude (2). Deactivation kinetics were generally well fitted by a single-exponential decay function and were quantitated by the time constant for decay (deactivation τ) (3).

Fig. 1. Fast desensitization occurs through all studied G protein-coupled receptors (GPCRs). A: example of a quinpirole-activated current in the Kir3.1/3.2/D2 cell line recorded at ~60 mV in the whole cell patch-clamp configuration. The 20-s application of a maximal dose of agonist (10 nM) is indicated by the horizontal bar (in this and all subsequent figures). Current desensitization (as indicated) was measured as shown by measuring current amplitudes at time points 1, 5, 10, and 20 s from the peak. Data are expressed as %desensitization from peak. B: summary of the current desensitization observed over 20 s in all receptor + channel cell lines studied. Data are from the following numbers of cells: HKIR3.1/3.2/A1, n = 32; HKIR3.1/3.2/α2A, n = 18; HKIR3.1/3.2/D2, n = 24; HKIR3.1/3.2/GBB, n = 26; HKIR3.1/3.2/M4, n = 9. HKIR3.1/3.2/A1, HEK-293 stable cell line expressing Kir3.1 + 3.2A channel complex and adenosine A1; NECA, 5′-(N-ethylcarboxamido)adenosine; HKIR3.1/3.2/D2, HEK-293 stable cell line expressing Kir3.1 + 3.2A channel complex and dopamine D2; HKIR3.1/3.2/α2, HEK-293 stable cell line expressing Kir3.1 + 3.2A channel complex and adrenergic α2.
Calcium imaging. HEK-293 cells were cultured on poly-l-lysine-coated coverslips and were loaded with 5 μM fura 2-AM for 30 min at 37°C. Cells were perfused with an extracellular buffer containing (in mM) 140 NaCl, 2.5 KCl, 0.5 MgCl₂, 1.2 CaCl₂, 10 HEPES, and 5 glucose. The solution was buffered at pH 7.4 with NaOH. Fluorescence microscopy was performed with a polychrome II monochromator as an epifluorescent light source (T.I.L.L. Photonics) connected to a Zeiss microscope. Images were collected and analyzed with a digital microscopy system (Openlab; Improvision, Coventry, UK). Cells were excited alternately at 340 and 380 nm, and intracellular calcium was assessed from the ratio of the respective emitted intensity of fluorescent signals at 470–550 nM (the dichroic was 400DCLP and the emission filter 510/80; Chroma Technology).

Data analysis. Membrane currents were measured at −60 mV, and all data are presented as means ± SE, where n indicates the number of cells recorded from. Data were analyzed for statistical significance with either Student’s t-test or one-way repeated-measures ANOVA with Bonferroni correction as appropriate.

Materials and drugs. Solutions were as follows (concentrations in mM): pipette solution: 107 KCl, 1.2 MgCl₂, 1 CaCl₂, 10 EGTA, 5 HEPES, 2 MgATP, and 0.3 Na₃GTP (KOH to pH 7.2; ~140 mM total K⁺); bath solution: 140 KCl, 2.6 CaCl₂, 1.2 MgCl₂, and 5 HEPES (pH 7.4). Cell culture materials were obtained from GIBCO BRL and Invitrogen. All chemicals were purchased from Sigma or Calbiochem. Drugs were made up as concentrated stock solutions and kept at −20°C: 5'- (N-ethylcarboxamido)adenosine (NECA), adenosine (9-bromo-6-ribofuranosyladenine), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), D-ribofuranosyladenine), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), baclofen hydrochloride, carbachol (carbamylcholine chloride), quinpirole, and (−)-norpinephrine hydrate (all obtained from Sigma Aldrich, Poole, UK).

RESULTS

Kir3.1 +3.2A desensitization after stimulation of a diverse array of Gᵢ/o-coupled receptors. We used a HEK-293 stable cell line that expresses the Kir3.1 +3.2A channel complex (HKIR3.1/3.2) and dual Gᵢ/o-coupled receptor plus channel stable lines (adenosine A1: HKIR3.1/3.2/A1, adrenergic α₂A: HKIR3.1/3.2/α₂, dopamine D₂S: HKIR3.1/3.2/D2, muscarinic M₁: HKIR3.1/3.2/M4, and the heterodimeric GABA₆₁₁₂: HKIR3.1/3.2/GGB) (27, 28). Cells were studied with the whole cell configuration of the patch-clamp technique, and drugs were applied with a rapid agonist application system (see MATERIALS AND METHODS). Figure 1A shows how current desensitization was measured during agonist application. We next investigated whether currents, which were activated via a diverse array of receptors with a saturating concentration of agonist (A₁: 1 μM NECA, α₂A: 3 μM norepinephrine, D₂S: 10 μM quinpirole, M₁: 10 μM carbachol, GABA₆₁₁₂: 100 μM baclofen), exhibited desensitization in a qualitatively and quantitatively similar fashion (representative recordings from the different channel + receptor cell lines are shown in Figs. 2 and 5). The current desensitization was quantified at the time points indicated, and the mean data are summarized in Fig. 1B and in Table 1. We previously (2) used radioligand binding to measure levels of receptor expression in the HKIR3.1/3.2/A1, HKIR3.1/3.2/α₂, and HKIR3.1/3.2/D2 cell lines and found that receptors were expressed to similar levels. It is clear that the rapid desensitization of currents was quantitatively similar for the α₂A and D₂S receptors, whereas the A₁ receptor exhibited more profound desensitization that was seemingly accounted for by an initial rapid phase not present with the other receptors. In the Kir3.1/3.2/A1 line we also saw a transient reactivation in current on removal of agonist. These unique kinetic effects were observed consistently in two different clonal isolates of A₁ and Kir3.1/3.2 channel-expressing cell lines and in recordings from HKIR3.1/3.2-expressing cells transiently transfected with the adenosine A₁ receptor (data not shown). A potential explanation for this behavior is given below.

Desensitization in our system did not seem to be accounted for by K⁺ accumulation in the cell and reduction of driving force. We measured the reversal potential (E_rev) before and after agonist application. There was only a small change: in the HKIR3.1/3.2/D2 line (10 μM quinpirole), ΔE_rev = −2.18 ± 6.6 mV (n = 5). We examined desensitization at another holding potential (−100 mV) and observed no statistical difference in the magnitude of the effect. In the HKIR3.1/3.2A/D2 line, using 10 μM quinpirole and measuring desensitization 10 s into the response, we found that at −60 mV there was 16.7 ± 1.9% desensitization (n = 19) compared with 12.8 ± 1.4% desensitization at −100 mV (n = 6; not significant).

Desensitization of currents is observed with both homomeric and heteromeric Kir3.0 channels. It was suggested recently that GIRQ4 homomeric channels do not desensitize, suggesting that the desensitization phenomenon reflects molecular processes at the channel level (1). We examined the potential role of different Kir3.0 channel subunits might have in the desensitization response by investigating desensitization in different channel formations. In addition to the “neuronal” Kir3.1 +3.2A channel-expressing cell line, we have also established a stable HEK-293 cell line expressing the “cardiac” channel subunits Kir3.1 +3.4 (hereafter referred to as HKIR3.1/3.4). We also made functional homomultimers of Kir3.1 and Kir3.4 by introducing point mutations (Kir3.1F137S and Kir3.4S143T) into the coding sequence (43) and transiently transfected these into HEK-293 cells. The M₁ muscarinic receptor was transiently transfected into the HKIR3.1/3.4 channel cell line and into the cells expressing the homomeric channel subunits and was stimulated with 10 μM carbachol. Figure 2A shows representative recordings from each of these channel formations, and quantification of the desensitization data is summarized in Fig. 2B and in Table 1. It is clear that, although there may be some differences in the relative degree of desensitization, the desensitization phenomenon itself is observed qualitatively for all subunits, Kir3.1, Kir3.2A, and Kir3.4, in both heteromeric and homomeric channel complexes.

**Table 1. Degree of desensitization present at point of drug removal**

<table>
<thead>
<tr>
<th>Receptor and Channel Isoform</th>
<th>%Desensitization</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA&lt;sub&gt;6&lt;/sub&gt;₁₁₁₂ + Kir3.1/3.2</td>
<td>41.11 ± 2.93</td>
<td>32</td>
</tr>
<tr>
<td>D₂S + Kir3.1/3.2</td>
<td>29.19 ± 1.86</td>
<td>26</td>
</tr>
<tr>
<td>α₂A + Kir3.1/3.2</td>
<td>19.54 ± 2.40</td>
<td>19</td>
</tr>
<tr>
<td>A₁ + Kir3.1/3.2</td>
<td>17.00 ± 2.49</td>
<td>10</td>
</tr>
<tr>
<td>M₁ + Kir3.1/3.2</td>
<td>27.07 ± 5.15</td>
<td>9</td>
</tr>
<tr>
<td>M₄ + Kir3.1</td>
<td>14.10 ± 2.07</td>
<td>12</td>
</tr>
<tr>
<td>M₄ + Kir3.3/4 (S143T)</td>
<td>23.14 ± 2.56</td>
<td>12</td>
</tr>
<tr>
<td>M₄ + Kir3.1/3.4</td>
<td>12.56 ± 3.64</td>
<td>4</td>
</tr>
</tbody>
</table>

Data are means ± SE of the degree of desensitization present at the point of drug removal (20-s time point) for the various conditions detailed in the text. The appropriate statistical comparisons are detailed in the text and figures.
activation of an inhibitory receptor-mediated G_{q/11} pathway (20) by using fura 2 ratiometric imaging to examine changes in intracellular Ca^{2+}, which may result from phospholipase Cβ activation and inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} mobilization. We investigated whether a change in intracellular Ca^{2+} could be detected when a maximal concentration of the appropriate agonist was applied to each of our HEK-293 cell lines as described above. In two of these cell lines (HKIR3.1/3.2/M4 and HKIR3.1/3.2/α2A2) we found a significant increase in Ca^{2+} on agonist application (Fig. 3A, top), suggesting that HEK-293 cells are likely to contain endogenous muscarinic (e.g., M_1 and M_3) and adrenergic receptors (e.g., α_1) receptors coupled to G_{q/11}. Another possibility is activation of phospholipase C by release of Gβγ (38). We further studied the carbachol-mediated Ca^{2+} rise and found that it occurred in all clonal HEK-293 cell isolates studied, and thus we used it as a positive control. In contrast, no Ca^{2+} response was detected in the A_1 or GABA_A cell lines after the application of NECA and baclofen, respectively (Fig. 3A, bottom). Figure 3B shows mean 340 nm-to-380 nm ratios from a number of cells in each of the cell lines. It is clear that cell lines that display desensitization of Kir3.1/3.2A currents need not necessarily coactivate phospholipase Cβ signaling pathways, as detected by intracellular Ca^{2+} mobilization.

G protein-independent inhibition of Kir3.1 +3.2A currents in HKIR3.1/3.2/A1 cell line. Recently there was a report describing channel inhibition by some receptors in a G protein-independent fashion (5). We investigated this finding further, and our data are shown in Fig. 4. With a whole cell patch-clamp configuration in which guanosine 5′-O-(3-thiotriphosphate) (GTPγS) replaced GTP in the pipette solution, inwardly rectifying currents steadily increased until a stable baseline was reached. In Fig. 4A we show that GTPγS-loaded cells of the HKIR3.1/3.2/A1 cell line exhibit a direct and reversible inhibition of current in response to NECA. As the concentration of NECA was increased the rate of inhibition increased, whereas it did not affect the relief of inhibition, suggesting a bimolecular reaction. Figure 4B shows that NECA applied to the HKIR3.1/3.2 cell line does not cause any inhibition in the absence of the A_1 receptor and that NECA applied to the HKIR3.1/3.2/D2 cell line during desensitization after quinuclidine application does not lead to any additional desensitization. In a similar experiment using the HKIR3.1/3.2/GGB cell line, we showed that baclofen (100 mM) applied to GTPγS-loaded cells does not induce current inhibition (Fig. 4C). Similarly, current mediated by GTPγS-loaded cells of the HKIR3.1/3.2/D2 and HKIR3.1/3.2/M4 cell lines did not display inhibition in response to agonists, either (data not shown). Finally, we showed that another agonist (adenosine) at the A_1 receptor can cause this phenomenon (Fig. 4D; n = 7 cells). This process can be blocked by the A_1 receptor antagonist DPCPX (n = 2; not shown). Thus our data are consistent with a recent report (5) but show that this phenomenon is confined to the A_1 receptor.

Desensitization is dependent on degree of channel activation. We next examined how receptor occupancy might affect the observed desensitization by varying the agonist concentration. We used the HKIR3.1/3.2/GGB and HKIR3.1/3.2/A1 cell lines and applied the relevant agonists at a low concentration (approximately EC_{50}) and at a high, saturating concentration. Example traces for stimulation of HKIR3.1/3.2/GGB cells with 1 and 100 μM are shown in Fig. 5A. It is clear that at the lower concentration the channel took longer to reach peak activation, and the extent of channel desensitization was also significantly reduced. The data are summarized in Fig. 5B. In the HKIR3.1/3.2/A1 line, the initial rapid phase of the fast desensitization appeared to be absent at a 30 nM NECA concentration, and the accompanying current reactivation effect—usually observed on removal of 1 μM NECA—was reduced. We also examined the relationship between the magnitude of GABA_A receptor stimulation (measured as peak current density, I_{max}) and the extent of current desensitization after 20-s exposure to varying concentrations of baclofen. It is clear that the intensity of receptor stimulation was well correlated with percent current desensitization (Fig. 5C, left). We then looked for a relationship between the time taken for current to reach peak amplitude and the extent of desensitization at different concentrations of baclofen (Fig. 5C, right). Interestingly, we found a good degree of inverse correlation between these two parameters (although
linear regression was not performed because of the saturating nature of this effect at 10 and 100 μM baclofen). Clearly, the slower the current activates the less it desensitizes, and the stronger the intensity of receptor stimulation the greater the extent of current desensitization. This is consistent with previous studies in native cardiac myocytes (24).

We investigated the effect of competitive inhibition of GDP-GTP exchange by including guanosine 5′-O-(2-thiodiphosphate) (GDPβS) in the pipette solution (in addition to the normal amount of GTP). This phosphorylation-resistant analog of GDP has been shown to attenuate many G protein-mediated signaling processes (23), probably by reversibly occupying the

![Graphs and figures showing calcium responses in HEK-293 cell stable lines.](image-url)
guanine nucleotide binding pocket on the Go subunit, thereby preventing binding of GTP and formation of active Go-GTP
and Goγ subunits. We used the HKIR3.1/3.2/GGB cell line to
examine the effect of GDP on signaling to the channel, and
representative current traces with different amounts of GDP
in the pipette solution are shown in Fig. 6A. We found that
desensitization was dramatically attenuated with 3 mM
GDP present in the pipette solution (Fig. 6B) and that the
rate of activation of the current was significantly slowed (Fig.
6C). Unexpectedly, the rate of deactivation of currents on
removal of agonist was also significantly enhanced in the
presence of GDP (Fig. 6C).

Thus, from the data shown in Figs. 5 and 6, it is apparent that
the degree of desensitization correlates with the rate of activa-
tion. Our previous studies (2) showed that activation kinetics
can also be affected by components of the whole ternary
complex (i.e., the unique combination of agonist, receptor, and
G protein isoform). To investigate the role of the specific G
protein α-subunit we used two Go subunits, Goα2 and Goα1,
which had been made resistant to the actions of PTX by the
mutation of the carboxy terminus cysteine residue to
glycine. Goα2C352G or GoαAC351G were transiently trans-
fected into the HKIR3.1/3.2/GGB cell line, and the cells were
subsequently treated with PTX to eliminate coupling to endog-
enous G proteins. We previously established (2) that activation with 100 μM baclofen application was significantly slowed via G_{i2} compared with G_{oA}. We found that baclofen-activated currents also exhibited significantly greater desensitization when mediated through G_{oA} compared with G_{i2} (Fig. 7).

**DISCUSSION**

Our data suggest that Kir3.0 channel desensitization is a general property of all receptors and channel isoforms. It depends on the intensity of the signal to the G protein and reflects the dynamics of the G protein cycle. Other factors may act to modulate this underlying response but are not central as a causative mechanism.

Since it was first described in atrial myocytes, there has been considerable interest in the fast desensitization phenomenon of Kir3.0 currents in both native and cloned systems (10, 20, 24, 32, 37, 45). Most of the previous work by other investigators has focused on the M2 receptor and the native and cloned cardiac channel. However, in this study we have attempted to systematically dissect the mechanisms for the phenomenon of fast desensitization by using cloned atrial, neuronal, and novel homomeric Kir3.0 channels and several GPCRs. We first showed that a large number of pharmacologically distinct GPCRs, when activated at saturating agonist concentration, can all lead to fast desensitization of the current. Although the GPCRs may be pharmacologically distinct, it is clear from the data shown in Fig. 1 that they all lead to a quantitatively similar magnitude of current desensitization. It would appear that desensitization is a general phenomenon that is related generically to GPCR activation rather than having a specific pharmacological context. This is supported by observations in both cloned and native systems (4, 10); however, desensitization in response to opioid receptor stimulation in locus ceruleus neurons was more compatible with a slow desensitization process (10). In hippocampal neurons, one of us (25) has demonstrated that baclofen activation of endogenous GABA_B receptors does lead to pronounced rapid desensitization similar to that observed in the current study. The dynamics and magnitude of the response were very similar. In addition, although we have looked at channel modulation via M4 in our heterologous system, our data are comparable to those obtained via M2 in atrial myocytes. Thus it is clear that desensitization is not an artifact of receptor and channel overexpression in the HEK-293 system. The phenomenon is qualitatively similar in native and heterologous systems.

Early studies in atrial myocytes found that desensitization only occurred at higher agonist concentrations. It was concluded that it was related to intrinsic properties of the G protein cycle (24), and this has been supported by studies on cloned channels (10). Here we show a similar agonist dependence in experiments using the A_1 and GABA_B heterodimeric receptors—desensitization was less profound at lower agonist concentrations—and thus demonstrate that desensitization is a general feature of agonist concentration and GPCR activation. Furthermore, we showed that fast current desensitization is...
channel activation. Why haven’t we seen regulation through the endogenously expressed receptor? Our pipette solution contains relatively little Ca$^{2+}$ (~20 nM) that is heavily buffered, and it is known that phospholipase C activity is dependent on Ca$^{2+}$ (38). Thus, to get significant enzymatic activity in the whole cell configuration, it seems likely that it is necessary to enhance signaling efficacy by increasing the levels of receptor expression.

A second possibility to account for desensitization is that a time-dependent decrease in external K$^+$ concentration occurs with agonist application. Measurement of $E_{rev}$ before and after agonist application revealed little change. In addition, performing the experiments at more hyperpolarized potentials where inhibited in our system by the inclusion of GDP$\beta$S, which retards GDP/GTP exchange and consequently inhibits G protein cycling from inactive to active states. Paradoxically, we found that the rate of current deactivation is also enhanced by GDP$\beta$S; the reasons for this are unclear. In addition, we found that the rate of current deactivation is also enhanced by GDP$\beta$S as indicated. B: summary of desensitization data obtained from experiments described in A. GDP$\beta$S acted to both decelerate channel activation and accelerate deactivation rates. *$P$ ≤ 0.05, **$P$ ≤ 0.001.

One of the more recent and controversial proposals is that concurrent activation of a G$\alpha_{q/11}$ receptor leads to PIP$_2$ depletion and thus current inhibition (9, 20, 32). A number of our observations tend to argue against such a mechanism being a broad one. First, GPCRs such as GABA$_B$ do not have a G$\alpha_{q/11}$-coupled counterpart, although it is conceivable that phospholipase C may be activated in these cell lines by G$\beta$$\gamma$ released from G$_{16}$ heterotrimers (38). Thus we formally investigated this possibility by looking for activation of such pathways in our cell lines with Ca$^{2+}$ imaging whereby the activation of phospholipase C and hydrolysis of PIP$_2$ resulting in the generation of IP$_3$ would lead to a rise in intracellular Ca$^{2+}$ as it is released from intracellular stores. We found that neither baclofen nor NECA stimulation of the GABA$_B$ and A$_1$ receptors mobilized intracellular Ca$^{2+}$. This argues against PIP$_2$ depletion as a general mechanism; instead, it may act to merely enhance desensitization. However, it is worth noting that even in the M$_4$ line, where carbachol can stimulate an endogenous G$\alpha_{q/11}$-coupled muscarinic receptor, the degree of desensitization was no more prominent than with the other receptors. In our previous studies we have generally had to overexpress muscarinic M$_1$ and M$_3$ receptors in HEK-293 cells to observe channel regulation. Why haven’t we seen regulation through the endogenously expressed receptor? Our pipette solution contains relatively little Ca$^{2+}$ (~20 nM) that is heavily buffered, and it is known that phospholipase C activity is dependent on Ca$^{2+}$ (38). Thus, to get significant enzymatic activity in the whole cell configuration, it seems likely that it is necessary to enhance signaling efficacy by increasing the levels of receptor expression.

i.e., overexpression of RGS proteins increases desensitization and signaling via RGS-insensitive Go subunits leads to less (although this is not statistically significant). In a previous study (2), we found that G protein levels simply controlled the amplitude of current response but not the channel kinetics or the desensitization; this finding supports our general hypothesis.
the currents are larger (and accumulation should be more pronounced) led to a similar level of desensitization. Finally, Chuang et al. (10) only observed desensitization in inside-out macropatch recordings when switching from GDP to GTP. Thus our data do not suggest a central role for K⁺ deactivation; however, we cannot exclude its importance in other situations or systems.

Here we also report an A₁-mediated channel inhibition that is independent of G protein activation that is revealed when HKIR3.1/3.2/A1 cells are dialyzed with GTPγS. This was a finding unique to the A₁ receptor—we did not observe this with any other receptors tested. Other investigators have observed a similar phenomenon after adenosinergic expression of the A₁ receptor in atrial myocytes (5). This is likely to account for the more profound desensitization observed with the A₁ receptor, and it may also underlie the transient current increase on agonist withdrawal. We suggest that it may represent a direct sequestration of Gβγ subunits by the A₁ receptor itself, but further studies are required to elucidate the underlying mechanism.

Recently, it was proposed that GIRK4 homomultimers do not desensitize and that desensitization reflects processes occurring at the level of the channel (1). It is worth noting that if channel activation by liberated free Gβγ is slow (relative to the kinetics of upstream events), recorded currents will not reflect the dynamics of the G protein cycle. If channel homomultimers in a given environment activate more slowly than heteromultimers then such an extrapolation cannot be made. To investigate this hypothesis we studied the desensitization of various hetero- and homomultimeric Kir3.0 channels in response to stimulation of M₄ receptors. In contrast to Bender et al. (1), we found that desensitization was a general property of all GIRK channels regardless of their subunit composition.

Thus our data support the hypothesis that the G protein cycle, and in particular the rate of entry into it, is of central importance to a general mechanism of desensitization that can occur with any Gsα-coupled GPCR at saturating agonist concentration. However, other processes such as PIP₂ depletion and receptor-dependent G protein-independent processes may be able to attenuate or potentiate this response.

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