

ION CHANNELS, TRANSPORTERS

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Christian Lüscher · Paul A. Slesinger**Endogenous RGS proteins enhance acute desensitization of GABA_B receptor-activated GIRK currents in HEK-293T cells**Received: 19 July 2004 / Revised: 14 September 2004 / Accepted: 22 October 2004 / Published online: 10 December 2004
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Abstract The coupling of GABA_B receptors to G-protein-gated inwardly rectifying potassium (GIRK) channels constitutes an important inhibitory pathway in the brain. Here, we examined the mechanism underlying desensitization of agonist-evoked currents carried by homomeric GIRK2 channels expressed in HEK-293T cells. The canonical GABA_B receptor agonist baclofen produced GIRK2 currents that decayed by $57.3 \pm 1.4\%$ after 60 s of stimulation, and then deactivated rapidly (time constant of 3.90 ± 0.21 s) upon removal of agonist. Surface labeling studies revealed that GABA_B receptors, in contrast to μ opioid receptors (MOR), did not internalize with a sustained stimulation for 10 min, excluding receptor redistribution as the primary mechanism for desensitization. Furthermore, heterologous desensitization was observed between GABA_B receptors and MOR, implicating downstream proteins, such G-proteins or the GIRK channel. To investigate the G-protein turnover cycle, the non-hydrolyzable GTP analogue (GTP γ S) was included in the intracellular solution and found to attenuate desensitization to $38.3 \pm 2.0\%$. The extent of desensitization was also reduced ($45.3 \pm 1.3\%$) by coexpressing a mutant form of the G α q G-protein subunit that has been designed to sequester endogenous

RGS proteins. Finally, reconstitution of GABA_B receptors with G α o G-proteins rendered insensitive to RGS resulted in significantly less desensitization ($28.5 \pm 3.2\%$). Taken together, our results demonstrate that endogenous levels of RGS proteins effectively enhance GABA_B receptor-dependent desensitization of GIRK currents.

Keywords Desensitization · GABA · GIRK · Kir3 · Potassium channels · RGS proteins

Introduction

Many inhibitory neurotransmitters exert their actions by stimulating G-protein-coupled neurotransmitter receptors (GPCR) that couple to pertussis-toxin (PTX)-sensitive G-proteins (G α i/o). Activation of G α i/o G-proteins leads to the inhibition of adenylyl cyclase, inhibition of voltage-gated Ca²⁺ channels (VGCC), and activation of G-protein-gated inwardly rectifying K⁺ channels (GIRK or Kir3) [25]. While the activation of GIRK channels localized on the soma and the dendrites inhibits the neuron by hyperpolarization [46], G-protein-mediated inhibition of VGCC or a direct inhibition of the release machinery downstream of Ca²⁺ entry is responsible for the presynaptic inhibition of transmitter release [65]. Together, activation of GIRK channels and inhibition of Ca²⁺ channels provides a powerful mechanism for suppressing electrical activity. Loss of GIRK channel activity results in susceptibility to seizures [61, 63], cardiac abnormalities [68] and changes in anxiety [3]. Four mammalian GIRK channel subunits (GIRK1–GIRK4) have been identified, which coassemble to form neuronal and cardiac GIRK channels [69]. Whereas most GIRK channels form heterotetramers (e.g. GIRK1/2, GIRK1/4, GIRK2/3), GIRK2 homotetramers may exist in certain regions of the brain, such as in the substantia nigra [27, 45].

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Stimulation of GPCRs that couple to PTX-sensitive G-proteins opens GIRK channels within milliseconds [25]. With persistent stimulation of the GPCR, however, the amplitude of current may decrease with time. This reduction in current level is referred to as “desensitization” and represents an adaptive response of the cell to prevent excessive G-protein signaling. Desensitization develops with different time courses, depending on the length of receptor stimulation [15]. For example, brief (minutes) stimulation of β 2Rs leads to phosphorylation of the GPCR by receptor kinases and subsequent uncoupling of the G-protein with the receptor. Prolonged stimulation (tens of minutes) can trigger receptor internalization, which by rapid recycling has been implicated in resensitization of the GPCR [14]. Even longer stimulation may lead to internalization followed by down-regulation of the receptor and/or effector. For GIRK channels, desensitization could develop anywhere along the signaling pathway, from the GPCR to the channel itself. Indeed, G-protein receptor kinases (GRK) [37, 58], RGS proteins [9, 31, 55], PIP₂ [36], and inhibitory G-proteins [1] have all been implicated in controlling the acute desensitization of GIRK channels.

Synaptic activation of GABA_B receptors in the dendrites of neurons produces a slow inhibitory postsynaptic potential (IPSP) that is carried by GIRK channels [46, 64]. Postsynaptic GABA_B receptors are postulated to be located extrasynaptically, requiring elevated levels of synaptic-released GABA, such as might occur during stimulation of multiple interneurons (“pooling”) [18, 29, 56]. It is therefore important to assess whether GABA_B receptors undergo desensitization during sustained activation. In periaqueductal gray neurons, for example, stimulation of GABA_B receptors produces little desensitization of GIRK currents, while stimulation of those in laterodorsal tegmental area results in >25% desensitization [7]. Likewise, in the VTA, desensitization to baclofen is only observed in dopamine neurons and is virtually absent in GABAergic neurons of the same nucleus [11]. Thus, the extent of GABA_B receptor desensitization is variable, depending on the type of neuron. GABA_B receptors are unique among GPCRs in that signaling requires the dimerization of two different GABA_B subunits (B1/B2) [32, 33, 67]. The existence of two splice variants of GABA_{B1} receptors, which differ only in the N-terminal domain, raises the possibility of diversity in signaling [18], though there are no apparent differences in agonist affinity with the different splice variants [34].

Several labs have attempted to study GABA_B receptor-dependent desensitization in heterologous expression systems. With brief (2 s) but repetitive stimulation of GABA_{B1/B2} receptors, the amplitude of GIRK current decreases ~60% over a period of 10–15 min and appears to internalize [10]. PKA phosphorylation of the C-terminal tail attenuated this decrease, suggesting that the stability of GABA_B receptors in the membrane is enhanced with PKA-dependent phosphorylation [10]. Prolonged stimulation (1 h) of GABA_B receptors

produces desensitization when the G-protein receptor kinase 4 (GRK4) is coexpressed with the receptor in HEK-293 cells [52]. Surprisingly, the kinase domain, and therefore phosphorylation via GRK4, was not required for internalization [52]. In CHO cells, long-term (>2 h) stimulation of GABA_B receptors also induces internalization [20]. Thus far, the effect of a sustained but brief (~60 s) stimulation of GABA_B receptors on GIRK currents has not been thoroughly studied. We reasoned that application of an agonist lasting seconds might be a more realistic representation of the pooling of GABA that is produced during synaptic activation [29, 56], particularly in response to tetanic stimulation or epileptic seizures. In the present study, we investigated the effects of acute 60-s stimulation of GABA_B receptors on GIRK currents reconstituted in mammalian cells (HEK-293T).

Materials and methods

Molecular biology and cell culture

The following cDNAs were used: rat GIRK1 in pcDNA3.1 [39], mouse GIRK2 in pcDNA3.1 or pIRES2-EGFP [44], rat GABA_{B1} (A splice-variant) and rat GABA_{B2} receptors (pcDNA3.1+) [48], and human MOR in pcDNA3 [5]. For internalization assays, hemagglutinin (HA: YPYDVDPDYA) tags were inserted into GABA_{B1} [48] and inserted after the third amino acid in MOR. G α_q -Q209L- Δ N (pcDNA3.1+) was constructed by using a 5' primer containing a lipid moiety from p60src (MGSSKSKPKDPSQRR) and amplifying A36 to K354 of Gqi5 (gift from Bruce Conklin). The PCR product was subcloned in HA-Gq and then subjected to whole-plasmid PCR to introduce the Q209L mutation. G α_o -PTXi M17526: (pcDNA3) contains C351G, and G α_o -PTXi/RGSi (pcDNA3) contains G184S/C351G (see [42]). All mutations and PCR products were confirmed by DNA sequencing. cDNAs purified using a Qiagen maxiprep kit were used for all transfections.

HEK-293T cells were cultured in DMEM supplemented with fetal bovine serum (10%), glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μ g/ml, GIBCO) in a humidified, 37°C incubator with 95% air/5% CO₂. Cells were plated onto 12-mm glass cover slips (Warner Instruments) coated with poly-D-lysine (20 μ g/ml, Sigma) and collagen (100 μ g/ml; BD Biosciences) in 24-well plates. After 1 day of proliferation, cells were transfected by calcium phosphate precipitation with cDNA for receptors, channels and, in some cases, G-proteins, along with the cDNA for YFP to identify transfected cells. cDNA was mixed in sterile deionized water with 2.5 M CaCl₂ to a final concentration of 2 ng/ μ L cDNA and 0.25 M CaCl₂. This mixture was combined 1:1 with HEPES buffered saline [280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM Glucose, 50 mM HEPES (pH 6.9) with ~1 N NaOH] and

then added to each well. HEK-293T cells were cultured for 16–32 h. For experiments with pertussis toxin, PTX (Sigma; 250 ng/ml) was added to the culture medium and incubated for 4 h at 37°C.

Electrophysiology

The whole-cell patch-clamp technique [22] was used to record macroscopic currents from HEK-293T cells. Borosilicate glass (Warner, P6165T) electrodes had resistances of 1–3 M Ω and were coated with Sylgard to reduce capacitance. Membrane currents were recorded with an Axopatch 200 or 200B (Axon Instruments) amplifier, adjusted electronically for cell capacitance and series resistance (80–100%), filtered at 2 kHz with an 8-pole Bessel filter, digitized at 5 kHz with a Digidata 1200 series interface (Axon Instruments), and stored on a laboratory computer. The extracellular solution contained (in mM): 140 NaCl, 20 KCl, 2 MgCl₂, 0.5 CaCl₂, and 10 HEPES/NaOH (pH 7.4). We used a low extracellular Ca²⁺ solution to minimize possible Ca²⁺-dependent toxicity during 30-min recordings. (\pm)-Baclofen (Sigma) was dissolved in water to make a 10-mM stock and then diluted directly in the extracellular solution. The intracellular solution contained (in mM): 20 NaCl, 130 KCl, 5.46 MgCl₂, 10 HEPES/NaOH (pH 7.4), and 5 EGTA/KOH (pH 7.4). On the day of recording, 2.56 mM K₂ATP was added with either 300 μ M Li₂GTP or 100 μ M GTP γ S to the intracellular solution. Data were acquired at room temperature (22–25°C) and analyzed with Clampfit 8.0 (Axon Instruments). The deactivation time constant (τ) was measured by fitting with a single exponential the decrease in current following removal of agonist. Unless indicated otherwise, HEK-293T cells were held at –100 mV. Current–voltage relations were determined using voltage ramps from –100 mV to +40 mV. Cells were superfused with the recording solution and rapidly exposed to different solution by means of a pinch-valve-controlled perfusion system (ValveLink8, Automate Scientific) connected to a 6–1 Teflon manifold (MM-6, Warner Instruments). The PE-10 output tubing was positioned at a 45° angle adjacent (~50 μ m) to the HEK-293T cell. All values are mean \pm SEM. One-way ANOVA, followed by Holm-Sidak's post hoc test; was used for multiple comparisons and Student's *t*-test for un-paired comparisons. Statistical significance was taken as $P < 0.05$.

Receptor internalization assay

HEK-293T cells grown on poly-D-lysine-coated glass coverslips at medium density at 37°C in a humidified incubator with 5% CO₂ were transfected with 0.5 μ g cDNA encoding for GIRK2 cDNA and either HA-GABA_{B1} receptor and GABA_{B2} receptor cDNA, or HA-MOR cDNA. After ~18 h of expression, the med-

ium was changed, and the cells were incubated with 2.5 μ g/ml of FITC conjugated anti-HA antibody (COVANCE) for 1 h on ice. The cells were then exposed to saturating concentration of the agonists (100 μ M baclofen for GABA_B receptors or 10 μ M DAMGO for MOR) for 10 or 20 min at 37°C. For negative control, cells were incubated with the vehicle only for 20 min at 37°C. The cells were placed on ice, quickly washed three times with ice-cold PBS, and then fixed for 20 min with 4% paraformaldehyde. Coverslips were rinsed three times (5 min each) with PBS and then mounted on slides in an anti-fading media Flucka (Sigma). Images were analyzed for receptor localization using an Olympus Fluoview confocal microscope.

Biochemistry

For RT-PCR, untransfected HEK293T cells were cultured at high density on 100-mm dishes, and the cytoplasmic RNA was isolated using an RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Total RNA was also prepared from fresh mouse brain tissue, using an RNeasy Mini Kit. Brain tissue was disrupted with mortar and pestle and then homogenized by ten passes through a 20-gauge needle. The purity of RNA was checked by denaturing-agarose gel electrophoresis and ethidium bromide staining. RT-PCR was performed using a Qiagen OneStep RT-PCR kit, according to the manufacturer's instructions. No template or omitting reverse transcriptase were included as negative controls. Q-solution from the kit was used only for the mouse brain total RNA. The RGS4 5' primers were 5'-TGTGCAAAGGGCTTGCAGG and 3'-GAGGCAC-ACTGAGGGACC.

For Western analysis, total proteins from HEK293T cells or cells transfected with RGS4 cDNA were homogenized in an ice-cold, 500- μ l hypotonic buffer (5 mM Tris-Cl/ 5 mM EDTA/ 5 mM EGTA, pH8.0) and centrifuged at 2,000 *g* for 10 min at 4°C. The supernatant was centrifuged again at 120,000 *g* for 30 min at 4°C, producing the cytosolic fraction (S1) and membrane pellet (P1). The P1 was solubilized in 200 μ l RIPA buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 1% triton, 0.1% SDS] and incubated for 1 h at 4°C before being centrifuged at 20,000 *g* for 20 min at 4°C. Fifteen microliters of either membrane or cytosolic fractions were denatured with SDS loading at 100°C for 3 min, separated on a 10% SDS-PAGE, and then transferred to nitrocellulose. Anti-RGS4 antibodies [no. sc-6203, RGS4(C-17) from Santa Cruz Biotechnology] were used for Western blot analysis and detected using donkey anti-goat (sc-2020, Santa Cruz Biotechnology) antibody and enhanced chemiluminescence. The RGS4 peptide (Santa Cruz) was used for control. Immunostaining using RGS4 antibody was performed as described previously [16].

Results

Homologous desensitization of GABA_B receptor-mediated activation of GIRK channels

To reconstitute GABA_B receptor-coupling to GIRK channels, we transfected HEK-293T cells with the cDNA for GABA_{B1} and GABA_{B2} receptors along with the cDNA for GIRK2. GIRK2 channels were used because of their propensity to form homotetramers in heterologous expression systems and in some neurons [27, 62]. We first determined the coupling efficiency of GABA_{B1/B2} receptor with GIRK2 channels expressed in HEK-293T cells, using increasing doses of the GABA_B receptor agonist, baclofen (Fig. 1a). The Hill plot shows an EC₅₀ = 1.73 ± 0.25 μM and a Hill coefficient = 1.20 ± 0.09 (*n* = 8) (Fig. 1b). Exposure of HEK-293T cells to PTX, which uncouples PTX-sensitive G-proteins

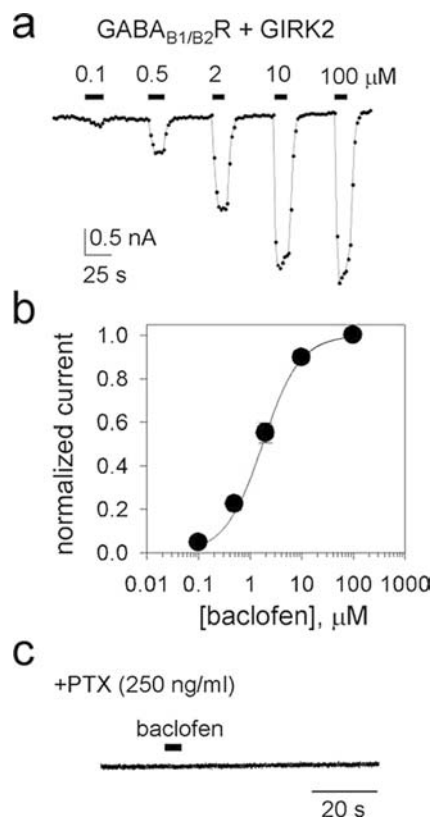


Fig. 1 GABA_B receptors activate GIRK2 channels expressed in HEK-293T cells. Unless indicated otherwise, HEK-293T cells were transfected with the cDNA for GABA_{B1} and GABA_{B2} receptors and GIRK2 channel in this and subsequent figures. **a** Whole-cell patch-clamp recording shows the increase in inward current with different doses of the GABA_B receptor agonist (\pm)-baclofen. Current was measured at -100 mV with voltage ramps from -100 to $+50$ mV. **b** Plot of the baclofen concentration as a function of normalized current. *Smooth curve* shows the best fit with the Hill equation: EC₅₀ = 1.73 ± 0.25 μM and the Hill coefficient = 1.20 ± 0.09 (*n* = 8). **c** Pre-treatment with pertussis toxin (PTX) (4 hr) uncouples PTX-sensitive G-proteins and prevents GABA_B receptor activation of GIRK2 channels. Current was recorded at a holding potential of -100 mV

from their cognate receptors, completely abrogated GIRK channel activation (Fig. 1c).

Having determined that 100 μM baclofen produces maximal activation, we next studied the effect of a 60-s stimulation of GABA_{B1/B2} receptors. With a short application of baclofen, GIRK current increased rapidly but then started to decay within seconds and attained a steady-state level of ~40% residual current (57.3 ± 1.4% desensitization, *n* = 40) (Fig. 2a). A 5-min baclofen treatment produced slightly more desensitization (Fig. 2b). The decrease in current during desensitization was well fit, with a single exponential having a time constant of 20.2 ± 0.89 s (*n* = 27); thus, the majority of desensitization occurred during the first 20 s of stimulation. The extent of GABA_B receptor-dependent desensitization with GIRK1/GIRK2 heteromeric channels (61.2 ± 5.2%, *n* = 3) was similar to that of GIRK2 homomultimers. We next examined the effect of intracellular cAMP on the desensitization produced during sustained a 60-s stimulation [10]. There was no statistical difference in the percentage desensitization (54 ± 4%, *n* = 8), suggesting that the dephosphorylation of the GABA_B receptor did not underlie the acute desensitization observed during the 60-s stimulation of GABA_B receptors (Fig. 2c).

We found that some desensitization occurred with repetitive, brief (5 s) receptor stimulation. For example, three 5-s saturating doses of baclofen separated by 35 s produced 16.0 ± 4.1% (*n* = 4) desensitization (Fig. 2d). Increasing the wash time to 60 s decreased the desensitization to 4.2 ± 1.5% (*n* = 8) (Fig. 2e). These results indicated that a wash of 60 s or longer was sufficient to allow receptors to recover from acute desensitization produced with 5-s pulses of baclofen. To study the recovery from desensitization produced by 60-s baclofen application, the GABA_B receptor-activated currents (5-s pulse) were measured at 15 s, 85 s, and 270 s, following the 60-s baclofen treatment (Fig. 2). The baclofen-induced current recovered ~30% of control at 15 s and nearly 80% of control after >4 min of wash (Fig. 2f). These experiments demonstrate that the GABA_B receptor-dependent desensitization of GIRK2 currents develops within seconds, recovers within minutes, and does not depend on intracellular cAMP.

Acute desensitization is not caused by GABA_B receptor internalization

Sustained stimulation of GPCRs can lead to receptor internalization [37], which could account for a decrease in GIRK current with time. To examine the possible internalization of GABA_B receptors, the movement of the receptor was monitored during receptor stimulation. GABA_{B1} receptors containing an extracellular HA tag (inserted into the N-terminal domain [47]) were coexpressed with GABA_{B2} receptor and GIRK2. The μ opioid receptor (MOR) was used

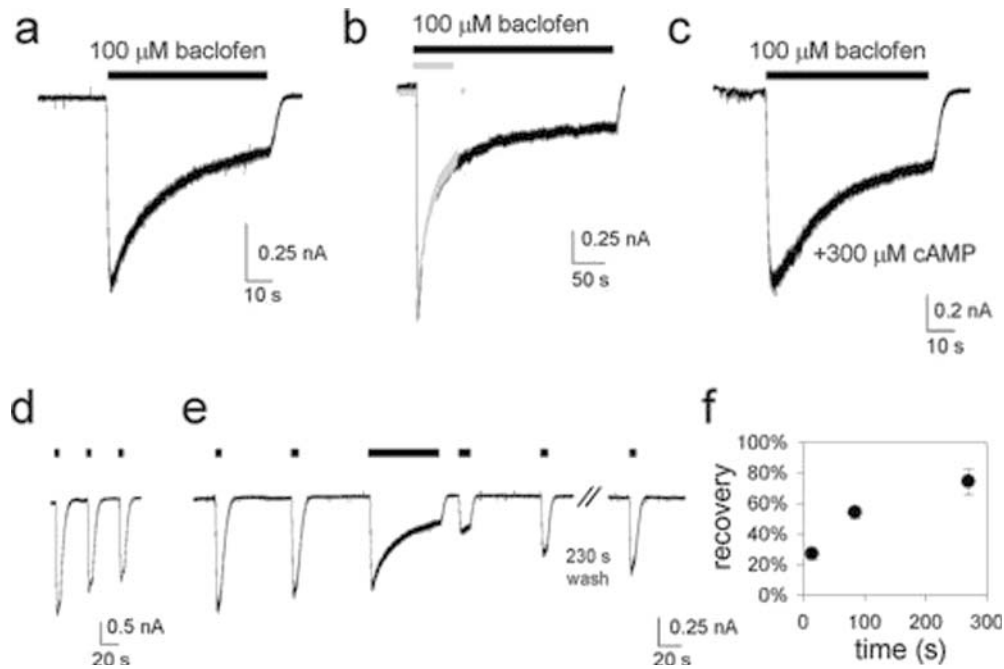


Fig. 2 Prolonged stimulation of GABA_B receptors produces acute desensitization of GIRK2 currents. **a** Whole-cell inward current recorded at -100 mV shows a significant relaxation toward zero current during a 60-s application of baclofen. On average, the current desensitized by $57.3 \pm 1.4\%$ ($n=40$). **b** A 5-min application of baclofen produces nearly the same extent of desensitization (*gray trace* is from **a**). **c** Inclusion of $300 \mu\text{M}$ cAMP in the intracellular solution did not significantly alter desensitization ($54.8 \pm 3.5\%$,

$n=8$). **d** Desensitization of baclofen-induced GIRK current is also produced with three, short (5 s) pulses of baclofen, separated by 35 s. Less desensitization was observed with a 60-s wash period. **e** Protocol used for measuring extent and recovery from desensitization. Holding potential was -100 mV. **f** The percentage recovery from desensitization is plotted a function of time after the 60-s stimulation with baclofen ($n=10$). Note $\sim 80\%$ recovery of the baclofen-induced current after several minutes of wash

as a positive control [37]. Using an anti-HA antibody with unpermeabilized cells, both HA-GABA_B receptors and HA-MOR were detected on the membrane surface (Fig. 3a, c). Ten-minute stimulation with baclofen did not produce any detectable internalization of GABA_B receptors (Fig. 3b). By contrast, 10-min stimulation with the MOR agonist DAMGO produced robust internalization of HA-MOR (Fig. 3d). Thus, GABA_B receptors did not internalize appreciably during sustained stimulation that was sufficient to induce strong desensitization of GIRK currents. The failure of GABA_B receptors to internalize in HEK cells could indicate that HEK cells lack a protein necessary for internalization or that the stimulation time was too short. GABA_B receptors have been reported to internalize in HEK-293 and CHO-K1 cells [10, 20] but only after long-term stimulation. We conclude that the acute desensitization produced during 60-s stimulation of the receptor is not caused by receptor internalization in these experiments.

Heterologous desensitization of GABA_B receptors and MOR

Although GABA_B receptors did not internalize during the 60-s stimulation period, it was possible that GABA_B receptors remained in the membrane but no longer

coupled to G-proteins. To determine if baclofen stimulation only uncoupled GABA_B receptors (homologous desensitization), we coexpressed GABA_B receptors with the MOR, which also couple to GIRK channels [37], to test for heterologous desensitization. In cells transfected

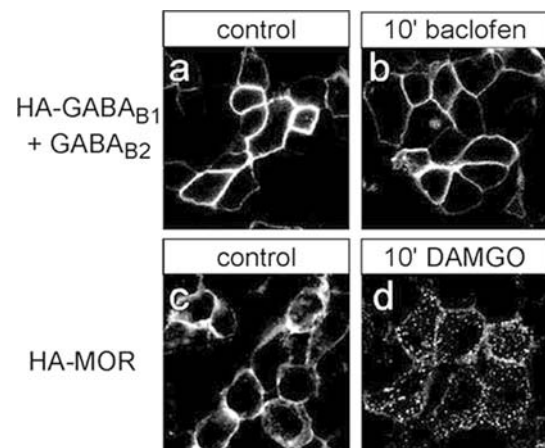


Fig. 3 μ Opioid receptors (MOR), but not GABA_B receptors, internalize following receptor stimulation. HEK-293T cells were transfected with HA-GABA_{B1}, GABA_{B2} and GIRK2, or HA-MOR and GIRK2. **a-d** Transfected cells were exposed to HA antibody (FITC-conjugated anti-HA) and then stimulated with vehicle (**a, c**), baclofen ($100 \mu\text{M}$) (**b**) or DAMGO ($10 \mu\text{M}$) (**d**) for 10 min at 37°C

with GABA_B receptors, MORs and GIRK2, both DAMGO (10 μ M) and baclofen (100 μ M) activated GIRK2 channels (Fig. 4a, b). We then measured the amplitude evoked by brief applications of DAMGO before and after 60 s of baclofen stimulation. Stimulation of the GABA_B receptor not only desensitized the baclofen-evoked current but also led to \sim 70% desensitization of the DAMGO-activated current (Fig. 4b, c). Conversely, a 60-s stimulation of MOR resulted also in \sim 45% desensitization of the currents evoked by brief baclofen applications (Fig. 4c). Plotting the heterologous desensitization as a function of the homologous desensitization falls along a 1:1 correlation (Fig. 4d). As a matter of fact, the degree of heterologous desensitization was at least as important as the homologous desensitization. These experiments demonstrate reciprocal homologous and heterologous desensitization between GIRK currents evoked by MORs and GABA_B receptors, which suggests that desensitization is expressed downstream of the GPCR in the signaling cascade. Interestingly, brief (60 s) stimulation of MOR with DAMGO (10 μ M) produced $49.6 \pm 1.6\%$ ($n=16$)

desensitization of current, whereas significant MOR internalization occurred only after a 10-min stimulation (Fig. 3d). Taken together, these findings suggest a similar mechanism of acute desensitization for GABA_B receptors and MOR.

GTP γ S attenuates acute desensitization

Both G-proteins and the channel are downstream of the GPCR and could therefore be involved in desensitization. G-protein turnover has been implicated previously in the desensitization of α 2R and opioid receptors [9, 31]. To test the role of G-proteins, we examined the effect of the non-hydrolyzable GTP analogue, GTP γ S, on the time course of GABA_BR-dependent desensitization. GTP γ S exchanges for GDP on the G α subunit, thereby liberating G $\beta\gamma$ and constitutively activating GIRK channels. If G-proteins are involved in the desensitization, we expect to observe an attenuation of desensitization. GABA_B receptors were stimulated for 60 s with baclofen to facilitate the exchange of GTP γ S for GDP

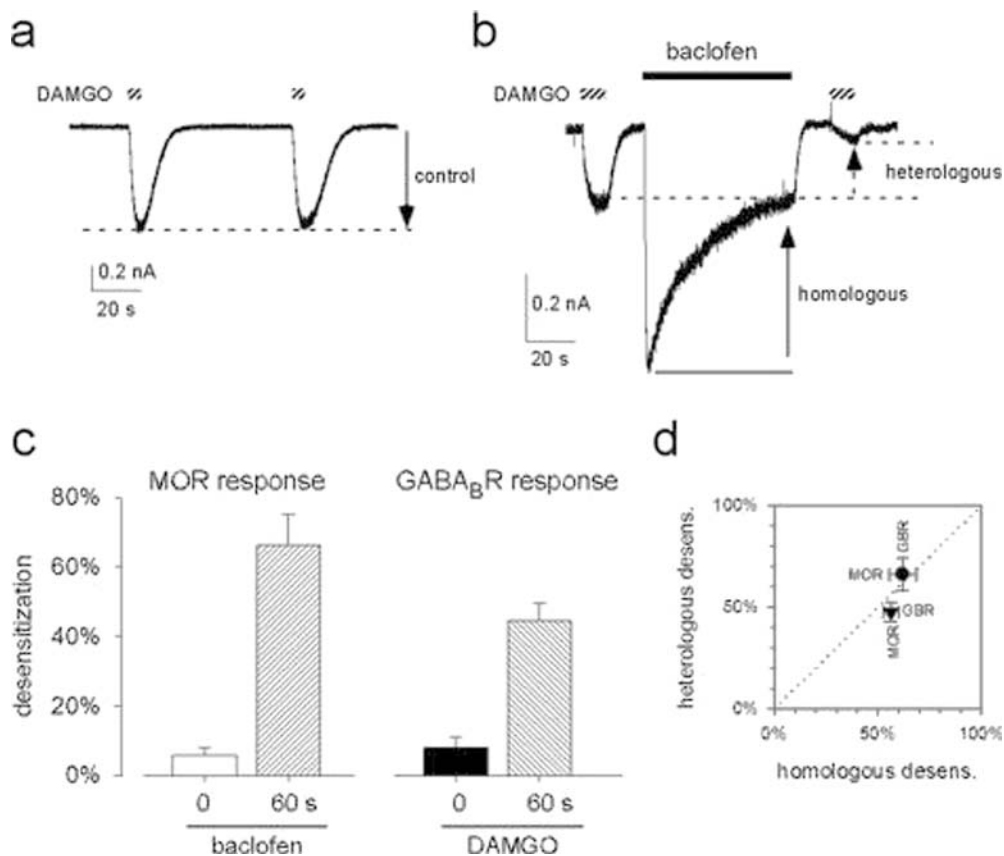


Fig. 4 GABA_B receptors exhibit heterologous desensitization with MOR expressed in HEK-293T cells. HEK-293T cells were transfected with the cDNA for GABA_{B1/B2} receptors, MOR and GIRK2. **a** Example of GIRK current activated with DAMGO (10 s, 10 μ M). **b** Example of heterologous desensitization protocol. The amplitude of DAMGO-activated current was measured before and then after 60-s stimulation of GABA_B receptor (baclofen, 100 μ M). Note the desensitization of both DAMGO (heterologous) and

baclofen (homologous) responses. **c** Bar graphs show the average desensitization of the MOR response with 60-s stimulation of GABA_B receptors (*left panel*) and the desensitization of the GABA_B receptor response with 60-s stimulation of MOR (*right panel*). **d** Plot of the homologous desensitization as a function of heterologous desensitization. Dashed line shows 1:1 correlation between homologous and heterologous desensitization ($n=5-11$)

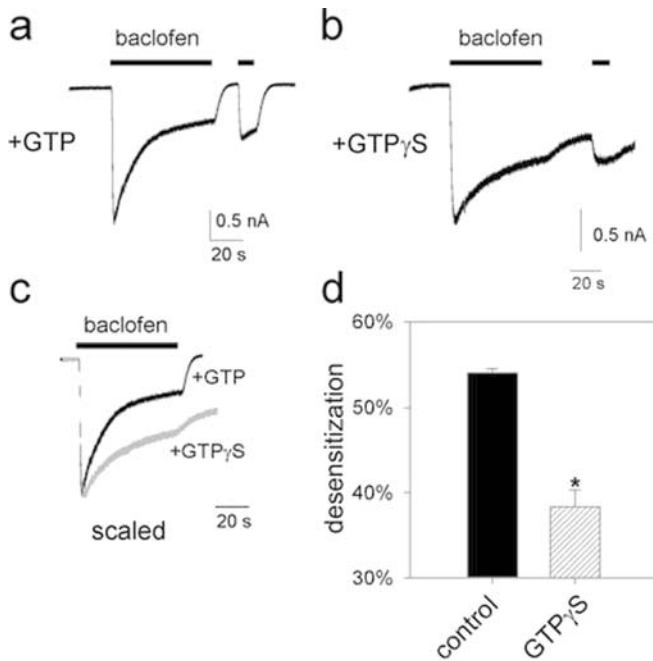


Fig. 5 Intracellular GTP γ S attenuates GABA $_B$ receptor-dependent desensitization of GIRK channels. **a, b** Whole-cell current traces show typical response with baclofen and either GTP (**a**) or GTP γ S (**b**) in the intracellular pipet. **c** Baclofen-induced currents scaled to show change in desensitization. **d** Bar graph shows less GABA $_B$ receptor-dependent desensitization of GIRK currents with intracellular GTP γ S. Asterisk indicates statistical difference using unpaired Student's *t*-test ($P < 0.05$, $n = 5-9$)

(Fig. 5a, b). As expected for constitutive activation of G-proteins, the currents did not deactivate completely following baclofen application (Fig. 5b). In addition, the GABA $_B$ receptor-activated GIRK2 currents showed less desensitization ($38.3 \pm 2.0\%$, $n=9$) in the presence of GTP γ S (Fig. 5c, d). The attenuation of GABA $_B$ R-dependent desensitization with GTP γ S (Fig. 5d) suggests that G-protein turnover is important for determining the extent of desensitization [9], though other factors may contribute to desensitization (see "Discussion").

Interference with endogenous RGS proteins attenuates acute desensitization

The reduction of desensitization with GTP γ S suggests that the turnover of G-proteins in HEK-293T cells is involved, in part, with producing acute desensitization of GABA $_B$ receptor-dependent GIRK currents. RGS4 has been shown to enhance desensitization of opioid receptors [9]. Since deactivation rates (time taken for channels to close after removing agonist) for GIRK channels expressed in HEK-293T cells were fast ($\tau = 3.9$ s, Fig. 7c), we predicted that HEK-293T cells possess endogenous RGS proteins. Untransfected HEK-293T cells were assessed for RGS4 mRNA and protein (Fig. 6). RT-PCR on mRNA isolated from HEK-293T cells and brain tissue revealed a band close to the

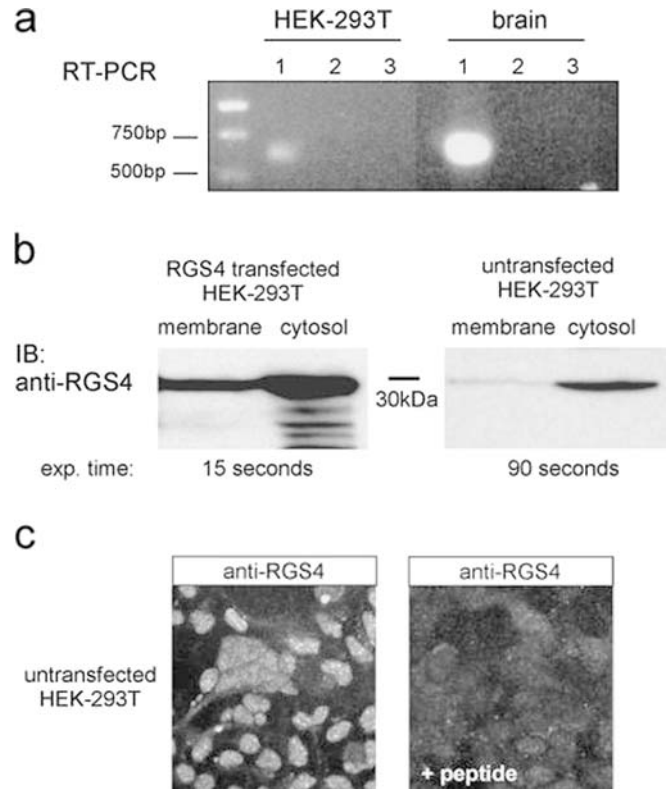


Fig. 6 HEK-293T cells express endogenous RGS4 mRNA and protein. **a** RT-PCR for RGS4, using mRNA isolated from untransfected HEK cells and brain tissue. Lane 1 contains RGS4 primers, reverse transcriptase (RT) and mRNA mixture, lane 2 has primers and mRNA only (-RT), and lane 3 contains primers and RT. **b** Immunoblot analysis, using antibody against RGS4 protein, of proteins isolated from untransfected HEK-293T cells or from HEK-293T cells transfected with RGS4 cDNA. Proteins were divided into cytosolic (15 μ l loaded out of 500 μ l) and membrane (15 μ l loaded out of 200 μ l) fractions. **c** Immunocytochemistry with untransfected HEK-293T cells, using the same RGS4 antibody. Inclusion of the RGS4 peptide during immunostaining reveals low background staining

predicted size of ~ 600 bp (Fig. 6a). Both cytosolic and membrane fractions of protein isolated from untransfected HEK-293T cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with a specific antibody against RGS4 (Santa Cruz). In both membrane and cytosolic fractions, a band was evident that migrated below 30 kDa, which is slightly higher than the predicted size of ~ 23 kDa for human RGS4. Transfecting HEK-293T cells with the cDNA for RGS4 increased the intensity of the band detected by the RGS4 antibody (Fig. 6b), supporting the conclusion that RGS4 migrates more slowly than predicted for its molecular weight. Using the RGS4 antibody, immunostaining of untransfected HEK-293T cells revealed a strong signal that was specifically competed with the RGS4 peptide (Fig. 6c). Taken together, we conclude that HEK-293T cells express RGS4 endogenously.

To study the effect of RGS proteins on GABA $_B$ receptor desensitization, we antagonized the endogenous RGS proteins, using two different strategies. First, we

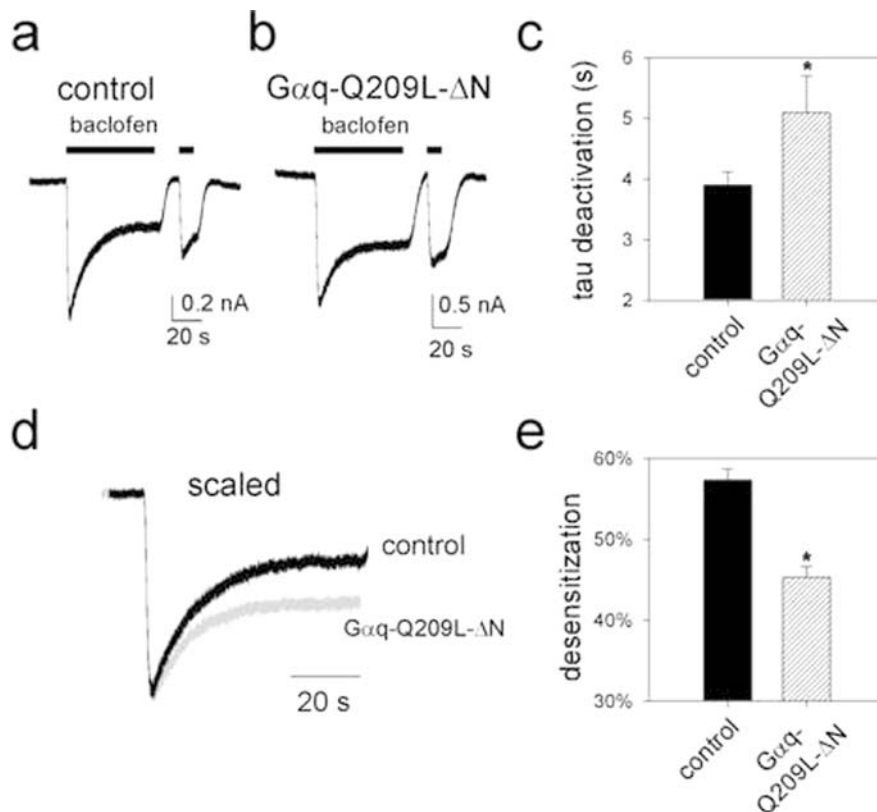


Fig. 7 Coexpression of $G\alpha_q$ -Q209L- ΔN attenuates $GABA_B$ receptor-dependent desensitization. **a**, **b** Examples of baclofen-induced currents recorded from HEK-293T cells in the absence and presence of coexpressed $G\alpha_q$ -Q209L- ΔN . **c** Baclofen-induced currents deactivate more slowly. *Bar graph* shows the average time constant (τ) for channel deactivation. **d** Baclofen-induced currents

are scaled to show change in desensitization. *Black trace* shows control. **e** *Bar graph* shows that coexpression of $G\alpha_q$ -Q209L- ΔN produces less desensitization than control. Asterisk indicates statistical difference using unpaired Student's *t*-test ($P < 0.05$, $n = 10$ for $G\alpha_q$ -Q209L- ΔN and $n = 40$ for control)

constructed a mutant $G\alpha$ G-protein that could, in principle, sequester endogenous RGS proteins. $G\alpha_q$ binds to RGS4 but does not couple to GIRK channels in native tissues. To create a putative RGS binding protein, we first introduced a Q209L mutation into $G\alpha_q$, which is homologous to the mutation in $G\alpha_s$ that slows the intrinsic GTPase activity [53]. We then deleted the N-terminal domain, to reduce the interaction with endogenous $G\beta\gamma$ subunits, and re-introduced a myristoylation site (p60src) into the N-terminal domain to anchor the mutant $G\alpha_q$ at the membrane ($G\alpha_q$ -Q209L- ΔN). We hypothesized that $G\alpha_q$ -Q209L- ΔN could serve as a chelating protein for endogenous RGS proteins and reduce the amount of RGS proteins available to act on the $G\alpha_i$ G-protein signaling pathway. Because RGS proteins accelerate the deactivation kinetics of GIRK channels [13], we first examined the deactivation kinetics in HEK-293T cells coexpressing the $GABA_B$ receptors, GIRK2 channels and $G\alpha_q$ -Q209L- ΔN . The deactivation kinetics reflect the influence of RGS proteins [13] – the absence of RGS leads to slower deactivation rates. Coexpression of $G\alpha_q$ -Q209L- ΔN slowed the deactivation time constant by nearly 1.5-fold, suggesting a reduction in the activity of endogenous RGS proteins (Fig. 7a–c). Coincident with the change in deactivation kinetics,

$GABA_B$ receptor-activated GIRK channels desensitized less than control (Fig. 7d): $45.3 \pm 1.3\%$ ($n = 13$) with $G\alpha_q$ -Q209L- ΔN versus $57.3 \pm 1.4\%$ ($n = 40$) for control.

In addition to expressing $G\alpha_q$ -Q209L- ΔN , we employed a different approach for antagonizing the endogenous RGS proteins. We tested the effect of a RGS-insensitive $G\alpha_o$ G-protein on $GABA_B$ receptor-dependent desensitization of GIRK currents. A glycine-to-serine mutation (G184S) in the $G\alpha_o$ subunit dramatically reduces the interaction with RGS proteins [40]. To exclusively study the coupling of ectopically expressed $G\alpha$ G-proteins, the $G\alpha_o$ -G184S (RGSi) was also rendered insensitive to PTX (PTXi: C351G mutation, see [42]). PTX treatment enables the selective coupling of the PTX-insensitive $G\alpha_o$ G-protein with $GABA_B$ receptors. In control cells, PTX treatment completely blocked all $GABA_B$ receptor-activated responses (Fig. 1c). GIRK channel activation was rescued by coexpression with $G\alpha_o$ -PTXi (Fig. 8a). Importantly, the extent of $GABA_B$ receptor-dependent desensitization was similar to that of controls (Fig. 8d). We next examined the effect of coexpressing $G\alpha_o$ -PTXi/RGSi with $GABA_B$ receptors and GIRK2 channels (Fig. 8b). The GIRK2 deactivation kinetics in cells coexpressing $G\alpha_o$ -PTXi/RGSi slowed by a factor of nearly 12-fold

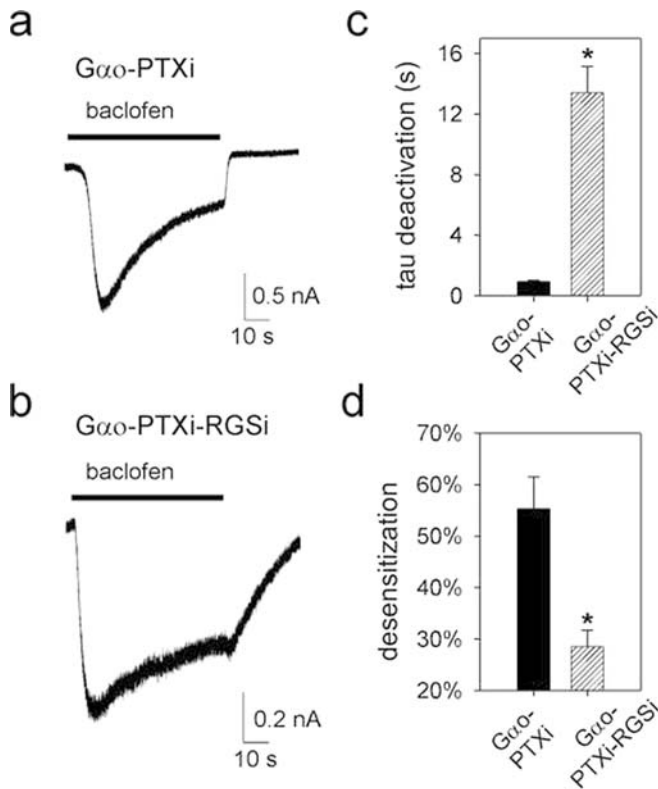


Fig. 8 Coexpression of RGS-insensitive $G_{\alpha o}$ G-protein produces less $GABA_B$ receptor-dependent desensitization of GIRK2 currents. HEK-293T cells were transfected with cDNA for $GABA_{B1/B2}$ receptors, GIRK2, and either $G_{\alpha o}$ -PTXi or $G_{\alpha o}$ -PTXi/RGSi and treated with PTX. PTX pre-treatment (4 h) occludes baclofen (100 μ M) activation of GIRK currents (see Fig. 1c). Expression of $G_{\alpha o}$ -PTXi (a) or $G_{\alpha o}$ -PTXi/RGSi (b) rescues $GABA_B$ receptor activation of GIRK currents in PTX-treated HEK-293T cells. Note less desensitization and slower deactivation kinetics with $G_{\alpha o}$ -PTXi/RGSi. **c** Bar graph shows average τ for deactivation with HEK-293T cells expressing $G_{\alpha o}$ -PTXi or $G_{\alpha o}$ -PTXi/RGSi. **d** Bar graph shows average desensitization measured at 60 s, following baclofen treatment. Asterisk indicates statistical difference using unpaired Student's *t*-test ($P < 0.05$, $n = 9$)

(Fig. 8c), consistent with reduction in the interaction of endogenous RGS proteins. Importantly, the extent of $GABA_B$ receptor-dependent desensitization was $\sim 25\%$ slower with $G_{\alpha o}$ -PTXi/RGSi (Fig. 8d): $55.3 \pm 6.2\%$ ($n = 9$) versus $28.5 \pm 3.2\%$ ($n = 9$). Taken together, these results support the conclusion that endogenous RGS proteins enhance acute desensitization as well as deactivation kinetics.

If the extent of desensitization is influenced by the levels of RGS proteins in the cell, we predicted that the deactivation time constant would correlate inversely with the extent of desensitization. In this summary plot, we also included data from experiments in which RGS4 was coexpressed with $GABA_B$ receptors and GIRK2 channels. Coexpression of RGS4 produced little change in the percentage desensitization and a small decrease in the deactivation time constant (Fig. 9a-b). Plotting the percentage of desensitization as a function of the deactivation time constant shows that the percentage of

desensitization decreases linearly as the deactivation time constant increases, (Fig. 9c, $r^2 = 0.90$).

Discussion

In this study, we examined the desensitization of GIRK currents evoked by $GABA_B$ receptors. We specifically focused on a stimulation paradigm that might mimic the pooling of GABA that occurs during sustained activation of GABAergic interneurons, such as following tetanic stimulation or epileptic seizures. With a 60-s sustained application of $GABA_B$ agonist, GIRK current desensitized nearly 60%. This acute desensitization was both homologous and heterologous, did not arise from receptor internalization, was reversible, and depended on the activity of endogenous RGS proteins.

We were surprised to discover that HEK-293T cells possess endogenous RGS proteins. The rapid deactivation kinetics for GIRK channels expressed without additional RGS proteins in HEK-293T cells provided the first clue that RGS may be present in HEK cells. Typically, overexpression of RGS proteins is required to accelerate the deactivation kinetics for GIRK channels heterologously expressed in *Xenopus* oocytes [13, 24, 54]. Consistent with our finding in HEK cells, Bünemann and Hosey found that coexpression of RGS4 did not alter the deactivation kinetics of GIRK1/4 channels expressed in HEK cells [4]. Interestingly, coexpression of RGS4 in CHO-K1 cells reduced the deactivation time by more than half, suggesting that CHO-K1 cells may express lower levels of endogenous RGS proteins. Here, we focused on RGS4, but HEK-293T cells may also express other RGS proteins. Preliminary RT-PCR experiments indicate that HEK-293T also express RGS2 but not RGS7 (data not shown). Recently, Krumins et al. identified endogenous RGS4 and RGS7 in PC12 and AtT20 cells [38].

Mechanism of acute desensitization of $GABA_B$ receptor-activated GIRK currents

Baclofen treatment for much longer times than were required to induce desensitization (10 min) did not produce appreciable internalization of $GABA_B$ receptors, though receptor internalization readily occurred with stimulation of MOR expressed in HEK-293T cells. In fact, receptor internalization of $GABA_B$ receptors may require a longer stimulation—2-hr stimulation of $GABA_B$ receptors expressed in CHO-K1 cells did lead to significant internalization [20]. In HEK-293 cells, Couve et al. also found that short-term treatments with baclofen did not induce internalization [10]. In contrast to our findings, however, 15-min stimulation with GABA was sufficient to produce $\sim 50\%$ internalization [10]. This difference might be due to differences among cell lines or perhaps the placement of the epitope tag. We also did

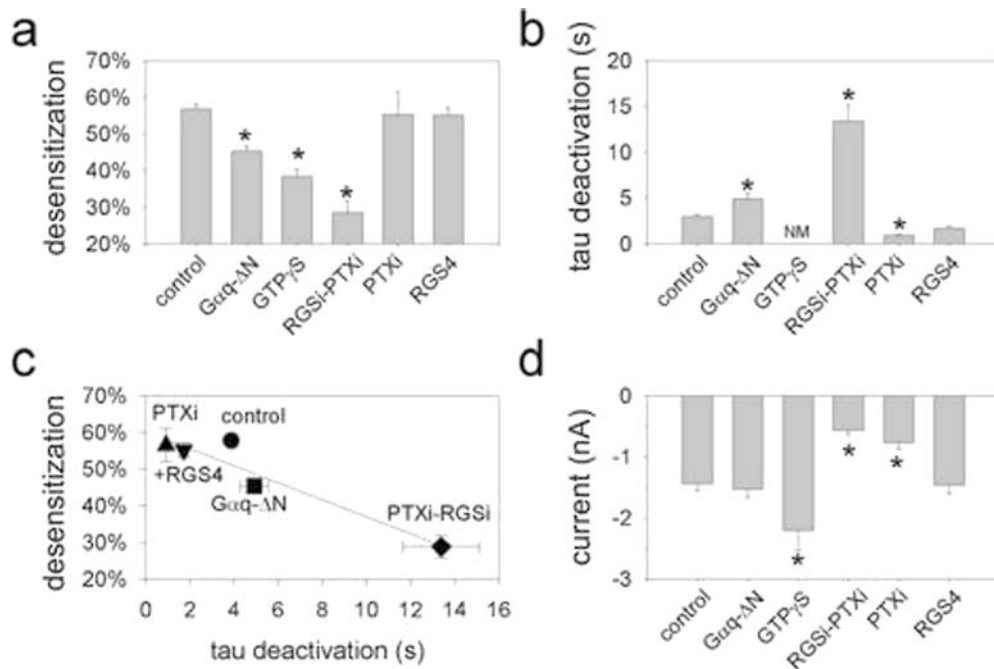


Fig. 9 Correlation between extent of desensitization and deactivation τ . **a** Average percentage desensitization measured at end of a 60-s baclofen treatment. **b** Average τ for deactivation kinetics. The deactivation τ for GTP γ S was not measured (NM). **c** Percentage desensitization is plotted as a function of deactivation τ . The line

shows the linear regression fit to the data ($r^2 = 0.90$). **d** Mean amplitude of baclofen-activated GIRK currents for different experiments ($n = 7-40$). Asterisk indicates statistical difference from control group (one-way ANOVA, followed by Holm-Sidak post hoc test for significance, $P < 0.05$)

not observe detectable internalization with 20-s stimulation of YFP-tagged GABA_B receptors (data not shown). Nonetheless, it is clear that receptor endocytosis cannot account for the acute desensitization of GABA_B receptor-activated GIRK currents reported here. Although not the focus of the present study, we believe that desensitization of MOR elicited currents also occurs independent of receptor internalization because in HEK cells desensitization was much faster than MOR internalization. Moreover, in acute slices, we observed no change in desensitization after interference with the endocytotic machinery [2].

A slower type of desensitization has been reported for GIRK channels and muscarinic receptors expressed in CHO cells but coexpression of a G-protein receptor kinase (GRK2) is required for this form of desensitization [57, 59]. The acute desensitization observed here did not require coexpression of GRKs, though we did not investigate the possibility of endogenous GRKs in HEK-293T cells.

The time course of GABA_B receptor-dependent desensitization of GIRK current in this study is similar to the acute desensitization of muscarinic receptor-activated GIRK currents in cardiac myocytes. Several different mechanisms have been proposed to explain acute desensitization of GPCR-activated GIRK channels in myocytes. Kobrinsky et al. provided evidence that stimulation of muscarinic receptors leads to two simultaneous effects [36]: activation of m2 (G α_i -linked) muscarinic receptors opens GIRK channels, while

activation of m1 (G α_q -linked) receptors stimulates that activity of PLC, which in turn hydrolyzes PIP₂, an essential co-factor for GIRK channel activation [26]. Thus, the decrease in GIRK current reflects a depletion of PIP₂ near the channel. In a PLC β 1 knockout mouse, however, activation of m1 and m3 muscarinic receptors still produced GIRK desensitization [8]. Furthermore, changes in PIP₂ hydrolysis and replenishment did not appear to alter acute desensitization [8]. Surprisingly, Kim and Bang found that exogenous PIP₂ inhibited acetylcholine-induced activation of GIRK channels in cardiac myocytes [35]. Although more studies are needed to clarify the role of PIP₂ in desensitization, changes in PIP₂ are unlikely to explain the desensitization observed with GABA_B receptors because GABA_B receptors couple to G α_i/o but not to G α_q G-proteins [17].

Having ruled out internalization and depletion of PIP₂, we investigated whether G-protein activity was important for GABA_B receptor-dependent desensitization of GIRK currents. Several lines of evidence implicate G-protein turnover in the acute desensitization of GIRK2 current in HEK-293 cells: (1) heterologous desensitization was observed with MOR and GABA_B receptors—this suggested that both GPCRs share a common pool of G-proteins and channels; (2) desensitization was attenuated with GTP γ S, implicating G-proteins; (3) coexpression of G α_q -Q209L-ΔN, a putative RGS binding proteins, slowed deactivation and desensitization; and (4) coupling of the GABA_B receptor with

a RGS-insensitive $G_{\alpha o}$ also reduced desensitization. If the activity of RGS proteins is coupled to the extent of desensitization, we would expect to observe a correlation between deactivation kinetics and extent of desensitization. Indeed, plotting the data obtained from different types of experiments, where RGS activity was increased or decreased, showed a linear correlation (Fig. 9c).

Interestingly, none of the manipulations that altered G-proteins and/or RGS proteins completely abrogated acute desensitization. In the $GTP\gamma S$ experiment, the remaining decaying current could indicate insufficient time to activate all of the endogenous G-proteins. Consistent with this, a second application of baclofen still elicited GIRK currents, indicating that $GTP\gamma S$ had not exchanged for all of the endogenous G-proteins (see Fig. 5b). Alternatively, $GTP\gamma S$ activation of $G_{\alpha q}$ G-proteins could decrease GIRK channel activity by depletion of PIP_2 [36]. In experiments designed to antagonize the endogenous RGS, desensitization was clearly slower but the remaining decaying current might reflect incomplete actions of the putative RGS chelator ($G_{\alpha q}$ -Q209L- ΔN) or residual RGS activity with the mutant RGSi- $G_{\alpha o}$ G-protein. In the latter case, the G184S mutation may impair but not completely eliminate the interaction with endogenous RGS. Alternatively, additional pathways may exist for GIRK2 current desensitization, such as PKA-dependent phosphorylation of GABA_B receptors [10].

In summary, an increasing number of studies have now established a link between RGS protein activity and desensitization. Coexpression of RGS proteins in oocytes enhances GIRK current desensitization following stimulation of opioid [9] and muscarinic receptors [55]. Similarly, ectopic expression of a RGS-insensitive $G_{\alpha o}$ G-proteins in sympathetic neurons dramatically reduced $\alpha 2R$ -dependent desensitization of GIRK currents [31]. In addition to GIRK channels, RGS proteins appear to modulate desensitization of GPCR-mediated inhibition of voltage-gated Ca^{2+} channels [6, 12, 66].

Recently, Ishii et al. discovered that RGS4 can modulate the voltage-dependent relaxation of GIRK currents that occurs upon hyperpolarization to negative membrane potentials [30]. The effect of RGS4 on the GIRK current relaxation required elevated intracellular Ca^{2+} and calmodulin, which enabled RGS4 to bind the G_{α} subunit. GABA_B-activated GIRK currents were recorded in our study with a high concentration of intracellular EGTA as well as high Mg^{2+} and low Ca^{2+} in the extracellular solution, suggesting that Ca^{2+} -dependent regulation of RGS4 did not contribute significantly to the acute desensitization.

While completing our studies, Leaney et al [43] showed that overexpression of two G_{α} subunits with different activation kinetics modulates the extent of GABA_B receptor-dependent desensitization. Although the role of endogenous RGS proteins in regulating desensitization was not directly investigated in this study, their conclusions are in agreement with our studies. Together, these results place a greater emphasis

on the importance of RGS proteins in regulating GPCR desensitization.

Similarity of acute desensitization in neurons and HEK-293T cells

Recently, several groups have reported GABA_B receptor-activated GIRK currents in neurons. In neurons of the ventral tegmental area, Cruz et al. noted that GABA_B receptor-activated GIRK currents desensitized in dopamine neurons but not in GABA neurons [11]. This type of desensitization in GABA neurons occurred over a slower time course (10–15 min) than the acute desensitization described in the current study and therefore likely involves a different mechanism. In laterodorsal tegmental nucleus, baclofen elicited strong desensitization within 5 min [7]. In cortical neurons, Sickman and Alzheimers [60] found that stimulation of GABA_B receptors produced two rates of desensitization, acute and intermediate, which each occurred within 60 s. Both types of desensitization were heterologous (with adenosine receptors) and were attenuated with intracellular $GTP\gamma S$, consistent with a role for G-proteins. GABA_B receptor-dependent desensitization of GIRK currents in acutely dissociated hippocampal neurons is similar in rate and extent as that observed in HEK cells [41]. Although not studied in detail, GABA_B receptor-activated GIRK currents in cerebellar granule cells also appear to desensitize to 50% within ~ 30 s [see Fig. 2A in ref. 23]. The time course and extent of GABA_B receptor desensitization, along with the fast deactivation kinetics, suggest that endogenous RGS proteins may be involved in GABA_B receptor-dependent desensitization of GIRK currents in neurons. Numerous types of RGS proteins, such as RGS2, RGS4, RGS5, RGS7, RGS13, and RGS14, are expressed in the hippocampus, cerebellum, cortex, and brain stem [19, 21, 28, 51].

RGS4 in the brain is of particular importance because of its implication in schizophrenia; transcripts encoding RGS4 are decreased in the prefrontal cortex of schizophrenic subjects [49, 50]. Although the link between the activity of RGS4 and schizophrenia is uncertain at this time, our studies suggest that reduced GIRK current desensitization could be one outcome of less RGS4 in neurons coexpressing GIRK channels. The development of more specific antagonists for RGS proteins should make it possible in the future to examine more directly the role of RGS proteins in receptor-dependent desensitization of GIRK channels and synaptic efficacy.

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