



Published in final edited form as:

J Biol Chem. 2002 September 6; 277(36): 32692–32696.

N-terminal Tyrosine Residues within the Potassium Channel Kir3 Modulate GTPase Activity of $G\alpha_i^*$

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Abstract

trkB activation results in tyrosine phosphorylation of N-terminal Kir3 residues, decreasing channel activation. To determine the mechanism of this effect, we reconstituted Kir3, trkB, and the mu opioid receptor in *Xenopus* oocytes. Activation of trkB by BDNF (brain-derived neurotrophic factor) accelerated Kir3 deactivation following termination of mu opioid receptor signaling. Similarly, overexpression of RGS4, a GTPase-activating protein (GAP), accelerated Kir3 deactivation. Blocking GTPase activity with GTP γ S also prevented Kir3 deactivation, and the GTP γ S effect was not reversed by BDNF treatment. These results suggest that BDNF treatment did not reduce Kir3 affinity for $G\beta\gamma$ but rather acted to accelerate GTPase activity, like RGS4. Tyrosine phosphatase inhibition by peroxyvanadate pretreatment reversibly mimicked the BDNF/trkB effect, indicating that tyrosine phosphorylation of Kir3 may have caused the GTPase acceleration. Tyrosine to phenylalanine substitution in the N-terminal domain of Kir3.4 blocked the BDNF effect, supporting the hypothesis that phosphorylation of these tyrosines was responsible. Like other GAPs, Kir3.4 contains a tyrosine-arginine-glutamine motif that is thought to function by interacting with G protein catalytic domains to facilitate GTP hydrolysis. These data suggest that the N-terminal tyrosine hydroxyls in Kir3 normally mask the GAP activity and that modification by phosphorylation or phenylalanine substitution reveals the GAP domain. Thus, BDNF activation of trkB could inhibit Kir3 by facilitating channel deactivation.

The family of G protein-activated potassium channels (Kir3 or GIRK)¹ is a principal effector mediating the actions of a wide range of pertussis toxin-sensitive, G protein-coupled receptors (GPCR) (1). Channel activation provides key regulation of both cardiac and neuronal excitability. The activity of this channel is controlled by a large number of modulators including phosphatidylinositol bisphosphate, Na⁺, eicosanoids, ATP, Mg²⁺, and phosphorylation (1–6). For example, in a previous study (7), we found that tyrosine phosphorylation of Kir3 resulted in channel inhibition. Brain-derived neurotrophic factor (BDNF) activation of trkB receptors caused the phosphorylation of specific tyrosine residues in the N-terminal domain of Kir3.1 and Kir3.4, reducing basal channel conductance. $G\beta\gamma$, released by opioid receptor activation, overcame the inhibition and evoked the original maximal effect. The results suggest that channel phosphorylation regulates the specific interaction with $G\beta\gamma$, but the mechanism of this effect is unclear. However, the finding may be physiologically significant because tyrosine kinase cascades initiated by neurotrophic factors such as BDNF and nerve growth factor are

*This work was supported by United States Public Health Service Grants DA11672 from the National Institute on Drug Abuse and GM07270 from NIGMS, National Institutes of Health.

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¹The abbreviations used are: Kir3 or GIRK, G protein-coupled inwardly rectifying potassium channel; GPCR, G protein-coupled receptor; BDNF, brain-derived neurotrophic factor; DAMGO, [D-Ala², N-MePhe⁴-Gly-Ol⁵]enkephalin; MOR, mu opioid receptor; ND96, low potassium vehicle; HK+, high potassium buffer; RGS4, regulator of G protein signaling, subtype 4; GAP, GTPase-activating protein; trkB, tyrosine kinase receptor for BDNF.

up-regulated under conditions such as inflammation (8). It is possible that tyrosine phosphorylation of Kir3 may mediate neuronal excitability in conjunction with GPCRs under conditions of inflammation. The goal of the present study was to define the mechanism responsible for BDNF inhibition of G protein regulation of Kir3 functioning.

Recent evidence shows that both arms of the heterotrimeric G protein complex, $G\alpha_i$ and $G\beta\gamma$, interact with Kir3 (9). Binding of the $G\beta\gamma$ subunit activates Kir3, whereas $G\alpha_i$ binding suppresses basal Kir3 activity and enhances G protein activation (9). This action of $G\alpha_i$ binding resembles the effect of BDNF-induced tyrosine phosphorylation on Kir3. Both reduce basal channel activity without blocking GPCR activation of Kir3, suggesting the possibility of mechanistic similarity. Thus, we explored the hypothesis that the underlying mechanisms were related and that tyrosine phosphorylation controls $G\alpha_i$ regulation of Kir3 response to GPCR activation. Understanding the basis for the modulation of Kir3 by tyrosine phosphorylation would provide additional insight into the processes regulating the activity of this physiologically significant channel and a molecular basis for interaction between GPCR and tyrosine kinase receptor signaling.

To test this hypothesis, we reconstituted the mu opioid receptor (MOR, a GPCR), Kir3, and trkB in *Xenopus* oocytes and investigated channel activation and deactivation kinetics. When current traces were fit to a simple exponential, trkB accelerated deactivation of Kir3. The kinetics resembled GTPase-activating protein (GAP)-mediated acceleration of channel deactivation, suggesting that trkB might stimulate GAP activity. Sequence alignment showed that Kir3 contains two signature GAP residues (glutamine and arginine) near both N-terminal domain tyrosines. These residues have been shown to promote catalytic activity of the GTPase domain (10–15). Phenylalanine substitution of the two N-terminal tyrosine residues within these tyrosine-arginine-glutamine sequences resulted in Kir3 with constitutively faster kinetics of deactivation. Further, BDNF treatment no longer accelerated deactivation kinetics of the mutant channels. These results suggest that phosphorylation of Kir3 unmasks a GAP domain embedded in the sequence of Kir3 itself, promoting GAP activity.

EXPERIMENTAL PROCEDURES

cRNA Preparation and Injection

Plasmid vectors containing cDNA for the following GenBank accession numbers were obtained from Drs. Lei Yu, Cesar Lebarca, Henry Lester, John Adelman, Mark Bothwell, and Nathan Dascal, respectively: MOR (L13069), Kir3.1 (U01071), Kir3.4 (X83584), trkB (M55293), and RGS4 (AF117211). Kir3.4 was point-mutated by PCR-based site-directed mutagenesis to create the functional homomers Kir3.4(S143T) and Kir3.4(S143T/Y32F/Y53F) (7). cRNA was synthesized from linearized plasmid vectors using RNA polymerases provided by the commercially available mMessage mMachine kit (Ambion Corporation, Austin, TX). The cRNA was subsequently micro-injected into stage V and VI oocytes harvested from mature anesthetized *Xenopus laevis* frogs. Oocytes were maintained in high salt buffer (ND96: 96 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 2.5 mM sodium pyruvate, 50 μg/ml gentamicin, pH 7.5).

Two-electrode Voltage Clamp Technique

Following 3–5 days of expression, the two-electrode voltage clamp technique was used to assess coupling efficiency of MOR to Kir3 in oocytes pretreated in BDNF or vehicle. Briefly, oocyte membranes were voltage-clamped at –80 mV by a microelectrode containing 3 M KCl (resistance between 0.4 and 2 MΩ) connected to a feedback amplifier (Axon Instruments, Inc.). A second microelectrode delivered current necessary to maintain the preset voltage. High potassium buffer (HK+: 2 mM NaCl, 96 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH

7.5) and the MOR-agonist [_D-Ala², *N*-MePhe⁴-Gly-ol⁵]enkephalin (DAMGO, diluted in high potassium buffer) were sequentially washed onto the oocytes, and changes in current were recorded and analyzed using pCLAMP 6 software.

Pharmacological Agents

DAMGO was obtained from Sigma and Peninsula Labs (San Carlos, CA). Naloxone was from Sigma. Sodium orthovanadate (Sigma) was activated in 3% hydrogen peroxide for 4–5 h prior to use. BDNF was a gift from AMGEN Corporation. All reagents were dissolved in water and then diluted in ND96 or high potassium buffer.

Data Analysis

Kinetics of Kir3 deactivation were calculated as reported previously by measuring time constant to reversal (τ_{off} , 16,17). Exponentials were fit to the naloxone reversal phases of the DAMGO-induced Kir3 currents. Cursors were positioned at ~20 and 80% of the maximal equilibrium conductance from current initiation and termination, respectively. Current traces between these points were fit to exponential Equation 1.

$$F(t) = \sum_{i=1}^n A_i e^{-t/\tau_i} + C \quad (\text{Eq. 1})$$

Time constants exceeding two standard deviations of the mean were excluded from the analysis. The membrane time constant (τ_{off}) was used to estimate kinetics of Kir3 deactivation. Statistical significance was determined using an unpaired Student's *t* test, in which a probability of $p < 0.05$ was considered statistically significant.

RESULTS

trkB Modulation of Kir3 Activation

We found previously that tyrosine kinase phosphorylation of Kir3 modulates its conductance (7). To investigate how tyrosine phosphorylation cascades initiated by trkB might regulate Kir3 activation by G protein receptors, we coexpressed cRNA for Kir3.1, Kir3.4, trkB, and MOR in *Xenopus* oocytes. Oocytes were pretreated in either vehicle (ND96) or 0.2 $\mu\text{g/ml}$ BDNF. Plasma membranes were clamped at -80 mV in two-electrode voltage clamp configuration. Perfusion of 96 mM potassium buffer (HK+) revealed an inwardly rectifying basal current (Fig. 1A). Pretreatment in BDNF suppressed basal current to $77 \pm 8\%$ ($p < 0.05$, $n = 35$) of vehicle controls in agreement with previous studies (Fig. 1B). Basal current was potentiated when the MOR agonist (DAMGO, 1 μM) was perfused into the recording chamber. BDNF had no effect on the amplitude of the DAMGO-induced response ($104 \pm 1\%$ of vehicle-treated controls, $n = 35$, Fig. 1B). MOR antagonist perfusion (naloxone, 1 μM) returned current back to basal amplitude, and ND96 perfusion returned the basal current to baseline (Fig. 1A). The rate of channel deactivation during naloxone perfusion appeared to increase after BDNF treatment. To quantify channel deactivation kinetics, we fit the portion of the trace between naloxone and final ND96 applications to a simple exponential. The time constant of deactivation (τ_{off}) was calculated using pCLAMP software as described under "Experimental Procedures." BDNF significantly accelerated channel deactivation rate; τ_{off} was reduced to $71 \pm 3\%$ of vehicle control ($p < 0.05$, $n = 35$, Fig. 1, A and B). Channel activation rate, as measured by τ_{on} , was not significantly accelerated in BDNF-treated oocytes (data not shown).

The decrease in the τ_{off} parameter was not a consequence of the reduced basal current amplitude; a regression analysis showed no correlation between DAMGO current amplitude and τ_{off} ($r^2 < 0.1$). Oocytes lacking the trkB receptor did not show significant acceleration of Kir3 deactivation or decrease in basal channel conductance following BDNF treatment (data

not shown). Thus, trkB activation by BDNF reduced the basal channel conductance and accelerated the deactivation kinetics of Kir3 without reducing the maximal DAMGO-evoked effect. Because Kir3 conductance is controlled predominately by binding to free $G\beta\gamma$, these results suggest that tyrosine phosphorylation affected the interaction between channel and $G\beta\gamma$.

trkB Acceleration of Kir3 Deactivation Is Mediated by Enhanced GAP Activity

The kinetic profile we describe for Kir3 deactivation resembles GAP-mediated acceleration of deactivation without concomitant decrease in DAMGO current amplitude or increase in τ_{on} (18). To illustrate the kinetic similarity, we injected 10 ng of cRNA for the GAP RGS4 into oocytes expressing Kir3, MOR, and trkB 12–18 h before recording. When traces were fit to a simple exponential, Kir3 deactivation (as measured by τ_{off}) was accelerated from 39 ± 3 s (Kir3.1/3.4, $n = 28$) to 27 ± 3 s (Kir3.1/3.4, $n = 36$) when oocytes not expressing RGS4 were pretreated in BDNF (Fig. 2). Injection of RGS4 accelerated channel deactivation to 12 ± 2 s (τ_{off} , $n = 7$). BDNF treatment reduced τ_{off} to 7 ± 1 s ($n = 11$). These results show that trkB modulation of deactivation kinetics is similar to the effect of the GAP RGS4, leading to the speculation that both operate by similar mechanisms.

To test the hypothesis that trkB activation increased GAP activity (rather than decreasing $G\beta\gamma$ binding affinity for Kir3) we inhibited GTP hydrolysis by injecting GTP γ S, a non-hydrolyzable analogue of GTP (19). If Kir3 deactivation rate was controlled solely by $G\beta\gamma$ dissociation and not $G\beta\gamma$ sequestration by $G\alpha_i$ -GDP, then BDNF-accelerated deactivation could result from a reduction in $G\beta\gamma$ affinity for the channel. Alternatively, if the deactivation rate was controlled by $G\beta\gamma$ sequestration by $G\alpha_i$ -GDP, then the accelerated deactivation rate would result from a BDNF-induced increased GTPase activity that would be blocked by GTP γ S. In oocytes expressing MOR, trkB, and Kir3, GTP γ S virtually eliminated Kir3 deactivation whether or not RGS4 was injected (Fig. 3, *a* and *b*). Pretreatment with BDNF did not accelerate deactivation kinetics of GTP γ S-injected oocytes (Fig. 3*c*). Because BDNF pretreatment did not even partially recover deactivation in the presence of GTP γ S, trkB-mediated acceleration of deactivation kinetics was probably not a result of a reduction in $G\beta\gamma$ affinity for the channel. These results suggest that trkB accelerates channel deactivation by facilitating GTP hydrolysis and subsequent $G\beta\gamma$ sequestration by $G\alpha_i$.

Tyrosine Phosphorylation of Kir3 Is the Mechanism of trkB-mediated GAP Activation

Previously, we identified tyrosine residues in the Kir3 N-terminal tail that were phosphorylated by trkB and modulated Kir3 gating parameters (7). To investigate whether phosphorylation state of Kir3 controlled channel deactivation, we pretreated oocytes with the phosphatase inhibitor peroxyvanadate ($100 \mu\text{M}$) before voltage clamp experimentation. Peroxyvanadate pretreatment reduced τ_{off} from 39 ± 3 s ($n = 4$) to 23 ± 5 s ($n = 4$). A 3-min perfusion with ND96 returned τ_{off} to 34 ± 2 s ($n = 4$), approaching vehicle-treated controls (Fig. 4, *A* and *C*). Conversely, when oocytes were pretreated first in vehicle followed by a 3-min treatment in peroxyvanadate, channel deactivation was accelerated (Fig. 4*B*). These results suggest that constitutive phosphorylation of Kir3 may result in enhanced GTP hydrolysis.

We hypothesized that BDNF/trkB-induced phosphorylation of specific N-terminal tyrosine residues accelerated Kir3 deactivation kinetics. Deactivation kinetics were compared for Kir3.4(S143T) (a variant able to form functional homomers as described) (20) and Kir3.4(S143T/Y32F/Y53F) (a homomeric channel with both N-terminal tyrosine residues point-mutated to phenylalanines) (7). trkB activation accelerated channel deactivation in oocytes expressing Kir3.4(S143T) (vehicle, $\tau_{off} = 26 \pm 4$ s, $n = 18$ and BDNF, $\tau_{off} = 17 \pm 1$ s, $n = 17$). However, trkB no longer accelerated deactivation in the double tyrosine to phenylalanine mutant (vehicle, $\tau_{off} = 15 \pm 1$ s, $n = 30$ and BDNF, $\tau_{off} = 15 \pm 1$ s, $n = 40$, Fig. 5*A*). These results

suggest that trkB-mediated phosphorylation of these tyrosine residues is important in facilitating GAP activity of the channel.

Interestingly, we found that the Kir3.4(S143T/Y32F/Y53F) mutations constitutively accelerated Kir3.4 kinetics (compare Kir3.4(S143T), $\tau_{\text{off}} = 26 \pm 4$ s, $n = 18$ with Kir3.4(S143T/Y32F/Y53F), $\tau_{\text{off}} = 15 \pm 1$ s, $n = 30$, Fig. 5A). These results suggest that the hydroxyl group in the tyrosine dampens GAP activity because phenylalanine substitution for tyrosine promotes GAP activity. A direct measure of GTPase activity of Kir3 was not possible because we would not have attained the high level of channel expression required (21).

Kir3 Has Potential GAP Activity

Recently, the crystal structures of G protein-GAP combinations such as Ras-Ras-GAP, Rho-Rho-GAP, and $G\alpha_i$ -RGS have been solved (10–12,14,22–24). A sequence alignment of the amino acids in the vicinity of Kir3.4 N-terminal tyrosine residues revealed that these tyrosines are part of a signature consensus sequence recurring in some GAPs to facilitate hydrolysis of GTP by directly inserting these amino acid residues into the catalytic binding pocket (Fig. 5B). RGS4 is also a GAP, but crystal structure data indicate that it does not contribute amino acid residues to the $G\alpha_i$ but instead holds the $G\alpha_i$ in a catalytically favored transition state (24). To determine whether trkB worked cooperatively with RGS4 to accelerate channel deactivation, we investigated the effect of BDNF pretreatment on the τ_{off} parameter in oocytes expressing RGS4, trkB, Kir3 mutants, and MOR. RGS4 accelerated channel deactivation in the Kir3.4(S143T/Y32F/Y53F) mutants ($\tau_{\text{off}} = 10 \pm 2$ s, $n = 20$, Fig. 5A), but BDNF had no additional effect ($\tau_{\text{off}} = 15 \pm 2$ s, $n = 18$). In fact, time constants resembled BDNF-pretreated oocytes lacking RGS4 (Fig. 5A). Similar acceleration of deactivation was seen in Kir3.4 (S143T) mutants (compare RGS4, $\tau_{\text{off}} = 13 \pm 3$ s, $n = 37$ with RGS4+BDNF, $\tau_{\text{off}} = 19 \pm 3$ s, $n = 8$). These results suggest an independent mechanism of Kir3 GAP activity for RGS4- and trkB-mediated Kir3 phosphorylation.

We conclude that Kir3.4 contains critical GAP-like amino acids and probably acts as a GAP for $G\alpha_i$. GAP activity is kept in check by the tyrosine hydroxyl. Either elimination of the hydroxyl by phenylalanine substitution or phosphorylation of the hydroxyl by tyrosine kinase signaling cascades results in potentiation of GAP activity. These results suggest a novel control mechanism of channel deactivation.

DISCUSSION

The principal finding of this study is that tyrosine phosphorylation of Kir3 by trkB/BDNF reduces channel activation by accelerating GTPase kinetics. Our data did not support the alternative hypothesis that the binding affinity of channel for $G\beta\gamma$ was reduced by channel phosphorylation. The concept that the channel regulates the GTPase activity of the $G\alpha_i$ subunit is novel, although the principle of an effector providing feedback regulation of its activator is well established (15,25).

Tyrosine phosphorylation has been described previously as a regulator of ion conductance of potassium and other ion channels (7,26–31). Tyrosine kinase cascades are ubiquitous, and they modulate signaling in cardiac myocytes and neurons among other physiological systems (32, 33). Our study provides a novel link between Kir3 tyrosine phosphorylation and G protein-mediated signaling. To our knowledge, this is the first demonstration that tyrosine phosphorylation of an ion channel regulates intrinsic GTPase accelerating capacity.

Recent evidence shows that $G\alpha_i$ binds directly to the Kir3 itself, suppressing basal $G\beta\gamma$ binding and basal current amplitude (9,34–38). Peleg and *et al.* (9) found that Kir3 $G\alpha_i$ interaction decreased $G\beta\gamma$ binding to the channel, and $G\alpha_i$ bound directly to the N-terminal domain

residues of Kir3. The tyrosine residues masking Kir3.4 GAP activity in our study are within this domain. Further, the arginine, glutamine, and tyrosine residues in the portion of Kir3 contacting $G\alpha_i$ are residues found in GTPase-activating proteins shown by crystallography to be critical in stabilizing the transition state of $G\alpha_i$, favoring catalysis (10,14,23). Glutamine and arginine residues in GAPs such as Ras-GAP, p50Rho-GAP, cdc42-GAP, and the yeast Ypt/Rab-GAP-Gyp1p are critical in mimicking residues in their respective G proteins to stabilize the transition phase of the $G\alpha_i$ binding pocket (10–12,14,23,39). The contribution of the tyrosine residue is less clear, although some studies imply that a tyrosine hydroxyl on the G protein itself (11,12,15,40) or the GAP in the case of the RGS4- $G\alpha_i$ interaction (24) is part of a network providing polar interaction between the G domain and GAP, facilitating catalysis. Our data support a role for the tyrosine hydroxyl in reducing rather than enhancing catalysis. Nevertheless, without crystal structure data for Kir3, speculations about the contributions of specific residues remain unverified.

The trkB facilitation of Kir3 deactivation kinetically resembles RGS4-mediated return to basal current (16,18). Our data conform kinetically to the properties of the known GTPase-activating protein RGS4 (18,21). Like kinetics after trkB activation, the RGS proteins elicit a similar acceleration of return to inactive heterotrimeric G protein state, presumably by mediating direct hydrolysis of GTP and resulting in a return of $G\alpha_i$ to the GDP-bound state (13,18,42,43). Besides, if trkB induced a decrease in the affinity of $G\beta\gamma$ for Kir3, we would have expected trkB/BDNF at least partially to overcome blockage of GTPase activity by enhancing $G\beta\gamma$ dissociation from Kir3, thereby restoring deactivation kinetics in oocytes injected with GTP γ S. Because BDNF/trkB did not restore GTPase activity in this experiment, we favor the hypothesis that trkB activation increases GTP hydrolysis.

CONCLUSIONS

This report provides a novel mechanism for Kir3.4 modulating its own kinetic parameters by dual GAP sequences in its N-terminal tail. GAP facilitation by phosphorylation may promote Kir3 deactivation, potentially providing a new mechanism for regulation of channel function. In opioid regulation of inflammatory pain, for example, endogenous opioids such as β -endorphin, methionine-enkephalin, and dynorphin-A are released from immune cells in inflamed tissue (44,45). Neurotrophic factors such as BDNF and nerve growth factor are up-regulated under these conditions as well (8,46). Further, in studies involving mice with mutant Kir3 channels (*weaver*), analgesia after opioid treatment is decreased, suggesting a role for Kir3 itself in opioid-mediated analgesia (41). Thus, inflammatory pain represents a scenario in which tyrosine kinase cascades coexist with G protein-coupled receptor stimulation in anatomical regions associated with pain transmission such as the dorsal horn of the spinal cord, potentially modulating neuronal excitability by a Kir3-dependent mechanism. Further, in the heart GPCRs such as angiotensin II type 1 receptor mobilize the tyrosine kinase Src, which plays a role in modulating excitability by increasing cellular calcium (32). A concomitant increase in GTPase activity following Src phosphorylation of Kir3 might likewise contribute to the increase in cardiac excitability. Thus, regulation of Kir3 functioning by tyrosine kinase cascades may provide an important means for physiologically regulating cardiac and neuronal excitability during processes such as development, inflammatory responses, synaptic plasticity, and cardiac excitability.

Acknowledgements

We thank Dr. Stephen Van Dien for advice on kinetic analyses. We also thank AMGEN for generously supplying BDNF.

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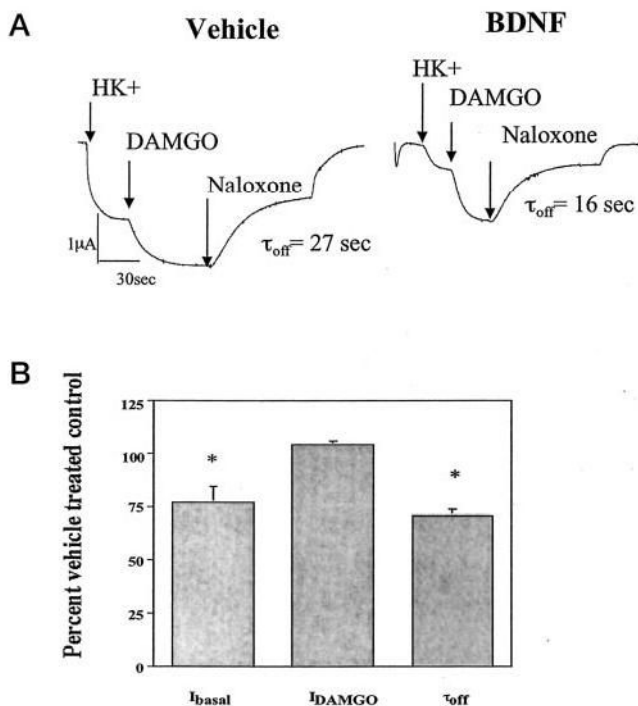


Fig. 1. trkB accelerates Kir3 deactivation

A, *Xenopus* oocytes were injected with cRNA for MOR, Kir3, and trkB and incubated at 19 °C for 2–4 days. On recording day, oocytes were pretreated in vehicle or 0.2 μ g/ml BDNF for 6–12 min. Oocytes were voltage-clamped in two-electrode configuration at -80 mV. At the *first arrow*, high (96 mM) potassium buffer (*HK+*) was perfused into the recording chamber until the current stabilized. Then, as noted by each subsequent *arrow* on the trace, 1 μ M DAMGO in *HK+*, 1 μ M naloxone in *HK+*, and vehicle (ND96) were sequentially perfused into the chamber. pCLAMP6 software was used to fit a simple exponential equation to the naloxone reversal phase of the trace, and time constant of deactivation (τ_{off}) was determined as described under “Experimental Procedures.” *B*, basal current, DAMGO-induced current, and time constant data were compiled from multiple batches of oocytes injected with Kir3.1/3.4 as potassium channel subunits. BDNF-treated oocytes were normalized to vehicle-treated controls. Data are expressed as percent of vehicle-treated controls following a 6–12-min preincubation in BDNF. trkB activation suppressed basal channel conductance and accelerated channel deactivation without affecting DAMGO-induced current amplitude.

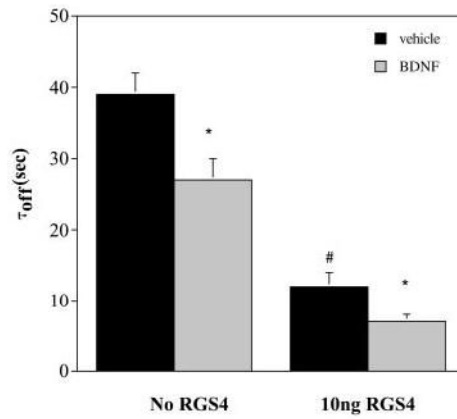


Fig. 2. *trkB* acceleration of Kir3 deactivation recapitulates RGS4 kinetics

Oocytes expressing Kir3.1, Kir3.4, *trkB*, and MOR were injected with 10 ng of RGS4 12–16 h prior to recording. RGS4 accelerated Kir3 deactivation, an effect enhanced by *trkB* activation. There was no statistical difference in the time constant parameter between batches. *, $p < 0.05$ of BDNF effect compared with vehicle-treated control. #, $p < 0.05$ for RGS4-expressing oocytes compared with oocytes not injected with exogenous RGS4.

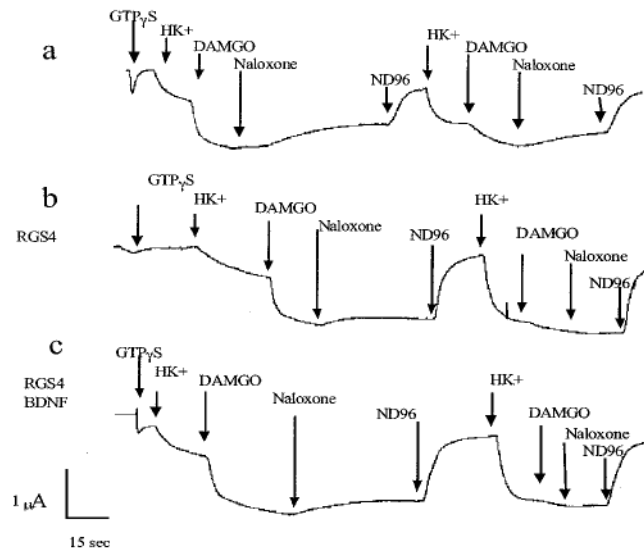


Fig. 3. GTP γ S blocks RGS4 and trkB acceleration of Kir3 deactivation

a, a representative oocyte expressing Kir3.1, Kir3.4, MOR, and trkB was injected with 120 pmol of GTP γ S. DAMGO-induced increase in current did not significantly reverse during naloxone perfusion, and subsequent applications of HK+ and then DAMGO showed that the activation of Kir3 did not reverse. *b*, a representative oocyte also expressing RGS4 showed a similar response. *c*, BDNF pretreatment of a representative oocyte had no effect on deactivation kinetics. RGS4 (10 ng) was injected 12–16 h before recording. Membranes were clamped at -80 mV and 120 pmol of GTP γ S was injected. High potassium buffer, DAMGO, naloxone, and vehicle were sequentially perfused as described. After current returned to baseline, the perfusion sequence was repeated. GTP γ S injection significantly blocked channel deactivation.

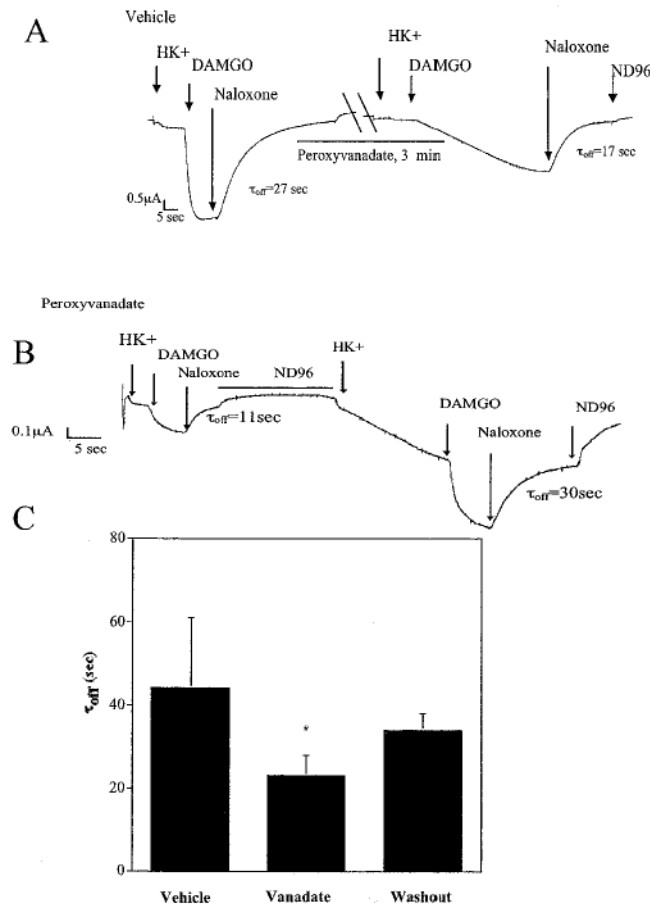
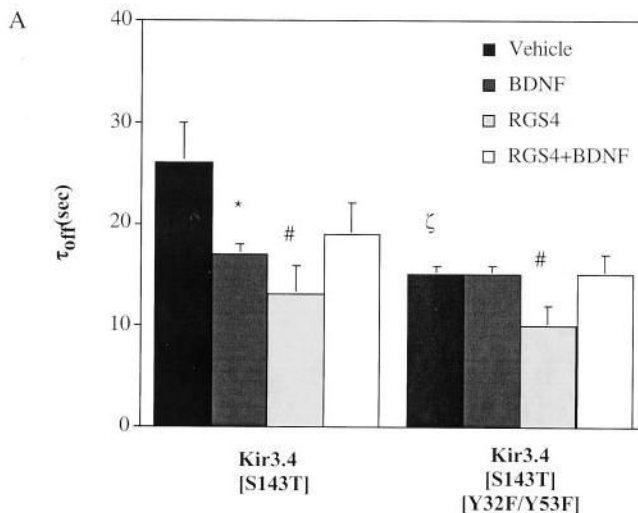


Fig. 4. Phosphatase inhibition accelerates Kir3 deactivation

Oocytes expressing MOR, trkB, Kir3.1, and Kir3.4 were pretreated 6–10 min in vehicle of 100 μ M sodium orthovanadate preactivated in 3% hydrogen peroxide for 4–5 h. Membranes were clamped at -80 mV and sequentially perfused with high potassium buffer, DAMGO, and naloxone (see *arrows* above trace). *A*, peroxyvanadate treatment accelerated channel deactivation. *B*, conversely, in peroxyvanadate-pretreated oocytes, naloxone reversal was initially accelerated. After 3 min of ND96 perfusion, channel 1 deactivation returned to control levels. *C*, these results were replicated in five oocytes, and data were summarized. *, $p < 0.05$ compared with vehicle-treated control.



B

Glutamine-arginine-tyrosine residues in Kir3.4 N terminal domain.

KKIPK**Q**ARDYIPI (23-35)
 KKPR**Q**RYMEKTG (47-58)

Fig. 5. Tyrosine phosphorylation in GAP motifs of the Kir3.4 N-terminal tail is the mechanism of trkB-mediated GAP activity

A, when tyrosines 32 and 53 were mutated to phenylalanines in Kir3.4(S143T) homomeric channels, channel deactivation was insensitive to trkB-mediated acceleration. Channel mutants were expressed in *Xenopus* oocytes, and two-electrode voltage clamp experiments were performed as described following pretreatment in vehicle or BDNF. Overexpression of 10 ng of RGS4 12–16 h prior to two-electrode voltage clamp recordings accelerated channel deactivation independently of Kir3 GAP activity conferred by N-terminal tyrosines. *, $p < 0.05$ of BDNF effect compared with vehicle-treated control. #, $p < 0.05$ for RGS4-expressing oocytes compared with oocytes lacking exogenously expressed RGS4. ζ, $p < 0.05$ for vehicle-treated oocytes expressing Kir3.4(S143T) compared with Kir3.4(S143T/Y32F/Y53F). B, Kir3.4 GAP-like tyrosine-arginine-glutamine motifs in the N terminus.