Facilitating channel deactivation. Thus, BDNF activation of trkB could inhibit Kir3 by or phenylalanine substitution reveals the GAP domain. GAP activity and that modification by phosphorylation phenylalanine substitution in the N-terminal domain of may have caused the GTPase acceleration. Tyrosine to effect, indicating that tyrosine phosphorylation of Kir3 pretreatment reversibly mimicked the BDNF/trkB ef- Tyrosine phosphatase inhibition by peroxyvanadate rather acted to accelerate GTPase activity, like RGS4. Tyrosine phosphatase inhibition by peroxyvanadate pretreatment reversibly mimicked the BDNF/trkB ef- fect, indicating that tyrosine phosphorylation of Kir3 may have caused the GTPase acceleration. Tyrosine to phenylalanine substitution in the N-terminal domain of Kir3 blocked the BDNF effect, supporting the hypo- these that phosphorylation of these tyrosines was responsible. Like other GAPS, Kir3.4 contains a tyrosine-argi- nine-glutamate motif that is thought to function by interacting with G protein catalytic domains to facil- itate GTP hydrolysis. These data suggest that the N-ter- minal 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.

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N-terminal Tyrosine Residues within the Potassium Channel Kir3 Modulate GTPase Activity of Gαi*

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The family of G protein-activated potassium channels (Kir3 or GIRQ) 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. Gβγ, 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 Gβγ, 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 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, Gαi and Gβγ, interact with Kir3 (9). Binding of the Gβγ subunit activates Kir3, whereas Gαi binding suppresses basal Kir3 activity and enhances G protein activation (9). This action of Gαi 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 Gαi 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 GTP- ase-activating protein (GAP)-mediated acceleration of channel deactivation, suggesting that trkB might stimulate GAPI 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 (AF17211). 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). The 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 m NaCl, 96 m KCl, 1 m CaCl2, 1 m MgCl2, 5 m 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**—BDNF 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 (τoff, 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.

\[
F(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} + C
\]

(Eq. 1)

Time constants exceeding two standard deviations of the mean were excluded from the analysis. The membrane time constant (τoff) 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 µg/ml BDNF. Plasma membranes were clamped at ~80 mV in two-electrode voltage clamp configuration. Perfusion of 96 m potassium buffer (HK+) revealed an inwardly rectifying basal current (Fig. 1A). Pretreatment in BDNF suppressed basal current to 77 ± 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 ± 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 (τoff) was calculated using pCLAMP software as described under “Experimental Procedures.” BDNF significantly accelerated channel deactivation rate; τoff was reduced to 71 ± 3% of vehicle control (p < 0.05, n = 35, Fig. 1, A and B). Channel activation rate, as measured by τon, was not significantly accelerated in BDNF-treated oocytes (data not shown).

The decrease in the τoff parameter was not a consequence of the reduced basal current amplitude; a regression analysis showed no correlation between DAMGO current amplitude and τoff (r² < 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 by binding to free Gβγ, these results suggest that tyrosine phosphorylation affected the interaction between channel and Gβγ.

**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 τon (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 \( \tau_{off} \)) was accelerated from 27 ± 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 (\( \tau_{off} \), \( n = 7 \)). BDNF treatment reduced \( \tau_{off} \) 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\( \gamma \)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\( \alpha \)-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\( \alpha \)-GDP, then the accelerated deactivation rate would result from a BDNF-induced increased GTPase activity that would be blocked by GTP\( \gamma \)S. In oocytes expressing MOR, trkB, and Kir3, GTP\( \gamma \)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\( \gamma \)S-injected oocytes (Fig. 3c). Because BDNF pretreatment did not even partially recover deactivation in the presence of GTP\( \gamma \)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\( \alpha \).

**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 peroxovanadate (100 \( \mu \)M) before voltage clamp experimentation. Peroxovanadate pretreatment reduced \( \tau_{off} \) from 39 ± 3 s (\( n = 4 \)) to 23 ± 5 s (\( n = 4 \)). A 3-min perfusion with ND96 returned \( \tau_{off} \) 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 peroxovanadate, channel deactivation was accelerated (Fig. 4B). 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/Y32F/Y53F) (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, \( \tau_{off} = 26 ± 4 \) s, \( n = 18 \) and BDNF, \( \tau_{off} = 17 ± 1 \) s, \( n = 17 \)). However, trkB no longer accelerated deactivation in the double tyrosine to phenylalanine mutant (vehicle, \( \tau_{off} = 15 ± 1 \) s, \( n = 30 \) and BDNF, \( \tau_{off} = 15 ± 1 \) s, \( n = 40 \), Fig. 5A). 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), \( \tau_{off} = 26 ± 4 \) s, \( n = 18 \) with Kir3.4(S143T/Y32F/Y53F), \( \tau_{off} = 15 ± 1 \) 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-GAP, Rho-RhoGAP, and GoG/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
A. Lacking RGS4 (Fig. 5A). In fact, time constants resembled BDNF-pretreated oocytes and naloxone (see arrows above trace). A, peroxynitrate treatment accelerated channel deactivation. B, conversely, in peroxynitrate-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.

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_i but instead holds the G_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 \( \tau_{off} \) parameter in oocytes expressing RGS4, trkB, Kir3.4 mutants, and MOR. RGS4 accelerated channel deactivation in the Kir3.4(S143T/Y32F/Y53F) mutants (\( \tau_{off} = 10 \pm 2 \text{ s}, n = 20 \)), but BDNF had no additional effect (\( \tau_{off} = 15 \pm 2 \text{ s}, n = 18 \)). 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, \( \tau_{off} = 13 \pm 3 \text{ s}, n = 37 \) with RGS4+BDNF, \( \tau_{off} = 19 \pm 3 \text{ s}, n = 8 \)). 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_i. GAP activity is kept in check by the tyrosine hydroxyl. Either elimination of the hydroxyl by phenyalanine 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_b was reduced by channel phosphorylation. The concept that the channel regulates the GTPase activity of the G_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_i binds directly to the Kir3 itself, suppressing basal G_b binding and basal current amplitude (9, 34–38). Peleg et al. (9) found that Kir3 G_i interaction decreased G_b binding to the channel, and G_b 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 Vpt/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 kinases 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_i for Kir3, we would have expected trkB/BDNF at least partially to overcome block of GTP\_\gamma_i, thereby restoring deactivation kinetics in oocytes injected with GTP\_\delta_i. 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 \beta-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 (\textit{weaver}), analgesia after opioid treatment is decreased, suggesting a role for Kir3 itself in opioid-mediated analgesia (41). Thus, inflammatory pain presents 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 GPCR\_\alpha_i 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.

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