Dominant-Negative Mutants Identify a Role for GIRK Channels in D3 Dopamine Receptor-mediated Regulation of Spontaneous Secretory Activity

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abstract The human D3 dopamine receptor can activate G-protein–coupled inward rectifier potassium channels (GIRKs), inhibit P/Q-type calcium channels, and inhibit spontaneous secretory activity in AtT-20 neuroendocrine cells (Kuzhikandathil, E.V., W. Yu, and G.S. Oxford. 1998. *Mol. Cell. Neurosci*. 12:390–402; Kuzhikandathil, E.V., and G.S. Oxford. 1999. *J. Neurosci*. 19:1698–1707). In this study, we evaluate the role of GIRKs in the D3 receptor-mediated inhibition of secretory activity in AtT-20 cells. The absence of selective blockers for GIRKs has precluded a direct test of the hypothesis that they play an important role in inhibiting secretory activity. However, the tetrameric structure of these channels provides a means of disrupting endogenous GIRK function using a dominant negative approach. To develop a dominant-negative GIRK mutant, the K^+ selectivity amino acid sequence -GYG- in the putative pore domain of the human GIRK2 channels was mutated to -AAA-, -GLG-, or -GFG-. While the mutation of -GYG- to -GFG- did not affect channel function, both the -AAA- and -GLG- GIRK2 mutants were nonfunctional. This suggests that the aromatic ring of the tyrosine residue rather than its hydroxyl group is involved in maintaining the pore architecture of human GIRK2 channels. When expressed in AtT-20 cells, the nonfunctional AAA-GIRK2 and GLG-GIRK2 acted as effective dominant-negative mutants and significantly attenuated endogenous GIRK currents. Furthermore, these dominant-negative mutants interfered with the D3 receptor-mediated inhibition of secretion in AtT-20 cells, suggesting they are centrally involved in the signaling pathway of this secretory response. These results indicate that dominant-negative GIRK mutants are effective molecular tools to examine the role of GIRK channels in vivo.

key words: potassium channel structure • calcium channels • selectivity filter • autoreceptor • FM1-43

INTRODUCTION

The molecular mechanisms underlying the dopaminergic regulation of mammalian neurotransmission are not well characterized. Among G protein–coupled dopamine receptors, the role of the D3 receptor in regulating secretory activity, in particular, is poorly understood. The D3 receptor has been proposed to function as an autoreceptor regulating the release of dopamine at nerve terminals (Rivet et al., 1994; Gobert et al., 1996). The relatively small size of dopaminergic terminals and the lack of D3-selective ligands and antibodies have precluded a direct test of this hypothesis in native neurons. Thus, existing studies have used heterologous expression systems to characterize D3 receptor function. Activation of D3 dopamine receptors heterologously expressed in a clonal mesencephalic line (MN9D) can modestly inhibit dopamine synthesis and release, albeit at high agonist concentrations (Tang et al., 1994; O'Hara et al., 1996). We have recently demonstrated that activation of heterologously expressed human D3 receptors can inhibit spontaneous secretory activity in AtT-20 mouse pituitary cells that release both acetylcholine and adrenocorticotropic hormone (ACTH)[1] (Kuzhikandathil and Oxford, 1999). Previous studies have demonstrated that the spontaneous secretory activity in AtT-20 cells is dependent upon the influx of extracellular calcium through voltage-dependent calcium channels (Luini et al., 1986; Wang and Greer, 1995; Kuzhikandathil and Oxford, 1999). However, the signaling mechanisms and, in particular, the relevant effectors through which D3 receptors might alter secretion in these systems remain unknown.

In the AtT-20 expression system, we have demonstrated that D3 receptors can efficiently couple to two different ion-channel effectors. D3 receptors can activate endogenous G protein–coupled inward rectifier potassium channels (GIRKs) with high efficacy (Kuzhikandathil et al., 1998). D3 receptor activation can also selectively inhibit P/Q-type voltage-gated calcium channels in these cells (Kuzhikandathil and Oxford, 1999). The coupling of D3 receptors to these two families of ion channels provides two possible mechanisms for the

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[1]*Abbreviations used in this paper:* ACTH, adrenocorticotropic hormone; CHO cell, Chinese hamster ovary cell; EGFP, enhanced green fluorescent protein; GIRK channel, G-protein–coupled inward rectifier potassium channel; SES, standard external solution.

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agonist-induced inhibition of secretion. The D3 receptor could inhibit calcium-dependent spontaneous secretion by the direct G protein–mediated inhibition of voltage-gated calcium channels. Alternately, D3 receptor-mediated activation of GIRK channels could trigger hyperpolarization of the cell, thereby reducing the open probability of voltage-dependent calcium channels and indirectly reducing the calcium influx that supports secretion. As these two possible pathways converge at the level of the calcium channel, molecular or pharmacological interventions at this level would be equally effective regardless of which pathway dominates. Thus, the underlying mechanisms can only be distinguished by selectively disrupting the function of GIRK channels. Given the absence of selective GIRK channel blockers, we chose to develop and employ an alternate molecular approach to test the involvement of GIRK channels in D3 receptor-mediated inhibition of secretion.

The GIRK subfamily consists of five major isoforms (GIRK1–GIRK5 or Kir3.1–Kir3.5). Functional GIRK channels are tetramers composed of either four identical (homomeric) or nonidentical (heteromeric) subunits. All known GIRKs form heteromultimers in vivo (Kofuji et al., 1995; Liao et al., 1996), though recent studies suggest that homomeric GIRK2 and GIRK4 channels might also occur in vivo (Corey and Clapham, 1998; Imanobe et al., 1999). The tetrameric composition of a functional GIRK channel provides a means to selectively disrupt native GIRK channel function, using a dominant-negative approach. In this approach, the coexpression of a nonfunctional mutant subunit disrupts the function of native GIRK channels by heteromultimerization. This approach has been used previously to study the function of the Kir2 family of inward rectifying potassium channels (Tinker et al., 1996).

We have previously shown, using reverse transcriptase PCR and Western blot analysis, that AtT-20 cells express only GIRK1 and GIRK2 isoforms (Kuzhikandathil et al., 1998). As a result, these cells are likely to express GIRK1–GIRK2 heteromultimers, as well as GIRK2 homomultimers. To target all the GIRK channels expressed in AtT-20 cells, we decided to generate a GIRK2 mutant, as this would disrupt both homomultimers and heteromultimers. To generate an effective dominant-negative subunit, we mutated the highly conserved -GYG- amino acid sequence present in the pore region of human GIRK2. Three mutants -GFG-, -GLG-, and -AAA- were generated and tested for function. The -GLG- and -AAA-GIRK2 mutants were not only nonfunctional, but were also effective dominant-negative mutants that ablated endogenous GIRK channel function in AtT-20 cells. Using these GIRK2 mutants, we present novel evidence that D3 dopamine receptors inhibit secretion predominantly by activating GIRK2 channels. Furthermore, our mutation studies suggest that the tyrosine residue in the pore region of human GIRK2 channels might contribute to pore architecture and function in a manner unlike the tyrosine in the recently described bacterial potassium channel pore region (Doyle et al., 1998).

MATERIALS AND METHODS

Generation of Mutant GIRK2 Constructs

The mutant GIRK2 constructs were generated by site-directed mutagenesis. The plasmid encoding the wild-type human GIRK2 gene was denatured and annealed to a selection primer (which converted a unique StuI restriction enzyme site in hGIRK2 to PpuMI) as well as the mutagenic primers (encoding either the GYG → AAA, GYG → GLG, or GYG → GFG mutations). Second-strand DNA synthesis from these annealed primers was carried out using T4 DNA polymerase (New England Biolabs, Inc.). The gaps in the modified plasmids were sealed using T4 DNA ligase (New England Biolabs, Inc.). StuI restriction enzyme was used to linearize unmodified plasmids while not affecting the modified plasmids (which had incorporated the selection and mutagenic primers). This step reduced the subsequent transformation efficiency of linear unmodified plasmids compared with the circular modified plasmids. The mixture of linear unmodified and circular modified plasmids were then transformed into *Escherichia coli* BMH 71-18 mutS (CLONTECH Laboratories, Inc.). This strain, being DNA mismatch repair deficient, allows the propagation of modified plasmids containing the selection and mutagenic primer. Plasmid DNA isolated from transformed *E. coli* BMH 71-18 mutS colonies was pooled and redigested with StuI to linearize unmodified plasmids and further enrich the population of circular modified plasmids. This mixture of plasmids was then transformed into *E. coli* DH5a (GIBCO BRL). Plasmid DNA was isolated from individual colonies and characterized by both restriction enzyme mapping and DNA sequencing. Clones that contained the desired mutations in the hGIRK2 gene were identified and subjected to further DNA sequencing to confirm that this was the only mutation incorporated into the hGIRK2 gene.

Generation of GIRK2–Enhanced Green Fluorescent Protein Fusion Constructs

Wild-type and mutant GIRK2 genes were fused in frame at the carboxyl terminal to the coding region of enhanced green fluorescent protein (EGFP) in the EGFP-N2 plasmid (CLONTECH Laboratories, Inc.). In brief, the carboxyl terminal region of the human GIRK2 constructs were amplified by PCR using an upper primer containing the unique BstEII restriction enzyme site and a lower primer that lacked the GIRK2 stop codon. The lower primer also incorporated a unique XmaI restriction enzyme site that allowed the in-frame introduction of the GIRK2 gene into the EGFP-N2 plasmid. The BstEII-XmaI PCR fragment was subcloned along with the remaining human GIRK2 sequence into the EGFP-N2 plasmid. As a result of the subcloning procedure, the recombinant GIRK2 fusion construct contains a linker region of nine amino acids (PGIHRPVAT) in between the terminal valine residue of human GIRK2 and the first methionine residue of EGFP.

Cell Culture

Chinese hamster ovary (CHO) cells were grown in Ham's F12 medium with 10% FCS and 10,000 U of penicillin/streptomycin. AtT-20 mouse pituitary cells were grown in Ham's F10 medium with 5% FBS, 20% heat-inactivated horse serum, 200 mM glutamine, and 1 mg/ml gentamicin. CHO and AtT-20 cells stably expressing human dopamine receptors were maintained in

200 mg/ml and 500 μg/ml of geneticin (G418), respectively. For transient transfections and subsequent electrophysiological characterization, cells were plated onto glass coverslips coated with 40 μg/ml poly l-lysine.

Transfection of Receptors and Channels into AtT-20 and CHO Cells

AtT-20 cells stably expressing the human D3 receptor were generated by clonal selection after a Pfx-2 reagent (Invitrogen Corp.)-mediated transfection. CHO-K1 cells stably expressing either the human D3 receptor (CHO-D3) or the human short isoform of the D2 receptor (CHO-D2S) were gifts from Dr. Tony Sandrasagra (Hoechst-Marion Roussel, Somerville, NJ). Transient-transfections into CHO cells were done using Lipofectamine (GIBCO BRL) and into AtT-20 cells using Pfx-2 (Invitrogen Corp.). To identify transfected cells, we used plasmids encoding either the EGFP (CLONTECH Laboratories, Inc.) or the CD4 membrane antigen (a gift from ICAgen Inc.). The latter marker was used in experiments with either fluo-3 or FM1-43 dyes (Molecular Probes, Inc.), since these compounds have excitation and emission wavelengths that overlap with EGFP. The cells expressing the CD4 membrane antigen were identified using Dynabeads® M-450 CD4 (Dynal). Transfection efficiency of 15–30% was routinely achieved.

Electrophysiology

Agonist-activated currents were measured in AtT-20 or CHO cells in the whole-cell configuration of the patch clamp using an Axopatch 200 amplifier (Axon Instruments, Inc.). Patch pipettes were constructed from N51A glass (Drummond), coated with dental wax (Kerr Sticky Wax), and polished on a homemade microforge at 600× magnification. Currents were elicited by ramp voltage commands (−120 to +40 mV), followed by a hyperpolarizing step (−100 mV) from holding potentials of −60 mV. The current responses were normalized to the cell capacitance (picoamperes per picofarad), to account for variation in cell size. The standard external solution (SES) used contained (mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose. The pipette solution contained (mM): 130 K-Aspartate, 20 NaCl, 10 HEPES, 10 glucose, 0.1 GTP, 5 Mg-ATP, 1 EGTA. To enhance inwardly rectifying K^+ currents in the voltage-clamp experiments, controls and drug exposures were performed in solutions with elevated extracellular [K^+] (50 mM) by substitution for Na^+. Quinpirole and somatostatin (RBI Chemicals) were used at 100 nM concentration, unless otherwise indicated. Drug solutions were delivered to cells via a multibarreled micropipette array (Drummond Microcaps, 3 μl).

Data Acquisition and Analysis

Whole-cell macroscopic currents in response to ramp and step commands were sampled via a Digidata 1200b interface using Axotape and pClamp 7.0 software (Axon Instruments, Inc.). Data files are then imported into SigmaPlot for display or analysis.

Intracellular Calcium Imaging

The cells on glass coverslips were rinsed in PBS and incubated at 37°C in 5 mM fluo-3 AM (Molecular Probes, Inc.) for 30 min. Cells were rinsed in SES and placed in a glass-bottom chamber on an inverted microscope stage (Nikon). Drug and control solutions were directly applied using a continuous flow (∼1 ml/min) bath system. After excitation at 485 nm, the fluorescence emission was band pass filtered at 535 nm, collected via a quartz phase objective (40× or 100×), amplified by a Videoscope KS-1381 intensifier, and passed to a Pentamax cooled CCD camera (Princeton Instruments). Video images were captured using the Metamorph software package (Universal Imaging Corp.). This software allows logging of fluorescence intensity versus time for several cells as a measure of intracellular [Ca^{2+}] changes (arbitrary units).

Imaging Vesicle Trafficking

The imaging procedures employed have been described previously (Kuzhikandathil and Oxford, 1999). In brief, FM1-43 dye (Molecular Probes, Inc.) was added to control or drug solutions at a final concentration of 2 μM and applied directly to the cells using a continuous flow bath exchange system in the experimental chamber described above. The dye and drug solutions were made up in SES that contained (mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose. Cells plated on glass coverslips were imaged as above in fluorescence mode (excitation at 480 nm, emission at 635 nm). To monitor and quantify changes in fluorescence, rectangular regions of interest corresponding to highly fluorescent areas of cell–cell contact were defined using Metamorph imaging software. Changes in fluorescence were monitored during cycles of solution exchange and represented in arbitrary intensity units.

Statistical Methods

Student's *t* test was performed on relevant data using SigmaPlot (SPSS Inc.). In the *t* test, the data was considered statistically different when the probability value was <0.05.

RESULTS

Generation and Characterization of Mutant GIRK2 Channels

To generate dominant-negative GIRK2 mutants, we mutated the -GYG- amino acid sequence in the putative pore region of the human GIRK2 channel to either GFG, GLG, or AAA (Fig. 1). The mutants were generated by site-directed mutagenesis as described in materials and methods. To determine whether the mutation affected expression and cellular localization, we also generated recombinant constructs in which both the wild-type and mutant GIRK2 proteins were fused in frame at the carboxyl terminal to the EGFP. These fusion constructs were individually transfected into CHO cells and their expression pattern determined by fluorescence microscopy. CHO cells transfected with the wild-type and mutant GIRK2::EGFP fusion constructs exhibit distinct organelle and membrane fluorescence staining patterns that are qualitatively different from the uniform fluorescence pattern observed in CHO cells transfected with control EGFP plasmid (data not shown). These results (and the functional studies below) suggest that both wild-type and mutant constructs are expressed on the surface of the cells and that mutation of the GYG sequence in the pore region does not detectably alter expression or localization of the GIRK2 channels.

Functional Characterization of GIRK2 Mutants in CHO Cells

Having shown that the mutant GIRK2 subunits are expressed, we next determined whether the expression

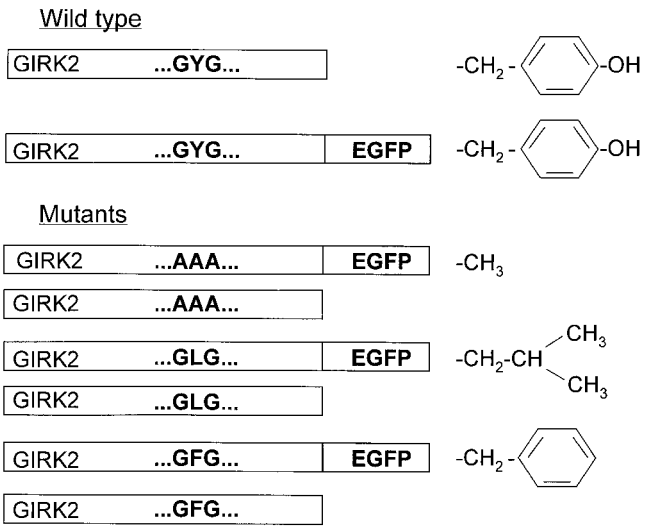

Figure 1. Wild-type and mutant EGFP-tagged human GIRK2 constructs were used in this study. The mutants were generated by site-directed mutagenesis and the EGFP fusion constructs were generated by PCR subcloning as described in material and methods. The highly conserved -GYG- selectivity sequence in the pore region was mutated to either -AAA-, -GLG-, or -GFG-. The amino acid structure for tyrosine (Y), alanine (A), leucine (L), and phenylalanine (F) are indicated to highlight the side groups that were substituted in the pore region of human GIRK2.

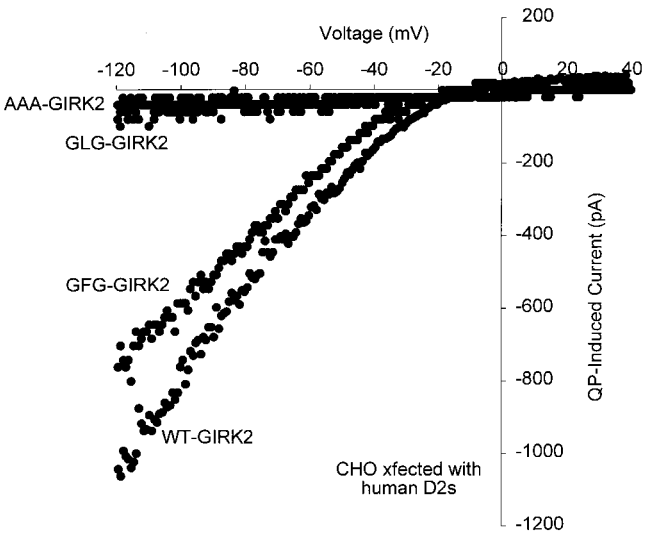

Figure 2. Comparison of wild-type and mutant human GIRK2 function in CHO cells. Representative quinpirole (QP) induced inward K^+ currents during a voltage ramp (−120 to +40 mV) from four different CHO cells coexpressing the human D2S receptor and different human GIRK2 constructs as indicated. The individual traces were obtained by subtracting the current response in 50 mM K^+ external solution (50K-ES) from current response in 100 nM quinpirole + 50 K-ES.

yielded functional channels. Wild-type and mutant GIRK2 channels (the non-EGFP–fused constructs) were transfected individually into CHO cells stably expressing either the human D2S or human D3 receptor, along with EGFP marker constructs. Transfected cells were identified by EGFP expression and whole-cell voltage clamp recording was performed as described in materials and methods. Fig. 2 shows representative quinpirole-induced inwardly rectifying currents elicited by voltage ramps applied to separate CHO cells, each coexpressing the human D2S receptor and a different human GIRK2 construct as indicated. As mentioned previously, the nontagged constructs were routinely employed to avoid interference with calcium or vesicle cycling measurements using similar fluorescence wavelengths; however, we have confirmed that similar responses occur in cells expressing the EGFP fusion constructs of each isoform. These records suggest that the GFG-GIRK2 mutant can make functional homomultimeric channels that are qualitatively indistinguishable from wild type, whereas neither GLG- nor AAA-GIRK2 mutants yield agonist-induced currents and are presumably nonfunctional.

In Fig. 3, we have plotted the mean normalized current densities at −100 mV in SES, 50K-ES, or 100 nM quinpirole (in 50K-ES) for the wild-type and the three mutant GIRK2 channels. The data indicate that wild-type GIRK2 and GFG-GIRK2 yield both constitutive and quinpirole-induced currents in CHO cells expressing either the human D2S (Fig. 3 A) or the human D3 (B) receptors. In contrast, the GLG-GIRK2 and AAA-GIRK2 mutants do not generate either constitutive or quinpirole-induced currents. Similar results were obtained with the GIRK2-EGFP fusion constructs shown in Fig. 1, suggesting that the carboxyl-terminal EGFP fusion does not affect function of these channels (data not shown). The remaining experiments in this report employed the non-EGFP–fused constructs for consistency.

The results of Figs. 2 and 3 indicate that while the mutation of the GYG sequence to GFG does not affect channel function, mutation to either GLG or AAA renders the human GIRK2 channel nonfunctional when expressed as a homomultimer.

The GLG- and AAA-GIRK2 Constructs Function as Dominant-Negative Mutants

To determine whether the GLG- and AAA-GIRK2 constructs could serve as dominant-negative elements, we assessed the ability of these mutants to disrupt the function of endogenous GIRK channels in AtT-20 mouse pituitary cells. We have previously shown that AtT-20 cells express only GIRK1 and GIRK2 isoforms and that the native channels couple to endogenous somatostatin receptors or transfected human D3 receptors (Kuzhikandathil et al., 1998). AtT-20 cells stably expressing the human D3 receptor were transfected with GFG-, GLG-, or AAA-GIRK2 mutant constructs and an EGFP marker construct. Agonist-induced inward currents in transfected cells are measured at −100 mV. Fig. 4 shows

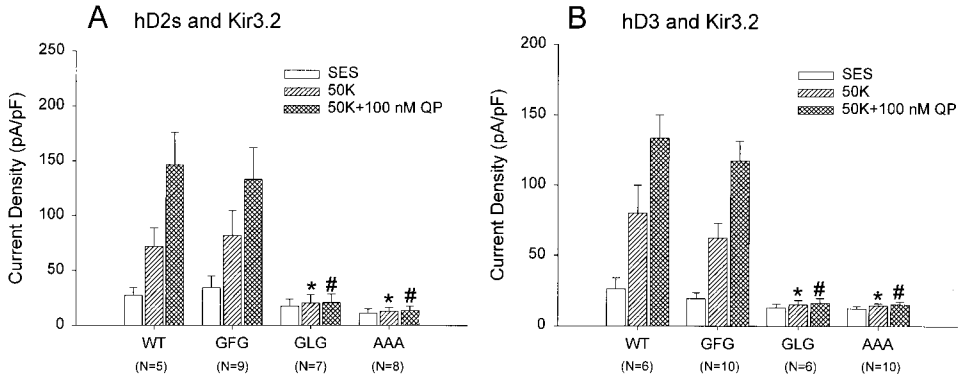

Figure 3. Functional characterization of GIRK2 mutants in CHO cells. Bars represent mean (±SEM) current densities at −100 mV from CHO cells transfected with the GIRK2 constructs and either the human D2S dopamine receptor (A) or the human D3 dopamine receptor (B). Wild-type and mutant GIRK2 constructs (non–EGFP-tagged forms) were cotransfected with a 10-fold excess of dopamine receptors. Whole-cell voltage-clamp recording was done 24–36 h after transfection. The numbers in parenthesis represent the number of cells tested in each case. The GLG and AAA mutants exhibited significantly reduced inward currents compared with either wild type (WT) or GFG human GIRK2 in an external solution containing either 50 mM potassium (50 K-ES, hatched bars) or dopamine receptor agonist quinpirole (100 nM QP, cross-hatched bars) ($^{*,\#}P < 0.01$, Student's *t* test).

mean current densities induced by activation of either somatostatin or D3 dopamine receptors (after subtraction of baseline current). While expression of the GFG-GIRK2 construct augments the responses, the agonist-induced currents are significantly attenuated in cells transfected with either GLG- or AAA-GIRK2 mutant constructs compared with untransfected AtT-20 cells. The augmentation of agonist-induced currents in GFG-GIRK2–transfected cells is expected since GFG-GIRK2 mutants form functional channels (Fig. 3). Conversely, the attenuation of agonist-induced currents by GLG- and AAA-GIRK2 strongly suggests that these mutants act as dominant-negative elements heteromultimerizing with endogenous wild-type GIRK subunits in AtT-20 cells to render the GIRK channel population nonfunctional.

Dominant-Negative GIRK2 Mutants Alter the Ability of D3 Receptors to Inhibit Spontaneous Action Potentials in AtT-20 Cells

AtT-20 cells are excitable and fire spontaneous action potentials (Surprenant, 1982; Adler et al., 1983; Kuzhikandathil and Oxford, 1999). Activation of either the stably expressed human D3 receptor or the endogenous somatostatin receptor in AtT-20 cells can inhibit spontaneous action potentials and hyperpolarize the cell (Fig. 5 A). Agonist-induced hyperpolarization and inhibition of spontaneous action potentials is also observed in AtT-20 cells transfected with the functional GFG-GIRK2 mutant (Fig. 5 B), suggesting that neither the transfection procedure nor the introduction of a benign functional subunit alters the basic response of these cells to agonists. In contrast, AtT-20 cells transfected with the dominant-negative AAA-GIRK2 mutants do not fire spontaneous action potentials, nor do they hyperpolarize in response to D3 or somatostatin receptor agonists (Fig. 5 C). Interestingly, the average resting membrane potential of AtT-20 cells transfected with the GFG-GIRK2 mutant measured during quiescent periods is significantly hyperpolarized, while that of AAA-GIRK2 mutant transfected cells is significantly depolarized compared with untransfected AtT-20 cells (Fig. 5 D). These results suggest that GIRK2 channels are important both for maintaining resting membrane potential and for mediating the somatostatin and D3 receptor-induced hyperpolarizations in AtT-20 cells.

AAA-GIRK2 Mutant Disrupts the Ability of D3 Receptor to Inhibit Spontaneous Calcium Influx

Using the calcium indicator dye Fluo-3, we demonstrated that the spontaneous action potentials in AtT-20 cells are

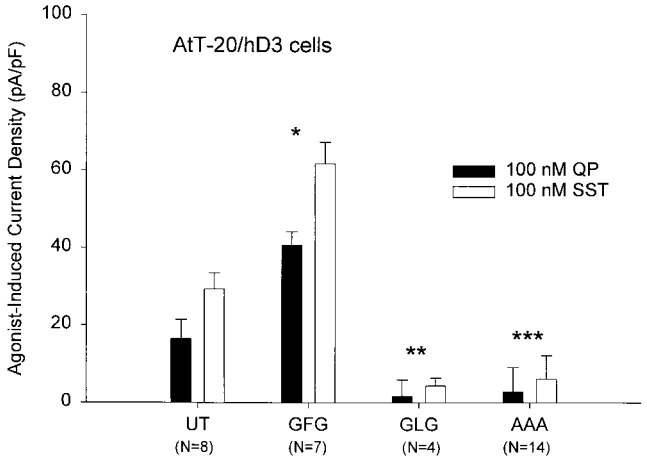

Figure 4. The nonfunctional GIRK2 mutants disrupt endogenous GIRK channel function in AtT-20 cells. Bars represent mean (±SEM) inward current density at −100 mV from AtT-20 cells stably expressing the human D3 receptor and transiently transfected with the different mutant GIRK2 constructs. Whole-cell voltage-clamp recording was performed on transfected cells 36–72 h after transfection. While the GFG mutant increased the agonist-induced inward currents ($^{*}P < 0.01$, Student's *t* test), both the GLG and AAA mutants significantly attenuated the somatostatin- (100 nM SST) and quinpirole- (100 nM QP) induced currents when compared with control cells ($^{**,***}P < 0.01$, Student's *t* test). The numbers in parenthesis represent the number of cells tested. Controls cells (UT) were transiently transfected with pUC19 and the EGFP marker plasmids.

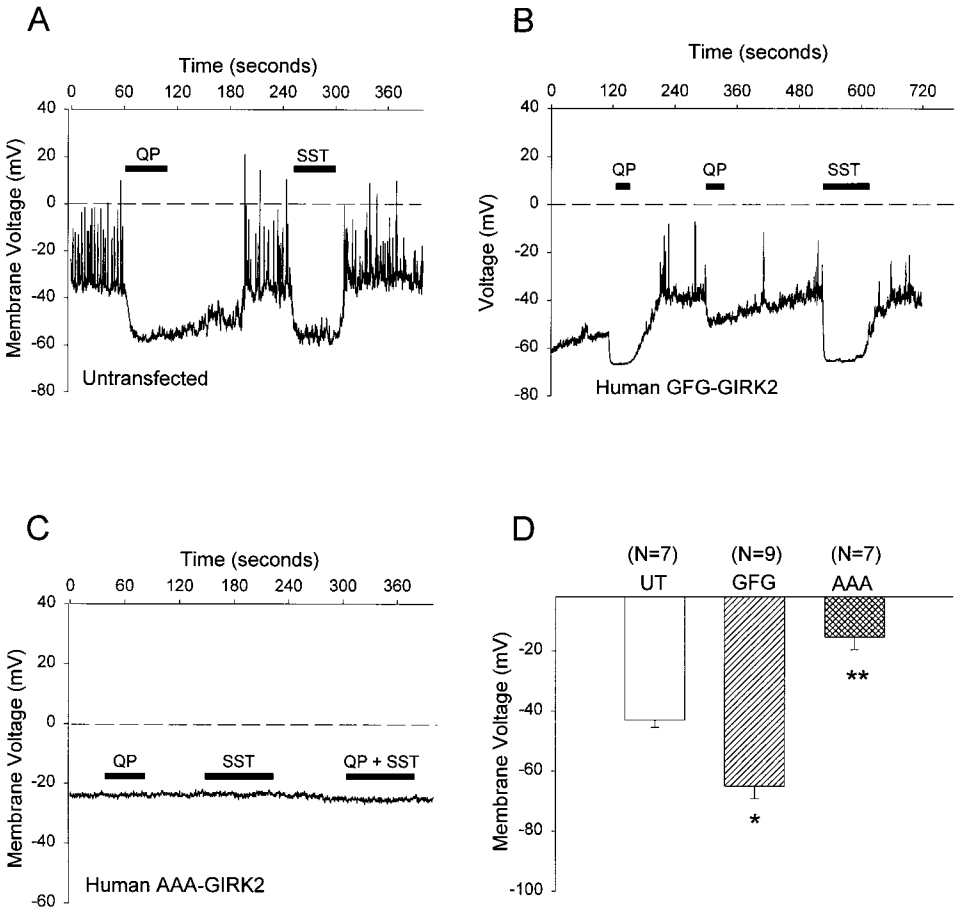

Figure 5. GIRK2 mutants alter resting membrane potential and the ability of D3 receptor to regulate membrane excitability. Representative whole-cell current-clamp recording measuring resting membrane potential from untransfected AtT-20 cells (A) and cells transfected with the GFG (B) or AAA (C) mutants. Untransfected AtT-20 cells and cells transfected with GFG mutant exhibit spontaneous activity that is inhibited by 100 nM quinpirole (QP) and/or 100 nM somatostatin (SST). The solid horizontal bars indicate the duration of agonist application. (D) Compared with untransfected control cells (UT, open bar), AtT-20 cells transfected with the GFG mutant (hatched bar) had a significantly hyperpolarized resting membrane potential, while the AAA mutant transfected cells (cross hatched bars) exhibited significantly depolarized resting membrane potentials. The numbers in parenthesis represent the number of cells tested in each case. The differences were statistically different (*,**$P < 0.01$, Student's *t* test).

accompanied by a calcium influx that can also be inhibited by activation of either endogenous somatostatin receptors or heterologously expressed D3 receptors (Kuzhikandathil and Oxford, 1999). As voltage-gated calcium channels are inhibited by D3 receptors in AtT-20 cells (Kuzhikandathil and Oxford, 1999), the suppression of calcium influx may reflect the direct inhibition of such channels. Alternatively, inhibition of calcium influx may occur indirectly through the activation of GIRK channels and subsequent membrane hyperpolarization (Kuzhikandathil et al., 1998). Since both mechanisms have voltage-gated calcium channels in common, we attempted to use the dominant-negative GIRK2 mutant to distinguish between the two alternatives. Representative Fluo-3 fluorescence (arbitrary units) recordings from two different AtT-20 cells, one expressing the GFG-GIRK2 mutant (●) and the other expressing the AAA-GIRK2 mutant (○) are shown in Fig. 6 A. In the cell expressing GFG-GIRK2, the intracellular calcium level is elevated at the beginning of the recording, presumably coinciding with the appearance of spontaneous electrical activity. Upon application of quinpirole, the calcium levels decline reversibly, as expected for a hyperpolarizing response that suppresses calcium-dependent action potential activity (e.g., Fig. 5, A and B). In contrast, in the cell expressing the AAA mutant, application of the agonist failed to reduce calcium levels. The results of all such observations are summarized in Fig. 6 B, where intracellular calcium levels are reduced on average by 45% by the activation of the human D3 receptor in GFG-GIRK2–transfected cells—a reduction comparable with that seen in untransfected cells (see also Kuzhikandathil and Oxford, 1999). In contrast, in cells transfected with the AAA-GIRK2 dominant-negative mutant, the D3 receptor is completely unable to inhibit the elevated intracellular calcium levels. Were the direct inhibition of P/Q type calcium channels by D3 receptor activation (Kuzhikandathil and Oxford 1999) sufficient to blunt calcium influx, it would be expected that some reduction would be observed even in cells expressing AAA-GIRK2. As this is not the case, these data suggest that the inhibition of spontaneous calcium influx by the activation of the D3 receptor is mediated indirectly through the activation of GIRK channels rather than through the direct inhibition of voltage-gated calcium channels.

AAA-GIRK2 Mutants Disrupt the Ability of D3 Receptors to Inhibit Spontaneous Secretory Activity in AtT-20 Cells

AtT-20 cells secrete ACTH and acetylcholine (Richardson and Schonbrunn, 1981; Carmeliet and Denef,

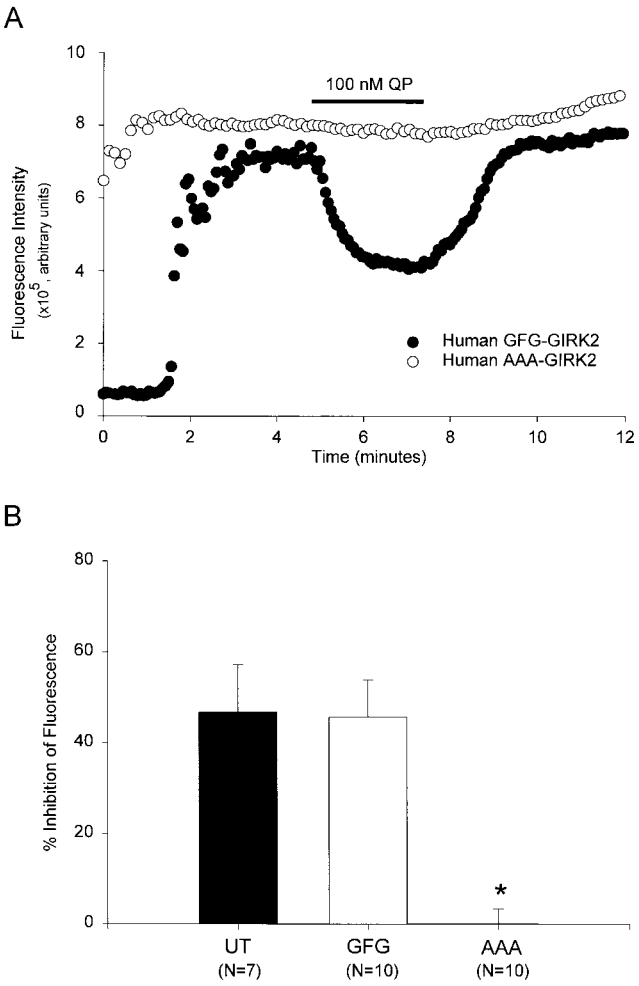

Figure 6. AAA-GIRK2 mutant disrupts the ability of D3 receptor to inhibit calcium influx in AtT-20 cells. (A) Representative measurements of fluo-3 fluorescence indicate that spontaneous calcium influx is inhibited by activation of the D3 receptor by 100 nM quinpirole (QP) in AtT-20 cells transiently transfected with the GFG mutant (●), but not the AAA mutant (○). Transfected cells were identified by coexpression of the CD4 antigen marker recognized by Dynabeads® coated with a monoclonal antibody for CD4. Fluorescence was measured as described in materials and methods. Cells transfected with the AAA mutant exhibited a higher steady state level of calcium compared with cells transfected with the GFG mutant, possibly reflecting the depolarized membrane potential in these cells. Note that the initial increase in fluorescence in the cell expressing the GFG mutant is spontaneous, reflecting the periodic nature of the action potential behavior in these cells. (B) Mean (±SEM) percent inhibition of fluorescence by 100 nM quinpirole in untransfected control cells (UT, filled bar) is comparable with cells expressing the GFG mutant (open bar), but significantly decreased in cells expressing the AAA-GIRK2 mutant (*$P < 0.001$, Student's t test). The percent inhibition values were obtained by comparing fluorescence intensity values before and after quinpirole application. The number of cells tested is indicated in parenthesis.

1989). Spontaneous secretory activity in AtT-20 cells is calcium dependent and can be monitored using FM1-43 dye. By measuring FM1-43 fluorescence changes concentrated at regions of cell–cell contact, we have found that activation of either endogenous somatostatin receptors or heterologously expressed human D3 receptors can inhibit this secretory activity (Kuzhikandathil and Oxford, 1999). As with G-protein–coupled receptor regulation of calcium levels in these cells, the regulation of secretory activity could arise from either inhibition of P/Q-type calcium channels or activation of GIRK channels. The dominant-negative AAA-GIRK2 mutant provides a tool to distinguish the relative role of GIRK channels in the inhibition of spontaneous secretory activity. AtT-20 cells stably expressing the human D3 receptor were again transfected with either the functional GFG-GIRK2 mutant or the nonfunctional AAA-GIRK2 mutant, but this time a marker plasmid encoding the CD4 membrane antigen rather than EGFP was cotransfected with the GIRK plasmids. This marker was used to avoid optical interference as the excitation and emission wavelengths of EGFP overlap those of the FM1-43 dye. The transfected AtT-20 cells were identified by incubating the cells with Dynabeads® coated with a primary monoclonal antibody specific for the CD4 antigen. FM1-43 fluorescence was monitored in bead-decorated cells as described in materials and methods. The results of the FM1-43 experiments are shown in Fig. 7 for cells transfected with the functional GFG-GIRK2 mutant (A and B) or the dominant-negative AAA-GIRK2 mutant (C and D). Fig. 7, A and C, illustrates the time course of FM1-43 fluorescence changes at cell–cell contacts as the dye is either introduced into or removed from the imaging chamber bath in either control SES (●) or the presence of 100 nM quinpirole (○). In AtT-20 cells transfected with the GFG-GIRK2 mutant, both the staining and destaining of FM1-43 fluorescence in the presence of 100 nM quinpirole are significantly inhibited compared with SES. The fluorescence intensity during the destaining process in SES or 100 nM quinpirole was normalized and replotted on a semilogarithmic scale and fit to a regression line for kinetic comparison (Fig. 7 A, inset). It is apparent that the destaining process was dramatically retarded by D3 receptor activation. Fig. 7 B shows cumulative destaining rates obtained from the slope of the regression line, indicating a significant decrease in the presence of 100 nM quinpirole. These results are essentially identical to our previous observations in untransfected AtT-20 cells stably expressing the human D3 receptor (Kuzhikandathil and Oxford, 1999). In contrast, in AtT-20 cells expressing the dominant-negative AAA-GIRK2 mutant, the inhibition of staining and destaining by 100 nM quinpirole is nearly abolished (Fig. 7 C and inset). In fact, the mean intensity decay rate during the destaining process in AAA-GIRK2–transfected cells is statistically indistinguishable in SES or 100 nM quinpirole ($P > 0.5$, Student's t test, Fig. 7 D). These results suggest that inhibition of spontane-

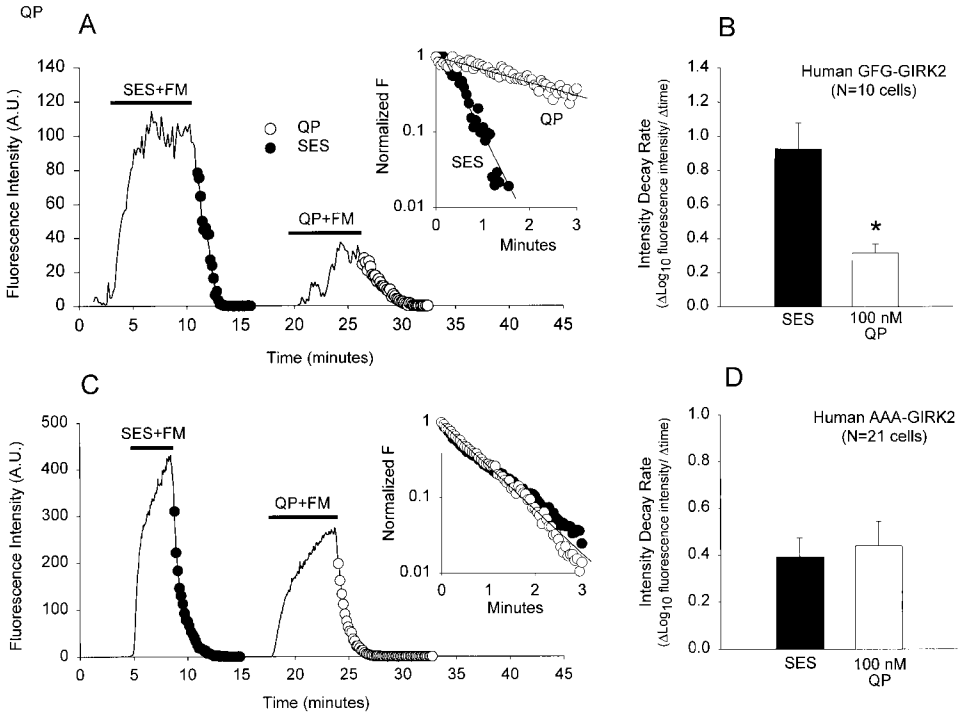

Figure 7. Inhibition of secretory activity by D3 receptors in AtT-20 cells is disrupted by the expression of AAA-GIRK2 mutant. Representative FM1-43 fluorescence traces from AtT-20 cells transfected with GFG (A) or AAA (C) mutant. Cells were stained with FM1-43 in either standard external solution (SES+FM) or in 100 nM quinpirole (QP+FM). The duration of staining in each case is indicated by the bar. After staining, cells were washed in the absence of FM1-43 in either standard external solution (●) or 100 nM quinpirole (○). Secretory activity, measured using FM1-43 fluorescence, is significantly inhibited by 100 nM quinpirole in cells transfected with GFG but not AAA-GIRK2 mutant. The fluorescence values during the washout (● and ○) were normalized and plotted on a semilogarithmic scale and fit to a regression line (A and C, insets). The slope of the regression lines were averaged and compared in SES and 100 nM quinpirole (QP) for cells expressing either GFG (B) or AAA-GIRK2 (D) mutant. Quinpirole significantly inhibited the intensity decay rate (or secretory activity) in GFG-transfected cells ($*P < 0.01$, $n = 10$ cells, Student's t test), but not in AAA-transfected cells ($P > 0.5$, $n = 21$ cells, Student's t test).

ous secretory activity by the D3 receptor in AtT-20 cells is mediated predominantly, if not exclusively, by its coupling to endogenous GIRK channels and that direct inhibition of calcium channels is insufficient in itself to effect regulation of the secretory process.

DISCUSSION

The present study provides novel evidence that GIRK2 channels play an important role in mediating D3 receptor modulation of secretory activity. The D3 dopamine receptor has been suggested to modulate dopamine release at nerve terminals functioning as inhibitory autoreceptors in dopaminergic neurons (Rivet et al., 1994). In MN9D cells, activation of heterologously expressed D3 dopamine receptors can modestly inhibit dopamine synthesis and release (Tang et al., 1994; O'Hara et al., 1996). In addition, D3 receptor activation inhibits secretory activity in AtT-20 cells (Kuzhikandathil and Oxford, 1999). The signaling mechanism underlying the inhibition of secretory activity by D3 receptors have been difficult to determine due to the absence of appropriate pharmacological tools. The robust coupling of D3 receptors to GIRK channels in CHO and AtT-20 cells (Kuzhikandathil, et al., 1998) leads us to explore the possibility that this signal transduction pathway might underlie the inhibition. The present study using the dominant-negative GIRK2 channels strongly suggests that D3 receptors exert their inhibitory effects on secretion by activating GIRK channels. Although the D3 receptor can also directly inhibit voltage-gated P/Q-type calcium channels in AtT-20 cells (Kuzhikandathil and Oxford, 1999), our data suggest that the activation of GIRK channels represents the dominant pathway for the inhibition of spontaneous secretory activity. The activation of GIRK channels by D3 receptors hyperpolarizes the cell (Fig. 5 A), thereby indirectly suppressing the activation of voltage-gated calcium channels. This subsequently decreases intracellular calcium levels (Fig. 6) and inhibits calcium-dependent secretory activity (Fig. 7).

The concept that activation of a potassium channel, rather than direct inhibition of a calcium channel, could be the major mechanism linking inhibitory neurotransmitters to reduction of secretion in neural and endocrine has been proposed previously. In the case of regulation of prolactin secretion by D2 receptor activation in pituitary lactotrophs, where direct calcium channel inhibition does not occur (Rendt and Oxford, 1994), activation of GIRK channels leading to hyperpolarization is consistently observed (Einhorn and Oxford, 1993) and likely underlies the functional inhibition of secretion. In another study, Lakhlani et al. (1996) sought a mechanism to discriminate GIRK- ver-

sus calcium-channel modulation by the α_{2A} adrenergic receptor. They found that a mutation of the α_{2A} receptor (D79N) previously shown to block coupling of the receptor to GIRK channels, but not to calcium channels, could blunt agonist-induced inhibition of ACTH secretion when expressed in AtT20 cells, suggesting a role for GIRK channels in the action of these exogenous receptors. Finally, a link between somatostatin receptors and potassium channels was found to underlie both hyperpolarization and suppression of calcium levels in GH4 cells (Koch et al., 1988).

While this study represents the first use of a dominant-negative approach to study GIRK function, one previous study has reported the over expression of wild-type GIRK channels in cultured hippocampal neurons using an adenovirus gene transfer method (Ehrengruber et al., 1997). In that study, the overexpression of wild-type GIRK1, GIRK2, and GIRK4 hyperpolarized the cell and inhibited action potential firing. These results are in agreement with our data on the functional GFG-GIRK2 mutant (Fig. 5) and further supports the role of GIRK channels in maintaining resting membrane potential and regulating cellular excitability.

Mice exhibiting the *weaver* phenotype have a G156S mutation in the GIRK2 gene (Patil et al., 1995). This pore region mutation increases Na^+ ion permeability, resulting in selective neuronal death and the appearance of the ataxic gait that characterizes the *weaver* phenotype (Slesinger et al., 1996). Interestingly, null mice lacking the GIRK2 gene do not appear to exhibit developmental abnormalities and the *weaver* phenotype (Signorini et al., 1997). However, the GIRK2 null mice are prone to spontaneous seizure activity. The impaired inhibition underlying this seizure phenotype was examined in neurons of hippocampal slices obtained from the GIRK2 null mice (Luscher et al., 1997). The average resting membrane potential of GIRK2$^{(-/-)}$ hippocampal neurons was observed to be depolarized by $\sim$7.9 mV and the currents evoked by agonists for $GABA_B$, 5HT1A, and adenosine A1 receptors were essentially absent. These results are consistent with our observations and confirm the coupling of G-protein–coupled receptors to GIRK channels and their role in maintaining resting membrane potential in vivo. However, the interpretations of the role of GIRK2 channel in modulating neuronal activity from studies of GIRK2 null mice are complicated by changes in expression of other GIRK channel isoforms (Signorini et al., 1997). To overcome such problems associated with developmental alterations in GIRK null mice, dominant-negative GIRK mutants as we have employed could be incorporated into viral vectors and introduced into select brain regions of adult animals. The use of EGFP-tagged GIRK mutants would facilitate the correlation of behavioral phenotype with altered neuronal function in specific brain regions. We are currently initiating such experiments to examine the role of GIRK2 channels in vivo.

The results from this study also raise some interesting questions about the role of certain amino acid residues of the selectivity filter in maintaining the pore architecture of GIRK2 channels. Based on the recent bacterial potassium channel crystal structure, Doyle et al. (1998) suggested that the hydroxyl group on the tyrosine residue in the selectivity filter interacts with tryptophan residues in the adjacent membrane helix to generate a pore architecture that allows K^+ but not Na^+ ion permeation. Our results suggest that, at least in the human GIRK2 channel, the substitution of the tyrosine residue in the selectivity filter by a phenylalanine residue (lacking the hydroxyl group) does not alter function. The GFG-GIRK2 mutant forms functional GIRK channels with no apparent change in permeability to Na^+ ions. The inward currents elicited in SES (with 5 mM K^+ and 145 mM Na^+) are comparable in wild type and GFG-GIRK2, as are the currents generated in 50 K-ES (with 50 mM K^+ and 100 mM Na^+) (Fig. 3). In contrast, the GLG-GIRK2 mutant is nonfunctional (Fig. 3), raising the possibility that the aromatic ring in the tyrosine residue, rather than the OH group, is involved in stabilizing GIRK2 pore structure and conferring selectivity. Interestingly, in the KcsA bacterial potassium channel, the signature tyrosine residue in the selectivity filter interacts via H-bonds with the two tryptophan residues present in the conserved FWW sequence in the pore region. However, in all inward-rectifying potassium channels, including GIRKs, this sequence is replaced by the conserved FLF sequence. The absence of conservation of the hydroxyl group interaction further suggests that the aromatic ring interactions might be more important in maintaining GIRK2 channel pore structure. Interestingly, substitution of the tyrosine residue by phenylalanine in GIRK1 does not affect channel function or selectivity, while an identical mutation in GIRK4 appears to alter selectivity without affecting other channel functions (Silverman et al., 1998). This suggests that the roles of amino acid residues in the selectivity filter are subtly different among the different GIRK isoforms.

In conclusion, this study has identified a role for GIRK channels in mediating the inhibition of secretory activity by the D3 receptor. While the D3 receptor can directly inhibit voltage-gated calcium channels, it appears that the coupling of the D3 receptor to GIRK channels primarily underlies the inhibition of spontaneous secretory activity in AtT-20 cells. The mutational studies presented in this report have also provided some insight into the role of the tyrosine residue in the selectivity filter region of human GIRK2-containing channels. Furthermore, given the lack of selective blockers for GIRK channels, the study shows that the dominant negative approach can be used to selectively

ablate GIRK function in vivo, thereby facilitating the study of their role in neuronal signaling.

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REFERENCES

Adler, M., B.S. Wong, S.L. Sabol, N. Busis, M.B. Jackson, and F.F. Weight. 1983. Action potentials and membrane ion channels in clonal anterior pituitary cells. *Proc. Natl. Acad. Sci. USA.* 80:2086–2090.

Carmeliet, P., and C. Denef. 1989. Synthesis and release of acetylcholine by normal and tumoral pituitary corticotorphs. *Endocrinology.* 124:2218–2227

Corey, S., and D.E. Clapham. 1998. Identification of native atrial G-protein regulated inwardly rectifying K^+ (GIRK4) channel homomultimers. *J. Biol. Chem.* 273:27499–27504.

Doyle, D.A., J.M. Cabral, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science.* 280:69–77.

Ehrengruber, M.U., C.A. Doupnik, Y. Xu, J. Garvey, M.C. Jasek, H.A. Lester, and N. Davidson. 1997. Activation of heteromeric G protein-gated inward rectifier K^+ channels overexpressed by adenovirus gene transfer inhibits the excitability of hippocampal neurons. *Proc. Natl. Acad. Sci. USA.* 94:7070–7075.

Einhorn, L.C., and G.S. Oxford. 1993. Guanine nucleotide binding proteins mediate D_2 receptor activation of a potassium channel in identified rat lactotrophs. *J. Physiol.* 462:563–578.

Gobert, A., F. Lejeune, J.M. Rivet, L. Cistarelli, and M.J. Millan. 1996. Dopamine D3 (auto) receptors inhibit dopamine release in the frontal cortex of freely moving rats in vivo. *J. Neurochem.* 66:2209–2212.

Imanobe, A., Y. Yoshimoto. Y. Horio, K.-I. Morishige, H. Hibino, S. Matsumoto, Y. Tokunaga, T. Maeda, Y. Hata, Y. Takai, and Y. Kurachi. 1999. Characterization of G-protein gated K^+ channels composed of Kir3.2 subunits in dopaminergic neurons of the substantia nigra. *J. Neurosci.* 19:1006–1017.

Koch, B.D., J.B. Blalock, and A. Schonbrunn. 1988. Characterization of the cyclic AMP-independent actions of somatostatin in GH cells: an increase in potassium conductance is responsible for both the hyperpolarization and the decrease in intracellular free calcium produced by somatostatin. *J. Biol. Chem.* 263:216–225.

Kofuji, P., N. Davidson, and H.A. Lester. 1995. Evidence that neuronal G-protein gated inwardly rectifying K^+ channels are activated by $G\beta\gamma$ subunits and functions as heteromultimers. *Proc. Natl. Acad. Sci. USA.* 92:6542–6546.

Kuzhikandathil, E.V., and G.S. Oxford. 1999. Activation of human D3 dopamine receptor inhibits P/Q-type calcium channels and secretory activity in AtT-20 cells. *J. Neurosci.* 19:1698–1707.

Kuzhikandathil, E.V., W. Yu, and G.S. Oxford. 1998. Human D3 and D2L receptors couple to inward rectifier potassium channels in mammalian cell lines. *Mol. Cell. Neurosci.* 12:390–402.

Lakhlani, P.P., D.M. Lovinger, and L.E. Limbird. 1996. Genetic evidence for involvement of multiple effector systems in α_{2A}-adrenergic receptor inhibition of stimulus-secretion coupling. *Mol. Pharmacol.* 50:96–103.

Liao, Y.J., Y.N. Jan, and L.Y. Jan. 1996. Heteromultimerization of G-protein gated inwardly rectifying K^+ channel proteins GIRK1 and GIRK2 and their altered expression in *weaver* brain. *J. Neurosci.* 16:7137–7150.

Luini, A., D. Lewis, S. Guild, G. Schoield, and F. Weight. 1986. Somatostatin, an inhibitor of ACTH secretion, decreases cytosolic free calcium and voltage-dependent calcium current in a pituitary cell line. *J. Neurosci.* 6:3128–3132.

Luscher, C., L.Y. Jan, M. Stoffel, R.C. Malenka, and R.A. Nicoll. 1997. G protein-coupled inwardly rectifying K^+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron.* 19:687–695.

O'Hara, C.M., A. Uhland-Smith, K.L. O'Malley, and R.D. Todd. 1996. Inhibition of dopamine synthesis by dopamine D2 and D3 but not D4 receptors. *J. Pharmacol. Exp. Ther.* 277:186–192.

Patil, N., D.R. Cox, D. Bhat, M. Faham, R.M. Myers, and A.S. Peterson. 1995. A potassium channel mutation in weaver mice implicates membrane excitability in granule cell differentiation. *Nat. Genet.* 11:126–129.

Rendt, J., and G.S. Oxford. 1994. Absence of coupling between D2 dopamine receptors and calcium channels in lactotrophs from cycling female rats. *Endocrinology.* 135:501–508.

Richardson, U.I., and A. Schonbrunn. 1981. Inhibition of adrenocorticotropin secretion by somatostatin in pituitary cells in culture. *Endocrinology.* 108:281–290.

Rivet, J.-M., V. Audinot, A. Gobert, J.-L. Peglion, and M.J. Millan. 1994. Modulation of mesolimbic dopamine release by the selective dopamine D3 receptor antagonist, (+)-S 14297. *Eur. J. Pharmacol.* 265:175–177.

Signorini, S., Y.J. Liao, S.A. Duncan, L.Y. Jan, and M. Stoffel. 1997. Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K^+ channel GIRK2. *Proc. Natl. Acad. Sci. USA.* 94:923–927.

Silverman, S.K., H.A. Lester, and D.A. Dougherty. 1998. Asymmetrical contributions of subunit pore regions to ion selectivity in an inward rectifier K^+ channel. *Biophys. J.* 75:1330–1339.

Slesinger, P.A., N. Patil, Y.J. Liao, Y.N. Jan, L.Y. Jan, and D.R. Cox. 1996. Functional effects of the mouse weaver mutation on G protein-gated inwardly rectifying K^+ channels. *Neuron.* 16:321–331.

Surprenant, A. 1982. Correlation between electrical activity and ACTH/beta-endorphin secretion in mouse pituitary tumor cells. *J. Cell Biol.* 95:559–566.

Tang, L., R.D. Todd, and K.L. O'Malley. 1994. Dopamine D2 and D3 receptors inhibit dopamine release. *J. Pharmacol. Exp. Ther.* 270:475–479.

Tinker, A., Y.N. Jan, and L.Y. Jan. 1996. Regions responsible for the assembly of inwardly rectifying potassium channels. *Cell.* 87:857–868.

Wang, X., and M.A. Greer. 1995. Blocking K^+ channels with TEA induces plasmalemma depolarization, increased $[Ca^{2+}]_i$, and ACTH secretion in AtT-20 cells. *Mol. Cell. Endocrinol.* 109:11–18.