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Receptor influences in GIRK current activation and desensitization

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
EGTA	Ethylene glycol tetraacetic acid
FBS	Fetal bovine serum
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine-5'-triphosphate
GTPγS	Guanosine 5'-O-[gamma-thio]triphosphate
h	Hill coefficient
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
K_d	Dissociation constant
Kir	Inwardly-rectifying potassium channel
mGluR	Metabotropic Glutamate receptor
PBS	Phosphate buffered saline

PIP₂	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase C
PVDF	Polyvinylidene fluoride
RIPA	Radio-Immunoprecipitation Assay
SDS-PAGE	Sodium dodecyl sulfate polycrylamide gel electrophoresis
T-TBS	mixture of Tris-Buffered Saline and Tween 20

ABSTRACT

Receptor influences in GIRK current activation and desensitization

By Gyu Tae Park, BSc.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
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Thesis Director: Diomedes E. Logothetis, Ph.D.

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G protein-coupled receptors (GPCRs) are seven-transmembrane domain receptors that sense extracellular signal and activate intracellular signaling pathways. Metabotropic glutamate receptor 2 (mGluR2) is one of the GPCRs coupled to Gi/o proteins whose G $\beta\gamma$ subunits stimulate G protein-gated inwardly rectifying K⁺ channels (GIRKs). Previous experiments demonstrated that in planar lipid bilayer both active forms of G proteins [G α (GTP γ S-stimulated) and G $\beta\gamma$ subunits] were required to activate GIRK channels in the absence of the receptor, but surprisingly, the G $\beta\gamma$ subunit alone could activate GIRK channel in the presence of GPCR. Currently, it is not clear whether GPCRs play a role beyond catalyzing the dissociation of G α and G $\beta\gamma$ subunits in the presence of extracellular agonist and intracellular GTP. Here we compare the G protein-stimulated GIRK currents in the presence and absence of mGluR2 by performing whole-cell patch clamp recordings on two types of cells: a HEK293 cell line stably expressing GIRK channels (HEK/GIRK) and HEK/GIRK cells with mGluR2 expressed transiently. Our experiments revealed that mGluR2 affects the behavior of G proteins even in the absence of the agonist. We show that intracellular application of GTP activated GIRK currents, and the GTP-induced GIRK currents became greater in the presence mGluR2. We also show that desensitization kinetics of the GTP-stimulated GIRK currents became greater and faster in the presence of mGluR2.

INTRODUCTION

G protein-coupled receptors (GPCRs) are a large family of transmembrane receptors that sense extracellular signals and transmit them to various intracellular downstream effectors. GPCRs are biological molecules that have been vigorously studied due to their involvement in many diseases (Filmore, 2004). In fact, GPCR is the target of approximately 30% of all modern medicinal drugs (Overington et al., 2006). The mechanism by which GPCRs signal to downstream effector state that when an agonist binds to an extracellular receptor binding site specific G protein signaling pathways are activated inside the cell (Oldham and Hamm, 2008). The first event that occurs upon activation of G protein signaling is that the G protein heterotrimer exchanges its bound GDP for a GTP. The heterotrimer can then dissociate into its component $G\alpha$ and $G\beta\gamma$ subunits and activate its downstream effectors. There are various subtypes of G proteins in the cell (e.g. G_i/o , $G_q/11$ and G_s) and each of them are coupled to their their specific subtypes of GPCRs (Wetschureck and Offermann, 2005).

One of the downstream effectors of GPCRs is G protein-gated inwardly rectifying potassium channels (GIRKs). GIRK channels are broadly distributed in the central nervous system and in the heart, controlling heart rate and neuronal excitability (Kobayash, et al., 1995;

Karschin, et al., 1996; Chen, et al., 1997; Krapivinsky, et al., 1995). Four mammalian isoforms comprise the GIRK subfamily: GIRK1-GIRK4 (Kubo et al., 1993; Dascal et al., 1993; Lesage et al., 1994; Kaprivinsky et al., 1995). GIRK1 channels function only as heteromers in association with one of the other subunits. For example, Some GIRK channels in cardiac tissue exist as heterotetramers composed of two GIRK1 and two GIRK4 subunits (Kaprivinsky et al., 1995). The other subunits (GIRK2-GIRK4) can function both as homotetramers and heterotetramers and are found in either form in several tissues. GIRK channels are inwardly-rectifying, which means they pass current (positive current) more easily in the inward direction (into the cell). Upon activation of Gi/o signaling pathway, G $\beta\gamma$ subunit directly interacts with GIRK channels, and this interaction causes the channel to be permeable to potassium ions, resulting in hyperpolarization of the cell (Logothetis et al. 1987).

Besides Gi/o signaling, GIRK channels are also activated via a G-protein-independent mechanism, which requires MgATP modification of channel activity (prolongation of the mean open time) for subsequent gating by intracellular sodium ions (Sui, et al., 1996). Hydrolysis of ATP sensitizes the channel to intracellular sodium ions and G protein gating through formation of Phosphatidylinositol-4,5-bisphosphate (PIP₂) from Phosphatidylinositol (PI), which controls both G $\beta\gamma$ and Na⁺ gating (Huang, et al., 1998; Sui, et al., 1998). The mechanism of Na⁺-induced GIRK activation is operative during intracellular Na⁺ accumulation, as with block of the Na⁺/K⁺ pump, and is likely to be involved in the electrophysiologic effects of cardiac glycosides, drugs widely used in heart failure or for improvement of the inotropic state of heart (Sui, et al., 1996).

Gi-mediated GIRK activation is regulated by intracellular PIP₂ level and GTPase activity (Kobrinisky, et al., 2000; Breitwieser and Szabo, 1998). Gq signaling activates phospholipase C, a class of enzymes that cleaves PIP₂ into diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). Previous studies demonstrated that PLC-mediated PIP₂ depletion in COS-1 cells results in rapidly desensitizing GIRK currents (Kobrinisky, et al., 2000). Rapid GTPase activity of G proteins also causes the stimulated GIRK currents to rapidly desensitize (Breitwieser and Szabo, 1998). Once G α -bound GTP is hydrolyzed into GDP, the G α subunit re-associates with the G $\beta\gamma$ subunit, inhibiting the interaction of G $\beta\gamma$ with GIRK channel. Earlier studies revealed that coexpression of certain regulators of G protein signaling (RGS) proteins that accelerate G

protein's GTPase activity, fully reconstitutes the GIRK activation and deactivation kinetics observed in native cells (Doupnik et al., 1997; Saitoh et al., 1997).

The metabotropic glutamate receptors (mGluRs) are members of the class 3 G protein-coupled receptor family, which includes the calcium sensing receptor and the GABA_B receptor, among others (Conn and Pin, 1997). There are eight known mammalian mGluR genes (mGluR1-8), which play diverse roles in the nervous system, including the modulating of synaptic transmission from both pre- and postsynaptic locations and regulation of synaptic plasticity. In addition, mGluRs also play a role in mediating sensory transduction (Bortolotto et al., 1994; Conn and Pin 1997; Wilsch et al., 1998). mGluRs have been divided into three groups based on sequence homology, sensitivity to pharmacological agents, and G protein –coupling specificity (De Blasi et al., 2001). Metabotropic Glutamate receptor 2 (mGluR2), the receptor employed in our study, is a member of group II that is known to couple exclusively to the Gi/o signaling pathway (Tanabe et al., 1992). It is considered a new target for potential anti-psychotic drugs and recently received attention in preclinical and clinical studies (Krystal et al., 2003). In our study, since the activity of GIRK is stimulated in response to the activation of the Gi/o signaling pathway, we measured GIRK current via whole-cell patch clamp recording to monitor the degree of mGluR2-mediated stimulation of Gi/o signaling.

Although G protein signaling, starting from the binding of receptor's ligands and ending with the induction of ion current, has long been studied by many researchers, we still lack structures of complexes revealing detailed interactions among GPCRs, G proteins and ion channels. Thus, the interactions among these components leading to modulation of channel function are not well understood. In 1987 Logothetis et. al. first demonstrated that the G $\beta\gamma$ subunits directly interact and activate GIRK channels (Logothetis et. Al., 1987). Increases in GIRK activity were observed when purified G $\beta\gamma$ subunits were applied to the intracellular surface of inside-out patches of chick embryo atrial cells. Excised membrane patches contained GPCRs and G proteins as well as GIRK channels, but it was not investigated whether GPCRs played a role beyond catalyzing the dissociation of G α and G $\beta\gamma$ subunits in the presence of extracellular agonist and intracellular GTP. A recent study from our laboratory published by Leal-pinto et al. demonstrated that in planar lipid bilayer experiments both active forms of G proteins [G α (GTP γ S-stimulated) and G $\beta\gamma$ subunits] were required to activate GIRK channels in

the absence of the receptor (Leal-pinto et al., 2010). Moreover, unpublished data (Leal-Pinto and colleagues) collected by our group indicates that the G $\beta\gamma$ subunit alone can only activate GIRK channel only in the presence of GPCR. Overall, these observations suggest a new role for GPCRs in the G $\beta\gamma$ -mediated GIRK activation.

In this study we tested whether the presence of GPCRs affects GIRK activation in a mammalian system. We demonstrated that the magnitude of GIRK stimulated currents and desensitization kinetics change due to the presence of mGluR2. Data were collected mainly by performing whole-cell patch clamp recordings. To differentiate between responses in the presence of mGluR2 from those in the absence of mGluR2, we employed two types of cells, a HEK/GIRK stable cell line and a HEK/GIRK with mGluR2 expressed transiently. In addition, we used different stimulators, e.g. GTP γ S, GTP and Glutamate, to distinguish between direct G protein stimulation from GPCR-influenced G protein activation.

MATERIALS AND METHODS

A) HEK-293 cells stably expressing GIRK1+GIRK4.

I assisted Dr. Lia Baki in stably expressing Kir3.1 (GIRK1) and Kir3.4 (GIRK4) in HEK-293 cells. To express the Kir3.1 and Kir3.4 subunits we used the mammalian bidirectional expression vector pBI-CMV1 (Clontech Laboratories, Madison, WI) which allows the simultaneous constitutive expression of two proteins of interest, driven by constitutively active, minimal human cytomegalovirus promoters, *PminCMV1* and *PminCMV2*, respectively. The Kir3.1 subunit was subcloned between BamH1-Not1 of the multiple cloning site MCS 1 (immediately downstream of *PminCMV1*) and the Kir3.4 subunit was subcloned between EcoRI-BglII of the MCS 2 (immediately downstream of *PminCMV2*). Subcloning was screened by restriction analysis and confirmed by sequencing before transfection into the mammalian cells. To allow for selection of stable clones we used the pPUR selection vector (Clontech Laboratories, Madison, WI) that confers puromycin resistance to eukaryotic cells.

DNA from the vectors (pPUR and pBI-CMV1 carrying Kir3.1 and Kir3.4) was prepared using the EndoFree plasmid Purification kit (QIAGEN, Hilden, Germany) and a mixture of both DNAs at 1:20 :ratio was transfected into HEK-293 cells using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

Screening of the clones was a two-step procedure. At first, all surviving clones were screened by Western Immunoblotting as described in C and the GIRK-positive clones were subjected to electrophysiological recordings (See D).

B) Cell Culture and Transient Transfection

The HEK293 cell line stably expressing GIRK1/4 (HEK/GIRK cells) was grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Inc., Lawrenceville, GA). The HEK/GIRK cells were transiently transfected with mGluR2. The HEK/GIRK cells were trypsinized and suspended in the Opti-MEM (Invitrogen, Carlsbad, CA) + 5% FBS and then plated on a 6-well plate one day prior to the transfection. Each wells on the plate contained different concentration of cells. On the next day, wells that show 90 to 95% confluency of cells were chosen to be transfected. 6 μ g of mGluR2 DNA and 250 μ L of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) were mixed with 250 μ L of serum-free Opti-MEM separately in different tubes and were left in room temperature for 30 minutes. In the case where two or more wells were to be transfected, the amount of DNA, Lipofectamine and serum-free Opti-MEM were multiplied by the number of wells. After 30 minutes, the DNA + Opti-MEM were transferred into the tube containing the mixture of Lipofectamine and Opti-MEM and gently mixed. The mixture was again left in RT for 30 minutes. After 30 minutes, 500 μ L of the mixture was directly pipetted into each wells and placed in the incubator. After 24 hours, the medium was replaced with fresh Opti-MEM containing 5% FBS.

C) Western Immunoblotting

I assisted Dr. Lia Baki in performing western blot to confirm the expression of GIRK and mGluR2 after transfections. Confluent cells, cultured in 60mm dishes, were washed once with PBS, and directly lysed on the culture dish by the addition of 300 ml RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA), containing Protease inhibitors (Roche Diagnostics, Basel, Switzerland). Lysed cells were scraped from the dish, transferred to microfuge tubes and sonicated at 4°C. Lysates were, mixed with 1/3 volume of 4X SDS-PAGE loading buffer and boiled for 10min before loading in 4-15% gradient polyacrylamide gels (Biorad, Hercules, CA).

Proteins were separated according to their electrophoretic mobility by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) and then transferred to PVDF membranes at 100V for one hour. The membranes were washed with T-TBS, blocked for two hours in NCS blocking solution and probed overnight at 4°C with the appropriate antibodies. For GIRK 1 detection we used the anti Kir3.1 polyclonal antibody H-145 (Santa Cruz Biotechnology, Santa Cruz, CA), for N-cadherin detection we used the BD Transduction Laboratories anti N-cadherin mouse monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ) and for detection of the HA-tagged mGluR2 we used 0.5 µg/ml of rat anti-HA antibody 3F10 (Boehringer Mannheim, Mannheim, Germany).

Following extensive washing with T-TBS (more than 2 hours at room temperature with frequent changes) the membranes were probed with the appropriate secondary antibodies (anti-rabbit for GIRK, anti-rat for mGluR2 and anti-mouse for N-cadherin) for 90 min at room temperature, washed in T-TBS (90 min at room temperature with frequent changes) and incubated for 5 min in Amersham ECL Plus Chemiluminescence detection reagent (GE Healthcare, Waukesha, WI) before being exposed to Autoradiography films.

D) Whole-cell patch clamp recordings and measurements

Whole-cell patch clamp recordings were obtained from HEK293 cells that overexpressed GIRK1/4 alone or overexpressed GIRK1/4 and mGluR2. Cells that had been plated on 100 mm dishes were trypsinized to resuspend them in medium containing DMEM (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA). The cells were aliquoted in 1mL tubes with tops open and were placed in the incubator for 3 hours for recovery. After 3 hours, cells were placed on poly-lysined coverslips that fit in the chamber of the patch-clamp setup. It usually took ~15 minutes for the cells to attach to the coverslips. When cells firmly anchored to the coverslip, the medium was replaced by perfusing an “External solution” that contained in mM: 140 Potassium gluconate, 2 CaCl₂, 5 EGTA/K, 10 Glucose, 10 HEPES-K and 1 MgCl₂. Electrodes were pulled to a resistance around 1.5~2.5MΩ. The electrodes were filled with “Internal solution” that contained in mM: 140 Potassium gluconate, 2 CaCl₂, 5

EGTA/K, 10 Glucose, 10 HEPES-K, 0.3 MgCl₂, 2 NaATP. The internal solution also contained different concentrations of GTPγS or GTP as indicated in different recordings. The electrode was placed so that it gently touched the exterior of the target cell and negative pressure was applied until the electrode sealed on the cell membrane achieving a gigaohm resistance seal. In order to establish the whole-cell mode, the membrane surrounded by the tip of electrode was disrupted by applying a gentle suction. Only the cells that retained the gigaohm resistance were subjected to the whole-cell recordings. A voltage ramp from -100mV to +100mV was applied once every 3 seconds. Currents corresponding to -95mV were plotted as a function of time to produce time course relationships. The inward potassium currents were blocked by placing 3mM BaCl₂ in the External solution to distinguish from contaminating leak currents. The recordings were obtained using an Axopatch amplifier and Axon 8.1 software (Axon Instruments, Union City, CA). Data were analyzed and graphed using Clampfit 9.2 (Molecular Devices, Sunnyvale, CA) and Excel software (Microsoft, Albuquerque, NM).

E) Statistical Analysis

All current values corresponding to -95mV in each ramps were obtained using Clampfit 9.2 (Molecular Devices, Sunnyvale, CA) and transferred to Excel software (Microsoft, Albuquerque, NM). The first few data points of the current versus time plot were averaged to obtain the Basal current. The agonist-induced currents were obtained by averaging several current values that appear immediately after the slope of the ramp current started to change. Similarly, the data points after Barium block were averaged. Barium-sensitive basal and agonist-induced currents were calculated by subtracting the current remaining after Barium block from basal and agonist-induced currents, determined as described above. These values were normalized to the membrane capacitance (pF) which correlates with the size of the cells. In the statistic analysis, basal and agonist-induced currents from each recording were again averaged. Error bars in the figures represent standard error. The standard deviations for each data set were divided by the square root of the number of recordings to get the standard errors. The *t* test was used to assess statistical significance (* indicates $p \leq 0.01$, ** indicate $p \leq 0.005$, *** indicate $p \leq 0.001$).

RESULTS

Application of GTP induces High GIRK activities in the presence of mGluR2.

As described in the Materials and Methods, a stable HEK cell line that expresses GIRK 1 and 4 was transiently transfected with mGluR2. Figure 1A shows a Western blot analysis GIRK1 levels in various clones of HEK/GIRK cells, whereas Figure 1B shows a Western blot analysis of mGluR2 levels in HEK/GIRK/mGluR2 cells, under different conditions of transient transfection. Both types of cells, HEK/GIRK and HEK/GIRK/mGluR2, were subjected to whole-cell patch clamp recordings. Different concentrations of GTP were added to the internal solution to stimulate GIRK activity. A 500 milisecond-long voltage ramp from -100mV to 100mV was applied to the cell at 3 second intervals, and the magnitude of the hyperpolarization-induced currents (at -95 mV) was plotted as a function of time (Figure 2A). The GIRK current increased as GTP entered the cytoplasm but it also desensitized. At the end of all recordings, 3 mM Barium Chloride was applied to make sure that the data collected came from the Barium-sensitive GIRK channel. Figure 2B shows a summary of GTP-induced GIRK currents in HEK/GIRK cells and HEK/GIRK/mGluR2 cells. In the cells that express mGluR2, higher concentrations of GTP induced greater GIRK activity. Notably, responses to 100 μ M GTP in HEK/GIRK/mGluR2 cells were significantly greater than ones in the HEK/GIRK cells. This observation suggested that the presence of receptors causes changes in the behavior of G proteins that, in turn, influence the activity of GIRK.

GTP γ S-induced GIRK current is greater than Glutamate-induced current.

The difference between GTP γ S-induced and Glutamate-induced GIRK activities was examined. Both cell types, HEK/GIRK and HEK/GIRK/mGluR2 were subjected to whole-cell patch clamp recordings. In order to make sure GIRK currents were maximally stimulated in

every recordings, we measured the activities of GIRK upon exposure to different concentrations of Glutamate (Figure 3A). The recordings displayed higher peak currents as we applied higher concentrations of Glutamate, and there was no further increase when the concentration exceeded 100 μ M. Figure 3B shows the hill plot that fits the data shown in panel A.

The GIRK currents responded to both GTP γ S and Glutamate as expected. The time-course traces are shown in Figure 3C. Upon exposure to 100 μ M GTP γ S, the GIRK activities in HEK/GIRK and HEK/GIRK/mGluR2 cells increased to a similar extent. The current increased to 36-42 pA/pF in HEK/GIRK cells and to 30-38 pA/pF in HEK/GIRK/mGluR2 cells (Figure 3D). In HEK/GIRK cells 100 μ M Glutamate failed to induce higher GIRK activity than basal level due to the absence of mGluR2. In the presence of mGluR2 GIRK currents clearly responded to 100 μ M Glutamate. The currents increased from 8-10 pA/pF to 18-23 pA/pF.

The GIRK response to Glutamate was significantly lower than the ones induced by GTP γ S in HEK/GIRK/mGluR2 cells. This observation is consistent with previous studies which have suggested that GPCRs, G proteins and ion channels exist as dynamically assembled macromolecular complexes that localize into discrete membrane compartments, rather than existing as individual proteins freely floating in a homogeneous sea of membrane phospholipids (Dounnik, 2008) (See Discussion).

In the presence of mGluR2, GTP-induced GIRK current displays greater and faster desensitization.

To further characterize mGluR2 influences on GIRK currents, both cell types (HEK/GIRK and HEK/GIRK/mGluR2) were again characterized using the whole-cell patch clamp technique) Desensitization kinetics were characterized following application of 100 μ M GTP. The time course was plotted for each recordings using the same method described above. The current value at steady state (point B in Figure 4A) was divided by the current value at the peak (point A in Figure 4A) and the calculated value was subtracted from 1 and multiplied by 100 to get the %Inhibition plotted in the ordinate. %Inhibition indicates the percentage of the peak current that was inhibited during desensitization.

The GTP-induced GIRK displayed greater desensitization in the presence of the receptor. The current desensitized 35-55 % of its peak current in HEK/GIRK cells but, in HEK/GIRK/mGluR2 cells, the degree of desensitization was 64-66 % (Figure 4B).

In addition to %Inhibition, the time it took to reach steady state was also measured to characterize desensitization. The values were obtained by subtracting the time value corresponding to the peak current (point A) from the time value corresponding to the beginning of the steady-state current, following desensitization (point B). The GTP-induced GIRK current took 130-150 seconds to completely desensitize in the absence of mGluR2 (Figure 4C). However, the presence of receptor in the system significantly reduced this time to 47-60 seconds.

Desensitization kinetics become greater and faster when the signaling involves greater stimulation of mGluR2.

Figure 4 showed that the presence of mGluR2 in the system allows for desensitization to be greater and faster. This observation motivated us to see whether activating less receptors would induce less and slower desensitization. If the amount of receptors being activated was the key to the changes in kinetics, activating less receptor should give less inhibition over a longer time period. HEK/GIRK/mGluR2 cells were subjected to whole-cell patch clamp recordings. Distinct mGluR2 levels could be stimulated by using different concentrations of Glutamate. Since the EC₅₀ for Glutamate was 43.6 μ M (Figure 3B), 25 μ M Glutamate was used to activate less amount of the receptor.

The %inhibition increased with a higher concentration of Glutamate. When the currents were stimulated with 25 μ M Glutamate, the degree of inhibition was 31-35% and it increased to 47-56% when 100 μ M Glutamate was used (Figure 5B). Also, changing the concentration from 25 μ M to 100 μ M shortened the amount of time it took for GIRK currents to desensitize from 95-100 to 77-97 seconds (Figure 5C), even though these two data sets are not significantly different according to the result from t-test. These observations further strengthened the idea that the presence of mGluR2 affects desensitization kinetics of GIRK currents and showed that the effect becomes more potent when the signaling involves greater receptor levels.

Characterization of recovery from desensitization

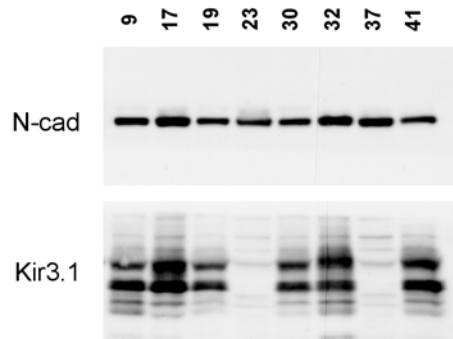
Previous recordings showed that the presence of mGluR2 makes desensitization kinetics of GTP γ S-induced and GTP-induced GIRK currents resemble the currents induced by Glutamate. These observations motivated us to monitor recovery kinetics of Glutamate-induced GIRK currents which can later be used for comparison with the recovery kinetics for GTP γ S-induced and GTP-induced GIRK currents in the HEK/GIRK/mGluR2 cells. Recordings were again performed using whole-cell patch clamp with the same methods and protocol.

As shown in Figure 6A, HEK/GIRK/mGluR2 cells displayed Glutamate-induced GIRK currents upon the first exposure to 100 μ M Glutamate. After the currents desensitized completely, Glutamate was washed away and the cells stayed in the external solution for various lengths of time. After a certain period of time, 100 μ M Glutamate was again applied to stimulate the second Glutamate-induced GIRK currents. Increasing time intervals for the second application following the first exposure to Glutamate gave increasingly greater currents (Figure 6B). The time-dependent recovery of Glutamate-induced GIRK currents shows it takes about 3 minutes to recover 53% and 4 minutes to recover fully from the previous exposure to Glutamate.

In the presence of mGluR2, GTP γ S-induced GIRK current displays faster but less pronounced desensitization.

We tested whether the presence of mGluR2 also changes desensitization kinetics of the GTP γ S-induced currents. Both cell types were again subjected to the whole-cell recording, and %inhibition and time values were obtained by the same method described above. In GTP-induced currents desensitization became greater and faster in the cells expressing mGluR2. However, the GTP γ S-induced currents showed a different trend. %Inhibition was decreased from 65-70 (in the absence of receptor) to 45-50 (in the presence of the receptor) (Figure 7B), while the time to reach steady-state was dramatically shortened. GTP γ S-induced GIRK current took 285-305 seconds to completely desensitize in the absence of mGluR2, and the mGluR2 in the system significantly reduced the time to 95-135 seconds (Figure 7C).

(A)



(B)

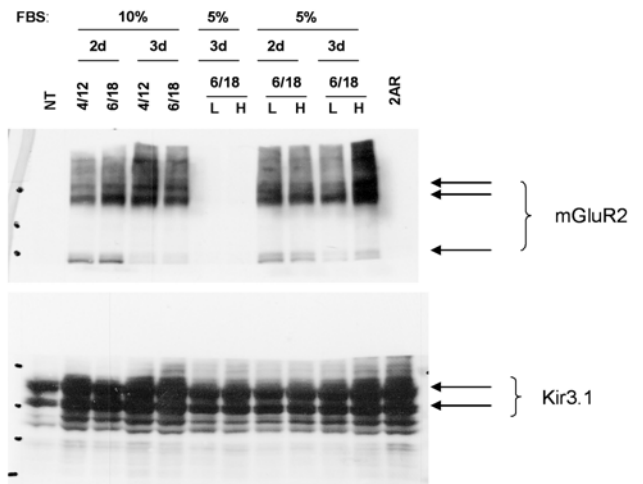
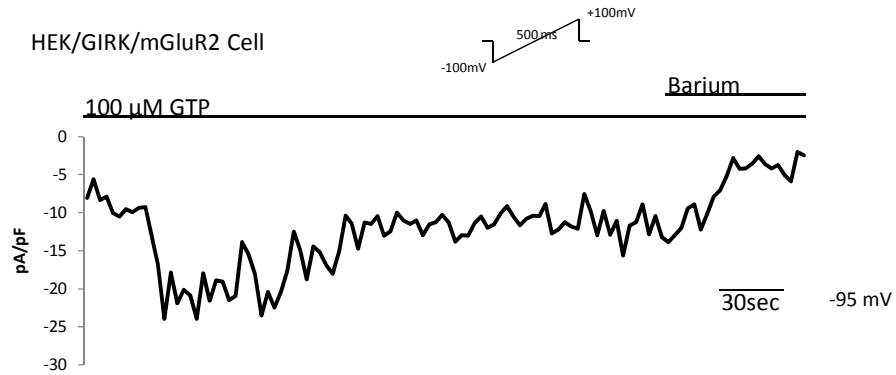


Figure 1. Western blot analysis of GIRK1 and mGluR2 levels in HEK/GIRK and HEK/GIRK/mGluR2 cells, respectively.

A. Western blot analysis GIRK1 levels in various antibiotic-resistant clones of HEK-293 cells, stably transfected with GIRK1+GIRK4. Probing of the upper part of the same membrane for N-cadherin served as an internal loading control. Numbers on the top indicate clone IDs. **B.** Western blot analysis of mGluR2 levels under different conditions of transient transfection of mGluR2 in HEK/GIRK cells. The lower part of the same membrane was probed for GIRK1.

(A) HEK/GIRK/mGluR2 Cell



(B)

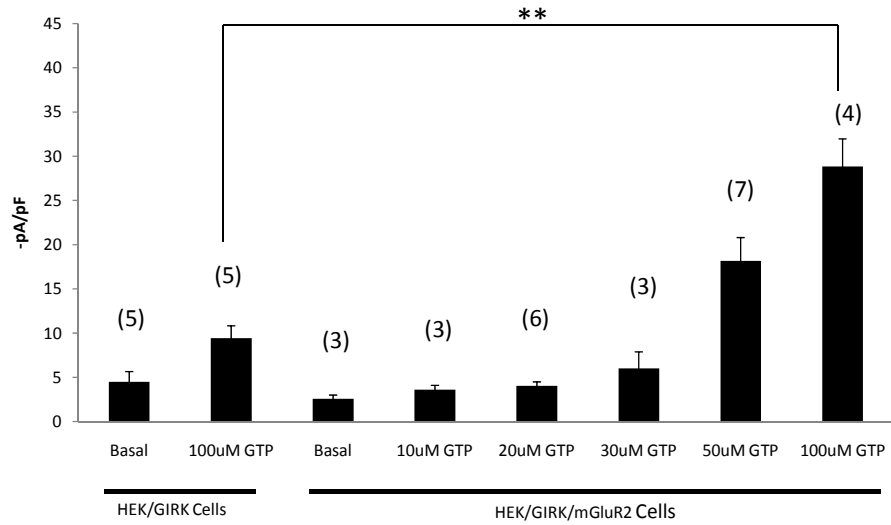


Figure 2. Effect of GTP in GIRK currents in HEK/GIRK cells and HEK/GIRK/mGluR2 cells

A. Representative time course of GIRK activity caused by 100 μ M GTP in a HEK/GIRK/mGluR2 cell obtained by whole-cell patch clamp. A 500 millisecond-long voltage ramp from -100mV to 100mV was applied to the cell at 3 second intervals, and the magnitudes of the hyperpolarization-induced currents (at -95 mV) divided by membrane capacitances (pF) were plotted as a function of time. At the end of each recording, the GIRK currents were completely abolished by 3 mM Barium Chloride. **B.** Summary of Barium-sensitive, basal and GTP-induced currents in HEK/GIRK and HEK/GIRK/mGluR2 cells, calculated as described under MATERIALS AND METHODS.

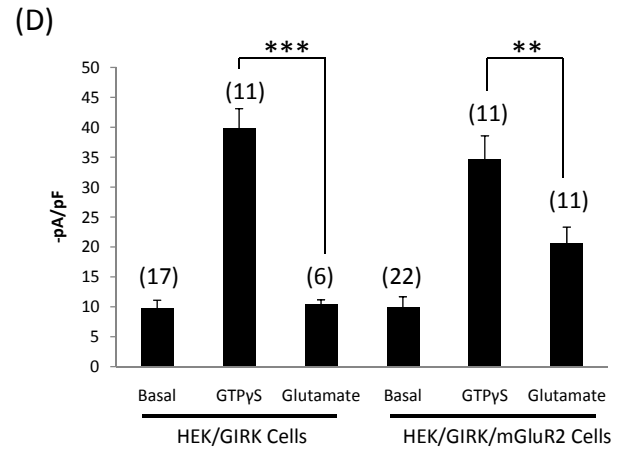
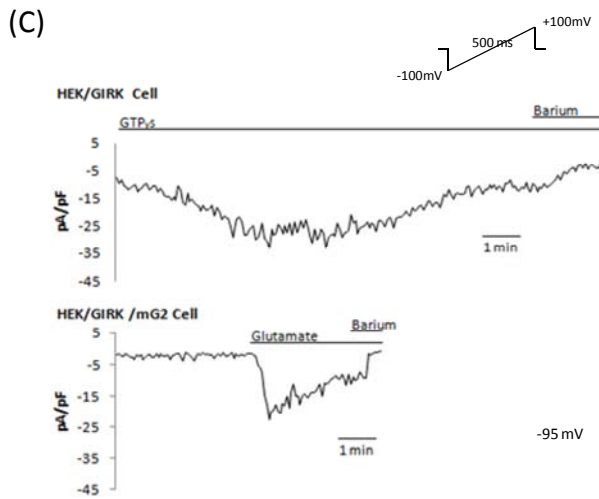
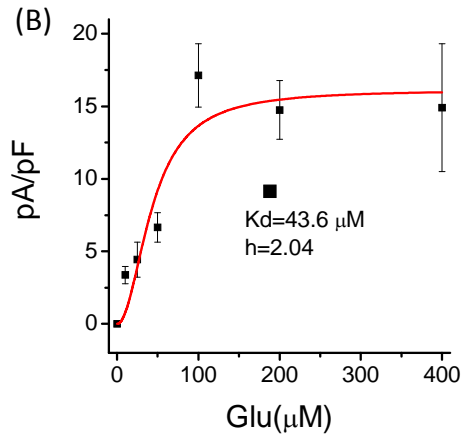
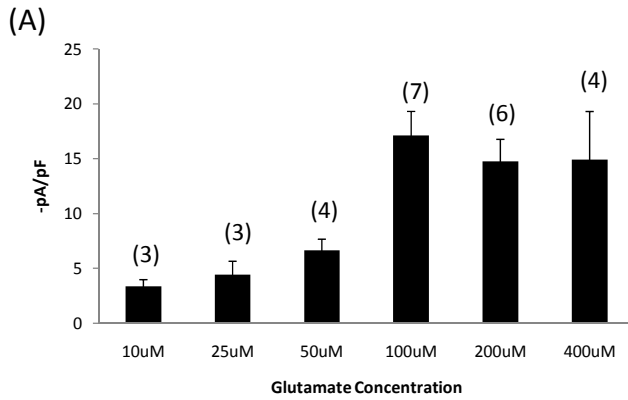


Figure 3. GTP γ S- and Glutamate-induced GIRK currents in HEK/GIRK cells and HEK/GIRK/mGluR2 cells

A. Summary of Glutamate titration data, collected by performing whole-cell patch clamp on HEK/GIRK/mGluR2 cells and taking values of the peak currents induced by Glutamate. The values collected were divided by membrane capacitances to normalize to the size of each cell. 10 μ M GTP was added to pipette solution to compensate for dilution of the intracellular GTP. Higher concentrations of Glutamate induced greater GIRK responses. **B.** Hill plot that fits the data shown in panel A. Dissociation constant for Glutamate is 43.6 μ M, and Hill coefficient is 2. **C.** Voltage ramp protocol and representative time-course traces of GIRK currents. Magnitudes of the hyperpolarization-induced currents (at -95 mV) divided by membrane capacitances (pF) were plotted as a function of time. **D.** Summary of basal currents and peak currents induced by 100 μ M GTP γ S and 100 μ M Glutamate in HEK/GIRK cells and HEK/GIRK/mGluR2 cells. Degrees of significance are indicated by the number of * labels (see Statistical Analysis under MATERIALS AND METHODS).

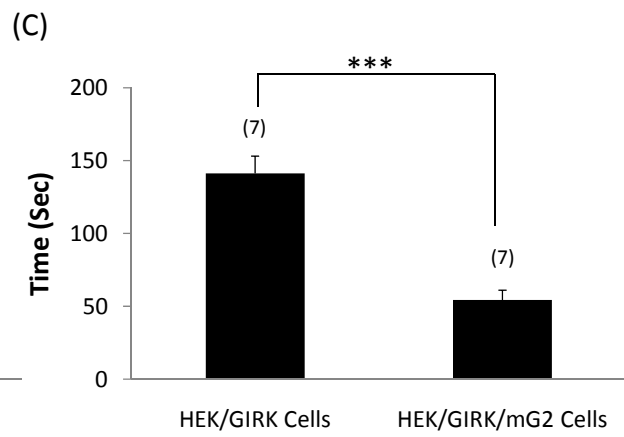
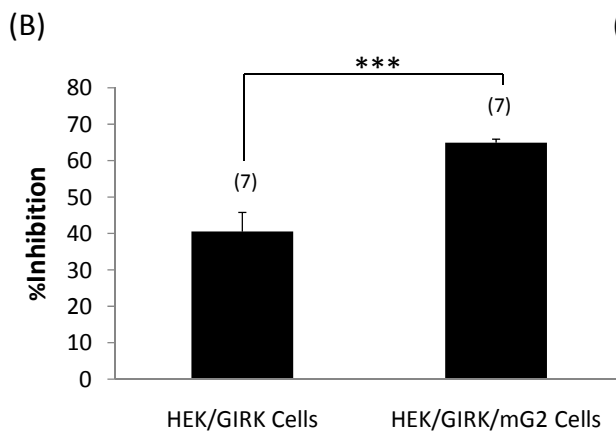
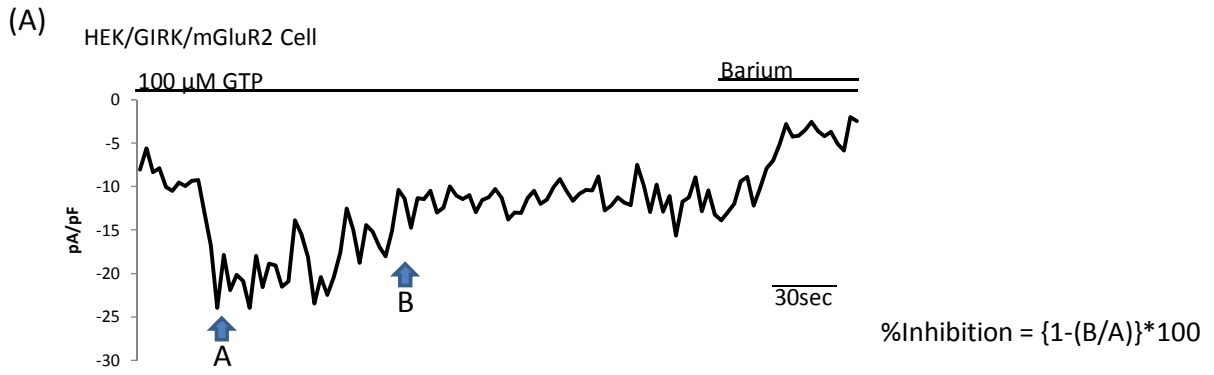


Figure 4. Difference in desensitization kinetics of GTP-induced GIRK current caused by the presence of mGluR2

A. Formula for calculating %Inhibition and the time-course trace. **B.** Summary of %Inhibition of GTP-induced GIRK currents in HEK/GIRK and HEK/GIRK/mGluR2 cells. GTP-induced currents displayed greater desensitization in the presence of mGluR2. **C.** Summary of observed lengths of time required for desensitization of GTP-induced GIRK currents in HEK/GIRK and HEK/GIRK/mGluR2 cells. The values were obtained by subtracting the time value corresponding to the beginning of steady current after desensitization (point B) from the time value corresponding to the peak current (point A). The currents desensitized faster in the presence of mGluR2.

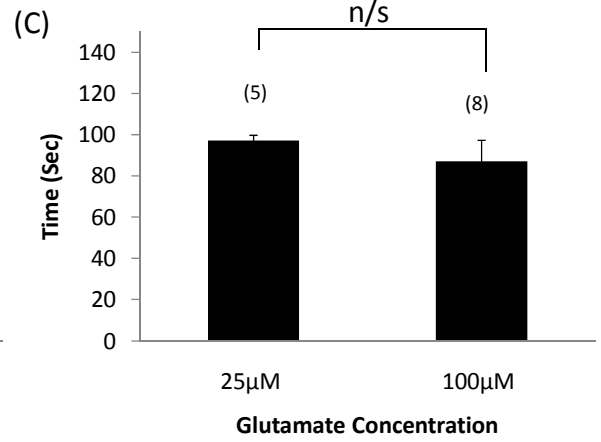
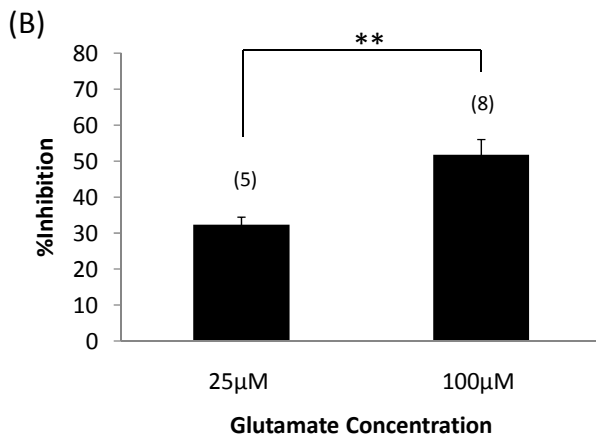
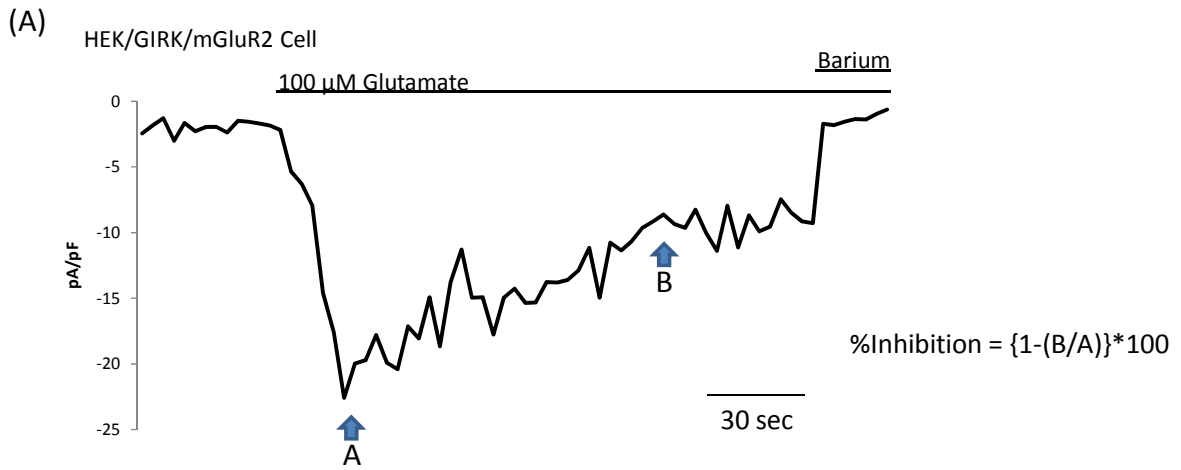
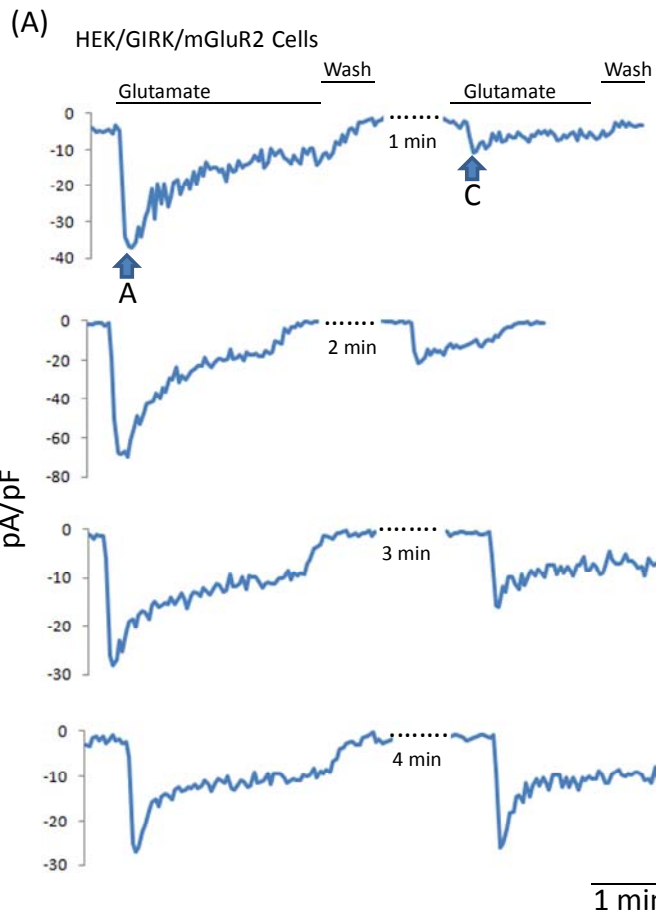


Figure 5. Differences in desensitization kinetics demonstrated by using different concentration of Glutamate

A. Formula for calculating %Inhibition and representative time-course trace of GIRK current induced by 100 μ M Glutamate. The recording was obtained by performing whole cell patch clamp. The method and protocol is same as described in Figure 2A. **B.** Summary of %Inhibition of GIRK currents induced by 25 μ M and 100 μ M Glutamate. 100 μ M Glutamate induced the current with greater desensitization. **C.** Summary of observed lengths of time required for desensitization of GIRK currents after activation induced by 25 μ M and 100 μ M Glutamate, calculated as described in Figure 4.



$$\% \text{Recovery} = (C/A) * 100$$

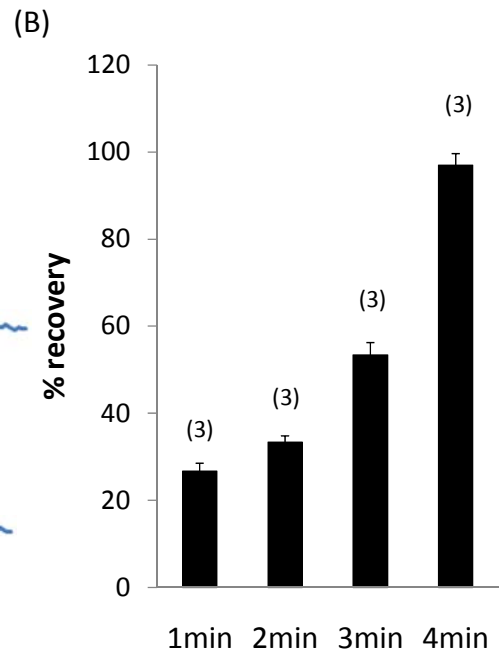


Figure 6. Time-dependent recovery of Glutamate-induced GIRK current

A. Representative time course traces of glutamate-induced GIRK currents in HEK/GIRK/mGluR2 cells. Traces were obtained by whole-cell patch clamp. Cells were perfused with External solution containing 100 μ M Glutamate a few seconds after recordings were initiated. As soon as Glutamate responses completely desensitized, cells were washed with normal External solution. When the currents returned to basal level, perfusion was stopped for certain period of time, and subsequently, cells were again exposed to 100 μ M Glutamate. Depending on the length of time between first and second Glutamate perfusions, magnitudes of second peak currents varied. The recoveries of currents were quantified by calculating for %Recovery as shown above. %Recovery indicates what percentage of first Glutamate response is present in the second response. **B.** Bar graph illustrating time-dependent recovery of GIRK currents. Longer waiting period showed greater currents upon second exposure to 100 μ M Glutamate.

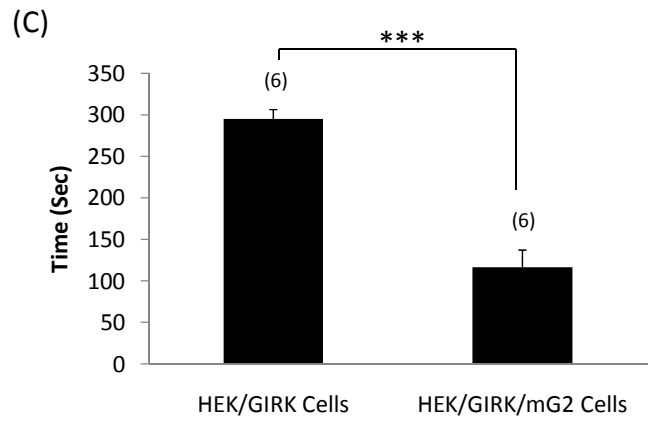
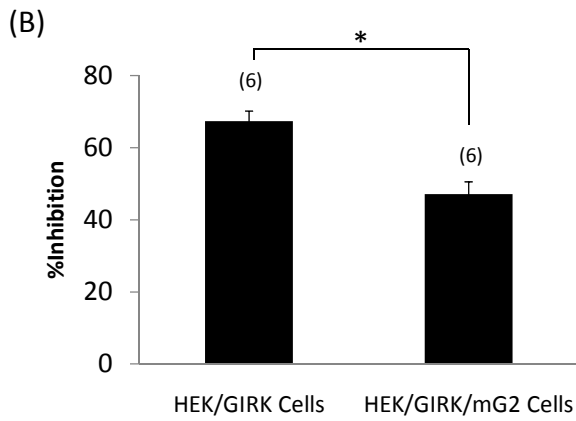
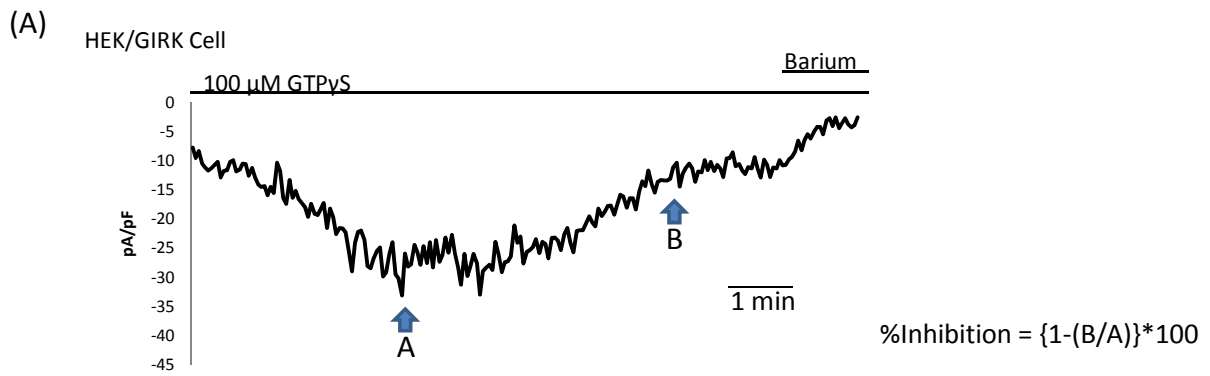


Figure 7. Difference in desensitization kinetics of GTP γ S-induced GIRK current caused by the presence of mGluR2

A. Formula for calculating %Inhibition and representative time-course trace of GIRK current induced by 100 μ M GTP γ S. The recording was obtained by performing whole cell patch clamp. The method and protocol is same as described in Figure 2A. **B.** Summary of %Inhibition of GTP γ S-induced GIRK currents in HEK/GIRK and HEK/GIRK/mGluR2 cells. GTP γ S-induced currents displayed greater desensitization in the absence of mGluR2. **C.** Summary of observed lengths of time GTP γ S-induced GIRK currents takes to desensitize in HEK/GIRK and HEK/GIRK/mGluR2 cells. The values were obtained by the same method described in Figure 4. The currents desensitized faster in cells expressing mGluR2.

DISCUSSION AND FUTURE DIRECTIONS

The aim of this study was to characterize the effect of mGluR2 in GIRK activity. This was achieved by comparing stimulated GIRK activities in the presence of mGluR2 with the activities in the absence of the receptor. The experimental system without mGluR2 was provided by the HEK293 cell line (HEK/GIRK cells) that stably expresses GIRK1 and GIRK4, and this cell line was then transiently transfected to include mGluR2 into this signaling system (HEK/GIRK/mGluR2 cells). Although it is well known that the amount of the protein expression is not consistent with each transient transfection, our data obtained from HEK/GIRK/mGluR2 transient transfections were quite stable with relatively tight error bars.

Our study shows that intracellular application of GTP (in the absence of extracellular agonist) stimulates GIRK currents. Initially, the preliminary result was obtained unintentionally during the process of creating HEK/GIRK/mGluR2 stable cell line. After making the HEK/GIRK stable cell line, we transiently transfected it with the mGluR2 and tested whether the GIRK channel responded to Glutamate using whole-cell patch clamp recordings. Although western blots confirmed the presence of mGluR2 in the cells (see Figure 1B), results from patch-clamp experiments were negative in yielding Glutamate-induced currents. In fact, the pipette solution did not contain GTP and, as it entered the cytoplasm, it was diluting the endogenous GTP concentration which needs to be kept high enough for G protein activation to open the GIRK channel. We next added 100 μ M GTP into the internal solution to support G protein activation. The high GTP concentration in the pipette induced a large Barium-sensitive GIRK current even before the cells were perfused with Glutamate (Figure 2A). Interestingly, the GTP effect was significantly smaller in HEK/GIRK than in HEK/GIRK/mGluR2 cells (Figure 2B). To

our knowledge, GTP acts on the G proteins which upon activation open GIRK channel. Yet, the exact mechanism by which GTP activates G proteins in the absence of mGluR2 agonist and the reason why it exerts a greater effect in the presence of mGluR2 are not clear. One possibility is that the molecular interactions between the basally active mGluR2 and G proteins are somehow causing structural changes of the G proteins which, in turn, increase their affinity to GTP. One experiment that will further test this idea will be to try an inverse-agonist of mGluR2, such as LY34, aiming to negate the effect of mGluR2 in the GTP-induced GIRK activation in HEK/GIRK/mGluR2 cells. If higher GTP-induced current is in fact mediated via a higher mGluR2 activity, simultaneous application of the extracellular LY34 with intracellular GTP should attenuate the mGluR2 effect yielding a lower current density, as if the experiments were performed in the absence of the receptor.

The extracellular glutamate can only activate Gi/o proteins that are coupled to the mGluR2, unlike GTP γ S that can non-specifically activate all G proteins in the cell. In the HEK/GIRK/mGluR2 cells the GIRK response to glutamate was significantly lower than the response to GTP γ S (Figure 3D). This result suggests that pool of G proteins able to activate the GIRK current (Gi/o) extends beyond the pool of G proteins associated with the mGluR2. This result was somewhat surprising because the transient transfection caused over-expression of mGluR2, but there were still excess amount of Gi/o proteins that were not associated with the receptor. The result shown in Figure 2 also suggested that the interaction between mGluR2 and the Gi/o protein exists even in the absence of the mGluR2 agonist. The studies done by Mirshahi et al. in 2002 showed that the basal GIRK activity, in addition to the stimulated GIRK activity, is also supported by the interaction between G $\beta\gamma$ subunit and GIRK (Mirshahi et al., 2002). These findings together strengthen the idea that GPCRs, G proteins and ion channels exist as dynamically assembled macromolecular complexes that localize into discrete membrane microdomains (Doupnik, 2008).

While we were recording data as those shown in Figure 2, we often recognized in the currents we obtained another distinguishable feature caused by the presence of mGluR2. Both cell types were first subjected to the whole-cell patch clamp recordings, showing that GIRK current induced by 100 μ M GTP displayed greater and faster desensitization kinetics in the presence of mGluR2 (Figure 4). Here again, we demonstrate that mGluR2 affects the behavior of

G proteins even in the absence of the agonist. Moreover, the data suggest that the role of the receptor is not only to transmit the extracellular signals to the cytoplasm, but also to make the signals briefer, serving as a limiting agent that prevents prolonged activation of the downstream pathway. The observed effect of mGluR2 in desensitization kinetics of GIRK current motivated us to test whether activating less receptor through signaling would give smaller and slower desensitization. The idea behind this experiment was that, if the amount of receptors being activated were the key to the changes in kinetics, activating less receptor should give less inhibition over a longer period of time. We activated less amount of the mGluR2 by using 25 μ M extracellular Glutamate which is less than EC50 shown in Figure 3B. Our data suggest that involving more receptors in the signaling (i.e. with 100 μ M Glutamate) would result in a greater and quicker desensitization (Figure 5). We confirmed that the changes in desensitization kinetics were indeed caused by the amount of mGluR2 that was activated. However, the mechanism by which the mGluR2 affects desensitization kinetics of GIRK current remains unclear. It is also not clear what causes the desensitization in the absence of mGluR2. It is possible that the GTP in the pipette solution was activating Gq protein pathway in addition to Gi pathway, which, in turn, would activate phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol-4,5- bisphosphate (PIP₂) that supports the GIRK activity. Previous studies showed that the Gq-mediated PIP₂ depletion contributed to GIRK current inhibition (Keselman et al, 2007). Although the data collected by Keselman et al. show greater and quicker desensitization, we should account for the fact that GTP is not as potent as acetylcholine on muscarinic receptors in Gq activation. Moreover, we showed that GTP gives a greater effect when the G proteins are associated with the receptors. The amount of Gq proteins associated with the receptors is likely to have been much less than the amount of Gi proteins associated with the over-expressed mGluR2. The idea that the smaller and slower desensitization of GIRK current in the absence of mGluR2 is carried out by Gq-mediated PIP₂ hydrolysis could be tested by using PLC blocker, such as U73122, that blocks the Gq pathway. If PLC involvement were true, the current inhibition would be much less or not happen at all in the presence of U73122.

We were motivated to characterize the recovery kinetics of the Glutamate-induced GIRK current. Waiting longer after the first exposure to 100 μ M glutamate resulted in greater recovery of current upon a second exposure, and it took 4 minutes to fully recover (Figure 6). It is still not

clear whether this characteristic is due to the mGluR2 or it is an intrinsic property of the G protein. To clarify this, the time-dependent recovery kinetics that we characterized should be compared with the recovery kinetics from GTP γ S- or GTP-induced GIRK currents, experiments that is not clear how to do using the whole-cell mode of the patch clamp technique.

We also examined how desensitization kinetics of GTP γ S-induced GIRK currents changed in the presence of mGluR2. Contrary to our expectation, the GTP γ S-induced current desensitized less in the presence of mGluR2, although the rate at which the current desensitized became much faster as expected (Figure 7). The different outcomes should be due to the difference between GTP and GTP γ S because those were the only variables between the two experiments. GTP can be hydrolyzed by the intrinsic GTPase activity of the G α subunits, thus allowing the G protein cycle to take place in a GPCR-dependent manner. In contrast GTP γ S once it binds, it cannot be hydrolyzed therefore not allowing for G protein cycling. We surmise that the presence of G protein cycling limits the time G proteins are activated to cause channel activation thus producing less current and this accounts for one aspect of desensitization, namely the faster component that is Gi-dependent, since it is only Gi proteins that activate GIRK currents. A second slower component of desensitization would result from activation of PLC as discussed above, hydrolyzing PIP₂ and limiting the time GIRK could be maintained open. Thus we have two ways in which we control current level: one through stimulating GIRK channels via a Gi-coupled mechanism that due to the cycling of G proteins shows a fast component of desensitization and another mechanism which manifests itself as a slower mechanism of desensitization by hydrolyzing PIP₂ and limiting the ability of the channel to stay open. Since the two mechanisms of controlling GIRK activity, namely the stimulatory Gi (with its relatively faster desensitization) and the inhibitory Gq (with its relatively slower desensitization), work in opposite ways, removing the Gi cycling with GTP γ S would cause the stimulation without the fast desensitization and now allow the second mechanism to inhibit without contribution from the Gi cycling mechanism. This may yield a lower level of inhibition and somehow affect the kinetics of the Gq inhibition to manifest themselves faster. Another possibility is that the greater potency of GTP γ S, compared to GTP, might have activated unknown factors that counteract desensitization.

Our results provide important information about the interactions of mGluR2 with G proteins and how the receptor influences GIRK activation and desensitization that are likely to stimulate additional studies in the laboratory to further investigate the mechanisms by which the GPCR controls activation and desensitization of GIRK currents. In addition, these studies have contributed to a better picture of how the molecules (GPCRs, G proteins, ion channels) are distributed in the plasma membrane likely forming preferential macromolecular complexes –that represent only a fraction of the overall activatable channel current.

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