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Functional Expression of a Novel Ginsenoside Rf Binding Protein from Rat Brain mRNA in *Xenopus laevis* Oocytes

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ABSTRACT

We have shown that ginsenoside Rf (Rf) regulates voltage-dependent Ca^{2+} channels through pertussis toxin (PTX)-sensitive G proteins in rat sensory neurons. These results suggest that Rf can act through a novel G protein-linked receptor in the nervous system. In the present study, we further examined the effect of Rf on G protein-coupled inwardly rectifying K^+ (GIRK) channels after coexpression with size-fractionated rat brain mRNA and GIRK1 and GIRK4 (GIRK1/4) channel cRNAs in *Xenopus laevis* oocytes using two-electrode voltage-clamp techniques. We found that Rf activated GIRK channel in a dose-dependent and reversible manner after coexpression with subfractions of rat brain mRNA and GIRK1/4 channel cRNAs. This Rf-evoked current was blocked by Ba^{2+}, a potassium channel blocker. The size of rat brain mRNA responding to Rf was about 6 to 7 kilobases. However, Rf did not evoke GIRK current after injection with this subfraction of rat brain mRNA or GIRK1/4 channel cRNAs alone. Other ginsenosides, such as Rb_1 and Rg_1, evoked only slight induction of GIRK currents after coexpression with the subfraction of rat brain mRNA and GIRK1/4 channel cRNAs. Acetylcholine and serotonin almost did not induce GIRK currents after coexpression with the subfraction of rat brain mRNA and GIRK1/4 channel cRNAs. Rf-evoked GIRK currents were not altered by PTX pretreatment but were suppressed by intracellularly injected guanosine-5′-(2-*O*-thio) diphosphate, a nonhydrolyzable GDP analog. These results indicate that Rf activates GIRK channel through an unidentified G protein-coupled receptor in rat brain and that this receptor can be cloned by the expression method demonstrated here.

Ginseng, the root of *Panax ginseng* C.A. Meyer, is well known as a folk medicine used as a tonic and restorative agent. Ginsenoside, which is also called ginseng saponin, is the one of the main molecular ingredients responsible for the actions of ginseng. Ginsenoside has a four-ring, steroid-like structure with sugar moieties attached, and a variety of ginsenosides have been isolated and identified from the root of *Panax* ginseng (Nah, 1997). Recent reports show that ginsenosides share a common signaling pathway with well defined neurotransmitters such as acetylcholine or opioids for their pharmacological or physiological actions (Watanabe et al., 1988; Nah and McCleskey, 1994; Choi et al., 2001). For example, ginsenoside Rf (20-*S*-protopanaxatriol-6-[*O*-β-D-glucopyranosyl (1→2)-β-glucopyranoside]) (Rf) inhibited voltage-dependent Ca^{2+} channels in sensory neurons to the same degree as opiates through PTX-sensitive G proteins. The inhibitory effect of Rf on Ca^{2+} channels was not blocked by various G protein-coupled receptor antagonists, providing a possibility that Rf acts through another G protein-coupled receptor (Nah et al., 1995). Furthermore, other ginsenosides, such as Rc and Re, were more effective than Rf in the inhibition of Ca^{2+} channel in rat chromaffin cells, suggesting that ginsenosides other than Rf also regulate Ca^{2+} channel through interaction with unidentified protein(s) (Kim et al., 1998).

To get further evidence that Rf acts through an unidentified PTX-sensitive G protein-coupled novel binding or receptor in the nervous system, it might be necessary to determine partial protein sequence on the Rf binding protein and to synthesize oligonucleotide probes, perhaps by cloning of most of the hormone or neurotransmitter receptors. However, the Rf binding protein has not been purified, and antibodies to the Rf binding protein have not been generated. Recent reports have shown that functional gene expression methods using *Xenopus laevis* oocytes could also be used as an alternative method for successful cloning of the novel or unidentified G protein-coupled receptors, ligand-gated receptors, or transporters (Masu et al., 1987; Julius et al., 1988; Snutch, 1988; Frech and Joho, 1992; Lustig et al., 1993; Brake et al.,

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ABBREVIATIONS: Rf, ginsenoside Rf; PTX, pertussis toxin; GIRK channel, G protein coupled inwardly rectifying K^+ channel; GDPβS, guanosine-5′-(2-*O*-thio) diphosphate; 5-HT, serotonin; ACh, acetylcholine.

1994), because *X laevis* oocytes efficiently and accurately translate exogenous genes.

On the other hand, G protein-coupled inwardly rectifying K^+ (GIRK) channels are known to open when most of the PTX-sensitive G protein coupled receptors are activated. Moreover, it has been well characterized that treatment of their respective agonists after coexpression of PTX-sensitive G protein-coupled receptors and GIRK channel genes activates GIRK channel (for review, see Dascal, 1997). Similarly, in the *X laevis* oocyte expression system, if Rf binds a novel protein expressed exogenously in *X laevis* oocytes and activates PTX-sensitive G proteins, it could be possible to observe an activation of GIRK channel after coexpression of GIRK channel gene. Thus, we used *X laevis* oocyte as an expression system, rat brain mRNA as a source of novel Rf binding protein, and GIRK channels as an indicator of cellular response. We coinjected subfractions of rat brain mRNA with GIRK cRNAs into *X laevis* oocytes and then examined the effect of Rf on GIRK channel activity using two-electrode voltage clamp techniques. We report here that coexpression of subfractions of the poly(A)$^+$ mRNA from rat brains, separated by sedimentation by sucrose gradient centrifugation, with GIRK channel genes leads to the activation of GIRK channel via a novel Rf binding protein in *X laevis* oocytes. Rf-evoked GIRK current was suppressed by intraoocyte injection of GDPβS but not by PTX pretreatment. Thus, these results indicate that Rf activates GIRK channel through interaction with an unidentified protein that is derived from rat brain via PTX-insensitive G protein, possibly endogenously present in *X laevis* oocytes.

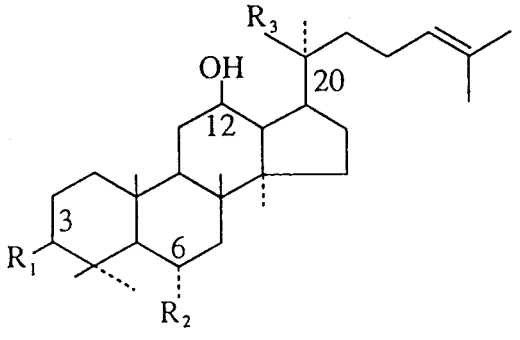
Experimental Procedures

Materials. Fig. 1 shows the structures of the five representative ginsenosides, including ginsenoside Rf. These ginsenosides were kindly provided by Korea Ginseng and Tobacco Research Institute (Taejon, Korea). 5-HT$_{1A}$, m2 muscarinic receptor, and GIRK1 and GIRK4 (GIRK1/4) channel cDNAs were kindly provided by Dr. N. Dascal (Tel Aviv University, Israel). Other agents were purchased from Sigma-Aldrich (St. Louis, MO).

mRNA Preparation and Size Fractionation. RNA was isolated from 2-week-old Sprague-Dawley rat brains by the LiCl-urea-SDS procedure (Dierks et al., 1981). Poly(A)$^+$ mRNA was prepared by column chromatography of oligo(dT)-cellulose (QIAGEN, Valencia, CA) and size-fractionation by centrifugation for 16 to 20 h at 2 to 4°C in a Beckman SW 40 Ti rotor (Beckman Coulter, Fullerton, CA) at 39,000 rpm through continuous gradients of sucrose (10 to 30%, w/v). To promote disruption of secondary structures the poly(A)$^+$ mRNA, before its application to the sucrose gradient, was heated at 70°C for 15 min and then placed on ice. After centrifugation, fractions were collected (about 2 ml) in sterile microcentrifuge tubes and mRNA of each fraction was precipitated by ethanol precipitation.

In Vitro Transcription of cDNAs. Recombinant plasmids containing 5-HT$_{1A}$ receptor, m2 muscarinic receptor, or GIRK1/4 channels cDNA insert were linearized by digestion with appropriate restriction enzymes. The cRNAs from linearized templates were obtained by in vitro transcription kit (mMessage mMachine; Ambion, Austin, TX) using a SP6 RNA, T3, or T7 polymerase. The RNA was dissolved in RNase-free water at 1 μg/μl, divided into aliquots and stored at −70°C until used.

Preparation of *X laevis* Oocytes and Microinjection. *X laevis* frogs were obtained from Xenopus I (Ann Arbor, MI). Their care and handling were in accordance with the highest standards of institutional guidelines. To isolate oocytes, frogs were operated on under anesthesia with an aerated solution of 3-aminobenzoic acid ethyl ester. Oocytes were separated by treatment with collagenase and agitation for 2 h in a Ca^{2+}-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stage V–VI oocytes were collected and stored in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 μg/ml gentamicin. This oocyte-containing solution was maintained at 18°C with continuous gentle shaking and changed everyday. Electrophysiological experiments with oocytes were performed within 5 to 6 days after their isolation. The drugs used in this study were bath-applied. One day after harvest, a 10-μl VWR microdispenser (VWR Scientific San Francisco, CA) fitted with a tapered glass pipette tip (15–20 μm in diameter) was used for injection of 40 nl of cRNAs into the animal or



Ginsenosides	R_1	R_2	R_3
Ginsenoside-Rb$_1$	-O-Glc^2-Glc	-H	-O-Glc^6-Glc
Ginsenoside-Rc	-O-Glc^2-Glc	-H	-O-Glc^6-Ara (pyr)
Ginsenoside-Re	-OH	-O-Glc^2-Rha	-O-Glc
Ginsenoside-Rf	-OH	-O-Glc^2-Glc	-OH
Ginsenoside-Rg$_1$	-OH	-O-Glc	-O-Glc

Fig. 1. Structures of the five representative ginsenosides. They differ at three side chains attached to the common steroid ring. Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara(pyr), arabinopyranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucose ring that links the two carbohydrates.

vegetable pole of each oocyte. Oocytes were injected with rat brain mRNA or GIRK1/4 channel cRNAs alone or in combination with GIRK1/4 channel cRNAs and rat brain mRNA. GDPβS solution (20 nl) was injected into oocytes to give calculated intracellular concentration of about 600 pmol.

Oocyte Recording. Two-electrode voltage-clamp recordings were obtained from single oocytes placed in a small plexiglas net chamber (0.5 ml), which was continuously superfused with the bathing medium (i.e., ND96). Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.2–0.7 MΩ) and voltage-clamped at -80 mV. After stabilization of oocytes with ND96, oocytes were then changed with a high K^+ solution (96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). In this solution, the K^+ equilibrium potential (E_K) was near 0 mV to enable K^+ inward currents to flow through inwardly rectifying K^+ channels at negative holding potentials. The electrophysiological experiments were done at room temperature with an oocyte clamp (OC-725C; Warner Instrument, Hamden, CT). Linear leak and capacitance currents were corrected with a leak subtraction procedure.

Data Analysis. All values are presented as mean ± S.E.M. The differences between means of control and treatment data were analyzed using unpaired t test. $p < 0.05$ was considered significant.

Results

Identification of Enriched mRNA Subfractions That Respond to Rf for GIRK Channel Activation. To confirm coexpression of 5-HT_{1A} receptors and GIRK channels in *X laevis* oocytes, we first coinjected GIRK1/4 cRNAs with 5-HT_{1A} receptor cRNA as a positive control (3 ng/oocyte of 5-HT_{1A} receptor cRNA). We could observe that serotonin (1 μM) produced large inward currents in the presence of high K^+ solution (Fig. 2A). Thus, this result indicates in our system that 5-HT_{1A} receptors were successfully coupled to GIRK channels in *X laevis* oocytes. Next, when we coinjected rat brain mRNA and GIRK 1/4 cRNAs into oocytes, we could observe that Rf induced a slight inward current in the presence of high K^+, suggesting that Rf binding protein might be present in the nervous system but needed to be enriched (data not shown). Therefore, we performed the size-fractionation of rat brain mRNA using 10 to 30% sucrose density gradient centrifugation to collect the enriched mRNA subfractions. Figure 2E shows sedimentation profiles of rat brain mRNA fractions and GIRK channel activity in response to Rf in each subfraction. Through coinjection experiments with each size-fractionated mRNAs and GIRK1/4 cRNAs, we could find that fractions 2 through 5 showed the large GIRK channel activity in response to Rf in the presence of high K^+ at a holding potential of -80 mV. The effect of Rf was reversible. Among them, fraction 3 showed the strongest GIRK channel activity in response to Rf in the presence of high K^+ (Fig. 2B). We also tested the effect of Rf in oocytes injected with mRNAs or GIRK1/4 channel cRNAs alone. As shown in Fig. 2, in oocytes injected with only GIRK1/4 cRNAs, we could observe a larger inward current than that of mRNA injection alone, but we could not observe Rf-evoked additional inward currents (Fig. 2C). In oocytes injected with mRNA of fractions 2, 3, 4, or 5 alone we could observe only a slight inward current after application of high K^+ at a holding potential of -80 mV. However, Rf with high K^+ did not induce further inward current (Fig. 2D). These results suggest that mRNA coding Rf binding protein could be separated and highly enriched among the whole fractionated rat brain mRNAs (Fig. 2).

Current-Voltage Relationship of Rf-Evoked GIRK Currents. GIRK channels usually display more inward current than outward current at voltages that are equally negative or positive from the reversal potential. To study whether Rf can activate this inwardly rectifying K^+ channel in this system, current-voltage relationships were produced. Figure 3A is a representative example of experiments among seven similar results, in which oocytes injected with fraction 3 and GIRK1/4 channel cRNAs were subjected to voltage ramps (-100 to + 40 mV) during treatment with only high K^+ or Rf and high K^+ together. This inwardly rectifying current activated under these conditions showed a reversal potential near 0 mV, as expected for a potassium current if we assume that intraoocyte $[K]_i$ is approximately 90 mM, as is extracellular $[K]_o$. Rf did not shift the potassium equilibrium potential of the GIRK current but increased its amplitude of inward current at negative potential rather than positive potential. Thus, Rf activates GIRK channel. The effect of Rf on GIRK current was concentration-dependent and reversible; the EC_{50} was 34 ± 3 μM, and the maximal effect was obtained at about 100 μM (Fig. 3B).

To provide further evidence of Rf-evoked GIRK current, we examined the effect of Ba^{2+}, a reversible blocker of GIRK channels, on Rf-evoked inward current. As expected, Ba^{2+} (300 μM) attenuated both high K^+- and Rf-evoked inward current in a reversible manner, but there was a slight inward current that was resistant to Ba^{2+} (Dascal et al., 1993a,b). These results indicate that Rf-evoked inward current is GIRK current (Fig. 4A).

We examined the effect of acetylcholine (ACh) or 5-HT in oocytes coinjected with GIRK1/4 channel cRNAs and size-fractionated mRNAs that responded to Rf, because we cannot exclude the possibility that Rf might activate GIRK channels via already known receptors existing in subfractions of mRNAs. As shown in Fig. 4B, Rf evoked a large inward current, whereas treatment of ACh (10 μM) showed a slight induction of inward current and serotonin (10 μM) had almost no effect on GIRK current in this oocyte.

We also examined the effect of other ginsenosides, such as Rb_1 and Rg_1, which are major constituents of ginsenosides, on GIRK current. As shown in Fig. 4C, inset, both Rb_1 and Rg_1 exhibited only a slight induction of GIRK current, suggesting that the induction of GIRK current might be Rf specific. As shown in Fig. 4, A and C, the repeated treatment of Rf did not induce desensitization and the effect of Rf was reproducible.

Previously, we demonstrated that ginseng root extract and Rf inhibited voltage-dependent Ca^{2+} channels via PTX-sensitive G proteins in rat sensory neurons (Nah and McCleskey, 1994; Nah et al., 1995). Hence, we tested the effect of PTX and of the nonhydrolyzable GDP analog GDPβS, which is known to inhibit the activity of both PTX-sensitive and -insensitive G proteins (Gilman, 1987), on the Rf-evoked GIRK current (Dascal et al., 1993a). As shown in Fig. 5, pretreatment of PTX (2 μg/ml, 24–48 h) did not depress the action of Rf, whereas pretreatment of the same concentration of PTX abolished the effect of ACh- or serotonin-evoked GIRK current (Dascal, 1997) (Fig. 5A). However, the intraoocyte injection of GDPβS (final concentration, 600 pmol) significantly inhibited the Rf action on GIRK current (Fig. 5, B and C).

Discussion

Ginseng has been used for hundreds of years as a treatment for a wide variety of ailments; some of these purported effects have been documented in the laboratory (Nah, 1997). However, the role of ginsenosides as active ingredients of ginseng is still elusive, because the signal pathways of ginsenosides are not clearly defined, unlike other natural medicines, such as opioids. Recent reports showed some progress in understanding the signal transduction of ginsenosides in cellular levels. For example, we have shown that Rf, one of the ginsenosides, transduces its signal through PTX-sensitive G proteins, suggesting that Rf binding protein might exist in nervous system and mediates the signal via interaction with Rf (Nah et al., 1995). In the present study, we provide further evidence that Rf regulates GIRK channel in

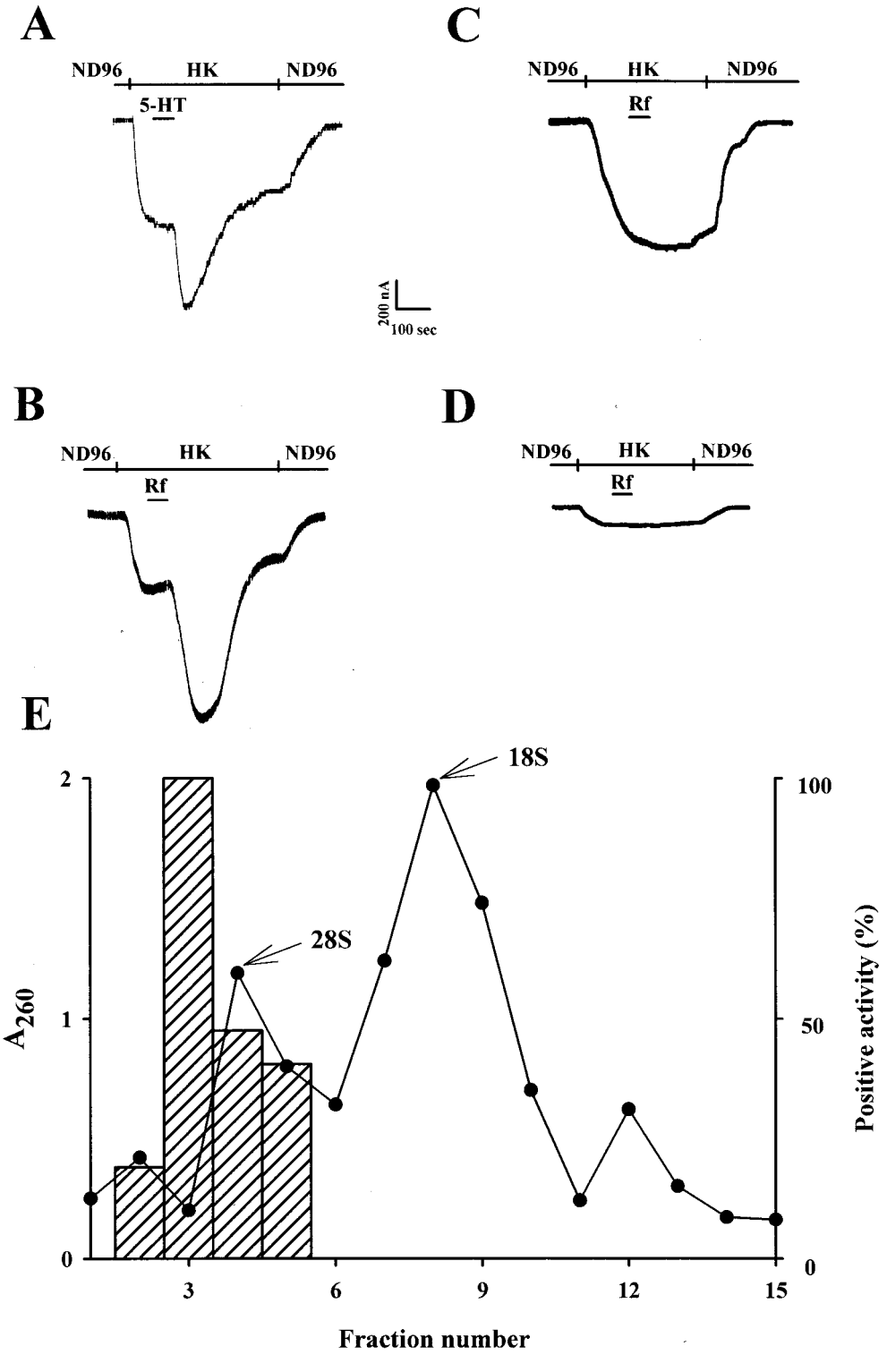


Fig. 2. Expression of Rf-evoked GIRK current with a subfraction of rat brain mRNA and GIRK1/4 cRNAs. A, 5-HT (1 μM) induced GIRK current in oocyte injected with 5-HT_{1A} receptor (3 ng) and GIRK1/4 channel cRNAs (2 ng) in the presence of high K^+ (HK). B, Rf (100 μM) induced inward current in oocyte coinjected with fraction 3 of brain mRNA (20 ng) and GIRK1/4 channel cRNAs (3 ng). C, the application of high K^+ solution to oocyte injected with GIRK1/4 channel cRNAs alone induced inward current but did not respond to Rf. D, Rf (100 μM) did not evoke GIRK current in oocytes injected with subfraction of mRNA (20 ng) (fraction 3) alone. There was only a small inward current under high K^+ (HK) solution but Rf (100 μM) does not evoke further inward current in the presence of high K^+ (HK) solution. Oocytes were voltage clamped at -80 mV in these experiments. E, size-fractionation of rat brain mRNA by sucrose gradient centrifugation for enrichment of mRNA coding Rf binding protein. After centrifugation, mRNA fractions were collected by 2 ml in sterile micro-centrifuge tubes and mRNA of each fraction was precipitated by ethanol. Fraction 1 indicates the bottom of the gradient (larger RNAs), and fraction 15 corresponds to the top of the gradient (smaller RNAs). Positive fractions 2, 3, 4, and 5 were identified by two-electrode voltage-clamp recordings using oocytes coinjected with GIRK1/4 channel cRNAs (3 ng) and each fractionated mRNA (20 ng).

X laevis oocytes through interaction with an unidentified membrane component derived from rat brain. As evidence for such an interaction of Rf with membrane protein in rat brain, we show that: 1) Rf evokes inward current only after coinjection of subfractions of rat brain mRNA and GIRK cRNA but not with subfractions of rat brain mRNA or GIRK cRNAs alone; 2) subfractions of mRNA responding to Rf could be enriched after size-fractionation of mRNA; 3) Rf-evoked current at oocytes coexpressed with the enriched mRNA fractions and GIRK1/4 channel cRNAs was blocked by Ba^{2+} and the effect of Ba^{2+} is reversible; 4) Rf-evoked currents at oocytes coexpressed with the enriched mRNA fractions and GIRK1/4 channel cRNAs were blocked by intraoocyte injection of GDPβS, a nonhydrolyzable GDP analog, but not by PTX pretreatment. Moreover, other ginsenosides showed a slight effect on inward currents after coinjection of subfrac-

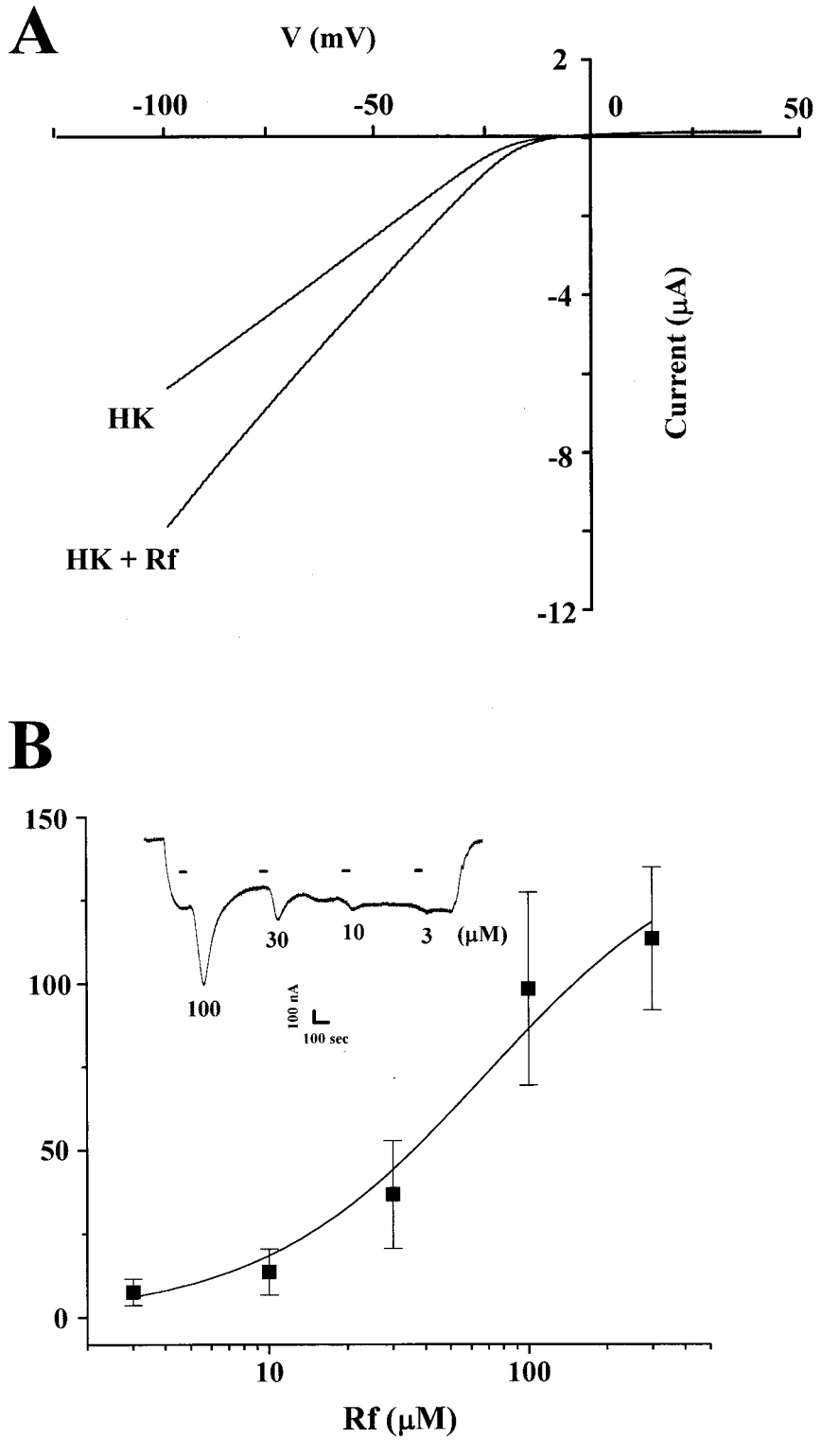


Fig. 3. Current-voltage and dose-response relationship for activation of GIRK current by Rf. A, current-voltage relationships were obtained using voltage ramps between −100 and +40 mV. Voltage ramps were applied before and after application of Rf (100 μM). Oocytes were coinjected with GIRK1/4 channel cRNAs (3 ng) and rat brain mRNA (20 ng) (fraction 3). B, oocytes were voltage clamped at −80 mV and each oocyte was tested with the indicated dose of Rf. The results are normalized from three independent experiments using three different batches of oocytes. Each point represents the mean ± S.E.M. (n = 9–12/group). Oocytes were coinjected with GIRK1/4 channel cRNAs (3 ng) and fraction 3 of rat brain mRNA (20 ng). Inset, GIRK currents from a typical cell exposed to the indicated concentration of Rf.

tions of rat brain mRNA and GIRK1/4 cRNA, suggesting that an unidentified protein expressed in oocytes selectively interacts with Rf.

As noted above, injection of foreign poly(A)$^+$ mRNA extracted from rat brain into *X. laevis* oocytes after size-fractionation by sucrose density gradients centrifugation shows ion channel activity or responses to various neurotransmitters (Sumikawa et al., 1984). Interestingly, the subfractions of mRNA responding to neurotransmitters or showing ion channel activity differ from each other, suggesting that the size of mRNA coding different receptors or ion channels is not the same (Sumikawa et al., 1984). In our experiments, the approximate molecular size of mRNA fraction showing the highest GIRK current enhancement after coinjection with

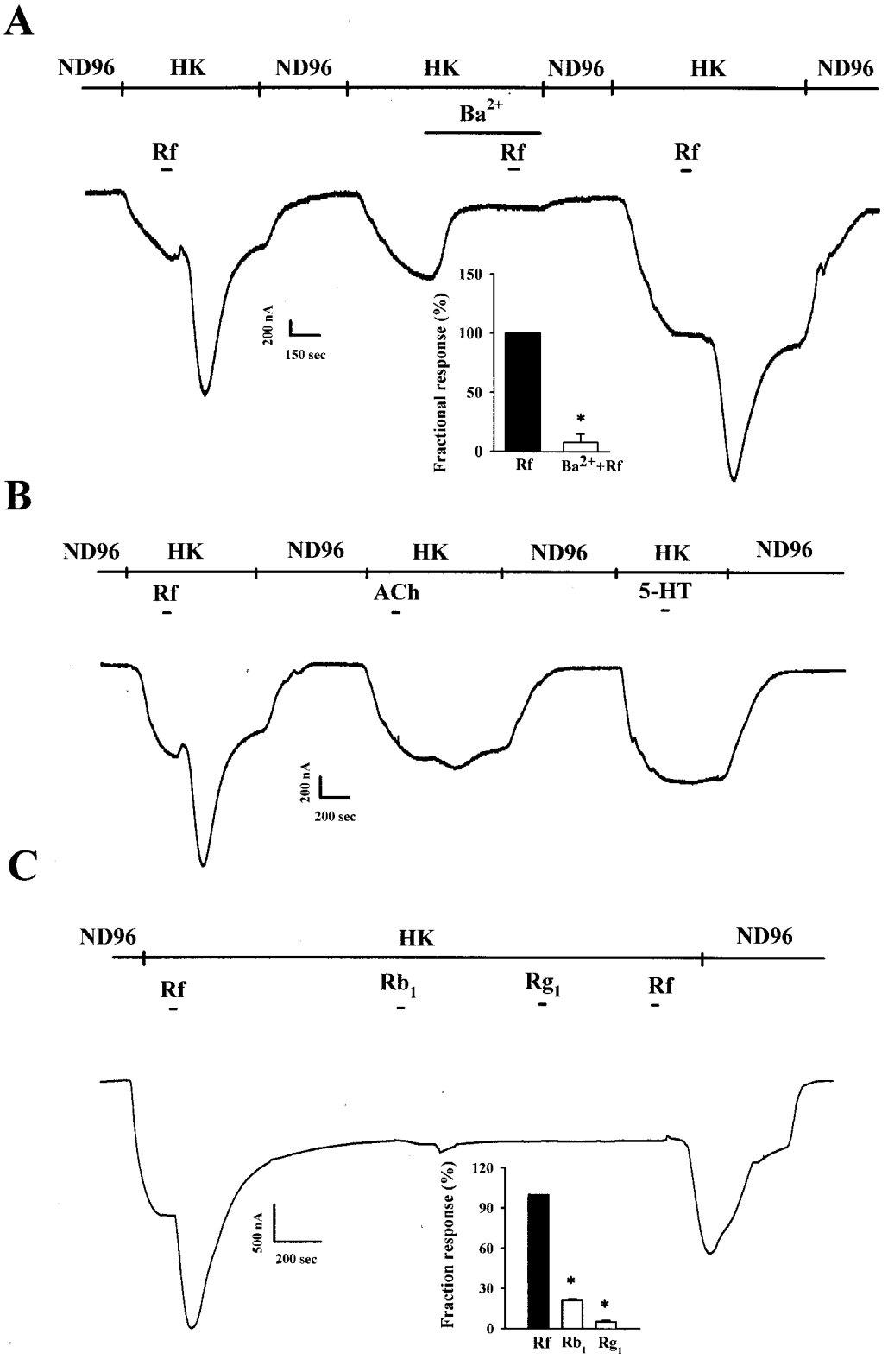


Fig. 4. Blockade of Rf-evoked GIRK current by Ba^{2+} and effect of ACh, 5-HT, ginsenoside Rb_1, or ginsenoside Rg_1 on GIRK channel activation in oocytes coinjected with rat brain mRNA (20 ng) (fraction 3) and GIRK1/4 channel cRNAs (3 ng). A, the application of 300 μM Ba^{2+} blocked the Rf- and high K^+-evoked inward currents and left a small, non-GIRK current mediated component of the inward current response to high K^+ (HK). The effect of Ba^{2+} was reversible. Inset, histograms of Rf-evoked GIRK currents in the absence or the presence of 300 μM Ba^{2+}. Each point represents the mean ± S.E.M. (n = 7/group). *$p < 0.01$ compared with Rf alone. Oocytes were coinjected with rat brain mRNA (20 ng) (fraction 3) and GIRK1/4 channel cRNAs (3 ng). B, acetylcholine (ACh, 10 μM) but not 5-HT (10 μM) induced a slight inward current. C, ginsenoside Rb_1 (100 μM) and ginsenoside Rg_1 (100 μM) showed a slight effect on GIRK channel activation in oocytes coinjected with rat brain mRNA (20 ng) (fraction 3) and GIRK1/4 channel cRNAs (3 ng). Inset, each point represents the mean ± S.E.M. (n = 5/group). Oocytes were voltage clamped at −80 mV. *$p < 0.05$ compared with Rf-induced inward currents.

GIRK1/4 channel cRNAs seemed to be near 28S ribosomal RNA. It could be 6 to 7 kilobases (Fig. 2). Similarly, previous reports showed that the size of mRNA subfractions corresponding to 5-HT_{1C} receptor is also near 5 to 7 kilobases (Julius et al., 1988). Thus, these results suggest that the size of mRNA coding an unidentified Rf binding protein might exist in this range. However, we cannot exclude the possibility that other ginsenoside-binding protein(s) with mRNA sizes different from those of Rf might also exist in other neural tissues, because ginsenosides other than Rf were more effective for the inhibition of Ca^{2+} channel in rat chromaffin cells (Kim et al., 1998).

On the other hand, GIRK channels play an important role in regulating cell excitability in both the heart and the nervous system (Dascal, 1997; Karschin, 1999). In the nervous system, GIRK channels are coupled to a variety of inhibitory neurotransmitter receptors through PTX-sensitive G proteins, including cannabinoid, γ-aminobutyric acid B, muscarinic, opioid, serotonin$_{1A}$, and somatostatin receptors (Dascal et al., 1993b; Chen and Yu, 1994; Kazutaka et al., 1995; Hans

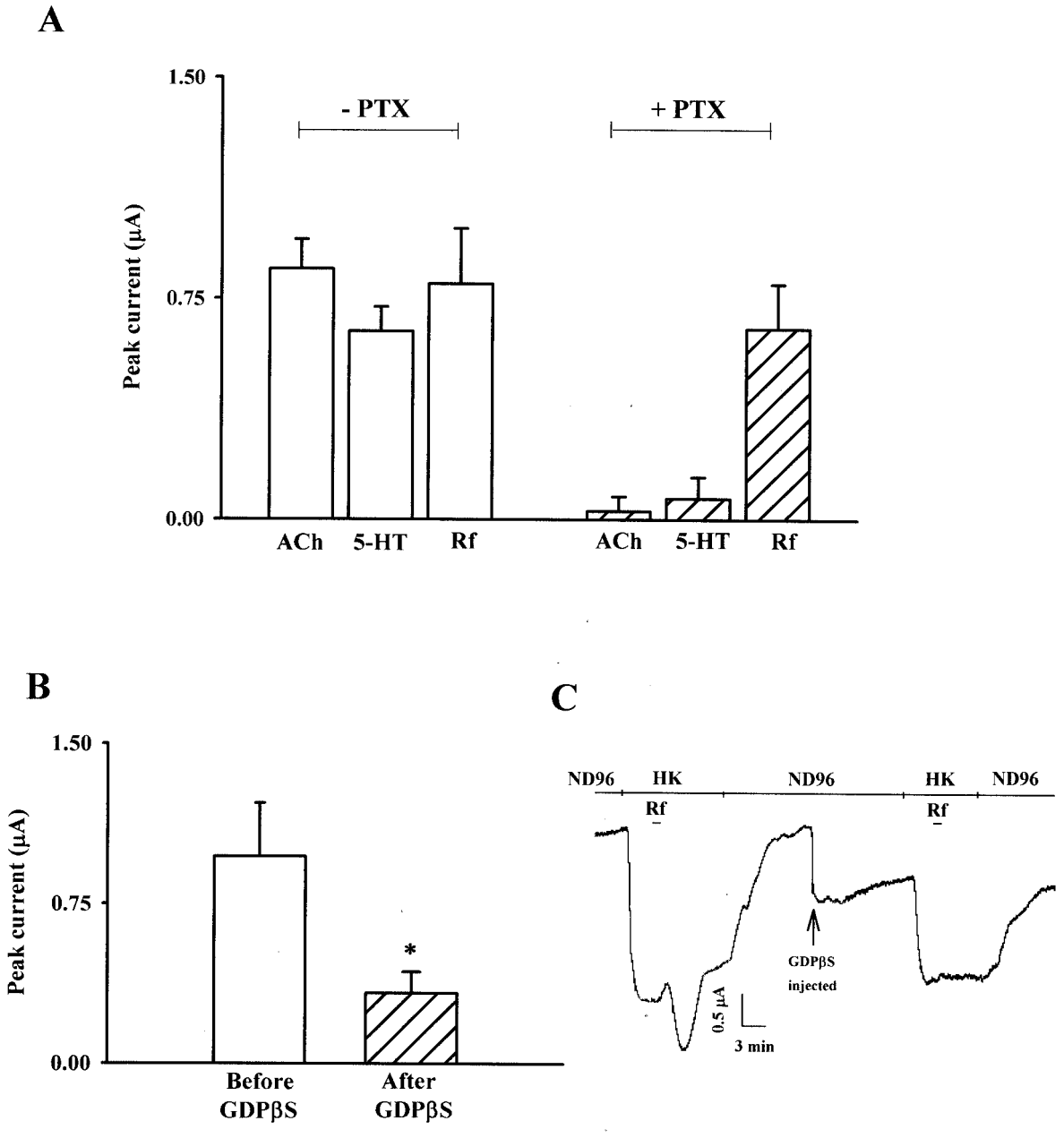


Fig. 5. Effect of PTX pretreatment or intracellular injection of GDPβS on Rf-evoked GIRK currents. A, the histograms show the GIRK currents evoked by Rf (100 μM), 5-HT (10 μM), or ACh (10 μM). Oocytes were coinjected with rat brain mRNA (20 ng) (fraction 3) + GIRK1/4 channel cRNAs (3 ng), 5-HT_{1A} receptor cRNA (2 ng) + GIRK1/4 channel cRNAs (3 ng), or m2 muscarinic receptor (2 ng) + GIRK1/4 channel cRNAs (3 ng). These oocytes were either untreated (−PTX) or incubated in PTX (2 μg/ml for 24–48 h). B, the histograms also show the GIRK currents evoked by Rf (100 μM) in oocytes coinjected with rat brain mRNA (20 ng) (fraction 3) and GIRK1/4 channel cRNAs (3 ng) before injection of GDPβS or after GDPβS (600 pmol). Each point represents the mean ± S.E.M. (n = 9–11/group). *, $p < 0.05$ significantly different from before GDPβS injection. C, the trace represents the typical cell for Rf (100 μM)-evoked GIRK current before GDPβS or after GDPβS. The arrow indicates the injection of GDPβS (600 pmol). Oocytes were voltage-clamped at −80 mV.

et al., 1997; McAllister et al., 1999). The mechanism through which inhibitory neurotransmitters interact to activate GIRK channels is well known. Thus, treatment of agonists that are coupled to $G_{\alpha i}/G_{\alpha o}$ catalyzes the turnover of heterotrimeric G proteins by releasing $G\beta\gamma$ subunits, which bind directly to the GIRK channels protein with consequent channel activation (Reuveny et al., 1994). *X laevis* oocytes have also been used for the functional characterization of GIRK channel coupled receptors after coinjection of specific G protein-coupled receptors and GIRK cRNA (Dascal et al., 1993a; Chen and Yu, 1994; Kazutaka et al., 1995; McAllister et al., 1999). In the present study, we also showed that Rf could regulate GIRK channels via unidentified proteins derived from rat brain via PTX-insensitive G protein**s** (Fig. 5). However, it is unlikely that Rf activates the GIRK channel directly without the mediation of an unidentified protein, because Rf did not activate GIRK channel in oocytes injected with GIRK channel cRNAs alone (Fig. 2). The activation of GIRK channel by Rf through interaction with unidentified Rf binding protein indicates that Rf may play an important role in regulation of neuronal cell excitability, because we also showed that Rf inhibits voltage-dependent Ca^{2+} channel in sensory neurons and chromaffin cells (Nah et al., 1995; Kim et al., 1998). Thus, these modulations of Ca^{2+} and K^+ channels provide additional evidence that Rf might act as a ligand for neuromodulation as do other hormones or neurotransmitters.

In summary, we found that Rf activated GIRK channel after coinjection of subfractions of rat brain mRNA with GIRK1/4 channel cRNAs in *X laevis* oocytes. These results show the possibility that Rf might interact with an unidentified protein derived from rat brain for its signal transduction. We are doing further investigation for cloning a novel Rf binding protein after preparation of cDNA library with subfractions of rat brain mRNA.

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