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Pharmacology

A trace component of ginseng that inhibits Ca^{2+} channels through a pertussis toxin-sensitive G protein

(saponins/ginsenosides/N-type Ca^{2+} channels/opioids/patch clamp)

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ABSTRACT **A crude extract from ginseng root inhibits high-threshold, voltage-dependent Ca^{2+} channels through an unknown receptor linked to a pertussis toxin-sensitive G protein. We now have found the particular compound that seems responsible for the effect: it is a saponin, called ginsenoside Rf (Rf), that is present in only trace amounts within ginseng. At saturating concentrations, Rf rapidly and reversibly inhibits N-type, and other high-threshold, Ca^{2+} channels in rat sensory neurons to the same degree as a maximal dose of opioids. The effect is dose-dependent (half-maximal inhibition: 40 μM) and it is virtually eliminated by pretreatment of the neurons with pertussis toxin, an inhibitor of G_o and G_i GTP-binding proteins. Other ginseng saponins—ginsenosides Rb1, Rc, Re, and Rg1—caused relatively little inhibition of Ca^{2+} channels, and lipophilic components of ginseng root had no effect. Antagonists of a variety of neurotransmitter receptors that inhibit Ca^{2+} channels fail to alter the effect of Rf, raising the possibility that Rf acts through another G protein-linked receptor. Rf also inhibits Ca^{2+} channels in the hybrid F-11 cell line, which might, therefore, be useful for molecular characterization of the putative receptor for Rf. Because it is not a peptide and it shares important cellular and molecular targets with opioids, Rf might be useful in itself or as a template for designing additional modulators of neuronal Ca^{2+} channels.**

Ginseng, the root of *Panax ginseng* C. A. Meyer (Araliaceae), is a mild oriental folk medicine that is reported to relieve a variety of ailments that might be considered physiological effects of stress (1). The molecule responsible for the effects of ginseng is unknown, as is the cellular basis of the action of ginseng. Extracts of ginseng mimic actions of opioids, without activating opioid receptors, in two important assays: inhibition of electrically evoked contraction of ileum smooth muscle (2) and inhibition of Ca^{2+} channels in sensory neurons through a pertussis toxin (PTX)-sensitive G protein (3). The suppression of neuronal Ca^{2+} channels by opioids (4), cannabinoids (5), and ginseng suggests that these various drugs share a common cellular action: presynaptic inhibition of Ca^{2+}-dependent neurosecretion.

The purpose of this study was to identify the compound within ginseng that inhibits Ca^{2+} channels. Ginseng contains both lipophilic components and saponins (6). All known ginseng saponins have a 4-ring, steroid-like structure with attached sugar moieties (7); these saponins appear responsible for most pharmacological effects (8). We tested a variety of purified ginseng saponins as well as a petroleum ether extract that should contain the lipophilic components of ginseng root. We found that ginsenoside Rf (Rf), a saponin that is present in ginseng in only trace amounts (9), reproduced all the effects of the crude extract, and no other compound was nearly as effective. Thus, the data describe the active ingredient in ginseng that mimics opioids in this cellular assay.

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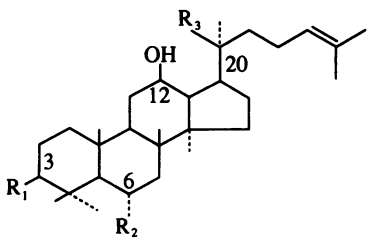

Ginsenosides	R_1	R_2	R_3
Ginsenoside-Rb_1	-O-Glc^2-Glc	-H	-O-Glc^6-Glc
Ginsenoside-Rc	-O-Glc^2-Glc	-H	-O-Glc^6-Ara (pyr)
Ginsenoside-Re	-OH	-O-Glc^2-Rha	-O-Glc
Ginsenoside-Rf	-OH	-O-Glc^2-Glc	-OH
Ginsenoside-Rg_1	-OH	-O-Glc	-O-Glc

FIG. 1. Structures of the five ginseng saponins studied. 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. Structures are as given in Kaku *et al.* (8).

MATERIALS AND METHODS

Materials. Fig. 1 shows the structures of the five ginseng saponins (ginsenosides) that were tested. The saponins and the petroleum ether extract of the root of *Panax ginseng* C. A. Meyer (Araliaceae) were obtained from the Korean Ginseng and Tobacco Research Institute (Taejon, Korea), which had purified the saponins as described in Paik *et al.* (6). Ginsenoside stocks of 100 mM were prepared in either 80% (vol/vol) ethanol (Rc, Rf, and Re) or in extracellular solution (Rb1 and Rg1) and were diluted directly into extracellular solution. Petroleum ether extract stock of 100 mg/ml in 80% ethanol was diluted into extracellular solution. The final concentration of ethanol (<0.1%) had no effect on Ca^{2+} currents.

Salts and chemicals were obtained from Sigma, except for: Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) and ω-conotoxin-GVIA (ω-Ctx-GVIA, Peninsula Laboratories); PTX (List Biological Laboratories, Campbell, CA); GTP (Aldrich); WIN 55,212-2 (Research Biochemicals, Natick, MA); 2.5S nerve growth factor (Biomedical Technologies, Stoughton, MA); medium and serum (GIBCO).

Cell Culture. Sensory neurons from dorsal root ganglia of adult (250 g) Sprague–Dawley rats were dissociated and maintained in culture as described in Schroeder *et al.* (10).

Abbreviations: Rb1, Rc, Re, Rf, Rg1, ginsenoside (ginseng saponin) Rb1, Rc, Re, Rf, and Rg1, respectively; DAMGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; PTX, pertussis toxin; ω-Ctx-GVIA, ω-conotoxin GVIA; I_{Ca}, Ca^{2+} current.

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Dissociated cells were plated on laminin-coated culture dishes and studied within the first 3 days in culture.

F-11 cells, a hybrid of dorsal root ganglia and mouse neuroblastoma cells (11), were obtained from Richard Miller (University of Chicago); culture conditions were similar to those of Boland and Dingledine (12). Cells were grown in a humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum/1% penicillin/streptomycin at 37°C and 5% CO_2/95% air. To induce differentiation, cells were plated in DMEM/1% penicillin/streptomycin with nerve growth factor at 5 ng/ml and no serum, and the medium was supplemented with 500 μM dibutyryl-cAMP for the first 2–3 days. Cells were used any time after 2 days of differentiation. Large cells with well-defined processes were selected for recording.

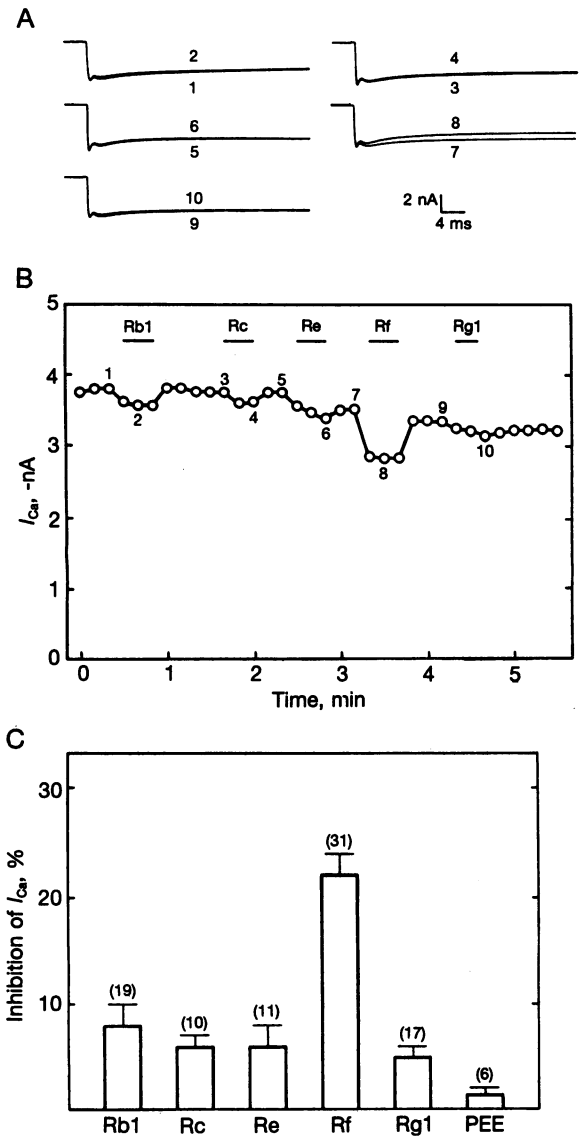

FIG. 2. Rf inhibits Ca^{2+} channels in adult rat sensory neurons. (*A*) Pairs of inward currents evoked by pulses to +10 mV from −70 mV at the times indicated in *B*. Leak and capacity currents were eliminated by subtracting records obtained in 1 mM Cd^{2+}, a Ca^{2+} channel blocker. (*B*) Graph of Ca^{2+} current (I_{Ca}) amplitude vs. time for the entire experiment. Solid bars indicate applications of the indicated ginsenosides, each at 100 μM. The gradual downward trend in the baseline is typical of Ca^{2+} channel run down in whole-cell patch clamp (14). (*C*) Average percentage inhibition (±SEM; number of cells in parentheses) of I_{Ca} by various ginsenosides (100 μM) and petroleum ether extract (PEE, 100 μg/ml) of ginseng root. Only Rf substantially inhibits I_{Ca}, and its effect differs significantly from the other compounds ($P < 0.0001$ in each case).

Recordings and Analysis. Whole-cell patch-clamp (13) experiments were done at room temperature using an Axopatch amplifier and PCLAMP software (both from Axon Instruments, Foster City, CA) for data acquisition and analysis. Records were filtered at 2 kHz with an 8-pole Bessel filter. Leak, capacity, and other contaminating currents were eliminated by subtracting recordings in 1 mM Cd^{2+}, a Ca^{2+} channel blocker. Current through Ca^{2+} channels was evoked by depolarizing pulses of 100-msec duration applied every 20 sec from a holding potential of −70 mV.

Ca^{2+} current was measured by averaging 10–20 msec of data at least 30 msec after pulse onset; the 30-msec delay allowed complete inactivation of voltage-gated Na^+ channels, which slightly contaminated subtracted currents because they were partially blocked by Cd^{2+}. Data are presented as means ± SEMs. Statistical significance was measured by a paired Student's *t* test. Least-squares curve fitting was done with the program NFIT (Island Products, Galveston, TX).

Experimental Solutions. Extracellular solution changes were made in ≈1 sec by moving a set of six 1-μl pipettes (Drummond "microcaps," VWR Scientific) glued together side-by-side, supported on a plastic coverslip, and mounted on a rod that was attached to a manipulator. The back ends of the pipettes were connected with polyethylene tubing to stopcocks and different solution reservoirs. The delivery end was positioned within 150 μm of the cell. Solution flowed over the cell at all times.

Unless otherwise indicated, the extracellular solution contained 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM Hepes, and 10 mM glucose, pH 7.3 (titrated with NaOH). The presence of Na^+ in the extracellular solution greatly diminished run down of Ca^{2+} channels. The pipette (intracellular) solution was 100 mM CsCl/1 mM Na_3ATP (equine)/0.3 mM GTP/10 mM EGTA/2.5 mM $MgCl_2$/2 mM $CaCl_2$/8.8 mM sodium phosphocreatine/0.08 mM leupeptin/40 mM Hepes, pH 7.0 (titrated with tetraethylammonium hydroxide).

RESULTS

Pairs of Ca^{2+} currents from a rat sensory neuron in the presence and absence of different ginseng saponins are shown

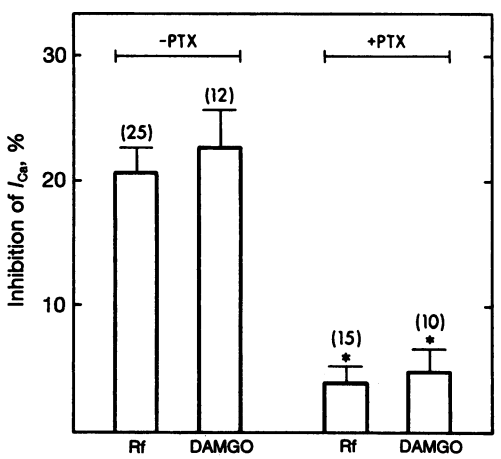

FIG. 3. PTX suppresses Ca^{2+} channel inhibition by Rf and by μ opioids. Average percentage inhibition (±SEM; number of cells in parentheses) of Ca^{2+} current by Rf (100 μM) and DAMGO (1 μM, a saturating dose, ref. 15) in neurons either untreated or incubated in PTX (250 ng/ml for 16 hr). Asterisk indicates significant difference from PTX-treated cells ($P < 0.0001$). Ca^{2+} currents are evoked by pulses to +10 mV.

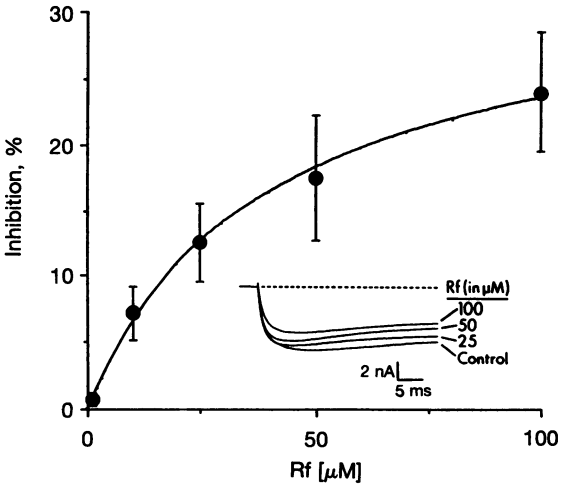

FIG. 4. Dose dependence of Ca^{2+} channel inhibition by Rf. Average percentage inhibition (±SEM; four to seven cells used for each data point) of I_{Ca} vs. Rf concentration. The curve is the best least-squares fit of the Michaelis–Menten equation: $y/y_{max} = [Rf]/([Rf] + K_{1/2})$, where y_{max} is the maximum inhibition (33 ± 2% SD), $K_{1/2}$ is the concentration for half-maximal inhibition (41 ± 6 μM SD), and [Rf] is the concentration of Rf. (*Inset*) I_{Ca} values from a typical cell exposed to the indicated concentrations of Rf; the order of application was from high to low concentration. I_{Ca} values are evoked by pulses to +10 mV.

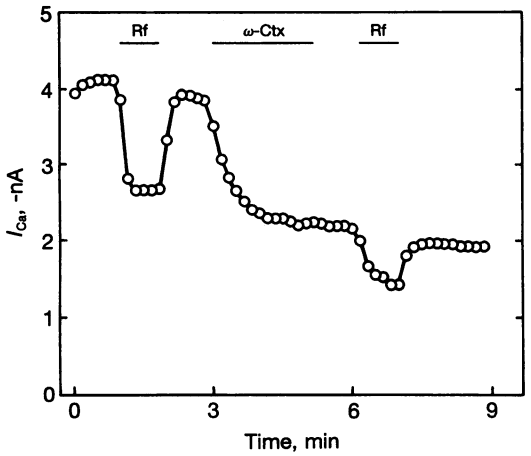

FIG. 5. Rf inhibits N-type Ca^{2+} channels. (*A*) Time course of the effects of 100 μM Rf on I_{Ca} amplitude before and after application of ω-Ctx-GVIA (1 μM), a selective, irreversible blocker of N-type Ca^{2+} channels. Because ω-Ctx-GVIA diminishes the amount of Rf-sensitive current, Rf inhibits N channels; because Rf remains active after this saturating dose of ω-Ctx-GVIA, another type of Ca^{2+} channel is also affected. Similar results were seen on each of the five cells tested. Currents are evoked by pulses to +10 mV.

in Fig. 2*A*. Currents were evoked by voltage steps to +10 mV applied at the times indicated in the graph of I_{Ca} amplitude vs. time (Fig. 2*B*). I_{Ca} clearly decreases upon application of 100 μM Rf (traces 7 and 8), but related ginsenosides have relatively little effect. Fig. 2*C* shows the average percentage inhibition of I_{Ca} in sensory neurons by the five ginsenosides (100 μM each) and by the lipophilic components of ginseng contained in the petroleum ether extract (100 μg/ml). Of the six compounds tested, only Rf inhibited Ca^{2+} channels >10%.

A maximal dose of Rf inhibits I_{Ca} as much as maximal activation of the μ opioid receptor by the agonist DAMGO (Fig. 3). Pertussis toxin, an inhibitor of two classes of G proteins (G_o and G_i), virtually eliminates effects of both Rf and DAMGO (Fig. 3). Inhibition of I_{Ca} by Rf is saturable and dose-dependent, with half-maximal inhibition occurring at ≈40 μM (Fig. 4).

N-type Ca^{2+} channels are responsible for electrically evoked neurotransmitter release from sensory neurons (16), so it is particularly important to determine whether this type of channel is affected. Rf inhibits N-type Ca^{2+} channels because the extent of inhibition is greatly diminished after application of ω-Ctx-GVIA, a selective blocker of N channels (Fig. 5). After a maximal dose of ω-Ctx-GVIA, the residual I_{Ca} remains sensitive to Rf; thus, Rf must inhibit other types of Ca^{2+} channels in addition to the N channel. DAMGO similarly targets multiple types of Ca^{2+} channels in these neurons, but the identity of the small, opioid-sensitive current remaining after ω-Ctx-GVIA is unclear (14, 17).

A variety of receptors suppress Ca^{2+} channels through G proteins in sensory neurons (18), so we considered whether Rf might act through one of these known receptors. We applied Rf with and without a mixture containing naloxone (10 μM), atropine (1 μM), yohimbine (1 μM), and phaclofen (10 μM), inhibitors of opioid, muscarinic, α_2-adrenergic, and γ-aminobutyric acid B receptors, respectively. Rf inhibits I_{Ca} equally well in the presence and absence of these drugs (data not shown; $n = 4$). The tempting conclusion that Rf acts at a different, undescribed receptor is premature because there are not good antagonists for all candidate receptors. Steroid and cannabinoid receptors are interesting possibilities because both suppress Ca^{2+} channels in certain cells (5, 19), and Rf has

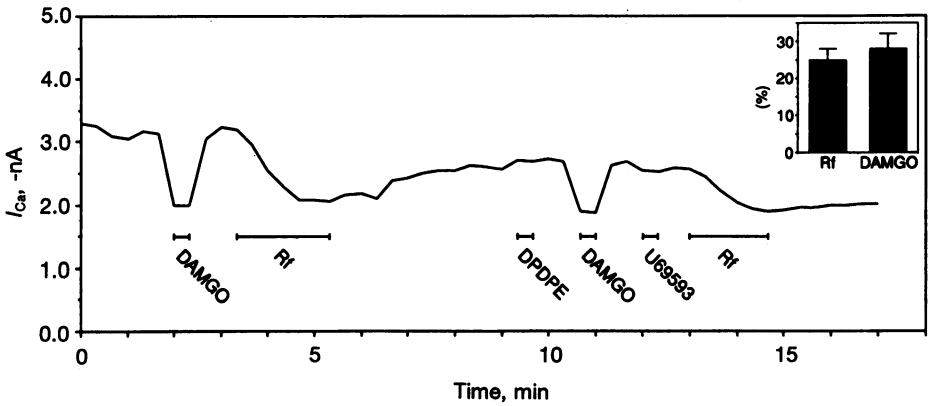

FIG. 6. Rf inhibits Ca^{2+} channels in the F-11 cell line. Time course from a differentiated F-11 cell showing the decrease in Ca^{2+} current amplitude by 1 μM DAMGO and 100 μM Rf, as well as the absence of inhibition by δ (DPDPE, [D-Pen^{2,5}]enkephalin) and κ (U69593) opioid agonists. (*Inset*) Average (±SEM) percentage inhibitions by DAMGO and Rf ($n = 15$) (Rf and DAMGO were both applied to each of the cells). Inhibition was measured upon reaching steady state, which occurs in F-11 cells more quickly with DAMGO than with Rf. Extracellular solution was as follows: 30 mM Ba^{2+} added to the usual extracellular solution (12). Currents were evoked by pulses to +10 mV.

a steroid backbone and, like cannabinoids, is lipid soluble. However, we found no evidence for Ca^{2+} channel modulation in these neurons by the cannabinoid, WIN 55,212-2 (5 μM) or a variety of steroids (10 μM corticosterone, 10 μM progesterone, 10 μM pregnenolone sulfate, 100 nM–50 μM β-estradiol, and 50 μM allotetrahydrocorticosterone) (data not shown).

If molecular methods are to be used to identify the receptor for Rf, it would be helpful to have a cell line that is sensitive to Rf. Fig. 6 demonstrates inhibition of Ca^{2+} channels by Rf in differentiated F-11 cells, a line that is a hybrid of rat dorsal root ganglia and mouse neuroblastoma cells (11). Saturating concentrations of Rf and DAMGO inhibit Ba^{2+} current through Ca^{2+} channels to the same final extent (Fig. 6 *Inset*). Unlike results in dorsal root ganglia neurons, Rf inhibits Ca^{2+} channels more slowly and reverses less completely than DAMGO (typical time course, Fig. 6). Rf does not inhibit I_{Ca} in undifferentiated F-11 cells.

DISCUSSION

Ginseng has mild physiological effects, indicating that the active ingredient(s) is either weak or scarce. Here, we demonstrate that a saponin within ginseng, Rf, has the same cellular effect as a maximal dose of opioids: both diminish high-threshold Ca^{2+} current in sensory neurons to the same degree using a G protein sensitive to PTX. Lipophilic components and other ginseng saponins had relatively little effect on I_{Ca}, demonstrating that Rf does not act through a nonspecific mechanism common to all saponins. Unlike other saponins, Rf is present within ginseng in very small amounts—too little, in fact, to detect in some species (9). Thus, ginseng root contains a trace compound with a potent action. Half-maximal suppression of Ca^{2+} current by Rf occurs at a high concentration (40 μM), although similarly high concentrations of morphine are required to inhibit Ca^{2+} channels under the same experimental conditions in the same primary sensory neurons (14).

Opioids inhibit Ca^{2+}-evoked neurotransmitter release from sensory neurons (20, 21), and this inhibition is a key element in spinal analgesia by opioids (22). N-type Ca^{2+} channels are the pathway for the Ca^{2+} entry that evokes neurotransmitter release in these cells (16). Inhibition of N channels by opioids (4, 14, 15) must be the essential molecular event in opioid suppression of neurotransmitter release in sensory neurons because opioids do not modulate K^+ channels in these cells (23–25). Thus, the ability of Rf to inhibit N channels to the same degree as opioids is clearly physiologically significant.

In the present assay, we measure 20–25% inhibition of I_{Ca} at maximal concentrations of either opioids or Rf. A 25% suppression of I_{Ca} should cause a 68% suppression of the postsynaptic potential because of the fourth-power relation between Ca^{2+} entry and neurotransmitter release (26, 27). Moreover, our measurement gives only a lower estimate of the magnitude of Ca^{2+} channel inhibition because maximal inhibition occurs immediately after the voltage step (14, 28), whereas we record current amplitudes 30 msec later. The time dependence of Ca^{2+} channel modulation results in far greater inhibition of Ca^{2+} entry during a brief action potential than is measured with voltage pulses (29).

Our results imply that Rf should have a major effect on neurosecretion from sensory neurons. Interestingly, a saponin purified from *Desmodium adscendens*, a medicinal herb used in Ghana, has been shown to open Ca^{2+}-activated K^+ channels (30). Ca^{2+}-activated K^+ channels are colocalized with Ca^{2+} channels at active zones of presynaptic terminals (31), so these two molecules provide particularly powerful means of modulating neurosecretion. That two unrelated plant saponins both target molecules that are critical to synaptic function suggests that it may be valuable to systematically test for saponin action on neurotransmission.

Extracts of ginseng cause analgesic (32–34) and anti-narcotic (35, 36) effects in whole animals. Our cellular studies cannot predict whether Rf has similar actions. Moreover, the fact that ginseng is free of side effects cannot be assumed if Rf is used in quantities greater than the very low levels present in ginseng. In general, saponins are nontoxic when ingested, but they damage red blood cells when injected directly into the blood stream. Rf and other ginseng saponins with glucose side chains exhibit relatively little hemolytic activity and, when injected into the peritoneum of mice, are lethal only at high doses (>1000 mg/kg, ref. 8). Because our results show that Rf has an action on single sensory neurons that is similar to opioids, further testing of the physiological and toxic effects of Rf in whole-animal studies would be valuable.

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