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# A novel activation of $Ca^{2+}$-activated $Cl^-$ channel in *Xenopus* oocytes by Ginseng saponins: evidence for the involvement of phospholipase C and intracellular $Ca^{2+}$ mobilization

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**1** The signal transduction mechanism of ginsenosides, the active ingredients of ginseng, was studied in *Xenopus* oocytes using two-electrode voltage-clamp technique. Ginseng total saponin (GTS), i.e., an unfractionated mixture of ginsenosides produced a large outward current at membrane potentials more positive than −20 mV when it was applied to the exterior of oocytes, but not when injected intracellularly. The effect of GTS was concentration-dependent ($EC_{50}$: 4.4 $\mu$g $ml^{-1}$) and reversible.

**2** Certain fractionated ginsenosides ($Rb_1$, $Rb_2$, Rc, Rf, $Rg_2$ and Ro) also produced an outward current in a concentration-dependent manner with the order of potency of $Rf > Ro > Rb_1 = Rb_2 > Rg_2 > Rc$. Other ginsenosides (Rd, Re and $Rg_1$) had little or no effect.

**3** The GTS effect was completely blocked by bath application of the $Ca^{2+}$-activated $Cl^-$ channel blocker niflumic acid and by intracellular injection of the calcium chelator BAPTA or the $IP_3$ receptor antagonist heparin. Also, the effect was partially blocked by bath-applied U-73122, a phospholipase C (PLC) inhibitor and by intracellularly injected GTP$\gamma$S, a non-hydrolyzable GTP analogue. Whereas, it was not altered by pertussin toxin (PTX) pretreatment.

**4** These results indicate that: (1) interaction of ginsenosides with membrane component(s) at the extracellular side leads to $Ca^{2+}$-activated $Cl^-$ channel opening in *Xenopus* oocyte membrane; and (2) this process involves PLC activation, the release of $Ca^{2+}$ from the $IP_3$-sensitive intracellular store and PTX-insensitive G protein activation.

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**Keywords:** Ginseng; ginsenosides; PLC; $Ca^{2+}$-activated $Cl^-$ channels; *Xenopus* oocytes

**Abbreviations:** ACh, acetylcholine; GTS, ginseng total saponins; PLC, phospholipase C; PTX, pertussis toxin

## Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a well-known folk medicine that has been shown to produce a variety of medicinal effects. Recent studies showed that ginseng saponins, which are also called ginsenosides, were the main molecular components responsible for the actions of ginseng. Ginsenosides have a four-ring, steroid-like structure with sugar moieties attached, and about 30 different ginsenosides have been isolated and identified from the root of *Panax* ginseng (Nah, 1997).

Several studies have demonstrated that ginsenosides can act on neuronal cells. They inhibit voltage-dependent $Ca^{2+}$ channels in chromaffin cells (Kim *et al*., 1998) and in sensory neurons *via* PTX-sensitive G proteins (Nah & McCleskey, 1994). They also inhibit acetylcholine (ACh)-stimulated catecholamine release from chromaffin cells *via* inhibition of $Na^+$ influx through nicotinic receptor-gated cation channels (Tachikawa *et al*., 1995; Kudo *et al*., 1998).

There is evidence that ginsenosides can act on non-neuronal cells as well. Chronic treatment with ginsenosides *in vivo* was shown to increase $^{32}PO_4$ incorporation into inositol phospholipids and to stimulate PLC activity in the mouse liver (Rim *et al*., 1997). Furthermore, ginsenosides was shown to increase intracellular $Ca^{2+}$ concentration in non-neuronal cells such as macrophages and NIH3T3 cells (Shin *et al*., 1996; Hong *et al*., 1998).

Despite numerous reports on the action of ginsenosides as described above, our understanding of their signal transduction mechanisms is relatively limited. It is particularly so in non-neuronal cells. In the present study we investigated the signalling pathway for ginsenosides using *Xenopus* oocytes, a convenient *in vitro* model which allows intracellular injection of putative second messengers or agents that interrupt the action of second messengers and thus substantially facilitates investigations of intermediate steps in signalling pathways (Parker & Yao, 1994; Hartzell, 1996). Here we present results suggesting that ginsenosides leads to the endogenous $Ca^{2+}$-activated $Cl^-$ channel opening *via* a well-defined signalling pathway, which involves PLC activation and $Ca^{2+}$ mobilization from $IP_3$-sensitive intracellular store.

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## Methods

### *Oocyte preparation*

*Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI, U.S.A.). Their care and handling were in accordance with the highest standards of institutional guidelines. To isolate oocytes, frogs were operated on under anaesthesia with an aerated solution of 3-amino benzoic acid ethyl ester. Oocytes were separated by treatment with collagenase and agitation for 2 h in a $Ca^{2+}$-free medium containing NaCl (82.5 mM), KCl (2 mM), $MgCl_2$ (1 mM), HEPES (5 mM), sodium pyruvate (2.5 mM), 100 units $ml^{-1}$ penicillin and 100 $\mu$g $ml^{-1}$ streptomycin. Stage V–VI oocytes were collected and stored in ND96 in mM: NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8 and HEPES 5, pH 7.5) supplemented with 0.5 mM theophylline and 50 $\mu$g $ml^{-1}$ gentamycin. This oocyte-containing solution was maintained at 16°C with continuous gentle shaking and changed everyday. Electrophysiological experiments with oocytes were performed within 4 days after their isolation.

### *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). The microelectrodes were filled with 3 M KCl and had a resistance of 0.2–0.7 MΩ. The electrophysiological experiments were performed at room temperature with Geneclamp 500 amplifier (Axon Instruments, CA, U.S.A.) or Oocyte Clamp (OC-725C, Warner Instrument, CT, U.S.A.). Linear leak and capacitance currents were corrected with leak subtraction procedure.

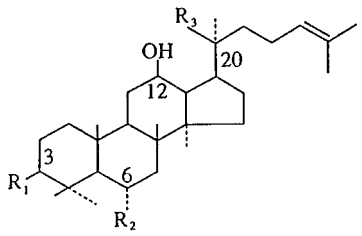
### *Drugs*

The drugs used in this study were either bath-applied or injected into oocytes with a Nanoject Automatic Oocyte Injector (Drummond Scientific, PA, U.S.A.). The injection pipette was pulled from glass capillary tubing, and its tip was broken to an outer diameter of about 20 $\mu$m. GTS, BAPTA, heparin and GTP$\gamma$S solutions (23–50 nl each) were injected into oocytes to give calculated intracellular concentrations of about 1 $\mu$g $\mu l^{-1}$, 1 mM, 1 $\mu$g $\mu l^{-1}$, and 150 $\mu$M, respectively.

Figure 1 shows the structures of the five representative ginsenosides. These ginsenosides and GTS were kindly obtained from Korea Ginseng and Tobacco Research Institute (Taejon, Korea). GTS contained $Rb_1$ (17.1%), $Rb_2$ (9.07%), Rc (9.65%), Rd (8.26%), Re (9%), Rf (3%), $Rg_1$ (6.4%), $Rg_2$ (4.2%), $Rg_3$ (3.8%), Ro (3.8%), Ra (2.91%) and other minor ginsenosides. BAPTA, heparin, niflumic acid and GTP$\gamma$S were obtained from Sigma. U-73122 (active PLC inhibitor) and U-73343 (inactive PLC inhibitor) were purchased from Calbiochem, and PTX was from List Biological Laboratories.

### *Data analysis*

All values are presented as mean ± s.e.mean. The differences between means of control and treatment data were analysed using unpaired *t*-test. $P<0.05$ was considered significant.




| *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 |

**Figure 1** Structures of the five representative ginsenosides. They differ at three side chains attached 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.

## Results

### *Enhancement of presumed $Ca^{2+}$-activated $Cl^-$ current by ginsenosides*

The oocytes recorded in this study had a resting membrane potential ($V_r$) of about −30 mV. Clamping these cells at a potential more negative than $V_r$ made an inward current flow. Whereas, clamping at a potential less negative than $V_r$ produced an outward current. Bath application of GTS increased these currents. To characterize the currents increased by GTS, the current-voltage (I–V) relationship was studied using the following protocol: at a holding potential of −80 mV, 500-ms voltage steps were applied from −60 to +60 mV in 20-mV increments. In the absence of GTS, the inward current at −60 mV was <0.1 $\mu$A and the outward current at +60 mV was 0.1–0.4 $\mu$A (Figure 2A). The addition of GTS to the bathing medium resulted in a slight increase in the inward current at −40 and −60 mV (Figure 2B). In contrast, at a potential more positive than −20 mV, GTS led to a large, voltage-dependent increase in outward current (Figure 2B). Typically, several microamperes of currents were recorded at +60 mV after treatment with 10 $\mu$g $ml^{-1}$ GTS. Figure 2C illustrates schematically the I–V relationship in the absence and presence of GTS. In both cases, the relationship was non-linear and the reversal potential was about −30 mV close to the calculated reversal potential of $Cl^-$ current. The analysis of the tail current (Figure 2D) elicited in the presence of GTS showed that the reversal potential of this current is also −30 mV, pointing to the possibility that the in- and outward currents increased by GTS were $Ca^{2+}$-activated $Cl^-$ current reported to be present in *Xenopus* oocytes (Barish, 1983; Miledi & Parker, 1984).

To provide further evidence for the notion above, we examined the effect of niflumic acid (NFA), a reversible blocker of $Ca^{2+}$-activated $Cl^-$ channels (White & Aylwin, 1990), on outward current. As expected, NFA (100 $\mu$M) slightly attenuated the baseline current, and it completely

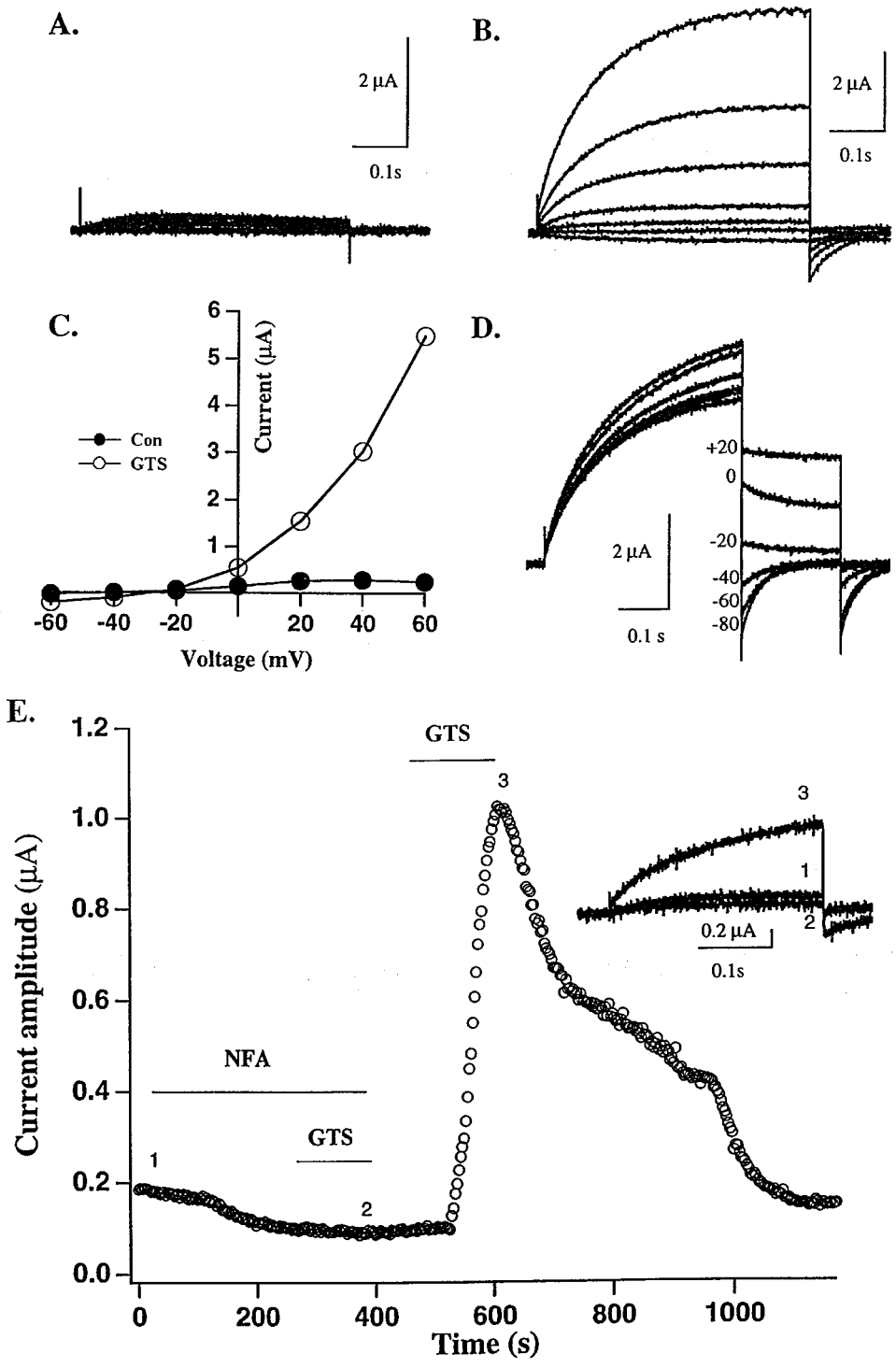



**Figure 2** Current produced by GTS and its blockade by the $Ca^{2+}$-activated $Cl^-$ channel blocker niflumic acid (NFA). (A) Current recorded in the absence of GTS. (B) Currents recorded in the presence of GTS (10 $\mu$g ml$^{-1}$) in the same cell in (A). (C) Current-voltage relationships. (D) Tail currents of the responses evoked in the presence of GTS (30 $\mu$g ml$^{-1}$). These currents were generated by first stepping the voltage to +40 mV from a holding potential of −80 mV and re-stepping the voltage to a less positive potential ranging from +20 to −80 mV with 20-mV decrements. Note that the reversal potential of the tail currents is somewhere between −20 and −40 mV. (E) Effect of NFA on GTS-evoked current. The amplitude of the outward current evoked every 5 s by a voltage step to +40 mV from a holding potential of −80 mV is plotted against time. Bars denote drug application (NFA: 100 $\mu$M, GST: 10 $\mu$g ml$^{-1}$). Inset: Traces of the current recorded in the presence of NFA (1), GTS (3) or both (2) are superimposed; the time points of the recording are indicated on the graph as 1, 2 and 3. This graph is representative of data obtained from six oocytes from three different frogs.

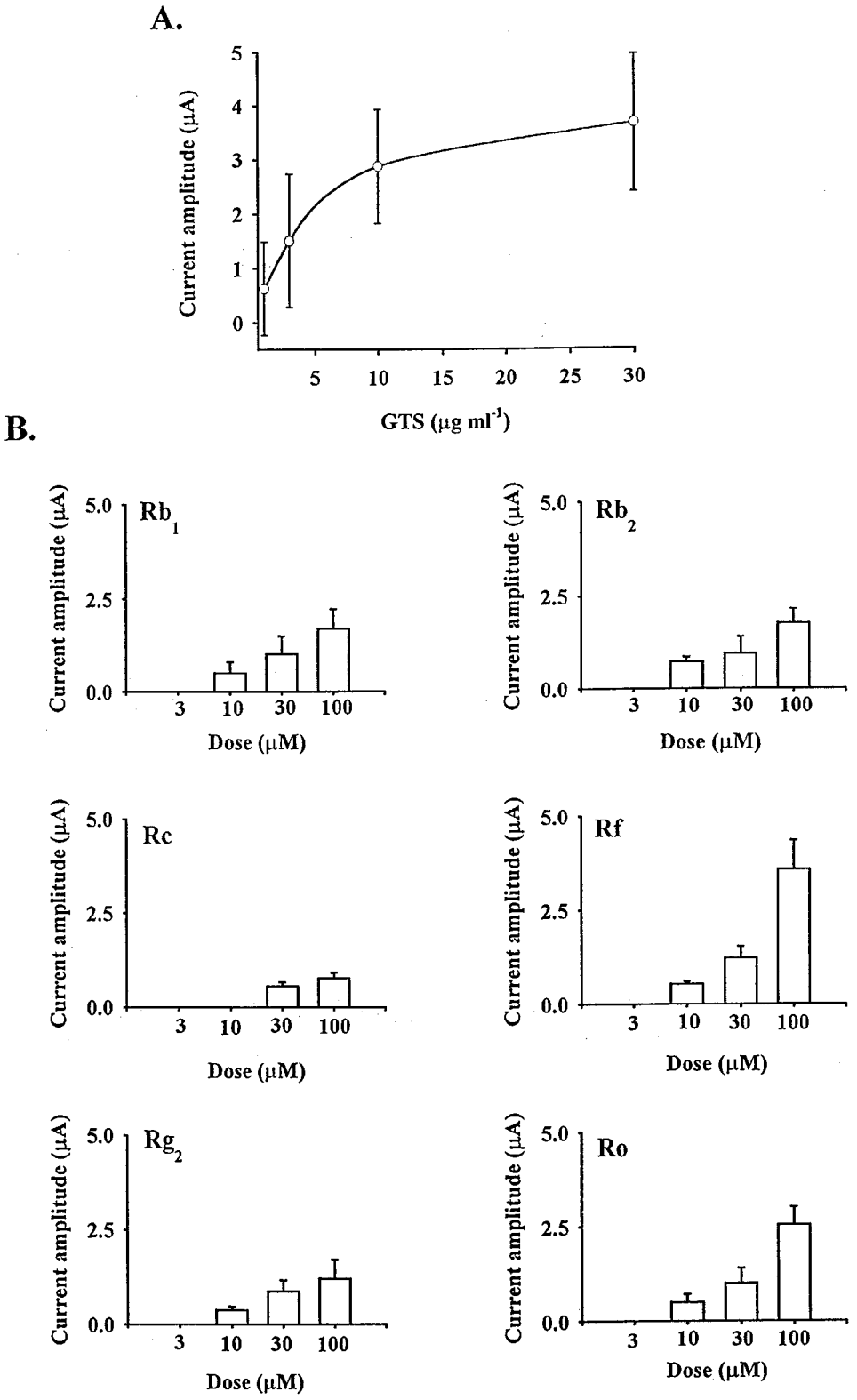



**Figure 3** Concentration dependence of the GTS (A) and fractionated ginsenoside (B) effects on outward current. In (A), a continuous curve was fitted according to the following equation: $I = I_{max}$ [GTS]/([GTS]) + $EC_{50}$, where $I_{max}$, the maximum current amplitude and $EC_{50}$, the concentration producing half-maximum activation, are equal to 4.6 $\mu$A and 4.4 $\mu$g $ml^{-1}$, respectively. In (B), the number of oocytes tested ranged from 7–10 for each ginsenoside.

prevented GTS from increasing outward current in a reversible fashion (Figure 2E).

The effect of GTS on the presumed $Ca^{2+}$-activated $Cl^-$ current was concentration-dependent; the $EC_{50}$ was $4.4 \pm 0.5$ $\mu g\ ml^{-1}$, and the maximal effect was obtained at about 10 $\mu g\ ml^{-1}$ (Figure 3A). Certain fractionated ginsenosides ($Rb_1$, $Rb_2$, Rc, Rf, $Rg_2$ and Ro) also had a concentration-dependent effect on the presumed $Cl^-$ current with the order of potency of $Rf > Ro > Rb_1 = Rb_2 > Rg_2 > Rc$ at 100 $\mu$M (Figure 3B). Whereas, other ginsenosides (Rd, Re and $Rg_1$ even at 100 $\mu$M-concentration) had little or no effect (data not shown).

### *Site of ginsenoside action*

Ginsenosides have steroid-like structure with sugar moieties attached (see Introduction). Therefore, it is unclear whether the effects produced by their bath application were mediated *via* cell surface binding site, intracellular site or both. To address this issue, we further examined the effects of GTS injected into oocytes. Unlike bath application (10 $\mu g\ ml^{-1}$), the intraoocyte injection ($n=6$, calculated concentration: 1 $\mu g\ \mu l^{-1}$) exerted essentially no effect (Figure 4), suggesting that the binding site(s) of ginsenosides is located on the surface of the oocyte.

### *Blockade of ginsenosides action by BAPTA and heparin*

The signal transduction mechanism underlying the effects of ginsenosides on the presumed $Ca^{2+}$-activated $Cl^-$ current was further studied. First, we examined whether the GTS effect on this current was dependent on external or internal $Ca^{2+}$. Removal of $Ca^{2+}$ from the bathing medium did not alter the effect of GTS (data not shown). By contrast, $Ca^{2+}$ chelation by intraoocyte injection of BAPTA almost completely abolished the effect of GTS (Figure 6). In addition, the BAPTA injection slightly reduced the baseline current recorded in the absence of GTS. Taken together, these observations suggest that GTS induce an elevation of $[Ca^{2+}]_i$ by mobilizing $Ca^{2+}$ from the intracellular store.

To see if $IP_3$ was involved in the action of ginsenosides, the $IP_3$ receptor antagonist heparin (Yao & Parker, 1993; Callamaras & Parker, 1994) was injected into oocytes. The heparin injection greatly attenuated the GTS effect (Figure 5). However, vehicle (distilled water) injection did not significantly alter the oocyte response to GTS. These results indicate that $IP_3$ plays a key role in the ginsenoside action.

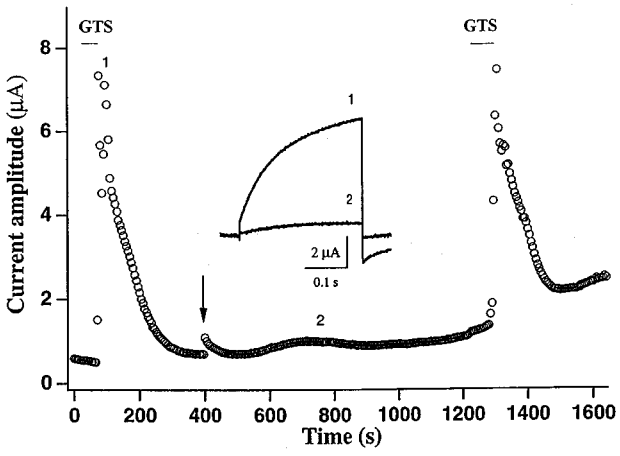



**Figure 4** Ginsenoside binding site(s) is located on the external surface of occyte membrane. The amplitude of the outward current evoked every 5 s by a voltage step to +40 mV from a holding potential of −80 mV is plotted against time. Bars denote bath application of GTS (10 $\mu g\ ml^{-1}$), and the arrow indicates intra-oocyte injection of 23 nl of GTS (oocyte concentration: 1 $\mu g\ \mu l^{-1}$). Inset: Traces of the current recorded at the time points 1 and 2 are superimposed. The graph is representative of data obtained from six oocytes from three different frogs.

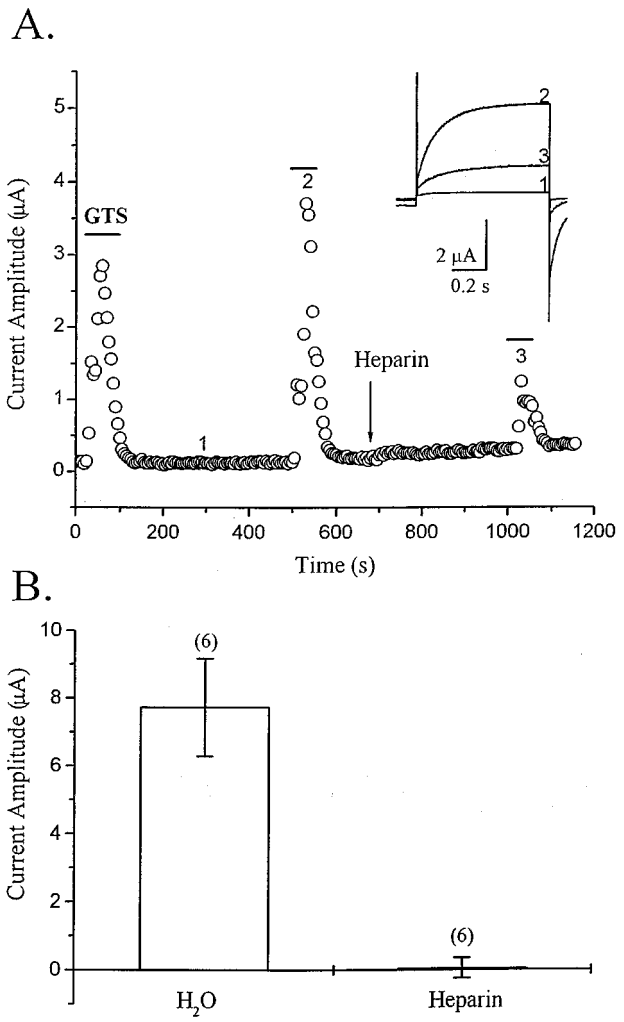



**Figure 5** Blockade of GTS action by intra-oocyte injection of heparin. (A) The amplitude of the outward current evoked every 5 s by a voltage step to +40 mV from a holding potential of −80 mV is plotted against time. Bars denote bath application of GTS (30 $\mu g\ ml^{-1}$), and the arrow indicates intra-oocyte injection of heparin (1 $\mu g$ final). Inset: Traces of the current recorded at the time points 1, 2 and 3 are superimposed. (B) The mean amplitudes of the outward currents recorded in the presence of GTS (100 $\mu g\ ml^{-1}$) following intra-oocyte injection (50 nl) of heparin (1 $\mu g$ final) or vehicle (i.e., $H_2O$) are plotted. A voltage step to +40 mV from a holding potential of −80 mV was used to evoke the outward currents. After heparin or $H_2O$ injection, oocytes were incubated for 20 min. The numbers in parentheses denote number of oocytes.

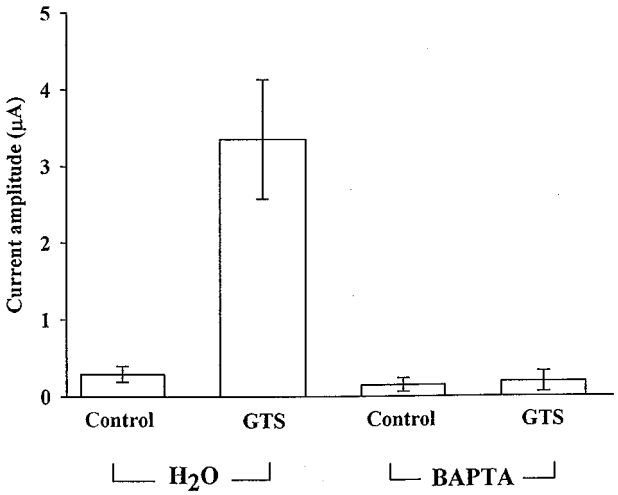



**Figure 6** Blockade of GTS action by intraoocyte injection of BAPTA. The mean amplitudes of the outward currents recorded in the absence and presence of GTS (30 $\mu$g ml$^{-1}$) following intra-oocyte injection (23 nl) of BAPTA (oocyte concentration: 1 mM) or vehicle (i.e., $H_2O$) are plotted. A voltage step to +40 mV from a holding potential of −80 mV was used to evoke the outward currents. After BAPTA or $H_2O$ injection, oocytes were incubated for 20 min. The numbers in parentheses denote number of oocytes.

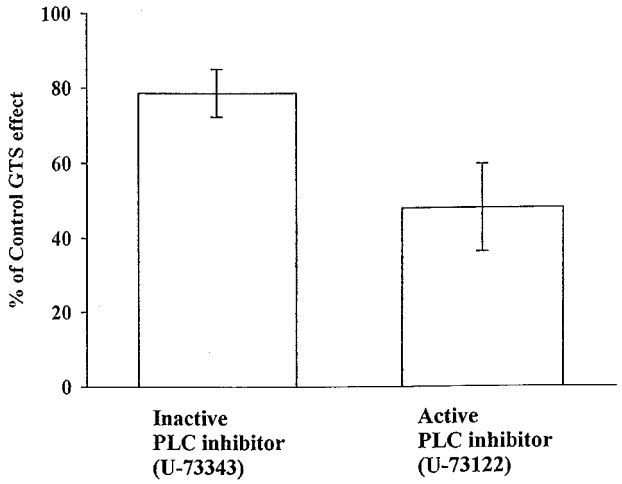



**Figure 7** Effects of active and inactive PLC inhibitors on GTS-induced outward current. The amplitudes of the currents recorded in GTS (30 $\mu$g ml$^{-1}$) before and during bath application of either the active (U-73122) or inactive inhibitor (U-73343) of PLC (1 $\mu$M) were measured, and their ratios (during/before) are plotted. The application of the PLC inhibitors preceded the GTS application by 5 min. The numbers in parentheses denote number of oocytes. Asterisk denotes statistical difference between the two data sets in the graph.

### *Blockade of ginsenoside action by PLC inhibitor*

Since the ginsenoside action involves the participation of $IP_3$, it may require PLC activation for the production of $IP_3$. To test this possibility, the effects of the active PLC inhibitor U-73122 and its inactive analogue U-73343 (Thompson *et al.*, 1991) were examined on GTS action. Bath application of U-73122, but not U-73343, significantly depressed the action of GTS: the GTS-induced current in U-73122 was 48.0±11.7% of the control, whereas the current in U-73343 was 78.6±6.4% of the control (Figure 7). These results indicate that the PLC inhibitor partially blocked the GTS action.

### *Effects of PTX and GTPγS on GTS action*

Previously, we demonstrated that ginseng root extract and ginsenoside Rf inhibited voltage-dependent $Ca^{2+}$ channels through PTX-sensitive G proteins in rat sensory neurons (Nah & McCleskey, 1994; Nah *et al.*, 1995). Hence, we tested the effects of PTX and of the non-hydrolyzable GTP analogue GTPγS on the GTS action in oocyte (Dascal *et al.*, 1984). As summarized in Table 1, pretreatment of PTX (2 $\mu$g ml$^{-1}$, 16 h) did not depress the action of GTS. However, the intra-oocyte injection of GTPγS (calculated concentration: 150 $\mu$M) significantly blocked the GTS effect on the oocyte current.

**Table 1** Effect of PTX pretreatment or intracullular injection of GTPγS on GTS-induced $Ca^{2+}$-activated $Cl^-$ currents

| | *$Ca^{2+}$-activated $Cl^-$ currents* ($\mu$A) | |
|---|---|---|
| *Treatment* | *Basal* | *GTS* (30 $\mu$g ml$^{-1}$) |
| −PTX | 1.25±0.20 (6) | 6.95±0.71 (6) |
| +PTX† | 1.16±0.05 (6) | 6.49±0.26 (6) |
| $H_2O$ | 0.22±0.04 (16) | 2.90±0.52 (16) |
| GTPγS$^{\#}$ | 0.48±0.06 (14) | 0.84±0.12* (14) |

*$P<0.001$ significantly different from $H_2O$ injected oocytes. †Current apmlitudes were measured after 16 h treatment with PTX (2 $\mu$g ml$^{-1}$). $^{\#}$Current amplitudes were measured after 10 min after intracellular injection of $H_2O$ or GTPγS (150 $\mu$M). The number of oocytes tested are shown in parenthesis.

## Discussion

To investigate the signalling pathway underlying ginsenoside action, we utilized *Xenopus* oocytes. The reason for employing this model was 2 fold. First, it allows the intracellular injection of putative second messengers or drugs that block the action of second messengers without much damaging the cells under investigation. Second, it exhibits various, well-studied ion channel activities which may be used as signal transduction markers.

In this study, we provided evidence that interaction of ginsenosides with their binding site(s) located at the cell surface increases the endogenous $Ca^{2+}$-activated $Cl^-$ current *via* PLC activation leading to $Ca^{2+}$ release from the intracellular store by $IP_3$ and this process involves PTX-insensitive G protein.

The effect of ginsenosides on $Ca^{2+}$-activated $Cl^-$ current was transient in some oocytes tested. The current produced by ginsenosides diminished spontaneously after reaching peak amplitude even in the continued presence of ginsenosides (Figure 5A). Thus, the $Cl^-$ channels seemed to be desensitized to ginsenosides. Moreover, the channels showed cross-desensitization to ginsenosides and ACh (data not shown). This finding, coupled with the reports that stimulation of oocyte muscarinic receptors by ACh activates PLC leading to intracellular $Ca^{2+}$ mobilization and activation

of $Cl^-$ current (Dascal *et al*., 1984; Berridge & Irvine, 1989; Lechleiter & Clapham, 1992), suggests that the ginsenosides binding site(s) shares a common signalling pathway with muscarinic receptor. However, this does not indicate that ginsenosides act on muscarinic receptors, since the muscarinic receptor antagonist atropine had no effect on the ginsenoside action (data not shown).

About 30 different ginsenosides have now been isolated and identified from *Panax ginseng*. Studies have shown that certain ginsenosides are more potent than others (Nah *et al*., 1995; Tachikawa *et al*., 1995). In this study, we examined the effects of nine different ginsenosides ($Rb_1$, $Rb_2$, Rc, Rd, Re, Rf, $Rg_1$, $Rg_2$ and Ro) and found that there was a clear difference in potency among these fractionated ginsenosides. Interestingly, we noted that GTS, i.e., an unfractionated mixture of ginsenosides, was more potent than any of the fractionated ginsenosides tested in the present study. This seemed to be due to a synergistic effect of the individual ginsenosides contained in GTS. However, other trace ingredients of ginseng such as alkaloids, carbohydrates, lipophilic components, phenolic components and $\beta$-sitosterol did not affect $Ca^{2+}$-activated $Cl^-$ channels in *Xenopus* oocytes (data not shown).

On the other hand, lysophosphatidic acid (LPA), trypsin, or hyaluronan also induces activation of the endogenous $Ca^{2+}$-activated $Cl^-$ channel in *Xenopus* oocytes in similar manner with ginsenosides (Tigyi & Miledi, 1992; Durieux *et al*., 1994; Fraser, 1997). However, higher concentration of trypsin or hyaluronan was required for the activation of $Ca^{2+}$-activated $Cl^-$ channels than that used in present study. Recently, the endogenous receptor against LPA has been cloned in *Xenopus* oocytes (Guo *et al*., 1996). In addition to *Xenopus* oocytes, other cell types also express the endogenous $Ca^{2+}$-activated $Cl^-$ channels. For example, lung epithelial cells possess $Ca^{2+}$-activated $Cl^-$ channels and they are involved in airway secretion (Ran & Benos, 1991). Interestingly, NFA inhibits the endogenous $Ca^{2+}$-activated $Cl^-$ channels in oocytes but not in lung epithelial cells (White & Aylwin, 1990; Ji *et al*., 1998). Moreover, treatment of phorbol ester, a protein kinase C activator, activates the endogenous $Ca^{2+}$-activated $Cl^-$ channels in lung endothelial cells but inhibits the endogenous $Ca^{2+}$-activated $Cl^-$ channels in *Xenopus* oocytes (Welsh, 1987; Boton *et al*., 1990). Thus, the regulatory modes on the endogenous $Ca^{2+}$-activated $Cl^-$ might be cell-type specific. Recently, the clonings of $Ca^{2+}$-activated $Cl^-$ channels in various species of tracheal endothelium have been reported (Cumningham *et al*., 1995; Gandhi *et al*., 1998; Gruber *et al*., 1998). However, it seems unlikely that this is the same channel investigated here because the channel kinetics and pharmacology differ (Ji *et al*., 1998).

In summary, we have obtained results suggesting that ginsenosides can increase $Ca^{2+}$-activated $Cl^-$ current in *Xenopus* oocytes *via* a signalling pathway linked to muscarinic ACh receptor, which involves G protein-coupled PLC activation and $Ca^{2+}$ mobilization from $IP_3$-sensitive intracellular store.

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