British Journal of Pharmacology (2004) 142, 585–593
www.nature.com/bjp

Ginseng saponins induce store-operated calcium entry in *Xenopus* oocytes

[1,4]Sang Min Jeong, [1,4]Jun-Ho Lee, [2]Sunoh Kim, [2]Hyewhon Rhim, [1]Byung-Hwan Lee, [1]Jong-Hoon Kim, [3]Jae-Wook Oh, [1]Sang-Mok Lee & *,[1]Seung-Yeol Nah

[1]Research Laboratory for the Study of Ginseng Signal Transduction and Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul 143-701, Korea; [2]Biomedical Research Center, KIST, Seoul 136-701, Korea; [3]Department of Anatomy College of Medicine, Chosun University, Kwangju 501-759, Korea

1 We investigated the effect of the active ingredients of *Panax* ginseng, ginsenosides, on store-operated Ca^{2+} entry (SOCE) using a two-electrode voltage clamp technique in *Xenopus* oocytes in which SOCE is monitored through Ca^{2+}-activated Cl^- currents.

2 Under hyperpolarizing voltage clamp conditions, treatment with ginsenosides produced a biphasic Ca^{2+}-activated Cl^- current consisting of a rapid transient inward current and a slowly developing secondary sustained inward current. The transient inward current was inactivated rapidly, whereas the sustained inward current persisted for nearly 10 min. The effect of ginsenosides on the biphasic current was dose-dependent and reversible. The EC_{50} was 42.8 ± 11.6 and $46.6 \pm 7.1\ \mu g\ ml^{-1}$ for the transient and sustained inward current, respectively.

3 In the absence of extracellular Ca^{2+} ginsenosides induced only a transient inward current but in the presence of extracellular Ca^{2+} ginsenosides induced the biphasic current. Magnitudes of the sustained currents were dependent on extracellular Ca^{2+} concentration. Sustained inward current induced by ginsenosides, but not transient inward current, and ginsenoside-induced store-operated Ca^{2+} (SOC) currents (I_{SOC}) were blocked by La^{3+}, a Ca^{2+} channel blocker, suggesting that the sustained inward current and I_{SOC} was derived from an influx of extracellular Ca^{2+}.

4 Treatment with 2-APB and heparin, which are IP_3 receptor antagonists, inhibited the ginsenoside-induced biphasic current. Treatment with the PLC inhibitor, U73122, also inhibited the ginsenoside-induced biphasic current. Intraoocyte injection of ATP-γS, but not adenylyl AMP-PCP, induced a persistent activation of ginsenoside-induced sustained current but did not affect the transient current.

5 In rat hippocampal neurons, ginsenosides inhibited both carbachol-stimulated intracellular Ca^{2+} release and intracellular Ca^{2+} depletion-activated SOCE.

6 These results indicate that ginsenoside might act as a differential regulator of intracellular Ca^{2+} levels in neurons and *Xenopus* oocytes.

British Journal of Pharmacology (2004) **142,** 585–593. doi:10.1038/sj.bjp.0705797

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

Abbreviations: AMP-PCP, adenylyl 5′-(β,γ-methylene)-diphosphonate; 2-APB, 2-aminoethxydiphenyl borate; ATP-γS, adenosine 5′-*o*-(3-Thiotriphosphate); BAPTA, bis (*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetracetic acid; GPCRs, G protein coupled receptors ginseng total saponins; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; SOC, store-operated Ca^{2+} channel; SOCE, store-operated Ca^{2+} entry

Introduction

Intracellular Ca^{2+} is a key molecule involved in many kinds of signal transduction pathways in a variety of cells. Increases in Ca^{2+} levels in cells regulate secretion, cell division, growth and differentiation, muscle contraction, and receptor internalization (Berridge *et al*., 1998). Free intracellular Ca^{2+} can be increased either by Ca^{2+} influx from extracellular fluid or through the release of stored Ca^{2+} from the intracellular compartment, endoplasmic reticulum (ER) (Parekh & Penner, 1997). In nonexcitable cells, an increase in cytoplasmic free Ca^{2+} is mediated via stimulation of receptors such as G protein coupled receptors (GPCRs), which are coupled to activation of the phospholipase C (PLC) pathway and production of inositol 1,4,5-triphosphate (IP_3). IP_3 then triggers an increase in the levels of cytosolic free Ca^{2+} from IP_3-sensitive ER, resulting in depletion of intracellular calcium stores. Depletion of intracellular Ca^{2+} by IP_3 causes Ca^{2+} entry through plasma membrane store-operated Ca^{2+} (SOC) channels via a process known as store-operated Ca^{2+} entry (SOCE) (Putney, 1992). This Ca^{2+} influx is important not only for refilling the depleted stores but also for modulating various Ca^{2+}-dependent intracellular signals after GPCR agonist stimulation (Yao & Parker, 1994).

Xenopus oocytes, with their large size and easy handling, are a useful model system for investigating the machinery of membrane signal transduction. The intracellular injection of putative second messengers or the introduction and expression of foreign genes can substantially facilitate investigations

*Author for correspondence; E-mail: synah@konkuk.ac.kr
[4]These authors contributed equally to this work.
Advance online publication: 17 May 2004

into the intermediate steps of signaling pathways. Moreover, *Xenopus* oocytes have endogenous Ca^{2+}-activated Cl^- channels that are well understood (Barish, 1983; Miledi & Parker, 1984), and which have been used for the study of both intracellular Ca^{2+} release and the SOCE process (Lechleiter & Clapham, 1992; Callamaras & Parker, 1994; Parker & Yao, 1994). For example, stimulation of oocyte muscarinic receptors by ACh leads to intracellular Ca^{2+} mobilization and activation of Ca^{2+}-activated Cl^- channels, resulting in depletion of calcium stores and initiation of SOCE (Dascal *et al*., 1984; Berridge & Irvine, 1989; Lechleiter & Clapham, 1992).

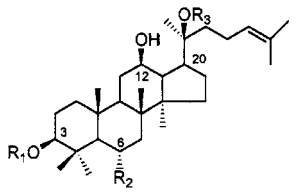
Choi *et al*. (2001b, c) demonstrated that, in *Xenopus* oocytes, ginsenosides enhanced the endogenous Ca^{2+}-activated Cl^- current via a $G\alpha_{q/11}$-PLC-β3 pathway coupled to IP_3-mediated intracellular Ca^{2+} release. Thus, treatment of *Xenopus* oocytes with ginsenosides might initiate two events. Firstly, ginsenoside-induced intracellular Ca^{2+} release, which is mediated through IP_3, coupled to activation of Ca^{2+}-activated Cl^- channels as reported in previous studies (Choi *et al*., 2001b, c). Secondly, ginsenoside-induced intracellular Ca^{2+} release followed by intracellular Ca^{2+} depletion might be responsible for activation of the process of SOCE. However, it is not yet proven whether or not ginsenoside-induced activation of Ca^{2+}-activated Cl^- channels is also coupled to subsequent activation of the SOCE process. Moreover, little is known about the signal transduction component(s) involved in SOCE activation by ginsenoside. In the present study, we investigated the signal transduction pathway and the relationship between activation of Ca^{2+}-activated Cl^- channels and SOCE in the presence or absence of ginsenosides using *Xenopus* oocytes, a convenient *in vitro* model system (Dascal, 1987). In addition, we investigated the effect of ginsenosides on SOCE in neuronal cells, since it has not yet been determined whether ginsenosides might also regulate SOCE in neuronal cells.

Here, we demonstrate that ginsenoside-induced intracellular Ca^{2+} release in *Xenopus* oocytes is coupled to activation of SOCE via a PLC-IP_3-extracellular Ca^{2+} influx pathway, in contrast to ginsenoside action in neurons whereby both receptor agonist-mediated intracellular Ca^{2+} release and depletion-activated SOCE are inhibited.

Methods

Drugs

Figure 1 shows the structures of the eight representative ginsenosides. These ginsenosides (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. GTS was diluted with bath medium ND96 before use. CNQX was purchased from Tocris (Ellisville, MO, USA). TTX was purchased from Alomone Labs (Jerusalem, Israel). 2-Aminoethxydiphenyl borate (2-APB), heparin, [adenosine 5-*O*-(3-Thiotriphosphate)] (ATP-[γS]), adenylyl 5′-(β,γ-methylene)-diphosphonate (AMP-PCP) and other chemicals were of analytical grade and purchased from Sigma (St Louis, MO, U.S.A.). The drugs used in this study were either bath-applied or injected into oocytes with a Nanoject Automatic Oocyte



Ginsenosides	R_1	R_2	R_3	PD or PT
Rb_1	-Glc_2-Glc	-H	-Glc_6-Glc	PD
Rb_2	-Glc_2-Glc	-H	-Glu_6-Ara(pyr)	PD
Rc	-Glc_2-Glc	-H	-Glc_6-Ara(fur)	PD
Rd	-Glc_2-Glc	-H	-Glc	PD
Re	-H	-O-Glc_2-Rha	-Glc	PT
Rf	-H	-O-Glc_2-Glc	-H	PT
Rg_1	-H	-O-Glc	-Glc	PT
Rg_2	-H	-O-Glc_2-Rha	-H	PT

Figure 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.

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 μm. In total, 23–50 nl of ATP-[γS], AMP-PCP, and heparin solutions were injected into oocytes to give a calculated intracellular concentration of about 100, 100, and 1 $\mu g\,\mu l^{-1}$, respectively.

Oocyte preparation

Xenopus laevis frogs were obtained from Xenopus I (Ann Arbor, MI, U.S.A.). Before oocyte extraction, they were kept in a temperature-controlled aquarium ($18 \pm 1^{\circ}C$) with a 12 : 12 h light–dark cycle, and food was given every 2 days. Oocytes were extracted under deep anesthesia, which was induced by immersing frogs in an aerated solution of 0.15% 3-amino benzoic acid ethyl ester. Following oocyte extraction, frogs were killed by anesthetic overdose. The extracted 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 U ml^{-1} penicillin and 100 $\mu g\,\mu l^{-1}$ streptomycin. Stage V–VI oocytes were collected and stored in ND96 (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 $\mu g\,\mu l^{-1}$ gentamicin. This oocyte-containing solution was maintained at 18°C with continuous gentle shaking and refreshed everyday.

Oocyte recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings. The chamber was constructed by milling two concentric wells into the chamber bottom (diameter/height for upper well: 8/3 mm, lower well: 6/5 mm) and gluing plastic meshes (~0.4-mm grid diameter) onto the bottom of the upper well. The perfusion inlet (~1-mm diameter) was formed through the wall of the lower well, and the suction tube was placed on the edge of the upper well. The oocyte was placed on the net that separates the upper and lower wells. The grids of the net served as dimples that kept the

oocytes in place during electrophysiological recordings. The oocytes were impaled with two microelectrodes filled with 3 M KCl (0.2–0.7 MΩ) and voltage-clamped at −40 mV. In our experiments, the membrane potential was held at −40 mV, stepped up to +40 mV for 1 s, down to −140 mV for 1 s, and then back to +40 mV for 1 s before returning to the holding potential. These steps were repeated every 10 s. For I_{SOC} current measurement, oocytes were held at −40 mV and voltage ramps were applied from −140 to +60 mV for 300 ms. For I_{SOC} current measurement, the external solution was 30 mM $CaCl_2$, 55 mM NaCl, and 10 mM HEPES (pH 7.5). The electrophysiological experiments were performed at room temperature with Oocyte Clamp (OC-725C, Warner Instrument, CT, USA) and both stimulation and data acquisition were controlled by pClamp 8 (Axon Instruments) (Choi *et al*., 2003a).

Hippocampal neuron preparation

Hippocampal cell cultures were prepared from neonatal (P1) Sprague–Dawley rats. The hippocampi were dissected and incubated with 0.05% papain in Leibovitz L-15 medium at 37°C for 20 min. Cells were then mechanically dissociated with fire-polished Pasteur pipettes by trituration and plated on poly-L-lysine coated coverslips in a 35-mm culture dish. Cells were maintained in Neurobasal/B27 medium containing 0.5 mM L-glutamine, 25 μM glutamate, 25 μM 2-mercaptoethanol, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cultures were fed twice a week with the same medium without glutamate. Experiments were carried out on neurons after 7–15 days in culture (Kim *et al*., 2002).

Intracellular Ca^{2+} imaging

The acetoxymethyl-ester form of fura-2 (Fura-2/AM; Molecular probes, Eugene, OR, U.S.A.) was used as fluorescent Ca^{2+} indicator. Hippocampal neurons were incubated for 40–60 min at 37°C with 5 μM Fura-2/AM and 0.001% Pluronic F-127 in normal HEPES-buffered medium composed of (in mM): 150NaCl, 5KCl, 1$MgCl_2$, 2$CaCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH. High K^+ buffered medium contained (in mM): 105NaCl, 50KCl, 1$MgCl_2$, 2$CaCl_2$, 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH. The cells were then washed with HEPES-buffered solution, placed on an inverted microscope (Olympus, Japan), and illuminated using a xenon arc lamp. Excitation wavelengths (340 and 380 nm) were selected by a computer-controlled filter wheel (Sutter Instruments, CA, USA). Data were recorded every 2 s but with a shutter in the light path between exposures to protect the cells from photo-toxicity. Emitter fluorescence light was reflected through a 515 nm long-pass filter to a frame transfer cooled CCD camera. Ratios of emitted fluorescence were calculated using a digital fluorescence analyzer and converted to indicate the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). All imaging data were collected and analyzed using Universal Imaging software (West Chester, PA, U.S.A.) (Kim *et al*., 2002).

Data analysis

To obtain a concentration–response curve of the effect of GTS on the transient or sustained Ca^{2+}-activated Cl^- current, the observed peak amplitudes were measured, plotted, and then fitted to the Hill equation below using Origin software (Northampton, MA, U.S.A.): $y/y_{max} = [A]^n/([A]^n + [EC_{50}]^n)$, where y is the transient or sustained Ca^{2+}-activated Cl^- current at a given concentration of GTS; y_{max} the maximal peak transient or sustained Cl^- current; EC_{50} is the concentration of GTS producing half-maximum effect of the control response to GTS; and $[A]$ is the concentration of GTS and n is the interaction coefficient. All values are presented as mean ± s.e.m. The differences between control and treatment data means were analyzed using an unpaired t-test, with $P < 0.05$ considered as significant.

Results

Effect of ginsenosides on two types of Ca^{2+}-activated Cl^- current in oocytes held in voltage clamp and subjected to multistep voltage cycles

In previous reports, Kuruma & Hartzell (1999) demonstrated fully that intraoocyte injection of IP_3 induced two distinct Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes using multisteps in a voltage clamp. Using this protocol they could distinguish Cl^- currents that are activated by intracellular Ca^{2+} release from Cl^- currents that are activated by extracellular Ca^{2+} influx (SOCE). Our group earlier reported that in *Xenopus* oocytes, ginsenosides enhanced endogenous Ca^{2+}-activated Cl^- currents evoked by depolarizing voltage clamps (Choi *et al*., 2001b, c). Here, we first tested whether treatment with ginsenosides also induces those distinct Ca^{2+}-activated Cl^- currents following the same protocol. For this, oocytes were held at −40 mV and stepped to +40 mV for 1 s, to −140 mV for 1 s, then back to +40 mV for 1 s before return to the holding potential (Figure 2a). The oocytes recorded in this study had a resting membrane potential (V_r) of about −20 to −30 mV. As shown in Figure 2b, ginsenoside treatment induced two distinct Ca^{2+}-activated Cl^- currents over different time periods during this voltage protocol, similar to those found by Kuruma & Hartzell (1999) in IP_3 injected oocytes. The $I_{Cl1\text{-}S}$ (activated by release of Ca^{2+} from intracellular Ca^{2+} stores) current first appeared at the end of the first 1 s step to +40 mV. The outward $I_{Cl1\text{-}T}$ (activated by external Ca^{2+} entry) current was measured as the peak time-dependent outward current during the second 1 s +40 mV step, which was preceded by a −140 mV pulse where the driving force for Ca^{2+} influx is higher than −40 mV. This preceding −140 mV step, before the +40 mV step up, also induced the inward I_{Cl2} (also activated by external Ca^{2+} entry). Thus, the $I_{Cl1\text{-}S}$ current (a) activated by intracellular Ca^{2+} release was recorded first and this was then followed by $I_{Cl1\text{-}T}$ (c) and I_{Cl2} (b), activated by external Ca^{2+} influx (Figure 2a). This suggests that treatment of oocytes with ginsenosides induced not only intracellular Ca^{2+} release but also Ca^{2+} influx from external media (Figure 2b and c).

Ginsenoside treatment induces biphasic inward currents following hyperpolarizing voltage steps in Xenopus *oocytes*

Parekh (1995) and Hartzell (1996) demonstrated, using a hyperpolarizing voltage step, that addition of 5-HT to oocytes

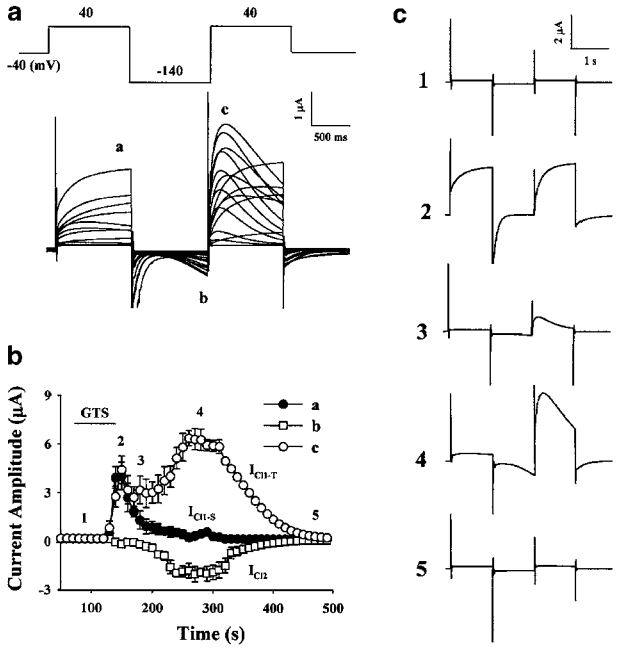


Figure 2 Effect of ginsenosides (GTS) on noninactivating outward Cl^- current ($I_{Cl1\text{-}S}$), slow inward Cl^- current (I_{Cl2}), and transient outward Cl^- ($I_{Cl1\text{-}T}$). Oocytes were voltage-clamped with two microelectrodes and GTS (50 $\mu g\,ml^{-1}$) was added to the bath solution following the voltage pulse every 10 s. (a) Current amplitudes recorded at every 10 s for 50 trials with a three-step pulse command were overlapped. Peak current values of three Cl^- current components were measured at the points *a* ($I_{Cl1\text{-}S}$), *b* (I_{Cl2}), and *c* ($I_{Cl1\text{-}T}$) indicated in the figure. (b) Time course of development of $I_{Cl1\text{-}S}$ (*a*), I_{Cl2} (*b*), and $I_{Cl1\text{-}T}$ (*c*) in response to GTS treatment of an oocyte bathed in ND96 (mean ± s.e.m; $n = 8$ oocytes). Steady-state outward current at the end of the first +40 mV pulse is $I_{Cl1\text{-}S}$, inward current during −140 mV pulse is I_{Cl2}, and transient outward current during second +40 mV pulse is $I_{Cl1\text{-}T}$. Current amplitudes recorded at points *a*, *b*, and *c* were illustrated as a function of time. The bar represents continuous application of GTS. (c) Five representative current traces of development and decay of $I_{Cl1\text{-}S}$, I_{Cl2}, and $I_{Cl1\text{-}T}$ at different times 1, 2, 3, 4, and 5 during the experiment. Times of traces are indicated by 1–5 in (b).

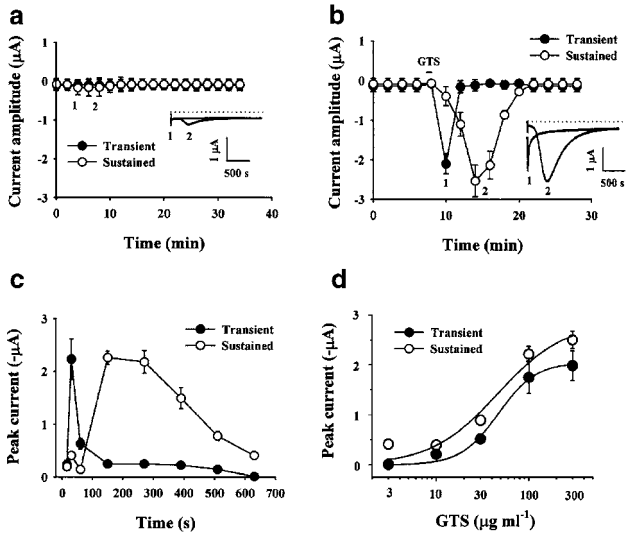


Figure 3 Treatment with GTS induces a biphasic inward Cl^- current under hyperpolarizing conditions. Oocytes were voltage-clamped at −40 mV and stepped to −140 mV for 5 s. The amplitude of the inward current evoked every 2 min is plotted against time in the absence (a) or presence of GTS (50 $\mu g\,ml^{-1}$). Inset: Traces of current recorded in the absence (a) or presence of GTS (b) with transient and sustained currents superimposed. The time points of the recording are indicated in the graph as 1 and 2. The graph is representative of data obtained from six oocytes from three different frogs. (c) Time course of appearance of biphasic currents. The inward current was recorded at 15, 30, 60, 150, 270, 390, 510 and 630 s after GTS (50 $\mu g\,ml^{-1}$) application. The peak current amplitude induced by GTS is plotted against time. These currents were generated by stepping to −140 mV from a holding potential of −40 mV, for 5 s. (d) Concentration-dependent effect of GTS on the transient and sustained currents. The illustrated histograms shows peak current response recorded during the treatment of GTS (3, 10, 30, 100, or 300 $\mu g\,ml^{-1}$). These data are shown as mean ± s.e.m. ($n = 10$–12 oocytes each).

that are heterologously expressing 5-HT_{2C} receptor, or intraoocyte injection of IP_3, both evoked a biphasic inward Cl^- current. This biphasic current consists of a rapid transient inward current, which is activated by release of Ca^{2+} from intracellular Ca^{2+} stores, followed by a slowly developing secondary sustained inward current, which is activated by external Ca^{2+} entry. We tested whether ginsenoside treatment induced a biphasic Cl^- current under hyperpolarizing voltage conditions. The oocytes were held (V_h) at −40 mV and stepped to −140 mV for 5 s with 2 min intervals. As shown in Figure 3a, in the absence of ginsenosides we could observe only a slight sustained current but not a transient current (Figure 3a). In contrast, we could observe a biphasic current following application of ginsenosides to the bath (50 $\mu g\,ml^{-1}$) for 60 s. The first phase was a rapid transient current and the second was a sustained inward current (Figure 3b, *inset*). The first transient phase of inward Cl^- current was rapidly inactivated and the second phase was persistent for about 10 min, even after removal of the ginsenosides by washing (Figure 3b). To further characterize the two kinds of Cl^- currents evoked by ginsenosides, we studied the currents over a time course. Figure 3c shows the time course for activation and inactivation of the biphasic current recorded after 15, 30, 60, 150, 270, 390, 510, and 630 s in the presence of ginsenosides. The transient inward current appeared 15 s after bath application of ginsenosides and was inactivated by 60 s. The sustained current appeared around 150 s after ginsenoside addition and persisted for more than 10 min. The effect of ginsenosides on both transient and sustained currents was concentration-dependent. The EC_{50} was 42.8 ± 11.6 and 46.6 ± 7.1 $\mu g\,ml^{-1}$ for the transient and sustained inward Cl^- currents, respectively (Figure 3d).

Effects of extracellular Ca^{2+} concentration and Ca^{2+} channel blocker on ginsenoside-evoked biphasic inward Cl^- current

The above results prompted us to formulate a working hypothesis that treatment with ginsenosides might activate the SOCE process following initial Ca^{2+} depletion from intracellular Ca^{2+} stores in *Xenopus* oocytes. This follows since the time course of appearance of the sustained inward Cl^- current was much slower than the transient inward Cl^- current. It suggests that the transient inward Cl^- current therefore reflects Ca^{2+} release from internal stores, whereas the sustained inward Cl^- current is derived from extracellular

Ca^{2+} influx via a pathway activated by IP_3-mediated internal store depletion as proven in previous reports (Choi *et al*., 2001b). As the first step toward testing this hypothesis, we investigated the effect of ginsenosides on changes in the biphasic Cl^- current at various extracellular Ca^{2+} concentrations. Thus, we recorded the ginsenoside-evoked biphasic current in ND96 plus various extracellular Ca^{2+} concentrations (0, 0.1, 1.8, and 20 mM). As shown in Figure 4a, the transient inward Cl^- current was not much changed in the range of external Ca^{2+} concentrations tested. However, the sustained inward Cl^- currents were abolished or significantly reduced when external Ca^{2+} was absent or lowered to 0.1 mM as shown in a previous report (Parekh, 1995). Moreover, when we raised external Ca^{2+} from 0.1 to 1.8 mM, we observed the sustained inward Cl^- currents (from 0.14 ± 0.04 to $0.51 \pm 0.09\,\mu$A, $^{**}P < 0.01$). We also tested whether or not the sustained inward Cl^- current evoked by ginsenoside treatment is affected by external La^{3+} treatment, a Ca^{2+}

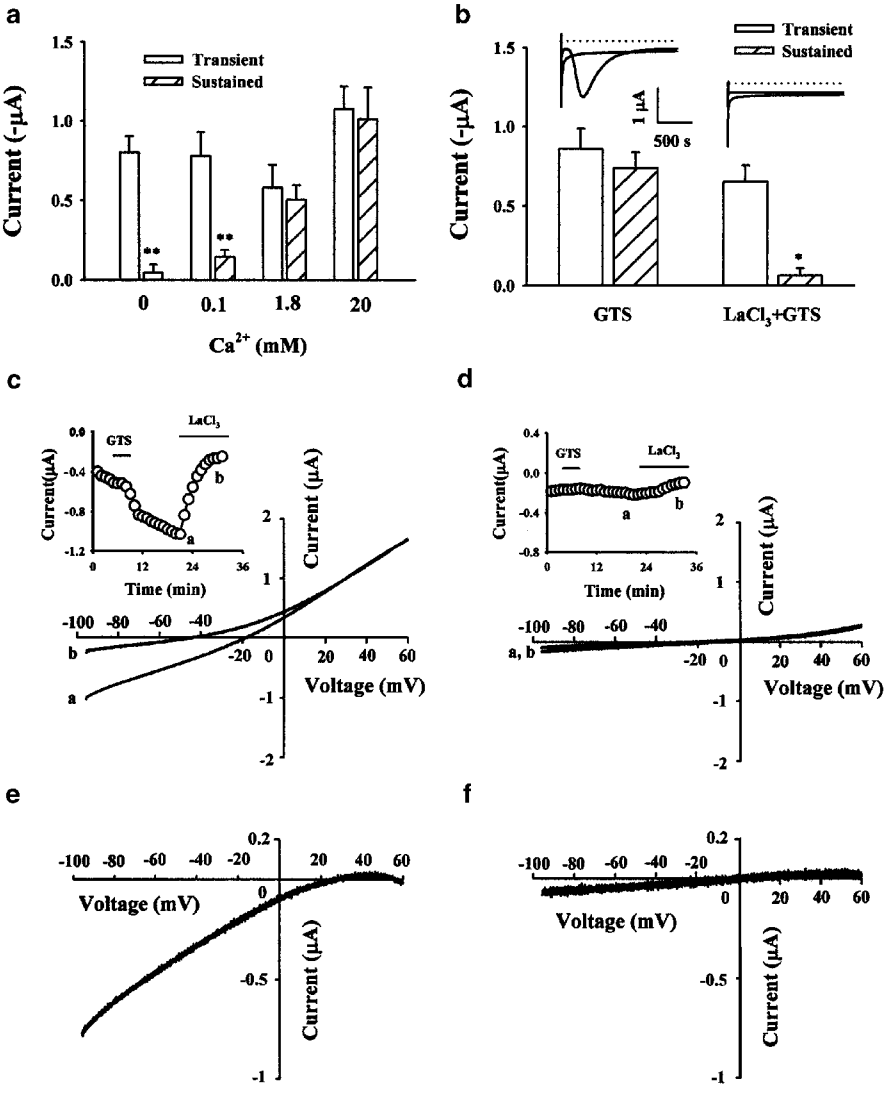


Figure 4 GTS-induced sustained inward Cl^- current and GTS-induced I_{SOC} current are dependent on extracellular Ca^{2+}. (a) Histograms illustrate the mean peak amplitude of GTS-induced transient and sustained inward Cl^- currents (mean $\pm$ s.e.m; $n = 8$–10 oocytes each). Oocytes were bathed in Ca^{2+}-free ND96 with various extracellular Ca^{2+} concentrations (0, 0.1, 1.8, or 20 mM). The other experimental procedures were performed as described in Figure 1. (b) Treatment with $LaCl_3$ (100 μM) blocks GTS-induced sustained inward Cl^- current. The histogram represents the peak mean amplitude of GTS-evoked inward Cl^- currents recorded in the presence or absence of $LaCl_3$ (mean $\pm$ s.e.m; $n = 16$–18 oocytes each). These current amplitudes were recorded by a voltage step to -140 mV from a holding potential of -40 mV for 5 s. Inset: Traces depicting the currents measured during the transient and sustained currents in the presence of GTS (50 μg ml^{-1}) or $LaCl_3$ + GTS. (c and d) Oocytes were preinjected with BAPTA to a final concentration of 20 mM and voltage clamped using the ramp shown in the inset once every 30 s in the absence (d) or presence (c) of extracellular Ca^{2+}. The external solution was 30 mM $CaCl_2$, 55 mM NaCl, 10 mM HEPES, pH 7.2. GTS treatment induced an inward current at -140 mV. To measure I_{SOC} current, 100 μM La^{3+} was added at the end of the experiment. I_{SOC} current was measured as the La^{3+}-inhibited current following GTS treatment. Current ramps before (a) and after (b) La^{3+} addition are shown. I_{SOC} current or current–voltage relationship was obtained as the difference between the currents before and after La^{3+} addition. Thus, GTS induced I_{SOC} current in the presence of extracellular Ca^{2+} (d) but not in the absence of extracellular Ca^{2+} (e). The graph is representative of data obtained from six oocytes from three different frogs.

channel blocker. As shown in Figure 4b, La^{3+} treatment (100 μM) slightly but not significantly reduced the transient inward Cl^- current (from 0.86 ± 0.12 to 0.72 ± 0.10 μA). However, treatment with La^{3+} abolished the sustained inward Cl^- current significantly (from 0.73 ± 0.1 to 0.06 ± 0.04 μA, $^*P < 0.01$).

Effect of ginsenosides on store-operated Ca^{2+} (I_{SOC}) current

To see whether or not I_{SOC} can be directly measured when oocytes are treated with ginsenosides, we first preinjected oocytes with bis (*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetracetic acid (BAPTA) (20 mM final) in order to inhibit endogenous Ca^{2+}-activated Cl^- current, as described by Yao & Tsien (1997) and Machaca & Haun (2000). I_{SOC} current was measured using a ramp voltage from -140 to $+60$ mV applied every 30 s to oocytes preinjected with BAPTA. As shown in Figure 4c, when we treated oocytes with ginsenosides in the presence of external 30 mM Ca^{2+}, we observed an inward current at -140 mV. As described in previous reports, I_{SOC} current is measured as the La^{3+}-inhibited current induced in response to ginsenosides. The I_{SOC} current–voltage relationship showed the typical inward rectifying character (Figure 4e) (Machaca & Haun, 2000). However, in the absence of external Ca^{2+}, we could not observe any ginsenoside-induced inward I_{SOC} current (Figure 4d and f), indicating that treatment with ginsenosides induces La^{3+}-sensitive inward I_{SOC} current in the presence of external Ca^{2+}.

Effects of IP_3 receptor antagonists on ginsenoside-evoked biphasic inward current

As a next step, we examined whether or not intracellular Ca^{2+} release via IP_3 receptors was responsible for ginsenoside-evoked biphasic inward currents. For this, we tested the effects of IP_3 receptor antagonists, such as membrane permeable 2-APB and heparin, on ginsenoside-evoked biphasic current (Parekh, 1995; Broad *et al*., 2001; Dobrydneva & Blackmore, 2001). As shown in Figure 5a, addition of 2-APB almost completely abolished both transient and sustained Cl^- currents induced by ginsenosides (for transient current from 1.05 ± 0.12 to 0.03 ± 0.01 μA and for sustained current from 1.01 ± 0.23 to 0.02 ± 0.07 μA; $^*P < 0.01$, $^{**}P < 0.001$). Intraoocyte injection of another IP_3 receptor antagonist, heparin (1 μg final), also attenuated both ginsenoside-induced phases of Cl^- current but a control H_2O injection did not significantly alter the oocyte response to ginsenosides (Figure 5a).

Effect of PLC inhibitor on ginsenoside-evoked biphasic inward current

We examined the effects of PLC inhibitors on ginsenoside-evoked biphasic inward current, since the ginsenoside effect on the biphasic inward current involves the participation of IP_3, which is produced via PLC activation. To test this, the effects of the active PLC inhibitor U-73122 and its inactive analog U-73343 (Thompson *et al*., 1991) on ginsensoside action were examined. Bath application of U-73122 significantly depressed the action of ginsenosides (for transient current from 0.93 ± 0.05 to 0.03 ± 0.04 μA and for sustained current from 0.83 ± 0.15 to 0.01 ± 0.04 μA, respectively), but U-73343 had no effect (Figure 5b). These results indicate that the active PLC inhibitor blocked the effect of ginsenoside on the biphasic inward Cl^- current.

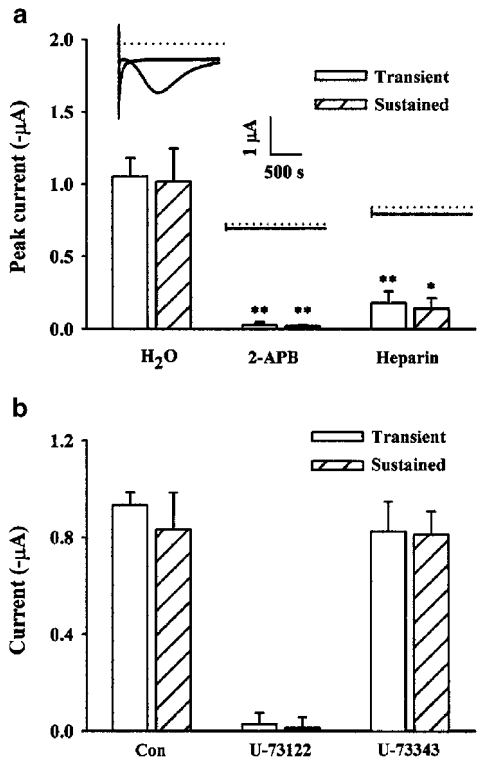


Figure 5 Effects of the IP_3 receptor antagonists, 2-aminoethoxydiphenyl borate (2-APB) and heparin, and U73122 (active PLC inhibitor) or U73343 (inactive PLC inhibitor) on GTS action. (a) Oocytes were injected with H_2O (as control) or heparin (1 μg, final concentration), or treated with 2-APB (100 μM) and incubated for 20 min. Histograms show the peak inward transient and sustained Cl^- currents (mean $\pm$ s.e.m; $n = 10$–12 oocytes each) evoked every 2 min by a voltage step of -140 mV from -40 mV holding potential. Inset: Traces showing the peak inward transient and sustained Cl^- currents. (b) Oocytes were treated with ND96 for control (Con) or ND96 plus U73122 or U73343 (1 μM) for 20 min. The peak inward currents are shown (mean $\pm$ s.e.m; $n = 10$–12 oocytes each) from experiments following the protocol as described in (a).

Intraoocyte injection of ATP-γS but not AMP-PCP prolongs ginsenoside-evoked sustained Cl^- currents

ATP-γS is an ATP analogue that can be used by protein kinases, and AMP-PCP is another ATP analogue that cannot be utilized by kinases. In a previous study, it was suggested that protein phosphorylation regulates capacitative Ca^{2+} influx following receptor stimulation (Parekh & Terlau, 1996). Hence, to examine whether the ginsensoside-induced biphasic current is affected by protein phosphorylation, we injected ATP-γS (100 μM, final concentration) or AMP-PCP (100 μM, final concentration) into *Xenopus* oocytes. As shown in Figure 6a, ATP-γS did not alter the ginsenoside-induced transient inward Cl^- current compared to controls, but the ginsenoside-induced sustained inward Cl^- current was not inactivated but was prolonged for more than 40 min. However, intraoocyte injection of AMP-PCP did not affect either phase of ginsenoside-induced of inward Cl^- current.

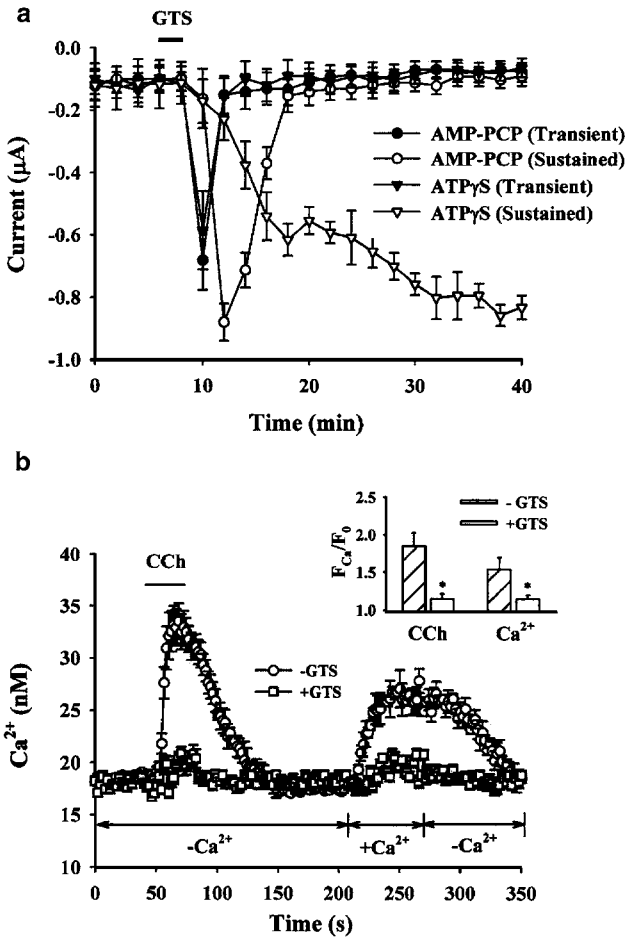


Figure 6 Effects of ATP-γS and AMP-PCP on GTS-induced biphasic Cl^- currents and effects of GTS on receptor agonist-induced intracellular Ca^{2+} release and SOCE in hippocampal neurons. (a) Oocytes were injected with ATP-γS (100 μM, final concentration) or AMP-PCP (100 μM, final concentration) and incubated for 20 min. Oocytes were voltage clamped at -40 mV and stepped to -140 mV for 5 s. The current amplitudes were cycled every 2 min and plotted against time. The graph is representative of data obtained from 10 oocytes from three different frogs. (b) Cytosolic Ca^{2+} changes $[Ca^{2+}]_i$ were measured by the Ca^{2+} indicator Fura-2/AM in the cell bodies of hippocampal neurons with well-defined neurites. The graph shows the $[Ca^{2+}]_i$ changes over time in the presence or absence of GTS (100 μM ml^{-1}) (mean $\pm$ s.e.m. from 10 to 15 neurons). The medium contained 0.4 μM TTX and 10 μM CNQX with or without 10 mM Ca^{2+}. TTX and CNQX were used to prevent the action potential-dependent entry of Ca^{2+} and the spontaneously occurring excitatory synaptic input, respectively. A measure of 200 μM carbachol (CCh) was added as indicated by the horizontal bar on the graph. Since we showed both 50 mM K^+-induced intracellular Ca^{2+} increase and the effect of GTS on high K^+-induced intracellular Ca^{2+} levels in our previous report, we did not present this data here (Kim *et al*., 2002). Inset, the peak Ca^{2+} fluorescence (F_{Ca}) was averaged and normalized as a ratio of the background fluorescence (F_0) taken before in the presence or absence of GTS, $^*P<0.01$ significantly different from carbachol or Ca^{2+} treatment alone.

Effect of ginsenosides on SOCE in hippocampal neurons

In order to characterize the effect of ginsenosides on SOCE in hippocampal neurons, we followed the intracellular Ca^{2+} imaging methods devised by Bouron (2000) but with slight modifications. Neurons were first depolarized with 50 mM KCl to load external Ca^{2+} into intracellular Ca^{2+} stores, necessary because the intracellular Ca^{2+} stores of cultured hippocampal neurons are empty at rest. Without prefilling Ca^{2+} by depolarization, treatment with the acetylcholine muscarinic receptor agonist, carbachol, does not induce intracellular Ca^{2+} releases in most neurons (Irving & Collingridge, 1998; Bouron, 2000). Thus, following high K^+-induced depolarization, neurons were stimulated with carbachol to deplete the intracellular Ca^{2+} stores in the absence of extracellular Ca^{2+} and were then treated with external medium containing Ca^{2+} in order to induce SOCE. As shown in Figure 6b, treatment of hippocampal neurons with carbachol following previous stimulation with 50 mM KCl induced transient increases in cytosolic free Ca^{2+} in the absence of external Ca^{2+}. After returning to basal Ca^{2+} levels, incubation with medium containing 10 mM Ca^{2+} induced sustained increases in intracellular Ca^{2+} levels. However, treatment with ginsenosides greatly attenuated both carbachol-stimulated intracellular Ca^{2+} release from intracellular Ca^{2+} stores, and SOCE by external Ca^{2+} influx (Figure 6b and inset). In this experiment, ginsenoside treatment did not affect the basal intracellular Ca^{2+} level in hippocampal neurons in normal medium as shown in a previous report by Kim *et al*. (2002).

Discussion

Although ginsenosides, the active ingredients of *Panax* ginseng, have been widely used as an invigorating agent for a long time, there have been few reports on ginsenoside-mediated signal transduction until now. Moreover, little is known about changes in intracellular Ca^{2+} signaling following exposure to ginsenosides. In previous studies, we demonstrated that ginsenosides interact with unidentified extracellular membrane components to induce a mobilization of Ca^{2+} from IP_3-sensitive intracellular stores via activation of PTX-insensitive $G\alpha_{q/11}$ proteins and the PLC pathway in *Xenopus* oocytes (Choi *et al*., 2001b, c). The present study aimed to confirm that ginsenoside-induced intracellular Ca^{2+} mobilization from IP_3-sensitive Ca^{2+} stores is coupled to the subsequent activation of the SOCE process. For this, we adapted the voltage clamp protocols of Parekh (1995) and Kuruma & Hartzell (1999), in which SOCE was indirectly monitored through measurement of biphasic inward currents. The biphasic current is composed of a transient Ca^{2+}-activated Cl^- current (activated by release of Ca^{2+} stores) followed by a sustained Ca^{2+}-activated Cl^- current (dependent upon external Ca^{2+} entry). In this study, we present four principal findings regarding ginsenoside action and the SOCE process. These are that (1) different time courses of biphasic inward Cl^- current, (2) external Ca^{2+} dependency, (3) IP_3 receptor activation, and (4) PLC activation are all involved in the main molecular mechanisms mediating ginsenoside-induced activation of the SOCE process in *Xenopus* oocytes.

First, in our hyperpolarizing voltage clamp protocol, ginsenosides induced a transient inward Cl^- current that was rapidly inactivated 15 s following ginsenoside treatment, followed by a second inward Cl^- current that appeared after the transient inward current was almost completely inactivated (2 min after ginsenoside treatment). The amplitude of the second inward Cl^- current gradually increased and was much

slower to reach the peak current than was the transient inward Cl^- current (Figure 3). Thus, this difference in the timings of the appearance of the biphasic inward Cl^- current following ginsenosides treatment might suggest that ginsenosides, in *Xenopus* oocytes, activate at least two types of Ca^{2+}-activated Cl^- current and that the first phase reflects Cl^- current activated from release of Ca^{2+} from internal stores and the second phase reflects Cl^- current induced by an influx of extracellular Ca^{2+} into the cell. Intraoocyte injection of IP_3, or agonist stimulation of a receptor such as 5-HT_{2C}, heterologously expressed in *Xenopus* oocytes, also exhibited similar patterns of inward Cl^- currents (Parekh, 1995; Kuruma & Hartzell, 1999), supporting the theory that treatment with ginsenosides induces activation of the SOCE process as well as intracellular Ca^{2+} release.

Secondly, we could only observe a ginsenoside-induced sustained inward Cl^- current when external Ca^{2+} was present. We also found that the magnitude of sustained inward Cl^- current was dependent on external Ca^{2+} concentrations, whereas the transient inward Cl^- current was independent of external Ca^{2+} levels. Moreover, pretreatment with the calcium channel blocker, La^{3+}, blocked most of the sustained inward Cl^- current and I_{SOC} but had no effect on the transient inward Cl^- current (Figure 4).

Evidence for a role for IP_3 receptor activation in SOCE induction by ginsenosides is demonstrated by treatment with 2-APB, or intraoocyte injection of heparin, whereby ginsenoside-induced transient and sustained inward Cl^- currents were both attenuated. It is known that 2-APB inhibits agonist-induced increases in intracellular free Ca^{2+} in platelets and neutrophils. 2-APB has been used extensively to inhibit the release of intracellular Ca^{2+} and 2-APB directly inhibits SOCE channels in human platelets (Dobrydneva & Blackmore, 2001). Heparin is a well-known IP_3 receptor antagonist (Callamaras & Parker, 1994; Yao & Parker, 1994). Parekh (1995) carried out injection of heparin to confirm that the depletion-activated Ca^{2+} influx pathway was responsible for the sustained Cl^- current. Thus, inhibition of intracellular Ca^{2+} mobilization by blocking of IP_3 receptors is also coupled to the inhibition of SOCE, resulting in a subsequent inhibition of the sustained inward Cl^- current in *Xenopus* oocytes.

The fourth piece of pharmacological evidence suggesting a role for PLC activation in ginsenoside-induced SOCE comes from experiments involving U73122, an active PLC inhibitor. The results of experiments blocking the ginsenoside effect of inducing the biphasic inward Cl^- current by U73122, suggest that production of polyphosphoinositides by PLC activation is a key factor in the induction of the biphasic inward Cl^- current by ginsenoside. Moreover, the present results are also consistent with the previous reports that U-73122 but not U73344 (inactive PLC inhibitor) blocked SOCE in epithelial cell and mast cell lines (Dobrydneva & Blackmore, 2001). These results suggest that PLC activation, which produces IP_3 from the precursor PIP_2, is required for ginsenoside-induced SOCE in *Xenopus* oocytes.

In addition, we studied the effects of protein phosphorylation on ginsenoside action in *Xenopus* oocytes. It was reported that SOCE, usually inactivated by 5-HT application after 15 min, was not inactivated but was rather prolonged by around two-fold when ATP-γS was injected into oocytes (Parekh & Terlau, 1996). Similarly, in our experiments, without intraoocyte injection of ATP-γS the sustained inward Cl^- current induced by ginsenosides was inactivated after about 10 min (Figure 3c). However, following injection of ATP-γS, an ATP analogue that is readily used by protein kinases, the sustained but not transient Cl^- current induced by ginsenosides was also prolonged more than 40 min (Figure 6a). What is the mechanism of the differential effect of ATP-γS on ginsenoside-induced biphasic Cl^- current? One possibility is that thiophosphorylation of oocyte membrane protein(s) by protein kinases, in which thiol groups are not easily removed by phosphatases (Eckstein, 1985) that are involved in SOCE induction, might cause a persistent activation of SOCE resulting in prolonged opening of the sustained Cl^- current without inactivation. The other possibility is that there might be two subtypes of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. One is not affected by phosphorylation and dephosphorylation processes because it is transiently activated by intracellular Ca^{2+} release from ER. The other is slowly activated by extracellular Ca^{2+} influx via SOCE processes. The phosphorylation and dephosphorylation of this Cl^- channel protein might affect the activation and inactivation process, respectively. Thus, ATP-γS-induced thiophosphorylation of Cl^- channel proteins might cause a persistent activation of the sustained Cl^- current as in oocytes expressing 5-HT_{1C} receptors in the study by Parekh & Terlau (1996). However, we could not entirely explain how ATP-γS injected into oocytes induced a persistent activation of the ginsenoside-induced sustained Cl^- current. Further studies will be needed to clarify the differential effect of ATP-γS on ginsenoside-induced biphasic Cl^- currents.

In contrast to the effect of ginsenoside on intracellular Ca^{2+} release and SOCE induction in *Xenopus* oocytes shown in previous reports and in the present study (Choi *et al*., 2001b, c), we have shown, using a Ca^{2+} imaging method, that ginsenosides inhibit both carbachol-stimulated intracellular Ca^{2+} release and intracellular Ca^{2+} depletion-induced SOCE in rat cultured hippocampal neurons (Figure 6b). Previous reports have shown that ginsenosides inhibit high threshold voltage-dependent Ca^{2+} channels via pertussis toxin-sensitive G proteins in several types of neuronal cells such as chromaffin cells and sensory neurons (Nah & McCleskey, 1994; Nah *et al*., 1995; Choi *et al*., 2001a; Rhim *et al*., 2002). Ginsenosides inhibited cation influx by blocking ligand-gated cation channel activity such as glutamate, 5-HT_{3A}, and muscle and neuronal nicotinic acetylcholine receptors (Choi *et al*., 2002; Kim *et al*., 2002; Sala *et al*., 2002; Choi *et al*., 2003b). Moreover, Lee *et al*. (2003) showed that in cultured rat cortical neurons, ginsenosides inhibited carbachol-stimulated turnover of phosphoinositides like IP_1, IP_2, and IP_3 which leads to intracellular $[Ca^{2+}]_i$ mobilization. Thus, both previous studies and the present study support the concept that ginsenosides might not only block external Ca^{2+} influx by inhibition of channel activity but also inhibit carbachol-stimulated intracellular Ca^{2+} mobilization and intracellular Ca^{2+} depletion-activated SOCE in neuronal cells (Figure 6b).

In summary, ginsenosides induced intracellular Ca^{2+} release from IP_3-sensitive internal stores and Ca^{2+} influx into the cell via the depletion-activated SOCE process in *Xenopus laevis* oocytes, whereas in neurons ginsenosides inhibited both intracellular acetylcholine muscarinic receptor-mediated Ca^{2+} release and SOCE. Further, these results highlight

the possibility that ginsenosides may act as differential regulators of intracellular Ca^{2+} level in neurons and non-neuronal cells.

This work was supported by the Ministry of Science and Technology through the Bio-Food and Drug Research Center at Konkuk University, Chungju, Korea.

References

BARISH, M.E. (1983). A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J. Physiol. (London),* **342,** 309–325.

BERRIDGE, M.J., BOOTMAN, M.D. & LIPP, P. (1998). Calcium – a life and death signal. *Nature (London),* **395,** 645–648.

BERRIDGE, M.J. & IRVINE, R.F. (1989). Inositol triphosphates and cell signalling. *Nature,* **341,** 197–205.

BOURON, A. (2000). Activation of a capacitative Ca^{2+} entry pathway by store depletion in cultured hippocampal neurons. *FEBS Lett.,* **470,** 269–272.

BROAD, L.M., BRAUN, F.-J., LIEVREMONT, J.-P., BIRD, G.S., KUROSAKI, T. & PUTNEY Jr, J.W. (2001). Role of the phospholipase C-inositol 1,4,5-triphosphate pathway in calcium release-activated calcium entry and capacitative calcium entry. *J. Biol. Chem.,* **276,** 15945–15952.

CALLAMARAS, N. & PARKER, I. (1994). Inositol 1, 4, 5-triphosphate receptors in *Xenopus laevis* oocytes: localization and modulation by Ca^{2+}. *Cell Calcium,* **15,** 66–78.

CHOI, S., JUNG, S.Y., KIM, C.H., KIM, H.S., RHIM, H., KIM, S.C. & NAH, S.Y. (2001a). Effect of ginsenosides on voltage-dependent Ca^{2+} channel subtypes in bovine chromaffin cells. *J. Ethnopharmacol.,* **74,** 75–81.

CHOI, S., JUNG, S.Y., LEE, J.H., SALA, F., ENGEL, A.G. & NAH, S.Y. (2002). Effect of ginsenosides, active components of ginseng, on nicotinic acetylcholine receptor expressed in *Xenopus* oocytes. *Eur. J Pharmacol.,* **442,** 37–45.

CHOI, S., KIM, H.J., KO, Y.S., JEONG, S.W., KIM, Y.I., SIMONDS, W.F., OH, J.W. & NAH, S.Y. (2001b). $G\alpha_{q/11}$ coupled to mammalian phospholipase C β3-like enzyme mediates the ginsenoside effect on Ca^{2+}-activated Cl^- current in the *Xenopus* oocyte. *J. Biol. Chem.,* **276,** 48797–48802.

CHOI, S., LEE, J.H., KIM, Y.I., KANG, M.J., RHIM, H., LEE, S.K. & NAH, S.Y. (2003a). Effects of ginsenoside on G protein-coupled inwardly rectifying K^+ channel activity expressed in *Xenopus* oocytes. *Eur. J. Pharmacol.,* **468,** 83–92.

CHOI, S.E., LEE, J.H., OH, S., RHIM, H., LEE, S.M. & NAH, S.Y. (2003b). Effects of ginsenoside Rg_2 on the $5\text{-}HT_{3A}$ receptor-mediated ion current in *Xenopus* oocytes. *Mol. Cells,* **15,** 108–113.

CHOI, S., RHO, S.H., JUNG, S.Y., KIM, S.C., PARK, C.S. & NAH, S.Y. (2001c). A novel activation of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes by ginseng saponins: evidence for the involvement of phospholipase C and intracellular Ca^{2+} mobilization. *Br. J. Pharmacol.,* **132,** 641–648.

DASCAL, N. (1987). The use of *Xenopus* oocytes for the study of ion channels. *CRC Crit. Rev. Biochem.,* **22,** 317–387.

DASCAL, N., YEKUEL, R. & ORON, Y. (1984). Acetylcholine promotes progesterone-induced maturation of *Xenopus* oocytes. *J. Exp. Zool.,* **230,** 131–135.

DOBRYDNEVA, Y. & BLACKMORE, P. (2001). 2-Aminoethoxydiphenyl borate directly inhibits store-operated calcium entry channels in human platelets. *Mol. Pharmacol.,* **3,** 541–552.

ECKSTEIN, F. (1985). Nucleoside phosphorothioates. *Annu. Rev. Biochem.,* **54,** 367–402.

HARTZELL, H.C. (1996). Activation of different Cl^- currents in *Xenopus* oocytes by Ca^{2+} liberation from stores and by capacitative Ca^{2+} influx. *J. Gen. Physiol.,* **108,** 157–175.

IRVING, A.J. & COLLINGRIDGE, G.L. (1998). A characterization of muscarinic receptor-mediated intracellular Ca^{2+} mobilization in cultured rat hippocampal neurons. *J. Physiol.,* **511,** 747–759.

KIM, S., AHN, K., OH, T.H., NAH, S.Y. & RHIM, H. (2002). Inhibitory effect of ginsenosides on NMDA receptor mediated-signals in rat hippocampus neurons. *Biochem. Biophys. Res. Comm.,* **296,** 247–254.

KURUMA, A. & HARTZELL, H.C. (1999). Dynamics of calcium regulation of chloride currents in *Xenopus* oocytes. *Am. J. Physiol.,* **276,** C161–C175.

LECHLEITER, J.D. & CLAPHAM, D.E. (1992). Molecular mechanisms of intracellular calcium excitability in *Xenopus* laevis oocytes. *Cell,* **69,** 283–294.

LEE, J.H., CHOI, S., KIM, J.H., KIM, J.K., KIM, J.I. & NAH, S.Y. (2003). Effects of ginsenosides on carbachol-stimulated formation of inositol phosphates in rat cortical cell cultures. *Neurochem. Res.,* **28,** 1307–1313.

MACHACA, K. & HAUN, S. (2000). Store-operated calcium entry inactivates at the germinal vesicle breakdown stage of *Xenopus* meiosis. *J. Biol. Chem.,* **275,** 38710–38715.

MILEDI, R. & PARKER, I. (1984). Chloride current induced by injection of calcium into *Xenopus* oocytes. *J. Physiol. (London),* **357,** 173–183.

NAH, S.Y. & MCCLESKEY, E.W. (1994). Ginseng root extract inhibits calcium channels in rat sensory neurons through a similar path, but different receptor, as μ-type opioids. *J. Ethnopharmacol.,* **42,** 45–51.

NAH, S.Y., PARK, H.J. & MCCLESKEY, E.W. (1995). A trace component of ginseng that inhibits Ca^{2+} channels through a pertussis toxin-sensitive G protein. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 8739–8743.

PAREKH, A.B. (1995). Interaction between capacitative Ca^{2+} influx and Ca^{2+}-dependent Cl^- currents in *Xenopus* oocytes. *Pflugers Arch.-Eur. J. Physiol.,* **430,** 954–963.

PAREKH, A.B. & PENNER, R. (1997). Store depletion and calcium influx. *Physiol. Rev.,* **77,** 901–930.

PAREKH, A.B. & TERLAU, H. (1996). Effects of protein phosphorylation on the regulation of capacitative calcium influx in *Xenopus* oocytes. *Pflugers Arch.-Eur. J. Physiol.,* **432,** 14–25.

PARKER, I. & YAO, Y. (1994). Relation between intracellular Ca^{2+} signals and Ca^{2+}-activated Cl^- current in *Xenopus* oocytes. *Cell Calcium,* **15,** 276–288.

PUTNEY JR, J.W. (1992). Inositol phosphates and calcium entry. *Adv. Second Messenger Phosphoprotein Res.,* **26,** 143–160.

RHIM, H., KIM, H., LEE, D.Y., OH, T.H. & NAH, S.Y. (2002). Ginseng and ginsenoside Rg_3, a newly identified active ingredient of ginseng, modulate Ca^{2+} channel currents in rat sensory neurons. *Eur. J. Pharmacol.,* **436,** 151–158.

SALA, F., MULET, J., CHOI, S., JUNG, S.Y., NAH, S.Y., RHIM, H., VALOR, L.M., CRIADO, M. & SALA, S. (2002). Effects of ginsenoside Rg2 on human neuronal nicotinic acetylcholine receptors. *J. Pharm. Exp. Ther.,* **301,** 1052–1059.

THOMPSON, A.K., MOSTAFAPOUR, S.P., DENLIGER, L.C., BLEASDALE, J.E. & FISHER, S.K. (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.,* **266,** 23856–23862.

YAO, Y. & PARKER, I. (1994). Ca^{2+} influx modulation of temporal and spatial patterns of inositol triphosphate-mediated Ca^{2+} liberation in *Xenopus* oocytes. *J. Physiol.,* **476,** 17–28.

YAO, Y. & TSIEN, R.Y. (1997). Calcium current activated by depletion of calcium stores in *Xenopus* oocytes. *J. Gen. Physiol.,* **109,** 703–715.

(Received November 24, 2003
Revised February 7, 2004
Accepted March 16, 2004)