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Nitric oxide-dependent modulation of the delayed rectifier K^+ current and the L-type Ca^{2+} current by ginsenoside Re, an ingredient of *Panax* ginseng, in guinea-pig cardiomyocytes

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1 Ginsenoside Re, a major ingredient of *Panax* ginseng, protects the heart against ischemia–reperfusion injury by shortening action potential duration (APD) and thereby prohibiting influx of excessive Ca^{2+}. Ginsenoside Re enhances the slowly activating component of the delayed rectifier K^+ current (I_{Ks}) and suppresses the L-type Ca^{2+} current ($I_{Ca,L}$), which may account for APD shortening.

2 We used perforated configuration of patch-clamp technique to define the mechanism of enhancement of I_{Ks} and suppression of $I_{Ca,L}$ by ginsenoside Re in guinea-pig ventricular myocytes.

3 *S*-Methylisothiourea (SMT, 1 μM), an inhibitor of nitric oxide (NO) synthase (NOS), and *N*-acetyl-L-cystein (LNAC, 1 mM), an NO scavenger, inhibited I_{Ks} enhancement. Application of an NO donor, sodium nitroprusside (SNP, 1 mM), enhanced I_{Ks} with a magnitude similar to that by a maximum dose (20 μM) of ginseonside Re, and subsequent application of ginsenoside Re failed to enhance I_{Ks}. Conversely, after I_{Ks} had been enhanced by ginsenoside Re (20 μM), subsequently applied SNP failed to further enhance I_{Ks}.

4 An inhibitor of guanylate cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), barely suppressed I_{Ks} enhancement, while a thiol-alkylating reagent, *N*-ethylmaleimide (NEM, 0.5 mM), clearly suppressed it. A reducing reagent, di-thiothreitol (DTT, 5 mM), reversed both ginsenoside Re- and SNP-induced I_{Ks} enhancement.

5 $I_{Ca,L}$ suppression by ginsenoside Re (3 μM) was abolished by SMT (1 μM) or LNAC (1 mM). NEM (0.5 mM) did not suppress $I_{Ca,L}$ inhibition and DTT (5 mM) did not reverse $I_{Ca,L}$ inhibition, whereas in the presence of ODQ (10 μM), ginsenoside Re (3 μM) failed to suppress $I_{Ca,L}$.

6 These results indicate that ginsenoside Re-induced I_{Ks} enhancement and $I_{Ca,L}$ suppression involve NO actions. Direct *S*-nitrosylation of channel protein appears to be the main mechanism for I_{Ks} enhancement, while a cGMP-dependent pathway is responsible for $I_{Ca,L}$ inhibition.
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Keywords: *Panax* ginseng; ginsenoside Re; herb medicine; potassium current; ion channel; cardiac; patch-clamp; nitric oxide; nitric oxide synthase; amphotericin B

Abbreviations: APD, action potential duration; DMSO, dimethylsulfoxide; DTT, di-thiothreitol; H89, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; HERG, human *ether-a-go-go-related* gene; $I_{Ca,L}$, the L-type Ca^{2+} current; I_{Kr}, the rapidly activating component of the delayed rectifier K^+ current; I_{Ks}, the slowly activating component of the delayed rectifier K^+ current; $I_{Ks,tail}$, tail current of I_{Ks}; LNAC, *N*-acetyl-L-cystein; LQT, long QT syndrome; NEM, *N*-ethylmaleimide; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, protein kinase G; SMT, *S*-methylisothiourea; SNAP, S-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside; V_h, holding potential; V_t, test potential

Introduction

The use of ethnobotanical drugs as complementary medicine is prevalent in Asia and is also gaining increasing popularity in the West. The most well-known herb traditionally used as a drug is the root of the ginseng species (Attele *et al*., 1999; Caron *et al*., 2003). *Panax* ginseng exhibits a variety of cardiovascular actions, including a vasorelaxing effect, protection against ischemia–reperfusion injury, and an antiarrhythmic effect (Lee *et al*., 1981; Chen *et al*., 1984; Chen 1996). We have previously reported that *Panax* ginseng shortens action potential duration (APD) and thereby reduces the amount of Ca^{2+} influx, which is suggested to account for cardiovascular protection against ischemia–reperfusion injury (Bai *et al*., 2003). APD shortening by *Panax* ginseng is mainly due to the enhancement of the slowly activating component of the delayed rectifier K^+ current (I_{Ks}) and the inhibition of the

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L-type Ca^{2+} current ($I_{Ca,L}$). Ginsenoside Re, the major ingredient of *Panax* ginseng (Gillis, 1997), is the main active component that is responsible for these cardiac actions (Bai *et al*., 2003). However, the underlying mechanism for these electrophysiological effects of ginsenoside Re in the heart is not completely clarified.

The delayed rectifier K^+ current is one of the major ion currents that determine the timing of repolarization of cardiac myocytes. The delayed rectifier K^+ current consists of two components: the rapidly activating (I_{Kr}) and the slowly activating component (I_{Ks}) (Sanguinetti & Jurkiewicz, 1990). Dysfunction of either I_{Kr} or I_{Ks} increases the risk of cardiac arrhythmias and sudden death. Long QT syndrome (LQT) is a typical example: certain forms of LQT are caused by mutation in one of the genes encoding the α-subunit of I_{Kr} (human *ether-a-go-go-related gene* (HERG)), β-subunit of I_{Kr} (KCNE2), α-subunit of I_{Ks} (KCNQ1), or β-subunit of I_{Ks} (KCNE1) (Keating, 1996; Sanguinetti, 1999). Several medicines including antiarrhythmic drugs, anti-allergic drugs, and antibacterial drugs suppress either I_{Kr} or I_{Ks}, resulting in drug-induced LQT (Kass & Cabo, 2000; Roden, 2001), which becomes a major concern in the development of new drugs. Although the medicines that reverse the QT prolongation are on the hunt, only a few drugs are found to have activating effects on I_{Ks} (Salata *et al*., 1998), and thus it is potentially important in the clinical setting that ginsenoside Re enhances I_{Ks}. In the present study, therefore, we further characterize the mode and mechanism of action of ginsenoside Re on I_{Ks}. In addition, we examine the mechanism of ginsenoside Re-induced inhibition of $I_{Ca,L}$, since $I_{Ca,L}$ is essential to the determination of APD and to the ginseng-mediated protection against ischemia–reperfusion injury. Our data suggest that enhancement of I_{Ks} and inhibition of $I_{Ca,L}$ by ginsenoside Re involve actions of nitric oxide (NO) *via* a distinct mechanism.

Methods

The investigation was conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Cell isolation

Single ventricular myocytes were harvested from hearts of adult guinea-pigs as previously reported (Bai *et al*., 2003). In brief, animals were anesthetized with intra-peritoneal injection of sodium pentobarbital (40–50 mg kg^{-1}). Using a Langendorff apparatus, we perfused excised heart with a Ca^{2+}-free Tyrode's solution containing 0.01% collagenase (Yakult, Tokyo, Japan) for 5–6 min. The single myocytes were obtained by gentle agitation of small pieces of ventricular tissues in a beaker containing a high K^+, low Cl^- solution (consisting of (in mM) 70 potassium glutamate, 15 taurine, 30 KCl, 10 KH_2PO_4, 0.5 $MgCl_2$, 11 glucose, 0.5 EGTA, pH 7.4). Cells were transferred to a recording chamber placed on the stage of an inverted phase-contrast microscope (Diaphot, TMD, Nikon Co., Tokyo, Japan).

Recording techniques

I_{Ks} and $I_{Ca,L}$ were recorded with perforated configuration of patch-clamp technique using an Axopatch 200B amplifier (Axon Instruments, Forster City, CA, U.S. A.). Analog signals of ionic currents were converted to digital signals with an analog-to-digital converter (Digidata 1200, Axon Instruments, Forster City, CA, U.S.A.) at a sampling rate of 2 kHz, and were stored in a personal computer (Physiotec Corp, Tokyo, Japan) for later analysis. The pCLAMP software (version 6.0, Axon Instruments) was used to generate voltage-pulse protocols, and to acquire and analyze data. I_{Ks} were elicited by 3.5-s-depolarizing pulses from a holding potential (V_h) of -40 mV to various test potentials (V_t) between -30 and $+50$ mV in a 10 mV increment at 0.1 Hz, and the amplitude of the tail current ($I_{Ks,tail}$) were measured by extrapolating from exponential fits. To elicit $I_{Ca,L}$, membrane potential was changed from a V_h of –80 to –40 mV for 100 ms, and then was stepped for 200 ms to various V_t between -40 and $+50$ mV in a 10 mV increment at 1 Hz. Suction pipettes made of borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, U.K.) were pulled by two steps using a vertical microelectrode puller (Model PP-830, Narishige Co., Tokyo, Japan). The resistance of electrodes was 1–3 MΩ when electrodes were filled with the internal solution. All experiments were performed at a temperature of 36 ± 1°C.

The external (bath) solution used for recording of I_{Ks} is the K^+-free Tyrode's solution (consisting of (in mM) 135 NaCl, 0.33 NaH_2PO_4, 1.8 $CaCl_2$, 0.53 $MgCl_2$, 5.5 glucose and 5.0 HEPES, pH 7.4 with NaOH) that is known to suppress I_{Kr} and enhance I_{Ks} (Sanguinetti & Jurkiewicz, 1992). Nisoldipine (3 μM), a selective $I_{Ca,L}$ channel blocker, and E-4031 (10 μM), a selective I_{Kr} channel blocker, were also added to the bath solution. The external (bath) solution for recording of $I_{Ca,L}$ contained (in mM) 140 tetraethylammonium (TEA)-Cl, 0.53 $MgCl_2$, 10 glucose, 2.0 $CaCl_2$, 10 HEPES, pH 7.4 with TEA-OH. The pipette solution contains (in mM): 110 aspartic acid, 30 KCl, 1.0 $CaCl_2$, 5.0 Mg-ATP, 5.0 creatine phosphate dipotassium salt, 5.0 HEPES, 10 EGTA, pH 7.25 with KOH. To achieve perforated configuration, antibiotic amphotericin B (Sigma) was used. Patch pipettes were initially front-filled by dipping them into pipette solution, and then back-filled with the pipette solution containing antibiotic amphotericin B (600 μg ml^{-1}).

Reagents

Ginsenoside Re was purchased from Wako (Osaka, Japan), E-4031 from Eisai Co. Ltd (Tokyo, Japan), and all other chemicals from Sigma. Ginsenoside Re was dissolved in the bath solution by vigorous stirring for 1 h on the day of experiments. *N*-acetyl-L-cystein (LNAC) was directly dissolved in the bath solution immediately before experiments. E-4031, propranolol, isoproterenol, chelerythrine, sodium nitroprusside (SNP), *S*-methylisothiourea (SMT), and di-thiothreitol (DTT) were prepared as a 5, 5, 0.5, 5 mM, 1 M, 1 mM, and 0.5 M stock solution in distilled water, respectively. *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), nisoldipine, and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ) were prepared as a 5, 10, and 20 mM stock solution in dimethylsulfoxide (DMSO), respectively. *N*-ethylmaleimide (NEM) was prepared as a 5 M stock solution in ethanol. They

were dissolved in the bath solution to achieve a final concentration described in the text. The final concentration of DMSO (0.05%) or ethanol (0.01%) was confirmed to have no significant effects on membrane currents.

Data analysis

All values are presented as mean ± s.e. Statistical significance was determined by repeated-measures analysis of variance (ANOVA). *P*-values less than 0.05 are considered to be significant.

Results

Concentration-dependent enhancement of I_{Ks} *by ginsenoside Re*

We examined effects of ginsenoside Re at a concentration between 0.3 and 20 μM on I_{Ks}. I_{Ks} currents were elicited by a 3.5-s depolarization pulse from a V_h of −40 mV to various V_t between –30 and +50 mV in a 10 mV step. Since the leak current, if any, was not subtracted, the amplitude of its tail current ($I_{Ks,tail}$) was measured upon return of membrane potential to a V_h (−40 mV). Figure 1a depicts representative current traces at +50 mV in the drug-free condition or in the presence of 0.3, 1, and 3 μM ginsenoside Re, which shows concentration–dependent enhancement of I_{Ks} by ginsenoside Re. In Figure 1b, concentration–response curves were constructed using the fractional increase in the amplitude of $I_{Ks,tail}$ at each concentration of ginsenoside Re relative to the value in the control state. Data were fit with the Hill's equation in the following formula using the least-squares method:

$$\text{Relative current} = A\{1 + (\text{EC}_{50} \times [\text{ginsenoside Re}])^{-n}\}^{-1} \quad (1)$$

where A is the maximum response, and n is the Hill coefficient. The maximum response (A) was 0.54 ± 0.03, n was 1.8 ± 0.3, and EC_{50} was 1.3 ± 0.1 μM ($n = 5$).

Effects on voltage-dependence of I_{Ks} *activation*

Effects of ginsenoside Re on the voltage dependence of I_{Ks} activation were examined by constructing the activation curve of $I_{Ks,tail}$ (Figure 1c). The amplitude of $I_{Ks,tail}$ was normalized relative to the maximum amplitude, plotted against the V_t, and was fit with the Boltzmann's function in the form:

$$(I_{Ks}, \text{tail})(\max I_{Ks,tail})^{-1} = 1 \times \{1 + \exp[(V_{0.5} - V_t) \times s^{-1}]\}^{-1} \quad (2)$$

where $V_{0.5}$ is the membrane voltage at which half-activation occurs, V_t is the test potential, and s is a slope factor. Ginsenoside Re at a concentration of 0.3–3 μM significantly shifted the voltage dependence of I_{Ks} activation to the negative direction: $V_{0.5}$ was +19.6 ± 2.7 mV in the control condition, +8.3 ± 1.1 mV at 0.3 μM, +3.6 ± 0.9 mV at 1 μM, and +2.8 ± 0.8 mV at 3 μM ($n = 5$, $P < 0.05$) (Figure 1d). At a higher concentration (10 and 20 μM) of ginsenoside Re, however, the negative shift of $V_{0.5}$ became less apparent, and the $V_{0.5}$ value was not significantly different from the control value. The s was not significantly changed at any of the concentrations of ginsenoside Re.

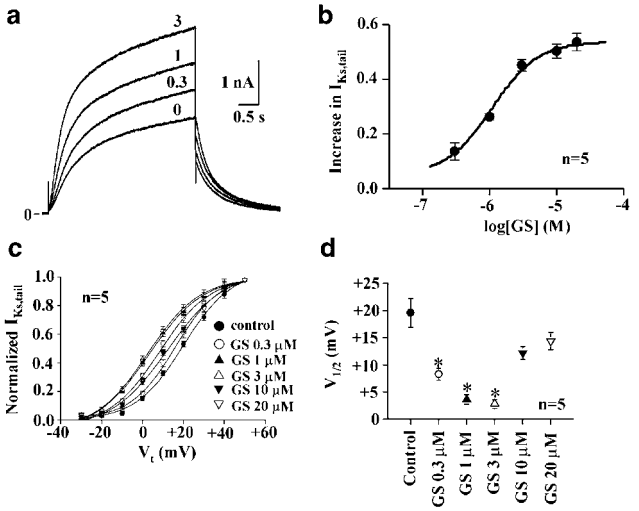

Figure 1 Effects of ginsenoside Re on I_{Ks} amplitude and the voltage dependence of its activation. (a) Representative superimposed current traces in the drug-free condition or in the presence of ginsenoside Re at a concentration of 0.3, 1, or 3 μM. In this and following figures, current traces of I_{Ks} are shown only at a V_t of +50 mV. (b) The concentration–response curve. The fractional increases in the amplitude of $I_{Ks,tail}$ relative to the value in the absence of drug are averaged from five experiments, and are plotted against the concentration of ginsenoside Re. In this and following figures, GS represents ginsenoside Re. Continuous line is the result of Hill's plotting in formula (1) in the text. (c) Activation curves are constructed by plotting $I_{Ks,tail}$ amplitude normalized to the maximum amplitude (at +50 mV) against the V_t at various concentrations of ginsenoside Re. Continuous curves are result of least-squares fit of Boltzmann distribution in formula (2) in the text. (d) The $V_{1/2}$ values at each concentration of ginsenoside Re. $^*P < 0.05$ vs control.

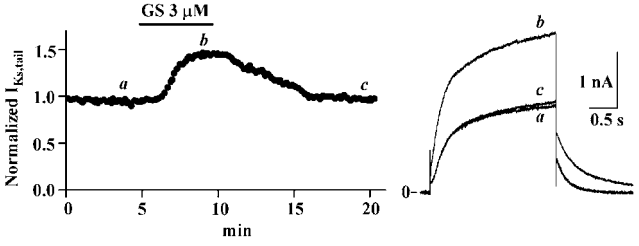

Figure 2 Reversible enhancement of I_{Ks} by ginsenoside Re. The left panels in this and the following figures represent averaged amplitude of $I_{Ks,tail}$ normalized to the value elicited by the first pulse against the time after start of experiments. The timing of the drug applications is shown on the top. Standard error bars are omitted to avoid crowdedness. The right panel depicts representative superimposed current traces recorded at the timing indicated by italic lower-case alphabets in the left panel.

To examine the reversibility of ginsenoside Re on I_{Ks}, I_{Ks} was elicited by continuously applying 3.5-s depolarizing pulses from a V_h of −40 mV to a V_t of +50 mV in a 10-s interval, and ginsenoside Re (3 μM) was applied for 5 min, followed by wash-out of the drug. $I_{Ks,tail}$ amplitude was measured, and is expressed as a fraction normalized to the value elicited by the first pulse (Figure 2). Ginsenoside Re increased $I_{Ks,tail}$ to 1.49 ± 0.04 ($n = 5$) relative to the first pulse, which was fully reversed by wash-out of the drug.

Effects of an inhibitor and an activator of β-adrenergic receptor

I_{Ks} is modulated by various signals, which include increase in $[Ca^{2+}]_i$ (Tohse, 1990; Nitta *et al*., 1994), β-adrenergic receptor stimulation (Duchatelle-Gourdon *et al*., 1989; Giles *et al*., 1989; Walsh *et al*., 1989; Duchatelle-Gouordon & Hartzell, 1990), protein kinase A (PKA) activation (Walsh & Kass, 1988), and protein kinase C (PKC) activation (Walsh & Kass, 1988; Tohse *et al*., 1990). We tested if effects of ginsenoside Re on I_{Ks} are mediated through one of these signals or not.

We first examined the effects of propranolol, a blocker of β-adrenergic receptor (Figure 3a). Treatment with 10 μM propranolol only slightly decreased $I_{Ks,tail}$ amplitude (0.82 ± 0.02, $n=5$), which was not significantly different from the change in $I_{Ks,tail}$ amplitude without drug application (0.83 ± 0.04, $n=5$) ($P=$ns). Subsequent application of 3 μM ginsenoside Re increased the amplitude of $I_{Ks,tail}$ to 1.50 ± 0.07 relative to the control ($n=5$). There was no significant difference in ginsenoside Re-induced $I_{Ks,tail}$ enhancement between in the absence (1.46 ± 0.13, $n=5$) and the presence (1.50 ± 0.07, $n=5$) of propranolol ($P=$ns).

We next examined the effects of isoproterenol, a β-adrenergic receptor stimulator, on I_{Ks} enhancement by ginsenoside Re (Figure 3b). When isoproterenol (30 nM) was added after the amplitude of $I_{Ks,tail}$ was increased by 1.51 ± 0.08-fold with ginsenoside Re (3 μM), isoproterenol still increased the amplitude of $I_{Ks,tail}$ further to 1.99 ± 0.13-fold from the control value ($n=5$, $P<0.05$ *versus* the value before isoproterenol application). These data suggest that ginsenoside Re enhances I_{Ks} through a pathway distinct from the isoproterenol-dependent one.

Effects of a PKA inhibitor and a PKC inhibitor

To examine if I_{Ks} enhancement by ginsenoside Re is *via* a pathway involving activation of PKA or PKC, the effects of H89, a selective PKA inhibitor, or chelerythrine, a potent PKC inhibitor, on I_{Ks} enhancement by ginsenoside Re were studied. After cardiomyocytes were pretreated with H89 (0.1 μM) for 20 min, application of ginsenoside Re (3 μM) still increased $I_{Ks,tail}$ amplitude to 1.45 ± 0.20 ($n=5$, $P<0.05$) relative to the control (Figure 3c). Similarly, in the presence of chelerythrine (5 μM), application of 3 μM ginsenoside Re increased $I_{Ks,tail}$ amplitude to 1.43 ± 0.16 relative to the control ($n=5$, $P<0.05$) (Figure 3d). There was no significant difference for the magnitude of ginsenoside Re-induced increase in $I_{Ks,tail}$ amplitude between in the presence (1.45 ± 0.20, $n=5$) and the absence (1.46 ± 0.13, $n=5$) of 0.1 μM H89 ($P=$ns) or between in the presence (1.43 ± 0.16, $n=5$) and the absence (1.46 ± 0.13, $n=5$) of 5 μM chelerythrine ($P=$ns).

Effects of a NOS inhibitor, a NO donor, and a NO scavenger

NO is shown to inhibit $I_{Ca,L}$ (Gallo *et al*., 2001), and protein kinase G (PKG), a downstream signal molecule of NO, enhances I_{Ks} (Shimizu *et al*., 2002). Since these actions of NO are in the same direction elicited by ginsenoside Re (Bai *et al*., 2003), we hypothesized a possible involvement of NO in the actions of ginsenoside Re. We first examined effects of SNP (1 mM), a NO donor, on I_{Ks} (Figure 4). Application of SNP increased the $I_{Ks,tail}$ amplitude in a concentration–dependent manner and maximum effects were attained by 1 mM SNP (Figures 4a, b). The maximum increase in $I_{Ks,tail}$ amplitude by 1 mM SNP (by 1.57 ± 0.19-fold, $n=4$) was comparable to the magnitude of I_{Ks} enhancement by a maximum dose (20 μM) of ginsenoside Re (1.54 ± 0.03, $n=5$) ($P=$ns). Subsequent application of a maximum dose (20 μM) of ginsenoside Re did not further increase the $I_{Ks,tail}$ amplitude: the $I_{Ks,tail}$ amplitude in the presence of both SNP and ginsenoside Re was 1.01 ± 0.13 relative to the value in the presence of SNP alone ($n=5$, Figure 4c). When a maximum dose (20 μM) of ginsenoside Re was initially applied followed by application of 1 mM SNP, SNP did not show any additional I_{Ks} enhancement beyond effects by ginsenoside Re: the $I_{Ks,tail}$ amplitude in the presence of both SNP and ginsenoside Re was 1.01 ± 0.10 relative to the value in the presence of ginsenoside Re alone ($n=5$, Figure 4d). Similar effects were observed using another NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP, 1 mM).

We next examined the effects of SMT, an inhibitor of NOS, on the effects of ginsenoside Re (Figure 5a). Application of SMT (1 μM) slightly decreased the amplitude of $I_{Ks,tail}$ to 0.89 ± 0.10 relative to the control ($n=5$). In the presence of

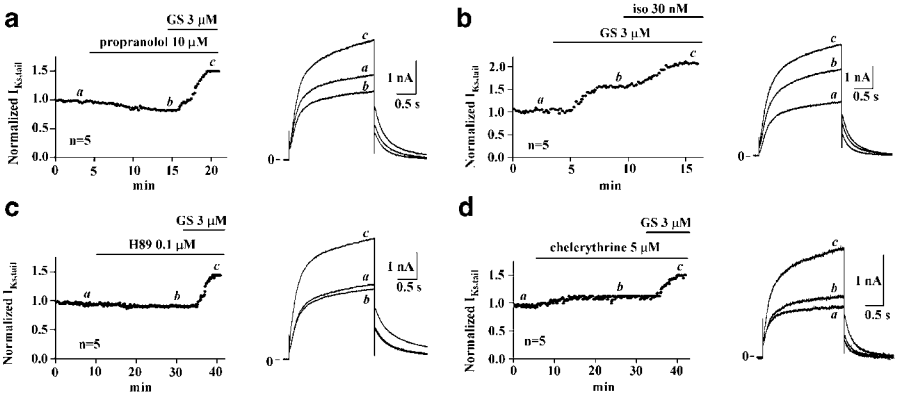

Figure 3 Effects of propranolol, isoproterenol, a PKA inhibitor, H89, and a PKC inhibitor, chelerythrine. (a) Effects of a blocker of β-adrenergic receptor, propranolol (10 μM), on the enhancement of I_{Ks} by ginsenoside Re. (b) Effects of a stimulator of β-adrenergic receptor, isoproterenol (iso, 30 nM), on the I_{Ks} that had been enhanced by ginsenoside Re. (c) Effects of a PKA inhibitor, H89 (0.1 μM), on the enhancement of I_{Ks} by ginsenoside Re. (d) Effects of a PKC inhibitor, chelerythrine (5 μM), on the enhancement of I_{Ks} by ginsenoside Re.

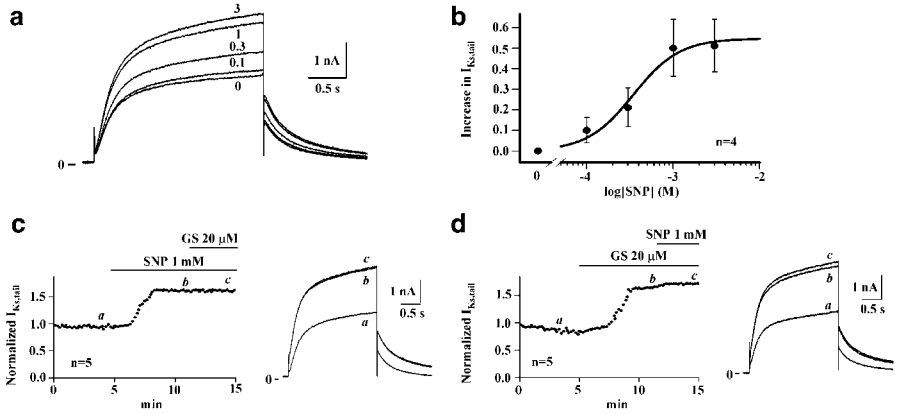

Figure 4 Effects of SNP. (a) Representative superimposed current traces in the drug-free condition or in the presence of SNP at concentrations of 0.1, 0.3, 1, or 3 mM. (b) The concentration–response curve. The fractional increases in the amplitude of $I_{Ks,tail}$ relative to the value in the absence of drug are averaged from five experiments, and are plotted against the concentration of SNP. Continuous line is the result of Hill's plotting in the form of (1) in the text. The maximum response (A) was 0.59 ± 0.05, n was 1.6 ± 0.9, and EC_{50} was 0.18 ± 0.17 mM. (c) Effects of an NO donor, SNP (1 mM), on the enhancement of I_{Ks} by a maximum dose (20 μM) of ginsenoside Re. (d) Effects of SNP (1 mM) on the I_{Ks} that had been enhanced by maximum dose (20 μM) of ginsenoside Re.

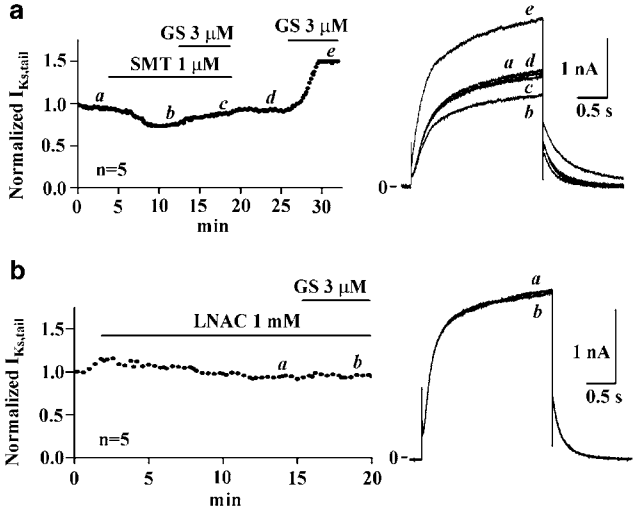

Figure 5 Effects of SMT and LNAC. (a) Effects of an NOS inhibitor, SMT (1 μM), on the enhancement of I_{Ks} by ginsenoside Re. (b) Effects of an NO scavenger, LNAC (1 mM), on the enhancement of I_{Ks} by ginsenoside Re.

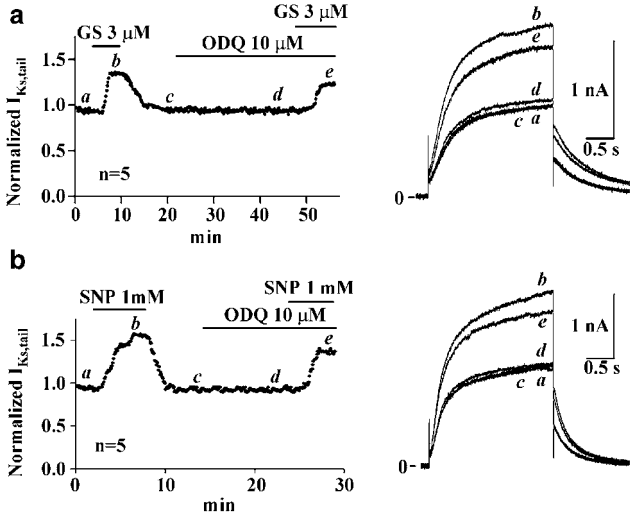

Figure 6 Effects of ODQ. (a) Effects of guanylate cyclase inhibitor, ODQ (10 μM), on the enhancement of I_{Ks} by ginsenoside Re. (b) Effects of guanylate cyclase inhibitor, ODQ (10 μM), on the enhancement of I_{Ks} by SNP.

SMT, addition of ginsenoside Re increased the amplitude of $I_{Ks,tail}$ only by 1.12 ± 0.11-fold from the value in the presence of SMT (1 μM) alone ($n = 5$). After washout of SMT, enhancement of I_{Ks} by application of ginsenoside Re (3 μM) was restored (see *e* in Figure 5a).

The involvement of NO was further examined using LNAC, an NO scavenger. After cells were incubated with 1 mM LNAC for 15 min, ginsenoside Re (3 μM) did not enhance I_{Ks} (Figure 5b).

Effects of a guanylate cyclase inhibitor and a thiol-alkylating reagent

NO activates guanylate cyclase and produces cGMP, which activates PKG. cGMP also inhibits cGMP-sensitive phosphodiesterase (PDE3), resulting in the increase in $[cAMP]_i$ and activation of PKA. We examined the contribution of cGMP in ginsenoside Re-induced I_{Ks} enhancement using an inhibitor of NO-sensitive guanylate cyclase, ODQ. In the presence of ODQ (10 μM), application of ginsenoside Re (3 μM) clearly increased the amplitude of I_{Ks}, even though its magnitude was slightly less (1.35 ± 0.12 relative to the control) compared to that in the absence of ODQ (1.46 ± 0.16 relative to the control) ($n = 5$, $P < 0.05$; please compare *b* and *e* in Figure 6a). To further confirm that the contribution of cGMP-dependent pathway to NO-dependent I_{Ks} modulation is not significant, a maximum dose of SNP (1 mM) was applied in the presence of ODQ (10 μM) (Figure 6b). In the presence of ODQ, application of SNP (1 mM) also clearly increased the amplitude of I_{Ks}, even though its magnitude was slightly less (1.40 ± 0.17 relative to the control) compared to that in the absence of ODQ (1.46 ± 0.16 relative to the control) ($n = 5$, $P < 0.05$; please compare *b* and *e* in Figure 6b).

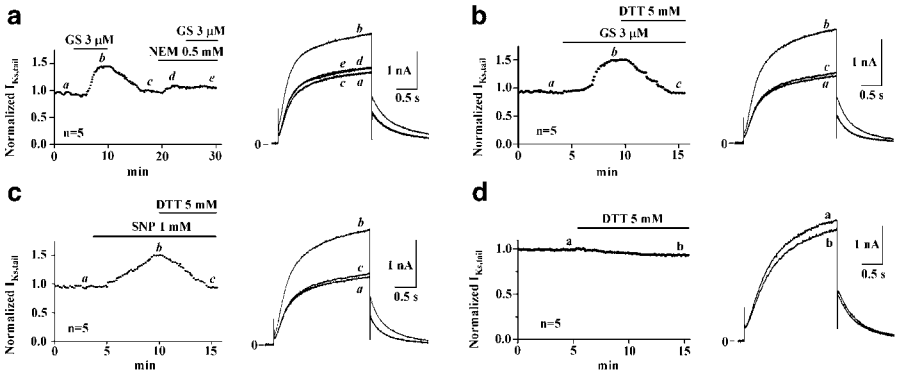

Figure 7 Effects of NEM and DTT. (a) Effects of a thiol-alkylating reagent, NEM (0.5 mM), on the enhancement of I_{Ks} by ginsenoside Re. (b) Effects of a reducing reagent, DTT (5 mM), on the I_{Ks} that had been enhanced by ginsenoside Re. (c) Effects of DTT (5 mM) on the I_{Ks} that had been enhanced by 1 mM SNP. (d) Effects of DTT (5 mM) alone on the I_{Ks} in the control state.

A known alternative pathway for biological effects of NO is direct *S*-nitrosylation of the thiol residue of target proteins. To test the involvement of *S*-nitrosylation, we pre-treated cells with a thiol-alkylating reagent, NEM, and then applied ginsenoside Re. In the presence of NEM (0.5 mM), ginsenoside Re (3 μM) increased I_{Ks} only to 1.01 ± 0.13 relative to the value in the presence of NEM alone ($n = 5$, Figure 7a), which suggests that the ultimate target of NO released by ginsenoside Re is a thiol residue. When DTT (5 mM) was applied after I_{Ks} had been enhanced by ginsenoside Re or SNP, it reversed both ginsenoside Re-induced (Figure 7b) and SNP-induced enhancement of I_{Ks} (Figure 7c). To exclude the possibility that these effects of DTT are due to its direct effects on I_{Ks} channel rather than effects on I_{Ks} channel modified by NO, we applied DTT (5 mM) in the control condition, which showed only a minor inhibition of I_{Ks} (Figure 7d). These findings further suggest the role of *S*-nitrosylation by NO on I_{Ks}.

Involvement of NO in ginsenoside Re-induced inhibition of $I_{Ca,L}$

$I_{Ca,L}$ were elicited by continuously applying 200-ms depolarizing pulses to +10 mV in a 1-s interval, the amplitude of the peak inward current was measured and is expressed as a fraction of the value elicited by the first pulse. It is known that the amplitude of $I_{Ca,L}$ decreased spontaneously during a long experiment (run-down). In our experimental condition, the amplitude of $I_{Ca,L}$ decreased to 0.86 ± 0.12 ($n = 5$) compared to the initial value of $I_{Ca,L}$ amplitude 20 min after the start of experiments (data not shown). Application of ginsenoside Re (3 μM) reduced $I_{Ca,L}$ to 0.34 ± 0.08 ($n = 5$), which was reversed to the level of the spontaneous run-down (0.83 ± 0.13) by the drug wash-out (Figure 8a). In the presence of a NOS inhibitor, SMT (1 μM) (Figure 8b), or after incubation with NO scavenger, LNAC (1 mM), for 15 min (Figure 8c), ginsenoside

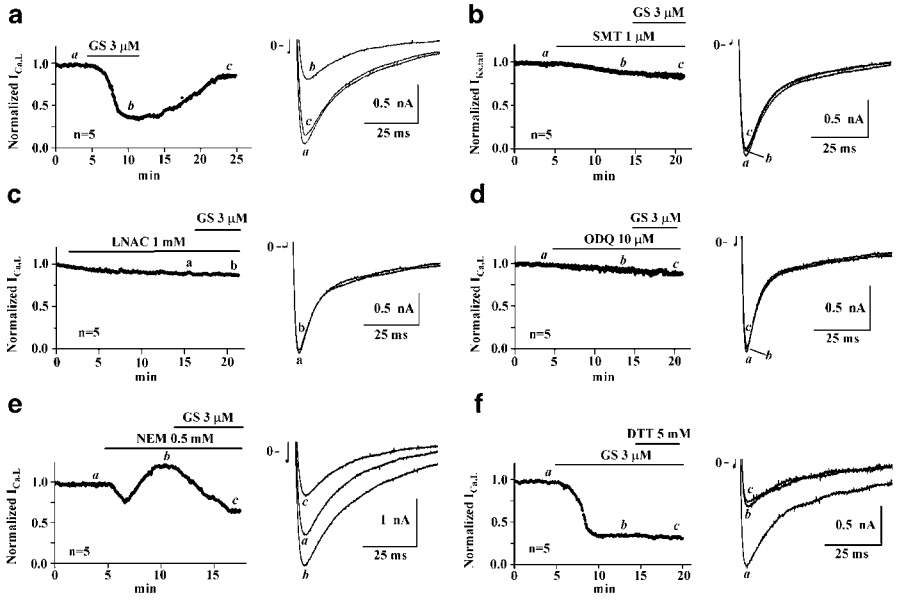

Figure 8 NO-dependent inhibition of $I_{Ca,L}$ by ginsenoside Re. Left panels in the figure represent the averaged amplitude of peak inward current of $I_{Ca,L}$ normalized to the value elicited by the first pulse plotted against the time after start of experiments. (a) Effects of ginsenode Re (3 μM). (b) Effects of an NOS inhibitor, SMT (1 μM), on the inhibition of $I_{Ca,L}$ by ginsenoside Re. (c) Effects of an NO scavenger, LNAC (1 mM), on the inhibition of $I_{Ca,L}$ by ginsenoside Re. (d) Effects of a NO-dependent guanylate cyclase inhibitor, ODQ (10 μM), on the inhibition of $I_{Ca,L}$ by ginsenoside Re. (e) Effects of a thiol-alkylating reagent, NEM (0.5 mM), on the inhibition of $I_{Ca,L}$ by ginsenoside Re. (f) Effects of a reducing reagent, DTT (5 mM), on the $I_{Ca,L}$ that had been inhibited by ginsenoside Re.

Re (3 μM) did not significantly change the amplitude of $I_{Ca,L}$ compared to the spontaneous run-down: 0.86 ± 0.12 ($n=5$) for SMT and 0.88 ± 0.16 ($n=5$) for LNAC.

We examined if NO suppressed $I_{Ca,L}$ *via* a cGMP-dependent or a cGMP-independent pathway, using a guanylate cyclase inhibitor, ODQ, and a thiol-alkylating reagent, NEM. In the presence of ODQ (10 μM), the amplitude of $I_{Ca,L}$ after treatment with ginsenoside Re (3 μM) was 0.89 ± 0.11 ($n=5$), which was not significantly different from that in the absence of ODQ (0.86 ± 0.12, $n=5$) ($P=$ ns) (Figure 8d). Application of NEM (0.5 mM) initially decreased $I_{Ca,L}$ to 0.76 ± 0.11 ($n=5$), and thereafter increased to 1.21 ± 0.15 ($n=5$). Subsequently applied ginsenoside Re (3 μM) clearly decreased $I_{Ca,L}$ to 0.62 ± 0.10 ($n=5$) ($P<0.05$) (Figure 8e). After ginsenoside Re diminished $I_{Ca,L}$ to 0.33 ± 0.07 ($n=5$), subsequent application of DTT (5 mM) did not show any reversal effects (0.30 ± 0.09, $n=5$) (Figure 8f).

Discussion

We have previously shown that *Panax* ginseng inhibits $I_{Ca,L}$, enhances I_{Ks}, and causes resultant shortening of APD, providing a possible explanation for cardiovascular protection against ischemia–reperfusion injury (Bai *et al*., 2003). At least 30 different ginsenosides have been identified as an ingredient of *Panax* ginseng. Ginsenoside Re is one of the ingredients with the highest content (0.15%) in *Panax* ginseng (Gillis, 1997), and we found that effects of *Panax* ginseng (1 mg ml^{-1}) on $I_{Ca,L}$ and I_{Ks} are roughly similar to those of ginsenoside Re at a corresponding concentration (1.9 μM) in *Panax* ginseng (1 mg ml^{-1}) (Bai *et al*., 2003). Therefore, in the present study, we investigated the underlying mechanisms of modulation of cardiac ion currents by ginsenoside Re, even though other types of ginsenosides (ginsenoside Rg3) are recently demonstrated to modulate Ca^{2+} current and NO production in rat sensory neurons (Rhim *et al*., 2002) and rat aortic ring (Kim *et al*., 2003). Data show that electrophysiological effects of ginsenoside Re on guinea-pig ventricular myocytes are *via* NO actions, and that NO appears to affect I_{Ks} by direct *S*-nitrosylation of the channel protein, and $I_{Ca,L}$ by a cGMP-dependent pathway.

I_{Ks} is shown to be modulated by various intracellular and extracellular signals, which include increase in $[Ca^{2+}]_i$ (Tohse, 1990; Nitta *et al*., 1994), β-adrenergic receptor stimulation (Duchatelle-Gourdon *et al*., 1989; Giles *et al*., 1989; Walsh *et al*., 1989; Duchatelle-Gouordon & Hartzell, 1990), PKA activation (Walsh & Kass, 1988), and PKC activation (Walsh & Kass, 1988; Tohse *et al*., 1990). None of these appears to be involved in ginsenoside Re-induced I_{Ks} enhancement, because there was no significant difference in ginsenoside Re-induced I_{Ks} enhancement between in the presence (1.54 ± 0.02, $n=5$) and the absence (1.51 ± 0.14, $n=5$) of a Ca^{2+} chelator, EGTA (10 mM) ($P=$ ns), and because no agonist nor antagonist of β-adrenergic receptor, or an inhibitor of PKA or PKC were able to suppress ginsenoside Re-induced I_{Ks} enhancement (Figure 3). In contrast, in the presence of an NOS inhibitor, SMT, enhancement of I_{Ks} by ginsenoside Re was abolished nearly completely. Application of an NO donor, SNP or SNAP, enhanced I_{Ks} by 1.57-fold, which is a comparable magnitude to the enhancement by a maximum dose of ginsenoside Re (1.53-fold). After application of SNP or SNAP, subsequently applied ginsenoside Re did not further enhance I_{Ks}. Treatment with LNAC, a NO scavenger, abolished enhancement of I_{Ks} by ginsenoside Re. All these data are in line with the suggestion that I_{Ks} enhancement by ginsenoside Re is *via* a pathway involving NO production.

NO modulates the Ca^{2+}-activated K^+ currents in vascular smooth muscle cells, and cGMP is suggested to play a principal role for the NO action, because of the following findings: (1) increase in intracellular cGMP level correlates with activation of the small-conductance Ca^{2+}-activated K^+ channels in pig aortic endothelial cells (Groschner *et al*., 1992); (2) application of a membrane-permeable cGMP or intracellular application of cGMP activate the Ca^{2+}-activated K^+ channels in cerebral artery smooth muscle cells (Robertson *et al*., 1993) or in rat pulmonary artery (Archer *et al*., 1994); and (3) an inhibitor of a cGMP-dependent guanylate cyclase, ODQ, abolished NO-dependent relaxation of rabbit isolated carotid artery (Plane *et al*., 1998). These are also reported in other types of K^+ channels in various cell types, including the ATP-sensitive K^+ channels in rabbit ventricular myocytes (Han *et al*., 2002) and the voltage-dependent K^+ current in carotid body glomus cells (Li *et al*., 2004). However, recent data suggest the contribution of the cGMP-independent pathway for NO-dependent regulation of the Ca^{2+}-activated K^+ currents (Chen *et al*., 1998; Lovren & Triggle, 2000), acethylcholine-activated K^+ currents (Abi-Gerges *et al*., 2002), and HERG channel (Taglialatela *et al*., 1999). Since NEM occluded NO-dependent activation of these K^+ currents, the direct production of reactive nitrogen intermediates, such as *S*-nitrosothiol by NO is suggested to be involved (Ahern *et al*., 1999). In the case of I_{Ks}, cGMP is shown to potentiate I_{Ks} in guinea-pig sino-atrial node cells (Shimizu *et al*., 2002). It is suggested that both activation of PKA *via* inhibition of cGMP-sensitive PDE3 and activation of PKG are involved in cGMP-dependent I_{Ks} potentiation. cGMP shifts the activation curve to the direction of negative potentials, which is supposed to be due to the addition of negatively charged P_i by PKA and PKG. On the other hand, I_{Ks} expressed in *Xenopus* oocytes is activated by the nitroso-donor *S*-nitroso-cysteine (Raber *et al*., 1995). In the latter study, since in the presence of the guanylate cyclase inhibitor LY-83,583 and the cGMP kinase inhibitor H8, *S*-nitroso-cysteine still enhances I_{Ks}, it is concluded that the effect of NO is *via* a pathway independent of a cGMP mechanism (Raber *et al*., 1995). In the present study, ginsenoside Re shifts the activation curve to the direction of negative potential at its relatively low concentrations, but not at its high concentrations (Figure 2). NEM is known to produce irreversible alkylation of thiol groups and, thereby, prevent the transfer of the NO group to protein thiolate anion (*S*-nitrosylation). NEM inhibited enhancement of I_{Ks} by about 80%, while ODQ, a guanylate cyclase inhibitor, did the same by about 20%. DTT that reduces the nitrosylated proteins reversed the enhancement of I_{Ks} by ginsenoside Re and SNP. Thus, direct *S*-nitrosylation of target protein is likely the main pathway of I_{Ks} enhancement by NO at least in guinea-pig ventricular myocytes.

We show that ginsenoside Re inhibits $I_{Ca,L}$ also *via* an NO action. There are several reports on inhibition of cardiac $I_{Ca,L}$ by NO, in which the cGMP-dependent pathway is suggested to be mainly responsible (Abi-Gerges, *et al*., 2001; Gallo *et al*., 2001). This is supported by the finding that PKG inhibits both native and cloned $I_{Ca,L}$ (Sumii & Sperelakis, 1995; Jiang *et al*.,

2000). However, NO is also shown to inhibit $I_{Ca,L}$ *via* a cGMP-indepenent mechanism. (Hu *et al*., 1997). In the present study, $I_{Ca,L}$ inhibition was not abolished by NEM or DTT, but was occluded by ODQ, suggesting a cGMP-dependent pathway as a main mechanism of $I_{Ca,L}$ inhibition at least in guinea-pig ventricular myocytes. The effects of NEM on $I_{Ca,L}$ are complicated, in that it initially decreased, and then increased $I_{Ca,L}$, which may indicate the presence of at least two target sites by NEM on $I_{Ca,L}$ channel, as suggested for ferret $I_{Ca,L}$ (Campbell *et al*., 1996).

The remaining issues to be addressed include: the identification of NOS that is activated by ginsenoside Re; and identification of the pathway linking ginsenoside Re to NOS activation. Both NOS1 and NOS3 are expressed in cardiac myocytes. NOS1 is localized to the sarcoplasmic reticulum and coimmunoprecipitates with ryanodine receptors, while NOS3 is localized to caveolae in the T-tubule and coimmunoprecipitates with caveolin-3 (Barouch *et al*., 2002; Khan *et al*., 2003). NO produced by NOS1 selectively activates ryanodine receptors, and NO produced by NOS3 inhibits $I_{Ca,L}$ (Barouch *et al*., 2002; Khan *et al*., 2003). Ginsenoside Re inhibits $I_{Ca,L}$, and we have previously shown that the β-subunit of the I_{Ks} channel, minK, localizes to the sarcolemma of the T-tubule (Furukawa *et al*., 2001); thus, it is likely that ginsenoside Re stimulates NOS3, rather than NOS1. Experiments to address these issues are currently under way.

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