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Effect of Ginseng Saponin on the Na^+, K^+-ATPase of Dog Cardiac Sarcolemma

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Abstract The effects of ginseng saponins on the sarcolemmal $Na,^+$ K^+-ATPase were compared to gypsophila saponin, sodium dodecylsulfate (SDS), and Triton X-100 to elucidate whether the effects are due to the membrane disruption, using a highly enriched preparation of cardiac sarcolemma prepared from dog ventricular myocardium. About 26% and 29% of vesicles in the preparation, enriched in ouabain-sensitive $Na,^+$ K^+-ATPase, ß-adrenergic and muscarinic receptors are rightside-out and inside-out in orientaton, respectively. Ginseng saponins (triol>total>diol) inhibited Na^+, K^+-ATPase activity, Na^+-dependent phosphorylation and [^{3}H]ouabain binding of sarcolemmal vesicles. However, gypsophila saponin, SDS (0.4$\mu g/\mu g$ protein) and Triton X-100 (0.6$\mu g/\mu g$ protein) caused about 1.35 and 1.40-fold increase in Na^+, K^+-ATPase activity and [^{3}H] ouabain binding, respectively. Especially, the activating effect of gypsophila saponin on membrane Na^+, K^+-ATPase was detected at gypsophila saponin to sarcolemmal protein ratios as high as 100. Low dose of ginseng saponin (3$\mu g/\mu g$ protein) decreased the phosphorylation sites and the concentration of ouabain binding sites (Bmax) without affecting the turnover number and affinity for ouabain binding, while gypsophila saponin, SDS (0.4ug/ug protein), and Triton X-100 (0.6$\mu g/\mu g$ protein) increased the Bmax. The results suggest that ginseng saponins cause a decrease in the number of active sites by interacting directly with Na^+, K^+-ATPase before disruption of membrane barriers of sarcolemmal vesicles.

Keywords Myocardium, Sarcolemma, Na^+, K^+-ATPase, Ginseng saponin, Gypsophila saponin, SDS.

Ginseng, most popular herb medicine in orient, has been widly used as tonics. Recently, Kim *et al.*[1] suggested that the inotropic effect of ginseng saponins might be related to the inhibition of Na^+, K^+-ATPase from the results that total ginseng saponin and ginsenoside Rb_1 increased the contractile force of rat ventricle and inhibited the activity of microsomal Na^+, K^+-ATPase prepared from rat ventricle. Other paper,[2] however, reported that total saponin or panax saponin C and ginsenoside Rb_1 increased and decreased the microsomal Na^+, K^+-ATPase activity of rat intestine, respectively. Thus, discrepancies exist between these reports in the effect of total saponin on microsomal Na^+, K^+-ATPase activity.

Saponin as a surfactant is often used to remove membrane lipid especially in preparation of chemical skinned smooth muscle[3-6] and in determination of membrane sidedness.[7] If ginseng saponins have the disruptive effect on membrane barriers and a microsomal preparation contains the mixed populations of vesicles (rightside-out and inside-out vesicles), it might increase the enzyme activity by the increase of ATP site without direct effect on Na^+, K^+-ATPase, since Na^+, K^+-ATPase is a membrane-bound enzyme primarily responsible for the maintenance of Na^+, and K^+ gradients in most animal cell.[8, 9]

In a previous report[10] we showed that total ginseng saponin, panaxadiol and panaxatriol inhibited the purified Na^+, K^+-ATPase isolated from sheep kidney. However, no studies have yet demonstrated whether ginseng saponins increase the accessibilities of substrates to their active sites on enzymes by obviating the permeability barriers of membrane vesicles. If ginseng saponins have the potent disruptive effect on membrane barriers, the inhibitory action on Na^+, K^+-ATPase may be meaningless *in vivo*.

In this study, the effects of ginseng saponins on Na^+, K^+-ATPase of sarcolemma prepared from canine ventricle were compared to gypsophila saponin, SDS and Triton X-100 to investigate what relationships the change of membrane Na^+, K^+-ATPase activity after ginseng saponins and detergents treatment have with the membrane disruption.

EXPERIMENTAL METHODS

Materials

Ginseng saponins prepared by the method of Han et al.[11] and Namba *et al.*[12] were kindly supplied by Dr. K.H. Bae of College of Pharmacy, Chungnam National University, Daejeon, Korea. Adenosine triphosphate disodium salt (vanadium free) was from Boehringer-Mannheim, Germany. Sodium dodecylsulfate (SDS) was from Bio-Rad Laboratories, Richmond, California, U.S.A. Ethylenediamine tetraacetic acid (EDTA), ethyleneglycol-bis-(ß-aminoethylether)-N,N′-tetraacetic acid (EGTA), L-histidine, nicotinamide adenine dinucleotide (reduced form, NADH), ouabain octahydrate, phosphoenolpyruvate tricyclohexylamine salt, pyruvate kinase lactic dehydrogenase (suspension), and gypsophila saponin were obtained from Sigma Chemical Co., St. Louis, Mo, U.S.A. [γ-^{32}P]ATP (25-45Ci/mmol),[45] $CaCl_2$ (10mCi/ml), [^{3}H] dihydroalprenolol (DHA, 35Ci/mmol), [^{3}H]ouabain (19.5Ci/mmol). [^{3}H]quinuclidinyl benzilate (QNB, 67Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. All other reagents were of reagent grade.

Preparation of cardiac sarcolemma

Dog cardiac sarcolemmal membranes were prepared by the method of Van Alstyne et al.[13] with only slight modifications. Dog cardiac muscle was cleaned of fat and connective tissue, cut into small pieces and homogenized in a sorvall omnimixer fully filled (to exclude air) with 30mM Tris- maleate buffer containing 0.3M sucrose, pH 7.0 (Medium A). The ratio (w/w) of muscle to Medium A was 1:4. Total homogenization time was 60sec with 15sec bursts and 30sec cooling intervals. The homogenized suspension was centrifuged at 5,500xg for 10min. The resultant pellet was resuspended in 4~5 volumes of Medium B (0.25M sucrose, 5mM NaN_3, 10mM $NaHCO_3$, pH 7.0) using a 250ml glass-teflon motor driven homogenizer (one pass with the reostat set at 80% of 140 volts). The suspension was centrifuged for 20min at 8,700xg and the pellet was resuspended in 6 volumes of Medium C (0.25M sucrose, 10mM Tris-Cl, pH 7.4) using 4 passes of the motor driven glass-teflon homogenizer (80% of 140 volts). This procedure releases a fraction of the sarcolemma (SL) membranes from the crude particulate fraction. The suspension was then centrifuged for 20min at 8,700xg and the supernatant containing the SL was saved. The pellet was re-extracted by resuspension in Medium C and recentrifugation at 8,700xg. The two supernatant were combined and centrifuged for 20min at 35,000xg. The pellet was resuspended in 10mM Tris-Cl, pH 7.4 using a hand driven 50ml glass teflon homogenizer (5 passes) · 10ml of this suspension was layered over 20ml of 24% (w/v) sucrose in 10mM Tris-Cl, pH 7.4, and centrifuged for 30min at 73,400xg. The interface between the sample and the 24% sucrose was collected, diluted to 50ml with Medium C, and centrifuged for 30min at 73,400xg. The pellet was resuspended in a small volume of Medium C, using a hand driven glass-teflon homogenizer to give a final Lowry protein concentration of 1.5 to 2.5mg/ml. Samples were either studied immediately or stored frozen in small aliquots at -80°C until used. Protein content was estimated by the method of Lowry *et al.*[14]

Assay of ATPase activity

Total and specific ATPase activities were measured by a spectrophotometric coupled-enzyme assay,[15] as previously described, in a reaction medium containing 100mM NaCl, 10mM KCl, 5mM $MgCl_2$, 5mM Na_2ATP, 0.4mM NADH, 1mM phosphoenolpyruvate, 20ul of a combind pyruvate kinase/lactic dehydrogenase suspension, and 25mM L-Histidine, pH 7.4, in a total volume of 2.5ml. Activity was measured by monitoring the decrease in absorbance at 340nm due to oxidation of NADH. Total ATPase activity was determined in the absence of inhibitors. ATPase activity presumably associated with mitochondria was determined by measuring the difference between total ATPase activity and the ATPase activity in the presence of 5mM NaN_3. Na^+, K^+ -ATPase activity was determined in the same cuvette by measuring the difference between azide-insensitive ATPase activity and activity present after addition of a 2mM ouabain. ATPase activity remaining after the further addition of 0.1mM EGTA represented a basal ATPase activity for the determination of calcium-dependent ATPase. Ca^{2+} -ATPase activity was determined by measuring the difference between this basal Mg^{2+} -APTase activity and ATPase activity after addition of 0.2mM $CaCl_2$.

Sodium dependent Phosphorylation

Sodium-dependent phosphorylation of sarcolemmal Na^+, K^+ -ATPase was carried out at 0°C by a filtration method,[16] as previously described. The reaction medium contained 30mM histidine (pH 7.4), 2mM $MgCl_2$, 100mM NaCl, 5mM NaN_3, 100μM EGTA, and 20ug of sarcolemma protein in a volume of 0.5ml. The reaction was initiated by the addition of [γ-^{32}P]ATP (100μM) and terminated after 15 seconds by the addition of 5ml of an ice-cold wash solution containing 10% trichloroacetic acid, 0.6mM Na_2ATP, and 0.6mM H_3PO_4. The tubes were immediately placed in an ice bath, and the contents filtered through 0.45um Gelman filters. The filters were washed three times with 5ml of the ice-cold wash solution and placed in 8ml of scintillation fluid for 12 hours before counting in a Beckman LS 200B liquid scintillation counter. Nonspecific radioactivity was measured by the inclusion of 5mM unlabeled ATP in the above medium and was subtracted from total.

[^{3}H] Ouabain binding

Binding of [^{3}H] ouabain was carried out by the Millipore filtration method[16] to seperate free from the membrane-bound ouabain. Unless otherwise states, the

binding media contained, in final concentration, 100mM NaCl, 10mM KCl, 5mM $MgCl_2$, 5mM Na_2ATP, 25mM L-histidine (pH 7.4), 0.4mM NADH and 1mM phosphoenolpyruvate. These ligands plus 100nM [^{3}H] ouabain were placed in a constant temperature of 37°C. After temperature equilibration was reached (10min), protein was added to initiate the reaction. At various time, aliquots of 0.9ml were removed and rapidly filtered through 0.45μm methylcellulose filters and washed three times with 5ml of cold deionized water.

The filters were placed in scintillation vials containing 8ml of scintillation fluid, and radioactivity was counted in a Beckman LS 200B scintillation counter. Nonspecific binding was measured in the same reaction medium as above, except that unlabeled ouabain (final concentration, 1mM) was added to the binding medium prior to the addition of protein. Dissociation of ouabain from the enzyme was measured by a chase method.[17] Binding of [^{3}H] ouabain (100 nM) was carried out at 37°C as described above for a sufficient time (90min) for equilibrium to be achieved. An excess of unlabeled ouabain (final 1mM) was then added and, at suitable times, aliquots were filtered, washed, and counted. Rate constants for association and dissociation reactions were determined by a procedure described by Wallick *et al*[18] For Scatchard analysis of [^{3}H] ouabain binding, sarcolemma were incubated for 90 minutes at 37°C in the same medium as described above and 1-500nM [^{3}H] ouabain. The dissociation constant (K_D) and maximal binding site concentration (Bmax) were estimated from Scatchard plots by linear regression analysis.

[^{3}H]Dihydroalprenolol (DHA) and [^{3}H]quinuclidinyl benzilate (QNB) binding

Binding of [^{3}H]DHA and [^{3}H]QNB was carried out in the presence of 50mM Tris-Cl (pH 7.4), 10mM $MgCl_2$, 20-25ug of sarcolemma and 10nM of [^{3}H]DHA or 200pM of [^{3}H] QNB. Temperature and incubation time were 30°C and 20min for [^{3}H]DHA, and 37°C and 60min for [^{3}H]QNB. Nonspecific binding was measured in the presence of 10^{-4}M alprenolol and 10^{-6}M atropine, respectively. After incubation, the reaction was stopped by the addition of 5ml ice-cold buffer containing 50mM Tris-Cl and 10mM $MgCl_2$ and immediately filtered through Whatman GF/F glass fiber filters. The filters were washed four times with 5ml of ice-cold buffer and placed in scintillation vials containing 8ml of scintillation fluid for count.

ATP dependent Ca^{2+} -uptake

To prevent Na^+/Ca^{2+} exchange, sarcolemmal vesicles were preloadedwith K^+ by incubation in the presence of 160mM KCl and 20mM MOPS/Tris-Cl, pH 7.4 (Medium I). The vesicles were pelleted by centrifugation, washed in Medium I and the final pellet (K^+-vesicles) was homogenized in small volume of Medium I and brought to a final concentration of 3mg/ml. For assay of total Ca^{2+}-uptake, K^+-vesicles (3-4ug) were incubated in Medium I containing 3mM $MgCl_2$, 10uM $^{45}CaCl_2$ and 2mM Tris-ATP in the presence or absence of 3mM potassium oxalate for 10min at 37°C, in final volume of 0.1ml. ATP dependent Ca^{2+} -uptake was calculated by subtraction of the Ca^{2+}-uptake in the absence ATP from the total Ca^{2+} - uptake. ATP-independent Ca^{2+}-uptake was about 10% of the total uptake.

Treatment of sarcolemma with saponin, SDS, or Triton X-100

Sarcolemma was incubated with various concentrations of ginseng saponin, gypsophila saponin, SDS or Triton X-100, for 10min at 37°C in a final volume of 0.32ml before experiment.

Statistical analysis was performed with student's t-test, and the level for significance was taken as a probability less than 5% ($p<0.05$).

RESULTS

Properties of the sarcolemma preparation

This procedure yields a highly enriched sarcolemma preparation from dog ventricles with the following characteristics: Sarcolemmal Na^+, K^+ -ATPase activity was $142.6 \pm 12.3\mu$mol pi/mg of protein/hr ($n=b$). The purification factor estimated by dividing sarcolemma enzyme activity by starting homogenate enzyme activity ($4.4 \pm 1.2\mu$mol pi/mg/hr, $n=6$) was 32.4 ± 3.8 ($n=6$). Equilibrium binding of [^{3}H] ouabain to homogenate and sarcolemmal fraction in the presence of 100nM [^{3}H]ouabain was 4.3 ± 0.3 and 181.4 ± 15.7pmol/mg ($n=6$), respectively, indicating a 42-fold enrichment of ouabain binding site in the sarcolemma fraction compared to the homogenate. [^{3}H]DHA and [^{3}H]QNB binding to ß-adrenoreceptor and muscarinic receptor were 1.92 ± 0.32pmol/mg ($n=4$) and 7.58 ± 0.62pmol/mg ($n=4$), respectively, in the sarcolemma fraction. These two sarcolemma markers also enriched about 48-fold in the sarcolemma fraction relative to the starting homogenate. In addition to Na^+, K^+ -ATPase activity low levels of azide sensitive and Ca^{2+} -ATPase activities were measured in the sarcolemma fraction. Their presence in this fraction may reflect contamination by vesicles from non-sarcolemma. In this regard, azide sensitive ATPase activity (3.5 ± 0.1 μmol pi/mg/hr) as a mitochondrial marker was less than 3% of total ATPase activity of sarcolemma fraction and Ca^{2+} -ATPase activity ($2.3 \pm 0.06\mu$mol pi/mg/hr) was referred to be associated with sarcolemma Ca^{2+} -ATPase because ATP-dependent Ca^{2+} -uptake of sarcolemmal fraction was not affected by oxalate (8.4 ± 0.4 and 7.8 ± 0.5nmol/mg, $n=3$, in the absence and presence of oxalate, respectively). Although small amounts of contaminating mitochondria may exist in this sarcolemma preparation, sarcolemma enrichment and the restriction

of Na^+, K^+-ATPase to the sarcolemmal membrane make meaningful assessment of our data possible without any suspicion of artifacts due to membrane impurities.

Effect of ginseng saponins on Na^+, K^+ -ATPase activity

Ginseng saponins used in this study decreased the Na^+, K^+ -ATPase activity of a typical preparation of cardiac sarcolemma, but the inhibition curve of total saponin showed different shape from those of panaxadiol and panaxatriol (Fig. 1). The inhibition sequences by ginseng saponins were triol>total>diol. Gypsophila saponin, however, increased the sarcolemma Na^+, K^+ -ATPase activity about 1.35-fold from a basal level of 103.8μmol pi/mg/hr to a maximum level of 140.1μmol pi/mg/hr. Maximal stimulation was occurred over a broad range of gypsophila saponin to protein ratio (w/w) between 1.0 to 6.0. No significant inhibition of enzyme activity was seen when gypsophila saponin per ug of sarcolemmal protein was increased to 200μg.

It is well known that plant saponins and chemical detergents increase the Na^+, K^+ -ATPase activity of sarcolemmal vesicles via their disruptive effect on membrane barriers.[19, 20] If our sarcolemma preparation has some of the sealed rightside-out vesicles the detectable Na^+, K^+ -ATPase activity of sarcolemma fraction should be increased by treatment with appropriate concentration of detergents. In order to investigate whether sarcolemma preparation contains rightside-out vesicles, sarcolemma was incubated with various concentrations of SDS and Triton X-100. As shown in Fig. 2, SDS (0.4μg/μg protein) and Triton X-100 (0.6μg/μg protein) both resulted in increase in the Na^+, K^+ -ATPase activity by 35%. Increase of detergent concentration to 1.2μg per ug of membrane protein decreased or diminished the enzyme activity. Activation of Na^+, K^+ -ATPase activity by these chemical detergents suggests that the preparations contained rightside-out vesicles whose membrane permeability barrier remained intact. The result before and after treatment with SDS from 6 seperate preparations are summarized in Table I. From 1.35-fold increase in enzyme activity after treatment with optimal concentration of SDS (0.4μg/μg protein), it was estimated that approx. 26% of the sarcolemmal vesicles are rightside-out in orientation.

If ginseng saponins acts by similar mechanism, it should increase the Na^+, K^+ -ATPase activity of this

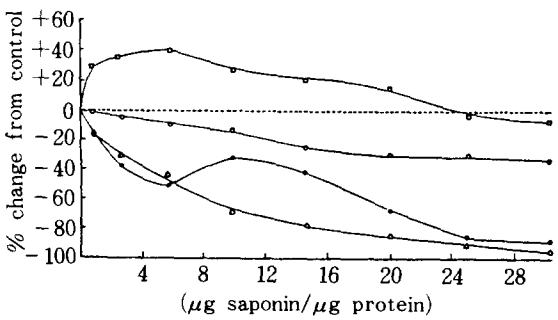

Fig. 1. Effect of ginseng and gypsophila saponins on Na^+, K^+ -ATPase activity of dog cardiac sarcolemma.

Sarcolemmal Na^+, K^+ -ATPase activity was determined using a spectrophotometric method described under Methods. Sarcolemma was incubated with a given concentration of total saponin (●), panaxadiol (○), panaxatriol (△), and gypsohpila saponin (□) for 10min at 37°C in a final volume of 0.32ml before measurement of enzyme activity. Control sarcolemmal Na^+, K^+ -ATPase activity was 105.6 ± 8.4 μmol pi/ mg protein/hr (n=6). Mean values of three different preparations are given.

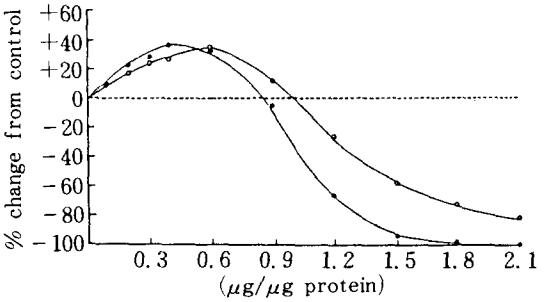

Fig. 2. Effect of SDS and Triton X-100 on the sarcolemmal Na^+, K^+ -ATPase activity.

●, SDS treated sarcolemma; ○, Triton X-100 treated sarcolemma. Average values for three experiments are shown. Other legends are the same as described in Fig. 1.

Table I. SDS effects on Na^+, K^+ -ATPase activity of and [^{3}H] ouabain binding to cardiac sarcolemmal vesicles

Parameter measured	Patent	Total	% from total
Na^+, K^+-ATPase activity (μmol pi/mg/hr)	105.6±8.4	142.6±12.3	74.1
[^{3}H] Ouabain binding (pmol/mg)	129.3±7.8	181.4±15.7	71.3

Sarcolemmal vesicles were treated with 0.4μg of SDS per μg protein as described in Methods. Total Na^+, K^+-ATPase activity and [^{3}H] ouabain binding represent the activity and binding of sarcolemma treated with SDS. Values are mean ± SEM of six different preparations.

preparation. However, we did not detect increase in enzyme activity at any concentration of ginseng saponins used here.

Na^+-dependent phosphorylation of sarcolemmal Na^+, K^+ -ATPase

The change in Na^+, K^+ -ATPase activity could result from a change in the number of Na^+, K^+ -ATPase molecules (sodium pump site) or a change in the turnover of Na^+, K^+ -ATPase. Sodium-dependent phosphorylation of Na^+, K^+-ATPase from gamma labeled [^{32}P]ATP was used to determine the number of active site of enzyme present in sarcolemma preparation. When sarcolemmal membranes were incubated with [r-^{32}P]ATP in the presence of Na^+ and Mg^{2+} there was rapid incorporation of ^{32}P into sarcolemma. All the ^{32}P incorporated was sensitive to 0.8M hydroxylamine, indicating the presence of acylphosphate intermediate of the Na^+, K^+ -ATPase (data not shown). Total saponin, panaxadiol, and panaxatriol inhibited the acyl phosphorylation of cardiac sarcolemma with the same inhibition sequence and curve as shown in Na^+, K^+ -ATPase activity (Fig. 3). The relationships between decreases in the activity and active site of sarcolemmal Na^+, K^+ -ATPase by ginseng saponin were illustrated in Table II. The number of phosphorylation sites in sarcolemma treated with low concentration of ginseng saponins (3μg/μg protein) was significantly lower than control sarcolemma. The turnover number which is

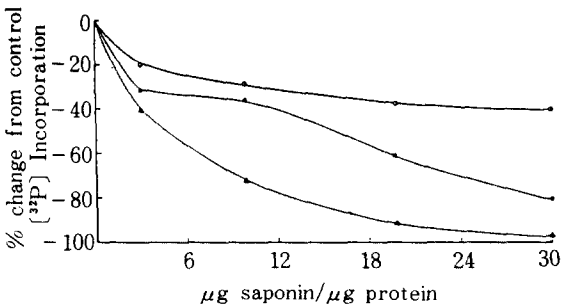

Fig. 3. Effect of ginseng saponins on the Na^+ -dependent phosphorylation of dog cardiac sarcolemma.

The [^{32}P] phosphoenzyme was formed in the presence of 2mM $MgCl_2$, 100mM NaCl, 5mM NaN_3, 100μg EGTA and 30mM histidine (pH 7.4) as described under Methods, in a volume of 0.5 ml. The amount of sarcolemma per assay was 20μg. ●, total saponin-treated sarcolemma; ○, panaxadiol-treated sarcolemma; ▲, panaxatriol-treated sarcolemma. Average values for three experiments are shown. [^{32}P]Incorporation of control sarcolemma was 254.2 ± 13.6pmol pi/mg protein (n=3).

Table II. Na-dependent phosphorylation of control and ginseng saponin treated dog cardiac sarcolemma

	[^{32}P] Bound (pmol pi / mg)	Turnover number ($\times 10^6 hr^{-1}$)
Control	254.2±13.6	0.42±0.04
Total	173.3±12.4*	0.43±0.04
Diol	204.7± 8.4*	0.47±0.03
Triol	147.6± 8.5*	0.46±0.03
Gypsophila	340.6±16.2*	0.42±0.05
SDS	332.5±13.8*	0.43±0.05

Phosphorylation of dog cardiac sarcolemma (20μg/0.5m*l*) was carried out at 0 ℃ in the presence of 100μM [r-^{32}P] ATP as described in Method. Dog cardiac sarcolemma was treated with 3μg of ginseng saponins, 6μg of gypsophila saponin or 0.4 μg of SDS per μg protein for 10min at 37℃ before phosphorylation experiment. Turnover number was calculated by equation, V=kE (k: turnover number, V : enzyme activity, E : active site concentration). Data were expressed as mean± SEM of three different preparations. Significantly different from corresponding value of control (*$p<0.05$).

ratio of enzyme activity to number of phosphorylation sites was the same for both control and saponin-treated preparations. Thus, the reduction in Na^+, K^+ -ATPase activity by low concentration of ginseng saponin is entirely explained by reduction in the number of accessible active sites. On the other hand, the treatment of sarcolemma with 6μg of gypsophila saponin and 0.4μg of SDS per μg protein increased the number of phosphorylation sites without affecting the turnover number.

[^{3}H]Ouabain binding studies

It is commonly accepted that digitalis glycosides specifically bind to Na^+, K^+ -ATPase and inhibit this enzyme system.[21, 22] To elucidate the effect of ginseng saponins on [^{3}H]ouabain binding to the sarcolemmal Na^+, K^+ -ATPase, sarcolemma was treated with various concentrations of ginseng and gypsophila saponins before binding assays. Since the rate of association of ouabain to the enzyme depends upon the presence and concentration of certain physiological ligands,[17] all ouabain binding assays were carried out under the same conditions at those employed to measure enzyme activity. As shown in Fig. 4, panaxadiol and panaxatriol inhibited the [^{3}H] ouabain binding to the sarcolemma in the concentration-dependent manner. Total saponin also inhibited the binding, but showed the triphasic dose-response curve. On the other hand, gypsophila saponin ranging from 1 to 30μg per ug sarcolemmal protein increased the ouabain binding. Treatment of the sarcolemma with 0.4μg of SDS

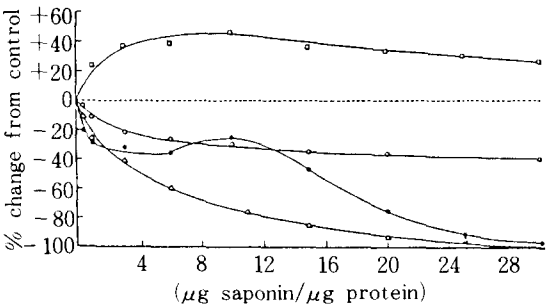

Fig. 4. Effect of ginseng and gypsophila saponins on [^{3}H] ouabain equilibrium binding to dog cardiac sarcolemma.

Saponin treatment and binding media are defined under Methods. The concentration of [^{3}H]ouabain was 10^{-7}M. All data are expressed as the percentage of that amount bound to control sarcolemma, which averaged (± SE) 129.3 ± 7.8pmol/mg (n=6). ●, total saponin; ○, panaxadiol; △, panaxatriol; □. gypsophila saponin. Mean values for three separate experiments are given.

or 0.6ug of Triton X-100 increased about 40% in the [^{3}H]ouabain binding (Fig. 5), indicating that 29% of the sarcolemmal vesicles were inside-out orientation (Table I). These results suggest that effect of ginseng saponins in the sarcolemmal Na^+, K^+ -ATPase is not due to the disruption of membrane vesicles.

Fig. 6-A shows time courses of [^{3}H]ouabain binding to the sarcolemma preparations. In the presence of 10^{-7}M [^{3}H]ouabain the equilibrium binding to then ative and total saponin-treated (3μg/μg protein) sarcolemma was 137.4±9.6 and 98.2±8.5 picomoles of [^{3}H]ouabain per miligram of protein (n=3), respectively. [^{3}H]Ouabain binding to the native sarcolemma follows first-order kinetics consistant with earlier reports[23, 24] and the rate of association of the saponin-treated sarcolemma, which were also first-order, was not significantly different from the rate of the native sarcolema (Fig. 6-B). Since the dissociation rate constant (K_{-1}) of ouabain to the Na^+, K^+ -ATPase is much smaller than association rate constant (K_1), the rate of dissociation was measured a "chase" techniques.[17] Binding of the tritium-labeled ouabain was allowed to reach equilibrium and an excess of unlabeled ouabain was then added. Under these condiontion, a first-order loss of redioactivity was observed and treatment with 3μg of

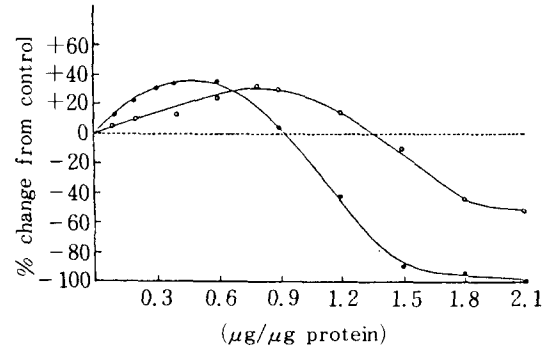

Fig. 5. Effect of SDS and Triton X-100 on [^{3}H] ouabain equilibrium binding to dog cardiac sarcolemma.

●, SDS treated sarcolemma; ○, Triton X-100 treated sarcolemma. Other legends are the same as described in Fig. 4.

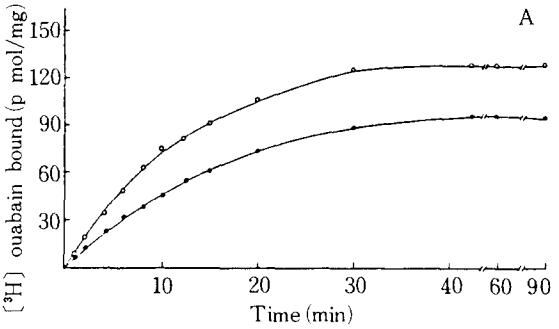

Fig.6-A [^{3}H]Ouabain binding to dog cardiac sarcolemma.

The vertial axis is the amount of [^{3}H] ouabain (pmole) bound to 1mg of sarcolemmal protein; horizontal axis is the time from start of [^{3}H]ouabain binding reaction. The concentration of [^{3}H]ouabain was 10^{-7}M.

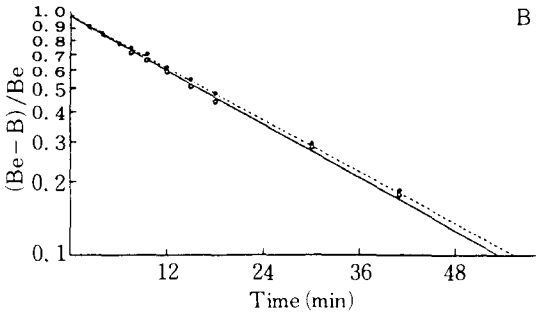

Fig.6-B Pseudofirst-order binding 0f [^{3}H] ouabain to dog cardiac sarcolemma replotted from data of Fig. 6A.

(Be-B)/Be (log scale) versus time is shown, where Be and B are radioligand bound to the sarcolemma at equilibrium and at time, t, respectively. ○, control sarcolemma; ●, sarcolemma treated with 3μg of total saponin per μg protein.

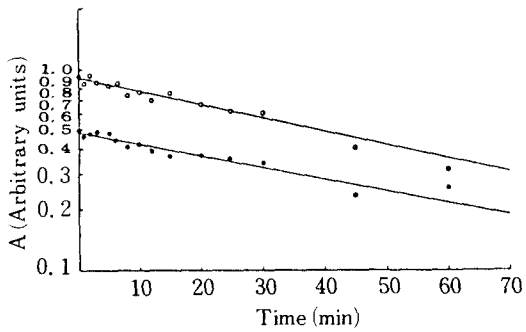

Fig. 7. Dissociation of [^{3}H]ouabain from enzyme-ouabain complex.

[^{3}H]Ouabain binding was carried out at 37°C in the presence of 10^{-7}M [^{3}H] ouabain under the same condition as described in Methods. Unlabeled ouabain was then added (final 10^{-4}M) and aliquots were removed and filtered as described under Methods. The amount of radioactivity associated with the enzyme, A, was plotted (log scale) versus time. The ordinate has been arbitrarily scaled. ○, control sarcolemma; ●, sarcolemma treated with 3μg of total saponin per μg protein.

total saponin per milligram protein did not appreciably affect the rate of breakdown of the enzyme-ouabain complex (Fig. 7). Since dissociation is measured under the same condition (except for the excess unlabeled ouabain) as association, the dissociation constant (K_D) can be calculated using equation, $K_D = K_{-1}/K_1$. The calculated K_D values were the same for the native sarcolemma and the sarcolemma treated with 3ug of total saponin per milligram protein (Table III).

Table Ⅲ. Kinetic constants for [^{3}H]ouabain binding of dog cardiac sarcolemma

	K_1^a ($min^{-1}M^{-1}\times10^{-3}$)	K_{-1}^b ($min^{-1}\times10^3$)	K_D^c (nM)
Control	374 ±31.1	15.7±0.7	42.0±5.4
SL+Saponin	351.3±28.2	16.5±0.5	47.0±6.7

a. Calculated from $K_1 = (K_{obs} - K_{-1}) / [I]$ where K_{obs} in the observed first-order approach to equilibrium derived from 3 studies of the type shown in Fig. 6-B.

b. Derived from 3 studies of the type shown in Fig. 7.

c. Calculated from $K_D = K_{-1}/K_1$. Rates of association and dissociation followed good first-order kinetics and were determined as described under experimental method. SL+Saponin: Dog cardiac sarcolemma was treated with total saponin (3μg saponin/μg protein). Values are mean±SEM of three assays.

Scatchard analysis of ouabain binding was carried out to test whether ginseng saponins decrease the ouabain binding sites. Fig. 8 shows a Scatchard plot of ouabain binding at various concentrations of [^{3}H]ouabain. The apparent K_D values calculated from the equation, $B = -K_D \cdot \frac{B}{F} + Bmax$, were the same for the native sarcolemma and sarcolemma treated with 3μg ginseng saponins within experimental error (Table IV), but the binding site concentration (Bmax) of ginseng saponin-treated sarcolemma was significantly lower than that of control.

As illustrated in Table IV, treatment with a optimal dose of gypsophila saponin (6μg), SDS (0.4μg) and Triton X-100 (0.6μg) which increased the Na^+, K^+-ATPase activity and ouabain binding increased the Bmax without any change of the K_D values for ouabain binding. However, high concentration of these chemical detergents and ginseng saponins decreased both the apparent affinity and number of the bindingsite. The binding site concentration of the sarcolemma treated with 10μg of total saponin was lower than that of control, but higher than that of the sarcolemma treated with 3μg total saponin. These results are similar to those described for the effect of total saponin on the sarcolemmal Na^+, K^+-ATPase activity.

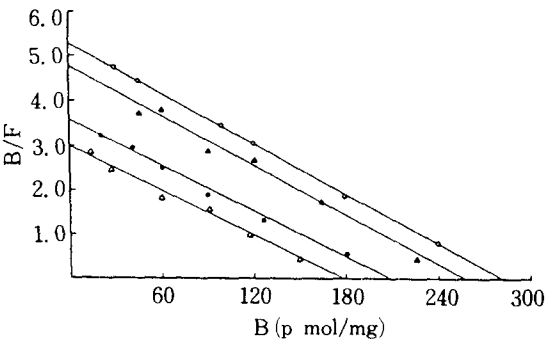

Fig. 8. Scatchard analysis of the inhibition of [^{3}H]ouabain binding to dog cardiac sarcolemma by ginseng saponins.

[^{3}H]Ouabain binding was performed in the presence of various concentrations of [^{3}H]ouabain. Saponin treatment and binding media are defined under Methods. Bound [^{3}H]ouabain/free [^{3}H]ouabain (B/F) is plotted as a function of specific [^{3}H]ouabain bound (B) to the sarcolemma. ○, [^{3}H]ouabain binding of control sarcolemma; ●, ▲, and △, [^{3}H]ouabain binding of total saponin (●), panaxadiol (▲), and panaxatriol (△)-treated sarcolemma (3μg saponon/μg sarcolemmal protein).

Table Ⅳ. K_D and Bmax of [^{3}H]ouabain binding to the sarcolemmal Na^+, K^+--ATPase

Treatment(n)	Dose ($\mu g/\mu g$ protein)	K_D(nM)	Bmax (pmol / mg)
Control (10)		54.2± 4.3	285.1±10.5
Total saponin (3)	3	58.1± 3.9	223.1±11.3**
	10	100.0± 8.6**	253.7±10.7*
Panaxadiol (3)	3	61.0± 5.6	250.3±12.5*
	10	67.9± 5.6*	219.5±10.5**
Panaxatriol (3)	3	59.4± 5.2	178.3±11.8**
	10	99.6±11.0**	98.1± 3.5**
Gypsophila saponin (3)	6	49.0± 3.8	393.3±16.9**
SDS (3)	0.4	57.3± 3.2	386.7±13.4**
Triton X-100 (3)	0.6	59.2± 4.5	383.5±14.8**

Cardiac sarcolemma was treated with the indicated dose of drugs as described in Methods before binding assay. K_D and Bmax were calculated from the equation, $B = -K_D \cdot \frac{B}{F} + Bmax$ (B: equilibrium binding at a given concentration of [^{3}H] ouabain, K_D: apparent affinity, F: free drug concentration, Bmax: binding site concentration). Other legends are the same as described in Fig. 8. Values are mean± SEM of 3 or 10 different preparations. Significantly different from corresponding value of control (*$p<0.05$, **$p<0.01$).

DISCUSSION

The main findings of this study are that the effect of low concentration of ginseng saponins on the sarcolemmal Na^+, K^+-ATPase differs from that of gypsophila and chemical detergents in that: (a) a increase in the number of available Na^+, K^+-ATPase active sites is not detected: and (b) the concentration of both phosphorylation and ouabain binding sites without affecting the turnover number and apparent affinity are reduced.

Since it is necessary to isolate the sarcolemma with the highest purity possible to compare the effect both of ginseng saponins and detergents on the sarcolemmal Na^+, K^+-ATPase, the purity of the sarcolemmal fraction isolated was evaluated in the first place on the basis of marker enzyme activity and receptor binding. The 32 to 42-fold enrichment of these sarcolemma markers strongly support the conclusion that our preparation is highly enriched with sarcolemma. Ca^{2+}-ATPase activity detected in the sarcolemma fraction was associated with the sarcolemma because ATP-dependent Ca^{2+}-uptake was not dependent on oxalate in contrast to the well-known oxalate-dependent Ca^{2+}-uptake in sarcoplasmic reticulum vesicles.[25] The presence of mitochondrial and residual ATPase activity, however, demonstrates that complete purification of the sarcolemma was not achieved.

The Na^+, K^+-ATPase activity of and ouabain binding to sarcolemmal vesicles are likely to be due to the fraction of vesicles in the inside-out and right-side-out orienation, respectively, since the phosphorylation sites of the Na^+, K^+-ATPase are presumed to be located on the cytoplasmic surface of the sarcolemmal membrane and the ouabain binding site of this enzyme is believed to face the extracellular space.[8, 26, 26] If the vesicles are made permeable, the substances which interact with these sites obtain access to these sites in the inside-out vesicles as well as the rightside-out vesicles. Thus, the total phosphorylation and ouabain sites could be unmasked. Several investigators have shown that SDS probably increases Na^+, K^+-ATPase activity of the sarcolemmal preparations by its disruptive effect on membrane barriers of sealed vesicles.[28, 29] The present results showing the effect of SDS and Triton X-100 provide indirect information on the orientation of cardiac sarcolemmal vesicles, although definitive determination of membrane sidedness will probably require some type of morphological analysis. SDS and Triton X-100 both increased 1.35-fold in the sarcolemmal Na^+, K^+-ATPase activity and 1.40-fold in the [^{3}H]ouabain binding to the sarcolemma. The values obtained suggest that about 26% and 29% of the vesicles are sealed rightside-out and inside-out vesicles, respectively, and about 45% of the vesicles are freely permeable.

It is well known that saponin has a high affinity toward cholesterol and causes the disruption of plasma membrane by removing cholesterol.[20] In our experiments, both gypsophila and SDS unmasked latent sarcolemmal Na^+, K^+-ATPase activity to nearly the same extent, and increased the number of ouabain binding sites without any change of the affinity for ouabain binding. These results suggest that the effect of gypsophila saponin on the sarcolemmal Na^+, K^+-ATPase was due primarily to the disruption of membrane barriers, so that formerly inaccessible enzymes could react with their substrates. Furthermore, at concentrations of SDS or Triton X-100 slightly greater than the activating concentration, activity decreased precipitously. In contrast, gypsophila saponin increased the Na^+, K^+-ATPase activity of and ouabain binding to the sarcolemmal vesicles over a broad range of concentrations, with no decrease in the activity and binding detected at gypsophila to protein ratios as high as 100. For this reason, gypsophila saponin could be an ideal unmasking agent for assaying the latent sites of sarcolemmal vesicles.

As mentioned above, comparison of the effects of ginseng saponins and gypsophila saponin on the sarcolemmal Na^+, K^+-ATPase showed very marked differences between two different sources of saponins. If

ginseng saponins have the disruptive effect on membrane barriers, Na^+, K^+ -ATPase activity and [^{3}H] ouabain binding in the sarcolemma preparations which contain both rightside-out and inside-out vesicles should be increased by the treatment with the appropriate concentrations of ginseng saponins. However, panaxadiol and panaxatriol inhibited the sarcolemmal Na^+, K^+ -ATPase activity in a dose-dependent manner. This effect cannot be accounted for by the disruption of membrane barriers. Therefore, it is assumed that the effect of ginseng saponin was due to a direct interaction of ginseng saponins with membrane-bound Na^+, K^+ -ATPase.

The reduction in Na^+, K^+ -ATPase activity is not due to a decrease in the intrinsic turnover number (molecular activity) of the enzyme, since the enzyme activity and the number of active sites estimated by Na^+ -dependent phosphorylation were both reduced by the same amount.

Many substances which are acting at an active site or allosteric site of Na^+, K^+ -ATPase, or even nonspecifically inhibit Na^+, K^+ -ATPase activity and [^{3}H] ouabain binding to the enzyme.[30-34] In case of ginseng saponins, it is quite likely that these saponins do not bind to the ouabain site, since no significant difference in the K_D values between control and saponin treated (3μg of ginseng saponins) sarcolemma was observed. Any agent which can prevent the ouabain binding would yield an increase of the apparent K_D values for ouabain binding or a decrease of the numbers of binding sites. If ginseng saponins do not affect the affinity for ouabain, one would expect a decrease in binding sites. A Scatchare analysis of ouabain binding clearly demonstrated a decrease in the Bmax without affecting the K_D values by a low concentration of ginseng saponins (3μg/μg protein), which is consistent with the observations of Lee et al.[10] who reported that inhibition of the purified sheep kidney Na^+, K^+ -ATPase by ginseng saponins is due to a decrease in the number of the ouabain binding sites. In contrast, low concentrations of SDS and Triton X-100 increased the Bmax as mentioned above. By these results, it is reasonable to presume that low doses of ginseng saponins inhibit the sarcolemmal Na^+, K^+ -ATPase without the disruptive effect on membrane barriers. However, high doses of ginseng saponins and chemical detergents used here decreased both Bmax and the apparent affinity for ouabain binding, indicating denaturation or solubilization of the enzyme.

Na^+, K^+ -ATPase activity and Bmax of sarcolemma treated with 10μg of total saponin were higher than those of sarcolemma treated with 3μg. In this regard, it should be noted that we do not eliminate the possibility that total ginseng saponin used here contains the constituents which obviate permeability barriers of sarcolemmal vesicles before enzyme inhibition. The identification of a specific ginseng saponin which has this effect remains to be investigated further.

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