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Alterations by saponins of passive Ca^{2+} permeability and Na^+-Ca^{2+} exchange activity of canine cardiac sarcolemmal vesicles

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Saponins can both permeabilize cell plasma membranes and cause positive inotropic effects in isolated cardiac muscles. Different saponins vary in their relative abilities to cause each effect suggesting that different mechanisms of action may be involved. To investigate this possibility, we have compared the effects of seven different saponins on the passive Ca^{2+} permeability and Na^+-Ca^{2+} exchange activity of isolated canine cardiac sarcolemmal membranes. Saponins having hemolytic activity reversibly increased the passive efflux of Ca^{2+} from sarcolemmal vesicles preloaded with $^{45}Ca^{2+}$ with the following order of potency: echinoside-A > echinoside-B > holothurin-A > holothurin-B > sakuraso-saponin. Ginsenoside-Rd and desacyl-jego-saponin, which lack hemolytic activity, had no significant effect on this variable. The saponins also stimulated Na^+-Ca^{2+} exchange activity measured as Na^+-dependent Ca^{2+} uptake by sarcolemmal vesicles. Ginsenoside-Rd and desacyl-jego-seponin, which did not affect passive Ca^{2+} permeability, stimulated the uptake, while in contrast, echinoside-A and -B only slightly increased or decreased this latter variable. Thus, the abilities of these compounds to enhance Na^+-Ca^{2+} exchange activity seem to be inversely related to their abilities to increase the Ca^{2+} permeability. Effects by the echinosides on Na^+-Ca^{2+} exchange may be masked by the loss of Ca^{2+} from the vesicles due to the increased permeability. These results suggest that the saponins interact with membrane constituent(s) that can influence the passive Ca^{2+} permeability and the Na^+-Ca^{2+} exchange activity of cardiac sarcolemmal membranes.

Introduction

The saponins, glycoside compounds widespread in plants and invertebrate animals, interact with cholesterol moieties in biological membranes altering membrane fluidity and permeability characteristics [1–5]. Formation of a saponin-cholesterol complex makes membranes containing the latter sterol permeable to ions and larger molecules and high concentrations of saponins have been used to selectively chemically permeabilize plasma membranes, which are relatively rich in cholesterol, of a variety of different cell types, including both cardiac and smooth muscles [6,7].

In a previous study [8], we found that at con-

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centrations lower than those needed to permeabilize cell membranes, several saponins exerted positive inotropic and depolarizing actions on isolated guinea pig cardiac muscles. These data suggested that different mechanisms might underlie the positive inotropy and the increase in membrane permeability caused by these agents and, at at these lower concentrations, the saponins might affect plasma membrane ion transport systems.

Saponins from various plant and animal sources differ in the structure of the aglycon and the associated sugar moieties and appear to exert qualitatively different effects (see, for example, Ref. 9). Therefore, in analyzing the effects of these agents on intact cells it may be necessary to consider more than one mechanism of action. The present study was undertaken to assess and compare the effects of seven different saponins on the passive Ca^{2+} permeability and Na^+-Ca^{2+} exchange activity of isolated cardiac sarcolemmal membranes and to investigate whether a common mechanism of action exists for effects on these two variables. A preliminary account of these studies has been presented [10].

Materials and Methods

Canine cardiac sarcolemmal vesicles were prepared from dog hearts according to the method described by Slaughter et al. [11]. Briefly, ventricular myocardium was cleaned of nonmuscle tissue, minced and homogenized in 2 vol. of mannitol buffer containing 0.25 M mannitol and 10 mM Mops-Tris (pH 7.4). The homogenate was centrifuged at $14000 \times g$ for 20 min. The pellet was resuspended in the same buffer and the suspension was centrifuged at $48000 \times g$ for 30 min. The resulting pellet was resuspneded in the mannitol buffer and 15 ml of the suspension was layered over 10 ml of a sucrose solution containing 0.64 M sucrose and 20 mM imidazol (pH 7.4) and centrifuged at $161000 \times g$ for 90 min. The membranes present at the sucrose interface were collected, diluted with 4 vol. of either K^+ medium (160 mM KCl, 20 mM Mops-Tris, pH 7.4) or Na^+ medium (160 mM NaCl, 20 mM Mops-Tris, pH 7.4) and centrifuged at $161000 \times g$ for 30 min. The pellet was resuspended in either K^+-medium (K^+-loaded vesicles) or Na^+-medium (Na^+-loaded vesicles) at a final concentration of 1–2 mg membrane protein/ml and stored in liquid nitrogen. Sarcolemmal membranes were thawed only once for experimental use and similar results were obtained with freshly prepared and frozen preparations. Protein concentrations were determined by the method of Lowry et al. [12].

Na^+-Ca^{2+} exchange was measured as Na_i^+-dependent Ca^{2+} uptake as described previously [13]. Ca^{2+} uptake was initiated by the dilution of 2 μl of Na^+-loaded vesicles into 100 μl of a reaction medium containing 15 μM $^{45}CaCl_2$, 160 mM KCl and 20 mM Mops-Tris (pH 7.4) at 37°C. When tested, the saponins were present in the reaction medium. Ca^{2+} uptake was terminated by the addition of 5 ml of an ice-cold terminating solution containing 200 mM KCl, 0.1 mM EGTA and 5 mM Mops-Tris (pH 7.4). The vesicles were harvested on Whatman GF/A filters and washed twice with two additional 5 ml aliquots of the terminating solution. The $^{45}Ca^{2+}$ retained on the filters was measured in liquid scintillation counter. All determinations were performed in triplicate. Ca^{2+} taken up by K^+-loaded vesicles was measured and used to correct for superficial Ca^{2+} binding and Na_i^+-independent influx of Ca^{2+} into the vesicles.

For measurement of membrane Ca^{2+} permeability, passive Ca^{2+} efflux was determined by first loading Na^+-loaded vesicles with Ca^{2+} for a 4-min period via Na^+-Ca^{2+} exchange as described above. The vesicle suspension was then diluted by the addition of 1 ml of an efflux medium containing 160 mM KCl, 100 μM EGTA and 20 mM Mops-Tris (pH 7.4) at 37°C. Ca^{2+} efflux was permitted to proceed for the times indicated in the figures and was then terminated as described above. When tested, the saponins were present in the efflux medium.

The saponins used in these studies were: holothurin-A and holothurin-B, from the sea cucumber *Holothuria leucospilota*; echinoside-A and echinoside-B from the sea cucumber *Actinopyga echinites*; sakuraso-saponin, from the primrose *Primula sieboldi*; desacyl-jego-saponin from the storax *Styrax japonica* and ginsenoside-Rd from the ginseng *Panax ginseng*. The structures of saponins are shown in Fig. 1. Holothurin-A, echinoside-A and sakuraso-saponin were dissolved in distilled

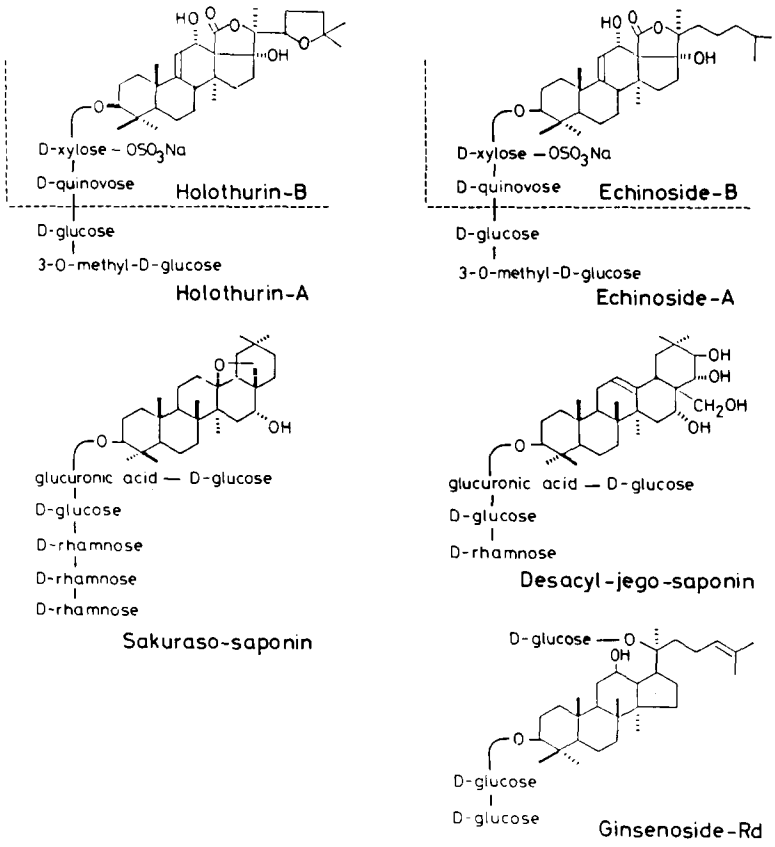

Fig. 1. Structure of saponins.

water at a concentration of 1 mM while the others were dissolved in dimethyl sulfoxide (DMSO) in the same concentration. When the highest concentration of saponin was used, DMSO in a reaction medium was 1%. In the case that DMSO was a solvent for a saponin, a similar concentration of DMSO was added to the reaction and efflux media for parallel control measurements and the data were corrected for the solvent effects.

Results

The time-course of the passive efflux of Ca^{2+} from sarcolemmal vesicles in the absence and presence of holothurin-A is shown in Fig. 2. Holothurin A enhanced this variable in a concentration-dependent manner with a threshold of 3 μM. In the presence of 10 μM holothurin-A approximately 90% of the vesicular Ca^{2+} was lost by 10 min, while in nontreated membranes more than 50% of the original Ca^{2+} content remained at this time.

The effects of the seven saponins on passive Ca^{2+} efflux from sarcolemmal vesicles measured during a 2 min interval are summarized in the upper panel of Fig. 3. At the maximum concentration used, DMSO (1%) alone decreased the vesicular content by 13% compared to that of nontreated controls. The data with echinoside-B, holothurin-B, desacyl-jego-saponin and ginsenoside-Rd were corrected for those solvent effects. The effects of these agents were concentration dependent and the following order of relative potency was observed: echinoside-A > echinoside-B > holothurin-A > holothurin-B > sakuraso-saponin > desacyl-jego-saponin > ginsenoside-Rd. The latter two saponins, which lack hemolytic activity [8], had no significant effect on passive Ca^{2+} efflux. Echino-

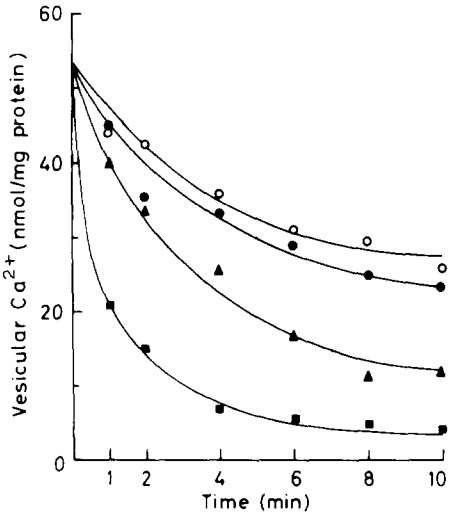

Fig. 2. Passive Ca^{2+} efflux from canine cardiac sarcolemmal vesicles in the absence or presence of holothurin-A. The Ca^{2+} remaining in the vesicles at the end of the efflux interval is expressed on the ordinate. ○, control values; ●, $3 \cdot 10^{-6}$ M; ▲, $8 \cdot 10^{-6}$ M; ■, $1 \cdot 10^{-5}$ M holothurin-A. The values shown are the means of three determinations.

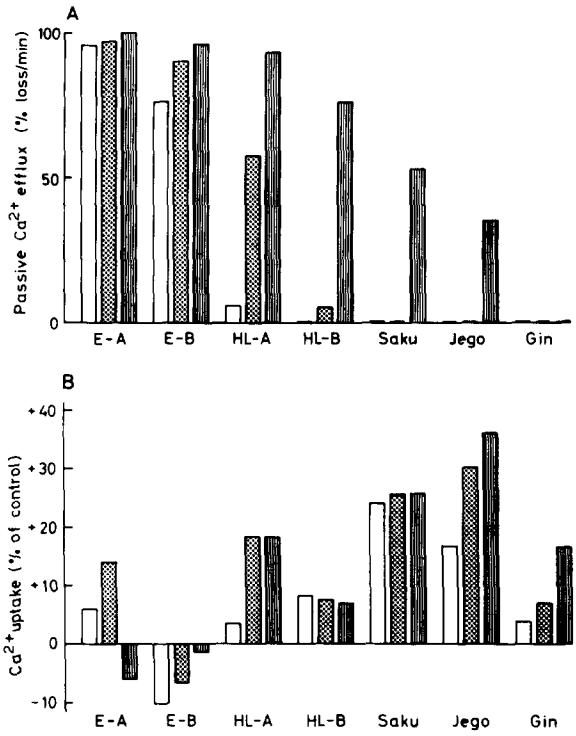

Fig. 3. Concentration dependence of the effects of the saponins on the passive Ca^{2+} permeability (upper panel) and Na_i^+-dependent Ca^{2+} uptake (lower panel) of cardiac sarcolemmal vesicles. (A) Passive Ca^{2+} permeability is expressed as the % loss of Ca^{2+} from saponin-treated vesicles during a 2 min efflux period. Percent loss of Ca^{2+} was calculated from the Ca^{2+} content remaining in nontreated membranes during the same interval. (B) Na_i^+-dependent Ca^{2+} content was measured 2 min after the initiation of Na^+-Ca^{2+} exchange. Control vesicles for echinosides-B, holothurin-B, desacyl-jego-saponin and ginsenoside-Rd were exposed to 1% DMSO. E-A, echinoside-A; E-B, echinoside-B; HL-A, holothurin-A; HL-B, holothurin-B; Saku, sakuraso-saponin; Jego, desacyl-jego-saponin; Gin, ginsenoside-Rd. Saponin concentrations are indicated as open columns, $1 \cdot 10^{-6}$ M; dotted columns, $5 \cdot 10^{-6}$ M; and hatched columns, $1 \cdot 10^{-5}$ M. The data represent the means of three determinations.

side-A, the most potent saponin, caused a complete loss of vesicular Ca^{2+} at the lowest concentration tested, 1 μM. We also tested the potency of a commercially available saponin preparation (Sigma Chemical Co.) on Ca^{2+} efflux. At a concentration of $1 \cdot 10^{-4}$ g/ml, the commercial saponin reduced the vesicular Ca^{2+} content to 25% of nontreated controls in 2 min, while a concentration of $1 \cdot 10^{-5}$ g/ml was without effect. On the weight basis, the potency of this saponin was less than desacyl-jego-saponin.

The reversibility of the increased sarcolemmal membrane Ca^{2+} permeability caused by the saponins was assessed by measuring passive Ca^{2+} efflux from sarcolemmal membranes that had been exposed to holothurin-A either during only the Ca^{2+} uptake period, during only the efflux period or during both intervals. As shown in Fig. 4, the increase in sarcolemmal membrane Ca^{2+} permeability was observed only when holothurin-A was present in the efflux medium and was not evident when the saponin was present only during the Ca^{2+} uptake period. These results indicate that in the latter case the effects of this agent were readily reversed when its concentration was reduced by the dilution of the membrane suspension with saponin-free efflux medium. It is unlikely that the conditions used for Na_i^+-dependent Ca^{2+} uptake precluded expression of the effects of holothurin-A, since it did enhance the uptake of Ca^{2+} by the vesicles during this interval (see below).

The results of the preceding experiment indicated that Na^+-Ca^{2+} exchange mediated by Ca^{2+} uptake into sarcolemmal vesicles was stimulated by the presence of $1 \cdot 10^{-5}$ M holothurin-A.

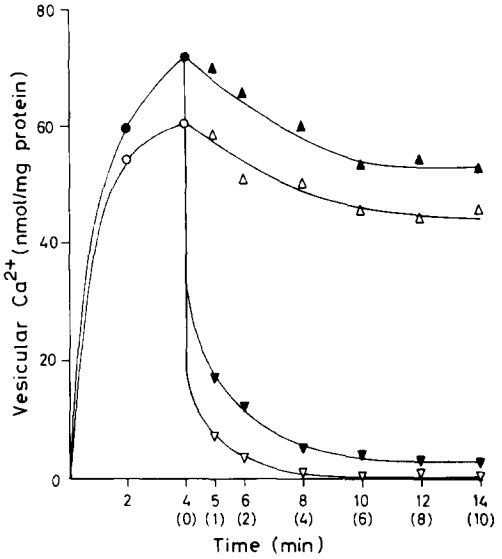

Fig. 4. Reversibility of the effects of the saponins on the passive Ca^{2+} permeability of sarcolemmal membranes. Sarcolemmal vesicles were loaded with $^{45}Ca^{2+}$ by Na^{+}-Ca^{2+} exchange in the presence (●) or absence (○) of $1 \cdot 10^{-5}$ M holothurin-A and then diluted in the efflux medium in the presence (▼, ▽) or absence (▲, △) of $1 \cdot 10^{-5}$ M holothurin-A. Filled triangles indicate the vesicles that were exposed to holothurin-A during the calcium uptake period.

Therefore, in the next series of experiments we examined the effects of the different saponins on this variable. As shown in the lower panel of Fig. 3, when tested over the concentration range of 1–10 μM, the seven saponins exerted variable

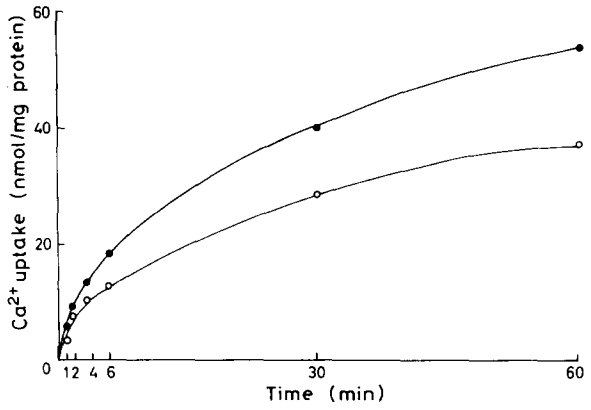

Fig. 5. Time-course of Na_i^{+}-dependent Ca^{2+} uptake by sarcolemmal vesicles in the absence (○) or presence (●) of $1 \cdot 10^{-5}$ M holothurin-A.

effects on Na_i^{+}-dependent Ca^{2+} uptake, that ranged from a small decrease to levels below those observed with nontreated vesicles caused by echinoside-A and -B, to small to moderate increases above control values caused by the other saponins. The actions by holothurin-B and sakuraso-saponin did not show a dependence on concentration over the range tested, whereas those of the other saponins did vary with concentration. The effects of 10 μM holothurin-A, which stimulated Ca^{2+} uptake, on the time-course of this event are shown in Fig. 5.

The effects by the saponins were specific for Na_i^{+}-dependent Ca^{2+} uptake, since the uptake of $^{45}Ca^{2+}$ into K^{+}-loaded vesicles, which in nontreated vesicles was less than 5% of that of Na^{+}-loaded vesicles, was not significantly affected by any of the saponins. When tested alone, DMSO, used as a solvent for echinoside-B, holothurin-B, desacyl-jego-saponin and ginsenoside-Rd, increased the uptake of $^{45}Ca^{2+}$ into otherwise nontreated vesicles by 20% or less. The data presented in Fig. 3 have been corrected for these solvent effects.

Discussion

In the present study we have found that saponins increase the passive Ca^{2+} permeability and enhance the Na^{+}-Ca^{2+} exchange activity of cardiac sarcolemmal vesicles. Only those saponins that have been found to have hemolytic activity [8] increased the passive Ca^{2+} permeability of sarcolemmal vesicles and their relative potencies on sarcolemmal membrane Ca^{2+} permeability were comparable to those found for their hemolytic activity. It is known that cholesterol reduces the permeability of cell membranes and artificial membranes [14,15] and that a formation of saponin-cholesterol complex increases the permeability [1,2]. Therefore, it appears that the action of saponins on Ca^{2+} permeability reflects a general disruption of the membrane, a result of interaction of saponins with membrane cholesterol.

The saponins tested are triterpene oligoglycosides. Holothurin-A and -B, or echinoside-A and -B have the same aglycone, but different number of sugar moieties. Holothurin-A and echinoside-A were more potent than holothurin-B and echino-

side-B, respectively, suggesting that an increase in the number of sugar moieties increases the potency of the effects by these agents on sarcolemmal membrane Ca^{2+} permeability. Consistent with this observation, holothurin-A and echinoside-A were found to be more potent hemolytic and cardiac inotropic agents than holothurin-B and echinoside-B, respectively [8]. These results are also consistent with the observations of Akiyama et al. [16], who reported that digitonin analogues with an increased number of sugar moieties form more rigid complexes with cholesterol and exhibit greater hemolytic activity. These latter workers postulated that the terminal sugar groups are important for the formation of a more stable complex between the saponin and cholesterol. Echinosides had more potent action on the Ca^{2+} permeability than holothurins. A difference of structure between them is a configuration at C-22 of the aglycon so that this may be important for the potency. Ginsenoside-Rd, which has two sugar chains, exhibited the weakest action on Ca^{2+} permeability among saponins tested. This is consistent with the general characteristics that the saponin with two sugar chains has little hemolytic activity [9].

The relative effects of the saponins to increase Na^+-Ca^{2+} exchange activity were in general inversely related to those on passive Ca^{2+} permeability and may be at least partially understood when viewed in the context of these latter changes. For example, echinoside-A and -B caused the greatest increase in passive Ca^{2+} permeability and decreased Na_i^+-dependent Ca^{2+} uptake to below control levels. Therefore in some instances the general increase in membrane Ca^{2+} permeability caused by the more potent saponins, such as echinoside-A and -B may have prevented observation of any actions on Na^+-Ca^{2+} exchange in the experimental system used for the present studies. This may explain why saponins with high potency on Ca^{2+} permeability did not exhibit dose-effect on Na^+-Ca^{2+} exchange and that those with less potency on the permeability exhibited a dose-effect on the exchange. In such cases the effects on sarcolemmal membrane permeability make it difficult to determine the actual relative potencies of the actions by the different saponins on Na^+-Ca^{2+} exchange activity.

In other cases though, there were nonparallel effects by the saponins on sarcolemmal membrane Ca^{2+} permeability and on Na^+-Ca^{2+} exchange activity. For example, echinoside-A decreased Na_i^+-dependent Ca^{2+} uptake at a concentration of $1 \cdot 10^{-5}$ M, but lower concentrations increased this variable, while all three concentrations of this agent produced large equivalent increases in sarcolemmal membrane Ca^{2+} permeability (Fig. 3A). In addition, desacyl-jego-seponin and ginsenoside-Rd, which had little or no effect on sarcolemmal membrane permeability, were capable of stimulating Na^+-Ca^{2+} exchange activity. These latter observations suggest that other factors may also be involved in producing these two effects. Whether the mechanism through which the saponins effect Na^+-Ca^{2+} exchange is different from that underlying their effects on sarcolemmal membrane permeability or if both effects share a common mechanism, but have different dependencies on saponin concentration is not clear. The latter possibility is consistent with the observation that the lowest concentrations of holothurin-B and sakuraso-saponin tested increased Na_i^+-dependent Ca^{2+} uptake, but did not affect sarcolemmal membrane Ca^{2+} permeability.

Although the relationship that exists between cholesterol and phospholipids in sarcolemmal membranes is not clearly understood, cholesterol is thought to be a determinant of the stability or fluidity of lipid bilayer membrane [17–19]. In fact, cholesterol affects the order of phospholipid acyl chain [20] and some actions of saponin depend on the kinds or length of the acyl chains [9,19]. Therefore, it is likely that the formation of a saponin-cholesterol complex could change the organization of sarcolemmal membrane phospholipids and alter ion transport mechanism of the membrane.

Changes in phospholipid organization and identity have been shown to stimulate Na^+-Ca^{2+} exchange activity in cardiac sarcolemmal membranes [21,22]. Phospholipase D, which breaks down phospholipids into phosphatidic acid, has been shown to stimulate Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles [22] and to activate Ca^{2+}-dependent action potentials in intact cardiac muscle [23]. Similar effects have been observed with the saponins (present results and Ref. 8).

Therefore, the formation of the saponin-cholesterol complex, alterations in the organization of sarcolemmal membrane phospholipids and the formation of phospholipid breakdown products, such as phosphatidic acid may all be factors involved in the actions of the saponins on the Ca^{2+} permeability and Na^{+}-Ca^{2+} exchange activity of cardiac sarcolemmal membranes. Further appropriate assays are required to accurately establish the interrelationship between these factors and the consequences of the actions by the saponins on sarcolemmal membrane characteristics.

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