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Effect of sub-skinning concentrations of saponin on intracellular Ca^{2+} and plasma membrane fluidity in cultured cardiac cells

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To determine the underlying mechanisms of the positive inotropic effect of sub-skinning concentrations of saponin, we studied changes in the intracellular Ca^{2+} ($[Ca^{2+}]_i$) and plasma membrane fluidity after exposure to digitonin (a representative saponin) in cultured cardiac cells. $[Ca^{2+}]_i$ was measured by use of the fluorescent calcium indicator Calcium Green-1. The membrane fluidity was evaluated by measuring the diffusion coefficient using the method of fluorescence recovery after photobleaching. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate was used as the fluorescent probe. Digitonin at a sub-skinning concentration (0.1 to 1 μM) produced an increase in cell motion and an augmentation of $[Ca^{2+}]_i$. Membrane fluidity which is evaluated by the diffusion coefficient (from $0.34 \cdot 10^{-8}$ to $0.28 \cdot 10^{-8}$ cm^2/s; $P < 0.05$), decreased in the presence of 0.2 μM digitonin while the cell maintained an augmented motion and an increased $[Ca^{2+}]_i$. The skinning concentration of digitonin (5 μM) produced a rapid contracture with a marked increase in $[Ca^{2+}]_i$. The membrane fluidity was further reduced (diffusion coefficient: $0.24 \cdot 10^{-8}$ cm^2/s; $P < 0.001$). These results suggest that saponin at the sub-skinning concentration also causes holes in the plasma membrane by interaction with cholesterol, as was shown with the skinning concentration, and it increases $[Ca^{2+}]_i$, which thereby induces a positive inotropic effect.

Introduction

Saponins are widely used to chemically skinned muscle preparations; at high concentrations saponins form insoluble complexes with cholesterol in the membrane and produce holes [1–4]. Recent studies have shown that saponins at low concentrations produce a positive inotropic action on cardiac muscles without manifestation of a skinning effect [5–7]. The underlying mechanisms of the positive inotropic action of saponin at sub-skinning concentrations have been proposed to be a modification in the Ca^{2+} channel [5], the stimulation of Na^+–Ca^{2+} exchange activity [6], or an increase in non-specific passive ion permeability [7]. However the intracellular Ca^{2+} ($[Ca^{2+}]_i$) with sub-skinning concentrations of saponin has not been evaluated yet. Furthermore, changes in the membrane characteristics have never been evaluated even though the interaction of saponin with membrane cholesterol is considered to alter membrane fluidity. The purpose of this study was to examine the effect of saponin on $[Ca^{2+}]_i$ and membrane fluidity at a concentration which induces an increased contractile state.

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Methods

Cell preparation

Cardiac cells in monolayer were prepared from mouse embryo hearts as described previously [8]. Briefly, 15 to 20 ventricles of 17- to 19-day-old mouse embryos were cut into 0.5 mm fragments in a Ca^{2+}- and Mg^{2+}-free solution of Saline A containing (mM) NaCl 140, KCl 5.4, glucose 5.5, and $NaHCO_3$ 4.2 (pH 7.4). The ventricular fragments were dissociated by adding 0.03% trypsin and 0.03% collagenase to Saline A at 37°C in four cycles of 10 min each. The supernatant suspensions containing dissociated cells were placed in a tube at 0°C. The suspensions were centrifuged at 1200 rpm for 10 min and then precipitated cells were resuspended in culture medium consisting of 10% heat-inactivated fetal calf serum, 45% M-199, 45% Ham F-12 (Sigma, MO, USA) and 0.1% penicillin streptomycin antibiotic solution (Gibco, USA). The cell suspension was diluted to $1 \cdot 10^6$ cells/ml and placed in plastic petri dishes containing 25 mm circular glass coverslips. The cells were incubated in a humidified 5% CO_2/95% air at 37°C for 2 days.

Measurement of contractility

A coverslip with adherent cells was placed in a chamber with inlet and exit ports facilitating rapid replacement of culture perfusate. The inlet of the chamber was connected to four parallel polyethylene tubes each of which was connected to a peristaltic pump. Flow rate was adjusted to 1 ml/min, which allows the complete exchange of solution within 5 s. The chamber was placed on a stage of an inverted phase contrast microscope (TM Nikon, Japan). The entire system was covered by a plastic box to maintain an atmosphere temperature of 37°C. The contractile state of the myocytes was assessed by the use of a phase-contrast microscopic video motion detector system. Briefly, using a 40 × objective lens, the image of cells was recorded by a low-light-level TV camera attached to the observation tube of the microscope using a 1 × lens. The TV camera provides the image which is composed of 525 raster lines. The motion detector monitors a selected raster-line segment and an image border of a single cardiac cell moving along the raster line. Data of a new position can be obtained every 32 ms. The analog voltage output from the motion detector was filtered at 10 Hz with a low-pass filter and recorded using an amplifier-recorder system.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was monitored by the use of the fluorescent calcium indicator, Calcium Green-1. Monolayer cells were exposed to 10 μM Calcium Green-1 acetoxymethyl ester (Molecular Probes, USA) in balanced salt solution (BSS) at 37°C for 15 min for loading. The cells were then washed twice with BSS and placed on the stage of an episcopic fluorescence microscope (Axioskop Zeiss, Germany). The fluorescent signal was measured with a fluorescence spectrophotometer (CAM 200 Japan Spectroscopic, Japan) attached to the microscope using a 40 × fluor-objective lens. The excitation wavelength was obtained using a 75-W xenon lamp with a band-pass filter of 488 nm. The fluorescence was collected by the objective lens and passed to a photomultiplier tube through a band-pass filter of 530 nm. The fluorescence intensity was used to estimate changes in $[Ca^{2+}]_i$.

Measurement of membrane fluidity

Membrane fluidity was evaluated by measuring the lateral diffusion coefficient using the method of fluorescence recovery after photobleaching (FRAP) [9,10]. The principle of the FRAP method is that a uniformly labeled membrane with a fluorescence probe is photobleached in a specific spot by an intensified laser beam and the recovery of fluorescence at that spot is monitored to measure the lateral diffusion of probe from the surrounding membrane into the spot. The diffusion coefficient (D) is calculated from D $(cm^2/s) = W^2$ $(cm^2)/(4 \cdot T_{1/2}$ (s)), where W is the radius of the laser beam, and $T_{1/2}$ is the half time of fluorescence recovery. A small diffusion coefficient represents low fluidity. The lipid analog 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiI, Molecular Probes, USA) was used as the fluorescent probe. The cells were exposed to 3 μg/ml of DiI in the BSS at 37°C for 10 min for loading. After washing DiI with BSS, the cells were placed on the microscope. A small spot (2 μm in radius) of the surface of a cardiac cell was irreversibly photobleached with an intensified Ar laser light (488 nm, 1 s in duration). Then the recovery of fluorescence in the bleached region was immediately monitored with a non-bleaching intensity of excitation laser light (1/1000 of the photobleaching light). All measurements were taken at 30°C and under constant BSS flow.

Solutions

BSS (pH 7.4) containing (mM) NaCl 137, KCl 5.4, $MgCl_2$ 1.0, glucose 5.5, and BES (Good's buffer; Sigma, USA) 0.5 were used in all experiments. The calcium concentration in experiments was 1.3 mM except for the low calcium studies in which the calcium concentration was 0.05 mM. Digitonin (Sigma, USA), used as a representative saponin, was dissolved in BSS. DiI was dissolved in DMSO with 3 mg/ml as a stock solution.

Statistical analysis

The significance of differences between groups was determined by Student's t-test.

Results

Effects of digitonin on contractility

Changes in the amplitude of cell motion were studied with various concentrations (0.1 to 5 μM) of digitonin, in order to determine the optimum digitonin concentration to produce a positive inotropic effect without causing the skinning effect. The effect of 0.2 μM digitonin on the cell motion is shown in Fig. 1. Exposure to 0.1 μM to 1 μM of digitonin produced an

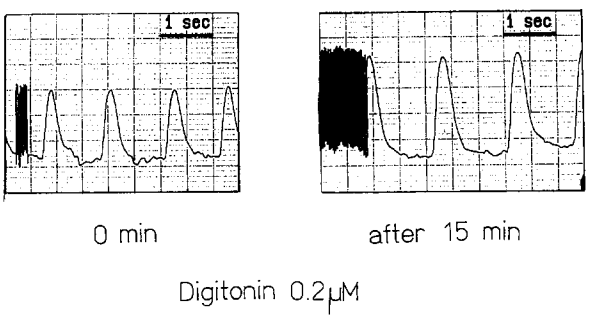

Fig. 1. The effect of 0.2 μM digitonin on cell motion. Left (Control); contraction in the absence of digitonin. Right; contraction 15 min after the addition of 0.2 μM digitonin.

TABLE I

Effects of digitonin on contraction of cultured heart cells

Values, in mean ± S.E. obtained from three to six determinations, represent the percentage change from control. C, contracture; –, arrest.

Digitonin	5 min	10 min	15 min	20 min
0.1 μM	103 ± 12	110 ± 12	111 ± 6	125 ± 7 [a]
0.2 μM	102 ± 10	108 ± 7	120 ± 7 [a]	121 ± 8
0.5 μM	110 ± 6	121 ± 12	124 ± 11 [a]	110 ± 5
1 μM	92 ± 6	128 ± 8 [a]	153 ± 26	108 ± 30
2.5 μM	C	–	–	–
5 μM	–	–	–	–

[a] Significant difference from the corresponding control value ($P < 0.05$).

increase in amplitude of the cell motion followed by a normalization of contraction after about 30 min (Table I). Times required to reach the significant increase in contraction were 20 min at 0.1 μM, 15 min at 0.2 μM and 10 min at 1 μM. At a concentration of 1 μM, the contraction ceased in 3 of 9 experiments. In all experiments contracture developed within 5 min at a concentration of 2.5 μM and within 1 min at 5 μM, indicating that 0.2 μM digitonin is appropriate as the sub-skinning concentration with a positive inotropic action. The beating rate remained unchanged at the low digitonin concentrations.

Effects of digitonin on $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured at the sub-skinning concentration of digitonin. The K_d of Calcium Green-1 is shown to be 189 nM which is lower than $[Ca^{2+}]_i$ of cardiac cells (about 282 nM), thus the rather high level of $[Ca^{2+}]_i$ during systole (about 1100 nM) is not reflected by the intensity of fluorescence [8]. Therefore, for the evaluation of alterations in $[Ca^{2+}]_i$ upon exposure to digitonin, $[Ca^{2+}]_i$ was measured at diastolic levels by reducing the Ca^{2+} concentration of the solution to 0.05 mM, which is not the physiological Ca^{2+} concentration. The effects of digitonin on $[Ca^{2+}]_i$ are shown in Fig. 2. During control perfusion with 1.3 mM Ca^{2+}, the cyclic changes in fluorescence represent $[Ca^{2+}]_i$ transients of the systolic and diastolic phases. On exposure to the 0.05 mM Ca^{2+} solution, the cells arrested in diastole and $[Ca^{2+}]_i$ was retained at the diastolic level, and then gradually declined further (Fig. 2A). With an addition of 0.2 μM digitonin, a slow but progressive increase in $[Ca^{2+}]_i$ was observed with the subsequent return of $[Ca^{2+}]_i$ toward the pre-digitonin level (Fig. 2B). Digitonin at 5 μM produced a marked elevation of $[Ca^{2+}]_i$ (Fig. 2C). The rapid decrease in fluorescence following the peak $[Ca^{2+}]_i$ depends on the leak of Calcium Green-1 from the cells though the digitonin-induced holes since the fluorescence level dropped to lower than the pre-digitonin level.

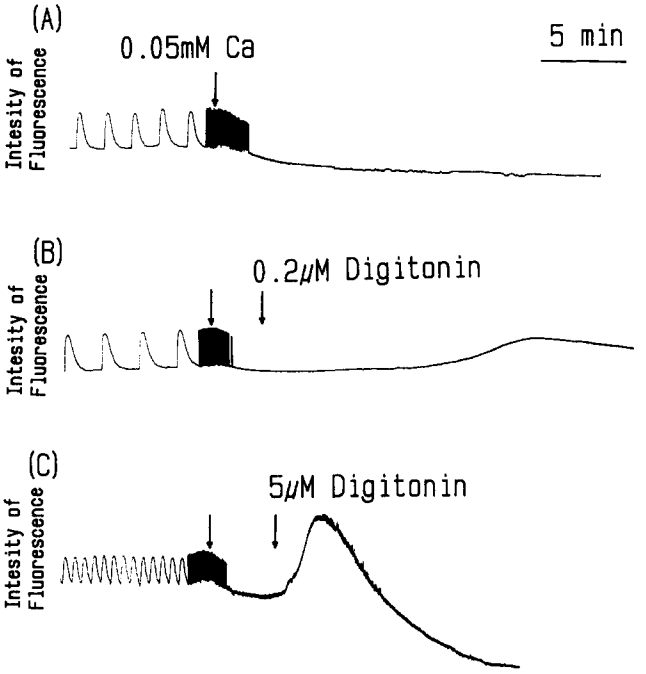

Fig. 2. Effects of digitonin on $[Ca^{2+}]_i$. After control perfusion (1.3 mM Ca^{2+}), the perfusate was changed to low Ca^{2+} solution (0.05 mM), as indicated by the first vertical arrow. Digitonin was added at the second vertical arrow (A: without digitonin; B: 0.2 μM digitonin; C: 5 μM digitonin). Recorder speed was briefly increased ×30 to display individual $[Ca^{2+}]_i$ transients. Representative tracings from four experiments are shown.

Effects of digitonin on membrane fluidity

Membrane fluidity was measured concomitantly with contractility experiments using cardiac cells obtained from the same series of cultured cells. Fig. 3 shows the effect of 0.2 μM digitonin on the fluorescence recovery curve after bleaching. The fluorescence recovery was slower in the presence of 0.2 μM digitonin compared with that of control (Fig. 3). The measurements were conducted under spontaneously beating conditions. The cell motions were reflected as minor oscillations in the

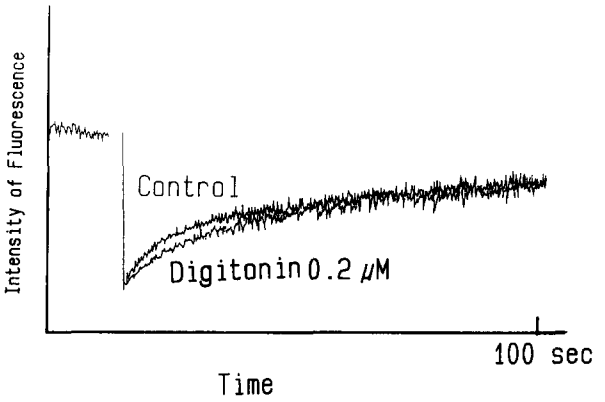

Fig. 3. The fluorescence recovery curves after photobleaching. The lower recording was obtained at 15 min after the addition of 0.2 μM digitonin.

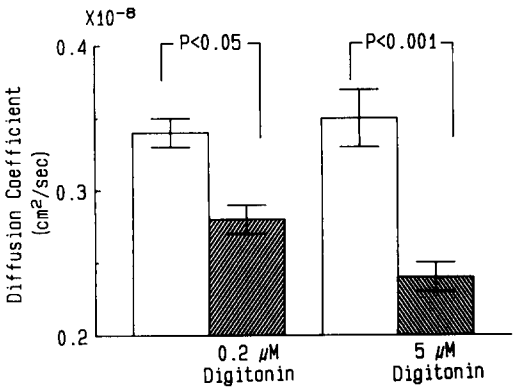

Fig. 4. Changes of diffusion coefficient of membrane, representing membrane fluidity, on exposure to 0.2 μM or 5 μM digitonin. Values are given as means ± S.E. of five or six samples. Both concentrations showed a significant difference from the corresponding control.

recovery curve. We confirmed that the cell motion did not interfere with the fluorescence recovery curve by comparing that with fibroblasts in various measurement conditions (data not shown). Changes in diffusion coefficients, derived by calculating using the half time of fluorescence recovery in the presence of digitonin, are shown in Fig. 4. The diffusion coefficient decreased from $(0.34 \pm 0.01) \cdot 10^{-8}$ to $(0.28 \pm 0.01) \cdot 10^{-8}$ cm^2/s (mean ± S.E., $P < 0.05$) with 0.2 μM digitonin even though the cell maintained an augmented motion. At 5μM of digitonin, the reduction in the diffusion coefficient was more marked (from $(0.35 \pm 0.02) \cdot 10^{-8}$ to $(0.24 \pm 0.01) \cdot 10^{-8}$ cm^2/s, $P < 0.001$). Thus, the decrease in membrane fluidity with a sub-skinning concentration of digitonin was shown.

Discussion

The results reported here showed that saponin at the sub-skinning concentration increases contractility concomitant with a rise in $[Ca^{2+}]_i$ and it decreases membrane fluidity in a dose-dependent manner. Regarding the mechanisms by which saponin increases $[Ca^{2+}]_i$, the following processes should be considered: activation of the Ca^{2+} channel, inhibition of Na^+-, K^+-ATPase, decreased Na^+–Ca^{2+} exchange, increased release or depressed uptake of Ca^{2+} in the sarcoplasmic reticulum (SR) and non-specific ion permeability in the plasma membrane. Activation of the Ca^{2+} channel appeared unlikely since $[Ca^{2+}]_i$ rose even when the $[Ca^{2+}]_i$ transients were eliminated by reducing the Ca^{2+} concentration of the solution to 0.05 mM and $[Ca^{2+}]_i$ was at the diastolic level during which the Ca^{2+} channels are in the resting stage (Fig. 2B). Regarding the possibility of inhibition of Na^+-, K^+-ATPase and a decreased Na^+–Ca^{2+} exchange, Noireaud et al. [7] reported that saponin does not interfere with Na^+-, K^+-ATPase at the sub-skinning concentration using papillary muscle preparation, even though the structure of saponin is remarkably similar to that of digoxin, a well-known inhibitor of Na^+-, K^+-ATPase. Yamasaki et al. [6] showed that the Na^+–Ca^{2+} exchange is rather enhanced at the sub-skinning saponin concentration in cardiac sarcolemmal vesicles. It appears to be less likely that saponin has an effect on SR, since the fraction of cholesterol in the SR, which is the target molecule of saponin, is only 1/10 of that in the plasma membrane [11]. Furthermore, Inamitsu and Ohtsuki [12] stated that saponin does not alter the Ca^{2+} uptake of SR. Therefore the non-specific ion permeability is the most plausible mechanism for the observed increase in $[Ca^{2+}]_i$. This explanation is in accordance with a previous study which showed an increase in the intracellular Na^+ activity and a decease in the intracellular K^+ activity using papillary muscle preparation in sub-skinning concentration of saponin [7]. The non-specific ion permeability implicates a leaky membrane due to the formation of holes. Therefore we postulate that even the sub-skinning concentration of saponin could form holes by the ring-structures of saponin-cholesterol complex as has been reported at the skinning concentration. To support this theory, we examined the changes in membrane characteristics since the ring-structure complexes occupy a large space in the membrane capacity, which might limit the lateral diffusion of the lipids. Using the FRAP method, we showed a dose-dependent decrease in the diffusion coefficient in the presence of saponin even at the sub-skinning concentration. The decrease of the diffusion coefficient is of the same order as that found for the decrease of the diffusion coefficient in artificial membranes taking place upon insertion of proteins. Thus artificial membrane, composed of pure phospholipids (radius 5–6 Å), gives a coefficient of 10^{-8} cm^2/s, whereas a membrane in which the fractions of phospholipids and proteins (radius 15–30 Å) are equal to the common biomembrane (1 : 1) gives a coefficient of 10^{-9} cm^2/s [13–15]. The size of the ring-structure complex is reported to be a radius of 40 Å [3]. Therefore the decrease in diffusion coefficient from $0.34 \cdot 10^{-8}$ cm^2/s to $0.28 \cdot 10^{-8}$ cm^2/s with the sub-skinning saponin is quite reasonable and in support of our theory.

We have observed partial recovery of cell motion and $[Ca^{2+}]_i$ with time even in the persistent presence of saponin (Table I and Fig. 2). This observation is consistent with a previous report which showed that an increase in the intracellular Na^+ activity and a decrease in the intracellular K^+ activity are reversible even in the presence of saponin [7]. A direct enhancement of the Na^+–Ca^{2+} exchange by saponin and secondary augmentation of the Na^+–Ca^{2+} exchange due to an increased $[Ca^{2+}]_i$ might explain the recovery of $[Ca^{2+}]_i$, although it remains to be proven.

In conclusion, the sub-skinning concentration of saponin produces holes in the plasma membrane by the formation of a saponin-cholesterol complex as is shown at the skinning concentration, and increases $[Ca^{2+}]_i$ which induces the positive inotropic effect.

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