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Specific perforation of muscle cell membranes with preserved SR functions by saponin treatment

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Summary

The effects of saponin on *Xenopus* and frog skeletal muscle fibres were examined. The twitch of *Xenopus* single fibres was first potentiated slightly and then irreversibly abolished by 5–10 μg/ml of saponin. Treatment with saponin at 5–10 μg/ml or higher concentrations for 30 min resulted in perforation of the muscle cell membrane, indicated by the following evidence. (i) Fibres became responsive to contractile activating solutions with a pCa–tension relationship similar to that of mechanically skinned fibres. (ii) Removal and re-introduction of MgATP became effective in bringing fibres into rigor and the relaxed state, respectively. (iii) After the saponin treatment large contractions due to Ca release from the SR could be elicited by substitution of Cl for methanesulphonate in the medium. (iv) The treatment decreased the optical path length across the fibre, indicating loss of soluble proteins. (v) The lattice spacing of myofilaments was wider after the treatment as in mechanically skinned fibres. Contractile response of mechanically skinned fibres and their SR responses such as Ca uptake, Ca-induced Ca release and Cl-induced Ca release were not affected by treatment with 50 μg/ml saponin for 30 min, while 150 μg/ml or higher concentrations severely impaired the SR functions. It is possible, therefore, to make chemically skinned skeletal muscle fibres in which the functions of the SR are preserved by applying 10–50 μg/ml saponin.

Introduction

Skinned skeletal muscle fibres introduced by Natori (1954) have proved to be a very useful preparation for the study of functions of the contractile proteins and of the sarcoplasmic reticulum (SR). Removal of the sarcolemma makes it possible to alter the ionic environment of the myofibrils and of the SR very easily, while these subcellular structures are still kept in a more or less physiological state. However, if muscle cells are small in size as in cardiac or smooth muscle, it is very difficult to remove the sarcolemma mechanically as in the original method of Natori. Use of detergents makes the cell membrane permeable to various substances, and thus it appears possible to

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obtain a preparation equivalent to skinned fibres with detergents, as far as the contractile protein system is concerned (Szent-Györgyi, 1949; Julian, 1971), provided that the detergents do not directly affect the contractile proteins. Detergents, however, usually destroy the internal membranes as well and, therefore, if one is interested in functions of the SR, detergent-treated fibres are of no use. In 1974, Dr Ohtsuki suggested to us that, since saponin perforates the membrane by combining with cholesterol molecules (Ohtsuki *et al.*, 1978), and since the content of cholesterol in the SR membrane is much less than that of the surface membrane (Martonosi, 1968; Waku *et al.*, 1971), saponin might specifically act on the surface membrane without affecting the SR. We report that this is in fact the case in amphibian skeletal muscle fibres. Preliminary accounts of this method have already appeared (Endo, 1976; Endo & Kitazawa, 1978).

Methods

Single fibres were isolated mainly from iliofibularis, but sometimes from tibialis anterior or toe muscles of African clawed toads, *Xenopus laevis*. When skinned fibres were necessary the single fibres were split into two long pieces in a relaxing solution (Endo & Nakajima, 1973). Small bundles of 10–20 fibres from *Xenopus* toe muscle were used for X-ray study. In some experiments, whole sartorius muscles of the frog, *Rana japonica*, were used.

Fibres were suspended in a trough through which solutions could be perfused rapidly. Isometric tension was measured with a strain gauge transducer (U-gauge, Shinkoh, Tokyo, Japan) at about 2° C and displayed on a pen recorder. Some experiments on intact fibres were conducted at about 20° C.

Normal external solution contained (mM) 120 Na, 2.5 K, 1.8 Ca, 121 Cl and 3 phosphate buffer (pH 7.4). The composition of *normal relaxing solution* was (mM) 111 potassium methanesulphonate, 4 magnesium methanesulphonate, 4 ATP-Na_2, 2 ethylene glycol-bis-(β-aminoethylether)-N-N′-tetra-acetic acid (EGTA), 20 tris-(hydroxymethyl)-aminomethane (Tris) and 20 maleic acid, brought to pH 6.8 with KOH. The concentration of EGTA was altered when necessary. In *calcium-containing solutions*, 10 mM total EGTA was used and a specified amount of calcium methanesulphonate was added. Free Ca^{2+} concentration was calculated by assuming an apparent association constant of 5×10^5 M^{-1} for CaEGTA (Ogawa, 1968). In all of the above alterations from normal relaxing solution, the ionic strength was kept constant by adjusting the concentration of potassium methanesulphonate. *Chloride-containing solutions* were prepared by replacing potassium methanesulphonate with an equivalent amount of KCl.

Saponin, purchased mainly from ICN Pharmaceuticals, Inc. (Cleveland, Ohio, U.S.A.), was dissolved in a suitable solution shortly before use.

A Zeiss interference microscope with transmitted light was used to compare the optical path difference across single fibres before and after the saponin treatment.

X-ray diffraction experiments were performed using a fine-focus rotating-anode generator (nominal focal size 1×0.1 mm, viewed at an angle of 6°) and mirror-monochromator focusing camera of the type described by Huxley & Brown (1967). A small bundle of fibres was placed vertically in a specimen cell with 'Milar' windows. The low angle equatorial diffraction patterns were recorded on Kodak X-ray film with a specimen to film distance of 45 cm. Exposure times were 1.5–2 h. The sarcomere length was determined by He–Ne laser light diffraction before and after obtaining each X-ray diffraction pattern recording.

Results

EFFECT OF SAPONIN ON THE SURFACE MEMBRANE

Effect of saponin on twitch

Treatment of *Xenopus* single fibres with 2.5 μg/ml saponin added to the Ringer solution for 30 min did not affect twitch responses at all, but with 5 μg/ml saponin at 20° C or 10 μg/ml at 1° C twitch tension was first increased slightly after some time and was then abolished as shown in Fig. 1. These effects of saponin were irreversible. A K-contracture could still be elicited (Fig. 1) at the time of the twitch disappearance. The effect of higher concentrations of saponin in normal Ringer was not examined systematically, but it was observed in one fibre that 1 mg/ml saponin dissolved in normal Ringer produced an immediate and irreversible contracture of the fibre.

Perforation of the surface membrane of muscle fibres by saponin

As shown in Fig. 2b, an intact single fibre cannot respond to the contractile activating solution containing 10^{-4} M free Ca. After 30 min treatment with 5 μg/ml saponin, the fibre still did not respond to 10^{-4} M Ca (Fig. 2c). However, a further 30 min treatment of the fibre with 10 μg/ml saponin made the same Ca solution effective in producing maximal tension, which could easily be abolished by reducing free Ca ion concentration in the medium (Fig. 2d). Further addition of 30 min treatment with 50 μg/ml saponin did not increase the rate of tension development on applying Ca nor that of tension decline on removal of Ca. The threshold saponin concentration for responsiveness to external Ca in this fibre on 30 min treatment was thus 10 μg/ml.

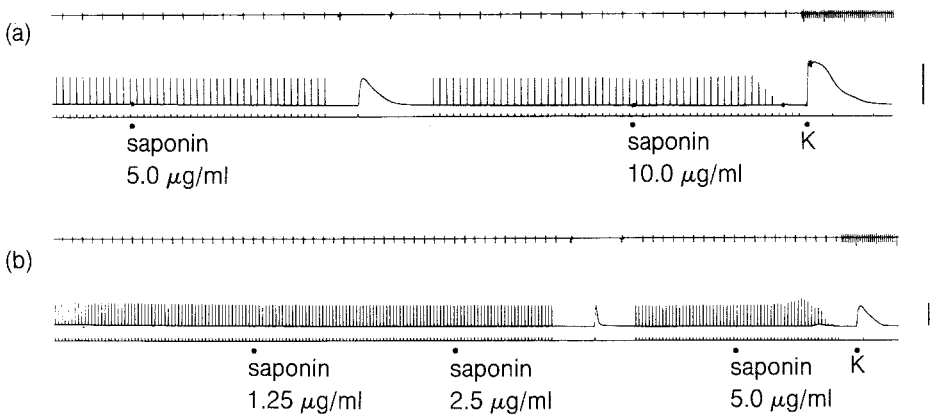

Fig. 1. Effect of saponin on twitch response of single *Xenopus* muscle fibres. After abolition of twitch, K-contracture could still be elicited on replacing normal Ringer by normal relaxing solution. (a) A fibre from toe muscle, at 1° C. Tension calibration 1 mN. (b) A fibre from tibialis anterior, at 20° C. Tension calibration 3 mN. Time (top traces) 1 min for series of twitches and 1 s for rapid trace of twitch and for K-contracture. The bottom traces were to monitor stimulator output.

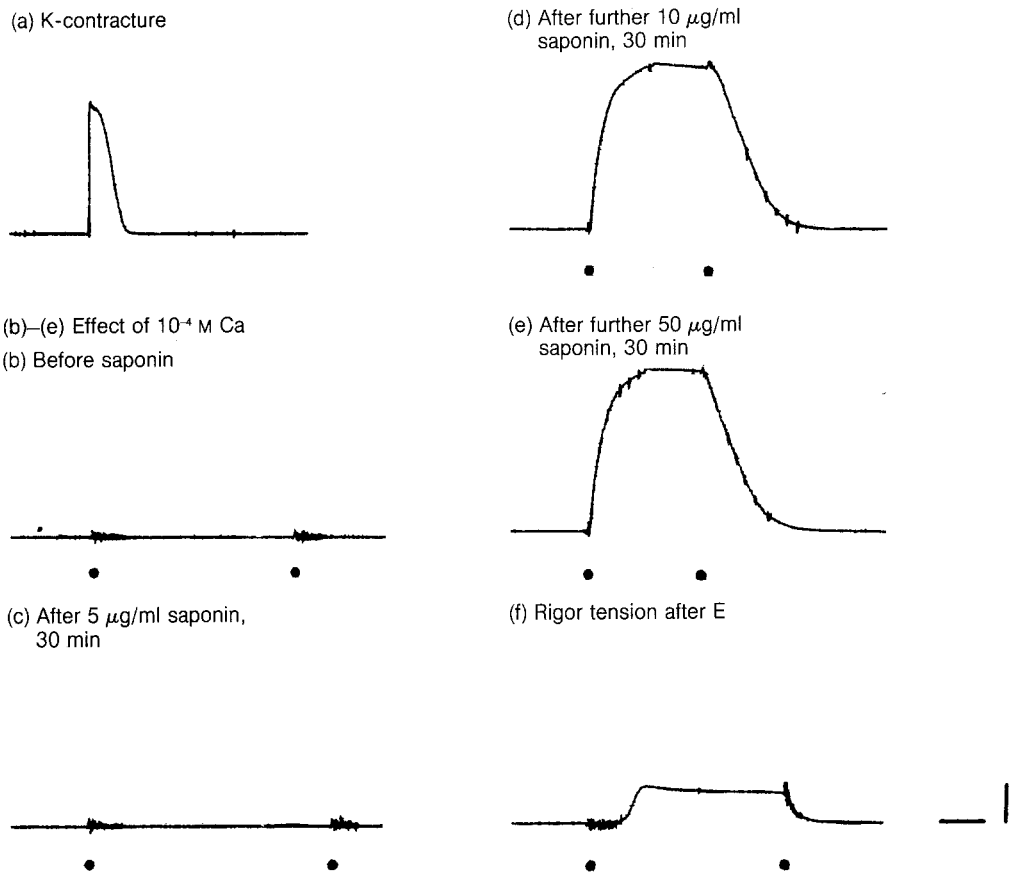

Fig. 2. Perforating effect of saponin on a single *Xenopus* muscle fibre at 2° C. (a) K-contracture induced by normal relaxing solution. In (b)–(e), a contractile activating solution containing 10^{-4} M Ca was applied at the first dot, and a relaxing solution containing 10 mM EGTA at the second dot. (b) Before saponin treatment. (c) After a treatment with 5 μg/ml saponin for 30 min. (d) After further treatment with 10 μg/ml saponin for 30 min. (e) After further treatment with 50 μg/ml saponin for 30 min. In (f), finally a solution of composition similar to normal relaxing solution except for omission of ATP was added at the first dot and a normal relaxing solution at the second dot. Calibrations: 1 mN and 24 s for (a) or 10 s for (b)–(f).

None of the fibres tested (n = 4) became responsive to external Ca after 30 min in 5 μg/ml saponin at 2° C, but all six fibres tested were responsive after 30 min in 10 μg/ml saponin. At 20° C, however, 5 μg/ml saponin was usually effective.

Another convenient way of determining the threshold saponin concentration is to add various amounts of saponin directly to the contractile activating solution, and to examine whether muscle fibres develop tension or not in these solutions. This was done with frog sartorius muscles and the threshold saponin concentration in this case was also 5–10 μg/ml (Fig. 3).

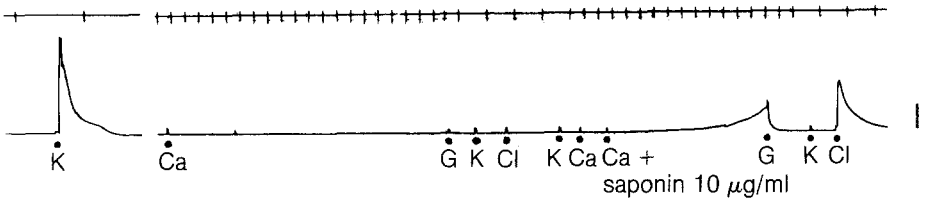

Fig. 3. Effect of saponin on frog sartorius. K, normal relaxing solution; Ca, contractile activating solution containing 5×10^{-6} M free Ca; G, relaxing solution containing 10 mM EGTA; Cl, normal relaxing solution of which methane sulphonate was replaced by chloride. Time 1 min, tension calibration 20 mN for initial K-contracture and 10 mN for the rest of the record.

Responses to Ca after the saponin treatment (Figs. 2, 3) could be interpreted as the result of perforation of the surface membrane, so that externally applied Ca ions had free access to the contractile protein system, rather than the result of a high sensitivity to external Ca acquired without perforation. The evidence for the perforation is as follows. (i) The relation between free Ca ion concentration and tension of the saponin-treated fibres was in the same general range of pCa axis as that of mechanically skinned fibres (Fig. 4). Such a coincidence is unlikely if the high sensitivity to extracellular Ca proposed above were the case. (ii) If Mg^{2+} and/or ATP were removed from a relaxing solution, saponin-treated fibres went into rigor, which could immediately be reversed by re-introducing MgATP (Fig. 2f). Thus, the surface membrane was apparently made permeable also to Mg and ATP. This always occurred whenever the fibres became responsive to external Ca; in other words, 10 μg/ml saponin for 30 min was enough to produce this effect. (iii) When the methane-sulphonate in a relaxing solution was replaced by Cl, saponin-treated fibres usually responded with a substantial transient contraction probably due to a change in membrane potential of the SR (Fig. 3). This also occurs with mechanically skinned fibres (Endo & Nakajima, 1973). If the surface membrane were intact, the only expected response to the above ionic replacement would have been hyperpolarization,

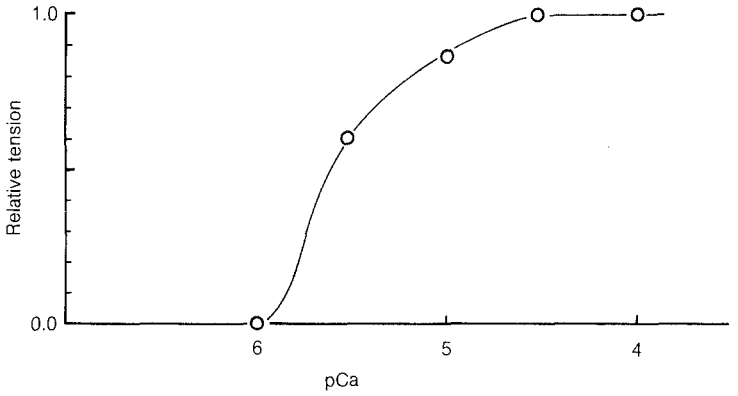

Fig. 4. pCa-tension relation of a *Xenopus* fibre treated with 50 μg/ml saponin for 30 min at 2° C.

which would not lead to contraction. In fact no contractions were evoked before the saponin treatment by the above ionic replacement (Fig. 3). (iv) Ohtsuki and his colleagues (Ohtsuki & Ozawa, 1977; Ohtsuki *et al.*, 1978) have shown that ferritin particles and various protein molecules can easily permeate through saponin-treated cell membranes. We have obtained an indication that, after a saponin treatment, soluble proteins appear to leak out of single muscle fibres. Fig. 5 shows interference microscopic pictures of a single fibre before (A) and after (B) a treatment with 50 μg/ml saponin as well as those of two long pieces of the same fibre obtained by subsequent mechanical splitting (C1 and C2). An appreciable reduction of optical path difference across the fibre after the saponin treatment is noticeable in the figure. For quantitative determination, a series of interference microphotographs were taken by gradually changing the compensation to obtain the accurate optical path difference at each part of the fibre. The optical path difference was then integrated graphically along the transverse direction of the fibre (Endo, 1967). In the fibre shown in Fig. 5, the integrated optical path difference decreased by 25% after the saponin treatment, because of a decrease in refractive index of the fibre, while the fibre width was slightly increased (5%). After subsequent splitting of the saponin treated fibre, no further decrease in integrated optical path difference was observed, indicating that most of the soluble proteins had already leaked out immediately after the saponin treatment. Similar results were obtained with three other single fibres. Although not measured quantitatively, treatment with 10 μg/ml saponin for 30 min also caused an appreciable reduction of optical path difference across the fibre, and was similar to Fig. 5. (v) Matsubara & Elliot (1972) found that the spacings between myofilaments measured by X-ray diffraction were larger in mechanically skinned fibres than in intact fibres. Yagi and Matsubara (personal communication) found that the spacings increased also after saponin treatment. We have confirmed this with an X-ray diffraction study of a small bundle from *Xenopus* toe muscle. For example, in one experiment, the centre to centre distance between thick filaments obtained from the spacing of the (1, 0) reflections was 41.1 nm and 46.8 nm at sarcomere length 2.2 μm, before saponin treatment in normal Ringer and after a treatment with 100 μg/ml saponin for 30 min in a relaxing solution, respectively. Since application of the relaxing solution without saponin treatment also increased the spacings but the magnitude was very small (at most 1 nm under the above conditions), it is concluded that the increase was primarily due to the saponin treatment. This seems to indicate that the environment of the myofilaments was completely altered in saponin-treated fibres as in mechanically skinned fibres. Although it is likely that 10 μg/ml saponin has essentially the same action if applied long enough, only a high concentration of saponin was used for the X-ray experiments. In these experiments, therefore, *all* the fibres in the bundle should be skinned, and unknown diffusion delays in bundles can be avoided.

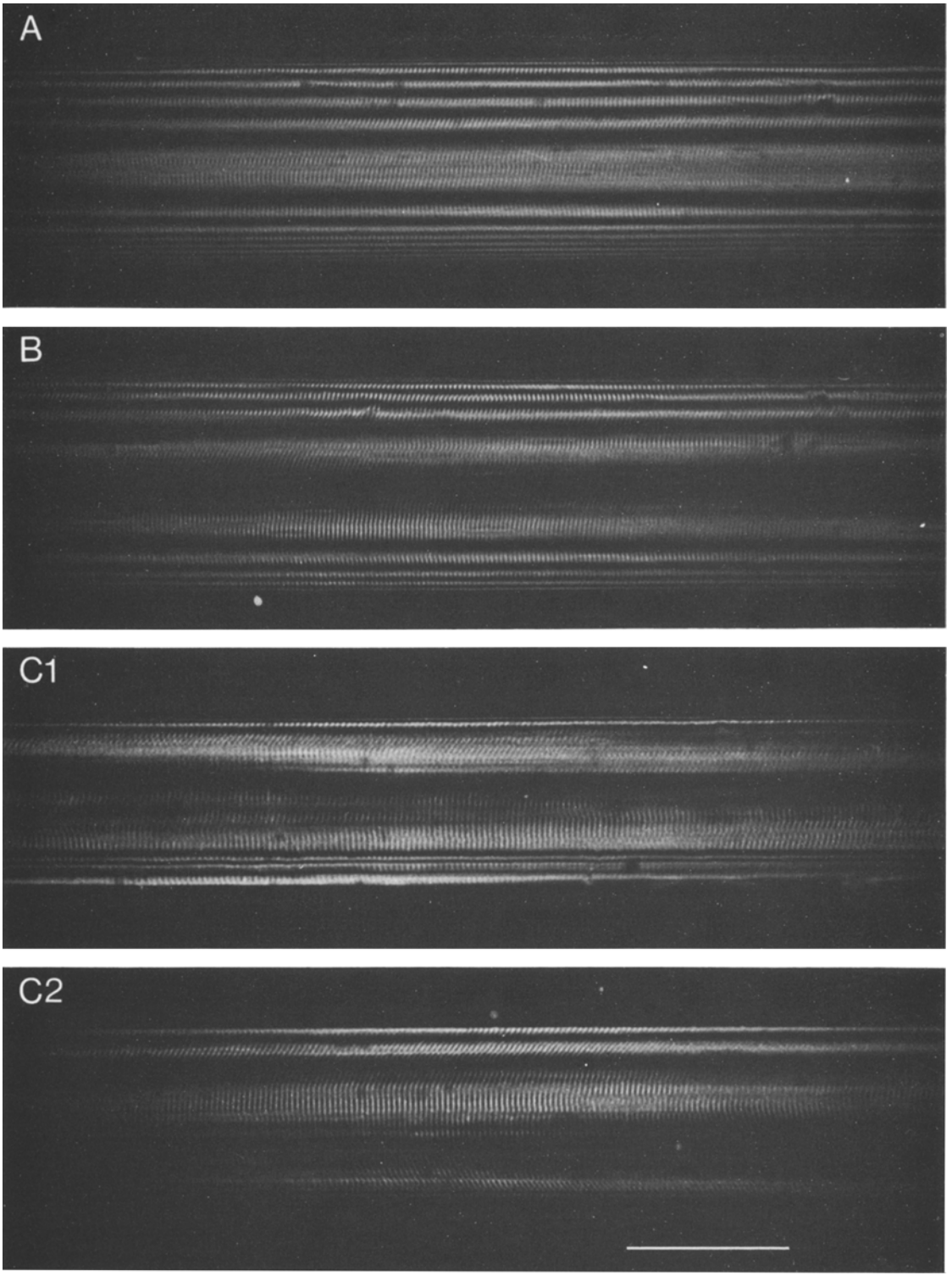

Fig. 5. Interference photomicrograph of a *Xenopus* fibre before (A) and after (B) treatment with 50 μg/ml saponin for 30 min, and after subsequent mechanical splitting (C1 and C2). The fibre was kept in a normal relaxing solution at room temperature. Scale bar: 100 μm.

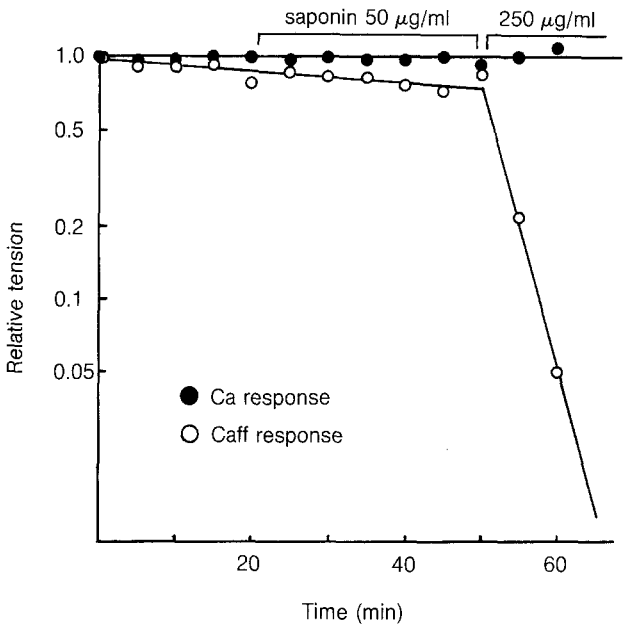

Fig. 6. Effect of saponin on the SR of a skinned *Xenopus* fibre. Peak tension produced by application of 3×10^{-6} M free Ca for 30 s (filled circles) and area under the subsequent caffeine contracture with 25 mM caffeine in normal relaxing solution (open circles) were plotted relative to the magnitude of the first pair of responses. During the intervals, the fibre was in normal or saponin-containing relaxing solution as indicated.

EFFECT OF SAPONIN ON THE SR MEMBRANE

In order to examine effects of saponin on the SR membrane and on the contractile protein system, mechanically skinned fibres were used at 2° C. A general idea about the effects of saponin was obtained by the type of experiments shown in Fig. 6. A skinned fibre underwent a pair of contractions comprising a Ca response (peak tension produced by 30 s application of 3×10^{-6} M free Ca) and a caffeine response (area under transient tension produced by subsequent application of 25 mM caffeine in normal relaxing solution) which was repeatedly elicited at 5 min intervals. Between test contractions, the fibre was kept in a relaxing solution containing either zero or various concentrations of saponin. Since 3×10^{-6} M Ca normally produces submaximal contraction, the Ca responses would be altered if the Ca sensitivity or the contractility of the myofibrils changed. From Fig. 6 it is clear that Ca responses were not affected by treatment with saponin at least up to 250 μg/ml. The caffeine response was a result of release of Ca which had accumulated in the SR during the preceding Ca response. It should be altered, therefore, if the ability of the SR to take up, retain or release Ca were altered. As shown in Fig. 6, the caffeine responses deteriorate gradually even in the absence of saponin, but the rate of deterioration does not seem to be altered by 50 μg/ml saponin. It is obvious, however, that 250 μg/ml saponin severely damaged the SR.

Table 1. Effect of various concentrations of saponin on SR responses.

Saponin concentration (μg/ml)	*Number of fibres*	*Ratio of SR response after to that before**
0	9	0.834 ± 0.042
50	11	0.892 ± 0.040
100	4	0.888 ± 0.081
150	10	0.283 ± 0.150†
250	6	0

*30 min treatment. For further details, see text. Mean ± SE.
†Seven fibres gave no response after the treatment.

To determine a threshold saponin concentration for the SR effect, experiments of a slightly different design were conducted. Responses to 25 mM caffeine after a 3 min Ca-loading period at 5×10^{-7} M Ca were compared before and after 30 min in relaxing solutions containing zero or various concentrations of saponin. The results are summarized in Table 1. It is clear that saponin did not affect the SR at all in a

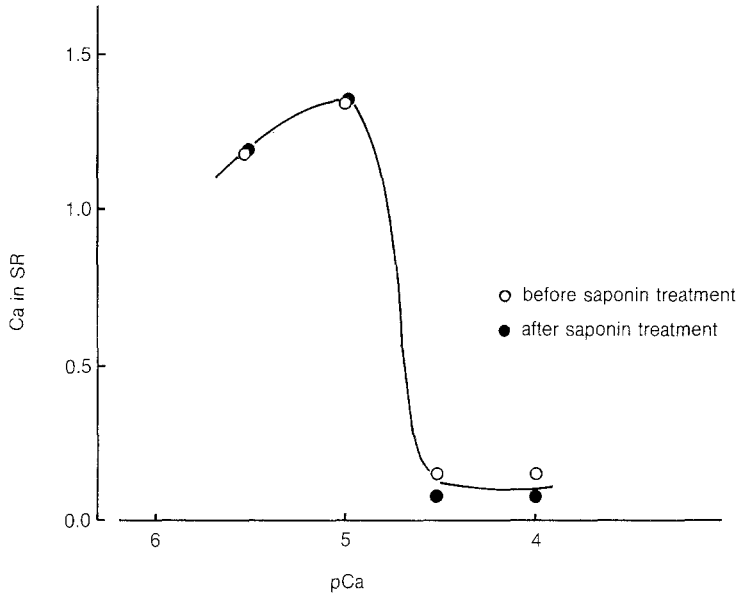

Fig. 7. Absence of effect of 50 μg/ml saponin on Ca-induced Ca release. The SR was loaded with 5×10^{-7} M Ca for 3 min and 'stimulated' for 10 s by various concentrations of free Ca plotted on the abscissa. The amount of Ca remaining after the above procedure was estimated by subsequent response to 25 mM caffeine in normal relaxing solution. The values relative to that without Ca 'stimulation' were plotted on the ordinate. Open circles, before and filled circles, after 30 min saponin treatment.

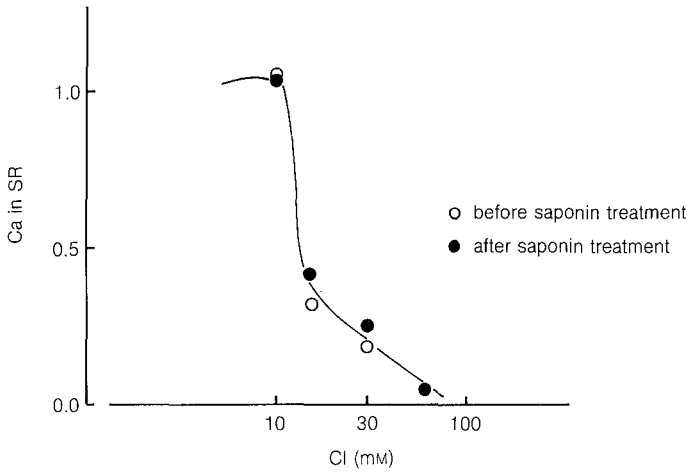

Fig. 8. Absence of effect of 50 μg/ml saponin on Cl-induced Ca release. Experimental procedures and symbols are the same as in Fig. 7, except that 'stimulation' was made not by applying Ca but by replacing various amounts of methane sulphonate with chloride (concentration plotted on the abscissa) in a relaxing solution containing 0.5 mM EGTA.

concentration of 50 μg/ml. The SR function of a small number of fibres was damaged by 100 μg/ml saponin, while that of most fibres was impaired by 150 μg/ml. The SR function of all fibres tested so far was severely affected by 250 μg/ml saponin.

The effects of treating skinned fibres with 50 μg/ml saponin for 30 min on Ca release mechanisms were also examined. It was found that neither the Ca-induced Ca release (Fig. 7) nor the chloride-induced Ca release (Fig. 8) was at all affected by the saponin treatment.

In conclusion, we could not detect any effects of saponin on the SR, at least up to 50 μg/ml for 30 min at 2° C.

Discussion

The present results clearly indicate that the surface membrane of amphibian skeletal muscles could be perforated by saponin at a concentration of 5–10 μg/ml or greater, if treated for 30 min. On the other hand, the SR membrane was shown to be unaffected by a treatment with 50 μg/ml of saponin for 30 min. Therefore, there is a range of saponin concentrations in which the surface membranes are chemically skinned, but the SR membranes are kept intact. Thus, it is possible to obtain a preparation almost equivalent to Natori's skinned fibre by using 10–50 μg/ml saponin. The only probable difference between saponin-treated fibres and mechanically skinned fibres is that diffusion of substances into and out of saponin-treated fibres, is slightly slower as judged by the time course of the rise and fall of tension (Fig. 2). However, the possibility cannot be excluded that saponin has some effects which escaped being detected in this study.

The specific perforating action of saponin on the surface membrane is especially useful when small muscle cells, such as cardiac or smooth muscle fibres, or many fibres in a bundle are to be skinned at the same time. Some applications of this technique to cardiac (Endo & Kitazawa, 1978) and smooth muscles (Endo *et al.*, 1977) have already been reported. The technique could also be applied to nerve or other kinds of cells, for example, Blaustein *et al.* (1978) have successfully applied saponin to a synaptosome preparation in order to study its calcium sequestration system. However, it is important to check if the SR of cardiac and smooth muscle or endoplasmic reticulum of nerve and other cells is not affected by saponin.

It is to be noted that perforation by saponin requires a certain time. For example, with 10 μg/ml saponin, exposure for about 20 min was usually necessary at 2° C to make single fibres responsive to a contractile activating solution. In the example shown in Fig. 1, after a short exposure to 10 μg/ml saponin, some membrane potential must have been retained, since a K-contracture could be elicited. The rate of action of saponin appears to be dependent on temperature: it is faster at 20° C than at 2°C. It is not clear at present if the observed difference in threshold saponin concentration for perforation with a 30 min treatment (5 μg/ml at 20° C; 10 μg/ml at 2° C) simply indicates the difference in rate or some other factor.

The initial effect of saponin was potentiation and then an abolition of the twitch (Fig. 1), which might well be a result of depolarization. Obviously, an electrophysiological study is required.

Ogawa (personal communication) recently revised the figure for apparent binding constant of CaEGTA, so that his preferred figure is 10^6 M^{-1} at pH 6.8 in agreement with that of other investigators (Allen *et al.*, 1977; Ashley & Moisescu, 1977). However, we used his older figure in this report, mainly to facilitate comparison of the results with our previously published data on mechanically skinned fibres. The sensitivities of the SR to Ca ion (Fig. 7) and to Cl (Fig. 8) in the present study are substantially higher than those we previously reported (Endo, 1975; Endo & Nakajima, 1973). One of the reasons appears to be sensitization both to Ca and to Cl during the 30 min rest period. Thus, when several series of Ca- or Cl-induced release experiments were made with 30 min rest period between each run, as in Figs. 7 and 8, the second and subsequent series were found to show substantially higher sensitivity to Ca or to Cl than that of the first series, irrespective of the presence or absence of saponin during the rest period. The second and the third series were used to measure the effect of saponin in Figs. 7 and 8. The reason for the sensitization is not known at present.

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