Journal of Physiology (1993), **470**, *pp.* 335–348
With 8 figures
Printed in Great Britain

EFFECTS OF RYANODINE ON THE PROPERTIES OF Ca^{2+} RELEASE FROM THE SARCOPLASMIC RETICULUM IN SKINNED SKELETAL MUSCLE FIBRES OF THE FROG

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(*Received 13 July 1992*)

SUMMARY

1. We studied the effects of ryanodine on the functions of the sarcoplasmic reticulum (SR) in skinned muscle fibres from *Xenopus laevis*.

2. Ryanodine treatment decreased the Ca^{2+} uptake capacity of the SR in a fixed Ca^{2+} loading condition. The extent of the decrease in the SR Ca^{2+} uptake capacity was closely correlated with the activity of the Ca^{2+}-induced Ca^{2+} release (CICR) during the ryanodine treatment. This suggests, in agreement with the previous biochemical results, that ryanodine acts on the CICR channels when they are open.

3. The rate of Ca^{2+} leakage from the SR increased after ryanodine treatment. However, the leakage rate constants were independent of the degree of the loss of SR Ca^{2+} uptake capacity by the ryanodine treatment. This is inconsistent with the notion that the SR is a single uniform compartment and that the decline in the SR Ca^{2+} uptake capacity is a result of the increase in the Ca^{2+} leakage.

4. Partial recovery of the Ca^{2+} uptake capacity of the ryanodine-treated SR was observed when Ca^{2+} loading was carried out in the presence of 10 mM procaine. This indicates that procaine partially blocks the open-locked channels.

5. After Ca^{2+} loading in the presence of procaine, removal of procaine induced a rapid release of Ca^{2+} from the SR through the open-locked channels. This rapid release was dependent both on the adenine nucleotide concentration and on the Ca^{2+} concentration. Thus, the 'open-locked' CICR channels are still regulated by Ca^{2+} and adenine nucleotides.

INTRODUCTION

Calcium release from the sarcoplasmic reticulum (SR) is an important step in the excitation–contraction coupling of skeletal muscle (Endo, 1977; Endo, 1985; Ebashi, 1991). Among the Ca^{2+} release mechanisms from the SR, the Ca^{2+}-induced Ca^{2+} release (CICR) has been best characterized (Endo, 1985). There are several lines of evidence, however, to indicate that physiological Ca^{2+} release from the SR is not mediated by CICR (Endo, 1985), but evoked by some direct effect of depolarization of the transverse tubules (Chandler, Rakowski & Schneider, 1976). On the other hand, it is generally believed that a common Ca^{2+} release channel is utilized both for

the physiological Ca^{2+} release and for the CICR (Lamb & Stephenson, 1990; Ebashi, 1991), although strict evidence for this idea of the common channel is still lacking.

Various pharmacological tools have been used to study the mechanism of Ca^{2+} release from the SR. Ryanodine, a neutral plant alkaloid causes irreversible contracture of skeletal muscle (Jenden & Fairhurst, 1969). The effects of ryanodine on the Ca^{2+} release mechanism have been extensively studied. The drug binds to a specific protein (ryanodine receptor) on the terminal cisternae of the SR (Fleischer, Ogunbunmi, Dixon & Fleer, 1985; Campbell *et al.* 1987; Lai, Erickson, Block & Meissner, 1987). The ryanodine receptor protein of rabbit has been purified (Imagawa, Smith, Coronado & Campbell, 1987; Inui, Saito & Fleischer, 1987), and its complementary DNA has been cloned and sequenced (Takeshima *et al.* 1989). The mode of the drug action has been extensively studied in fragmented SR (Fleischer *et al.* 1985; Meissner, 1986) and in single Ca^{2+} release channels incorporated into planar lipid bilayers (Rousseau, Smith & Meissner, 1987; Lai, Erickson, Rousseau, Liu & Meissner, 1988). These studies suggested that ryanodine binds to the Ca^{2+} release channels of the SR and locks the channels in an open state.

Since ryanodine is such a well-characterized, specific drug, it may be useful as a tool for the differential characterization of physiological Ca^{2+} release and CICR. As a necessary step to reach this goal, we closely examined the effects of ryanodine on the properties of the SR in skinned frog muscle fibres, which are more physiological preparations than fragmented SR. The results indicated that the ryanodine-sensitive Ca^{2+} release channels are the same as, or at least they include, the CICR channels. Our results also suggest that the SR is not a single homogeneous compartment but is composed of many functional compartments and that the Ca^{2+} channels 'open-locked' by ryanodine still retain properties of the native channels. Preliminary results have been reported elsewhere (Oyamada, Iino & Endo, 1988, 1990).

METHODS

Preparation of skinned muscle fibres

Skinned fibres were prepared from the fast fibres in the ileofibularis muscle of male adult *Xenopus laevis* (30–50 g), which were stunned by a blow to the head and pithed. The muscles were dissected to obtain small bundles of a few fibres under a stereomicroscope (JM, Olympus, Japan) in a Ringer solution (118 mM NaCl, 2·5 mM KCl, 2 mM $Ca(Ms)_2$, 2 mM Hepes, pH 7·2 adjusted with NaOH at room temperature). (For abbreviations, see the end of the Methods section.) The external solution was then replaced by a relaxing solution (solution G2, Table 1) and a single muscle fibre (100–200 μm in diameter, about 1·5 cm in length) was isolated and split into two (or more) longitudinal pieces to obtain skinned fibres (50–100 μm in diameter).

Force measurement and recording

Both ends of a skinned fibre segment (2–5 mm in length) were tied with single silk thread to a pair of tungsten hooks, one of which was connected to a strain-gauge transducer element (AE801, Akers, Norway) and the other was fixed on a micromanipulator (Narishige, Japan). The length of the skinned fibre was adjusted to 1·28 times the slack length. Solution changes were carried out using the 'bubble plate' (Horiuti, 1988), a metal plate with sixteen round wells into which solutions were poured to such an extent that the solutions made dome-shaped protrusions ('bubbles') above the top surface of the plate. The skinned fibre was placed horizontally just above the bubble plate. By moving the plate horizontally, we could transfer the skinned fibre from one bubble to another in a fraction of a second to change solutions. We controlled the temperature of the solutions at 1·0–2·0 °C by running chilled water beneath the plate and turning small magnetic stirrer bars in the wells.

TABLE 1. Composition of the solutions

	$[Mg^{2+}]$ (mM)	$[MgATP^{2-}]$ (mM)	$[Ca^{2+}]$ (pCa)	$[Mg]_{tot}$ (mM)	$[ATP]_{tot}$ (mM)	$[EGTA]_{tot}$ (mM)	$[Ca]_{tot}$ (mM)	[KMs] (mM)	Others
Relaxing solutions									
G2	1	4	—	5·01	5·86	2	0	92·1	—
G10	1	4	—	5·04	5·81	10	0	68·3	—
Ca^{2+} loading solution									
L	1	4	6·7	5·03	5·81	10	2·52	63·3	—
Rigor solutions									
G2R	1	0	—	1·01	0	2	0	125·9	—
G10R	1	0	—	1·04	0	10	0	101·8	—
G2RMg0	0	0	—	0	0	2	0	128·9	—
G10RMg0	0	0	—	0	0	10	0	104·8	—
pCa5·5RMg0	0	0	5·5	0	0	10	8·44	88·1	—
Stop solution*	10	0	—	10·4	0	10	0	64·7	10 mM procaine
Assay solutions									
G0·5	1	4	—	5·00	5·87	0·5	0	96·7	—
G0·5caf	1	4	—	5·00	5·87	0·5	0	96·7	35 mM caffeine
G0·5TX	1	4	—	5·00	5·87	0·5	0	96·7	1% Triton X-100

* Stop solution for rapid termination of CICR. All solutions contain 20 mM Pipes (pH 7·0 at 1 °C) and their ionic strength was 0·17 M. Test solutions contained 10 mM EGTA and were modified for the purpose of each experiment (see the legends of each figure).

The output of the strain gauge was amplified by a strain meter (DSA-601B, Shinkoh, Japan), and was fed into a pen-recorder (SR6211, Graphtec, Japan). The isometric tension signal was also collected digitally at 10 Hz by an analog-to-digital converter (Analog-Pro, Canopus Denshi, Japan) equipped in a microcomputer (PC-9801VF2, NEC, Japan) and the data were stored on floppy disks for later analysis.

Assay of Ca^{2+} in the SR and the measurement of CICR rates

In order to assay the amount of Ca^{2+} in the SR, the caffeine contracture method (Endo, 1977; Endo & Iino, 1988) was used. SR of the skinned fibre was loaded with a fixed amount of Ca^{2+} by incubation for 2 min in the Ca^{2+} loading solution (Table 1). After washing out the loading solution or after various test procedures, the EGTA concentration of the bathing solution was lowered to 0·5 mM (G0·5, Table 1). Then, Ca^{2+} stored in the SR was discharged by the application of 35 mM caffeine in the assay solution (G0·5caf, Table 1). The tension–time integrals of the resulting contracture curves were used as the index of Ca^{2+} content in the SR.

In order to measure the Ca^{2+} release rate from the SR, following Ca^{2+} loading we applied a test procedure to induce Ca^{2+} release in the absence of ATP to avoid simultaneous Ca^{2+} uptake, and the Ca^{2+} remaining in the SR after the test procedure was assayed. The amount of decline in the Ca^{2+} content remaining in the SR should correspond to the amount of Ca^{2+} released during the test procedure. The Ca^{2+} release rate constant was calculated assuming single-exponential decay in the Ca^{2+} content of the SR (Endo & Iino, 1988).

Solutions

All the solutions used were listed in Table 1 which shows the ionic conditions as calculated in a previous paper (Horiuti, 1986) by employing the stability constants compiled by Smith & Martell (1974–1989). The pH of the solutions was adjusted at room temperature with KOH to such values that it would become pH 7·0 at 1·0 °C, allowing for the temperature dependence of pK_a of Pipes, $-0{\cdot}00669\ K^{-1}$. The value was calculated using the van't Hoff equation and the ΔH value of $-11{\cdot}4\ kJ\ mol^{-1}$ for the protonation of the ligand (Smith & Martell, 1974–1989). The ionic strength of all the solutions was set to 0·17 M by adding an appropriate amount of KMs. (Abbreviations: Ms, methanesulphonate; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetate; Hepes, *N*-2-hydroxyethylpiperazine-ethanesulphonate; Pipes, piperazine-*N,N'*-bis-ethylenesulphonate.)

RESULTS

Decrease in the Ca^{2+} uptake capacity of the SR following ryanodine treatment

Figure 1*A* shows the effect of ryanodine treatment on the caffeine contracture in a skinned fibre. If the ryanodine treatment (10 μM) was carried out with Ca^{2+} (pCa 5·5) under the rigor condition (absence of MgATP) to avoid simultaneous Ca^{2+} uptake, there was little reduction in the caffeine contracture after a constant Ca^{2+} loading procedure (Fig. 1*A*, left *vs.* centre). But when the fibre was treated with ryanodine in the presence of caffeine (25 mM) in addition to Ca^{2+}, the size of caffeine contracture was significantly reduced (Fig. 1*A*, right). This effect could be due either to a reduced Ca^{2+} loading of the SR or to a blockade of caffeine-induced Ca^{2+} release. The latter possibility was ruled out by the following experiment in which Triton X-100 was used to destroy the membrane of the SR and to discharge Ca^{2+}. In the ryanodine-treated fibres the application of 1 % Triton X-100 (G0·5TX, Table 1) after the caffeine application failed to elicit any contracture indicating that no substantial amount of Ca^{2+} was left unreleased in the SR (Fig. 1*A* right). On the other hand, an application of 1 % Triton X-100 induced a large contracture after the Ca^{2+} loading in ryanodine-untreated fibres (Fig. 1*B*), but it failed to induce a contraction after the caffeine application (Fig. 1*C*). We, therefore, conclude that the ryanodine treatment reduced the Ca^{2+} uptake capacity of the SR in skinned fibres. The solution with

Triton X-100 was not stirred because stirring caused bubble formating owing to decreased surface tension. The resulting delay in the diffusion of Ca^{2+} out of the fibre space partly accounts for the greater and more prolonged contracture with the detergent than with caffeine.

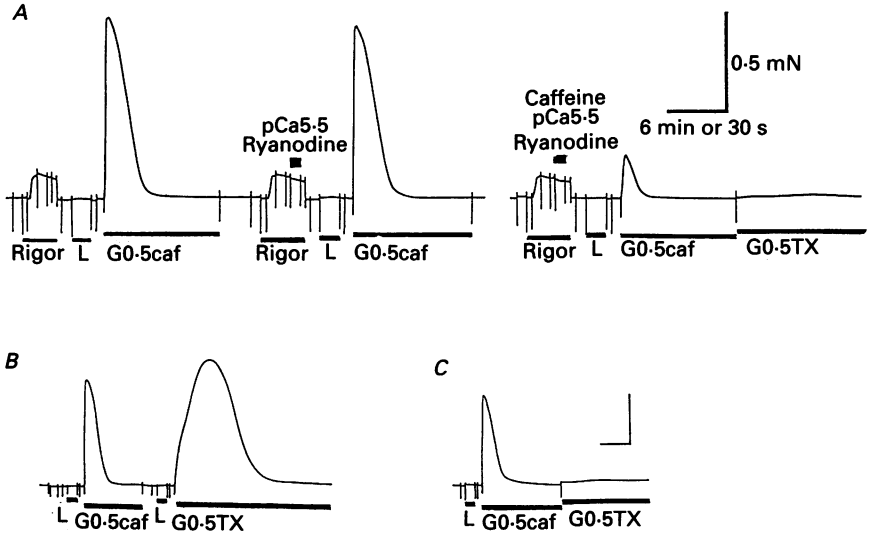

Fig. 1. Reduction of Ca^{2+} uptake capacity of the SR after ryanodine treatment. *A*, comparison of the effectiveness of ryanodine treatment under two different conditions. Treatment with 10 μM ryanodine at pCa 5·5 in the absence of both Mg^{2+} and ATP (pCa5·5RMg0, Table 1; note the development of rigor tension) did not induce appreciable change in the caffeine-induced contracture (centre) following the Ca^{2+} loading by immersing the fibre in a medium containing 0·2 μM Ca^{2+} for 2 min (L) as compared with the control run (left) without ryanodine treatment. If the ryanodine was treated with 25 mM caffeine at pCa 5·5, there was a significant reduction in the following caffeine contracture (right) after the same Ca^{2+} loading. Subsequent application of Triton X-100 induced no contracture. The same result was obtained in another fibre. *B* and *C*, control experiments in ryanodine-untreated skinned fibres. Triton X-100 at 1% induced a large contracture after the Ca^{2+} loading (*B*; $n = 2$), but it failed to do so after Ca^{2+} release with 35 mM caffeine (*C*; $n = 2$). The chart speed was increased 12-fold during the caffeine and Triton applications. Scale bars the same for *A*, *B* and *C*.

Since ryanodine has been shown to lock the Ca^{2+} release channels in an open state (Fleischer *et al.* 1985; Meissner, 1986), the reduced SR Ca^{2+} uptake capacity is likely to be due to the increased loss of Ca^{2+} through the 'open-locked' channels. First, however, we have to rule out the possibility that ryanodine has an inhibitory effect on the Ca^{2+} pump ATPase. As shown in Fig. 2 (lower trace), if 10 mM procaine was added during the Ca^{2+} loading as well as the period immediately before the application of the assay solution, the reduction of SR Ca^{2+} uptake capacity due to ryanodine treatment was partially reversed. Since procaine had a small inhibitory effect on the Ca^{2+} loading in the control condition (Fig. 2, upper trace), these results exclude the possibility that the reduction of SR Ca^{2+} uptake capacity by ryanodine treatment was primarily due to a reduced Ca^{2+} pump activity. Similar

effects were observed when we used 10 mM Mg^{2+} instead of procaine (data not shown). The partial recovery of the SR Ca^{2+} uptake capacity by procaine or Mg^{2+} suggests that the open-locked Ca^{2+} channels can be partially blocked by these inhibitors of CICR (Ford & Podolsky, 1972; Thorens & Endo, 1975; Endo, 1975).

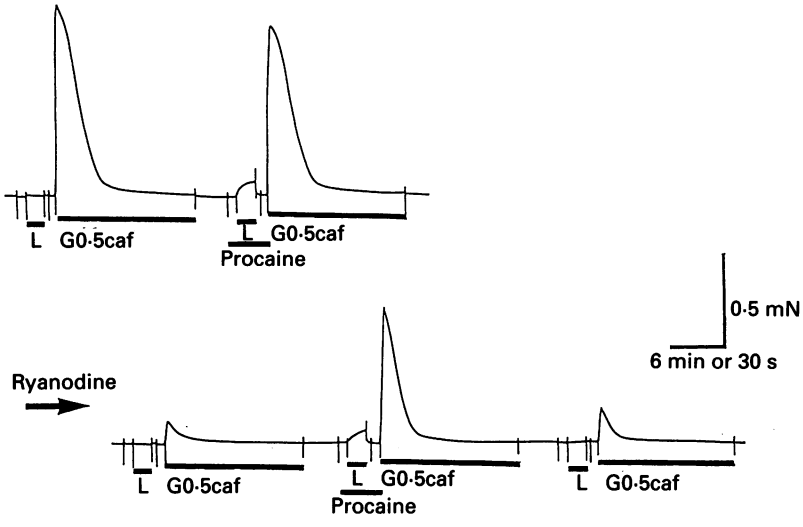

Fig. 2. Tension records showing the apparent recovery of the Ca^{2+} uptake capacity of the SR by procaine (10 mM) in a ryanodine-treated fibre. Upper trace is the control record before ryanodine treatment to show that procaine reduced the Ca^{2+} uptake by the SR. Lower trace is the record after the 10 μM ryanodine treatment (in G0·5caf for 1 min) to show that procaine increased the Ca^{2+} uptake capacity in this condition. Owing to the procaine-dependent increase in the Ca^{2+} sensitivity of the contractile system, there was a small rise in tension during the Ca^{2+} loading with procaine. The figure is a representative record of four experiments. The chart speed was increased 12-fold during caffeine applications.

Effects of ryanodine have been reported to be biphasic, and very high concentrations of ryanodine were shown to block rabbit Ca^{2+} release channels (Meissner, 1986). Blockade of Ca^{2+} release channels is expected to result in the recovery of the SR Ca^{2+} uptake capacity. However, even after treatment with 1·0 mM ryanodine for several hours with 25 mM caffeine at pCa 5·5, we could not observe any contracture by applying 1 % Triton X-100 to the skinned fibres after the Ca^{2+} loading procedure (not shown). This is different from the results obtained in rabbit skinned muscle fibres (Su, 1987).

Development of ryanodine effect under various conditions

As shown in Fig. 1, the effect of ryanodine on the SR Ca^{2+} uptake capacity was dependent on the presence of caffeine. Caffeine enhances the CICR mechanism in SR (Endo, 1975). To examine whether there was a correlation between the rate of development of the ryanodine effect and the magnitude of CICR activation, we treated the fibres with 10 μM ryanodine for 1 min under various conditions. Figure 3 plots the SR Ca^{2+} uptake capacity after the ryanodine treatment for 1 min against

the rate of CICR during the treatment which was varied by changing the concentrations of Ca^{2+} (pCa > 9–4·1), caffeine (0–10 mM) and β,γ-methylene adenosine triphosphate (AMPOPCP), a non-hydrolysable ATP analogue (0 or 1·0 mM). The greater the rate of CICR during the ryanodine treatment, the greater

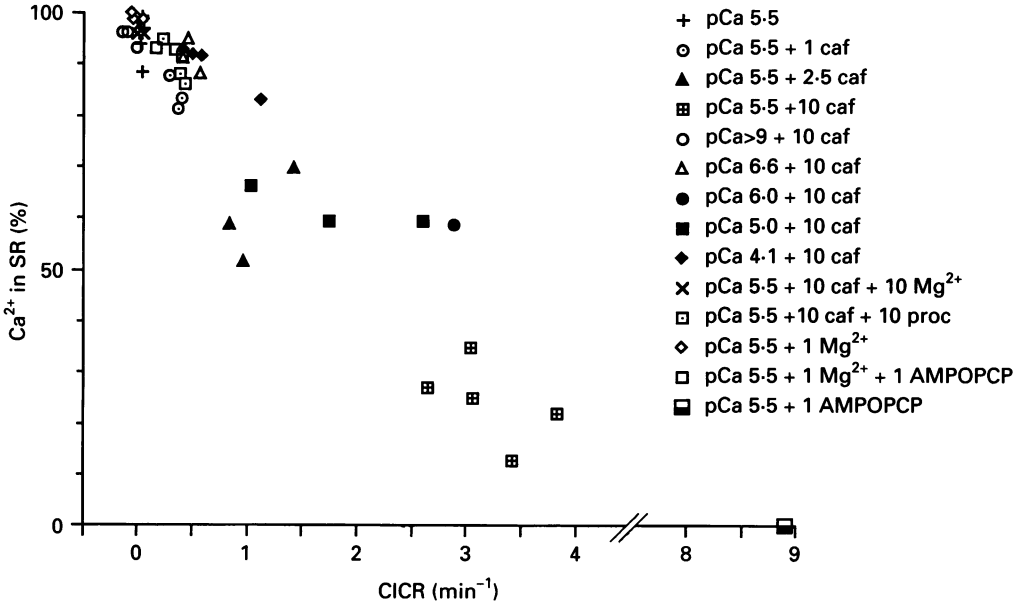

Fig. 3. The relation between the rate of CICR during the ryanodine treatment and the rate of reduction of Ca^{2+} uptake capacity. Magnitude of caffeine contracture (relative to the control caffeine contracture) after the treatment with ryanodine (10 μM) for 1 min in various conditions is plotted against the rate constant of CICR (min^{-1}) during the ryanodine treatment. The rate constants of CICR were varied by changing the concentrations of Ca^{2+} (pCa > 9–pCa 4·1), Mg^{2+} (0–10 mM), caffeine (caf; 0–10 mM), AMPOPCP (0 or 1 mM), or procaine (0 or 10 mM) as shown in the inset. Each symbol represents an experiment in one fibre.

was the degree of the reduction of the SR Ca^{2+} uptake capacity. Development of the effect was inhibited by procaine (10 mM), Mg^{2+} (10 mM) and a high concentration of Ca^{2+} (pCa 4·1).

These results are consistent with the notion that ryanodine preferentially binds to the active CICR channels and locks these channels in an open state.

Rate constants of Ca^{2+} leakage from the SR vs. the extent of the ryanodine effect

The above results strongly suggest that the reduced SR Ca^{2+} uptake capacity after ryanodine treatment can be explained by the increase in the Ca^{2+} leakage through the open-locked channels in the SR. If this were the case and if the SR were a single compartment with multiple Ca^{2+} release channels, then we should expect to see the correspondence between the rate of Ca^{2+} leakage from the SR and the degree of reduction of SR Ca^{2+} uptake capacity. Therefore, we studied the Ca^{2+} leakage rate from the SR which had been treated with ryanodine.

Figure 4 shows the experimental protocol. In each run a constant Ca^{2+} loading (L) was carried out first. Then, the fibre was left in a Ca^{2+}-free solution (G10, Table 1) for 30 s to 6 min before the amount of Ca^{2+} remaining in the SR was examined by the caffeine application. Before ryanodine treatment, there was little Ca^{2+} leakage from

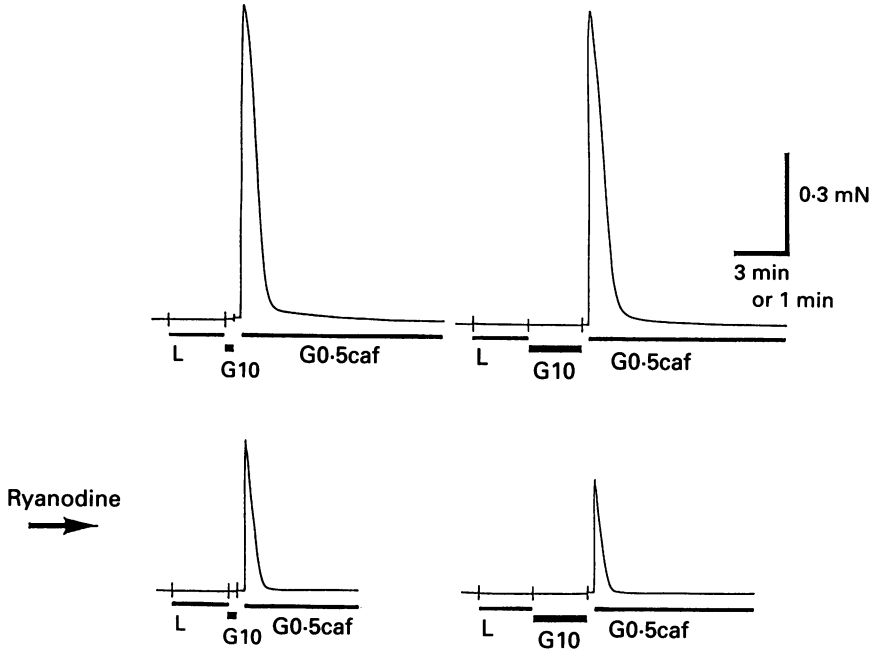

Fig. 4. Tension records showing the increase in Ca^{2+} leakage after ryanodine treatment. Upper traces are the control records before ryanodine treatment to show that there was little Ca^{2+} leakage from the SR in the Ca^{2+}-free solution containing 10 mM EGTA (G10) following the Ca^{2+} loading (L). Lower traces are the records after the ryanodine treatment (1 μM ryanodine with 10 mM caffeine at pCa 5·5 for 30 s) to show the reduction of the Ca^{2+} uptake capacity of the SR and the increment of Ca^{2+} leakage from the SR in the G10 solution. The chart speed was increased 3-fold during the caffeine applications. The figure is a representative result from more than forty experiments of this kind.

the SR in the G10 solution for 3 min (Fig. 4, upper panel, left *vs.* right). However, after the ryanodine treatment the SR Ca^{2+} uptake capacity was decreased to about 35% in this fibre and the Ca^{2+} leakage was significantly increased in the G10 solution (Fig. 4, lower panel, left *vs.* right).

We carried out the ryanodine treatment to various extents so that the SR Ca^{2+} uptake capacity varied from about 70 to 15% of the control and examined the time course of Ca^{2+} leakage (Fig. 5*A*) using the same protocol as shown in Fig. 4. In order to compare the Ca^{2+} leakage rate constants with different degrees of the ryanodine effect, all the data points were expressed as values relative to the first point in each curve in Fig. 5*B*.

Quite contrary to our expectation, the rate constant of Ca^{2+} leakage was almost independent of the SR Ca^{2+} uptake capacity, although the leakage rate constant in the ryanodine-treated fibres was significantly greater than that of the control. Similar results were obtained when the Ca^{2+} loading durations were adjusted so that

the amount of Ca^{2+} in the SR at zero time was almost the same despite the difference in the extent of the ryanodine effect (not shown). These results suggest that the SR of skinned fibres cannot be regarded as a single compartment with multiple Ca^{2+} release channels but seems to be composed of multiple compartments and that the

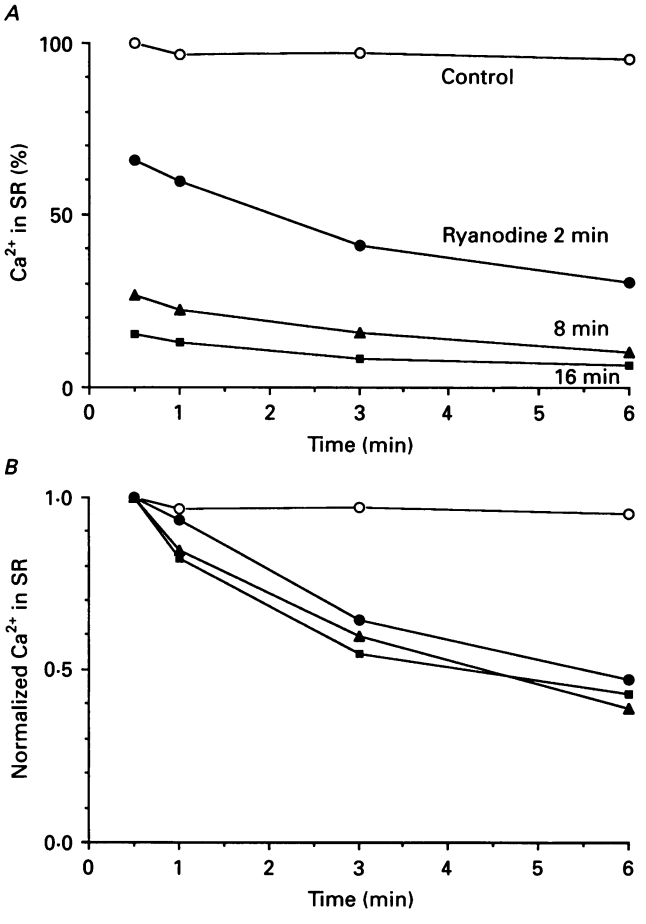

Fig. 5. The time course of Ca^{2+} leakage from the SR after ryanodine treatment (at 1 μM for 2, 8 or 16 min). The experimental protocol was the same as that in Fig. 4 and the duration of the incubation period in G10 (Time) after the Ca^{2+} loading was varied. *A*, amount of Ca^{2+} remaining in the SR plotted against the duration of Ca^{2+} leakage in a relaxing solution (G10). The amount of Ca^{2+} in the SR is expressed as a value relative to that before the ryanodine treatment. *B*, the data points in *A* are normalized by the first point of each curve to compare the Ca^{2+} leakage rate constants. The ryanodine treatment was carried out in the rigor condition containing 1 μM ryanodine and 1 mM caffeine at pCa 6 without Mg^{2+}. The data set was obtained from one fibre, and the ryanodine treatment period indicated in the figure shows the cumulative time period in the ryanodine-containing solution. These are representative results of five other experiments.

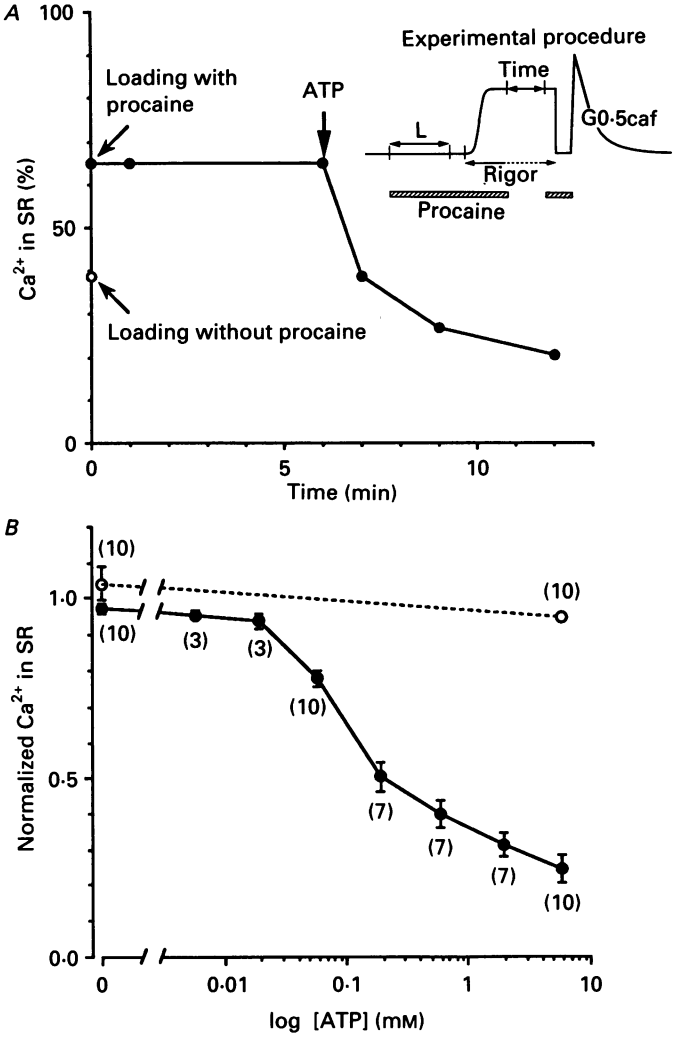

Fig. 6. The effect of ATP on the Ca^{2+} leakage from the ryanodine-treated SR. *A*, the Ca^{2+} loading was carried out in the presence of 10 mM procaine (●) to allow Ca^{2+} uptake by the ryanodine-affected SR. Following the Ca^{2+} loading, the Ca^{2+} leakage procedure was carried out at 1 mM Mg^{2+} with 10 mM EGTA for the duration indicated on the abscissa, and the Ca^{2+} remaining in the SR was assayed by the caffeine contracture afterwards (see the inset). Therefore, each data point was obtained from each run in which the duration and the condition of Ca^{2+} leakage was varied. The $MgATP^{2-}$ concentration during the Ca^{2+} leakage was raised from 0 mM (G10R) to 4 mM (G10) at the arrow. The SR had been treated with 10 μM ryanodine for 1 min with 10 mM caffeine at pCa 5·5 in the absence of MgATP (pCa5·5RMg0). On the ordinate, 100% represents the SR Ca^{2+} content before the ryanodine treatment. The same result was obtained in another fibre. *B*, dependence of Ca^{2+} leakage from the ryanodine-treated SR on the total ATP concentration. The same protocol as in *A* was used. On the ordinate is plotted the amount of Ca^{2+} remaining in the SR after 3 min incubation in the Ca^{2+}-free solutions (10 mM EGTA, 1 mM Mg^{2+}, 0–5·8 mM ATP) normalized by that without the leakage procedure. Results from control fibres, ○, ryanodine-treated fibres, ●. Numbers by the symbols indicate the number of determinations. Means ± S.E.M.

reduced SR Ca^{2+} uptake capacity may reflect the increase in the fraction of compartments of the SR affected by ryanodine.

Effect of nucleotides and Ca^{2+} on the open-locked Ca^{2+} release channels

As already shown in Fig. 2, the open-locked channels seem to be partially blocked by procaine. Indeed, we observed a decreasing effect of procaine on the rate of Ca^{2+}

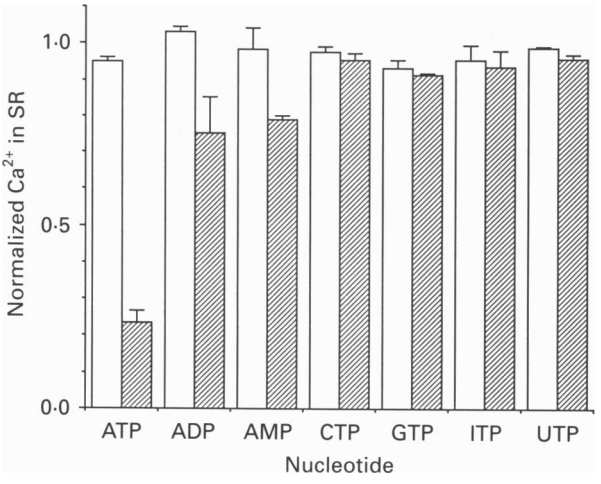

Fig. 7. Effects of various kinds of nucleotides on the Ca^{2+} leakage from the ryanodine-treated SR. The protocol, data representation and ryanodine treatment are the same as in Fig. 6. Open and hatched columns show data from the control fibres and from the fibres after the ryanodine treatment, respectively. Ca^{2+} leakage was carried out in the presence of 1 mM Mg^{2+} and 10 mM EGTA for 3 min. For the nucleotide triphosphates (NTP), $MgNTP^{2-}$ concentration was adjusted to 4 mM; and for ADP and AMP, total concentration was adjusted to 5·8 mM (same as ATP). Total NTP concentrations were (in mM): ITP, 4·9; UTP, 4·5; GTP, 4·8; CTP, 4·6. Means ± S.E.M. ($n = 3$).

leakage from the ryanodine-treated SR (not shown). This provided us with the opportunity to study the rate of Ca^{2+} leakage from the ryanodine-affected SR which had been loaded with Ca^{2+} in the presence of 10 mM procaine (for protocol, see Fig. 6*A* inset). Ca^{2+} loading with procaine resulted in the partial recovery of SR Ca^{2+} uptake capacity (Fig. 6*A*, open *vs.* filled circle at zero time). In the absence of both Ca^{2+} and ATP there was hardly any Ca^{2+} leakage even after the removal of procaine. Only after the addition of ATP was the Ca^{2+} leakage rate markedly increased (Fig. 6*A*), and to a level even faster than that after Ca^{2+} loading in the absence of procaine (cf. Fig. 5). The rate of Ca^{2+} leakage was dependent on the ATP concentration added during the leakage procedure (Fig. 6*B*).

To test whether our nucleotides are also effective on the Ca^{2+} leakage from the ryanodine-affected SR, other nucleotides were used in place of ATP. We saw a similar enhancement of Ca^{2+} leakage by AMPOPCP (not shown). Smaller effects were found with AMP and ADP, but other nucleotides hardly had any effect on the rate of Ca^{2+} leakage from the ryanodine-affected SR (Fig. 7).

We then studied whether there was a Ca^{2+} concentration dependence in the rate

of Ca^{2+} release from ryanodine-treated SR. As shown in Fig. 8, the Ca^{2+} leakage from the ryanodine-treated SR (hatched columns) initially increased (pCa 7·0–5·5) and then decreased with a higher concentration of Ca^{2+} (pCa 4·1), just as seen in the native Ca^{2+} release channels (open columns).

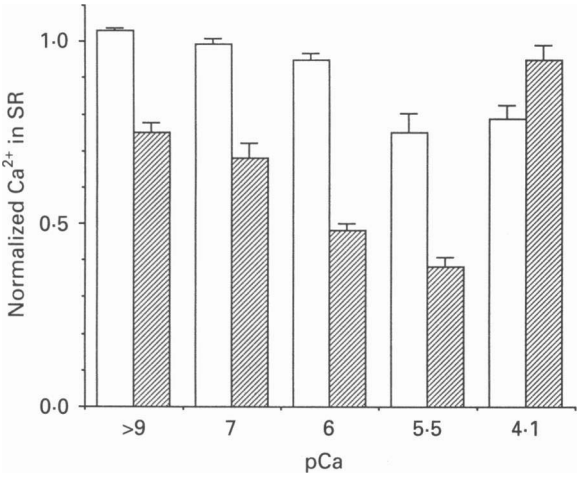

Fig. 8. The Ca^{2+} concentration dependence of the Ca^{2+} leakage from the ryanodine-treated SR. Similar experiments to those in Fig. 6. Open and hatched columns show the amount of Ca^{2+} in the SR after incubation at the indicated pCa for 6 min in the control fibres and for 2 min in the ryanodine-treated fibres, respectively. Both Mg^{2+} and ATP were absent from the Ca^{2+} leakage test solutions. Means ± S.E.M. ($n = 3$).

DISCUSSION

Ryanodine has been shown to bind to the Ca^{2+} release channels in the SR when they are open and to lock the channels in an open state (Fleischer *et al.* 1985; Meissner, 1986; Pessah, Stambuk & Casida, 1987; Rousseau *et al.* 1987). From the experimental conditions reported, the channels affected by the drug appear to be CICR channels. In skinned fibres these properties of the effect of ryanodine were confirmed and extended, and the drug reduced the Ca^{2+} uptake capacity of the SR by increasing Ca^{2+} leakage through the 'open-locked' Ca^{2+}channels. The close correlation between the rate of development of the ryanodine effect and the magnitude of CICR activation during the ryanodine treatment (Fig. 3) clearly indicates that the drug affects the CICR channels. Whether ryanodine also affects the Ca^{2+} release channels opened by the physiological stimulation (depolarization of T-tubules), and if so, its affinity and number of binding sites per channel are required to be elucidated. Lamb & Stephenson (1990) presented results which show that ryanodine acts on the Ca^{2+} release channels opened by the solution change that is expected to depolarize resealed T-tubules in skinned fibres. However, this remains to be confirmed in intact fibres.

An unexpected result of this study was the finding that the rate of Ca^{2+} leakage in the ryanodine-treated skinned fibres was not a function of the SR Ca^{2+} uptake capacity. After ryanodine treatment to a level at which the SR Ca^{2+} uptake capacity

decreased to about 70%, the Ca^{2+} leakage rate showed a significant increase. However, the leakage rate constant was almost independent of the further reduction in the SR Ca^{2+} capacity to 30–15% (Fig. 5). These results are difficult to explain, if we assume the SR to be a single compartment. In this case, we would expect to see a correspondence between the rate constant of Ca^{2+} leakage and the reduction in the SR Ca^{2+} uptake capacity. Therefore, we must assume that the SR is composed of multiple compartments which can be individually affected by ryanodine to lose Ca^{2+} uptake capacity. The enhanced leakage rate constant might represent the translocation of Ca^{2+} from intact compartments to ryanodine-affected compartments. Further study is required for the morphological identification of the compartments.

The last series of experiments dealt with the regulatory mechanism of the open-locked channels. In these experiments the SR (including the ryanodine-affected compartments) was loaded with Ca^{2+} with the help of 10 mM procaine to block open-locked channels, and the rates of Ca^{2+} leakage were measured after the removal of procaine. The leakage rate constants were found to be much higher than those of intact skinned fibres and those of ryanodine-treated fibres loaded in the absence of procaine, if the measurements were carried out in the presence of ATP (Fig. 5 *vs.* Fig. 6). However, the Ca^{2+} leakage through these open-locked channels showed almost the same slow rate as normal channels in the absence of both ATP and Ca^{2+} (Fig. 6). An expansion of these findings was presented in Figs 7 and 8. Although the Ca^{2+} release channels can be phosphorylated by protein kinases (Seiler, Wegenar, Whang, Hathaway & Jones, 1984; Hymel, Schindler, Yang, Inui, Reif & Fleischer, 1989), the regulation of the open-locked channels is not due to phosphorylation because AMPOPCP, ADP and AMP were also effective. These results, therefore, demonstrate that open-locked Ca^{2+} release channels are still under the control of Ca^{2+} and adenine nucleotides as in the native channels (Endo, 1977, 1985). Therefore, ryanodine does not induce permanent opening of the channels as implied by the term open-locked but rather a shift towards a state that favours the opening of the channels.

We thank Miss H. Iida and Miss N. Shimizu for their technical assistance. This work was supported in part by Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

REFERENCES

Campbell, K. P., Knudson, C. M., Imagawa, T., Leung, A. T., Sutko, J. L., Kahl, S. D., Raab, C. R. & Madson, L. (1987). Identification and characterization of the high affinity [^{3}H]ryanodine receptor of the junctional sarcoplasmic reticulum Ca^{2+} release channel. *Journal of Biological Chemistry* **262**, 6460–6463.

Chandler, W. K., Rakowski, R. F. & Schneider, M. F. (1976). A non-linear voltage dependent charge movement in frog skeletal muscle. *Journal of Physiology* **254**, 245–283.

Ebashi, S. (1991). Excitation–contraction coupling and the mechanism of muscle contraction. *Annual Review of Physiology* **53**, 1–16.

Endo, M. (1975). Conditions required for calcium-induced release of calcium from the sarcoplasmic reticulum. *Proceedings of the Japan Academy* **51**, 467–472.

Endo, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiological Reviews* **57**, 71–108.

Endo, M. (1985). Ca^{2+} release from sarcoplasmic reticulum. *Current Topics in Membranes and Transport* **25**, 181–230.

ENDO, M. & IINO, M. (1988). Measurement of Ca^{2+} release in skinned fibers from skeletal muscle. *Methods in Enzymology* **157**, 12–26.

FLEISCHER, S., OGUNBUNMI, E. M., DIXON, M. C. & FLEER, E. A. M. (1985). Localization of Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Proceedings of the National Academy of Sciences of the USA* **82**, 7256–7259.

FORD, L. E. & PODOLSKY, R. J. (1972). Calcium uptake and force development by skinned muscle fibres in EGTA buffered solutions. *Journal of Physiology* **223**, 1–23.

HORIUTI, K. (1986). Some properties of the contractile system and sarcoplasmic reticulum of skinned slow fibres from *Xenopus* muscle. *Journal of Physiology* **373**, 1–23.

HORIUTI, K. (1988). Mechanism of contracture on cooling of caffeine-treated frog skeletal muscle fibres. *Journal of Physiology* **398**, 131–148.

HYMEL, L., SCHINDLER, H. G., YANG, S.-D., INUI, M., REIF, S. & FLEISCHER, S. (1989). Protein kinase/phosphatase modulation of purified skeletal muscle calcium release channel activity in planar bilayers. *Biophysical Journal* **55**, 307a.

IMAGAWA, T., SMITH, J. S., CORONADO, R. & CAMPBELL, K. P. (1987). Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca^{2+}-permeable pore of the calcium release channel. *Journal of Biological Chemistry* **262**, 16636–16643.

INUI, M., SAITO, A. & FLEISCHER, S. (1987). Purification of the ryanodine receptor and identity with feet structure of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *Journal of Biological Chemistry* **262**, 1740–1747.

JENDEN, D. J. & FAIRHUST, A. S. (1969). The pharmacology of ryanodine. *Pharmacological Reviews* **21**, 1–25.

LAI, F. A., ERICKSON, H. P., BLOCK, B. A. & MEISSNER, G. (1987). Evidence for a junctional feet–ryanodine receptor complex from sarcoplasmic reticulum. *Biochemical and Biophysical Research Communications* **143**, 704–709.

LAI, F. A., ERICKSON, H. P., ROUSSEAU, E., LIU, Q.-Y. & MEISSNER, G. (1988). Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature* **331**, 315–319.

LAMB, G. D. & STEPHENSON, D. G. (1990). Control of calcium release and the effect of ryanodine in skinned muscle fibres of the toad. *Journal of Physiology* **423**, 519–542.

MEISSNER, G. (1986). Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *Journal of Biological Chemistry* **261**, 6300–6306.

OYAMADA, H., IINO, M. & ENDO, M. (1988). Open lock by ryanodine of the Ca-induced Ca release channels in skeletal muscle fibers requires the channel activation. *Japanese Journal of Pharmacology* **46**, 312P.

OYAMADA, H., IINO, M. & ENDO, M. (1990). Modulation of 'open-fixed' ryanodine-bound Ca release channels by ATP. *Japanese Journal of Pharmacology* **52**, 373P.

PESSAH, I. N., STAMBUK, R. A. & CASIDA, J. E. (1987). Ca^{2+}-activated ryanodine binding: Mechanisms of sensitivity and intensity modulation by Mg^{2+}, caffeine and adenine nucleotides. *Molecular Pharmacology* **31**, 232–238.

ROUSSEAU, E., SMITH, J. S. & MEISSNER, G. (1987). Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *American Journal of Physiology* **253**, C361–368.

SEILER, S., WEGENAR, A. D., WHANG, D. D., HATHAWAY, D. R. & JONES, L. R. (1984). High molecular weight proteins in cardiac and skeletal muscle junctional sarcoplasmic reticulum vesicles bind calmodulin are phosphorylated, and are degraded by Ca^{2+}-activated protease. *Journal of Biological Chemistry* **259**, 8550–8557.

SMITH, R. S. & MARTELL, A. E. (1974–1989). *Critical Stability Constants*, vols 1–6. Plenum Press, New York.

SU, J. Y. (1987). Effects of ryanodine on skinned skeletal muscle fibers of the rabbit. *Pflügers Archiv* **410**, 510–516.

TAKESHIMA, H., NISHIMURA, S., MATSUMOTO, T., ISHIDA, H., KANGAWA, K., MINAMINO, N., MATSUO, H., UEDA, M., HANAOKA, M., HIROSE, T. & NUMA, S. (1989). Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature* **339**, 439–445.

THORENS, S. & ENDO, M. (1975). Calcium-induced calcium release and 'depolarization'-induced calcium release: their physiological significance. *Proceedings of the Japan Academy* **51**, 473–478.