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Enhancing effect of calmodulin on Ca^{2+}-induced Ca^{2+} release in the sarcoplasmic reticulum of rabbit skeletal muscle fibres

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1. We analysed the effect of calmodulin on Ca^{2+}-induced Ca^{2+} release (CICR) in the sarcoplasmic reticulum (SR) using chemically skinned fibres of rabbit psoas muscle. Ca^{2+} release was measured using fura-2 microfluorometry.
2. In saponin-skinned fibres, calmodulin potentiated Ca^{2+} release at low Ca^{2+} concentrations (< 3 μM), while it showed an inhibitory effect at high Ca^{2+} concentrations (3–30 μM).
3. Co-application of ryanodine and calmodulin at 0·3 μM Ca^{2+}, but not ryanodine alone, induced a decline in the Ca^{2+} uptake capacity of the SR, an effect expected from the open-lock of active CICR channels by ryanodine. Thus, potentiation of Ca^{2+} release by calmodulin at low Ca^{2+} concentrations can be regarded as a result of the activation of the ryanodine receptor.
4. Greater concentrations of calmodulin were required for potentiation of CICR at low Ca^{2+} concentrations (1 μM) than for inhibition at high Ca^{2+} concentrations (10 μM).
5. In β-escin-permeabilized fibres in which intrinsic calmodulin was retained, the rates of CICR were similar to those measured in the presence of 1 μM calmodulin in saponin-permeabilized fibres.
6. These results suggest that calmodulin plays an important role in the regulation of CICR channels in intact skeletal muscle fibres.

Physiological contraction of skeletal muscle is initiated by Ca^{2+} release from the sarcoplasmic reticulum (SR) following depolarization of the transverse tubule membrane (Endo, 1977, 1985; Ebashi, 1991). It has been shown that ryanodine, a neutral plant alkaloid, binds to a specific protein (the ryanodine receptor) on the terminal cisternae of the SR and locks the Ca^{2+} release channel in an open state (Fleischer, Ogunbunmi, Dixon & Fleer, 1985). The ryanodine receptor protein of rabbit skeletal muscle has been purified using drug-specific binding techniques (Imagawa, Smith, Coronado & Campbell, 1987; Lai, Erickson, Rousseau, Liu & Meissner, 1988; Saito, Inui, Radermacher, Frank & Fleischer, 1988), and its complementary DNA has been cloned and sequenced (Takeshima *et al.* 1989). Upon incorporation into planar lipid bilayers, the ryanodine receptor displayed channel activities (Imagawa *et al.* 1987; Lai *et al.* 1988; Hymel, Inui, Fleischer & Schindler, 1988; Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988) which were similar to those of the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism extensively studied in skinned fibres (Endo, 1985) and isolated SR vesicles (Meissner, 1984; Meissner, Darling & Eveleth, 1986; Smith, Coronado & Meissner, 1986). These results demonstrate that the ryanodine receptor is functionally identical with the CICR channel protein.

There are several lines of evidence, however, which indicate that physiological Ca^{2+} release from the SR is not mediated by the CICR mechanism (Endo, 1985), but is directly controlled by voltage sensors situated on the transverse tubule membrane which transmit the signal of action potentials to the Ca^{2+} release channels on the SR membrane (Schneider & Chandler, 1973). On the other hand, it is generally believed that a common Ca^{2+} release channel is utilized for both physiological Ca^{2+} release and CICR (Lamb & Stephenson, 1990; Ebashi, 1991; Endo, 1992). Indeed, mice with a targeted mutation of the skeletal muscle ryanodine receptor gene lack excitation–contraction coupling (Takeshima *et al.* 1994). As a necessary step to clarifying the nature of the physiological Ca^{2+} release mechanism, it is important to study functional domains and modulator sites on the CICR channel.

Calmodulin (CaM) is a ubiquitous intracellular protein which regulates a multitude of enzymes in a Ca^{2+}-dependent manner (Cheung, 1980). It has been shown to cause inhibition of Ca^{2+} release from isolated skeletal and

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cardiac SR vesicles at concentrations of a few micromoles in a non-catalytic manner (Meissner, 1986; Meissner & Henderson, 1987). The open probability of the channels, as measured by the planar lipid bilayer technique, was also reduced by CaM (Smith, Rousseau & Meissner, 1989). In addition, several potential CaM-binding sites have been predicted in the primary sequence of the ryanodine receptors (Takeshima *et al.* 1989; Zorzato *et al.* 1990; Otsu, Willard, Khanna, Zorzato, Green & MacLennan, 1990; Nakai, Imagawa, Hakamata, Shigekawa, Takeshima & Numa, 1990; Hakamata, Nakai, Takeshima & Imoto, 1992). A recent binding study showed that there were multiple CaM-binding sites on the ryanodine receptor protein (Yang, Reedy, Bruke & Strasburg, 1994). It is therefore possible that CaM could be a physiological regulator of the Ca^{2+} release channel in intact skeletal muscle fibres, because it is present in rabbit skeletal muscle at a concentration of about 2 μM (Yagi & Yazawa, 1978). We therefore analysed the effects of CaM on CICR using chemically skinned rabbit psoas muscle fibres, in which the physiological state of the Ca^{2+} release mechanism is better preserved than in isolated SR vesicles. Our results demonstrate that CaM has both inhibiting and potentiating effects on CICR, in contrast to previous reports on SR vesicles, in which only the inhibitory effect was observed. Our results raise the possibility that CaM is an important regulatory factor of the Ca^{2+} release mechanism, not only in skeletal muscle fibres but also in other types of cells which express ryanodine receptors.

Part of this work has appeared elsewhere in abstract form (Ikemoto, Iino, Takano-Ohmuro & Endo, 1994).

METHODS

Preparation of skinned muscle fibres

Male New Zealand White rabbits weighing 2–2·5 kg were anaesthetized with 50 mg kg^{-1} sodium pentobarbitone (I.V.) and exsanguinated. Skinned fibres were prepared from psoas muscle bundles (width 2–3 mm) which had been treated with saponin (50 μg ml^{-1}) for 45 min or with β-escin (5 μM) for 10 min at room temperature in a relaxing solution (mM: ATP, 4·76; magnesium methanesulphonate ($MgMs_2$), 5·54; potassium methanesulphonate (KMs), 108·6; NaN_3, 20; Pipes, 20; EGTA, 1; pH 7·0) to permeabilize the surface membrane (Endo & Iino, 1980; Kobayashi, Kitazawa, Somlyo & Somlyo, 1989; Konishi & Watanabe, 1995). After washout of the detergent, thin bundles of two or three muscle fibres, each with a diameter of ~70 μm, were carefully dissected in the relaxing solution and tied with silk filaments at both ends to a stainless-steel wire (100 μm in width). The length of the preparation between knots was ~5 mm. The skinned fibres attached to the metal wire were inserted into a microcuvette with an internal diameter of 400 μm and a length of 32 mm.

Experimental apparatus

A microfluorometric method was used to measure CICR rates and Ca^{2+} content of the SR in skinned muscle fibres mounted in the microcuvette (Iino, 1989). The microcuvette containing the skinned fibres was placed on the stage of an epifluorescence microscope (BHS-RFK, Olympus, Tokyo, Japan). One end of the microcuvette was connected to a step-motor-controlled multi-position (16 channels) valve (ECSD-16P, Valco, Houston, TX, USA) for the selection of perfusing solution. The other end was linked to two peristaltic pumps arranged in parallel. One of the pumps (501U, Watson Marlow Ltd, Falmouth, UK) was used to suck 180 μl of solutions (three times the dead space) in 1 s to rapidly change the solution in the microcuvette. The other pump (Minipuls 2, Gilson France S.A., Villiers le Bel, France) was used to induce a slow continuous flow (1 $\mu l\ s^{-1}$) through the microcuvette. Fura-2, a fluorescent Ca^{2+} indicator (Grynkiewicz, Poenie & Tsien, 1985) was used to measure the amount of Ca^{2+} released from the SR. Fluorescence intensity of fura-2 was measured at 510 nm with alternating 340 and 380 nm excitations (400 Hz) through an objective lens (Olympus, DPlan Apo × 20 UV) using a light source and photometer system (CAM-200, Nihon Bunko Kogyo, Tokyo, Japan). Illumination spot size was 0·8 mm in diameter. The fluorescence signals at the two excitation wavelengths were electronically separated and low-pass-filtered with a 0·1 s time constant. Data collection by an analog-to-digital converter board, as well as control of the valve and pumps, were carried out using a personal computer (PC9801VM, NEC, Japan).

Experimental protocol

To measure activity of CICR in the SR, we took the following points into consideration. (1) In the presence of MgATP, released Ca^{2+} from the SR will activate the Ca^{2+} pump and partly be taken up again. Ca^{2+} release was therefore observed in the absence of MgATP. (2) Direct monitoring of Ca^{2+} concentration outside the SR using fluorescent indicators requires weakly Ca^{2+}-buffered conditions, since no Ca^{2+} signal is obtained with a strong Ca^{2+} buffer. However, under such conditions Ca^{2+} released through the CICR channels would further activate the channels. For this reason, direct cytoplasmic Ca^{2+} monitoring is not suitable for quantitative measurement of the Ca^{2+} release process (Endo & Iino, 1988). Activity of the CICR channels was therefore estimated by measurement of Ca^{2+} remaining in the SR subsequent to induction of Ca^{2+} release under strongly Ca^{2+}-buffered conditions.

An example of the experimental protocol (Endo & Iino, 1988) is shown in Fig. 1*A* and *B*. The recordings show the relative fluorescence intensity at 510 nm with 340 nm excitation. Fura-2 (35 μM) was added to the solution during the time periods indicated by the hatched horizontal bars. The microcuvette was illuminated only when fura-2 was present in the solution, to avoid unnecessary irradiation of the skinned fibres with the UV light. The protocols used were as follows. Step 1 (loading): after 60–90 s pre-incubation in the relaxing solution, the SR was loaded with Ca^{2+} at pCa 6·7 for 90 s in the presence of 4 mM MgATP. Step 2 (wash): both Ca^{2+} and MgATP were removed by flushing with a solution containing 1 mM EGTA, 1·5 mM Mg^{2+} and no ATP and perfusion with the same solution for 30 s. This procedure was repeated twice. Step 3 (test): a pretest solution which contained 1 mM EGTA was flushed and perfused for 30 s. This was repeated twice. Then a test solution of various concentrations of Ca^{2+} buffered with 10 mM EGTA was applied to induce CICR for 10–360 s. The pretest and test solutions contained no ATP, to avoid reuptake of Ca^{2+} into the SR. Application of test solution was included only in the test run (Fig. 1*B*). Mg^{2+} concentration of the test and pretest solutions was varied between 0 and 1·5 mM depending on the type of experiment. When the effect of CaM on CICR was to be tested, CaM was added to the second pretest and test solutions (filled horizontal bar in Fig. 1*B*). Then the test solution was removed by flushing and perfusion with a solution containing 10 mM EGTA and 10 mM Mg^{2+}, and no Ca^{2+} to

deactivate the CICR. Step 4 (assay): fura-2 was then introduced in a solution which contained 1 mM EGTA and 1·5 mM Mg^{2+} for 30 s. Then both EGTA and Mg^{2+} were removed by flushing and perfusion with a EGTA- and Mg^{2+}-free solution in the continued presence of fura-2 (30 s). Finally, the assay solution containing 50 mM caffeine and 22·5 mM AMP was applied in the presence of fura-2 to induce complete release of Ca^{2+} remaining in the SR and to measure its amount. Step 4 was also carried out in the absence of MgATP.

To obtain the baseline Ca^{2+} level of the assay solution, steps 2–4 were repeated without the Ca^{2+} loading. The resulting Ca^{2+} signal (a' and b' of Fig. 1) was subtracted from that of the initial assay (a and b of Fig. 1) to obtain the amount of Ca^{2+} released from the SR (Fig. 1*C*). Figure 1*C* shows typical superimposed recordings used to determine the amount of Ca^{2+} released from the SR, as measured by the ratio of the fluorescence intensity change (F_{340}/F_{380}) at 510 nm with alternating excitations at 340 (F_{340}) and 380 nm (F_{380}). The amount of Ca^{2+} released during the test procedure was estimated by comparing assays with and without the test procedure ($a - a'$ *vs.* $b - b'$ in Fig. 1*C*). Released Ca^{2+} from the SR during assays may diffuse away along the microcuvette. Although Ca^{2+} concentration at the end of the fibres may decline rapidly, that near the centre should change very slowly. Because only a 0·8 mm long portion in the centre of a 5 mm long fibre was illuminated for data collection, the decrease in the Ca^{2+} signal due to longitudinal diffusion should be minimal and was not taken into account in the analysis of data. This was supported by a calculation of diffusion of Ca^{2+}, and there is indeed no sign of rapid decline in the fluorescence signals (e.g. Fig. 1*C* and Fig. 2*B*).

The amount of Ca^{2+} remaining in the SR measured with fura-2 declined exponentially to zero with increasing duration of the test procedure (Fig. 3) in accordance with results obtained by another method, which determined the size of caffeine-induced contracture of skinned fibres (Endo & Iino, 1988). Activity of CICR was therefore expressed in terms of the decay rate constants, based on the amount of Ca^{2+} in the SR at $t = 0$ (t_0) and t_1. Duration of the test procedure (t_1) was such that the amount of remaining Ca^{2+} (A_1) was ~30–50% of the control (A_0). The rate constant (R) was determined as:

$$R = -[\ln (A_1/A_0)]/t_1.$$

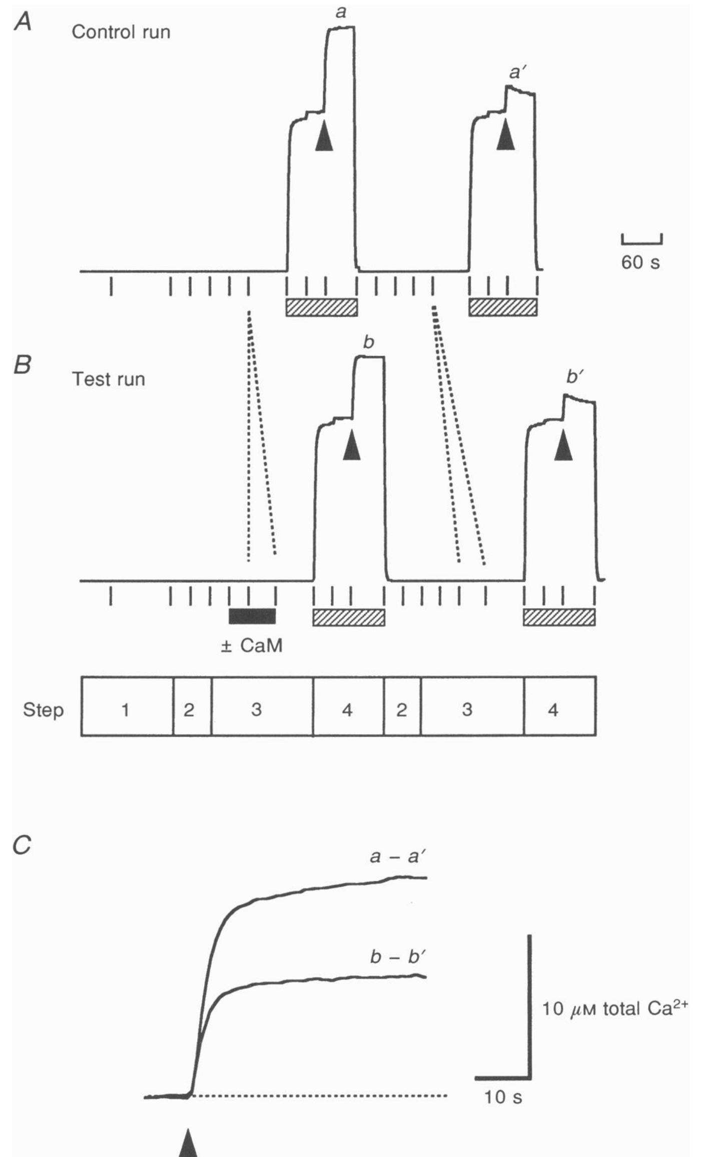

Figure 1. Typical recordings of an experiment for measuring Ca^{2+} release rate

Shown are the relative fluorescence intensities with 340 nm excitation (*A* and *B*) and the ratio of the fluorescence intensities with alternating excitations at 340 and 380 nm (*C*). Small vertical bars below the trace indicate the timing of solution changes. Fura-2 (35 μM) was added to the solutions only during assay, as indicated by the hatched bars, during which the capillary cuvette was illuminated. Assay solution was applied at time points indicated by arrowheads. *A*, control run without test procedure. *B*, test run with a 45 s application of test solution at pCa 4·5. Steps in the protocols are: 1, loading; 2, wash; 3, test; 4, assay. *C*, amount of Ca^{2+} released during assay in the control ($a - a'$) and in the test run ($b - b'$). For further explanation see text.

For measurement of the Ca^{2+} uptake capacity of the SR, the protocol in Fig. 1*A* was used. The Ca^{2+} content of the SR was estimated by the magnitude of caffeine response in the presence of fura-2 during assay ($a - a'$). At the end of each experiment, the fluorescence intensities of assay solutions containing fura-2 supplemented with either 100 μM (saturating) Ca^{2+} or 1 mM EGTA were obtained separately. The difference between these measurements was used to calibrate the increase in the total Ca^{2+} concentration within the cuvette due to Ca^{2+} release from the skinned muscle fibres (Iino, 1989). Statistical significance was tested using Student's paired *t* test. Compositions of the solutions, further details of the protocol, and experimental apparatus were as previously described (Iino, 1989). All experiments were done at room temperature (20–22 °C).

Materials

Na_2ATP was obtained from Boehringer Mannheim, saponin from ICN Pharmaceuticals Inc. (Cleveland, OH, USA), fura-2 from Molecular Probes, Inc., and EGTA from Dojindo Laboratories (Kumamoto, Japan). AMP, β,γ-methyleneadenosine-5′-trisphosphate (AMPOPCP), β-escin, and CaM from bovine brain were purchased from Sigma. Concentrations of CaM were calculated assuming 98% purity and a molecular weight of 16700. All the other chemicals were of the highest reagent grade available.

RESULTS

Effect of CaM on the Ca^{2+} release in the absence of Mg^{2+}

Figure 2*B* shows the time course of caffeine-induced Ca^{2+} release from the SR in the absence of both Mg^{2+} and ATP after Ca^{2+} loading of the SR at pCa 6·7 for 90 s. Without caffeine, Ca^{2+} was only slowly released from the SR (Fig. 2*A*). To our surprise, when CaM (1 μM) was applied in place of caffeine, it induced Ca^{2+} release from the SR to the same extent as caffeine (Fig. 2*C*), although the time course of Ca^{2+} release was slower than that of caffeine. After the Ca^{2+} release by CaM for 90 s, 50 mM caffeine did not induce further Ca^{2+} release (data not shown). These results suggest that CaM is able to enhance Ca^{2+} release from the SR under these conditions.

We therefore examined the effect of CaM (1 μM) on the rate of CICR in the absence of Mg^{2+} using the protocol described in Methods. Figure 3 shows the time course of CICR at pCa 6·0. The amount of Ca^{2+} in the SR declined with increasing duration of the test procedure in both the

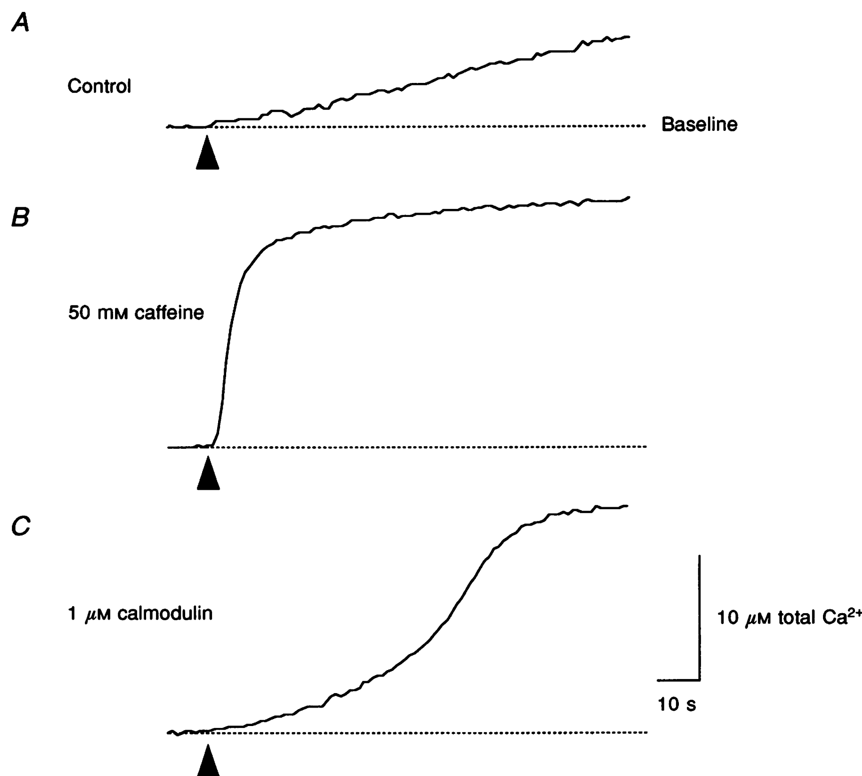

Figure 2. Caffeine- and CaM-induced Ca^{2+} release from the SR

As in protocol 1 (Fig. 1), SR was loaded with Ca^{2+} and the time course of Ca^{2+} release was subsequently measured using 35 μM fura-2 in the absence of Mg^{2+}, ATP and EGTA. Three different Ca^{2+}-releasing stimuli were tested on the same fibre. *A*, slow leakage of Ca^{2+} from the SR in the absence of Ca^{2+} release stimuli. *B* and *C*, 50 mM caffeine (*B*) or 1 μM CaM (*C*) was applied at the time point indicated by the arrowhead.

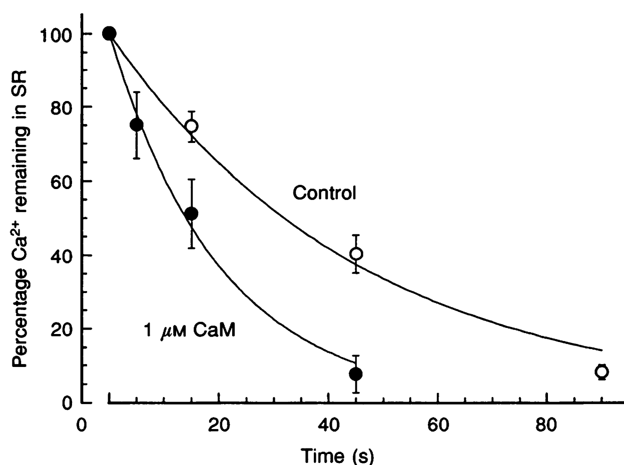

Figure 3. Effects of CaM on the time course of Ca^{2+} release from the SR in saponin-skinned fibres

Starting from a fixed amount of Ca^{2+} in the SR, fibres were treated at pCa 6·0 in the presence (●) or absence (○) of 1 μM CaM for the period of time shown on the abscissa. The Ca^{2+} remaining in the SR after the treatment was plotted on the ordinate. Means ± S.E.M. ($n = 4$). Exponential curves were fitted by a non-linear least squares method.

absence and presence of CaM (1 μM), but the Ca^{2+} release rate was significantly increased by CaM. The open and filled circles in Fig. 4 show the Ca^{2+} release rate constants at various Ca^{2+} concentrations in the control condition and in the presence of 1 μM CaM, respectively. Although CaM binds Ca^{2+}, its effect on Ca^{2+} buffering is negligible due to the presence of EGTA at a concentration four orders of magnitude greater (10 mM). The presence of CaM potentiated the Ca^{2+} release at low Ca^{2+} concentrations (pCa 7–6), while it exhibited no effect on the Ca^{2+} release at pCa 5·5. On the other hand, the presence of CaM inhibited the Ca^{2+} release at the highest Ca^{2+} concentration (pCa 4·5).

Reduction of Ca^{2+} uptake capacity of the SR after co-application of ryanodine and CaM

To clarify whether potentiation of Ca^{2+} release by CaM resulted from elevation of CICR channel activity or activation of a different Ca^{2+} pathway in the SR, we used ryanodine, which preferentially binds to the activated CICR channels and locks them in an open state. Open-lock of the CICR channels by ryanodine results in the irreversible reduction of Ca^{2+} uptake capacity of the SR (Oyamada, Iino & Endo, 1993). Therefore, if CaM is able to activate the CICR channel, simultaneous application of ryanodine and CaM should cause a reduction of the Ca^{2+} uptake capacity of the SR.

Results shown in Fig. 5 were from experiments carried out to observe the change in the Ca^{2+} uptake capacity of the SR before and after ryanodine treatment with or without CaM. The amount of Ca^{2+} loaded in the SR within 90 s at pCa 6·7 was measured three times before the ryanodine application (runs 1, 2 and 3, Fig. 5). There was only a slight decrease in the Ca^{2+} uptake capacity of the SR in these control runs. Before runs 4 and 5, when the SR was not yet loaded with Ca^{2+}, 30 μM ryanodine and/or 5 μM CaM was applied for 120 s at pCa 6·5 in the absence of ATP. When either ryanodine alone or CaM alone was applied, little decrease in Ca^{2+} uptake capacity was observed in the subsequent runs (○ and □, runs 4 and 5, Fig. 5). In contrast, after

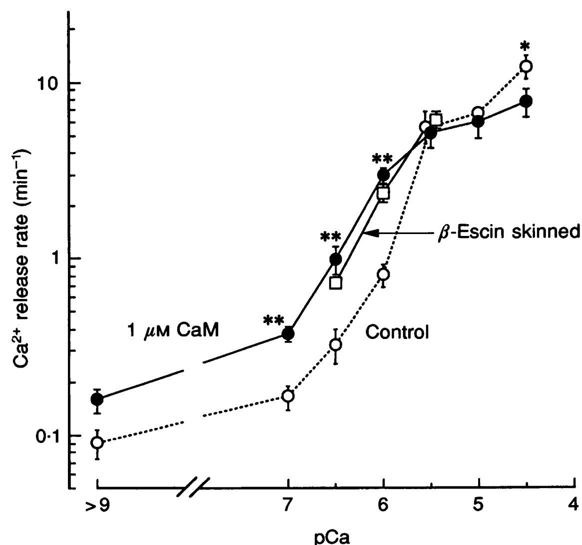

Figure 4. Effect of CaM on rates of CICR plotted against Ca^{2+} concentration in the absence of Mg^{2+}

Saponin-skinned fibres ($n = 3$–5) with (●) or without (○) 1 μM CaM. □, β-escin-skinned fibres without addition of CaM ($n = 5$). Symbols at pCa 5·5 were slightly offset horizontally for clarity. ** $P < 0{\cdot}01$; * $P < 0{\cdot}05$, means ± S.E.M.

simultaneous application of ryanodine and CaM, the Ca^{2+} uptake capacity of the SR was significantly decreased (●, runs 4 and 5, Fig. 5). Application of a lower concentration of ryanodine (5 μM) also decreased the Ca^{2+} uptake capacity only in the presence of CaM (5 μM), although the degree of reduction of Ca^{2+} content was lower than with 30 μM ryanodine ($n = 4$, data not shown). These results suggest that channel activity under the condition of pCa 6·5 was not sufficient for ryanodine to bind to the CICR channel proteins to a detectable amount within 120 s, whereas CaM elevated the CICR channel activity so that some of the channels were locked in an open state by ryanodine. These results are consistent with the idea that CaM enhances CICR channel activity.

Effects of CaM on CICR in the presence of Mg^{2+} and nucleotide

We then studied the effect of CaM in the presence of 0·5 mM Mg^{2+} (Fig. 6). In comparison with the rates of CICR in the absence of Mg^{2+} (Fig. 4 *vs.* Fig. 6, ○), control values (in the absence of CaM) of the CICR rates were much lower due to inhibition by Mg^{2+} of the Ca^{2+} release mechanism (Endo, 1985). CaM at the same concentration as in Fig. 4 also increased the rate of CICR twofold at pCa 6·0. However, the rates of CICR were significantly reduced by about 40% at the high Ca^{2+} concentrations (pCa 5·0–4·5). Note that the ordinate in Fig. 6 as well as in Fig. 4 is a logarithmic scale to show the difference at both high and low Ca^{2+} release rates. The extent of CaM-induced inhibition of CICR under these conditions (about 40% reduction) was much the same as for isolated SR vesicles (Meissner, 1986). At pCa 5·5, interestingly, the rate of CICR was not affected by CaM, as was also the case in the absence of Mg^{2+}. The CICR rate at pCa 7·0 was almost the same with or without CaM, unlike the result in the absence of Mg^{2+}.

We also carried out experiments under quasi-physiological conditions, i.e. in the presence of Mg^{2+} (1·5 mM) and an adenine nucleotide, both of which are key modulators of CICR (Endo, 1977, 1985). To avoid both reuptake of Ca^{2+} into the SR and contraction of muscle fibres, we used β,γ-methyleneadenosine-5′-trisphosphate (AMPOPCP), a non-hydrolysable ATP analogue, in place of ATP. The rate of CICR was higher with AMPOPCP (Fig. 7 *vs.* Fig. 6), being consistent with enhanced activity of the Ca^{2+} release channel in the presence of adenine nucleotides. The rate of CICR at pCa 6·0 with 2·0 mM AMPOPCP was further increased more than twofold by 1 μM CaM, whereas at pCa 5·0 with 0·2 mM AMPOPCP it was reduced by about 40% in the presence of CaM (Fig. 7). Thus, we were able to observe similar biphasic effects of CaM in the presence of the ATP analogue in addition to Mg^{2+}.

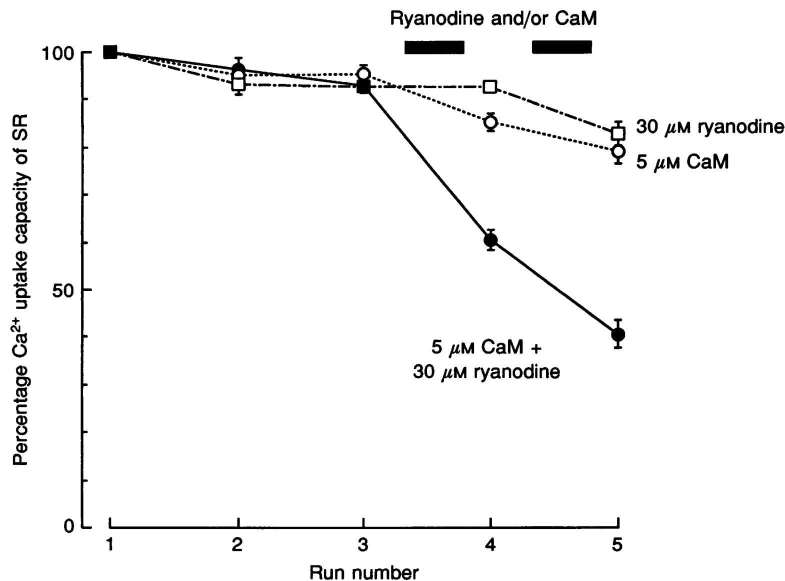

Figure 5. Reduction of the Ca^{2+} uptake capacity of the SR after co-application of ryanodine and CaM

The SR was loaded with Ca^{2+} at pCa 6·7 for 90 s and the amount of Ca^{2+} released by subsequent application of 50 mM caffeine was measured. Five successive measurements were obtained from the same preparations. The data were normalized by the magnitude of caffeine response in the first run. Between runs, muscle fibres were treated with Ca^{2+} (pCa 6·5) for 120 s in the absence of Mg^{2+} and adenine nucleotide, to which solution either CaM (5 μM) alone (○), ryanodine (30 μM) alone (□) or both ryanodine and CaM (●) were added before the fourth and fifth runs, indicated by the filled bars. Means ± S.E.M. ($n = 4$ for each symbol).

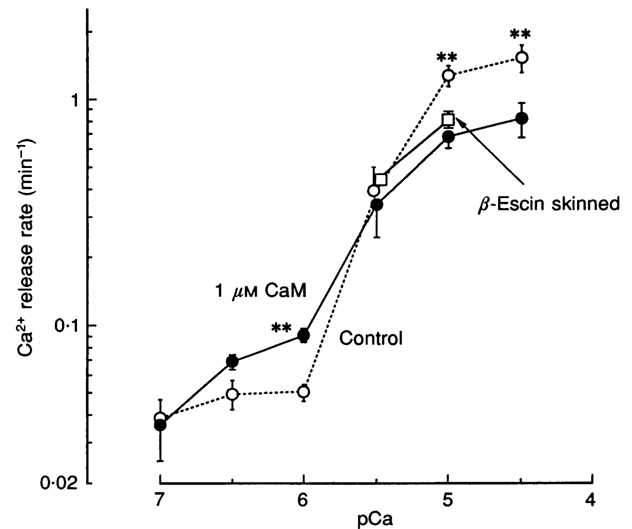

Figure 6. Effect of CaM on the rates of CICR in the presence of 0·5 mM Mg^{2+}

Saponin-skinned fibres ($n = 4$–8) with (●), or without (○) 1 μM CaM. □, β-escin-skinned fibres without addition of CaM ($n = 3$). Symbols at pCa 5·5 were slightly offset horizontally for clarity. ** $P < 0{\cdot}01$, means ± S.E.M.

Dependence of the rate of CICR on CaM concentration

Dependence of the rate of CICR on the CaM concentration was determined in the presence of Mg^{2+} at both high and low Ca^{2+} concentrations, where the respective inhibiting and potentiating effects are observed. All the data points in Fig. 8*A* and *B* were expressed as values relative to the rate of CICR in the absence of CaM. At pCa 6·0, the CICR rate was increased by CaM in a dose-dependent manner (0·2–5 μM) and the effect did not seem to level off even at 5 μM (Fig. 8*A*). On the other hand, low concentrations of CaM were sufficient for the maximum inhibitory effect at pCa 5·0 (Fig. 8*B*). Thus, the relationship between CaM concentration and the potentiating effect was different from the relationship between CaM concentration and the inhibiting effect.

Rates of CICR in β-escin-skinned muscle fibres

It is difficult to determine what proportion of intrinsic CaM is lost in saponin-skinned fibres. Thus there remains ambiguity as to whether the results with or without extrinsic CaM correspond to the behaviour of the CICR in living cells. β-Escin, one of the saponin esters, has been used to obtain permeabilized smooth muscle fibres which retain their responsiveness to receptor activation (Kobayashi *et al.* 1989). It has been shown that intracellular proteins as well as fluorescence-labelled dextran (~10 kDa) injected into the cytoplasm were retained well within the cell in β-escin (5 μM)-treated single skeletal muscle fibres (Konishi & Watanabe, 1995). We therefore permeabilized the fibres using a mild treatment of β-escin (5 μM) for 10 min, and compared the rates of CICR in β-escin-skinned muscle fibres with those in saponin-skinned fibres. As shown by the open squares in Figs 4 and 6, the rates of CICR in β-escin-skinned fibres in the absence of CaM were almost the same as those in saponin-skinned fibres with added CaM (1 μM). Furthermore, exogenously added CaM (1 μM) did not cause any change of the CICR rate in β-escin-skinned fibres (data not shown). These results suggest that intrinsic CaM was retained within β-escin-skinned skeletal muscle fibres and regulated the CICR mechanism.

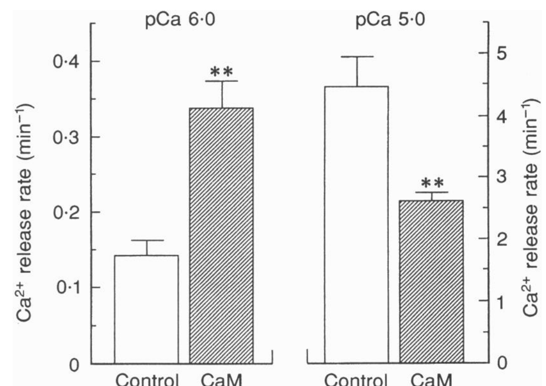

Figure 7. Effect of CaM on the rates of CICR in saponin-skinned fibres in the presence of 1·5 mM Mg^{2+} and AMPOPCP (quasi-physiological conditions)

The concentration of AMPOPCP was 2·0 mM at pCa 6·0 and 0·2 mM at pCa 5·0. ** $P < 0{\cdot}01$, means ± S.E.M. ($n = 4$). Note the difference in the scales.

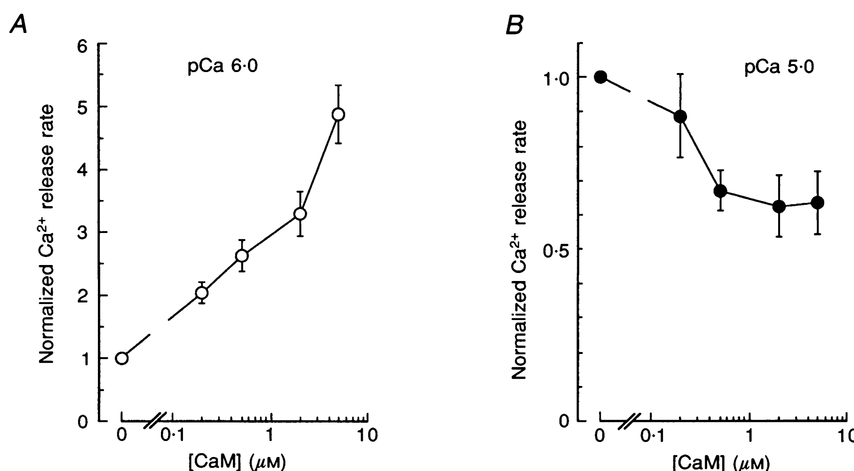

Figure 8. Dependence of the rate of CICR in saponin-skinned fibres on CaM concentration

The Ca^{2+} release rates were measured at pCa 6·0 (*A*) or pCa 5·0 (*B*) in the presence of 0·5 mM Mg^{2+} and the absence of adenine nucleotide. The results were normalized by the control value (in the absence of CaM) within each preparation. Means ± S.E.M. ($n = 4$ for *A*, $n = 4$–6 for *B*).

DISCUSSION

In this paper we have described the biphasic effect of CaM on CICR in saponin-skinned skeletal muscle fibres: at low Ca^{2+} concentrations (below 3 μM), CaM potentiated the CICR, while it inhibited the Ca^{2+} release at higher Ca^{2+} concentrations. The inhibitory effect of CaM on CICR is similar to that reported in isolated SR vesicles (Meissner, 1986) and in channels incorporated in planar lipid bilayers (Smith *et al.* 1989). Quite contrary to the previous reports, in which CaM inhibited the Ca^{2+} release at or above 1 μM Ca^{2+} concentration, we observed that the rates of Ca^{2+} release were potentiated by CaM at low Ca^{2+} concentrations in skinned muscle fibres (Figs 4 and 6).

Supporting evidence for the enhancing effect of CaM on Ca^{2+} release was provided by the direct measurement of Ca^{2+} release induced by CaM (Fig. 2). We estimated the total Ca^{2+} contamination in the assay solution to be 3·4 μM, using the technique described in Methods. Assuming the dissociation constant of fura-2 to be 220 nM (Grynkiewicz *et al.* 1985), the free Ca^{2+} concentration immediately after application of CaM in Fig. 2*C* was calculated to be about 23 nM. This is within the range of the Ca^{2+} concentration at which the enhancing effect of CaM on the Ca^{2+} release is observed (Fig. 4). Therefore, the increase in CICR rates by CaM is likely to be the cause of Ca^{2+} release observed in Fig. 2*C*. Furthermore, the reduction of the Ca^{2+} uptake capacity of the SR following co-application of ryanodine and CaM (Fig. 5) supports the hypothesis that the target of CaM is the CICR channel (the ryanodine receptor).

Since both potentiating and inhibitory effects of CaM on the CICR were observed in the absence of MgATP, the effects are unlikely to be mediated by CaM-dependent phosphorylation. In accordance with this notion, the effects of CaM on the CICR were not blocked by CaM antagonists (*N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide (W-7), chlorpromazine and CaM-dependent protein kinase II peptide fragment 290–309; T. Ikemoto & M. Iino, unpublished observation) which are known to inhibit activity of Ca^{2+}-CaM-modulated enzymes. These results suggest that CaM directly binds to the ryanodine receptor and modulates its activity.

The CaM concentration required to obtain half-maximal potentiation of CICR at low Ca^{2+} concentrations is higher than 2 μM (Fig. 8*A*). However, at high Ca^{2+} concentrations, CICR was almost maximally inhibited even at 0·5 μM CaM (Fig. 8*B*). Therefore, the CaM concentration–effect relationships are quite different for the potentiating and inhibiting effects on the CICR. These results suggest that there may be two distinct CaM-regulated sites which control the CICR in opposite ways. Alternatively, the affinity for the binding site may differ between Ca^{2+}-free CaM and Ca^{2+}-bound CaM, and the Ca^{2+} dependence of the ryanodine receptor is altered upon CaM binding. The potentiating effect of CaM on the CICR has not been reported in isolated SR vesicles or isolated ryanodine receptor proteins incorporated into planar lipid bilayers, although the effects of CaM on CICR activity has not been extensively studied at concentrations below 1 μM Ca^{2+}. In the light of the present results, it is important to reinvestigate the effects of CaM on the isolated preparations at lower Ca^{2+} concentrations.

In β-escin-skinned fibres, the rates of CICR were similar to those of saponin-skinned fibres in the presence of 1 μM CaM (Figs 4 and 6), and there was no effect of extrinsic

CaM on the Ca^{2+} release rates. These results indicate that intrinsic CaM is retained in β-escin-skinned fibres due to limited diffusion of CaM through the sarcolemma, while it is partially lost in saponin-skinned fibres, and the results obtained in the presence of extrinsic CaM better represent the characteristics of the CICR in the living cells. Therefore, the biphasic effect of exogenously applied CaM in saponin-skinned fibres is an important regulatory mechanism of CICR in intact skeletal muscle fibres.

Although it is widely accepted that the primary mechanism of Ca^{2+} release in the physiological excitation–contraction coupling in vertebrate skeletal muscle is the allosteric regulation of the Ca^{2+} release channel by the voltage sensor in the T-tubule membrane, the possibility has been raised that CICR is secondarily involved in physiological Ca^{2+} release (Ríos & Pizarro, 1991; Schneider, 1994). It has also been shown that CICR and physiological Ca^{2+} release share a common channel protein (Lamb & Stephenson, 1990; Ebashi, 1991; Endo, 1992). CaM may, therefore, have a possible regulatory effect on the excitation–contraction coupling of skeletal muscle.

It has been reported that CaM inhibits the CICR in isolated cardiac SR vesicles (Meissner & Henderson, 1987; Smith *et al.* 1989). Although the enhancing effect of CaM has not been reported in these preparations, it needs to be examined in more physiological preparations such as skinned cardiac cells. Such a study would be particularly important because the CICR is thought to be an important mechanism in the excitation–contraction coupling of cardiac muscle cells (Näbauer, Callewaert, Cleemann & Morad, 1989).

McPherson & Campbell (1993) reported that ryanodine binding to purified ryanodine receptor protein from brain was strongly inhibited by CaM at a high Ca^{2+} concentration (0·1 mM Ca^{2+}). This suggests that the Ca^{2+} release channels in brain are negatively regulated by CaM, which is abundantly expressed in the brain. Again, the present results raise the possibility that the activity of Ca^{2+} release channels may be potentiated by CaM under different conditions. It will therefore be important to study how the Ca^{2+} release channels in brain are regulated by CaM at lower, more physiological Ca^{2+} concentrations.

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