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Properties of Ca^{2+} release induced by clofibric acid from the sarcoplasmic reticulum of mouse skeletal muscle fibres

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1 To characterize the effect of clofibric acid (Clof) on the Ca^{2+} release mechanism in the sarcoplasmic reticulum (SR) of skeletal muscle, we analysed the properties of Clof-induced Ca^{2+} release under various conditions using chemically skinned skeletal muscle fibres of the mouse.

2 Clof (>0.5 mM) released Ca^{2+} from the SR under Ca^{2+}-free conditions buffered with 10 mM EGTA (pCa >8).

3 Co-application of ryanodine and Clof at pCa >8 but not ryanodine alone reduced the Ca^{2+} uptake capacity of the SR. Thus, Ca^{2+} release induced by Clof at pCa >8 must be a result of the activation of the ryanodine receptor (RyR).

4 At pCa >8, (i) Clof-induced Ca^{2+} release was inhibited by adenosine monophosphate (AMP), (ii) the inhibitory effect of Mg^{2+} on the Clof-induced Ca^{2+} release was saturated at about 1 mM, and (iii) Clof-induced Ca^{2+} release was not inhibited by procaine (10 mM). These results indicate that Clof may activate the RyR-Ca^{2+} release channels in a manner different from Ca^{2+}-induced Ca^{2+} release (CICR).

5 In addition to this unique mode of opening, Clof also enhanced the CICR mode of opening of RyR-Ca^{2+} release channels.

6 Apart from CICR, a high concentration of Ca^{2+} might also enhance the unique mode of opening by Clof.

7 These results suggest that some features of Ca^{2+} release activated by Clof are similar to those of physiological Ca^{2+} release (PCR) in living muscle cells and raise the possibility that Clof may be useful in elucidating the mechanism of PCR in skeletal muscle.

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Abbreviations: CICR, Ca^{2+}-induced Ca^{2+} release; Clof, clofibric acid; PCR, physiological Ca^{2+} release; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

Introduction

Control of the intracellular Ca^{2+} concentration is an important mechanism of regulation of function in a wide variety of cells. Ryanodine receptor (RyR)-mediated Ca^{2+} release is one of the most important mechanisms in the regulation of cytosolic Ca^{2+} concentrations (Berridge, 1993). Ca^{2+}-induced Ca^{2+} release (CICR), a release mechanism from the sarcoplasmic reticulum (SR) extensively studied in skinned fibres (Endo, 1977; 1985) and in isolated SR vesicles (Meissner, 1984; Meissner *et al*., 1986; Smith *et al*., 1986), has been shown to be a function of the RyR (Imagawa *et al*., 1987; Lai *et al*., 1988; Hymel *et al*., 1988; Smith *et al*., 1988).

In skeletal muscle fibres, however, there are several lines of evidence indicating that physiological Ca^{2+} release (PCR) from the SR is not mediated by CICR (Endo, 1985), but is directly controlled by the voltage sensors in the transverse tubule membrane. The voltage sensors transmit the action potential signals to the Ca^{2+}-release channels on the SR membrane (Schneider & Chandler, 1973; Ríos & Pizarro, 1991; Schneider, 1994). It is generally believed that a common Ca^{2+}-release channel is utilized for both PCR and CICR (Lamb & Stephenson, 1990; Endo, 1992). Indeed, mice with a targeted mutation of the skeletal muscle RyR (RyR1) gene lack excitation-contraction coupling (Takeshima *et al*., 1994). Thus, RyR1 apparently functions at least in two opening modes, CICR and PCR.

Various activators and inhibitors of CICR have been reported, including well-known agents such as caffeine, adenine nucleotides, and procaine (Endo, 1985), as well as many new ones (Xu *et al*., 1998), including 4-chloro-*m*-cresol (Zorzato *et al*., 1993; Herrmann-Frank *et al*., 1996) and amentoflavone (Suzuki *et al*., 1999). However, no agents have been reported to activate the RyR1 by a mode other than CICR.

Clofibric acid (Clof) was reported to increase the probability of opening of the SR Ca^{2+}-release channel incorporated into lipid bilayers, with a complex effect on the function of SR (Sukhareva *et al*., 1994). In the present study, we analysed the effect of Clof on Ca^{2+} release in skinned skeletal muscle fibres. Our results demonstrate for the first time that Clof is an agent capable of causing Ca^{2+} release from the SR *via* the RyR in a mode quite different from that of CICR.

Methods

Preparation of skinned muscle fibres

Male mice (ICR, 5–9 weeks) were anaesthetized with 50–80 mg kg^{-1} sodium pentobarbital (i.p.) and killed by

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decapitation. Skinned fibres were prepared from extensor digitorum longus muscle bundles (EDL, width 2–3 mm) that were treated with saponin (50 μg ml^{-1}) for 45 min in a relaxing solution (in mM: ATP, 4.76; Mg methanesulfonate [$MgMs_2$] 5.54; potassium methanesulfonate [KMs] 108.6; NaN_3 20; piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) [PIPES] 20; and EGTA 1; pH 7.0) to permeabilize the surface membrane (Endo & Iino, 1980; Kobayashi *et al*., 1989). After washout of saponin, thin bundles of three or four fibres were carefully dissected in the relaxing solution and were tied with silk filaments at both ends to a stainless steel wire (100 μm in width). 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.

Measurement of Ca^{2+} release in skinned fibres

The Ca^{2+} released from the SR of the skinned fibres was quantified using Fura-2 (Grynkiewicz *et al*., 1985). Details of the method are described elsewhere (Iino, 1989; Ikemoto *et al*., 1995; 1996). Briefly, thin bundles fixed in a capillary cuvette were mounted on the stage of a microscope (BHS-RFK, Olympus, Tokyo, Japan) equipped with a fluorometer (CAM-200, Nihon Bunko Kogyo, Tokyo, Japan) for the measurement of the fluorescence intensity of Fura-2 at 510 nm with alternating 340 and 380 nm excitations.

The SR of skinned fibres was actively loaded with Ca^{2+} for 60 s in a loading solution (pCa 6.7 and 4 mM MgATP), and then the external solution was changed to a Ca^{2+}-free (pCa >8) or Ca^{2+} solution buffered with 10 mM EGTA (pCa 7.0–4.5, test procedure) for induction of Ca^{2+} release in the absence of ATP for 4–270 s. A test solution containing Clof or other modulators was then applied for a certain period of time to cause Ca^{2+} release. After washout of the test solution, the external solution was replaced with an assay solution containing 50 mM caffeine, 25 mM AMP, and 35 μM Fura-2 to induce complete release of the Ca^{2+} remaining in the SR and to measure its amount (Endo & Iino, 1988). In this way, the Ca^{2+} remaining in the SR after treatment with a test solution can be determined from the fluorescence intensity change of Fura-2 (Iino, 1989; Ikemoto *et al*., 1995; 1996). The time course of the amount of Ca^{2+} remaining in the SR was adequately described by a single exponential fall with increasing duration of application of a test solution with or without Clof (Ikemoto *et al*., 1995; 1996). Therefore, the activity of Ca^{2+} release was expressed in terms of the decay rate constants based on the amount of Ca^{2+} remaining in the SR (Iino, 1989; Ikemoto *et al*., 1995; 1996).

For the direct measurement of Ca^{2+} release in response to Clof or caffeine, as shown in Figure 1, the fluorescence intensity change of 35 μM Fura-2 after constant Ca^{2+} loading was continuously observed in the absence of Mg^{2+}, ATP, and EGTA and in the presence or absence of Ca^{2+}-releasing stimuli (Ikemoto *et al*., 1995).

For measurement of the Ca^{2+}-uptake capacity of the SR, the Ca^{2+} content of the SR was also estimated by the magnitude of the response to caffeine in the presence of Fura-2 after a standard loading procedure but without the test procedure mentioned above (Ikemoto *et al*., 1995). All experiments were carried out at room temperature (21–23°C). Statistical

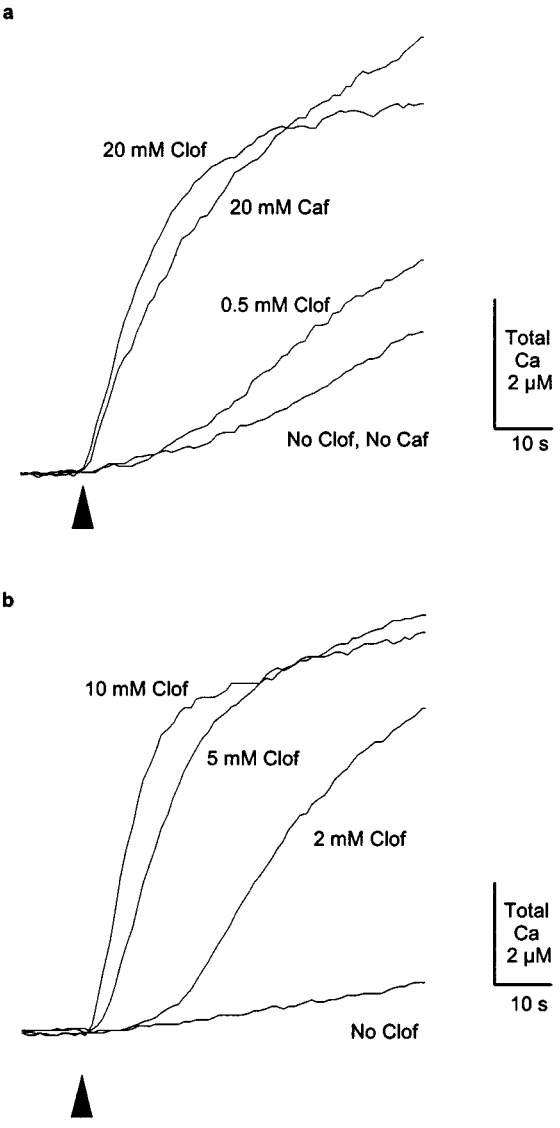

Figure 1 Clofibric acid- and caffeine-induced Ca^{2+} release from the SR. The SR was loaded with Ca^{2+}, and the time course of Ca^{2+} release was subsequently measured using 35 μM Fura-2 in the absence of Mg^{2+}, ATP, and EGTA. (a) Release of Ca^{2+} from the SR in the absence and presence of Ca^{2+}-releasing stimuli. Either clofibric acid (Clof, 0.5 and 20 mM) or 20 mM caffeine (Caf) was applied at the time indicated by the arrowhead. (b) Clof (2–10 mM) was applied at the time indicated by the arrowhead. The data in each panel were obtained from different preparations.

significance was tested by analysis of variance followed by Fisher's protected least significant difference (PLSD) or by the paired *t*-test for comparisons between data with and without drug in the same preparation (Figures 6, 8c, and 9).

Experimental solutions

The basic composition of all experimental solutions used in this study, including the test solutions and other solutions, was as previously described (Iino, 1989). Solutions of various pCa's ($\leqslant 8$) were obtained by mixing a 10 mM EGTA-containing and a 10 mM CaEGTA-containing solution. The pH of these solutions was adjusted to 7.0 with KOH. Clof was dissolved directly in these solutions up to 40 mM by

neutralizing with KOH. Thus, the concentration of K^+ in Clof-containing solutions was higher than those without Clof, and their ionic strength was higher. In order to exclude the possibilities that rather high concentrations of Clof might appreciably bind divalent cations and that a higher ionic strength or K^+ concentration in the Clof-containing solutions might affect the divalent ion concentrations, the free Mg^{2+} and the free Ca^{2+} concentrations in the presence of Clof were measured by the fluorescence change of Mag-Fura-2 (Raju *et al*., 1989) and of Fura-2, respectively. The presence of 10 mM Clof did not alter the free Ca^{2+} concentration, except for a possible small change at pCa 5. Nor did the presence of up to 20 mM Clof appreciably alter the free Mg^{2+} concentration. Since the possibilities mentioned above are thus excluded, the effects of addition of Clof may be regarded as the genuine effect of the agent.

Materials

Na_2ATP was obtained from Boehringer Mannheim (FRG), saponin from ICN Pharmaceuticals Inc. (U.S.A.), Fura-2 and Mag-Fura-2 from Molecular Probes, Inc. (U.S.A.), and EGTA from Dojindo Laboratories (Japan). AMP, *p*-aminobenzoic acid diethylaminoethyl ester (procaine, Proc) hydrochloride, and 2-(*p*-chlorophenoxy)-2-methylpropionic acid (clofibric acid, Clof) were purchased from Sigma Chemical Co. (U.S.A.). All other chemicals were of the highest reagent grade available.

Results

Effect of clofibric acid on Ca^{2+} release

After Ca^{2+} loading of the SR, Clof (0.5–20 mM) induced Ca^{2+} release from the SR in the absence of Mg^{2+}, ATP, and EGTA. The rate of release at 20 mM was comparable to that of caffeine at the same concentration (Caf, 20 mM), (Figure 1). Without caffeine or Clof, Ca^{2+} was only slowly released from the SR (Figure 1a,b). After Ca^{2+} release by Clof (>10 mM) for 90 s, 50 mM caffeine did not induce further Ca^{2+} release (data not shown). These results indicate that, under these conditions, Clof is able to induce Ca^{2+} release from the same Ca^{2+} store in the SR that is influenced by caffeine.

Direct monitoring of the Ca^{2+} concentration outside the SR, as shown in Figure 1, is not suitable for the quantitative measurement of the Ca^{2+} release rate, because the CICR channels would be further activated at the later times by the released Ca^{2+} under such weakly buffered conditions (Endo & Iino, 1988). We therefore examined the effect of Clof (10 mM) on the rate of Ca^{2+} release using the protocol described in Methods. Figure 2 shows the time course of Ca^{2+} release at pCa >8 (10 mM EGTA and no added Ca^{2+}). The amount of Ca^{2+} in the SR decreased by about 30% in 120 s due to leakage of Ca^{2+} from the SR (Figure 2a, open circles). On the other hand, 10 mM Clof strongly potentiated the release of Ca^{2+} from the SR (Figure 2a, filled circles). The Ca^{2+} release rate was significantly and dose-dependently increased by Clof (Figure 2b). Mg^{2+} at 0.5 mM significantly suppressed Clof-induced Ca^{2+} release at all Clof concentrations as well as in the absence of Clof (Figure 2b).

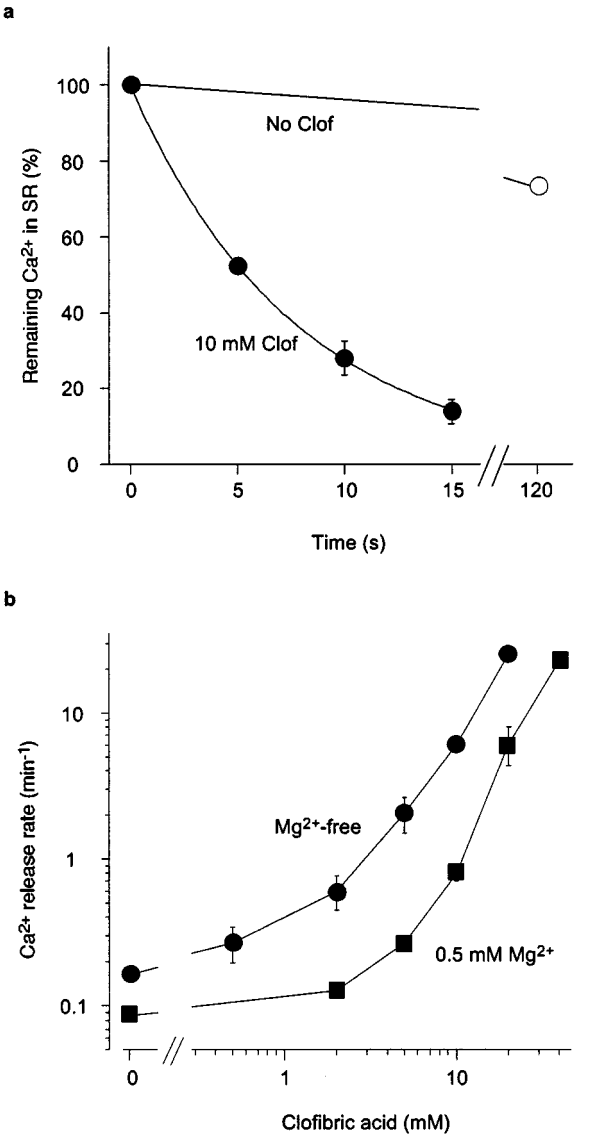

Figure 2 Ca^{2+}-releasing effect of Clof in the absence of Ca^{2+} and its inhibition by Mg^{2+}. (a) With a fixed amount of Ca^{2+} in the SR at the start, fibres were treated in the absence of Mg^{2+} with 10 mM EGTA (no added Ca^{2+}) in the presence or absence of 10 mM Clof for the period of time shown on the abscissa. The Ca^{2+} remaining in the SR after the treatment was plotted. An exponential curve was fitted by a nonlinear least square method ($n=3$). (b) The Ca^{2+} release rates were measured at pCa >8 and plotted against the Clof concentrations applied in the absence or presence of 0.5 mM Mg^{2+} (mean ± s.e.mean, $n=3-9$). Error bars smaller than the symbols are not shown.

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

A question then arises as to whether the Ca^{2+} release induced by Clof is through the RyR (CICR) channel or through a different Ca^2-release pathway in the SR. We used ryanodine to resolve this question. Ryanodine preferentially binds to activated RyR channels and locks them in an open state. An open-lock of the RyR channels by ryanodine results in the irreversible reduction of the Ca^{2+}-uptake capacity of the SR (Oyamada *et al*., 1993, Ikemoto *et al*., 1995). Therefore, if

Clof activates the RyR, simultaneous application of ryanodine and Clof should cause a reduction of the Ca^{2+}-uptake capacity of the SR.

The effect of ryanodine treatment with or without Clof (10 mM) on the Ca^{2+}-uptake capacity of the SR was examined (Figure 3). First, the amount of Ca^{2+} loaded in the SR for 60 s at pCa 6.7 in the presence of ATP was measured (100%). Before the next measurement, ryanodine was applied for 90 s in the absence of ATP at either pCa >8, 6.5, or 5.5. After washout of ryanodine, the amount of Ca^{2+} loaded in the SR under the same condition as the first measurement was measured in the same fibres. The Ca^{2+}-uptake capacity in the second measurement changed very little after ryanodine treatment at pCa >8.0, whereas it was significantly reduced at pCa 6.5 and 5.5 (Figure 3, open columns). Without ryanodine, the Ca^{2+}-uptake capacity in the second measurement was not significantly reduced, even at pCa 6.5 or 5.5 (data not shown), as in agreement with a previous report (Ikemoto *et al*., 1995). Thus, the effect of ryanodine treatment on the Ca^{2+}-uptake capacity was enhanced in a Ca^{2+}-dependent manner. These results suggest that the channel activity under the Ca^{2+}-free condition (pCa >8) was not sufficient for ryanodine to bind to the RyR to a detectable extent within 90 s, while the Ca^{2+}-release channel was activated in a Ca^{2+}-dependent manner. However, after treatment with ryanodine and with 10 mM Clof, even under the Ca^{2+}-free condition, the Ca^{2+}-uptake capacity was significantly reduced (Figure 3, hatched column), indicating that Clof activated the RyR, and it was the cause of the Ca^{2+} release from the SR in the absence of Ca^{2+}. These results are consistent with the result using the lipid bilayer method in which Clof increased the open channel probability of the RyR (Sukhareva *et al*., 1994).

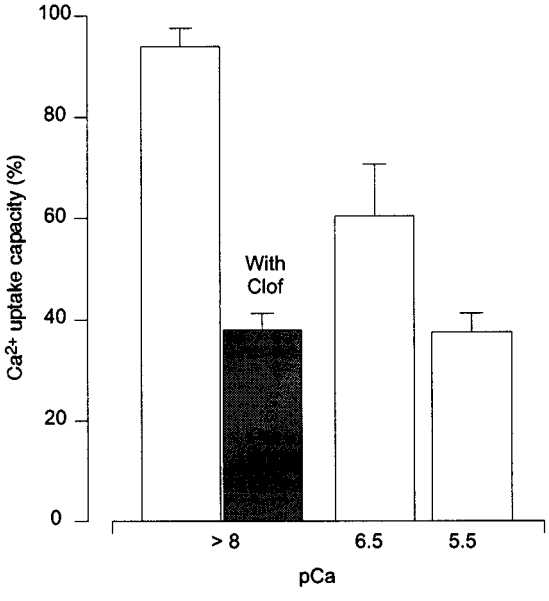

Figure 3 Effect of Clof on the open-lock activity of ryanodine on the SR. The SR was loaded with Ca^{2+} using the constant procedure, and the amount of Ca^{2+} released by the subsequent application of 50 mM caffeine was measured. Two successive measurements were made from the same preparations. The data were normalized by the magnitude of the caffeine response in the first run. Between the runs, muscle fibres were treated with Ca^{2+} (pCa >8, 6.5, 5.5) for 90 s in the absence of Mg^{2+} and adenine nucleotide, to which solution either ryanodine (30 μM) alone (open column) or both ryanodine and 10 mM Clof (hatched column) were added (mean ± s.e.mean, $n=3-4$).

Different effect of modulators of CICR on Clof-induced Ca^{2+} release

As shown in the previous sections, Clof can cause intensive Ca^{2+} release through RyR channels in the absence of Ca^{2+}. This might suggest that Clof-induced opening of RyR channels is achieved by a mode different from that of CICR. The fact that some modulators of CICR affect Clof-induced Ca^{2+} release in a quite different manner as described below supports this notion.

Effect of AMP ATP and other adenine compounds are well-known potentiators of CICR, and this was confirmed in our preparations as well. We used AMP, which strongly increased rates of Ca^{2+} release induced by 20 mM caffeine in the absence of Ca^{2+} and Mg^{2+} (Figure 4, open squares). However, as shown in the same figure, AMP clearly decreased rather than increased the rates of Ca^{2+} release induced by 20 mM (open circles) and 10 mM Clof (filled circles). A similar inhibitory effect was observed with ATP (data not shown).

The rate of Ca^{2+} release with 20 mM caffeine in the absence of AMP in Figure 4 (0.9 min^{-1}) is much lower than that reported previously under a similar condition (0.9 s^{-1}) (Rousseau *et al*., 1988). The reason for the difference is not known at present, but it may well reflect the different preparations used – skinned fibres in the present study and isolated SR vesicles in the previous report.

Effect of Mg^{2+} It is well known that Mg^{2+} inhibits CICR. As shown in Figure 2b, Clof-induced Ca^{2+} release is also

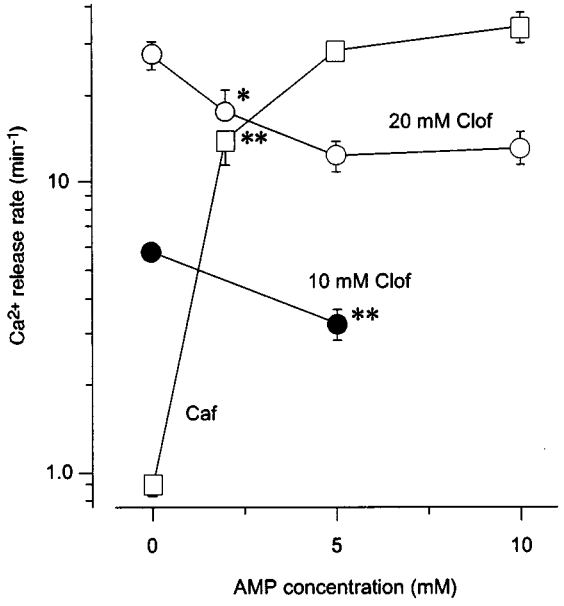

Figure 4 Effect of AMP on Clof- or caffeine-induced Ca^{2+} release. Dependence on AMP (0–10 mM) of the rate of Ca^{2+} release was determined with 20 mM and 10 mM Clof or caffeine (Caf) at pCa >8 under Mg^{2+}-free conditions (mean ± s.e.mean, $n=4-5$ for 20 mM Clof, $n=3$ for 10 mM Clof, $n=3-4$ for Caf). Error bars smaller than the symbols are not shown. $^*P<0.05$, $^{**}P<0.01$ (vs no AMP in each line).

inhibited by Mg^{2+}. However, we found that the concentration dependence of the inhibitory effect of Mg^{2+} on Clof-induced Ca^{2+} release is quite different from that on CICR. The inhibitory effect of Mg^{2+} on Clof-induced Ca^{2+} release saturates at about 1 mM, whereas that on caffeine-induced Ca^{2+} release increases continuously up to 10 mM (Figure 5, open circles vs open squares). One might argue that the difference in the inhibitory effect of Mg^{2+} may be related to the difference in Ca^{2+} concentration between Clof-induced Ca^{2+} release (pCa >8) and caffeine-induced Ca^{2+} release (pCa 6), especially since one of the mechanisms of inhibition by Mg^{2+} is competition with Ca^{2+} at the activation site. However, even if caffeine-induced Ca^{2+} release was examined at pCa >8 (in this case 20 mM AMP was added to the caffeine-containing medium in place of Ca^{2+}, because in the absence of Ca^{2+}, the rate of caffeine-induced Ca^{2+} release was too low to examine the effect of inhibitors), 10 mM Mg^{2+} still exerted a significantly stronger inhibitory effect than 1 mM Mg^{2+} (data not shown). Considering the fact that there is no appreciable alteration in Mg^{2+} concentration in Clof-containing solutions as described in Methods, one must conclude that the difference in Mg^{2+} concentration dependence described above between Clof-induced and caffeine-induced Ca^{2+} release is genuine.

Effect of procaine Procaine is a well-known inhibitor of CICR, and we could also demonstrate an inhibitory effect with procaine. Caffeine-induced Ca^{2+} release was clearly inhibited by procaine in the absence of Ca^{2+}, Mg^{2+}, and AMP (Figure 6b). However, Clof-induced Ca^{2+} release under similar conditions was not inhibited by the same concentration of procaine (Figure 6a).

Effect of Clof on CICR

The experiments with Clof described thus far were all conducted in the practical absence of Ca^{2+}. We also examined the effect of Clof in the presence of various concentrations of Ca^{2+}, and compared the results with those

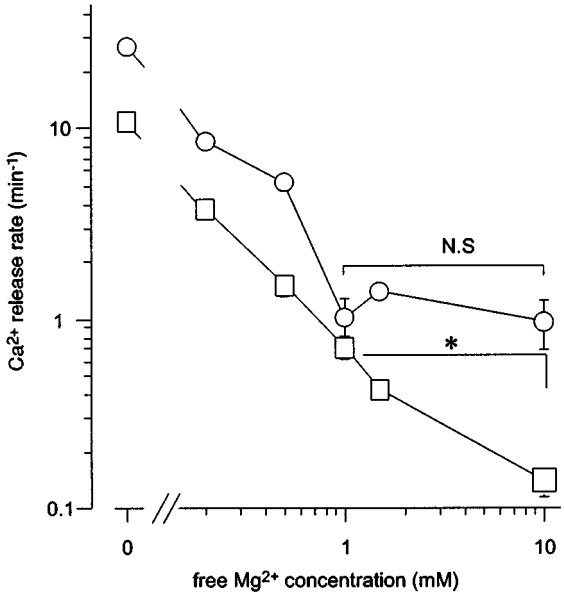

Figure 5 Effect of Mg^{2+} on Clof- or caffeine-induced Ca^{2+} release. Dependence on Mg^{2+} (0–10 mM) of the rate of Ca^{2+} release was determined with 20 mM Clof at pCa >8 or with caffeine (Caf) at pCa 6.0 in the absence of AMP. The concentration of total Mg (added as $MgMs_2$) in the 0.2, 0.5, 1.0, 1.5, and 10 mM free Mg^{2+} test solutions at pCa >8 (with Clof) were 0.27, 0.67, 1.33, 1.99, and 12.55 mM, respectively. The concentration of total Mg in the 0.2, 0.5, 1.0, 1.5, and 10 mM free Mg^{2+} test solutions at pCa 6.0 (with caffeine) were 0.22, 0.55, 1.10, 1.64, and 10.88 mM, respectively. Caffeine was used at pCa 6.0 because it could not cause an appreciable Ca^{2+} release at pCa >8.0 (mean$\pm$s.e.mean, $n=5$). *$P<0.05$. N.S., not significant. Error bars smaller than the symbols are not shown.

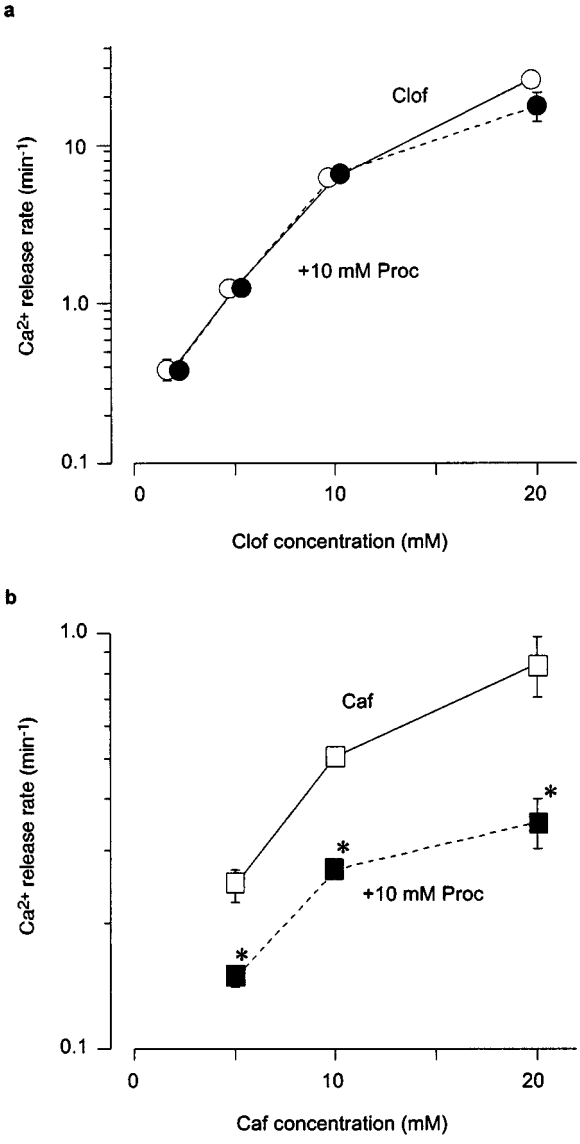

Figure 6 Effect of procaine on Clof- or caffeine-induced Ca^{2+} release. Under Mg^{2+}-, AMP-, and Ca^{2+}-free conditions (pCa >8), the rate of Ca^{2+} release with 2–20 mM Clof (a) or 5–20 mM Caf (b) was measured in the presence or absence of 10 mM procaine (Proc) (mean$\pm$s.e.mean, $n=3-5$ for Clof, $n=3$ for Caf). The paired data with procaine or without procaine were obtained from the same preparation at each Clof or Caf concentration. *$P<0.05$. Symbols at 2–10 mM Clof were slightly offset horizontally for clarity. Error bars smaller than the symbols are not shown. Note the difference in the scales.

of caffeine. Experiments were conducted in the presence of 0.5 mM Mg^{2+}. The presence of Clof (10 mM) potentiated the Ca^{2+} release at all Ca^{2+} concentrations tested (pCa >8, pCa 7–4.5, Figure 7a, open triangles vs filled circles). The rate of Ca^{2+} release by Clof at higher Ca^{2+} concentrations (7.15 min^{-1} at pCa 5.0, filled circles) is very much greater than the sum of the Ca^{2+} release rate by Clof at pCa >8 (0.78 min^{-1} at pCa >8, filled circles) and that by Ca^{2+} alone (0.59 min^{-1} at pCa 5.0, open triangles). This indicates that the model of independent Ca^{2+}-release channels for Clof-induced Ca^{2+} release and for CICR should be abandoned. These results suggest that Clof potentiates CICR. The presence of caffeine (10 mM) also potentiated the Ca^{2+} release at low Ca^{2+} concentrations (pCa 6.5), while it exhibited no effect in the absence of Ca^{2+} (pCa >8) or at the high Ca^{2+} concentrations (pCa 5.0 and 4.5, Figure 7a, open circles vs open triangles).

We also carried out similar experiments in the presence of AMP. AMP itself potentiated Ca^{2+} release only at higher Ca^{2+} concentrations (Figure 7a, open triangles vs Figure 7b, filled triangles), which is in marked contrast to that of caffeine, which increased the calcium sensitivity of CICR (Figure 7a, open triangles vs open circles; Endo, 1977; 1985). Co-application of caffeine and AMP significantly increased the rate of Ca^{2+} release at pCa <7 (Figure 8b, open circles). On the other hand, the rate of Ca^{2+} release with Clof at low Ca^{2+} concentrations (pCa >5.5) was significantly reduced in the presence of AMP, while it was increased at the higher Ca^{2+} concentrations (pCa <5.0, filled circles, Figure 7a vs b). The inhibitory effect of AMP on Clof-induced Ca^{2+} release at the low Ca^{2+} concentrations is consistent with the results shown in Figure 4. The potentiating effect of AMP on Clof-induced Ca^{2+} release at the high Ca^{2+} concentrations supports the notion proposed in the previous paragraph that Clof also potentiates CICR. Indeed, unlike at low Ca^{2+} concentrations, modulators of CICR now exert their usual effects on Clof-induced Ca^{2+} release at high Ca^{2+} concentrations. Thus, AMP dose-dependently enhanced Clof-induced Ca^{2+} release (Figure 8a), in accordance with the result of Figure 7. The inhibition by Mg^{2+} is not saturated at 1 mM, as much stronger inhibition was obtained at 10 mM (Figure 8b). Furthermore, 10 mM procaine now clearly inhibits Clof-induced Ca^{2+} release (Figure 8c).

Effect of high concentrations of Ca^{2+} on Clof-induced Ca^{2+} release independent of CICR

Finally, we examined the effect of various concentrations of Ca^{2+} on Clof-induced Ca^{2+} release under maximum inhibition of CICR. For this purpose, we used 10 mM Mg^{2+} together with 10 mM procaine (Figure 9). Under this condition, quite contrary to the results at low Mg^{2+} concentrations (open triangles in Figure 7a), the rates of Ca^{2+} release without Clof were decreased at pCa <6 (Figure 9, open squares). The results with 10 mM Mg^{2+} alone were much the same (data not shown). Thus, CICR activity was strongly inhibited in the presence of 10 mM Mg^{2+}. However, Clof still caused a greater increase in the rate of Ca^{2+} release at pCa 5.0 than at high pCas (Figure 9, filled squares). These results suggest that a high concentration of Ca^{2+} might also enhance Clof-induced Ca^{2+} release independent of CICR, although it is difficult to exclude completely the possibility that some CICR activity still remained under this condition.

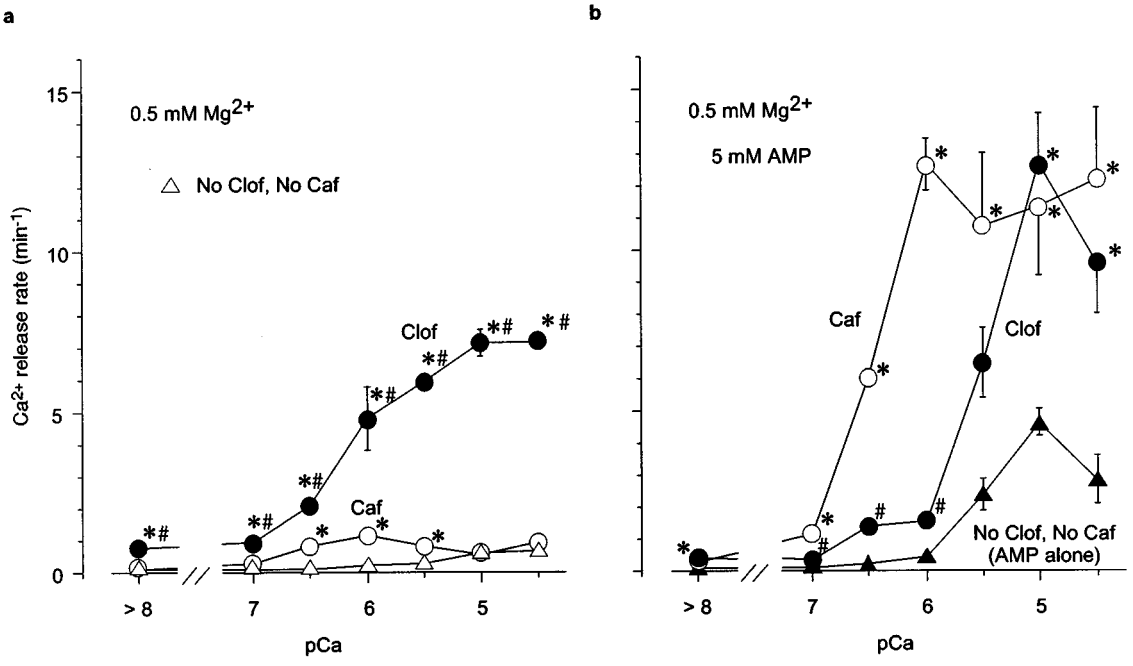

Figure 7 Effect of Clof on the rates of Ca^{2+} release under various Ca^{2+} concentrations. In the presence of 0.5 mM Mg^{2+}, the rate of Ca^{2+} release was measured at a range of pCa >8–4.5 with 10 mM Clof, 10 mM Caf, or without both agents (CICR activity without any agents), in the absence (a) or presence of 5 mM AMP (b). The data with AMP alone are shown in b (mean ± s.e.mean, $n=3-9$ for each panel). *$P<0.05$ (vs control or AMP alone), #$P<0.05$ (vs Caf). Error bars smaller than the symbols are not shown.

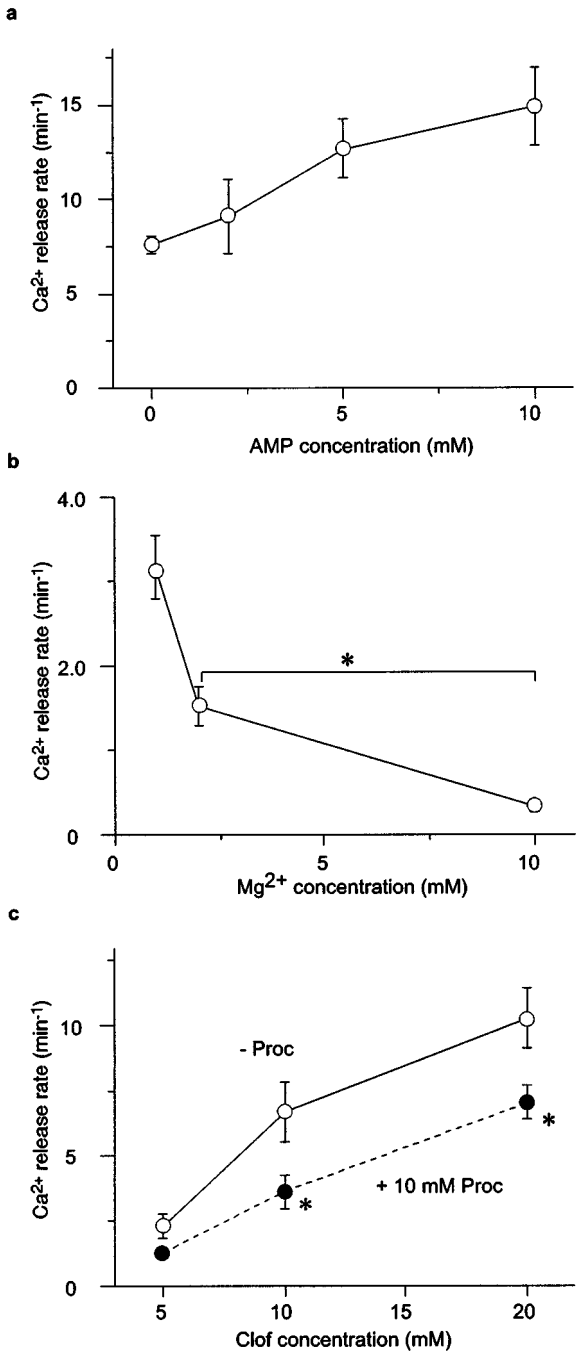

Figure 8 Effects of modulators of CICR on Clof-induced Ca^{2+} release at a high Ca^{2+} concentration. The rates of Ca^{2+} release with Clof (20 mM in a and b, 5–20 mM in c) were measured in the presence of AMP (0–10 mM, a, $n=3$), Mg^{2+} (1–10 mM, b, $n=3$), or 10 mM procaine (Proc, c, $n=4$) at pCa 5.0. The data in a and c were obtained at 0.5 mM Mg^{2+}. The experiments in b and c were carried out under AMP-free conditions (mean ± s.e.mean). In c, the paired data with procaine or without procaine were obtained from the same preparation at each Clof concentration. $^{*}P<0.05$. Note the difference in the scales.

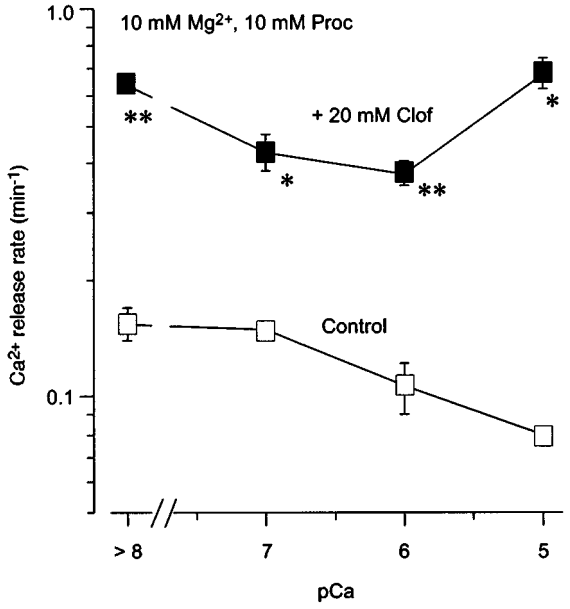

Figure 9 Enhancing effect of Clof on Ca^{2+} release in the presence of a high concentration of Mg^{2+} and procaine. The rates of Ca^{2+} release with or without 20 mM Clof were measured in the presence of 10 mM Mg^{2+} and 10 mM procaine (Proc) (mean ± s.e.mean, $n=3$). The paired data with or without Clof were obtained from the same preparation at each Ca^{2+} concentration. $^{*}P<0.05$, $^{**}P<0.01$.

Discussion

Unique mode of opening of RyR by Clof

In this study, we described the unique effects of Clof on the Ca^{2+} release in saponin-skinned skeletal muscle fibres. There were several major differences between CICR and Clof-induced Ca^{2+} release. First, Clof strongly increased the rate of Ca^{2+} release in the absence of Ca^{2+}. Second, AMP, an adenine nucleotide, inhibited the Clof-induced Ca^{2+} release, quite contrary to its effect on CICR. Third, the inhibitory effect of Mg^{2+} was saturated at about 1 mM, again contrary to its effect on CICR. Finally, procaine, a CICR inhibitor, failed to inhibit the Clof-induced Ca^{2+} release. This unique mode of Ca^{2+} release by Clof is a result of opening of the RyR-Ca^{2+}-release channel, because ryanodine applied together with Clof at pCa >8 could lock the Ca^{2+}-release channel in an open state, thereby bringing about the reduction of the Ca^{2+}-uptake capacity of the SR (Figure 3).

Activation of CICR by Clof

Apart from the unique mode of opening, the Ca^{2+}-dependent increase of the rate of Clof-induced Ca^{2+} release (Figure 7a) suggested that Clof also has an enhancing effect on CICR, and this was supported by the facts that, unlike in the absence of Ca^{2+}, at higher Ca^{2+} concentrations modulators of CICR exert their usual effects on Clof-induced Ca^{2+} release: it is enhanced by AMP, more strongly inhibited by 10 mM Mg^{2+} than by 1 mM, and inhibited by procaine (Figure 8). The enhancing effect of Clof on CICR is similar to that of adenine compounds in that, unlike the effect of caffeine, the Ca^{2+} sensitivity of CICR was not altered, so that the shape of the curve plotted against Ca^{2+} concentrations in the range of pCa <5.5 is similar with and without Clof (Figure 7).

Thus, Clof exerts enhancing effects on both CICR and some other activating mode of RyR simultaneously. The dual effects of Clof in the presence of Ca^{2+} can explain the discrepancy that the effects of Clof seem to level off even at

Table 1 Comparison of Clof-induced Ca^{2+} release with PCR and CICR

Mode of opening	*Stimulus to cause Ca^{2+} release*	*Ca^{2+} requirement*	*Adenine compounds potentiation*	*Ryanodine open-fixation*	*Mg^{2+} inhibition 10 mM >1 mM*	*Procaine*
PCR	Depolarization of T-tubule	−	− ? + ?	+	+	− ? + ?
Clof-induced* Ca^{2+} release	(>mM)	−	Inhibition	+	−	−
CICR	Increase in cytoplasmic Ca^{2+} concentration	+	+	+	+	+

+: positive; −: negative. *The properties of Clof-induced Ca^{2+} release are confined to those at lower Ca^{2+} concentration. For further explanations, see text.

low concentrations (10 mM, Figure 1) but that it is still increasing at higher concentrations (Figure 2b). Under the weakly buffered conditions of the experiment shown in Figure 1, Ca^{2+} release is enhanced by the effect of Clof on both CICR (with released Ca^{2+}) and some other mechanism, while Clof cannot activate the CICR without Ca^{2+} (pCa >8 buffered with 10 mM EGTA, Figure 2b). Therefore, Clof is more effective under weakly buffered conditions.

Whether these effects of Clof are a result of direct activation of the RyR-Ca^{2+}-release channel or of an indirect mechanism *via* protein(s) binding to the RyR, for example, such as the FK-506 binding protein (Marks, 1996) or triadin (Fan *et al*., 1995) is an important problem to be solved.

Effect on intact muscle

Although Clof could effectively induce Ca^{2+} release, as caffeine can in our experimental condition (Figure 1), it has not been reported that Clof releases Ca^{2+} from the SR in intact skeletal muscle fibres to cause contracture as caffeine does. The action of Clof was inhibited by AMP at the Ca^{2+} concentration in resting living cells (around pCa 7), and a similar inhibitory effect was observed with ATP (pCa >8, unpublished observation). The rate of Ca^{2+} release induced by Clof in the presence of AMP was much lower than that induced by caffeine at pCa >6 (Figure 7b). Since ATP is present in millimolar concentrations in living cells, it is not surprising that, unlike caffeine, Clof cannot cause Ca^{2+} release from the SR of intact muscles. In addition, high concentrations of Clof may have different effects on intact cells, e.g. inhibition of Cl^- channels (De Luca *et al*., 1992). Therefore, effects of Clof on intact cells are likely to be complex.

Opening modes of Clof-induced Ca^{2+} release and physiological Ca^{2+} release in skeletal muscles

In the Introduction, it is pointed out that CICR and PCR are two different modes of opening of the RyR-Ca^{2+}-release channel in skeletal muscles. In this study, we demonstrated that at lower Ca^{2+} concentrations, Clof induced an opening of RyR-Ca^{2+}-release channels of skeletal muscle in a mode quite different from that of CICR. A question then arises as to whether the mode of Clof-induced opening of RyR channels is the same as that of PCR.

As summarized in Table 1, Clof-induced Ca^{2+} release had some properties similar to those of PCR. It was reported that PCR is not inhibited but enhanced in the presence of a high concentration of Fura-2 (Baylor & Hollingworth, 1988; Hollingworth *et al*., 1992; Pape *et al*., 1993). Thus, Ca^{2+} is not required for PCR activation, but it only inhibits PCR. As shown in the present report, Clof-induced Ca^{2+} release was also activated in the absence of Ca^{2+} (pCa >8).

In contrast to its effect on CICR, ATP does not appear to potentiate PCR. In cut fibres, when ATP was removed so that CICR activity was strongly attenuated, PCR was practically unchanged (M. Iino, personal communication). Furthermore, whereas adenine, an inhibitor of CICR in the presence of ATP, was shown to inhibit caffeine contracture of living skeletal muscle fibres, it did not inhibit the twitch of the same fibres (Ishizuka *et al*., 1983). This indicates that either adenine compounds do not affect PCR, or at least the effects of ATP and adenine on PCR are similar in magnitude, unlike those on CICR. Clof-induced Ca^{2+} release was inhibited by AMP (Figure 4), and this is at least in common with PCR in that no potentiation by adenine compounds is observed. It should be noted that Lamb and his colleagues reported that reducing the ATP concentration from 8 to 0.5 mM reduced PCR of the toad and the rat (Owen *et al*., 1996; Blazev & Lamb, 1999). Since the range of ATP concentrations in which CICR is most affected is between 0 and 0.5 mM, the effect of ATP demonstrated by Lamb's group on PCR seems to be much weaker than that on CICR. However, clearly further investigation is necessary.

Mg^{2+}, which exerts an inhibitory effect on CICR, appears to inhibit PCR as well, since in skinned fibres of the toad and the rat T-tubule, depolarization is reported to cause Ca^{2+} release from the SR in low (1 mM) but not high (10 mM) concentrations of Mg^{2+} (Lamb & Stephenson, 1991; 1994). In this regard, PCR may be similar to CICR rather than to Clof-induced Ca^{2+} release at low Ca^{2+} concentrations, since the inhibition of the latter by Mg^{2+} is saturated at about 1 mM (Figure 5). However, it is common to all Ca^{2+}-release mechanisms that qualitatively, Mg^{2+} inhibits Ca^{2+} release.

Procaine is a well-known inhibitor of CICR, and this was confirmed in the present report (Figure 6). The effect of procaine on PCR is somewhat controversial. While 10 mM procaine was reported not to inhibit PCR (Thorens & Endo, 1975; Donaldson, 1985), it was also reported that procaine inhibits PCR in both amphibian and mammalian fibres (Klein *et al*., 1992; Garcia & Schneider, 1995). However, even in the report that procaine inhibits PCR, the inhibitory effect of procaine on CICR appears stronger than that on PCR: while 60% of PCR remained, CICR was abolished in the

presence of 1 mM procaine (Klein *et al*., 1992). Clof-induced Ca^{2+} release in the absence of Ca^{2+} was not inhibited by procaine (Figure 6).

Thus, Clof-induced Ca^{2+} release in the skinned skeletal muscle fibres has some features in common with those of PCR: it can be induced in the absence of Ca^{2+}; they are not potentiated by adenine compounds; and inhibitory effects of procaine are weak or absent, although they are not exactly the same. In this regard, it is interesting that in the accompanying study (Ikemoto *et al*., 2001, accompanying study), another parallelism between Clof-induced Ca^{2+} release and PCR was shown concerning the inhibitory action of dantrolene and its newly synthesized derivatives.

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