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Effect of calmodulin antagonists on calmodulin-induced biphasic modulation of Ca^{2+}-induced Ca^{2+} release

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1 Calmodulin (CaM) has a biphasic effect on Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR): potentiation and inhibition at low (pCa > 6.0) and high (pCa 5) Ca^{2+} concentrations, respectively. To characterize the mode of action of CaM, we studied the effect of CaM antagonists on the CICR in skinned muscle fibres of the rabbit. Ca^{2+} release was measured by microfluorometry with Fura-2.

2 A CaM antagonist, trifluoperazine (TFP), potentiated the CICR in a dose-dependent manner (10–300 μM) at pCa 6, where a simple reversal of the CaM effect would be inhibition of the CICR. Furthermore, 100 μM TFP sensitized the CICR to Ca^{2+}. A similar effect was produced by other CaM antagonists that were tested: chlorpromazine, W-7, mastoparan, and peptide fragment of CaM-binding residues of CaM-dependent protein kinase II.

3 The biphasic effect of CaM on the CICR was observed even in the presence of high concentrations of CaM antagonists or CaM-binding peptides.

4 From these results we suggest that CaM has a unique mode of action on the CICR which is quite different from the effect of CaM on known enzymes.

Keywords: Calcium release; calmodulin; calmodulin antagonists; skinned fibres

Introduction

The ryanodine receptor (RyR) plays an important role in Ca^{2+} release from the sarcoplasmic reticulum (SR) for the initiation of striated muscle contraction. Indeed, it has been demonstrated that mice which do not express type 1 RyR due to a targeted mutation in its gene do not show excitation-contraction coupling (Takeshima *et al.*, 1994). In the physiological excitation-contraction coupling, the Ca^{2+} release via RyR is regulated by the voltage-sensor molecules, now known to be dihydropyridine receptors on the transverse-tubule membrane (Ríos & Pizzaro, 1991; Schneider, 1994). On the other hand, the Ca^{2+}-induced Ca^{2+} release (CICR) mechanism, which is activated by micromolar Ca^{2+} concentrations and inhibited by millimolar Ca^{2+} concentrations, has been well characterized as the mechanism of Ca^{2+} release from the SR, although it is thought that the CICR is not the primary mechanism of physiological Ca^{2+} release (Endo, 1985). Recent data obtained from the study of the channel properties of RyR incorporated into lipid bilayers have revealed that the RyR is functionally identical to the CICR channel protein (Imagawa *et al*, 1987; Lai *et al.*, 1988; Hymel *et al.*, 1988; Smith *et al.*, 1988). Thus, it is now believed that the RyR has two modes of action, the physiological Ca^{2+} release mode and the CICR mode (Lamb & Stephenson, 1990; Ebashi, 1991; Endo, 1992). To clarify the molecular mechanisms of physiological Ca^{2+} release, it is important to study the functional domains and modulator sites of the CICR channel.

Calmodulin (CaM) is a ubiquitous intracellular protein which regulates a multitude of enzymes in a Ca^{2+}-dependent manner (Cheung, 1980) and has been reported to inhibit the CICR from isolated SR vesicles (Meissner, 1986; Meissner & Henderson, 1987; Smith *et al.*, 1989). In a previous study we demonstrated that CaM has a potentiating effect on the CICR at low Ca^{2+} concentrations, although it does inhibit it at higher Ca^{2+} concentrations with a transition point near 3 μM Ca^{2+} (Ikemoto *et al* 1995). Similar results were obtained in recent bilayer experiments (Tripathy *et al.*, 1995). Several potential CaM-binding sites have been predicted in the primary sequence of the RyR (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Otsu *et al.*, 1990; Nakai *et al.*, 1990; Hakamata *et al.*, 1992) and a binding study showed that there were multiple CaM-binding sites on the RyR protein (Yang *et al.*, 1994). These results suggest that the biphasic effect of CaM on the CICR is caused by direct binding to the RyR, but the precise mechanism remains to be elucidated. Therefore, in this study, we investigated whether the biphasic effect of CaM on the CICR is affected by CaM antagonists which inhibit the activity of CaM-dependent enzymes in a Ca^{2+}-dependent manner (Kobayashi *et al.*, 1979; Tanaka & Hidaka, 1980). We found that several CaM antagnonists, including CaM-binding peptides, do not suppress the biphasic effect of CaM on the CICR in skinned fibres and suggest that the effect of CaM is mediated by a novel CaM-dependent mechanism.

Methods

Male New Zealand White rabbits weighing 2–2.5 kg were anaesthetized with 50 mg kg^{-1} sodium pentobarbitone and exsanguinated. Skinned fibres were prepared from psoas muscle which had been treated with saponin (50 μg ml^{-1}) for 45 min in a relaxing solution (in mM: ATP 4.67, $MgMs_2$ 5.54, KMs 108.6, NaN_2 20, PIPES 20, EGTA 1, pH 7.0) to perforate the plasma membrane (Endo & Iino, 1980). After washing out of detergents, two or three muscle fibres were carefully dissected in the relaxing solution and tied with silk filaments at both ends to stainless-steel wire. The length of the preparation between the knots was ~5 mm. The skinned fibre attached to the wire was inserted into a microcuvette with an internal diameter of 400 μm.

Experimental apparatus

The microfluorometric method for the measurement of CICR rates and Ca^{2+} contents of the SR in skinned muscle fibres has been described elsewhere (Iino, 1989; Ikemoto *et al.*, 1995). In brief, the microcuvette containing the skinned fibres was placed on the stage of an epifluorescence microscope (BHS-

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RFK, Olympus, Tokyo, Japan). One end of the microcuvette was connected to peristaltic pumps so that the solution could be rapidly changed (Minipuls 2, Gilson France S.A., Villiers le Bel, France). The other end was linked to a step-motor controlled multi-position (16 channels) valve (ECSD-16P, Valco, Houston, TX, U.S.A.) for the selection of perfusing solution. 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 protocols

Diagrammatic representations of the experimental protocols are shown in Figure 1. Briefly, for the measurement of CICR rates we first carried out Ca^{2+} loading under constant conditions (pCa 6.7 in the presence of 4 mM $MgATP^{2-}$ for 90 s). After washing out of Ca^{2+} and $MgATP^{2-}$, as a test procedure, Ca^{2+} (buffered with 10 mM EGTA) was applied to induce CICR. When the effect of CaM and/or CaM antagonists on CICR was to be tested, these agents were added prior to the application of test solution, i.e., in both pretest and test solutions (black horizontal bar in Figure 1). Ca^{2+} and EGTA were then washed out and 30 or 35 μM Fura-2, a fluorescent Ca^{2+} indicator (Grynkiewicz *et al.*, 1985), was introduced. Assay solution containing both 50 mM caffeine and 25 mM AMP was then applied in the presence of Fura-2 to induce the release of Ca^{2+} remaining in the SR, the amount of which was measured by the fluorescence intensity change at 510 nm with alternating excitation at 340 and 380 nm (400 Hz). Since both methylxanthines and adenine nucleotides potentiate the CICR (Endo, 1985), caffeine and AMP were applied together to induce cooperative enhancement of CICR. The amount of Ca^{2+} released during the test procedure was estimated by comparing the results of assays with and without the test procedure (protocol 2 vs. protocol 1 in Figure 1). The amount of Ca^{2+} remaining in the SR measured with Fura-2 declined exponentially to zero with increasing duration of the test procedure in protocol 2 (Figure 2). Therefore, the rate of CICR was expressed in terms of the rate constants. We vigorously washed out MgATP with three flushes before the test procedure to avoid simultaneous activation of the Ca^{2+} pump. The absence of Ca^{2+} pump activity was confirmed by the control experiments in which no Ca^{2+} reuptake was observed upon application of Ca^{2+} and Mg^{2+} alone after the ATP washout procedure. Statistical significance was determined using the paired t test. Compositions of the solutions, further details of the protocol and experimental apparatus are described elsewhere (Iino, 1989; Ikemoto *et al.*, 1995). All experiments were performed at room temperature (20–22°C).

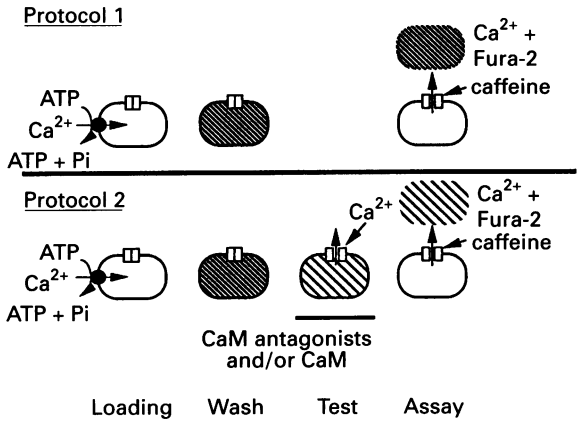

Figure 1 Experimental protocols for measurement of CICR rate. Rounded boxes represent SR. Loading: *In situ* Ca^{2+} pump ATPase was used to load the SR in the presence of 4 mM $MgATP^{2-}$ at pCa 6.7 for 90 s. Wash: $MgATP^{2-}$ and Ca^{2+} were washed out in the presence of 10 mM EGTA. Test: Test solution (various Ca^{2+} concentrations, buffered with 10 mM EGTA and no ATP) was applied to induce CICR in the presence or absence of CaM. This procedure was omitted in protocol 1. Assay: After washing out of the test solution, Fura-2 (30 or 35 μM) was introduced and then the release of Ca^{2+} remaining in the SR was induced by addition of the assay solution containing high concentrations of caffeine (50 mM) and AMP (25 mM) in the presence of Fura-2. The difference in the amounts of Ca^{2+} assayed between the two types of protocol was taken to represent the amount of Ca^{2+} released during the Test procedure in protocol 2. CaM and CaM antagonists were applied during the Test period.

Materials

Na_2ATP was obtained from Boehringer Mannheim (Germany), saponin from ICN Pharmaceuticals Inc. (U.S.A.), Fura-2 from Molecular Probes, Inc. (U.S.A.), EGTA from Dojindo Laboratories (Japan), N-(-6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7) from Seikagaku Kogyo Co. (Japan), chlorpromazine hydrochloride (CPZ) from Nacalai Tesque, Inc. (Japan), mastoparan from BACHEM, Inc. (U.S.A.), and AMP, trifluoperazine (TFP), calmodulin (CaM) from bovine brain, calmodulin-dependent protein kinase II fragment 290-309 ($CaMPKII_{290-309}$) from Sigma (U.S.A.). Concentrations of CaM was calculated assuming 98% purity and a molecular weight of 16,700. All the other chemicals were of the highest reagent grade availabe.

Results

Effect of trifluoperazine on the Ca^{2+} release from SR

It has been reported that some CaM antagonists potentiate the Ca^{2+} release from the SR vesicles of skeletal muscle (Meissner, 1986; Palade, 1987; Wyskovsky *et al.*, 1988; Vale, 1990). Therefore we first examined the effect of trifluoperazine (TFP) on the Ca^{2+} release in skinned fibres. Figure 2 shows the time course of the Ca^{2+} release from the SR at pCa 6.0 in the presence of 0.5 mM Mg^{2+}. In the absence of TFP, Ca^{2+} was slowly released from the SR (Figure 2, open circles). In the presence of TFP (100 μM), Ca^{2+} was released much faster from the SR than in the absence of TFP (Figure 2, filled circles). The rate constant at pCa 6.0 in the presence of 100 μM

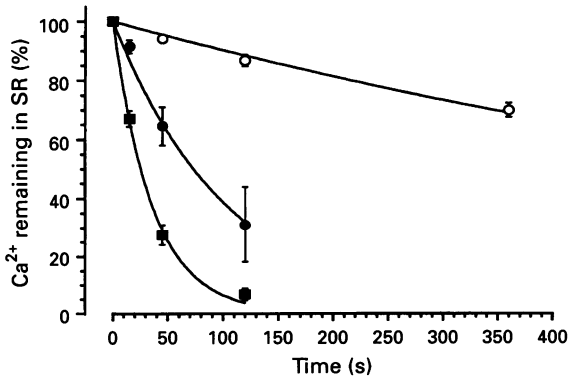

Figure 2 Effect of TFP on the time course of CICR from the SR in saponin-skinned fibres. Fibres with fixed amount of Ca^{2+} in the SR were treated with pCa 6.0 in the presence (●) or absence (○) of 100 μM TFP for the period of time shown on the abscissa scale; 0.5 mM Mg^{2+} was also present. The same type of experiments with co-application of TFP and CaM produced the results shown by (■). The percentages of Ca^{2+} remaining in the SR after the treatment were plotted (mean ± s.e.mean, n = 4–5). Exponential curves were fitted by a nonlinear least squares method.

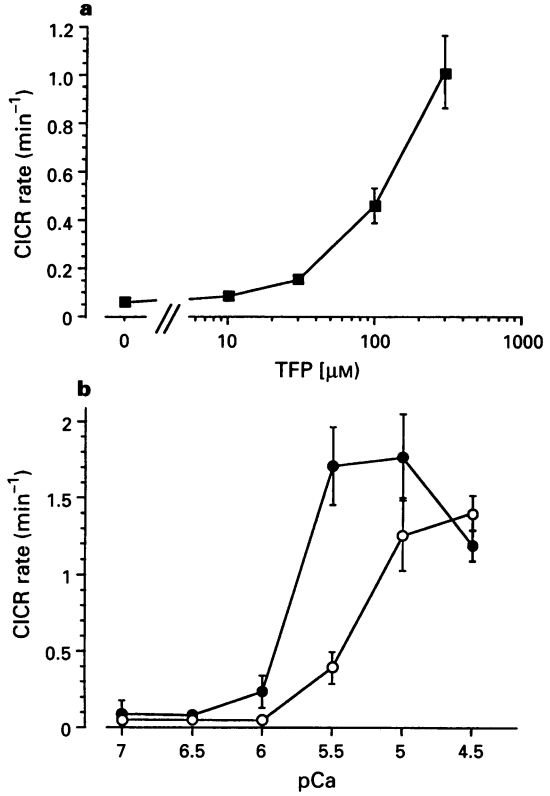

Figure 3 Dependence of TFP concentration of the rate of CICR at pCa 6.0 and the effect of 100 μM TFP on the rate of CICR at various Ca^{2+} concentrations. (a) The CICR rates were measured at pCa 6.0 in the presence of 0.5 mM Mg^{2+} ($n=5$). (b) Each value represents the rate of CICR with (●) or without (○) 100 μM TFP (mean ± s.e.mean, $n=4-6$).

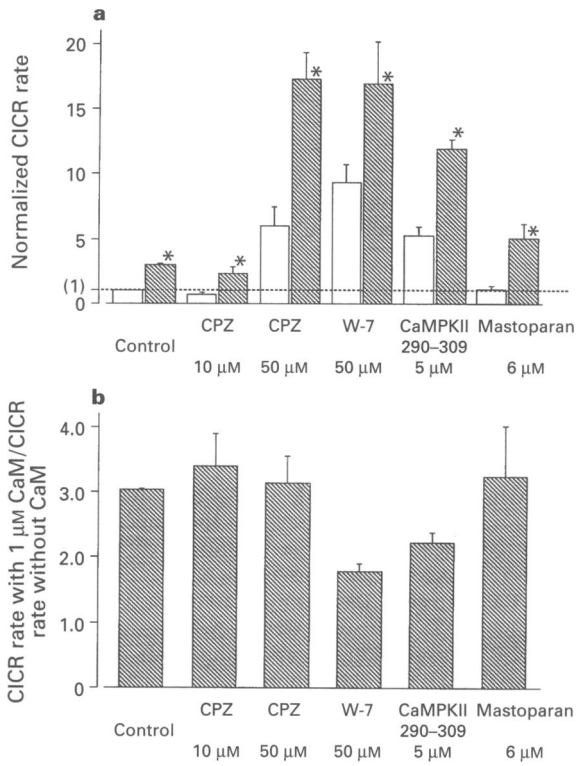

Figure 4 Effect of CaM antagonists and CaM-binding peptides on enhancing effect of CaM on the CICR. Effects of CaM antagonists and CaM-binding peptides on the rates of CICR were measured at pCa 6.5 in the presnce (1 μM, hatched column) or absence (open column) of CaM. (a) The results were normalized by the control value (in the absence of both CaM and CaM antagonists) within each preparation (mean ± s.e.mean, $n=4-7$). (b) Each value in the presence of both CaM and CaM antagonists in (a) (hatched column) was normalized by the value in the presence of CaM antagonist alone (open column). *$P<0.05$.

TFP was about 6 fold higher than that in the absence of TFP. At pCa 6.0, the Ca^{2+} release rate was increased by TFP in a dose-dependent manner (10–300 μM) and the effect did not seem to level off even at 300 μM (Figure 3a).

We then examined the effect of TFP (100 μM) on the rate of Ca^{2+} release at various Ca^{2+} concentrations in the presence of 0.5 mM Mg^{2+}. Figure 3b shows the Ca^{2+} release rates in the presence (filled circles) or absence (open circles) of 100 μM TFP. The presence of TFP significantly potentiated the Ca^{2+} release at pCa 6.0 and 5.5 (about 5–6 fold), while it exhibited little effect on the Ca^{2+} release at low and high Ca^{2+} concentrations (pCa $\geq$ 6.5 and pCa 4.5). These resullts suggest that TFP potentiated the Ca^{2+} release from the SR mainly as the result of enhancement of the Ca^{2+} sensitivity of the CICR mechanism.

The effect of CaM antagonists and CaM-binding peptides on CaM-induced potentiation of CICR

We examined whether the enhancing effect of CaM (Ikemoto *et al.*, 1995) on the CICR is affected by CaM antagonists. Although TFP (100 μM) alone potentiated the CICR at pCa 6.0, CaM (1 μM) further enhanced it even in the presence of TFP (Figure 2, squares). We have already shown that CaM alone potentiates the rate of Ca^{2+} release in the absence of TFP (Ikemoto *et al.*, 1995). A 100 μM concentration of TFP was sufficient to inhibit CaM-dependent phosphorylation of cardiac SR ($IC_{50}=28$ μM, Wyskovsky *et al.*, 1988), indicating that TFP does not antagonize the enhancing effects of CaM on the CICR.

We then studied the effect of other CaM antagonists on the CaM-induced enhancement of CICR. These experiments were carried out in Mg^{2+}-free conditions, where the enhancing effect of CaM on the CICR is most clearly observed (Ikemoto *et al.*, 1995). Figure 4 shows the effect of other CaM antagonists and CaM-binding peptides on the CICR at pCa 6.5. Chlorpromazine (CPZ) or W-7 increased the rate of CICR. Simultaneous application of CaM (1 μM) and these agents further increased the rate of CICR (Figure 4a).

A peptide fragment derived from CaM-dependent protein kinase II residues 290-309 ($CaMPKII_{290-309}$) also binds to CaM and inhibits the activity of CaM-dependent enzymes (Payne *et al.*, 1988). As seen in Figure 4a, this peptide fragment enhanced the CICR to nearly the same extent as the CaM antagonists and the rate of CICR in the presence of $CaMPKII_{290-309}$ was doubled by adding CaM. In the presence of a high-affinity CaM-binding peptide, mastoparan (6 μM, Malencik & Anderson, 1983), CaM also increased the CICR rate at pCa 6.5 (Figure 4a).

The magnitude of CaM-induced potentiation of CICR in the presence of these agents at pCa 6.5 was compared with that in their absence (Figure 4b). Roughly the same extent of potentiation (two to three fold) was observed in the presence of CaM antagonists or CaM-binding peptides.

The effect of CaM antagonists and CaM-binding peptides on CaM-induced inhibition of CICR

We then examined the effect of CaM antagonists on the CaM-induced inhibition of CICR which is observed at higher Ca^{2+} concentrations. At pCa 5.0 the rate of CICR was decreased by adding CaM (Figure 5a, control). At the same Ca^{2+} concentration, TFP (100 μM) alone significantly increased the rate of CICR, but CaM (1 μM) decreased it even in the presence of

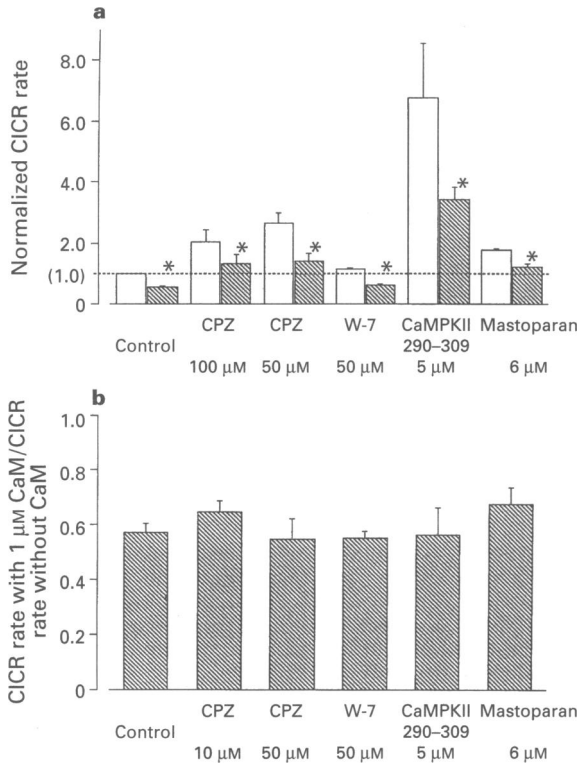

Figure 5 Effect of CaM antagonists and CaM-binding peptides on inhibitory effect of CaM on the CICR. Effect of these agents on the rates of CICR were measured at pCa 5.0 (0.5 mM Mg^{2+}) in the presence (1 μM, hatched column) or absence (open column) of CaM. The results were normalized by the control value (in the abscence of both CaM and CaM antagonists) within each preparation (mean ± s.e.mean, $n=4-5$). (b) Each value in the presence of both CaM and CaM antagonists in (a) (hatched column) was normalized by the value in the presence of CaM antagonist alone (open column). *$P<0.05$.

TFP (Figure 5). CaM also decreased the rate of CICR in the presence W-7 (50 μM) or CPZ (50 μM, Figure 5a). In addition, even in the presence of a higher concentration of W-7 (200 μM), CaM inhibited the CICR under the same conditions (data not shown).

$CaMPKII_{290-309}$ (5 μM) and mastoparan (6 μM) also increased the CICR rate on their own. However they did not suppress the inhibitory effect of CaM on the CICR at pCa 5.0 (Figure 5a).

The extent of CaM-induced inhibition was compared in the presence of different drugs and peptides (Figure 5b). The presence of 1 μM CaM always decreased the rate of CICR at pCa 5.0 by ~40%. Thus CaM is able to inhibit the CICR even in the presence of CaM antagonists or CaM-binding peptides.

Discussion

Our main conclusion is that the CaM-dependent modulation of CICR from the SR of skeletal muscle fibres is insensitive to CaM antagonists. We previously reported that CaM (1 μM) increased the rate of CICR by about two to three fold at low Ca^{2+} concentrations (<3 μM), while the rate of CICR was markedly reduced, by about 40%, at high Ca^{2+} concentrations (>3 μM, Ikemoto *et al.*, 1995). The extent of CaM-induced enhancement and inhibition of CICR was much the same even in the presence of CaM antagonists (Figures 4b and 5b). Thus, we were able to observe a similar biphasic effect of CaM in the presence of CaM antagonists.

All the CaM antagonists and CaM binding peptides we tested potentiated the Ca^{2+} release from the SR. Several CaM antagonists were reported to enhance the CICR from isolated SR vesicles (Meissner, 1986; Palade, 1987; Wyskovsky *et al.*, 1988; Vale, 1990) and the open probability of a Ca^{2+} release channel incorporated into a lipid bilayer was increased by the presence of a high concentration of W-7 (Smith *et al.*, 1989). The Ca^{2+}-dependent enhacing effect of TFP (Figure 2b) also suggests that TFP potentiates the Ca^{2+} release from the SR as a result of activation of the CICR channel rather than via a nonspecific effect on the SR membrane. Not only TFP but also W-7 (200 μM) increased Ca^{2+} sensitivity of CICR (unpublished observation). CPZ and CaM-binding peptides were not tested at a wide range of Ca^{2+} concentrations; however, at both pCa 6.5 and pCa 5.0 these agents increased the rate of CICR (Figures 4a and 5a). Thus CICR is enhanced by many CaM antagonists and CaM-binding peptides. It will be interesting to see if the CaM antagonists have any effect on the twitch contractions in intact muscle.

CaM is present in the cytoplasm of skeletal muscle cells at a concentration of about 2 μM (Yagi & Yazawa, 1978), and a part of CaM might be retained in skimmed fibres. However, CaM antagonist-induced potentiation of CICR was not related to recovery from the inhibitory effect of the intrinsic CaM on CICR, because CaM antagonists at concentrations close to the IC_{50} for Ca^{2+}-CaM activated phosphodiesterase activity (Hidaka *et al.*, 1979) were not able to reverse the inhibitory effect of CaM at a high Ca^{2+} concentration (Figure 5). Even in the presence of higher concentrations of TFP (100 μM) or W-7 (200 μM) these agents did not affect the inhibitory effect of exogenous CaM. Furthermore, the CaM antagonists potentiated the CICR at low Ca^{2+} concentrations (Figure 4a), where inhibition of the CaM effect would be expected to induce inhibition of CICR. These results indicate that the CaM antagonists we tested have a direct potentiating effect on CICR, which seems to be independent of the antagonism between these agents and CaM. Although both CaM and CaM antagonists have a potentiating effect at low Ca^{2+} concentrations (<3 μM), at a higher Ca^{2+} concentration (10 μM) CaM has little or rather an inhibitory effect on the CICR (Ikemoto *et al.*, 1995; see also Figure 5a) while CaM antagonists still potentiate the Ca^{2+} release mechanism (Figure 5). Thus CaM and CaM antagonists have different modes of action. Further study is required to elucidate the mechanism of the enhancing effect of CaM antagonists.

It is thought that CaM antagonists bind to the effector binding sites on CaM in the presence of Ca^{2+} and inhibit interaction between CaM and the enzyme in question (Tanaka & Hidaka, 1980). Since the biphasic effect of CaM on the CICR was not blocked by these conventional CaM antagonists and CaM-binding peptides, the effects of CaM on CICR are not likely to be induced via the same mechanism as the classical Ca^{2+}-CaM-dependent effect. The absence of effect of the $CaMPKII_{290-309}$ may suggest that CaM binds to the effector molecule at a different site from that used for binding to CaM-dependent protein kinase II.

Recent ligand overlay experiments on RyR fusion proteins indicated that the skeletal muscle RyR contains multiple regions to which CaM binds in the presence or absence of Ca^{2+} (Chen & MacLennan, 1994; Menegazzi *et al.*, 1994). Since CaM potentiates the CICR even at submicromolar Ca^{2+} concentrations, the regions to which CaM binds in the absence of Ca^{2+} may be important in the potentiating effect of CaM. However, we cannot at present rule out the alternative possibility that CaM influences the CICR via the binding of another regulatory protein to the RyR.

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