Effects of dantrolene and its derivatives on Ca^{2+} release from the sarcoplasmic reticulum of mouse skeletal muscle fibres

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1 We analysed the effect of dantrolene (Dan) and five newly synthesized derivatives (GIFs) on Ca^{2+} release from the sarcoplasmic reticulum (SR) of mouse skeletal muscle.

2 In intact muscles, GIF-0185 reduced the size of twitch contraction induced by electrical stimulation to the same extent as Dan. GIF-0082, an azido-functionalized Dan derivative, also inhibited twitch contraction, although the extent of inhibition was less than that of Dan and of GIF-0185.

3 In skinned fibres, Dan inhibited Ca^{2+}-induced Ca^{2+} release (CICR) under Mg^{2+}-free conditions at room temperature. In contrast, GIF-0082 and GIF-0185 showed no inhibitory effect on CICR under the same conditions.

4 Dan-induced inhibition of CICR was not affected by the presence of GIF-0082, whereas it was diminished in the presence of GIF-0185.

5 GIF-0082 and GIF-0185 significantly inhibited clofibric acid (Clof)-induced Ca^{2+} release, as did Dan.

6 Several Dan derivatives other than GIF-0082 and GIF-0185 showed an inhibitory effect on twitch tension but not on the CICR mechanism. All of these derivatives inhibited Clof-induced Ca^{2+} release.

7 The magnitudes of inhibition of Clof-induced Ca^{2+} release by all Dan derivatives were well correlated with those of twitch inhibition. This supports the notion that the mode of Clof-induced opening of the RyR-Ca^{2+} release channel may be similar to that of physiological Ca^{2+} release (PCR).

8 These results indicate that the difference in opening modes of the RyR-Ca^{2+} release channel is recognized by certain Dan derivatives.

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Introduction

Ryanodine receptor (RyR)-mediated Ca^{2+} release from the sarcoplasmic reticulum (SR) is an essential step in excitation-contraction coupling (E-C coupling) in skeletal and cardiac muscles. Purified RyR proteins incorporated into lipid bilayers show properties of the Ca^{2+}-induced Ca^{2+} release (CICR) channel (Meissner, 1994). While CICR is thought to be the mechanism of Ca^{2+} release in cardiac E-C coupling (Näbauer *et al.*, 1989; Bers & Perez-Reyes, 1999), it is not considered to be the mechanism of physiological Ca^{2+} release (PCR) in skeletal muscles E-C coupling (Endo, 1985; Schneider, 1994), because, for example, inhibitors of CICR such as procaine and adenine (in the presence of ATP) do not inhibit PCR (Thorens & Endo, 1975; Ishizuka *et al.*, 1983). However, since mice with a targeted mutation of the skeletal muscle RyR (RyR1) gene lack E-C coupling (Takeshima *et al.*, 1994), it must be concluded that PCR takes place through RyR1. This means that RyR1 can open in two different modes, PCR and CICR. In order to elucidate a molecular or submolecular mechanism of PCR that

is distinct from CICR, specific inhibitors of PCR at the level of RyR1 would be very useful. However, no agents have been shown to inhibit PCR without also affecting CICR. Some specific inhibitors of CICR mentioned above, and other agents that inhibit both PCR and CICR, such as tetracaine or ruthenium red, are known (Almers & Best, 1976; Ohnishi, 1979; Smith *et al.*, 1986).

Dantrolene sodium (Dan) was originally reported to inhibit E-C coupling of skeletal muscle (Ellis & Bryant, 1972; Ellis & Carpenter, 1972). It was then found to be an effective agent for the treatment of porcine and human malignant hyperthermia (MH), a pathophysiological state in susceptible individuals caused by an excessive release of Ca^{2+} through the RyR1 activated by inhalation anaesthetics (Harrison, 1975; Kolb *et al.*, 1982; MacLennan & Phillips, 1992). MH was shown to be a consequence of an excessive CICR (Endo *et al.*, 1983), and it was demonstrated that Dan, in addition to inhibiting PCR, can effectively inhibit CICR at 37°C (Ohta & Endo, 1986; Ohta *et al.*, 1989). Interestingly, it was then found that at room temperature, Dan does not inhibit CICR, although it inhibits E-C coupling at 37°C and at room temperature equally (Ohta &

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Endo, 1986; Kobayashi & Endo, 1988; Ohta *et al.*, 1990). The results described in this study are somewhat different from these reports. However, they do indicate that under a certain experimental condition, Dan somehow preferentially inhibits PCR at room temperature.

In the present study, we examined the effects of several newly synthesized Dan derivatives with the goal of finding some agents that more clearly distinguish between PCR and CICR than Dan. We tested these agents for inhibition of twitch tension of intact muscle fibres and inhibition of CICR in saponin-treated skinned muscle fibres. We also tested for their effects on clofibric acid (Clof)-induced Ca^{2+} release, which has certain characteristics similar to those of PCR (Ikemoto & Endo, 2001, accompanying study). We found that some Dan derivatives indeed distinguish PCR from CICR more clearly than Dan itself. In addition, the data demonstrate further similarity of the mode of opening of RyR-Ca^{2+} release channels activated by Clof to the mode of PCR.

## Methods

Preparation of muscle fibres

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

The extensor digitorum longus muscles (EDL) were excised. One of the four heads of EDL was carefully isolated in a physiological saline solution (PSS, in mM: NaCl 150; KCl 4; CaCl$_2$ 1.8; MgCl$_2$ 1; 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES) 5; glucose 5.6; pH 7.4). The bundles were tied at both ends with silk threads and mounted between a pair of stainless steel rods that attached to a force transducer (AE801, Akers, Norway or UL-10GR, NMB, Japan) and a micromanipulator (Narishige, Japan). The muscle preparation was immersed in PSS.

Preparation of skinned fibres was described previously (Ikemoto & Endo, 2001, accompanying study). All experiments were carried out at room temperature (21–23°C).

Measurement of tension in intact fibres

Supramaximal field electrical stimulation (20 V, 0.5 ms duration) was delivered by a pair of platinum wires placed on both sides of the preparation. After twitch tension became constant in PSS containing D-tubocurarine (25 μM) and 0.2% dimethyl sulphoxide (DMSO), the external solution was changed to a PSS containing dantrolene (Dan) or its derivatives, stock solutions of which were made by dissolving the agents in DMSO as described below. After the twitch tension became constant during a 15–30 min treatment period, it was compared
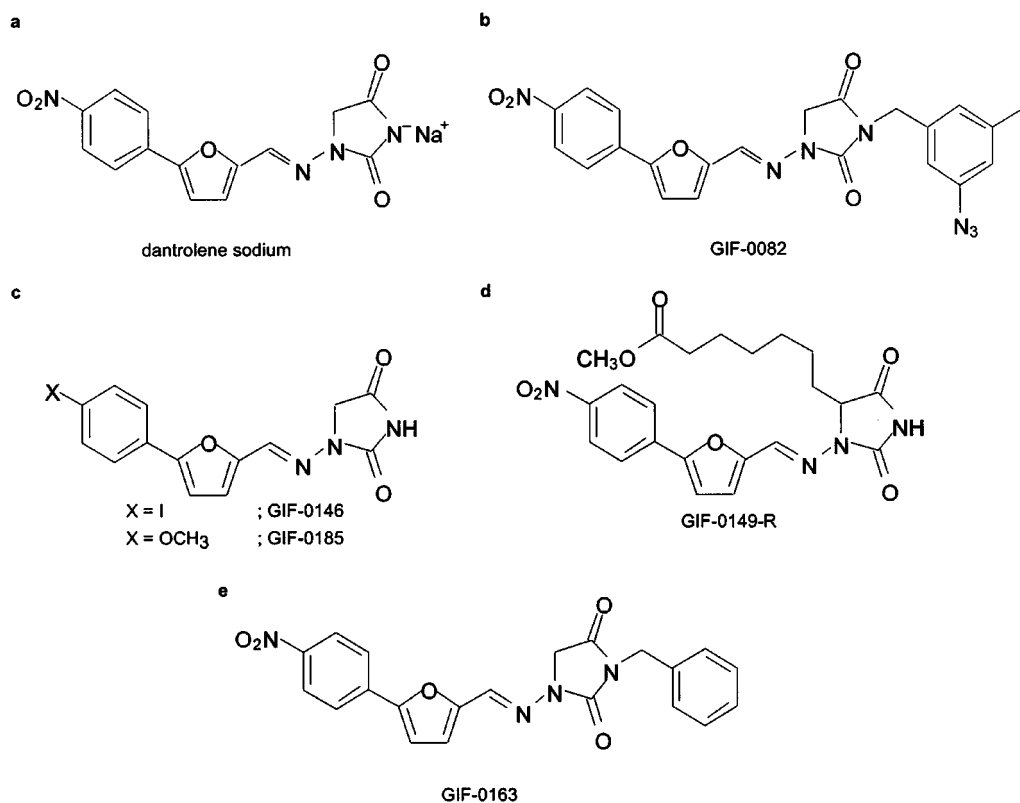
Figure 1 Structures of dantrolene and its derivatives. (a) The structure of dantrolene sodium (Dan). (b) *N*-(3-azido-5-iodo)benzylated Dan derivative, which is referred to as GIF-0082, was designed as a photoaffinity probe by incorporation of azido group as a photoreactive unit and iodine. (c) GIF-0146 and GIF-0185 are Dan analogues, in which the substituent on the phenyl ring is changed from an electron-withdrawing nitro group to electron-donating iodo and methoxy groups, respectively. (d) GIF-0149-R is another type of Dan derivative in which an alkyl chain is anchored on the hydantoin ring. (e) GIF-0163, *N*-benzyldantrolene, is the structurally more simplified compound of GIF-0082.

with that measured before treatment. D-tubocurarine was present throughout these experiments. Statistical significance was tested by Dunnett's test for multiple comparisons (Figure 2a).

Measurement of Ca²⁺ release in skinned fibres

The Ca^{2+} released from the skinned fibres was quantified using Fura-2 as described previously (Ikemoto & Endo, 2001, accompanying study). Briefly, the protocol for the measurement of the Ca^{2+} release rate consisted of (1) loading the SR with Ca^{2+}, (2) test (Ca^{2+}-releasing stimuli are given), and (3) determination of the Ca^{2+} remaining in the SR. During the test period, various concentrations of Ca^{2+} buffered with 10 mM EGTA, sometimes together with Clof or CICR activators (caffeine or AMP), were given, and the effects of further addition of Dan or its derivatives were examined. The activity of Ca^{2+} release was expressed in terms of the decay rate constants based on the amount of Ca^{2+} in the SR. Stock solutions of Dan or its derivatives were dissolved in DMSO to obtain concentrations of 0.1– 25 mM (Dan, GIF-0082, and GIF-0185) or 25 mM (other derivatives). All test solutions with or without these agents were adjusted to contain 0.2% DMSO except for those used in the experiments performed to determine the effects of GIFs on Dan-induced inhibition of CICR activity; in those experiments, the concentration of DMSO in the test solution was kept at 0.4%. Statistical significance was tested using the paired *t*-test.

Synthesis of Dan derivatives

Dan derivatives, which we referred to as GIF-0082 (Figure 1b) and GIF-0163 (Figure 1e), were prepared by coupling Dan (Figure 1a) with (3-azido-5-iodo)benzyl bromide or benzyl bromide, respectively. GIF-0146 (Figure 1c), which is an iodo-congener of dantrolene, was prepared from 4-iodoaniline by the conventional method reported by Snyder *et al.* (1967). The methoxy analogue of Dan, GIF-0185 (Figure 1c), was synthesized by the Stille reaction (Stille, 1986) of 4-iodoanisole and 5-(tri-*n*-butylstannyl)-2-furaldehyde (Denat *et al.*, 1992), followed by condensation with 1-aminohydantoin (Traube & Hoffa, 1898). GIF-0149-R (Figure 1d) was obtained by condensation of 5-(4-nitrophenyl)-2-furaldehyde with racemic 1-amino-5-(6-methoxycarbonylhexyl)hydantoin (Barraclough *et al.*, 1994). All of the newly synthesized GIF compounds were purified by recrystallization (>99% purity) and fully characterized by ¹H- and ¹³C-NMR (nuclear magnetic resonance) spectroscopy, IR (infrared) spectroscopy, UV (ultraviolet) spectroscopy, and combustion analysis. The details of the synthesis of GIFs will be reported elsewhere (manuscript in preparation).

Other materials

Dantrolene sodium was a gift from Yamanouchi Pharmaceutical Co., Ltd. (Japan) or purchased from Nacalai Tesque, Inc. (Japan). D-tubocurarine chloride was purchased from Nacalai Tesque, Inc. All the other chemicals were as described in the previous paper (Ikemoto & Endo, 2001, accompanying study).

Results

Effects of Dan and GIFs on Ca²⁺ release in intact fibres and on CICR in skinned fibres

We designed and synthesized *N*-(3-azido-5-iodo)benzylated dantrolene, referred to as GIF-0082, which carries an azido group as a photoreactive unit (Kotzyba-Hibert *et al.*, 1995). As shown in Figure 2a, GIF-0082 inhibited the twitch tension of intact skeletal muscle in a dose-dependent manner (0.2– 50 μM, open squares), although the extent of inhibition was less than that produced by Dan (Figure 2a, filled circles). GIF-0185, another Dan derivative, also inhibited the twitch tension to the same extent as Dan (Figure 2a, open triangles). Since GIF-0082 and GIF-0185 showed no inhibitory effect on the Ca^{2+} uptake activity in the SR or on the relationship between pCa and tension in skinned fibres (data not shown) as was the case with Dan (Ohta *et al.*, 1990), these data indicate that both of these GIFs inhibit the physiological Ca^{2+} release (PCR) from the SR in intact muscles.

Figure 2b shows the effects of Dan (open circles), GIF-0082 (filled squares), and GIF-0185 (filled triangles) on the Ca^{2+}-induced Ca^{2+} release (CICR) in skinned fibres of mouse skeletal muscles. The rates of Ca^{2+} release were significantly decreased by the application of Dan, although it exhibited little effect on Ca^{2+} release at high Ca^{2+} concentrations (open triangles vs open circles). The rates of Ca^{2+} release at pCa 6.0 were decreased by approximately 50% in the presence of high concentrations of Dan (20–50 μM, Figure 4a, open circles). These data indicated that Dan had an inhibitory effect on CICR even at room temperature, in apparent disagreement with a previous report (Ohta *et al.*, 1990). In contrast, the rates of CICR were not significantly changed by the GIFs (50 μM) at any Ca^{2+} concentration (Figure 2b, open triangles vs filled squares and filled triangles). Thus, these GIFs can distinguish CICR from PCR much more clearly than Dan.

Effect of Dan and GIF-0082 on Clof-induced Ca²⁺ release in skinned fibres

We have reported that the opening mode of the RyR channel activated by Clof in skinned fibres has some features similar to those of PCR, but different from those of CICR (Ikemoto & Endo, 2001, accompanying study). If the similarity applies also to GIF-0082 and GIF-0185, they may inhibit Clof-induced Ca^{2+} release as well. Therefore, in skinned fibres, we examined the effect of GIF-0082 on Clof-induced Ca^{2+} release at pCa > 8. As shown in Figure 3a, the rates of Clof (>10 mM)-induced Ca^{2+} release were significantly decreased by GIF-0082 (50 μM). On the other hand, GIF-0082 exerted no effect on the Ca^{2+} release rate in the presence or absence of caffeine (Figure 3b), in accordance with the absence of effects on CICR (Figure 2b), while Dan significantly decreased the Ca^{2+} release rate under the same conditions (Figure 3b). The results were made clearer by normalizing the rate with GIF-0082 or Dan by the rates without these inhibitors in the same fibres (Figure 3c). Although the AMP-stimulated Ca^{2+} release rate was not significantly inhibited by Dan, its normalized rate may suggest that it is reduced by Dan. It is also seen that the extent of inhibition by GIF-0082

was greater, at higher Clof concentrations. GIF-0185 also inhibited Clof-induced Ca^{2+} release, as described in the next section.

Effects of GIF-0082 and GIF-0185 on Dan-induced inhibition of Ca^{2+} release

It is not surprising that while GIF-0082 and GIF-0185 do not inhibit CICR at all, they inhibit PCR, because it is thought

that the PCR from the SR is not mediated by the CICR mechanism (Endo, 1985; Schneider, 1994). On the other hand, it is possible that in preparations of skinned fibres, molecule(s) responsible for the action of GIFs may have been lost. This is very unlikely, however, since Clof-induced Ca^{2+} release in skinned fibres is well affected by GIFs as just
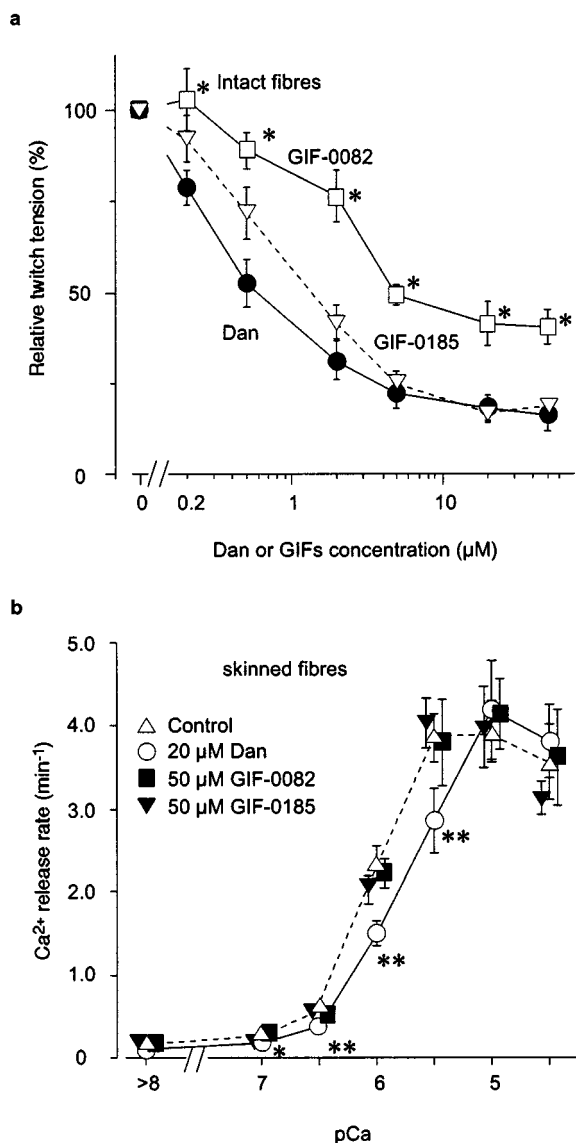
Figure 2 Effects of Dan, GIF-0082, and GIF-0185 on Ca^{2+} release. (a) In intact fibres, after treatment with dantrolene (Dan), GIF-0082, or GIF-0185 in the concentration range of 0.2–50 μM, the size of twitch contraction was compared with that before the treatment. Relative tensions were plotted against the concentrations. (mean± s.e.mean, $n=9$ for Dan, $n=3-6$ for GIF-0082, $n=3-6$ for GIF-0185). *$P<0.05$ (Dunnett's test). (b) The rates of Ca^{2+} release were obtained in saponin-skinned fibres with 20 μM Dan ($n=5$), 50 μM GIF-0082 ($n=3-7$) or 50 μM GIF-0185 ($n=3-4$) in the absence of Mg^{2+} and adenine nucleotide at pCa>8–4.5. Open triangles show the rates without these agents (control, $n=11-16$). The symbols for GIF-0082 and GIF-0185 were slightly offset horizontally for clarity (mean±s.e.mean). *$P<0.05$, **$P<0.01$ (vs control). Error bars smaller than the symbols are not shown.
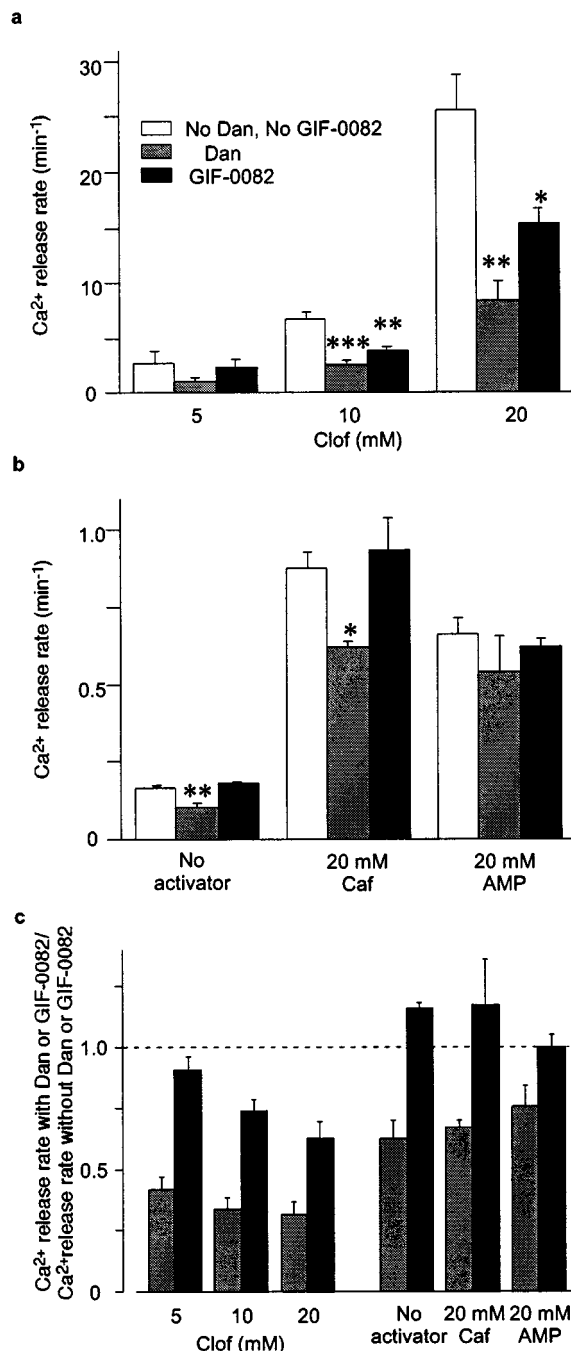
Figure 3 Effects of Dan and GIF-0082 on caffeine- and clofibric acid-induced Ca^{2+} release in skinned fibres. The rates of Ca^{2+} release with 20 μM Dan or 50 μM GIF-0082 were measured under Mg^{2+}- and Ca^{2+}-free conditions (buffered with 10 mM EGTA) with 5–20 mM clofibric acid (Clof, Figure 3a, mean±s.e.mean, $n=3-6$) or with or without CICR activators (caffeine, Caf; AMP; Figure 3b, $n=5-7$). Note the difference in the scales. (c) The results in a and b were normalized to the control value (without Dan or GIF-0082) within each preparation. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

described. The following experiments also provide support that this theory is unlikely.

Dan decreased the rates of CICR in skinned fibres in a dose-dependent manner (2–50 μM, Figure 4a, open circles). Although GIF-0082 and GIF-0185 could not inhibit the CICR as shown in Figure 2b, the inhibitory action of low concentrations of Dan (2–20 μM) were completely abolished by the presence of GIF-0185 (Figure 4a, filled triangles). On the other hand, Dan-induced inhibition of CICR was not affected by the presence of GIF-0082 (Figure 4a, open squares). The abolition of the Dan effect on CICR by GIF-

0185 in skinned fibres clearly shows that CICR in skinned fibres retains some sensitivity to GIF-0185.

We further studied the effect of co-applications of GIF-0082 or GIF-0185 and Dan on Clof-induced Ca²⁺ release. As shown in Figure 4b, both GIF-0082 (25 μM) and GIF-0185 (5 μM) significantly inhibited Clof-induced Ca²⁺ release. We confirmed that these GIFs showed no inhibitory effect on CICR at the same concentrations (data not shown). Dan (2 and 5 μM) also inhibited Clof-induced Ca²⁺ release (Figure 4b). Simultaneous applications of GIF-0082 or GIF-0185 and Dan showed additive inhibitory effects (Figure 4b, filled column).

Effect of other Dan derivatives on the Ca²⁺ release

Next we examined the effect of Dan derivatives other than GIF-0082 and GIF-0185. Among the many derivatives we tested, GIF-0146 and GIF-0149-R inhibited twitch tension in intact fibres, while GIF-0163 had little effect (Figure 5a). The magnitude of inhibitory action of these derivatives and Dan on twitch tension was in the following sequence: Dan (20 μM)=GIF-0185 (50 μM)>GIF-0082 (50 μM)>GIF-0146 (50 μM)⩾GIF-0149-R (50 μM)>GIF-0163 (50 μM) (Figures 2a and 5a). On the other hand, in skinned fibres, the rates of CICR at pCa 6 were not decreased with any of these derivatives (Figure 5b).

We also tested the effect of these derivatives on the Clof-induced Ca²⁺ release in skinned fibres. In order to mimic the physiological condition, Clof was applied at pCa 7 in the presence of 0.5 mM Mg²⁺ and 10 mM AMP. As shown in Figure 6a, all of the derivatives that showed the inhibitory effect on twitch contraction decreased the rates of Clof
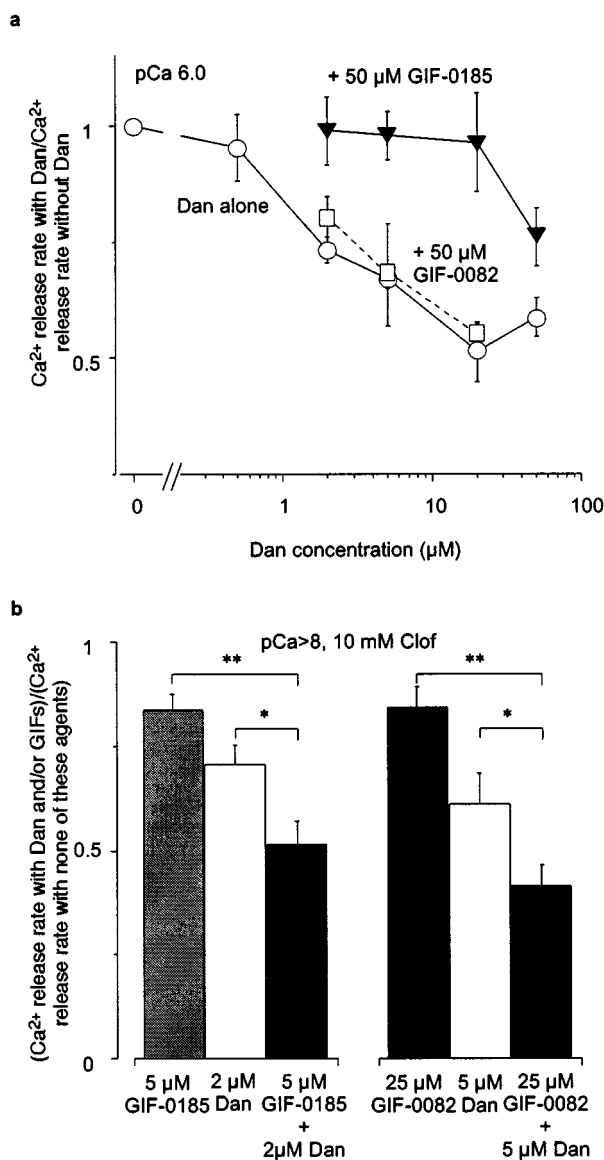

Figure 4 Different effect of GIF-0082 and GIF-0185 on Ca²⁺ release. (a) In skinned fibres, the rates of Ca²⁺ release measured with Dan (0–50 μM) at pCa 6.0 under Mg²⁺-free conditions were normalized to the control value (without Dan) obtained in the same preparation in the absence (Dan alone, mean±s.e.mean, *n*=3–6) or presence of 50 μM GIF-0082 (*n*=3) or GIF-0185 (*n*=5). (b) The rates of Ca²⁺ release by 10 mM Clof in the presence of Dan (2 or 5 μM, mean±s.e.mean) and/or either GIF-0185 (5 μM) or GIF-0082 (25 μM, *n*=4) under Ca²⁺, Mg²⁺-free conditions (buffered with 10 mM EGTA) were normalized to the control value (without these agents) within each preparation. *P<0.05, **P<0.01.
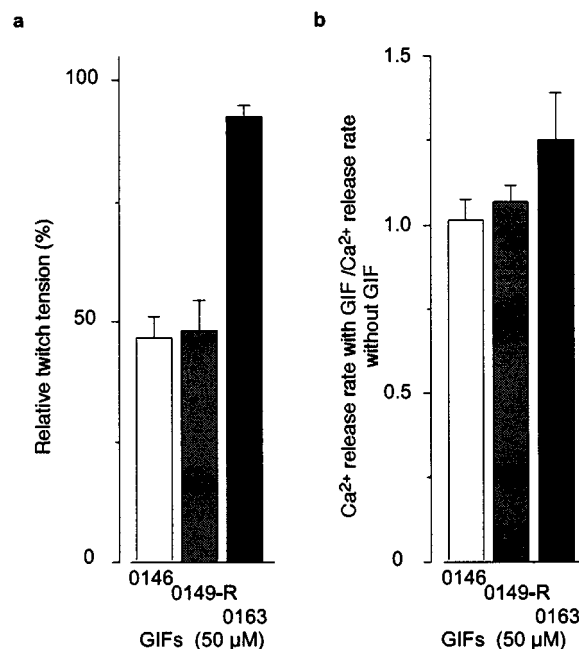
Figure 5 Effects of some Dan derivatives on Ca²⁺ release. (a) The sizes of twitch contraction after treatment of GIF-0146, GIF-0149-R, GIF-0163, or GIF-0185 (50 μM) were compared with those before treatment in intact muscle. (b) In skinned fibres, the rates of Ca²⁺ release measured with these derivatives at pCa 6.0 in the absence of Mg²⁺ were normalized to the control value (without the derivatives) within each preparation (mean±s.e.mean, *n*=3–4 for a, *n*=3 for b).

(20 mM)-induced Ca²⁺ release (Figure 6a). On the other hand, caffeine-induced Ca²⁺ release (Caf, 20 mM) was not significantly inhibited by these derivatives, although the rates appeared slightly reduced (Figure 6b).
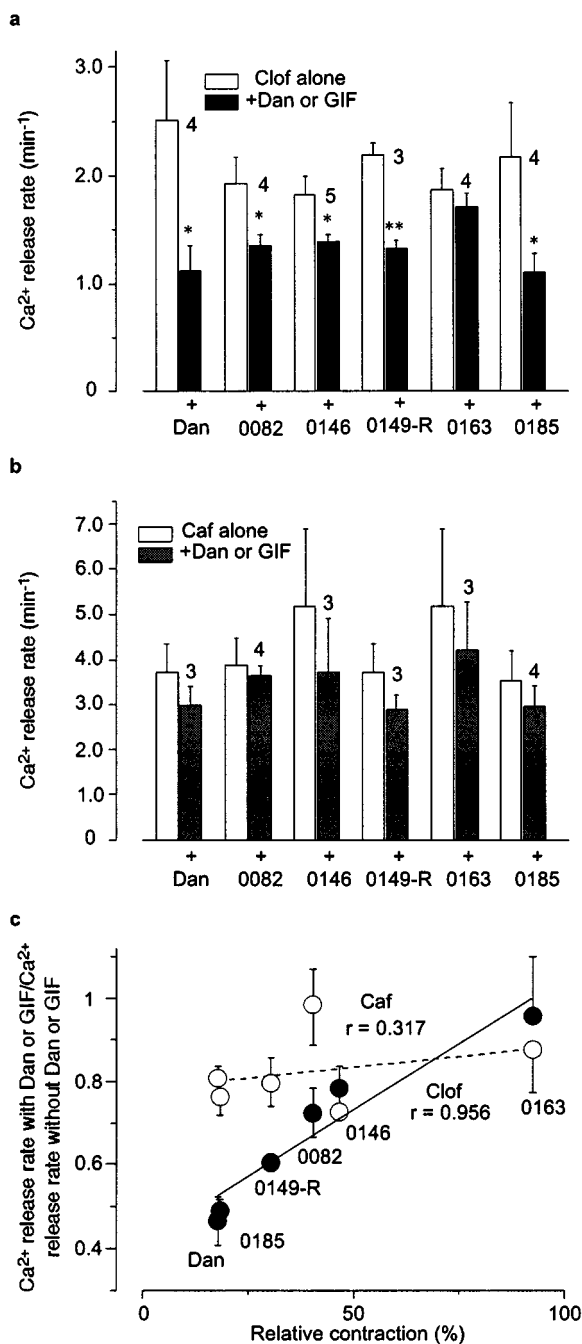
Figure 6 Correlation between the rates of Ca²⁺ release in skinned fibres and twitch contraction in the presence of Dan derivatives. In skinned fibres, the rates of Ca²⁺ release with Dan (20 μM) or GIFs (50 μM) were measured at pCa 7.0 with 20 mM Clof (a) or caffeine (Caf, b) in the test solution containing 0.5 mM Mg²⁺ and 10 mM AMP (mean±s.e.mean, $n=3-5$ for a, $n=3-4$ for b). (c) These results were normalized to the control value (without Dan or its derivatives) and plotted against the mean value of relative twitch contraction at 20 μM Dan or 50 μM other derivatives from Figures 2a and 5a. The data for Caf or Clof were fitted by a least square method, and the correlation coefficients were 0.317 and 0.956, respectively. *$P<0.05$, **$P<0.01$.

The extent of reduction of the rates of Clof-induced or caffeine-induced Ca²⁺ release in skinned fibres by Dan and its derivatives was compared with that of the twitch tension in intact fibres (Figure 6c). The magnitude of inhibition of twitch contraction was in good agreement with that of the inhibition of Clof-induced Ca²⁺ release (Figure 6c, filled circles), while no correlation was seen between the twitch tension and the rate of caffeine-induced Ca²⁺ release (Figure 6c, open circles).

In conclusion, we showed that some Dan derivatives specifically inhibited PCR and Clof-induced Ca²⁺ release without appreciable inhibition of CICR.

Discussion

We analysed the effects of Dan and some newly synthesized derivatives on twitch tension (indicating PCR) of intact fibres and CICR in skinned fibres of mouse skeletal muscle. We demonstrated that several Dan derivatives did not inhibit CICR at all, but definitely inhibited twitch tension (Figures 2 and 4). Since twitch inhibition of these Dan derivatives is most likely to be the result of their action on the Ca²⁺-release mechanism of the SR, they appear to distinguish PCR and CICR clearly.

The notion that these Dan derivatives directly affect the Ca²⁺ release mechanism of the SR was supported by the fact that they inhibited Clof-induced Ca²⁺ release in skinned fibres (Figures 3, 4, and 6). In the accompanying study, we demonstrated that Clof-induced Ca²⁺ release has some features similar to those of PCR (Ikemoto & Endo, 2001, accompanying study). The results of the present report provide a further similarity: the magnitude of twitch inhibition by Dan and its derivatives that is most likely to indicate the magnitude of PCR inhibition is very closely correlated with the magnitude of inhibition of Clof-induced Ca²⁺ release, but not with that of CICR inhibition (Figure 6).

Difference of Dan effect on CICR at room temperature between the present and previous results

We showed that the CICR in skinned fibres from mouse skeletal muscle was significantly inhibited by Dan (20 μM) at room temperature, which is in disagreement with the previous report (Ohta *et al.*, 1990). The inhibitory effect of Dan on CICR was reduced by increasing Mg²⁺ concentrations, and it was abolished in the presence of 0.5 mM Mg²⁺ (unpublished observation), and some of the previous results were obtained in presence of Mg²⁺ (>0.5 mM) (Ohta & Endo, 1986; Ohta *et al.*, 1990). However, Ohta *et al.* (1990) reported that Dan (50 μM) showed no inhibitory effect on CICR in skinned fibres of skeletal muscles from guinea-pigs even in the absence of Mg²⁺ at room temperature (20°C). The reason for the discrepancy is presently unknown. It may be a consequence of the difference in animal species used.

Usefulness of Dan and its derivatives on the study of Ca²⁺ release

Recently, Dan has been used in a wide variety of cells other than skeletal muscles as an inhibitor of intracellular Ca²⁺

release (Hui, 1983; Shoshan-Barmatz *et al.*, 1991; Partridge & Valenzuela, 1999; Seutin *et al.*, 2000). However, the exact mechanism of action of Dan has remained unclear, not only in skeletal muscles, but also in other types of cells. For example, it is not clear whether the inhibitory action of Dan on Ca^{2+} release results from direct binding to the RyR (Parness & Palnitkar, 1995; Palnitkar *et al.*, 1997; Fruen *et al.*, 1997). Therefore, it is important to elucidate the molecular mechanism of Dan-induced inhibition of the Ca^{2+} release *via* the RyR.

Some of the Dan derivatives we synthesized inhibited PCR without an inhibitory effect on CICR. They inhibited Clof-induced Ca^{2+} release in skinned fibres, an effect that was significantly correlated with their inhibitory effect on twitch tension (Figure 6c). In addition, GIF-0185 diminished the inhibition by Dan on CICR, although it showed no inhibitory effect on CICR (Figures 2b and 3a). These results

indicate that the target(s) of Dan and its derivatives are clearly retained in skinned fibres. Among these specific PCR inhibitors at room temperature ($21-23°C$), GIF-0082, with its azido-functionalized and iodinated structure, is applicable to photo-affinity and its binding experiment on target molecule(s). Since GIF-0082 does not interfere with the inhibitory action of Dan on CICR (unlike GIF-0185), it might well be more specific to PCR than GIF-0185. We, therefore, think that this agent may be a useful probe to study PCR of skeletal muscles in various preparations including isolated SR membranes and the purified RyR.

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