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4, 6-Dibromo-3-hydroxycarbazole (an analogue of caffeine-like Ca^{2+} releaser), a novel type of inhibitor of Ca^{2+}-induced Ca^{2+} release in skeletal muscle sarcoplasmic reticulum

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1 4,6-Dibromo-3-hydroxycarbazole (DBHC) was synthesized as an analogue of bromoeudistomin D (BED), a powerful Ca^{2+} releaser, and its pharmacological properties were examined.

2 In Ca^{2+} electrode experiments, DBHC (100 μM) markedly inhibited Ca^{2+} release from the heavy fraction of sarcoplasmic reticulum (HSR) induced by caffeine (1 mM) and BED (10 μM).

3 DBHC (0.1 to 100 μM) inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+} from HSR in a concentration-dependent manner.

4 DBHC (100 μM) abolished $^{45}Ca^{2+}$ release induced by caffeine (1 mM) and BED (10 μM) in HSR.

5 Inhibitory effects of calcium-induced calcium release (CICR) blockers such as procaine, ruthenium red and Mg^{2+} on $^{45}Ca^{2+}$ release were clearly observed at Ca^{2+} concentrations from pCa 7 to pCa 5.5, and were decreased at Ca^{2+} concentrations higher than pCa 5.5 or lower than pCa 7. However, DBHC decreased Ca^{2+} release induced by Ca^{2+} over the wide range of extravesicular Ca^{2+} concentrations.

6 [^{3}H]-ryanodine binding to HSR was suppressed by ruthenium red, Mg^{2+} and procaine, but was not affected by DBHC up to 100 μM.

7 [^{3}H]-ryanodine binding to HSR was enhanced by caffeine and BED. DBHC antagonized the enhancement in a concentration-dependent manner.

8 9-[^{3}H]-Methyl-7-bromo-eudistomin D, an ^{3}H-labelled analogue of BED, specifically bound to HSR. Both DBHC and caffeine increased the K_D value without affecting the B_{max} value, indicating a competitive mode of inhibition.

9 These results suggest that DBHC binds to the caffeine binding site to block Ca^{2+} release from HSR. This drug is a novel type of inhibitor for the CICR channels in SR and may provide a useful tool for clarifying the Ca^{2+} releasing mechanisms in SR.

Keywords: 4,6-Dibromo-3-hydroxycarbazole; bromoeudistomin D; caffeine; skeletal muscle; sarcoplasmic reticulum; Ca^{2+}-induced Ca^{2+} release; ruthenium red; procaine; Mg^{2+}; ryanodine binding

Introduction

Ca^{2+} release from the sarcoplasmic reticulum (SR) plays a key role in excitation-contraction coupling (EC-coupling) in skeletal muscle (Ford & Podolsky, 1972; Endo, 1977; Endo *et al.*, 1981; Kirino & Shimizu, 1982; Ebashi, 1991). It is well known that ryanodine, a plant alkaloid, promotes Ca^{2+} release from skeletal and cardiac SR and interferes with the inactivation of Ca^{2+}-induced Ca^{2+} release (CICR) from SR (Meissner, 1986). The alkaloid binds with high affinity to a receptor localized in the heavy fraction of sarcaplasmic reticulum (HSR) (Fleischer *et al.*, 1985). The purified ryanodine receptor (Inui *et al.*, 1987; Imagawa *et al.*, 1987; Lai *et al.*, 1988; Wagenknecht *et al.*, 1989) is identical in morphology with the 'feet' structures that span the transverse tubule-SR junction and form caffeine-sensitive Ca^{2+} channels. It has been reported that ryanodine locks the Ca^{2+} release channels of SR in an open state and that its high affinity binding site is localized in terminal cisternae of SR (Fleischer *et al.*, 1985). These studies revealed that the ryanodine receptor is identical with CICR channels of SR (McPherson & Campbell, 1993; Sorrentino & Volpe, 1993). One of the useful approaches that may achieve a better understanding of the molecular mechanism of Ca^{2+} release is the application of specific drugs that affect the releasing mechanisms.

It has been reported that caffeine increases the Ca^{2+} sensitivity of CICR channels (Nagasaki & Kasai, 1984; Endo, 1985) and the open probability of the channels at saturating Ca^{2+} concentrations (Rousseau *et al.*, 1988). Numerous studies using skinned skeletal muscle fibres and isolated SR membrane preparations have revealed the presence of a caffeine-sensitive Ca^{2+} release pathway through CICR channels (Kim *et al.*, 1983). However, the characterization of the caffeine receptor site in Ca^{2+} release channels has not been possible because of its low affinity and the detailed molecular mechanism of Ca^{2+} release from SR remains unresolved. We have reported that bromoeudistomin D (BED), a derivative of eudistomin D isolated from the Caribbean tunicate *Eudistoma olivaceum*, induces Ca^{2+} release from the heavy fraction of SR (HSR) (Nakamura *et al.*, 1986). Our pharmacological studies indicate that BED is approximately 500 times more potent than caffeine in Ca^{2+} releasing activity. For the purpose of finding the inhibitor in order to investigate the function of CICR channels, numerous analogues of BED were synthesized (Kobayshi *et al.*, 1984; 1989; Takahashi *et al.*, unpublished observations). Here we present the first report that 4,6-dibromo-3-hydroxycarbazole (DBHC), one of these analogues, is a novel type of CICR-channel inhibitor having properties different from those of typical inhibitors such as procaine, ruthenium red and Mg^{2+}.

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Methods

Preparation of SR vesicles

HSR was prepared from skeletal muscle of male rabbits (Japanese white rabbits weighing about 2–3 kg), according to the method of Kim *et al.* (1983) in the presence of protease inhibitors: aprotinin (76.8 mM), *p*-APMSF (0.1 mM) and benzamidine (0.83 mM). Rabbit white muscle was homogenized in 5 volumes of 5 mM Tris-maleate (pH 7.0) and centrifuged at 5000 *g* for 15 min. The supernatant was centrifuged at 12 000 *g* for 30 min. The pellet was suspended in 5 mM Tris-maleate (pH 7.0) containing 90 mM KCl and centrifuged at 70 000 *g* for 40 min. The pellet (the HSR) was resuspended. These procedures were performed at 0–4°C. HSR was used within four days. The protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Ca^{2+} electrode experiments

The concentration of extravesicular Ca^{2+} in the HSR suspension was measured at 30°C with a Ca^{2+} electrode as described previously (Seino *et al.*, 1991). The Ca^{2+} electrode showed Nernstian response (slope, 27–29 mV pCa^{-1} unit) in the calibration buffer containing Ca^{2+}-EGTA between pCa decreased from 6 to 4. The assay solution (final volume, 1 ml) contained 0.05 mM $CaCl_2$, 90 mM KCl, 0.25 mM $MgCl_2$, 50 mM MOPS-Tris (pH 7.0), 1 mg ml^{-1} of HSR, 5 mM creatine phosphate, 0.13 mg ml^{-1} of creatine kinase and 0.5 mM ATP. The Ca^{2+} uptake reaction was initiated by simultaneous addition of creatine kinase and ATP.

$^{45}Ca^{2+}$ release measurement

$^{45}Ca^{2+}$ release from HSR passively preloaded with $^{45}Ca^{2+}$ was measured at 0°C according to the method of Nakamura *et al.* (1986). HSR (20 mg ml^{-1}) was preincubated in a solution containing 5 mM $^{45}CaCl_2$ at 0°C over 12 h. $^{45}Ca^{2+}$ release was started by diluting the solution 100 fold with the reaction medium containing various concentrations of Ca^{2+} or different reagents. Free Ca^{2+} concentration was controlled with Ca^{2+}-EGTA buffer. The calculation of free Ca^{2+} was accomplished by a computer programme (Fabiato *et al.*, 1979). The reaction was stopped by adding the solution containing 5 mM $LaCl_3$ and 5 mM $MgCl_2$ at an appropriate time interval. The mixture was filtered through Millipore Filters (HAWP type, 0.45 μm pore size) and washed with 10 times its volume solution containing 5 mM $LaCl_3$ and 5 mM $MgCl_2$. The amounts of $^{45}Ca^{2+}$ in HSR were measured by counting the radioactivity remaining on the filters. The reaction medium contained (mM): $CaCl_2$ 0.5, KCl 90, 4-morpholinepropanesulphonic acid (MOPS) 50 at pH 7.0.

[^{3}H]-ryanodine and 9-[^{3}H]-methyl-7-bromoeudistomin D binding assay

[^{3}H]-ryanodine binding to HSR was measured according to the method of Seino *et al.* (1991). HSR (300 μg ml^{-1}) was incubated with 10 nM [^{3}H]-ryanodine in the reaction medium at 37°C for 45 min. Bound and free [^{3}H]-ryanodine were separated by filtration through glass fibre filters (Whatman) under reduced pressure. The filters were washed five times with 2 ml of ice-cold water. Radioactivity that remained on the filter was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM unlabelled ryanodine. The reaction medium contained (mM): sucrose 300, NaCl 1000, dithiothreitol 2, $CaCl_2$ 0.2, EGTA 0.192, *p*-APMSF 0.1 and HEPES/Tris 20 at pH 7.4 and 37°C.

9-[^{3}H]-methyl-7-bromoeudistomin D ([^{3}H]-MBED) binding experiments were performed by the method of Fang *et al.* (1993). HSR (300 μg ml^{-1}) was incubated with various concentrations of [^{3}H]-MBED in the reaction medium at 0°C for 45 min. Separation of bound and free [^{3}H]-MBED was carried out as described above. Nonspecific binding was determined in the presence of 5 μM unlabelled MBED. The reaction medium contained (mM): sucrose 300, dithiothreitol 2, $CaCl_2$ 0.2, EGTA 0.192, *p*-APMSF 0.1 and HEPES/Tris 20 at pH 7.4 and 37°C.

Drugs

DBHC (Figure 1) was synthesized as follows. 3-Methoxycarbazole was synthesized by bromination of carbazole with bromine in pyridine followed by methoxylation with NaOMe/MeOH and dimethylformamide catalyzed by CuI. DBHC was synthesized by demethylation of 3-methoxycarbazole with BBr_3 in CH_2Cl_2 followed by bromination with NBS in acetic acid. ^{1}H NMR (acetone-d_6) δ10.56

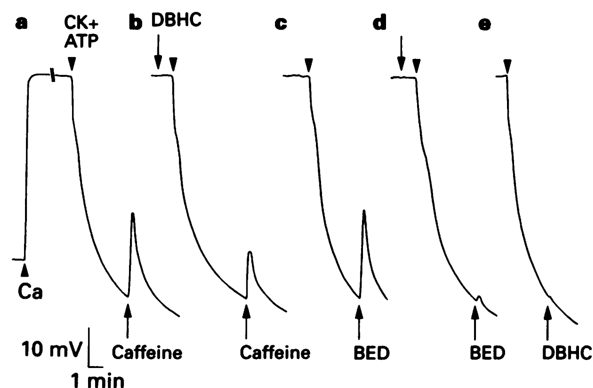

Figure 2 Effects of the preincubation of HSR with DBHC on Ca^{2+} release induced by caffeine (1 mM) and BED (10 μM). The concentrations of extravesicular Ca^{2+} were monitored at 30°C with a Ca^{2+} electrode in the assay solution containing 0.05 mM $CaCl_2$, 90 mM KCl, 0.25 mM $MgCl_2$, 50 mM MOPS-Tris (pH 7.0), 1 mg ml^{-1} of HSR, 5 mM creatine phosphate, 0.13 mg ml^{-1} of creatine kinase (CK) and 0.5 mM ATP. After addition of 0.05 mM $CaCl_2$, the reaction of Ca^{2+} uptake was started by a simultaneous addition of CK and ATP. Vertical calibration bar indicates a response for voltage change (10 mV) corresponding to 0.5 pCa unit. In (b) to (d) DBHC (100 μM) was added before addition of CK and ATP. For abbreviations in this and subsequent figures, please see text.

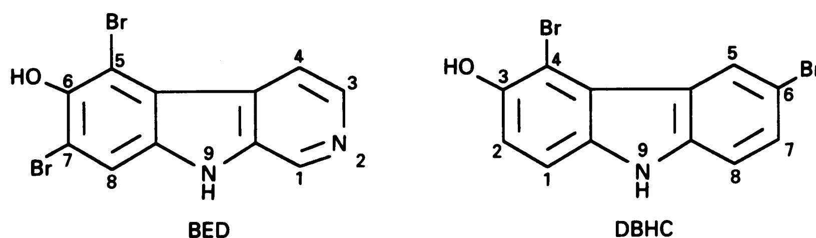

Figure 1 Chemical structure of bromoeudistomin D (BED) and 4,6-dibromo-3-hydroxycarbazole (DBHC).

(1H, br, s), 8.82 (1H, s), 7.55 (3H, m) and 7.18 (1H, d, $J = 8.8$ Hz); EIMS m/z 343, 341 and 339 (M+); Found m/z 338.8899, Calcd for $C_{12}H_7ON^{79}Br_2$: M, 338.8895. BED and MBED were synthesized as reported previously (Kobayashi *et al.*, 1988). [^{3}H]-MBED was synthesized as described by Fang *et al.* (1993). $^{45}CaCl_2$ (1 Ci ml^{-1}) and [9, 21-^{3}H(N)]-ryanodine (54.7 Ci mmol^{-1}) were purchased from Du Pont New England Nuclear, Boston, MA, U.S.A. Ryanodine was purchased from S.B. Penick Company, New York, NY, U.S.A. Caffeine, procaine hydrochloride, and ruthenium red were purchased from Wako Pure Chemical Industries, Osaka, Japan. Other reagents used were of analytical grade.

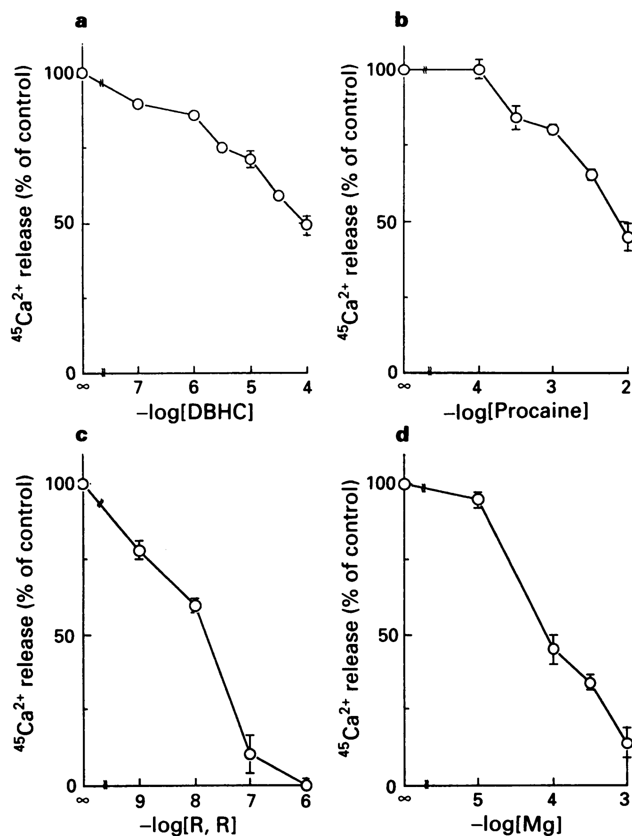

Figure 3 Effects of CICR inhibitors on $^{45}Ca^{2+}$ release induced by Ca^{2+} from skeletal muscle HSR. $^{45}Ca^{2+}$ release was measure at pCa 7.0, as described under Methods. The $^{45}Ca^{2+}$ content in HSR was measured at 0°C by the filtration method after 100 fold dilution of HSR (20 mg ml^{-1}) passively preloaded with $^{45}CaCl_2$ (5 mM) into Ca^{2+}-EGTA buffer medium. The initial $^{45}Ca^{2+}$ content of HSR was obtained by using the release medium containing 5 mM $LaCl_3$ and 5 mM $MgCl_2$. The Ca^{2+} release activity was calculated from the decrease in the $^{45}Ca^{2+}$ content in HSR vesicles during 1 min after dilution. $^{45}Ca^{2+}$ release activity was normalized against that in the absence of the inhibitors. (a) DBHC, (b) procaine, (c) ruthenium red (RR) and (d) Mg^{2+}. Data are mean ± s.e.mean (bars) values ($n = 6$).

Statistical comparison

Results of the experiments are expressed as mean ± s.e.mean. Student's t test and paired t test were used for statistical analysis of the results.

Results

Effects of DBHC on Ca^{2+} release from skeletal muscle HSR

In the course of our survey of CICR inhibitors in natural products and their derivatives, we have succeeded in finding DBHC. In electrode experiments, preincubation of DBHC

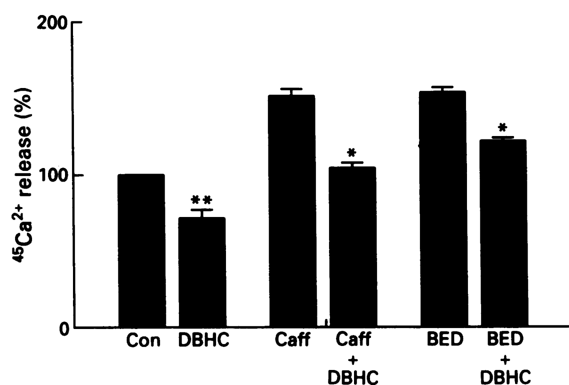

Figure 5 Effects of DBHC on $^{45}Ca^{2+}$ release induced by Ca^{2+}, caffeine (caff) and BED. $^{45}Ca^{2+}$ release was measured as described under Methods. The amount of released $^{45}Ca^{2+}$ was calculated from the decrease in the $^{45}Ca^{2+}$ content in HSR during 1 min after dilution at pCa 7.0. The concentrations of caffeine, BED and DBHC were 1 mM, 10 μM and 100 μM, respectively. Data are mean ± s.e.mean ($n = 6$). **Significantly ($P < 0.05$) different from the control values. *Significantly ($P < 0.05$) different from the values in the presence of caffeine or BED alone.

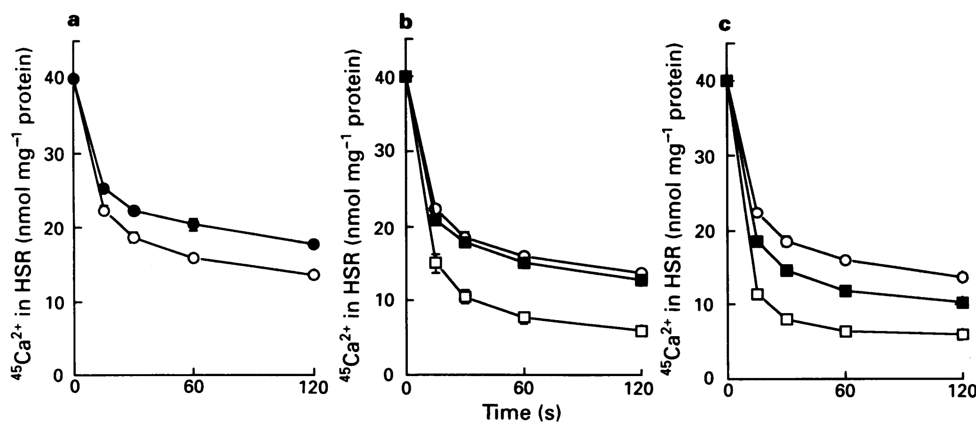

Figure 4 Effects of DBHC on the time course of the $^{45}Ca^{2+}$ release induced by Ca^{2+} (a), caffeine (b) and BED (c) from skeletal muscle HSR at pCa 7.0. The time course of the decrease in the $^{45}Ca^{2+}$ content in HSR was measured after 100 fold dilution of HSR (20 mg ml^{-1}) passively preloaded with $^{45}CaCl_2$ (5 mM) into Ca^{2+}-EGTA buffer medium. (a) Control (○), 100 μM DBHC (●); (b) control (○), 1 mM caffeine (□), 1 mM caffeine and 100 μM DBHC (■); (c) control (○), 10 μM BED (□), 10 μM BED and 100 μM DBHC (■). Data are mean ± s.e.mean values ($n = 6$).

(100 μM) with HSR resulted in the marked decrease in Ca^{2+} release induced by caffeine (1 mM) or BED (10 μM) (Figure 2a–2d). DBHC alone (100 μM) did not induce Ca^{2+} release (Figure 2e). Interestingly, DBHC was synthesized as an analogue of BED, a powerful inducer of CICR with caffeine like properties (Nakamura et al., 1993). Therefore, the inhibitory effect of DBHC was investigated in comparison with the representative CICR inhibitors such as procaine, ruthenium red and Mg^{2+}. As shown in Figure 3, DBHC and these CICR inhibitors inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+} from HSR in a dose-dependent manner. Their IC_{50} values were 30 μM, 3.5 mM, 15 nM and 58 μM for DBHC,

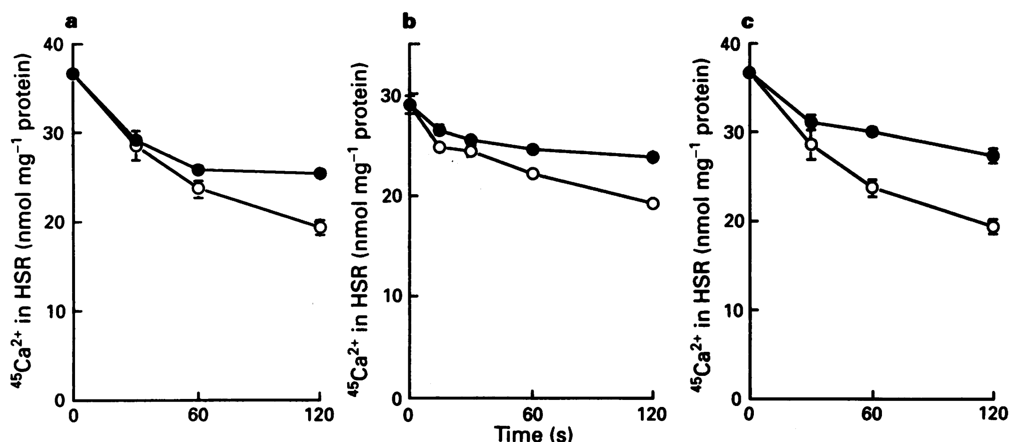

Figure 6 Effects of CICR inhibitors in the time course of the $^{45}Ca^{2+}$ release induced by Ca^{2+} from skeletal muscle HSR at pCa 7.0. The time course of the decrease in the $^{45}Ca^{2+}$ content in HSR vesicles was measured after 100 fold dilution of 20 mg ml^{-1} HSR preloaded with 5 mM $^{45}CaCl_2$ into Ca^{2+}-EGTA buffer medium: (○) control; (●) 3 mM procaine (a) or 30 nM ruthenium red (b) or 100 μM Mg^{2+} (c). Data are mean ± s.e.mean ($n = 6$).

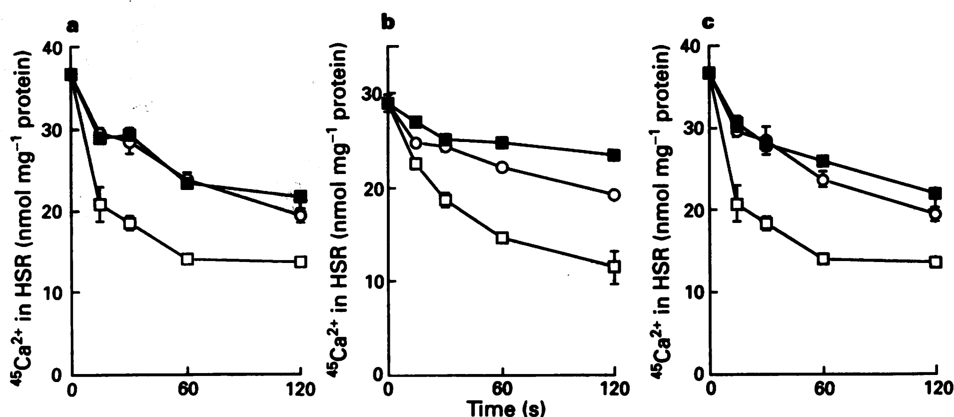

Figure 7 Effects of CICR inhibitors on the time course of the $^{45}Ca^{2+}$ release induced by caffeine from skeletal muscle HSR at pCa 7.0. Experimental protocol was similar to that described in Figure 3: (○) control; (□) 1 mM caffeine; (■) 3 mM procaine and 1 mM caffeine (a) or 30 nM ruthenium red and 1 mM caffeine (b) or 100 μM Mg^{2+} and 1 mM caffeine (c). Data are mean ± s.e.mean ($n = 3$).

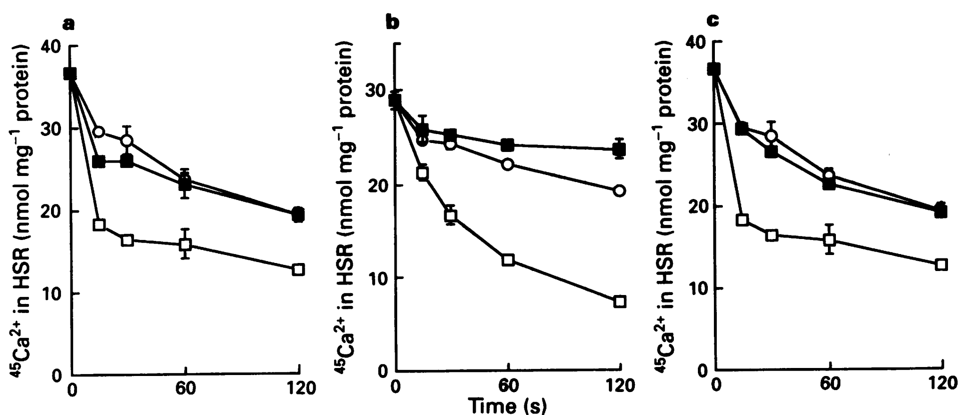

Figure 8 Effects of CICR inhibitors on the time course of the $^{45}Ca^{2+}$ release induced by BED from skeletal muscle HSR at pCa 7.0. Experimental protocol was similar to those described in Figure 3: (○) control; (□) 10 μM BED; (■) 3 mM procaine and 10 μM BED (a) or 30 nM ruthenium red and 10 μM BED (b) or 100 μM Mg^{2+} and 10 μM BED (c). Data are mean ± s.e.mean ($n = 3$).

procaine, ruthenium red and Mg^{2+}, respectively. Figure 4 depicts the time courses of inhibitory effects of DBHC on $^{45}Ca^{2+}$ release induced by Ca^{2+} (Figure 4a), caffeine (Figure 4b) or BED (Figure 4c) at the extravesicular Ca^{2+} concentration of 0.1 μM. The maximum inhibitory response to DBHC (100 μM) of $^{45}Ca^{2+}$ release induced by Ca^{2+} (0.1 μM) was obtained at 30 s and those by caffeine (1 mM) and BED (10 μM) at 15 s. Figure 5 shows that DBHC significantly inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+}, caffeine and BED by approximately 25–35% at 60 s. CICR inhibitors, such as procaine (3 mM), ruthenium red (30 nM) and Mg^{2+} (100 μM) also inhibited $^{45}Ca^{2+}$ release induced by Ca^{2+} (Figure 6), caffeine (Figure 7) or BED (Figure 8).

DBHC (30 μM) maintained its inhibitory effect on $^{45}Ca^{2+}$ release induced by Ca^{2+} over the wide range from pCa 7.0 to pCa 4.0 (Figure 9a). On the other hand, procaine (3 mM), ruthenium red (30 nM) and Mg^{2+} (30 μM) powerfully suppressed Ca^{2+} release Ca^{2+} concentrations from pCa 7 to pCa 5.5, whereas the effects were decreased at Ca^{2+} concentrations higher than pCa 5.5 or lower than pCa 7 (Figure 9b–d).

Effects of CICR inhibitors on [³H]-ryanodine binding to HSR

[³H]-ryanodine binding to HSR was inhibited by procaine (Figure 10b), ruthenium red (Figure 10c) and Mg^{2+} (Figure 10d) in a concentration-dependent manner with IC_{50} values of 8.4 mM, 1.5 μM and 34 mM, respectively. However, DBHC had no effect on [³H]-ryanodine binding to HSR up to 100 μM (Figure 10a). It is well known that [³H]-ryanodine binding is dependent on Ca^{2+} concentrations. Figure 11 depicts the effects of DBHC and procaine on [³H]-ryanodine binding to HSR at various Ca^{2+} concentrations. In the absence of these inhibitors, [³H]-ryanodine binding increased with increase in Ca^{2+} concentrations and reached a plateau at about pCa 5. Procaine suppressed the binding over a wide range of Ca^{2+} concentrations (pCa 8–pCa 5), whereas DBHC had no inhibitory effect.

Ca^{2+} releasers such as caffeine and MBED increase the amount of [³H]-ryanodine bound to CICR (Seino *et al.*, 1991). [³H]-ryanodine binding increased by 21% and 41% in the presence of 2 mM caffeine and 5 μM BED, respectively (Figure 12). DBHC antagonized the enhancement of the binding in a concentration-dependent manner, suggesting that the DBHC binds to the caffeine/BED binding site in HSR.

Effects of DBHC on [³H]-MBED binding to HSR

We have recently reported that 9-[³H]-methyl-7-bromoeudistomin D ([³H]-MBED), an ³H-labelled analogue of BED shares the same binding site as that of caffeine in terminal cisternae of skeletal muscle SR (Fang *et al.*, 1993). As shown in Figure 13a, specific binding of [³H]-MBED to HSR was saturable and of high affinity. Scatchard analysis showed that [³H]-MBED bound to a high affinity receptor site with $K_D = 39.5$ nM and $B_{max} = 6.1$ pmol mg^{-1} (Figure 13b). Both

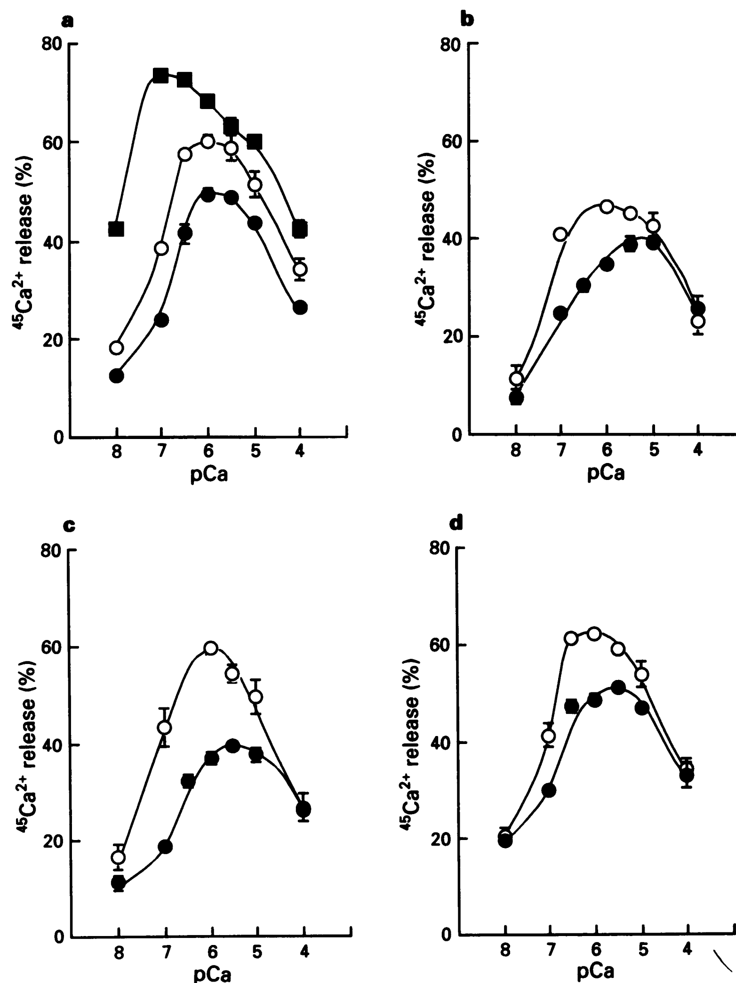

Figure 9 Inhibitory effects of CICR inhibitors on $^{45}Ca^{2+}$ release at various Ca^{2+} concentrations. $^{45}Ca^{2+}$ release at various concentrations of free Ca^{2+} was measured during 1 min after dilution. Each value was normalized against the amount of $^{45}Ca^{2+}$ in HSR at zero time. (a) Control (○), 100 μM DBHC (●), 10 μM BED (■). (b) Control (○), 3 mM procaine (●). (c) Control (○), 30 nM ruthenium red (●). (d) Control (○), 30 μM Mg^{2+} (●). Data are mean ± s.e.mean ($n = 6$).

caffeine (0.5 mM) and DBHC (50 μM) increased the K_D from 39.5 to 64.7 and 94.2 nM, respectively, without affecting the B_{max} value, indicating a competitive mode of interaction between [^{3}H]-MBED and either caffeine or DBHC. These results suggest that DBHC binds to the caffeine/BED-binding site to reduce Ca^{2+} release from HSR.

Discussion

Many compounds are known to inhibit CICR (McPherson & Campbell, 1993). Procaine, ruthenium red and Mg^{2+} are representative inhibitors of CICR. Inhibitory effects of procaine, ruthenium red and Mg^{2+} on $^{45}Ca^{2+}$ release from HSR were dependent on the extravesicular Ca^{2+} concentrations, whereas that of DBHC was not dependent on them. It has been reported that procaine binds to the site which influences the Ca^{2+} sensitivity of the Ca^{2+} regulatory site and that Mg^{2+} inhibits the Ca^{2+}-gated open state of the channels by direct competition with Ca^{2+} at the Ca^{2+}-regulatory site (Pessah et al., 1987). Ruthenium red having a large positive charge (+6) has been reported to bind to the Ca^{2+}-binding site of SR (Corbalan-Garcia et al., 1992), suggesting that the ruthenium red binding site is the Ca^{2+}-binding site in CICR

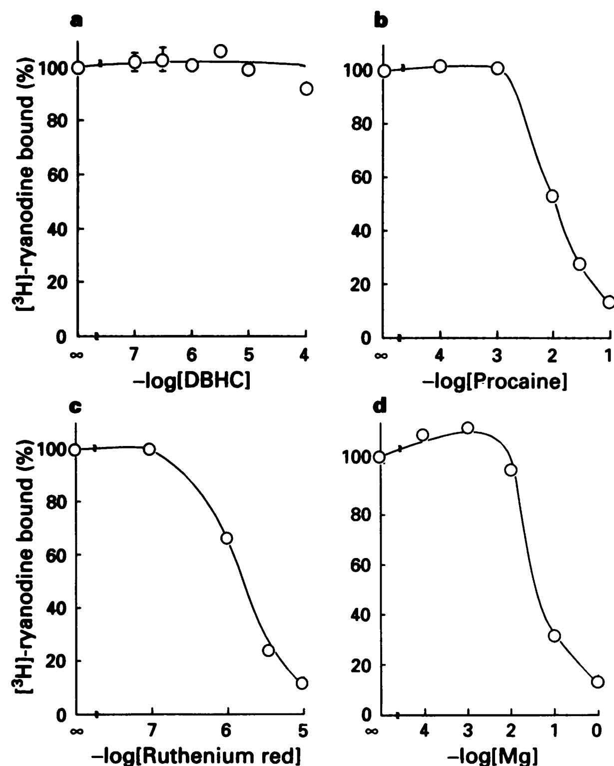

Figure 10 Effects of CICR inhibitors on [^{3}H]-ryanodine binding to skeletal muscle HSR. HSR (300 μg ml^{-1}) was incubated with 10 nM [^{3}H]-ryanodine and CICR inhibitors at 37°C for 45 min in a solution containing 0.3 M sucrose, 1 M NaCl, 10 μM $CaCl_2$, 2 mM DTT, 100 μM p-APMSF and 20 mM HEPES-Tris (pH 7.4). Nonspecific binding was determined in the presence of 10 μM unlabelled ryanodine. (a) DBHC, (b) procaine, (c) ruthenium red, (d) Mg^{2+}. Data are mean ± s.e.mean (n = 4).

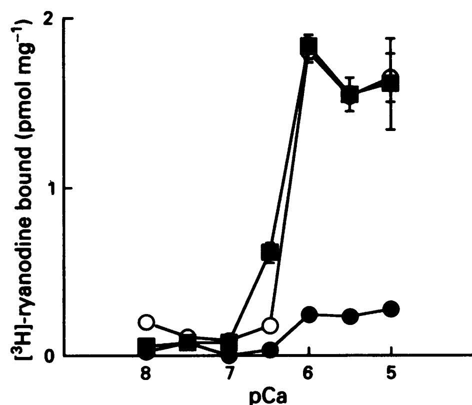

Figure 11 Effects of DBHC and procaine on [^{3}H]-ryanodine binding at various Ca^{2+} concentrations. HSR (300 μg ml^{-1}) was incubated with 10 nM [^{3}H]-ryanodine for 45 min at various Ca^{2+} concentrations in the absence (■) or presence of DBHC (100 μM) (○) or procaine (10 mM) (●). Data are mean ± s.e.mean (n = 6).

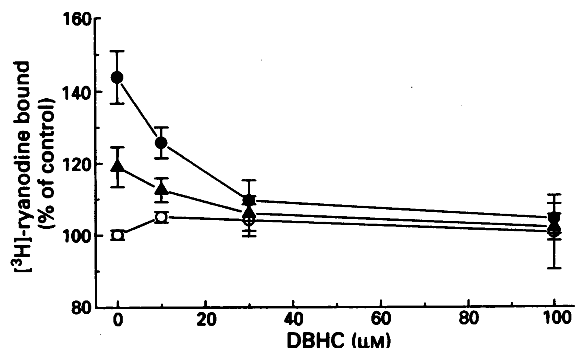

Figure 12 Effects of DBHC on the [^{3}H]-ryanodine binding to HSR enhanced by caffeine (▲) or BED (●). HSR (300 μg ml^{-1}) were incubated with 10 nM [^{3}H]-ryanodine at 37°C for 45 min in the presence or absence of either caffeine (2 mM) or BED (5 μM) in the solution containing 0.3 M sucrose, 1 M NaCl, 10 μM $CaCl_2$, 2 mM DTT, 100 μM p-APMSF and 20 mM HEPES-Tris (pH 7.4).

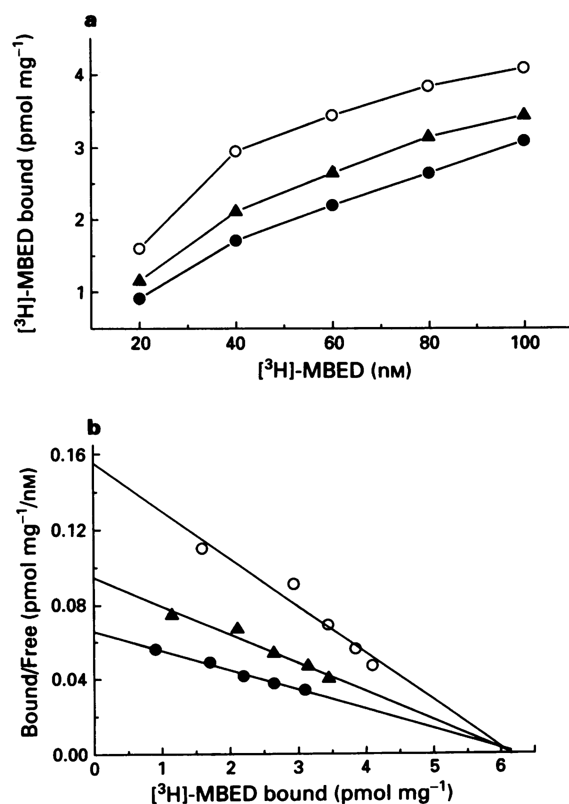

Figure 13 Effects of DBHC and caffeine on [^{3}H]-MBED binding. HSR (300 μg ml^{-1}) was incubated with increasing concentrations of [^{3}H]-MBED from 20 to 100 nM for 45 min at 0°C. (a) [^{3}H]-MBED binding was measured in the presence or absence (○) of 50 μM DBHC (●) or 0.5 mM caffeine (▲) and is plotted. (b) [^{3}H]-MBED binding in (a) is presented as a Scatchard plot.

channels of SR. These reports and our results suggest that the inhibitory effects of ruthenium red, procaine and Mg^{2+} on CICR are modified at high extravesicular Ca^{2+} concentrations because these drugs bind to the Ca^{2+} binding site or the site influenced by Ca^{2+} and that the binding site of DBHC which is thought to be the caffeine binding site is different from those of procaine, ruthenium red and Mg^{2+}. This may be the reason why the inhibitory effects of procaine, ruthenium red and Mg^{2+} on CICR channels are suppressed at high Ca^{2+} concentrations. DBHC is a novel type of CICR inhibitors having unique pharmacological properties.

The activity of the ryanodine receptor/Ca^{2+} releasing channels are modulated by various compounds. Ryanodine and Ca^{2+} have dual effects, *i.e.*, they activate the channels at low concentrations and inhibit them at high concentrations (Meissner, 1986). The binding of [^{3}H]-ryanodine to the Ca^{2+} release channels is enhanced by the compounds which activate the channels and inhibited by other agents which reduce the channel activity (Pessah *et al.*, 1987; Michalak *et al.*, 1988). Our results are in agreement with a previous observation that CICR activators such as caffeine and BED increase [^{3}H]-ryanodine binding to HSR and CICR inhibitors such as procaine, ruthenium red and Mg^{2+} decrease it (Imagawa *et al.*, 1987; Pessah *et al.*, 1987; Seino *et al.*, 1991). The inhibitory effect on [^{3}H]-ryanodine binding was strongly suppressed by Ca^{2+} (Figure 11). However, DBHC (0.1 to 100 μM) did not affect [^{3}H]-ryanodine binding. Enhancement of [^{3}H]-ryanodine binding to HSR by caffeine or BED was inhibited by DBHC, probably indicating the interference of caffeine/BED binding by DBHC.

It has been recognised that caffeine activates CICR channels of skeletal muscle SR (Endo, 1977). Caffeine potentiates Ca^{2+} sensitivity of CICR channels (Endo, 1975), increases [^{3}H]-ryanodine binding to HSR (Pessah *et al.*, 1987) and induces a contraction of the chemically skinned fibres from skeletal muscle (Nakamura *et al.*, 1986). We have found that BED is approximately 500 times more potent than caffeine in Ca^{2+}-releasing activity (Nakamura *et al.*, 1986). BED has pharmacological properties similar to those of caffeine in its action such as potentiating Ca^{2+} sensitivity (Figure 9a), increasing [^{3}H]-ryanodine binding (Figure 12) and inducing the contractions of skinned fibres (Nakamura *et al.*, 1986). Interestingly, our present studies indicate that DBHC, an analogue of BED inhibits $^{45}Ca^{2+}$ release from HSR induced Ca^{2+}, caffeine or BED and that DBHC blocks [^{3}H]-MBED binding in a competitive mode. On the basis of our observations, it is suggested that DBHC binds to the caffeine/BED binding site to block Ca^{2+} release from HSR. DBHC has been revealed to be a CICR inhibitor with novel pharmacological properties and may provide valuable information about the functions of CICR channels and excitation-contraction (E-C) coupling of skeletal muscle.

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