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Structure–Activity Relationships for the Ca^{2+}-releasing Activity of 6-Hydroxy-β-carboline Analogues in Skeletal Muscle Sarcoplasmic Reticulum—The Effects of Halogen Substitution at C-5 and C-7

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Abstract

This study of structure–activity relationships of 6-hydroxy-β-carboline analogues has been performed on the basis of quantitative measurement of Ca^{2+}-releasing activity in the sarcoplasmic reticulum of skinned fibres of skeletal muscle.

Substitution of halogens for hydrogens at the C-5 and C-7 positions and further introduction of a methyl group into the N-9 position of 6-hydroxy-β-carboline resulted in Ca^{2+}-releasing activity. The 50% effective concentrations of 5,7-dibromoeudistomin D, 5,7-dichloroeudistomin D, 5,7-diiodoeudistomin D, 9-methyl-5,7-dibromoeudistomin D, 9-methyl-5,7-dichloroeudistomin D, 9-methyl-5,7-diiodoeudistomin D, and caffeine were $5{\cdot}6 \times 10^{-6}$, $6{\cdot}3 \times 10^{-6}$, $7{\cdot}8 \times 10^{-6}$, $2{\cdot}1 \times 10^{-6}$, $2{\cdot}0 \times 10^{-5}$, $3{\cdot}7 \times 10^{-5}$, and $4{\cdot}7 \times 10^{-4}$ M, respectively, indicating that these analogues are 10–200 times more potent than caffeine. Substitution of bromine by chlorine or iodine at the C-5 and C-7 positions markedly reduced the activity of the analogues with a methyl group at the N-9 position.

These results suggest that halogens at the C-5 and C-7 positions in the β-carboline skeleton are essential for Ca^{2+}-releasing activity and that an N-9 methyl group also affects the activity of these analogues. Thus, these 6-hydroxy-β-carboline analogues might become powerful tools for studying the molecular mechanism of Ca^{2+} release in the sarcoplasmic reticulum.

Contraction of skeletal muscle is triggered by the rapid release of Ca^{2+} from the ryanodine receptor located in the terminal cisternae of the sarcoplasmic reticulum (Meissner 1994; Zucchi & Ronca-Testoni 1997). The purified ryanodine receptor is identical in morphology with the base structures that span the transverse tubule-sarcoplasmic reticulum junction and form caffeine-sensitive Ca^{2+} channels (Shoshan & Ashley 1998). Numerous studies using fragmented sarcoplasmic reticulum preparations and sarcoplasmic reticulum from skinned fibres of skeletal muscle have revealed the presence of a caffeine-sensitive Ca^{2+}-release pathway (Coronado et al 1994), although characterization of the caffeine-binding site in the Ca^{2+}-release channels of the sarcoplasmic reticulum has not been possible because of its low affinity. The detailed molecular mechanism of caffeine-induced Ca^{2+} release from the sarcoplasmic reticulum remains unresolved.

In the course of our survey of Ca^{2+} release in the sarcoplasmic reticulum, 6-hydroxy-β-carboline analogues derived from eudistomin D, a marine natural product isolated from a Caribbean tunicata *Eudistoma olivaceum*, have been found to be powerful Ca^{2+} releasers in skeletal muscle sarcoplasmic reticulum (Kobayashi et al 1984, 1989; Nakamura et al 1986). It has been demonstrated that 9-methyl-5,7-dibromoeudistomin D shares the same binding site as caffeine in the skeletal muscle

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sarcoplasmic reticulum and induces Ca^{2+} release in the same manner as caffeine (Seino et al 1990; Fang et al 1993).

This paper reports a quantitative study of structure–activity relationships for the Ca^{2+}-releasing activity of 6-hydroxy-β-carboline analogues (Figure 1) in the sarcoplasmic reticulum of skinned muscle fibres. We examined the effect on Ca^{2+}-releasing activity of halogen substitution at positions C-5 and C-7 (5,7-dibromoeudistomin D, 5,7-dichloroeudistomin D, and 5,7-diiodoeudistomin D) and further introduction of a methyl group at the N-9 position (9-methyl-5,7-dibromoeudistomin D, 9-methyl-5,7-dichloroeudistomin D, and 9-methyl-5,7-diiodoeudistomin D) of 6-hydroxy-β-carboline, and report that these analogues are 10–200 times more potent than caffeine.

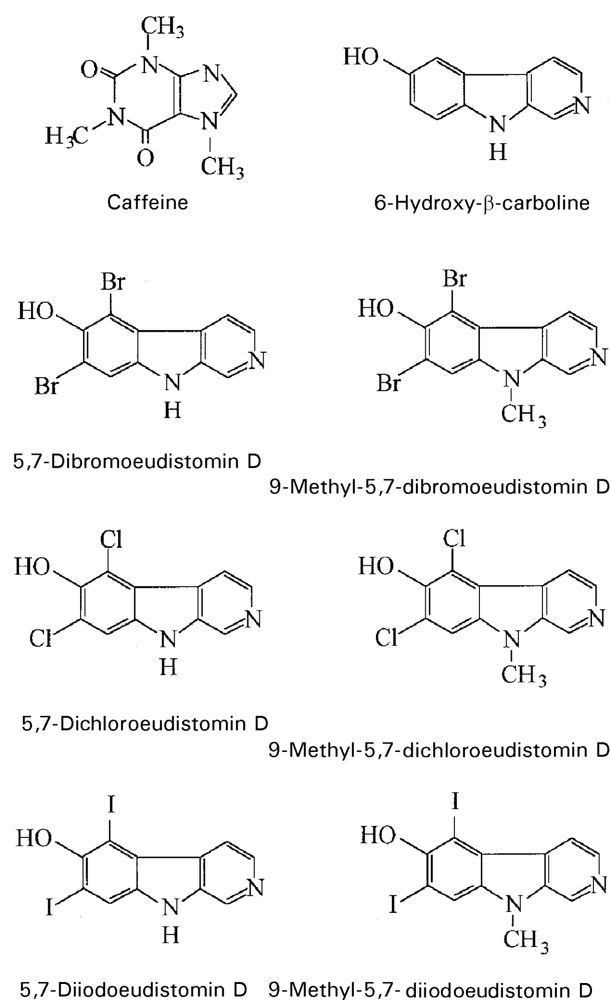

Figure 1. The chemical structures of caffeine, 6-hydroxy-β-carboline, 5,7-dibromoeudistomin D, 5,7-dichloroeudistomin D, 5,7-diiodoeudistomin D, 9-methyl-5,7-dibromoeudistomin D, 9-methyl-5,7-dichloroeudistomin D and 9-methyl-5,7-diiodoeudistomin D.

Materials and Methods

Synthesis of 6-hydroxy-β-carboline analogues

The 6-hydroxy-β-carboline analogues were synthesized as previously described (Kobayashi et al 1988, 1989). 5,7-Dibromoeudistomin D was synthesized by bromination of 6-methoxypyrido[3,4-*b*] indole with *N*-bromosuccinimide in acetic acid then demethylation with BBr_3. 5,7-Dichloroeudistomin D was prepared by the same procedure but with *N*-chlorosuccinimide. 5,7-Diiodoeudistomin D was synthesized by iodination of 6-methoxypyrido[3,4-*b*]indole with iodine and HIO_4 in acetic acid then demethylation with BBr_3. 5,7-Dibromoeudistomin D, 5,7-dichloroeudistomin D, and 5,7-diiodoeudistomin D were O-acetylated by treatment with acetic anhydride; methylation with methyl iodide was then followed by saponification to give 9-methyl-5,7-dibromoeudistomin D, 9-methyl-5,7-dichloroeudistomin D, and 9-methyl-5,7-diiodoeudistomin D, respectively.

Ca^{2+} release from the sarcoplasmic reticulum of skinned muscle fibres

The isometric tension of chemically skinned fibres from guinea-pig psoas muscle was measured at 23°C as previously described (Endo & Iino 1988; Seino et al 1990). If the concentration of EGTA, magnesium methanesulphonate, and calcium methanesulphonate in a solution are denoted X, Y and Z, respectively, the solution denoted "GXMYCZ" contained X mM EGTA, Y mM magnesium methanesulphonate, Z mM calcium methanesulphonate, 4 mM ATP, and 20 mM PIPES–KOH (pH 7·0). The concentration of Mg^{2+}ATP was adjusted to 3·5 mM (for G2M5C0, G0M5C0, G10M5C7 and G0.2M5C0) or 1 mM (for G2M1C0 and G0.2M1C0). In each solution the ionic strength was fixed at 0·2 M by adding an appropriate amount (90–130 mM) of potassium methanesulphonate (Endo & Iino 1988).

A small bundle (0·1 mm diam., 3 mm length, approx.) of muscle fibres was fixed on the needles of a force displacement transducer (AE 801, SensoNor, Norway) in G2M5C0 solution ($[Mg^{2+}]_{free}$ (free Mg^{2+} ion) = 1·5 mM) and the resting sarcomere length was set to 2·8 μm. The fibre was treated for 30 min with G2M5C0 solution containing 50 μg mL^{-1} saponin (ICN Pharmaceuticals, Cleveland, OH), and then washed with a G2M5C0 solution.

It has been reported that ions or compounds can immediately enter the intracellular contractile system of skinned fibres prepared by this procedure (Endo & Iino 1988). Sufficient perforation of the

cell membrane was indicated by the rapid response of the fibres to changes of extracellular Ca^{2+} concentrations and the rigour state of the fibres on removal of Mg^{2+}ATP; this could immediately be reversed by re-introducing Mg^{2+}ATP (Endo & Iino 1980; Horiuti 1986). When the fibres were immersed in G0M5C0 solution ($[Mg^{2+}]_{free} = 1{\cdot}5$ mM), spontaneous contractions occurred repeatedly at constant intervals of 10 to 15 min.

The amount of Ca^{2+} released by the drug was then measured. After immersion in the G10M5C7 solution ($[Ca^{2+}]_{free}$ (free Ca^{2+} ion) $= 1\ \mu$M, $[Mg^{2+}]_{free} = 1{\cdot}5$ mM) for 2 min to load sarcoplasmic reticulum with Ca^{2+}, loading was stopped by washing with G2M5C0 solution for 0·5 min. The fibres were then treated for 1 min with G2M1C0 solution ($[Mg^{2+}]_{free} = 0{\cdot}09$ mM) containing the test substance. After washing with G0.2M5C0 solution ($[Mg^{2+}]_{free} = 1$ mM) for 0·5 min, G0.2M1C0 ($[Mg^{2+}]_{free} = 0{\cdot}09$ mM) solution containing 40 mM caffeine was applied to the fibres to cause contractions that resulted from the release of Ca^{2+} remaining in the sarcoplasmic reticulum. The amount of Ca^{2+} released by the drugs in the G2M1C0 solution was determined from the difference between the amounts of contraction in the presence and absence of the drugs.

Results and Discussion

We have previously reported that 6-hydroxy-β-carboline has no stimulatory effect on Ca^{2+}-releasing activity in skeletal muscle sarcoplasmic reticulum, whereas brominated derivatives of this compound induce Ca^{2+} release and have properties similar to those of caffeine (Nakamura et al 1986; Seino et al 1990; Ohizumi 1997).

In this study the Ca^{2+}-releasing activity of six 6-hydroxy-β-carboline analogues (5,7-dibromoeudistomin D, 5,7-dichloroeudistomin D, 5,7-diiodoeudistomin D, 9-methyl-5,7-dibromoeudistomin D, 9-methyl-5,7-dichloroeudistomin D, and 9-methyl-5,7-diiodoeudistomin D) and of caffeine were examined in chemically skinned muscle fibres. Spontaneous contractions, repeated at constant intervals of 5 to 15 min, were recorded for skinned fibres the sarcoplasmic reticulum of which had been loaded with Ca^{2+} (Figure 2). The addition of 5,7-dibromoeudistomin D, 5,7-dichloroeudistomin D or 5,7-diiodoeudistomin D (10^{-5} M) just after relaxation induced immediate tension then contractions of higher frequency. The subsequent application of procaine (3 mM), an inhibitor of Ca^{2+}-induced Ca^{2+} release, reversed the effect of the analogues and markedly reduced the frequency

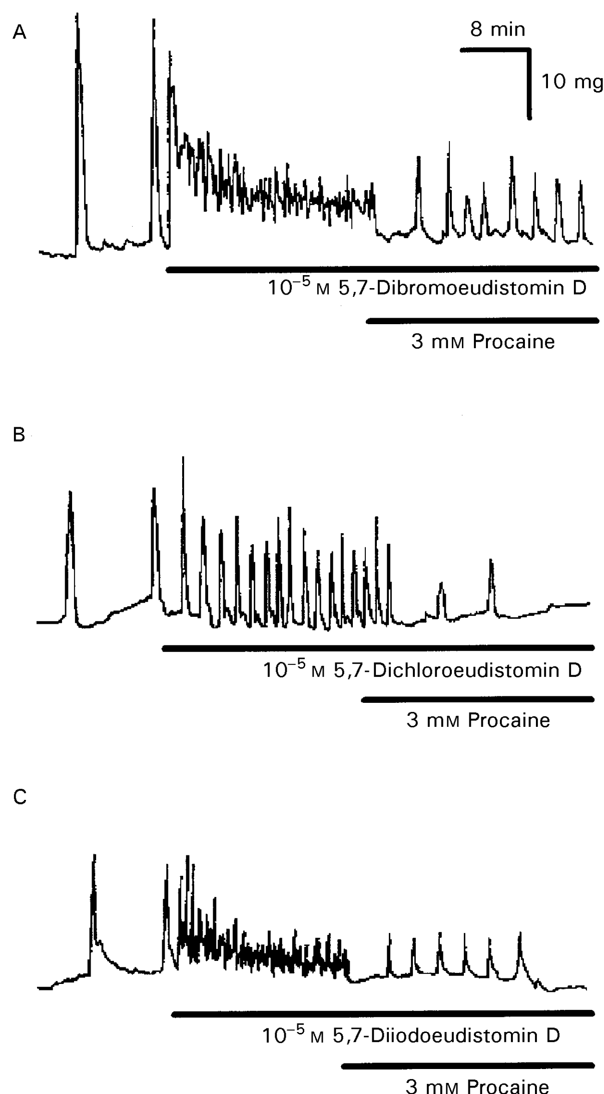

Figure 2. Contractile response to 6-hydroxy-β-carboline analogues of chemically skinned fibres from skeletal muscle. The 6-hydroxy-β-carboline analogues (10^{-5} M) and procaine (3 mM) were applied just after relaxation from the contraction. A. 5,7-dibromoeudistomin D; B. 5,7-dichloroeudistomin D; C. 5,7-diiodoeudistomin D.

of contractions. Similar contractions were observed after addition of 9-methyl-5,7-dibromoeudistomin D, 9-methyl-5,7-dichloroeudistomin D, 9-methyl-5,7-diiodoeudistomin D (10^{-5} M) or caffeine (10^{-3} M) (data not shown).

These results suggest that these analogues act on Ca^{2+}-induced Ca^{2+}-release channels of the sarcoplasmic reticulum in a similar manner to caffeine. The Ca^{2+}-releasing activity of the 6-hydroxy-β-carboline analogues on the sarcoplasmic reticulum of skinned fibres was examined by quantitative measurement of the amount of Ca^{2+} released. Figure 3A shows typical recording traces from the measurement of Ca^{2+} release from the sarcoplasmic reticulum of skinned fibres. In the control,

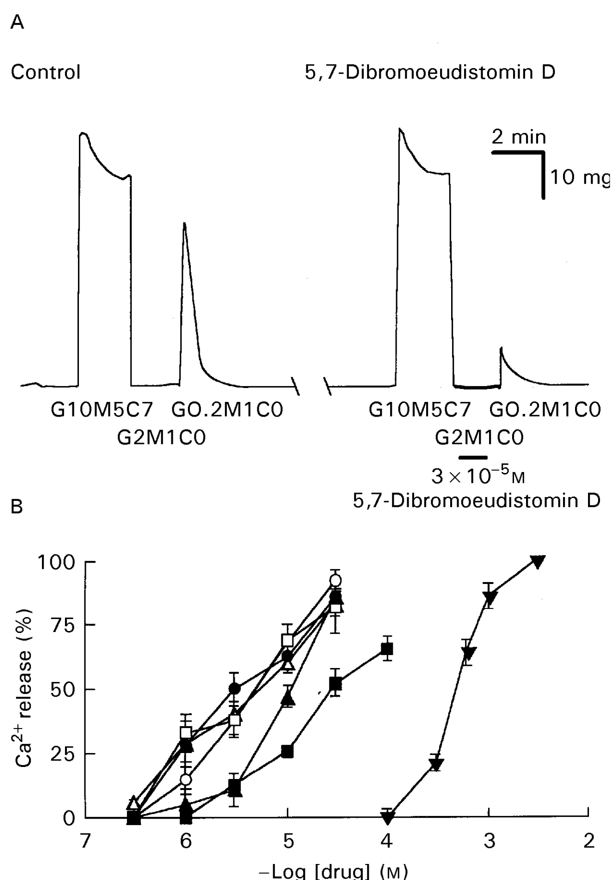

Figure 3. Stimulatory effect of 6-hydroxy-β-carboline analogues or caffeine on Ca^{2+} release from the sarcoplasmic reticulum of skinned skeletal muscle fibres. A. Typical recording traces. After 2 min Ca^{2+} loading in a Ca^{2+}-containing solution (G10M5C7), the fibres were treated with a G2M5C0 solution for 0·5 min, a releasing solution (G2M1C0) with or without 3×10^{-5} M 5,7-dibromoeudistomin D for 1 min, a G0.2M5C0 solution for 0·5 min, and a contraction-inducing solution containing 40 mM caffeine (G0.2M1C0). B. Concentration-dependent stimulation of Ca^{2+} release from the sarcoplasmic reticulum of skinned muscle fibres: ○, 5,7-dibromoeudistomin D; △, 5,7-dichloroeudistomin D; □, 5,7-diiodoeudistomin D; ●, 9-methyl-5,7-dibromoeudistomin D; ▲, 9-methyl-5,7-dichloroeudistomin D; ■, 9-methyl-5,7-diiodoeudistomin D; ▼, caffeine. Data are means ± s.e.m., n = 4–10.

the addition of 40 mM caffeine to the fibres induced a transient contraction as a result of the rapid release of Ca^{2+} accumulated in the sarcoplasmic reticulum. Pretreatment of the fibres with 5,7-dibromoeudistomin D (3×10^{-5} M) markedly reduced the caffeine (40 mM)-induced contraction, suggesting that Ca^{2+} released by 5,7-dibromoeudistomin D is chelated by EGTA, reducing the amount of Ca^{2+} remaining in the sarcoplasmic reticulum. As shown in Figure 3B, the 6-hydroxy-β-carboline analogues enhanced Ca^{2+} release from sarcoplasmic reticulum of skinned fibres in a concentration-dependent manner.

The 50% effective concentrations (EC50) of 5,7-dibromoeudistomin D, 5,7-dichloroeudistomin D, 5,7-diiodoeudistomin D, 9-methyl-5,7-dibromoeudistomin D, 9-methyl-5,7-dichloroeudistomin D, 9-methyl-5,7-diiodoeudistomin D, and caffeine in Ca^{2+}-releasing action are shown in Table 1. The results indicate that the 6-hydroxy-β-carboline analogues were 10–200 times (approx.) more potent than caffeine in sarcoplasmic reticulum from skinned muscle fibres. Substitution of the bromine of 5,7-dibromoeudistomin D by chlorine or iodine slightly increased the EC50 value, but it was not significantly different from that of 5,7-dibromoeudistomin D. The EC50 value was increased 10 and 20 times (approx.) by the substitution of the bromine of 9-methyl-5,7-dibromoeudistomin D by chlorine or iodine.

These results suggest that the presence of halogens at the C-5 and C-7 positions of the β-carboline skeleton is essential for the development of the Ca^{2+}-releasing activity. They also suggest that substitution of bromine by chlorine or iodine at the C-5 and C-7 positions reduced the activity of analogues in which a methyl group has been introduced into the N-9 position. In addition, the slopes of the concentration–response curves for 9-methyl-5,7-dichloroeudistomin D, 9-methyl-5,7-diiodoeudistomin D and caffeine are slightly different from those for 5,7-dibromoeudistomin D, 5,7-dichloroeudistomin D, 5,7-diiodoeudistomin D and 9-methyl-5,7-dibromoeudistomin D (Figure 3B), suggesting that the pharmacological behaviour of 9-methyl-5,7-dichloroeudistomin D, 9-methyl-5,7-diiodoeudistomin D or caffeine are different from those of the other 6-hydroxy-β-carboline analogues.

It has been shown that the ryanodine receptor is identical with the Ca^{2+}-induced Ca^{2+}-release channels of the sarcoplasmic reticulum (Meissner

Table 1. The 50% effective concentration (EC50) values of 6-hydroxy-β-carboline analogues on Ca^{2+}-releasing activity in skinned fibre sarcoplasmic reticulum.

Compound	n	EC50 (M)
5,7-Dibromoeudistomin D	6	$5{\cdot}6\ (3{\cdot}5–9{\cdot}1) \times 10^{-6}$
5,7-Dichloroeudistomin D	6	$6{\cdot}3\ (3{\cdot}7–10{\cdot}7) \times 10^{-6}$
5,7-Diiodoeudistomin D	6	$7{\cdot}8\ (3{\cdot}2–18{\cdot}2) \times 10^{-6}$
9-Methyl-5,7-bromoeudistomin D	7	$2{\cdot}1\ (0{\cdot}79–5{\cdot}8) \times 10^{-6}$
9-Methyl-5,7-dichloro-eudistomin D	4	$2{\cdot}0\ (0{\cdot}83–47{\cdot}9) \times 10^{-5}$
9-Methyl-5,7-diiodoeudistomin D	4	$3{\cdot}7\ (2{\cdot}7–5{\cdot}0) \times 10^{-5}$
Caffeine	10	$4{\cdot}7\ (4{\cdot}0–5{\cdot}4) \times 10^{-4}$

The EC50 values were calculated from the concentration–response curves in Figure 3. Values are means (95% confidence limits in parentheses).

1994; Zucchi & Ronca-Testoni 1997; Shoshan & Ashley 1998). Although caffeine is a typical activator of the ryanodine receptor/Ca^{2+} release channels, because of its low affinity and multiple effects it cannot be used as a tool for clarifying its binding site on the ryanodine receptor (Sawynok & Yaksh 1993). The pharmacological properties of 9-methyl-5,7-dibromoeudistomin D have been investigated and have provided useful information (Ohizumi 1997). In this study we have shown that 6-hydroxy-β-carboline analogues act on Ca^{2+}-induced Ca^{2+}-release channels of the sarcoplasmic reticulum and that their Ca^{2+}-releasing activity in skinned muscle fibres is 10–200 times more potent than that of caffeine.

Not only 9-methyl-5,7-dibromoeudistomin D but also the other analogues described here might provide promising tools for studying the Ca^{2+}-release process in excitation–contraction coupling of skeletal muscle.

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