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Properties of the binding sites of [^{3}H]9-methyl-7-bromoeudistomin D in bovine aortic smooth muscle microsomes

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Abstract—[^{3}H]9-Methyl-7-bromoeudistomin D ([^{3}H]MBED), a powerful caffeine-like Ca^{2+} releaser, binds to the caffeine binding site of terminal cisternae of skeletal muscle sarcoplasmic reticulum and activates Ca^{2+}-induced Ca^{2+} release. Properties of the binding site of [^{3}H]MBED were investigated in aortic smooth muscle. The specific activity was higher in microsomes than in other fractions. [^{3}H]MBED binding sites in smooth muscle microsomes were of a single class with a high affinity (K_D 50 nM), comparable with that in skeletal muscle sarcoplasmic reticulum. Caffeine competitively inhibited [^{3}H]MBED binding, indicating MBED shares the same binding site with caffeine. Solubilization and fractionation of the microsomes gave two fractions of [^{3}H]MBED binding activities. These results suggest that, in smooth muscle, there are multiple binding sites of [^{3}H]MBED and caffeine, which might correspond to different pharmacological actions of caffeine on smooth muscle. Therefore, [^{3}H]MBED, which binds to the different binding sites of caffeine, is useful as a probe for investigation of the actions of caffeine at the molecular level.

The physiological role of Ca^{2+} as a second messenger has been studied in a variety of tissues (Abdel-Latif 1986). Caffeine has been used extensively as an inducer of Ca^{2+}-induced Ca^{2+} release (Endo 1977), which not only plays a key role in excitation-contraction coupling of skeletal or cardiac muscle (Ebashi 1991), but also is involved in cellular responses of other tissues including smooth muscle (Iino et al 1988; Herrmann-Frank et al 1991; Berridge 1993; Sorrentino & Volpe 1993).

We have recently reported that 9-methyl-7-bromoeudistomin D (MBED), a derivative of eudistomin D isolated from the Caribbean tunicate *Eudistoma olivaceum*, induces Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle in a caffeine-like manner (Nakamura et al 1986; Kobayashi et al 1989; Seino et al 1991). Furthermore, we synthesized [^{3}H]MBED and clarified that the ligand shares a binding site with caffeine in skeletal muscle sarcoplasmic reticulum (Fang et al 1993). [^{3}H]MBED has become an essential tool for investigation of the sarcoplasmic reticulum caffeine binding site at the molecular level.

Pharmacological actions of caffeine or its target are not restricted to an activation of Ca^{2+}-induced Ca^{2+} release (Sawynok & Yaksh 1993). The finding that [^{3}H]MBED bound to the caffeine binding site of sarcoplasmic reticulum led to the idea that the ligand could detect other binding sites of caffeine different from that in skeletal sarcoplasmic reticulum. [^{3}H]MBED binds to the caffeine binding site in brain microsomes (Furukawa et al, unpublished results). In this study, we have shown for the first time the existence of the [^{3}H]MBED binding site in bovine aortic smooth muscle, and investigated its properties.

Materials and methods

Materials. [^{3}H]MBED (Fig. 1) was synthesized as previously described (Fang et al 1993) with specific radioactivity 10·2 Ci mmol^{-1}, and was dissolved in dimethylsulphoxide of which the final concentration was kept at <2% (v/v) in all experiments. Aprotinin, benzamidine hydrochloride, pepstatin A and rotenone were purchased from Sigma Chemical Company (St Louis, MO). (9*p*-Amidinophenyl) methanesulphonyl fluoride (*p*-APMSF), (±)-dithiothreitol (DTT) and iodeacetamide were from Wako Pure Chemical Industries (Osaka, Japan). Nonanoyl-*N*-methylglucamide (MEGA-9) was from Dojin Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade.

Preparation of bovine aortic smooth muscle microsomes. Bovine aorta, transferred to the laboratory in ice-cold saline, were cut open and connective tissues and fat were removed. Tunica media were collected, cut into pieces, frozen in liquid nitrogen and stored at −80°C. Frozen tissue was thawed and homogenized in 10 vol ice-cold homogenizing buffer (0·25 M sucrose, 0·6 M KCl, 20 mM MOPS-KOH, pH 7·0, 76·8 mM aprotinin, 0·1 mM *p*-APMSF, 0·83 mM benzamidine hydrochloride, 1 mM iodoacetamide, leupeptin, 0·7 μM pepstatin A) with a Polytron (Kinematica). All subsequent procedures were at 0–4°C. Homogenate was centrifuged (1000 *g*, 10 min) and the supernatant was designated as post-nuclear supernatant (PNS). PNS was centrifuged (12 000 *g*, 10 min) and the pellet (heavy membranes) was obtained. The supernatant was again centrifuged (100 000 *g*, 45 min) and the microsomal pellet was resuspended in storing buffer (0·25 M sucrose, 20 mM MOPS-KOH, pH 7·0, supplemented with protease inhibitors) and frozen in liquid nitrogen. In some experiments, microsomes were washed with storing buffer supplemented with 1 mM Mg ATP and 0·1 mM EGTA, and centrifuged (100 000 *g*, 45 min) before resuspending in storing buffer. The protein concentration was determined by the method of Bradford (1976) using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

[^{3}H]MBED binding assay. [^{3}H]MBED binding assay was performed as described previously (Fang et al 1993) with the following modifications. NaCl was omitted from the incubation buffer and the glass filter was pretreated with polyethyleneimine (3%) for 45 min. Nonspecific binding in the presence of 2–5 μM unlabelled MBED was subtracted from the readings.

Solubilization and fractionation of microsomes. Microsomes (10–20 mg) were suspended (1–2 mg mL^{-1}) in solubilizing buffer containing 0·3 M sucrose, 20 mM Tris-HCl (pH 7·4,

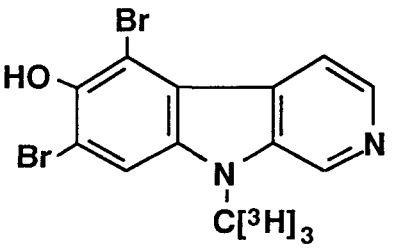

FIG. 1. Chemical structure of tritium-labelled 9-methyl-7-bromoeudistomin D ([^{3}H]MBED).

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Table 1. [^{3}H]MBED binding activities of fractions from bovine aortic smooth muscle.

	Binding activity in fraction	
	Total (% post-nuclear supernatant)	Specific (pmol mg^{-1})
Post-nuclear supernatant	100	10
Heavy membranes	4	17
Soluble fraction	68	7
Microsomes	29	21

Homogenate of bovine aortic smooth muscle was fractionated by differential centrifugation into post-nuclear supernatant (1000 *g*), heavy membranes (12000 *g*), soluble fraction (100000 *g*) and microsomes (100000 *g*) and [^{3}H]MBED binding (50 nM, 0°C, 45 min) was determined.

0°C), 2 mM DTT, 0·1 mM *p*-APMSF, 1–2% MEGA-9 (MEGA-9 : protein, 10 w/w) at 20°C for 20 min. The suspension was centrifuged (100000 *g*, 30 min) and the supernatant was loaded (1 mL min^{-1}) on to Mono Q column (Pharmacia) equilibrated with buffer A (20 mM Tris-HCl, pH 7·0) connected to fast protein liquid chromatography (FPLC) (Pharmacia) or a biocompatible HPLC system (Gilson). The column was then washed with 5 mL buffer A and eluted with a linear gradient of NaCl (0–0·75 M, 15 mL) formed by mixing with buffer B (buffer A plus 1 M NaCl). Fractions (1 mL) were stored on ice and their [^{3}H]MBED binding activities were assayed.

Results

Specific binding of [^{3}H]MBED was detected in each fraction from bovine aortic smooth muscle (Table 1). The specific activity of microsomes was higher than in those of heavy membranes or the soluble fraction, although the total amount of the binding sites was largest in the soluble fraction. Hence [^{3}H]MBED binding sites in microsomes were investigated in the following experiments.

Binding of [^{3}H]MBED to microsomes of aortic smooth muscle was inhibited by unlabelled MBED and caffeine (Fig. 2) in a concentration-dependent manner with IC50 values of 0·8 and 120 μM, respectively. The [^{3}H]MBED binding sites in aortic smooth muscle microsomes were of a single class with a high affinity (K_D 50 nM), which is comparable with that in skeletal muscle sarcoplasmic reticulum (K_D 40 nM (Fang et al 1993)) as revealed by Scatchard analysis (Fig. 3). Caffeine (0·1 mM) increased K_D without affecting B_{max} (20 pmol mg^{-1}), indicating that the mode of inhibition by caffeine was competitive as in skeletal muscle sarcoplasmic reticulum (Fang et al 1993) and brain microsomes (Furukawa et al, unpublished results), indicating that [^{3}H]MBED shares the same binding sites with caffeine in microsomes of aortic smooth muscle.

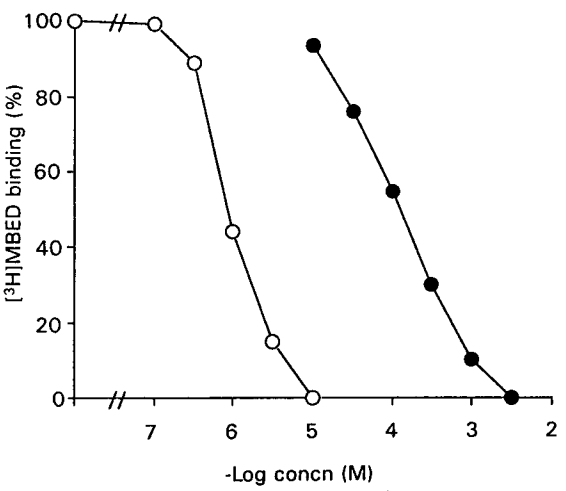

FIG. 2. Effects of unlabelled MBED and caffeine on [^{3}H]MBED binding to microsomes of bovine aortic smooth muscle. Microsomes of bovine aortic smooth muscle were incubated with 50 nM [^{3}H]MBED at 0°C for 45 min in the absence and presence of unlabelled MBED (○) and caffeine (●) at concentrations indicated. Non-specific binding was subtracted from the results.

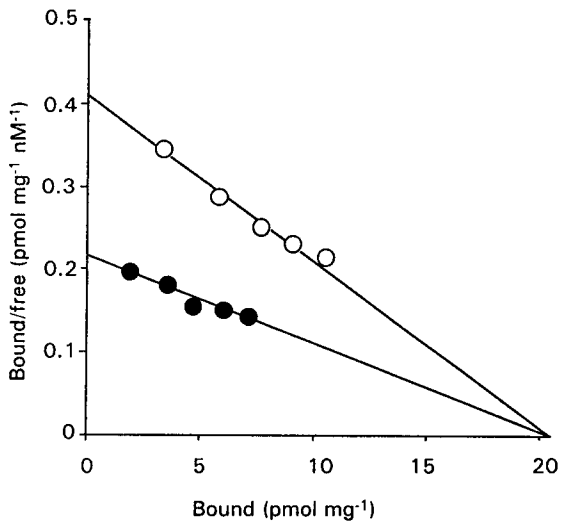

FIG. 3. Scatchard analysis of [^{3}H]MBED binding to microsomes of bovine aortic smooth muscle in the absence and presence of caffeine. Microsomes of bovine aortic smooth muscle were incubated with 10–50 nM [^{3}H]MBED at 0°C for 45 min in the absence (○) and presence (●) of 0·1 mM caffeine. Results are represented as Scatchard plots, which gave K_D values of 50 nM (control) and 100 nM (0·1 mM caffeine) and B_{max} of 20 pmol mg^{-1}.

For further characterization of the [^{3}H]MBED binding sites, bovine aortic microsomes were solubilized with a nonionic detergent, MEGA-9, and fractionated with anion-exchange chromatography on Mono Q (Fig. 4). When eluted with a linear gradient of NaCl (0–0·75 M), [^{3}H]MBED binding activities were eluted as two peaks at ~0·2 and ~0·5 M, respectively.

Discussion

We have shown the existence of [^{3}H]MBED binding sites in aortic smooth muscle. The specific activity of the [^{3}H]MBED binding was highest in microsomal fraction in which sarcoplasmic reticulum is included. The [^{3}H]MBED binding site in microsomes was of a single class with a high affinity comparable to that in skeletal muscle sarcoplasmic reticulum, and ligand binding to this site was inhibited by caffeine. The mode of inhibition by caffeine was competitive, suggesting that the

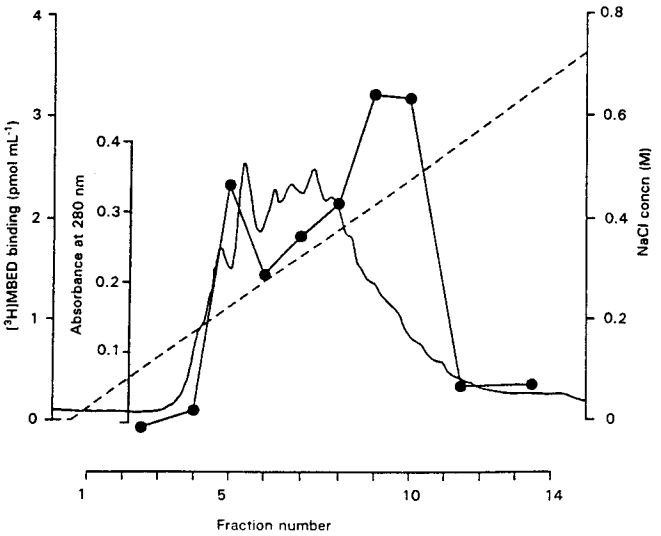

FIG. 4. Fractionation of [^{3}H]MBED binding activities in solubilized microsomes of bovine aortic smooth muscle with ion-exchange chromatography on Mono Q. Microsomes of bovine aortic smooth muscle (16·8 mg) were solubilized with 1·7% MEGA-9. Solubilized supernatant (9 mL) was loaded on to a Mono Q column equilibrated with buffer A (20 mM Tris-HCl, pH 7·0, 1% MEGA-9). The column was washed with buffer A and eluted with a linear gradient (0-0·75 M, 15 mL) of NaCl (broken line) in buffer A. Absorbance at 280 nm (solid line) and [^{3}H]MBED binding (pmol mL^{-1} ●) of each fraction (1 mL) are indicated.

[^{3}H]MBED binding site in smooth muscle sarcoplasmic reticulum is the same as that of caffeine, as in skeletal muscle sarcoplasmic reticulum and brain microsomes. Fractionation of detergent solubilized microsomes with anion-exchange chromatography gave two peaks of the [^{3}H]MBED binding activity, suggesting the existence of at least two binding proteins with similar affinities in microsomes. The Ca^{2+}-releasing action of caffeine from sarcoplasmic reticulum has been reported not only in skeletal or cardiac muscle but also in smooth muscle (Iino et al 1988; Herrmann-Frank et al 1991), therefore it is possible that one of the [^{3}H]MBED binding sites in smooth muscle microsomes is the counterpart of that in skeletal muscle sarcoplasmic reticulum.

Besides Ca^{2+}-release channels (ryanodine receptors), caffeine is known to interact with several other enzymes or receptors such as phosphodiesterases, 5′-nucleotidase, adenosine receptors and GABA/benzodiazepine receptors (Sawynok & Yaksh 1993). There seem to be at least two [^{3}H]MBED binding sites in smooth muscle microsomes and, moreover, the [^{3}H]MBED binding activity in the soluble fraction is not negligible since the total amount of soluble binding sites exceeds that of microsomes. Multiple binding sites of MBED and caffeine in aortic smooth muscle might be explained by these known targets of caffeine. We conclude that [^{3}H]MBED is useful as a probe for investigation of the pharmacological actions of caffeine at the molecular level in a variety of tissues.

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