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Tissue- and subcellular-distribution of the binding site of [^{3}H]9-methyl-7-bromoeudistomin D, a potent caffeine-like Ca^{2+} releaser, in rabbits

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Abstract—Tissue and subcellular distribution of the binding site of ^{3}H-labelled 9-methyl-7-bromoeudistomin D ([^{3}H]MBED), a powerful caffeine-like Ca^{2+} releaser, were investigated in rabbits. The order of specific activities of total homogenates was liver > brain > other tissues. All binding was completely suppressed by 10 mM caffeine, indicating that all [^{3}H]MBED binding sites are modulated by caffeine. [^{3}H]MBED binding sites distributed mainly in membrane fractions rather than soluble fractions in most tissues. In lung and liver, [^{3}H]MBED binding was enriched in microsomes. [^{3}H]MBED may be useful as a probe to investigate the actions of caffeine at the molecular level not only in muscles but also in a variety of tissues including liver, kidney and lung.

Caffeine has been used extensively as an inducer of Ca^{2+}-induced Ca^{2+} release (CICR), which plays a key role not only in excitation-contraction coupling (Ebashi 1991) of skeletal or cardiac muscle, but also in cellular responses of other tissues (Sorrentino & Volpe 1993).

In the course of our survey for biochemical probes on the molecular mechanism of Ca^{2+} movement, we have recently found that derivatives of eudistomin D isolated from the Caribbean tunicate *Eudistoma olivaceum*, induce Ca^{2+} release from sarcoplasmic reticulum (SR) of skeletal muscle (Nakamura et al 1986; Kobayashi et al 1989; Seino et al 1991). Of these derivatives, 9-methyl-7-bromoeudistomin D (MBED) was the most powerful caffeine-like Ca^{2+} releaser (Seino et al 1991). [^{3}H]MBED has become a useful tool for investigation of the SR caffeine binding site at the molecular level (Adachi et al 1994).

The properties of the hepatic binding site are different from those of muscle SR. To estimate the physiological role of these [^{3}H]MBED binding sites, and to seek an abundant source for purification, we have been investigating the tissue- and subcellular-distribution of the [^{3}H]MBED binding sites. In this study, we investigated the tissue- and subcellular-distribution of [^{3}H]MBED binding sites in rabbits.

Materials and methods

Materials. ^{3}H-Labelled MBED (Fig. 1) was synthesized as previously described (Fang et al 1993) with a specific radioactivity of 10·2 Ci mmol^{-1}. [^{3}H]MBED was dissolved in dimethylsulphoxide of which the final concentration was kept at < 2% (v/v) in all the experiments. All other chemicals were of reagent grade.

Preparation of tissue homogenates and fractionation. Tissues were obtained from male albino rabbits weighing about 3 kg. Excised tissues were washed with ice-cold 0·3 M sucrose, freed of connective tissue and fat, cut into pieces, snap-frozen in liquid nitrogen and stored at −80°C until use. Tissues from two or three rabbits were thawed in 0·3 M sucrose, minced finely with scissors and homogenized in 10 vol ice-cold homogenizing buffer (0·25 M sucrose, 20 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS)-NaOH, pH 7·0, 2 mM (±)-dithiothreitol (DTT), 76·8 mM aprotinin, 0·1 mM (*p*-amidinophenyl) methanesulphonyl fluoride (*p*-APMSF), 0·83 mM benzamidine, 1 mM iodoacetamide and 0·7 μM pepstatin A) with a motor-driven Teflon-glass homogenizer (in the case of brain, thymus, liver and spleen, 2000 rev min^{-1}, 4 strokes) or with a Waring blender (lung, heart, kidney and stomach, 15 000 rev min^{-1}, 20 s, twice). The homogenate was centrifuged (1000 *g*, 10 min) and the post-nuclear supernatant (PNS) was used as a total homogenate. PNS was centrifuged (8000 *g*, 20 min) and the pellet was designated as the heavy membrane fraction. Supernatant was again centrifuged (100 000 *g*, 60 min) to obtain microsomes (pellet) and soluble fraction (supernatant). Pellets were resuspended in homogenizing buffer. Fractions were snap-frozen in liquid nitrogen and stored at −80°C until use. 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 by Fang et al (1993) with the following modifications: 1 M NaCl was omitted from the incubation buffer and glass-fibre filters were pretreated with polyethyleneimine (3% for 45 min).

Enzyme assays. Activities of several marker enzymes were determined in PNS, heavy membranes and microsomes. Succinate-cytochrome c reductase activity, a marker for mitochondrial inner membranes, and NADPH-cytochrome c reductase activity, a putative marker of endoplasmic reticulum (ER), were measured by the method of Tisdale (1967) and Sottocasa et al (1967), respectively. 5′-Nucleotidase activity, the plasma membrane marker, was assayed by the method of Song & Bodansky (1967), except inorganic phosphate measurement, which was by the method of Chan et al (1986).

Results

All the tissues tested showed [^{3}H]MBED binding, and their specific activities varied over a 5-fold range (4·5–23·1 pmol mg^{-1}). The specific activity was highest in liver and brain. The contents of binding sites were almost equal in other tissues (Table 1).

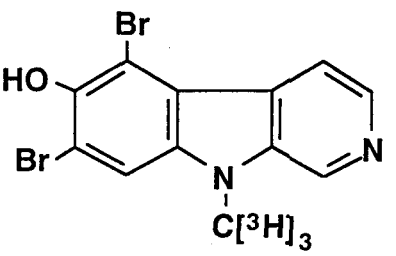

FIG. 1. Chemical structure of [^{3}H]9-methyl-7-bromoeudistomin D.

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Table 1. [^{3}H]MBED binding to total homogenates of various tissues of rabbit. Tissues were homogenized in homogenizing buffer (0·25 M sucrose, 20 mM MOPS, pH 7·0) in the presence of protease inhibitors and postnuclear supernatants (PNS; 1000 *g*, 10 min) were used as total homogenates. [^{3}H]MBED binding was determined in the absence (control) or presence of the drugs (10 mM) indicated. Results are represented as specific activity (pmol mg^{-1}, mean ± s.e.) and percent of control value (in parentheses).

	[^{3}H]MBED binding to PNS		
Tissues	Control	Caffeine	Procaine
Brain	12·6 ± 0·0	0·1 ± 0·1 (1)	5·6 ± 2·0 (44)
Lung	5·1 ± 0·7	−0·6 ± 0·6 (0)	2·8 ± 0·6 (54)
Thymus	6·6 ± 0·4	−0·2 ± 0·6 (0)	2·7 ± 0·3 (40)
Heart	4·6 ± 1·6	−0·2 ± 0·2 (0)	2·3 ± 0·5 (50)
Liver	23·1 ± 0·5	0·6 ± 0·2 (3)	17·9 ± 0·7 (77)
Spleen	4·9 ± 0·7	0·2 ± 0·1 (4)	2·2 ± 0·4 (44)
Kidney	4·5 ± 0·4	0·2 ± 0·1 (5)	2·9 ± 0·6 (65)
Stomach	6·7 ± 1·3	0·2 ± 0·2 (3)	2·9 ± 0·1 (44)

Table 2. Subcellular distribution of [^{3}H]MBED binding in various tissues. PNS of various tissues were further fractionated to heavy membranes (8000 *g*), microsomes (100 000 *g*) and soluble fractions (100 000 *g* supernatant) by differential centrifugation and [^{3}H]MBED binding was determined. Results are represented as specific activity (pmol mg^{-1}, mean ± s.e.) and percentage of total activity present in each fraction (values in parentheses). Total activity recovered in three fractions was taken as 100%.

	[^{3}H]MBED binding		
Tissues	Heavy membranes	Microsomes	Soluble fraction
Brain	5·1 ± 0·0 (39)	9·5 ± 0·1 (35)	5·9 ± 1·9 (26)
Lung	6·6 ± 0·1 (32)	14·3 ± 0·5 (58)	1·0 ± 0·4 (10)
Thymus	11·0 ± 1·2 (24)	12·5 ± 1·0 (24)	5·8 ± 0·8 (51)
Heart	5·9 ± 0·2 (92)	17·1 ± 2·2 (8)	−0·9 ± 1·0 (0)
Liver	60·8 ± 4·1 (23)	105·9 ± 3·6 (69)	3·3 ± 1·1 (8)
Spleen	10·4 ± 0·3 (47)	8·8 ± 1·6 (27)	2·8 ± 0·2 (26)
Kidney	14·6 ± 0·1 (54)	17·5 ± 0·1 (38)	1·4 ± 0·1 (8)
Stomach	19·0 ± 2·0 (51)	16·0 ± 5·4 (37)	2·4 ± 0·5 (13)

[^{3}H]MBED binding in all the tissues tested was completely suppressed by caffeine (10 mM). Procaine, a representative inhibitor of CICR, inhibited [^{3}H]MBED binding in tissues by 40–60% except in liver which was inhibited by only 20%. When each PNS was fractionated into three fractions most of the binding sites existed in membrane fractions, although the distributions within the two fractions (heavy membranes and microsomes) were different compared with tissues (Table 2). Proportions of the binding activities in soluble fractions were generally low in all the tissues except thymus.

In three representative tissues, lung, liver and kidney, distribution of the [^{3}H]MBED binding sites were compared with those of membrane marker enzymes (Table 3). In lung the extent of enrichment of [^{3}H]MBED binding activities in each fraction was different from those of marker enzyme of mitochondria, and was closely correlated with those of ER and plasma membrane markers. In liver, [^{3}H]MBED binding and ER marker were more enriched in microsomes, than in heavy membranes enriched with mitochondrial marker. For kidney, distribution of [^{3}H]MBED binding did not correlate with any single marker.

Discussion

Our previous reports (Seino et al 1991; Fang et al 1993) strongly suggested that binding of 9-methyl-7-bromoeudistomine D (MBED) to the caffeine binding site effected Ca^{2+} release from SR. [^{3}H]MBED has become a useful tool for investigation of the caffeine-binding site in SR at the molecular level. The finding that the binding site of [^{3}H]MBED in SR is the same as that of caffeine suggests that the ligand may bind to other caffeine binding sites different from that in SR. In this report, we have shown the presence of [^{3}H]MBED binding sites in tissues other than skeletal muscle. Ligand binding to all of these sites was completely suppressed by caffeine, suggesting that these [^{3}H]MBED binding sites are at least related to those of caffeine. It is likely that there are at least two types of [^{3}H]MBED binding sites differing in their mode of inhibition by caffeine, and in sensitivity to procaine, an inhibitor of CICR. One type comprises the sites competitively inhibited by caffeine and sensitive to procaine, in rabbit skeletal muscle SR (Fang et al 1993), in aortic smooth muscle SR (Adachi et al 1994) and in guinea-pig brain microsomes (unpublished observations); the second type comprises the sites inhibited by caffeine non-competitively and less sensitive to procaine compared with the former, in rabbit liver microsomes (unpublished observations).

The [^{3}H]MBED binding sites existed mainly in membrane fractions. In lung and liver, both the binding sites and the ER (and plasma membrane) markers were enriched in the microsomal fraction. In the case of kidney, [^{3}H]MBED binding sites were distributed in both of the membrane fractions. The ER marker also distributed in both fractions. Hence, [^{3}H]MBED binding sites in the heavy membrane fraction of kidney might be explained by contamination with ER, or by localization of the binding sites in kidney mitochondria.

Caffeine induces Ca^{2+} release not only in muscles but also in non-muscle cells such as pancreatic exocrine cells (Schmid et al 1990) and adrenal chromaffin cells (Cheek et al 1991). MBED,

Table 3. Comparison of the distributions in two membrane fractions between [^{3}H]MBED binding and marker enzymes.

	Lung			Liver			Kidney		
	Postnuclear fraction	Heavy membranes	Microsomes	Postnuclear fraction	Heavy membranes	Microsomes	Postnuclear fraction	Heavy membranes	Microsomes
Binding	1	1·3	2·8	1	2·7	4·7	1	3·3	3·9
Marker enzymes									
Succinate-cytochrome c reductase (mitochondrial)	1	2·2	0·4	1	7·7	0·9	1	3·2	0·3
NADPH-cytochrome c reductase (endoplasmic reticulum)	1	0·7	2·3	1	1·3	3·6	1	1·5	1·8
5′-Nucleotidase (plasma membrane)	1	1·1	1·9	–	–	–	–	–	–

All values are expressed as the mean ± s.e.-fold enrichment of the activity in the postnuclear supernatant fraction. 5′-Nucleotidase in liver and kidney was not measurable because of poor recovery (< 20%) in the membrane fractions.

like caffeine, may therefore induce Ca^{2+} release in these cells and tissues. However, the Ca^{2+}-releasing action of caffeine is only a part of its diverse actions. Several enzymes or receptors are putative targets of caffeine, including phosphodiesterases, 5′-nucleotidase, adenosine receptors and γ-aminobutyric acid/benzodiazepine receptors (Sawynok & Yaksh 1993), and novel actions have been reported recently (Toescu et al 1992; Mcnulty & Taylor 1993). It is also possible that [^{3}H]MBED binds to these target proteins of caffeine.

An important feature of [^{3}H]MBED, that the compound shares the same binding site with caffeine, was first demonstrated by us in skeletal muscle SR (Fang et al 1993). Abolition of [^{3}H]MBED binding by caffeine in all the tissues tested including skeletal SR suggests that these [^{3}H]MBED binding sites are identical with the binding site of caffeine or are allosterically modulated by it. The studies on [^{3}H]MBED binding sites may lead to resolution of the molecular mechanism of action of caffeine.

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