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[^{3}H]9-Methyl-7-bromoeudistomin D, a caffeine-like powerful Ca^{2+} releaser, binds to caffeine-binding sites distinct from the ryanodine receptors in brain microsomes

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Abstract [^{3}H]9-Methyl-7-bromoeudistomin D ([^{3}H]MBED), the most powerful Ca^{2+} releaser from sarcoplasmic reticulum, specifically bound to the brain microsomes. Caffeine competitively inhibited [^{3}H]MBED binding. [^{3}H]MBED binding was markedly blocked by procaine, whereas that was enhanced by adenosine-5′-(β,γ-methylene)triphosphate. The B_{max} value was 170 times more than that of [^{3}H]ryanodine binding. The profile of sucrose-density gradient centrifugation of solubilized microsomes indicated that [^{3}H]MBED binding protein was different from [^{3}H]ryanodine binding protein. These results suggest that there are MBED/caffeine-binding sites in brain that are distinct from the ryanodine receptor and that MBED becomes an essential molecular probe for characterizing caffeine-binding protein in the central nervous system.

Key words: Caffeine; Ryanodine receptor; cGMP; Brain; 9-Methyl-7-bromoeudistomin D

1. Introduction

Pharmacological actions of caffeine have been extensively studied by numerous investigators because it has many interesting actions on the central nervous system including analeptic and analgesic effects [1]. One of the most important pharmacological actions of caffeine is related to calcium movements. In neuronal tissues, release of stored Ca^{2+} from endoplasmic reticulum (ER) triggers several physiological processes including neutrotransmitter release [2] and changes in the cytoskeleton [3]. Although Ca^{2+} release from the store has been well characterized with inositol 1,4,5-trisphosphate (IP_3), one of the most important second messengers [4–7], the presence of IP_3-resistant Ca^{2+} stores has been suggested [8–11]. The Ca^{2+}-induced Ca^{2+} release (CICR) channel is a possible candidate for the pathway of Ca^{2+} release from the IP_3-resistant Ca^{2+} stores and has been identified as the [^{3}H]ryanodine receptor in brain [12–15]. Caffeine triggers Ca^{2+} release from not only sarcoplasmic reticulum (SR) but also from ER and through CICR channels [16–21]. However, direct binding studies of caffeine have been impossible because of its low affinity. Natural bioactive substances such as ryanodine have been essential as biochemical tools for characterizing and purifying functional proteins [22–26]. It has been reported that 9-methyl-7-bromoeudistomin D (MBED), a derivative of eudistomin D isolated from the Caribbean tunicate *Eudistoma olivaceum*, having caffeine-like properties, powerfully induces Ca^{2+} release from SR in skeletal muscle [27,28]. Recently, we have successfully synthesized ^{3}H-labeled MBED with a high specific radioactivity and revealed the properties of MBED binding sites [29]. [^{3}H]MBED interacts competitively with caffeine in binding at the same site on the skeletal muscle Ca^{2+}-release channels (ryanodine receptor). The site differs from the ryanodine-, adenine nucleotide- or Ca^{2+}-binding site on the same receptor [29–31]. In the central nervous system, we have more recently found that MBED exhibits effects similar to that of caffeine (S. Kaneko, T. Tadano, K. Matsunaga, K. Kisara and Y. Ohizumi, unpublished observation). In this paper we successfully characterized the caffeine-binding sites in mammalian brain using [^{3}H]MBED as a specific ligand for the caffeine-binding site for the first time. [^{3}H]MBED has become an essential tool not only for studying caffeine-binding sites but also for Ca^{2+}-release channels in SR/ER.

2. Materials and methods

Guinea pig whole brain without the brainstem or dissected brain regions were homogenized in Buffer A containing 0.32 M sucrose, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)/Tris (pH 7.6), 10 mM EGTA and protein inhibitors (100 μM *p*-aminophenylmethylsulfonylfluoride, 0.5 mg/ml aprotinin, 0.5 ng/ml leupeptin, 0.83 mM benzamidine). Homogenates were centrifuged twice at $11,000 \times g$ for 20 min, and supernatants were centrifuged at $100,000 \times g$ for 60 min. Pellets were resuspended in Buffer A and freezed with liquid nitrogen and stored at −80°C. These procedures were carried out below 4°C. Protein was assayed by the modified Lowry method [32] with bovine serum albumin as a standard.

Brain microsomes were suspended at 5 mg of protein/ml in Buffer B containing 2.5% 3-[(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 1% phosphatidylcholine, 1 M NaCl, 20 mM Tris-HCl (pH 7.4). After 90 min of gentle stirring at 4°C, the mixture was centrifuged for 30 min at $310,000 \times g$ and supernatant was collected as the CHAPS-solubilized sample.

The CHAPS-solubilized sample was layered onto a 8 ml linear density gradient of sucrose (5–20%) in buffer containing 1.25% CHAPS, 0.5% phosphatidylcholine, 50 mM NaCl, 20 mM Tris-HCl (pH 7.4) and centrifuged for 16 h in a Beckman SW-41 rotor at $100,000 \times g$ using slow acceleration and deceleration profiles. After centrifugation, each fraction (1 ml) was collected from the top, then diluted with water to

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Abbreviations: MBED, 9-methyl-7-bromoeudistomin D; B_{max}, amount of maximum binding; K_d, dissociation constant; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; CICR, Ca^{2+} induced Ca^{2+} release; HEPES, 2[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; AMPPCP, adenosine-5′-(β,γ-methylene)triphosphate; PKG, cyclic GMP-dependent protein kinase.

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decrease the concentration of sucrose below 0.32 M, and then sedimented by centrifugation for 90 min in a Beckman Ty-42.1 rotor at 100,000 × g. Pellets were resuspended in Buffer B.

^{3}H-labeled MBED was synthesized by *N*-methylation of 6-O-acetylbromoeudistomin D with [^{3}H]methyl iodide, followed by deacethylation as reported previously [28,33]. The labeled MBED was purified by reversed phase and silica gel thin-layer chromatography. The specific radioactivity was 10.2 Ci/mmol.

Brain microsomes or solubilized samples (0.02–0.2 mg/ml) were incubated with 5–70 nM [^{3}H]MBED at 0°C or 2–30 nM [^{3}H]ryanodine (Du Pont-New England Nuclear) at 37°C in Buffer C containing 1 M NaCl, 20 mM Tris-HCl (pH 7.0), 0.3 M sucrose, 2 mM DTT, and protease inhibitors for 45 min in the presence or absence of 1 μM MBED for [^{3}H]MBED binding and 10 μM ryanodine for [^{3}H]ryanodine binding. [^{3}H]MBED binding reached a plateau within 30 min at 0°C. The amounts of both radio-ligands bound to microsomes or solubilized samples were determined by membrane filtration as previously described [29].

cGMP-dependent protein kinase (PKG) was purified from bovine lung by the method of Lincoln [34]. Microsomes (1 mg/ml) were incubated with PKG (5 mg/ml) plus 1 mM ATP and 10 μM 8-bromo-cGMP for 30 min at 20°C in phosphorylation buffer (0.3 M sucrose, 1 M NaCl, 10 mM $MgCl_2$, 100 μM $CaCl_2$, 20 mM Tris-HCl, pH 7.4). After 20-times dilution with Buffer B, [^{3}H]MBED and [^{3}H]ryanodine binding was assayed as described above.

3. Results

Fig. 1 shows a concentration-dependent inhibition of [^{3}H]MBED binding by caffeine. [^{3}H]MBED binding was nearly abolished by 5 mM caffeine (Fig. 1A). Lineweaver–Burk plot of the data indicates the IC_{50} value of 1 mM (Fig. 1B). On the other hand, the IC_{50} value for replacement of bound [^{3}H]MBED by non-labeled MBED was 0.18 μM. Therefore, [^{3}H]MBED binds to the sites with about 5000 times higher affinity than that of caffeine. Fig. 2 shows a saturation analysis and a corresponding Scatchard analysis from a typical binding

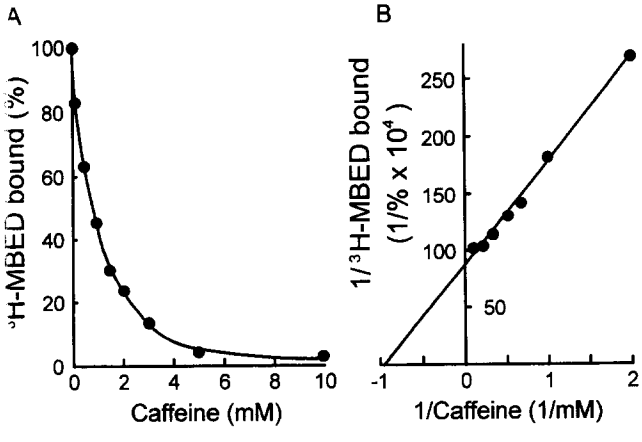

Fig. 1. Concentration-dependent inhibition of [^{3}H]MBED binding by caffeine. (A) [^{3}H]MBED to microsomes in the presence of various concentrations of caffeine were measured. (B) Lineweaver–Burk plot of the data in (A). Microsomes were incubated with 50 nM [^{3}H]MBED for 45 min at 0°C, as described in section 2. Specific binding was derived by subtracting non-specific binding determined in the presence of 1 μM unlabeled MBED. Results are representative from three experiments. The amount of [^{3}H]MBED binding in the absence of caffeine was 22 ± 2.1 pmol/mg. The concentration of caffeine which reduced the amount of bound [^{3}H]MBED to one-half the maximum value was 1 mM.

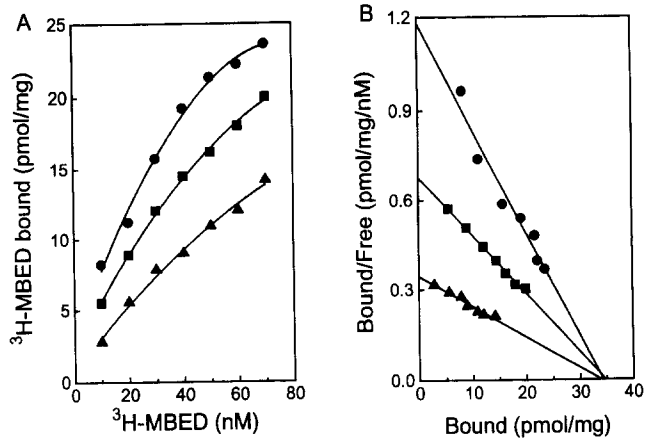

Fig. 2. Kinetical analysis of [^{3}H]MBED binding to brain microsomes in the presence or absence of caffeine. Microsomes were incubated with various concentrations of [^{3}H]MBED (10–70 nM) in the presence or absence of caffeine for 45 min at 0°C, as described in Fig. 1. The caffeine concentrations were 0 (●), 0.3 (■) and 0.8 mM (▲). Results are representative from three experiments. K_d values for [^{3}H]MBED in the presence of 0.3 mM and 0.8 mM caffeine were 51 and 102 nM, respectively, and that in the absence of caffeine was 29 nM. Caffeine did not affect the B_{max} value (34.5 pmol/mg).

experiment, respectively. Specific binding was saturable and of high affinity. Values for K_d and B_{max} were 29 nM and 34.5 pmol/mg, respectively. Caffeine (0.3 and 0.8 mM) increased the K_d value for [^{3}H]MBED from 29 to 51.3 and 102 nM, respectively, without affecting the B_{max} value, indicating a competitive mode of interaction between [^{3}H]MBED and caffeine at the same binding site. The specific binding of [^{3}H]MBED completely disappeared by treatment of the microsomes with trypsin (data not shown). These results suggest that [^{3}H]MBED binds to caffeine-binding sites in brain microsomes with higher affinity than that of caffeine and that the site is presumably formed with proteins.

Fig. 3 shows the effects of various drugs on [^{3}H]MBED binding. The [^{3}H]MBED binding was stimulated by AMPPCP, an unhydrolyzable ATP-analog in a concentration-dependent manner (0.1–100 μM), and reached a peak at 10 μM AMPPCP. However, ryanodine and inositol 1,4,5-trisphosphate up to 10 μM did not affect the binding. Procaine inhibited the binding with an IC_{50} value of 1.1 mM, whereas Ruthenium red up to 10 μM failed to suppress the binding. [^{3}H]MBED binding was not affected by Mg^{2+} up to 1 mM or Ca^{2+} up to 0.1 mM. Adenosine receptor antagonists such as 8-cyclopentyl-1,3-dimethylxanthine and 3,7-dimethyl-1-propargylxanthine had no effect on the binding (data not shown).

It is well known that plant-alkaloid ryanodine binds with high affinity to a receptor of SR in skeletal muscle [35–37]. Recently, ryanodine-binding sites have been demonstrated in brain [12–15]. The [^{3}H]ryanodine binding to our microsomes was saturable and of high affinity and the values for K_d and B_{max} were 13 nM and 240 fmol/mg, respectively. Although both of the K_d values for [^{3}H]ryanodine and [^{3}H]MBED were almost the same, the B_{max} value for [^{3}H]MBED was extremely larger than that for [^{3}H]ryanodine (about 140 times).

The distribution of [^{3}H]MBED binding within the central

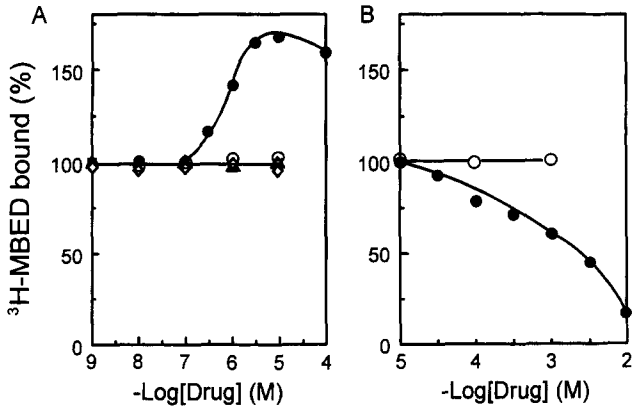

Fig. 3. Effects of various drugs on [^{3}H]MBED binding to brain microsomes. (A) Microsomes were incubated with 30 nM [^{3}H]MBED in the presence of various concentrations of ryanodine (▲), inositol-1,4,5-trisphosphate (◇), Ruthenium red (□), AMPPCP (●) or Ca^{2+} (○). (B) Microsomes were incubated with 30 nM [^{3}H]MBED in the presence of various concentrations of procaine (●) or Mg^{2+} (○). Specific binding of [^{3}H]MBED was measured as described as in Fig. 1. The amount of [^{3}H]MBED bound to microsomes was expressed as percentages of that obtained in control experiment (17 pmol/mg). Results are representative from three experiments.

nervous system was investigated. Olfactory bulb, brainstem, cerebellum and hippocampus had 42 ± 10.6, 87 ± 7.8, 113 ± 12.1 and 135 ± 9.4% binding of the whole brain. The highest binding was observed in cerebral cortex (148 ± 6.2%).

Brain microsomes were solubilized and fractionated by linear sucrose-density gradient centrifugation and the profiles of [^{3}H]MBED and [^{3}H]ryanodine binding activities of the fractions were shown in Fig. 4. Both profiles were different from each other. The peak of [^{3}H]MBED binding activity (55% of the total binding activity) was found in fraction 6 and there was no [^{3}H]ryanodine binding activity, whereas the peak of [^{3}H]ryanodine binding activity (52% of the total binding activity) was found in fraction 8 and there was only 8% of the [^{3}H]MBED binding activity. These results suggest that caffeine-binding proteins are different from ryanodine-binding proteins.

PKG modulates [^{3}H]ryanodine binding to cardiac muscle SR [38]. PKG may play also an important role in neuronal tissues [39,40]. Therefore, effects of PKG on both [^{3}H]MBED and [^{3}H]ryanodine binding were investigated (Table 1). Binding of both was markedly suppressed by treatment of microsomes with PKG in the presence of 8-bromo-cGMP and ATP. [^{3}H]MBED binding was not affected by ATP alone and/or 8Br-cGMP in the absence of PKG (data not shown).

Table 1
Effect of cGMP-dependent phosphorylation on [^{3}H]MBED and [^{3}H]ryanodine binding to brain microsomes

	Control	PKG
MBED (pmol/mg)	8.58 ± 0.42	1.83 ± 0.06*
Ryanodine (fmol/mg)	140 ± 24	38.5 ± 3.6*

Brain microsomes were treated with PKG in the presence of ATP and 8-bromo-cGMP for 30 min. Then, [^{3}H]MBED and [^{3}H]ryanodine binding were assayed as described in section 2.
*Significantly ($P < 0.01$) different from the control value.

4. Discussion

Caffeine has been extensively used as a pharmacological tool in various tissues including muscle and brain. However, the details of its binding sites are still unknown, because of its low affinity for binding. It has been reported that [^{3}H]MBED shares the same binding site as that of caffeine in skeletal muscle SR [29]. In the present experiment we successfully demonstrated that [^{3}H]MBED bound to proteins in brain microsomes with about 5000 times higher affinity than that of caffeine in a replaceable and saturable manner. [^{3}H]MBED binding was almost completely inhibited by 5 mM caffeine. Kinetic analysis indicates a competitive mode of interaction between [^{3}H]MBED and caffeine. These results suggest that even in neuronal tissue [^{3}H]MBED shares the same binding site as that of caffeine but with extremely higher affinity than that of caffeine. Therefore, [^{3}H]MBED is an essential biochemical tool for characterization of caffeine-binding sites in mammalian brain at a molecular level.

AMPPCP, the strongest stimulator of Ca^{2+} release among adenine compounds, potentiated the [^{3}H]MBED binding and procaine suppressed it, whereas ryanodine, Ruthenium red and divalent cations such as Ca^{2+} and Mg^{2+} did not affect [^{3}H]MBED binding. These characteristics of [^{3}H]MBED binding sites in brain microsomes are quite similar to those in skeletal SR membrane [29]. Caffeine is known to affect IP_3 receptors [13]. However, IP_3 did not affect [^{3}H]MBED binding, and the B_{max} value for [^{3}H]MBED in guinea pig brain is about 400 times more than that for [^{3}H]IP_3 obtained in rat brain (0.098 pmol/mg for [^{3}H]IP_3 [41] and 34.5 pmol/mg for [^{3}H]MBED, respectively). Therefore, it is unlikely that [^{3}H]MBED binds to IP_3 receptors. It is possible that caffeine and MBED bind to adenosine receptors in the plasma membrane because of their structural similarity to adenosine [1]. However, the B_{max} value

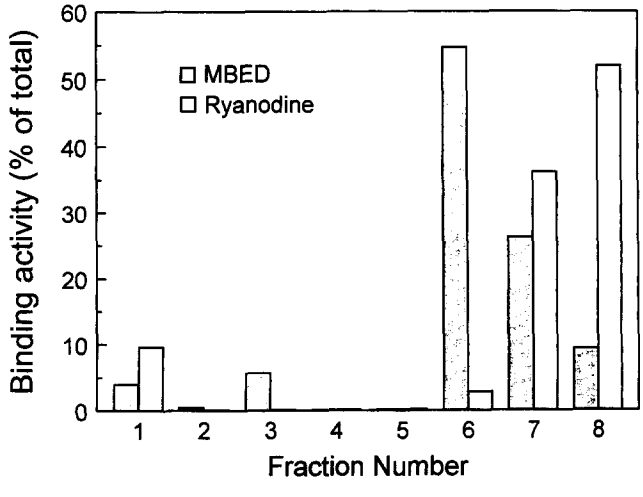

Fig. 4. Sucrose density-gradient centrifugation of solubilized brain microsomes. CHAPS-solubilized microsomes (5 mg) were fractionated by linear sucrose density-gradient centrifugation as described in section 2. The amounts of [^{3}H]MBED and [^{3}H]ryanodine bound to each fraction were measured and expressed as percentage of those of the solubilized microsomes applied to the gradient.

for [^{3}H]MBED in guinea pig brain is 90–200 times more than that for [^{3}H]1,3-dipropyl-8-cyclopentylxanthine, an adenosine receptor antagonist, obtained in porcine brain (0.17–0.40 pmol/mg for [^{3}H]1,3-dipropyl-8-cyclopentylxanthine [42]). Furthermore, antagonists for A_1 and A_2 subtypes of receptor did not affect the binding of [^{3}H]MBED. Therefore, it would seem that [^{3}H]MBED binds to major caffeine-binding proteins in brain microsomes but not to adenosine receptors. Interestingly, we have recently found that MBED, like caffeine markedly stimulates locomotor activities of mice at a low dose (see Introduction). Detailed investigation concerning the role of MBED binding protein in the mechanism of its action on the central nervous system is underway.

Recently, [^{3}H]ryanodine binding sites have been demonstrated in brain microsomes [24]. At least 3 subtypes of ryanodine receptors are expressed in brain. The amount of [^{3}H]MBED binding sites is almost equal to that of [^{3}H]ryanodine binding sites, suggesting that [^{3}H]MBED binds to the ryanodine receptor in skeletal SR membrane [29]. However, in the present experiment there is a serious discrepancy between the amount of specific binding of [^{3}H]MBED and that of [^{3}H]ryanodine to brain microsomes (34.5 pmol/mg for [^{3}H]MBED and 240 fmol/mg for [^{3}H]ryanodine). The distribution of [^{3}H]MBED binding within the central nervous system distinct from that for [^{3}H]ryanodine binding in rabbit [13]. Binding of [^{3}H]MBED but not [^{3}H]ryanodine was enriched in the cerebral cortex in relation to the whole brain. Furthermore, judging from the profile of both [^{3}H]MBED and [^{3}H]ryanodine binding to fractions of sucrose density-gradient centrifugation, [^{3}H]MBED binding protein seems to be different from the ryanodine receptor (Fig. 4). An attractive interpretation of all these observation is that [^{3}H]MBED binds to a novel Ca^{2+} release channel or protein related to the channel in brain. However, the possibility that [^{3}H]MBED binding protein represents ryanodine receptor monomers which fail to bind [^{3}H]ryanodine cannot be excluded.

The important role of PKG in neuronal tissues has been recognized [39,40]. Elevation of cGMP level may prevent neuronal excitotoxicity by lowering the cytosolic calcium concentration. Addition of exogenous PKG with ATP and 8Br-cGMP decreased both [^{3}H]MBED and [^{3}H]ryanodine binding. These results suggest that cGMP-dependent phosphorylation is involved in the regulation of the functions of major caffeine-binding proteins and Ca^{2+}-release channels in neuronal tissues. It is consistent with the protection against the neuronal excitotoxicity by cGMP.

A physiological ligand for the activation of the CICR channel (ryanodine receptor) is unknown except Ca^{2+} in some tissues. A novel NAD^+ metabolite, cyclic ADP-ribose has been reported as a candidate for such a role not only in sea urchin egg cells but also in mammalian cells [43]. Relationship among 3 ligands, i.e. cyclic ADP-ribose, caffeine and ryanodine remains to be elucidated, and further investigation should reveal the detailed mechanism of CICR and the action of caffeine in neural system. [^{3}H]MBED will provide fruitful information about them.

In summary, [^{3}H]MBED specifically binds to the major caffeine-binding protein in brain microsomes with extremely high affinity and becomes an essential biochemical tool to clarify the function of MBED/caffeine binding protein in neuronal tissues.

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