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A Derivative of Bisbenzylisoquinoline Alkaloid is a New and Potential Calmodulin Antagonist

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A new derivative of bisbenzylisoquinoline (berbamine type): O-(4-ethoxylbutyl) berbamine (EBB) was found to possess powerful and specific calmodulin (CaM) inhibitory properties. It inhibited CaM-stimulated Ca^{2+}-Mg^{2+}-ATPase in human erythrocyte membrane with IC_{50} value of 0.35 μM compared to that of 60 μM of berbamine. CaM-independent basal Ca^{2+}-Mg^{2+}-ATPase, Na^{+}-K^{+}-ATPase and Mg^{2+}-ATPase were not effect at 1.0 μM of EBB at which CaM-dependent Ca^{2+}-Mg^{2+}-ATPase was already potently inhibited. The inhibition of CaM-dependent Ca^{2+}-Mg^{2+}-ATPase was competitive with respct to CaM. Higher amount of CaM reversed the inhibition caused by higher concentration of EBB.

Using dansyl-CaM (D-CaM), it was shown that EBB binds directly to CaM and caused a conformational change of CaM polypeptide chain. From fluorescence titration curve we obtained evidence that in the presence of Ca^{2+}, CaM has two specific binding sites for EBB and additional unspecific binding sites. The Ca^{2+}-dependent binding sites of EBB on CaM were novel region different from the binding sites for TFP.

Calmodulin (CaM) is a ubiquitous intracellular Ca^{2+}-binding protein and plays a fundamental role in regulation of many physiological events and enzyme systems. The actions of CaM are influenced by a wide range of chemically unrelated substances. Of the most potent inhibitors of CaM reported thus far were calmidazolium and compound 48/80 with IC_{50} value of about 0.3-0.8 μM.[1][2] Among the natural inhibitors, in addition to vinca alkaloid belonging to indole derivative,[3] we have reported previously that tetrandrine (Tet), belonging to isoquinoline alkaloid, was a new CaM antagonist.[4] It inhibited CaM-stimulated Ca^{2+}-Mg^{2+}-ATPase in human erythrocyte membrane with IC_{50} value of 40 μM.

In search for the relationship between structure and its activities of CaM-antagonists we have shown that a derivative of bisbenzylisoquinoline: O-(4-ethoxylbutyl)-berbamine (EBB) possesses potential inhibitory ability of CaM-dependent Ca^{2+}-Mg^{2+}-ATPase with IC_{50} value of 0.35 μM similar to that of calmidazolium. The results of fluorescence study demonstrated that on CaM exist 2 Ca^{2+}-dependent binding sites of EBB different from that of TFP.

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Materials and Methods

All reagents were of the highest purity available. Berbamine kindly was provided by Dong Sheng pharmaceutica, Peiking.

1. EBB was synthesized from berbamine and 1,4-dibromobutane by the method of Williamson.[5] Purity of the compound was checked by thin-layer chromatography using silica gel plate. The solvent used is chloroform/methanol 5:1. The mass spectrum has a molecular ion at m/e=708.6. The peaks are at m/e= 395.2 due to double benzylic cleavage (E.F) which is base peak, m/e=101.1 due to $-CH_2CH_2CH_2CH_2OCH_2CH_3$.

The structure of EBB is shown in Fig.1.

2. CaM and CaM-deficient RBC membrane was prepared and Ca^{2+}-Mg^{2+}-ATPase, Na^+-K^+-ATPase Mg^{2+}-ATPase activities were determined as described previously.[8]

Protein was determined by the method of Brandford with bovine serum albumin as standard.[7]

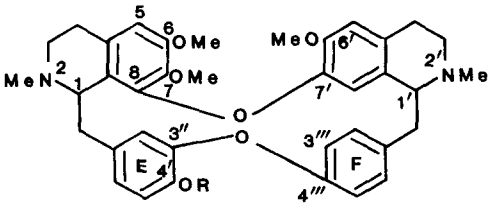
3. Dansyl-CaM (D-CaM) was previously prepared by us.[6] D-CaM retained basically the biological activity of native CaM and contained average 1.3 mole of dansyl per mole of CaM.

Fluorescence spectra and its intensities were recorded with Shimadzu RF 54 fluorescence spectrophotometer at room temperature. All fluorescence experiments were carried out in the standard buffer (0.1M KCl, 10mM Mops. pH 7.0) and indicated concentration of EGTA or Ca^{2+}. The titration was carried out at a fixed excitation and emission wavelength of 340 nm and 498 nm for D-CaM, respectively. The corresponding bandwidths were 5 and 10 nm.

Results

1. Effect of EBB on Ca^{2+}-Mg^{2+}-ATPase Na^+-K^+-ATPase, Mg^{2+}-ATPase activities in RBC membrane.

The stimulation of erythrocyte Ca^{2+}-Mg^{2+}-ATPase by CaM and antagonism of the activation by EBB were demonstrated in Fig.2. The specific activities of CaM-dependent Ca^{2+}-Mg^{2+}-ATPase and basal activities are about 60, 15 nmol/mg.min, respectively. The EBB dramatically inhibited the CaM induced activity of Ca^{2+}-Mg^{2+}-ATPase at concentration 1.0 μM up to which no significant inhibition of basal activity was apparent. The IC_{50} value for inhibiting CaM was approximately 0.35 μM (fig.2). On the other hand, Na^+-K^+-ATPase and Mg^{2+}-ATPase was almost not affected by EBB in the concentration less than 1.0 μM(fig.2). At the higher concentration (1.0 μM), the activity of the basal Ca^{2+}-Mg^{2+}-ATPase (CaM-independent), Na^+-K^+-ATPase and Mg^{2+}-ATPase enzyme was also inhibited. IC_{50}value of EBB for basal Ca^{2+}-Mg^{2+}-ATPase, Na^+-K^+-ATPase and Mg^{2+}-ATPase were about 30 μM, 20 μM and 50 μM, respectively.



R = $-CH_2CH_2CH_2CH_2-OCH_2CH_3$

Fig.1. The chemical structure of EBB.

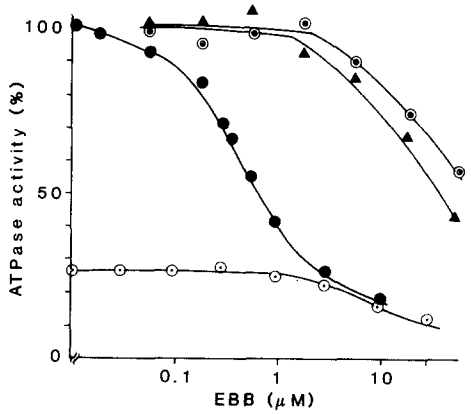


Fig.2. Inhibition of the Ca^{2+}-Mg^{2+}-ATPase, Na^{+}-K^{+}-ATPase and Mg^{2+}-ATPase activity by EBB.

-⊙- basal, CaM-independent Ca^{2+}-Mg^{2+}-ATPase

-●- CaM-stimulated Ca^{2+}-Mg^{2+}-ATPase (in the presence of 24 nM CaM)

-▲- Na^{+}-K^{+}-ATPase

-◎- Mg^{2+}-ATPase

100% activity for Ca^{2+}-Mg^{2+}-ATPase = 60 nmoles/mg.min

100% activity for Na^{+}-K^{+}-ATPase = 6 nmoles/mg.min

100% activity for Mg^{2+}-ATPase = 3 nmoles/mg.min

each point is the mean of three determinations.

In the presence of different concentration of EBB, the activation of Ca^{2+}-Mg^{2+}-ATPase by CaM was investigated. The result was shown in fig.3(a). In the absence of the EBB about 3.6 nM CaM required for half-maximal activation of the CaM-dependent fraction of Ca^{2+}-Mg^{2+}-ATPase. In the presence of 0.18 μM and 0.54 uM EBB the dose effect curve of CaM was shifted to the right. The concentration of CaM required for half-maximal activation was increased approximately to 8.4 and 24 nM, respectively.(fig.3(a)) The double-reciprocal plot demonstrated that the activation of erythrocyte CaM-stimulated Ca^{2+}-Mg^{2+}-ATPase is antagonized by EBB according to a competitive mechanism(fig. 3(b)). Increasing concentration of CaM completely reverse the inhibition caused by higher concentration of EBB.

2. The fluorescence study on the interaction of EBB with CaM

In the presence of EGTA the fluorescence spectrum of D-CaM exhibited a a maximum at 512 nm and a lower fluorescence intensity(fig.4,A). In contrast, in the presence of Ca^{2+}, the fluorescence spectra of D-CaM undergo blue shift.

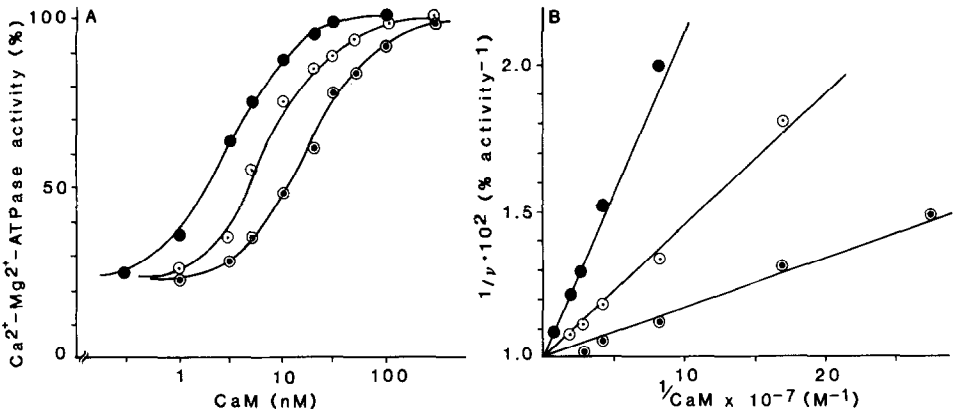
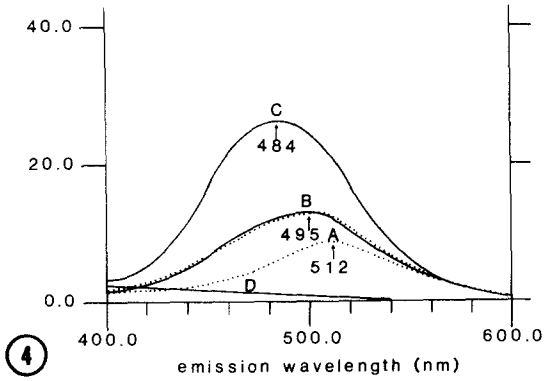


Fig.3 (A) Antagonism of CaM-stimulated Ca^{2+}-Mg^{2+}-ATPase activity by EBB.
basal Ca^{2+}-Mg^{2+}-ATPase is stimulated by increasing amount of CaM in the absence (-●-) and presence of the EBB at concentration of 0.18 μM (-⊙-) and 0.54 μM (-◎-)
(B) Double-reciprocal plot of dependence of rate(ν) of Ca^{2+}-Mg^{2+}-ATPase reaction on CaM. represents % activity as given in Fig.3(A)

The wavelength of the emission maximum decreased from 512 to 495 nm and fluorescence intensity increased at 495 nm by 1.6 fold(fig.4,B).

Subsquent addition of EBB to a solution of Ca^{2+}-CaM leads to increase in Dansylfluorescence intensity accompanied by a progressive blue shift (from 495 to 484 nm). Titration of EBB to CaM solution in the presence of EGTA induced also blue shift and fluorescence intensity change, although these alterations were much weaker than in the presence of Ca^{2+}. (The result does not shown)

The interaction of CaM and EBB was further investigated by stoichimetric titrating D-CaM (1 μM) either in the presence or absence of Ca^{2+} with EBB and motitoring the fluorescence intensity changes of D-CaM at 484 nm. Because the protein concentration used was far higher than the dissociation constant (Kd is about 10 nM), under these condition added EBB was assumed to be totally bound to the protein. Fig.5 illustrates the increase in fluorescence intensity as a function of EBB/CaM concentration. The titration plot exhibited two slops (fig.5). The endpoints of the titration can be calculated from the change in the slop of the plots. The results indicate that CaM contains 2 binding sites for EBB. In the presence of EGTA, a gradual increase in fluorescence intensity with the increase of EBB concentration was observed, with a slope similar to that of the nonspecific EBB binding in the presence of Ca^{2+}. These results clearly demonstrated that EBB interacts even with



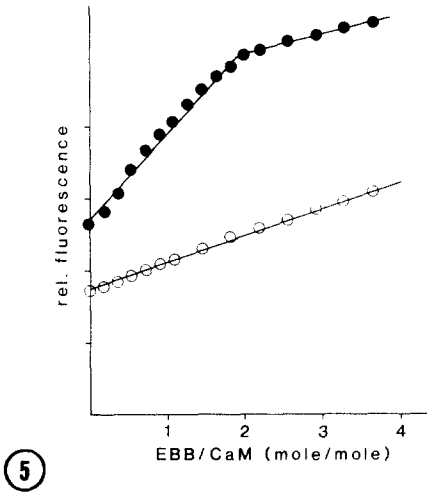


Fig.4 Effects of Ca^{2+} and EBB binding to CaM on fluorescence spectrum of D-CaM.
the spectra of 1.0 μM solution of D-CaM were recorded in the standard buffer contained 10 mM Mops(pH 7.0) 0.1 M KCl. Excitation 340 nm.
curve A: in the presence of 0.1 mM EGTA
B: in the presence of 0.1 mM Ca^{2+}
C: in the presence of 0.1 mM Ca^{2+} and 1.8 μM EBB.
D: only buffer plus 1.8 μM EBB in the absence of D-CaM

Fig.5 Fluorescence intensity of D-CaM as a function of EBB concentration (mole/mole of CaM). Irradiation was performed at 340 nm. The fluorescence intensity of 1.0 μM D-CaM was measured at 484 nm in standard buffer succesive additions of EBB in the presence of Ca^{2+} 0.1 mM -●- and EGTA -○- .

Ca^{2+}-free CaM probably due to nonspecific hydrophobic adsorption of EBB by CaM. However, Ca^{2+}-CaM involves extensive binding of EBB to CaM which gives far greater changes in Dansyl-fluorescence than Ca^{2+}-free CaM.

4. Effect of Ca^{2+}-CaM and EBB on fluorescence of NPN

It had been shown that the fluorescence of hydrophobic probes (such as NPN, ANS, TNS) are significally enhanced in the presence of CaM in a Ca^{2+}-dependent manner.(9) When excited at 350 nm, in the absence of CaM, NPN exhibited a maximum emission wavelength at 458 nm(fig.6,A). After addition of CaM(1 μM), maximum emission of NPN shifted to 415 nm and fluorescence intensity was about increased 7 fold. (fig.6,B) TFP depressed this enhanced fluorescence and show less blue shifting extent.(fig.6,D) In contrast, however, EBB increased further the fluorescence of NPN and much blue shift.(fig.6,C)

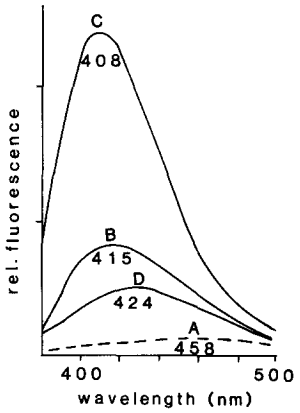


Fig.6 Effect of EBB, TFP on the fluorescence of NPN in the presence of CaM. (excitation 350 nm)
curve A: emission fluorescence of NPN alone (1.0 uM), 0.1 mM Ca^{2+}.
maximal emission 458 nm.
B: NPN plus 1.0 uM CaM. max 414 nm.
C: NPN, 1.0 uM CaM plus 1.8 uM EBB. max 408 nm.
D: NPN, 1.0 uM CaM plus 3.0 uM TFP. max 424 nm.

Discussion

CaM inhibitors have one common feature and may be classified as cationic amphiphiles.[10] Hidaka et al reported the correlation between the hydrophobicity of Naphthalenesulfonamide derivatives and their potency in inhibiting Ca^{2+}-CaM through a hydrophobic interaction.[11] We have reported previously that the Tetrandrine is a new CaM antagonist.[4] In order to study the relationship of activities-structure of tetrandrine-analogues we synthesized a serious derivatives of bisbenzylisoquinoline with various lengths of alkyl chain. Our results clearly demonstrate the close correlation between the hydrophobicity of each derivatives and their potency in inhibiting CaM-dependent Ca^{2+}-Mg^{2+}-ATPase.[12] Among them the EBB is a most potent CaM antagonist. The fact that the inhibition of CaM-dependent Ca^{2+}-Mg^{2+}-ATPase by EBB was 10 fold more potent than that by O-butylberbamine suggests that in addition to hydrophobic and electrostatic interaction between CaM and its antagonist, the fine structure of antagonist, the fine structure of antagonist can dramatically affect their potency of antagonism.[12]

The action of EBB on Ca^{2+}-Mg^{2+}-ATPase seems to be rather specific with respect to other ATPase of the membrane. EBB has a higher specific coefficient (the ratio of the IC_{50} value of basal Ca^{2+}-Mg^{2+}-ATPase activity to the IC_{50} value of the CaM-dependent fraction of the enzyme's activity) (29 for calmidazolium, 85 for EBB)[13]. From this point EBB seems to be a outstanding CaM

antagonist and proposed to be a promising tool for studying CaM dependent processes.

The fluorescence of D-CaM provides a convenient means for examing its interaction with the CaM antagonist[8]. The further increase in the spectral shift fluorescence intensity of D-CaM observed when EBB was added in the presence of Ca^{2+} may be interpreted as result from the formation of EBB-CaM complex and alteration of the protein comformation. Our results have also shown that even in the absence of Ca^{2+} EBB interact with CaM probaly through a non-specific hydrophobic adsorption of EBB by CaM although this interaction is weaker than in the presence of Ca^{2+}. The nonspecific adsorption sites could not be quantified in our experiment. NPN is weakly fluorescence in aqueous solution and its fluorescence intensity is increased when it binds to Ca^{2+}-CaM. The spectral modifications are due to the appearance of hydrophobic region on the CaM surface following conformational change in CaM[14]. TFP depressed this enhanced fluorescence as a result of competitive interaction of TFP and NPN on same binding sites of CaM. In contrast, however, EBB increased the fluorescence of NPN on Ca^{2+}-CaM complex. These results demonstrated that the binding of EBB on CaM increased its affinity for NPN suggesting that allosteric interactions occur among different hydrophobic binding sites on CaM[15] and the interaction of EBB with CaM through a manner that differ from the interaction of CaM with TFP. The binding of EBB may expose more hydrophobic region on CaM as evidenced by the further binding of NPN.(either number and/or affinity of NPN binding sites)

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