The effect of berbamine derivatives on activated Ca^{2+} -stimulated Mg^{2+} -dependent ATPase in erythrocyte membranes

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Ca^{2+} -stimulated Mg^{2+} -dependent ATPase ($\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase) stimulated by calmodulin, by partial proteolysis or by oleic acid in erythrocyte membranes was inhibited by various derivatives of the naturally occurring alkaloid berbamine. The ability of these derivatives to inhibit trypsin-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase correlated well with their ability to inhibit the calmodulin-stimulated enzyme. Inhibition of the trypsin-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase by *O*-4-(ethoxybutyl)berbamine (EBB) was competitive with respect to ATP. The K_i for inhibition was about $8 \mu\text{M}$. These results suggest that the binding site of EBB on the activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase may bear structural similarity to that on calmodulin, and may be closely related to the ATP-binding site on the enzyme.

INTRODUCTION

It has recently been shown that several CaM-dependent enzymes, including cyclic AMP phosphodiesterase, $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase, myosin light-chain kinase and NAD^+ kinase, are stimulated by partial tryptic digestion in a Ca^{2+} -independent manner or by anionic amphiphiles, such as acidic phospholipids and unsaturated fatty acids [1]. It is of interest, also, that many CaM antagonists, such as trifluoroperazine, penfluridol and calmidazolium, inhibit $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activated by trypsin treatment, acidic phospholipids or unsaturated fatty acids [2]. This implies that $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase might contain a similar structural domain to that on CaM.

Recently we reported that many bisbenzylisoquinoline compounds possess anticalmodulin activities [3,4]. Tetrandrine and berbamine are among the better CaM inhibitors among the natural isoquinoline alkaloids. EBB is a potentially and particularly useful CaM antagonist among the synthetic derivatives of berbamine, as it has a high specific coefficient for inhibition [4]. In order to assess the regulatory mechanism of $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase in the membrane we have investigated the effects of various berbamine derivatives on the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity activated in a number of different ways.

MATERIALS AND METHODS

All reagents were analytical-reagent grade or the highest purity available. Berbamine and tetrandrine were provided by Dong Sheng (Beijing, China) and Jing-hua Pharmaceutica (Zhejiang Province, China) respectively. Trypsin (204 units/mg) was a product of Biochemical Pharmaceutica (Shanghai, China). Ouabain and soya-bean trypsin inhibitor were obtained from Sigma Chemical Co. Preparations of CaM and CaM-deficient erythrocyte membranes were obtained as described previously [5].

$\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity was determined at 37°C

by measuring the rate of liberation of P_i by the Malachite Green dye method of Lanzatta [6] as modified slightly by us [7]. The assay medium contained $60 \mu\text{g}$ of membrane protein/ml, 25 mM-Tris/maleate buffer, pH 7.0, 100 mM-NaCl, 2 mM- MgCl_2 , 0.1 mM-ouabain, 1 mM-ATP and $36 \mu\text{M}$ - Ca^{2+} (as a $0.2 \text{ mM-Mg}^{2+}/0.2 \text{ mM-Ca}^{2+}/0.4 \text{ mM-EDTA}$ buffer).

CaM-deficient erythrocyte membranes were treated with 0.2 mg of trypsin/mg of protein. After 5 min incubation at 37°C the reaction was terminated by addition of a 5-fold (w/w) excess of soya-bean trypsin inhibitor. This procedure activated the enzyme up to 90% of the value obtained with CaM.

Oleic acid microdispersions were prepared by sonication in a W-220 Sonicator (Heat Systems-Ultrasonics) for 3 min on output control 7 in 0.1 mM-EGTA/5 mM-Mops buffer, pH 7.0, under a stream of N_2 . Oleic acid also stimulated the enzyme activity up to 90% of the CaM-dependent activation.

The various berbamine derivatives were synthesized as described previously [3].

Protein was determined by the method of Bradford [8], with bovine serum albumin as a standard.

RESULTS

The effect of EBB on $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activity activated by CaM, by tryptic proteolysis or by oleic acid is shown in Fig. 1. IC_{50} values for the three forms of activated enzyme were $0.35 \mu\text{M}$, $13 \mu\text{M}$ and $15.5 \mu\text{M}$ respectively. That is, the inhibitory effect of EBB on CaM-stimulated enzyme was much more potent than that on the trypsin-activated and oleic acid-activated enzymes.

Table 1 summarizes the IC_{50} values of various berbamine derivatives on $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activated by the three methods described above. We have already reported that the inhibition of CaM-dependent $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase by various derivatives of berbamine

Abbreviations used: $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase, Ca^{2+} -stimulated Mg^{2+} -dependent ATPase; CaM, calmodulin; EBB, *O*-4-(ethoxybutyl)berbamine.

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Fig. 1. Inhibition of activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase by EBB
●, CaM-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase (assayed in the presence of $0.4 \mu\text{g}$ of CaM/ml and $36 \mu\text{M}$ free Ca^{2+}); ▲, trypsin-treated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase; ○, oleic acid-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase. For experimental details see the text. Each point represents the mean for three determinations. The 100% activity of CaM-activated enzyme corresponds to $60 \text{ nmol/min per mg}$ of protein.

Fig. 2. Correlation between IC_{50} values of berbamine derivatives for CaM-activated and trypsin-treated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase
For experimental details see the text. The abbreviated names of the berbamine derivatives are explained in Table 1.

Table 1. Effect of various derivatives of berbamine on $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activated by CaM, by trypsin treatment or by oleic acid
 IC_{50} values were calculated as the concentration producing 50% inhibition of activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase.

Structure of berbamine derivatives:				
R	Abbreviated name	$\text{IC}_{50} (\mu\text{M})$ of activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase by:		
		CaM	Trypsin treatment	Oleic acid
-OH (berbamine)	B-0	60 ± 5	320	300
-OCH ₃ (tetrandrine)	B-1	40 ± 7	140	80
-OC ₂ H ₅	B-2	20	80 ± 10	160
-OC ₄ H ₉ (n)	B-4	6.8	13	22
-OC ₈ H ₁₇ (n)	B-8	2.5	14	22
-O(CH ₂) ₄ -OC ₂ H ₅	BBB	0.35	13	15.5
-O-C(=O)-CH ₃	AB	18	90	190
-O-C(=O)-C ₆ H ₅	BB	5.0	4.8	100
-SO ₂ -	DB	1.7	24	19

Fig. 3. Inhibition of trypsin-treated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase by EBB at different concentrations of ATP

For experimental details see the text.

correlated with the length of the hydrocarbon chain [4]. As shown in Table 1, $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activated by the other two methods (trypsin treatment and oleic acid) was also inhibited by berbamine derivatives, and the inhibition also depends on the length of the alkyl chain, as in the case of the CaM-dependent enzyme.

Fig. 2 shows the IC_{50} values of various derivatives of berbamine for the inhibition of CaM-stimulated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase plotted against the corresponding values for trypsin-activated enzyme. The results show that the ability of the berbamine derivatives to inhibit the enzyme activated by proteolysis correlates well with the potency for inhibition of the CaM-activated enzyme.

In order to study the relationship between the binding site of EBB on $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase and the active centre of the enzyme, the inhibition by EBB was investigated with respect to different concentrations of ATP. Fig. 3 shows the kinetic analysis of the inhibition of trypsin-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase by EBB by a Dixon plot [9]. As can be seen from this plot, the inhibition by EBB of the trypsin-activated enzyme was competitive with respect to ATP. The K_i value was about $8 \mu\text{M}$. This result suggests that EBB has a somewhat higher affinity for enzyme exposed through proteolysis, and the binding site(s) may be close to the ATP-binding site(s) on the enzyme.

DISCUSSION

The extensive work of Hidaka *et al.* [10] on the structure-activity relationship of compound W-7 and its naphthalenesulphonamide analogues has shown that the length of the hydrocarbon chain is an important determinant of anti-CaM activity. Similar results were obtained by us from a study of berbamine derivatives [4]. On the other hand, the length of the alkyl chain also determines the activity of these derivatives for inhibition of the trypsin-activated and oleic acid-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase. This result suggests that the activations of the enzyme by CaM and the other treatments share some common mechanism [11]. The hydrophobic interaction

between CaM and its target enzyme or with CaM antagonists is an important factor in both activation and inhibition phenomena in CaM-regulated enzymes. The observed inhibition of the trypsin-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase by berbamine derivatives at concentrations similar to those that inhibit CaM activation led to the assumption that these compounds interact with CaM as well as with the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase [11]. It may be speculated that the binding site for EBB on the enzyme activated by proteolytic treatment may have similar features to that on CaM, although the affinity of EBB for CaM is higher than that of binding sites on trypsin-activated enzyme.

The binding site of EBB on $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase appears to be at or near the active centre, as EBB inhibited the trypsin-activated enzyme in a competitive fashion with respect to ATP (Fig. 2). Itoh & Hidaka [12] reached a similar conclusion from the study of the inhibition of CaM-dependent phosphodiesterase by a series of W-7 analogues. This suggests that similar structural features seem to exist among different CaM-activated enzymes.

It may be noted that the different kinds of berbamine derivatives can be divided into two groups with regard to their inhibition of CaM- or trypsin-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase. One group is an *O*-alkylated series, and the other is an *O*-acylated group. By comparison, trypsin-activated $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase is more sensitive to the alkylated derivatives than to the acylated derivatives. In contrast, CaM-dependent $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase is more sensitive to acylated berbamine derivatives. The one exception to this is EBB. Although this compound belongs to the alkylated series of berbamine derivative, it shows a very potent inhibitory effect and exceptional selectivity for the CaM activation. Half-maximal inhibition of the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase activated by partial proteolysis or oleic acid required more than 35-fold higher concentrations of EBB than that for CaM antagonism. This suggests that besides the hydrophobic and electrostatic interaction between CaM and its antagonists the stereoarrangement of the atoms in the antagonist molecule is also an important determinant of anti-CaM activity and specificity.

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