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# INTERACTION OF BERBAMINE COMPOUND $E_6$ AND CALMODULIN-DEPENDENT MYOSIN LIGHT CHAIN KINASE

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**Abstract**—The interaction of the berbamine compound $E_6$ and calmodulin (CaM)-dependent myosin light chain kinase (MLCK) has been studied. The experimental results showed that the inhibition of MLCK activity was increased with increasing amounts of $E_6$ and was overcome completely by the addition of excessive CaM. The stimulatory activity of MLCK induced by CaM was gradually inhibited by the increasing concentrations of compound $E_6$, showing that the inhibition of MLCK activity by compound $E_6$ was concentration dependent; and the $K_i$ was 0.95 $\mu$M. Compound $E_6$ diminished the fluorescence intensity of dansyl-labeled CaM and the intensity was increased gradually by the addition of different amounts of CaM. Compound $E_6$ had no effect on the activity of MLCK fragments produced by limited trypsinization, and it is a novel and considerably potent calmodulin antagonist.

Seeing that (i) some berbamine compounds (E‡) markedly inhibit enzymes that are calmodulin (CaM) dependent [1], (ii) CaM-dependent myosin light chain kinase (MLCK) plays a key role in the contraction of smooth muscle, and (iii) the regulation of MLCK activity might be abnormal in some cardiovascular diseases, a specific inhibitor of MLCK activity may provide a new drug for the treatment of some of these diseases. $R_0$ 22-4839 [2], one of the isoquinoline compounds used in clinical trials as a new cerebral circulation improver, is one notable example. MLCK is not only regulated by CaM, but also is affected by cAMP-dependent protein kinase. To increase the specificity of inhibition for MLCK activity, Kemp *et al.* [3] synthesized a peptide fragment conjugated between MLCK and CaM, which is a competitive inhibitor for binding between MCLK and CaM, similar to a CaM antagonist. It has been reported that berbamine compounds, a type of benzylisoquinoline compound, are active in cardiovascular abnormalities such as antiarrhythmia and decreased blood pressure. Compound $E_6$ inhibits the activity of CaM-dependent MLCK and phosphodiesterase (PDE) with $IC_{50}$ values of 8 and 0.53 $\mu$M, respectively; its $IC_{60}$ values for MLCK and PDE, which is also CaM dependent, are four times smaller than those of trifluoperazine (TFP) [4]. In the present study, we have explored further the interaction between compound $E_6$ and CaM/MLCK in order to probe the probable relationship and action on the $Ca^{2+}$–CaM–MLCK system.

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‡ Abbreviations: E, berbamine compound; MLCK, myosin light chain kinase; MLC, myosin light chain; CaM, calmodulin; TFP, trifluoperazine; EGTA, ethylene glycol bis ($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

## MATERIALS AND METHODS

Berbamine compounds were synthesized in our laboratory. Their purity was confirmed by spectral analysis and also by elemental analysis. The structure of compound $E_6$ is shown in Fig. 1. Trypsin was obtained from Difco. Trypsin inhibitor (from the Sigma Chemical Co.) was donated by Dr. Wai Yiu Cheung (Department of Biochemistry, St. Jude Children's Research Hospital, U.S.A.). ATP was obtained from the Kyona Hakko Kogyo Co. Ltd., and [$\gamma$-$^{32}$P]ATP, specific activity 500 Ci/mmol, from the Xing Tong Biotechnical Research Institute. 5-Dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride was from Sigma. Phenylmethylsulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were obtained from the Shanghai Biochemical Institute. Standard proteins for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were from Pharmacia. Other chemicals were of reagent grade, and water was deionized.

CaM was purified to homogeneity from porcine brain using a phenyl-Sepharose CL 4B column [5] and DEAE-ion exchange column chromatography [6].

The MLCK was prepared from fresh chicken gizzards by the method of Adelstein and Klee [7]. Due to the limitations of the experimental conditions, we were obliged to change the centrifugation conditions (10,000 ~ 12,000 *g* for 20 or 30 min) and to make use of a single enzyme inhibitor, PMSF; however, we did obtain homogeneous MLCK as verified by SDS–PAGE.

The 20,000 Da myosin light chain from gizzards ($MLC_{20}$), which was used as a substrate for the MLCK, was prepared according to the method of Hathaway and Haeberle [8], with some modifications. Fresh chicken gizzards were treated at 0–4° to remove fat and connective tissue. Muscle was washed to a white color with buffer A (containing

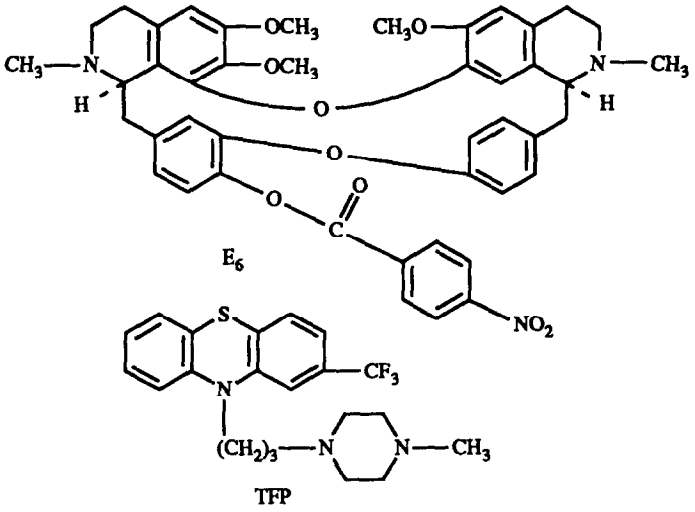


Fig. 1. Structures of a berbamine compound ($E_6$) and trifluoperazine (TFP).

20 mM Tris–HCl, pH 6.8, 20 mM KCl, 2 mM 2-mercaptoethanol, 1 mM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM $MgCl_2$, with or without 0.2% Triton X-100). The white myofibrils were homogenized in buffer B (containing 0.5 M KCl, 15 mM Tris–HCl, 5 mM 2-mercaptoethanol, 1 mM EGTA, pH 7.5). Following sedimentation, the supernatant was dialyzed and sedimented. The pellet was sonicated in 2 vol. buffer (containing 20 mM Tris–HCl, pH 7.0, 5 mM 2-mercaptoethanol); solid urea, guanidine hydrochloride, 20% SDS and ethanol were added, followed by the addition of deionized water and sedimentation. The supernatant was dialyzed to remove ethanol, urea and other denaturants. The light chain enriched supernatant was adjusted to pH 7.5 with 1 M Tris base; in addition, solid sodium chloride was added to 0.6 M. The mixture was loaded onto a phenyl-Sepharose 4B column which was equilibrated with high salt buffer and washed with 10 column volumes of the same buffer. $MLC_{20}$ was eluted with 10 mM Tris–HCl buffer, pH 7.0, containing 1 mM 2-mercaptoethanol.

The contents of $MLC_{20}$ and $MLC_{17}$ in our preparation were 54 and 46%, respectively, as determined by SDS–PAGE and thin-layer gel scanning with a Shimadzu CS-930 at 560 nm.

Dansyl-labeled CaM was prepared according to the method of Ronca-Testoni *et al.* [9]. Trypsinization of MLCK was performed as described by Tanaka *et al.* [10].

*Assay of MLCK activity.* MLCK activity was measured essentially as previously described [11]. The enzyme activity was assayed in a total volume 0.1 mL of buffer (containing 25 mM Tris–HCl, pH 7.5, 4 mM $MgCl_2$, 60 mM KCl, 0.2 mM $CaCl_2$ or 2 mM EGTA, with or without 12 nmol CaM, 0.34 mg/mL MLC and 25 μg/mL MLCK). The assay was initiated by the addition of 10 μL of 1 mM [$\gamma$-$^{32}$P]ATP (50–80 cpm/ppm) and incubated at 25° for 2 min. An aliquot of the mixture was applied to 2 × 2 cm Xin Hua 2* filter paper and the filter paper was placed immediately into 25% trichloroacetic acid–2% sodium pyrophosphate solution to terminate the reaction. Next, the filter paper was washed with the same solution to remove the excess [$\gamma$-$^{32}$P]-ATP, and then by anhydrous ethanol; the filter paper was immersed in 4 mL of toluene scintillation liquid and the radioactivity was counted in a Beckman 5801 Scintillation Spectrophotometer.

When measuring the effect of the berbamine compound on the MLCK activity, different concentrations of the compound were added individually into the assay system and the mixture was incubated.

*Detection of the interaction between CaM and the berbamine compound.* Measurements were conducted in 3 mL buffer (containing 10 mM Tris–HCl, 0.4 μM dansyl-CaM, 90 mM KCl and 2 mM EGTA, pH 7.5). The excitation and emission wavelengths were 340 and 490 nm, respectively. Both excitation and emission spectral bandwidths were 6 nm. The fluorescence intensities were determined in the presence and absence of $Ca^{2+}$ and the different concentrations of berbamine compound at excitation 340 and emission 490 nm with a HITACHI MPF-4 Fluorescence Spectrophotometer. All relative fluorescence was determined by setting a blank control each time.

SDS–PAGE was performed in 0.1% SDS–12.5% or –7.5% polyacrylamide gel for MLC and CaM or for MLCK using the buffer system of Laemmli [12].

Protein determination was carried out according to Lowry *et al.* [13]. Using BSA as a standard.

All assay procedures were performed two or three times.

## RESULTS AND DISCUSSION

The stimulatory activity of MLCK induced by CaM was gradually inhibited by increasing concentrations of compound $E_6$. The inhibition could be abolished by adding an excess of CaM, indicating

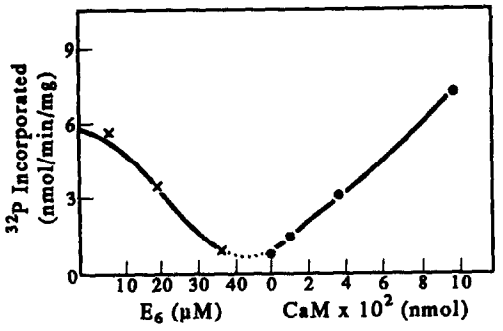


Fig. 2. Effects of increasing concentrations of CaM on the inhibition of MLCK activity by $E_6$. The activity was measured as described in Materials and Methods. Key: (×—×) effects of $E_6$ on the activity of MLCK activated by CaM, and (●—●) effects of increasing concentrations of CaM on the inhibition of MLCK activity by $E_6$.

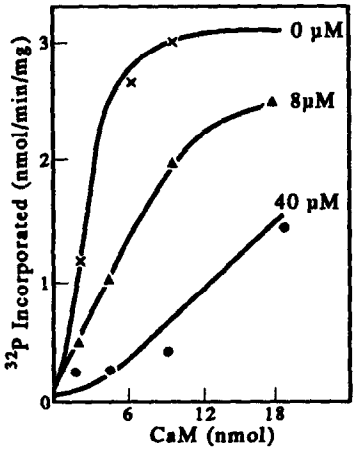


Fig. 3. Effects of different concentrations of $E_6$ on the inhibition of MLCK activity.

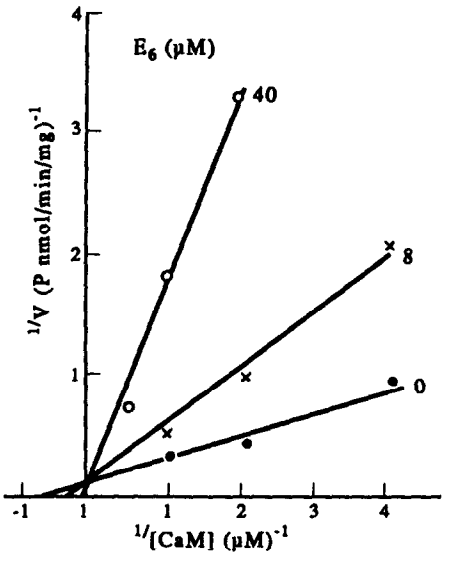


Fig. 4. Double-reciprocal plots of the inhibition of CaM-activated MLCK by $E_6$. MLCK activity was determined as described in Materials and Methods.

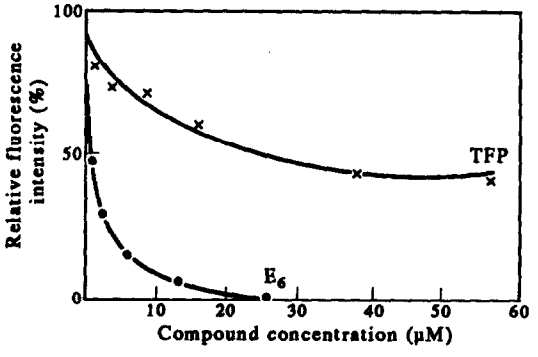


Fig. 5. Effects of compound $E_6$ and TFP on fluorescence intensities of dansylated CaM.

that the inhibition of MLCK activity stimulated by CaM was produced as a result of the action of compound $E_6$ and that it may produce its effect by acting directly on CaM (Fig. 2). Vice versa, when measuring the stimulation curve of MLCK, the inhibition of MLCK activity decreased with decreasing concentrations of compound $E_6$, showing a concentration-dependent effect of the compound (Fig. 3). This result suggested indirectly that compound $E_6$ may bind to CaM. By increasing the amount of CaM or decreasing the amount of compound $E_6$, free CaM in the reaction system was relatively increased; thus, the MLCK activity that was inhibited by compound $E_6$ was restored. $E_6$ acted as a competitive inhibitor to CaM. The inhibition constant ($K_i$) was determined from the data shown in Fig. 3 using a double-reciprocal plot; the $K_i$ was 0.95 μM (Fig. 4).

To elucidate the direct interaction between compound $E_6$ and CaM, the effects of compound $E_6$ and TFP on the fluorescence intensity of the dansyl fluorescence probe labeled CaM were observed (Fig. 5). The data shown in Fig. 5 appear to indicate a dissociation constant for $E_6$ binding to dansyl-CaM of approximately 1 μM, using a Scatchard plot. The value of this dissociation constant was approximately the same as the $K_i$ value. It would be helpful to determine the $K_i$ value for MLCK inhibition. Both compound $E_6$ and TFP diminished the fluorescence intensity of dansyl-CaM, but the potency of the former was stronger than that of the latter. In the presence of 2 mM EGTA, the relative maximum fluorescence intensity of dansyl-CaM was 9.0. When the concentration of $CaCl_2$ was 0.15 mM in excess of EGTA, the relative maximum fluorescence intensity of dansyl-CaM was equivalent to 17.5. This value was almost 2-fold greater than that of the former. The addition of the berbamine compound $E_6$ to the assay system diminished the

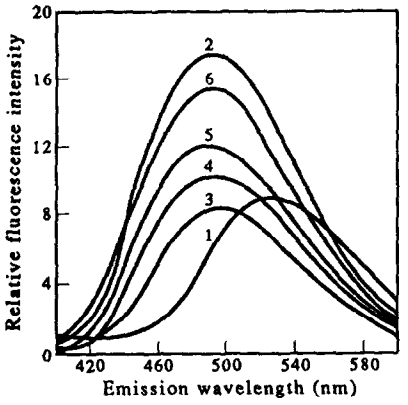


Fig. 6. Effects of increasing concentrations of CaM on the inhibition of fluorescence intensity of dansyl-CaM elicited by $E_6$. Key: (1) D-CaM + 2 mM EGTA; (2) D-CaM + 0.15 mM $CaCl_2$; (3) D-CaM + 0.15 mM $CaCl_2$ + 2.5 $\mu$M $E_6$; (4) D-CaM + 0.15 mM $CaCl_2$ + 2.5 $\mu$M $E_6$ + 0.2 $\mu$M CaM; (5) D-CaM + 0.15 mM $CaCl_2$ + 2.5 $\mu$M $E_6$ + 0.4 $\mu$M CaM; and (6) D-CaM + 0.15 mM $CaCl_2$ + 2.5 $\mu$M $E_6$ + 1.2 $\mu$M CaM.

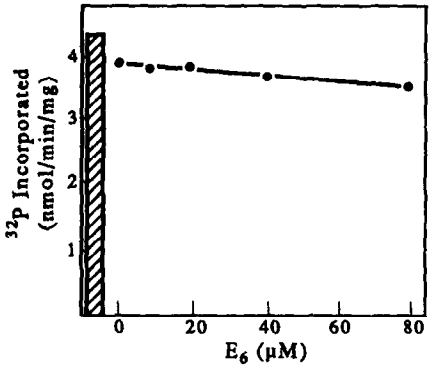


Fig. 7. Effect of $E_6$ on the activity of trypsin-digested MLCK. The assay of digested MLCK was performed in the presence of 2 mM EGTA. Key: (▨) MLCK activity determined in the presence of 0.2 mM $CaCl_2$ and 19 nmol CaM.

relative fluorescence intensity of dansyl-CaM, but the change of wavelength at the point of maximum emission was not obvious. With the addition of CaM, the position of the fluorescence curve was near that of the fluorescence curve in the absence of compound $E_6$ (Fig. 6). $E_6$ had no effect in the presence of 2 mM EGTA, suggesting that compound $E_6$ effected directly on CaM, and was dependent on calcium.

Though the experiments above indicated that CaM and compound $E_6$ interact directly, another question was whether compound $E_6$ can also interact with MLCK in the reaction system.

A characteristic for many CaM-dependent enzymes is that limited trypsinization of these enzymes increases their catalytic activity. An example is the native CaM-dependent protein phosphatase which is stimulated by $Ca^{2+}$ and CaM. Limited trypsinization of this phosphatase and its catalytic subunit A increases its catalytic activity to the level observed with CaM; this activity is further stimulated by the regulatory subunit B but not by CaM [14]. It is known that MLCK is absolutely dependent on CaM. Like CaM-dependent protein phosphatase, limited trypsinization of MLCK increases its activity to the level determined with CaM and this activity is not stimulated by $Ca^{2+}$/CaM [10]. We obtained a similar result.

Taking advantage of this characteristic of MLCK after limited proteolysis, we observed the effect of compound $E_6$ on the activity of an MLCK fragment. The experiment showed that compound $E_6$ did not influence the activity of the MLCK fragment (Fig. 7). This result showed in another way that compound $E_6$ inhibited the activity of MLCK in the presence of $Ca^{2+}$ because of its antagonism to CaM. If there is a similar regulation both *in vitro* and *in vivo* by compound $E_6$, its physiological significance will be elucidated.

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