Potent Stimulation of Myofilament Force and ATPase Activity of Skeletal Muscle by Eudistomin M, a Novel Ca\(^{++}\)-Sensitizing Agent from a Caribbean Tunicate\(^1\)

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ABSTRACT

In the course of our survey of biologically active compounds from natural sources, eudistomins were isolated from a Caribbean tunicate Eudistoma olivaceum. In the present experiments, eudistomin M (Eud-M, \(>10^{-5}\) M) caused a concentration-dependent increase in the contractile response of skinned fibers from guinea pig skeletal psoas muscles to Ca\(^{++}\). The superprecipitation and ATPase activity of myosin B from fast skeletal muscles of rabbit back and leg were potentiated by this compound \((>10^{-5}\) M) in a concentration-dependent manner. In skinned fibers, superprecipitation and the ATPase activity of myosin B, Eud-M shifted the concentration-response curve for Ca\(^{++}\) to the upper direction. Ca\(^{++}\), K\(^+-\)EDTA- or Mg\(^{++}\)-ATPase was not affected by Eud-M. This compound had no effect on the ATPase activity of actomyosin reconstituted from actin and myosin in the presence or absence of troponin. However, the ATPase activity of actin-myosin-troponin-tropomyosin reconstituted system was increased significantly by Eud-M. These results suggest that Eud-M increases the Ca\(^{++}\) sensitivity of the contractile apparatus in skeletal muscles at least partially mediated through troponin-tropomyosin system and thus enhances the ATPase activity of myosin B and the contractile force of myofilament.

Binding of Ca\(^{++}\) to the Ca\(^{++}\)-specific sites of troponin C alters the interactions between troponin C, troponin I and troponin T. This in turn alters the troponin I-actin and troponin I- and troponin T-tropomyosin interactions in a manner that strengthens the actin-myosin interaction and results in a remarkable enhancement of the actomyosin ATPase activity. Concomitant with these changes is the well known shift of tropomyosin in the groove of F-actin (Huxley, 1971). The superprecipitation of actomyosin is generally accepted to be basically the same phenomenon \textit{in vitro} as a contraction in skeletal muscle cells (Szent-Györgyi, 1951). Numerous marine natural products have been useful as tools for physiological and biological studies because of their actions on specific sites of functional protein (Ohizumi, 1997). In the course of our survey on biologically active substances from marine sources, much attention has been given to compounds affecting the contractile apparatus. Recently, we have isolated several natural products that affect myosin and actin functions, such as purealin which modulates myosin ATPase activity (Takito \textit{et al.}, 1986; Nakamura \textit{et al.}, 1987), xestoquinone which modulates the specific sulfhydryl groups of myosin (Kobayashi \textit{et al.}, 1991a, b; Sakamoto \textit{et al.}, 1995) and goniodomin A which induces modulation of actomyosin ATPase activity mediated through conformational change of actin (Furukawa \textit{et al.}, 1993). In further research into marine natural products, eudistomins having \(\beta\)-carboline skeleton were isolated from a Caribbean tunicate (Kobayashi \textit{et al.}, 1984). In our structure-activity relationship studies of eudistomin derivatives we found that MBED induced Ca\(^{++}\) release from the skeletal muscle SR about 1000 times more potent than caffeine (Seino \textit{et al.}, 1991) and bound the same binding site the same as that of caffeine (Fang \textit{et al.}, 1993). In our continuous screening program for bioactive substances from natural resources, Eud-M (fig. 1) has been shown to potentiate the ATPase activity of myosin B. It is of interest whether the potentiation by Eud-M is due to the direct effect on the myosin molecules or to the modulatory effect on the interaction between actin and myosin. We present the first report indicating that Eud-M potentiates the contractile system of skeletal muscles.

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ABBREVIATIONS: Eud-M, eudistomin M; MBED, 9-methyl-7-bromoeudistomin D; SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis (\(\beta\)-aminoethyl ether)-N, N’-tetraacetic acid; Ms, methanesulfonate.
Materials and Methods

Materials. Eud-M was isolated from a Caribbean tunicate *Eudistoma olivaceum* as previously reported (Kobayashi et al., 1984). In the biochemical experiment, fast skeletal muscles of male rabbit (3 kg) back and leg were used to obtain much amount of experimental materials. Myosin B, actin, myosin, troponymosin and troponin were prepared as described by Szent-Györgyi (1951), Spudich and Watt (1971), Weeds and Taylor (1975), Ebashi et al. (1968) and Kohama (1979), respectively. In skinned fiber experiment, psoas muscles of male guinea pig (250-300 g) and male rabbit (3 kg) were used (Endo and Iino, 1979) and were immediately transferred into relaxing solution containing (mM): NaCl, 150; KCl, 2; CaCl₂, 2; glucose, 5.5; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 (pH 7.4) and were immediately transferred into relaxing solution containing (mM): K-Ms, 74.7; Mg-Ms₂, 5.4; ATP, 4; EDTA; 10; and piperazine-N, 9-bis(2-ethanesulfonic acid)-KOH, 20 (pH 7.0). A small muscle bundle of 4-5 fibers (ca. 0.1 mm in diameter and ca. 3 mm in length) was dissected from the psoas muscle. One end of the fiber was secured to the tissue holder by a ligature and the other end was connected to a force-displacement transducer (Acers AE801; Horten, Norway), the compliance of tension measurement system being approximately 0.5 mg/mm for measurement of isometric contraction of the fiber at 20 to 23°C. Fibers were treated with the relaxing solution containing 50 µg/ml saponin for 30 min and then with a 0.5% Triton X-100 solution for 15 min (Endo and Iino, 1980; Horii, 1986). Various solutions for skinned fiber experiments were prepared as described elsewhere (Kobayashi et al., 1991a). The maximal tension in response to high Ca²⁺ concentration was similar to the values in the literature (Endo and Iino, 1980; Horii, 1986). The survival of the preparation was at least 5 hr.

**Superprecipitation assay.** The superprecipitation was induced by adding 0.4 mM ATP in 0.3 mg/ml myosin B, 0.76 mM CaCl₂, 1 mM EGTA, 2 mM MgCl₂, 50 mM KCl and 20 mM Tris-HCl at pH 6.8 and 25°C, and the change in the absorbance at 660 nm was followed.

**Enzyme assay.** The reaction mixture for each ATPase was as follows (mM): 0.3 mg/ml myosin B; ATP, 2; EGTA, 1; MgCl₂, 2; CaCl₂, 0.76; KCl, 50; and Tris-HCl, 20 (pH 6.8) for myosin B ATPase; 0.1 mg/ml actin; 0.1 mg/ml myosin; ATP, 1; CaCl₂, 0.76; EGTA, 2; KCl, 50; MgCl₂ 2; and Tris-HCl, 20 (pH 6.8); for the ATPase activity of actomyosin reconstituted from actin and myosin; 0.1 mg/ml actin; 0.1 mg/ml myosin; 0.1 mM ATP; 10 mM Hepes; 2 mM MgCl₂; 20 mM Tris-HCl, 20 (pH 6.8); for the ATPase activity of actomyosin reconstituted from actin, myosin and tropomyosin; 0.1 mg/ml actin; 0.1 mg/ml myosin; 0.2 mg/ml tropomycin-tropomyosin complex; ATP, 2; EGTA, 1; MgCl₂, 2; KCl, 50; and Tris-HCl, 20 (pH 6.8); for the ATPase activity of actomyosin reconstituted from actin, myosin and tropomyosin-tropomyosin complex; 0.15 mg/ml myosin; ATP, 2; CaCl₂, 10; KCl, 500; and Tris-HCl, 50 (pH 7.4); for the Ca⁺⁺-ATPase activity of myosin, 0.015 mg/ml myosin; ATP, 2; EDTA-Tris, 5; KCl, 500; Tris-HCl, 50; for the K⁺-EDTA-ATPase activity of myosin; 1.5 mg/ml myosin; ATP, 2; MgCl₂, 5; KCl, 500; and Tris-HCl, 50 (pH 7.4); for the Mg⁺⁺-ATPase activity of myosin. The mixture preincubated in the absence of Eud-M and ATP at 30°C for 5 min, followed by the addition of Eud-M and further preincubation. Eud-M was dissolved in dimethyl sulfoxide and a final concentration of dimethyl sulfoxide did not exceed 1%. Less than 1% dimethyl sulfoxide had little effect on the ATPase activities. The reaction was started by the addition of ATP and stopped by adding an equal volume of cold 10% trichloroacetic acid. The amount of inorganic phosphate liberated during the 5 min incubation was determined by the method of Martin and Doty (1949).

**Statistical analysis of the data.** The data are expressed as means ± S.E.M. Statistical comparisons were made by using Student's *t* test. *P* < .05 was considered significant.

Results

Contractile response of skinned fibers. To measure the contractile force of skinned fibers under the direct influence of Ca⁺⁺ concentration, the fibers were prepared from guinea pig and rabbit skeletal muscles by sufficient treatment with detergents to destroy the function of both the cell membrane and SR membrane. Caffeine (40 mM) did not cause any contraction of skinned fibers, suggesting destruction of SR membrane (Nakamura et al., 1986). Figure 2 shows the typical recording trace of contractile response of skinned fibers of guinea pig skeletal muscle before and after exposure to Eud-M (10⁻⁶ M) in the presence of Ca⁺⁺ (3 × 10⁻⁷ M). The effect of Eud-M was abolished after wash out. Also similar recording trace was obtained in rabbit skeletal muscle skinned fibers (data not shown). As shown in figure 3, Eud-M (3 × 10⁻⁶ to 3 × 10⁻⁴ M) produced a concentration-dependent enhancement of the contractile response of skinned fibers to Ca⁺⁺. At high Ca⁺⁺ concentrations above 3 × 10⁻⁷ M, Eud-M (3 × 10⁻⁵ M) increased the contractile response of skinned fibers to Ca⁺⁺ (fig. 4). The maximum response to Ca⁺⁺ was increased by 20% by it (fig. 4).

**Superprecipitation of myosin B.** The effect of Eud-M was examined on the superprecipitation of skeletal myosin B, an *in vitro* model reaction of muscle protein contraction, by monitoring the turbidity change. After the addition of ATP, clearing occurred and then the turbidity increased for 20 min. Eud-M at 10⁻⁶ M or more enhanced the increase in turbidity without affecting clearing. Figure 5 shows the representative trace of the effects of various concentrations of Eud-M on the superprecipitation of myosin B prepared from rabbit skeletal muscles. As shown in figure 6, Eud-M caused a concentration-dependent increase in the maximum turbid-
ity change 20 min after application. Eud-M (10^{-6}-10^{-4} M) enhanced the superprecipitation activity of myosin B in a concentration-dependent manner (figs. 5 and 6). The Ca^{++} concentration-activity relationship curve for superprecipitation was shifted to the upper direction by Eud-M (3 \times 10^{-5} M, fig. 7).

**Myosin B ATPase and other enzymes.** The ATPase activity of rabbit skeletal myosin B was measured in the presence of various concentrations of Eud-M. As shown in figure 8, Eud-M caused a concentration-dependent increase in the myosin B ATPase activity. In the Ca^{++} concentration-
activity relationship curve for myosin B ATPase. The maximum response to Ca\(^{+}\) (10\(^{-6}\)-10\(^{-4}\) M) was increased by Eud-M (3 \times 10^{-5} M, fig. 9). Eud-M also increased the ATPase activity of actomyosin reconstituted from actin, myosin and troponin-tropomyosin complex (table 1) and the Ca\(^{+}\) sensitivity was increased (data not shown). Furthermore, Eud-M did not affect the activities of Ca\(^{+}\)-, K\(^{+-}\)-EDTA- or Mg\(^{++}\)-ATPase of myosin, ATPase of actomyosin reconstituted from actin and myosin in the presence or absence of troponin as well as SR Ca\(^{2+}\)-ATPase (table 1).

**Discussion**

The ATPase activity is related functionally to the shortening velocity of unloaded muscle, whereas the isometric tension is related to the number of cross-bridge complexes (Barany, 1967; Barany and Close, 1971). The formation of force-generating cross-bridges depends not only on the presence of Mg\(^{++}\)-ATP, but also on the free energy change in ATP hydrolysis. For this process the energy is provided by the ATPase located in the cross-bridges which is activated upon complexation of myosin with actin (Lynn and Taylor, 1971; Eisenberg et al., 1980; Stein et al., 1981). The troponintropomyosin interaction is thought to be a crucial part of the protein interactions that regulate the actomyosin ATPase activity of skeletal muscles (Huxley, 1971; Morris and Lehrer, 1984; Ingraham and Swenson, 1985). It is well known that superprecipitation of skeletal natural actomyosin is an in vitro model reaction of muscle protein contraction (Szent-Györgyi, 1951). In the present experiment, Eud-M enhanced Ca\(^{+}\)-induced tension development of skinned fibers, superprecipitation and ATPase activity of myosin B. The concentration dependence of Eud-M in the tension development of skinned muscle fibers, superprecipitation and the ATPase activity of myosin B were closely correlated. These observations suggest that an increase in the ATPase activity of myosin B by Eud-M brings about the enhancement of superprecipitation of myosin B and tension development of skinned fibers.

Contraction of skeletal muscle is switched on and off by Ca\(^{++}\) over the concentration range of 10\(^{-7}\) to 10\(^{-4}\) M. Troponin is a Ca\(^{+}\)-binding protein in thin filament of skeletal and cardiac muscles. It is generally accepted that in skeletal muscles Ca\(^{++}\) binding to troponin results in shifting the position of tropomyosin on skeletal thin filament, leading to the contraction of muscle fibers. Troponin confers Ca\(^{++}\) sensitivity on the contractile system of skeletal muscle (Farah and Reinach, 1995; Gagne et al., 1997). It has been reported that Ca\(^{++}\) sensitizing substances increase skeletal or cardiac muscle contraction by increasing the responsiveness of the contractile proteins to Ca\(^{++}\) rather than by increasing the free Ca\(^{++}\) ion concentration (Strauss et al., 1994). In our experiments, Eud-M potentiated Ca\(^{++}\)-induced tension development of skinned fibers. The superprecipitation and ATPase activity of myosin B were stimulated by Eud-M in the same concentration range. Eud-M increased Ca\(^{++}\) sensitivity of skinned fibers, superprecipitation and ATPase activity of myosin B. The activities of Ca\(^{++}\)-, K\(^{+-}\)-EDTA- or Mg\(^{++}\)-ATPase of myosin and ATPase of actomyosin reconstituted from actin and myosin were not affected by Eud-M, suggesting elimination of possible involvements of direct stimulation of myosin ATPase or actin-myosin interaction on the Eud-M-induced enhancement of superprecipitation and ATPase activity of myosin B. Eud-M significantly potentiates the ATPase activity of actin-myosin-troponin-tropomyosin reconstituted system. As previously reported, Eud-M did not cause Ca\(^{++}\) release from SR (Nakamura et al., 1986). These observations suggest that Eud-M increases Ca\(^{++}\) sensitivity of contractile protein system, resulting in stimulation of myosin B ATPase activity and thus enhances contractility of skinned fibers. It is also suggested that an increase in Ca\(^{++}\) sensitivity of the contractile protein system is caused at least partially mediated through tropinin-tropomyosin complex. Eud-M even at high concentration of 10\(^{-4}\) M did not affect myosin ATPase or SR Ca\(^{2+}\)-ATPase, suggesting a highly selective Ca\(^{++}\)-sensitizing agent. Eud-M has become an useful tool to study the molecular regulatory mechanism of Ca\(^{++}\) sensitivity of the contractile protein system.

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**References**


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**TABLE 1**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Change in Enzyme Activity (%)</th>
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<tbody>
<tr>
<td>Myosin K(^{+-})-EDTA-ATPase</td>
<td>-8.8 (\pm) 2.2</td>
</tr>
<tr>
<td>Ca(^{++})-ATPase</td>
<td>10.5 (\pm) 2.7</td>
</tr>
<tr>
<td>Mg(^{++})-ATPase</td>
<td>-5.7 (\pm) 3.0</td>
</tr>
<tr>
<td>Myosin + actin</td>
<td>-2.2 (\pm) 0.5</td>
</tr>
<tr>
<td>Myosin + actin + troponin</td>
<td>5.1 (\pm) 3.4</td>
</tr>
<tr>
<td>Myosin + actin + troponin+ tropomyosin</td>
<td>28.8 (\pm) 0.7k</td>
</tr>
<tr>
<td>SR Ca(^{2+})-ATPase</td>
<td>-5.4 (\pm) 1.3</td>
</tr>
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* Mean \(\pm\) S.E.M. (n = 3).
* \(b\) Significantly higher than the absence of eudistomin M, P \(<\) .05.


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