III. 1. Mastoparan Causes $\text{Ca}^{2+}$ Release from Skeletal Muscle Sarcoplasmic Reticulum through its Binding to a 97 kDa Protein

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The $^{45}\text{Ca}^{2+}$ release from the heavy fraction of sarcoplasmic reticulum (HSR) was accelerated by mastoparan (MP), radiolabeled $[\text{Tyr}^3]\text{MP}$ or caffeine in a concentration-dependent manner. The $EC_{50}$ values for MP, $[\text{Tyr}^3]\text{MP}$ and caffeine were approximately 2.0 $\mu$M, 7.7 $\mu$M and 1.8 mM, respectively. We succeeded in the synthesis of $[^{125}\text{I-\text{Tyr}}^3]\text{MP}$ with a high specific activity. $[^{125}\text{I-\text{Tyr}}^3]\text{MP}$ bound specifically to HSR with the $K_p$ $4.0 \mu$M and the $B_{max}$ of 3.0 nmol/mg. Furthermore, $[^{125}\text{I-\text{Tyr}}^3]\text{MP}$ specifically cross-linked to the 97 kDa protein without direct binding to ryanodine receptor. The protein was not triadin or Ca$^{2+}$-pump, because anti-triadin antibody or anti-Ca$^{2+}$-pump antibody did not immunoprecipitate the protein. These results suggest that the 97 kDa MP binding protein may have an important role in the excitation-contraction coupling of skeletal muscle.

Introduction

Ryanodine receptor (RyR) is highly enriched in endings of the skeletal muscle sarcoplasmic reticulum (SR) called terminal cisternae, which permit allosteric coupling between plasmalemmal dihydropyridine receptor and RyR$^1$. The N-terminal domain of the RyR is a major site of interaction with regulatory proteins of the channel function$^2$. However, the detailed mechanism of the modulation of RyR by the regulatory proteins containing SR intrinsic proteins remains to be solved.

MP, a tetradecapeptide from wasp venom, is originally found as a histamine releaser from mast cells$^3$. Recently, MP-induced Ca$^{2+}$ release from SR has been reported by two groups$^4,5$. However, its detailed mechanism of action is unknown.

To clarify the molecular basis of action of MP, we synthesized $[^{125}\text{I-\text{Tyr}}^3]\text{MP}$ with a high specific activity. Using the pharmacological probe, we showed for the first time that MP bound specifically to a 97 kDa protein in HSR of rabbit skeletal muscles.
Experimental Procedures

Materials.

MP (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Leu-Ala-Leu-Lys-Lys-Ile-Leu-NH₂) and [Tyr⁴]MP (Ile-Asn-Tyr-Lys-Ala-Leu-Ala-Leu-Ala-Leu-Lys-Lys-Ile-Leu-NH₂) were synthesized by a peptide synthesizer. HSR was prepared from rabbit skeletal muscle⁹. Synthesis of [¹²⁵I-Tyr⁴]MP was performed by the chloramine-T method⁷.

⁴⁵Ca²⁺ release experiments.

The ⁴⁵Ca²⁺ release from HSR passively preloaded with ⁴⁵Ca²⁺ was measured at 0°C as described previously⁸.

¹²⁵I-Tyr³]MP binding assay.

[¹²⁵I-Tyr³]MP binding was examined as follows. HSR (200 µg/ml) was incubated with 0.1-100 µM [¹²⁵I-Tyr³]MP for 15 min at 0°C 90 mM KCl, 50 mM MOPS-Tris (pH 7.0) and pCa 7. The amount of [¹²⁵I-Tyr³]MP bound was determined by filtration using Whatman GF/B filters under reduced pressure. Nonspecific binding was determined in the presence of 0.5-1 mM unlabeled [Tyr³]MP.

Cross-linking experiments.

The heterobifunctional photoreactive cross-linking agent Sulfo-SANPAH (10 mM) was reacted first with primary amines of [¹²⁵I-Tyr³]MP (25 µM) to form a succinimidyl linkage at 0°C in the dark in buffer containing 50 mM HEPES-Na (pH 7.4), 90 mM KCl, and pCa 7. The modified [¹²⁵I-Tyr³]MP was coupled with free amino groups of HSR proteins (1 mg/ml) by photoactivation. The photoactivation was performed by exposing with long wave (254 nm) and short wave (360 nm) for 4 min at 0°C. After ultrafiltration, the sample was incubated in the SDS sample buffer overnight at room temperature. After the samples were subjected to SDS-PAGE, analysis of [¹²⁵I-Tyr³]MP binding to HSR proteins was performed by using an image analyzer (Molecular Imager GS-363, Bio-Rad laboratories).

Immunoprecipitation experiments.

HSR proteins or HSR proteins cross-linked with [¹²⁵I-Tyr³]MP were solubilized for 1h on ice at a protein concentration of 1 mg/ml in buffer containing 3% (w/v) CHAPS, 1.0 M NaCl, 1 mM dithiothreitol, 20 mM Tris-HCl (pH 7.4), and protease inhibitors. CHAPS-solubilized HSR proteins were diluted 10-fold in 20 mM Tris-HCl (pH 7.4) to reduce the high salt and detergent concentrations. Monoclonal (mouse) anti-triadin antibody (1: 50), monoclonal (mouse) anti-SR Ca²⁺-pump antibody (1: 50) or monoclonal (mouse) anti-RyR antibody (1: 50) was added to the supernatants, and the samples were incubated for 2 h at 4°C, followed by further incubation with protein A-Sepharose 4B beads (0.27 mg/ml) for 2 h at
4°C. Immunoprecipitates were washed two or three times with buffer containing 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.3% CHAPS and pCa 7. The samples were subjected to SDS-PAGE after the SDS sample buffer was added.

**Results**

**Ca\(^{2+}\) release from HSR induced by MP or [Tyr\(^3\)]MP.**

The effects of MP, [Tyr\(^3\)]MP and caffeine on \(^{45}Ca^{2+}\) release from HSR vesicles were measured under the conditions in which the Ca\(^{2+}\)-pump did not work. The \(^{45}Ca^{2+}\) release was accelerated by MP, [Tyr\(^3\)]MP or caffeine in a concentration-dependent manner, but the maximum response to MP or [Tyr\(^3\)]MP was larger than that to caffeine (Fig. 1). The EC\(_{50}\) values for MP, [Tyr\(^3\)]MP and caffeine were approximately 2.0 μM, 7.7 μM and 1.8 mM, respectively.

**\(^{125}\)I-Tyr\(^3\)]MP Binding to HSR.**

We succeeded in the synthesis of a radio-labeled MP analogue \([^{125}\)I-Tyr\(^3\)]MP with a high specific activity (1.3 kBq /pmol). Fig. 2 shows a saturation curve and a corresponding Scatchard plot of \([^{125}\)I-Tyr\(^3\)]MP binding to HSR. Specific binding of \([^{125}\)I-Tyr\(^3\)]MP to HSR was saturable (Fig. 2A). Scatchard analysis revealed that \([^{125}\)I-Tyr\(^3\)]MP bound to a single binding site with a \(K_d\) of 4.0 μM and \(B_{max}\) of 3.0 nmol/mg (Fig. 2B). The \(K_d\) value was close to the EC\(_{50}\) value for [Tyr\(^3\)]MP in Ca\(^{2+}\) release.

**Identification of 97 kDa protein bound to \([^{125}\)I-Tyr\(^3\)]MP.**

In order to identify the binding protein(s) for \([^{125}\)I-Tyr\(^3\)]MP in HSR vesicles, we performed cross-linking experiments with using Sulfo-SANPAH. We found that \([^{125}\)I-Tyr\(^3\)]MP did not bind to RyR but to another protein of 97 kDa (Fig. 3B). \([^{125}\)I-Tyr\(^3\)]MP binding to the 97 kDa protein was inhibited by MP or unlabeled [Tyr\(^3\)]MP (500 μM) (Fig. 3C). To examine whether the 97 kDa protein is triadin or Ca\(^{2+}\)-pump, CHAPS-solubilized HSR proteins cross-linked with \([^{125}\)I-Tyr\(^3\)]MP were immunoprecipitated with anti-triadin monoclonal antibody or anti-SR Ca\(^{2+}\)-pump monoclonal antibody. These immunoprecipitated proteins were identified as triadin and Ca\(^{2+}\)-pump by immunoblotting using corresponding antibodies. (Fig. 4A and B). However, the 97 kDa \([^{125}\)I-Tyr\(^3\)]MP binding protein was not immunoprecipitated with anti-triadin monoclonal antibody or anti-SR Ca\(^{2+}\)-pump monoclonal antibody (Fig. 4C). The 97 kDa protein was still detected in the supernatant after the immunoprecipitation (Fig. 4C).

**Discussion**

Under the conditions lacking the activity of the Ca\(^{2+}\)-pump at 0°C, MP or [Tyr\(^3\)]MP, like caffeine, induced \(^{45}Ca^{2+}\) release from the \(^{45}Ca^{2+}\)-preloaded HSR in a concentration-dependent manner. MP is 4-times more potent than [Tyr\(^3\)]MP in Ca\(^{2+}\) releasing activity.
The low sensitivity of [Tyr]$^3$MP would be the results from the replacement of Leu$^3$ by Tyr in the structure. However, it is likely that [Tyr]$^3$MP causes Ca$^{2+}$ release with the same mechanism as MP, because both drugs showed the same maximum response.

We succeeded in the synthesis of $^{125}$I-Tyr$^3$]MP to characterize the MP binding site. We found that [Tyr]$^3$MP bound to HSR in a replaceable and saturable manner, indicating the existence of a specific binding site. This site was of a single class with the $K_D$ value of 4.0 $\mu$M, which was similar to the EC$_{50}$ value for [Tyr]$^3$MP in $^{45}$Ca$^{2+}$ release. Therefore, the binding site of [Tyr]$^3$MP might be functionally important for the $^{45}$Ca$^{2+}$ release.

We found that [Tyr]$^3$MP specifically cross-linked with a 97 kDa protein, and the cross-linking was inhibited by MP or unlabeled [Tyr]$^3$MP. An immunoprecipitation by anti-RyR antibody did not recognize [Tyr]$^3$MP-bound RyR, then we can rule out a possibility that MP binds directly to RyRs (data not shown). These observations suggest that the 97 kDa protein interacts directly or indirectly with RyRs.

There are some proteins that have molecular mass of around 97 kDa in HSR, such as triadin$^{9}$, Ca$^{2+}$-pump$^{10}$ and the 90 kDa$^{11}$. We examined whether the 97 kDa [Tyr]$^3$MP binding protein is triadin or not. However, the immunoprecipitated protein with anti-triadin monoclonal antibody was not [Tyr]$^3$MP binding protein, showing that the 97 kDa protein was not triadin. Furthermore, the immunoprecipitated protein with anti-SR Ca$^{2+}$-pump monoclonal antibody was not the 97 kDa [Tyr]$^3$MP binding protein.

In conclusion, MP induces Ca$^{2+}$ release through RyR from HSR vesicle without directly binding to RyR. We identified a 97 kDa protein as the target protein for MP in HSR vesicle. The 97 kDa protein may have an important role in the excitation-contraction coupling of skeletal muscle. MP is a useful pharmacology probe for elucidating the functional role of the 97 kDa protein.

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Fig. 1. Concentration-dependent acceleration of $^{40}$Ca$^{2+}$ release from HSR by MP, [Tyr]$^3$MP and caffeine at pCa 7. The content of $^{40}$Ca$^{2+}$ in HSR was measured at 0°C by the filtration method. Each value was calculated as difference in the amount of released $^{40}$Ca$^{2+}$ measured in the presence and absence of the test substance. caffeine (○); MP (●); [Tyr]$^3$ MP (■). Values are means ± S.E.M. (n=3).

Fig. 2. Saturation (A) and Scatchard (B) plots of $^{125}$I-Tyr$^3$MP binding to HSR. HSR (200 µg/ml) was incubated with $^{125}$I-Tyr$^3$MP (0.1-100 µM) at 0°C for 15 min. The amount of $^{125}$I-Tyr$^3$MP bound was measured, as described in Experimental Procedures. Values are means ± S.E.M. (n=3).
Fig. 3. Identification of the 97 kDa $[^{125}]$Tyr$^3$ MP binding protein. A, a coomassie blue-stained HSR proteins. B, 97 kDa protein cross-linked with $[^{125}]$Tyr$^3$MP using Sulfo-SANPAH. C, Inhibition of $[^{125}]$Tyr$^3$MP binding to the 97 kDa protein by MP (500 µM) or unlabeled Tyr$^3$MP (500 µM). The relative density of 97 kDa protein cross-linked with $[^{125}]$MP (T) was analyzed in the presence of MP or Tyr$^3$MP.

Fig. 4. Immunoprecipitation with anti-triadin or anti-Ca$^{2+}$-pump antibody of solubilized HSR proteins cross linked with $[^{125}]$Tyr$^3$MP. A, a Coomassie blue-staining. Native HSR proteins (HSR, lane 1), immunoprecipitate with anti-triadin monoclonal antibody (TRN-IP, lane 2) and immunoprecipitate of anti-SR Ca$^{2+}$-pump monoclonal antibody (Ca$^{2+}$-pump-IP, lane 3). B, immunoblotting of immunoprecipitated material with anti-triadin monoclonal antibody (Anti-TRN, lane 4) or anti-SR Ca$^{2+}$-pump monoclonal antibody (Anti-Ca$^{2+}$-pump, lane 5). C, the CHAPS extract prepared from HSR proteins cross-linked with $[^{125}]$Tyr$^3$MP was incubated with anti-triadin monoclonal antibody (TRN) or with anti-SR Ca$^{2+}$-pump monoclonal antibody (Ca$^{2+}$-pump). $[^{125}]$Tyr$^3$MP binding protein was analyzed with an image analyzer. The CHAPS extract (E, lane 6), immunoprecipitate with anti-triadin monoclonal antibody (P, lane 7) or anti-SR Ca$^{2+}$-pump monoclonal antibody (P, lane 9), and the corresponding supernatants (S, lane 8, 10).