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Identification of a 97-kDa Mastoparan-Binding Protein Involving in Ca^{2+} Release from Skeletal Muscle Sarcoplasmic Reticulum

MOLECULAR PHARMACOLOGY

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ABSTRACT

Mastoparan (MP) and radiolabeled [Tyr^3]MP caused a transient Ca^{2+} release from the heavy fraction of sarcoplasmic reticulum, which was inhibited by ryanodine. MP enhanced [^{3}H]ryanodine binding in a concentration-dependent manner with an EC$_{50}$ value of approximately 0.3 μM. The ^{45}Ca^{2+} release was accelerated by MP, [Tyr^3]MP, or caffeine in a concentration-dependent manner. The EC$_{50}$ values for MP, [Tyr^3]MP, and caffeine were approximately 2.0 μM, 7.7 μM, and 1.8 mM, respectively. MP, like caffeine, shifted the stimulatory limb of a bell-shaped curve of Ca^{2+} dependence to the left. ^{45}Ca^{2+} release induced by caffeine was completely inhibited by typical blockers of Ca^{2+}-induced Ca^{2+} release, such as Mg^{2+}, ruthenium red, or procaine. However, ^{45}Ca^{2+} release induced by MP was completely inhibited by Mg^{2+}, but it was only partially inhibited by ruthenium red or procaine. The rate of ^{45}Ca^{2+} release induced by MP was further increased in the presence of caffeine, showing that the MP binding site is different from that of caffeine on Ca^{2+} release channels. We succeeded in the synthesis of ^{125}I-[Tyr^3]MP with a high specific activity. ^{125}I-[Tyr^3]MP bound specifically to heavy fraction of sarcoplasmic reticulum with a K_d value of 4.0 μM and a B_{max} value of 3.0 nmol/mg. Furthermore, ^{125}I-[Tyr^3]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 antitriadin antibody and 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.

Cells maintain a rigid control over the intracellular level of Ca^{2+}, thus ensuring that the level is kept low in the resting condition. To use Ca^{2+} as a messenger, cells overcome this tight homeostatic control by using sophisticated mechanisms to release Ca^{2+} in brief bursts using either inositol 1,4,5-trisphosphate receptor or ryanodine receptor (RyR) (Berridge, 1997). In skeletal muscle, RyR is highly enriched in endings of the sarcoplasmic reticulum (SR) called terminal cisternae, which are closed to transverse tubules. Triads, consisting of paired terminal cisternae juxtaposed to transverse tubules, permit allosteric coupling between plasmalemmal dihydropyridine receptor and RyR in the skeletal muscle SR (Rios et al., 1993). RyR is suggested to have a small cytoplasmic C terminus and a large cytoplasmic (~80% of structure) N terminus. This N-terminal domain of the RyR is a major site of interaction with regulatory proteins of the channel function (MacKrill, 1999). However, the detailed mechanism of the modulation of RyR by the regulatory proteins containing SR intrinsic proteins remains to be solved.

Molecular probes that specifically interact with the RyR or the regulatory proteins are useful tools to analyze mechanisms how RyR and the regulatory proteins work in the excitation-contraction coupling. RyR is a binding protein of neutral plant alkaloid ryanodine, a toxin used extensively in the biochemical and functional characterization of the Ca^{2+} channel protein (Coronado et al., 1994). [^{3}H]Ryanodine binding is used as an indirect indicator of the RyR channel activity, because several ligands influence not only the opening of the Ca^{2+} channel but also the [^{3}H]ryanodine binding (Coronado et al., 1994). Recently, we reported that Ca^{2+} release induced by myotoxin a, a peptide toxin from prairie rattlesnake, is mediated through the RyR with a distinct mecha-

ABBREVIATIONS: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; MP, mastoparan; HSR, heavy fraction of sarcoplasmic reticulum; SR Ca^{2+}-pump, sarco/endoplasmic reticulum Ca^{2+}-ATPase; Sulfo-SANPAH, sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Fluo-3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Ck, creatinine kinase.

nism from caffeine (Furukawa et al., 1994). Myotoxin a specifically binds to 30-kDa protein but not purified RyR, indicating that myotoxin a-induced Ca^{2+} release is not a direct stimulation of RyR but is mediated with the associated protein molecule (Hirata et al., 1999).

Mastoparan (MP), a tetradecapeptide from wasp venom, is originally found as a histamine releaser from mast cells (Hirai et al., 1979). By subsequent analysis of the mechanism in histamine release, it has been shown that MP stimulates G proteins in a manner strikingly analogous to that of agonist-bound receptors (Sukumar et al., 1997). Conformation change of MP to an amphiphilic α-helix in a lipid environment is essential for the activity of MP, and this structure is maintained when MP is directly bound to G-protein (Sukumar and Higashijima 1992). Although MP is a useful molecular tool for studying receptor-G protein interaction, it has been shown that MP has several additional pharmacological activities, such as arachidonic acid release (Nakahata et al., 1996), activation (Gusovsky et al., 1991) and inhibition (Nakahata et al., 1990) of phospholipase C, and inhibition of calmodulin (Ohki et al., 1991). These pharmacological actions are not always explained by its activation of G proteins. Recently, MP-induced Ca^{2+} release from SR has been reported by two groups (Ikemoto et al. 1996; Longland et al., 1998). However, its detailed mechanism of action is unknown.

Here we demonstrated that MP-induced Ca^{2+} release from heavy fraction of SR(HSR), with a different mechanism from caffeine. To clarify the molecular basis of action of MP, we synthesized ^{125}I-[Tyr^3]MP with a high specific activity. Using the pharmacological probe, we showed for the first time that MP binds specifically to a 97-kDa protein in HSR of rabbit skeletal muscles.

Experimental Procedures

Materials. MP (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH$_2$) was purified from wasp venom. The materials used in this work were purchased from the sources indicated: Fluo-3 from Dojindo Laboratories (Kumamoto, Japan); ryanodine from S.B. Penick Co (New York, New York); caffeine from Wako Pure Chemical Industries (Osaka, Japan); Na^{125}I (95.3 kBq/pmol), [^{3}H]ryanodine (2.29 MBq/pmol), and $^{45}CaCl_2$ (25.9 kBq/pmol) from DuPont New England Nuclear (Boston, MA), monoclonal (mouse) anti-RyR antibody (clone 34-C), monoclonal (mouse) anti-triadin antibody (clone GE 4.90), and monoclonal (mouse) anti-sarco/endoplasmic reticulum Ca^{2+}-ATPase (SR Ca^{2+}-pump) antibody (clone IIH11) from Affinity BioReagents, Inc. (Golden, CO); and sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino] hexanoate (Sulfo-SANPAH) from Pierce Chemical Co. (Rockford, IL). Other chemicals or drugs were of reagent grade or highest quality available.

Radioiodination of [Tyr^3]MP. [Tyr^3]MP (Ile-Asn-Tyr-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH$_2$) was synthesized by a peptide synthesizer. Synthesis of ^{125}I-[Tyr^3]MP was performed by the chloramine T method (Ohkura et al., 1995). The reaction was performed by three additions of 10 μl of 3.8 mM chloramine T to the mixture of 10 μl of 10 mM [Tyr^3]MP with 80 μl of 0.4 M sodium phosphate buffer (pH 7.5) containing 37 MBq of Na^{125}I. After 2 min, iodination was terminated by adding 100 μl of 2.5 mM Na$_2$S$_2$O$_5$. ^{125}I-[Tyr^3]MP with a high specific activity (1.3 kBq/pmol) was purified by a Sephadex G-10 (Pharmacia Biotech, Uppsala, Sweden) column (1.5 × 5.8 cm).

Preparation of HSR from Skeletal Muscle and Partial Purification of RyR. HSR was prepared from rabbit skeletal muscle

(Seino et al., 1991) with a minor modification. The back muscle (250 g) was homogenized four times in five volumes of 5 mM Tris-maleate, pH 7.0, for 30 s with 30-s intervals. The homogenate was centrifuged at 5000g for 15 min. The supernatant fraction was filtered through the four layers of cheesecloth, and the filtrate was centrifuged again at 12,000g for 30 min. The pellet was resuspended in a solution containing 90 mM KCl and 5 mM Tris-maleate, pH 7.0, 76.8 μM aprotinin, and 0.83 μM benzamidine (buffer A), and centrifuged at 70,000g for 40 min. The obtained HSR was stored in buffer A containing 0.3 M sucrose at $-80°C$ until use. The protein concentration was determined by dye-binding method (Bradford, 1976) with BSA as a standard.

HSR proteins were solubilized for 1 h on ice at a protein concentration of 3 mg/ml in buffer B (1.0 M NaCl, 2 mM dithiothreitol, 20 mM Tris-HCl, pH 7.4) containing 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) and protease inhibitors (1 μg/ml aprotinin, 10 μM leupeptin, and 2 μM pepstatin). The sample was incubated on ice for 1 h and centrifuged at 100,000g for 30 min. The CHAPS-solubilized HSR proteins were obtained as the supernatant. The supernatant was layered onto 5 to 20% linear sucrose density gradient in buffer B containing 1% (w/v) CHAPS and protease inhibitors, and centrifuged at 100,000g for 16 h. Resulting fractions were analyzed for protein composition by SDS-polyacrylamide gel electrophoresis (PAGE). The partially purified RyR proteins were stored at $-80°C$ until use.

Fluorescent Ca^{2+} Indicator Experiments. The change in the extravesicular free Ca^{2+} concentration was monitored by the intensity of 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)   ethane-N,N,N',N'-tetraacetic acid (Fluo-3) fluorescence at 30°C. The assay mixture (final volume, 0.8 ml) contained 3 μM Fluo-3, 50 μM CaCl$_2$, 90 mM KCl, 0.5 mM MgCl$_2$, 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-Tris, pH 7.0, 0.75 mg/ml HSR, 5 mM creatine phosphate, 0.1 mg/ml creatine kinase (CK) and 0.5 mM ATP. The reaction of Ca^{2+} uptake was started by a simultaneous addition of CK and ATP. Once the extravesicular free Ca^{2+} concentration was reduced to the steady state level, MP or [Tyr^3]MP followed by caffeine was then added. The change in 530-nm fluorescence of Fluo-3 at an excitation wavelength of 488 nm was measured by a fluorescence spectrophotometer (Hitachi F-2000).

$^{45}Ca^{2+}$ Release Experiments. The $^{45}Ca^{2+}$ release from HSR passively preloaded with $^{45}Ca^{2+}$ was measured at 0°C as described previously (Furukawa et al. 1994). After 12 h preincubation of HSR (20 mg/ml) at 0°C in a solution containing 5 mM $^{45}CaCl_2$, 90 mM KCl, and 5 mM Tris-maleate, pH 7.0, the HSR (5 μl) was diluted with 500 μl of an ice-cold reaction medium containing 500 μM CaCl$_2$ with various concentrations of EGTA, 90 mM KCl, and 50 mM MOPS-Tris, pH 7.0 (buffer B) in the presence or absence of the test substance. For measurement of the amount of $^{45}Ca^{2+}$ in HSR at time 0, HSR was diluted with the reaction medium containing 5 mM LaCl$_3$. At an appropriate time, 5 mM LaCl$_3$ was added to terminate the reaction. The reaction mixture was then filtered through Millipore filters (HAWP type, 0.45 μm pore size; Millipore Corp, Bedford, MA) and washed with 5 ml of an ice-cold solution containing 5 mM LaCl$_3$, 90 mM KCl, 5 mM MgCl$_2$, and 50 mM MOPS-Tris, pH 7.0. The amount of $^{45}Ca^{2+}$ remaining in HSR was measured by counting the radioactivity on the washed filters.

[^{3}H]Ryanodine Binding Assay. [^{3}H]Ryanodine binding was examined as described previously (Seino et al., 1991) with slight modification. HSR (200 μg/ml), was incubated with 1 nM [^{3}H]ryanodine in the 0.3 M sucrose, 0.35 M KCl, CaCl$_2$ equivalent to 0.1 μM free Ca^{2+} (Ca^{2+}-EGTA buffer), 100 μM (p-amidinophonyl)methanesulfonyl fluoride hydrochloride and 20 mM Tris-HCl, pH 7.4, for 2 h at 37°C. The amount of [^{3}H]ryanodine bound was determined by membrane filtration through Whatman filters (GF/B) under reduced pressure. The filters were washed twice with 12.5 time-volumes of ice-cold 50 mM MOPS-HCl, pH 7.4. Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine.

The partially purified RyR proteins (3 μg/ml) were incubated with 3 nM [^{3}H]ryanodine in 0.5% lecithin, 0.3 M sucrose, 1 M KCl, CaCl$_2$ equivalent to 0.1 μM free Ca^{2+} (Ca^{2+}-EGTA buffer), 100 μM (*p*-amidinophonyl)methanesulfonyl fluoride hydrochloride, and 20 mM Tris-HCl, pH 7.4, for 2 h at 37°C. The amount of [^{3}H]ryanodine bound was determined by membrane filtration through Millipore filters (HAWP type, 0.45 μm pore size) under reduced pressure. The filters were washed twice with 12.5 time-volumes of ice-cold 50 mM Tris-HCl, pH 7.4. Nonspecific binding was determined in the presence of 20 μM unlabeled ryanodine.

^{125}I-[Tyr ^{3}]MP Binding Assay. ^{125}I-[Tyr^3]MP binding was examined as follows. HSR (200 μg/ml) was incubated with 0.1 to 100 μM ^{125}I-[Tyr ^{3}]MP for 15 min at 0°C in buffer B. The amount of ^{125}I-[Tyr ^{3}]MP bound was determined by filtration using Whatman GF/B filters under reduced pressure. The filters were washed twice with 12.5 time-volumes of ice-cold 90 mM KCl, 50 mM MOPS-HCl, pH 7.0. Nonspecific binding was determined in the presence of 0.5 to 1 mM unlabeled [Tyr^3]MP.

Cross-Linking Experiments. The heterobifunctional, photoreactive, cross-linking agent Sulfo-SANPAH (10 mM) was reacted first with primary amines of ^{125}I-[Tyr ^{3}]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 (500 μM CaCl$_2$ and 612 μM EGTA). After the removal of the unreacted cross-linker by gel filtration in the dark, the modified ^{125}I-[Tyr ^{3}]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.

SDS-PAGE was conducted according to Laemmli (1970). After electrophoretic separation on 7.5% resolving gel with a 4% stacking gel, analysis of ^{125}I-[Tyr^3]MP binding to HSR proteins was performed by using with an image analyzer (Molecular Imager GS-363; Bio-Rad Laboratories, Hercules, CA).

Immunoprecipitation Experiments. Immunoprecipitation was conducted by the method of Zhang et al. (1997) with a minor modification. HSR proteins or HSR proteins cross-linked with ^{125}I-[Tyr^3]MP were solubilized for 1 h 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 (10 μM leupeptin and 2 μM pepstatin). CHAPS-solubilized HSR proteins were diluted 10-fold in 20 mM Tris-HCl, pH 7.4, to reduce the high

salt and detergent concentrations. The dilution buffer also contained Ca^{2+}-EGTA to give 100 nM free Ca^{2+}. The diluted supernatant was preincubated with protein A-Sepharose 4B beads (Zymed Laboratories, Inc., San Francisco, CA) for 2 h at 4°C with rotary shaking and then sedimented to eliminate nonspecific binding. Monoclonal (mouse) anti-triadin antibody (1:50), monoclonal (mouse) anti-sarco/endoplasmic reticulum Ca^{2+}-ATPase (SR Ca^{2+}-pump) antibody (1:50), or monoclonal (mouse) anti-RyR antibody (1: 50) was added to the precleared supernatants. After 2 h at 4°C, 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, and 0.3% CHAPS including Ca^{2+}-EGTA to give 100 nM free Ca^{2+}. The samples were subjected to SDS-PAGE after the SDS sample buffer was added.

Immunoblotting. After SDS-PAGE was performed, the separated proteins were electrophoretically transferred on to polyvinylidene difluoride membranes, at 120 mA for 1 h. The membranes were washed five times by TBST [Tris-buffered saline (0.1 M NaCl and 10 mM Tris-HCl, pH 7.5) containing 0.05% Tween 20] and blocked by a blocking buffer TBS containing 1% (w/v) BSA for 2 h. After washing in TBST, the membranes were incubated for 2 h with 1000-fold diluted anti-triadin antibody or 2500-fold diluted anti-SR Ca^{2+}-pump antibody. The membranes were washed again in TBST, and were incubated with alkaline phosphatase-conjugated anti-mouse monoclonal antibody (Bio-Rad Laboratories) diluted 3000-fold in TBS containing 1% BSA for 2 h to detect the primary antibodies. After the blots were exposed to enhanced chemiluminescence reagents (Bio-Rad Laboratories) for 30 min, they were then exposed to Hyper Film-enhanced chemiluminescence (Amersham, Buckinghamshire, England) for 1 to 5 min.

Results

Activation by MP of a Ryanodine-Sensitive Ca^{2+} Channels in HSR Vesicles. The Ca^{2+} mobilizing action of MP or [Tyr^3]MP on HSR can be visualized clearly by monitoring the intensity of Fluo-3 fluorescence at 30°C (Fig. 1). On the addition of 0.1 mg/ml CK and 0.5 mM ATP, the extravesicular free Ca^{2+} concentration decreased gradually because of Ca^{2+} uptake by SR Ca^{2+}-pump. When the concentration of Ca^{2+} was reduced to steady-state level, the addition of 5 μM
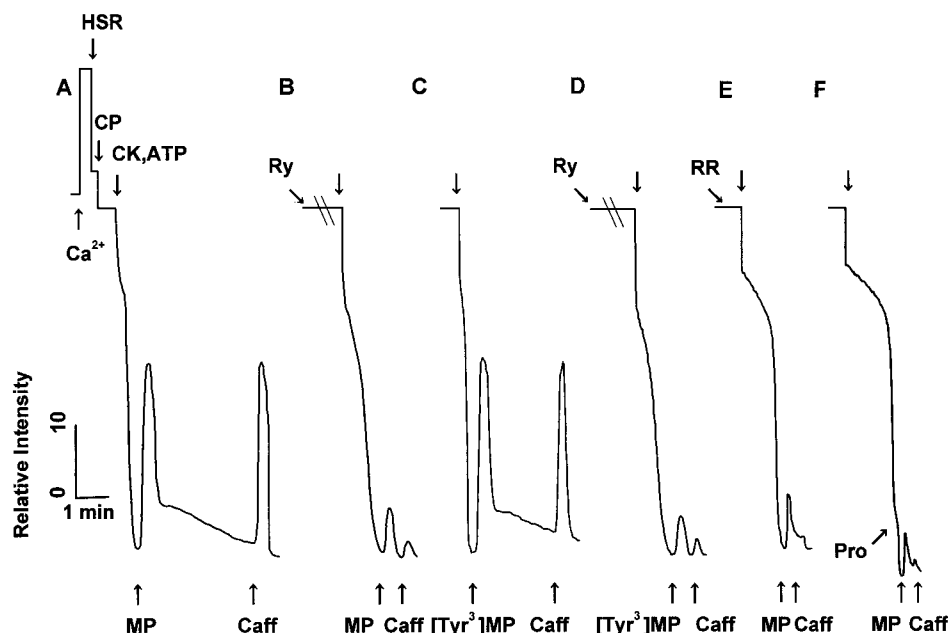

Fig. 1. Ca^{2+} release by MP, [Tyr^3]MP, and caffeine from HSR in Fluo-3 experiments. Typical recording traces in separate preparations from three animals. Experimental protocols are described under *Experimental Procedures*. Ca^{2+} uptake was initiated by the addition of 0.1 mg/ml creatine kinase (CK) and 0.5 mM ATP as indicated by arrow. In B to F, the traces are shown after the addition of creatine phosphate (CP). A, MP (5 μM) followed by caffeine (Caff; 1 mM); B, [Tyr^3]MP (10 μM) followed by caffeine (Caff; 1 mM) after treatment with ryanodine (Ry, 10 μM) for 5 min; C, MP (5 μM) followed by Caff (1 mM); D, [Tyr^3]MP (10 μM) followed by Caff (1 mM) after treatment with ryanodine (Ry, 10 μM) for 5 min; E, MP (10 μM) followed by Caff (1 mM) after treatment with ruthenium red (RR, 2 μM); F, MP (10 μM) followed by Caff (1 mM) after treatment with procaine (Pro, 5 mM).

MP or 10 μM [Tyr^3]MP to Ca^{2+}-filled HSR induced an immediate Ca^{2+} release followed by Ca^{2+} reuptake (Fig. 1, A and C). Caffeine at 1 mM also caused a transient Ca^{2+} release. Ryanodine at 10 μM inhibited Ca^{2+} release induced by 5 μM MP, 10 μM [Tyr^3]MP, or 1 mM caffeine (Fig. 1, B and D). The almost complete block of MP- or [Tyr^3]MP-induced Ca^{2+} release was demonstrated when 2 μM ruthenium red or 5 mM procaine was added (Fig. 1, E and F). To determine the involvement of RyR in MP-induced Ca^{2+} release, the effects of MP on the binding of [^{3}H]ryanodine to HSR vesicles were examined. MP clearly enhanced [^{3}H]ryanodine binding in a concentration-dependent manner (Fig. 2). The EC$_{50}$ values for MP and caffeine were approximately 0.3 μM and 3 mM, respectively, indicating that MP is 10,000 times more potent than caffeine. These results demonstrate that Ca^{2+} release induced by MP is mediated through the RyR.

Characteristics of Ca^{2+} Release from HSR Induced by MP or [Tyr^3]MP. To evaluate the properties of Ca^{2+} release induced by MP or [Tyr^3]MP from HSR, we examined the ^{45}Ca^{2+} release compared with caffeine. HSR vesicles were passively loaded with 5 mM ^{45}Ca^{2+} and then they were diluted into a medium with or without test substance at 0°C. 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. 3). The EC$_{50}$ values for MP, [Tyr^3]MP, and caffeine were approximately 2.0 μM, 7.7 μM, and 1.8 mM, respectively, indicating that MP and [Tyr^3]MP are 900 and 234 times more potent than caffeine in Ca^{2+} releasing activity.

Figure 4 shows basal, MP-, or caffeine-induced ^{45}Ca^{2+} release during 1 min after dilution into the medium under several free Ca^{2+} concentrations. Ca^{2+} accelerated ^{45}Ca^{2+} release in the low micromolar range of Ca^{2+} concentration
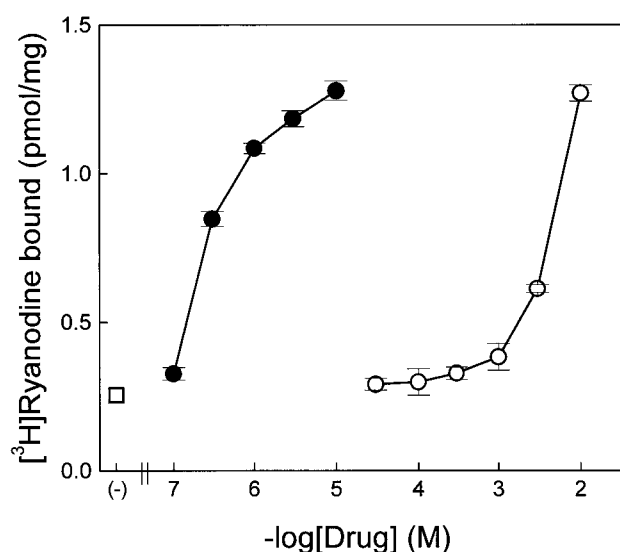
but it inhibited ^{45}Ca^{2+} release in a concentration range above 10 μM, showing a bell-shaped concentration-response curve for Ca^{2+} (Fig. 4). MP at 3 μM and caffeine at 1 mM shifted the stimulatory limb of the Ca^{2+} curve to the left (Fig. 4). Thus, MP and caffeine sensitize RyR to Ca^{2+}.

Fig. 5 shows the effect of typical inhibitors of Ca^{2+} release on MP- and caffeine-induced ^{45}Ca^{2+} release. ^{45}Ca^{2+} release induced by 1 mM caffeine was completely inhibited by Mg^{2+}, ruthenium red, and procaine. Interestingly, ^{45}Ca^{2+} release induced by 3 μM MP was completely inhibited by Mg^{2+} but only partially inhibited by ruthenium red or procaine.

The interrelations between the Ca^{2+} releasing actives of MP and caffeine were examined by measuring the ^{45}Ca^{2+} release from HSR. The rate of ^{45}Ca^{2+} release induced by MP at 30 μM was further increased in the presence of caffeine at 30 mM (Fig. 6).
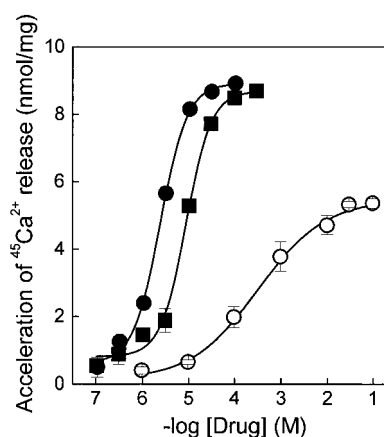

Fig. 3. Concentration-dependent acceleration of ^{45}Ca^{2+} release from HSR by MP, [Tyr^3]MP, and caffeine at pCa 7. Experimental protocols are described under *Experimental Procedures*. The content of ^{45}Ca^{2+} in HSR was measured at 0°C by the filtration method. Each value was calculated as difference in the amount of released ^{45}Ca^{2+} measured in the presence and absence of the test substance. ○, caffeine; ●, MP; ■, [Tyr^3]MP. Values are means ± S.E. ($n = 3$).

Fig. 2. Concentration-dependent effects of MP on [^{3}H]ryanodine binding to HSR. HSR (200 μg/ml) was incubated with 1 nM [^{3}H]ryanodine in the presence of various concentrations of MP or caffeine for 2 h at 37°C, as described under *Experimental Procedures*. Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine. □, basal; ●, MP; ○, caffeine. Values were expressed as percentages to the presence of 10 μM unlabeled ryanodine. Data are means ± S.E. ($n = 3$).
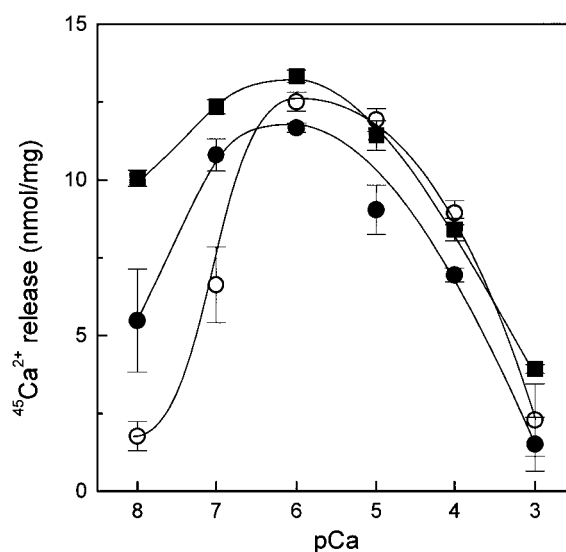

Fig. 4. Effect of free Ca^{2+} concentrations on ^{45}Ca^{2+} release induced by MP and caffeine from HSR. Experimental protocols were the same as those for Fig. 3. Each value was the amount of ^{45}Ca^{2+} released from HSR for 1 min after dilution into the medium with or without drugs under several free Ca^{2+} concentrations (pCa 8–3). ○, basal; ■, 3 μM MP; ●, 1 mM caffeine. Values are means ± S.E. ($n = 3$).

[125]I-[Tyr[3]]MP Binding to HSR. We succeeded in the synthesis of a radiolabeled MP analog [125]I-[Tyr[3]]MP with a high specific activity (1.3 kBq/pmol). [125]I-[Tyr[3]]MP binding to HSR was inhibited by unlabeled [Tyr[3]]MP (0.1–500 μM) in a concentration-dependent manner at 0°C under the same conditions for $^{45}Ca^{2+}$ release experiments (data not shown). Figure 7 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. 7A). Scatchard analysis revealed that [125]I-[Tyr[3]]MP bound to a single biding site with a K_D value of 4.0 μM and B_{max} value of 3.0 nmol/mg

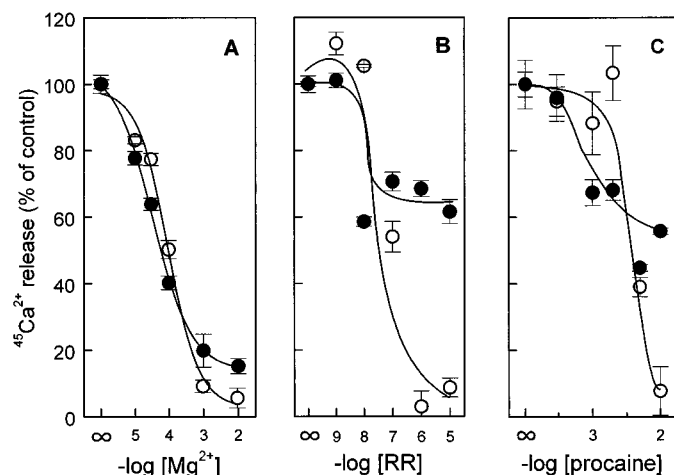**Fig. 5.** Effects of Ca^{2+} release channel inhibitors for $^{45}Ca^{2+}$ release on MP- and caffeine-induced $^{45}Ca^{2+}$ release from HSR. Experimental protocols were the same as those for Fig. 3. Concentration-dependent effects of Mg^{2+} (A), ruthenium red (RR, B) and procaine (C) on $^{45}Ca^{2+}$ release induced by 3 μM MP and 1 mM caffeine were investigated. ●, 3 μM MP; ○, 1 mM caffeine. Data are expressed as percentages to the $^{45}Ca^{2+}$ release in the absence of inhibitors. Values are means ± S.E. (n = 3).
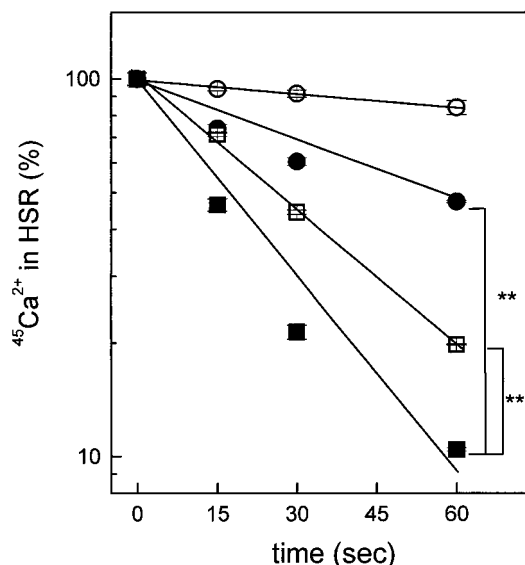

Fig. 6. Interrelations between the Ca^{2+}-releasing activities of MP and caffeine. Experimental protocols were the same as those for Fig. 2 except for at pCa 8. ○, control ; □, 30 μM MP; ●, 30 mM caffeine; ■, 30 μM MP plus 30 mM caffeine. Values are means ± S.E. (n = 3). The rates of caffeine-, MP-, and MP+caffeine-induced Ca^{2+} release were 0.32 ± 0.05/min, 0.71 ± 0.02/min, 1.03 ± 0.14/min, respectively. **P < .01, significantly different from the rate of MP plus caffeine-induced Ca^{2+} release (two-way ANOVA).

(Fig. 7B). 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. To identify the binding protein(s) for [125]I-[Tyr[3]]MP in HSR vesicles, we performed cross-linking experiments using Sulfo-SANPAH. We found that [125]I-[Tyr[3]]MP did not bind to RyR but to another protein of 97 kDa (Fig. 8B). [125]I-[Tyr[3]]MP binding to the 97-kDa protein was inhibited by MP or unlabeled [Tyr[3]]MP (500 μM) (Fig. 8 C). 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. 9A 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. 9C). The 97-kDa protein was still detected in the supernatant after the immunoprecipitation (Fig. 9C).

The Effects of MP on RyR Proteins. To determine whether MP interacts with the RyR directly or not, we first tested the effect of [^{3}H]ryanodine binding in partially purified RyR preparations. Although caffeine (10 mM) increased [^{3}H]ryanodine binding by 1.5-fold, MP (10 μM) did not affect [^{3}H]ryanodine binding in partially purified RyR preparations. (Fig. 10 A), in clear contrast to the results obtained using HSR (Fig. 2). Second, we examined whether CHAPS-solubilized HSR proteins cross-linked with [125]I-[Tyr[3]]MP were immunoprecipitated with anti-RyR monoclonal antibody or not. As shown in Fig. 10 B, RyR was immunoprecipitated by anti-RyR antibody, but [125]I-[Tyr[3]]MP did not directly bind to RyR. However, the 97-kDa [125]I-[Tyr[3]]MP-binding protein were coprecipitated with RyR.

Discussion

In the present study, we show that MP and [Tyr[3]]MP, a radiolabelable MP analog, induce Ca^{2+} release mediated through the RyR. In the experiments using the fluorescent Ca^{2+} indicator, MP or [Tyr[3]]MP induced a transient Ca^{2+} release from HSR, as with caffeine. The transient Ca^{2+} release induced by MP or [Tyr[3]]MP was inhibited by typical
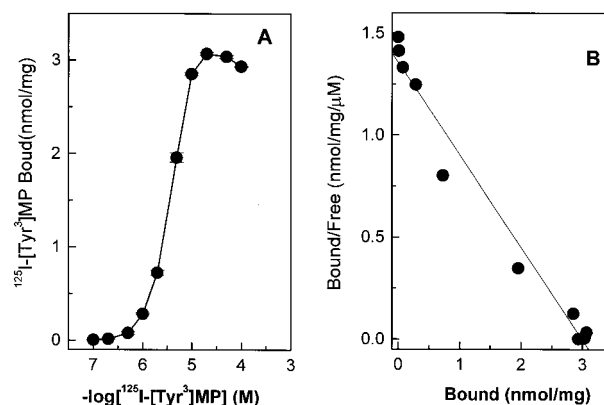

Fig. 7. Saturation (A) and Scatchard (B) plots of [125]I-[Tyr[3]]MP binding to HSR. HSR (200 μg/ml) was incubated with [125]I-[Tyr[3]]MP (0.1–100 μM) at 0°C for 15 min. The amount of [125]I-[Tyr[3]]MP bound was measured, as described under *Experimental Procedures*. Values are means ± S.E. (n = 3).

blockers of Ca^{2+}-induced Ca^{2+} release channels, such as ryanodine, ruthenium red, or procaine. Ryanodine interacts in a complex manner with sites thought to be localized within the C-terminal domain (Witcher et al., 1994). Ryanodine at a high concentration inactivates SR Ca^{2+} channels when Ca^{2+} release is measured under conditions in the presence of ATP and a regenerating system. The inactivated channels no longer respond to caffeine, MP, or [Tyr³]MP. These observations suggest that MP or [Tyr³]MP causes Ca^{2+} release mediated via RyR. This suggestion is supported by the observation that MP increased [³H]ryanodine binding to HSR.

Under the conditions lacking the activity of the Ca^{2+}-pump at 0°C, MP or [Tyr³]MP, like caffeine, induced $^{45}Ca^{2+}$ release from the $^{45}Ca^{2+}$-preloaded HSR in a concentration-dependent manner. These results are consistent with the observa-

Fig. 8. Identification of the 97-kDa ^{125}I-[Tyr³]MP-binding protein. A, Coomassie Brilliant blue-stained HSR proteins. B, 97-kDa protein cross-linked with ^{125}I-[Tyr³]MP using Sulfo-SANPAH. See details under *Experimental Procedures*. C, inhibition of ^{125}I-[Tyr³]MP binding to the 97-kDa protein by MP (500 μM) or unlabeled [Tyr³]MP (500 μM). The relative density of the 97-kDa protein cross-linked with ^{125}I-MP (T) was analyzed in the presence of MP or [Tyr³]MP.
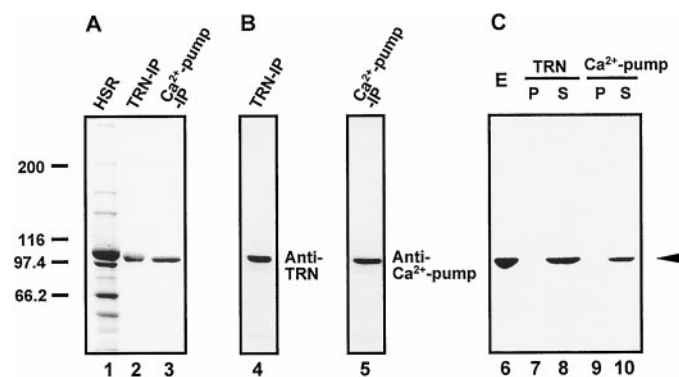
Fig. 9. Immunoprecipitation with anti-triadin or anti-Ca^{2+}-pump antibody of solubilized HSR proteins cross-linked with ^{125}I-[Tyr³] MP. A, Coomassie Brilliant blue-staining. Native HSR proteins (HSR, lane 1), immunoprecipitate with anti-triadin monoclonal antibody (TRN-IP, lane 2) and immunoprecipitate 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}I-[Tyr³] MP was incubated with anti-triadin monoclonal antibody (TRN) or with anti-SR Ca^{2+}-pump monoclonal antibody (Ca^{2+}-pump) as described under *Experimental Procedures*. ^{125}I-[Tyr³]MP-binding protein was analyzed with an image analyzer (Molecular Imager GS-363; Bio-Rad). 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, lanes 8 and 10).

tion that MP-induced rapid release of Ca^{2+} from SR is not caused by the inhibition of Ca^{2+}-pump (Longland et al., 1998). The EC_{50} values of MP and [Tyr³]MP were approximately 2.0 and 7.7 μM, respectively. Thus, MP is four times more potent than [Tyr³]MP in Ca^{2+}-releasing activity. The low sensitivity of [Tyr³]MP would result from the replacement of Leu³ by Tyr in the structure. However, it is likely that [Tyr³]MP causes Ca^{2+} release with the same mechanism as MP, because both drugs showed the same maximum response. In contrast, MP- or [Tyr³]MP-induced maximum $^{45}Ca^{2+}$ efflux was approximately 1.7 times higher than that of caffeine. It is known that RyR was sensitized to Ca^{2+} when the affinity of the Ca^{2+} sensor in RyR was increased by caffeine (Meissner et al., 1997). MP, like caffeine, shifted the stimulatory limb of the Ca^{2+} bell-shaped curve to the left. Therefore, it is suggested that the Ca^{2+}-dependent mechanism of MP-induced $^{45}Ca^{2+}$ release from HSR may be common to caffeine. Mg^{2+}, ruthenium red, and procaine have been used extensively as the inhibitors of Ca^{2+}-induced Ca^{2+} release (CICR) (McPherson and Campbell, 1993). The inhibition by Mg^{2+} results from competitive displacement of Ca^{2+} from its activating high-affinity site (Pessah et al., 1987) or by binding to the inhibitory low-affinity Ca^{2+} binding site (Meissner and el-Hashem, 1992). We examined the effects of these inhibitors on $^{45}Ca^{2+}$ release induced by MP or caffeine. Interestingly, caffeine-induced $^{45}Ca^{2+}$ release was inhibited by these blockers in a concentration-dependent manner, whereas MP-induced $^{45}Ca^{2+}$ release was completely inhibited by Mg^{2+}, but was only partially inhibited by ruthenium red or procaine. However, in the Ca^{2+} release with a fluorescent assay at 30°C, MP-induced Ca^{2+} release was almost completely inhibited by ruthenium red or procaine, suggesting that the experimental conditions (e.g., temperature) influence the effects of the inhibitors on MP-induced Ca^{2+} release. Recently, Xu et al. (1999) suggested that ruthenium red inhibits RyR in a noncompetitive manner through the inhibition of Ca^{2+} regulatory sites of RyR. It is possible that ruthenium red- or procaine-resistant Ca^{2+} re-
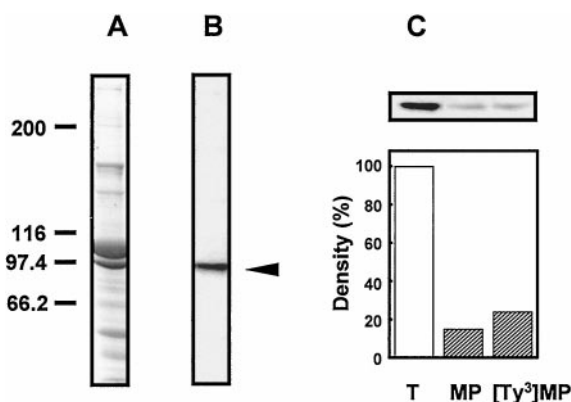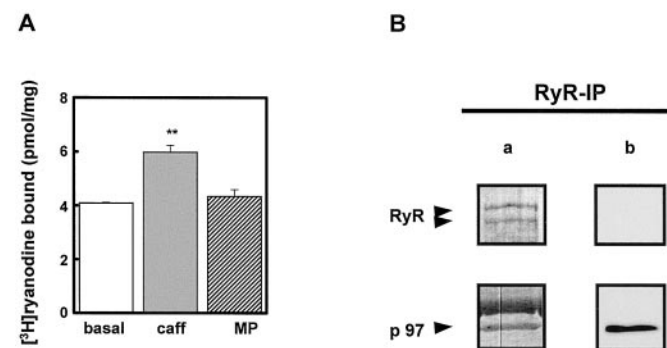

Fig. 10. The effects of MP on RyR. A, the effect of MP on [³H] ryanodine binding to partially purified RyR (RyR). Partially purified RyR (3 μg/ml) was incubated with 3 nM [³H]ryanodine in the presence of 10 mM caffeine (caff) or 10 μM MP (MP) for 2 h at 37°C, as described under *Experimental Procedures*. Significantly different from basal, **$P < .05$ (Student's t test). Data are means ± S.E. ($n = 3$). B, immunoprecipitation of solubilized HSR proteins cross-linked with ^{125}I-[Tyr³]MP with anti-RyR antibody. The CHAPS solubilized HSR proteins cross-linked with ^{125}I-[Tyr³]MP were immunoprecipitated with RyR monoclonal antibody (RyR-IP). Experimental protocols were the same as those for Fig. 9. a, Coomassie Brilliant Blue-staining; b, radioluminography. ^{125}I-[Tyr³]MP did not cross-link with RyRs (RyR) but the 97-kDa protein coimmunoprecipitated with it (p 97).

lease induced by MP is mediated through Ca^{2+} release channels with novel mechanisms. Furthermore, it is assumed that MP-induced Ca^{2+} release may have two components (i.e., RyR-dependent and RyR-independent). One possible interpretation of the results is that the Ca^{2+} released by MP through a RyR-independent pathway may then activate the RyR-dependent pathway through the mechanism of CICR. On the other hand, these observations indicate that the MP binding site is different from that of caffeine. This is supported by the fact that MP further enhanced the rate of Ca^{2+} release in the presence of the maximal concentration of caffeine.

We succeeded in the synthesis of ^{125}I-[Tyr^3] MP to characterize the MP binding site. We found that ^{125}I-[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 μM, which was similar to the EC_{50} value for [Tyr^3]MP in $^{45}Ca^{2+}$ release. Therefore, the binding site of ^{125}I-[Tyr^3]MP might be functionally important for the $^{45}Ca^{2+}$ release.

We found that ^{125}I-[Tyr^3]MP specifically cross-linked with a 97-kDa protein, and the cross-linking was inhibited by MP or unlabeled [Tyr^3]MP. Because MP has no effect on [^3H]ryanodine binding to partially purified RyRs, and an immunoprecipitation by anti-RyR antibody did not recognize ^{125}I-[Tyr^3]MP-bound RyR, then we can rule out the possibility that MP binds directly to RyRs. Interestingly, anti-RyR monoclonal antibody coimmunoprecipitated the 97-kDa MP-binding protein, suggesting that the 97-kDa protein interacts directly or indirectly with RyRs.

Some proteins have a molecular mass of around 97-kDa in HSR, such as triadin (Caswell et al., 1991), Ca^{2+}-pump (MacLennan et al., 1997), and the 90-kDa protein (Guo et al., 1994). Triadin is a major transmembrane glycoprotein in the junctional SR, linking the voltage-sensing dihydropyridine receptor $\alpha 1$-subunit to RyR (Brandt et al., 1992). Guo and Campbell (1993) have shown that triadin binds to calsequestrin and RyR in a Ca^{2+}-dependent manner. Moreover, it is reported that triadin inhibits the RyR activity in the cytoplasmic side and that RyR is regulated by both triadin and calsequestrin (Ohkura et al., 1998; Groh et al., 1999). Because triadin has attracted attention as a candidate protein for playing an important role in excitation-contraction coupling, we examined whether the 97-kDa ^{125}I-[Tyr^3]MP-binding protein is triadin or not. However, the immunoprecipitated protein with antitriadin monoclonal antibody was not ^{125}I-[Tyr^3]MP-binding protein, showing that the 97-kDa protein was not triadin. Recently, Longland et al. (1999) have demonstrated using purified SR Ca^{2+}-pump (Ca^{2+}-ATPase) that the MP inhibits its activity, decreases the affinity of the pump for Ca^{2+}, and abolishes the cooperativity of Ca^{2+} binding. However, the immunoprecipitated protein with anti-SR Ca^{2+}-pump monoclonal antibody was not the 97-kDa ^{125}I-[Tyr^3]MP-binding protein. It seems likely that this contradiction is caused by differences in experimental condition, such as temperature. However, we found that MP induced Ca^{2+} release from HSR under the conditions lacking the activity of the Ca^{2+}-pump at 0°C. The 90-kDa protein in SR has been recently reported by two groups (Guo et al., 1994; Froemming et al., 1999). Guo et al. (1994) have been shown that the 90-kDa protein is specifically expressed in skeletal muscle but not in cardiac muscle or brain, and it is not recognized by anti-triadin antibody. Froemming et al. (1999) have shown that the 90-kDa junctional SR protein forms an integral part of a supramolecular triad complex in skeletal muscle. Since the 90-kDa protein is not recognized by anti-triadin antibody, further study is necessary to determine whether MP-binding protein is identical with the 90-kDa 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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