Mastoparan binds to glycogen phosphorylase to regulate sarcoplasmic reticular Ca^{2+} release in skeletal muscle

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The ryanodine receptor, a Ca^{2+} -releasing channel in sarcoplasmic reticulum (SR), plays an important role in the excitation–contraction coupling of skeletal muscle. In a previous study [Hirata, Nakahata and Ohizumi (2000) *Mol. Pharmacol.* **57**, 1235–1242], we reported that mastoparan caused Ca^{2+} release through ryanodine receptor from the heavy fraction of SR (HSR) isolated from rabbit skeletal muscle, and that it specifically bound to a 97 kDa protein which was distinct from Ca^{2+} -pump or triadin. The present study was undertaken to identify and characterize the 97 kDa mastoparan-binding protein. The 97 kDa protein was purified from solubilized HSR by DEAE-Sepharose column chromatography and preparative SDS/PAGE. The partial amino acid sequence of the purified 97 kDa protein was matched with that of glycogen phosphorylase (GP).

The proteolytic cleavage pattern of the 97 kDa protein was identical with that of GP. Furthermore, [¹²⁵I-Tyr³]mastoparan specifically bound to GP. Interestingly, mastoparan-induced Ca^{2+} release was inhibited by exogenous addition of GP-a, and mastoparan dissociated GP from HSR. These results indicate that the 97 kDa mastoparan-binding protein is GP, which negatively regulates Ca^{2+} release from HSR. There may be a functional cross-talk between Ca^{2+} release from HSR and glycogenolysis for energy supply mediated through GP in skeletal muscles.

Key words: Ca^{2+} -release channel, [¹²⁵I-Tyr³]mastoparan, ryanodine receptor.

INTRODUCTION

Ca^{2+} regulates diverse cellular functions as an intracellular messenger, and its movements are controlled mainly by a variety of Ca^{2+} channels in the plasma membrane and intracellular Ca^{2+} store. In skeletal-muscle cells, excitation–contraction (E–C) coupling requires the conversion of the depolarization signal of the transverse (T) tubule to Ca^{2+} release from the sarcoplasmic reticulum (SR) without entry of extracellular Ca^{2+} , and the Ca^{2+} released from the SR causes the muscle contraction [1]. Signal transduction occurs at the triad junction, which contains two components essential for E–C coupling, the dihydropyridine receptor (DHPR) in the T-tubule and the ryanodine receptor (RyR) in the SR. DHPR is thought to act as the voltage sensor for transmitting the electrical signal to the SR [2]. RyR can release Ca^{2+} from SR, probably triggered by a conformational change of the DHPR [3].

RyR has a small cytoplasmic C-terminus and a large cytoplasmic N-terminus, which have a major domain of interaction with regulatory proteins of the channel function [4]. DHPR and RyR appear to be mechanically linked to each other at the triad junction, where the T-tubule is facing the SR membrane on both sides. However, when DHPR and RyR were expressed in cells using cloned cDNAs, the cells did not show Ca^{2+} release in response to depolarization [5]. Therefore it is suggested that some unknown molecules, other than DHPR and RyR, may be necessary for the mechanical coupling between DHPR and RyR.

To understand E–C coupling at the molecular level, it seems important to examine protein components of the triad junction. It has been shown that the triad is composed of functional molecules such as DHPR, RyR, calsequestrin, triadin, junctin, sorcin, FKBP-12 (FK506-binding protein 12) and mitsugumin29 [6–12]. Triadin is a major transmembrane glycoprotein in the junctional SR, linking DHPR α 1-subunit to RyR [13]. It is demonstrated that triadin binds to calsequestrin and RyR in a Ca^{2+} -dependent manner on the cytoplasmic side [14], resulting in the inhibition of the RyR activity [15,16]. Sorcin, a penta-EF-hand Ca^{2+} -binding protein, inhibits cardiac RyR activity in a Ca^{2+} -independent manner, by binding at a site distinct from calmodulin binding. The phosphorylation of sorcin by protein kinase A reduces the inhibition of cardiac RyR activity [10]. The interaction between FKBP-12 and RyR has been shown to involve four FKBP-12 molecules binding to each tetrameric RyR channel complex [11]. FK506 or rapamycin disrupts the interaction, thereby increasing the open probability of the channel and resulting in an enhanced sensitivity of ⁴⁵ Ca^{2+} release to caffeine [17,18]. Previous studies [12,19] indicated that mitsugumin29, identified as a synaptophysin family member, is essential for both effective E–C coupling and the accurate formation of junctional complexes between SR network and triad junction in developing skeletal-muscle cells. However, the detailed mechanism of the modulation of RyR by the regulatory proteins remains unknown.

Mastoparan is a wasp venom toxin with the structure Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Ile-Leu-NH₂,

Abbreviations used: CK, creatine kinase; DHPR, dihydropyridine receptor; E–C, excitation–contraction; FKBP-12, FK506-binding protein 12; 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; GP, glycogen phosphorylase; HSR, heavy fraction of SR; PP-1, protein phosphatase-1; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; SR, sarcoplasmic reticulum; sulpho-SANPAH, sulphosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate; T-tubule, transverse tubule.

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which releases histamine from mast cells [20]. It is known that mastoparan stimulates G proteins in a manner strikingly analogous to agonist-bound receptors [21]. However, it has been shown that mastoparan has several additional pharmacological activities, such as arachidonic acid release [22], activation [23] and inhibition [24] of phospholipase C, and inhibition of calmodulin [25]. These pharmacological effects were not always explained by its activation of G proteins. Previous studies [24,27] have shown that mastoparan induces Ca^{2+} release from SR. Ikemoto et al. [26] reported that mastoparan influenced the biphasic effect of calmodulin on the Ca^{2+} -induced Ca^{2+} release from SR. Independently, we have shown that mastoparan and [^{125}I -Tyr³]mastoparan induce Ca^{2+} release from a heavy fraction of SR (HSR) vesicles mediated via RyR. Furthermore, we found that [^{125}I -Tyr³]mastoparan specifically cross-linked with a 97 kDa protein [28]. There are some proteins with a molecular mass of approx. 97 kDa in HSR, such as the Ca^{2+} -pump [29] and triadin [30]. Longland et al. [27,31] reported that mastoparan inhibited the activity of purified sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) pump, decreased its affinity for Ca^{2+} , and abolished the co-operativity of Ca^{2+} binding. However, we showed that the 97 kDa mastoparan-binding protein was not a Ca^{2+} -pump using an immunoprecipitation and cross-link technique. In a similar immunoprecipitation and cross-link technique, we could exclude the possibility that the 97 kDa mastoparan-binding protein is triadin. In the present study, we tried to identify and characterize the 97 kDa mastoparan-binding protein in HSR vesicles of rabbit skeletal muscles.

EXPERIMENTAL

Radioiodination of [^{125}I -Tyr³]mastoparan

Synthesis of [^{125}I -Tyr³]mastoparan was performed by the chloramine-T method [28], using [Tyr³]mastoparan (Ile-Asn-Tyr-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH₂). The reaction was performed by adding 10 μl of 3.8 mM chloramine-T three times to a mixture of 10 μl of 10 mM [Tyr³]mastoparan and 80 μl of 0.4 M sodium phosphate buffer (pH 7.5) containing 74 MBq of Na^{125}I . After 2 min, iodination was terminated by adding 100 μl of 2.5 mM $\text{Na}_2\text{S}_2\text{O}_5$. [^{125}I -Tyr³]mastoparan was purified by a Sephadex G-10 (Pharmacia Biotech, Uppsala, Sweden) column (1.5 cm $\times$ 5.8 cm) to high specific radioactivity (1.3 MBq/ μmol).

Preparation of HSR from skeletal muscle

HSR was prepared from rabbit skeletal muscle as in [32,33] with a minor modification. The back muscle (250 g) was homogenized four times in 5 vols of 5 mM Tris/maleate (pH 7.0) for 30 s at 30 s intervals. The homogenate was centrifuged at 5000 g for 15 min. The supernatant fraction was filtered through four layers of cheese-cloth, and the filtrate was centrifuged again at 12000 g for 30 min. The pellet was resuspended in a solution containing 90 mM KCl, 5 mM Tris/maleate (pH 7.0), 76.8 μM aprotinin and 0.83 μM benzamidine (Buffer A), and centrifuged at 70000 g for 40 min. The obtained HSR was stored in Buffer A containing 0.3 M sucrose at -80°C until use.

Purification of the 97 kDa mastoparan-binding protein

HSR proteins (20 mg) were incubated for 1 h on ice at a protein concentration of 4 mg/ml in a buffer containing 4% (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). The supernatants were obtained by centrifugation at 70000 g for 40 min and dialysed against a solution containing 0.1% CHAPS and 20 mM Tris/HCl (pH 7.4) (Buffer B) to reduce the high salt and detergent concentration. The sample was loaded over a 15 ml column of DEAE-Sepharose pre-equilibrated with Buffer B. The flow-through fraction (15 ml) was collected, and the fractions were obtained by 15 ml washes of Buffer B containing 0.1–0.5 M NaCl applied in 0.1 M NaCl concentration steps. Aliquots of the column fractions were electrophoresed as described below. The flow-through fraction containing the partially purified 97 kDa protein was pooled and concentrated, and the sample was subjected to preparative SDS/PAGE using an ATTO Maxyield-NP (ATTO Co., Tokyo, Japan).

Analysis of the amino acid sequence

The 97 kDa protein (20 μg) was digested by 2 μg of protease V8 in a 4% (w/v) stacking gel of SDS/PAGE, and separated on a 15% (w/v) resolving gel for 1 h. The protein in the gel was transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using a semi-dry transfer system. After staining of the membrane by Amido Black, the stained band was cut and the amino acid sequence of the peptide was determined from the N-terminus by using a Shimadzu Protein Sequencer PSQ-1 System (Shimadzu, Tokyo, Japan).

Cross-linking of [^{125}I -Tyr³]mastoparan

Cross-linking of [^{125}I -Tyr³]mastoparan was performed as described in our previous paper [28]. The heterobifunctional photoreactive cross-linking agent sulphosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sulpho-SANPAH, 10 mM) was allowed to react first with primary amines of [^{125}I -Tyr³]mastoparan (25 μM , 1.3 MBq/ μmol) to form a succinimidyl linkage at 0°C in the dark in Buffer C containing 50 mM Hepes/Na (pH 7.4), 90 mM KCl, 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 -Tyr³]mastoparan was coupled with free amino groups of HSR proteins (1 mg/ml) at 0°C for 1 h, with the purified 97 kDa protein and with glycogen phosphorylase (GP, 1 mg/ml) by photoactivation. The photoactivation was performed by exposing to long-wave (360 nm) and short-wave (254 nm) radiation for 4 min at 0°C . After ultrafiltration, the sample was incubated in the SDS sample buffer overnight at 20°C . SDS/PAGE was conducted as described by Laemmli [34]. After electrophoretic separation on 7.5% resolving gel with a 4% stacking gel, analysis of [^{125}I -Tyr³]mastoparan binding to HSR proteins was performed by using an image analyser (Molecular Imager GS-363; Bio-Rad Laboratories).

Trypsin digestion of the purified 97 kDa protein and GP

The purified 97 kDa protein (10 μg), GP-a and GP-b (both 10 μg) were incubated with trypsin (0.5 μg) in Buffer C at 30°C for 30 min. These samples were analysed by SDS/PAGE completed on a 15% resolving gel, and the gels were stained with Coomassie Brilliant Blue.

Ca^{2+} measurement using a fluorescent indicator

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 [35]. 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 Mops/Tris (pH 7.0), 0.75 mg/ml of HSR, 5 mM phosphocreatine, 0.1 mg/ml of 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, mastoparan or caffeine was added after adding GP. 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).

Dissociation of GP from HSR by mastoparan

HSR was incubated at a final concentration of 0.2 mg/ml in Buffer C containing mastoparan (0–100 μM) at 0 °C for 1 h, and then centrifuged at 100 000 *g* for 30 min. The supernatants were separated by SDS/PAGE, and the GP content in the supernatants was determined by Coomassie Brilliant Blue staining and Western blotting using anti-GP antibody. Proteins were transferred electrically from the gel on to a PVDF membrane (Millipore Corporation, Bedford, MA, U.S.A.) by a semidry blotting method. The blots were blocked for 2 h with 1 % BSA in Tris-buffered saline at 25 °C, and incubated with anti-GP antibody (500 times dilution; Funakoshi, Tokyo, Japan). The blots were washed several times and incubated with a 1:10 000 dilution of horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) in Tris-buffered saline containing 1 % BSA at 25 °C for 2 h. Blots were developed by chemiluminescence from the membrane to the Hyperfilm.

Protein determination and data analysis

The protein concentration was determined by the dye-binding method [36] with BSA as a standard. The statistical differences ($P < 0.05$) of values were determined with Student's *t* test.

Materials

Mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu- NH_2) was obtained from Peptide Institute Inc. (Osaka, Japan). [^{125}I]Mastoparan (Ile-Asn-Tyr-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu- NH_2) was synthesized by a peptide synthesizer. The materials used in the present study were purchased from the sources indicated: ryanodine (S. B. Penick Co., New York, NY, U.S.A.), trypsin and protease V8 (Wako Pure Chemical Industries, Osaka, Japan), GP-a and GP-b (Sigma, St. Louis, MO, U.S.A.), anti-GP antibody (Funakoshi, Tokyo, Japan), horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Pharmacia Biotech), Na^{125}I (95.3 GBq/ μmol) (DuPont New England Nuclear, Boston, MA, U.S.A.). All other chemicals or drugs were of the reagent grade or the highest quality available.

RESULTS

Purification and identification of the 97 kDa mastoparan-binding protein

For purification of the 97 kDa mastoparan-binding protein, the detergent extract from the HSR was loaded over a DEAE-

Figure 1 Purification of the 97 kDa mastoparan-binding protein

(A) Coomassie Brilliant Blue staining of the flow-through (FT) fraction and 0.1–0.5 M NaCl fractions eluted from the DEAE-Sepharose column. The staining of HSR proteins is also shown as control. Equivalent volumes of all fractions were loaded on the gel. (B) Radioluminogram of HSR and FT samples cross-linked with [^{125}I -Tyr 3]mastoparan. See the Materials and methods section for details. Arrow indicates the 97 kDa protein.

Sepharose column (Figure 1A). The bulk of the 97 kDa mastoparan-binding protein was eluted mainly in the flow-through fraction. By column chromatography, the 97 kDa mastoparan-binding protein was separated from the Ca^{2+} -pump (approx. 105 kDa) in the 0.2–0.3 M NaCl fractions and calsequestrin (63 kDa) in the 0.4 M NaCl fractions (Figure 1A). The 97 kDa mastoparan-binding protein in the flow-through fraction was cross-linked with [^{125}I -Tyr 3]mastoparan, using a cross-linker sulpho-SANPAH (Figure 1B).

After pooling and concentrating the flow-through fraction from the DEAE-Sepharose column and one additional step of preparative SDS/PAGE, the 97 kDa mastoparan-binding protein was purified to homogeneity. The purified 97 kDa protein was digested by protease V8. Figure 2(A) shows the result of SDS/PAGE of the purified 97 kDa protein (left lane), its digested fragments with protease V8 (middle lane) and protease V8 alone (right lane). Since protease V8 proteins were detected as 27–32 kDa bands on SDS/PAGE, the purified 97 kDa protein was digested mainly into two fragments whose molecular masses were approx. 14.5 and 16 kDa respectively. The partial amino acid sequence of the 16 kDa fragment is shown in Figure 2(B). As a result of the homology search, the partial amino acid sequence of the 16 kDa fragment was matched with the region between residues 707 and 716 of rabbit skeletal muscle GP (the amino acid sequence of rabbit skeletal muscle GP can be accessed through Swiss Protein Database under Swiss-Prot No. P00489).

To compare the proteolytic digestions of the purified 97 kDa protein and commercial GP-a and GP-b, these proteins were subjected to trypsin proteolysis (Figure 3). Several degradation products of 6.5–31 kDa were generated by trypsin treatment. The tryptic cleavage pattern of the purified 97 kDa protein was identical with that of GP. To confirm further that the 97 kDa protein is GP, we performed [^{125}I -Tyr 3]mastoparan cross-linking to commercial GP using sulpho-SANPAH. We found that [^{125}I -Tyr 3]mastoparan cross-linked to GP-a and GP-b and that the

Figure 2 Proteolysis of the purified 97 kDa protein and determination of the partial amino acid sequence of the fragment

(A) The purified 97 kDa protein, obtained from preparative gel electrophoresis of FT fraction in Figure 1, was incubated in the presence or absence of protease V8 (2 μ g) for 1 h in the 4% stacking gel of SDS/PAGE, and separated on a 15% resolving gel. The gel was stained with Coomassie Brilliant Blue. Left lane, purified 97 kDa protein (+) without protease V8 (-); middle lane, the 97 kDa protein (+) with protease V8 (+); right lane, protease V8 (+) without the 97 kDa protein (-). (B) The partial amino acid sequence of the 16 kDa fragment (arrow in A) obtained by digestion of 97 kDa protein (97 kDa). The amino acid sequence at positions 702–722 of rabbit skeletal-muscle GP is also shown.

Figure 3 Trypsin digestion of the purified 97 kDa protein and GP

The purified 97 kDa protein (left lane: 10 μ g), GP-a (middle lane: 10 μ g) and GP-b (right lane: 10 μ g) were incubated with 0.5 μ g of trypsin at 30 °C for 30 min in 90 mM KCl, 0.1 μ M free Ca^{2+} , 50 mM Hepes (pH 7.4). The samples were separated by SDS/PAGE on a 15% resolving gel, and the gel was stained with Coomassie Brilliant Blue.

Figure 4 Cross-linking of [^{125}I -Tyr 3]mastoparan with GP-a and GP-b

GP-a (left lane) and GP-b (right lane) were cross-linked with [^{125}I -Tyr 3]mastoparan (1 μ M) using sulphy-SANPAH in the absence (-) or presence (+) of unlabelled mastoparan (MP, 500 μ M). After SDS/PAGE on 7.5% resolving gel, [^{125}I -Tyr 3]mastoparan binding to GP-a or GP-b was analysed by an image analyser.

cross-linking was completely inhibited by mastoparan (500 μ M) (Figure 4). These results suggest that the 97 kDa mastoparan-binding protein is GP.

Effect of GP on mastoparan-induced Ca^{2+} release

To determine the involvement of GP in mastoparan-induced Ca^{2+} release, the effect of GP on Ca^{2+} release was compared with that of BSA as a negative control. Mastoparan-induced Ca^{2+} release from HSR can be visualized clearly by monitoring the intensity of Fluo-3 as Ca^{2+} indicator (Figure 5A). After the addition of 0.1 mg/ml CK and 0.5 mM ATP, the extravesicular free Ca^{2+} concentration was reduced to the steady-state level because of Ca^{2+} uptake by SERCA pump. Mastoparan induced an immediate Ca^{2+} release from the Ca^{2+} -filled HSR vesicles followed by Ca^{2+} uptake (Figure 5A, left panel), which was inhibited by 200 μ g/ml GP-a by approx. 50% (Figure 5A, right panel). Furthermore, we examined the inhibition by GP of the mastoparan-induced Ca^{2+} release compared with caffeine as an activator of RyR. GP-a significantly inhibited mastoparan-induced Ca^{2+} release over a concentration range of 50–200 μ g/ml, but GP-b and BSA did not show inhibition (Figure 5B). Interestingly, GP-a also inhibited the Ca^{2+} release by caffeine in a concentration-dependent manner, whereas GP-b and BSA did not show inhibition (Figure 5C). Taken together, the results suggest that GP acts as a negative regulator of Ca^{2+} release through the RyR.

Effect of mastoparan on the dissociation of GP from HSR

HSR (0.2 mg/ml) was incubated with various concentrations of mastoparan at 0 °C for 1 h, and then centrifuged for 30 min at 100000 g. The major protein released into the supernatant was a 97 kDa protein as determined by Coomassie Brilliant Blue staining after SDS/PAGE (Figure 6A). Densitometric analysis indicated that the 97 kDa protein was dissociated from HSR by treatment with mastoparan in a concentration-dependent manner (Figure 6B). To confirm that the released 97 kDa protein is GP, Western blotting was performed by using anti-GP antibody. The 97 kDa protein was recognized by anti-GP antibody (Figure 6C).

Figure 5 Effect of GP on Mastoparan-induced Ca^{2+} release from HSR

(A) Typical recording traces of mastoparan (MP)-induced Ca^{2+} release. Experimental methods were described in the Materials and methods section. Ca^{2+} uptake was initiated by the addition of 0.1 mg/ml CK and 0.5 mM ATP as indicated by the arrow in the presence of phosphocreatine. Once the extravesicular free Ca^{2+} concentration was reduced to the steady-state level, 4 μM MP was added (left). Pretreatment with 200 $\mu\text{g/ml}$ of GP-a reduced MP-induced Ca^{2+} increase (right). (B) Concentration-dependent effect of GP on 4 μM MP-induced Ca^{2+} release. Δ , BSA; $\bullet$, GP-a; $\blacksquare$, GP-b. Values are means $\pm$ S.E.M. ($n = 3$). (C) Concentration-dependent effect of GP on 1.5 mM caffeine-induced Ca^{2+} release. Δ , BSA; $\bullet$, GP-a; $\blacksquare$, GP-b. Values are means $\pm$ S.E.M. ($n = 3$) and are significantly different from BSA treatment (* $P < 0.05$).

Furthermore, mastoparan increased the release of the immunoreactive 97 kDa protein from HSR in a concentration-dependent manner (Figure 6D).

DISCUSSION

Mastoparan, a tetradecapeptide from wasp venom, induces Ca^{2+} release through RyR from SR [26,27] without direct binding to RyR [28]. Previously, we found that mastoparan bound specifically to 97 kDa protein in skeletal-muscle HSR vesicles, which was not a triadin or Ca^{2+} pump [28]. Since the 97 kDa mastoparan-binding protein interacted directly or indirectly with RyR [28], we assumed that the 97 kDa protein might have an

important role in the Ca^{2+} release through RyR. In the present study, we purified the 97 kDa protein and succeeded in its identification as GP (EC 2.4.1.1) by using partial amino acid sequence analysis (Figures 1 and 2). The identification was confirmed by the identical peptide maps of the 97 kDa protein and GP (Figure 3), and by the fact that [^{125}I -Tyr 3]mastoparan specifically cross-linked to GP (Figure 4).

GP, a rate-limiting enzyme in the regulation of glycogen metabolism, catalyses the degradation of glycogen to glucose 1-phosphate. In the striated muscles, glucose 1-phosphate is immediately utilized via glycolysis to generate metabolic energy. GP is regulated by reversible enzymic phosphorylation [37] and it exists in two interconvertible forms, a less active b-form and a

Figure 6 Dissociation of GP from HSR by mastoparan (MP)

Release of GP from HSR on incubation with 0–100 μM MP in 90 mM KCl, 500 μM CaCl₂, 612 μM EGTA, 50 mM Hepes/Na (pH 7.4). The supernatant obtained after incubation with 0–100 μM MP at 0 °C for 1 h and centrifugation for 30 min at 100 000 *g* was separated by SDS/PAGE. (A) Coomassie Blue staining of SDS/PAGE. Arrow indicates 97 kDa protein (GP). (B) Density of 97 kDa protein in Coomassie Blue staining as percentage of the density without MP. Values are means ± S.E.M. (*n* = 3). (C) Western blotting by anti-GP antibody. Arrow indicates GP. (D) The density of immunoreactive 97 kDa protein as percentage of the density without MP. Values are means ± S.E.M. (*n* = 3) and are significantly different from those obtained without MP (**P* < 0.05).

more active a-form in glycogenolysis. GP-b is converted into the a-form on phosphorylation of Ser¹⁴ by phosphorylase kinase, whereas dephosphorylation of GP-a to the b-form is catalysed by protein phosphatase-1 (PP-1), which is a serine/threonine-specific phosphatase [38].

Glycogen particles are anchored to the SR by a glycogen-binding protein (termed G_M). G_M interacts directly with PP-1 and SR, because it contains a PP-1-binding domain in the vicinity of the N-terminus of this 124 kDa protein, the glycogen-binding domain, with the SR-binding domain being located near the C-terminus [38,39]. On the surface of glycogen particles, the GP-b is bound in the catalytic site and then the absorbed GP-b is covered by the phosphorylase kinase binding to glycogen particles [40]. Therefore GP is reversibly associated with the SR via its binding to the glycogen particles.

Since the SERCA pump is the major component of SR as a Ca²⁺ store and its activity may be regulated by ATP supplied through the enzymic breakdown of glycogen by GP, it has been focused whether or not GP affects SERCA. Cuenda et al. [41] found that the binding of GP-b to SR affected the conformation of SERCA. However, they reported that GP was unable to bind to SERCA and had no effect on SERCA activity

[42]. They did not discuss the role of GP in the Ca²⁺ release through RyR from SR. We found that GP-a inhibited the Ca²⁺ release through RyR from HSR. Interestingly, GP-a inhibited mastoparan-induced Ca²⁺ release in a concentration-dependent manner, whereas similar concentrations of GP-b failed to cause any inhibition. Furthermore, mastoparan released GP from HSR in a concentration-dependent manner. These observations suggest that GP acts as a negative regulator of Ca²⁺ release from HSR. We therefore propose that mastoparan binds to GP and induces Ca²⁺ release from HSR, accompanied by the release of GP-a as a negative regulator.

Caffeine is widely known to activate Ca²⁺ release through RyR from SR by increasing the apparent affinity of the Ca²⁺ sensitization site of RyR for Ca²⁺ [43]. We previously suggested that mastoparan, like caffeine, increased the apparent affinity of the Ca²⁺ sensitization site of RyR for Ca²⁺. Therefore the Ca²⁺-sensitive mechanism for the effect of mastoparan on Ca²⁺ release from HSR through RyR might be similar to that for caffeine. In the present study, GP-a inhibited not only mastoparan-induced Ca²⁺ release but also caffeine-induced release in a concentration-dependent manner, although GP-b failed to cause any inhibition. Therefore it is suggested that GP-a may be involved in the

common mechanism of mastoparan- and caffeine-induced Ca^{2+} release. It is also assumed that the effective concentration of caffeine at the caffeine-binding site of RyR is reduced after caffeine binds to GP [44].

Caffeine has been shown as an allosteric inhibitor of GP and binds to the inhibitory site, which is located at the entrance to the catalytic site. The binding of caffeine to the inhibitory site promotes conversion of the active R-state into the inactive T-state in the conformational equilibrium by stabilizing the location of the 280s loop in the inactive T-state [44,45]. Therefore it is assumed that caffeine may have a novel mechanism of Ca^{2+} release by acting on GP in addition to the direct effect on the Ca^{2+} sensitization site of RyR. One possible interpretation of these observations is that the caffeine binds to GP and induces Ca^{2+} release from HSR.

In conclusion, mastoparan caused Ca^{2+} release from HSR of skeletal muscles through RyR by acting on GP. The binding of mastoparan to GP dissociates this enzyme from HSR, resulting in the disappearance of the inhibition of GP to RyR activity. Our present study provides the first evidence that RyR is regulated negatively by GP. As the activity by GP of glycogenolysis is regulated by Ca^{2+} released from SR [46,47], the ability of GP to inhibit the gating of RyR provides a hypothesis that there may be a functional cross-talk between Ca^{2+} release from SR and glycogenolysis for energy supply mediated through GP.

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