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Himehabu Lectin, a Novel Inducer of Ca^{2+}-release from the Venom of the Snake *Trimeresurus okinavensis*, in Sarcoplasmic Reticulum

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Abstract

The lectin himehabu lectin (HHL) has recently been isolated from crude venom of the snake *Trimeresurus okinavensis*.

Ca^{2+}-electrode and fluorescent Ca^{2+}-indicator experiments showed that HHL induced release of Ca^{2+} from the heavy fraction of skeletal muscle sarcoplasmic reticulum (HSR). The release of Ca^{2+} induced by caffeine from HSR was abolished by ryanodine, Mg^{2+} and ruthenium red, typical inhibitors of Ca^{2+}-release channels, whereas that induced by HHL was only partially reduced by these inhibitors. HHL, unlike caffeine, had no effect on [^{3}H]ryanodine binding to HSR.

These results suggest that HHL induces release of Ca^{2+} which is at least partially mediated through Ca^{2+}-release channels with novel pharmacological properties.

Ryanodine receptors on the sarcoplasmic reticulum, a type of intracellular Ca^{2+}-release channel, are the largest ion channels known. They are centrally important in excitation–contraction coupling, which occurs in specialized regions where the sarcoplasmic reticulum and the transverse-tubule form a junction (Wagenknecht & Radermacher 1997). Released Ca^{2+} is taken up by the sarcoplasmic reticulum via the Ca^{2+} pump and stored mainly by binding to calsequestrin (Wang et al 1998). Despite their significance, the detailed regulatory mechanisms of ryanodine receptors remain unknown. Several natural products such as imperatoxins (Valdivia et al 1992), 9-methyl-7-bromoendistomin D (MBED) (Seino et al 1991), myotoxin a (Furukawa et al 1994) and mastoparan (Longland et al 1998) have been shown to modulate the release of Ca^{2+} mediated through ryanodine receptors in the sarcoplasmic reticulum.

In the course of our survey of natural products, we have recently isolated himehabu lectin (HHL) from the crude venom of the snake *Trimeresurus okinavensis* and found that HHL powerfully induces release of Ca^{2+} from the heavy fraction of skeletal muscle sarcoplasmic reticulum (HSR). Here we demonstrate for the first time that HHL induces release of Ca^{2+} from HSR with novel properties. HHL might serve as a useful probe for elucidating the regulatory mechanism of release of Ca^{2+} in skeletal muscle HSR.

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Materials and Methods

Materials

Lyophilized crude venom of the snake *Trimeresurus okinavensis*, from Okinawa, was purchased from the Japan Snake Institute (Gunma, Japan). Sepharose 4B was obtained from Pharmacia Biotech (Uppsala, Sweden). [^{3}H]Ryanodine (60 Ci mmol^{-1}) was purchased from DuPont NEN Research Products (Boston, MA). Caffeine, ruthenium red and procaine hydrochloride were purchased from Wako (Osaka, Japan). Other chemicals were of analytical grade.

Purification and homogeneity of himehabu lectin (HHL)

Crude venom (1 g) from the snake *Trimeresurus okinavensis* was dissolved at a concentration of

10% in 0·01 M Tris(hydroxymethyl) aminomethane (Tris)–HCl buffer (pH 7·4) containing NaCl (0·1 M). The soluble portion was chromatographed on a Sepharose 4B column with the same buffer containing lactose (0·15 M) as mobile phase to give pure HHL (0·004 g) (Figure 1A). When rabbit blood cells were used to determine the haemagglutinating activity of the purified HHL, it was strongly agglutinated. HHL was electrophoretically homogeneous in disc polyacrylamide-gel electrophoresis (pH 8·3) (Figure 1B). When antivenin-*Trimeresurus okinavensis* venom was tested against purified HHL by immunodiffusion precipitation only one line was observed, indicating further confirmation of the homogeneity of HHL. The molecular weight of HHL was determined to be 29 000 by sodium dodecyl sulphate (SDS)–polyacrylamide-gel electrophoresis. The amino acid analysis of newly isolated HHL polypeptide was Cys_9, Aps_{12}, Asn_5, Ser_8, Glu_{11}, Gln_5, Gly_6, Ala_6, Val_3, Met_4, Leu_{11}, Tyr_8, Phe_5, Lys_{13}, His_4, Arg_4, Trp_7.

Preparation of HSR

HSR enriched in Ca^{2+}-release activity was prepared from rabbit skeletal muscle by the method of Seino et al (1991) with a minor modification. White muscle was homogenized four times, for 30 s with 30-s intervals, in 5 vol. Tris-maleate (5 mM; pH 7·0). The homogenate was centrifuged at 5000 *g* for 15 min. The supernatant was filtered through cheesecloth and the filtrate was centrifuged again at 12 000 *g* for 30 min. The pellets were resuspended in a solution (buffer A) containing KCl (90 mM), Tris-maleate (5 mM; pH 7·0), aprotinin (76·8 μM), and benzamidine (0·83 μM) and centrifuged at 70 000 *g* for 40 min. The HSR obtained was stored in buffer A containing sucrose (0·3 M) at −80°C until use. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

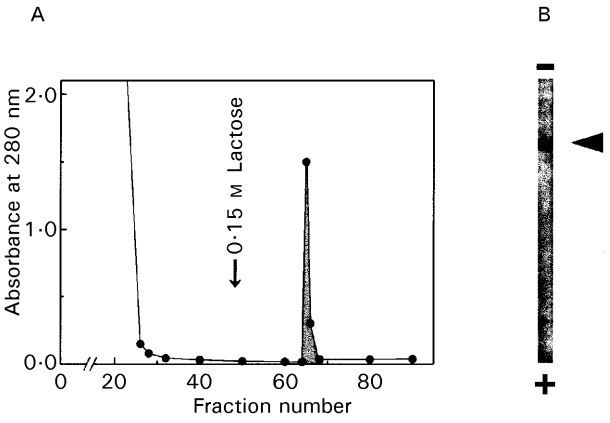

Figure 1. A. Elution profile from Sepharose 4B column. The crude venom was dissolved (10%) in 0·01 M Tris–HCl buffer (pH 7·4) containing NaCl (0·1 M). The solution was applied to a Sepharose 4B column which was developed with lactose (0·15 M), as shown. The shaded area indicates the fraction designated himehabu lectin (HHL). B. HHL was electrophoretically homogeneous in disc polyacrylamide-gel electrophoresis (pH 8·3). The arrowhead indicates HHL.

Ca^{2+}-electrode experiments

The extravesicular Ca^{2+} concentration of HSR suspension was measured at 30°C with a Ca^{2+} electrode as described previously (Nakamura et al 1986; Seino et al 1991). The Ca^{2+} electrode had a Nernstian response (slope 27–29 mV/pCa unit) in calibration solutions containing Ca^{2+}[ethylenebis-(oxyethylenenitrilo)]tetraacetic acid (EGTA) between pCa 3 and 6·5. The time for 90% response was approximately 0·6 s when pCa decreased from 6 to 4. The assay mixture (final volume 1 mL) contained $CaCl_2$ (50 μM), KCl (90 mM), $MgCl_2$ (0·5 mM), 3-(*N*-morpholino)propanesulphonic acid (MOPS)–Tris (50 mM pH 7·0), HSR (0·75 mg mL^{-1}), creatine phosphate (5 mM), creatine kinase (0·1 mg mL^{-1}) and ATP (0·5 mM). The addition of HSR (0·75 mg mL^{-1}) with Ca^{2+} contained in the HSR solution caused an increase in the Ca^{2+} concentration. Creatine phosphate (5 mM) and Mg^{2+} (10 mM) brought about a decrease and an increase of the Ca^{2+} concentration, respectively, probably by a formation of Ca^{2+}- and Mg^{2+}-creatine phosphate complexes. Ca^{2+} uptake was started by a simultaneous addition of ATP and creatine kinase. Ryanodine (10 μM) was added 5 min before addition of creatine kinase, because optimum Ca^{2+} concentrations for ryanodine binding to HSR were in the range 1–50 μM. Mg^{2+} (10 mM) was added just before addition of creatine kinase. Mg^{2+} (10 mM) but not ruthenium red (10 μM) affected the concentration of extravesicular free Ca^{2+}.

Experiments with a fluorescent Ca^{2+} indicator

The change in the extravesicular free Ca^{2+} concentration was monitored by measurement of the intensity of the fluorescence 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′-tetra-acetic acid (Fluo-3) at 30°C—the change in 530 nm fluorescence at an excitation wavelength of 488 nm was measured with a fluorescence spectrophotometer (Hitachi F-2000). The assay mixture (final volume, 0·8 mL) contained Fluo-3 (3 μM), $CaCl_2$ (50 μM), KCl (90 mM), $MgCl_2$ (0·5 mM), MOPS–Tris (50 mM; pH 7·0), HSR (0·75 mg mL^{-1}), creatine phosphate (5 mM), creatine

kinase (0·1 mg mL^{-1}) and ATP (0·5 mM). Addition of HSR (0·75 mg mL^{-1}) caused a reduction in the intensity of Fluo-3 fluorescence (artefact). Creatine phosphate (5 mM) and Mg^{2+} (10 mM) brought about a decrease and an increase, respectively, in the Ca^{2+} concentration. Ca^{2+} uptake was started by simultaneous addition of ATP and creatine kinase.

Ryanodine (10 μM) was added 5 min before addition of creatine kinase, because optimum concentrations of Ca^{2+} for ryanodine binding to HSR were in the range 1–50 μM. Mg^{2+} (10 mM) was added just before addition of creatine kinase. Mg^{2+} (10 mM) but not ruthenium red (10 μM) affected the concentration of extravesicular free Ca^{2+}.

[^{3}H]Ryanodine binding assay

[^{3}H]Ryanodine binding was examined as described elsewhere (Ohkura et al 1996) with slight modification. HSR (200 μg mL^{-1}) was incubated with [^{3}H]ryanodine (5 nM) for 2 h at 37°C in a solution containing sucrose (0·3 M), (*p*-amidinophenyl) methanesulphonyl fluoride hydrochloride (0·1 mM), $CaCl_2$ (100 μM), KCl (0·35 M) and MOPS–HCl (20 mM; pH 7·4). The amount of [^{3}H]ryanodine bound was determined by membrane filtration through Whatman filters (GF/B). Non-specific binding was determined in the presence of unlabelled ryanodine (10 μM).

Statistical analysis

The results obtained are expressed as means ± s.e.m. (standard error of the mean) and differences between results were evaluated for significance by use of Student's *t*-test, with $P < 0.05$ being regarded as indicative of significance.

Results

The effect of HHL on the Ca^{2+}-mobilizing activity of HSR can be visualized clearly by monitoring extravesicular Ca^{2+} concentrations of HSR directly with a Ca^{2+} electrode. Figure 2A shows that upon addition of ATP, free Ca^{2+} concentrations decreased gradually owing to Ca^{2+} uptake by the energized Ca^{2+} pump. When the concentration of Ca^{2+} was reduced to submicromolar levels, apparent Ca^{2+} uptake became slower. At this point, the addition of HHL (5 μM) or caffeine (1 mM) to the Ca^{2+}-filled HSR induced a marked release of Ca^{2+} (Figure 2A, a). Pretreatment of HSR with ryanodine (10 μM) and ruthenium red (10 μM) had no effect on the amount of Ca^{2+} uptake by HSR (Figure 2A, b and d). Addition of Mg^{2+} caused an increase in the rate of Ca^{2+} uptake but had no effect on the amount (Figure 2A, c). The release of Ca^{2+} induced by HHL was inhibited markedly (by 43·6 ± 1·5%) by ryanodine (10 μM), whereas that induced by caffeine was nearly abolished (Figures

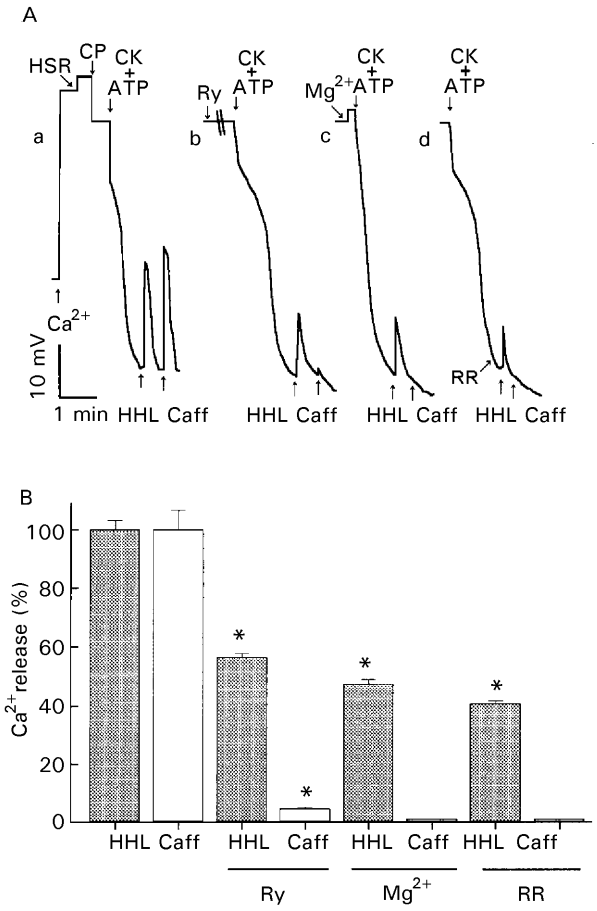

Figure 2. Results from Ca^{2+} electrode experiments performed to measure HHL-induced release of Ca^{2+} in the presence or absence of ryanodine (Ry), Mg^{2+} and ruthenium red (RR). A. Typical traces from separate preparations from three animals. The extravesicular Ca^{2+} concentration was monitored at 30°C in an assay mixture containing $CaCl_2$ (50 μM), KCl (90 mM), $MgCl_2$ (0·5 mM), MOPS–Tris (50 mM; pH 7·0), HSR (0·75 mg mL^{-1}), creatine phosphate (CP; 5 mM), creatine kinase (CK; 0·1 mg mL^{-1}) and ATP (0·5 mM). Ca^{2+} uptake was started by simultaneous addition of CK and ATP. The vertical calibration bar indicates a voltage-change (10 mV) corresponding to 0·3 pCa units. In b–d the traces are shown after addition of CP. a. Addition of HHL (5 μM) then caffeine (Caff, 1 mM). b. Effect of ryanodine (Ry, 10 μM) on the release of Ca^{2+} induced by HHL (5 μM); ryanodine was added 5 min before the addition of CK. c. Effect of Mg^{2+} (10 mM) on the release of Ca^{2+} induced by HHL (5 μM); Mg^{2+} was added just before addition of CK. d. Effect of ruthenium red (RR, 10 μM) on the release of Ca^{2+} induced by HHL (5 μM); ruthenium red was added just before addition of HHL. B. Inhibition by ryanodine, Mg^{2+} and ruthenium red of HHL-induced release of Ca^{2+}. The data are expressed as percentages of control values (means ± s.e.m., n = 3). * $P < 0.05$ compared with controls.

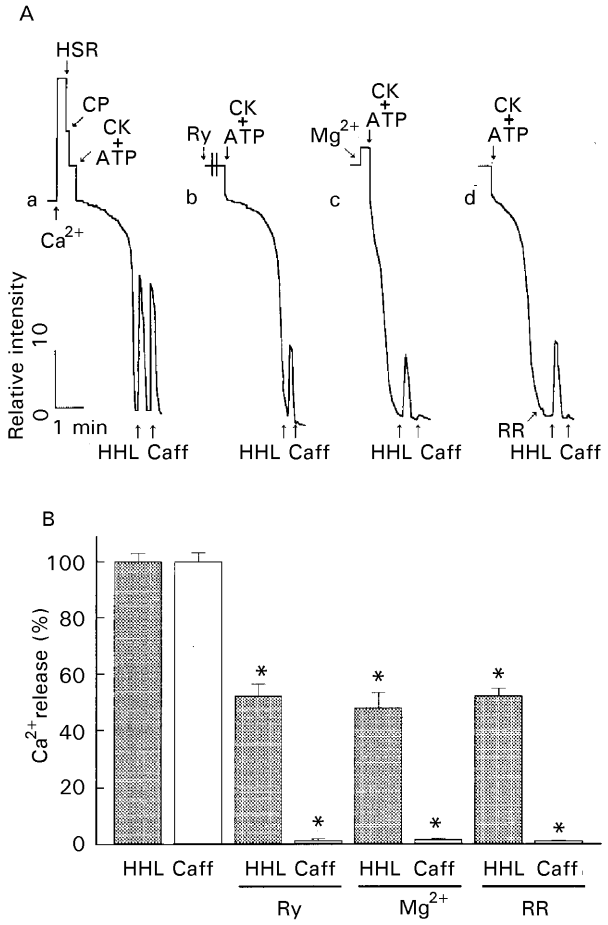

Figure 3. Results from experiments with fluorescent Ca^{2+} indicator performed to measure HHL-induced release of Ca^{2+} in the presence or absence of ryanodine (Ry), Mg^{2+} and ruthenium red (RR). A. Typical traces from separate preparations from three animals. Extravesicular Ca^{2+} concentrations were monitored at 30°C with a fluorescence spectrophotometer in an assay mixture containing $CaCl_2$ (50 μM), KCl (90 mM), $MgCl_2$ (0·5 mM), MOPS–Tris (50 mM pH 7·0), HSR (0·75 mg mL^{-1}), creatine phosphate (CP; 5 mM), creatine kinase (CK; 0·1 mg mL^{-1}) and ATP (0·5 mM). Ca^{2+} uptake was started by the simultaneous addition of CK and ATP. In b–d the traces are shown after addition of CP. a. HHL (5 μM) then caffeine (Caff, 1 mM); b. Effect of ryanodine (Ry, 10 μM) on the release of Ca^{2+} induced by HHL (5 μM); ryanodine was added 5 min before the addition CK. c. Effect of Mg^{2+} (10 mM) on the release of Ca^{2+} induced by HHL (5 μM); Mg^{2+} was added just before addition of CK. d. Effect of ruthenium red (RR, 10 μM) on the release of Ca^{2+} induced by HHL (5 μM); ruthenium red was applied just before the addition of HHL. B. Inhibition by ryanodine, Mg^{2+} and ruthenium red of HHL-induced release of Ca^{2+}. The data are expressed as percentages of control values (means ± s.e.m., n = 3). * $P < 0.05$ compared with controls.

2A, b–d and 2B). Similarly, a concentration of Mg^{2+} (10 mM), which completely inhibited the caffeine-induced release of Ca^{2+}, resulted in marked inhibition (52·6 ± 1·6%) of the release of Ca^{2+} induced by HHL (Figures 2A, c and 2B). Ruthenium red (10 μM) abolished the release of Ca^{2+} induced by caffeine and substantially reduced the release of Ca^{2+} induced by HHL (by 59·3 ± 1·0%) (Figures 2A, d and 2B).

Other fluorescent Ca^{2+} indicator experiments examining Ca^{2+}-release activity involved determination of extravesicular free Ca^{2+} concentration by measurement of the intensity of Fluo-3 fluorescence. HHL (5 μM) or caffeine (1 mM) induced marked release of Ca^{2+} from the Ca^{2+}-filled HSR (Figure 3A, a). The effects of ryanodine, Mg^{2+} and ruthenium red on the rate and amount of Ca^{2+} uptake of HSR were similar to those obtained in experiments with the Ca^{2+} electrode (Figure 3A, b–d). HHL-induced release of Ca^{2+} was markedly inhibited (by 47·8 ± 4·3%) in the presence of ryanodine (10 μM) which nearly abolished caffeine-induced release of Ca^{2+} (Figures 3A, b–d and 3B). Similarly, a concentration of Mg^{2+} (10 mM) which completely inhibited caffeine-induced release of Ca^{2+} resulted in marked inhibition (52·1 ± 5·5%) of HHL-induced release of Ca^{2+} (Figures 3A, c and 3B). Ruthenium red (10 μM) abolished caffeine-induced release of Ca^{2+} and substantially reduced HHL-induced release of Ca^{2+} (by 47·9 ± 2·8%; Figures 3A, d and 3B).

[^{3}H]Ryanodine binding to HSR was examined in the presence of HHL (0·1–30 μM) or caffeine (1 mM). Caffeine (1 mM) caused a 115·6 ± 1·2% (n = 4) increase in [^{3}H]ryanodine binding to HSR but HHL had no effect (n = 3) (data not shown).

Discussion

It has been shown that ryanodine receptors are identical with the Ca^{2+}-induced Ca^{2+}-release channels of the sarcoplasmic reticulum (McPherson & Campbell 1993). Many compounds, e.g. ryanodine (Meissner 1985), imperatoxin (Valdivia et al 1992), MBED (Seino et al 1991), myotoxin a (Furukawa et al 1994) and mastoparan (Longland et al 1998), have been extensively studied by numerous investigators to define the mechanisms of Ca^{2+}-releasing action in the sarcoplasmic reticulum. The use of these compounds as chemical probes has revealed the molecular regulatory mechanisms of ryanodine receptors.

Lectins are a structurally diverse class of protein, their only common features being the ability to bind carbohydrates specifically and reversibly (Sharon 1993). Recently, puff adder lectin was the first lectin reported to cause release of Ca^{2+} from HSR (Ohkura et al 1996). In work with a Ca^{2+} electrode and a fluorescent Ca^{2+} indicator we have

shown for the first time that a lectin named himehabu lectin (HHL), from the snake *Trimeresurus okinavensis*, induces release of Ca^{2+} from HSR. It is of interest that puff adder lectin and HHL both recognize sugars and cause release of Ca^{2+} from HSR.

HHL-induced release of Ca^{2+} from HSR was only partially reduced by treatment with inhibitors of Ca^{2+}-induced release of Ca^{2+}, e.g. ryanodine and ruthenium red, and Mg^{2+} at high concentrations, whereas release of Ca^{2+} stimulated by caffeine was completely inhibited. HHL, unlike caffeine, had no effect on [^{3}H]ryanodine binding to HSR. Therefore, it is probable that release of Ca^{2+} evoked by HHL has two components. These observations suggest that the component reduced by inhibitors of Ca^{2+}-release channels is the release of Ca^{2+} through Ca^{2+}-induced Ca^{2+}-release channels, whereas the component resistant to these inhibitors is possibly mediated through Ca^{2+}-release channels with novel pharmacological properties. There are three possibilities for the insensitive component of release of Ca^{2+} induced by HHL, i.e. HHL induces release of Ca^{2+} by binding:

to an unknown binding site on Ca^{2+}-induced Ca^{2+}-release channels;

to a regulatory protein of Ca^{2+}-induced Ca^{2+}-release channels; or

to novel channels which release Ca^{2+} and which are distinct from Ca^{2+}-induced Ca^{2+}-release channels.

HHL might serve as a useful pharmacological tool for clarifying the regulatory mechanism of release of Ca^{2+} in skeletal muscle.

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