Bisprasin, a Novel Ca\textsuperscript{2+} Releaser with Caffeine-Like Properties from a Marine Sponge, *Dysidea* spp., Acts on Ca\textsuperscript{2+}-Induced Ca\textsuperscript{2+} Release Channels of Skeletal Muscle Sarcoplasmic Reticulum\textsuperscript{1}

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ABSTRACT

Bisprasin, a unique bromotyrosine derivative containing a disulfide linkage, was isolated from a marine sponge of *Dysidea* spp. This compound caused a concentration-dependent (from 10 to 30 \(\mu\)M) increase in the \(^{45}\text{Ca}^{2+}\) release from the heavy fraction of skeletal muscle sarcoplasmic reticulum (HSR) of rabbit skeletal muscle in the same way as does caffeine. The 50% effective concentrations of bisprasin and caffeine were approximately 18 \(\mu\)M and 1.2 mM, respectively, indicating that the \(^{45}\text{Ca}^{2+}\) -releasing activity of bisprasin was approximately 70 times more potent than that of caffeine in HSR. The bell-shaped profile of \(\text{Ca}^{2+}\) dependence for bisprasin was almost the same as that for caffeine. Typical blockers of \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release channels, such as Mg\textsuperscript{2+}, procaine, and ruthenium red, inhibited markedly bisprasin- and caffeine-induced \(^{45}\text{Ca}^{2+}\) release from HSR. This compound, like caffeine, significantly enhanced \[^3\text{H}\]ryanodine binding to HSR. Scatchard analysis of \[^3\text{H}\]ryanodine binding to HSR revealed that bisprasin and caffeine decreased the \(K\text{D}\) value without affecting the \(B\text{max}\) value, suggesting that both the drugs facilitate the opening of ryanodine receptor channels. The bisprasin- and caffeine-induced increases in \[^3\text{H}\]ryanodine binding were further enhanced by adenosine-5’-(\(\beta,\gamma\)-methylene)triphosphate. These results suggest that the pharmacological properties of bisprasin are almost similar to those of caffeine, except for its 70-fold higher potency. Here, we present the first report on the pharmacological properties of bisprasin, which, like caffeine, induces \(\text{Ca}^{2+}\) release from skeletal muscle SR mediated through the ryanodine receptor.

The ryanodine receptor, which functions as a \(\text{Ca}^{2+}\) release channel of sarcoplasmic reticulum (SR), is postulated to play a key role in excitation-contraction coupling in the muscle (McPherson and Campbell, 1993; Coronado et al., 1994; Sutko and Airey, 1996). Genes encoding ryanodine receptors have been referred to as ryanodine receptors 1, 2, and 3. Ryanodine receptors 1 and 2 appear to be expressed predominantly in skeletal muscle and heart, respectively, whereas ryanodine receptor 3 is expressed in brain, smooth muscle, and epithelial cells (McPherson and Campbell, 1993; Sutko and Airey, 1996). Several compounds, such as amentoflavone (Suzuki et al., 1999), 2-hydroxycarbazole (Tovey et al., 1998), bastadins (Mack et al., 1994), and 9-methyl-7-bromoeudistomin D (MBED; Seino et al., 1991), have been shown to induce \(\text{Ca}^{2+}\) release from skeletal muscle SR mediated by the ryanodine receptor. Our previous reports indicated that myotoxin \(a\) (Ohkura et al., 1995), puff adder lectin (Ohkura et al., 1996a), and quinolidomicin A\(_1\) (Ohkura et al., 1996b) induced \(\text{Ca}^{2+}\) release from the heavy fraction of fragmented SR (HSR) with novel properties.

Numerous marine natural products have been isolated and given much attention as useful tools for pharmacological and biological studies because of their actions on the specific sites of functional proteins (Ohizumi, 1997; Moriya et al., 1998; Strachan et al., 1999). In the course of our survey of pharmacologically active substances from natural resources, we have devoted our attention to the occurrence of natural compounds possessing \(\text{Ca}^{2+}\) releasing activity from skeletal muscle SR, because these compounds are useful as chemical probes to elucidate the functional ryanodine receptor. Recently, we successfully isolated bisprasin (Fig. 1) from a

**ABBREVIATIONS:** SR, sarcoplasmic reticulum; HSR, heavy fraction of fragmented skeletal muscle sarcoplasmic reticulum; LSR, light fraction of fragmented skeletal muscle sarcoplasmic reticulum; MBED, 9-methyl-7-bromoeudistomin D; MOPS, 3-(N-morpholino)propanesulfonic acid; AMP-PCP, adenosine-5’-(\(\beta,\gamma\)-methylene)triphosphate.
marine sponge of *Dysidea* spp. collected at Palau. This compound is a unique brominated tyrosine-derived metabolite containing a disulfide linkage. Here, we present the first report on the pharmacological properties of bisprasin, which induces Ca\(^{2+}\) release from skeletal muscle SR mediated through the ryanodine receptor in the same way as does caffeine.

**Experimental Procedures**

**Materials.** Bisprasin was isolated from a marine sponge of *Dysidea* spp. Briefly, *Dysidea* spp. (500 g) was extracted with acetone/methanol (1:1). The extract was concentrated under reduced pressure and the residue was partitioned between ethyl acetate and water. The ethyl acetate-soluble fraction (3.4 g) was chromatographed over silica gel with a stepwise gradient of chloroform/methanol as eluant. The fraction (1.2 g) eluted with chloroform/methanol (9:1) was subjected to the HPLC with methanol/water as eluant, resulting in the isolation of active compound (380 mg). The chemical structure of this active compound was elucidated to be bisprasin on the basis of physicochemical data such as NMR, mass, and infrared spectra. The structure of this active compound was elucidated to be bisprasin on the basis of physicochemical data such as NMR, mass, and infrared spectra.

**Preparation of SR Vesicles from Skeletal Muscle.** HSR enriched in Ca\(^{2+}\)-induced Ca\(^{2+}\) release activity was prepared from rabbit skeletal muscle as previously reported (Seino et al., 1991) with slight modification. Male rabbits (Japanese White; weight, 3 kg) were anesthetized by i.v. injection of pentobarbital sodium, and the white muscle was removed. The animals used in this study were treated in accordance with the principles and guidelines of Tohoku University Council on Animal Care. All solutions used to prepare SR membranes included protease inhibitors 76.8 mM aprotinin and 0.83 mM benzamidine. White muscle was homogenized four times with a National Sigma Chemical Co. (St. Louis, MO). 4\(^{6}\)CaCl\(_2\) (0.7 Ci/mmol) and 4\(^{3}\)H]ryanodine (60 Ci/mmol) were purchased from NEN Life Science Products. All other chemicals were of analytical grade.

**Preparation of SR Vesicles from Skeletal Muscle.** HSR enriched in Ca\(^{2+}\)-induced Ca\(^{2+}\) release activity was prepared from rabbit skeletal muscle as previously reported (Seino et al., 1991) with slight modification. Male rabbits (Japanese White; weight, 3 kg) were anesthetized by i.v. injection of pentobarbital sodium, and the white muscle was removed. The animals used in this study were treated in accordance with the principles and guidelines of Tohoku University Council on Animal Care. All solutions used to prepare SR membranes included protease inhibitors 76.8 mM aprotinin and 0.83 mM benzamidine. White muscle was homogenized four times with a National MX-915C mixer in 5 volumes of 5 mM Tris-maleate (pH 7.0) for 30 s at 30-s intervals. The homogenate was centrifuged at 5000g for 15 min. The supernatant was filtered through the cheesecloth, and the filtrate was centrifuged again at 12,000g for 30 min. The pellets were suspended in a solution containing 90 mM KCl and 5 mM Tris-maleate (pH 7.0) and centrifuged at 70,000g for 40 min. The pellets were suspended in a solution containing 90 mM KCl, 5 mM Tris-maleate (pH 7.0), and 0.3 M sucrose.

The light fraction of fragmented skeletal muscle SR (LSR) was prepared from rabbit skeletal muscle as described by Seino et al. (1991). White muscle was homogenized four times with a National MX-915C mixer in 5 volumes of 5 mM Tris-maleate (pH 7.0) for 30 s at 30-s intervals. The homogenate was centrifuged at 5000g for 15 min. The supernatant was filtered through the cheesecloth, and the filtrate was centrifuged at 10,000g for 30 min. The supernatant was centrifuged again at 70,000g for 50 min. The pellets were suspended in a solution containing 0.6 M KCl, 5 mM Tris-maleate (pH 7.0), and 0.3 M sucrose, and this suspension was centrifuged at 100,000g for 70 min. This suspension/centrifugation cycle was repeated twice. The resultant pellets were washed with a solution containing 0.1 M KCl and 5 mM Tris-maleate (pH 7.0) and resuspended in the same solution to provide LSR suspension.

The obtained SR vesicles were stored at −80°C until use. The protein concentration was determined according to the method of Bradford (1976) with BSA as a standard.

**45Ca\(^{2+}\) Release Experiments.** 45Ca\(^{2+}\) release from the vesicular HSR was passively preloaded with 45Ca\(^{2+}\) was measured at 0°C as described previously (Nakamura et al., 1986) with slight modification. After a 12-h preincubation of 20 mg/ml HSR with 5 mM 45CaCl\(_2\) in a solution containing 90 mM KCl and 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-Tris (pH 7.0) at 0°C, the suspension was diluted with 100 volumes of an ice-cold reaction medium containing 0.4 mM CaCl\(_2\) with varying concentrations of EGTA, 90 mM KCl, and 50 mM MOPS-Tris (pH 7.0). For measurement of the amount of 45Ca\(^{2+}\) in HSR at time 0, the suspension was diluted with the reaction medium containing 5 mM LaCl\(_3\). At an appropriate time, 5 mM LaCl\(_3\) was added to stop 45Ca\(^{2+}\) release. The reaction mixture was then filtered with a Millipore filter (HAMP type, 0.45 mm pore size) and washed with 5 ml of a solution containing 5 mM LaCl\(_3\), 5 mM MgCl\(_2\), 90 mM KCl, and 50 mM MOPS-Tris (pH 7.0). The amount of 45Ca\(^{2+}\) remaining in HSR vesicles was measured by counting the radioactivity on the washed filters.

The free Ca\(^{2+}\) concentration was maintained by using Ca\(^{2+}\)-EGTA buffer (0.5 mM CaCl\(_2\) plus various concentrations of EGTA) and was estimated by using a microcomputer program that took into account the binding constant for Ca\(^{2+}\)-EGTA, pH, and the concentration of K\(^+\), Mg\(^{2+}\), and nucleotides (Silburn and Martell, 1964, 1971).

**Binding Assays.** 3H]Ryanoctine binding to HSR was examined as described previously (Furukawa et al., 1994) with slight modification. HSR (100 μg/ml) was incubated with 1 to 20 nM 3H]Ryanoctine at 37°C for 2 h in a solution containing 0.3 M sucrose, 0.3 M KCl, 100 μM CaCl\(_2\), and 20 mM Tris-HCl (pH 7.4). The amount of 3H]Ryanoctine bound was determined by membrane filtration through Whatman filters (GF/B). Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine.

**Mechanical Response.** The procedure for preparing the diaphragm and the technique of measurement of contractile response was performed as described previously (Ohizumi et al., 1986). Hemi-diaphragm preparations were isolated from male mice (ddys; weight, 25–30 g) and mounted in an organ bath containing 5 ml of Krebs-Ringer-bicarbonate solution of 120 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl\(_2\), 1.3 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 25.2 mM NaHCO\(_3\), and 5.8 mM glucose (pH 7.4) and were aerated with 95% O\(_2\)/5% CO\(_2\) at 37°C. A resting tension of 1 g was applied to each preparation. Isometric contractions were measured by a force-displacement transducer and recorded on a polygraph. Preparations were stimulated directly with 5-ms pulses (supramaximal voltage) at a frequency of 0.1 Hz.

**Statistical Analysis.** The data are expressed as means ± S.E. Statistical comparisons were made by using Student’s t test for unpaired data. The n number for statistical analysis presented the number of different preparations. P < .05 was considered significant.

**Results**

**45Ca\(^{2+}\) Release from SR Vesicles by Bisprasin and Caffeine.** The effects of bisprasin and caffeine on 45Ca\(^{2+}\) release from HSR vesicles were measured under the conditions in which the Ca\(^{2+}\) pump did not work. Figure 2 shows the time course of the change in 45Ca\(^{2+}\) content in HSR after the addition of bisprasin. The 45Ca\(^{2+}\) release was markedly

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accelerated by bisprasin over the concentration range of 10 to 100 μM. The concentration-response curve of $^{45}\text{Ca}^{2+}$ release from HSR for bisprasin and caffeine is shown in Fig. 3. $^{45}\text{Ca}^{2+}$ release was increased by bisprasin ($EC_{50} = 18 \text{ μM}$) and caffeine ($EC_{50} = 1.2 \text{ mM}$) in a concentration-dependent manner. In LSR, $2.1 \pm 0.4$ and $11.2 \pm 2.5\%$ of $^{45}\text{Ca}^{2+}$ release from HSR were induced by bisprasin (30 μM) and caffeine (1 mM), respectively.

**Effects of Bisprasin and Caffeine on $^{45}\text{Ca}^{2+}$ Release from HSR over a Wide Range of Free $\text{Ca}^{2+}$ Concentrations.** $^{45}\text{Ca}^{2+}$ release was increased in a linear fashion at the free $\text{Ca}^{2+}$ concentration from 0.1 to 1 μM ($EC_{50} = 0.25 \text{ μM}$), reached a maximal response at 3 μM, and decreased when the free $\text{Ca}^{2+}$ concentration was increased further from 10 to 100 μM (Fig. 4). Bisprasin (20 μM) and caffeine (1 mM) caused a parallel left shift of the concentration-response curve for $^{45}\text{Ca}^{2+}$ release plotted against the external $\text{Ca}^{2+}$ concentration (from 1 nM to 3 μM) without affecting the maximal response (Fig. 4). At a free $\text{Ca}^{2+}$ concentration of 100 μM, bisprasin (20 μM) stimulated $^{45}\text{Ca}^{2+}$ release, but caffeine (1 mM) did not affect it.

Effects of Typical Inhibitors of $\text{Ca}^{2+}$-Induced $\text{Ca}^{2+}$ Release Channels on Bisprasin- and Caffeine-Induced $^{45}\text{Ca}^{2+}$ Release. $^{45}\text{Ca}^{2+}$ release induced by 20 μM bisprasin and 1 mM caffeine was inhibited by ruthenium red (Fig. 5), Mg$^{2+}$ (Fig. 6), and procaine (Fig. 7) in a concentration-dependent manner. It is probable that under the present experimental conditions, there was a component refractory to the inhibition by procaine (Fig. 7).

$[^3H]$Ryanodine Binding to HSR. $[^3H]$Ryanodine binding to HSR was examined in the presence or absence of bisprasin and caffeine. As shown in Fig. 8, $[^3H]$ryanodine binding was increased by bisprasin (10 and 20 μM) and caffeine (30 mM). The degree of enhancement by bisprasin (by approximately 25%) was comparable to that of caffeine (by 25%) and MBED (by 20%, Seino et al., 1991). Figure 9 shows a saturation curve (A and C) and a corresponding Scatchard plot (B and D) of $[^3H]$ryanodine binding in the

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**Fig. 2.** The time course of stimulatory effects of bisprasin on $^{45}\text{Ca}^{2+}$ release from HSR. The $^{45}\text{Ca}^{2+}$ content in HSR vesicles was measured at 0°C by the Millipore filtration method. ○, control; ●, 10 μM bisprasin; □, 20 μM bisprasin; ■, 30 μM bisprasin; ▲, 100 μM bisprasin. **P < .01, statistically significant difference from the control.

**Fig. 3.** Concentration-dependent acceleration of $^{45}\text{Ca}^{2+}$ release from HSR by bisprasin (●) and caffeine (○). $^{45}\text{Ca}^{2+}$ release was measured at a $\text{Ca}^{2+}$ concentration of 0.1 μM. The amount of $^{45}\text{Ca}^{2+}$ release was calculated from the decrease in the $^{45}\text{Ca}^{2+}$ content in HSR vesicles during 1 min after dilution. Each value was obtained by subtracting the amount of $^{45}\text{Ca}^{2+}$ release measured in the absence of bisprasin from that measured in its presence. Values are means ± S.E. (n = 3). **P < .01, statistically significant difference from the control.

**Fig. 4.** Effects of free $\text{Ca}^{2+}$ concentration on the $^{45}\text{Ca}^{2+}$ release from HSR in the absence or presence of bisprasin and caffeine. ○, control. ●, 1 mM caffeine. □, 20 μM bisprasin. $^{45}\text{Ca}^{2+}$ release was expressed as a percentage against a maximum release (100%) in the absence of bisprasin and caffeine. *P < .05, **P < .01, statistically significant difference from the control.

**Fig. 5.** Effects of ruthenium red on the $^{45}\text{Ca}^{2+}$ release induced by bisprasin and caffeine from HSR. Data are expressed as the difference between $^{45}\text{Ca}^{2+}$ release in the presence and in the absence of bisprasin and caffeine. Values are means ± S.E. (n = 3). ○, 1 mM caffeine. ●, 20 μM bisprasin. **P < .01, statistically significant difference from the control (100%).
presence or absence of bisprasin (A and B) or caffeine (C and D). The $K_D$ value was decreased from 8.33 ± 0.48 (n = 3) to 4.17 ± 0.52 (n = 3) and from 6.20 ± 0.88 (n = 3) to 4.80 ± 0.73 (n = 3) by adding bisprasin and caffeine, respectively, whereas the $B_{\text{max}}$ value was unaffected. As shown in Fig. 10, bisprasin (20 μM)- and caffeine (30 mM)-induced increases in [$^3$H]ryanodine binding to HSR were further increased with adenosine-5′-(β,γ-methylene)triphosphate (AMP-PCP; 100 μM) by 20 and 30%, respectively.

**Effects of Bisprasin on Contractile Response of Isolated Mouse Hemidiaphragm.** Tetrodotoxin (1 μM), a Na$^+$ channel blocker, nearly abolished the contraction of hemidiaphragm induced by direct electrical stimulation, whereas bisprasin (30 μM) had no or little effect on it. KCl (50 mM)-induced contracture of hemidiaphragm was not affected by bisprasin (30 μM).

**Discussion**

The ryanodine receptor, which is generally known as a Ca$^{2+}$-induced Ca$^{2+}$ release channel of SR (Ebashi, 1991; Sutko and Airey, 1996), may be the machinery of excitation-contraction coupling in skeletal muscle (Ford and Podolsky, 1970; Endo, 1977). Ryanodine was reported to selectively bind to its receptor in an open state (McPherson and Campbell, 1993). The Ca$^{2+}$ channel has been purified using [$^3$H]ryanodine as a specific ligand (Inui et al., 1987; Hymel et al., 1988; Wagenknecht et al., 1989). Not only ryanodine but also a variety of natural products, such as imperatoxin (Valdivia et al., 1992) and MBED (Seino et al., 1991), have attracted the attention of pharmacologists, physiologists, and biochemists because they act on their specific binding sites in the ryanodine receptor with high affinity. The function of Ca$^{2+}$ release channels is inhibited by several inhibitors, such as procaine, Mg$^{2+}$, ruthenium red, and spermine (Palade, 1987; McPherson and Campbell, 1993).
hemidiaphragm induced by direct electrical stimulation. However, even at high concentrations, bisprasin had no or little effect on it. KCl-induced contracture of hemidiaphragm was not affected by bisprasin. These results suggest that bisprasin might be a specific Ca\(^{2+}\) releaser that has no effect on Na\(^+\) or Ca\(^{2+}\) channels. On the other hand, it is well known that LSR has far fewer ryanodine receptors than HSR (Liu et al., 1994). Bisprasin, like caffeine, slightly caused 45Ca\(^{2+}\) release from LSR, suggesting that both of the drugs are preferential Ca\(^{2+}\) releasers in HSR.

In conclusion, the pharmacological properties of bisprasin-induced Ca\(^{2+}\) release from HSR are very similar to those of caffeine, except for its 70-fold higher potency. This is the first report on the pharmacological properties of bisprasin, which is a caffeine-like Ca\(^{2+}\) releaser in HSR that may serve as a useful biochemical tool to clarify the regulatory mechanism of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in HSR.

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References


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