Activation of the Ryanodine Receptor Ca\textsuperscript{2+} Release Channel of Sarcoplasmic Reticulum by a Novel Scorpion Venom*  

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We identified a peptide fraction from the venom of the scorpion Buthurus hottentota that stimulated binding of \textsuperscript{[\textit{H}]}ryanodine to ryanodine receptors of skeletal and cardiac sarcoplasmic reticulum and brain microsomes in a highly specific manner. Activity was concentrated in a peptide fraction of 5,000–8,000. Assuming a single active peptide in this fraction, we estimated a dissociation constant of 20–30 nM for the interaction of the peptide with the ryanodine receptor. The whole venom and the purified fraction activated skeletal ryanodine receptor Ca\textsuperscript{2+} release channels incorporated into planar lipid bilayers. The venom produced a 10-fold increase in the mean open time and induced the appearance of a long lasting subconductance state not seen in controls. Changes were reversible and could be induced by the partially purified venom fraction. This novel scorpion venom should be helpful in establishing the role of ryanodine receptors in the initiation of intracellular Ca\textsuperscript{2+} release in striated muscle and in nonmuscle cells containing functional ryanodine receptors such as neurons and secretory cells.

Activation of muscle cells by voltage evokes a release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores which in turn triggers muscle contraction (1). A major type of intracellular Ca\textsuperscript{2+} channel controlling Ca\textsuperscript{2+} mobilization in the sarcoplasmic reticulum (SR)\textsuperscript{1} of striated muscle serves as a binding site for ryanodine (2–4), an alkaloid with strong paralyzing effects in neurons and secretory cells. Ryanodine receptors mediate the caffeine-sensitive release of Ca\textsuperscript{2+} from the SR of cardiac and skeletal muscle (5). Ryanodine receptors mediate Ca\textsuperscript{2+}, adenine nucleotide, and caffeine-sensitive release of Ca\textsuperscript{2+} from the SR of cardiac and skeletal muscle (6–8).

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‡ The abbreviations used are: SR, sarcoplasmic reticulum; Pipes, a polybasic buffer; CaCl\textsubscript{2}, calcium chloride; KCl, potassium chloride; [\textit{H}]-ryanodine (\textit{dpm}), \textit{dpm} per milligram; M, molar; LMW, low molecular weight; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

EXPERIMENTAL PROCEDURES

Scorpion Venom—Venom from the African scorpion B. hottentota was obtained from Latoxan (Rosans, France). Approximately 100 mg of lyophilized venom were resuspended in 3 ml of deionized water and centrifuged at 10,000 \textit{x} g for 15 min to remove mucoid material. The supernatant was saved, and the pellet was extracted twice in 5 ml of deionized water. The combined supernatants were filtered through a nitrocellulose filter with a pore size of 0.66 \textmu m (Millipore, Bedford, MA) and lyophilized until use.

Preparation of SR and Brain Membranes—Heavy SR was prepared from rabbit back and leg white muscle, and separately from bovine ventricle, as described elsewhere (19). SR was stored frozen at −80 °C in 0.32 m sucrose, 0.1 m KCl, and 5 mm Na-Pipes, pH 7.0. Brain microsomes were prepared from rat brain cortex. Five cortices of Sprague-Dawley rats were removed and homogenized in ice-cold 0.32 m sucrose with five strokes of a motor-driven Teflon/glass homogenizer. The resulting homogenate was sedimented at 1,000 \textit{x} g for 15 min, and the supernatant was used in binding experiments. Homogenizations of muscle and brain tissues were carried out in the presence of the following protease inhibitors: pepstatin A (1 \mu m), iodoacetic acid (1 m), phenylmethylsulfonyl fluoride (0.1 m), leupeptin (1 \mu m), and benzamidine (1 m).

Binding Assay—\textsuperscript{[\textit{H}]}Ryanodine binding was carried out for 90 min at 36 °C in 0.1 m of 0.2 m KCl, 1 mm Na-Pipes, pH 7.2. The calculated free Ca\textsuperscript{2+} was 10 \mu m. Other concentrations of Ca\textsuperscript{2+} and KCl are indicated in the text and figure legends. Free Ca\textsuperscript{2+} concentrations were calculated with a computer program using affity constants of Fabiato (20). \textsuperscript{[\textit{H}]}Ryanodine (60 mCi/mmol) was purchased from Du Pont-New England Nuclear and was diluted directly in the incubation medium to achieve concentrations in the saturable range of 1–30 nm. During incubation, skeletal SR (0.2–0.3 mg/ml), cardiac SR (0.3–0.5 mg/ml), or brain microsomes (0.6–0.8 mg/ml) were the last reagent to be added to the medium. Samples were filtered on Whatman GF/B glass fiber filters and washed twice with 5 ml of distilled water.

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curred during the binding assay, control incubations were carried out in the presence or absence of the protease inhibitors pepstatin A (1 μM), iodoacetamide (1 mM), phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (1 μM), and benzamidine (1 mM). Addition of the protease inhibitor mixture did not result in a significant change in the site density or the affinity of [3H]ryanodine binding.

Planar Bilayer Recording—Planar bilayers were composed of brain phosphatidylethanolamine and brain phosphatidylserine at a 1:1 weight ratio (Avanti Polar Lipids, Birmingham, AL) dissolved in decane (Aldrich). Recordings were filtered through a low-pass Bessel filter (Frequency Devices, Haverhill, MA) at 2 kHz and digitized at 4 kHz for analysis (19).

Others—SDS-polyacrylamide gel electrophoresis was performed according to the Laemmli method. Samples were incubated for 15 min at 80°C in 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 10 mM Tris (pH 6.8) and run on a 6–15% linear polyacrylamide gradient. Gels were stained with 0.05% Coomassie Blue R in 10% acetic acid. Molecular weight standards were: myosin, M, 200,000; β-galactosidase, M, 116,000; phosphorylase b, M, 97,400; bovine serum albumin (BSA), M, 68,000; ovalbumin, M, 43,000; carbonic anhydrase, M, 31,000; and cytochrome c, M, 12,000. Protein concentration was determined according to Bradford, using BSA as a standard.

RESULTS AND DISCUSSION

Studies have shown that ryanodine binds preferentially to the open conformational state of the Ca²⁺ release channel, since ligands that reversibly stimulate [3H]ryanodine binding (ATP, Ca²⁺, caffeine) also open release channels, whereas ligands that inhibit [3H]ryanodine binding (Mg²⁺, ruthenium red, acid pH) also close or block release channels (3, 21, 22). Thus, for the initial screening of various scorpion venoms we used a standard [3H]ryanodine binding assay based on the assumption that changes in binding activity produced by a given venom were likely to reflect activation or inhibition of Ca²⁺ release channel activity. Fig. 1A shows the specific binding of [3H]ryanodine to rabbit skeletal muscle heavy SR at a fixed time of 90 min and 37°C in an incubation medium containing 0.2 M KCl, 10 mM Na-Pipes, pH 7.2, and 10 μM free Ca²⁺. In the range of 2–30 nM, [3H]ryanodine was bound to a single class of receptor sites with an apparent dissociation constant (Kd) of 8.2 nM and an apparent receptor site density (Bmax) of 4.2 pmol/mg protein. Other data (not shown) indicated that the apparent Bmax of [3H]ryanodine binding could be increased by agonists of ryanodine receptors such as 5 mM ATP (8.7 pmol/mg), 10 mM caffeine (5.3 pmol/mg), pH 8.5 (7.6 pmol/mg), or 0.5 M KCl (7.1 pmol/mg). As shown by the filled circles of Fig. 1A, a fixed concentration of B. hottentota venom (80 μg/ml) increased the specific binding at all [3H]ryanodine concentrations. Only venom from the Buthotus genera, out of eight genera of scorpion venoms tested, stimulated [3H]ryanodine binding. The main effect of the venom was to increase the apparent Bmax to 7.8 pmol/mg with a slight modification of the Kd to 3.4 nM. Thus, the venom was likely to have produced a noncompetitive type of interaction with the alkaloid binding site. Fig. 1B shows that the stimulation of [3H]ryanodine binding produced by Buthotus venom was not restricted to skeletal SR. The venom also increased [3H]ryanodine binding to bovine cardiac heavy SR and to rat cerebral cortex microsomes. Binding in the absence of venom in control medium containing 7 nM [3H]ryanodine was labeled 100% and was 1.2, 0.5, and 0.1 pmol/mg in skeletal SR, cardiac SR, and brain microsomes, respectively. At saturating concentrations of venom (>100 μg/ml) there was a 4-fold increase of binding to the skeletal receptor but only a 2-fold increase for cardiac or brain receptors. This difference could be explained if under the conditions of the assay (i) a larger proportion of cardiac and brain receptors were in a conformationally open state and (ii) [3H]ryanodine binds to the receptor faster than the toxin. In such a case [3H]ryanodine could alter the gating state of the channel and conceivably prevent toxin binding. Alternatively, there could be a tissue-specific interaction of the venom with one of the two isoforms described for the ryanodine receptor. A cardiac isoform is expressed in heart and brain, whereas the skeletal muscle isoform is expressed in both fast- and slow-twitch skeletal muscle (23). In such a case, the preferential effect of Buthotus venom on the skeletal receptor could be an indication of a structural difference between the venom binding sites in the two isoforms.

Since caffeine is a known agonist of ryanodine receptors (22), we compared the effect of caffeine and Buthotus venom. Fig. 2 shows the biphasic Ca²⁺ dependence of [3H]ryanodine binding consisting of an increase in the range of pCa 9–5 and a decrease in the range of pCa 4–2. This dual effect of Ca²⁺ gave rise to a bell-shaped curve which was similar to those described previously (3, 19). In the absence of caffeine (open circles), binding had a threshold for detection at pCa 7 and peaked at pCa 5. Caffeine, at a concentration of 10 mM, reduced the threshold for Ca²⁺-dependent stimulation to pCa 8 and, in addition, shifted the ascending part of the curve toward lower Ca²⁺ by approximately a factor of 6. The latter is in agreement with previous reports that describe caffeine as a positive modulator at submicromolar Ca²⁺ (24). The agonist effect of Buthotus venom was markedly different from that of caffeine. Venom, at a concentration of 100 μg/ml (open triangles), increased binding in both the ascending and de-
scending limbs of the pCa curve. The combined effect of caffeine and Buthotus venom (filled triangles) was equally interesting since there was a dramatic synergism. For example, binding at pCa 9 increased from 0.5 pmol/mg for each compound alone to 4.2 pmol/mg for both combined. No synergism was observed, however, at pCa 4 or lower, which is consistent with the idea that caffeine is ineffective at high Ca2+ (24). Ca2+ binding to a high affinity site increases the density of sites available for [3H]ryanodine binding whereas Ca2+ binding to a low affinity site decreases the number of binding sites (24). Within this scheme, the major effect of Buthotus venom could be explained by an action of the venom on both high and low affinity sites for Ca2+.

The direct interaction of Buthotus venom with the Ca2+ release channel was investigated in planar bilayer recordings in the absence of ryanodine (19). Fig. 3A shows control recordings of Ca2+ release channels at +20 mV and 3 μM free Ca2+ present in solution as contaminant Ca2+. Channels displayed a characteristic mean open time of approximately 1 ms and a predominant conductance state of 640 pS (19, 25). In each of four recordings, addition of Buthotus venom to the myoplasmic solution produced a marked stimulation of the control activity. Open probability averaged 0.24 in control (Fig. 3A) and increased to 0.89 upon the addition of 100 μg/ml Buthotus venom (Fig. 3B). The onset of activation was fast and reached steady state within 30 s of addition. The most significant kinetic effects were an increase in the burst time and in the mean open time. This is best shown in Fig. 3C where 678 events collected during the control period were fit to a single exponential with a time constant of 1.1 ms. In the presence of venom, two exponentials with time constants of 1.2 and 10.8 ms were required to fit 4,237 events collected during a similar period. The venom also induced the appearance of a subconductance state (see arrows) that was virtually absent in the control period. The current histogram of Fig. 3D shows that the subconductance state comprised 40% of the total open events and had a mean amplitude of 180 pS. The open time histogram of the subconductance state analyzed separately (not shown) was fit to a single exponential with a time constant of 68 ms. The increase in mean open time and the stabilization of a long lived subconductance state are consistent with the view that the stimulation of [3H]ryanodine binding produced by Buthotus venom results from an overall increase in the open probability, which in turn favors the binding of the alkaloid.

To identify the component(s) responsible for channel activation we fractionated the venom peptides by gel filtration (26). Fig. 4A shows the chromatographic profile of Buthotus venom in a column of Sephadex G-50. The elution volume of BSA (68 kDa), cytochrome c (12 kDa), and NaCl (58.4 Da) are indicated on the top. Five fractions were pooled as indicated by the horizontal bars and assayed for activation of [3H]ryanodine binding. The bulk of the stimulatory activity (>65%) was concentrated in Fraction III although some stimulation was also observed in Fractions I and IV but none in Fraction V. The stimulation by Fraction I was discarded since it was not always present. The inset of Fig. 4A shows that fraction III was composed of peptides of M, 5,000–8,000, which was consistent with the active component being a peptide with a size similar to that of other channel-specific scorpion toxins (26). However, unlike other scorpion toxins (26), Fraction III could be inactivated by boiling for 2 min (not shown).

Fig. 4B shows that Fraction III mimicked the effect of whole venom. Open probability averaged 0.16 in a control channel of skeletal SR (top traces) and increased to 0.61 after the addition of 10 μg/ml Fraction III (middle traces). Similar to the effect of whole venom, Fraction III increased the mean open time and induced the appearance of the 180 pS subconductance state which, in this case, accounted for 40% of the openings. Bottom traces were taken immediately after perfusion of the cis chamber with 30 ml of a peptide-free cis solution. Following removal of Fraction III, open probability decreased to 0.18, and the subconductance state accounted for only 5% of the openings, indicating an almost complete reversibility. The effect of Fraction III on the binding of [3H]ryanodine is shown in Fig. 4C. Increasing concentrations of Fraction III increased [3H]ryanodine binding in a sigmoidal manner with an ED50 of approximately 1.5 μg/ml. Fraction

Fig. 3. Activation of Ca2+ release channel by Buthotus venom. A, control single channel recordings at +20 mV in 250 mM cis-CsCl as the current carrier and 3 μM free Ca2+. The trans solution was 50 mM CsCl. Open probability averaged 0.24. B, traces from the same channel in A after the addition of 100 μg/ml Buthotus venom to the cis solution. Open probability averaged 0.89. The arrows point to a subconductance level not seen in controls. C, open events of duration time, t, or longer are plotted as a function of time, t. Number of observations was 678 (control) or 4,237 (+ Buthotus venom). D, three data files of 65,000 bins each from control and following addition of venom were collected and plotted as occurrences (y axis) versus current amplitude (x axis). Occurrences of 0 pA (baseline) and occurrences of 11 pA (subconductance state) were 148,200 and 9,750 for control and 78,127 and 60,010 after venom addition, respectively.
III represented a 14-fold or 70-fold enrichment in activity depending on whether the starting material was considered to be the water-soluble components of the whole venom or the water-soluble plus the water-insoluble mucoid material (see "Experimental Procedures"). Based on the half-maximally effective concentration (1.5 μg/ml) and the M, of Fraction III (5,000–8,000), we estimated a Kd of 300–200 nM for Fraction III as a whole. Since Fraction III is composed of at least 10 different peptides, a single active peptide in this fraction would have an estimated Kd of 20–30 nM. The specificity of Fraction III for ryanodine receptors was tested by its ability to displace radiolabeled ligands for other transport proteins present in muscle or brain. Concentrations of Fraction III of up to 20 μg/ml failed to modify the binding of [3H]ryanodine, [3H]quinoxalinyl benzylate, [3H]ouabain, and [3H]PN200–110 to the brain inositol (1,4,5)-triphosphate receptor, cardiac muscarinic receptor, and skeletal muscle Na+/K+ pump and dihydropyridine receptor, respectively (not shown). These results revealed that Fraction III had a high degree of specificity for the SR Ca2+ release channel. Although information on the structural and molecular properties of ryanodine receptors has accumulated rapidly (23, 27, 28), the functional role of this protein in excitation-contraction coupling remains unknown. Both the reversibility and high specificity of Buthotus venom clearly makes it more advantageous than ryanodine as a chemical probe of excitation-contraction coupling.

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FIG. 4. Fractionation of active component of Buthotus venom. A, 12 mg of soluble venom was loaded onto a column (1.5 × 120 cm) of Sephadex G-50 fine, equilibrated and run in 20 mM sodium acetate (pH 4.7) at a flow rate of 10 ml/h. Individual fractions (5 ml each) were pooled as indicated by horizontal bars and assayed for activity. Binding of 7 nM [3H]ryanodine to skeletal SR was performed in the presence of 5 μg/ml Fractions I-V. The specific binding in the absence of venom was taken as unity, and the percent of activation above control produced by Fractions I-V is indicated by vertical bars. Inset, SDS-polyacrylamide gel electrophoresis of soluble venom (lane 1) and Fraction III (lane 2). Lane 1, 30 μg of soluble venom; lane 2, 12 μg of Fraction III. Arrows indicate M, markers. B, single channel recordings of skeletal Ca2+ release channels. Open probability was 0.16 in control, increased to 0.61 after the addition of 10 μg/ml Fraction III to the cis chamber, and decreased to 0.18 after perfusion of the cis chamber with 30 ml of peptide-free cis solution. C, dose-response curve for the stimulation of [3H]ryanodine binding by Fraction III. The binding of 7 nM [3H]ryanodine in the absence of Fraction III (control, 100%) was 2.6 pmol/mg protein.