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Inhibition of the Ca^{2+}-activated K^+-channel by sapecin B, an insect antibacterial protein

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Abstract

Sapecin is an antibacterial protein of the flesh fly and sapecin B is its homologue structurally similar to charybdotoxin of scorpion venom, which is known to be a K^+ channel inhibitor. We found that, like charybdotoxin, sapecin B inhibits part of the voltage pulse-induced K^+ currents of rat cerebellar Purkinje cells. We suggest that this effect is due to inhibition of the Ca^+-activated K^+ channel. Probably, sapecin B is a naturally occurring K^+ channel inhibitor as well as an antibacterial protein.

Key words: Sapecin B; Antibacterial protein; Charybdotoxin; K^+ channel inhibitor; Purkinje cell

1. Introduction

In dipteran insects, antibacterial proteins are induced in the hemolymph in response to bacterial infection and/or body injury [1–5]. Sapecin, one of these antibacterial proteins of *Sarcophaga peregrina* (flesh fly), has potent bactericidal activity against Gram-positive bacteria [6,7]. Previously, we suggested that sapecin may play independent roles in both defense and development [8]. As evidence for the latter role, we demonstrated that sapecin stimulated the proliferation of NIH-Sape-4 cells, an embryonic cell line of *Sarcophaga* [9]. We also found that during their development, imaginal discs synthesize and secrete sapecin when cultured in the presence of 20-hydroxyecdysone [10].

In ^1H NMR studies, Bontems et al. found that charybdotoxin, a scorpion venom toxin acting on the Ca^{2+}-activated K^+ channel, and sapecin share common structural motifs of an α-helix and β-sheet linked by three intramolecular disulfide bonds [11]. Recently, we isolated two homologues of sapecin from the culture medium of NIH-Sape-4 cells [12]. One of them termed sapecin B was found to be more similar to charybdotoxin than sapecin in terms of the number of its amino acid residues between the first and second Cys residues and its overall amino acid sequence. Therefore, we examined if sapecin B inhibited the K^+ channel like charybdotoxin. We found that it inhibited the Ca^{2+}-activated K^+ channel of rat Purkinje cells significantly at the same concentration as charybdotoxin. Possibly, sapecin B modulates the K^+ channel of *Sarcophaga*, if any, during the developmental stages of this insect.

2. Materials and methods

2.1. Sapecin B

Sapecin B was purified from the culture medium of NIH-Sape-4 cells as described previously [12]. About 0.1 mg of sapecin B was obtained from 1 liter of culture medium. The preparation used for experiments gave a single peak on HPLC, and had a single amino-terminal amino acid residue, Leu, indicating that it was pure enough to use in subsequent experiments.

2.2. Electrophysiology of Purkinje cells in rat cerebellar slices

Experiments were performed with thin slices of cerebellum obtained from anesthetized rats. The patch-clamp technique used for study of neurons in slices was essentially as described by Edwards et al. [15]. Briefly, slices of vermis of 125 μm width were prepared with a vibrating tissue slicer. The slices were perfused continuously with an external solution (113 mM NaCl, 3 mM KCl, 10 mM glucose, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$ and 0.4 μM tetrodotoxin (Sankyo, Tokyo)) gassed with O_2/CO_2 (95%:5%). Purkinje cells were identified using Nomarski optics with a × 40 water-immersion lens. A tight seal was achieved with a Sylgard-coated patch pipette which was filled with an intracellular solution (10 mM Hepes/KOH buffer (pH 7.3), containing 122.5 mM K^+ gluconate, 17.5 mM KCl, 9 mM NaCl, 1 mM $MgCl_2$ and 0.2 mM EGTA) with a resistance of 4–6 MΩ, and then the membrane inside the pipette was broken to achieve the whole cell configuration. Throughout experiments, the membrane potential was clamped at −60 mV unless otherwise stated. Potassium currents were evoked with test pulses (160 ms duration) between −60 mV and +20 mV with 10 mV intervals. Sapecin B and charybdotoxin (CTX, Peptide Institute, Osaka) were added directly to the experimental chamber.

2.3. Measurement of intracellular Ca^{2+}

Cerebellar slices were treated with 10 μM fura-2/AM (Dojin, Tokyo) at 32°C for 1 h in the external solution gassed with O_2/CO_2 (95%:5%).

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They were then placed in a recording chamber superfused with the external solution (20°C, 3 min). After stimulation with a high K^+ concentration (50 mM), Ca^{2+} responses of Purkinje cells were examined using an inverted fluorescence microscope (Zeiss Axiovert, Germany) equipped with a silicon-intensified target video camera (Hamamatsu Photonics C2400-80, Japan) and imaging processor (Hamamatsu Photonics ARGU-200, Japan) according to the improved F_{340}/F_{380} method of Kudo et al. [14,15]. This method has the advantage of detecting the only portion that responds to stimulation of fluorescence.

3. Results

To determine whether sapecin B affects the Ca^{2+}-activated K^+ channel of rat cerebellar Purkinje cells, we compared the effects of sapecin B and CTX on the K^+ currents induced by depolarizing voltage pulses. Under the conditions used, both voltage-dependent K^+ and Ca^{2+} channels are opened, Ca^{2+} flowing in from the medium and K^+ flowing out from the cells. Accumulated intracellular Ca^{2+} opens the Ca^{2+}-activated K^+ channel, and K^+ also flows out through this K^+ channel, resulting in the induction of Ca^{2+}-activated K^+ currents [16]. Therefore, we measured the sum of two K^+ currents. When Purkinje cells were treated with 10^{-8} M CTX for 7 min, about 35% of the voltage pulse-induced K^+ currents were inhibited and this inhibition was not restored appreciably by perfusion for 20 min with a CTX-free solution, as shown in Table 1. These results suggest that about 35% of the total K^+ current was due to the Ca^{2+}-activated K^+ channel. When the same experiment was performed with 10^{-8} M sapecin B, about 25% of the total K^+ current was inhibited. However, unlike with CTX, the percentage inhibition was increased to nearly 60% by perfusion with sapecin B-free solution. Probably the voltage-dependent K^+ currents were also inhibited in some way by this perfusion. This was the only significant difference between results with CTX and sapecin B.

However, we assume that the inhibition of K^+ currents by sapecin B detected before the perfusion was due to inhibition of the Ca^{2+}-activated K^+ channel from the following evidence.

(1) As shown in Fig. 1, typical current records obtained with 10^{-8} M sapecin B and CTX had almost the same

Table 1
Effects of sapecin B and CTX on voltage pulse-induced K^+ currents of rat Purkinje cells

Addition	Percentage response	
	7 min after application	after 20 min perfusion
None (control)	100	–
Sapecin B	74.8 ± 5.3	42.3 ± 5.8 (n = 5)
CTX	64.2 ± 2.6	79.4 ± 9.5 (n = 3)

Percentage responses relative to the control were determined using currents recorded with a test pulse at 0 mV. Test compounds were added at 10^{-8} M.

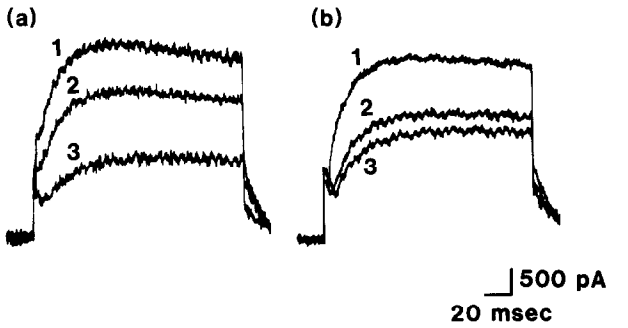

Fig. 1. Effects of sapecin B (a) and CTX (b) on the voltage pulse-induced K^+ currents of rat cerebellar Purkinje cells. Membrane K^+ currents were measured under a whole-cell voltage clamp (V = −60 mV). Records of K^+ currents detected with a voltage pulse at 0 mV are shown. (1) Before application of the test compound; (2) 7 min after application of the compound; (3) after perfusion with substance-free solution for 20 min.

shapes, and the inhibitions of the K^+ currents reached a plateau in about 40 ms.

(2) Although the specific activity of sapecin B was about 10 times less than that of CTX, these two compounds gave very similar dose-dependent curves for inhibition of voltage pulse-induced K^+ currents, as shown in Fig. 2.

(3) The curves for the I–V relations of sapecin B and CTX were very similar, as shown in Fig. 3.

(4) Sapecin B did not have an additive effect on inhibition by CTX, as shown in Table 2. All these results suggest that sapecin B inhibited the Ca^{2+}-activated K^+ channel like CTX.

Although the above results suggested that sapecin B inhibits the Ca^{2+}-activated K^+ channel, the possibility remained that it inhibits the depolarization-induced Ca^{2+}

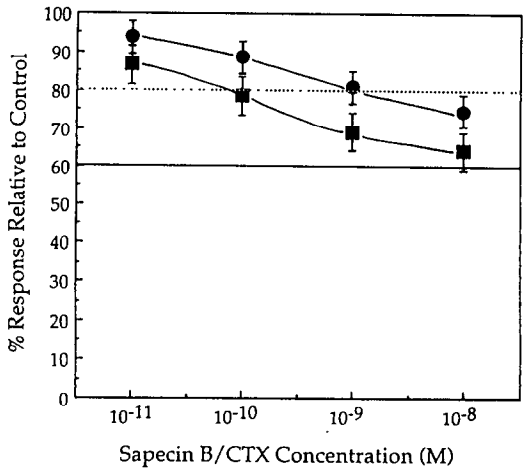

Fig. 2. Comparison of dose-dependence curves of effects of sapecin B and CTX on voltage pulse-induced K^+ currents. After recording the induced K^+ currents with a voltage pulse at 0 mV, increasing amounts of test compounds were added to the chamber. Inhibitory activity is expressed as the ratio of the currents with and without test compounds. Values are means ± S.D. (●) Sapecin B; (■) CTX.

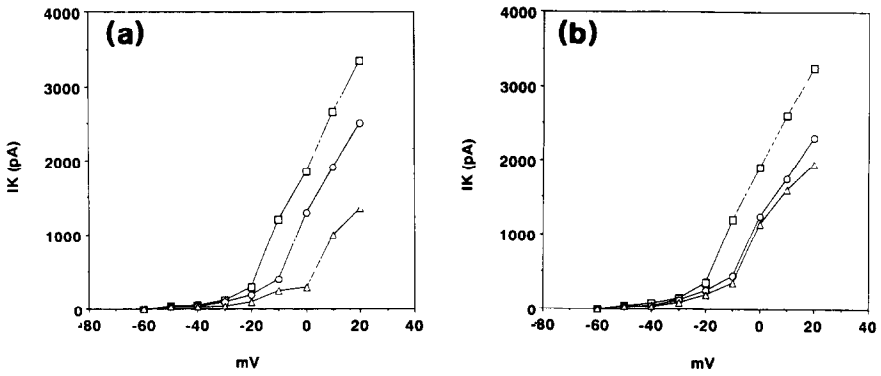

Fig. 3. *I–V* relation curves for sapecin B and CTX. K^+ currents were measured by changing the test pulse (160 ms) from −60 mV to 20 mV of 10 mV intervals. (a) Sapecin B; (b) CTX. (□) Before application of test compound; (○) 7 min after application of test compound; (△) after perfusion with solution free of the compound for 20 min.

response, namely the voltage-dependent flow of Ca^{2+}, resulting in inhibition of opening of the K^+ channel. To examine this possibility, we measured the increase of intracellular Ca^{2+} in response to stimulation with 50 mM K^+ in the presence and absence of 10^{-8} M sapecin B. Intracellular Ca^{2+} increased irrespective of the presence of sapecin B when Purkinje cells were stimulated with K^+. However, this increase was not detected in Ca^{2+}-free external medium. These results suggest that sapecin B does not interfere with the depolarization-dependent Ca^{2+} response (Table 3).

4. Discussion

We expected that sapecin B would inhibit the Ca^{2+}-activated K^+ channel like CTX because of the structural similarity of these two compounds. In fact, we demonstrated that sapecin B partly inhibited the voltage-dependent K^+ currents of rat Purkinje cells consisting of voltage-activated K^+ currents and Ca^{2+}-activated K^+ currents, and our results suggested that it inhibited the latter current. The effect of sapecin B on the voltage-dependent K^+ currents was not exactly the same as that of CTX, the percentage inhibition being increased significantly after perfusion with sapecin B-free solution. We assume that this apparent increase of the percentage inhibition was due to a complex reaction, and may not be explained simply by inactivation of voltage-dependent K^+ channels. However, the inhibition of the K^+ currents detected 7 min after treatment of the cells with sapecin B seemed likely to be due to inhibition of the Ca^{2+}-activated K^+ channel.

Unlike CTX, sapecin B is a protein in the hemolymph of *Sarcophaga*, and it is induced as an antibacterial protein in the acute phase and also as a growth factor in the embryonic and pupal stages of this insect [8]. If sapecin B acts as a K^+ channel blocker during the development of *Sarcophaga*, it should affect the same K^+ channel when it is induced as an antibacterial protein in the acute phase. This may be harmful to the insect. How does this insect overcome the paradoxical problem? Resolution of this problem requires studies on whether sapecin B affects the Ca^{2+}-activated K^+ channel of *Sarcophaga* as well as that of rat Purkinje cells.

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Table 2
Effects of CTX with and without sapecin B

Addition	Percentage response
None (control)	100
CTX	72.9 ± 5.9 (n = 5)
Sapecin B + CTX	65.6 ± 5.3 (n = 5)

Percentage responses were calculated from the currents obtained 7 min after application of compounds. Test compounds were added at 10^{-8} M.

Table 3
Effect of sapecin B on the depolarization-induced intracellular Ca^{2+} concentration

	Relative intracellular Ca^{2+}
Control	100
Sapecin B	94.1 ± 3.2 (n = 6)
Ca^{2+}-free external solution	43.1 ± 2.5 (n = 5)

Relative increase in intracellular Ca^{2+} in response to stimulation with a high K^+ concentration (50 mM) was determined using fura-2. Percentage responses were determined 7 min after application of 10^{-8} M sapecin B.

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