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Functional and structural features of γ-zeathionins, a new class of sodium channel blockers

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Abstract γ1- and γ2-zeathionins (γ1-Z and γ2-Z) are members of a family of small and basic peptides involved in plant protection. These plant defensins exhibit remarkable structural similarity to scorpion neurotoxins and insect defensins. In the present report, we used the whole-cell patch clamp technique to investigate the inhibition of the sodium current (I_{Na}) by γ1-Z and γ2-Z in the GH3 cell line. Both γ1-Z and γ2-Z rapidly and reversibly inhibited I_{Na} without changing the kinetics or voltage dependence of activation or inactivation. To our knowledge, this is the first example of a plant protein that inhibits the sodium channel. From structural comparisons with the μ-conotoxins, a family of peptides that block the sodium channel, we detected some similar features that could provide the basis of inhibition of sodium channels by γ-zeathionins.

Key words: Plant defensin; γ-Thionin; Sodium channel; μ-Conotoxin; Patch clamp; GH3 cell line

1. Introduction

Plants are continuously exposed to many pests and pathogens and in self-defense they produce many compounds with toxic properties. One group of proteins probably involved in plant resistance against fungal and bacterial pathogens is the γ-thionin family [1]. These proteins form a group of small (5 kDa), highly basic and disulfide-rich polypeptides isolated from several monocots [2–6]. We have recently reported the complete amino acid sequences of two γ-thionins from maize (*Zea mays* L.) called γ1- and γ2-zeathionins (γ1-Z and γ2-Z) [7]. γ1-Z and γ2-Z are clearly homologous with 31% identity in their amino acid sequences and four disulfide bridges.

The γ-zeathionins exhibit a high degree of homology with other γ-thionins whose three-dimensional structures have been established by 2D NMR and resemble neurotoxins found in scorpion venom which bind with high affinity and specificity to voltage-gated ion channels [8,9]. Among other conserved characteristics, they present a common structural motif formed by an α-helix connected to an extended structure by disulfide bridges, originally named CSH (cysteine-stabilized α-helix) motif [10], but currently defined as CSαβ (cysteine-stabilized α-helix β-sheet) motif [11]. Another group of toxins that act on ion channels are the μ-conotoxins, a class of three related 22-residue peptide amides, each with three disulfide bonds, isolated from the venom of the piscivorous sea snail, *Conus geographus* L. [12–15]. These peptides specifically block muscle-type sodium channels [16]. Structural similarities between γ-thionins and μ-conotoxins suggest that these peptides may share a similar mechanism of action.

In the present study, we employ the whole-cell patch clamp technique [17] to investigate the effect of γ1- and γ2-zeathionins on voltage-gated ion channels in the GH3 cell line.

2. Materials and methods

2.1. Materials

γ1-Z and γ2-Z were purified from maize (*Zea mays* L.) seeds by saline extraction, ammonium sulfate precipitation, pseudo-affinity and RP-HPLC chromatographies as previously described [7]. Cell culture sera and antibiotics were from Gibco BRL. Other chemicals were from Sigma, unless otherwise specified.

2.2. Cell culture

GH3 cells were purchased from American Type Culture Collection (Rockville, MD). The cells were kept in Ham's F-10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were fed three times per week and were maintained in a 5% CO_2 atmosphere at 37°C. For experiments, cells were plated onto sterile plastic dishes and used 1–10 days later.

2.3. Electrophysiology

The whole-cell patch clamp technique [17] was employed at room temperature (23–25°C) to measure ionic currents. Pipettes were pulled from glass capillaries and had a resistance of 1–3 MΩ when filled with pipette solution. We measured currents in two sets of solutions, one designed to isolate the Na^+ current and the other to measure total cellular currents, as described below.

To isolate the Na^+ current, we used a reversed sodium gradient [18]. Extracellular solution contained (in mM): choline chloride 150, $CaCl_2$ 2, $CdCl_2$ 0.2, HEPES 10 pH 7.3 (adjusted with CsOH). Pipette solution contained (in mM): NaF 100, NaCl 30, EGTA 10, HEPES 5 pH 7.3 (adjusted with CsOH). We used positive pressure in the pipette during the approach to the cell, a common practice used to keep the pipette tip clean of debris. A note of caution is warranted, however, when using this combination of solutions. We often experienced salt precipitation at the pipette tip, which we assume is CaF_2 (solubility at 18°C is about 0.2 mM), formed when the pipette solution mixed with the extracellular solution. This problem was alleviated by reducing the amount of positive pressure in the pipette.

To measure total cellular currents, we used solutions designed to mimic physiologic intra- and extracellular ionic concentrations. Extracellular solution contained (in mM): NaCl 150, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, glucose 10, HEPES 5 pH 7.4 (adjusted with NaOH). Pipette solution contained (in mM): KCl 140, NaCl 10, EGTA 5, HEPES 10 pH 7.4 (adjusted with KOH).

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Abbreviations: γ1-Z and γ2-Z, γ1- and γ2-zeathionins; 2D NMR, two-dimensional nuclear magnetic resonance spectroscopy; RP-HPLC, reverse-phase high-performance liquid chromatography; TTX, tetrodotoxin; STX, saxitoxin

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The patch clamp amplifiers used were either an Axopatch 200B with a Digidata 1200 A/D converter controlled by pClamp 6.01 software (Axon Instruments) or an EPC-9 with Pulse software (HEKA). Capacitive currents were electronically compensated and a P/4 protocol [19] was used for linear leak and capacitance subtraction. Na^+ currents were low-pass filtered at 4 kHz and sampled at 20 kHz or low-pass filtered at 17 kHz and sampled at 50 kHz. Total cellular currents (used to examine K^+ currents) were low-pass filtered at 2 kHz, then sampled at 5 kHz. The bandwidths are given in the figure legends. Series resistance compensation was used as necessary to keep the error in membrane potential to below 2 mV.

Cells were held at a potential of −70 or −80 mV between voltage pulses. Voltage pulse protocols are given in the text and figure legends.

Perfusion was accomplished using a micropipette (inner diameter ~500 μm) positioned within 100 μm of the cell under study. The solution was gravity fed at ~0.1 ml/min, and a solenoid valve allowed the operator to choose between two solutions with a dead time of < 10 s. Cells under study were perfused continuously after whole-cell patch clamp was achieved until currents had stabilized (usually ~5 min), at which point experiments were started.

Statistical values are given as means ± 95% confidence limits.

3. Results and discussion

The experiment of Fig. 1 shows the effect of γ1-Z (100 μM) and γ2-Z (50 μM) on positive sodium currents, elicited by stepping the membrane potential from −100 mV to +50 mV in the presence of a reversed sodium gradient [18] (see Section 2). Inhibition of I_{Na} was rapid, reversible and repeatable. The large graphs (A and D) show the time course of I_{Na} during two cycles of application and washout of γ-zeathionin. The cell under study was perfused continuously and I_{Na} sampled every 1.4 s. At the times indicated by the bars, the toxin was included in the perfusing solution. Representative currents are shown (B and E) from different regions of the time course curve as indicated by the numbers. γ1-Z and γ2-Z did not affect the kinetics of activation or inactivation of I_{Na} because normalized currents before, during and after treatment had essentially the same time course, as shown in parts C and F.

A dose-response curve for inhibition of I_{Na} by γ1-Z was constructed. At concentrations of 400 μM or greater, γ1-Z abolished I_{Na}. At four lower concentrations, inhibition of I_{Na} by γ1-Z was dose-dependent (Fig. 2). Fitting a first-order dose-response curve to the data on γ1-Z gave an IC_{50} of 62 μM. At a concentration of 50 μM, γ2-Z inhibited 60 ± 18% of I_{Na} ($n = 3$ cells). Assuming first-order binding, this indicates that the IC_{50} of γ2-Z is ~33 μM.

To determine if γ1-Z inhibits the Na^+ current by changing the voltage dependence of activation or inactivation, the current–voltage (I–V) relationship, and the voltage dependence of steady-state inactivation were measured before and during application of 50 μM γ1-Z, a concentration that inhibited I_{Na} by 25.0 ± 2.6%. I_{Na} was measured as the peak inward current during voltage steps to test potentials ranging from −60 mV to +70 mV in increments of 10 mV (Fig. 3). The parameters of voltage dependence of activation and the reversal potential were obtained by fitting a Boltzmann activation/Ohm's law equation (given in the figure legend) to the peak current at each test potential. Activation and reversal of I_{Na} were not significantly modified by γ1-Z (curve-fitting parameters given in the figure legend).

To measure the effect of γ1-Z on the voltage dependence of steady-state inactivation, cells were held at pre-pulse voltages ranging from −100 mV to −30 mV, then stepped to 0 mV for 50 ms to elicit currents (Fig. 4). The peak inward current was

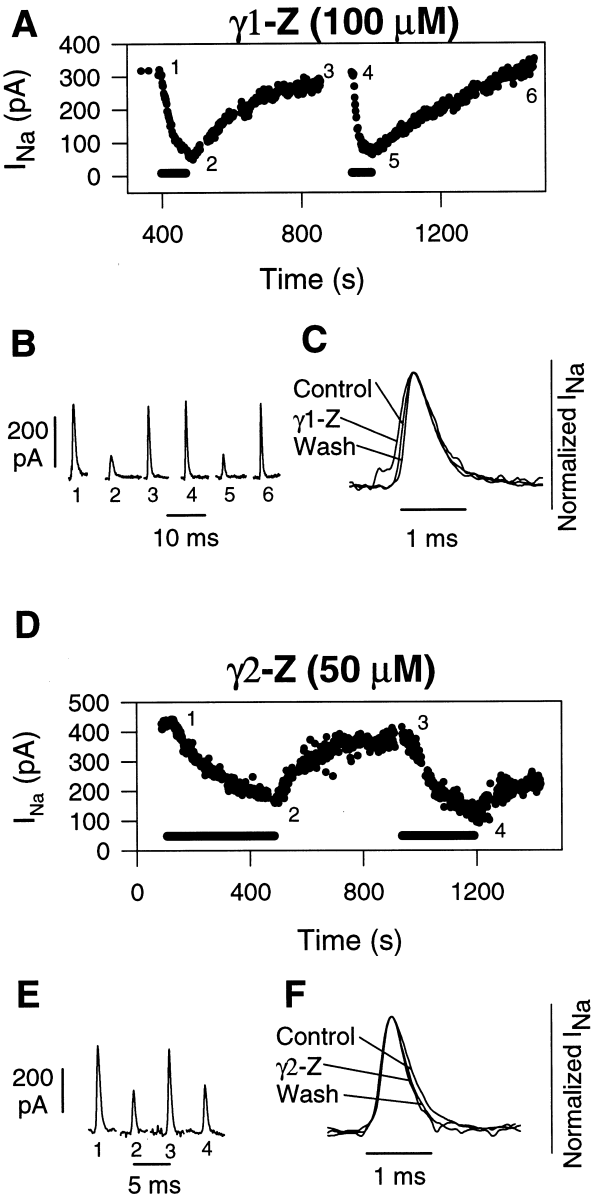

Fig. 1. Inhibition of I_{Na} by γ-zeathionins. Whole-cell patch clamp was used with reversed sodium gradient solutions (see Section 2) to measure I_{Na}. A: Time course of inhibition and recovery. I_{Na} was measured at 1.4 s intervals by hyperpolarizing the cell to −100 mV for 200 ms to remove fast inactivation followed by a 50 ms step to +50 mV. Cells were continuously perfused with control extracellular solution. At the times indicated by the bars, 100 μM γ1-Z was added to the perfusing solution. B: Representative currents from the regions indicated by the numbers (1–6). C: Normalized currents. D–F: Same as A–C except with 50 μM γ2-Z. Currents were filtered at 17 kHz and sampled at 50 kHz.

plotted versus the test potential, and a Boltzmann relationship was fit to the data to determine the potential at which I_{Na} is half-inactivated ($V_{0.5}$) and the steepness factor (K). $V_{0.5}$ was −59.0 ± 8.2 mV in control solution and −65.0 ± 8.8 mV after application of γ1-Z ($n = 4$). Thus, due to cell-to-cell variation, there was no statistically significant shift in the average value of $V_{0.5}$ among cells. However, the average shift of $V_{0.5}$, calculated by subtracting the value of $V_{0.5}$ after application of γ1-Z from the value obtained from the same cell in control solu-

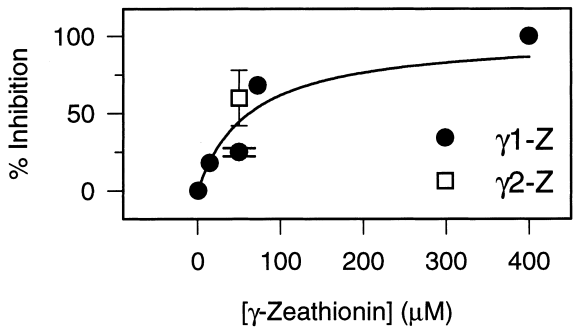

Fig. 2. Dose response of inhibition of I_{Na} by (●) γ1-Z or (□) γ2-Z. Na^+ currents were measured during pulses to 0 mV before and during perfusion with γ-zeathionin. Inhibition was calculated as $100\% \times (I_C - I_Z)/I_C$, where I_C and I_Z are the currents measured in control solution and in the presence of γ-zeathionin, respectively. Sample size (number of cells tested) varied as follows: 50 μM γ1-Z, $n=4$; 50 μM γ2-Z, $n=3$; 400 μM γ1-Z, $n=4$; other concentrations, $n=1$. Where shown, error bars are 95% confidence limits. Smooth curve is the best fit of the equation $I = C/(C+IC_{50})$, using only the data for γ1-Z.

tions, and then calculating statistics from the resultant shifts, was -7.0 ± 2.2 mV. Likewise, the average value of the steepness constant (K) among cells was not changed by γ1-Z (5.5 ± 0.6 mV versus 6.4 ± 1.1 mV) but the average increase in K, calculated as above by comparing each cell with itself before and after application of γ1-Z, was 0.93 ± 0.61 mV. These shifts in the voltage dependence of inactivation, while statistically significant, do not explain the observed inhibition of I_{Na}. To measure inhibition of I_{Na}, a pre-pulse to −100 mV for 200 ms was applied to remove fast inactivation. The net effect of the negative shift in $V_{0.5}$ and the increase in K would be a slight increase in steady-state inactivation at −100 mV. Given the numerical values presented above however, one can calculate that this will result in an apparent inhibition of only ~1%, whereas we observed 25% inhibition at this concentration of γ1-Z.

In order to determine what effect γ-zeathionins have on K^+ channels, whole-cell currents were measured before and during application of 400 μM γ1-Z, a concentration that eliminated I_{Na} (Fig. 5). Part A shows the currents elicited during voltage steps from −70 and +60 mV in intervals of 10 mV. Application of 400 μM γ1-Z eliminated the sodium current. In Part B, the I–V relationship of the peak inward current (i.e. I_{Na}) and the steady-state current (i.e. I_K) is shown. I_{Na} was eliminated whereas I_K appeared unchanged.

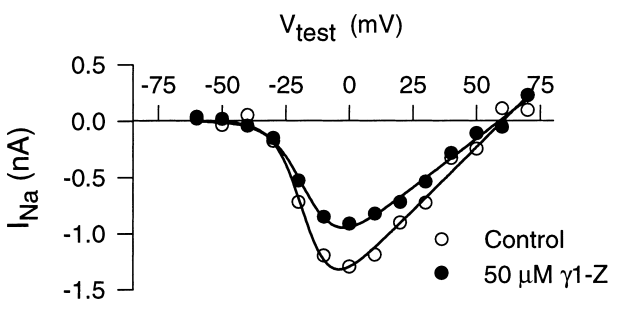

Fig. 3. Voltage dependence of I_{Na} in the presence of 50 μM γ1-Z. Cells were held at −80 mV then stepped to −100 mV for 200 ms to remove fast inactivation followed by steps to voltages ranging from −60 mV to +70 mV in 10 mV increments. The peak current during control perfusion (○) and during perfusion with 50 μM γ1-Z (●) was plotted versus voltage. The smooth line represents the best fit of the equation $I = g \times (V - V_{rev})/[1+\exp(-(V-V_{0.5})/K)]$. Best fit parameters were as follows. Control: $g = 22.5$ nS, $V_{rev} = +60$ mV, $V_{0.5} = -17$ mV, $K = 5.6$ mV; γ1-Z: $g = 17.1$ nS, $V_{rev} = +59$ mV, $V_{0.5} = -16$ mV, $K = 6.7$ mV. Shown is one typical experiment of three from different cells (see text for statistics). Solutions: physiologic (see Section 2). Filtered at 2 kHz, sampled at 10 kHz.

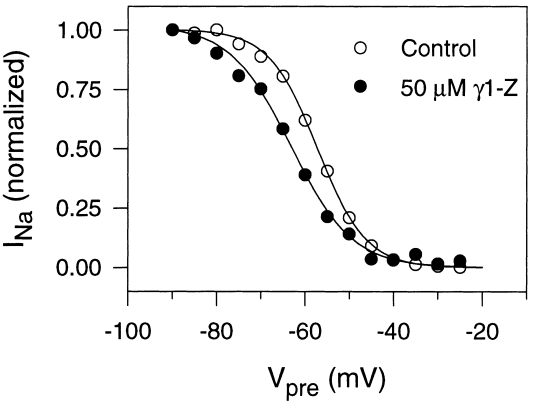

Fig. 4. Voltage dependence of steady-state inactivation of I_{Na} in the presence of 50 μM γ1-Z. Cells were held at −80 mV, then stepped for 200 ms to pre-pulse voltages ranging from −90 mV to −25 mV in 5 mV increments, followed by a test pulse to 0 mV for 50 ms during which currents were recorded. The normalized peak current measured during control perfusion (○) and during perfusion with 50 μM γ1-Z (●) was plotted versus pre-pulse voltage. The smooth line represents the best fit of the equation $I = I_{max}/[1+\exp(-(V-V_{0.5})/K)]$. Best fit parameters were as follows. Control: $V_{0.5} = -57$ mV, $K = 5.8$ mV; γ1-Z: $V_{0.5} = -63$ mV, $K = 7.1$ mV. Shown is one typical experiment of four from different cells. Statistics are discussed in the text. Solutions: physiologic (see Section 2). Filtered at 2 kHz, sampled at 10 kHz.

The μ-conotoxins, one of many neurotoxins that target sodium channels [20], compete for the guanidinium toxins tetrodotoxin and saxitoxin binding site and prevent sodium permeation [21–23]. In addition to functional similarity, γ-thionins and μ-conotoxins share structural similarities which suggest a common mechanism of action.

Many studies have been carried out to identify which residues of μ-conotoxins are involved in the binding and blocking activity [24–27]. One important characteristic required for blockage of Na^+ conductance is the presence of a positively charged region on the neurotoxin since there is a negative charge at the mouth of the pore and the resultant electrostatic forces contribute to the attachment of the toxin to its binding site on the channel [28]. This basic profile is also found in the γ-thionin family, whose members are characterized by their relative abundance of Arg and Lys residues in their sequences (Fig. 6), producing a net positive charge ($pI > 8$) on these molecules [2–7].

Several derivatives of μ-conotoxin GIIIA were synthesized and functional studies have demonstrated that toxin residue Arg-13 is the most important residue for its blocking activity [24–27]. Arg-13 interacts strongly with Glu-758 and less strongly with Glu-403 on the channel [27] in a way similar to that of the guanidinium moieties of TTX and STX [29]. This critical Arg residue is conserved among the γ-thionin members and it is located in a cluster of positively charged amino acids on the C-terminal region of these molecules [7]. An identical distribution of basic residues is also present on μ-conotoxin molecules, as illustrated in Fig. 6.

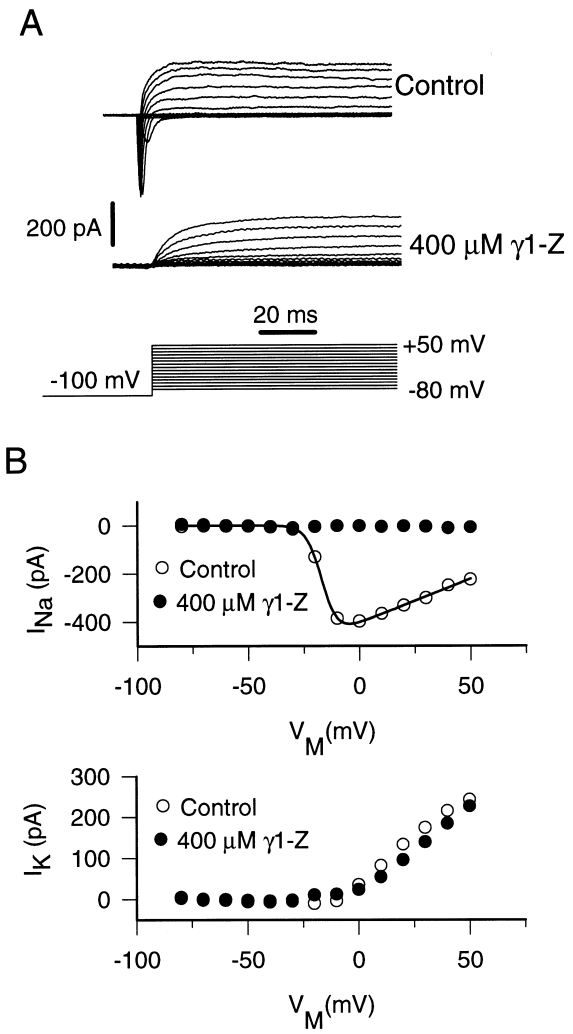

Fig. 5. Effect of 400 μM γ1-Z on I_{Na} and I_K. Cells were held at −80 mV, stepped to −100 mV for 20 ms then stepped to test voltages ranging from −80 to +50 mV in 10 mV increments. A: Currents and voltage protocol. B: *I–V* relationship of I_{Na} (the peak inward current) and I_K (the steady-state outward current measured 80 ms after the onset of the test pulse). Solutions: physiologic (see Section 2). Filtered at 1 kHz, sampled at 2.5 kHz.

The three-dimensional structures of μ-conotoxins GIIIA [30] and GIIIB [31] have been determined and these peptides adopt a common global fold consisting of a compact structure stabilized by three disulfide bridges, two of which connect the helix and the small β-hairpin, forming a structural core with similarities to the cysteine-stabilized αβ (CSαβ) motif. The CSαβ motif could be represented as a βαββ (or αββ) type sequence and it is shared by several proteins and peptides with different toxic properties, including γ-thionins [11]. The same characteristics are found in GIIIB, but the strand and the helix are connected in the opposite direction [31].

Recently, the 3D structure of a small antimicrobial peptide isolated from seeds of *Impatiens balsamina* has been reported, and this structure was compared with those of α-conotoxins, paralytic peptides isolated from *Conus* venom. Although their disulfide connectivities are similar, their folds are not. No similar active sites were found between these peptides [32]. Thus to our knowledge, the present report is the first example of structural similarities between conotoxins and plant defensins.

The potencies of γ1-Z and γ2-Z are similar to those of toxins from plants that affect Na^+ channels, such as aconitine and veratridine and to batrachotoxin from frog skin, which are effective in the 1–100 μM range [33]. However, these lipid-soluble Na^+ channel toxins open the channel and depolarize the membrane, a mechanism different from the conductance-blocking activity we propose for γ-zeathionins. It is thus more appropriate to compare γ-zeathionins with scorpion, spider and *Conus* peptide toxins (effective at concentrations < 1 μM [34,35]). Differences between the species may account for some of the difference in potencies between γ-zeathionins and the other Na^+ channel peptide toxins. Animals that hunt require potent toxins to immobilize their prey; plants need only impart a chronic inhibition of some critical function (e.g. sodium channels) in order to gain an evolutionary advantage. Alternatively, since the overall structure of ion channels is conserved among eukaryotes [33], the γ-zeathionins may prove to be more potent inhibitors of channels from insects, which may be their native target.

In summary, we have shown that γ1-Z and γ2-Z reversibly block the voltage-gated sodium channel. We observed no effects on the voltage dependence of inactivation or steady-state inactivation that could explain this inhibition, and thus suggest γ-zeathionins block the conductance of the sodium channel (i.e. either reduce the open channel conductance or decrease the number of channels that open during a voltage step). To our knowledge, this is the first example of a plant protein that blocks the sodium channel in animal cells.

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GIIIA   ----------RDCCTP-----PKKCK--DRQCKPQRCCA
GIIIB   ----------RDCCTP-----PRKCK--DRRCKPMKCCA
GIIIC   ----------RDCCTP-----PKKCK--DRRCKPLKCCA
SIα1    RVCMGKSQHHSFPCISDRLCSNECVKEEGGWTAGYCH--LRYCRCQKAC-
γ2-Z    RVCMGKSQHHSFPCISDRLCSNECVKEDGGWTAGYCH--LRYCRCQKAC-
γ1-P    KICRRRSAGFKGPCMSNKNCAQVCQQE--GWGGGNCDGPFRRCKCIRQC-
γ1-H    RICRRRSAGFKGPCVSNKNCAQVCMQE--GWGGGNCDGPLRRCKCMRRC-
γ2-P    KVCRQRSAGFKGPCVSDKNCAQVCLQE--GWGGGNCDGPFRRCKCIRQC-
SIα3    RVCRRRSAGFKGLCMSDHNCAQVCLQE--GWGGGNCDGVIRQCKCIRQC-
γ1-Z    RVCRRRSAGFKGVCMSDHNCAQVCLQE--GYGGGNCDGIMRQCKCIRQC-
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Fig. 6. Sequence alignment between μ-conotoxins and γ-thionins. Gaps are included to maximize sequence similarity. γ1-P and γ2-P, γ1-purothionin and γ2-purothionin [2]; γ1-H, γ1-hordothionin [3]; SIα1 and SIα3, α-amylase sorghum inhibitor 1 and α-amylase sorghum inhibitor 3 [4]; γ1-Z and γ2-Z, γ1-zeathionin and γ2-zeathionin [7]; GIIIA, GIIIB and GIIIC, μ-conotoxin GIIIA, μ-conotoxin GIIIB and μ-conotoxin GIIIC [14]. P = hydroxyproline and Arg-13 in bold.

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