Cellular responses to *Pyrularia* thionin are mediated by Ca^{2+} influx and phospholipase A_2 activation and are inhibited by thionin tyrosine iodination

(hemolysis/neuromuscular junction/lymphocytes/membrane potential)

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ABSTRACT *Pyrularia* thionin, isolated from nuts of *Pyrularia pubera*, is a strongly basic peptide of 47 amino acids. The amino acid sequence and configuration of its four disulfide bonds place this plant peptide, known to be hemolytic, cytotoxic, and neurotoxic, among the thionins. We report and compare several cellular responses mediated by *Pyrularia* thionin: hemolysis of human erythrocytes, activation of an endogenous phospholipase A_2 in Swiss 3T3 cells, cytotoxicity toward HeLa and mouse B16 melanoma cells in culture, viability of rat hepatocytes and lymphocytes measured by trypan blue exclusion, and lethality in mice. Cellular responses related to ion movement include a toxin-mediated influx of Ca^{2+} into mouse P388 cells measured by Fura-2 fluorescence, depolarization of mouse P388 plasma membrane measured by fluorescence of bis(1,3-diethylthiobarbituric acid)trimethine oxonol (bisoxonol), and depolarization of frog (*Rana pipiens*) sartorius muscle determined by direct measurement of membrane potential. Graded iodination of *Pyrularia* thionin leads to a related loss of activity for hemolysis, phospholipase A_2 activation, cytotoxicity, and lethality in mice. The mediated Ca^{2+} influx into and depolarization of P388 cells require Ca^{2+} in the external medium and are inhibited by $100\ \mu\text{M}$ Ni^{2+} . Depolarization of sartorius muscle by *Pyrularia* thionin also requires a functional Ca^{2+} channel, as shown by verapamil inhibition. This muscle depolarization also involves phospholipase A_2 activation because dexamethasone and quinacrine, but not indomethacin, protect against depolarization. The IC_{50} values for viability of rat hepatocytes and splenic lymphocytes measured by trypan blue exclusion were 0.17 and $40\ \mu\text{M}$, respectively. The general response of cells to *Pyrularia* thionin involves a membrane alteration leading to depolarization and a channel-mediated influx of Ca^{2+} . There is a related activation of phospholipase A_2 that results in loss of membrane integrity, hemolysis in the case of erythrocytes, and eventually cell death. Iodination of *Pyrularia* thionin leads to a corresponding inhibition of all three cellular responses, which indicates an essential role for tyrosine in either maintenance of peptide structure or interaction of the peptide with cellular membranes.

Nuts of the parasitic plant *Pyrularia pubera* contain a bioactive peptide of 47 amino acids that is hemolytic, cytotoxic, and neurotoxic (1, 2). The amino acid sequence and configuration of four disulfide bonds designate it a member of the family of plant thionins, with which it has a high degree of sequence homology (1). Related plant thionins were previously isolated from wheat (purothionins $\alpha 1$, $\alpha 2$, and β) (3, 4), barley (hordothionins α and β) (5, 6), oats (7), and rye (avenothionin) (8). Similar toxic peptides have been isolated from *phoradendron* (phorotoxin) (9) and mistletoe (visco-

toxins A_2 , A_3 , and B) (10–12). Like *Pyrularia* (*P*) thionin, the cereal grain thionins have eight cysteine residues covalently linked by four disulfide bridges (13). Although the viscotoxins contain only three disulfide bonds (14), they have slightly more sequence homology with *P* thionin than do the other thionins (1). This homology may reflect the fact that *Pyrularia* is in the same order (*Santalales*) as mistletoe. Another related peptide of 46 amino acids is crambin, a hydrophobic protein isolated from Abyssinian cabbage (*Crambe abyssinica*) (15). The amino acid sequences of these peptides are compared in ref. 1.

The thionins have been widely studied for their toxicity in several animal cells as well as in brewer's yeast (16–18). These thionins act on cell membranes, causing leakage of nucleotides, amino acids, and ions from yeast cells (19) and baby hamster kidney cells (16). Some biological responses to *P* thionin have been reported, including its ability to induce hemolysis of erythrocytes (1, 20, 21), inhibit growth of cultured mammalian cells (2), affect the chemosensitive synapse (22), and activate an endogenous phospholipase A_2 (PLA_2) in Swiss 3T3 cells (23). *In vivo* *P* thionin is lethal to mice, inducing muscular shaking followed by death (2). This paper extends the previous investigations and includes mouse P388 cells, reporting on the effect tyrosine iodination has on these activities. All data indicate the cellular responses of *P* thionin stem from a stimulated Ca^{2+} influx coupled to depolarization of the plasma membrane, which then leads to activation of an endogenous PLA_2 . An essential role of tyrosine in *P* thionin activity is also shown.

MATERIALS AND METHODS

Iodo-Gen was purchased from Pierce. *P* thionin was prepared as described (1). [^3H]Arachidonic acid was purchased from New England Nuclear. Bis(1,3-diethylthiobarbituric acid)-trimethine oxonol (bisoxonol), Fura-2, and the acetoxy-methyl ester derivative Fura-2AM were purchased from Molecular Probes. Dexamethasone, verapamil, quinacrine, indomethacin, and trypsin-EDTA solution ($1\times$) were purchased from Sigma.

Hemolysis. Venous blood was drawn into EDTA vacutainers and used the same day. Five samples were pooled for each series of assays after diluting and washing each sample three times ($1000\times g$ for 10 min) in 10 vol of 150 mM NaCl. The buffy layer of lymphocytes was discarded. For one experiment 0.10 ml of packed red blood cells was diluted to 100-ml final vol with 150 mM NaCl/5 mM $\text{Na}_2(\text{PO}_4)$, pH 7.4. Three aliquots were used for each assay (run in triplicate), which consisted of 1-hr incubation at 37°C of gentle shaking with the drug to be tested. Cells were sedimented at $150\times g$ for 15

min, and absorbance of the supernatant fluid was determined at 416 nm.

PLA₂. PLA₂ activity was measured by release of [³H]arachidonic acid according to the method of Shier and Durkin (24). Briefly, Swiss 3T3 cells were incubated with [³H]arachidonic acid for 1 day, after which compounds to be tested for phospholipase activation were added and incubated for 1 hr. The released [³H]arachidonic acid was extracted in ethyl acetate, and the radioactivity was determined by scraping off the arachidonic acid band from a TLC plate used in separation.

Cytotoxicity. Cytotoxicity tests with mouse B16 and HeLa cells were done essentially according to Geran *et al.* (25). Mouse B16 melanoma cells and HeLa cells were plated out in 96-well plates (5×10^3 cells per well) and allowed to grow for 24 hr in Eagle's minimal essential medium. Native or iodinated *P* thionin (see below) was added in concentrations from 0.1 to 30 μ g/ml or 100 μ g/ml for the iodinated *P* thionin. After 48 hr B16 cells were confluent; wells were washed twice with physiological saline, and 50 μ l of trypsin-EDTA solution (1 $\times$) was added. After 10 min at 37°C, 200 μ l of physiological saline was added, and the well contents were transferred to 10 ml of Isoton (Scientific Products) for counting in the Coulter counter. HeLa cells were counted directly after the 48-hr growth period. Cell counts were compared to controls to determine percentage growth. Hepatocytes and lymphocytes were tested for viability based on their ability to exclude trypan blue.

Ca²⁺ Influx. Mouse P388 cells were grown in RPMI 1640 medium at 37°C. Cells were washed twice and then suspended in Hepes buffer solution (145 mM NaCl/1 mM MgSO₄/0.4 mM sodium phosphate/10 mM Hepes/5 mM glucose/1.5 mM CaCl₂, pH 7.3). The cells were loaded with Fura-2AM according to Grynkiewicz *et al.* (26) for 30 min, washed twice with 10 mM Hepes buffer, pH 7.3, suspended in buffer at 1×10^6 cells per ml, and transferred to a quartz cuvette. Temperature was maintained at 37°C, and a stirring bar was used to keep cells suspended. Fluorescence was measured in a Perkin-Elmer LS3 fluorescence spectrophotometer (excitation λ 340 nm and fluorescence λ 510 nm). Ca²⁺ concentrations were determined from a standard calibration curve prepared with the water-soluble Fura-2 as described (26).

P388 Membrane Potential. The fluorescence intensity of the dye bisoxonol was measured essentially as described by Tatham *et al.* (27)—excitation λ 540 nm and emission λ 580 nm. The dye in dimethyl sulfoxide was added to the cuvette containing the washed P388 cells, as described above, to final concentration of 0.123 nM.

Iodination. Iodinated *P* thionin (nonradioactive) was prepared by using NaI and Iodo-Gen (Pierce) that had been

dissolved in dichloromethane and deposited on the walls of test tubes after drying as described (20). Extent of the iodination was controlled by the concentrations of NaI and Iodo-Gen in the iodination reaction mixture. For samples A, B, and C (Table 1) concentrations of Iodo-Gen were 180, 120, and 180 μ g, and the concentrations of NaI were 0.25, 0.45, and 0.45 μ M, respectively. A measure of the extent of iodination of *P* thionin is the absorbance at 312 nm, which corresponds to diiodotyrosine concentration. This measurement cannot be used directly, however, because monoiodotyrosine absorbs slightly at this wavelength. By using known molar absorbances of these two species at 312 and 300 nm, a set of equations was derived that allows close approximation of the concentrations of the two iodinated species. With these equations the ratios of monoiodotyrosine/*P* thionin and diiodotyrosine/*P* thionin, respectively, were calculated for the samples reported in Table 1, yielding values of 0.81 and 0.45 for sample A, 0.56 and 0.93 for sample B, and 0.62 and 1.43 for sample C. These results show that both tyrosines were completely iodinated in either the monoiodo or diiodo form for sample C.

Electrophysiology. All experiments with sartorius muscle from *Rana pipiens* were done at room temperature (20–22°C). To record membrane potential and miniature endplate potentials of the muscle, tetrodotoxin at 300 nM was freshly dissolved and added to Ringer's solution (115 mM NaCl/2.0 mM KCl/1.3 mM Na₂HPO₄/0.7 mM NaH₂PO₄/3.6 mM CaCl₂, pH 7.2). Muscle-twitch experiments were done with sciatic nerve-sartorius muscle preparations. Indirect muscle twitches were evoked by passing supramaximal square-wave pulses of 3-msec duration through a platinum bipolar electrode attached to the sciatic nerve (22).

Hepatocytes and Lymphocytes. The technique of hepatocyte isolation from rat liver followed in principle the procedure of Berry and Friend (28) as modified by Krebs (29). After isolation the hepatocytes were incubated in a Krebs-Henseleit-bicarbonate buffer solution with continuous gassing (5% CO₂/95% O₂ at 4°C) before exposure to *P* thionin at 37°C. Lymphocytes were isolated from rat spleen by centrifugation through Ficoll-Paque (Pharmacia) and subsequently washed with Hanks' balanced salt solution at room temperature (30). Isolated lymphocytes were exposed to *P* thionin at 37°C. Viability of the exposed lymphocytes was measured by the trypan blue (0.1%) exclusion technique (31).

RESULTS AND DISCUSSION

Table 1 lists known biological responses of *P* thionin. The hemolytic, cytotoxic, and neurotoxic activities of *P* thionin have been reported (1, 2) as well as its ability to activate an endogenous PLA₂ activity in Swiss 3T3 cells (23). The *P*

Table 1. Effect of graded iodination of *P* thionin on four biological activities of this peptide

Thionin sample	Iodination*	Hemolysis,† %	PLA ₂ activity‡	IC ₅₀ , μ g/ml		Mice surviving¶	Time to death, min
				B16§	HeLa cells		
Native	None	50 $\pm$ 0.48	2129 $\pm$ 489	5.0 $\pm$ 0.89	14 $\pm$ 0.84	0	4.5 $\pm$ 0.47
A	0.51	41 $\pm$ 1.0	1329 $\pm$ 241	22 $\pm$ 1.6	16 $\pm$ 0.75	0	6.8 $\pm$ 1.9
B	0.97	21 $\pm$ 1.2	139 $\pm$ 51	40 $\pm$ 6.4	25 $\pm$ 0.80	1	14 $\pm$ 11.2
C	1.4	0	0	Not toxic	Not toxic	All	

Samples A, B, and C were progressively iodinated; sample C was the most highly iodinated. Values reported are averages $\pm$ SEM of three experiments with the same samples of iodinated *P* thionin (except for hemolysis experiments, which numbered five).

*Numbers given for the three iodinated samples are A₃₁₂ units for 1 mg/ml protein, which are proportional to the extent of diiodotyrosine formation. See *Materials and Methods* for discussion and calculated values for monoiodotyrosine and diiodotyrosine.

†Human erythrocytes were used. Thionin was present at 20 μ g/ml. Complete hemolysis gave an A₄₁₆ of 2.20.

‡Activity is expressed as counts per min for [³H]arachidonic acid released above control. Thionin was present at 100 μ g/ml.

§Cultured mouse B16 melanoma cells were used to determine cytotoxicity.

¶*n* = 5 for each group. Each mouse was injected with 100 μ g of native or iodinated *P* thionin. For native *P* thionin this amount is 3 $\times$ LD₅₀ value.

Mice alive after 24 hr were counted as survivors.

|| Average time to death for nonsurviving mice; all mice in group C lived.

thionin contained no contaminating PLA₂ activity and had no such activity by itself (20). The general response seen for all activities was a graded decrease in activity as *P* thionin became more iodinated. At the highest level of iodination, all responses were completely inhibited. Mice injected with lethal doses of *P* thionin began shaking, leading to rapid death. This toxicity was completely absent when the extensively iodinated *P* thionin was injected. Correlations of all measured biological phenomena to the iodination level of *P* thionin indicate some common mode of action for these reactions.

Fig. 1 shows that P388 cells respond to added *P* thionin with increased intracellular Ca²⁺; reference to a calibration curve allowed calculation of intracellular Ca²⁺ concentrations of 1.3×10^{-4} mM before and 10.6×10^{-4} mM after addition of *P* thionin for this experiment. The response of the least iodinated *P* thionin was somewhat larger than for the native *P* thionin. Further iodination caused complete loss of this activity. As shown below, the *P* thionin-mediated depolarization of sartorius muscle also depended upon Ca²⁺ influx.

Fig. 1 data also show that increase in internal Ca²⁺ relates to Ca²⁺ influx because treatment of the cells with *P* thionin without Ca²⁺ in the external medium caused no change in internal Ca²⁺. Activation of a Ca²⁺ channel is probable because addition of 100 μ M Ni²⁺, an inhibitor of Ca²⁺ channels (32, 33), inhibited the Ca²⁺ response. The initial decrease in fluorescence upon adding Ni²⁺ is unrelated to a Ca²⁺ change because examination of the fluorescence emission at 510 nm with excitation from 300 to 400 nm showed a general displacement, not the characteristic crossover change seen for Ca²⁺ responses. Data of Fig. 1 were obtained with the same cell culture and iodinated *P* thionins to allow direct comparison of all activities. Data from five experiments gave average cellular Ca²⁺ concentrations of 192 ± 60 nM before and 772 ± 395 nM after the Ca²⁺ influx induced by *P* thionin.

Adding *P* thionin to P388 cells also depolarized the plasma membrane, as measured by changes in fluorescence of the indicator dye bisoxonol (Fig. 2). Repeating the experiments in Ca²⁺-free medium decreased membrane depolarization to $\approx 70\%$ of that normally seen. A similar decrease in depolar-

FIG. 1. Mediation of Ca²⁺ influx into P388 cells by *P* thionin: inhibition by iodination of the thionin and indication of Ca²⁺ channel involvement. At times indicated by arrows, 20 μ g of *P* thionin (curve A) or the iodinated derivatives (curves B, C, and D) were injected into the reaction cuvette containing 1×10^6 cells and Fura-2 fluorescence measured at 510 nm and 37°C. For curve A the Ca²⁺ concentration changed from 1.3×10^{-7} to 10.6×10^{-7} M inside the cell, measured from calibration curves of fluorescence excited by 300- to 400-nm light (26). Samples of *P* thionin used for curves B–D were the same iodinated samples designated A–C, respectively, in Table 1. For curve E the medium Ca²⁺ was replaced with Na⁺, and 1 mM EGTA was present. For curve F NiCl₂ was added at the first arrow to give 100 μ M Ni²⁺, followed by *P* thionin addition at the second arrow.

FIG. 2. Depolarization of mouse P388 cells induced by *P* thionin. Membrane potential was determined by fluorescence of bisoxonol at 580 nm and 37°C as described. At arrows 20 μ g of *P* thionin was added. Curves: A, *P* thionin in regular medium with Ca²⁺ at 1.5 mM; B, cells were washed and resuspended in Ca²⁺-free medium as described for Fig. 1; C, the reaction included 100 μ M Ni²⁺; and D, 50 nM gramicidin D was added for *P* thionin.

ization was seen when 100 μ M Ni²⁺ was present. For comparison, the membrane depolarization caused by gramicidin D is also shown. The ratio of these two changes in fluorescent intensities is 0.48. In another experiment ratios of 0.26 and 0.51 were obtained with 10 and 50 μ g of *P* thionin per ml, respectively.

The biological assay involving membrane potential of frog sartorius muscle reveals a dramatic depolarization of the muscle cells by *P* thionin (Fig. 3). Separate experiments on the dose dependency of the reaction showed an IC₅₀ of 17 μ M for the toxin. Also shown in Fig. 3 are the effects of dexamethasone and quinacrine, inhibitors of PLA₂. Both inhibitors protected the membrane against the effects of *P* thionin, which indicates the depolarization resulting from *P* thionin addition was caused by thionin-induced PLA₂ action on the membrane. Addition of indomethacin, a cyclooxygenase inhibitor, potentiated the effect of *P* thionin, indicating that depolarization was facilitated by the arachidonic acid released from membrane by activated PLA₂.

The powerful effect of *P* thionin on membrane potential of frog sartorius muscle also involves a Ca²⁺ influx, similar to the response shown above for P388 cells. Fig. 4 shows that the channel blocker verapamil (10 μ M) blocked the action of *P* thionin on the membrane potential of sartorius muscle. A similar protection was seen when the muscle was suspended in Ca²⁺-free Ringer's solution (22). These data indicate that

FIG. 3. Effects of inhibitors of PLA₂ (dexamethasone and quinacrine) and cyclooxygenase (indomethacin) on *P* thionin-induced depolarization of frog sartorius muscle. Each preparation except the control was preincubated with the inhibitors for 1–3 hr before adding 60 μ M *P* thionin. Each point represents the mean of at least 10 fibers. $\circ$, Control; $\bullet$, 100 μ M dexamethasone; Δ , 100 μ M quinacrine; and $\blacktriangle$, 100 μ M indomethacin.

FIG. 4. Verapamil protects against *P* thionin-induced depolarization of surface fibers of frog sartorius muscles. Each preparation was preincubated with 10 μ M verapamil for 60 min, followed by verapamil plus *P* thionin. The muscle was subsequently washed for 60 min with frog Ringer's solution. Changes in the experimental conditions are indicated by arrows. *P* thionin alone at 60 μ M induced a depolarization in membrane potential to 45% of control. Each point represents a mean from at least 30 surface muscle fibers of more than three muscle preparations.

the initial effect of *P* thionin in both P388 cells and sartorius muscle is activation of a Ca^{2+} channel and that the increased concentration of Ca^{2+} inside the cell stimulates PLA_2 activity, which causes the observed depolarization.

P thionin also affects the viability of rat lymphocytes and hepatocytes (22). By measuring trypan blue exclusion we calculated an IC_{50} of 40 μ M for rat lymphocytes, showing that lymphocytes are less sensitive to *P* thionin than the other cells tested (2). Similar experiments with rat hepatocytes yielded an IC_{50} value of 0.17 μ M. These results coupled with previous data (2) show that the toxin is active with a wide variety of cells.

Some general features of *P* thionin cytotoxicity become apparent from this study. Because *P* thionin is active on many different cell types, its interaction with and effect on cell membranes probably involves general membrane components and mechanisms that operate for all cells tested. The ability of *P* thionin to induce a Ca^{2+} influx and the related activation of PLA_2 would explain the observed biological effects. These two phenomena are related because both must take place for depolarization of sartorius muscle. Although not shown in Table 1, the release of arachidonic acid induced by *P* thionin in Swiss 3T3 cells is also inhibited in Ca^{2+} -free medium, with counts of released [^3H]arachidonic acid going from 5053 for control to 3935 for cells treated with no Ca^{2+} in a suspending medium also containing 10 mM EGTA. The data also suggest that the Ca^{2+} influx involves a channel because responses are blocked by verapamil with sartorius muscle cells and by Ni^{2+} with mouse P388 cells.

Also indicative of the general nature of *P* thionin interaction with cells are the facts that viscotoxin is also cytotoxic and depolarizes muscle cells (34) and cardiotoxins isolated from cobra venoms show the same cellular responses as does *P* thionin. Cardiotoxins have no intrinsic PLA_2 activity (35), are hemolytic—reacting synergistically with PLA_2 in the hemolysis assay (35, 36), are cytotoxic (37), trigger an influx of Ca^{2+} into skeletal muscle (38), induce endogenous PLA_2 activity in cultured Swiss 3T3 cells (39), and cause depolarization and contraction of muscle cells (40). *P* thionin shows all these activities, and both toxins appear to act by the same or similar mechanisms. Recent evidence (21) shows that *P* thionin and cardiotoxin bind to the same site on erythrocytes: iodinated *P* thionin competitively inhibits the hemolytic activity of cardiotoxin from *Naja naja kaouthia*.

The common features of *P* thionin interaction with cells (as with cardiotoxin) include an influx of Ca^{2+} , membrane de-

polarization, and activation of PLA_2 , which leads to membrane alteration and finally to cell death. Iodination of the peptide inhibits all three activities in concert, showing the essential nature of one or both tyrosines in the molecule. Treatment of *P* thionin with staphylococcal V8 protease followed by reduction of the disulfide bonds with mercaptoethanol yields two fragments, each containing one of the two tyrosines found in *P* thionin (41). This fragmentation allows a determination of the iodination sequence in *P* thionin and coupling this information with activity measurements will indicate which of the two tyrosines are essential for the observed biological activities.

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