Purification, Sequence, and Pharmacological Properties of Sea Anemone Toxins from *Radianthus paumotensis*.[1] A New Class of Sea Anemone Toxins Acting on the Sodium Channel†

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ABSTRACT: Four new toxins have been isolated from the sea anemone *Radianthus paumotensis*: Rp_I, Rp_{II}, Rp_{III}, and Rp_{IV}. They are polypeptides comprised of 48 or 49 amino acids; the sequence of Rp_{II} has been determined. Toxicities of these toxins in mice and crabs are similar to those of the other known sea anemone toxins, but they fall into a different immunochemically defined class. The sequence of Rp_{II} shows close similarities with the N-terminal end (up to residue 20) of the previously sequenced long sea anemone toxins, but most of the remaining part of the molecule is completely different. Like the other sea anemone toxins, *Radianthus* toxins are active on sodium channels; they slow down the inactivation process. Through their Na^+ channel action, *Radianthus* toxins stimulate Na^+ influx into tetrodotoxin-sensitive neuroblastoma cells and tetrodotoxin-resistant rat skeletal myoblasts. The efficiency of the toxins is similar in the two cellular systems. In that respect, *Radianthus* toxins behave much more like scorpion neurotoxins than sea anemone toxins from *Anemonia sulcata* or *Anthopleura xanthogrammica*. In binding experiments to synaptosomal Na^+ channels, *Radianthus* toxins compete with toxin II from the scorpion *Androctonus australis* but not with toxins II and V from *Anemonia sulcata*.

The voltage-sensitive Na^+ channel plays a major role in nerve and muscle action potentials. Electrophysiological studies have identified a number of classes of specific neurotoxins that interact with the Na^+ channel at discrete receptor sites and the use of these toxins has permitted detailed elucidation of the structural and functional characteristics of the channel protein. To date, six classes of specific neurotoxins have been described (Catterall, 1980; Lazdunski & Renaud, 1982). (i) Tetrodotoxin (TTX) and saxitoxin (STX) block Na^+ entry through the Na^+ channel (Narahashi, 1974; Ritchie & Rogart, 1977; Ritchie, 1980). (ii) Lipophilic toxins such as veratridine and batrachotoxin stabilize a permanently open form of the Na^+ channel (Ulbricht, 1969; Albuquerque & Daly, 1976). (iii) Pyrethroids, a class of highly active insecticides, prolong the lifetime of the open form of the Na^+ channel (Narahashi, 1976; Jacques et al., 1980; Vijverberg et al., 1982). (iv) Polypeptide toxins from North African and North American scorpions and from sea anemones slow down the inactivation process of the Na^+ channel (Romey et al., 1975, 1976; Bergman et al., 1976). (v) Polypeptide toxins from South and Central American scorpions (Wheeler et al., 1982, 1983; Meves et al., 1982; Jaimovich et al., 1982; Jover et al., 1984) act differently. A very potent representative of this class is toxin γ from *Tityus serrulatus*, which produces repetitive firing in neuroblastoma cells, due to a shift in the voltage dependence of Na^+ activation to more negative potentials (Vijverberg et al., 1984; Barhanin et al., 1984). (vi) Ciguatoxin and brevetoxin depolarize excitable membranes by selectively opening a class of sodium channels (Bidard et al., 1984; Huang et al., 1984).

Sea anemone toxins have been particularly well studied (Bêress et al., 1975; Norton et al., 1976; Romey et al., 1980; Vincent et al., 1980; Barhanin et al., 1981; Schweitz et al., 1981; Frelin et al., 1984). Although they have an amino acid sequence that is completely different from that of scorpion toxins from *Androctonus australis*, *Leiurus quinquestriatus*, and *Buthus eupeus*, they seem to act in a very similar way by prolonging action potentials due to their action in slowing down the inactivation of the Na^+ channel. The affinity of sea anemone toxins for Na^+ channels varies with the animal species, the tissue, and the state of innervation (Frelin et al., 1984). The most active sea anemone toxins have affinities for Na^+ channels in some tissues that correspond to dissociation constants of the toxin–Na^+ channel complex of 0.15–5 nM (Frelin et al., 1984). The purpose of this paper is to report the purification, the immunological properties, the sequence, and the pharmacological properties of a new class of sea anemone toxins extracted from *Radianthus paumotensis*.

Materials and Methods

Sea Anemones. Radianthus paumotensis were collected at Moorea, French Polynesia, shipped in 50% ethanol, and then stored at −20 °C until extraction. Toxins II and V from *Anemonia sulcata* (As_{II} and As_V) and toxins I and II from *Anthopleura xanthogrammica* (Ax_I and Ax_{II}) were prepared

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[1] Variety of *Heteractis magnifica*.

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as previously described (Schweitz et al., 1981).

Toxicity Measurements. Toxicities were measured in both 10–20-g *Carcinus meanas* crabs and 20–30-g Swiss mice as described by Schweitz et al. (1981) and Schweitz (1984).

Purification of Sea Anemone Toxins. Eight kilograms of wet, drained sea anemones was extracted, and the toxins were purified according to the method previously described for other sea anemone toxins (Schweitz et al., 1981) with some modifications. After gel filtration on Sephadex G-50 (Pharmacia), four toxins were separated by chromatography on SP-Sephadex C-25 (Pharmacia) with 100 mM ammonium acetate and a pH gradient from 4.5 to 5.8. They were called after their order of elution, Rp_I, Rp_{II}, Rp_{III} and Rp_{IV}. Rp_I and Rp_{II} were further purified on QAE-Sephadex A-25 (Pharmacia) with 100 mM ammonium acetate at pH 8.5 and 9.0, respectively. Final purification of the four toxins was achieved by high-pressure liquid chromatography (HPLC) (Beckman, Model 332 gradient liquid chromatography and Model 160 absorbance detector) under the conditions described in Figure 1.

For routine amino acid analyses, toxins were hydrolyzed in 6 N HCl for 24, 48, and 76 h at 110 °C in sealed evacuated tubes. Half-cysteine residues were oxidized into cysteic acid with performic acid. Tryptophan residues were obtained from alkaline hydrolyses. Hydrolysates were analyzed in a Beckman 120C automatic amino acid analyzer. Molecular weights of toxins were determined from the results of amino acid analysis according to Delaage (1968).

Radioimmunoassays. Immune serum was prepared from a female New Zealand rabbit (Lessieux, 95710 Bray-Lu, France) as described elsewhere for the bee venom toxin apamin (Schweitz & Lazdunski, 1984). Here, the antigen used was unmodified Rp_{III} (200 μg for each injection).

Toxin III from *R. paumotensis* was iodinated by the peroxidase method (Morrison & Bayse, 1970) and filtered through a Sephadex G-15 column (5 mL), to remove free iodine. The excluded volume contained the [^{125}I]Rp_{III} at a specific radioactivity of 1800 Ci/mmol, 76% of it being precipitable by the Rp_{III} antiserum. The toxicity in mice of this iodinated toxin is about 60% that of the native toxin. The adequate dilution of the antiserum for the radioimmunoassay (1:50000) was determined as described for apamin (Schweitz & Lazdunski, 1984). Competition experiments between [^{125}I]Rp_{III} and different concentrations of unlabeled Rp_{III}, or other unlabeled sea anemone toxins, were also carried out as previously described for apamin (Schweitz & Lazdunski, 1984).

Cell Cultures. Cells from the N1E 115 neuroblastoma clone were grown as previously described (Jacques et al., 1978) and allowed to differentiate for 2 days in a medium containing 1.5% Me_2SO and 1% fetal calf serum (GIBCO). Cells were labeled with L-[^{3}H]leucine (0.2 μCi/mL, Commissariat â l'-Energie Atomique, Saclay, France) for 2 days before the uptake experiments to determine protein recovery by measuring ^{3}H-labeled counts.

Primary cultures of skeletal muscle myoblasts were prepared from newborn rat thigh muscle as previously described (Frelin et al., 1983). Cells were grown in Ham's F_{12} culture medium (GIBCO) supplemented with 10% fetal calf serum, 10% horse serum (GIBCO), and antibiotics and used at the myoblast stage.

$^{22}Na^+$ Uptake Experiments. $^{22}Na^+$ uptake experiments were performed under previously described conditions (Honerjager et al., 1982). The incubation medium contained 0.1 mM veratridine (Sigma), 10 mM Na^+, 0.2 mM ouabain (Sigma), and 2 μCi/mL $^{22}Na^+$ (Amersham). Cell proteins were determined according to Hartree (1972) with bovine serum albumin as a standard.

Electrophysiology. For membrane potential measurements and voltage-clamp experiments, 35-mm culture dishes containing neuroblastoma cells (clone N1E 115), differentiated according to the procedure described by Jacques et al. (1978), were placed on the warm stage on an inverted phase-contrast microscope, and the culture medium was replaced by 2 mL of the appropriate external solution. Measurements were performed by a suction pipette method as previously described by Vijverberg et al. (1984) at a temperature of 30 ± 1 °C unless indicated otherwise.

For membrane potential measurements, the external solution contained 115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, 5 mM glucose, and 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes)/NaOH buffer at pH 7.4. The osmolarity was adjusted to 310 mosM/L with sucrose. The pipette solution contained 10 mM NaOH, 115 mM potassium glutamate, 10 mM Hepes, and the pH was adjusted to 7.1 with L-glutamate. In voltage-clamp experiments the external solution contained 120 mM NaCl, 25 mM tetraethylammonium chloride, 1.8 mM $CaCl_2$, 0.4 mM $MgCl_2$, and 25 mM Hepes/tetramethylammonium hydroxide buffer at pH 7.4. The osmolarity was adjusted to 310 mosM/L with tetramethylammonium chloride. The pipette solution contained 20 mM NaOH, 120 mM CsF, 25 mM Hepes, and L-glutamic acid to adjust the pH at 7.2. The osmolarity of both pipette solutions was adjusted to 290 mosM/L with sucrose. Rp_{III} was applied by adding a small amount (5–20 μL) of concentrated stock solution of the toxin in distilled water to the external solution in the culture dish at a site distant from the cell under investigation.

Binding Experiments. Androctonus australis toxin II (Aa_{II}) was iodinated by the lactoperoxidase–H_2O_2 method as previously described (Morrison & Bayse, 1970). The specific radioactivity of [^{125}I]Aa_{II} was 1200 Ci/mmol. Lactoperoxidase was from Boehringer; Na^{125}I (2000 Ci/mmol) was from Amersham.

Rat brain synaptosomes, prepared according to Abita et al. (1977), were preincubated at 4 °C for 45 min with the different *Radianthus* toxins in the binding medium containing 140 mM choline chloride, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, and 0.1% BSA. Then 7.5×10^{-10} M [^{125}I]Aa_{II} was added to the medium, and the incubation was continued for 60 min. The amount of [^{125}I]Aa_{II} bound was determined by filtering aliquots through Sartorius 0.22-μm filters under reduced pressure. Nonspecific binding was determined in parallel incubations containing 2×10^{-7} M of unlabeled Aa_{II}.

Sequence Determination of Radianthus paumotensis Toxin II. S-Carboxymethylation was performed according to Scheffler et al. (1982) with ^{14}C-labeled iodoacetic acid (1.14 μCi/μmol, New England Nuclear).

(*A*) *Chymotryptic Digestion.* Carboxymethylated (CM) toxin (100 nM) was digested with α-chymotrypsin (50:1 w/w; Worthington Biochemicals) in a 0.1 M pyridine–collidine acetate buffer at pH 8.1 at 37 °C for 16 h. The digest was chromatographed on a SP-Sephadex C-25 column (Pharmacia) equilibrated at pH 2.5 with a 0.1 M pyridine–formate buffer. Elution was performed with a gradient of increasing pH and ionic strength as described in the caption of Figure 1 (Isobe et al., 1977). Elution was monitored by amino acid analysis of aliquots after complete hydolysis and by scintillation counting. Further purification of peptides was carried out by

Table I: Amino Acid Composition of *Radianthus paumotensis* Toxins

amino acid	Rp_I	Rp_{II}	Rp_{III}	Rp_{IV}
Asx	6.02 (6)	6.90 (7)	5.79 (6)	5.04 (5)
Thr	1.84 (2)	4.23 (5)[d]	1.97 (2)	2.00 (2)
Ser	1.95 (2)	2.98 (3)	1.97 (2)	1.95 (2)
Glx	4.19 (4)	2.07 (2)	4.09 (4)	4.18 (4)
Pro	2.70 (3)	1.86 (2)	2.61 (3)	2.58 (3)
Gly	5.62 (6)	2.98 (3)	4.64 (5)	4.85 (5)
Ala	3.03 (3)	4.09 (4)	3.08 (3)	3.33 (3)
Cys	5.82 (6)	4.97 (6)	4.66 (6)	5.27 (6)
Val	2.09 (2)	4.10 (4)	2.01 (2)	2.05 (2)
Met	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Ile	1.01 (1)	0.00 (0)	1.00 (1)	0.94 (1)
Leu	2.07 (2)	0.00 (0)	2.03 (2)	2.03 (2)
Tyr	3.99 (4)	0.93 (1)	4.05 (4)	4.98 (5)
Phe	0.00 (0)	1.98 (2)	0.00 (0)	0.00 (0)
Lys	4.73 (5)	4.96 (5)	4.88 (5)	4.96 (5)
His	0.00 (0)	0.00 (0)	0.00 (0)	0.00 (0)
Arg	2.06 (2)	2.05 (2)	1.99 (2)	2.05 (2)
Trp	1.00 (1)	1.80 (2)	0.98 (1)	0.92 (1)
total[a]	49	48	48	48
M_r[b]	5392	5292	5335	5383
$A^{1\%}_{1cm}$ at 280 nm	20.0	25.2	20.7	22.5
yield[c]	2.4	13.8	15.2	1.8

[a] Total number of amino acids calculated from the nearest integers. [b] Molecular weight calculated from the nearest integers. [c] The yield in pure toxin is given in milligrams of toxin per kilogram of wet sea anemone. [c] Value obtained from the sequence.

gel filtration on Bio-Gel (Bio-Rad Laboratories) P6, P4, or P2 columns (200–400 mesh, 0.9 × 100 cm) using 70% HCOOH according to the size of peptides.

(*B*) *Tryptic and Chymotryptic Digestion.* Only the bond between residues 46 and 47 of CM toxin could be cleaved by trypsin. However, the chymotryptic digests of the CM toxin (from 50 nmol) were further digested with trypsin (25:1 w/w; Worthington Biochemicals) in the same buffer at 37 °C for 8 h. The digest was then chromatographed successfully on SP-Sephadex and on Bio-Gel as described above.

(*C*) *Amino Acid Analysis.* Peptides were hydrolyzed in a mixture of CF_3CO_2H/HCl (1:2 v/v) at 166 °C for 25 and 50 min (Tsugita & Scheffler, 1982). For cystine analysis the peptides were oxidized with performic acid and hydrolyzed (Hirs, 1956). For tryptophan analysis, hydrolysis was made by a microscale modification (Maeda, J. J. Scheffler, and A. Tsugita, unpublished results) of 3 N mercaptoethanesulfonic acid (Penke et al., 1974).

(*D*) *N-Terminal Sequence.* ^{14}C-labeled CM toxin (50 nmol) was sequenced 3 times with a Beckman 890C sequenator according to Scheffler et al. (1982). Manual sequencing of peptides was carried out as described in Schenkman et al. (1984). The thiazolinone derivatives were converted into phenylthiohydantoin amino acids with a mixture of acetyl chloride/methanol (7:1 v/v). The resulting phenylthiohydantoin amino acids were analyzed by high-pressure chromatography on a RP-18 reversed-phase column.

(*E*) *C-Terminal Sequence.* Carboxypeptidases A and/or B (5 μg) (Sigma diisopropyl fluorophosphate treated) was added to 1–2 nmol of the CM toxin or peptide and incubated at 37 °C for various times (Scheffler et al., 1982; Tsugita et al., 1979). The buffer for both digestions was 0.1 N pyridine–collidine acetate at pH 8.3, and the digest was dried and directly analyzed for amino acids.

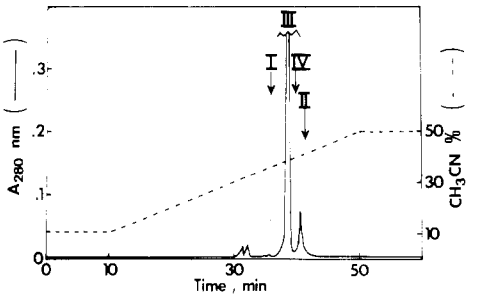

FIGURE 1: Last step of purification of *Radianthus paumotensis* toxins. This step, performed by HPLC, is illustrated with Rp_{III}. The semipreparative column [reverse-phase Lichrosorb RP-18 (Merck) precolumn 4 × 20 mm (40 μm) and 10 × 250 mm (7 μm)] was equilibrated with the following solvents: (A) 50 mM ammonium acetate buffer, pH 6.2, and (B) acetonitrile (B/A, 10/90 v/v). Flow rate was 4 mL/min. Load was 10 μl of Rp_{III} (500 μg) coming from the SP-C25 chromatography. After 10 min of elution with the above mobile phase, a gradient from 10 to 50% of B/(A + B) was applied for 40 min. Absorbance was determined at 280 nm. The toxicity, determined on crabs, was found only in the main absorbance peak, named III, which was used in this work. Arrows show the retention times of the three other *Radianthus paumotensis* toxins (I, II, and IV) obtained from similar chromatographies.

RESULTS

The last step of purification of *Radianthus paumotensis* toxins was performed on RP-18 reverse-phase HPLC. Figure 1 illustrated the purification of Rp_{III}, the most abundant of the four toxins, by this step. This step was also necessary for the final purification of the other three toxins, although two of them, Rp_I and Rp_{II}, were more pure than Rp_{III} prior to this HPLC step because they had been submitted to anion-exchange chromatography on QAE-Sephadex A-25 after the usual cation-exchange chromatography.

The amino acid composition of the four toxins is presented in Table I together with their molecular weight, their absorbance at 280 nm, and the yield of their purification. Amino acid analyses show the purity of the toxins. They are all devoid of three or four amino acids.

The toxic properties of the four toxins were estimated by injection in mice (via intraperitoneal and intracisternal route) and in crabs. Results presented in Table II show that all toxins are highly toxic to mice by the intracisternal route and to crabs. Rp_{II} has a low toxicity when injected intraperitoneally in mice.

Immunochemical cross-reactivity among different sea anemone toxins was analyzed by radioimmunoassay. Figure 2 shows the variation of the ratio of bound to total Rp_{III} in the incubation medium as a function of the concentration of unlabeled toxins. Rp_I and Rp_{IV} have the same affinity as Rp_{III} for Rp_{III} antibodies ($IC_{50} = 4 \times 10^{-10}$ M). Rp_{II} has a lower affinity ($IC_{50} = 2 \times 10^{-9}$ M), and the four other sea anemone

Table II: Toxicities and Biochemical Properties of *Radianthus paumotensis* Toxins

	toxicity			$^{22}Na^+$ uptake by cultured cells		
toxin	LD_{50} ip in mice (μg/kg)	LD_{50} ic in mice (μg/kg)	LD_{50} in crabs (μg/kg)	N1E 115, ED_{50} (μM)	rat skeletal myotubes, ED_{50} (μM)	protection of [^{125}I]Aa_{II} binding to rat brain synaptosomes, $K_{0.5}$ (μM)
Rp_I	145	1.5	36	3.7	4	0.9
Rp_{II}	4200	12	15	30	10	>100
Rp_{III}	53	2.4	10	2.1	2.1	0.3
Rp_{IV}	40	2.0	90	1	0.9	10

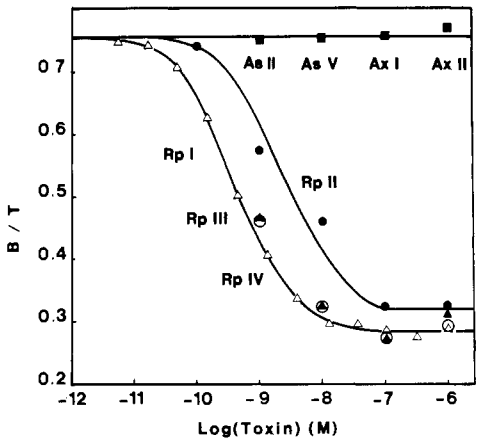

FIGURE 2: Competition between [^{125}]Rp_{III} and different sea anemone toxins for association with Rp_{III} antibodies. The Rp_{III} antiserum was used at a dilution of 1:50 000. (Ordinate) Ratio of bound Rp_{III} (B) (obtained by directly counting radioactivity in the pellets) to total precipitable Rp_{III} of the medium (T) (obtained by precipitation with a large excess of antiserum). (○) Rp_I, (●) Rp_{II}, (△) Rp_{III}, (▲) Rp_{IV}, and (■) As_{II}, As_V, Ax_I, and Ax_{II}.

Table III: Automatic Edman Degradation of the ^{14}C-Labeled Carboxymethylated Toxin (40 nmol)

cycle	residue	yield (nmol)	cycle	residue	yield (nmol)
1	Ala	46.0	15	Ala	34.0
2	Ser	3.5	16	Thr	2.3
3	Cys	^{14}C labeled	17	Phe	21.0
4	Lys	34.2	18	Thr	2.0
5	Cys	^{14}C labeled	19	Gly	8.0
6	Asp	29.4	20	Thr	1.6
7	Asp	36.0	21	Val	20.0
8	Asp	43.2	22	Asp	18.1
9	Gly	37.5	23	Phe	20.0
10	Pro	36.0	24	Asp	16.0
11	Asp	34.0	25	Gly	15.5
12	Val	34.2	26	Glu	14.3
13	Arg	14.9	27	Lys	12.8
14	Ser	2.5			

toxins (As_{II}, As_V, Ax_I, and Ax_{II}) do not recognize antibodies directed against the *Radianthus* toxin.

One of the *Radianthus* toxins, Rp_{II}, was submitted to sequence analysis. Carboxypeptidase A digestion of the CM toxin resulted in the liberation of 2 mol of lysine/mol of toxin. Both carboxypeptidases A and B further released 1 mol of arginine and lysine, and the kinetics showed the C-terminal sequence to be Lys-Arg-Lys-Lys. Repeated automatic Edman degradation from 40 nmol provided the sequence in 27 steps (Table III). ^{14}C-Labeled (carboxymethyl)cysteine was found at positions 3 and 5 (Figure 3).

The ^{14}C-labeled CM toxin was digested with chymotrypsin, and the resulting peptides were separated by ion-exchange chromatography on SP-Sephadex C-25 (Figure 4). Peptide fractions were further purified by gel filtration. Altogether, four major peptides and three minor peptides were recovered; their amino acid compositions are given in Table IV. Each peptide was completely or partially sequenced by manual Edman degradation and carboxypeptidase digestion (Figure 3). The three minor peptides, C-33–39, C-33–42, and C-43–48 were shown to be the result of partial splits of C-33–48 judging from amino acid compositions and partial sequences. Peptide C-1–17 was shown to be the *N*-terminal peptide because of the identity with the N-terminal sequence of the toxin. Peptides C-33–48 and C-43–48 were considered to be the C-terminal peptides because they share a common C-terminal sequence with the intact toxin. Three peptides, C-1–17, C-18–23, and C-24–32, were ordered from the data of the N-terminal sequence of the toxin. There is no direct evidence showing the connection between C-24–32 and C-33–48. However, the amino acid composition of the protein and compositions of the peptides clearly indicate that a direct connection between them exists with no intervening amino acid(s) or peptide(s) (see Table I and Table IV).

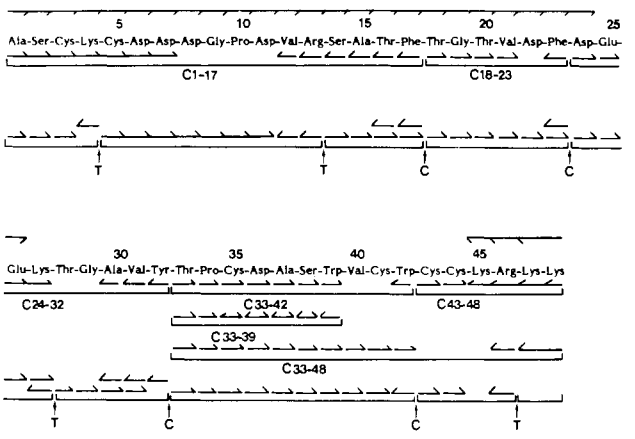

FIGURE 3: Determination of the Rp_{II} sequence showing (i) automatic Edman degradation of the toxin (777), (ii) carboxypeptidase digestion of the toxin (ττττ), (iii) manual Edman degradation of peptides (λλλ), (iv) carboxypeptidase digestion of peptides (LLL), and (v) cleavage sites of the protein with trypsin and chymotrypsin (indicated by T and C, respectively).

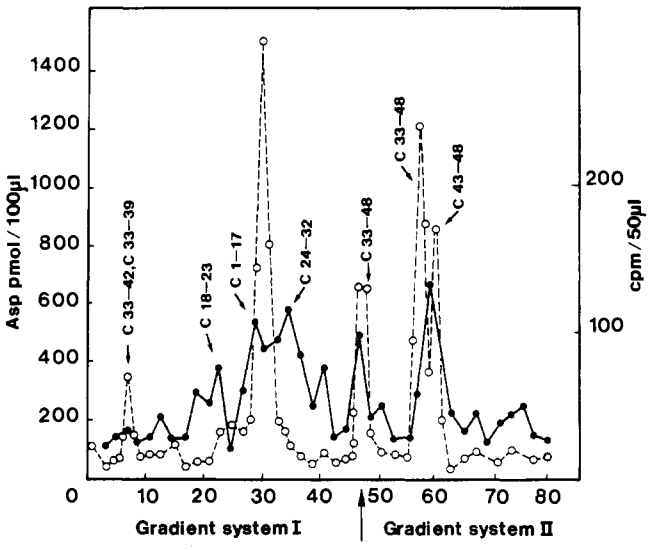

FIGURE 4: Isolation of the chymotryptic peptides of Rp_{II} on SP-Sephadex C-25. The chymotryptic digest (from 100 nM ^{14}C-labeled CM Rp_{II}) was applied to a SP-Sephadex C-25 column (0.6 cm × 10 cm) and eluted with a gradient of pyridine–acetate buffers established with a nine-chamber Varigrad apparatus. The gradient was made with 20 mL of each buffer as follows: chambers 1 and 2, 0.1% (v/v) in pyridine–acetate (pH 3.8); chambers 3 and 4, 0.25% (pH 3.97); chambers 5 and 6, 0.75% (pH 4.25); chamber 7, 1.5% (pH 4.47); chamber 8, 2.5% (pH 4.65); chamber 9, 5% (pH 4.87). The elution was carried out at a flow rate of 8 mL/h at room temperture, and fractions of 1.5 mL were collected. The final elution was carried out with a second gradient [pyridine–acetate, 5% (pH 4.87) to 30% (pH 6.0)]. Eluents were analyzed for radioactivity and for amino acids after complete hydrolysis. (○) cpm in 50 µl; (●) picomoles of Asp in 100 µL.

The toxin was digested with chymotrypsin and then with trypsin in order to confirm the detailed sequences. As shown in Figure 3, nine fragments were isolated; the amino acid compositions and sequences of these fragments covered the entire sequence of the toxin.

The summary of the sequencing procedure is as follows. C-1–17 (2 nmol) was sequenced by manual Edman degra-

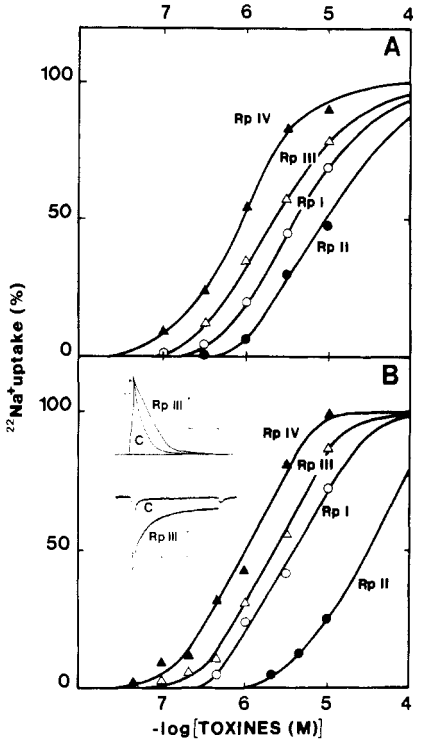

FIGURE 5: Sea anemone toxin induced increase in the rate of $^{22}Na^+$ uptake by rat skeletal myoblasts (A) and N1E 115 neuroblastoma cells (B). Uptake experiments were performed as described under Materials and Methods at 37 °C in the presence of 100 μM veratridine, 10 mM Na^+, and increasing concentrations of sea anemone toxins. Dose–response curves are for Rp_I (○), Rp_{II} (●), Rp_{III} (△), and Rp_{IV} (▲). The toxin-stimulated $^{22}Na^+$ uptakes were fully inhibited by 100 nM tetrodotoxin (N1E 115 cells) or 10 μM TTX (rat muscle cells). The basal rate of $^{22}Na^+$ uptake, measured in the presence of veratridine (100 μM) alone, has been substracted. The ordinate scale has been normalized (%) to the action of 1 μM *Anemonia sulcata* toxin V and 0.1 mM veratridine; i.e., 100% = 40 and 105 nmol min^{-1} mg^{-1} for neuroblastoma cells and muscle myoblasts, respectively. (Inset) (Upper) Action potentials in a neuroblastoma cell elicited by a depolarizing stimulus from a steady hyperpolarized level of −80 mV. The rapid control action potential (*C*) is evoked just before and the slow action potential 6 min after application of 5×10^{-7} M Rp_{III} in the same cell. The arrow indicates the control action potential amplitude, and the dotted line represents the zero potential level. Horizontal calibration was 50 ms; vertical calibration was 20 mV. (Lower) Na^+ currents obtained from a neuroblastoma cell under voltage clamp by depolarizing the membrane to 20 mV for 30 ms following a conditioning hyperpolarization at −110 mV for 100 ms. Holding potential was −70 mV. The rapid control current (*C*) was obtained just before and the slow current 12 min after application of 4.5×10^{-6} M Rp_{III}. Horizontal calibration was 10 ms; vertical calibration was 10 nA.

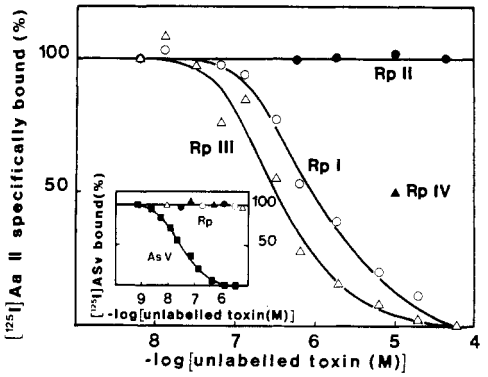

FIGURE 6: (Main panel) Inhibition of $[^{125}I]Aa_{II}$ binding to Na^+ channels in synaptosomes by *Radianthus paumotensis* toxins. Rat brain synaptosomes (0.85 mg of protein/mL) were incubated with different concentrations of Rp_I (○), Rp_{II} (●), Rp_{III} (△), and Rp_{IV} (▲) during 45 min. Then, 7.5×10^{-10} M $[^{125}I]Aa_{II}$ was added and the radioactivity bound to membranes determined as described under Materials and Methods after a further incubation of 60 min. A value of 100% of $[^{125}I]Aa_{II}$ specifically bound corresponds to 29 fmol/mg of protein. (Inset) Competition between $[^{125}I]AS_V$ and unlabeled toxins for binding to synaptosomes. $[^{125}I]AS_V$ (10 nM) was incubated with rat brain synaptosomes (1 mg of protein/mL) in the presence of increasing concentrations of unlabeled AS_V (■), Rp_I (○), Rp_{II} (●), Rp_{III} (△), or Rp_{IV} (▲). Radioactivity bound to synaptosomes was determined after an incubation of 1 h at 4 °C.

dation: 1, Asp (1.9), 2, Ser (0.5), 3. Cys (^{14}C), 4, Lys (1.8), 5, Cys (^{14}C), 6, Asp (1.2), and 7, Asp (0.6), where the numbers in parentheses are in nanomoles and ^{14}C shows ^{14}C count of ^{14}C-labeled (carboxymethyl)cysteine. After the eighth step, data became unclear probably because of the repetition of aspartic acid residues, which may disturb the coupling reaction in the present manual method. The C-terminal sequence Ser-Ala-Thr-Phe was obtained from kinetics of carboxypeptidase A digestion and the additional Val-Arg with carboxypeptidases A and B. Isolation and sequence of the tetrapeptide Ser-Ala-Thr-Phe confirmed the C-terminal sequence. The structure of the nonapeptide Cys-Asp-Asp-Asp-Gly-Pro-Asp-Val-Arg gave the structure of the middle part of C-1–17. C-18–23 was isolated from both the chymotryptic digest and chymotryptic and tryptic digests. The sequence was established by manual Edman degradation of the peptide (1 nmol): 1, Thr (0.8), 2, Gly (0.7), 3, Thr (0.3), 4, Val (1.0), 5, Asp (0.7), and 6, Phe (0.7). Carboxypeptidase A digestion resulted in the liberation of phenylalanine only but quantitatively. C-24–32 (1.1 nmol) was sequenced by manual degradation: 1, Asp (1.1), 2, Glu (0.6), 3, Glu (0.7), and 4, Lys (0.3). The poor yield from the Edman degradation may be due to the presence of Lys and to the hydrophobicity of the peptide (the benzene extraction may wash out the peptide). Two peptides, which compose C-24–32 resulting from tryptic action, were isolated. The complete sequencing of these two peptides provided the complete sequence of C-24–32. C-33–48 (2 nmol) was sequenced by automatic sequencing: 1, Thr (1.4), 2, Pro (1.4) 3, Cys (^{14}C), 4, Asp (1.7), 5, Ala (1.5), 6, Ser (0.5), 7, Trp (1.0), 8, Val (0.9), 9, Cys (^{14}C), and 10, Trp (1.0). Chymotryptic peptides C-33–42, C-33–34, and C-43–48 and two tryptic peptides resulting from C-43–48 were isolated. The compositions and partial sequences of these peptides provided the complete sequence of C-33–48. All these results taken together have provided the final sequence of the toxin.

Action of Radianthus Toxins on the Na^+ Channel. The upper inset of Figure 5 shows that Rp_{III} prolongs the action potential in neuroblastoma cells. Voltage-clamp experiments show that actions of Rp_{III} (lower inset of Figure 5) and of toxin II from *Anemonia sulcata* (Lazdunski et al., 1979) are similar. Both of them increase the Na^+ current amplitude and slow down the inactivation step.

The efficiency of sea anemone toxin and of other polypeptide toxins on the Na^+ channel can be easily evaluated with $^{22}Na^+$ flux studies (Jacques et al., 1978; Schweitz et al., 1981; Frelin et al. 1984). Sea anemone toxins from *Radianthus* produce a marked increase in the rate of $^{22}Na^+$ uptake (measured in the presence of 100 μM veratridine) by rat skeletal myoblasts and N1E 115 neuroblastoma cells (main panel of Figure 5). This increase in the rate of $^{22}Na^+$ uptake through the Na^+ channel is completely abolished by 100 nM TTX in neuroblastoma cells and by 10 μM TTX in rat myoblasts. Dose–response curves presented in Figure 5 have given ED_{50} values that are listed in Table II. The order of potency of the four sea anemone toxins tested is $Rp_{IV} > Rp_{III} > Rp_I > Rp_{II}$. The potency of the toxins (ED_{50}) is very similar in neuronal and muscle cells (Table II).

Table IV: Amino Acid Composition of Peptides Obtained by Chymotryptic Cleavage of *Radianthus paumotensis* II

	C-1–17	C-18–23	C-24–32	C-33–42	C-43–48	C-33–48
Asx	3.9 (4)	1.2 (1)	1.2 (1)	1.0 (1)		0.9 (1)
Thr	1.0 (1)	2.1 (2)	1.3 (1)	1.0 (1)		0.8 (1)
Ser	2.2 (2)		0.3 (0)	0.7 (1)		1.8 (1)
Glx			1.8 (2)			0.2 (0)
Pro	1.0 (1)			1.0 (1)		1.0 (1)
Gly	1.4 (1)	1.4 (1)	1.4 (1)			0.2 (0)
Ala	2.0 (2)	0.1 (0)	1.0 (1)	1.0 (1)		1.0 (1)
[^{14}C]CM-Cys	1.7 (2)			1.5 (2)	1.4 (2)	2.1 (4)
Val	0.8 (1)	0.5 (1)	0.7 (1)	0.8 (1)		0.6 (1)
Tyr			0.8 (1)			
Phe	0.7 (1)	0.8 (1)	0.2 (0)			
Lys	0.9 (1)		0.9 (1)		2.8 (3)	2.8 (3)
Arg	0.7 (1)		0.1 (0)		1.0 (1)	1.1 (1)
Trp				+ (2)		+ (2)
total	(17)	(6)	(9)	(10)	(6)	(16)

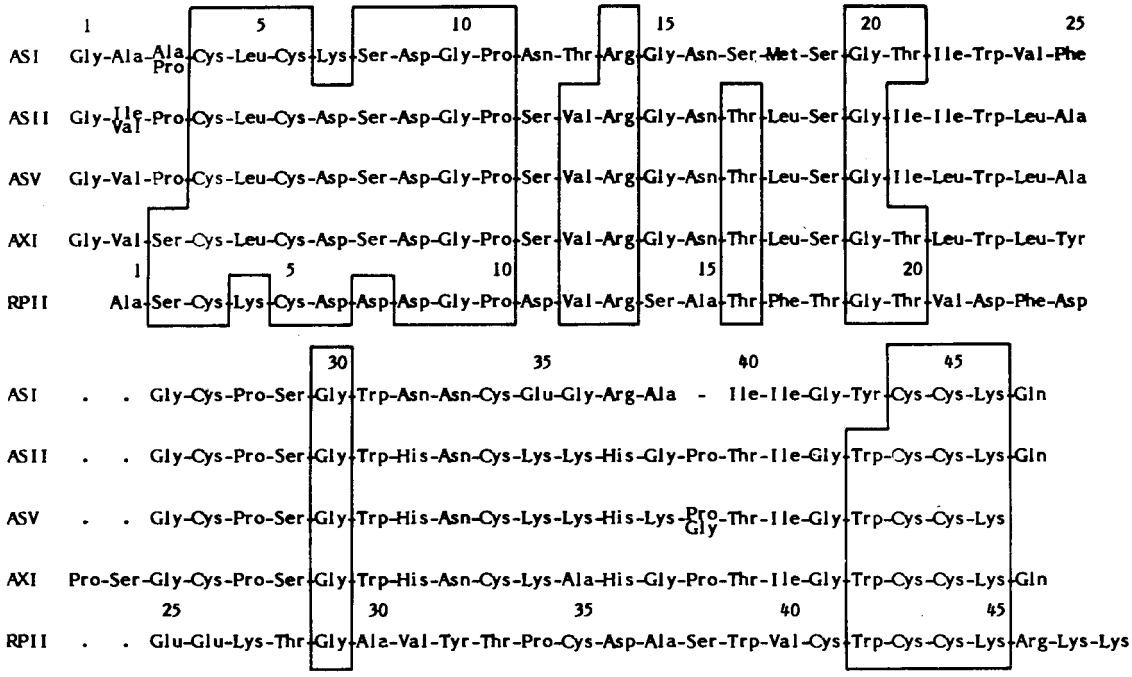

FIGURE 7: Homologies and differences in the amino acid sequences from different sea anemone toxins: toxins As_I, As_{II}, and As_V from *Anemonia sulcata*, toxin Ax_I from *Anthopleura xanthogrammica*, and toxin Rp_{II} from *Radianthus paumotensis*.

The inset of Figure 6 shows that none of the four *Radianthus* toxins has any significant effect on the binding of radioiodinated derivatives of toxins II and V from *A. sulcata* to synaptosomal Na^+ channels. The main panel of Figure 6 shows that among the four *R. paumotensis* toxins only two of them, Rp_I and Rp_{III}, interacted with the receptor site of the scorpion toxin Aa_{II}. Concentrations that give half-maximal inhibition of [^{125}I]Aa_{II} binding to synaptosomes, $K_{0.5}$, are 9 $\times 10^{-7}$ and 3 $\times 10^{-7}$ M for Rp_I and Rp_{III}, respectively (Table II). The other toxins have no (Rp_{II}) or nearly no effect (Rp_{IV}) on [^{125}I]Aa_{II} binding.

Binding to synaptosomes of ^{125}I-labeled *Tityus* γ-toxin, another scorpion toxin specific for the Na^+ channel (Vijverberg et al., 1984; Barhanin et al., 1982), is unaffected by the presence of high concentrations (up to 10 μM) of the different *Radianthus* toxins (not shown).

Discussion

Sea anemone toxins have been very useful tools to analyze the functioning of the Na^+ channel (Narahashi, 1974; Bergman et al., 1976; Romey et al., 1976; Jacques et al., 1978; Low et al., 1979; Vincent et al., 1980; Neumcke et al., 1980; Romey et al., 1980; Schweitz et al., 1981; Ulbricht & Schmidtmayer, 1981; Warashina & Fujita, 1983; Frelin et al., 1984; Erxleben & Rathmayer, 1984). The best known toxins are those extracted from *Anemonia sulcata* (Béress et al., 1975; Schweitz et al., 1981) and from *Anthopleura xanthogrammica* (Norton et al., 1976; Tanaka et al., 1977; Schweitz et al., 1981). This paper describes the purification of four new toxins extracted from the sea anemone *Radianthus paumotensis* that are toxic both to mice and crabs as are the above toxins.

A. sulcata toxins and *A. xanthogrammica* toxins all belong to the same immunological class. Double-diffusion experiments (Ouchterlony) have shown that rabbit antibodies directed against As_{II} and As_V precipitate As_{II}, As_V, Ax_I, and Ax_{II} (not shown). Conversely, *R. paumotensis* toxins form an immunologically distinct class of sea anemone toxins. Rp_I, Rp_{II}, Rp_{III}, and Rp_{IV} all recognize antibodies against Rp_{III} (Figure 2), but none of them are precipitated by antibodies raised against As_{II} or As_V in double-diffusion experiments (not shown). Moreover, none of As_{II}, As_V, Ax_I, and Ax_{II} recognize antibodies raised against Rp_{III} (Figure 2).

The large differences in immunological properties are explained by large differences in sequence. Figure 7 compares the sequence of Rp_{II} with the sequence of other sea anemone toxins known. The sequence of Rp_{II} is clearly atypical, as compared to other sequences. Similarities are observed in the N-terminal sequence up to residue 20. They are also observed

at the C-terminal end: Trp_{42}-Cys-Cys-Lys_{45}. Similarities in the N-terminal structure are particularly interesting since residues that are known to be essential for the activity of As_{II} are Arg-14 and at least one of the Asp residues in positions 7 and 9 (Barhanin et al., 1981). All these residues are conserved in the Rp_{II} sequence. One even observes a cluster of Asp residues in this region of the N-terminal part of the Rp_{II} structure. The sequence Trp(or Tyr)-Cys-Cys-Lys is also the same in all toxins at the extreme C-terminal part of the sequence, which probably means that this region of the molecule is important for the structure and/or function of all sea anemone toxins. The Rp_{II} sequence is atypical due to the presence of a cluster of very positively charged residues in the four last positions (Lys-Arg-Lys-Lys). The rest of the Rp_{II} sequence is very different from that of other sea anemone toxins, and no attempt has been made in Figure 7 to find any particular common feature.

At the present time, and simply in terms of sequence, there appears to be three different classes of sea anemone toxins. The "long" toxins with amino acids that are represented in Figure 7 comprise two classes of toxins, *R. paumotensis* toxins on one hand and As_I, As_{II}, and As_V and Ax_I and Ax_{II} toxins on the other hand. The third class of toxins is that of "short" sea anemone toxins comprising less than 30 amino acids (Bêress et al., 1975; Martinez et al., 1977; Ishikawa et al., 1979), of which the prototype is As_{III} (Martinez et al., 1977).

In spite of their differences in sequence with *A. sulcata* and *A. xanthogrammica*, *Radianthus* toxins are also active on Na^+ channels as shown in Figure 5. Electrophysiological experiments including voltage-clamp data indicate that, like As_{II} (Romey et al., 1976), they slow down the inactivation on the Na^+ channel. By prolonging the time during which the Na^+ channel stays open, these toxins also stimulate $^{22}Na^+$ influx into neuroblastoma cells and rat skeletal muscle cells in culture. The order of potency of the different *Radianthus* toxins is $Rp_{IV} > Rp_{III} > Rp_I > Rp_{II}$; the least active toxin is Rp_{II}, which is also the least toxic in mice. These toxins with ED_{50} between 1 and 30 μM are much less active on Na^+ channels from neuroblastoma cells and from rat myoblasts than toxins from *A. sulcata* and *A. xanthogrammica* for which ED_{50} values range between 0.15 and 150 nM (Frelin et al., 1984). The most striking difference between *Radianthus* toxins and the other class of toxins from *A. sulcata* and *A. xanthogrammica* is that *Radianthus* toxins have nearly the same ED_{50} for Na^+ channels in rat skeletal myotubes and in neuroblastoma cells (Table II). It has been found previously (Frelin et al., 1984) that tetrodotoxin-resistant Na^+ channels ($K_{0.5}$ = 1 μM) that are present in rat skeletal myotubes are much more sensitive (a factor of 10–50) to *A. sulcata* and *A. xanthogrammica* toxins than tetrodotoxin-sensitive Na^+ channels ($K_{0.5}$ = 5 nM) that are present in neuroblastoma cells. Scorpion neurotoxins have been found to have nearly the same affinity for TTX-sensitive and TTX-resistant channels (Frelin et al., 1984). In this respect therefore, *Radianthus* toxins would have a behavior that would more closely resemble that of scorpion toxins, like Aa_{II}. Binding experiments to Na^+ channels in synaptosomes confirm this conclusion. It has been shown previously that although sea anemone toxins (Catterall, 1980; Vincent et al., 1980) prevent the binding of [^{125}I]Aa_{II} to Na^+ channels in synaptosomes, the reverse situation is not true: Aa_{II} does not prevent the binding of [^{125}I]As_V to Na^+ channels in synaptosomes. Figure 6 clearly shows that *Radianthus* toxins prevent the binding of [^{125}I]Aa_{II} to synaptosomes but they do not prevent the binding of [^{125}I]As_V.

In conclusion, among sea anemone toxins, the class of *Radianthus* toxins has different immunological properties and a different type of sequence. Although these toxins like other sea anemone toxins (Romey et al., 1976) and like some scorpion toxins (Romey et al., 1975; Meves et al., 1984) clearly act on the Na^+ channel by slowing down the inactivation step, they behave in that respect much more like scorpion neurotoxins than sea anemone toxins from *A. sulcata* or *A. xanthogrammica*.

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Registry No. Sodium, 7440-23-5; toxin II (*Radianthus paumotensis*), 96427-20-2.

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