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High Affinity Binding of Pyrethroids to the α Subunit of Brain Sodium Channels

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SUMMARY

Na^+ channels are the primary molecular targets of the pyrethroid insecticides. Na^+ channels consisting of only a type IIA α subunit expressed in Chinese hamster ovary cells responded to pyrethroid treatment in a normal manner: a sustained Na^+ current was induced progressively after each depolarizing pulse in a train of stimuli, and this Na^+ current decayed slowly on repolarization. These modified Na^+ channels could be reactivated at much more negative membrane potentials ($V_{0.5}$ = −139 mV) than unmodified Na^+ channels ($V_{0.5}$ = −28 mV). These results indicate that pyrethroids can modify the functional properties of the Na^+ channel α subunit expressed alone by blocking their inactivation, shifting their voltage dependence of activation, and slowing their deactivation. To demonstrate directly the specific interaction of pyrethroids with the α subunit of voltage-gated Na^+ channels, a radioactive photosensitive derivative, [^{3}H]RU58487, was used in binding and photolabeling studies. In the presence of a low concentration of the nonionic detergent Triton X-100, specific pyrethroid binding to Na^+ channels in rat brain membrane preparations could be measured and reached 75% of total binding under optimal conditions. Binding approached equilibrium within 1 hr at 4°, dissociated with a half-time of ~10 min, and had K_D values of ~58–300 nM for three representative pyrethroids. Specific pyrethroid binding was enhanced by ~40% in the presence of 100 nM α-scorpion toxin, but no allosteric enhancement was observed in the presence of toxins acting at other Na^+ channel receptor sites. Extensive membrane washing increased specific binding to 89%. Photolabeling with [^{3}H]RU58487 under these optimal binding conditions revealed a radiolabeled band with an apparent molecular mass of 240 kDa corresponding to the Na^+ channel α subunit. Anti-peptide antibodies recognizing sequences within the α subunit were able to specifically immunoprecipitate the covalently modified channel. Together, these results demonstrate that the pyrethroids can modify the properties of cells expressing only the α subunit of Na^+ channels and can bind specifically to a receptor site on the α subunit.

Pyrethroids are synthetic insecticidal compounds that resemble pyrethrins, natural toxins that are contained in the flowers of *Chrysanthemum* sp. Although the biological effects of pyrethroids include inhibitory effects on nicotinic acetylcholine receptors, γ-aminobutyric acid receptors, Ca^{2+}/Mg^{2+}-ATPases, and voltage-gated Ca^{2+} channels, the primary cause of toxicity is their stimulatory effect on the voltage-gated Na^+ channels of insects and vertebrates (for reviews, see Refs. 1 and 2). Pyrethroids such as deltamethrin and tetramethrin shift activation to more negative potentials and inhibit inactivation, resulting in persistent activation of Na^+ channels at the resting membrane potential. Because of this persistent activation of Na^+ channels, pyrethroids enhance steady state $^{22}Na^+$ influx through Na^+ channels present in cultured insect and mammalian neuronal cells (3). Binding studies that measure the ability of pyrethroids to displace radiolabeled neurotoxin derivatives from their specific Na^+ channel receptor sites indicate that pyrethroids do not act at sites previously defined for other Na^+ channel toxins (3). This has led to the suggestion that pyrethroids may act at a novel receptor site on the Na^+ channel protein.

Voltage-gated Na^+ channels from rat brain are complexes composed of three glycoprotein subunits: a 260-kDa α subunit that is covalently linked to a 33-kDa β2 subunit and noncovalently interacting with a 36-kDa β1 subunit (for a review, see Ref. 4). Neurotoxins have been shown to interact with at least five distinct receptor sites on the Na^+ channel, four of which are present on the α subunit. Although these multiple neurotoxin receptor sites on Na^+ channels are topologically distinct, there are strong allosteric interactions

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ABBREVIATIONS: LqTx, α-scorpion toxin from *Leiurus quinquestriatus*; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

among them. This allostery is demonstrated by the pyrethroids deltamethrin and cypermethrin and the insecticide dichlorodiphenyl-trichloroethane (chlorphenothane; DDT), each of which has been shown to increase the binding affinity of batrachotoxin, acting at Na^+ channel receptor site 2 (5). Similar synergy has been demonstrated among a synthetic pyrethroid (RU39568), sea anemone toxin II acting at receptor site 3, and *Ptychodiscus brevis* toxin-2, acting at receptor site 5, which together enhance binding of batrachotoxin to synaptosomes ~100-fold (3). The combination of RU39568 and *P. brevis* toxin-1 causes a 1000-fold enhancement of batrachotoxin binding to purified Na^+ channels reconstituted in phospholipid vesicles (6).

To provide direct evidence of the interaction of pyrethroids with the subunits of the Na^+ channel and to identify the pyrethroid receptor site, conditions must be developed for the detection of specific binding of radiolabeled pyrethroids to Na^+ channels. However, even the most active tritiated pyrethroid derivatives used in previous binding studies have exhibited a major component of nonspecific binding to rat brain synaptosomes and insect neuronal membranes that prevented the detection of specific binding (3). In the current study, we show that high affinity pyrethroids can modify the gating of Na^+ channels consisting of only an α subunit. Specific binding of the photosensitive, radioactive pyrethroid [^{3}H]RU58487 to rat brain synaptosomes is observed in the presence of the nonionic detergent Triton X-100, and specific covalent labeling of the α subunit of the Na^+ channels is detected under these conditions. Our results show that the functional pyrethroid receptor site is located on the α subunit of the Na^+ channels, and we provide the first direct detection of this new receptor site.

Experimental Procedures

Materials. Ecolume and Solvable were from DuPont-New England Nuclear (Boston, MA). Phosphatidyl choline and phosphatidyl ethanolamine used for reconstitution were from Avanti Polar Lipids (Birmingham, AL). Prestained molecular weight marker proteins were from Life Technologies (Grand Island, NY). Triton X-100 and glycerol formal were from Sigma Chemical (St. Louis, MO). α-Scorpion toxin LqTx V was purified from the venom of *Leiurus quinquestriatus quinquestriatus* (Latoxan, Rosans, France) (7, 8).

Electrophysiological Studies. Electrophysiological studies were performed on cultured Chinese hamster ovary cells expressing the wild-type rat brain IIA α subunit (9). Whole-cell patch-clamp recording was carried out on cells in 200 μl of bath solution (9) with the application of 20 μl of RU39568 stock in glycerol formal to yield final concentrations of 1–10 μM.

Synaptosome binding experiments. Synaptosomes were prepared from the brains of Sprague-Dawley rats according to the method described by Dodd *et al.* (10) and stored at −70° until use. They were diluted to give a final concentration of 100 μg/ml in standard binding medium (130 mM choline chloride, 50 mM HEPES, adjusted to pH 7.4 with Tris base, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, with no added bovine serum albumin; Ref. 11) containing 0.05% Triton X-100. [^{3}H]RU58487 was added to the above solution at a final concentration of 1–20 nM in the presence or absence of 100 nM LqTx to give a final volume of 1 ml. Some samples were incubated with 20 μM RU51049 for the determination of nonspecific binding. In competitive displacement assays, we used serial dilutions of RU39568 or RU51049 as an unlabeled competitor of [^{3}H]RU58487 for its specific binding site. Experimental samples were incubated at 4° for 1 hr in the dark, collected on glass-fiber filters (GF/C; Whatman, Fairfield, NJ) under vacuum, and washed three times with wash medium (12). Samples prepared for immunoprecipitation were collected by centrifugation in microcentrifuge tubes.

Photolabeling of purified Na^+ channels. Rat brain Na^+ channels were purified and reconstituted into phosphatidyl choline/phosphatidyl ethanolamine vesicles as described previously (13, 14). Reconstituted Na^+ channels or synaptosomes were incubated with [^{3}H]RU58487 and toxins as described above at 4° for 1 hr in the dark and irradiated at 4° for 30 min using a germicidal lamp (λ_{max} = 254 nm; UVP, Inc, Upland, CA) that was placed 5 cm from the sample on a rotating shaker.

Sequence-directed antibodies. Polyclonal antisera were raised in rabbits against synthetic Na^+ channel peptides (SP) corresponding to the sequences of the rat brain type IIA α subunit (15, 16) with lysine and tyrosine residues added to the amino terminus. Peptide synthesis, peptide coupling to bovine serum albumin, and antibody production have been described previously (17–19). The antibodies used in this study were directed against sequences within three of the four Na^+ channel α subunit domains: SP13, KY-PDCDPEKDH-PGSSVKGDCGN (1729–1748); SP19, K-TEEQKKYYNA-n-KKLG-SKK (1491–1508); and SP20, KY-PIALGESDFENLNTEEFSSE (1106–1126) (n is norleucine).

Preparation of protein A-Sepharose-bound antibodies. Protein A-Sepharose was swollen for 20 min at room temperature in 0.1 M sodium phosphate, pH 8.1, to give a final concentration of 100 mg/ml. For each 100 μl of swollen protein A-Sepharose, 75 μl of antiserum was added, and both reagents were mixed by rotation for 1 hr at room temperature or at 4° overnight. Supernatants were removed, and the pellets were washed five times with 10 volumes of buffer S (10 mM Tris, adjusted to pH 7.4 with HCl, 150 mM NaCl, 1 mM EDTA). The pellet was resuspended in 1 volume of buffer S and used for immunoprecipitation of photolabeled Na^+ channels.

Immunoprecipitation of photolabeled Na^+ channels from synaptosomes. Binding was performed as described above. Before photolabeling, synaptosomes were washed three times by centrifugation and resuspended in 1 ml of wash medium containing 0.05% Triton X-100 and 1 mg/ml bovine serum albumin. After photolabeling, SDS was added to each reaction tube to give a final concentration of 0.1%. Samples were incubated for 10 min at 37° and cooled to room temperature. A final concentration of 1% Triton X-100, 150 mM NaCl, and 1 mg/ml globulin-free BSA was added and incubated for 10 min at 4°. The samples were mixed by rotation with protein A-Sepharose-bound antibody overnight at 4°. Supernatants were removed, and the pellets were washed two times with 5 volumes of buffer S. The proteins were solubilized from the pellet by incubation with 8% SDS and analyzed by scintillation spectroscopy.

SDS-PAGE and gel slicing. For analysis of [^{3}H]RU58487 covalently bound to purified and reconstituted Na^+ channels, a 7% porous reducing gel system was used according to Doucet *et al.* (20). Prestained molecular mass standards were used to determine the molecular mass of the protein of interest. To determine protein-bound radioactivity, individual gel lanes were manually cut into 3-mm slices, and radioactivity was eluted in 5% (v/v) Soluene in Ecolume according to the manufacturer's instructions.

Results

Electrophysiological effects of RU39568. The functional effects of RU39568 (see Fig. 1 for structures) were tested electrophysiologically in cultured Chinese hamster ovary cells expressing the wild-type rat brain IIA Na^+ channel α subunit (CNaIIA-1 cells; Ref. 9). In an untreated cell, Na^+ currents activate rapidly in response to a depolarization to 0 mV and then inactivate almost completely within 2 msec (Fig. 2A). After exposure to 1 μM RU39568 for 6 min at −90 mV with infrequent depolarizations, there was a small decrease in peak current. However, in response to a 10-Hz train of pulses to the same potential (Fig. 2B), peak current was

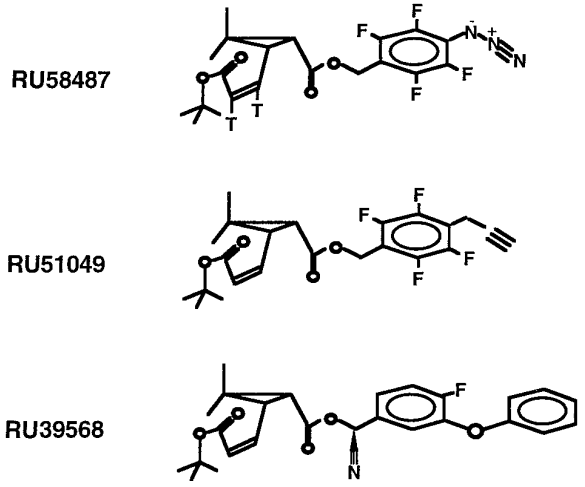

Fig. 1. Structures of the pyrethroids RU51049 and RU39568 and the photoactivatable, ^3H-labeled derivative, RU58487. *T*, Position of ^3H.

decreased further and a noninactivating component of Na^+ current progressively developed. In addition, a fraction of channels closed extremely slowly after repolarization, resulting in a pulse-wise increase in inward Na^+ current at the holding potential (Fig. 2B). The voltage dependence of activation of the Na^+ conductance during depolarizing pulses was unaffected in the presence of RU39568 (Fig. 2C), which is consistent with the conclusion that only unmodified Na^+ channels open rapidly and contribute to the peak Na^+ current. Thus, during repetitive depolarizing pulses, Na^+ channels bind the pyrethroid and are progressively converted from channels that activate rapidly on depolarization to channels that are open persistently at the holding potential.

The sustained Na^+ currents observed after repolarization to the holding potential (i.e., tail currents) must be the result of RU39568-modified Na^+ channels that fail to close because they are absent in control recordings. The increment in magnitude of these sustained tail currents after each pulse therefore indicates the number of channels that became modified during the preceding depolarization. To quantify sustained tail currents, Na^+ currents were measured at 200 μsec after the end of depolarizations of different size and duration. The magnitude of the sustained tail current increased steeply as a function of the depolarization potential (Fig. 3A) and as a function of conditioning pulse duration (Fig. 3B). Thus, the number of RU39568-modified channels open at the end of depolarizing pulses increased with the extent and duration of Na^+ channel activation.

Because the slowly decaying tail currents were entirely due to RU39568-modified channels, it was possible to measure the voltage dependence of closing of these modified channels. A population of RU39568-modified Na^+ channels was created by applying a 10-Hz train of depolarizations (Fig. 2B). After the final pulse of the train, the membrane potential was set to a variable potential to determine the voltage dependence of channel closing. Examples of normalized tail currents observed using such a protocol are shown in Fig. 4A. The magnitudes of such normalized tail currents were measured 400 msec after repolarization (Fig. 4A, *arrow*) to the indicated potential and plotted as a function of repolarization potential (Fig. 4B). The fitted curve describing the isochronal

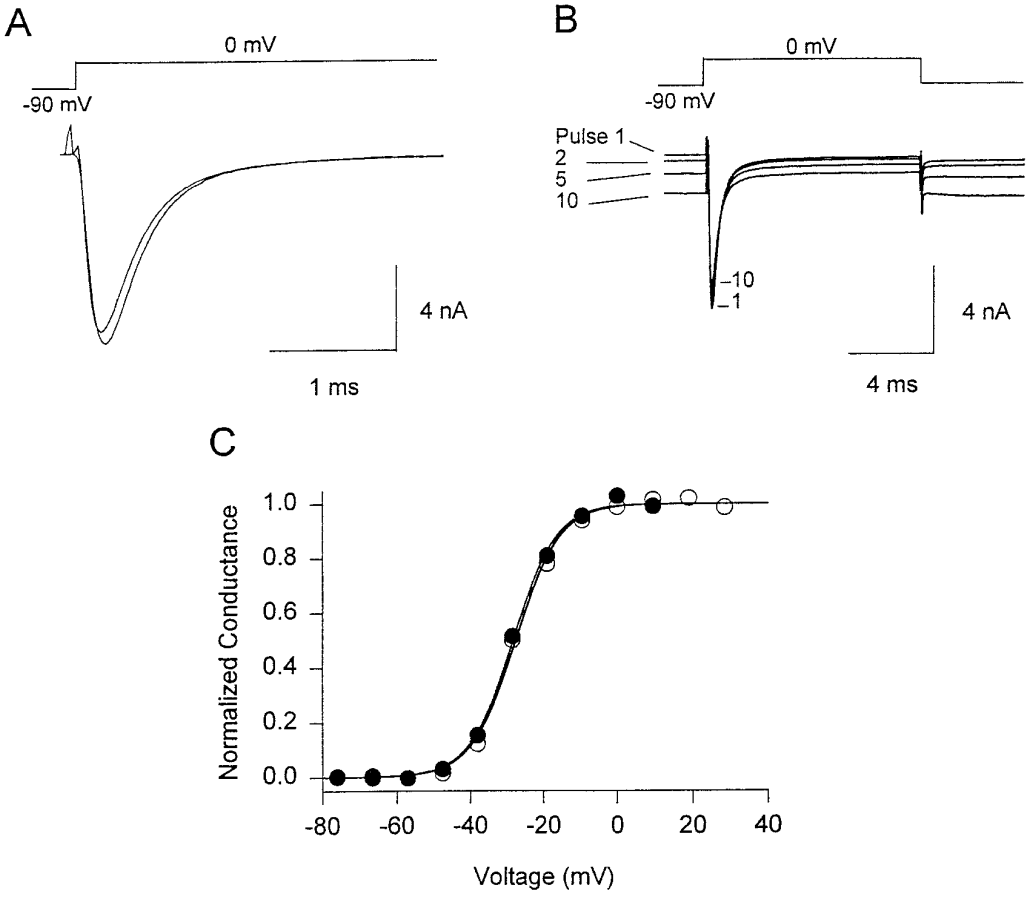

Fig. 2. Response of Na^+ currents to RU39568. A, Sodium currents in response to a pulse to 0 mV from a holding potential of −90 mV were recorded in control (*large trace*) and 6 min after the introduction of 1 μM RU39568 into the bath (*small trace*). B, Current traces recorded in response to the 1st, 2nd, 5th, and 10th pulse of a 50-Hz train of 10-msec-long pulses. Changes in current at the holding potential and at the peak in response to each pulse of the train are indicated. C, Conductance-voltage relationships determined from the peak Na^+ currents measured during pulses to the indicated potentials in control solutions (○) and in the presence of 10 μM RU39568 (●). Conductance was calculated as Peak $I/(V - V_{rev})$, where V_{rev} is the reversal potential. The fitted curves were made on the basis of $1/\{1 + \exp[(V - V_{0.5})/k]\}$, where $V_{0.5}$ is the half-activation voltage and *k* is a steepness factor. $V_{0.5}$ was −27.6 mV for control and −28.3 mV for RU39568.

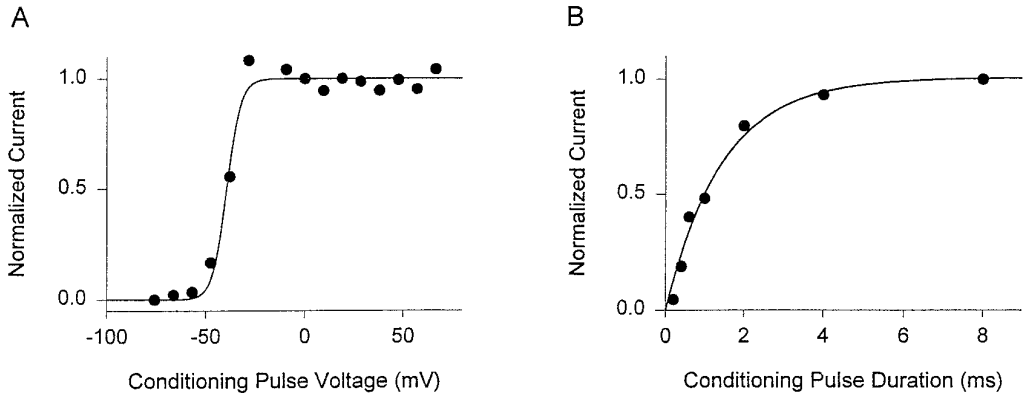

Fig. 3. Effects of the magnitude and duration of the depolarization on the number of RU39568-modified channels. A, Effect of conditioning pulse voltage on the number of RU39568-modified channels. Tail current magnitude in the presence of 10 μM RU39568 was measured 200 μsec after return to the holding potential of −90 mV after 10-msec-long pulses to the indicated potential. B, Effect of conditioning pulse duration on the number of RU39568-modified channels. Tail current magnitudes were measured as described above after pulses to 0 mV of the indicated durations.

voltage dependence of closure of pyrethroid-modified channels has a voltage for half-maximal closure of −139 mV (Fig. 4B), indicating the requirement for a very negative membrane potential to rapidly close modified channels. In contrast, the voltage for half-maximal opening (or closure) of unmodified type IIA Na^+ channels was −28 mV (Fig. 2C). This isochronal measurement does not represent the true steady state voltage dependence because channel closure did not reach steady state during these 400-msec repolarizing pulses. For example, channels closed nearly completely at −100 mV but >30 sec was required. Thus, RU39568-modified channels undergo voltage-dependent closure that is extremely slow, and the apparent voltage dependence is shifted to strongly negative potentials in comparison to unmodified channels. Once closed, RU39568-modified channels cannot be reopened by depolarizations from −160 mV to potentials negative to the normal resting potential (data not shown). Therefore, it seems likely that toxin molecules must leave their binding sites before the channels can close and that once closed, the channels revert to unmodified gating properties. This behavior is consistent with effects of other pyrethroids that prevent channels from inactivating during depolarizations and from closing on repolarization (for a review, see Ref. 2). Because CNaIIA-1 cells express only the α subunit of the Na^+ channel, these effects of RU39568 must reflect modification of the function of the Na^+ channel α subunit.

Specific binding of the pyrethroid [^{3}H]RU58487 to synaptosomes. The studies of pyrethroid action described above implicate the α subunit of the Na^+ channel as the target of pyrethroid action. To directly demonstrate specific pyrethroid binding to the α subunit, we measured binding of the radiolabeled photoreactive pyrethroid [^{3}H]RU58487 to Na^+ channels in synaptosomes. Pyrethroid binding to Na^+ channel preparations has been difficult to study because their hydrophobicity results in high nonspecific binding relative to specific binding. We found that this problem can be circumvented using the nonionic detergent Triton X-100 to keep both the hydrophobic pyrethroids and the rat brain membrane preparations in solution. No specific binding of [^{3}H]RU58487 is observed in the absence of detergent (not shown) or when 0.01% Triton X-100 is used in the binding medium (Fig. 5). In fact, nonspecific binding in the presence of unlabeled RU51049 exceeds "total" binding in its absence, probably because of coprecipitation of the two hydrophobic pyrethroids in the absence of detergent. In contrast, specific binding reaches a maximum of 75% of total binding when 0.05% Triton X-100 is included in the binding medium (Fig. 5). This likely results from the ability of this detergent to maintain the pyrethroid in solution and to prevent substan-

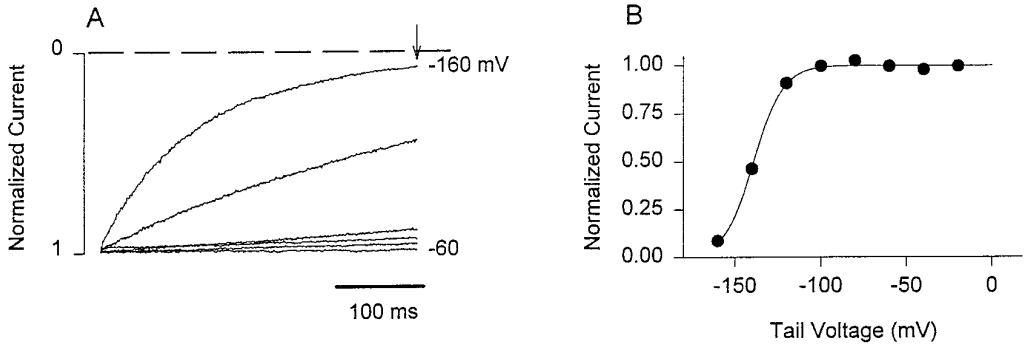

Fig. 4. Voltage dependence of closing of RU39568-modified channels. A, Normalized tail currents were measured at voltages ranging from −160 to −40 mV in 20-mV increments after a 10-Hz train of 10-msec-long pulses to 0 mV from a holding potential of −90 mV. The absolute magnitude of the tail currents varied due to the open channel conductance properties of the modified channels at the tail voltage. B, The voltage dependence of RU39568-modified channels. The magnitude of the tail current was measured 400 msec after stepping to the tail potential. *Line*, fit of $1/\{1 + \exp[(V - V_{0.5})/k]\}$, with the half-activation voltage, $V_{0.5}$, = −139 mV and the steepness, k = −8.5 mV.

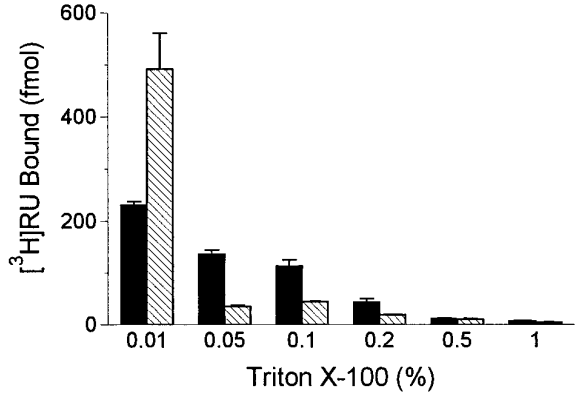

Fig. 5. Effect of Triton X-100 concentration on specific [^{3}H]RU58487 binding. [^{3}H]RU58487 binding was measured as described under "Experimental Procedures" at the Triton X-100 concentrations shown in the presence (▨) and absence (■) of 20 μM RU51049.

tial partitioning of the ligand into the hydrophobic membrane. At this low detergent concentration, the Na^+ channel is not solubilized, so the binding activity remains in the membrane fraction. At higher detergent concentrations, progressively less specific binding is observed in the membrane fraction (Fig. 5), probably because of solubilization of the Na^+ channels. Therefore, binding of [^{3}H]RU58487 to synaptosome preparations in the presence of 0.05% Triton X-100 was used for further characterization of the pyrethroid receptor site.

Kinetics of pyrethroid binding to Na^+ channels. [^{3}H]RU58487 binding to synaptosomes approaches equilibrium after ~1 hr at 4° (Fig. 6A). The calculated observed rate constant (k_{obs}) was 0.075 min^{-1}. Taking into account the [^{3}H]RU58487 concentration of 6 nM, an association rate constant (k_{+1}) of 0.0011 min^{-1} nM^{-1} can be calculated. The dissociation of [^{3}H]RU58487 from synaptosomes is complete after 20 min (Fig. 6B). An estimated dissociation rate constant (k_{-1}) of 0.064 min^{-1} can be calculated from the half-time for dissociation. Using the equation $K_D = k_{-1}/k_{+1}$, a K_D value of 58 nM for RU58487 is estimated from these kinetic data. The small amount of RU58487 available did not allow a study of saturation binding with this ligand.

Competitive inhibition of specific binding of [^{3}H]RU58487 by saturation of the pyrethroid receptor site with unlabeled RU51049 or RU39568 occurs over a broad range of concentrations, with half-maximal inhibition at ~300 nM for these two pyrethroids (Fig. 7). Both unlabeled competitors displaced a similar fraction of the radioactive pyrethroid from its specific binding site, as expected for the binding of all three compounds to a common pool of high affinity pyrethroid receptor sites. The broad competition curve may result from the difficulties of maintaining the pyrethroids in solution at higher concentrations, even in the presence of 0.05% Triton X-100.

Allosteric effects of other neurotoxins. Because several pyrethroids have been shown to enhance binding of both batrachotoxin and brevetoxin to their specific receptor sites on the Na^+ channel α subunit (3, 6), we tested the reciprocal ability of these neurotoxins to allosterically enhance pyrethroid binding. In the presence of 100 nM LqTx, which acts at Na^+ channel receptor site 3, maximal enhancement of specific pyrethroid binding above control levels is ~40% (Fig. 6A). No enhancement of pyrethroid binding is observed in the presence of veratridine or brevetoxin, which act at Na^+ channel receptor sites 2 and 5, respectively (data not shown). Evidently, only binding of neurotoxins at receptor site 3 leads to allosteric modulation of pyrethroid binding under the conditions of our experiments.

Reduction of nonspecific binding. Elevated nonspecific binding is a common problem encountered when working with hydrophobic photoaffinity labels due to the presence of a highly reactive photosensitive group that can covalently react with the lipid bilayer. Nonspecific binding of this hydrophobic photoaffinity probe was reduced by the use of BSA, a scavenger protein that provides the photoaffinity derivative with numerous sites for nonspecific attachment without interfering with high affinity binding and photoreaction with specific binding sites. Four washes with 1% BSA by dilution and centrifugation resulted in a reduction of nonspecific binding and a substantial increase in specific binding to 89% of total binding (Fig. 8). All subsequent immunoprecipitation experiments with the photoaffinity label used four washes with BSA before irradiation and precipitation to maximize specific labeling of the receptor protein.

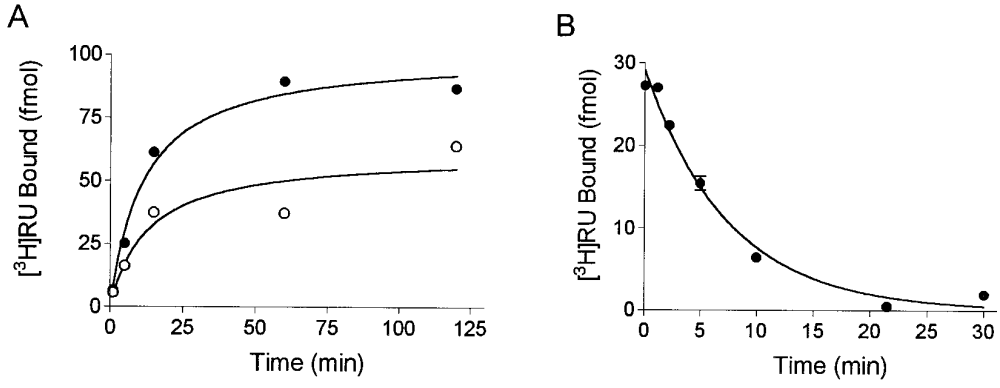

Fig. 6. Time course of [^{3}H]RU58487 association and dissociation from rat brain synaptosomes. A, Association of [^{3}H]RU58487 binding was measured as described in Experimental Procedures in the presence (●) and absence (○) of 100 nM LqTx. At each time point, nonspecific binding was measured in parallel samples incubated in the presence of 20 μM RU51049. *Data points*, specific binding values determined as the difference between total and nonspecific binding. B, [^{3}H]RU58487 binding was measured as described in Experimental Procedures for 1 hr at 4°. After this time, a final concentration of 100 μM RU51049 in one half of the initial volume of binding buffer was added, and the solution was mixed gently. Samples were filtered at the designated times.

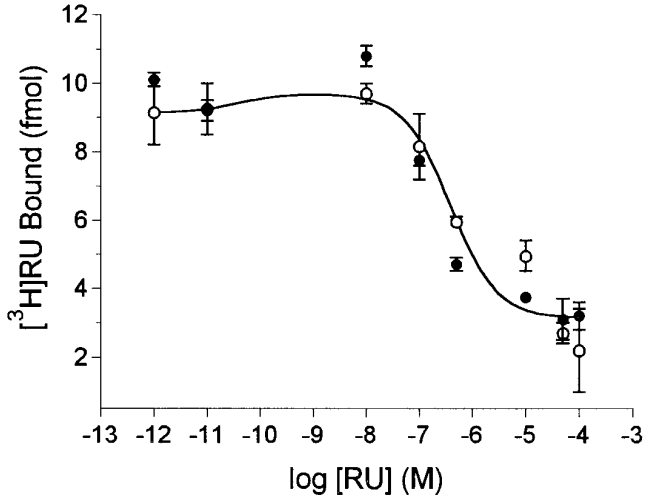

Fig. 7. Competitive displacement of [^{3}H]RU58487 bound to rat brain synaptosomes by unlabeled pyrethroids. [^{3}H]RU58487 binding was measured as described in Experimental Procedures in the presence of increasing concentrations of RU39568 (●) or RU51049 (○).

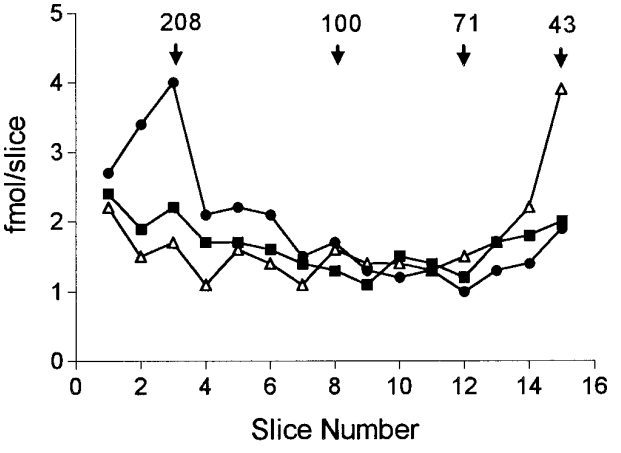

Fig. 9. SDS-PAGE analysis of the pyrethroid receptor covalently labeled with [^{3}H]RU58487. Purified and reconstituted Na^+ channels were photolabeled with [^{3}H]RU58487 in the presence (■) or absence (●) of 20 μM RU51049 and then analyzed by SDS-PAGE as described in Experimental Procedures. [^{3}H]RU58487 alone ran as a large band on the bottom of the gel (△).

Specific photolabeling of Na^+ channels by [^{3}H] RU58487. An analysis of specifically labeled, reconstituted Na^+ channels by SDS-PAGE, gel slicing, and scintillation counting revealed a single broad band of covalently incorporated [^{3}H]RU58487 with an apparent molecular mass of 240 kDa (Fig. 9, ●). In contrast, samples labeled in the presence of an excess of unlabeled RU51049 did not contain any [^{3}H]RU58487 covalently attached to protein (Fig. 9, ■). [^{3}H]RU58487, in the absence of Na^+ channel protein, ran on SDS-PAGE as a broad peak that spread to the electrophoretic dye front (Fig. 9, △).

To demonstrate that this peak of specific radioactivity detected on SDS-PAGE gels is indeed the Na^+ channel α subunit, sequence-directed anti-peptide antibodies recognizing different regions of the α subunit were used to immunoprecipitate Na^+ channels solubilized from a specifically photolabeled synaptosome preparation. These antibodies, which recognize sequences within three of the four Na^+ channel α subunit domains, immunoprecipitate significantly more of the [^{3}H]RU58487-labeled protein than does the preimmune serum control (Fig. 10). These results further confirm that the 240-kDa protein labeled by [^{3}H]RU58487 is the α subunit of the Na^+ channel.

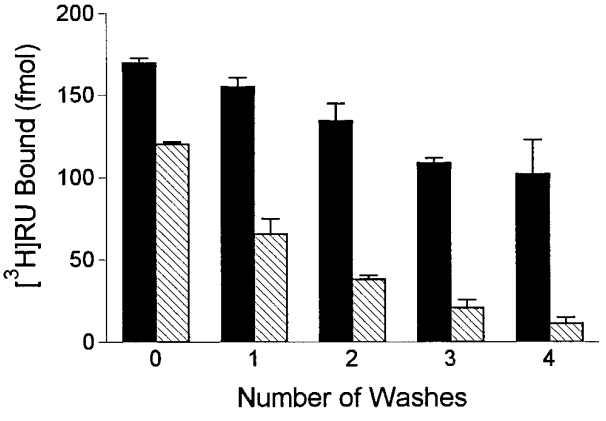

Fig. 8. Reduction in nonspecific binding by centrifugation and resuspension in wash solution containing 1% BSA. [^{3}H]RU58487 binding was measured as described in Experimental Procedures in the presence (▨) or absence (■) of 20 μM RU51049. Samples were incubated for 1 hr at 4° in microcentrifuge tubes and then centrifuged at 14,000 × *g* for 3 min. Supernatants were aspirated and resuspended in 1 ml wash medium containing 1% BSA. The centrifugation and pellet resuspension were repeated up to four times. Pellets were assayed for radioactivity by scintillation spectroscopy.

Discussion

Functional effects of pyrethroids on the Na^+ channel α subunit. Pyrethroids cause repetitive firing of nerve cells. Much of this activity results from their actions on voltage-dependent Na^+ channels (1). Pyrethroids act on voltage-dependent Na^+ channels by decreasing peak conductance and preventing inactivation during depolarizations. After repolarization, pyrethroid-modified channels close extremely slowly, resulting in the steady flow of depolarizing current at potentials where the channels are normally closed. Modification of Na^+ channels is strongly enhanced by depolarization, presumably reflecting rapid and high affinity

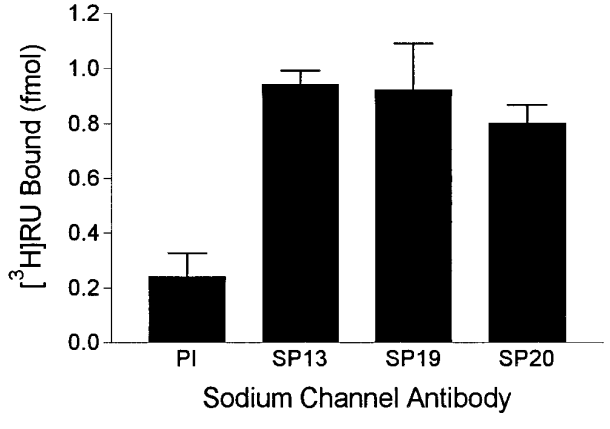

Fig. 10. Immunoprecipitation of the covalently labeled pyrethroid receptor. Rat brain synaptosomes covalently labeled with [^{3}H]RU58487 were immunoprecipitated as described in Experimental Procedures. Three specific Na^+ channel antibodies, designated anti-SP13, anti-SP19, and anti-SP20, and a control preimmune serum (*PI*), were used.

binding to the activated state of the channel (for a review, see Ref. 2). All of these effects of pyrethroids were observed in CNaIIA-1 cells expressing only the type IIA rat brain Na^+ channel α subunit, demonstrating that the functional effects of pyrethroids require only the Na^+ channel α subunit.

Pyrethroid binding to the Na^+ channel α subunit. Receptors for different classes of toxins acting on the Na^+ channel have been characterized using tritiated or iodinated toxin derivatives in radioligand binding assays and have been covalently labeled by photoreactive toxin derivatives. Similar biochemical identification of the pyrethroid receptor site has not been possible due to the prohibitively high levels of nonspecific binding measured in assays using radiolabeled pyrethroids (21, 22). In the current study, we describe the use of the nonionic detergent Triton X-100 for measurement of pyrethroid specific binding. In the presence of this detergent, binding equilibrium was reached within 1 hr at 4°, and K_D values in the range of 58 nM for RU58487 and 100 nM for the unlabeled pyrethroids RU39568 and RU51049 were measured. These results provide the first direct detection of a high affinity pyrethroid receptor site on the Na^+ channel.

The optimal binding conditions that we developed also allowed detection of reproducible and specific covalent labeling of the Na^+ channel α subunit by the pyrethroid [^{3}H]RU58487. This radiolabeled, photoreactive pyrethroid derivative is incorporated specifically into the rat brain Na^+ channel α subunit when it is photoactivated while bound to the pyrethroid receptor site. Anti-peptide antibodies recognizing amino acid sequences in three of the four Na^+ channel α subunit domains are each able to specifically immunoprecipitate the labeled channel. These results demonstrate that pyrethroids bind specifically with high affinity to the α subunit of the voltage-gated Na^+ channel.

Allosteric interaction of pyrethroids with α-scorpion toxin. Insecticides are known to stabilize the Na^+ channel open state by slowing or preventing the transition to a closed state. These insecticide-modified channels result in enhanced binding of alkaloid toxin activators, suggesting allosteric coupling of the pyrethroid and alkaloid toxin receptor sites. However, a reciprocal allosteric effect of alkaloid-activated channels on pyrethroid binding was not observed in our experiments. In contrast, an allosteric interaction is observed between the α-scorpion binding site 3 and the site of pyrethroid interaction under the conditions of our experiments. These results indicate that pyrethroids may bind to a region of the Na^+ channel that is sensitive to conformational changes allosterically induced by α-scorpion toxin acting at receptor site 3 but not by toxins binding at sites 2 and 5.

Mapping of the pyrethroid binding site. There is evidence of species-specific differences in the membrane-depolarizing properties of pyrethroid insecticides (23), some of which are also toxic to mammals. A comparison of insect and mammalian Na^+ channels may give insight into the regions of the channel primary structure that are responsible for the differing sensitivities of mammals and insects to a variety of pyrethroids. The radioligand binding and covalent labeling methods developed in this study may provide the first steps toward molecular mapping of the pyrethroid binding site on the Na^+ channel.

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