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MODIFICATION BY PYRETHROIDS AND DDT OF PHOSPHORYLATION ACTIVITIES OF RAT BRAIN SODIUM CHANNEL

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Abstract—The effects of pyrethroids and DDT on the α-subunit protein of the rat brain sodium channel were studied by using both native and exogenously added cAMP-dependent protein kinases. For this purpose, the sodium channel was partially purified, using the method of Hartshorne and Catterall [*J Biol Chem* **259**: 1667–1675, 1984], and ^{32}P-phosphorylated using [γ-^{32}P]ATP and exogenously added catalytic subunit of cAMP-dependent protein kinase. By comparing the phosphorylation patterns of the isolated sodium channel to those of the partially purified or unpurified (i.e. intact synaptosomes) preparations, it was concluded that the α-subunit of the voltage-sensitive sodium channel protein is the only phosphorylatable protein present at the 260 kD molecular weight range on the sodium dodecyl sulfate–polyacrylamide gel electrophoretogram. Phosphorylation of the α-subunit was induced by depolarization, and this process was inhibited by 10^{-6} to 10^{-10} M 1*R*-deltamethrin, but not by 1*S*-deltamethrin, the latter being an inactive enantiomer of the former. DDT produced a similar effect, but only at a higher concentration range. By using lysed synaptosomal membranes, it was possible to study the direct effects of these compounds on the α-subunit, which were similar to those produced by depolarization of intact synaptosomes.

Voltage-sensitive sodium channels are known to be solely responsible for the changes in sodium conductance during the generation of the action potential in all excitable membranes (for review, see Refs 1–3). In recent years, there have been remarkable advances in the biochemical understanding of various sodium channels following the initial isolation and identification of [^{3}H]saxitoxin binding proteins [2, 4–6]. The central subunit, α-subunit, is found in various tissues from all phyla studied thus far and is regarded as the channel proper [2]. There appear to be at least three, and probably as many as six, major binding sites within the α-subunit for various pharmacologically active drugs, naturally occurring venoms, and toxins that are known to affect sodium channels. It has been shown by Costa *et al.* [7] and Costa and Catterall [8] that the rat brain synaptosomal sodium channels are phosphorylated by protein kinases during depolarization and that such activities are influenced by agents known to affect sodium channel operations.

Pyrethroids are one of the most potent groups of insecticidal chemicals marketed successfully [9]. One of their major sites of action appears to be the sodium channel [10–12], where these chemicals cause prolonged opening of the ion gates. That DDT acts in a similar manner on the same channel has been pointed out (see, for example, Refs 13 and 14). Their similarities are pronounced, particularly on the sensory neurons where characteristic "repetitive discharges" are produced by both groups of chemicals.

Meanwhile, it has been shown by our research team that DDT and some pyrethroids affect membrane phosphorylation activities initiated by the addition of [γ-^{32}P]ATP [15, 16]. In view of their potent actions on the sodium channel and protein kinases, we examined their effects on the phosphorylation processes of the α-subunit protein. We found this protein to be especially sensitive to the influence of this group of chemicals with regard to its phosphorylation–dephosphorylation processes.

MATERIALS AND METHODS

Chemicals. 8-Bromo-cyclic-AMP (8-Br-cAMP), veratridine, cAMP, A23187, and cAMP-dependent protein kinase (holoenzyme and catalytic subunit) were obtained from the Sigma Chemical Co. (St Louis, MO). [γ-^{32}P]Adenosine triphosphate (3000 Ci/mmol) was purchased from Amersham. Sea anemone toxin II (ATX-II) was from the Calbiochem Co. DEAE-Sephadex and WGA-Sepharose CL-4B were products of Pharmacia Inc. Deltamethrin (1R-deltamethrin) and its inactive enantiomer (1*S*-deltamethrin) were gifts from ROUSSEL UCLAF. All other chemicals were commercial products of the highest purity available.

Preparation of synaptosomal fraction (P_2). The P_2 fraction was obtained using basically the method of Gray and Whittaker [17]. The rat brain was homogenized in a Teflon–glass homogenizer rotated at 1000 rpm in 20 ml of 0.3 M sucrose, containing 5 mM sodium phosphate buffer, pH 7.4, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 1000 *g* for 10 min using a Sorvall centrifuge with an SS-34 rotor. The super-

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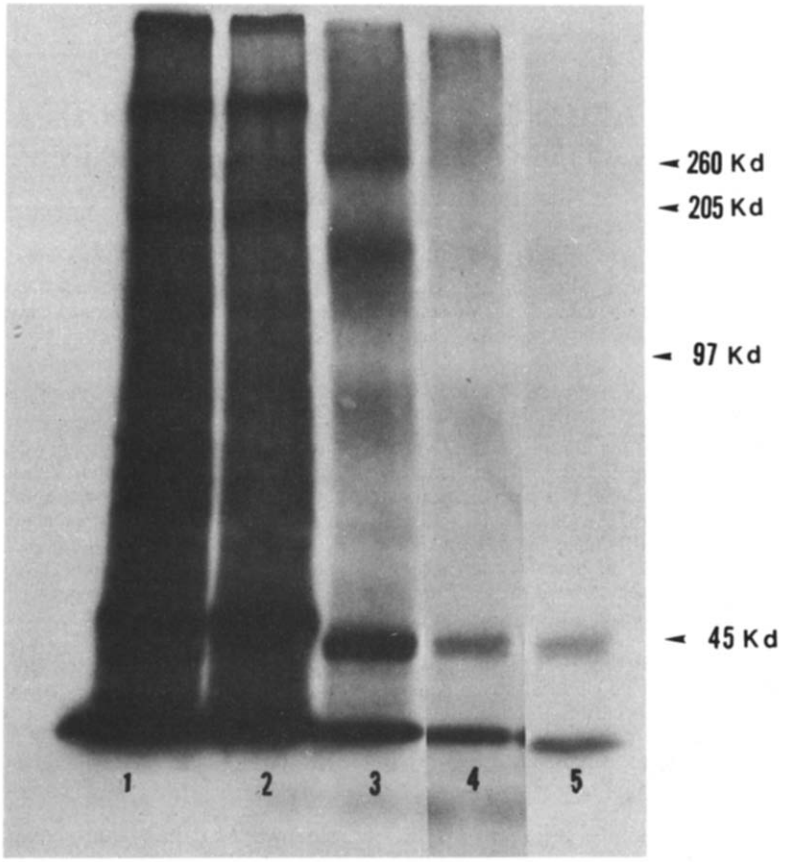

Fig. 1. Radioautogram of 5% SDS–polyacrylamide gel electrophoresis (PAGE) of partially purified sodium channel labeled with [γ-^{32}P]ATP and the catalytic subunit of cAMP-dependent protein kinase. Lane 1: intact synaptosomes treated in a manner identical to lane 2, but without 8-Br-cAMP; lane 2: intact synaptosomes first treated with 8-Br-cAMP, lysed using 0.1% SDS, diluted with 1% Triton X-100, and ^{32}P-phosphorylated using cAMP-dependent protein kinase and [γ-^{32}P]ATP; lane 3: ^{32}P-phosphorylated sodium channel purified from lysed membrane according to Hartshorne and Catterall [1] up to the wheat germ affinity column step and ^{32}P-phosphorylated using catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP; lane 4: same as for lane 3 but treated with 1 μM 1*R*-deltamethrin (84.4% inhibition of phosphorylation on the sodium channel); and lane 5: same as for lane 3 but treated with 0.1 mM veratridine (97.8% inhibition).

natant fraction was saved, and the pellet was resuspended in the same volume of buffer, rehomogenized, and centrifuged at 1000 *g* for 10 min. The two supernatant fractions were combined and centrifuged at 15,000 *g* for 30 min. The pellet was resuspended in 8 ml of high Na^+ buffer (140 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose, 20 mM Hepes*–Tris, pH 7.4). When the effect of Ca^2 concentration was tested, 3 mM $CaCl_2$ was added with 0.5 mM ethyleneglycol*bis* (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA).

Phosphorylation by endogenous ATP and protein kinases. Intact P_2 fraction in high Na^+ buffer was first incubated at 30° for 30 min to equilibrate the phosphorylation–dephosphorylation process within the synaptosomes. Either 250 μl of high Na^+ buffer (for the non-depolarization condition) or 250 μl of high K^+ buffer (for the depolarization condition, 5 mM NaCl, 140 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20 mM Hepes/Tris, pH 7.4) was placed into a microcentrifuged tube (1.5 ml), and chemicals to be tested were dissolved in this buffer. Next, 250 μl of P_2 fraction in high Na^+ buffer was added to the tube to start the reaction and, after different incubation times (15 sec to 5 min) at room temperature (25°), 50 μl of 1% SDS (final concn 0.1%) was added; the mixture was vortexed vigorously and boiled immediately for 2 min to stop all enzymatic reactions. For 8-Br-cAMP tests, P_2 fraction, which had been preincubated as above, was incubated with this cyclic nucleotide analog at 30° for an additional 10 min. After cooling, samples were centrifuged at 16,000 *g* for 5 min, and 500 μl of the supernatant fraction was taken out and used for partial purification on DEAE-Sephadex and/or WGA-Sepharose column chromatography. For the application of this sample to WGA-Sepharose columns, Triton X-100 was added to a final concentration of 1% and then diluted ten times with buffer prior to application.

* Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and SDS, sodium dodecyl sulfate.

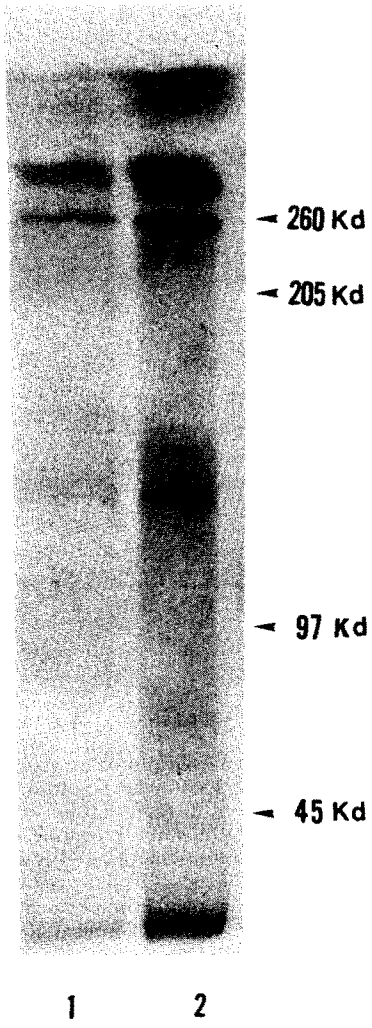

Fig. 2. Radioautogram of SDS–PAGE of ^{125}I-labeled sea anemone toxin (^{125}I-labeled ATX-II) bound to synaptic membrane proteins. After binding, ^{125}I-labeled ATX-II was chemically immobilized using disuccinimidyl suberate as a crosslinking agent. Lane 1: lysed synaptic membrane proteins partially purified using a Sephadex G-150 column after solubilization; and lane 2: solubilized lysed synaptic membranes.

DEAE-Sephadex chromatography. DEAE-Sephadex columns (0.5 ml) were made in 1-ml tuberculin syringe barrels and equilibrated with buffer A (100 mM KCl, 20 mM histidine/HCl, pH 6.5, 0.1% Triton X-100). Samples were mixed with the same volume of 50 mM histidine/HCl, pH 6.5, applied to the columns, washed with 10 ml of buffer A, and eluted with 2 ml of 400 mM KCl in buffer A. The eluate was either concentrated by ultrafiltration with a Centricon 30 (Amicon Co., mol. wt cutoff 30,000 kD) according to the method provided by the company, or applied to the WGA-Sepharose column directly.

WGA-Sepharose column chromatography. WGA-Sepharose (0.3 ml) was packed in a 1-ml tuberculin syringe barrel, equilibrated, and washed with 30 ml of 0.15 M NaCl, 0.1% Triton X-100, 50 mM Hepes–Tris buffer, pH 7.4. Samples were loaded and washed with 20 ml of the same buffer, and then eluted with 2 ml of 100 mM *N*-acetylglucosamine in the same buffer. Eluate was concentrated by ultrafiltration with a Centricon 30 and rephosphorylated as described below.

Rephosphorylation by exogenous cAMP-dependent protein kinase. To the concentrate from the Centricon 30 (*ca.* 40 μl) was added 10 μl of 100 μM cAMP, 50 mM $MgCl_2$, and a 30-μl solution of cAMP-dependent protein kinase (PKA, 15 μg). A 20-μl aliquot of [γ-^{32}P]ATP solution (7 μCi in distilled water) was added to start phosphorylation, and the mixture was incubated at 30° for 10 min. The reaction was stopped by the addition of 80 μl of an electrophoresis sample treatment buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% mercaptoethanol, with bromphenol blue) and boiled for 2 min. The sample was applied directly to the wells of the electrophoresis gel without further treatment.

Total purification of the α-subunit. For verification of the α-subunit protein phosphorylation, total purification steps were carried out exactly as described by Hartshorne and Catterall [1] up to the step of WGA-Sepharose column chromatography. The final purified fraction was concentrated using a Centricon 30, phosphorylated, and analyzed by electrophoresis. [^3H]Saxitonin binding tests were conducted by using the method employed in Ref. 1.

SDS–Polyacrylamide gel electrophoresis. Samples were resolved on a discontinuous electrophoresis system by the method of Laemmli [18] using a 5% separating gel and a 3% stacking gel. The gels were stained with Coomassie brilliant blue R-250, destained, dried over thick filter paper, and exposed to Kodak X-omat AR-5 film.

Radioiodination of sea anemone toxin II (ATX-II). ATX-II from *Anemonia sulcata* was radioiodinated as follows: toxin (10 μg in 100 μl of 20 mM sodium phosphate buffer, pH 7.0) and an IODO-BEAD (Pierce Co.), which had been prewashed with the phosphate buffer, were placed in a microcentrifuge tube and 1 mCi $Na^{125}I$ was added. After a 20-min incubation at room temperature, the liquid was transferred into a dialysis bag (mol. wt cut off 1000, Spectra-Por 7), and dialysed against 20 mM sodium phosphate buffer, pH 7.0 for 2 days with four changes of the buffer. The specific activity obtained was 1.6 Ci/mmol.

ATX-II coupling. ^{125}I-Labeled ATX-II was covalently coupled to the sodium channel using disuccinimidyl suberate (DSS) as a crosslinker according to the method of Barhanin *et al.* [19] and Vincent *et al.* [20]. Synaptosomes (1.0 mg/ml, 200 μl) were prepared in Na^+-free buffer with 0.1% bovine serum albumin (BSA) (140 mM choline chloride, 5 mM KCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20 mM Hepes–Tris, pH 7.4, 0.1% BSA), and incubated with 300,000 cpm ^{125}I-labelled ATX-II and 100 μM veratridine for 40 min on ice. Synaptosomes were sedimented by centrifugation (30 sec, 16,000 *g*) washed once in the Na^+-free buffer without BSA, and resuspended at the initial concentration. DSS (7 mM in dimethyl sulfoxide) was added to a final concentration of 0.07 mM, and the mixture was incubated for 15 min on ice. Glycine (20 mM) was added

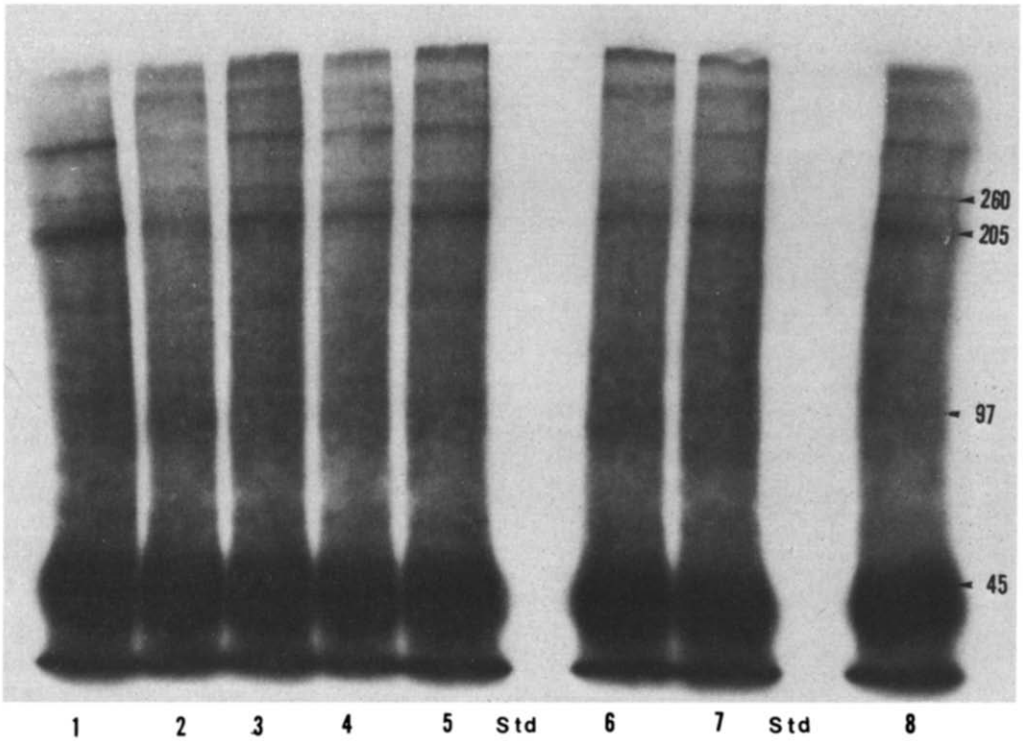

Fig. 3. Radioautogram of SDS–PAGE of labeled phosphoproteins from intact synaptosomes treated with various agents *in situ*. After the treatments the synaptosomes were dissolved by heating with an SDS-containing solution, cooled, diluted using Triton X-100 to block actions of SDS, labeled with [γ-^{32}P]ATP and cAMP-dependent protein kinases, and analysed on SDS–PAGE. Lane 1: control, no treatment; lane 2: treated with 8-bromo-cAMP; lane 3: treated with 10^{-4} M veratridine; lane 4: 15-sec treatment with A23187; lanes 5–8: treated with high K^+ for (5) 15 sec, (6) 30 sec, (7) 1 min, and (8) 5 min. Note that, in this mode of labeling, ^{32}P-phosphorylation of the sodium channel is reduced, since it is already phosphorylated with the endogenous protein kinases *in situ* before the ^{32}P-phosphorylation treatment. Std is where a molecular weight standard protein mixture was placed.

to neutralize excess crosslinker, and the synaptosomes were sedimented by centrifugation and washed twice in the Na^+-free buffer.

^{32}P-*Phosphorylation of lysed synaptic membranes.* The effects of pesticides on the phosphorylation process in lysed synaptic membrane were studied by incubating 500 μl of membrane suspension containing 1.7 mg protein in 5 mM Tris–HCl buffer (with 1 mM EDTA, 1 mM EGTA and 0.1 mM PMSF) with a pesticide (added directly to each glass test tube already containing the membrane with 1 μl ethanol) for 5 min at 4°. [γ-^{32}P]ATP (6 μCi/tube, final concn 6.7 mM) was added and the mixture was incubated for 1 min at the same temperature; the reaction was stopped using 50 μl of 1% SDS and boiling for 1 min. After cooling to 0°, 55 μl of 10% Lubrol PX was added; the system was incubated for 30 min to further solubilize the membranes and then centrifuged at 16,000 *g* for 5 min; 500 μl of the supernatant fraction was used for the electrophoresis experiment (see Fig. 5). The method to quantify the level of total protein phosphorylation, using trichloroacetic acid, was essentially identical to the one published previously [16].

RESULTS

To verify the identity of the phosphorylated sodium channel we first repeated the experiment of Costa *et al.* [7]. For this purpose the rat brain synaptosomes were treated in a manner identical to that described by Hartshorne and Catterall [1] up to the WGA-Sepharose chromatography step. The resulting, partially purified, sodium channel was phosphorylated using [γ-^{32}P]ATP in the presence and the absence of exogenously added catalytic subunit of cAMP-dependent protein kinase (PKA). The results of the electrophoresis and the radioautographic experiments indicate that there was only one ^{32}P-labeled band with molecular weight corresponding to 260 kD in the lane (Fig. 1).

To ascertain that this phosphoprotein is the sodium channel, the intact synaptosomes were first reacted with ^{125}I-labeled ATX-II toxin, chemically crosslinked, and solubilized; the resulting soluble proteins were purified on a Sephadex column and analyzed by SDS–polyacrylamide gel electrophoresis. The ^{125}I-labeled ATX-II linked protein was detected using radioautography. The ^{125}I-labeled band position was found to coincide with the ^{32}P-labeled sodium channel (Fig. 2).

The effects of depolarization of the freshly prepared intact synaptosomes on the level of phosphorylation of the sodium channel were studied next. This was done by first depolarizing the synaptosomal

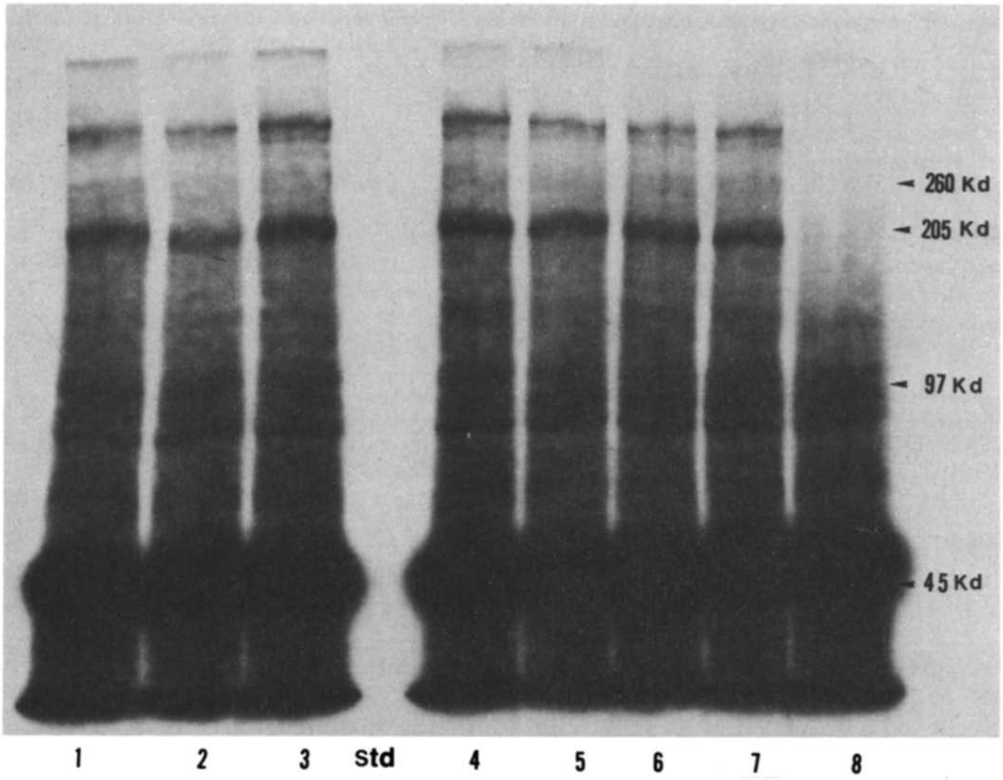

Fig. 4. Radioautogram of SDS–PAGE of phosphoproteins from intact synaptosomes treated with 1*R*- and 1*S*-deltamethrin (see Fig. 3 and Table 1 for additional explanation). Lane 1: control; lane 2: treated with 8-Br-cAMP; lane 3–7: treated with high K^+ for 30 sec in the presence of (3) 10^{-10} M, (4) 10^{-9} M, (5) 10^{-8} M, (6) 10^{-7} M, and (7) 10^{-6} M 1*R*-deltamethrin; lane 8: treated with high K^+ for 30 sec in the presence of 1*S*-deltamethrin.

membrane using high K^+ or veratridine, stopping with SDS and, after dilution with Triton X-100 to reduce the protein-denaturing effects of SDS, reacting with [γ-^{32}P]ATP, cAMP and PKA as described by Costa and Catterall [8]. In this approach the proteins that were not phosphorylated at the time of reaction with endogenous protein kinases appear as the ^{32}P-labeled band, while those phosphorylated by the endogenous kinases during depolarization do not go through ^{32}P-phosphorylation. As can be seen in Fig. 3, the phosphoprotein band at 260 kD disappeared during depolarization induced by high K^+, as expected. Such an effect of depolarization was maximal 15 sec after the additional high K^+ (Table 1). This same phenomenon was reproduced, as expected, when the synaptosomes were pretreated with 8-Br-cAMP to stimulate the endogenous protein kinase. The effect of veratridine was intermediate. This compound, however, is known to both depolarize and inhibit phosphorylation. A23187, a known calcium ionophore, also caused the disappearance of this band.

In the next experiment, we examined the effects of 1*R*-deltamethrin and its inactive enantiomer, 1*S*-deltamethrin, on sodium channel phosphorylation processes that are triggered by depolarization (Fig. 4). The results indicate that the expected, high K^+-induced disappearance of the 260 kD band at 30 sec took place in the synaptosomes treated with 1*S*-deltamethrin, but not in those treated with 1*R*-deltamethrin. The same experiment was repeated, and the results were analysed using densitometry on the autoradiograms (Table 2). The results clearly confirmed the above visual observation. Thus, it may be concluded that 10^{-10} to 10^{-6} M 1*R*-deltamethrin has the ability to inhibit the process of depolarization-coupled phosphorylation of the sodium channel.

In the next series of experiments, lysed synaptosomal membranes were used for direct ^{32}P-phosphorylation with [γ-^{32}P]ATP using endogenous protein kinases. In this mode the highest phosphorylation activities are expressed as the darkest band appearing in the radioautogram (Fig. 5). Veratridine (10^{-4} M) was the most potent inhibitor of this phosphorylation process followed by DDT (10^{-5} M) and 1*R*-deltamethrin (10^{-6} M). Surprisingly, DDE (10^{-5} M) showed some inhibitory properties. However, side-by-side comparisons revealed that DDT was a relatively more potent inhibitor than DDE at 10^{-5} M.

To quantify the extent of total protein phosphorylation, 1/20 of the above ^{32}P-phosphorylation products was taken out of each tube, and proteins were precipitated with trichloroacetic acid, washed and radioassayed. The total ^{32}P-labeling was 1980, 2296, 2504, 1820, and 1896 dpm/tube, respectively, for veratridine-treated, 1*R*-deltamethrin-treated, control, DDT-treated and DDE-treated membrane

Table 1. Densitometric readings of the band intensities of the ^{32}P-phosphorylated sodium channel from intact synaptosomes

Lane no. as in Fig. 3	Depolarization treatments	Relative peak area in each lane (lane 1 = 100)	Relative peak area at 260 kD (lane 1 = 100)	% Radioactivity found at 260 kD (each lane as 100)
1	Control (non-depolarized)	100	2.93 ± 0.12	2.93 ± 0.12
2	8-Bromo-cAMP	78.2 ± 0.1	1.33 ± 0.75	1.70 ± 0.95
3	Veratridine	121.8 ± 46.7	1.78 ± 0.08	1.67 ± 0.57
4	A23187	94.1 ± 14.8	1.65 ± 0.16	1.83 ± 0.46
5	High K^+, 15 sec	95.1 ± 11.7	2.65 ± 0.42	2.38 ± 0.27
6	High K^+, 30 sec	93.6 ± 16.2	3.71 ± 1.34	3.14 ± 0.07
7	High K^+, 60 sec	69.7 ± 12.3	3.32 ± 0.78	4.39 ± 2.5
8	High K^+, 300 sec	81.0 ± 12.8	3.48 ± 0.28	4.35 ± 1.15

Synaptosomes were treated with various depolarizing conditions as shown in the legend of Fig. 3. The proteins were phosphorylated (nonradioactive) by endogenous protein kinases. After stopping the reaction, remaining unphosphorylated proteins were phosphorylated using [γ-^{32}P]ATP and exogenously added protein kinase A. Each value is the average (±SD) of four readings of two radioautograms from two independent experiments.

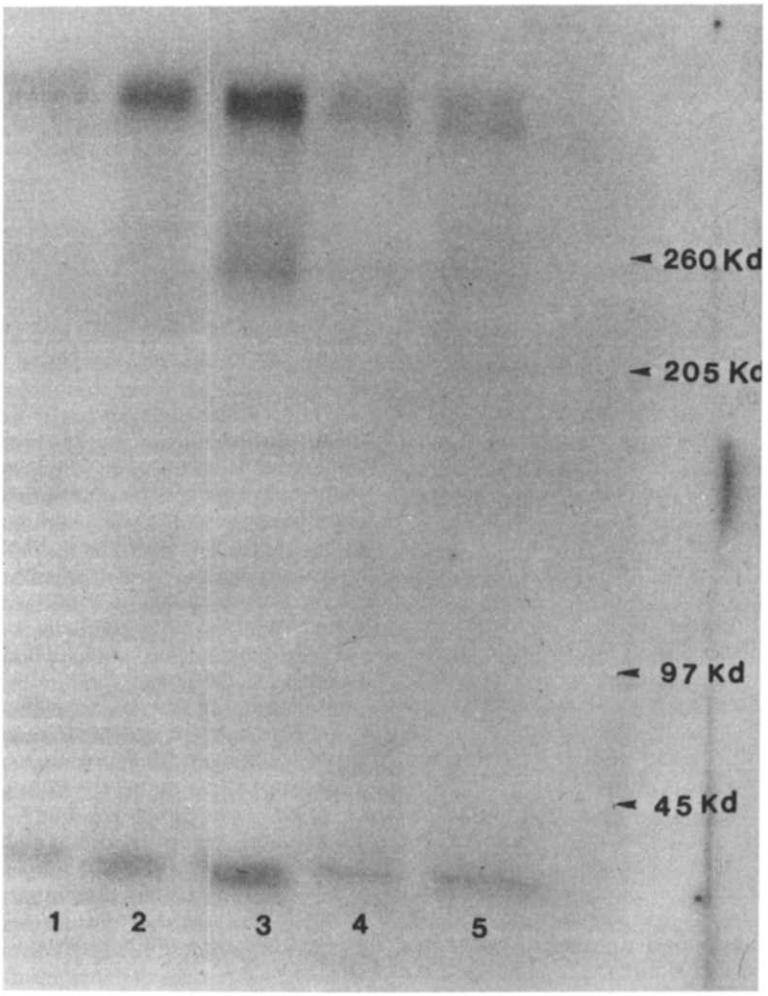

Fig. 5. Radioautogram of SDS–PAGE of ^{32}P-labeled lysed synaptosomal membrane sodium channel. Lane 1: treated with 10^{-4} M veratridine; lane 2: treated with 10^{-6} M 1*R*-deltamethrin; lane 3: control, no treatment; lane 4: treated with 10^{-5} M DDT: and lane 5: treated with 10^{-5} M DDE.

Table 2. Densitometric readings of the band intensities of the ^{32}P-phosphorylated sodium channels from intact synaptosomes treated with various concentration of deltamethrin

Lane no. as in Fig. 4	Treatments	% Radioactivity found at 260 kD (each lane = 100)
1	Control (nondepolarized)	8.28 ± 3.91
2	8-Bromo-cAMP	2.58 ± 0.62
3*	1*R*-Deltamethrin, 10^{-10} M	7.02 ± 0.69
4*	1*R*-Deltamethrin, 10^{-9} M	8.65 ± 1.05
5*	1*R*-Deltamethrin, 10^{-8} M	10.01 ± 1.10
6*	1*R*-Deltamethrin, 10^{-7} M	7.06 ± 0.76
7*	1*R*-Deltamethrin, 10^{-6} M	9.56 ± 0.95
8*	1*S*-Deltamethrin, 10^{-6} M	3.76 ± 0.56

The results are expressed in percent radioactivity found at the 260 kD band of the total radioactivities per lane. Values are averages (±SD) of two independent experiments, N = 6 scans (see Fig. 4 for more details). Note that depolarization is expected to cause a decline in phosphorylation.

* Depolarized using high K^+.

preparations. The intensity of the labeled protein bands on the radioautogram of the gel was also monitored using a densitometer connected to an integrator. The results generally confirm the above radiometric assay data, that both DDT and DDE are good inhibitors of protein phosphorylation followed by veratridine and 1*R*-deltamethrin under these experimental conditions.

DISCUSSION

In this work, initial efforts were made to identify the 260 kD phosphoprotein. The evidence that it solely represents the α-subunit of the sodium channel is: (a) molecular weight, (b) voltage sensitivity, (c) voltage-dependent binding to ^{125}I-labeled ATX-II anemone toxin, (d) sensitivity to veratridine in terms of phosphorylation, and (e) [^3H]saxitoxin binding behavior. Furthermore, as shown by Costa *et al.* [7], this is the only phosphoprotein band in this molecular weight range that can be phosphorylated by both endogenous and exogenously added PKA after the WGA-Sepharose column chromatography purification treatment.

As to the functional meaning of the interference of these pesticides with the phosphorylation and dephosphorylation processes, the best information available is that produced by Costa and Catterall [8]. These workers studied the effect of 8-Br-cAMP on $^{22}Na^+$ influx into the intact rat brain synaptosomes in the presence and absence of veratridine and batrachotoxin. In the absence of 8-Br-cAMP (i.e. nonphosphorylated α-subunit), the rate of $^{22}Na^+$ influx is consistently higher (approximately 30%) than in the presence of this cAMP analog, indicating that phosphorylation induces a reduction in Na^+ influx through the sodium channel. Both veratridine and batrachotoxin increase $^{22}Na^+$ influx (I_{50} in the neighborhood of 10^{-6} M), an . ıch actions are facilitated by the presence of a 200 nM concentration of scorpion venom from *Leiurus quinquestriatus*. Since electrophysiological evidence indicates that DDT and pyrethroids also increase the depolarization-evoked Na^+ influx, the overall logic points to the possibility of a similar functional consequence of increased Na^+ influx as the result of inhibition of α-subunit phosphorylation by these pesticides. However, critical evidence is lacking as to whether phosphorylation of the α-subunit is directly or even indirectly related to the hypothetical "gate operation" processes. Also, there are qualitative differences among these neuroactive agents in their actions on the sodium channel as shown by electrophysiological means [3, 21]. Furthermore, there are several sites for phosphorylation within the α-subunit of the sodium channel [2]. Judging by the action patterns of these insecticides, they could bind to site 2, which is susceptible to lipid soluble toxins [22–24]. However, the results from cultured neuroblastoma experiments suggest that pyrethroids might be attaching to yet another site independent from any other sites identified thus far [25, 26]. Therefore, much more work would be needed to understand the precise meaning of the inhibition of phosphorylation processes by these pesticides.

While the functional meaning of phosphorylation activities on sodium channel itself remains unanswered, there are two ion channel systems where the meaning of such activities is known. In the case of potassium channels, phosphorylation of the channel protein by cAMP-dependent protein kinase has been known to control K^+ passage [27]. In the case of *Aplysia* sensory neurons, such phosphorylation activity results in either blockage of K^+ conductance of the serotonin-sensitive K^+ channel [28] or its increase [29]. In the case of acetylcholine receptor, phosphorylation of its receptor protein affects the rate of the desensitization process of the receptor [30]. In the case of K^+ channels of leech Retzius cells, Leake *et al.* [31] have already shown that the effects of pyrethroids are likely mediated through their action on the cAMP-dependent protein kinase, indicating that alteration of the state of phosphorylation on the channel proteins by these pesticides is likely the cause for their interference on K^+ channel functions.

As to the biochemical cause(s) of such effects by these insecticides, three major components participating in phosphorylation processes must be first considered as potential action sites. They are: protein kinases, phosphatases and the substrate proteins, in this case the α-subunit, as compared to other proteins. According to Costa and Catterall [32], the α-subunit is an excellent substrate for both PKA and protein kinase C. No determination can be made from our current research as to which of the endogenous protein kinases is most affected by the insecticides in intact synaptosomes. As for phosphatases, at least in the case of one model system, where partially purified sodium channels were directly reacted with [γ-^{32}P]ATP and PKA, there were only two active proteins for deltamethrin's action, i.e. PKA and the sodium channel. Therefore, the question on phosphatases appears to be moot.

As to the question on the target protein, we have noted that depolarization caused changes in phosphorylation on a few selected bands of proteins, but not in all of them. Among those showing significant changes, the α-subunit exhibited outstanding sensitivity. The same thing applies to its sensitivities to other neuroactive agents such as veratridine. Such selective actions tend to support the view that the specific action of deltamethrin on the sodium channel is more important than that on protein kinases, though no definite conclusion may be made at this stage.

In the current study we have firmly established that DDT and pyrethroids affect the phosphorylation processes in the α-subunit of the sodium channel proper. While more work is needed to understand the precise functional meaning of such actions of these pesticides, the finding provides supporting evidence that many of the agents known to affect Na^+ permeability through the Na^+ channel have the ability to affect the voltage-sensitive processes of phosphorylation and dephosphorylation in the α-subunit protein.

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