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Modulation of Levels of Free Calcium within Synaptosomes by Organochlorine Insecticides[1]

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ABSTRACT

Effects of the organochlorine insecticides chlordecone, mirex, 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethane and 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane on free intrasynaptosomal Ca^{2+} ($[Ca^{++}]_i$), synaptosomal ^{45}Ca uptake and synaptosomal plasma and mitochondrial membrane potentials *in vitro* were studied. Chlordecone (10–50 μM) increased $[Ca^{++}]_i$ from the resting level of 370 nM in a dose- and time-dependent manner to above 1.5 μM. This took place in the presence of 1 mM extrasynaptosomal Ca^{++} but not in nominally Ca^{++}-free medium. Verapamil, a voltage sensitive Ca^{++} channel blocker, inhibited the initial increase of $[Ca^{++}]_i$ caused by chlordecone, by 40%. Chlordecone also elevated $[Ca^{++}]_i$ in synaptosomes in which mitochondrial Ca^{++} uptake had been abolished by valinomycin. Chlordecone depolarized partially the synaptosomal plasma membrane and, to a lesser extent, the potential of mitochondria within synaptosomes. However, chlordecone appeared to inhibit synaptosomal K^+-stimulated and unstimulated $^{45}Ca^{++}$ uptake by 20 to 30%. Inasmuch as chlordecone also stimulated release of $^{45}Ca^{++}$ and the fluorescent dye fura-2 from preloaded synaptosomes, the apparent inhibition of uptake might be due to lysis of some synaptosomes by chlordecone. The effect of chlordecone on $[Ca^{++}]_i$ decreased when the total amount of tissue in incubations was increased. $[Ca^{++}]_i$ was only elevated marginally by mirex at the same concentration range. The results suggest that chlordecone increases free intrasynaptosomal Ca^{++} mainly by increasing influx of extrasynaptosomal Ca^{++}. The principal mechanism appears to be a nonspecific leakage of Ca^{++} through the plasma membrane but some Ca^{++} may pass through voltage-sensitive Ca^{++} channels due to chlordecone-induced membrane depolarization.

The neurotoxicity of many organochlorine and pyrethrin insecticides is believed to be due at least in part to modulation of ion fluxes across the neuronal plasma membrane. In the case of *p,p'*-DDT and pyrethrins, there is reliable evidence for a delayed closing of the sodium channel after depolarization (Vijverberg and van den Berken, 1982; Woolley, 1982; Narahashi, 1985). There is other evidence that some organochlorine insecticides may cause neuronal hyperexcitability by a different mechanism. Whereas an intraventricular injection of $CaCl_2$ diminishes tremor in *p,p'*-DDT-treated rats, tremor is exacerbated in chlordecone-treated rats (Herr *et al.*, 1987).

The anticonvulsant phenytoin is believed to act primarily by inhibition of the sodium channel (MacDonald, 1983; Willow *et al.*, 1984). This drug alleviates *p,p'*-DDT or permethrin-induced tremor but aggravates chlordecone-induced tremor (Tilson *et al.*, 1985), again suggesting chlordecone does not act at the same locus as *p,p'*-DDT or permethrin. Alteration of calcium homeostasis by chlordecone has been postulated (Carmines *et al.*, 1979; End *et al.*, 1979, 1981; Hoskins and Ho, 1982; Philips and Eroschenko, 1985). There is also evidence that other organochlorine insecticides may affect Ca^{++} distribution. Lindane (γ-hexachlorohexane) (Woolley *et al.*, 1985; Lievremont *et al.*, 1984), toxaphene (Trottman *et al.*, 1985) and dieldrin (Singh and Singh, 1984) have been suggested to alter free levels of Ca^{++} within the neuron. Many of these studies utilized $^{45}Ca^{++}$ in order to examine the uptake and release of Ca^{++} by mitochondria or synaptosomes. The prevention of damage in the presence of high concentrations of Mg^{++} also implies indirectly that organochlorine insecticide neurotoxicity may be Ca^{++}-related (Singh and Singh, 1984; Woolley *et al.*, 1985).

However, no direct evaluation of the effects of these insecticides upon the concentration of free ionic Ca^{++} within neuronal cytoplasm has hitherto been made. Fluxes in $^{45}Ca^{++}$ do not

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ABBREVIATIONS: *p,p'*-DDT, 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane; $[Ca^{++}]_i$, free intrasynaptosomal calcium concentration; *o,p'*-DDT, 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; fura-2/AM, acetoxymethyl ester of fura-2; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; TPP, tetraphenylphosphonium; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; ATPase, adenosine triphosphatase.

reflect this critical parameter accurately. These Ca^{++} values are less than 0.1% of extracellular calcium concentration or total mitochondrial calcium content (Brinley, 1980). The small size of this free Ca^{++} pool enables it to undergo rapid changes in concentration and these values cannot be determined readily by indirect means. Recently, methods have become available that allow the direct quantification of Ca^{++} within undisrupted living cells (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985). These methods have been adapted to synaptosomes (Ashley *et al.*, 1984; Nachshen, 1985; Komulainen and Bondy, 1987a).

This report deals with the effects of various organochlorine insecticides upon $[Ca^{++}]_i$. Such data have been contrasted with results derived from the use of $^{45}Ca^{++}$ in order to study fluxes across the plasma membrane. Changes in mitochondrial or plasma membrane potential have also been determined. The results suggest that the mechanism of action of chlordecone toxicity may be distinct from that of mirex, *p,p'*-DDT or *o,p'*-DDT.

Materials and Methods

Preparation of synaptosomes. Adult male Fisher 344-strain rats (Charles River Breeding Laboratories, Inc., Wilmington, MA), 4 to 5 months old weighing 290 to 330 g were used. Rats were decapitated, the brains excised quickly on ice and the whole brain except the cerebellum and pons-medulla dissected out. Synaptosomes were made by the modification of Dodd *et al.* (1981) of the method of Gray and Whittaker (1962). Briefly, after homogenization in 10 volumes of cold 0.32 M sucrose, the homogenate was centrifuged (1500 × *g*, 10 min, 0–4°C) and the supernatant laid over 1.2 M sucrose (8 ml). After high speed centrifugation (50,000 rpm, 10 min, Beckman model L5-65, rotor Ti 60) the layer at the interface was collected, diluted 2.5-fold with 0.32 M sucrose and laid over 0.8 M sucrose. After centrifugation again at high speed, the synaptosomal pellet was suspended in HEPES buffer, pH 7.4, to give the tissue concentration 0.15 g-equivalent/ml (about 1.6 mg/ml of protein). The composition of HEPES buffer was (millimolar): NaCl, 125; KCl, 5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 5; glucose, 6; $CaCl_2$, 1; and HEPES, 25.

Assay of $[Ca^{++}]_i$. $[Ca^{++}]_i$ was measured using the fura-2/AM; 1-ml aliquots of the synaptosome suspension were incubated with 5 μM fura-2/AM added in DMSO (final concentration of DMSO, 0.5%) at 37°C for 20 min. HEPES buffer (9 ml) at 37°C was then added and the incubation continued for 25 min. Synaptosomes were sedimented (12300 × *g*, 5 min), resuspended in 5 ml of HEPES buffer and kept on ice. For fluorescence measurement 500-μl aliquots of the suspensions were centrifuged rapidly in Eppendorf-tubes in a microcentrifuge (13000 × *g*, 30 sec) and the synaptosomal pellet was resuspended in 2 ml of warm (37°C) HEPES buffer, pH 7.4. This buffer did not contain $NaHCO_3$ or NAH_2PO_4 in order to prevent precipitation of high levels of Ca^{++} used subsequently. The emitted fluorescence of the sample was measured in a thermostatted (37°C) cuvette using Aminco SPF-500 spectrofluorometer at the excitation wavelengths of 340 and 380 nm (bandpass, 1 nm) and at 510 nm for emission (bandpass, 8 nm). Samples contained 140 to 160 μg of protein and were allowed to equilibrate for 10 min before addition of chemicals. Mixing was carried out with a magnetic stirrer 30 to 60 sec before reading fluorescence. Extrasynaptosomal fura-2 was quenched by 40 μM $MnCl_2$ at the end of each incubation. It was added 15 to 20 sec before recording the fluorescence. This Mn^{++} concentration corrected for extrasynaptosomal fura-2 (Komulainen and Bondy, 1987a). In order to calculate $[Ca^{++}]_i$ *before* addition of insecticides, a separate average Mn^{++} correction was made for each batch of synaptosomes. This correction was around 6% of the fluorescence at 340 nM and was very stable from one synaptosomal suspension to another (table 2). Autofluorescence of synaptosomes was always recorded and subtracted before calculation of fluorescence ratios of fura-2. This constituted around 15% of total fluorescence. For calibration of the synaptosomal fura-2-Ca^{++} signal (R), R_{min} (the ratio of fluorescence at 340 nm/380 nm in the absence of Ca^{++}) and R_{max} (the ratio when all fura-2 of the sample was saturated with Ca^{++}) were determined for each batch of fura-2-loaded synaptosomes. In order to determine R_{min} synaptosomes were lyzed with 0.1% sodium dodecyl sulfate and Ca^{++} and Mn^{++} chelated with 5 mM alkaline EGTA and 10 μM diethylenetriaminepentaacetic acid. R_{max} was determined by the addition of 7 mM $CaCl_2$. $[Ca^{++}]_i$ was calculated using the formula (Grynkiewicz *et al.*, 1985):

$$[Ca^{++}]_i = K_d \frac{(R - R_{min})}{(R_{max} - R)} \frac{(Sf_2)}{(Sb_2)}$$

where K_d (224 nM) is the dissociation constant of fura-2-Ca^{++}-complex and Sf_2 and Sb_2 denote fluorescence of fura-2 at zero Ca^{++} and Ca^{++} saturation, respectively, at the excitation wavelength 380 nm.

$^{45}Ca^{++}$ transport

K^+-stimulated uptake. A synaptosomal (0.1 ml) suspension (150–170 μg of protein) was mixed with 0.9 ml of the Tris-buffer, pH 7.4, containing (millimolar): NaCl, 150; KCl, 5; $CaCl_2$, 1.5; $MgSO_4$, 1.4; glucose, 25; and Tris-HCl, 40 and incubated at 37°C for 12 min. Then 0.1 ml of $^{45}Ca^{++}$ (0.5 μCi, 55.9 Ci/mol) in either 0.5 M KCl or 0.5 M NaCl was mixed into the suspension and incubation continued for a further 15 sec. When indicated, the insecticides were added in DMSO (the final concentration not exceeding 1%) 15 min or 15 sec before $^{45}Ca^{++}$. DMSO was also present in control tubes. $^{45}Ca^{++}$ uptake was stopped by the addition of 5 ml of ice-cold wash buffer (Tris-buffer omitting $CaCl_2$ and containing 1 mM EGTA) followed by rapid filtration on glass fiber filters (Type A/E, Gelman Sciences, Inc., Ann Arbor, MI) (Wu *et al.*, 1982). The filter discs were washed with 2 × 5 ml of buffer and accumulated label counted in 10 ml of Aquasol (New England Nuclear, Boston, MA) in a liquid scintillation counter. The difference in ^{45}Ca uptake between incubation in the presence of 50 mM KCl or 50 mM NaCl was taken to represent depolarization-triggered Ca^{++} entry. Blank tubes contained synaptosomes that had been sonicated and heated to 100°C for 10 min and were thus incapable of Ca^{++} transport. These blanks representing binding of $^{45}Ca^{++}$ into membranes were subtracted from the results.

Unstimulated uptake. In other experiments, synaptosomes (about 80 μg of protein) were incubated simultaneously with $^{45}Ca^{++}$ (added in Tris-buffer) and chlordecone for 10 min without preincubation with chlordecone and incubation terminated as described above.

Spontaneous release. Synaptosomes were preloaded with $^{45}Ca^{++}$ by incubating 3.0 ml of the synaptosome suspension in Tris-buffer with $^{45}Ca^{++}$ (7.5 μCi) at 37°C for 30 min. Particulate material was then centrifuged down (12300 × *g*, 10 min) and the supernatant removed. The synaptosomal pellet was then resuspended in 3.0 ml of Tris-buffer and 0.1-ml aliquots (about 70 μg of protein) were incubated with chlordecone in Tris-buffer at 37°C for 10 min. $^{45}Ca^{++}$ remaining in synaptosomes was then assayed by filtration and washing of the synaptosomes as described above.

Membrane potential. Membrane potential was assayed by estimation of the concentration gradient of a lipophilic cation, TPP^+, that is accumulated by organelles with a potential across their external membrane (Lichtenstein *et al.*, 1979). This method has been applied successfully to synaptosomes (Ramos *et al.*, 1979).

Synaptosomes in HEPES buffer were incubated (10 min, 37°C) in a 1.0-ml volume containing 120 μg of synaptosomal protein, 2 μM TPP^+ containing 0.2 μCi of [phenyl-^{3}H]TPP^+ (35.5 Ci/mmol) and 5 μM valinomycin in order to eliminate the mitochondrial contribution to membrane potential. Synaptosomes were then collected by filtration through glass-fiber filters (Type A/E, Gelman Sciences, Inc.), washed three times with 5 ml of 0.2 M NaCl and counted. By ascertaining input counts and by taking intrasynaptosomal fluid volume to be 3.6 μl/mg of protein (Marchbanks, 1975), it was then possible to calculate the ratio of TPP^+ concentration within synaptosomes (TPP^+_i) to that

remaining in the extrasynaptosomal medium (TPP^+_o). The membrane potential ψ was calculated as $-61 \log \frac{(TPP^+_i)}{(TPP^+_o)}$ (Ramos *et al.*, 1979). Synaptosomes subjected to 100°C for 10 min and to sonication were used as blanks.

The membrane potential of mitochondria within synaptosomes was determined by a similar incubation to that described above, with the omission of valinomycin. The additional radioactivity taken up under these circumstances represented mitochondrial change. By taking a value of 12% as the proportion of synaptosomal volume occupied by mitochondria (Scott and Nicholls, 1980), it was possible to determine the concentration of TPP^+ within mitochondria over that in the synaptic cytoplasm when valinomycin was present. The ability of free mitochondria prepared from the P_2 fraction of brain homogenates (Dodd *et al.*, 1981) to accumulate TPP^+ was estimated to be only around 2% of synaptosomal mitochondria. This is probably due to their being damaged by the preparative medium which is not designed for mitochondrial stabilization. Thus, the contribution of any contaminating free mitochondria in the synaptosomal preparation to calculated mitochondria potential could be discounted. The substitution of 1 μM of the oxidative phosphorylation uncoupler CCCP for valinomycin, gave essentially identical inhibition of TPP^+ uptake, confirming that mitochondrial potential had been disrupted selectively by either agent.

Protein determination. Protein content of synaptosomal suspensions were assayed with the method of Lowry *et al.* (1951) using bovine albumin as a reference.

Statistics. Results were analyzed using Fisher's Least Significant Difference Test after one-way analysis of variance. Throughout the results, the symbol (*) means $P < .05$ or lower using a two-tailed t distribution.

Chemicals. Fura-2/AM was obtained from Molecular Probes (Junction City, OR) and $^{45}CaCl_2$ and [phenyl-^{3}H]TPP bromide from New England Nuclear. Chlordecone was obtained from Radian Corp. (Austin, TX) and other organochlorine insecticides from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals including valinomycin and CCCP were from Sigma Chemical Corp. (St. Louis, MO).

Results

The resting level of $[Ca^{++}]_i$ was 368 ± 5 nM (n = 75) when 1 mM Ca^{++} was present in the incubation medium. When calcium was omitted from the medium $[Ca^{++}]_i$ was significantly depressed to 208 ± 8 nM (n = 23). Incubation of synaptosomes together with chlordecone elevated $[Ca^{++}]_i$ in a dose-dependent manner (fig. 1). At 10 μM chlordecone $[Ca^{++}]_i$ was raised significantly by 40% and at 30μM chlordecone by 320%. In contrast, mirex increased $[Ca^{++}]_i$ by only 30% (fig. 1) at the highest concentration studied (30 μM). *o,p'*- and *p,p'*-DDT at 10 μM were ineffective in raising $[Ca^{++}]_i$ (table 1).

The effect of 20 μM chlordecone upon $[Ca^{++}]_i$ was rapid, increasing its value by 75% within 60 sec (fig. 2). The Ca^{++} level continued to increase thereafter. When extrasynaptosomal Ca^{++} was low (<0.01 mM), chlordecone only had a very minor effect on $[Ca^{++}]_i$ (fig. 3), suggesting that much of the source of the elevated $[Ca^{++}]_i$ was from the incubation medium. This idea was supported further by a 40% inhibition (fig. 4) of the initial chlordecone-induced increase of $[Ca^{++}]_i$ by 200 μM verapamil, a blocker of a group of voltage-sensitive Ca^{++} channels (Nachshen and Blaustein, 1979). Verapamil alone did not affect the resting $[Ca^{++}]_i$ or interfere with fura-2 fluorescence.

The contribution of mitochondrial Ca^{++} to the chlordecone-induced elevation of $[Ca^{++}]_i$ was studied by preincubation of synaptosomes for 5 min at 37°C with 5 μM valinomycin, a K^+ and proton ionophore. This caused an elevation of $[Ca^{++}]_i$ by 50 to 60 nM in the presence of 1 mM extrasynaptosomal Ca^{++},

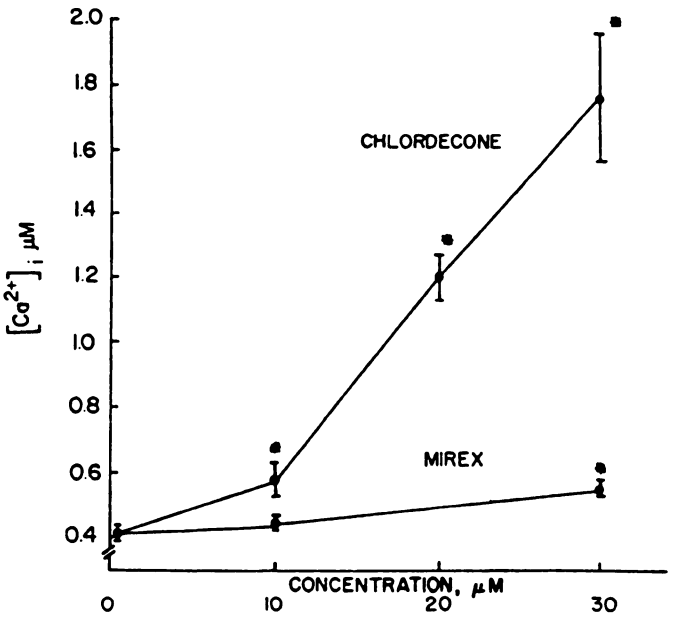

Fig. 1. Effect of chlordecone and mirex on $[Ca^{++}]_i$ *in vitro*. Fura-2-loaded synaptosomes (about 240 μM fura-2, 150 μg of protein) were incubated with these agents in a cuvette at 37°C for 10 min and $[Ca^{++}]_i$ determined as described under "Materials and Methods." Results are mean ± S.E. of six to eight separate determinations. *Differs significantly ($P < .05$) from control value.

TABLE 1
Effect of *p,p'*-DDT[a] and *o,p'*-DDT on $[Ca^{++}]_i$

Agent	$[Ca^{++}]_i$
	nM
0.5% DMSO (control)	417 ± 20[b]
p,p'-DDT, 10 μM	433 ± 21
o,p'-DDT, 10 μM	477 ± 25

[a] Synaptosomes (about 150 μg of protein) were incubated with these agents for 10 min.
[b] Mean ± S.E., n = 6.

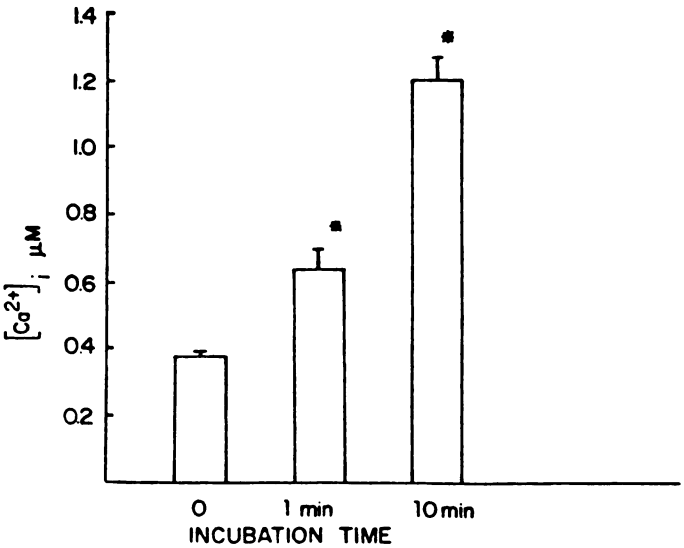

Fig. 2. Time course of elevation of $[Ca^{++}]_i$ by chlordecone. Incubation conditions as in figure 1 except incubation time with chlordecone (20 μM) varied. Mean ± S.E. of 6 to 12 determinations. *$P < .001$ as compared to all other values.

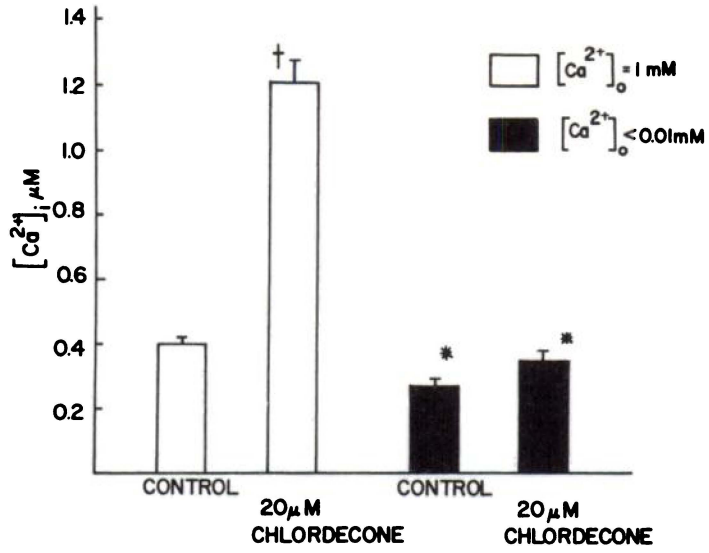

Fig. 3. Effect of extrasynaptosomal Ca^{++} on $[Ca^{++}]_i$ and on its elevation by chlordecone. Synaptosomes (about 150 μg of protein) were incubated with chlordecone (20 μM) in HEPES buffer containing 1 mM $CaCl_2$ ($[Ca^{++}]_o$, 1 mM) or $CaCl_2$ omitted ($[Ca^{++}]_o$ < .01 mM) for 10 min. Mean ± S.E. of five determinations. †Differs significantly (P < .001) from corresponding control value. *Differs significantly (P < .01) from the respective value in the presence of 1 mM Ca^{++}.

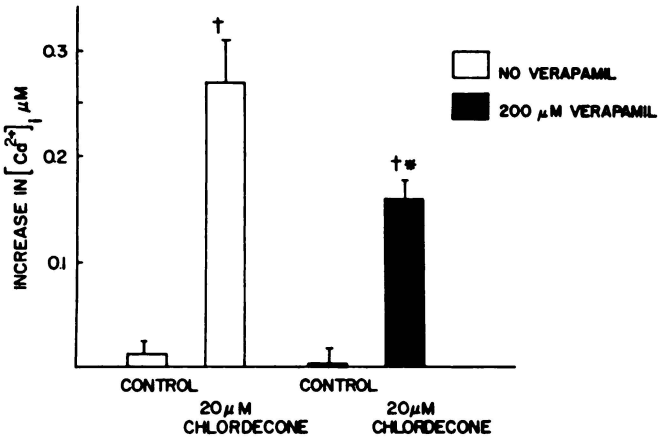

Fig. 4. Effect of verapamil on chlordecone-induced elevation of $[Ca^{++}]_i$. Verapamil (200 μM) was added 10 min before chlordecone (20 μM) and the incubation continued for 1 min. Mean ± S.E. of six to seven determinations. †Differs significantly (P < .001) from the respective control. *Differs significantly (P < .05) from the corresponding value in the absence of verapamil.

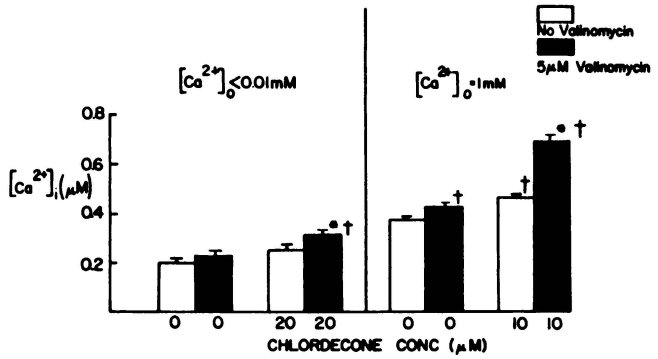

Fig. 5. Modulation of $[Ca^{++}]_i$ by valinomycin and chlordecone. Synaptosomes (about 150 μg of protein) were incubated either in HEPES buffer containing 1 mM $CaCl_2$ (right panel) or in nominally Ca^{++}-free buffer (left panel). Valinomycin (5 μM) was added 5 min before chlordecone or the incubation continued without valinomycin. The final incubation time with indicated chlordecone concentrations (CONC) was 10 min. Mean ± S.E. of four measurements. †Differs significantly (P < .05) from the control value (no valinomycin, no chlordecone). *Differs significantly (P < .05) from the corresponding value in the presence of 5 μM valinomycin, alone.

probably due to inhibition of mitochondrial uptake of Ca^{++} and stimulated Ca^{++} release. A subsequent addition of 10 μM chlordecone elevated further $[Ca^{++}]_i$ in what appeared to be a synergistic manner (fig. 5, right panel). However, no further increase in $[Ca^{++}]_i$ was caused by the addition of valinomycin to synaptosomes incubated with 20 μM chlordecone (results not shown). A synergistic effect could also be observed when the extrasynaptosomal medium was nominally calcium-free (fig. 5, left panel). In this case, both 5 μM valinomycin and 20 μM chlordecone together were needed in order to produce a significantly higher $[Ca^{++}]_i$.

The total protein content of synaptosomes incubated with chlordecone was found to be very critical. When the amount of synaptosomes incubated was doubled the effect of 20 μM chlordecone on $[Ca^{++}]_i$ was abolished (fig. 6). A threshold ratio of chlordecone to tissue content may be more critical than the concentration of chlordecone used, in order to detect an elevation of $[Ca^{++}]_i$. The critical value of this ratio was between 0.13 and 0.26 μmol of chlordecone per mg of synaptosomal protein. Above this threshold, 10 μM chlordecone significantly depolarized the synaptosomal plasma membrane from 67 ± 4 to 28 ±

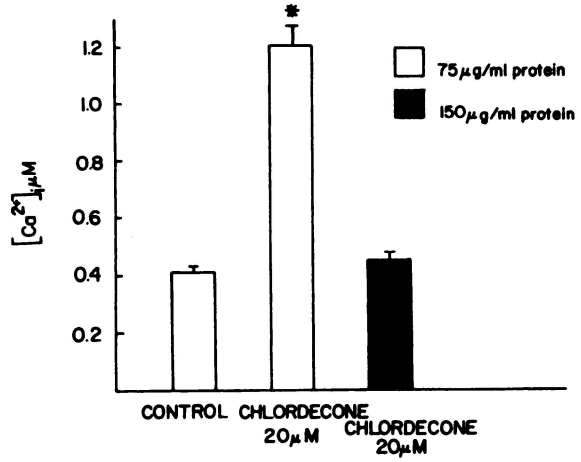

Fig. 6. Effect of varying synaptosomal protein on response of $[Ca^{++}]_i$ to chlordecone. Synaptosomes either containing 75 μg/ml of protein (total protein, 150 μg) or 150 mg/ml of protein (total protein, 300 μg) were incubated with chlordecone (20 μM) for 10 min and $[Ca^{++}]_i$ determined. Mean ± S.E. of five to six determinations. *Differs significantly (P < .001) from control value.

3 mV (n = 6) (fig. 7). This reduction of membrane potential was not much greater when higher concentrations of chlordecone were used (fig. 7). Ten micromolar mirex, p,p'- or o,p'-DDT did not have any effect on plasma membrane or mitochondrial potential (fig. 8). However, the membrane potential of the mitochondria within synaptosomes (177 ± 3 mV, n = 6) was also significantly depressed down to 136 ± 4 mV (n = 6) by 20 μM chlordecone (fig. 7).

Another parameter that the use of fura-2 allowed us to examine was the permeability of the synaptosomal membrane, as any extrasynaptosomal fura-2-related fluorescence could be quenched rapidly with 40 μM Mn Cl_2 (Komulainen and Bondy, 1987a). At chlordecone concentrations of 20 μM and above, the amount of fura-2 leaking out of the synaptosomes increased from control values of around 6% to 10 to 30% (table 2). This was interpreted as lysis of some synaptosomes or damage to their external membranes. Mirex and the DDT-isomers did not show evidence of a similar disruption (table 2).

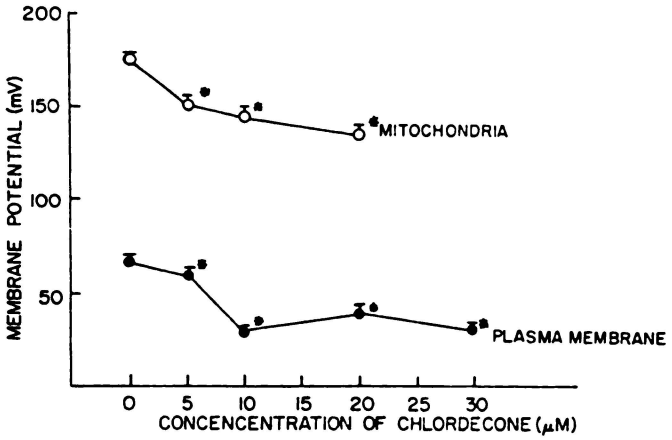

Fig. 7. Effect of chlordecone upon plasma and mitochondrial membrane potential in synaptosomes. Synaptosomes (about 120 μg of protein) were incubated with 2 μM [^{3}H]TPP$^+$ in the absence or presence of 5 μM valinomycin in order to obtain total potential and plasma membrane potential, respectively, and various concentrations of chlordecone in HEPES buffer for 10 min. Membrane potentials were calculated from the distribution of [^{3}H]TPP$^+$ between synaptosomes and medium as described under "Materials and Methods." Mean ± S.E. of six determinations. *Differs significantly ($P < .05$) from control value.

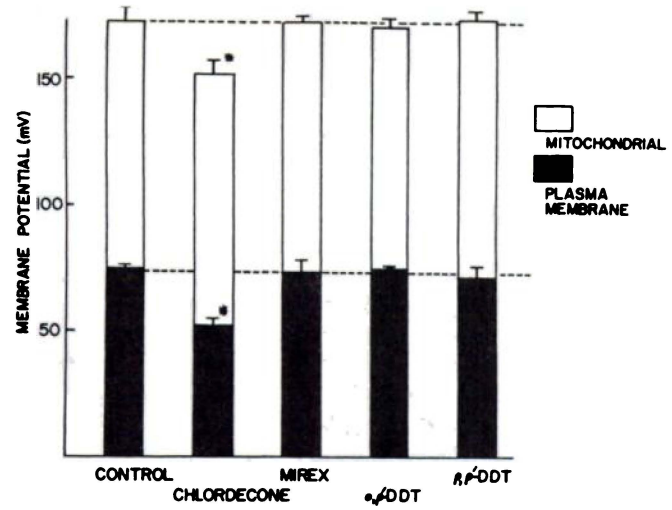

Fig. 8. Effects of mirex, o,p'- and p,p'-DDT on synaptosomal plasma and mitochondrial membrane potentials. Synaptosomes (about 120 μg of protein) were incubated with 10 μM concentrations of these agents as in figure 7. Chlordecone was included as a reference. Mean ± S.E. of six determinations. *Differs ($P < .05$) significantly from control value.

Chlordecone (50 μM) inhibited K^+-stimulated uptake of $^{45}Ca^{++}$ by 25 to 30% after 15 sec or 15-min preincubation (fig. 9). It also inhibited $^{45}Ca^{++}$ uptake during a 10-min incubation under nondepolarizing conditions (fig. 10). Using a similar incubation of ^{45}Ca-loaded synaptosomes with chlordecone, chlordecone was also shown to stimulate ^{45}Ca release (table 3). This effect was dependent on chlordecone concentration and also on protein content. Chlordecone (50 μM) released 48% of $^{45}Ca^{++}$ when synaptosomal protein was 150 μg/ml but 85% of $^{45}Ca^{++}$ at a protein concentration of 75 μg/ml (table 3). Under these latter conditions, 1.34 μmol of chlordecone were present per mg of protein. Mirex, at concentrations as high as 50 μM, did not significantly affect $^{45}Ca^{++}$ uptake, but both p,p'- and o,p'-DDT inhibited K^+-stimulated $^{45}Ca^{++}$ uptake by 30 to 40% (fig. 9).

Discussion

The elevation of free Ca^{++} within synaptosomes by chlordecone could occur by one or more of several mechanisms. Our data suggest a major route for this elevation is by increased influx of extrasynaptosomal Ca^{++}. This is supported by the finding that chlordecone could increase $[Ca^{++}]_i$ only in the presence of extrasynaptosomal Ca^{++}. When Ca^{++} was omitted from the incubation buffer, the effect of chlordecone was abolished. The basic mechanisms of action are not known at present but at high concentrations chlordecone had nonspecific membrane effects (discussed later). Hence, chlordecone might simply increase Ca^{++} leakage through the plasma membrane. Verapamil at 200 μM concentration inhibited the initial chlordecone-induced increase of $[Ca^{++}]_i$ by 40 to 50%, to the same extent as verapamil inhibits the increase caused by 50 mM extrasynaptosomal K^+ (Komulainen and Bondy, 1987a). This suggests that part of the chlordecone-induced increase in $[Ca^{++}]_i$ might also occur *via* voltage-regulated calcium channels. Other experiments indicated that chlordecone induces a partial depolarization of the plasma membrane and such a membrane depolarization is expected to increasingly open Ca^{++} channels. Chlordecone is known to inhibit Na^+-K^+-ATPase *in vitro* (Desaiah, 1981), but inhibition of this enzyme alone can not induce such a rapid and high increase in $[Ca^{++}]_i$ (Komulainen and Bondy, 1987b). Chlordecone could also increase $[Ca^{++}]_i$ in synaptosomes in which mitochondrial Ca^{++} uptake was inhibited and Ca^{++} release facilitated by 5 μM valinomycin which depolarizes mitochondria but does not alter plasma membrane potential (Scott and Nicholls, 1980). The increase in $[Ca^{++}]_i$ that was obtained with valinomycin (maximum 200 nM), was very similar to that reported by Nachshen (1985). Hence, it is unlikely that the enormous increase of $[Ca^{++}]_i$ caused by chlordecone could be attributed largely to mitochondrial inhibition. Such an effect on mitochondria by chlordecone has been proposed (Carmines *et al.*, 1979; End *et al.*, 1979). However, chlordecone did reduce mitochondrial membrane potential and is known to inhibit synaptosomal Mg^{++}-Ca^{++} ATPase (Ca^{++} pump) *in vitro* (Mishra *et al.*, 1980). Inhibition of mitochondrial and plasma membrane mechanisms for removal of cytosol Ca^{++} could account for the prolonged increase of $[Ca^{++}]_i$ with time caused by chlordecone. The partial depolarization of mitochondria by chlordecone is much less than the complete depolarization effected by valinomycin and does not support the con-

TABLE 2

Synaptosomal damage, estimated by quenching[a] of extracellular fura-2 by 40 μM Mn^{++}

Numbers in parentheses, number of determinations.

Agent	% Decrease in Fluorescence at 340 nM			
	0	10 μM	20 μM	30 μM
Chlordecone	6.4 ± 0.6 (14)	9.0 ± 1.0[b,*] (12)	21.0 ± 1.0* (11)	28.5 ± 0.9* (6)
Mirex		8.0 ± 0.6 (6)		5.3 ± 0.7 (6)
o,p'-DDT		7.0 ± 1.5 (6)		
p,p'-DDT		7.6 ± 1.5 (6)		

[a] Mn^{++} (40 μM) was added after a 10-min incubation of synaptosomes wih the agents indicated.
[b] Mean ± S.E.
* P < .05 or lower.

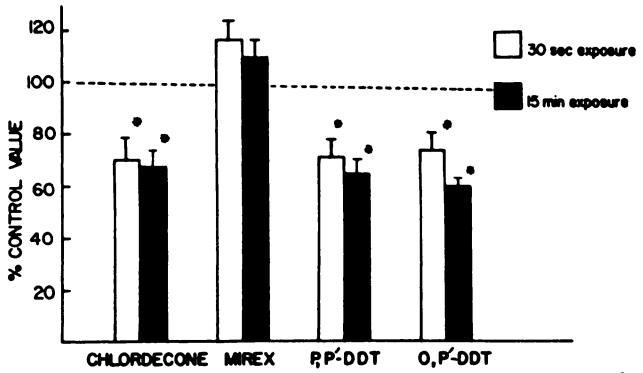

Fig. 9. Effect of organochloride insecticides on K^+-stimulated Ca^{++} uptake in synaptosomes. Synaptosomes (160 μg of protein) were preincubated with the insecticides (50 μM) in Tris-buffer containing 1.5 mM $CaCl_2$ either for 15 sec or for 15 mi. Then 0.5 μCi of $^{45}Ca^{++}$ was added together with 50 mM K^+ and the incubation continued for 15 sec. Ca^{++} uptake in control samples was 5.0 ± 0.3 nmol/mg of protein per 15 sec and this was taken as 100%. *The value differs significantly (P < .05) from control value. Mean ± S.E. of 12 determinations.

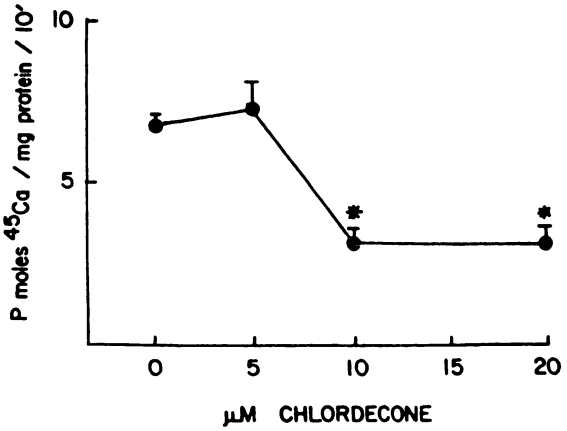

Fig. 10. Effect of chlordecone on unstimulated uptake of Ca^{++} by synaptosomes. Synaptosomes (70 μg of protein) were incubated as in figure 9 except $^{45}Ca^{++}$ was added in Tris-buffer (5 mM K^+) at the same time with chlordecone and the samples were incubated for 10 min. Mean ± S.E. of six experiments. *Differs significantly (P < .05) from control value.

cept of mitochondrial disruption and consequent massive Ca^{++} release.

The increase of $[Ca^{++}]_i$ and decrease of uptake of ^{45}Ca caused by chlordecone appeared at first to be paradoxical. However, the demonstration of elevated ^{45}Ca release from synaptosomes by chlordecone suggested the apparent inhibition of uptake to be due to synaptosomal lysis. Two independent observations converge on this possibility: the apparently reduced uptake and release of ^{45}Ca by chlordecone-treated synaptosomes and the

TABLE 3

Effect of varying concentrations of chlordecone on release of $^{45}Ca^{++}$ accumulated previously by synaptosomes

Chlordecone Conc.	% $^{45}Ca^{++}$ Remaining within Synaptosomes[a] (Control = 100%)	
	Synaptosomal protein concentration 75 μg/ml	150 μg/ml
μM		
0	100 ± 1.1[b]	100 ± 0.6
10	95.0 ± 1.7	89.4 ± 2.5*
20	92.9 ± 0.7*	87.4 ± 2.5*
50	16.2 ± 1.7*	47.8 ± 2.1*

[a] Synaptosomes were incubated with varying concentrations of chlordecone for 10 min at 37°C. Control tissue was incubated with 0.5% DMSO vehicle.
[b] Mean ± S.E., $n = 3$.
* P < .05 that value differs significantly from corresponding control value.

increased extrasynaptosomal fura-2 found after such an incubation. The magnitude of the ^{45}Ca-flux effects and free fura-2 effects of chlordecone are very similar (25–30%). The assay of $[Ca^{++}]_i$ and of ^{45}Ca fluxes differ considerably from a methodological point of view. $[Ca^{++}]_i$ measurement is independent of synaptosomal number whereas ^{45}Ca fluxes diminish if some of the synaptosomes are disrupted. Thus, interpretation of data showing reduced ^{45}Ca uptake *in vitro* must be cautious, as Ca^{++} entry blockage and synaptosomal lysis can give identical results. Some previously noted contraindications between chlordecone-caused inhibition of synaptosomal calcium uptake and stimulation of neurotransmitter release (End *et al.*, 1979) can be accounted for in terms of synaptosomal rupture. One should note that leakage of fura-2 out of synaptosomes by chlordecone also creates a potential methodological source of error for $[Ca^{++}]_i$ when this extrasynaptosomal fura-2 has not been corrected. All liberated fura-2 is saturated with Ca^{++} (1 mM extracellularly) and gives a significant Ca^{++} signal. In the present experiments extrasynaptosomal fura-2 was quenched by Mn^{++} at the end of incubation and such an artifact does not contribute to $[Ca^{++}]_i$.

Mirex was practically inactive throughout these studies at the concentration range where chlordecone was toxic; this is in concert with the fact that it is much less neurotoxic than chlordecone (Desaiah *et al.*, 1981; Fujimori *et al.*, 1982). Our study shows that chlordecone is more potent than mirex in disrupting synaptosomes. This difference may be quantitative rather than qualitative. Neither p,p'-DDT nor the less neurotoxic o,p'-DDT affected $[Ca^{++}]_i$ at 10 μM concentration and chlordecone was the only organochlorine examined that reduced plasma membrane or mitochondrial potentials at low concentration. However, 50 μM of either DDT-isomers apparently inhibited ^{45}Ca uptake.

The effect of chlordecone on $[Ca^{++}]_i$ appeared to be strictly

dependent on the total amount of synaptosomes present. Chlordecone is highly lipophilic and binds almost completely to biological membranes (End *et al.*, 1979). Extra tissue can attenuate the effects of a given amount of chlordecone, perhaps by an increased number of available binding sites. Thus, extrapolation of effects from *in vitro* studies to the living organism should be guarded and pharmacokinetics of chlordecone be considered. Also comparisons of results from different studies may not be relevant when differing amounts of tissue are used. In that respect, the present assays are comparable to each other.

Chlordecone has the potential to increase $[Ca^{++}]_i$ and this property is not shared by several other organochlorine insecticides. The relevance of this distinctive property to the neurotoxicity of chlordecone remains to be definitely demonstrated. Although similar overall concentrations of chlordecone to those used here may be reached after an *in vivo* exposure (Fujimori *et al.*, 1982), the final subcellular distribution of chlordecone is not known. The phenomena that we have described here may especially apply to a vulnerable subpopulation of susceptible nerve endings. Chlordecone, by effecting localized increase of $[Ca^{++}]_i$, might stimulate Ca^{++}-dependent events in such nerve endings. This could account for the higher activity reported specifically for serotonergic and noradrenergic neurons, as deduced by accumulation of neurotransmitter metabolites (Hong *et al.*, 1984; Gandolfi *et al.*, 1984; Chen *et al.*, 1985).

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References

Ashley, R. H., Brammer, M. J. and Marchbanks, R.: Measurement of intrasynaptosomal free calcium by using the fluorescent indicator quin-2. Biochem. J. **219:** 149–158, 1984.

Brinley, F. J.: Regulation of intracellular calcium in squid axons. Fed. Proc. **39:** 2778–2882, 1980.

Carmines, E. L., Carchman, R. A. and Borzelleca, J. F.: Kepone: Cellular sites of action. Toxicol. Appl. Pharmacol. **49:** 543–550, 1979.

Chen, P. H., Tilson, H. A. Marbury, G. D., Karoum, F. and Hong, J. S.: Effect of chlordecone (Kepone) on the rat brain concentration of 3-methoxy-4-hydroxyphenylglycol: Evidence for a possible involvement of norepinephrine system in chlordecone-induced tremor. Toxicol. Appl. Pharmacol. **77:** 158–164, 1985.

Desaiah, D.: Interaction of chlordecone with biological membranes. J. Toxicol. Environ. Health **8:** 719–730, 1981.

Dodd, P. R., Hardy, J. A., Oakley, A. E., Edwardson, J. A. Perry, E. K. and Delaunoy, J. P.: A rapid method for preparing synaptosomes: Comparison with alternative procedures. Brain Res. **226:** 107–118, 1981.

End, D. W., Carchman, R. A., Ameen, R. and Dewey, W. L.: Inhibition of rat brain mitochondrial calcium transport by chlordecone. Toxicol. Appl. Pharmacol. **51:** 189–196, 1979.

End, D. W., Carchman, R. A. and Dewey, W. L.: Neurochemical correlates of chlordecone neurotoxicity. J. Toxicol. Environ. Health **8:** 707–718, 1981.

Fujimori, K., Benet, H., Mehendale, H. M. and Ho, I. K.: Comparison of brain discrete area distributions of chlordecone and mirex in the mouse. Neurotoxicology **3:** 125–130, 1982.

Gandolfi, O., Cheney, D. L., Hong, J. S. and Costa, E.: On the neurotoxicity of chlordecone: A role of γ-aminobutyric acid and serotonin. Brain Res. **303:** 117–123, 1984.

Gray, E. G. and Whittaker, V. P.: The isolation of nerve endings from brain: An electron microscope study of cell fragments derived by homogenization and centrifugation. J. Anat. (Lond.) **96:** 79–88, 1962.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y.: A new generation of Ca^{++} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260:** 3440–3450, 1985.

Herr, D. W., Gallus, J. A. and Tilson, H. A.: Pharmacological modification of tremor and enhanced acoustic startle by chlordecone and p,p'-DDT. Psychopharmacology **91:** 320–325, 1987.

Hong, J. S., Tilson, H. A., Uphouse, L. L., Gerhart, J. and Wilson, W. E.: Effects of chlordecone exposure on brain neurotransmitters: Possible involvement of the serotonin system in chlordecone-elicited tremor. Toxicol. Appl. Pharmacol. **73:** 336–344, 1984.

Hoskins, B. and Ho, I. K.: Chlordecone-induced alterations in content and subcellular distribution of calcium in mouse brain. J. Toxicol. Environ. Health **9:** 535–544, 1982.

Komulainen, H. and Bondy, S. C.: The estimation of free calcium within synaptosomes and mitochondria with fura-2; comparison to quin-2. J. Neurochem. Int. **10:** 55–64, 1987a.

Komulainen, H. and Bondy, S. C.: Increased free intrasynaptosomal Ca^{++} by neurotoxic organometals: Distinctive mechanisms. Toxicol. Appl. Pharmacol., in press, 1987b.

Lichtenstein, D., Kaback, H. R. and Blume, A. J.: Use of a lipophilic cation for determination of membrane potential in neuroblastoma-glioma hybrid cell suspensions. Proc. Natl. Acad. Sci. U.S.A. **76:** 650–654, 1979.

Lievremont, M., Barnier, J. V. and Potus, J.: γ-Hexachlorohexane inhibition of the calcium fluxes at the densensitized mouse neuromuscular junction. Toxicol. Appl. Pharmacol. **76:** 280–287, 1984.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with Folin phenol reagent. J. Biol. Chem. **193:** 265–275, 1951.

Macdonald, R. L.: Barbiturate and hydantoin anticonvulsant mechanisms of action. *In* Basic Mechanisms of Neuronal Hyperexcitability, pp. 361–387, Alan Liss, Inc., New York, 1983.

Marchbanks, R. M.: The chloride content, anion deficit and volume of synaptosomes. J. Neurochem. **25:** 463–470, 1975.

Mishra, S. K., Koury, M. and Desaiah, D.: Inhibition of calcium ATPase activity in rat brain and muscle by chlordecone. Bull. Environ. Contam. Toxicol. **25:** 262–268, 1980.

Nachshen, D. A.: Regulation of cytosolic calcium concentration in presynaptic nerve endings isolated from rat brain. J. Physiol. (Lond.) **363:** 87–101, 1985.

Nachshen, D. A. and Blaustein, M. P.: The effects of some organic "calcium antagonists" on calcium influx in presynaptic nerve terminals. Mol. Pharmacol. **16:** 579–586, 1979.

Narahashi, T.: Nerve membrane ionic channels as the primary target of pyrethroids. Neurotoxicology **6:** 3–22, 1985.

Phillips, D. E. and Eroschenko, V. P.: Effects of the insecticide chlordecone on the ultrastructure of mouse skeletal muscle. Neurotoxicology **6:** 45–52, 1985.

Ramos, S., Grollman, E. F., Lazo, P., Dyer, S. A., Habig, W. H., Hardegree, M. C., Kaback, H. R. and Kohn, L. D.: Effect of tetanus toxin on the accumulation of the permeant lipophilic cation tetraphenylphosphonium by guinea pig brain synaptosomes. Proc. Natl. Acad. Sci. U.S.A. **76:** 4783–4787, 1979.

Scott, I. D. and Nicholls, D. G.: Energy transduction in intact synaptosomes. Biochem. J. **186:** 21–33, 1980.

Singh, G. J. P. and Singh, B.: Cellular lesions in the central nervous system of *Periplaneta Americana* following insecticide treatment *in vitro* and *in vivo*. I. Nerve cell bodies and the neuropile. Neurobehav. Toxicol. Teratol. **6:** 201–213, 1984.

Tilson, H. A., Hong, J. S. and Mactutus, C. F.: Effects of 5,5-diphenylhydantoin (phenytoin) on organochlorine insecticides and permethrin. J. Pharmacol. Exp. Ther. **233:** 285–289, 1985.

Trottman, C. H., Rao, K. S. P., Morrow, W., Uzodinma, J. E. and Desaiah, D.: *In vitro* effects of toxaphene on mitochondrial calcium ATPase and calcium uptake in selected rat tissues. Life Sci. **36:** 427–433, 1985.

Tsien, R. Y., Pozzan, T. and Rink, T. J.: Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new intracellularly trapped fluorescent indicator. J. Cell. Biol. **94:** 325–334, 1982.

Vijverberg, H. P. M. and van den Berken, J.: Action of pyrethroid insecticides on the vertebrate nervous system. Neuropathol. Appl. Neurobiol. **8:** 421–440, 1982.

Willow, M., Kvenzel, E. A. and Catterall, W. A.: Inhibition of voltage-sensitive sodium channels in neuroblastoma cells and synaptosomes by the anticonvulsant drugs diphenylhydantoin and carbamazepine. Mol. Pharmacol. **25:** 228–234, 1984.

Woolley, D. E.: Neurotoxicity of DDT and possible mechanisms of action. *In* Mechanisms of Actions of Neurotoxic Substances, ed. by K. N. Prasad and A. Vernadakis, pp. 95–141, Raven Press, New York, 1982.

Woolley, D. E., Zimmer, L., Dodge, D. and Swanson, K.: Effects of lindane-type insecticides in mammals: Unsolved problems. Neurotoxicology **6:** 165–192, 1985.

Wu, P. H., Phillis, J. W. and Thierry, D. L.: Adenosine receptor agonists inhibit K^+-evoked Ca^{++} uptake by rat brain cortical synaptosomes. J. Neurochem. **39:** 700–708, 1982.

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