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ROLE OF ION CHANNELS AND INTRATERMINAL CALCIUM HOMEOSTASIS IN THE ACTION OF DELTAMETHRIN AT PRESYNAPTIC NERVE TERMINALS

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Abstract—Using a continuous perfusion system, synaptosomes prepared from rat brain released [^{3}H]norepinephrine in a Ca^{2+}-dependent manner when pulse depolarized by briefly elevating external potassium concentrations. Tetrodotoxin (10^{-7} M), a sodium channel blocker, inhibited 48% of this pulsed release, and D595 (10^{-5} M), a phenethylamine-type calcium channel blocker, inhibited 21%. In combination, these two specific ion channel antagonists appear to function independently of each other in an additive fashion. Addition of deltamethrin to this preparation resulted in an enhanced release of [^{3}H]norepinephrine which occurred in a biphasic fashion. At 10^{-7} M, deltamethrin produced a 42% enhancement in the first or initial peak of [^{3}H]norepinephrine release and a 100% enhancement in the second or tailing peak. Addition of deltamethrin to tetrodotoxin-pretreated synaptosomes resulted in a net 37% enhancement of the initial peak release and a net increase of 277% in the tailing peak. Addition of deltamethrin to D595-pretreated synaptosomes produced no significant effect on enhanced [^{3}H]norepinephrine release from either peak. Since tetrodotoxin is a specific sodium channel blocker, deltamethrin may be enhancing [^{3}H]norepinephrine release by increasing the uptake of Ca^2 via other voltage-gated channels (e.g. calcium) or exchange mechanisms in addition to its action at voltage-gated sodium channels. To determine whether deltamethrin may also have an effect on intraterminal Ca^{2+} homeostasis, external Ca^{2+} was replaced with Ba^{2+} and synaptosomes were depolarized with pentylenetetrazole (PTZ). At 10^{-5} M, deltamethrin produced a 66% increase in neurotransmitter release over that produced by PTZ alone. An estimated EC_{50} value of deltamethrin for PTZ-induced release was calculated to be 2.4×10^{-10} M.

The pharmacology of pyrethrum insecticides has held our interest ever since their direct stimulatory effect on nerve tissue was reported by Lowenstein [1]. These natural compounds, which were originally isolated from the flower head of *Chrysanthemum cinerariaefolium*, have potent insecticidal properties but are environmentally benign and practically non-toxic to mammals. The need for photostability led to the development of a vast array of structurally dissimilar "synthetic" pyrethroids which could be used in agricultural and public health settings as replacements for organophosphate and carbamate insecticides. Along with increased longevity, some pyrethroids were found to have greatly enhanced toxicity, particularly in aquatic organisms, and to produce distinctly different types of symptomology of poisoning in mammals. It is now generally agreed that toxic pyrethroid esters that possess both a halogenated acid component and an alpha cyano 3-phenoxybenzyl alcohol (i.e. type II) will induce choreoathetotic writhing and salivation (CS syndrome). These type II pyrethroids are usually more photostable and more potent neurotoxins. Toxic pyrethroids which lack either or both of these two entities (type I) induce tremor (T syndrome) [2–8].

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It has been almost 25 years since the classic electrophysiological studies first described the mode of action of pyrethroids on voltage-dependent sodium channels [9]. In the interval which followed, this approach was enlarged upon using a variety of preparations with essentially the same result [10]. Thus, there is little disagreement that pyrethroids have an action at or near the sodium channel in nerve. Recently, a number of additional mechanisms have been suggested which include the perturbation of other voltage-dependent ion channels such as the voltage-dependent calcium channels [10–15]. Also, from an organismal viewpoint, the variety of toxic symptoms that are produced by pyrethroids makes it difficult to ascribe all of them to a single mechanism [2, 13, 16–18].

The lack of repetitive activity in nerve axons poisoned by the more potent neurotoxic type II pyrethroids, however, seems in contradiction to their obvious convulsive nature [10]. In previous studies from this laboratory [15, 19], it was suggested that type II pyrethroids have a highly sensitive action at presynaptic nerve terminals in the CNS. Using a functional bioassay which consists of a Ca^{2+}-dependent, K^+-stimulated pulsed release of norepinephrine ([^{3}H]NE) from rat brain synaptosomes, type II pyrethroids caused a substantial enhancement of [^{3}H]NE release which was not apparent when type I pyrethroids were applied. The EC_{50} dose of deltamethrin which resulted in half-maximal enhanced [^{3}H]NE release (i.e. 2.9×10^{-9} M) correlated well with the EC_{50} dose of deltamethrin which resulted

Table 1. Representative buffer compositions of external solutions

Solution	NaCl	KCl	D-Glucose	$CaCl_2$ (mmol/liter)	$SrCl_2$	$BaCl_2$	$MnCl_2$
NSM	128	5	16	1	—	—	—
DM	77	56	16	1	—	—	—
SrNSM	128	5	16	—	1	—	—
SrDM	77	56	16	—	1	—	—
BaNSM	128	5	16	—	—	1	—
BaDM	77	56	16	—	—	1	—
MnNSM	128	5	16	—	—	—	1
MnDM	77	56	16	—	—	—	1

In addition to the above, all solutions contained 12 μM nialamide and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES). The solutions were all adjusted to pH 7.35 with Tris base. Abbrevations: NSM, normal superfusion medium; DM, depolarization medium; SrNSM, strontium containing normal superfusion medium; SrDM; strontium depolarization medium; BaNSM, barium containing normal superfusion medium; BaDM, barium containing depolarization medium; MnNSM, manganese containing normal superfusion medium; and MnDM, manganese containing depolarization medium.

in half-maximal enhanced $^{45}Ca^{2+}$ uptake (i.e. 2.4×10^{-9} M). Enhanced release was only evident during pulsed membrane depolarization which indicates that this is a use-dependent phenomenon [19–22]. The relative ability of various type II pyrethroids to enhance monoaminergic neurotransmitter release correlated well with their mammalian oral toxicity and their ability to prolong time constants which describe the exponential decay rate of sodium tail currents via voltage-gated sodium channels [19].

Because release of norepinephrine is highly dependent on Ca^{2+} entering the cytosol of the synaptosome, it is assumed that deltamethrin may act on entities which transport Ca^{2+}, such as the voltage-gated sodium and calcium channels [23, 24] and the Na^+/Ca^{2+} exchanger [24]. In similar studies, norepinephrine was released from rat brain cortex slices by electrical stimulation. This release was shown to be absolutely dependent on extracellular calcium and was partially blocked not only by tetrodotoxin (TTX) but also by the phenethylamine calcium channel antagonist, D600, and by manganese [25].

Likewise, it was shown that acetylcholine is depleted significantly from rat brain after oral administration of deltamethrin. The ability of cismethrin to deplete this neurotransmitter from the CNS is greatly reduced, while DDT results in no depletion whatsoever [26]. Recently, it was found that only the toxic s-acid, s-alcohol isomer of fenvalerate evokes an enhanced neutrotransmitter release in rabbit brain striatal slices [27]. In related studies on insect neuromuscular preparations [28], type II pyrethroids were very potent in increasing the rate of miniature excitatory postsynaptic potentials, but DDT and type I pyrethroids were much reduced in this ability. Furthermore, deltamethrin causes almost complete depletion of synaptic vesicles from presynaptic motor nerve terminals [29].

Finally, depletion of brain norepinephrine stores has been shown to be highly correlated to the convulsive state [30, 31]. Thus, there appears to be a correlation of enhanced [^{3}H]NE release, depletion of presynaptic stores of NE, and the CS convulsive state which are all caused by the action of deltamethrin and other type II pyrethroids.

The present study details those experimental conditions that were used to investigate the relative importance of Ca^{2+} uptake via sodium and calcium channels and what role intraterminal calcium homeostasis may play in the action of deltamethrin at the presynaptic nerve terminals which results in enhanced [^{3}H]NE release.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats, 56- to 60-days-old (250 g), were obtained from the Charles River Breeding Laboratories Inc. (Wilmington, MA).

Chemicals. Deltamethrin ((*S*)-alpha cyano 3-phenoxybenzyl-*cis*-(1*R*,3*R*)-2,2-dimethyl-3-(2,2-dibromovinyl)cyclopropanecarboxylate) was obtained from Dr J. Martel, Centre de Researches, Roussel Uclaf, Romainville, France. D595 (5-[3,4-dimethylphenyl)methyl-amino]-2-(3,4-dichlorophenyl)-2-isopropylvaleronitrile) was a gift of Prof. Kretzchmar, Knoll AG, D-67 Ludwigshafen A/Rhein, West Germany. Pentylenetetrazole (PTZ), tetrodotoxin (TTX), and all other organic or inorganic chemicals were obtained from the Sigma Chemical Co. (St Louis, MO).

Preparation of synaptosomes. Synaptosomes were prepared from whole brains by the method of Whittaker *et al.* [32]. The P_2 fraction (crude mitochondrial fraction) was resuspended in 5.0 ml of 0.32 M sucrose, layered onto a discontinuous sucrose density gradient of 0.32, 0.8, and 1.2 M sucrose, and centrifuged at 50,000 *g* for 1 hr. The material at the 0.8–1.2 M interface was collected by aspiration [33]. This was returned to a more physiological normotonic environment by addition of small volumes of ice-cold normal superfusion medium (NSM, Table 1) over a 30-min period to a final volume equal to four times the collected synaptosomal volume [34]. The equilibrated synaptosomes were then pelleted at 15,000 *g* for 10 min and resuspended in ice-cold NSM. The concentration of protein was set typically between 8 and 11 mg/ml [35].

Analysis of [^{3}H]*NE efflux.* Uptake and release of [^{3}H]NE were accomplished exactly as previously

Table 2. Effects of Ca^{2+} replacement ions on [^{3}H]NE release from rat brain synaptosomes

Solution	[^{3}H]NE release (summation of fractional rate constants)		
	Non-depolarlized fraction total	Depolarized fraction total	Difference due to depolarization
$CaCl_2$	33.4 ± 1.2	43.9 ± 0.3	10.5 ± 1.5
$SrCl_2$	30.1 ± 0.3	42.0 ± 1.0	11.9 ± 0.7
$MnCl_2$	64.5 ± 0.5	69.4 ± 0.9	4.9 ± 0.2*
$BaCl_2$	31.4 ± 5.8	34.5 ± 5.0	3.1 ± 0.8*

Solutions were NSM or DM in which 1 mM of the listed salt replaced 1 mM $CaCl_2$. Non-depolarized fraction total represents a summation of the fractional rate constants for fractions 12 through 20 of the synaptosomes that were not subjected to K^+ depolarization. Depolarized fraction total represents a summation of the fractional rate constants for fractions 12 through 20 of the synaptosomes that were subjected to K^+ depolarization. The fractional rate constant is the amount of [^{3}H]NE released in each 1-min fraction (i.e. 0.5 ml) as a percentage of the radioactivity remaining in synaptosomes during the preceding minute. Difference due to depolarization is the difference in the release of [^{3}H]NE by depolarized synaptosomes compared to non-depolarized synaptosomes in the presence of the same salt. Means (±SE) represent an average of two experiments with four replicates for each salt.

* Significantly different from the percent difference for $CaCl_2$ (i.e. 10.5 ± 1.5) ($P < 0.05$, Student's one-tailed t-test).

described by Brooks and Clark [19]. To study the effect of replacing Ca^{2+} with other divalent cations (Ba^{2+}, Mn^{2+} and Sr^{2+}), the 1 mM $CaCl_2$ in NSM and DM (Table 1) was replaced with either 1 mM $BaCl_2$, $MnCl_2$ or $SrCl_2$. Synaptosomes were then washed, perfused, and K^+-pulse depolarized with these replacement media.

The effects of pretreatment with TTX or D595 were analyzed by adding various TTX or D595 concentrations so their amounts were adjusted to give a final assay concentration when 1.0 μl was added to the 0.1-ml aliquot. Aliquots were pre-equilibrated in this fashion for 10 min on ice prior to addition of [^{3}H]NE.

In some cases, synaptosomes were loaded with [^{3}H]NE, washed, and perfused exactly as above except that Ca^{2+} was replaced with Ba^{2+} and the synaptosomal aliquots were never treated with high K^+-depolarizing medium (DM, Table 1). Instead at min 6 (fraction 6), selected synaptosomal aliquots received BaNSM containing various concentrations of PTZ. After 3 min, PTZ was removed and perfusion continued with BaNSM until 24 fractions (i.e. 0.5 ml per fraction) were collected. Synaptosomal aliquots serving as controls received BaNSM without PTZ for the entire assay. To analyze any synergistic effect between PTZ and deltamethrin, synaptosomes were incubated at 0° with various concentrations of deltamethrin for 10 min prior to loading with [^{3}H]NE. After loading, the synaptosomes were washed and perfused with BaNSM. After 6 min (fraction 6), selected aliquots received BaNSM containing 5×10^{-5} M PTZ. After 3 min, these aliquots received BaNSM without PTZ. Some deltamethrin-treated synaptosomes received only BaNSM without PTZ for the entire assay and served as non-depolarized controls for those assays.

Calculation of [^{3}H]NE released. Release of [^{3}H]NE was expressed as either a fractional rate constant, as fractional rate constant differences, or as a summation of fractional rate constant differences [19]. The fractional rate constant is the amount of [^{3}H]NE released in each 1-min fraction (i.e. 0.5 ml) as a percentage of the radioactivity remaining in synaptosomes during the preceding minute [25, 36–38]. After determining fractional rate constants for all barrels, a fractional average of all fractional rate constants from similarly treated barrels was calculated. The fractional average determined for non-depolarized or non-PTZ-treated barrels was then subtracted from the fractional average determined for K^+-pulse depolarized or PTZ-treated barrels to give a fractional average difference. The fractional average differences for fractions 12 through 20 were summed to give a summation of fractional average differences (e.g. summation of [^{3}H]NE released). These values were used to compare the effects of various nerve convulsants (deltamethrin, PTZ), ion channel blockers (TTX, D595) and divalent cationic calcium replacements (Ba^{2+}, Mn^{2+}, Sr^{2+}).

Addition of ion channel blockers and nerve convulsants. In experiments that used deltamethrin or water-insoluble agents, compounds were solubilized in 95% ethanol. Their amounts were adjusted to give a final assay concentration when 1.0 μl was added to a 0.1-ml synaptosomal aliquot.

Synaptosomes were pre-equilibrated for 10 min on ice prior to the addition of [^{3}H]NE. Ethanol was included in all control tubes and accounted for not more than 1% of the total value. Tetrodotoxin was solubilized in 6.0×10^{-3} M sodium citrate (pH 4.8). This sodium citrate solution was also added to control tubes.

In experiments with deltamethrin, incubation tubes were coated with Carbowax PEG 20000. Insecticides were added to Carbowax-treated tubes and incubated under standard conditions [39]. Solutions were transferred to noncoated tubes prior to loading

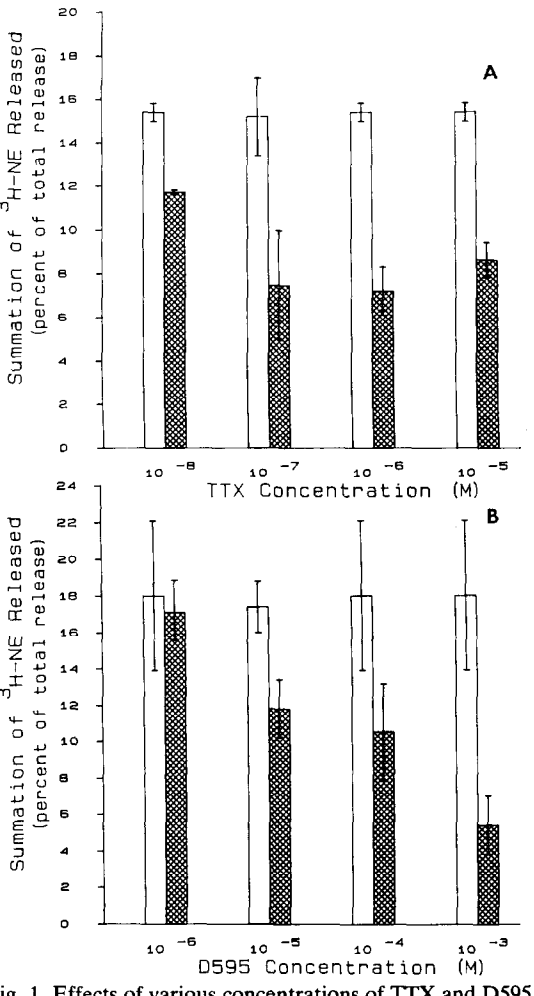


Fig. 1. Effects of various concentrations of TTX and D595 on release of [^{3}H]NE. Synaptosomal aliquots were incubated with either TTX (hatched bars, Fig. 1A), D595 (hatched bars, Fig. 1B) or 1.0 μl of 95% ethanol (open bars, both panels) prior to addition of 0.139 μM [^{3}H]NE. Aliquots were subsequently washed and superfused with NSM. After 6 min (fraction 6), selected aliquots were K^+-pulse depolarized with DM for 2 min. Results are expressed as a summation of [^{3}H]NE released ± SE. Summations exressed are an average of two experiments per concentration, with each experiment consisting of four replicates.

with [^{3}H]NE. This transfer was necessary in that Carbowax (polyethylene glycol) readily absorbs amines such as norepinephrine.

Statistics. *T*-tests and least-squares regression analyses were performed on the Cyber CDC mainframe computer located at the University of Massachusetts using the Statistical Package for the Social Sciences (SPSS).

RESULTS

Cationic specificity of [^{3}H]*NE release.* As illustrated in Table 2, several ions can be used as Ca^{2+} replacements. Sr^{2+} replaced Ca^{2+} effectively showing no significant ($P > 0.05$) reduction in [^{3}H]NE release during K^+-pulsed depolarization. Although Mn^{2+} appeared to significantly ($P < 0.05$) inhibit [^{3}H]NE release (4.9 ± 0.2 compared to 10.5 ± 1.5 for Ca^{2+}), synaptosomes that were not pulse depolarized by high K^+ also released [^{3}H]NE. This is evident in the summation totals for Mn^{2+}-treated, non-depolarized synaptosomes compared to Ca^{2+}-treated synaptosomes. The Mn^{2+} non-depolarized controls released nearly twice as much (64.5 ± 0.5) [^{3}H]NE as Ca^{2+} non-depolarized controls (33.4 ± 1.2). This suggests that Mn^{2+} replacement of Ca^{2+} affected synaptosomal integrity, resulting in "leaky" synaptosomes. Replacement with Ba^{2+} inhibited [^{3}H]NE release by 70% compared to controls (3.1 ± 0.8 vs 10.5 ± 1.5 for Ca^{2+}) but without causing release of [^{3}H]NE from non-depolarized synaptosomes (31.4 ± 5.8 compared to 33.4 ± 1.2 for Ca^{2+}). Therefore, it appears that replacement of Ca^{2+} with Ba^{2+} allows normal [^{3}H]NE uptake but is inhibitory to Ca^{2+}-dependent, K^+-pulse depolarized [^{3}H]NE release.

Effects of channel antagonists on [^{3}H]*NE release.* The results in Fig. 1A show that TTX, a sodium channel blocker, inhibited Ca^{2+}-dependent, K^+-pulse depolarized [^{3}H]NE release. Release of [^{3}H]NE dropped from 15.4 ± 0.4 for controls to 11.7 ± 0.1, 7.5 ± 2.5, 7.2 ± 1.1 and 8.6 ± 0.8 for TTX concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M respectively. TTX inhibition appeared to have reached saturation at 10^{-7} M.

D595, a phenethylamine-type calcium channel blocker, was also found to inhibit [^{3}H]NE release from K^+-pulse depolarized synaptosomes (Fig. 1B). At 10^{-5} M, D595 significantly ($P < 0.05$) inhibited release of [^{3}H]NE, dropping it from 17.4 ± 1.4 for controls to 11.8 ± 1.6 for treated synaptosomes. Increasing the concentration of D595 did not produce any greater inhibition of [^{3}H]NE release until a concentration of 10^{-3} M was achieved. Usually phenethylamines such as verapamil and D600 block calcium currents in nerve at concentrations between 10^{-5} and 10^{-4} M with inhibition of sodium currents occurring at still higher concentrations [40]. The increased potency of D595 on the inhibition of neurotransmitter release from presynaptic terminals may be correlated to its strong negative inotropic effect on cardiac muscle. In myocardium, D595 has been shown to be the most potent negative inotropic agent with an EC_{50} value of 7.9×10^{-7} M compared to 3.5×10^{-6} M for verapamil (i.e. approximately five-times more potent) [41]. It is also interesting that D595 is the only halogen-substituted phenethylamine-type calcium channel blocker of the verapamil class and thus bears this structural similarity with the halogen-substituted type II pyrethroids such as deltamethrin.

The abilities of TTX and D595 to collectively block [^{3}H]NE release during K^+-pulsed depolarization are detailed in Table 3. TTX at 10^{-7} M inhibited 48% of [^{3}H]NE release compared to control synaptosomes. This level of TTX inhibition is consistent with previous findings [42]. At 10^{-5} M, D595 inhibited 21% of [^{3}H]NE release versus control values. Addition of both TTX (10^{-7} M) and D595 (10^{-5} M) inhibited [^{3}H]NE release by 74% compared to controls. Thus,

Table 3. Effect of deltamethrin on [^{3}H]NE release from rat brain synaptosomes treated with TTX or D595

Treatment	[^{3}H]NE release (summation of fractional average differences)		Percentage inhibited
	Control	Treated	
D595	16.7 ± 1.4	13.2 ± 0.6	21 ± 4
D595 + deltamethrin	16.7 ± 1.4	12.6 ± 1.4	24 ± 8
TTX	12.1 ± 2.5	6.3 ± 0.3	48 ± 3
TTX + deltamethrin	12.1 ± 2.5	12.7 ± 3.0	—*
D595 ± TTX	19.8 ± 1.1	5.1 ± 1.4	74 ± 7

Non-treated controls received 1.0 μl ethanol only. Release of [^{3}H]NE from control and treated synaptosomes is expressed as a summation of fractional average differences for fractions 12 through 20 and is calculated as follows: (1) fractional rate constants are determined from the amount of [^{3}H]NE released in each fraction as a percentage of the radioactivity remaining in synaptosomes during the preceding minute; (2) a fractional average of all fractional rate constants from similarly treated barrels is determined; (3) the fractional average determined from non-depolarized barrels is then subtracted from the fractional average determined for K^+-pulse depolarized barrels to give a fractional average difference; (4) the fractional average differences for fractions 12 through 20 are summed to give a summation of fractional average differences. Percentage inhibited refers to the difference between the [^{3}H]NE released by treated synaptosomes compared to control synaptosomes. Values are means (±SE) of two experiments with four replicates. Assay concentrations: D595, 10^{-5} M; TTX, 10^{-7} M; and deltamethrin, 10^{-7} M.

* No significant difference between control and treated ($P > 0.05$, one-tailed t-test).

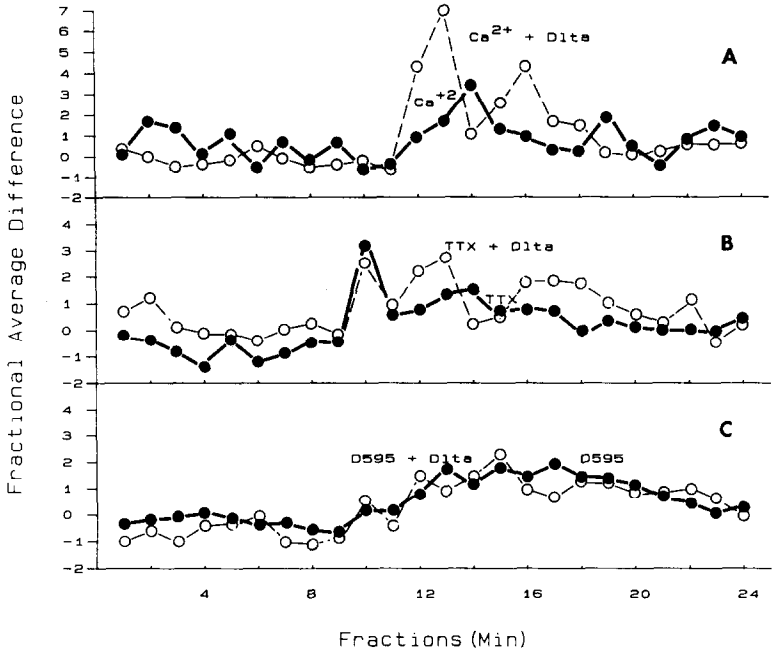


Fig. 2. Effect of deltamethrin on TTX- and D595-influenced [^{3}H]NE release patterns. In panel A, synaptosomal aliquots were incubated with either 10^{-7} M deltamethrin (open circles) or 95% ethanol (solid circles) for 5 min. In panel B, synaptosomal aliquots were incubated with 10^{-7} M TTX for 5 min. In panel C, synaptosomal aliquots were incubated with 10^{-5} M D595 for 5 min. At the conclusion of this period, synaptosomal aliquots in panel A were incubated an additional 5 min while those in panels B and C received either 10^{-7} M deltamethrin (open circles) or 1.0 μl of 95% ethanol (solid circles) for 5 min. Aliquots were then incubated with [^{3}H]NE for 15 min, washed, and perfused with NSM. After 6 min (fraction 6), selected aliquots were pulse depolarized with DM for 2 min. Results are expressed as rate constant differences and represent the average of two experiments, each consisting of four replicates.

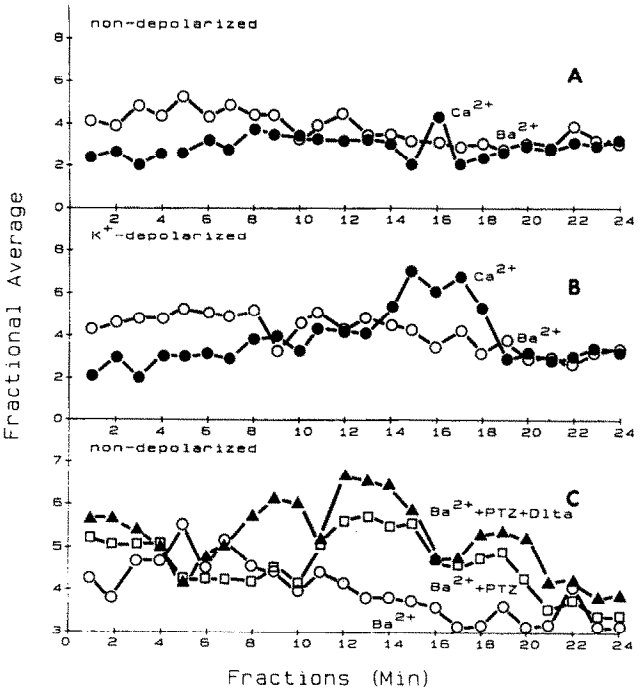


Fig. 3. Effects of Ba^{2+} and Ca^{2+} on K^+-pulse depolarized [^{3}H]NE release and the deltamethrin on PTZ-induced [^{3}H]NE release. Panel A: synaptosomes loaded with [^{3}H]NE were effluxed with either NSM (Ca^{2+}, solid circles) or BaNSM (Ba^{2+}, open circles) for 24 min (24 fractions). Panel B: synaptosomes loaded with [^{3}H]NE were effluxed and pulse depolarized for 2 min with either DM (Ca^{2+}, solid circles) or BaDM (Ba^{2+}, open circles) at min 6 (fraction 6). Panel C: synaptosomes were pretreated (5 min) with either 10^{-5} M deltamethrin (solid triangles) or ethanol (open squares, open circles) before being loaded with [^{3}H]NE and effluxed with BaNSM. At min 6 (fraction 6), selected synaptosomes were pulsed for 2 min with 5×10^{-5} M PTZ (open squares, solid triangles). Other synaptosomes received BaNSM for the entire 24 min collection (24 fractions, open circles).

these agents at the concentrations tested apparently block sodium and calcium channels in an additive fashion independent of each other. Further, it is shown in Table 3 that deltamethrin failed to enhanced release of [^{3}H]NE from D595-pretreated synaptosomes (i.e. from 13.2 ± 0.6 to 12.6 ± 1.4) but did increase [^{3}H]NE release from TTX-pretreated synaptosomes (i.e. from 6.3 ± 0.3 to 12.7 ± 3.0). This amounts to a net increase of 101% of [^{3}H]NE release due to deltamethrin enhancement in the presence of TTX.

The overall release pattern of [^{3}H]NE in the presence and absence of TTX and D595 is graphically illustrated in Fig. 2. Deltamethrin, which enhanced [^{3}H]NE release from untreated (i.e. no TTX or D595 pretreatment) synaptosomes upon depolarization (Fig. 2A), was unable to alter the rate constant difference of D595-pretreated synaptosomes (Fig. 2C) but clearly enhanced the rate of [^{3}H]NE release despite the presence of TTX (Fig. 2B). As in the absence of TTX (Fig. 2A), the addition of deltamethrin in the presence of a saturating concentration of TTX enhanced both the intial peak at fractions 12–15 as well as producing substantial tailing in the following peak (i.e. tailing peak) through fractions 16–20 (Fig. 2B). If each of the two peaks are compared quantitatively (e.g. femtomoles [^{3}H]NE released per milligram of protein per peak), a pattern of enhanced [^{3}H]NE release becomes apparent in which the effect of deltamethrin is most obvious on the increased magnitude of the second or tailing peak. In untreated K^+-pulse depolarized synaptosomes, the initial peak was calculated to be 43 ± 4 fmol [^{3}H]NE released/mg protein and the tailing peak 27 ± 8 fmol (solid circles, Fig. 2A). Addition of deltamethrin (10^{-7} M) resulted in an initial peak of 61 ± 4 fmol (net increase of 42%) and a tailing peak of 54 ± 3 fmol (net increase of 100%) (open circles, Fig. 2A). Pretreatment of synaptosomes with TTX (10^{-7} M) reduced the initial peak to 19 ± 8 fmol (a 56% reduction) and the tailing peak to 13 ± 9 fmol (a 52% reduction) (solid circles, Fig. 2B). Addition of deltamethrin to TTX-treated synaptosomes (open circles, Fig. 2B) enhanced the initial peak to 26 ± 3 fmol (a net increase of 37%) and the tailing peak to 49 ± 5 fmol (a net increase of 277%).

Pretreatment of synaptosomes with D595 (10^{-5} M) reduced the initial peak to 23 ± 10 fmol (a 47% reduction) and the tailing peak to 20 ± 11 fmol (a 26% reduction) (solid circles, Fig. 2C). Addition of deltamethrin to D595-treated synaptosomes (open circles, Fig. 2C) resulted in an initial peak of 24 ± 11 fmol (a net increase of 4%) and a tailing peak of 20 ± 8 fmol (no net change). Consequently, the addition of deltamethrin to D595-treated synaptosomes produced no significant ($P > 0.05$) effect on

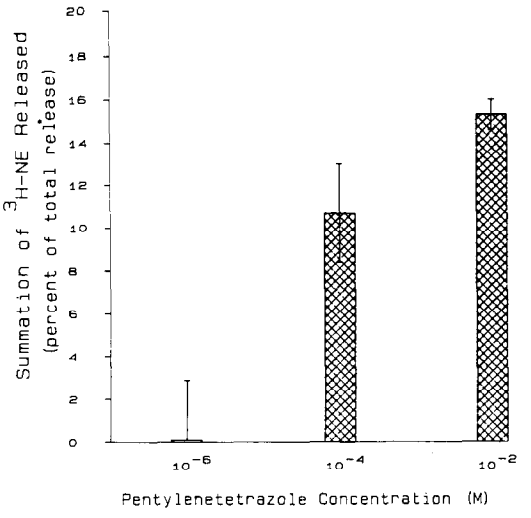


Fig. 4. Effect of PTZ on release of [^{3}H]NE. Synaptosomal aliquots were loaded with [^{3}H]NE, washed, and perfused with medium which replaced Ca^{2+} with Ba^{2+} (BaNSM). At 6 min (fraction 6), selected aliquots were pulsed for 2 min with BaNSM containing various concentrations of PTZ. Results are expressed as a summation of [^{3}H]NE released ± SE (i.e. summation of fractional average differences between barrels receiving PTZ and those which only received BaNSM). Summations are an average of two experiments, each consisting of four replicates.

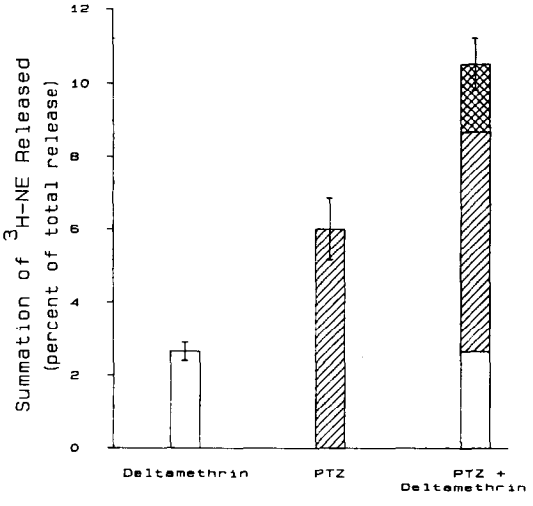


Fig. 5. Effect of deltamethrin on PTZ-induced release of [^{3}H]NE. Synaptosomal aliquots were incubated with either 10^{-5} M deltamethrin or 1.0 μl of 95% ethanol prior to loading with [^{3}H]NE. Aliquots were subsequently washed and perfused with BaNSM. After 6 min (fraction 6), selected aliquots were pulsed for 2 min with BaNSM containing an EC_{50} of PTZ (i.e. 5×10^{-5} M). Results are expressed as a summation of [^{3}H]NE released ± SE. The summation value is an average of two experiments, each consisting of four replicates.

enhanced [^{3}H]NE release. Since TTX is a specific sodium channel blocker, deltamethrin may be enhancing [^{3}H]NE release by increasing the uptake of Ca^{2+} via other voltage-gated channels or exchange mechanism in addition to TTX-sensitive sodium channels. Also, it should be noted that the femtomole values given above compare favorably to the summation of rate constant difference values given in Table 3. The differences in the percentages between the two sets of values are due to the fact that rate constant values take into account variables in synaptosomal load and integrity while the actual femtomole values do not.

Pentylenetetrazole-induced [^{3}H]NE release. To determine whether deltamethrin has an effect on intraterminal Ca^{2+} homeostasis, the convulsant drug PTZ was used as a depolarization agent [43–45]. Besides its well established ability to produce paraoxysmal depolarization shifts in neurons, PTZ also has been implicated in various Ca^{2+}-related cytoplasmic reactions resulting in the release of Ca^{2+} from intracellular stores causing PTZ-induced bursting activity [46–48]. In the present experiments, external Ca^{2+} was replaced with Ba^{2+}. As detailed above, Ba^{2+}-treated synaptosomes load [^{3}H]NE normally but do not release it under these protocols. Thus, any enhancement of [^{3}H]NE release would be expected to be dependent upon the mobilization of intraterminal Ca^{2+} stores necessary for the Ca^{2+}-dependent release of neurotransmitter [23].

The results of these experiments are graphically illustrated in Fig. 3. Comparison of panels A and B shows that the structural integrity of the synaptosomes was maintained during the perfusion period regardless of the substitution of Ba^{2+} for Ca^{2+}. Upon K^+-pulsed depolarization (Fig. 3B), the two characteristic Ca^{2+}-dependent peaks of [^{3}H]NE release occurred (fractions 12–20, solid circles) but were absent in Ba^{2+} substituted buffers (open circles). Addition of PTZ to synaptosomes resuspended in BaNSM (Fig. 3C) induced a released of [^{3}H]NE (fractions 10–21, open squares). This PTZ-induced release of [^{3}H]NE was enhanced if synaptosomes were pretreated with deltamethrin prior to [^{3}H]NE uptake (solid triangles). At 10^{-5} M, deltamethrin produced a 66% increase in [^{3}H]NE release over PTZ-induced release (10.5 ± 0.7 fractional average difference in the presence of deltamethrin vs 6.0 ± 0.9 for PTZ-treated only).

The results in Fig. 4 show that pulsed depolarization with PTZ in perfusion medium where Ba^{2+} had been substituted for Ca^{2+} (i.e. BaNSM) released synaptosomal [^{3}H]NE in a concentration-dependent manner (0.25 ± 2.9 for 10^{-6} M, 10.7 ± 2.3 for 10^{-4} M and 15.3 ± 0.7 for 10^{-2} M). A least-square regression analysis of these data gave an r^2 value of 0.95 with a calculated EC_{50} for PTZ of approximately 5×10^{-5} M. The EC_{50} value is an extrapolated value which woulud release a summation value of 9.0. On a fmol [^{3}H]NE/mg protein basis, a concentration of 5×10^{-5} M PTZ released a total of 49 ± 0.1 fmol [^{3}H]NE.

Pretreatment with deltamethrin (10^{-5} M) resulted in a significantly ($P < 0.05$) greater release of [^{3}H]NE (10.5 ± 0.7) than the additive releases measured in the presence of either PTZ (6.0 ± 0.9) or deltamethrin (2.7 ± 0.3) individually (Fig. 5). This indicates a synergistic action of deltamethrin on PTZ-induced release. Increasing the concentration of deltamethrin increased the amount of PTZ-induced

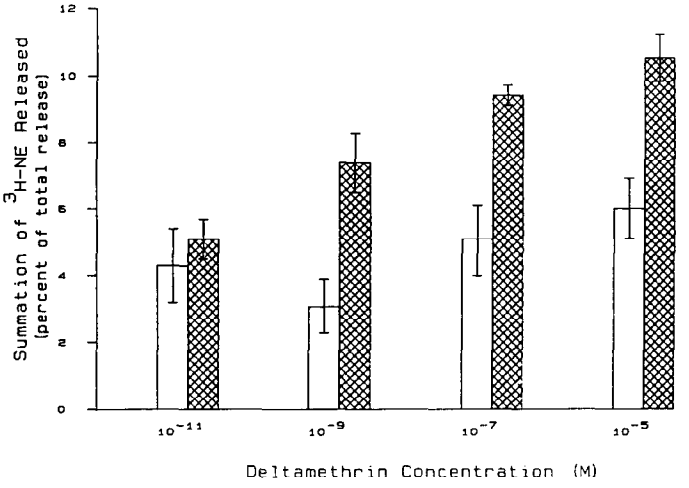


Fig. 6. Enhancement of PTZ-induced release of [^{3}H]NE by increasing concentrations of deltamethrin. Synaptosomal aliquots were incubated with various concentrations of deltamethrin (hatched bars) or with 1 μl of 95% ethanol (open bars) prior to loading with [^{3}H]NE. Aliquots were subsequently washed and perfused with BaNSM. After 6 min (fraction 6), selected aliquots were pulsed for 2 min with BaNSM containing 5×10^{-5} M PTZ. Results are expressed as a summation of [^{3}H]NE released ± SE. Summations are an average of two experiments, each consisting of four replicates.

[^{3}H]NE release in a dose-dependent manner (Fig. 6). Although no significant effect on release ($P > 0.05$) was noted for deltamethrin at concentrations of 10^{-11} M or less (5.1 ± 0.6 compared to 4.3 ± 1.1 for controls), a significant enhancement was noted at 10^{-9} M (7.4 ± 0.9 compared to 3.1 ± 0.8 for controls). Increasing the concentration of deltamethrin above 10^{-9} M failed to change significantly ($P > 0.05$) the difference in PTZ-induced release, indicating saturation above this concentration. From this, the maximum effect of deltamethrin (10^{-9} M) on PTZ-induced [^{3}H]NE release was calculated to be a summation value of 4.08 or an enhanced release of 27 ± 0.1 fmol of [^{3}H]NE/mg protein. The summation values of enhanced [^{3}H]NE release above untreated PTZ-induced values, for various concentrations of deltamethrin (from 5×10^{-11} to 10^{-9} M), were then subjected to a least-squares regression analysis. The regression gave an r^2 value of 0.94 with an estimated EC_{50} value calculated for deltamethrin of 2.4×10^{-10} M. The EC_{50} value is calculated as the extrapolated value which would release a summation value of control plus 2.04.

DISCUSSION

The role of both voltage-gated sodium and calcium channels in the stimulus–secretion coupling mechanism of neurotransmitter release is well established [49]. Voltage clamp experiments have shown that Ca^{2+} enters the neuron in two phases. The early phase is TTX-sensitive and follows the time–course of fast Na^+ current via sodium channels. However, the permeability of the sodium channel for Ca^{2+} is only about 1% of that for Na^+. The latter phase of Ca^{2+} influx is not sensitive to either TTX or tetraethylammonium and, if it undergoes inactivation, it does so more slowly. It is this latter phase of Ca^{2+} entry that is responsible for neurotransmitter release [50].

Invasion of action potentials onto the synaptolemma of presynaptic nerve terminals apparently alters the configuration of channel gating proteins, allowing increased inward Na^+ currents via fast sodium channels and transient membrane depolarization. As the synaptolemma becomes increasingly depolarized, the gating proteins of the "late or slow" calcium channels also become altered, and Ca^{2+} flux increases raising the cytosolic Ca^{2+} concentration. The calcium binding protein, calmodulin, binds Ca^{2+} allowing it to activate a Ca^{2+}/calmodulin-dependent protein kinase (Ca/CaM kinase II). In the presence of ATP, the activated kinase phosphorylates a synaptic vesicle-binding protein, synapsin I, in the tail region which liberates the vesicle. Vesicles freed in this manner can now interact with the synaptolemma and release their neurotransmitter content into the synaptic gap via exocytosis [51].

As previously described, the action of deltamethrin on the enhancement of monoaminergic neurotransmitter release (i.e. [^{3}H]NE) correlates well with $^{45}Ca^{2+}$ uptake into synaptosomes [19] and the present work indicates that other voltage-gated channels (e.g. calcium channel), in addition to the TTX-sensitive sodium channel, may also be involved in this process. Because of this, it was proposed that deltamethrin and other type II pyrethroids may interact with entities which regulate Ca^{2+} flux across the synaptolemma, and possibly on those that control intraterminal calcium regulation (e.g. endoplasmic reticulum and mitochondria). In doing so, deltamethrin and related pyrethroids could produce the convulsive CS syndrome of poisoning by depleting specific brain regions of the CNS of monoamine neurotransmitters in a manner similar in many aspects to epileptic or PTZ-induced seizures. Virtually all of the noradrenergic pathways that have

been studied at this time are efferent pathways of the locus ceruleus to cerebral cortex, cerebellum and hippocampus. The major effect of stimulating these pathways is an inhibition of spontaneous discharges [52]. Depletion of monoamines in the presynaptic processes of these neurons by type II pyrethroids would then be convulsive in nature as spontaneous discharges increase. Recently, a direct excitatory role for released NE on hippocampal pyramidal cells has been established resulting in hyperexcitation by diminishing the slow after hyperpolarization via a Ca^{2+}-activated K^+ channel. Interestingly, the hippocampus plays a key role in epilepsy [53].

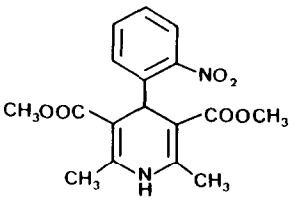
The convulsant drug PTZ has been used extensively in elucidating the mechanism of epileptogenesis and has been shown recently to be involved in the phosphorylation of synapsin I associated with Ca^{2+} influx into synaptosomes [54] and thus could be involved in enhancing the release of neurotransmitters. In experiments that examined the pharmacological effects of deltamethrin on live rats, it was found to prolong the convulsive seizures induced by PTZ and to potentiate PTZ toxicity [55]. The CS syndrome of poisoning produced by type II pyrethroids (e.g. deltamethrin) has many similarities with an epileptic seizure (salvation, choreoathetotic writhing, etc.). Although the exact mode of action has yet to be determined at the cellular level, two mechanisms have been proposed which directly relate to the enhancement of monoaminergic neurotransmitter release by deltamethrin in PTZ-treated synaptosomes. The first possibility is that PTZ is involved in the release of intracellular Ca^{2+} from dense lysosome-like granules [46] or mitochondria [56] during bursting activity. PTZ acts to deplete the granules of Ca^{2+} and alters their structure from dense type to lamella type. In our experiments with PTZ-induced neurotransmitter release, external Ca^{2+} was replaced by Ba^{2+} which serves as a mimic for Ca^{2+} as a charge carrier through voltage-gated calcium channels. Barium, however, does not support the phosphorylation of synapsin I [57] and hence does not support Ca^{2+}-triggered neutrotransmitter release from synaptosomes. Thus, any Ca^{2+}-triggered neurotransmitter release induced by PTZ is assumed to be due to the mobilization of intraterminal stores of Ca^{2+} possibly by a mechanism similar to that described above. Enhancement of PTZ-induced neutrotransmitter release by deltamethrin under these experimental restrictions may indicate an action of type II pyrethroids at cytoplasmic reactions that regulate intraterminal free Ca^{2+} concentrations (e.g. Ca^{2+}-pumps, Na^+/Ca^{2+} exchange, etc.) [58]. Failure to rapidly sequester or efflux Ca^{2+} liberated by the action of PTZ would result in elevated cytosolic Ca^{2+} concentrations and prolonged neurotransmitter release.

A second possibility is that PTZ acts at the voltage-gated sodium channel producing proxysmal depolarizing shifts [44]. This causes increased Na^+ conductance and results in membrane depolarization which allows Ba^{2+} to influx into presynaptic nerve terminals via voltage-gated calcium channels [54]. Increased cytosolic Na^+ and Ba^{2+} are now available for exchange with intraterminal stores of Ca^{2+} (e.g. mitochondria) via Na^+/Ca^{2+} and Ba^{2+}/Ca^{2+} exchangers [59–62]. Increased cytosolic Ca^{2+} is then available to induce neurotransmitter release. In this case, enhanced neurotransmitter release induced by deltamethrin could be explained by an action at either the sodium or calcium channels or both.

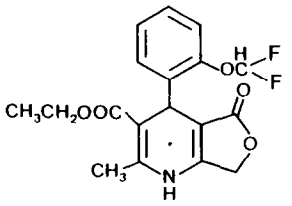
The ability of TTX and D595 to block K^+-stimulated, Ca^{2+}-dependent [^{3}H]NE release in an additive fashion indicates that both sodium and calcium channels are involved in this process and that these two channel antagonists are acting independently of each other at the concentrations tested. It is well established that TTX almost totally abolishes Ca^{2+} uptake and protein phosphorylations stimulated by the addition of veratridine but not by high K^+ or A23187 [42]. Clearly, other ion channels and transporters are being activated by K^+ depolarization that are not activated by veratridine. Further, the stimulatory action of high K^+ on Ca^{2+} uptake occurs almost immediately and is essentially complete within the first 30 sec. The effect of veratridine on Ca^{2+} uptake is slower in onset and requires longer for completion [63]. The addition of deltamethrin in the presence of a saturating concentration of TTX enhanced both the initial peak of [^{3}H]NE release as well as producing highly significant tailing in the second peak. These results are similar to the original observation of Brooks and Clark of the enhancement of [^{3}H]NE release by deltamethrin made in the absence of TTX [19]. Since TTX is an extremely specific sodium channel blocker, deltamethrin is apparently enhancing the uptake of external Ca^{2+} via other channels or exchange mechanisms. The absence of enhancement of [^{3}H]NE release by deltamethrin in the presence of D595, particularly the lack of enhancement of the tailing peak, supports the contention of a significant role in this process of the calcium channel.

It is possible that the Ca^{2+} agonism elicited by deltamethrin as evidenced by enhanced $^{45}Ca^{2+}$ uptake and [^{3}H]NE release is due to its shared structural similarity with D595. There are a number of instances where slight structural alterations reverse the action of a compound from antagonism to agonism. A case in point is the L-type calcium channel antagonist, nifedipine, and its structurally-related dihydropyridine agonist, CGP 28392 (Fig. 7). Whereas CGP 28392 has been shown to increase cytosolic Ca^{2+} levels and enhance cardiac contractability (a positive inotropic effect), nifedipine is completely antagonistic of these actions (a negative inotropic effect) [64]. Apparently the conversion of the antagonistic action of nifedipine to the agonistic action of CGP 28392 on calcium channels is due to an increased electronegatively caused by the addition of halogen atoms (i.e. fluorine) to the benzene ring and to increased bulkiness by the addition of a furan moiety to the pyridine ring of CGP 28392 [65].

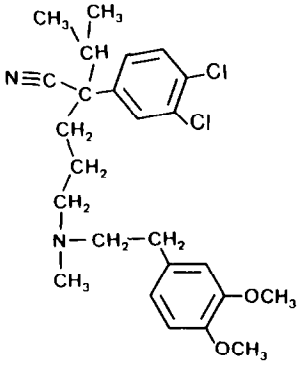
Addition of the structurally-dissimilar calcium channel antagonists, verapamil (phenethylamine-type) and diltiazem (benzothiazepine-type), does not after the effect of CGP 28392, indicating separate sites of action for these different classes of calcium channel antagonists [64]. Also unlike nifedipine, verapamil and other phenethylamine analogs such as D600 and D595 are use-dependent compounds which exert their potent negative inotropic effect



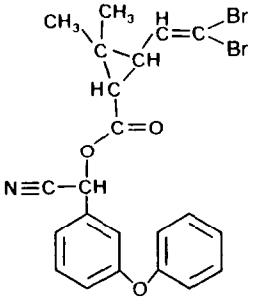
Nifedipine



CGP 28392



D595



Deltamethrin

Fig. 7. Structural configurations of the calcium channel antagonists, nifedipine and D595; the Ca^{2+} channel agonist, CGP 28392; and the pyrethroid insecticide, deltamethrin.

only after depolarization [66]. Deltamethrin also acts as a use-dependent compound in the enhancement of [^{3}H]NE which is in full agreement with other studies [20–22] but has been shown to cause a positive intotropic effect in atrial muscle [12]. The ability of the phenethylamine derivatives to function as calcium channel blockers depends on the inclusion of two benzene or similar carbon ring structures at either end of the molecule and a tertiary amine spaced two carbons from one of the benzyl or ring groups (i.e. phenethylamine). Quarternization of this nitrogen group results in total loss of antagonistic action, whereas substitution within the benzene rings causes a decrease in potency [67].

Overall, there is a great deal of structural and chemical similarity between type II pyrethroids and phenethylamine-type calcium channel antagonists as exampled by verapamil, D600 or D595 (Fig. 7). Although not classically a tertiary amine, the inclusion of a nitrile on the alpha carbon of the phenoxybenzyl alcohol (i.e. alpha cyano group) of the type II pyrethroids does satisfy the tertiary amine requirement of verapamil analogs. Upon close inspection, all type II pyrethroids are, in fact, phenethylamine derivatives of the same class of compounds as the verapamil analogs. Thus, although structurally similar to D595, a phenethylamine-calcium channel blocker which has an extemely potent negative inotropic action, deltamethrin, may act as a Ca^{2+} channel agonist due to the inclusion of a strongly electronegative dibromovinyl moiety and increased bulkiness afforded by its alpha cyano 3-phenoxybenzyl alcohol component in a fashion much the same as discussed above for the dihydropyridine agonist, CGP 28392 [66].

Indeed as pointed out by Triggle [65], only phenethylamine-type organic calcium channel blockers such as verapamil and D595 have a strong, class III action at conducting and nodal tissues. The other three major types of organic calcium channel blockers (e.g. nifedipine, diltiazem and lidoflazine) are greatly reduced in their action on nerve tissues. Additionally, only phenethylamine-type blockers have strong class I action at myocardium and strong class III action at cerebral vasculature. It has been shown previously that deltamethrin has an agonistic action at each of the three sites. Deltamethrin is a potent neurotoxic enhancer of transmitter release from CNS presynaptic nerve terminals [15, 19, 27]. It produces a positive inotropic action at myocardium [12] and results in increased blood flow in cerebral vasculature [68]. Finally, of the three types of calcium channels of chick dorsal root ganglion cells in culture, only the non-inactivating L-type channel is stimulated by the dihydropyridine agonist, Bay K 8644 [69, 70]. In the same nerve preparation, the fast inactivating T-type calcium channel was insensitive to both the dihydropyridine, nifedipine, and the phenethylamine, verapamil. Nevertheless, verapamil is antagonistic to the slowly inactivating N-type calcium channel but nifedipine has no effect [71]. These findings are supportive of a specific interaction

of phenethylamine-type compounds at N-type calcium channels in nerve.

Recently using whole cell patch clamp techniques with neuroblastoma cells (N1E-115), Narahashi [72] showed that type I pyrethroids block both Type I (T-type calcium channels) and Type II (L-type calcium channels) calcium currents. Type II pyrethroids had no effect on either of these two types of calcium channel in the neuroblastoma preparation. Although the action of type I pyrethroids on calcium channels of neuroblastoma cells correlates with our initial findings that type I pyrethroids are not potent enhancers of neurotransmitter release from rat brain synaptosomes [19], they are at odds concerning the action of type II pyrethroids at presynaptic nerve terminals presented in this work. A possible explanation for the discrepancy may lie in the fact that N1E-115 neuroblastoma cells have only L-type and T-type calcium channels and not the intermediately or slowly inactivating N-type calcium channel [73]. In many fully differentiated and functioning neurons, neurotransmitter release is regulated by a Ca^{2+}-triggered event that undergoes slow inactivation, is sensitive to Cd^{2+}, but insensitive to dihydropyridines, which are the same characteristics of the N-type calcium channels [74]. Recently, Hirning *et al.* [75] reported a dominant role of N-type calcium channels in the depolarization-dependent release of NE from sympathetic neurons. They also present the possibility that Ca^{2+} entry through N-type calcium channels may dominate the release of small synaptic vesicles containing cholinergic and monoaminergic neurotransmitters, whereas Ca^{2+} flux via L-type calcium channels may dominate the release of large dense core vesicles containing peptide and hormonal neurotransmitters. If the release of NE is controlled by N-type calcium channels, the lack of effect of type II pyrethroids in the above neuroblastoma preparation may be the result of the apparent absence of N-type channels.

The fact that type II pyrethroids may interact with both the sodium and calcium voltage-gated channels should not be that contradictory to the existing sodium channel theory in that there appears to be a great deal of structural homology between them. As Curtis and Catterall [76, 77] point out, both the isolated sodium channel ionophore [78] and the dihydropyridine calcium antagonist receptor complex of the voltage-gated calcium channel [79] are large membrane glycoproteins of 200–300 kD which consist of one large subunit and two smaller subunits. Indeed, Tanabe *et al.* [80] have reported close structural and primary sequence similarities of the dihydropyridine receptor of the voltage-gated calcium channel to the voltage-gated sodium channel in support of this contention. Such overall structural similarities indicate similar requirements for rapid movement of ions across membranes via voltage-gated ionophores and may indicate similar binding regions for pyrethroids. This apparent commonality in action has already been extended to the phenethylamine class of calcium channel blockers which also have an inhibitory action at other voltage-gated channels including the sodium channel, although at much higher dosages [81–84].

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