PRESYNAPTIC ACTIONS OF INSECTICIDAL DIHYDROPYRAZOLES IN MAMMALIAN BRAIN

by

Aiguo Zhang

B.Sc. Beijing Normal University, 1986

M.Sc. Chinese Academy of Sciences, 1989

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

November 1996

ISBN 0-612-17193-0

SIMON FRASER UNIVERSITY

DEPARTMENT OF BIOLOGICAL SCIENCES

BURNABY, BRITISH COLUMBIA V5A 1S6
Telephone: (604) 291-4475
Fax: (604) 291-3496

Phone: (604) 291-4107
Fax: (604) 291-3496
E Mail: russell_nicholson@sfu.ca

Academic Press, Inc.
Pesticide Biochemistry & Physiology
Copyright Department
Harcourt Brace & Company
525 B Street, Suite 1900,
San Diego, California, 92101-4495
USA

Sept. 13 1996

Dear Sir/Madam,
My graduate student Aiguo Zhang is now writing up his thesis entitled **"Presynaptic Actions of Insecticidal Dihydropyrazoles in Mammalian Brain"** which he will defend for the degree of Ph. D. later this semester (probably in mid-late November). During the course of his research we have published two papers in **Pesticide Biochemistry and Physiology** and a third is in the galley proof preparation stage, as follows:-

A. Zhang and R. A. Nicholson: The Dihydropyrazole RH-5529 Blocks Voltage-Sensitive Calcium Channels in Mammalian Synaptosomes, **45**, 242-247 (1993).

A. Zhang, P. Towner and R. A. Nicholson: Dihydropyrazole Insecticides: Interference with Depolarization-Dependent Phosphorylation of Synapsin I and Evoked Release of L-Glutamate in Nerve Terminal Preparations from Mammalian Brain, **54**, 24-30 (1996).

A. Zhang and R. A. Nicholson: Presynaptic Inhibition of [^{3}H]Nitrendipine Binding and Modification of Membrane Properties by Insecticidal Dihydropyrazoles in Mammalian Brain, (MS # 96-018, in galley proof preparation stage).

For each of the articles above I have signed the copyright to Academic Press Incorporated. I request authorization for A. Zhang to reproduce this copyrighted material in his thesis as detailed below.
The Simon Fraser University Library has also requested that I obtain written permission to allow reproduction of the copyright information in the thesis, and the National Library of Canada to make copies of this thesis available for loan or sale to interested users on request.

PBP 45, 242-247 (1993)
1. Written extract of approximately 90% of Materials and Methods
2. Table 1
3. Fig. 1 (with major modifications)
4. Fig. 3 (with major modifications)
5. Fig. 4 (with minor modifications)

PBP 54, 24-30 (1996)
1. Materials and Methods (entire sections on "Release of L-Glutamate from Synaptosomes", "Labeling of Synaptosomal Proteins using 32Pi" and "Electrophoresis of Synaptosomal Proteins".
2. Fig. 1. (graph only)
3. Fig.2. (graph and histogram to be displayed as two separate figures in thesis)
4. Table 1.

MS#96-018
1. Materials and Methods
2. Figures 1-5 inclusive
3. Tables 1 and 2

Reprints of the published material is enclosed. I look forward to receiving favorable desisions on these requests at your earliest convenience.

Yours Sincerely

Russell A. Nicholson
Associate Professor
Pesticide Toxicology

HARCOURT BRACE

Harcourt Brace & Company
6277 Sea Harbor Drive
Orlando, FL 32887
Tel 407-345-2000

September 20, 1996

VIA FAX ONLY AND REGULAR MAIL

Aiguo Zhang
c/o Russell A. Nicholson, Associate Professor
Pesticide Toxicology, Department of Biological Sciences
Simon Fraser University
Burnaby, B.C. V5A 1S6
CANADA

Re: Request for Permission

Dear Ms. Mr. Zhang:

PERMISSION GRANTED to use articles you have co-written in our publication **Pesticide Biochemistry and Physiology, Volume 45, 242-247 (1993); Volume 54, 24-30 (1996) Article No. 0005; and MS#96-018, in galley proof preparation,** in your doctoral dissertation, provided that 1) complete credit is given to the source, including the Academic Press copyright notice; 2) the material to be used has appeared in our publication without credit or acknowledgment to another source; and 3) if commercial publication should result, you must contact Academic Press again.

We realize that The National Library of Canada must have permission to sell copies of your thesis, and we agree to this. However, we must point out that we are not giving permission for separate sale of your article.

Date: 9/20/96

Ana D. Merced
Paralegal Group
Academic Press, Inc. (Permissions)
Orlando, Florida
(407)345-3994

SIMON FRASER UNIVERSITY

DEPARTMENT OF BIOLOGICAL SCIENCES

BURNABY, BRITISH COLUMBIA V5A 1S6
Telephone: (604) 291-4475
Fax: (604) 291-3496

Phone: (604) 291-4107
Fax: (604) 291-3496
E Mail: russell_nicholson@sfu.ca

ACS Books
1155 Sixteenth Street, N. W.
Washington D.C. 20036
USA

Sept. 13 1996

Dear Sir/Madam,
My graduate student Aiguo Zhang is now writing up his thesis entitled **"Presynaptic Actions of Insecticidal Dihydropyrazoles in Mammalian Brain"** and he will defend for the degree of Ph.D. later this semester (probably in mid-late November). During the course of his research we published an invited paper entitled **"Presynaptic Actions of Dihydropyrazoles"** in the **ACS Symposium Series No. 591, Edited by J. Marshall Clark,** which appeared on pages 44-55 (photocopy enclosed). *Molecular Action of Insecticides on Ion Channels 1995*

For this article I signed the copyright to American Chemical Society, and request authorization for A. Zhang to reproduce copyrighted material in his thesis as detailed below:-

1. Table II
2. Figure 3
3. Figure 6
4. Figure 7
5. Figure 8
6. Table III

The Simon Fraser University Library has also requested that I obtain written permission to allow reproduction of the copyright information in the thesis, and the National Library of Canada to make copies of this thesis available for loan or sale to interested users on request.
I look forward to receiving favorable desisions on these requests at your earliest convenience.

Yours sincerely,

Russell A. Nicholson
Associate Professor
Pesticide Toxicology

TAR
9/24

SIMON FRASER UNIVERSITY

DEPARTMENT OF BIOLOGICAL SCIENCES

BURNABY, BRITISH COLUMBIA V5A 1S6
Telephone: (604) 291-4475
Fax: (604) 291-3496

Phone: (604) 291-4107
Fax: (604) 291-3496
E Mail: russell_nicholson@sfu.ca

Elsevier Science Ltd
Copyright Office
The Boulevard
Langford Lane, Kidlington,
Oxford, OX5 1GB, England

Sept. 13 1996

Dear Sir/Madam,
My graduate student Aiguo Zhang is now writing up his thesis entitled **"Presynaptic Actions of Insecticidal Dihydropyrazoles in Mammalian Brain"** which he will defend for the degree of Ph. D. later this semester (probably in mid-late November). During the course of his research we have published a paper in **Comparative Biochemistry and Physiology** as follows:-

A. Zhang and R. A. Nicholson: RH-3421, a potent dihydropyrazole insecticide, inhibits depolarization-stimulated rises in free [Ca2+] and 45Ca2+ uptake in mammalian synaptosomes, CBP, **108C**, 307-310 (1994).

For this article I signed the copyright to Elsevier Science Ltd. and request authorization for A. Zhang to ~~reproduce copyrighted material in his~~ thesis as detailed below:-

1. Materials and Methods ("45Ca2+ Fluxing Assay" section only)
2. Table 1
3. Fig. 1
3. Fig. 2
4. Fig. 3 (graph only)

The Simon Fraser University Library has also requested that I obtain written permission to allow reproduction of the copyright information in the thesis, and the National Library of Canada to make copies of this thesis available for loan or sale to interested users on request.
A reprint of the paper in question is enclosed. I look forward to receiving favorable desisions on these requests at your earliest convenience.

Yours sincerely,

Russell A. Nicholson
Associate Professor
Pesticide Toxicology

Pergamon Press

Proper Credit Line:

Reprinted by permission of the publisher from (title and author), name of journal, Vol. No., pp. Copyright 19~~9~~7 by Elsevier Science Inc.

Permission to reprint as requested is granted by
Elsevier Science Inc.,
655 Avenue of the Americas, New York, NY 10010.

10/8/96
Date

~~V. Lopez~~
Rights & Permissions

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

PRESYNAPTIC ACTIONS OF INSECTICIDAL DIHYDROPYRAZOLES IN MAMMALIAN BRAIN

Author: _____
(signature)

_____
(name)

DECEMBER 9, 1996
(date)

ABSTRACT

Dihydropyrazoles possess broad spectrum insecticidal activity (Mulder *et al.*, Naturwissenschaften, **62**: 531-532, 1975) and dihydropyrazole RH-3421 is extremely active (Jacobson, In *Advances in the Chemistry of Insect Control*, Oxford, England, 1989). In mammals RH-3421 inhibits sodium channel-dependent release of the neurotransmitter γ-aminobutyric acid (Nicholson and Merletti, Pestic. Biochem. Physiol., **37**: 30-40, 1990), sodium channel-dependent sodium influx (Deecher and Soderlund, Pestic. Biochem. Physiol., **39**: 130-137, 1991) and causes a delayed neurological syndrome (Salgado, Mol. Pharmacol., **41**: 120-126, 1992). At present, there is no toxicological information available in mammals for RH-5529, a less insecticidal analog. I examined the action of these two dihydropyrazoles (RH-3421 and RH-5529) on changes induced by opening sodium channels (with veratridine) or calcium channels (with K^+) in synaptosomes isolated from mouse brain. My studies on evoked release of L-glutamate showed dihydropyrazoles not only to block veratridine-induced release effectively but also to suppress the K^+-mediated (tetrodotoxin-insensitive) component of neurotransmitter efflux. Moreover, both dihydropyrazoles inhibited veratridine-stimulated increases in free [Ca^{++}] and phosphorylation of synapsins Ia and Ib. Potassium-induced rises in synaptosomal free [Ca^{++}] and phosphorylation of synapsins, both of which require external Ca^{++} and are unaffected by tetrodotoxin, are also blocked by RH-3421 and RH-5529 in a dose-dependent fashion. K^+-stimulated $^{45}Ca^{++}$ uptake by synaptosomes is not changed by tetrodotoxin, but is suppressed by Co^{++} and inhibited by RH-3421 and RH-5529. In non-depolarized synaptosomes, RH-3421 and RH-5529 do not change the concentration of free Ca^{++}, basal $^{45}Ca^{++}$ uptake, L-glutamate release, or the level of phosphorylation of synapsin I. I used a radioligand binding technique to show that dihydropyrazoles

displace [^{3}H]nitrendipine (an L-type Ca^{++} channel probe) in synaptosomal preparations by a non-competitive mechanism, however, kinetic experiments confirmed that allosteric interactions were not involved. Fluorescence assays with region specific probes suggest that dihydropyrazole-induced perturbations of surface lipids in synaptosomal membranes may influence the density of [^{3}H]nitrendipine binding sites on L-type calcium channels.

This investigation therefore provides several lines of evidence to support the hypothesis that in addition to their established inhibitory effect on sodium channels, the dihydropyrazoles RH-3421 and RH-5529 block calcium channels in mammalian brain. These dihydropyrazoles affect calcium channels and disturb neuronal membrane lipids at higher concentrations than those required to inhibit sodium channels. Nevertheless, the cellular targets identified in this study may be important in explaining the unusual neurological syndrome observed in mammals exposed to RH-3421.

DEDICATION

TO MY PARENTS AND MY WIFE

ACKNOWLEDGMENTS

I wish to express my gratitude and appreciation to Dr. Russell A. Nicholson, my indefatigable senior supervisor, for patiently guiding me and encouraging me through this project. I would like to thank Dr. Kerry R. Delaney, Dr. A. H. Jay Burr and Dr. Peter Belton, my committee supervisors, for their valuable advice on matters relating to research.

I also wish to express my sincere gratitude to Dr. Jeffrey R. Bloomquist and Dr. Christopher J. Kennedy for their kindness in evaluating my thesis and serving on my thesis defense committee as external examiner and public examiner, respectively.

I would like to thank Dr. Victor L. Bourne for his kind assistance with the electron microscope work and the preparation of photographs for my thesis.

Special thanks go to my wife, Rebecca Hong Lu, for her continuous encouragement and support all the way through my Ph. D. program.

Acknowledgments are extended to the following organizations for permission to reproduce our published materials as list below:-

a) Figs 2.15, 4.4, 4.5, 4.7, 5.1, 5.2, 5.3, 5.5, 6.1, 6.2 and
Tables 3.1, 6.1, 6.2; copyright to Academic Press.

b) Figs 2.16 and 2.17; copyright to Pergamon Press.

c) Figs 2.8, 2.9, 2.10, 2.14 and Table 2.2; copyright to ACS Press.

TABLE OF CONTENTS

Chapter 2

The Effect of Dihydropyrazoles on Depolarization-Induced Changes to Intraterminal Free [Ca^{++}] and Depolarization-Coupled Uptake of $^{45}Ca^{++}$ in Mouse Brain Synaptosomes

Chapter 3

Interference with Depolarization-Dependent Phosphorylation of Synapsins Ia and Ib by Dihydropyrazoles

Chapter 4

Blockade of Depolarization-Coupled Release of L-glutamate from Synaptosomes Isolated from Mammalian Brain

Chapter 6

Alteration of Synaptic Plasma Membrane Properties by Insecticidal Dihydropyrazoles

Chapter 7

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BTX	batrachotoxin
BTX-B	batrochotoxinin A 20-α-benzoate
DMSO	dimethylsulfoxide
DPH	1,6-diphenyl-1,3,5-hexatriene
EC_{50}	concentration that is effective in producing a 50% change in response
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-*bis*-(β-aminoethylether) N, N, N', N' -tetraacetic acid
GABA	γ-aminobutyric acid
IC_{50}	concentration required to caused 50% inhibition
Me	CH_3
NOEL	no observable effect level
NSF	N-ethylmaleimide sensitive fusion protein
SASR	slowly adapting stretch receptor
SDS	sodium dodecyl sulfate
SNAPS	synaptosomes-associated protein
TMA-DPH	1-(4-trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene
TTX	tetrodotoxin
VTD	veratridine

Chapter 1. Introduction

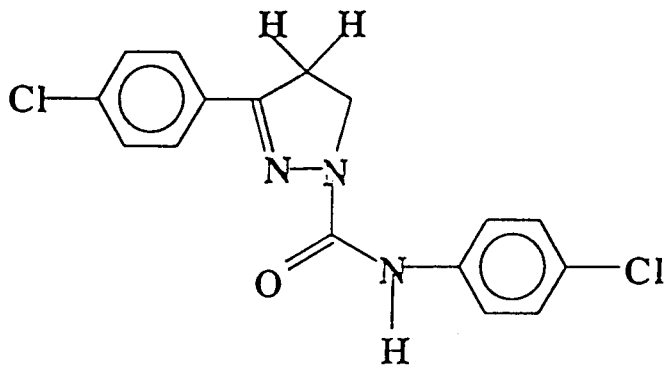
1.1 The Development of Insecticidal Dihydropyrazoles

The recent focus on the dihydropyrazole series of insecticides by the Rohm and Haas Laboratories can be traced to the original discovery by Mulder and co-workers of the Philips-Duphar Laboratories that addition products of 3-aryl-2-pyrazolines and phenyl isocyanates possess high insecticidal activity (Mulder *et al.*, 1975).

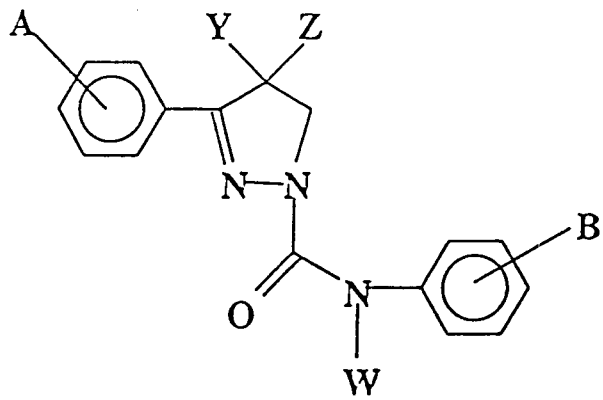
Within the group of early pyrazolines developed by Philips-Duphar, PH 60-41 (Fig. 1a) stood out as a broad spectrum insecticide having particular efficacy against lepidopterous and coleopterous species (Mulder *et al.*, 1975; Wellinga *et al.*, 1977; Van Hes *et al.*, 1978). The impaired posture and coordination reported in insects poisoned with PH 60-41 or other pyrazoline analogs (Mulder *et al.*, 1975; Wellinga *et al.*, 1977) suggested that these compounds may be neuroactive.

Laboratory tests demonstrated PH 60-41 to be highly insecticidal both by contact and as a stomach poison, however, no evidence for systemic activity in plants was observed (Mulder *et al.*, 1975). In spite of a very encouragingly low acute mammalian toxicity (LD_{50} > 3160 mg/kg by oral or intraperitoneal route) and a total absence of phytotoxicity (Mulder *et al.*, 1975), two problems with early pyrazolines were found which made the series unsuitable for commercial development. Firstly, the pyrazoline ring was shown to be highly susceptible to photoaromatization under field conditions, rendering these compounds vulnerable to photodegradation with concomitant loss of biological activity (Sheele, 1980). Secondly, other investigations revealed that when incorporated into soil the dissipation rate of pyrazolines can be unacceptably long (for example $t_{1/2}$ = 17 months, Fuhr *et al.*, 1980).

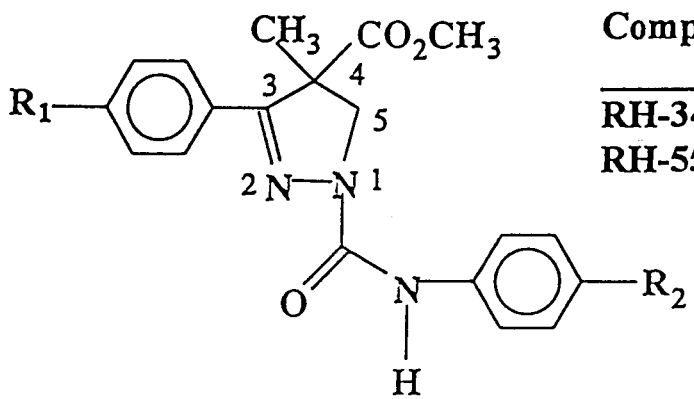
a)



b)



c)



Compound	R_1	R_2
RH-3421	Cl	CF_3
RH-5529	Cl	H

Fig. 1 a) The structure of the pyrazoline PH 60-41
b) Regions of substitution (**A**, **B**, **W**, **Y** and **Z**) assigned to the molecule to assist optimizing of insecticidal activity (Jacobson, 1989)
c) The structure of dihydropyrazoles RH-3421 and RH-5529.

Encouraged by the exceptional potency of some of the Philips-Duphar pyrazolines as agents for the control of arthropods, Jacobson and colleagues at the Rohm and Haas Laboratories embarked on a program of synthetic chemistry aimed at exploring structure/insecticidal activity relationships in greater depth. In addition, they hypothesized that photoaromatization could be eliminated by disubstitution at carbon-4 of the nitrogen-containing ring.

The finding that there was minimal interaction between the five regions assigned for substitution on the molecule (**A**, **B**, **W**, **Y** and **Z**, Fig. 1b), greatly facilitated the investigation (Jacobson, 1989). This meant that the substituent that gave the best insecticidal activity in a particular region remained the best substituent as other regions of the molecule were optimized. At the iso-cyanate-derived phenyl ring (ring **B**), a single electron withdrawing substituent at the *para* position produced the highest insecticidal activity. *p*-CF_3 and *p*-OCF_3, for example, supported high insecticidal potency, followed by *p*-Cl. Insecticidal activity was greatly diminshed or lost with *ortho*- or *meta*-substitution, 3,4- or 3, 5-disubstitution and also when no substituent was present. Insecticidal activity could be maintained with a variety of different substituents at ring **A**, including electron withdrawing and donating groups, however, *para*-chloro-substitution was superior. Data on the amide nitrogen-substituted analogs (**W** position) indicated that insecticidal activity could be retained with a variety of functional groups, although contact activity is lower compared to the unsubstituted compounds. At **Y** only small substituents (e. g. H and Me) were tolerated. Analogs with a carbo-methoxy group at **Z** were the most significant in terms of eliminating the photo-aromatization problem, promoting good insecticidal activity, and imparting a faster degradation rate in soil compared to the PH 60-41 prototype. Optimization by Jacobson (1989) resulted in RH-3421 (Fig. 1c.), a dihydropyrazole, which had a soil $t_{1/2}$ of approximately 30 days.

1.2 Toxicology and Mechanism of Action of Dihydropyrazoles

1.2.1 Insecticidal Properties

RH-3421 shows high activity against a variety of insect species including Lepidoptera, Coleoptera, Diptera, Orthoptera, Hymenoptera and Homoptera. Two economically important pests, the Southern Army Worm (*Spodoptera eridania*) and Mexican Bean Beetle (*Epilachna varivestis*), are particularly sensitive to RH-3421 as indicated by LC_{50}s of 1.9 and 0.16 ppm respectively. RH-3421 is also particularly toxic towards beneficial insects such as bees (LD_{50} = 60 ng/insect); however, this compound is considerably less acutely toxic to *Daphnia*, a representive aquatic arthropod (Jacobson, 1989).

A more detailed examination of the toxicity of dihydropyrazoles in insects has been conducted by Salgado (1990). When RH-3421 (4.4 μg) is administered to *Periplaneta americana* by injection it elicits unusual and complex poisoning symptoms, which initially involve incoordination and tremor. After a few hours, very little movement is observed and the insect eventually develops a condition which has the outward appearance of full paralysis however, during this phase, tactile stimulation will elicit an intense tremor with leg extensions. This quasi-paralysis continues until death occurs between four and six days after the initial exposure (Salgado, 1990). At lower injected doses (0.5 μg of RH-3421) an identical sequence of poisoning occurs, however symptoms are delayed by several hours. RH-5529 (Fig. 1c) is a weaker insecticide than RH-3421 as indicated by studies conducted in our laboratory that show injection of RH-5529 at 10 μg and above into *Acheta domesticus* causes dose-dependent mortality which is preceded by the same poisoning symptoms described for RH-3421 (Zhang and Nicholson, 1993).

Compared to other neurotoxic insecticides, poisoning with dihydropyrazoles and the closely related pyrazolines is particularly slow in insects (Salgado 1990;

Mulder *et al.*, 1975). It remains to be established whether the extended time to end point is due to a novel action of dihydropyrazoles or a slow accumulation at critical sites of action in the nervous system.

1.2.2 The Mechanism of Dihydropyrazole Poisoning in Invertebrates

There is good evidence that loss of spontaneous activity in sensory and central neurons is responsible for the paralysis observed during poisoning. *In vitro* recordings from the crural nerve, cercal nerve and connectives running between the meso- and meta-thoracic ganglia reveal a total inhibition of spontaneous activity three hours after injection of 4.4 μg RH-3421 (Salgado, 1990). When all spontaneous traffic in the crural nerve had been blocked by RH-3421, physical stimulation of the trochanter was still capable of inducing motor spikes even in insects that had been paralyzed for long periods. It is therefore clear that certain reflex loops remain functional when all spontaneous activity has ceased. This condition has been suggested as an explanation for limb and appendage movement in quasi-paralysed insects (Salgado, 1990).

Important insights into the mechanism of dihydropyrazole-induced nerve blockade in invertebrates were provided by experiments on the crayfish slowly adapting stretch receptor (SASR) and crayfish giant axons (Salgado, 1990). Current clamp recordings made on the cell body of the SASR confirmed that dihydropyrazoles did not affect the resting potential but caused an inhibition of spike initiation (induced by current injection), suggesting that the sodium current was blocked (Salgado, 1990). The fact that blockade was voltage-dependent led to the proposal by Salgado (1990) that dihydropyrazoles act in a way similar to sodium channel-directed local anesthetics, Class I anticonvulsants and Class I antiarrhythmics. This possibility was reinforced by later experiments using crayfish giant axons under voltage clamp conditions (Salgado, 1992) which

showed that dihydropyrazoles bind to an inactivated state of the sodium channel (as has been proposed for some local anesthetics), although association kinetics were considerably slower.

1.2.3 Vertebrate Toxicology of Dihydropyrazoles

A moderate amount has been published on the toxicology of the more prominent dihydropyrazoles in vertebrates. As previously stated, the highly insecticidal pyrazoline PH 60-41 is only weakly toxic to mice when acutely administered by gavage or intraperitoneal injection (LD_{50} > 3160 mg/kg, Mulder *et al.*, 1975). A low acute oral and acute dermal toxicity was subsequently reported for RH-3421 in the rat and rabbit where LD_{50}s exceed 5,000 and 1,000 mg/kg respectively (Jacobson, 1989). Unfortunately no information is available on the symptoms or the timecourse of poisoning in mammals following acute dosing with RH-3421. The potential of RH-3421 to cause eye or skin irritation is very low and RH-3421 has no mutagenic or teratogenic effects (Jacobson, 1989). This compound appears to be moderately toxic to birds (LC_{50} > 500 ppm), however fish are extremely susceptible to RH-3421 under static test conditions (96 hr LC_{50} = 0.04 - 0.11 mg/kg). Without doubt, the single most important factor preventing the development of RH-3421 and close analogs as commercial insecticides has been their unfavorable toxicities to mammals when administered sub-acutely in the diet. For example, rats exposed to dietary RH-3421 (400 ppm) eventually develop a variety of symptoms including ataxia, lethargy and pacificity, with occasional tremorigenic and convulsive episodes (Salgado, 1992; Dr. V. Salgado personal communication to Dr. R. Nicholson, 1990). At 400 ppm, RH-3421 is lethal to rats, with death occuring within one to two weeks. The poisoning has been classified as a "delayed onset neurological syndrome" (Salgado, 1992). In sub-acute investigations using rodents the No Observable Effect Level (NOEL) of dietary

RH-3421 has been estimated at 10 ppm (Dr. V. Salgado personal communication to Dr. R. Nicholson, 1990). While it is possible that the NOEL could be considerably lower than 10 ppm in dietary trials of more extended duration, it is clear that RH-3421 has the potential to act as chronic cumulative toxicant in situations where mammals are receiving continuous dietary exposure. The sub-acute toxicological profile of RH-3421 is entirely consistent with this compound's high lipophilicity and limited potential for biodegradation and elimination in mammals.

1.2.4 The Interaction of Dihydropyrazoles with Mammalian Brain

Using superfused synaptosomes prepared from mammalian brain, Nicholson and Merletti (1990) found that release of the inhibitory neurotransmitter (γ-aminobutyric acid) was blocked by dihydropyrazoles (RH-3421 and RH-5529) when elicited by the sodium channel activators veratridine (VTD), *Leiurus quinquestriatus* venom and deltamethrin. In these experiments the dihydropyrazoles inhibited release of γ-aminobutyric acid (GABA) in a similar fashion to tetrodotoxin (TTX), a highly selective blocker of voltage-sensitive sodium channels (Narahashi, 1974). Blockade of activator-induced release of neurotransmitter by dihydropyrazoles occured at sub-micromolar concentrations and in all cases RH-3421 was more effective as an inhibitor than RH-5529. These results were the first to implicate the voltage-sensitive sodium channel in mammalian brain as an important site of action for these insecticides. In these studies Nicholson and Merletti, (1990) also noted that 1 μM RH-3421 and RH-5529 slightly reduced spontaneous release of γ-aminobutyric acid from superfused resting synaptosomes.

In mammals, the uptake of ^{22}Na into brain microvesicles that occurs during activation of voltage-sensitive sodium channels is also inhibited by RH-3421

(Deecher *et al.*, 1991a). In subsequent binding experiments with [^{3}H]batrachotoxinin A 20-α-benzoate (BTX-B) and mouse brain vesicles, Deecher and colleagues discovered that RH-3421 interacts with a site on the voltage-sensitive sodium channel that is allosterically coupled to the BTX-B binding site (Site 2 activator recognition site). This result, together with the non-competitive nature of dihydropyrazole-mediated inhibition of the Site 2 ligand, suggested that RH-3421 had a binding profile similar to local anesthetics, class I anticonvulsants and class I antiarhythmics at the sodium channel (Deecher *et al.*, 1991b).

As stated by Salgado (1992) it is not certain to what extent the inhibition of sodium channels contributes to the delayed onset neurological syndrome in mammals. As far as the author is aware, there are no literature reports of this syndrome occuring in animals receiving anesthetic or therapeutic drugs which allosterically block BTX-B binding to Site 2 on the sodium channel. Clearly, the delayed onset neurological syndrome is complicated and there is a real possibility that interactions with other molecular and cellular targets (in addition to voltage-sensitive sodium channels) contribute to neurotoxic symptoms in mammals.

An indication of an alternative target for insecticidal dihydropyrazoles was provided by studies on the release of GABA by synaptosomes (Nicholson and Merletti, 1990), where it was found that dihydropyrazoles were not acting as totally selective sodium channel blockers and may be interfering with the operation of voltage-sensitive calcium channels or the transmitter release process at a stage subsequent to calcium influx.

1.2.5 Experimental Approach and Objectives of the Present Research

My experimental approach throughout the investigations decribed in this thesis has been to use synaptosomes (pinched-off nerve endings) isolated from mamma-

lian brain. Synaptosomes display many of the properties attributed to nerve terminals of functionally intact brain (De Belleroche and Bradford, 1975; Marchbanks and Campbell, 1976; Dodd *et al.*, 1981). Moreover, the relative ease with which specific cellular processes involved in the release of neurotransmitters can be pharmacologically activated in synaptosomal preparations makes this preparation ideal for investigating the presynaptic actions of inhibitory compounds like the dihydropyrazoles. Also, being virtually cellular entities, synaptosomes contain an abundance of possible neuronal sites of attack by neurotoxic chemicals. It could be argued that this feature increases the chances of identifying novel, toxicologically relevant sites of action, so offering advantages over less complex systems.

The underlying aim of the research described in this thesis was to examine the hypothesis that voltage-sensitive calcium channels in nerve terminals derived from mammalian brain are affected by insecticidal dihydropyrazoles. Particular emphasis has been given to assessing this possibility by indirect and direct approaches. The former utilizes functional biochemical assays and provides the opportunity to establish whether interference with critical cellular processes involved in transmitter release by dihydropyrazoles is (or is not) consistent with a primary action on voltage-sensitive calcium channels. On the other hand, direct approaches provide the means to derive specific information on the identity of the molecular target (e.g. calcium channel type) involved and on the particular mechanism(s) by which dihydropyrazole-induced pertubations to the target occur. So, with the philosophy behind the general aims of this research outlined, the following specific experimental objectives were formulated:-

1) Define the effects of dihydropyrazoles on the depolarization-dependent rise in intraterminal free calcium ion ($[Ca^{++}]_{free}$) concentration and depolarization-stimulated radiocalcium ($^{45}Ca^{++}$) uptake into mouse brain synaptosomes.

2) Investigate the effects of dihydropyrazoles on depolarization-coupled and Ca^{++}-dependent phosphorylation of synapsin I in synaptosomes isolated from mammalian brain.

3) Determine the action of dihydropyrazoles on sodium- and calcium-channel dependent release of L-glutamate from synaptosomes.

4) Characterize the binding of dihydropyrazoles to calcium channels in mouse brain synaptosomes and establish the mechanism involved.

5) Examine the changes in membrane fluidity and lipid order following exposure of synaptic membranes to dihydropyrazoles.

6) Use biochemical and pharmacological data to develop a coherent explanation for the actions of dihydropyrazoles on presynaptic nerve endings and the toxicological relevance of the molecular/cellular targets proposed.

1.3 The Interactions of Natural Toxins, Pesticides and Drugs with Sodium and Calcium Channels

1.3.1 Sodium Channels

The voltage-dependent sodium channel is responsible for the generation of action potentials in most neurons and many other excitable cells (Hille, 1992). All sodium channels possess an α subunit that is approximately 1,800-2,000 amino acids in length (Catterall, 1993). Over the past decade, it has become evident that brain sodium channels have a heterotrimeric composition with α, β-1 and β-2 subunits. Furthermore, it has now been clearly shown that the protein structure forming the α subunit is sufficient to form a functional Na^+-specific pore and contains a number of pharmacologically-specific receptor sites (Catterall, 1993;

Strichartz *et al.*, 1987; Clark, 1995). Of particular interest are the findings that a variety of neurotoxins and drugs affect one or more of the three essential functional properties of sodium channels namely voltage-dependent activation, inactivation, or selective ion conductance. Some of these substances bind with high affinity and specificity to recognition sites on sodium channels, and some have consequently been exploited as molecular probes to identify the protein components of sodium channels (Catterall, 1988). Thus far, at least five neurotoxin groups have been identified which act at distinct recognition sites on the Na^+ channel (Catterall, 1988).

Site 1, on the extracellular opening of the channel, is affected by water soluble heterocyclic guanidines, including TTX and saxitoxin, leading to block of neuronal Na^+ ion conductance (Narahashi *et al.*, 1966; Catterall, 1988). TTX occurs normally in the ovaries and liver of puffer fish of the suborder *Gymnodontes* (Brown and Mosher, 1963). A selective action of TTX has also been reported in myelinated nerve, in skeletal muscle, and in a number of other excitable cells in which inward Na^+ currents are important in the generation of action potentials (Narahashi *et al.*, 1974). The inhibition of sodium currents by TTX is slowly reversible (Nakamaura *et al.*, 1965). Saxitoxin is biosynthesized by dinoflagellates of the genus *Gonyaulax* (Kao, 1966) and is commercially available in radioligand form. Binding data have established that this toxin inhibits sodium channels by associating with the same receptor site as TTX (Barnola *et al.*, 1973).

Site 2, which is thought to be localized on the inside region of the sodium channel, binds several lipid-soluble toxins, such as batrachotoxin (BTX), VTD, grayanotoxin and aconitine, all of which elicit persistent activation of voltage-sensitive sodium channels at resting membrane potential by blocking sodium channel inactivation (Catterall, 1988; Hille, 1992). It is clear that the alkaloid neurotoxins have the ability to depolarize resting nerve by causing sodium

channels to open and remain open, although precise mechanisms are subtly different. BTX, which is isolated from the skin of the Colombian frog, (*Phyllobates aurotaemia*), modifies Na^+ channels irreversibly in nerve and muscle preparations (Albuquerque and Daly 1976; Khodorov and Revenoko, 1979). At resting membrane potentials, BTX binds to Na^+ channels extremely slowly, however, this interaction occurs much faster when the membrane is depolarized (Khodorov and Revenko, 1979) leading to Na^+ channel activation. In addition to modifying gating kinetics, BTX reduces ion selectivity in favour of increased Ca^{++} flow (Khodorov, 1978). Recently, a radiolabeled BTX derivative, BTX-B has been discovered as a new radioligand for the voltage-dependent Na^+ channel (Brown *et al.*, 1981). VTD is isolated from plants of family of *Lilaceae* and is one of the more potent alkaloid compounds acting on sodium channels (Ulbricht, 1969). The modification of Na^+ channels by VTD is quite different from BTX. It causes Na^+ channels to open under rest conditions and the effect becomes more pronounced as the membrane depolarizes (Ulbricht, 1969; Catterall, 1975). Inactivation of the channels modified by VTD is found to be slowly reversible (Leicht *et al.*, 1971; Sutro, 1986).

Neurotoxin binding Site 3 is located on a region of the sodium channel that undergoes a conformational change during voltage-dependent channel activation. Polypeptide α-scorpion toxins and sea anemone toxins selectively bind to Site 3 resulting in a progressive block of sodium channel inactivation (Catterall, 1977; Catterall, 1992) which in turn increases sodium permeability and prolongs the duration of action potentials (Adam *et al.*, 1966). The binding of scorpion toxin to its receptor site is highly voltage-dependent (Stallcup, 1977). As one might predict α-scorpion toxins derived from North African scorpions (Rochat *et al.*, 1970) are capable of causing secretion of neurotransmitters, arrhythmias in heart, and repetitive firing and depolarization in nerve (Catterall, 1980).

Site 4 is located in a similar region to receptor site 3 of sodium channel but binds a different class of scorpion toxin (β-scorpion toxins) (Angelides *et al.*, 1985; Catterall, 1992). Without changing Na^+ channel inactivation, these toxins shift the voltage-dependent activation to more negative membrane potentials (Couraud *et al.*, 1982; Catterall, 1992). β-Scorpion toxin was orignally found in crude venom from *Centruroides sculpturatus* scorpion (Cahalan, 1975). The β-toxin acts on depolarized nerves to accelerate Na^+ channel activation (Couraud *et al.*, 1982).

A fifth receptor site on the sodium channel which binds the brevetoxins and ciguatoxins has been characterized by direct binding studies using a radioligand derivative of brevetoxin (Poli *et al.*, 1986; Catterall and Gainer, 1985). Electrophysiological experiments demonstrate that by shifting the voltage-sensitivity of Na^+ channel activation, these toxins cause repetitive neuronal firing and block of Na^+ channel inactivation (Huang *et al.*, 1984; Catterall, 1995).

As stated above, the major groups of lipid-soluble toxins affecting Na^+ channels have been reported include the alkaloid neurotoxins, which act on receptor site 2 on sodium channels, such as BTX, VTD, aconitine, and grayanotoxin, and the brevetoxins which act on receptor site 5 of sodium channels. It has been proposed that due to their lipophilic nature, these toxins most likely associate with the channel at sites buried in the matrix of the lipid bilayer (Albuquerque and Daly, 1976; Brown *et al.*, 1981). In support of this, the lipid-soluble toxins produce their effects from both the extracellular and the cytoplasmic compartment of the nerve (Strichartz, 1987). The toxins of this group have the ability to interfere with virtually every aspect of sodium channel physiology including voltage-dependent gating, ion selectivity, and single channel conductance (Strichartz, 1987).

Certain classes of lipophilic insecticides including the pyrethroids and DDT have been found to act on sodium channels (Narahashi *et al.*, 1966; Narahashi, 1979; Narahashi *et al.*, 1985). Pyrethroids are synthetic analogs of the pyrethrins, neurotoxins isolated from the flower heads of *Chrysanthemum cinerariafolium.* Many pyrethroids are lipophilic esters. From the chemical perspective, pyrethroids can be divided into two groups: Type II which posess an α-cyano group and Type I which have no cyano substituent (Gammon and Casida, 1981). Binding to open sodium channels, pyrethroids produce a steady-state slow Na^+ current and this modified current decays slowly (Lund and Narahashi, 1983). Currents modified by Type I pyrethroids decay much faster than those modified by Type II pyrethroids (Vijverberg *et al.*, 1983). Type I pyrethroids were found to cause repetitive firing of sensory neurons in the cockroach trochanter nerve (Narahashi, 1962). Furthermore, repetitive firing appears to be a consistent feature of Type I pyrethroid action occuring in numerous sensory, motor and CNS neurons (Narahashi, 1971; Wouters and Van den Bercken, 1978). In contrast, Type II pyrethroids depolarize nerves without repetitive firing (Lund and Narahashi, 1983). The symptoms of DDT poisoning in insects are characterized by hyperactivity at low doses, and tremor and convulsions at higher doses (Narahashi and Yamasaki, 1960). In common with the Type I synthetic pyrethroids, DDT acts directly on the axon to slow sodium channel closure after opening. This has the effect of increasing inward movement of sodium ions which results in prolongation of the action potential and repetitive discharges (Narahashi *et al.*, 1979). Although the recognition sites for pyrethroids and DDT are clearly distinct from other neurotoxin binding sites on the sodium channel, their precise location(s) remain(s) to be established.

1.3.2 Ca^{++} channels

In neurons, Ca^{++} channels vary in their cellular location, biophysical and pharmacological properties and modulation (Olivera *et al.*, 1994; Tsien *et al.*, 1995). Voltage-sensitive Ca^{++} channels are one of the most extensively studied classes of cation channels. A single neuron typically contains multiple types of Ca^{++} channels, which are fundamental to the integration and expression of neuronal activity (Carbone and Lux, 1984; Armstrong and Matteson, 1985; Nowycky *et al.*, 1985; Dunn, 1988). The current classification divides voltage-sensitive Ca^{++} channels into L-, N-, T-, and P-types according to their pharmacological and physical properties (Catterall, 1994; Olivera *et al.*, 1994; Clark, 1995). More recently attention has been directed toward another class of Ca^{++} channels, termed Q-type Ca^{++} channels (Zhang *et al.*, 1993; Randall and Tsien., 1995) and no doubt others will be added to the list.

L-type Ca^{++} channels are ubiquitous in excitable tissue, where they form the major pathway for voltage-gated Ca^{++} entry (Rane *et al.*, 1987). Calcium ion fluxing through L-type calcium channels typically results in long-lasting calcium currents, hence L-type calcium channels play a major role both in activating the contraction of heart and other kinds of smooth muscle, and also in the control of transmitter release from endocrine cells and sensory neurons (Rane *et al.*, 1987). Studies have shown that L-type Ca^{++} channels play an important role in the central nervous system. It is known that BAY K 8644, a dihydropyridine calcium channel activator, augments K^+-stimulated release of the neurotransmitter serotonin from rat frontal cortex slices. Nifedipine, verapamil and diltiazem were found to be potent inhibitors of this response indicating that L-type calcium channels are also involved in neurotransmitter release (Middlemiss and Spedding, 1985). Similar results have been obtained more recently by Sabria *et al.* (1995) who showed that BAY K 8644 enhancement of K^+ evoked [^{3}H]noradrenaline release is antagonized

by 1,4-dihydropyridines in synaptosomes isolated from mammalian cerebral cortex. Furthermore, high affinity binding sites for dihydropyridines have been demonstrated on synaptosomes (Turner and Goldin, 1985; Dunn, 1988; Dooley *et al.*, 1987), and it has been estimated that about 15% of the total Ca^{++} current in rat cerebellar granule neurons can be attributed to L-type Ca^{++} channels (Randall and Tsien, 1995). N-type voltage-sensitive calcium channels are almost completely restricted to neurons (Tsien *et al.*, 1988) and show a tendency for fast inactivation at depolarizing holding potentials (Lemos and Nowycky, 1989). N-type calcium channels are widely recognized to be critical in the action of neurotransmitter release at presynaptic nerve terminals (Hess, 1990). The N-type voltage-sensitive calcium channel blocker ω-conotoxin markedly reduced the K^+-evoked [^{3}H]noradrenaline release in nerve terminals, indicating that both L- and N-type voltage-sensitive calcium channels participate in neurotransmitter release in mammalian brain (Sabria *et al.*, 1995). N-type calcium channels are activated by relatively large voltage changes in way similar to L-type channels but differ in their inactivation characteristics which are much more rapid than those of L-type channels (Sher and Clementi, 1991; Catterall, 1993; Clark, 1995). T-type calcium channels are low voltage-activated Ca^{++} channels, which can be opened by small depolarizations. They were originally reported in vertebrate sensory neurons (Carbone and Lux, 1984). Recently, these channels have been found in numerous excitable and non-excitable cells from a variety of organisms (Hess, 1990; Catterall, 1993). The most obvious function of T-type channels is to support pacemaker activity, or Ca^{++} entry at more negative membrane potentials (Tsien *et al.*, 1988). P-type channels are high voltage-activated Ca^{++} channels. They have been found in Purkinje cells of the cerebellum, at the squid giant synapse and in several other types of neurons where the channel plays a role in transmitter release (Mintz *et al.*, 1992; Mori *et al.*, 1993). Q-type Ca^{++} channels have been reported

recently in mammalian central neurons, and appear to be distinct from P-type Ca^{++} channels in inactivation properties (Zhang *et al.*, 1993; Randall and Tsien, 1995).

Molecular biological investigations have revealed that the different calcium channel types are all part of a family of multisubunit ion channels (Catterall, 1993; Clark, 1995; Randall and Tsien, 1995). It has been established that the skeletal dihydropyridine-sensitive L-type calcium channel is composed of two distinct high molecular weight subunits $\alpha 1$ and $\alpha 2$, and three smaller subunits β, γ and δ (Campbell *et al.*, 1988). Recently, cDNAs of $\alpha 1$ subunits of dihydropyridine receptors have been cloned from rat brain (Koch *et al.*, 1989). Immunoprecipitation with specific antibodies against $\alpha 2\delta$ subunits revealed a complex of polypeptides with sizes corresponding to $\alpha 1$, $\alpha 2\delta$, and β subunits of dihydropyridine-sensitive L-type calcium channels in brain (Westenbroek *et al.*, 1995; Ahlijanian *et al.*, 1991). The 175 KDa form of $\alpha 1$ subunit has been demonstrated to be the predominant species which is capable of acting as a voltage sensor (Tanabe *et al.*, 1988). ω-Conotoxin-sensitive N-type calcium channels purified from rat brain contain an $\alpha 1$ subunit, a 140 KDa $\alpha 2$-like subunit, and β subunits of 60 KDa to 70 KDa (Sakamoto and Campell, 1991; Ahlijanian *et al.*, 1991). The $\alpha 1$ subunit of L-type channels, which contains dihydropyridine binding site, has a high level of homology with the α subunit of the voltage-dependent sodium channel and can function independently as a voltage-sensitive calcium channel when expressed in *Xenopus* oocytes or mammalian cells (Catterall, 1993; Clark, 1995). Although molecular cloning approaches have revealed an ever-increasing number of Ca^{++} channel subunits which have a wide distribution in the CNS (Tsien *et al.*, 1991; Miller, 1993), the matchup between many cloned subunits and Ca^{++} channels in neurons is incomplete. Some Ca^{++} channel subunits appear to correspond well with established channel types, whereas others are difficult to identify as components of neuronal Ca^{++} channels (Randall and Tsien, 1995).

Several lipid soluble compounds have been found to act as Ca^{++}-antagonists and Ca^{++}-agonists. The L-type Ca^{++} channel appears identical to the so-called dihydropyridine-sensitive Ca^{++} channel, which is highly sensitive to blockade by dihydropyridines, such as nifedipine and nitrendipine (Ehlert *et al.*, 1982; Catterall, 1993). Verapamil (Ehlert *et al.*, 1982; Murphy *et al.*, 1983) and diltiazem (Yamamura *et al.*, 1982) also inhibit L-type Ca^{++} channels preferentially. Much more recently, the antiepileptic drug felbamate (2-phenyl-1,3-propanediol dicarbamate) has been reported to inhibit L-type Ca^{++} channels in mammalian central neurons (Stefani *et al.*, 1996). The demonstration that epileptic episodes can be controlled with L-type Ca^{++} channel blockers provides more evidence that L-type calcium channels play an important functional role in mammalian brain. In contrast, BAY K 8644 has been demonstrated to act as a potent agonist at L-type Ca^{++} channels (Woodward and Leslie, 1986). The investigations of Cruz & Olivera (1986) and Kasai *et al.* (1987) have established that N-type Ca^{++} channels are blocked by ω-conotoxin and are insensitive to dihydropyridines. P-type Ca^{++} channels are not sensitive to either dihydropyridines or ω-conotoxin, however, they are blocked by a low molecular weight toxin (FTX) purified from funnel web spider venom (Llinas *et al.*, 1989; Mintz *et al.*, 1992). T-type Ca^{++} channels are not affected by dihydropyridines or ω-conotoxin. Interestingly some sensitivity to amiloride and tetramethrin has been reported however, none of these ligands are particularly selective for T-type Ca^{++} channels (Hess, 1990).

As previously stated there is strong evidence that DDT and the synthetic pyrethroids interfere with the gating kinetics of voltage-sensitive sodium channels in nerve at low concentrations. Some time ago however, Orchard and Osborne (1979) reported that synthetic pyrethoids depolarize neurosecretory neurons of the stick insects, where the inward current is carried exclusively by calcium ions. Within the last few years studies conducted by Clark and associates (1995) reveal

that DDT and the α-cyanopyrethroid 1R-deltamethrin activate voltage-sensitive calcium channels of the ciliary membrane of *Paramecium tetruralia* producing multiple spiking and depolarization. It is significant that DDE and 1S-deltamethrin which are non-toxic analogs have no such action. The ciliary membrane channel was also found to be sensitive to phenylethylamines and dihydropyridines, but was unaffected by ω-conotoxin GVIA (Clark *et al.*, 1995), indicating an L-type pharmacology. An additional action of the phenylethylamine L-type calcium channel blocker D595 on voltage-gated sodium channels has already been established although higher concentrations are required (Atlas and Adler, 1981; Miledi and Parker, 1980). Thus, in addition to establishing a precedent for the interaction of certain neuroactive substances with voltage-sensitive sodium and L-type calcium channels, these observations also indicate a degree of structural similarity in the molecular architecture of these channels. This will be discussed in relation to dihydropyrazole specificity later in the thesis.

1.4 Ca^{++} and Intracellular Events in Mammalian Brain

Ca^{++}, which has larger radius and more flexible coordination number than other physiological divalent ions (Hodgkin and Keynes, 1957; Carafoli, 1987), imposes a smaller physical constraint on the surrounding protein. These properties make Ca^{++} ideally suited to be complexed by irregularly shaped cavities in protein macromolecules. Ca^{++} has therefore evolved as a more important intracellular messenger compared with other ions present in the biological environment (Carafoli, 1987). In neurons, the intracellular concentration of free calcium ions is about 10^{-7} M, which is over four orders of magnitude lower than the excellular free Ca^{++} ion concentration (McBurney and Neering, 1987). This remarkable transmembrane concentration gradient is essential for maintaining cell integrity (McBurney and Neering, 1987). The cellular mechanisms for regulating Ca^{++}

entry are remarkably diverse. Voltage-gated Ca^{++} channels are extremely important in this respect and consequently have been extensively studied (Tsien and Tsien, 1990). A variety of other Ca^{++} channels including receptor-operated Ca^{++} channels (Collingridge and Bliss, 1987), second messenger-operated Ca^{++} channels (Meldolesi and Pozzan, 1987), mechanically operated Ca^{++} channels (Lansman *et al.*, 1987), tonically active Ca^{++} channels (Benham and Tsien, 1987) and gap junction channels (Cornell-Bell *et al.*, 1990) are also known to participate in the control of intracellular free [Ca^{++}] in different types of cells. In neurons, cytosolic [Ca^{++}] is also regulated by supply from and removal by intracellular stores (Tsien and Tsien, 1990). Although neurons must maintain low intracellular levels of Ca^{++} in the resting situation, tightly controlled changes in $[Ca^{++}]_i$ permit calcium ions to play their role as intracellular activators or regulators of neuronal function (McBurnay and Neering, 1987).

Ca^{++} binds to a family of small heat-stable proteins the major ones including troponin C and calmodulin, to exert its main intracellular effects (Hardie, 1991). In striated muscles, troponin C is responsible for activating contraction in response to Ca^{++} influx (Hardie, 1991). In contrast, calmodulin is an abundant protein of ubiquitious distribution. In the presence of Ca^{++}, calmodulin activates many different enzymes, the most important of which include the protein kinases (Pallen *et al.*, 1985). Ca^{++}-transporting ATPase in the plasma membranes are also activated by Ca^{++}/calmodulin to render the system much more sensitive to rises of intracellular free [Ca^{++}] (Pallen *et al.*, 1985). The adenylate cyclase is activated by calmodulin to produce the second messenger cyclic AMP, offering the flexibility by which first messengers can mediate their effects (Krupinski *et al.*, 1989). Obviously, these enzymes are regulated by direct binding of Ca^{++}-binding proteins. Nevertheless, most actions of Ca^{++} are mediated indirectly, through

protein phosphorylation catalyzed by calmodulin-activated protein kinase (Anderson *et al.*, 1990).

One of the more prominent brain specific substrates for protein kinases is synapsin I. Its dephosphorylated form binds to the surface of synaptic vesicles securing them within the cytoskeletal framework of the presynaptic terminal by crosslinks with actin and fodrin (Trimble *et al.*, 1991). The rise in cytosolic free [Ca^{++}] triggers synapsin phosphorylation (Kelly, 1988) which in turn causes release of synaptic vesicles from the cytoskeleton, allowing them to approach the plasma membrane, thus facilitating neurotransmitter release (Greengard *et al.*, 1993; Zimmermann, 1993).

A number of synaptic vesicle-specific proteins have been identified which appear to be intimately involved in docking and fusion with active zones. For instance, the fusion of vesicles with the presynaptic membrane during exocytosis is partly regulated by the vesicular proteins synaptophysin and synaptophorin, which can be phosphorylated by CaM-kinase II and may play a role in the formation of the exocytotic fusion pore (Zimmermann, 1993). Synaptobrevin, another abundant synaptic vesicle protein, appears to be essential for docking the vesicle with discrete sites on the plasma membrane (Sudhof *et al.*, 1993). Synaptobrevin in fact binds a protein complex containing N-ethylmaleimide sensitive fusion protein (NSF) and synaptosome-associated proteins (SNAPS), proteins that facilitate the fusion of all types of intracellular vesicles with their target membranes (Littleton and Bellen, 1995). Significantly the NSF-SNAP complex is also capable of binding to the active zone of axon terminals, and thus the synaptobrevin-NSF-syntaxin complex may attach the vesicles to the plasma membrane so that they can fuse together when intraterminal free [Ca^{++}] is increased (Walch-Solimena *et al.*, 1993; Zimmermann, 1993; Littleton and Bellen, 1995).

The synaptic vesicle protein synaptotagmin contains two binding sites for Ca^{++} on a cytosolic projection and is thought to be a key Ca^{++}-sensing protein (Littleton and Bellen, 1995). It is significant in this regard that Ca^{++}-stimulated vesicle exocytosis is inhibited when an antibody specific for the cytosolic domain of synaptotagmin is injected into the nerve (Tully, 1987). Synaptotagmin apparently binds to the complex of synaptobrevin and syntaxin, another docking protein located on the presynaptic membrane (Littleton and Bellen, 1995). When Ca^{++} is not bound, synaptotagmin blocks the binding of the fusion protein α-SNAP to the complex and this prevents vesicle fusion. When synaptotagmin binds Ca^{++}, it is displaced from the complex, allowing α-SNAP to bind and thus initiate membrane fusion (Walch-Solimena *et al.*, 1993; Littleton and Bellen, 1995).

Protein phosphorylation has become the most widely recognized mechanism mediating the downstream effects of first messengers. It can provide a large amplification of the input signal, allows a very sensitive response, and also enables great versatility to be built into the control system (Lodish, *et al*, 1995).

Drugs which act at many of the sites reviewed above can be thought of as modulators of neurotransmitter release. In most cells, elevations in $[Ca^{++}]_i$ arise from Ca^{++} entry through Ca^{++} channels in the surface membrane, Ca^{++} discharge from internal stores, or both. However, Ca^{++} channels are thought to be the major pathway (Tsien and Tsien, 1990). Therefore, drugs, which affect Ca^{++} channels in mammalian brain should alter the $[Ca^{++}]_i$ and thus affect the intracellular events such as protein phosphorylation and neurotransmitter release. Agents known to increase Ca^{++} transport across the plasma membranes of nerve terminals have also been found to stimulate the phosphorylation of specific endogenous proteins in synaptosomes isolated from rat brain. It has been reported that VTD and K^+ (Robinson and Dunkley, 1983), which increase Ca^{++} channel-dependent Ca^{++} influx, as well as the calcium ionophore A 23187 (Krueger *et al.*, 1977), markedly

stimulate the incorporation of $^{32}P_i$ into synapsins Ia and Ib. By demonstrating that all three agents were unable to stimulate the phosphorylation of synapsin I in calcium-free medium, Krueger concluded that Ca^{++} influx from the extracellular compartment was a prerequisite. The sodium channel blocker TTX inhibited the stimulation of protein phosphorylation by VTD (a Na^+ channel activator) but not by K^+ or A 23187 (a Ca^{++} channel activator and ionophore respectively) (Krueger *et al.*, 1977). This finding agrees with results obtained by Forn and Greengard (1978) who performed similar experiments with rat cerebral cortex slices.

As mentioned previously, synapsin I is a neuro-specific phosphoprotein which is intimately associated with synaptic vesicles in the nerve endings (Navone *et al.*, 1984). The phosphorylation status of synapsin I is recognized as playing a pivotal role in the control of transmitter release from the nerve ending (Navone *et al.*, 1984). When phosphorylated synapsin I is injected into the presynaptic compartment of the squid giant terminal, it has been found to increase the rate of rise, increase the amplitude and decrease the latency of the postsynaptic potential as a result of increased neurotransmitter release (Llinas *et al.*, 1985). Conversely, injection of dephosphorylated synapsin I inhibits both spontaneous and evoked neurotransmitter release (Llinas *et al.*, 1985). In agreement with these data, microinjection of CaM kinase II which stimulates the phosphorylation of synapsin I induces a 3-fold to 7-fold increase in the number of neurotransmitter quanta released in response to presynaptic depolarization (Llinas *et al.*, 1985).

As one would predict from the strict dependence of neurotransmitter release on calcium influx, K^+-induced release of glutamate is inhibited by the L-type Ca^{++} channel inhibitor verapamil (Sanchez-Prieto *et al.*, 1987). Dihydropyridines also block release of [^{3}H]noradrenaline when evoked by high K^+ (Sabria *et al.*, 1995). Peptide neurotoxins such as ω-conotoxins, the N-type calcium channel blockers,

are also effective inhibitors of K^+-stimulated transmitter release from nerve endings (Olivera *et al.*, 1985).

The transmitter releasing action of pyrethroids was first demonstrated in mammalian synaptosomal preparations (Nicholson *et al.*, 1983). This result has since been confirmed by other researchers (Brooks and Clark, 1987). Under some experimental conditions, pyrethroid responses are totally suppressed by TTX, suggesting a significant action on voltage-sensitive sodium channels (Nicholson *et al.*, 1983; Berlin *et al.*, 1984). In other situations, pyrethroid-induced transmitter release is only partially sensitive to TTX (Brooks and Clark, 1987; Matsumura, 1987). Nevertheless, there is good evidence that depolarization-induced release of neurotransmitters by the α-cyanopyrethroid deltamethrin is also associated with an enhanced uptake of $^{45}Ca^{++}$ into synaptosomes (Brooks and Clark, 1987) and an increase in the level of phosphorylation of several intraterminal proteins including synapsin I (Matsumura and Clark, 1988).

Previous investigations on protein phosphorylation provided valuable insights into how insecticidal neurotoxicants disrupt nerve terminal function (Matsumura and Clark, 1988; Enan and Matsumura, 1992; Kanemoto *et al.*, 1992). I reasoned that studies on depolarization-coupled phosphorylation of synapsin I and neurotransmitter release would serve to define the effects of dihydropyrazoles on an intracellular protein regulator of critical importance and also offer the opportunity to investigate the extent to which dihydropyrazoles interfered with depolarization-coupled phosphorylation of synapsin I and transmitter release via Na^+ or Ca^{++} channel blockade.

1.5 Physical Disturbance of Synaptosomal Membrane Properties by Neuroactive Chemicals

Many biochemical and biophysical events in the cell membrane rely on strict structural and dynamic organization. The phospholipids present in cell membranes are known to consist of two physicochemically distinct regions: a phosphate-containing head group which being either polar or negatively charged is highly soluble in water (hydrophilic) and two fatty acyl hydrocarbon chains that are attached to the headgroup which are practically insoluble in water (hydrophobic) (Cevc and Marsh, 1987). Lipids with short or unsaturated fatty acyl chains undergo phase transition at lower temperatures than lipids with long or saturated chains (Singer and Nicolson, 1972). This is because short fatty acyl chains have less surface area to form Van der Waals interactions with each other compared to long fatty acyl systems. Because the gel state is stabilized by these interactions, short-chain lipids will melt at lower temperatures than long-chain lipids. Likewise, the kinks in unsaturated fatty acyl chains renders them less able to form stable Van der Waals associations, causing unsaturated lipids to adopt a more random, fluid state at lower temperatures than lipids with saturated fatty acyl chains (Singer and Nicolson, 1972). All cell membranes contain a mixture of different fatty acyl chains and are fluid at the temperature at which the cell is grown. Any homeostatic changes to membrane lipid structure and composition are almost completely restricted to the hydrocarbon layers, without any substantial change in the lipid headgroup region (Singer and Nicolson, 1972; Seelig and Seelig, 1980). Therefore, the lipid hydrocarbon layer is the region most sensitive to physical and chemical effects and maintenance of lipid microviscosity at a precise level is essential for normal physiological functioning (Chapman, 1975). It is clear that the synaptic membrane, like most plasma membranes, has a complex lipid composition with position, order and motional freedom of the various hydrocarbon moieties (Seelig

and Seelig, 1980). Membrane lipid fluidity can be affected by a variety of ambient physical factors, such as temperature, volume, pressure, and acidity (Seelig and Seelig, 1980; Hitzemann *et al.*, 1983). Formation of an electric potential across a lipid bilayer causes a small but significant increase in microviscosity in natural membranes (Georgesauld and Duclohier, 1978). The reverse relationship, namely an increase in lipid fluidity upon decrease the membrane potential has also been reported (Cord *et al.*, 1982).

Treatment of membranes *in vitro* with drugs, alcohol, or anesthetics often leads to an increase in lipid fluidity (Jahnig, 1984; Shmeeda *et al.*, 1984). Upon chronic exposure to pharmacologically relevant doses of ethanol, anesthetics, and other drugs, a tolerance occurs which involves a partial reduction of lipid fluidity as a result of increase biosynthesis and incorporation of cholesterol and saturated fatty acids into membranes (Wodtke, 1978).

Alcohol and barbiturates have a disording effect on synaptic membranes (Richards *et al.*, 1978; Hitzemann *et al.*, 1984). Harris and Schroeder (1982) demonstrated that low, pharmacologically relevant concentrations of alcohol (20 to 100 mM) have significant effects on synaptic membrane lipid order. Ethanol increases fluidity of the hydrophobic core of synaptic membranes and reduces the excitability of membranes. Harris and Bruno (1985) also investigated the relationship between synaptosomal Na^+ and Ca^{++} fluxes and synaptic membrane disordering caused by anesthetic drugs. They found that a structurally diverse group of the membrane perturbants including ethanol, pentobarbital, chloroform, diethylether, and phenytoin all decreased the fluorescence polarization of the synaptic membrane. This indicated that these compounds increased the membrane fluidity and disordered the membrane lipids. More pronounced effects were found in the membrane core than at the membrane surface and all of the compounds produced a dose-related inhibition of sodium and calcium ion influx. Drug-related

inhibitory effects on sodium channels were closely correlated with effects on lipid order within the hydrophobic core of the membrane. These results suggested that the inhibitory effects of intoxicant-anesthetic drugs on neuronal sodium flux may be the result of their capacity to disorder lipids. In contrast, the effects of drugs on voltage-gated calcium channels were not obviously related to the ability of these agents to disorder membrane lipids (Harris and Bruno, 1985).

For the lipophilic dihydropyrazoles, it is clearly important to clarify whether they affect the physical properties of synaptic membranes (i.e. membrane fluidity and lipid order), and further more to establish how closely this effect is related to interference with Ca^{++} channels in mammalian brain.

1.6 Classes of Synapses within the Central Nervous System and the *in vitro* Synaptosomal System

Most neurons contain four distinct regions with differing functions: the cell body, the dendrites, the axon, and the axon terminals. The axon terminal from the presynaptic cell sends signals that are picked up by postsynaptic cells. Synapses, are specialized regions where neurons send and receive information. Two different types of synapses have been described, electrical and chemical, which differ in both morphology and function (Kandel and Siegelbaum, 1985; Bennett *et al.*, 1991; Bennett and Scheller, 1993; Shepherd, 1994). In electrical synapses the cytoplasm of pre- and postsynaptic elements is interconnected by bridge junctions (gap junctions) (Kandel and Siegelbaum, 1985). In many cases, the presence of these junctions is associated with a low-resistance electrical pathway between the two neurons (Shepherd, 1994). These gap junctions are thought to facilitate the passage of the electrical impulse from the presynaptic to postsynaptic cell without a delay (Kennedy, 1994). Ions and small molecules can pass through the junction, supporting the idea that the spanning structures enclose cytoplasm that is

continuous with pre- and postsynaptic cells (Kandel and Siegelbaum, 1985). In chemical synapses, the cytoplasm is not bridged and the neurons are separated by a cleft. This separation, 30-50 nm, is much wider than the gap between junctional plasma membranes of electrical synapses (2 nm) (Kandel and Siegelbaum, 1985). Chemical synapses are clearly the more complicated form of junction in the nervous system (Shepherd, 1994). For example, the presynaptic and postsynaptic membranes are often morphologically specialized, and the presynaptic terminals contain localized distributions of vesicles (Kandel and Siegelbaum, 1985). The vesicle-containing presynaptic process releases a chemical messenger which interacts with postsynaptic receptors following a diffusional delay of up to 50 μsec. As a result, the presynaptic process modulates the interaction between the transmitter and the receptor molecule in the postsynaptic cells (Shepherd, 1994; Kandel and Siegelbaum, 1985). It is this interaction which ultimately leads to the activation of ion-selective channels and current flow, which results in various postsynaptic potentials (Kandel and Siegelbaum, 1985).

A useful way of classifying chemical synapses in CNS is according to the identity and action of their principle chemical transmitter. In excitatory synapses, the chemical transmitter activates receptors which cause depolarization of the postsynaptic cell membrane. In this context, ion channels are opened for the influx of cations such as K^+, Na^+ or Ca^{++}. The amino acid L-glutamate is thought to be a major excitatory neurotransmitter in mammalian brain (Kennedy, 1994). In contrast, inhibitory synapses are those at which the chemical transmitter activates receptors such as $GABA_A$ and $GABA_B$ to increase the conductance of Cl^- (influx) and K^+ (efflux) across the membrane (Shepherd, 1994). GABA is the principle inhibitory transmitter in the CNS of vertebrates (Curtis and Johnston, 1974). In addition, a large number of neurotransmitters, such as noradrenaline, dopamine and serotonin, initiate biochemical events that modulate synaptic transmission or

membrane excitability rather than excite or inhibit postsynaptic neurons directly. This group of synapses in the CNS is called modulatory synapses (Kennedy, 1994).

Synaptosomes are pinched-off, resealed nerve endings, obtained by controlled homogenization of nerve tissue (Whittaker *et al.*, 1964). Because synaptosomes display the essential features of intact nerve endings, they offer an invaluable system for studying neurochemical processes *in vitro* (Whittaker *et al.*, 1964; Luo and Bodnaryk, 1987). Nerve endings from various brain regions appear to survive the rigors of the preparative techniques with sufficient biochemical and morphological preservation to provide highly functional preparations of nerve terminals for *in vitro* studies. The functional integrity of the synaptosomal membrane is demonstrated by its ability to transport a variety of biomolecules including Na^+ (Ling and Abdel-Latif, 1968), K^+ (Marchbanks, 1967; Escueta and Appel, 1969), choline (Marchbanks , 1968), and GABA (Weinstein *et al.*, 1965). The development of methods to isolate synaptosomes has meant that the synthesis, storage, and depolarization-evoked release of transmitters have become amenable to biological study, together with the actions of pharmacological agents on these processes. Synaptosomes remain viable for several hours after isolation, during which time they display a variety cellular functions related to the processes of neurotransmission and neurosecretion (Bradford, 1975). Recently, the development of a rapid Percoll-based method to isolate synaptosomes from rat brain in high purity and with minimal osmotic stress was reported (Dunkley *et al.*, 1986). For experiments on the presynaptic actions of dihydropyrazoles described in this thesis, I prepared synaptosomes from the whole brains of CD1 mice using the method of Dunkley *et al.* (1986).

Chapter 2. The Effect of Dihydropyrazoles on Changes to Intraterminal Free [Ca^{++}] and Depolarization-Induced Uptake of $^{45}Ca^{++}$ in Mouse Brain Synaptosomes

2.1 Introduction

The evidence for dihydropyrazoles acting on voltage-dependent sodium channels has already been discussed in Section 1.2.4. During the course of their investigations using the [^{3}H]GABA release assay, Nicholson and Merletti (1990) obtained pharmacological data for RH-5529 that was not entirely consistent with the theory that dihydropyrazoles act as selective inhibitors of sodium channel-dependent transmitter release. The main argument was the finding that when transmitter release was stimulated by direct depolarization of the synaptosomal membrane with a high concentration of potassium ions, which open voltage-sensitive calcium channels (Nachshen and Blaustein, 1980), RH-5529, in contrast to TTX, consistently produced inhibition (Nicholson and Merletti, 1990). The effect of dihydropyrazoles was not totally clearcut, because in these experiments RH-3421 was a poor inhibitor of K^+-induced release of [^{3}H]GABA. Nevertheless, the opportunity to pinpoint and characterize an additional molecular target for insecticidal dihydropyrazoles in mammalain brain, and recognition that the findings might provide new biochemical and physiological reasons for the "delayed-onset neurological syndrome" in mammals, provided the initial impetus for the research described in this thesis. This phase of the study was undertaken to examine the possibility that dihydropyrazoles affect depolarization-related changes in $[Ca^{++}]_{free}$ in the cytosolic compartment and $^{45}Ca^{++}$ uptake in the nerve ending through a specific action on voltage-sensitive calcium channels. Clearly, if this were the case, it would help to eliminate the possibility that other molecular recognition targets for dihydropyrazoles exist downstream in the signalling

pathway for transmitter release and strengthen the rationale for exploring a direct action of dihydropyrazoles on voltage-sensitive calcium channels.

Since synaptosomes were to play a major role in this investigation, electron microscopical studies were carried on the purified synaptosomal fraction at an early stage to verify its composition.

2.2 Materials and Methods

2.2.1 Chemicals

TTX, Percoll, the acetoxymethylester of fura-2, glutaraldehyde, osmium tetroxide, uranyl acetate and lead citrate were purchased from Sigma (St. Louis, MO). VTD was a gift from Dr. Botham of the Wellcome Research Laboratories (Berkhamsted, UK.) and the dihydropyrazoles RH-3421 and RH-5529 (Fig.1) were supplied by Rohm and Haas Co. (Spring House, PA). Radiocalcium was purchased from ICN Pharmaceutucals Canada Ltd., (Montreal).

2.2.2 Isolation of Synaptosomes

The complete brain was rapidly removed from one male CD1 mouse (25-30 g) and homogenized using a motor-driven pestle (6 excursions; 0.33 mm pestle to tube clearance) in 20 ml of ice-cold 0.32 M sucrose (pH 7.4) containing 1 mM EDTA (ethylenediaminetetraacetic acid) and 5 mM Tris (tris(hydroxymethyl)-aminomethane). Synaptosomes were isolated on Percoll gradients as previously described by Dunkley *et al.* (1986) (Fig. 2.1). All fractionation procedures were carried out at 4 ^{o}C using a Beckman J2-HS centrifuge. The pure synaptosomal fraction (band 4) was carefully removed from the percoll gradient, resuspended in physiological saline [NaCl (125 mM), KCl (5 mM), NaH_2PO_4 (1.2 mM),

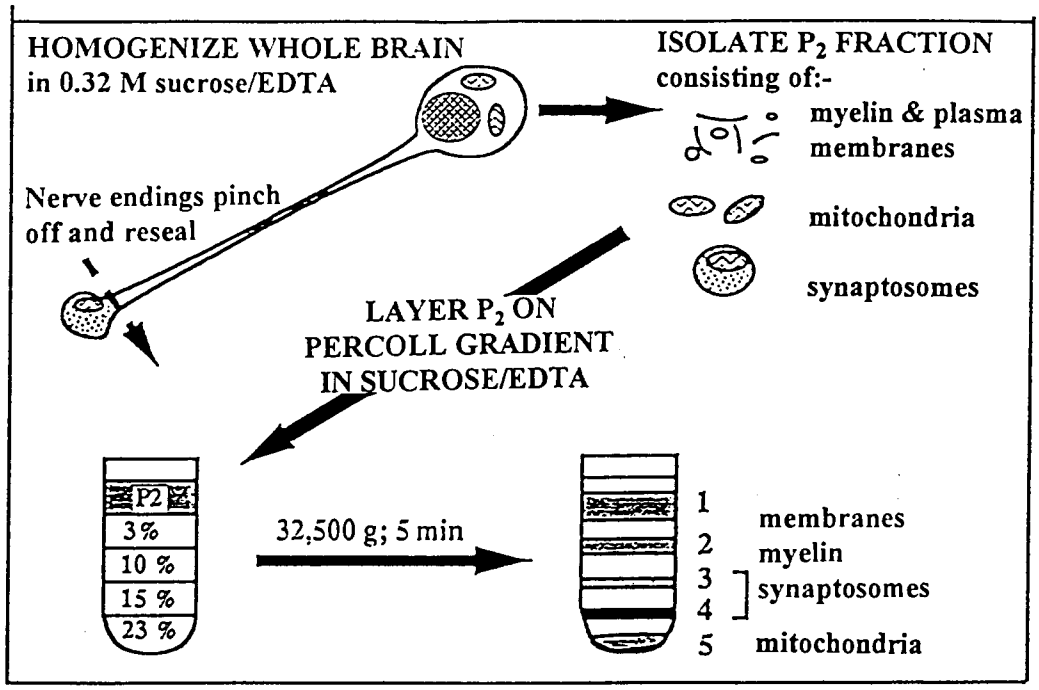


Fig. 2.1 Diagrammatic representation of the procedure for isolation of synaptosomes (pinched-off nerve endings) using an iso-osmotic Percoll gradient as described by Dunkley, *et al.* (1986).

$MgCl_2.6H_2O$ (1.2 mM), $NaHCO_3$ (5 mM), glucose (10 mM), and Hepes (20 mM); adjusted to pH 7.4 with NaOH], and centrifuged at 20,000g for 10 min. The pellet was dispersed in the saline and held on ice prior to assay.

2.2.3 Electron Microscopy

Purified synaptosomes were fixed in phosphate-buffered 8% glutaraldehyde for 1 hour. The pellets were then rinsed in 0.2 M phosphate buffer (pH 7.4) and postfixed in OsO_4 (1% in phosphate buffer, w/v). The pellets were rinsed again in phosphate buffer and dehydrated in graded ethanol, transferred to propylene oxide and infiltrated and embedded in Epon/Araldite. Thin sections (80-100 nm) were cut with diamond knives on a Reichert OM U2 microtome and mounted on non-coated copper grids (200 mesh). The sections were stained in 5% uranyl acetate for 20 min and then stained in 2.6 % lead citrate for 15 min after rinsing in distilled water (Loscher *et al.*, 1985; Lou and Bodnaryk, 1987).

2.2.4 Determination of Intrasynaptosomal Free Calcium

Synaptosomes isolated from one CD1 mouse were resuspended in physiological saline [NaCl (125 mM), KCl (5 mM), NaH_2PO_4 (1.2 mM), $MgCl_2.6H_2O$ (1.2 mM), $NaHCO_3$ (5 m), glucose (10 mM), and Hepes (20 mM); adjusted to PH 7.4 with NaOH] to a protein concentration of 1.6 mg/ml and then loaded with the fluorescent indicator fura-2. These incubations were carried out according to the procedure of Komulainen and Bondy (1987), except that loading was carried out in physiological saline to which no calcium had been added. Briefly, 5 µl of the acetoxymethylester of fura-2 in dimethylsulfoxide (DMSO; 1 µ g/µl) was added to 1 ml of the synaptosome suspension. The suspension was incubated for 20 min at 37 ^{o}C in the dark with shaking. At this point 9 ml of saline (37 ^{o}C) was added and the incubation continued for a further 25 min. The

synaptosomes loaded with fura-2 were then centrifuged at 9,000g for 5 min. The pellet was then carefully resuspended in 5 ml of ice-cold physiological saline using gentle vortexing and the resulting suspension was held on ice in darkness prior to assay.

Fluorescence measurements were conducted on synaptosomal suspensions at 37 ^{o}C using a Perkin-Elmer LS 50 fluorescence spectrophotometer linked to an IBM 50 Z computer. The excitation wavelengths were 340 and 380 nm (slit width 2.5 nm) and the emission wavelength was set at 510 nm (slit width 8 nm). A 0.5-ml aliquot of the fura-2-loaded synaptosomes was centrifuged (Beckman microfuge). After resuspending the pellet in 3 ml of physiological saline containing calcium (2 mM) using gentle homogenization, the sample was transferred to a cuvette. Immediately before assay dihydropyrazoles, TTX or solvent controls, as appropriate, were added to the synaptosomal suspension by microsyringe followed by thorough mixing using a pasteur pipette. Activators, VTD (50 μM), ionic potassium (60 mM), or the solvent control were added as required about 120 sec after the start of recording. VTD and dihydropyrazoles were added to the cuvette in DMSO. The concentration of the carrier did not exceed 0.1%. Calibrations were performed using alkaline EGTA (ethyleneglycol-bis-(β-aminoethylether) N, N, N', N'-tetraacetic acid) followed by SDS (sodium dodecyl sulfate) to establish the R_{min} ratio and at a saturating concentration of calcium to establish R_{max}. Fluorescence values were corrected for autofluorescence using synaptosomal samples containing no probe. The concentration of intraterminal-free calcium ($[Ca^{++}]_i$) was calculated according to Grynkiewicz *et al.* (1985, see equation below) assuming a dissociation constant (K_d) of the fura-2: calcium complex of 224 nM.

$$[Ca^{++}]_i = K_d \left[(R - R_{min})/(R_{max} - R)\right] S_{f2}/S_{b2}$$

(where R = fluorescence ratio of 340/380; R_{min} = ratio of 340/380 when no Ca^{++} is present; R_{max} = ratio when probe is fully saturated with Ca^{++}; S_{f2} = fluorescen-ce of the probe at 380 nm excitation in zero Ca^{++} and S_{b2} = fluorescence at 380 nm when the probe is fully saturated with Ca^{++}). Data for cytosolic-free $[Ca^{++}]$ for each treatment were averaged over fixed periods of time and expressed as means ± standard deviation. The calibration solutions and physiological salines were filtered through a 0.45 μm filter prior to use.

2.2.5 Assay of $^{45}Ca^{++}$ Uptake into mouse Brain Synaptosomes

This assay was essentially carried out according to a method established by Mendelson *et al.* (1984). Fifty microliter aliquots, containing 421 ± 31 μg synaptosomal protein, were preincubated at 30 °C for 30 min with dihydropyra-zoles, TTX, or solvent controls in saline (0.75 ml) [NaCl (125 mM), NaH_2PO_4 (1.2 mM), $MgCl_2.6H_2O$ (1.2 mM), $NaHCO_3$ (5 mM), glucose (10 mM), and Hepes (20 mM); adjusted to pH 7.4 with NaOH] containing 2 mM $CaCl_2$. Influx was initiated by the addition of depolarizing solution (1 M KCl; 50 μl) containing 10 μ Ci/ml $^{45}Ca^{++}$ followed by immediate vortexing. The control (non-depolarizing) solution consisted of 50 μl 1 M NaCl containing 10 μCi/ml $^{45}Ca^{++}$. Influx was terminated after 5 sec by rapid filtration on 0.8 μm membrane filters. Synaptosomes then received two washings with 4 ml ice-cold saline containing $CaCl_2$ (2 mM) and were dissolved in 1 ml of Triton X-100 (8%). Radiocalcium associated with the synaptosomes was quantitated by liquid scintillation counting.

2.2.6 Protein Determination

The protein concentration of synaptosomal suspensions was estimated according to the method of Lowry as adapted by Peterson (Peterson, 1977) with bovine serum albumin as the reference standard.

2.3 Results

2.3.1 Electron Microscopy

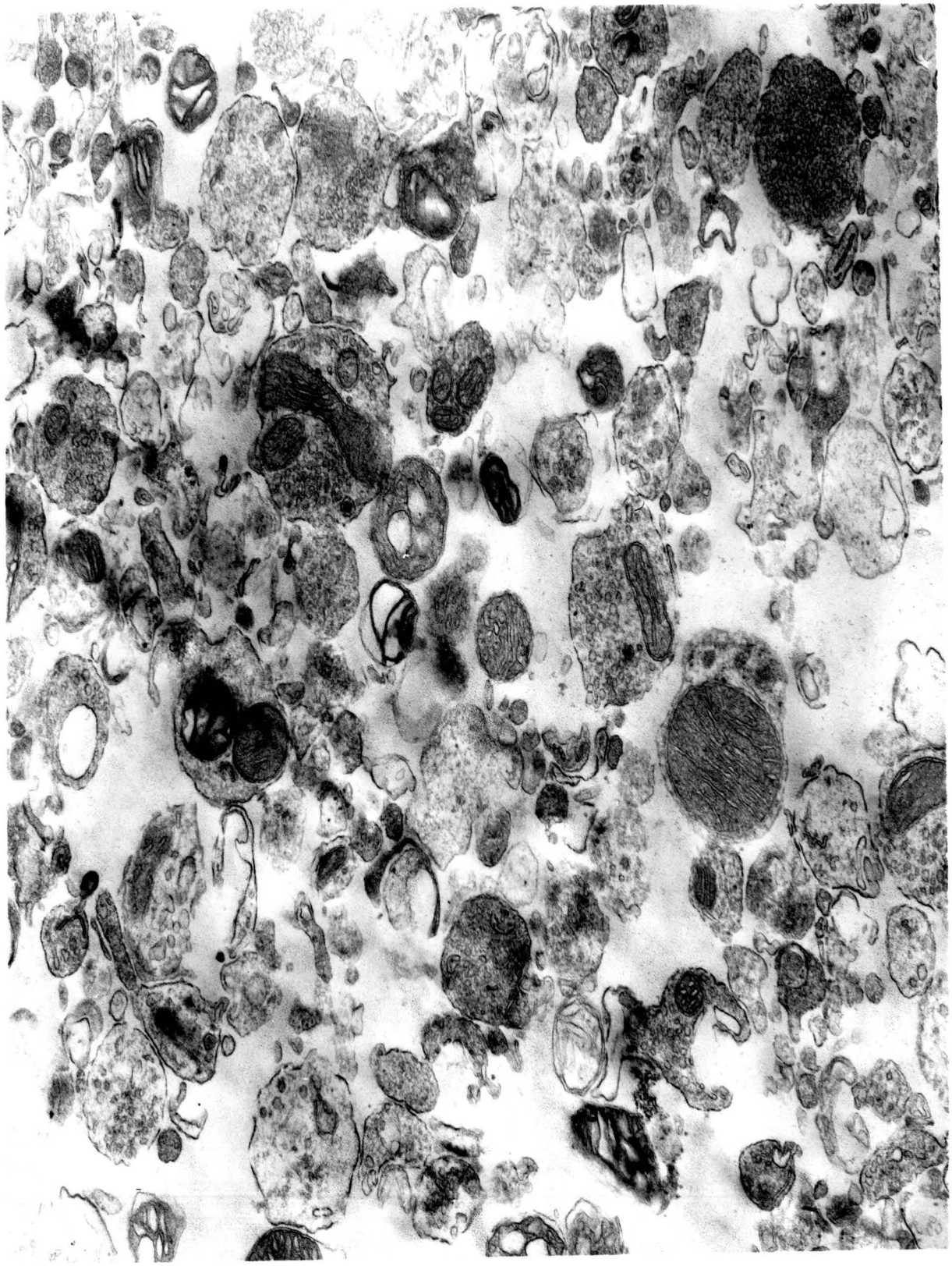
Electron microscopical examination of the band 4 prepared from CD1 mouse brain demonstrated that this fraction consists predominantly of synaptosomal profiles displaying good morphological preservation. Within the synaptosomes, numerous synaptic vesicles are observed and a large proportion of the isolated nerve endings contain mitochondria (Fig. 2.2).

2.3.2 The Effects of Insecticidal Dihydropyrazoles on Intraterminal Free [Ca^{++}]

The resting concentrations of synaptosomal free [Ca^{++}] averaged 260 nM (Table 2.1) and were not affected by high concentrations of either RH-5529 (Fig. 2.3; Table 2.1), RH-3421 (Fig. 2.4; Table 2.1) or TTX (Fig. 2.5; Table 2.1). Exposure of synaptosomes to 50 μM VTD produced a dramatic rise in intraterminal-free [Ca^{++}] (Fig. 2.6) amounting to 350 ± 24 nM above control values (Table 2.1). VTD-stimulated increases in synaptosomal-free [Ca^{++}] were totally prevented by the sodium channel blocker TTX at 3 μM (Fig. 2.6; Table 2.1). No significant rise in synaptosomal-free [Ca^{++}] was observed with 50 μM VTD when calcium ions were removed from saline (Fig. 2.7; Table 2.1).

The dihydropyrazoles also inhibited the rise in synaptosomal-free [Ca^{++}] produced by VTD (Table 2.1). The [Ca^{++}] profiles clearly show that the inhibitory effects of dihydropyrazoles on VTD-induced increase occur in a dose-dependent fashion (Fig. 2.8 and Fig. 2.9). There was a large (approximately 15 fold) difference in potency between the two compounds. The IC_{50} of RH -5529 was

Fig. 2.2 Electron micrograph of band 4 isolated from the Percoll gradient (Bar = 1 μm).



37b

Table 2.1 Effects of dihydropyrazoles on the intraterminal free [Ca^{++}] in resting and depolarized mouse brain synaptosomes. The basal concentration of free [Ca^{++}] in resting (non-depolarized) synaptosomes was estimated to be 260 ± 29.2 nM. Changes in intraterminal free [Ca^{++}] were estimated as described in Figure 2.10 and Figure 2.14.

Treatments [a]	Changes in intraterminal free [Ca^{++}] nM [b]
RH-5529 (10 μM)	28.0 ± 20.4
RH-3421 (40 μM)	25.0 ± 20.8
Tetrodotoxin (3 μM)	3.6 ± 11.8
Veratridine (50 μM)	342.5 ± 34.2
Veratridine (50 μM) (no Ca^{++} plus EGTA at 2.5 mM)	11.6 ± 15.0
Veratridine (50 μM) plus tetrodotoxin (3 μM)	4.9 ± 8.2
Veratridine (50 μM) plus RH-5529 (10 μM)	1.4 ± 4.0
Veratridine (50 μM) plus RH-3421 (1 μM)	3.67 ± 3.0
Potassium ions (60 mM)	172.3 ± 9.9
Potassium ions (60 mM) (no Ca^{++} plus EGTA at 2.5 mM)	6.9 ± 11.5
Potassium ions (60 mM) plus tetrodotoxin (3 μM)	164.5 ± 17.4
Potassium ions (60 mM) plus RH-5529 (10 μM)	0.7 ± 6.4
Potassium ions (60 mM) plus RH-3421 (10 μM)	2.0 ± 2.5
Potassium ions (60 mM) plus RH-5529 (10 μM) plus tetrodotoxin (3 μM)	4.5 ± 9.8
Potassium ions (60 mM) plus RH-3421 (10 μM) plus tetrodotoxin (3 μM)	7.1 ± 28.5

[a] Calcium ions present in saline except where otherwise indicated.
[b] Values represent means ± SD of the three independent experiments.

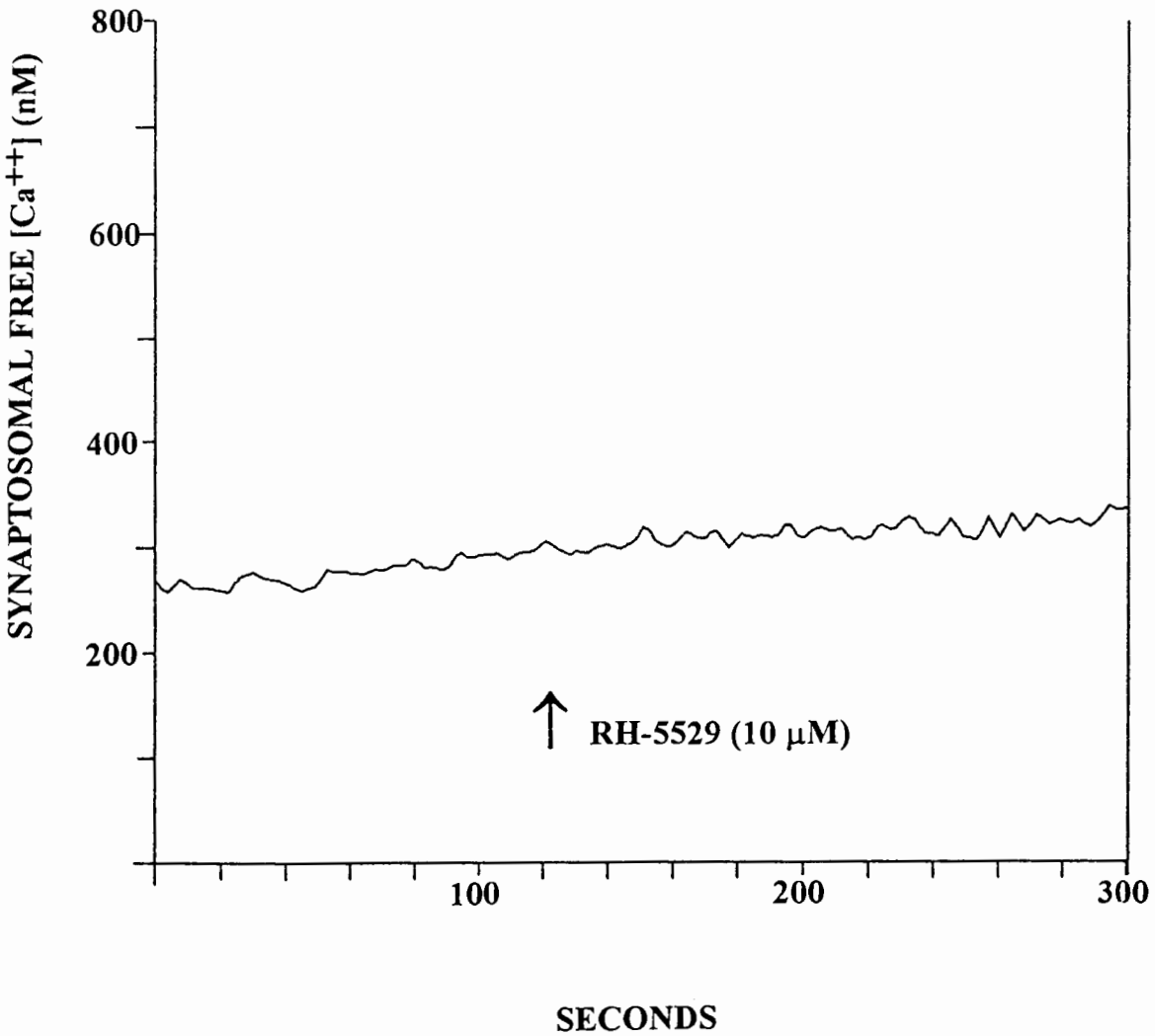


Fig. 2.3 The lack of effect of RH-5529 (10 μM) on intraterminal free [Ca^{++}] in resting (non-depolarized) synaptosomes. The record shows results for one synaptosomal preparation. Two additional preparations were examined and similar results were obtained.

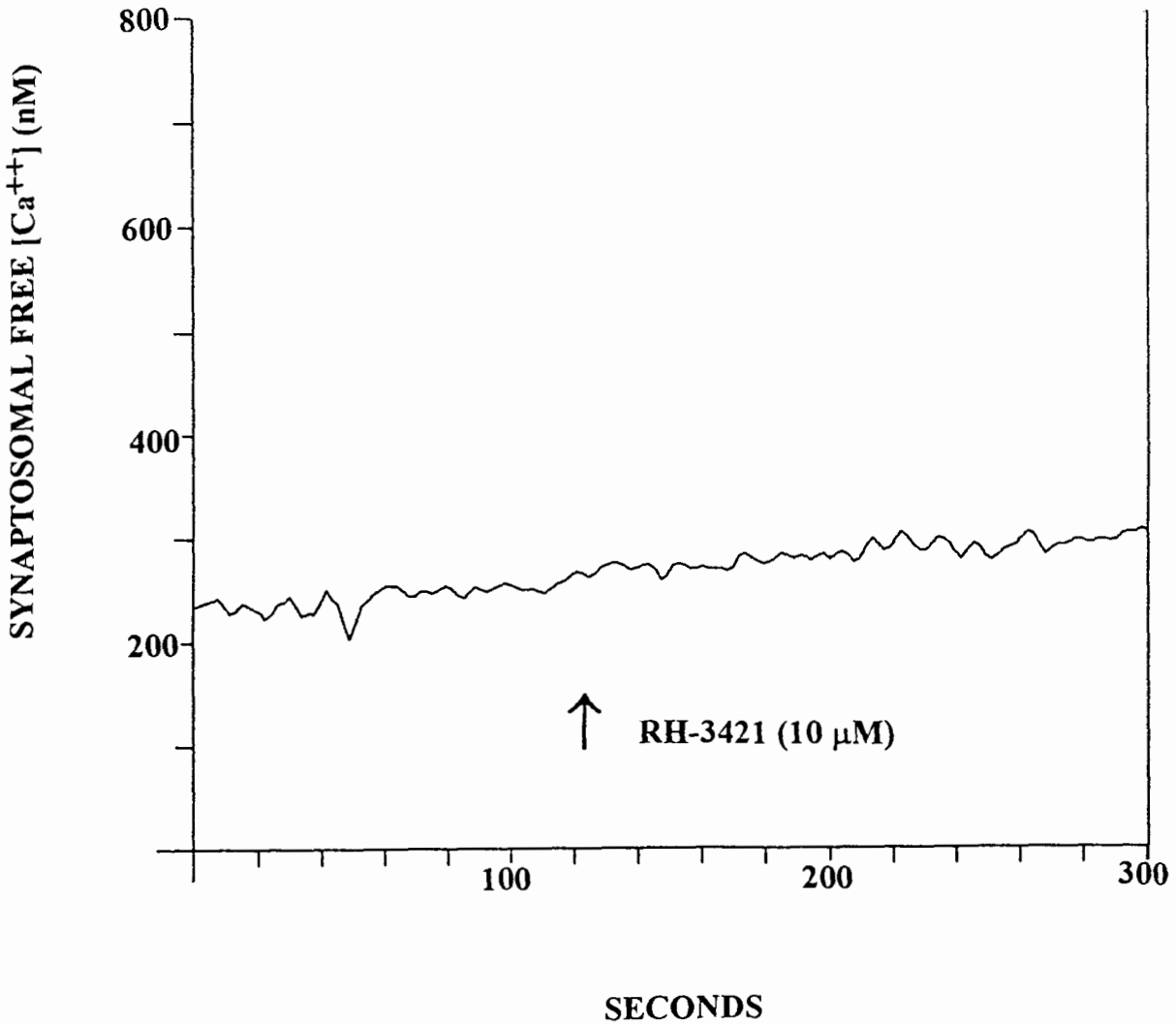


Fig. 2.4 The lack of effect of RH-3421 (10 μM) on intraterminal free [Ca^{++}] in resting (non-depolarized) synaptosomes. The record shows results for one synaptosomal preparation. Two additional preparations were examined and similar results were obtained.

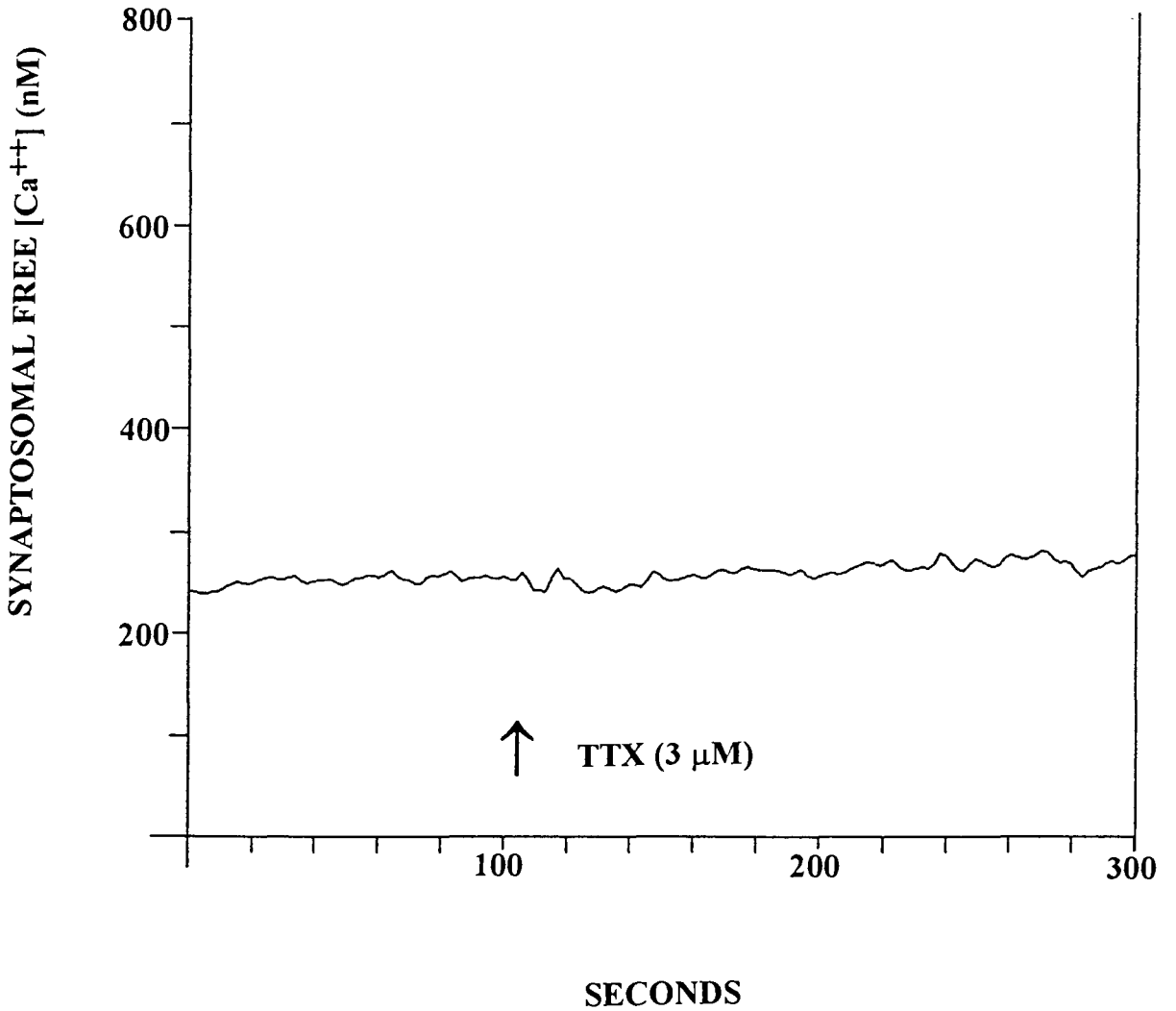


Fig. 2.5 The lack of effect of TTX (3 μM) on intraterminal free [Ca^{++}] in resting (non-depolarized) synaptosomes. The record shows results for one synaptosomal preparation. Two additional preparations were examined and similar results were obtained.

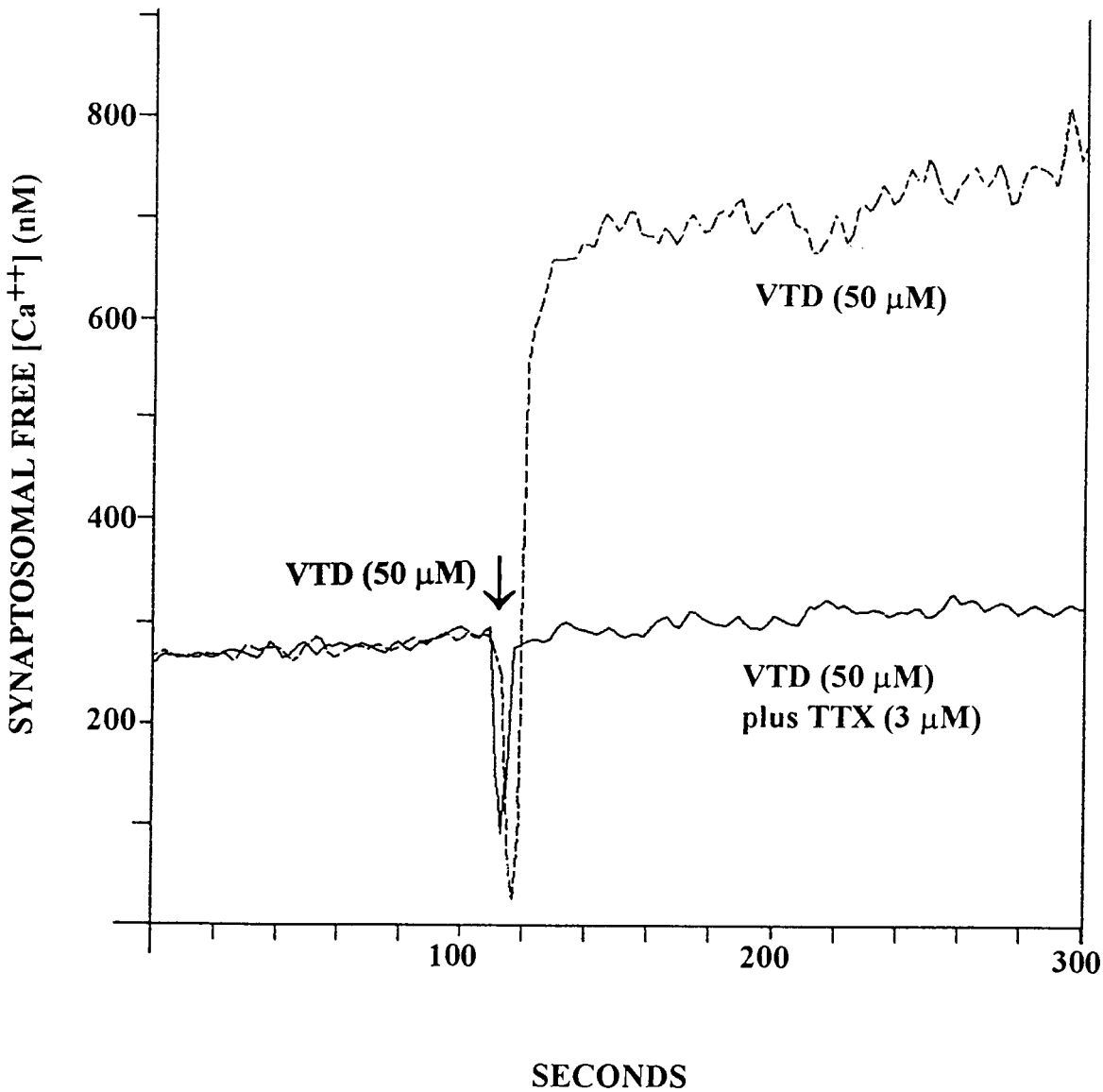


Fig. 2.6 The increase in intraterminal free [Ca^{++}] by VTD and the inhibition of this response by TTX. VTD (50 μM) was added at the arrow. TTX (3 μM) was added to synaptosomes before VTD. These records show results from synaptosomes prepared from the same brain. Two more synaptosomal preparations were examined and these showed similar responses.

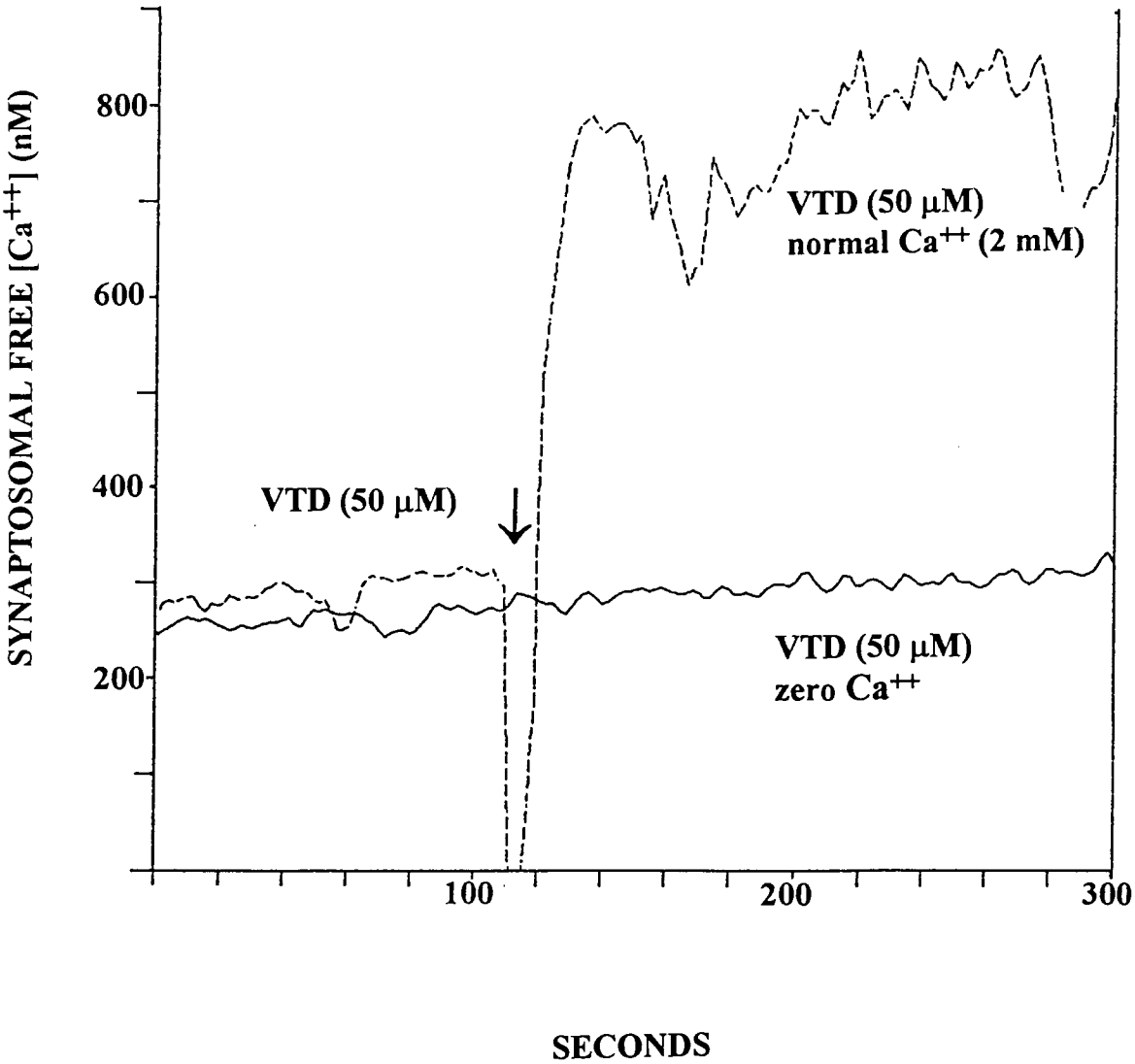


Fig. 2.7 The effect of removal of calcium from the saline on ability of VTD to increase intraterminal free [Ca^{++}]. VTD (50 μM) was added at the arrow and zero Ca^{++} saline contained EGTA (2.5 mM). These records show results from synaptosomes prepared from the same brain. Two more synaptosomal preparations were examined and these showed similar responses.

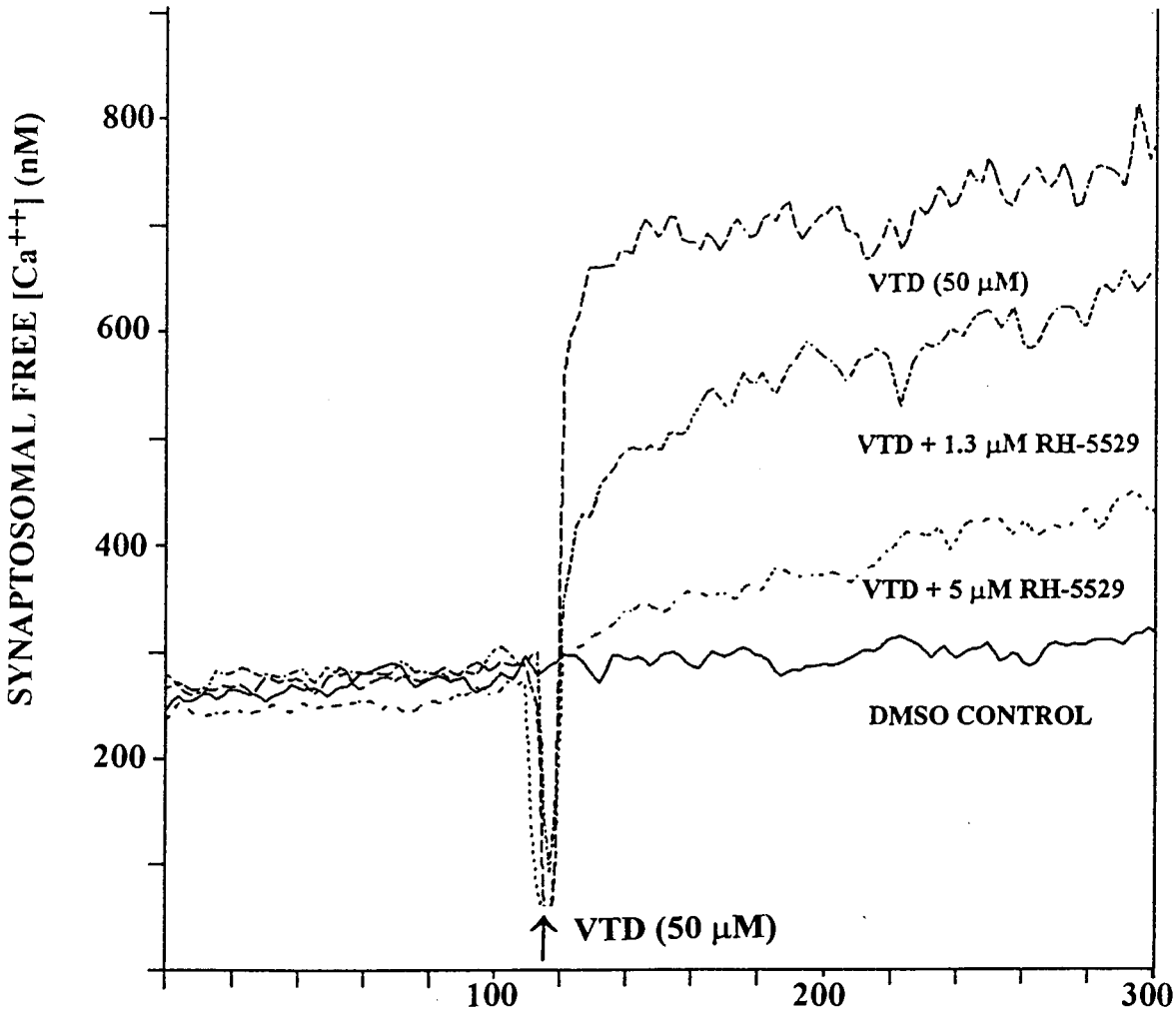


Fig. 2.8 Inhibition of the VTD-stimulated rise in intraterminal free [Ca^{++}] by RH-5529. VTD (50 μM) or solvent control was added at the arrow. RH-5529 was added to synaptosomes before VTD. These records show results from synaptosomes prepared from the same brain. Two more synaptosomal preparations were examined and these showed similar responses.

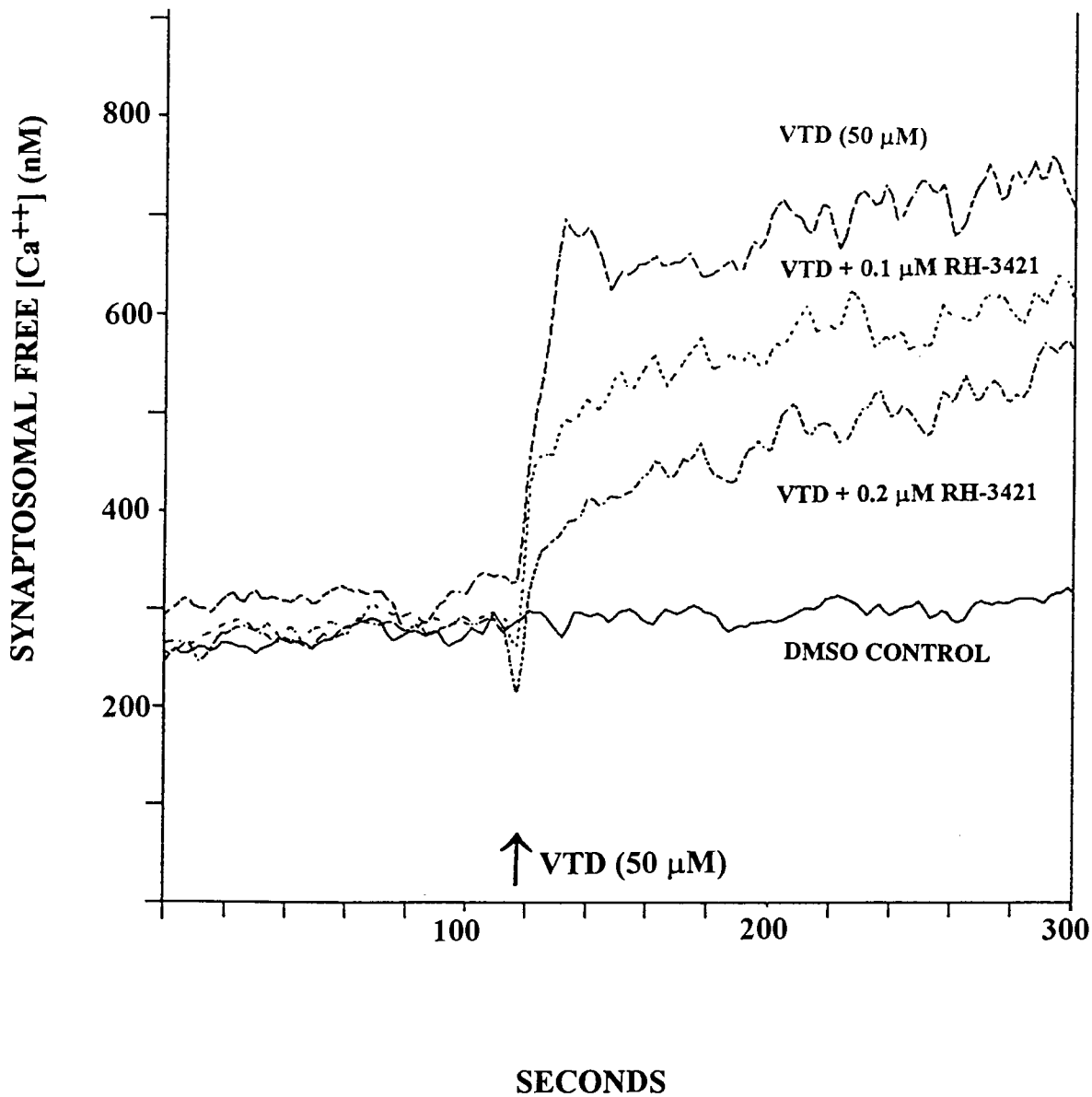


Fig. 2.9 Inhibition of the VTD-stimulated rise in intraterminal free [Ca^{++}] by RH-3421. VTD (50 μM) or solvent control was added at the arrow. RH-3421 was added to synaptosomes before VTD. These records show results from synaptosomes prepared from the same brain. Two more synaptosomal preparations were examined and these showed similar responses.

about 3 μM (Fig. 2.10), significant inhibitory effects were detected at 1.3 μM and full suppression of the VTD response was observed at concentrations approaching 10 μM. However, the IC_{50} for RH-3421 was much lower, at about 0.2 μM. Significant inhibitory effects were detected at 0.045 μM and full suppression of the response was observed at concentration of 1 μM (Fig. 2.10).

Ionic potassium increased intraterminal-free [Ca^{++}] by 172 ± 10 nM at 60 mM (Table 2.1). When synaptosomes were incubated with 3 μM TTX, it failed to influence the K^+-induced rise in synaptosomal free [Ca^{++}] (Fig. 2.11; Table 2.1).

However, both RH-5529 (Fig. 2.12; Table 2.1) and RH-3421 (Fig. 2.13; Table 2.1) blocked the rise in intraterminal free [Ca^{++}] stimulated by K^+ and very clear dose-related effects were observed for the two compounds (Fig. 2.14). Compared to VTD, there is no obvious difference between RH-3421 and RH-5529 in their ability to inhibit the increase in $[Ca^{++}]_i$ induced by K^+ at their saturating concentrations. Maximum inhibition of the K^+-response with RH-5529 was about 10 μM (Fig. 2.14), almost the same as RH-3421 (approximately 10 μM) (Fig. 2.14). But at low concentrations, RH-3421 shows greater potency than RH-5529 in inhibiting the rise of free calcium in synaptosomes stimulated by K^+. There was almost no effect of RH-5529 at the concentration of 1.32 μM. In contrast, at this concentration, RH-3421 showed about 30% inhibition. The IC_{50} of RH-3421 was about 1 μM, 3-fold less than that of RH-5529, which was about 3 μM (Fig. 2.14).

Furthermore, when synaptosomes were exposed to RH-5529 or RH-3421 with TTX, the dihydropyrazoles still fully inhibited the K^+-stimulated rise in intraterminal-free [Ca^{++}] (Fig. 2.15; Table 2.1). As has been found by others, when calcium was omitted from the saline and EGTA (2.5 mM) was included there was no discernible increase in synaptosomal free [Ca^{++}] with 60 mM K^+ as the depolarizing treatment (Fig. 2.15; Table 2.1).

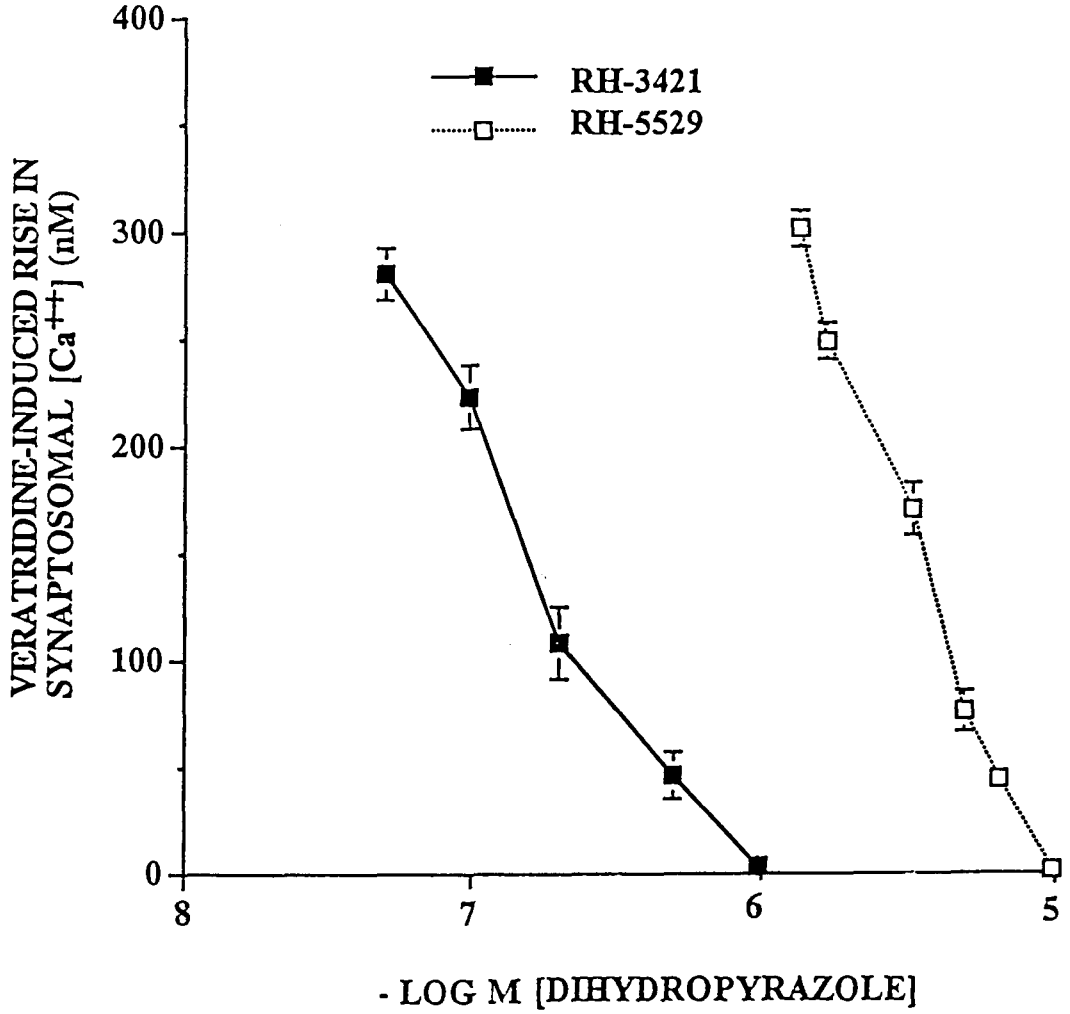


Fig. 2.10 Concentration response relationships for the inhibition of veratridine-stimulated increase in intraterminal free [Ca^{++}] by RH-3421 and RH-5529. In the absence of dihydropyrazoles the rise in free [Ca^{++}] with VTD (50 μM) averaged 342.5 ± 34.2 nM. Intraterminal free [Ca^{++}] was estimated from spectrophotometric measurements of synaptosomal preparations loaded with fura-2 (see Methods, section 2.2.4). Three replicate measurements of the average [Ca^{++}] from 160-300 seconds after addition of VTD (e.g. Figs. 2.8, 2.9) were combined to produce the data shown here (mean ± SD; n=3).

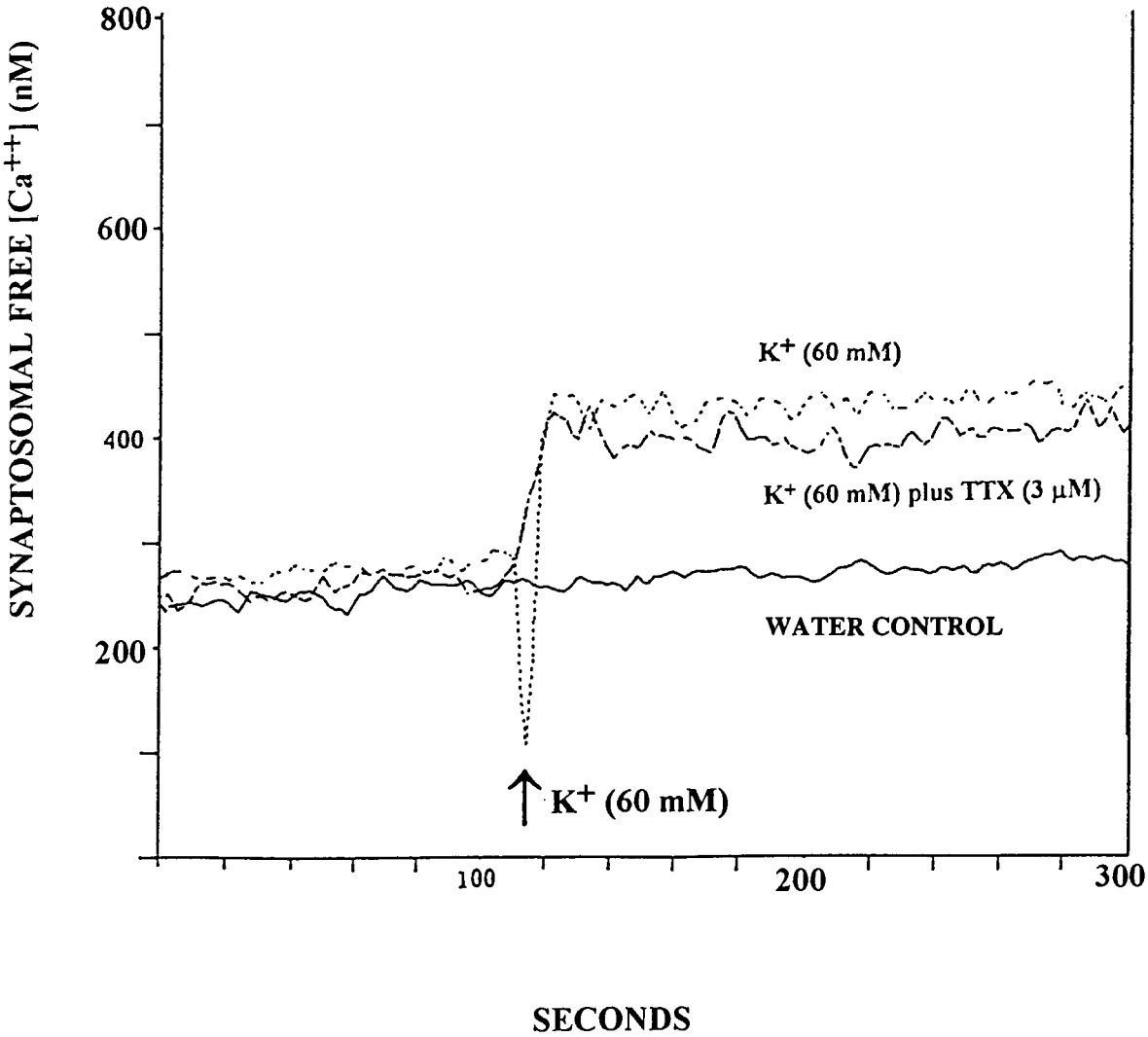


Fig. 2.11 Increase of intraterminal free [Ca^{++}] evoked by K^+ in the absence and presence of TTX. K^+ (60 mM) or solvent control was added at the arrow. Synaptosomes were suspended in TTX (3 μM) before and during challenge with K^+. These records show results from synaptosomes prepared from the same brain. Two more synaptosomal preparations were examined and these showed similar responses.

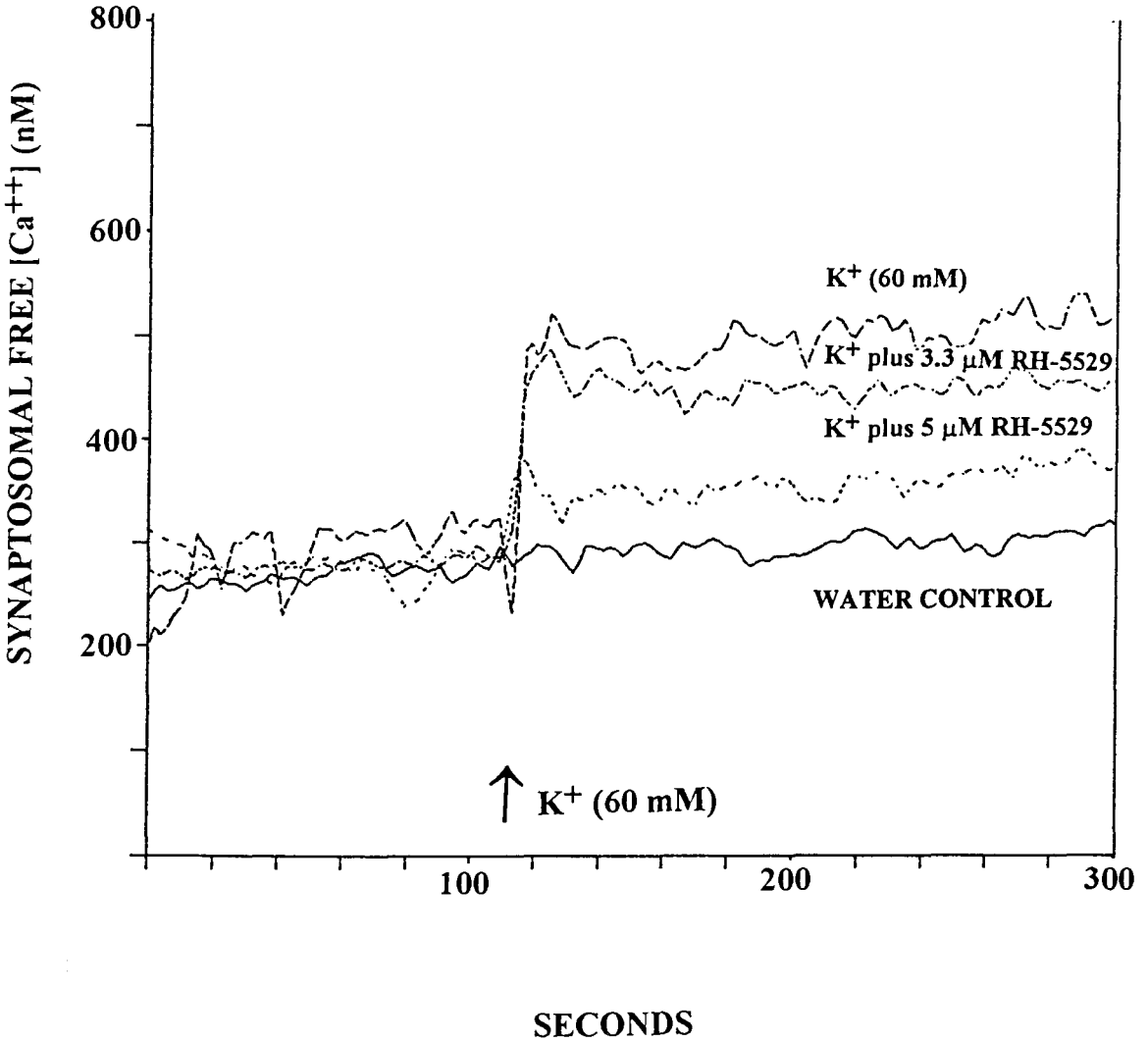


Fig. 2.12 Inhibition of K^+-stimulated increases in intraterminal free [Ca^{++}] by RH-5529. K^+ (60 mM) or solvent control was added at the arrow. Synaptosomes were suspended in RH-5529 before and during challenge with K^+. These records show results from synaptosomes prepared from the same brain. Two more synaptosomal preparations were examined and these showed similar responses.

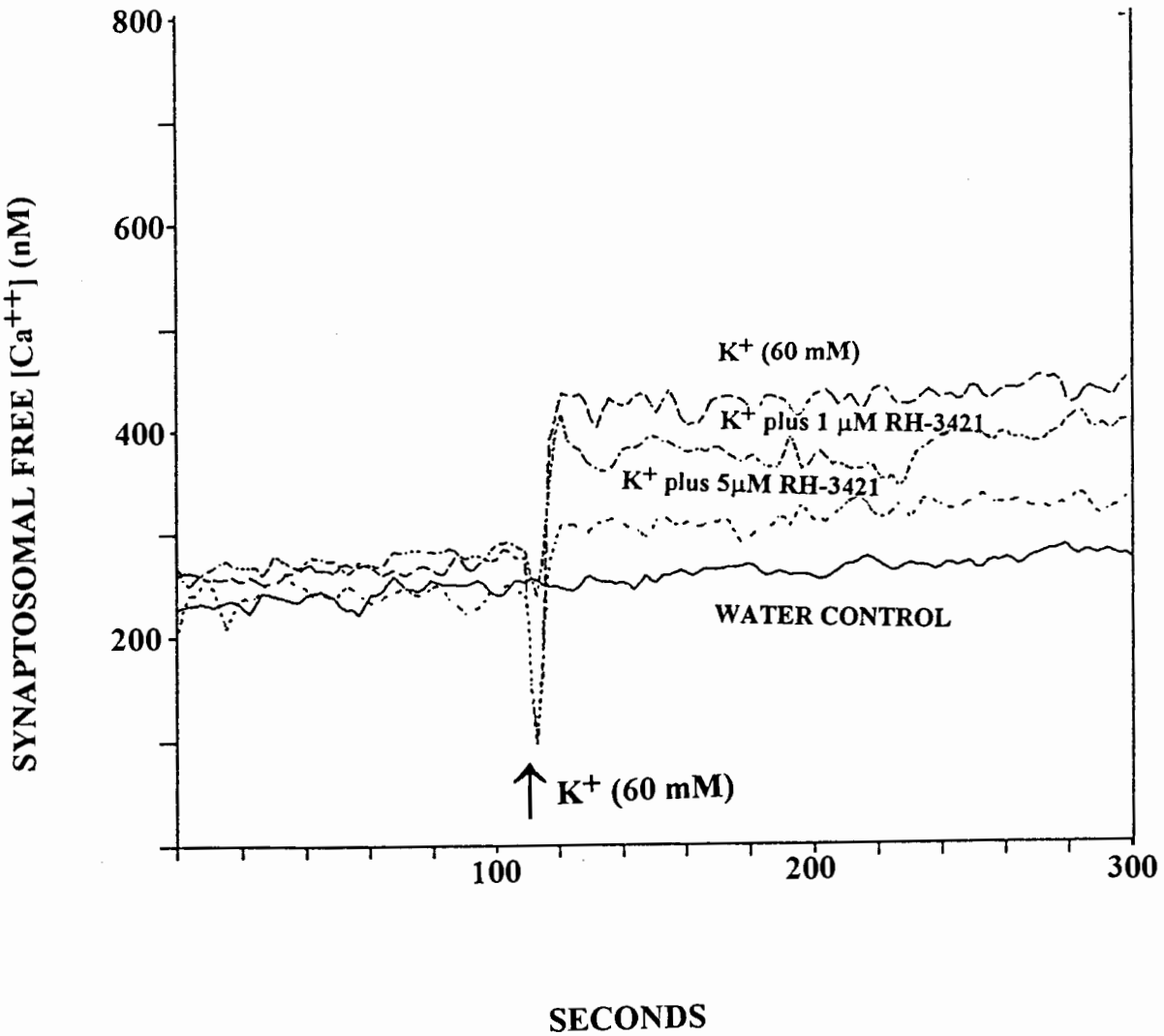


Fig. 2.13 Inhibition of K^+-stimulated increase in intraterminal free [Ca^{++}] by RH-3421. K^+ (60 mM) or solvent control was added at the arrow. Synaptosomes were suspended in RH-3421 before and during challenge with K^+. These records show results from synaptosomes prepared from the same brain. Two more synaptosomal preparations were examined and these showed similar responses.

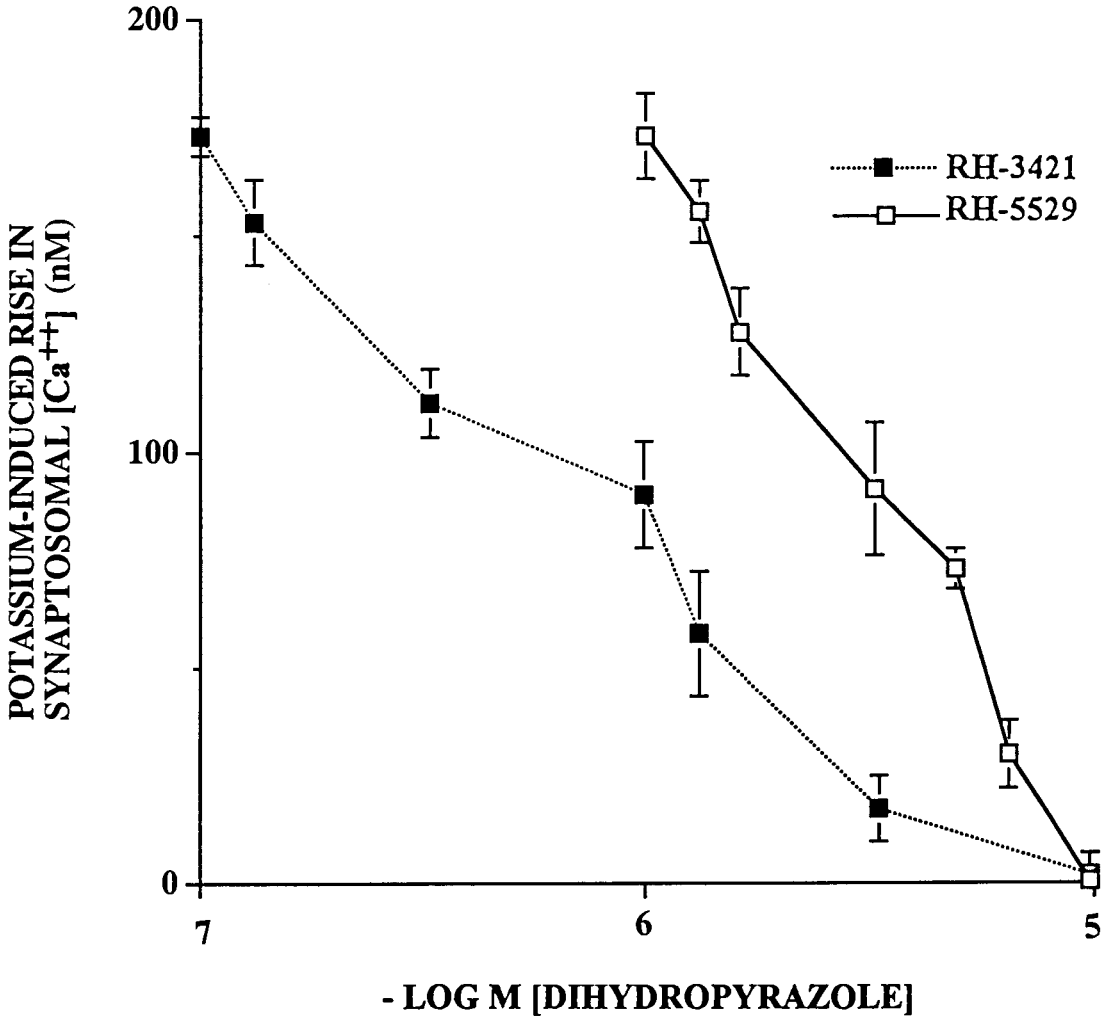


Fig. 2.14 Concentration-dependent inhibition by RH-3421 and RH-5529 of the rise in intraterminal free [Ca^{++}] by 60 mM K^+. In the absence of dihydropyrazoles, 60 mM K^+ increased free [Ca^{++}] by172.3 ± 9.9 nM. Intraterminal free [Ca^{++}] was estimated from spectrophotometric measurements of synaptosomal preparations loaded with fura-2 (see Methods, section 2.2.4). Three replicate measurements of the average [Ca^{++}] from 140-300 seconds after addition of K^+ ions (e.g. Figs. 2.12, 2.13) were combined to produce the data shown here (mean ± SD; n=3).

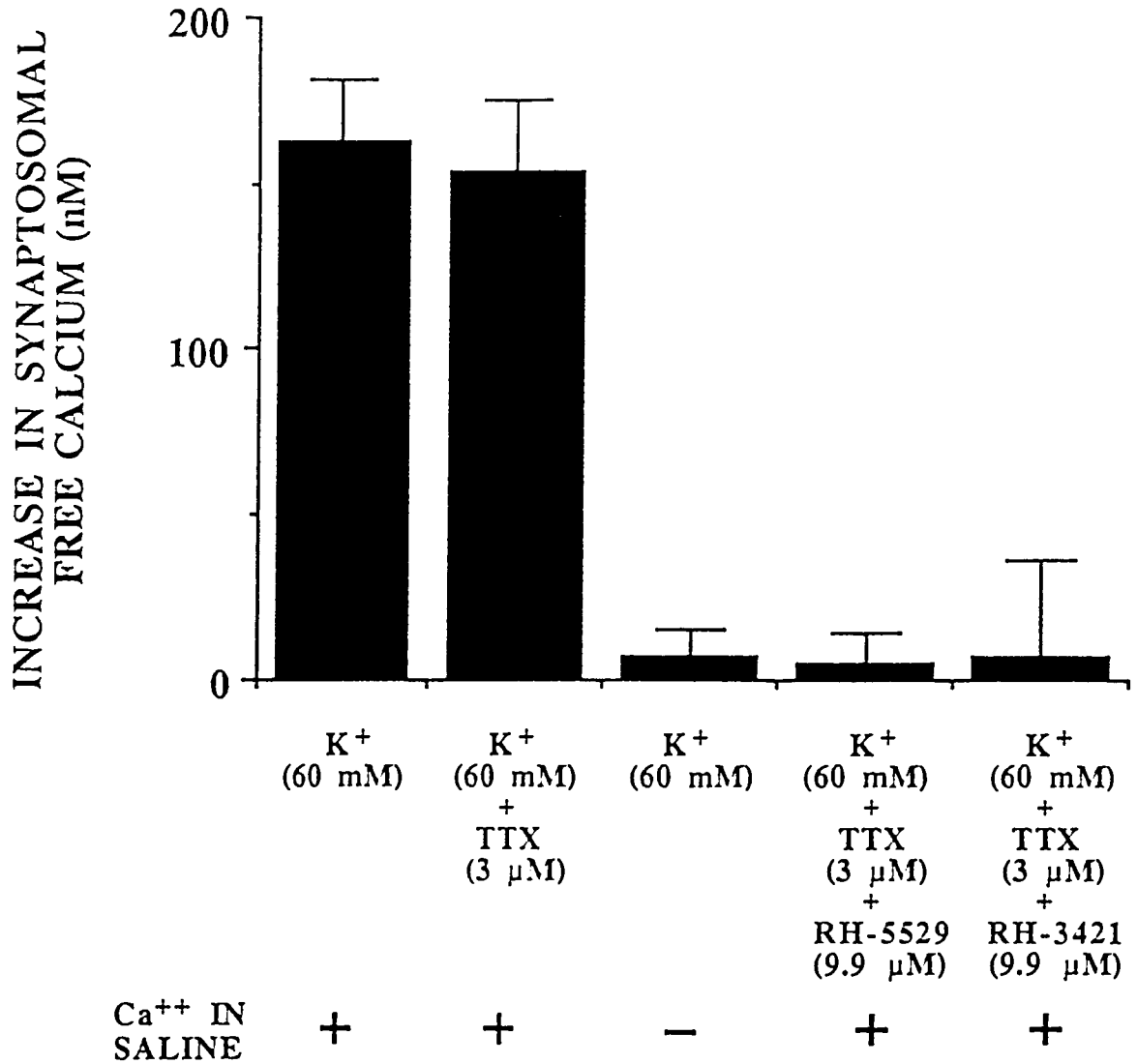


Fig. 2.15 K^+-induced rise in intraterminal free [Ca^{++}] in the presence and absence of external Ca^{++} and the effect of RH-3421 and RH-5529 on the K^+-induced rise in intraterminal free [Ca^{++}] in the presence of TTX (3 μM).Values are means ± SD of three separate synaptosomal preparations.

2.3.3 Effects of Insecticidal Dihydropyrazoles on Depolarization-Dependent $^{45}Ca^{++}$ Uptake in Mouse Brain Synaptosomes

To complement the fluorescence work, I carried out a series of experiments to investigate the actions of dihydropyrazoles on $^{45}Ca^{++}$ uptake by synaptosomes isolated from mouse brain. Under non-depolarizing (resting) conditions, RH-5529 and RH-3421 had no effect on radiocalcium influx at high concentrations (Table 2.2). Activating calcium channels with K^+ (60 mM), typically produces 2,251 ± 253 cpm/mg protein of $^{45}Ca^{++}$ influx above control value (Table 2.2).

However, K^+-stimulated $^{45}Ca^{++}$ uptake by the synaptosomal fraction is inhibited by the dihydropyrazoles (Fig. 2.16). Threshold inhibition with each analogue was detected at concentrations approaching 1 μM. Full inhibition was observed at 100 μM for the two compounds and inhibition curves were quantitatively similar (IC_{50}s: RH-3421 & RH-5529 ~ 11 μM). Under the same experimental conditions, ionic cobalt, a known inhibitor of calcium channels, fully blocked K^+-evoked $^{45}Ca^{++}$ influx (Fig. 2.17). Also in agreement with previous findings on fura-2, the dihydropyrazoles are capable of inhibiting K^+-stimulated $^{45}Ca^{++}$ influx in the presence of a saturating concentration of TTX (Fig. 2.17).

2.4 Discussion

The electron micrograph (Fig. 2.1) demonstrates that synaptosomes isolated from mouse brain using the Percoll procedure, possess a morphology and purity that compares favorably with that of Percoll fraction 4 described by Dunkley *et al.* (1986). The fact that the synaptosomes from fraction 4 support depolarization-induced increases in cytosolic free [Ca^{++}], $^{45}Ca^{++}$ uptake, synapsin I phosphorylation and L-glutamate release (see this and later chapters) confirmed they possess a high level of functional competence.

Table 2.2 Lack of effect of RH-3421 and RH-5529 on $^{45}Ca^{++}$ uptake in resting (non-depolarized) synaptosomes.

Treatment	$^{45}Ca^{++}$ influx into synaptosomes [ab] (cpm / mg protein)
Control	2,266 ± 140
RH-3421 (100 μM)	2,472 ± 275
RH-5529 (100 μM)	2,452 ± 255

[a] Values as mean corrected cpm ± standard error of three determinations

[b] K^+-stimulated influx of $^{45}Ca^{++}$ into synaptosomes was 2,251 ± 253 cpm/mg protein above control value.

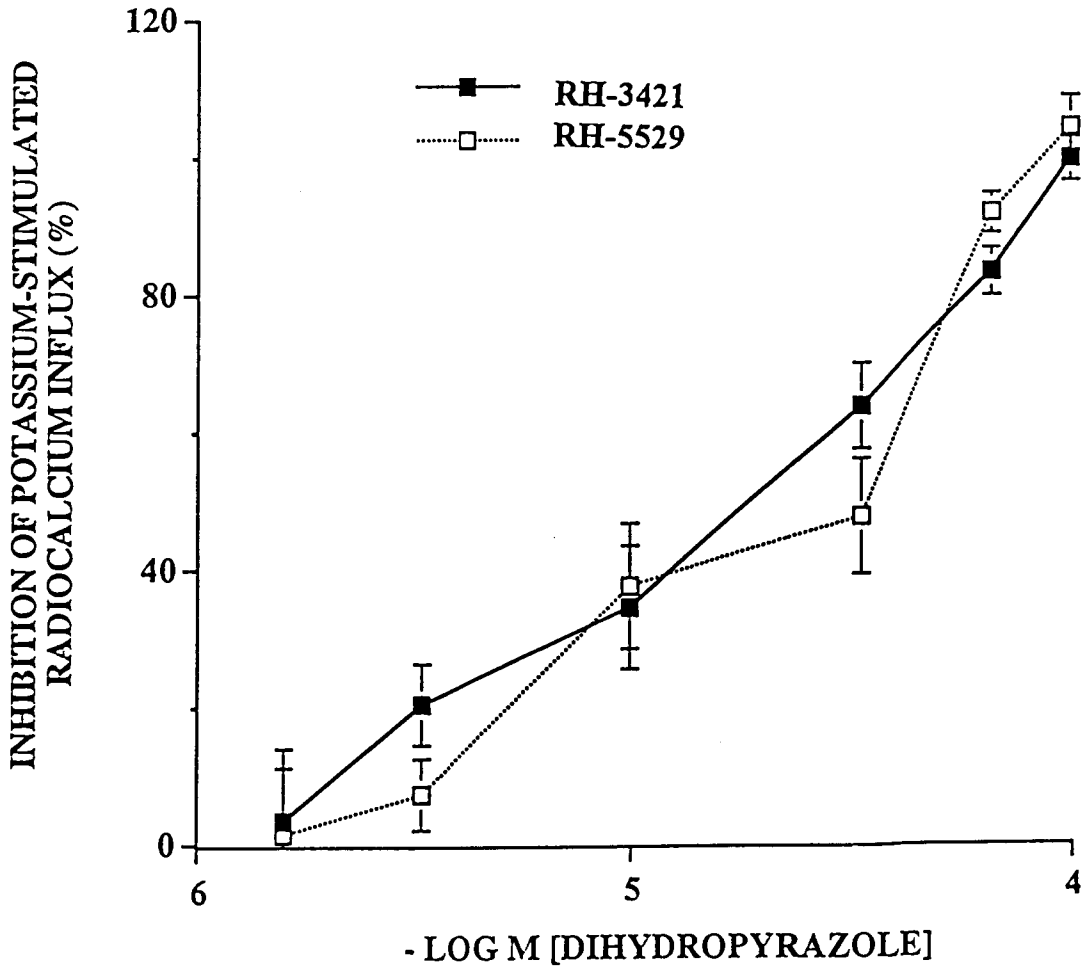


Fig. 2.16 Inhibition of K^+-stimulated $^{45}Ca^{++}$ uptake by RH-3421 and RH-5529 in synaptosomes isolated from mouse brain. Values are means ± SD of three to six independent experiments.

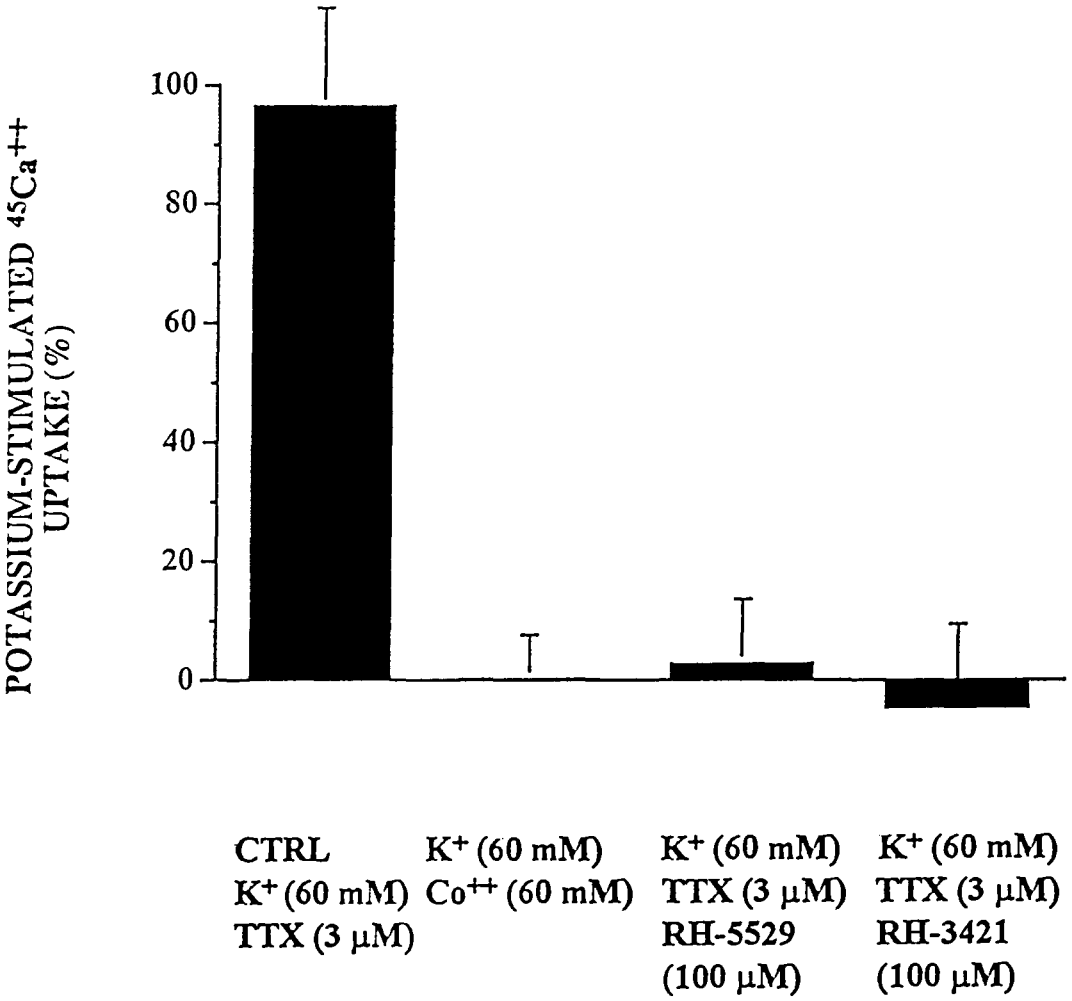


Fig. 2.17 Lack of effect of TTX (3 μM) on the K^+-induced $^{45}Ca^{++}$ uptake and the effect of RH-3421 and RH-5529 on the K^+-stimulated $^{45}Ca^{++}$ uptake in the presence of TTX (3 μM). Co^{2+} fully suppressed $^{45}Ca^{++}$ uptake induced by K^+ at the same conditions. Values are means ± SD of three to six independent experiments.

An elevated concentration of potassium ions is widely used as an experimental treatment to rapidly depolarize the nerve terminal. Depolarization under these circumstances directly activates voltage-sensitive calcium channels resulting in Ca^{++} influx (Blaustein and Goldring, 1975; Scott and Nicholls, 1980). Veratridine is also used to depolarize the nerve terminal, but this acts an a different way, by selectively opening voltage-sensitive sodium channels (Blaustein, 1975). The entry of sodium ions depolarizes the nerve membrane, which then in turn activates voltage-gated calcium channels, permitting calcium entry.

The fura-2 experiments demonstrate that the increase in intraterminal free [Ca^{++}] produced by VTD is fully suppressed by the sodium channel blocker TTX (Fig. 2.6; Table 2.1), thus confirming the sodium channel dependence of this alkaloid's action. In agreement with the results of others (Heemskirk *et al.*, 1991), the rise in intracellular free [Ca^{++}] following K^+ challenge is not affected by TTX (see Figs. 2.11 and 2.15), and the response is highly dependent on extrasynaptosomal Ca^{++} (Fig. 2.15; Table 2.1).

Clearly, a significant finding of this part of the investigation is that RH-3421 and RH-5529 are capable of fully suppressing a rise in intraterminal free [Ca^{++}] when elicited not only by VTD but also high [K^+]. Any possible involvement of sodium channels in the inhibitory action of dihydropyrazoles during the K^+-activated rise in [Ca^{++}] (e.g. low level influx of calcium ions through sodium channels, Hille, 1992) was essentially eliminated by including TTX at 3 μM in the assay (Fig. 2.15; Table 2.1). The conclusions of the fura-2 experiments are essentially substantiated by the $^{45}Ca^{++}$ uptake results which clearly show that both RH-3421 and RH-5529 block K^+-induced entry of $^{45}Ca^{++}$ (Fig. 2.16) and this occurs with TTX present in the assay (Fig. 2.17).

The observation that RH-3421 and RH-5529 (10-100 μM) have no effect on intraterminal free [Ca^{++}] or $^{45}Ca^{++}$ uptake into synaptosomes under resting

conditions is in close agreement with previous work which shows that dihydropyrazoles fail to influence the resting potential of invertebrate or vertebrate nerve preparations at high concentrations (Salgado, 1990; Nicholson, 1992). These results however are apparently at variance with a study which found that low micromolar concentrations of these insecticides reduce spontaneous efflux of [^{3}H]GABA from synaptosomes (Nicholson and Merletti, 1990). However, because this effect was also observed with TTX, it may indicate a small amount of sodium channel instability in synaptosomes held under superfusion conditions. Certainly no resting perturbations were observed with dihydropyrazoles and TTX in the present experiments or for that matter in the L-glutamate release and phosphorylation experiments (see later), all of which used synaptosomes in suspension.

Data obtained in these fura-2 experiments allowed conclusions to be drawn on the potencies of dihydropyrazoles as inhibitors of sodium channel- (VTD-) dependent and calcium channel- (K^+-) dependent responses. The IC_{50}s for RH-5529 were similar at 3 and 3.5 μM for VTD and K^+-stimulated increases respectively. Compared to RH-5529, RH-3421 is 15-fold more potent as a sodium channel blocker (IC_{50} = 0.2 μM) and approximately 3-fold more effective as an inhibitor of calcium channels (IC_{50} = 1 μM). The inhibitory potencies of dihydropyrazoles against K^+-evoked responses are somewhat lower in the $^{45}Ca^{++}$ uptake assay (IC_{50}s for RH-3421 and RH-5529 = 11 μM), however these differences are not unreasonable given the fact Ca^{++} measurements necessitate longer assay times than fluxing measurements and unavoidable exchange of $^{45}Ca^{++}$ during the filtration and wash stage may lower sensitivity.

In conclusion, the results of this phase of the work provide compelling evidence that RH-3421 and RH-5529 interact with the nervous system through a mechanism that is independent of their previously documented actions on sodium

channels. Since it has been demonstrated that the response to elevated K^+ depends on external Ca^{++}, it is reasonable to conclude that an important alternative site (of which there are more than one type) of action for dihydropyrazoles is voltage-sensitive calcium channels in the nerve terminal membrane.

Chapter 3. Interference with Depolarization-Dependent Phosphorylation of Synapsins Ia and Ib by Insecticidal Dihydropyrazoles

3.1 Introduction

It could be argued that the next logical step after completing the fura-2 and $^{45}Ca^{++}$ analyses would be to examine the interaction of dihydropyrazoles with synaptosomal calcium channels using a binding assay. However, I felt that there were aspects of the action of dihydropyrazoles on subsequent biochemical processes involved in presynaptic stimulus-secretion coupling and transmitter release that warranted further study before starting the binding work. The phosphorylation experiments on synapsin I described in this chapter and the study on L-glutamate release in the next chapter were undertaken primarily for two reasons. Firstly, studying the effects of dihydropyrazoles on these calcium-activated signalling pathways offered an additional (indirect) means to check our emerging theory that these neurotoxicants block voltage-sensitive calcium channels in the nerve ending. Secondly, data derived would help to define experimentally the sequence of cellular perturbations that could occur after nerve endings are exposed to dihydropyrazoles which likely lead to overt symtoms of poisoning in the whole animal.

The phosphorylation status of synaptosomal proteins is suggested to play a pivotal role in the control of transmitter release from nerve endings, because it can provide an important molecular link between depolarization-coupled entry of calcium ions and association of transmitter vesicles with release sites on the presynaptic membrane (Valorta *et al.*, 1992; Sikorski *et al.*, 1991, Shepherd, 1994). After synapsin I is phosphorylated by calcium-dependent protein kinases, neurotransmitter vesicles are freed from cytoskeleton and so can potentially undergo fusion with the presynaptic membrane (Lodish *et al.*, 1995).

Pharmacological treatments known to increase Ca^{++} transport across the plasma membranes of nerve terminals have been found to stimulate the phosphorylation of synapsin I in intact mammalian synaptosomes (Krueger *et al.*, 1977; Robinson and Dunkley, 1983; Robinson and Dunkley, 1985). As outlined in Chapter 1, the depolarizing agents VTD and K^+ have been shown to markedly stimulate the incorporation of $^{32}P_i$ into synapsins Ia and Ib (Krueger *et al.*, 1977; Robinson and Dunkley, 1983). It has been established that the phosphorylation status of synapsin I depends strongly on extracellular calcium ions (Robinson and Dunkley, 1983; Robinson and Dunkley, 1985). Since dihydropyrazoles have been demonstrated to block Ca^{++} influx induced by VTD and K^+ (Chapter 2), these compounds should affect depolarization-coupled phosphorylation of synapsins Ia and Ib in mouse brain synaptosomes. Investigations at this level of cellular control are considered important to gaining a more comprehensive understanding of how insecticidal neurotoxicants interfere with synaptic function (Clark and Matsumura, 1991).

3.2 Materials and Methods

3.2.1 Chemicals

Radiolabeled orthophosphate (10 mCi/ml) was purchased from Amersham Canada Ltd. (Oakville, Ontario, Canada). VTD was supplied by Wellcome Foundation (Berkhamsted, UK). SDS was obtained from ICN Pharmaceuticals Inc. (Montreal, Canada) and all other chemicals for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO). RH-3421 and RH-5529 were provided by Rohm and Haas Co. (Philadelphia, PA). The remaining chemicals employed in this study were analytical grade.

3.2.2 Labeling of Synaptosomal Proteins Using $^{32}P_i$

After receiving two washes in Ca^{++}- and PO_4- free physiological saline, the synaptosomal fraction was resuspended in a total volume of 450 µl of this medium. Radiolabeled orthophosphate (30 µl) was slowly added and the mixture incubated for 20 min at 37 °C. Portions of this suspension (55 µl) were then placed in tubes containing inhibitors (dihydropyrazoles or TTX) or control preincubation treatments as appropriate, dissolved in saline (55 µl). The suspension was mixed thoroughly. After 10 min preincubation, 55 µl aliquots of these samples were transferred to tubes with saline (55 µl) containing calcium chloride (1 mM), the activators [VTD (50 µM) or K^+ (70 mM) final concentrations] both with and without inhibitors, inhibitors alone, or solvent controls, followed by rapid vortex mixing. Incubations were continued for precisely 15 sec, whereupon "stopping buffer" (110 µl) containing glycerol (10%), SDS (10%), β-mercaptoethanol (0.05 mM), and bromophenol blue (0.05%) was added. The mixture was then vigorously vortexed, and the tubes were transferred to ice. After all samples had been processed in this way, they were heated in a boiling water bath for 2-5 min prior to electrophoresis.

3.2.3 Electrophoresis of Synaptosomal Proteins

Synaptosomal proteins (20 µg total protein per lane) were separated on 10% polyacrylamide sodium dodecyl sulfate gels (Takacs, 1979) with a 4% stacking gel using a Mini-Protean II dual-slab cell (Bio-Rad). Proteins were fixed and stained with 0.1% Coomassie blue R-250 in methanol + acetic acid (4 + 1 by vol). Gels were then dried under reduced pressure at 80 °C for 120 min. Phosphoproteins were located by autoradiography using Kodak X-Omat RP XRP-1 film and intensifier screens. Incorporation of radiophosphorus into synaptosomal proteins was quantitated by digital scanning of the autoradiographs and then applying a

densitometric analysis (Macintosh Quadra 650 using NIH Image 1.44 software). The apparent molecular weights of phosphoproteins in question were estimated by coelectrophoresis of reference standards. Our confirmation in the present study that $^{32}P_i$-labeling of two synaptosomal phosphoproteins of apparent molecular weights 80 and 75 kDa is enhanced by depolarization, when calcium ions are present in external saline (Figs. 5.2 and 5.3, and Table 5.1), correlates with the findings of other researchers (Gomez-Putertas *et al.*, 1991; Robinson and Dunkley, 1983) and suggests that the phosphoproteins examined in this study are synapsins Ia and Ib.

3. 3 Results

3.3.1 The Effect of Insecticidal Dihydropyrazoles on the Phosphorylation of Synapsins Ia and Ib

The protein extracts obtained from mouse brain synaptosomes were separated by SDS-polyacrylamide gel electrophoresis and Fig. 3.1 shows a typical separation. The molecular weights of the phosphoproteins in question were estimated after autoradiography by comparison with standard proteins of known molecular weight.

Under resting conditions, neither dihydropyrazoles nor TTX are able to change the level of incorporation of $^{32}P_i$ into synapsins Ia and Ib (Fig. 3.2; Table 3.1). The depolarizing treatments, VTD and K^+, were unable to increase the incorporation of $^{32}P_i$ into synapsin when calcium ions were omitted from the saline and EGTA included (Fig. 3.2; Table 3.1).

However, in the presence of calcium ions, both VTD and K^+ strongly increased the incorporation of $^{32}P_i$ into the 75k and 80k dalton proteins (Fig. 3.3; Table 3.1), indicating that the two phosphoproteins in question are synapsins Ia and Ib. The increase in phosphorylation of synapsin induced by VTD was virtually

Fig. 3.1 Proteins separated from mouse brain synaptosomes compared with reference standards (Coomassie blue stain).

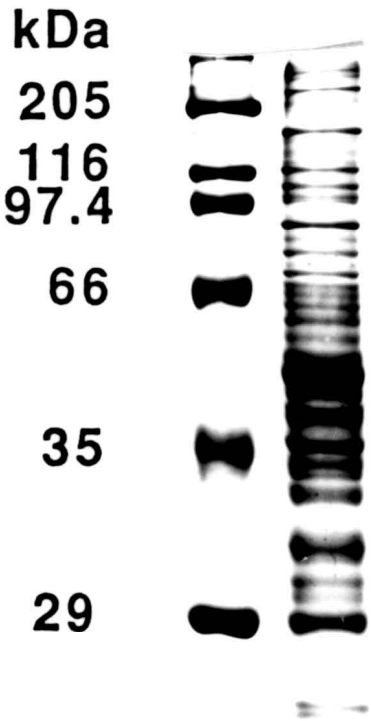

kDa
205
116
97.4
66
35
29

Fig. 3.2 Representative autoradiograph showing the effect of TTX and dihydropyrazoles RH-3421 and RH-5529 on the level of phosphorylation of synapsins Ia and Ib in resting synaptosomes. Depolarizing agents failed to increase the phosphorylation of synapsins Ia and Ib when calcium ions were removed from the assay.

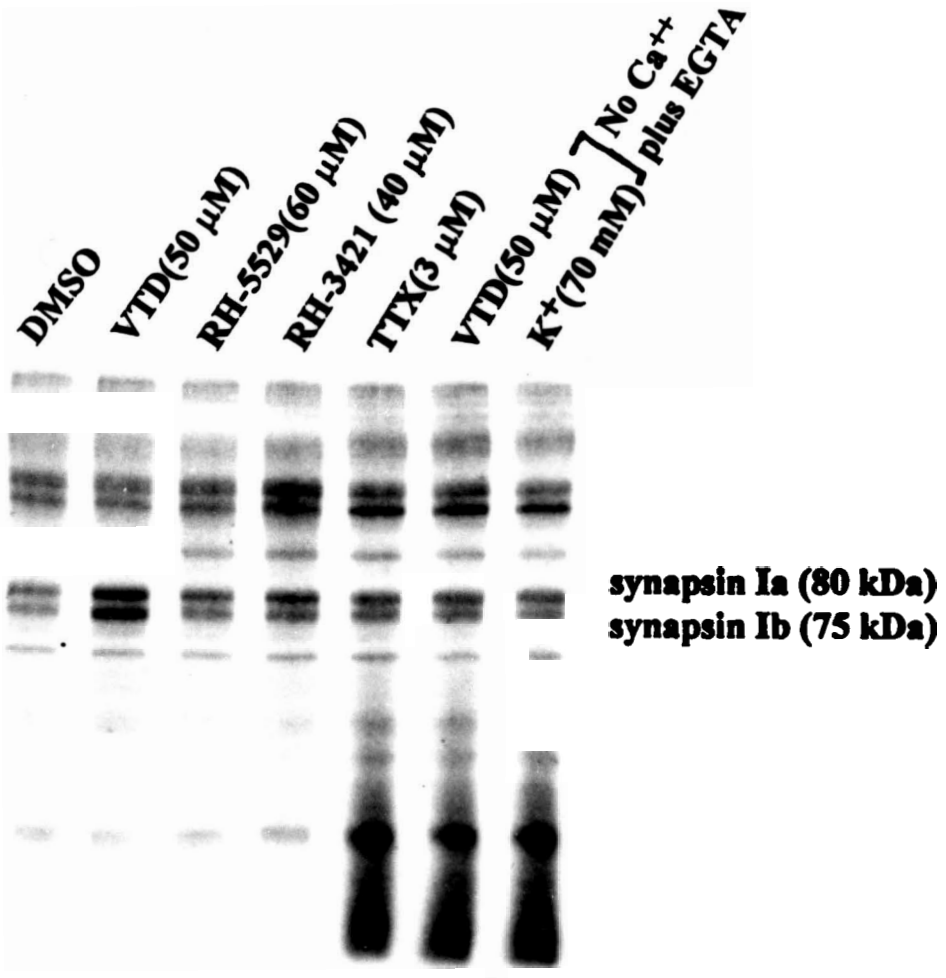

DMSO
VTD(50 μM)
RH-5529(60 μM)
RH-3421 (40 μM)
TTX(3 μM)
VTD(50 μM)
K+(70 mM)
No Ca++
plus EGTA
synapsin Ia (80 kDa)
synapsin Ib (75 kDa)

Table 3.1 Effects of dihydropyrazoles on the phosphorylation of synapsin I [a] in resting and depolarized mouse brain synaptosomes

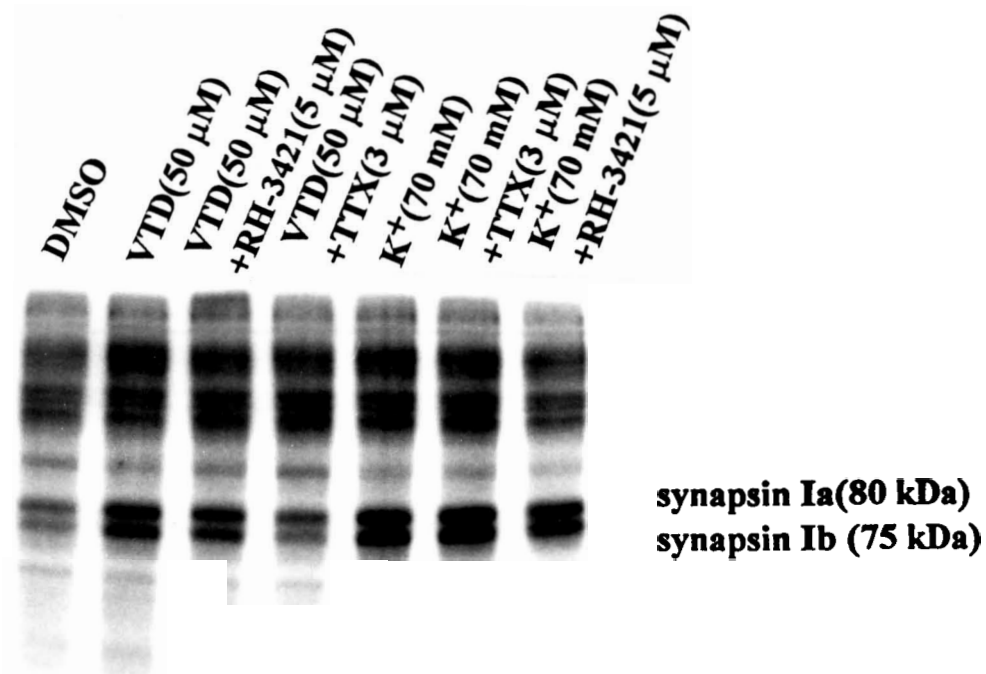
Treatments [b]	Percentage change in phosphorylation of synapsin I relative to basal activity [c]	% Inhibition
Tetrodotoxin (3 μM)	1.2 ± 1.9	
RH-3421 (40 μM)	2.1 ± 3.1	
RH-5529 (60 μM)	2.2 ± 5.0	
Veratridine (50 μM)	73.9 ± 3.8	
Potassium ions (70 mM)	82.1 ± 7.2	
Veratridine (50 μM) plus tetrodotoxin (3 μM)	-7.9 ± 3.2	110.6
Potassium ions (70 mM) plus tetrodotoxin (3 μM)	68.3 ± 3.5	16.8
Veratridine (50 μM) plus RH-3421 (5 μM)	51.0 ± 2.1	30.9
Veratridine (50 μM) plus RH-3421 (40 μM)	2.5 ± 3.2	96.6
Potassium ions (70 mM) plus RH-3421 (5 μM)	52.9 ± 2.4	35.6
Potassium ions (70 mM) plus RH-3421 (40 μM)	2.6 ± 2.8	96.8
Potassium ions (70 mM) plus RH-3421 (40 μM) plus tetrodotoxin (3 μM)	7.9 ± 1.7	90.4
Veratridine (50 μM) plus RH-5529 (5 μM)	53.0 ± 2.8	28.3
Veratridine (50 μM) plus RH-5529 (60 μM)	0.1 ± 7.3	99.9
Potassium ions (70 mM) plus RH-5529 (5 μM)	54.8 ± 1.9	33.3
Potassium ions (70 mM) plus RH-5529 (60 μM)	8.0 ± 3.5	90.3
Potassium ions (70 mM) plus RH-5529 (60 μM) plus tetrodotoxin (3 μM)	0.4 ±8.0	100.6
Veratridine (50 μM) (no Ca^{++} plus EGTA at 2.5 mM)	4.5 ± 2.4	93.9
Potassium ions (70 mM) (no Ca^{++} plus EGTA at 2.5 mM)	8.0 ± 5.2	90.3

[a] As total of synapsins Ia and Ib.

[b] Calcium ions present in saline except where otherwise indicated.

[c] Values represent means ± SE of three to six independent experiments.

Fig. 3.3 Typical autoradiograph showing the effect of TTX on VTD- and K^+-induced phosphorylation of synapsins Ia and Ib.


DMSO
VTD(50 μM)
VTD(50 μM) +RH-3421(5 μM)
VTD(50 μM) +TTX(3 μM)
K+(70 mM)
K+(70 mM) +TTX(3 μM)
K+(70 mM) +RH-3421(5 μM)
synapsin Ia(80 kDa)
synapsin Ib (75 kDa)

abolished by the sodium channel blocker TTX (Fig. 3.3; Table 3.1). In contrast, K^+-stimulated increase in phosphorylation of synapsin was largely unaffected by TTX at the same conditions (Fig. 3.3; Table 3.1).

At 60 μM, RH-5529 caused full blockade of the increase in phosphorylation of synapsin produced by VTD and approximately 30% inhibition was observed at a concentration of 5 μM (Fig. 3.4; Table 3.1). K^+-evoked increase in phosphorylation of synapsin was also strongly inhibited (> 90%) by RH-5529 at 60 μM. At lower concentrations (5 μM) a 33% decrease of the incorporation of $^{32}P_i$ into the phosphoproteins was observed (Fig. 3.4; Table 3.1).

Likewise RH-3421 prevented the increase in phosphorylation of synapsin evoked by VTD causing greater than 95% inhibition at a concentration 40 μM (Fig. 3.5; Table 3.1). At this concentration, RH-3421 also effectively suppressed K^+-stimulated increases in the incorporation of $^{32}P_i$ into synapsins Ia and Ib in mouse brain synaptosomes (Fig. 3.5; Table 3.1).

In agreement with the effects of dihydropyrazoles on calcium mobilization (Chapter 2), the depolarization-stimulated rise in phosphorylation of synapsins Ia and Ib produced by elevated $[K^+]$ is also blocked when TTX is included in the assay to ensure flux of sodium ions or small quanities of calcium ions through sodium channels does not occur (Fig. 3.6 and Table 3.1).

3.4 Discussion

The investigation using mouse brain preparations demonstrates that concentrations of RH-3421, RH-5529, and TTX as high as 40, 60 and 3 μM, respectively, do not significantly affect the level of phosphorylation of synapsins Ia and Ib in resting synaptosomes. This observation is in good agreement with the finding that dihydropyrazoles have no effect on basal uptake of sodium and

Fig. 3.4 Typical autoradiograph of the effect of RH-5529 on the increases in phosphorylation of synapsins Ia and Ib induced by VTD and K^+.

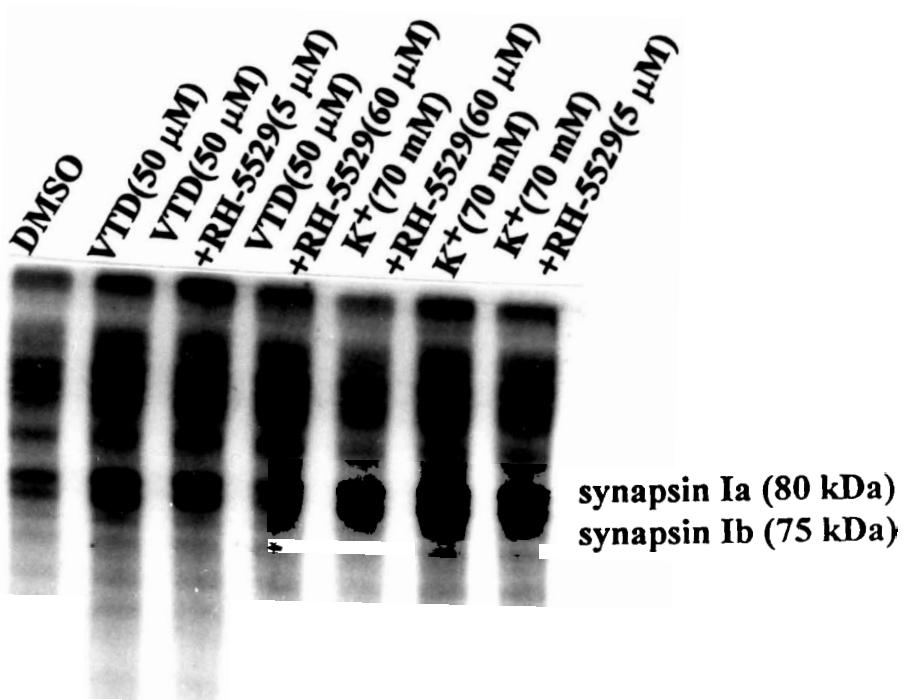

DMSO
VTD(50 μM)
VTD(50 μM) +RH-5529(5 μM)
VTD(50 μM) +RH-5529(60 μM)
K+(70 mM)
K+(70 mM) +RH-5529(60 μM)
K+(70 mM) +RH-5529(5 μM)
synapsin Ia (80 kDa)
synapsin Ib (75 kDa)

Fig. 3.5 Typical autoradiograph of the effect of RH-3421 on increases in phosphorylation of synapsins Ia and Ib induced by VTD and K^+.

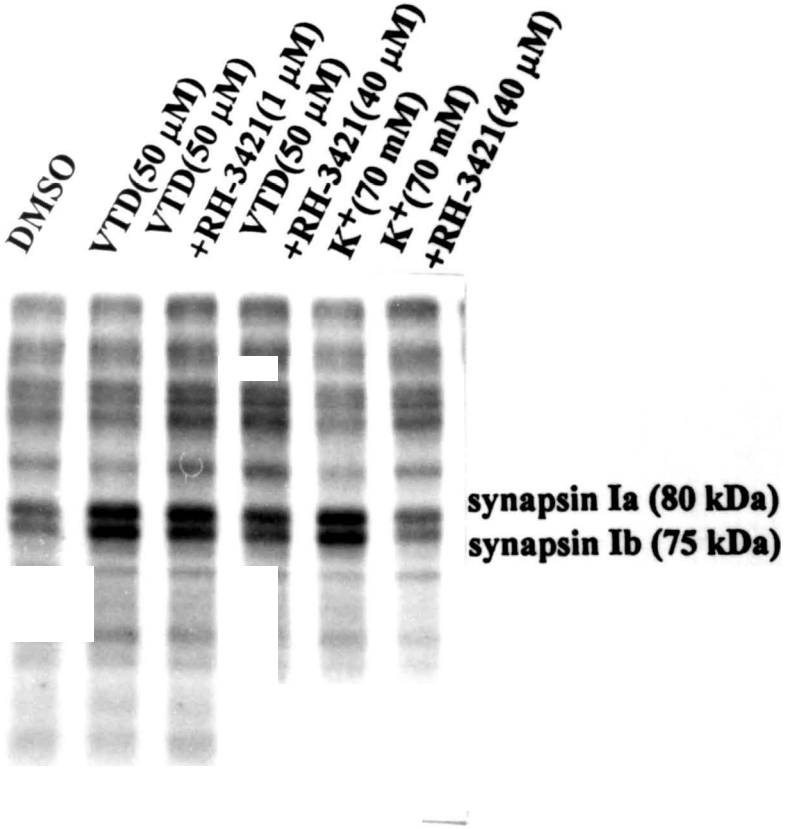
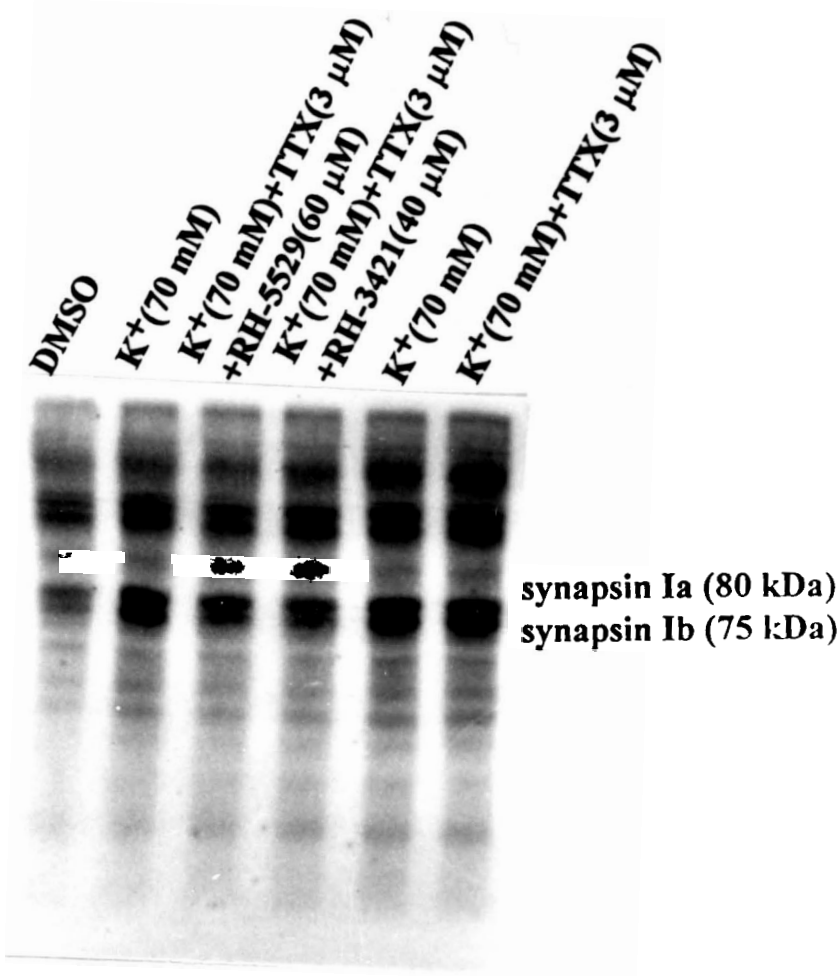

DMSO
VTD(50 μM)
VTD(50 μM)
+RH-3421(1 μM)
VTD(50 μM)
+RH-3421(40 μM)
K+(70 mM)
K+(70 mM)
+RH-3421(40 μM)
synapsin Ia (80 kDa)
synapsin Ib (75 kDa)

Fig. 3.6 Typical autoradiograph showing the effect of dihydropyrazoles RH-3421 and RH-5529 on the increases in phosphorylation of synapsins Ia and Ib induced by VTD and elevated K^+ in the presence of TTX.


DMSO
K+(70 mM)
K+(70 mM)+TTX(3 μM)
+RH-5529(60 μM)
K+(70 mM)+TTX(3 μM)
+RH-3421(40 μM)
K+(70 mM)
K+(70 mM)+TTX(3 μM)
synapsin Ia (80 kDa)
synapsin Ib (75 kDa)

calcium ions (Deecher *et al.*, 1991a; Chapter 2), resting cytosolic free calcium levels (Chapter 2) and resting membrane potential (Nicholson, 1992). The depolarizing agents veratridine and K^+ increase the phosphorylation of synapsin only in the presence of extracellular free calcium ions, a phenomenon that has also been observed in my experiments using the fura-2 assay. This is consistent with data published by other researchers which show that Ca^{++}-dependent protein phosphorylation is prevented by the addition of sufficient EGTA to chelate all free extracellular calcium ions (Krueger *et al.*, 1977; Robinson and Dunkley, 1985).

At concentrations ranging from low micromolar to 40 μM (RH-3421) and 60 μM (RH-5529), the dihydropyrazoles clearly act as effective inhibitors of depolarization-dependent increase in phosphorylation of synapsins Ia and Ib, when either VTD or an elevated K^+ concentration is used as the activator. With VTD as the depolarizing treatment, about 30% inhibition occurred with 5 μM RH-3421, and 5 μM RH-5529 (Table 3.1). When K^+ is used as the activator, approximately 35% inhibition was found with 5 μM RH-3421, while about 33% inhibition occurred with 5 μM RH-5529 (Table 3.1). The results closely accord with data from the $^{45}Ca^{++}$ influx assay, where similar levels of inhibition against K^+-induced $^{45}Ca^{++}$ influx in mouse brain synaptosomes were observed. Nevertheless, it is difficult to explain why RH-3421 is not more effective as an inhibitor of VTD- than K^+-stimulated phosphorylation of synapsin I and why RH-3421 is not more effective than RH-5529 in the former assay as would logically be predicted from my data on intraterminal free Ca^{++}.

The overall pharmacological profile of the dihydropyrazoles, however, is fundamentally different to that of TTX, which blocks only the VTD-induced increase in phosphorylation of synapsins. Again, the blockade of ionic potassium-stimulated synapsins Ia and Ib phosphorylation by dihydropyrazoles also occurs when sodium channels are rendered non-operational by inclusion of TTX in the

assay (Fig. 3.6). These results therefore provide an alternative line of evidence to show that dihydropyrazoles are not specific for the sodium channels of central nerve endings, lending support to our previous proposal that these neurotoxicants act at a second site namely the calcium channel in the nerve membrane.

Chapter 4. Blockade of Depolarization-Coupled Release of L-Glutamate from Synaptosomes Isolated from Mammalian Brain

4.1 Introduction

There is, for the most part, a high level of agreement between the actions of dihydropyrazoles on sodium- and calcium channel-dependent responses in the fura-2 and $^{45}Ca^{++}$ experiments and also in the GABA release assay. Nevertheless, there are two inconsistencies. Firstly, RH-3421, RH-5529 and TTX reduced the resting efflux of GABA from superfused synaptosomes at 1 μM, but failed to affect either the resting concentration of intraterminal free [Ca^{++}] or resting $^{45}Ca^{++}$ uptake at higher concentrations. The other being the fact that RH-3421, in contrast to RH-5529, was unable to influence K^+-dependent release of γ-aminobutyric acid at 10 μM, but blocked K^+-dependent increases in intraterminal free [Ca^{++}] between 0.33 μM and 10 μM and produced 30% and 100% inhibition of K^+-dependent uptake of $^{45}Ca^{++}$ at 10 μM and 100 μM respectively. Moreover, other preliminary experiments carried out at this time by my supervisor indicated that RH-3421 was an effective blocker of K^+-evoked release L-glutamate from mammalian brain synaptosomes and neither RH-3421, RH-5529 or TTX appeared to influence resting release of this excitatory amino acid transmitter. The possibility that the sensitivities of GABAergic nerve endings may not be representative of nerve endings releasing other neurotransmitters thus required futher exploration. An advantage of examining L-glutamate was that this assay offered the opportunity to more thoroughly investigate the effects of dihydropyrazoles on evoked release of endogenously stored neurotransmitter (Nicholls *et al.*, 1987). This contrasts with the GABA system where a proportion of the exogenously loaded [^{3}H]GABA that is released originates from a cytosolic rather than the vesicular compartment (Haycock *et al.*, 1978).

The other basis for investigating the effects of dihydropyrazoles on release of L-glutamate from mouse brain synaptosomes stems from a symptomological consideration, since reduced evoked release of GABA (an abundant inhibitory neurotransmitter in mammalian brain) (Curtis and Johnston, 1974) should cause a poisoning symptomology in which convulsions dominate, unless a concomitant reduction in the release of excitatory neurotransmitters occured. While this latter possibility is very likely given the known sodium channel blocking actions of dihydropyrazoles, it was considered informative from the toxicological perspective to establish this experimentally for a candidate excitatory neurotransmitter.

4.2 Materials and Methods

4.2.1 Chemicals

VTD was supplied by the Wellcome Foundation (Berkhamsted, UK). TTX, percoll, and glutamate dehydrogenase (Type II, from bovine liver), were purchased from Sigma Chemical Co. (St. Louis, MO). RH-3421 and RH-5529 were provided by Rohm and Haas Co. (Philadelphia, PA). The remaining chemicals employed in this study were of analytical grade.

4.2.2 Determination of L-glutamate Release

The effects of dihydropyrazoles on the release of endogenous L-glutamic acid from synaptosomes were determined using a fluorescence technique described by Nicholls *et al.* (1987). Briefly, purified synaptosomes resuspended in saline to a protein concentration of 1.6 mg/ml and synaptosomes (50 µl) of were introduced into physiological buffered saline (3 ml) containing NADP (0.9 mM). This mixture was allowed to preincubate for 10 min with inhibitors or solvent controls. The sample was then transferred to a fluorescence spectrophotometer (Perkin-Elmer LS 50) and recording commenced. The excitation wavelength was 360 nm (slit width

5 nm) and the emission was 460 nm (slit width 10 nm). Calcium chloride (1.5 mM final concentration) was added immediately followed by glutamate dehydrogenase (30 units) at 50 sec. After baseline release was established, activators [VTD (50 μ M) or potassium chloride (33 mM)] were introduced at approximately 450 sec. Assays were normally terminated at 750 sec. Release of L-glutamate from synaptosomes was calculated on a nmol/mg synaptosomal protein basis after calibration with standard L-glutamic acid.

4.3 Results

4.3.1 Effect of Dihydropyrazoles on L-Glutamate Release in Resting Synaptosomes and the Effect of Calcium Ions on Evoked Release of Transmitter

In resting synaptosomes, the basal release of endogenous L-glutamate was not affected by either dihydropyrazole or TTX at high concentrations (Fig. 4.1). Depolarizing agents, VTD and potassium ions, markedly increased the release of L-glutamate from mouse brain synaptosomes in the presence of calcium ions (Fig. 4.2 a and b). However, when calcium ions were removed from the external saline, both of the depolarizing agents were unable to stimulate the release of L-glutamate from synaptosomes (Fig. 4.2 a and b).

4.3.2 Veratridine-induced L-Glutamate Release: Inhibition by Insecticidal Dihydropyrazoles

Veratridine-induced release of L-glutamate from mouse brain synaptosomes was suppressed by the sodium channel blocker TTX (Fig. 4.3 c). The dihydropyrazoles RH-3421 and RH-5529 also inhibited the release of L-glutamate when evoked by VTD (Figures 4.3 a & b). Inhibition of VTD-evoked transmitter release by the dihydropyrazoles was concentration-dependent (Fig. 4.4).

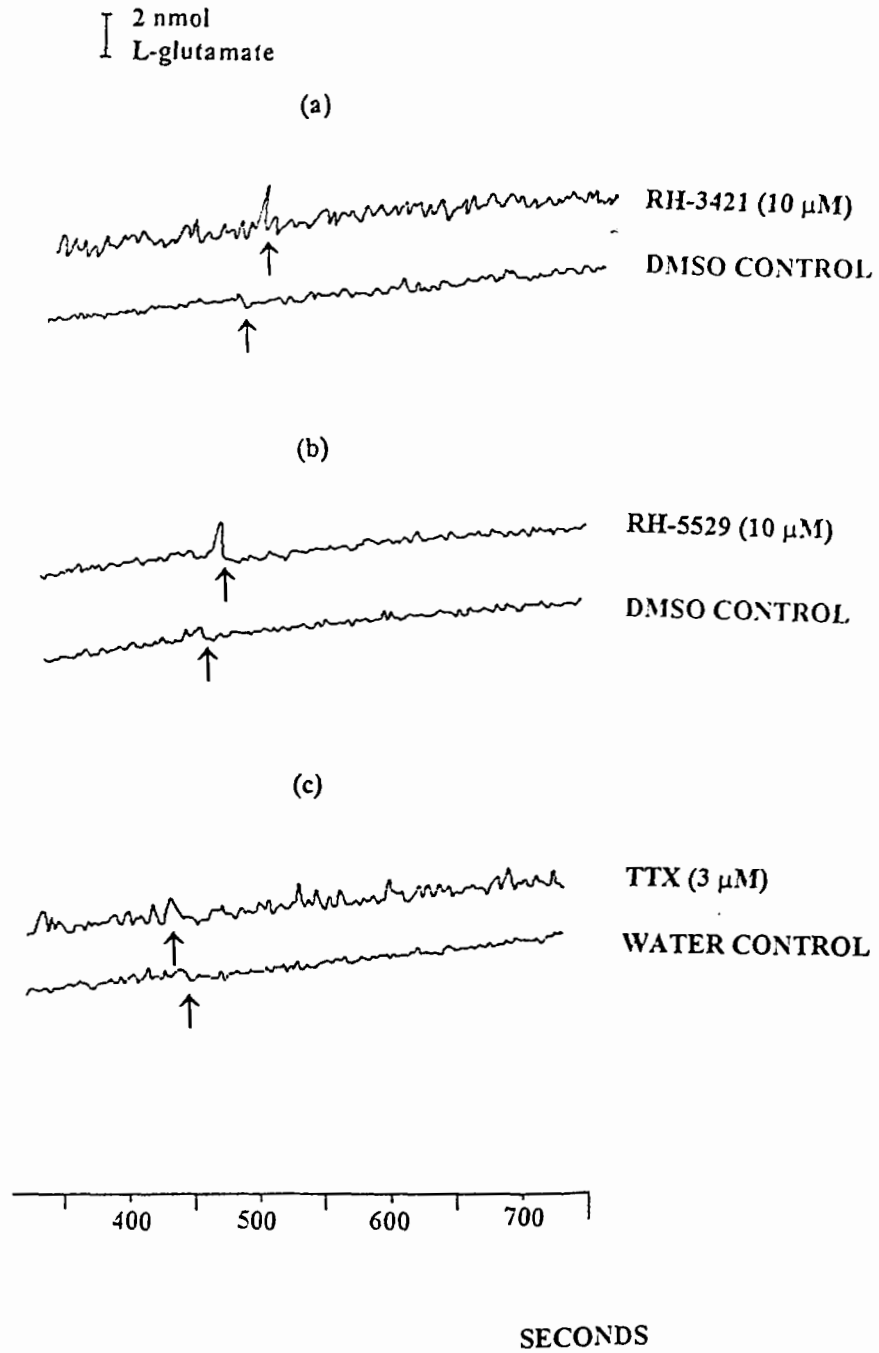


Fig. 4.1 The lack of effect of dihydropyrazoles and TTX on release of L-glutamate from resting synaptosomes. Continuous recording are shown. Neurotoxicants or solvent controls were added at the arrow.

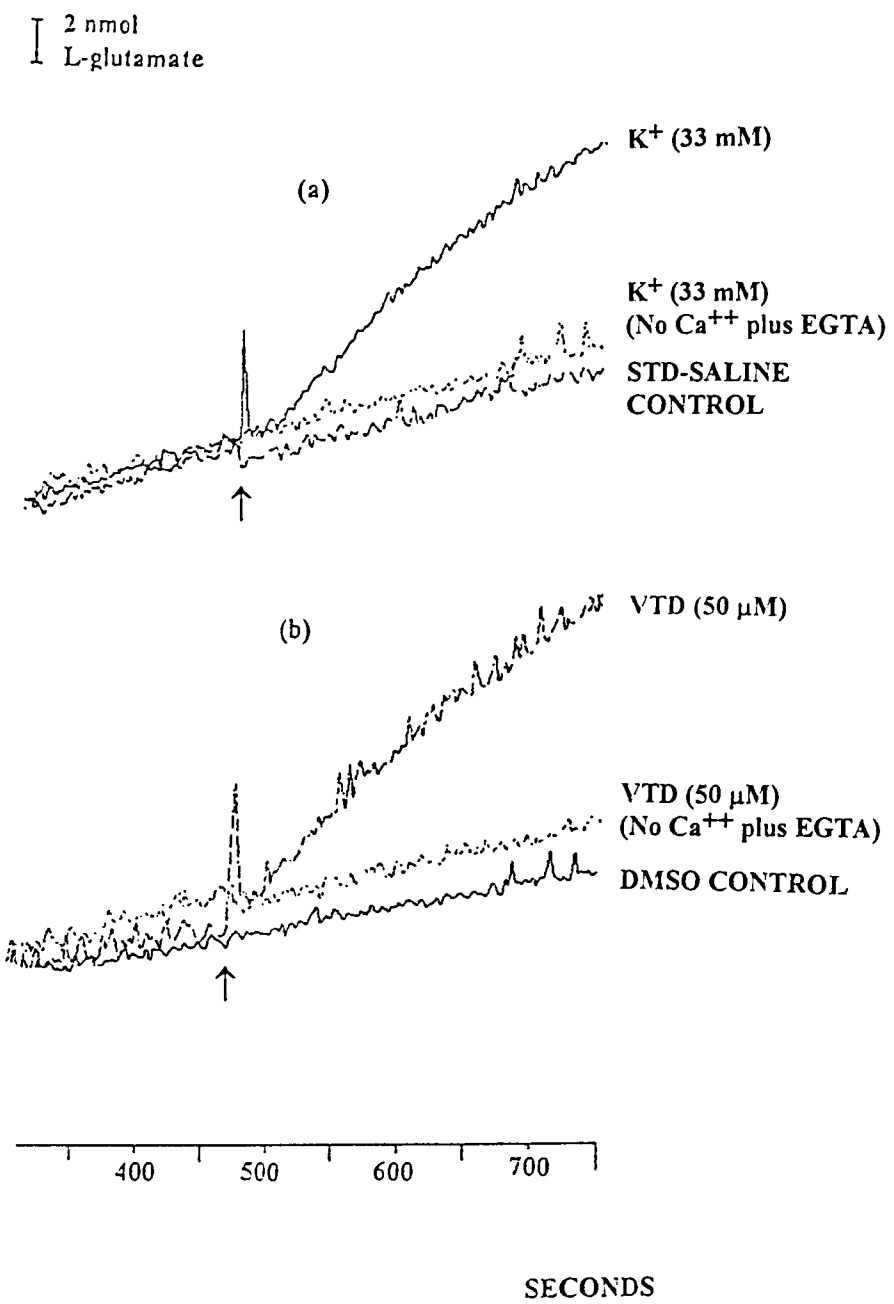


Fig. 4.2 Stimulation of endogenous L-glutamate release by depolarizing agents and effect of removing extrcellular calcium ions. Depolarizing agents of solvent controls were added at the arrow.

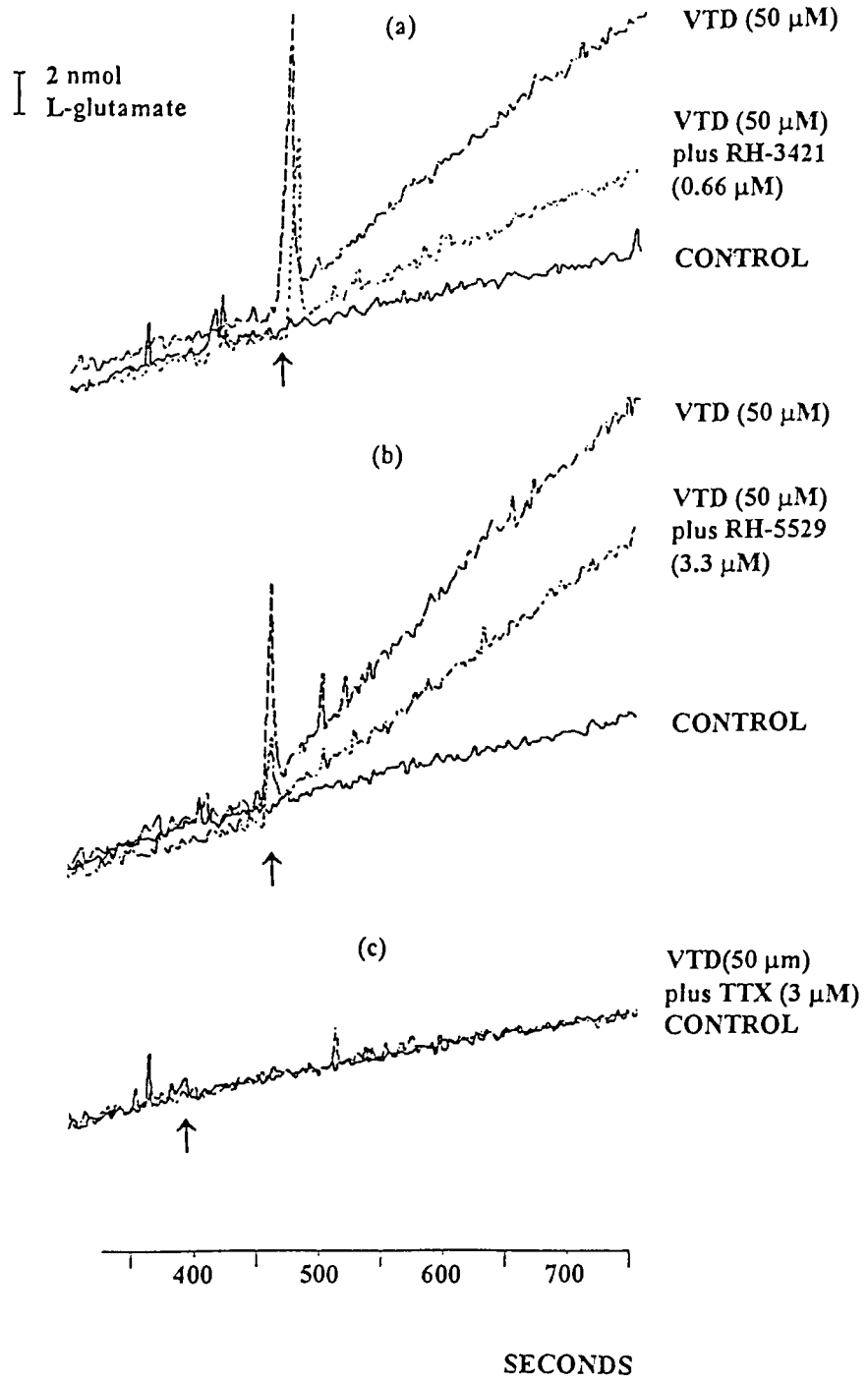


Fig. 4.3 Inhibition of VTD-induced release of L-glutamate from synaptosmes by a) RH-3421, b) RH-5529 and c) TTX. VTD (50 μM) or solvent control was added at the arrow. Synaptosomes were exposed to the dihydropyrazoles or TTX prior to challenge with VTD.

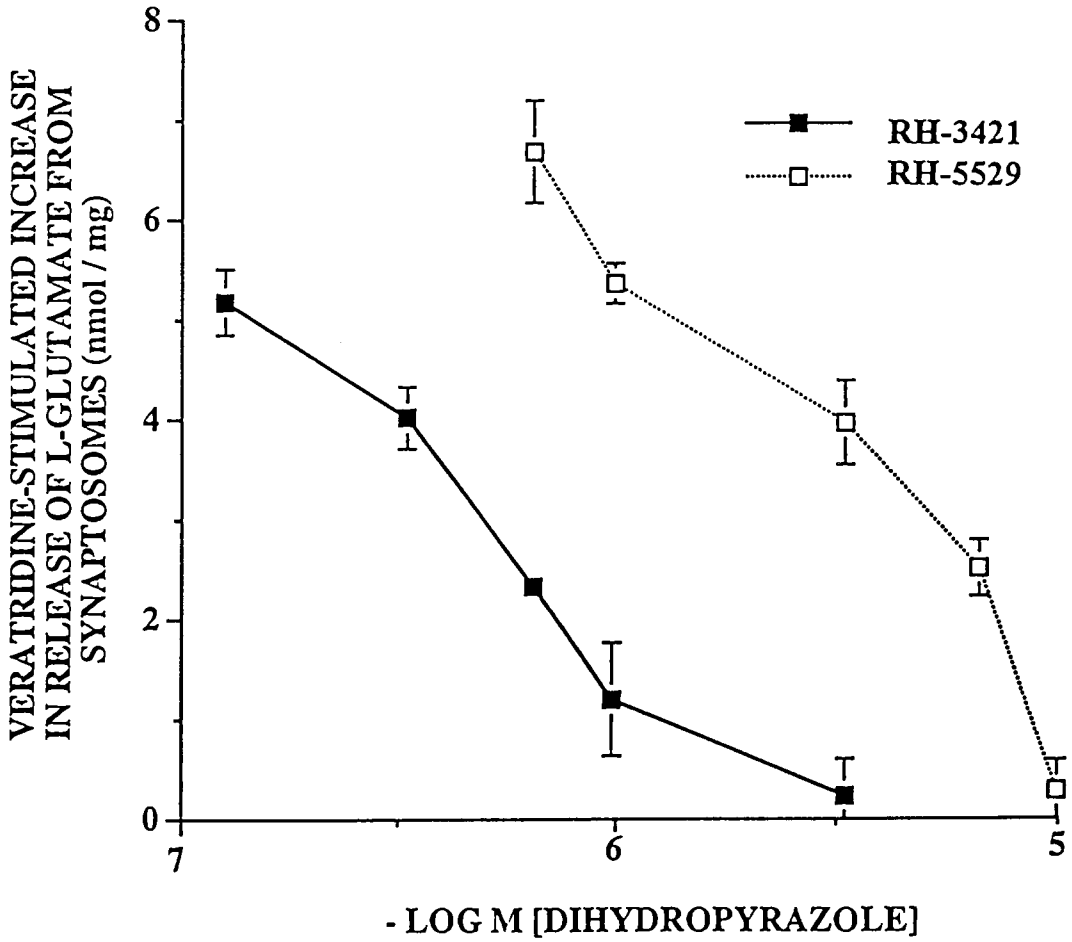


Fig. 4.4 Effect of RH-3421 and RH-5529 on VTD-dependent release of L-glutamate from synaptosomes isolated from mouse brain. Measurements were made at 750 seconds. Values represent means ± SD of three to five independent experiments.

The IC_{50} of RH-5529 (4.7 μM) is greater than that of RH-3421 (0.5 μM), demonstrating RH-3421 to be more potent as an inhibitor of VTD-dependent release of L-glutamate from nerve endings.

4.3.3 K^+-elicited L-glutamate Release: Inhibition by Insecticidal Dihydropyrazoles

Continuous recordings demonstrate that depolarization with K^+, which directly activates Ca^{++} channels, enhances release of L-glutamate from mouse brain synaptosomes. The response is the blocked by RH-3421 and RH-5529 (Fig. 4.5 a & b), however, in contrast, TTX at a concentration of 3 μM had no such inhibitory effect (Fig. 4.5 c and Fig. 4.6). The dihydropyrazoles were still capable of causing pronounced inhibition of K^+-evoked release of L-glutamate when TTX was included in the assay to ensure that sodium channels could not participate in the action of these insecticides (Fig. 4.6). Further experiments showed that the inhibition of K^+-induced release of L-glutamate by dihydropyrazoles is strictly concentration-dependent (Fig. 4.7). However, no discernable difference between inhibitory potency of these analogs was detected (IC_{50}s = 10 μM for each dihydropyrazole)

4.4 Discussion

The data presented in this study essentially confirm that RH-3421 and RH-5529 have the ability to block both VTD- and K^+-evoked release of the excitatory neurotransmitter L-glutamate. These results therefore support the hypothesis that dihydropyrazole-sensitive sodium and calcium channels are present in mammalian central nerve endings. Moreover, a greater sensitivity of sodium channels is strongly suggested by the finding that RH-3421 and RH-5529 inhibitory potencies are 20- and 2-fold higher respectively against VTD-versus K^+-induced release of

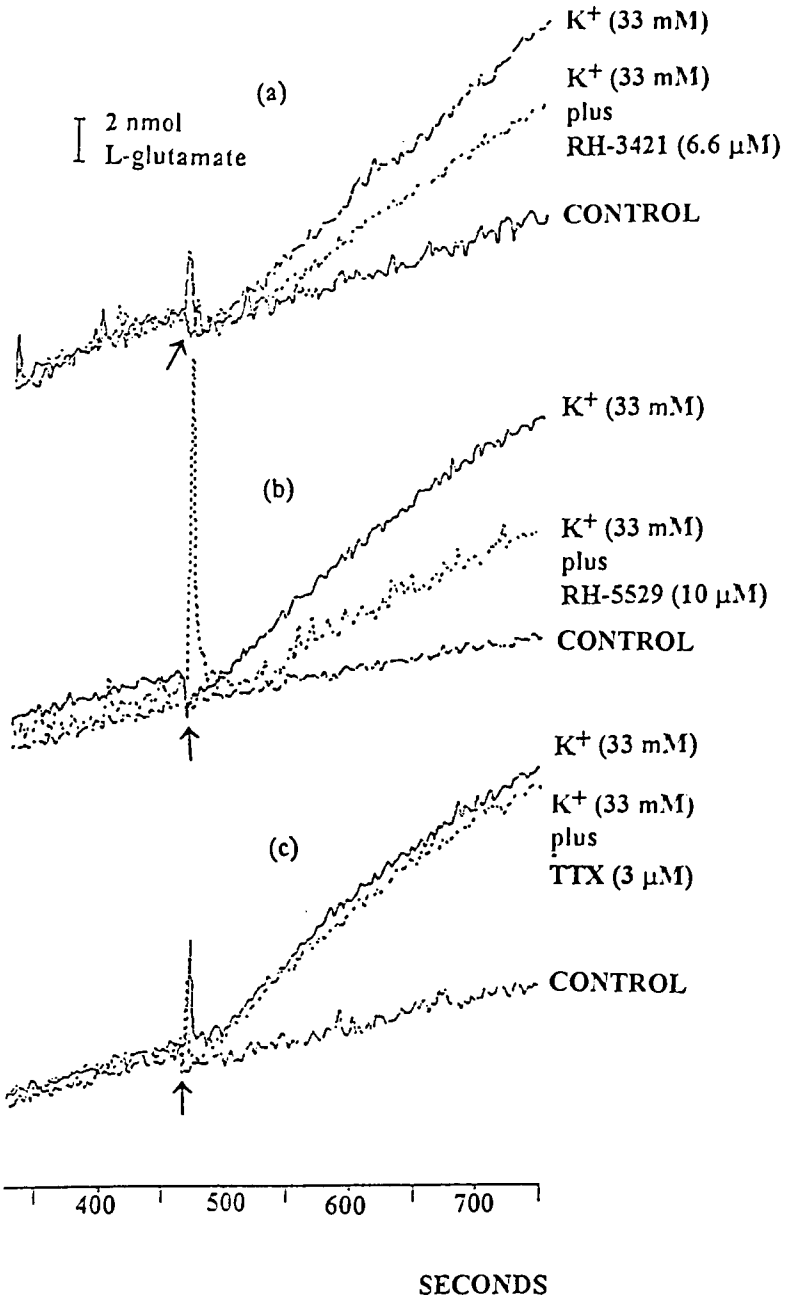


Fig. 4.5 Inhibition of K^+-evoked release of L-glutamate from synaptosomes by a) RH-3421, b) RH-5529 and c) TTX. K^+ (33 mM) or solvent control was added at the arrow. Synaptosomes were exposed to neurotoxicants prior to depolarizing challenge.

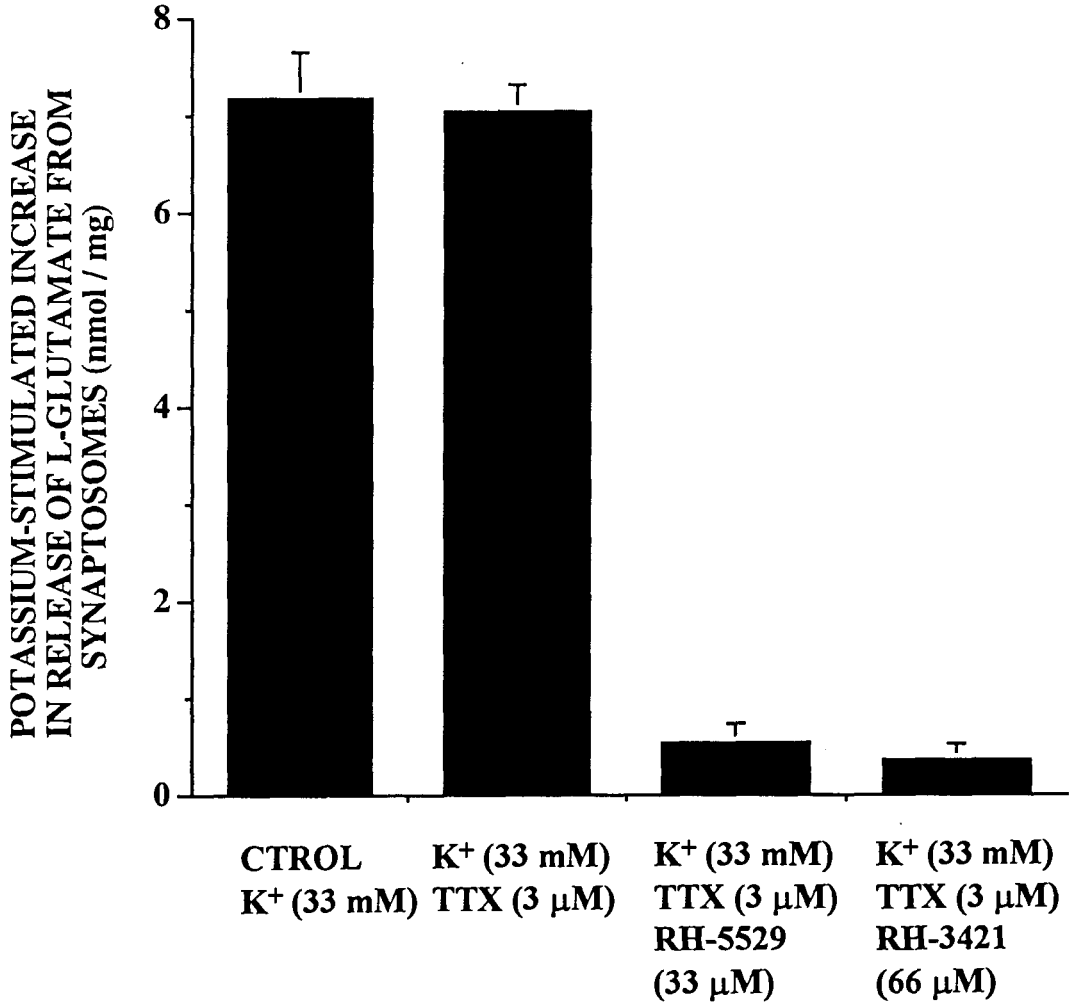


Fig. 4.6 Effect of RH-3421 and RH-5529 on K^+-stimulated release of L-glutamate from synaptosomes isolated from mouse brain in the presence of TTX (3 μM). TTX (3 μM) alone failed to affect K^+-stimulated release of L-glutamate. Values are means ± SD of three to five independent experiments.

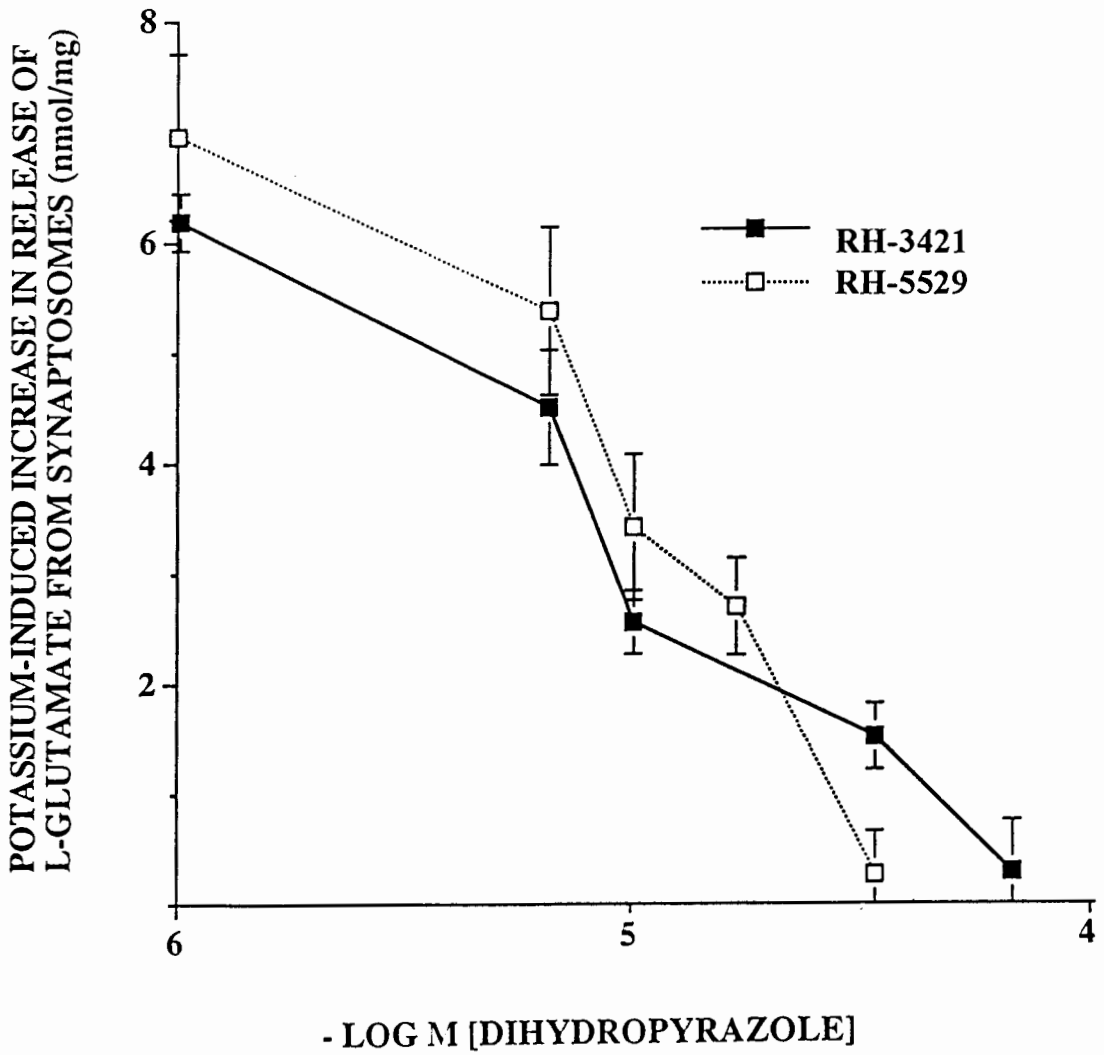


Fig. 4.7 Effect of RH-3421 and RH-5529 on K^+-dependent release of L-glutamate from synaptosomes isolated from mouse brain. Values are means ± SD of three to five independent experiments.

L-glutamate. It is also evident that RH-3421 exhibits a 10-fold greater inhibitory potency compared to RH-5529 against sodium channel-mediated release, based on IC_{50} values of 0.5 μM (RH-3421) and 5 μM (RH-5529). Similar differentials, (although higher overall potencies) were seen for these compounds against sodium channel-dependent release of exogenously loaded [^{3}H]GABA from guinea pig brain synaptosomes (Nicholson and Merletti, 1990). However at 10 μM, RH-3421 produced minimal inhibition of K^+-evoked release of [^{3}H]GABA, in contrast to RH-5529. This raises the interesting possibility that either the vulnerability of calcium channels to RH-3421 may be greater or coupling between $[Ca^{++}]_i$ and release may be tighter in nerve endings releasing L-glutamic acid compared to those releasing GABA.

The proposal that RH-3421 and RH-5529 are unable to influence the functional properties of resting nerve (Salgado, 1990; Nicholson, 1992), is clearly supported by the observation that these insecticides (in common with tetrodotoxin), fail to modify the basal (resting) release of L-glutamate. However in a previous study from this laboratory, as previously mentioned, both dihydropyrazoles reduced basal release of [^{3}H]GABA from synaptosomes isolated from guinea pig brain (Nicholson and Merletti, 1990). In this context, attention should be drawn to the fact that TTX also reduced the spontaneous release of [^{3}H]GABA from superfused guinea pig brain synaptosomes (Nicholson and Merletti, 1990; Nicholson *et al.*, 1983). Species differences may account for this, but most likely this reflects a certain amount of functional instability of sodium channels under superfusion conditions and possibly therefore, a greater physiological and biochemical relevance of the L-glutamate data.

Lethargy and pacificity are cardinal symptoms following sub-chronic dietary adminstration of RH-3421 to rodents (Salgado, 1992). The observation that dihydropyrazoles block depolarization-coupled release of L-glutamate provides a

basis for predicting that excitation mediated via glutamatergic neurons (and likely other excitatory transmitter-specific neurons) will be attenuated in brain during poisoning. Concomitant inhibition of the release of inhibitory and excitatory neurotransmitters in brain would clearly facilitate the development of such poisoning features.

Chapter 5. Inhibition of [^{3}H]Nitrendipine Binding by Insecticidal Dihydropyrazoles in Mammalian Brain

5.1 Introduction

The investigations described in Chapter 2, 3 and 4 produced data which support the previously documented action of dihydropyrazoles on voltage-sensitive sodium channels and provide novel evidence that these insecticidal neurotoxicants are also capable of interfering with the operation of voltage-sensitive calcium channels in presynaptic nerve endings prepared from mammalian brain.

Radioligand binding studies are an extremely useful way of characterizing the pharmacological properties of recognition sites for chemical insecticides in the nervous system. For example, this experimental approach has been instrumental in explaining the mechanism of insecticide action at two of the most widely exploited ion channel targets for chemical control agents in the nervous system, namely the voltage-sensitive sodium channel which contains DDT, dihydropyrazole and N-alkylamide recognition sites (Payne and Soderlund, 1989; Deecher *et al.*, 1991b; Ottea *et al.*, 1989) and the GABA-gated chloride channel complex which possesses discrete binding sites for the avermectins, trioxabicyclooctanes, phenylpyrazoles and polychlorocycloalkanes (Deng and Casida, 1992; Konno and Scott, 1991; Casida *et al.*, 1988; Cole *et al.*, 1993; Lawrence and Casida, 1984). We knew from previous experiments conducted with rat brain membranes (R. A. Nicholson, unpublished observations) that competitive displacement of [^{3}H]RH-3421 by unlabeled RH-3421 in a conventional binding assay was marginal at best. Similar difficulties had been experienced by Dr. Soderlund's group at Cornell University (D. M. Soderlund, personal communication to R. A. Nicholson, April, 1991). The failure to demonstrate a specific binding signal however was not unanticipated and was consistent with RH-3421's high lipophilicity, a property

which would be expected to facilitate efficient partitioning of any [^{3}H]RH-3421 displaced from recognition sites into neuronal membrane lipids. It became clear therefore that a more realistic approach would be to examine the ability of dihydropyrazoles to influence the binding of calcium channel selective radioligands. The question was "which calcium channel type should the focus be on ?". Our initial experimental approach was to obtain basic information on the susceptibility of K^+-evoked release of L-glutamate to blockade by specific L-type and N-type calcium channel inhibitors. With this data, it became possible to identify a potential calcium channel type and a suitable candidate radioligand with which to conduct initial displacement experiments with RH-3421 and RH-5529.

After establishing simple competitive displacement curves, Scatchard analysis was used to experimentally determine equilibrium binding constants (K_ds) and binding maxima (B_{max}s), as described by Bennett (1978). Finally, conventional kinetic analysis of the effects of dihydropyrazoles on the rate of association and dissociation of radioligand was conducted (Bennett, 1978). These data permitted conclusions to be reached on whether these neurotoxicants acted by competitive or non-competitive mechanisms and if allosterism is involved in dihydropyrazole action.

5.2 Materials and Methods

5.2.1 Chemicals

Percoll was purchased from Sigma (St. Louis, MO). Nifedipine and diltiazem were obtained from ICN Pharmaceuticals Canada Ltd. (Montreal) and [^{3}H]nitrendipine was supplied by DuPont NEN Canada Inc.. Dihydropyrazoles RH-3421 and RH-5529 were gifts from Vincent L. Salgado, Rohm and Haas Co. (Spring House, PA). ω-Conotoxin and verapamil were obtained from Sigma (St. Louis, MO).

5.2.2 Isolation of Synaptosomes and Binding Assay Using [^{3}H]Nitrendipine

Synaptosomes were prepared from whole brains of four male CD1 mice (25-30g) under isoosmotic conditions using Percoll gradient centrifugation according Dunkley *et al.* (1986). Prior to assay synaptosomal pellets were washed twice with ice-cold saline [NaCl (145 mM), KCl (5 mM), $MgCl_2$ (1.2 mM), $NaHPO_4$ (2.4 mM), HEPES (10 mM); adjusted to pH 7.4 with Tris base], resuspended in buffer at a concentration of 1.5 mg protein / ml and stored on ice. Binding experiments with [^{3}H]nitrendipine were carried out essentially as described by others who have used mammalian synaptosomes (Turner *et al.*, 1985; Gould *et al.*, 1982). Synaptosomes (150 μg protein) were incubated with 0.2 nM [^{3}H]nitrendipine (83 Ci/mmol) and other compounds where appropriate, in a total volume of 1 ml of buffered saline. Incubations were carried out in the dark for 60 min at 25 ^oC. Binding was terminated by adding 4 ml ice-cold saline and rapid filtration of the suspension on Whatman glass fiber filters (GF/C). Synaptosomes retained by filters were washed twice with 4 ml ice-cold buffered saline and then dissolved in 1 ml of Triton X-100 (8%). Radioactivity was quantified by liquid scintillation spectrophotometry. Non-specific binding was measured in the presence of 5 μM nifedipine and subtracted from total binding values to give specific binding. The protein concentration of synaptosomal suspensions was determined using the Lowry method as adapted by Peterson (Peterson, 1977) with bovine serum albumin as the reference standard.

5.3 Results

5.3.1 Selection of an Appropriate Calcium Channel-Specific Radioligand

Table 5.1. shows the effects of the three calcium channel-specific blockers (which could be obtained commercially in radioligand form) on K^+-stimulated release of L-glutamate from mouse brain synaptosmes. The results indicate that the

Table 5.1 Inhibition of K^+-stimulated (calcium channel-dependent) release of L-glutamate from mouse brain synaptosomes.

Treatment	K^+-stimulated release of L-glutamate (% inhibition) [a]
ω-Conotoxin (100 μM)	4.67 ± 2.62
Verapamil (80 μM)	84.46 ± 2.34
Nifedipine (60 μM)	97.61 ± 2.62

[a] Values represent means ± SD deviation of three independent experiments.

N-type calcium channel antagonist ω-conotoxin has very little capacity to block the K^+-response, inhibition being no greater than 5% at the relatively high concentration of 100 μM. In contrast, L-type calcium channel ligands are much more effective inhibitors of K^+-evoked release of L-glutamate, as demonstrated by 84.46 and 97.61% inhibition of the response by verapamil (80 μM) and nifedipine (60 μM) respectively. These experiments therefore established that L-type Ca^{++} channel blockers are clearly effective at interfering with calcium channel function in mouse brain synaptosomes.

5.3.2 Displacement of Specifically Bound [^{3}H]Nitrendipine by Insecticidal Dihydropyrazoles

Under standard assay conditions, both RH-3421 and RH-5529 inhibited the specific binding of [^{3}H]nitrendipine to mouse brain synaptosomes and for both analogs the effect was concentration-dependent (Fig. 5.1). The potency of RH-3421 as an inhibitor of radioligand binding was marginally greater than that of RH-5529 according to IC_{50} estimates of 30 μM (RH-3421) and 48 μM (RH-5529). The displacement curves indicate that approximately 15% of the specifically bound radioligand cannot be displaced by RH-3421 and RH-5529.

5.3.3 Scatchard Analysis

The effects of dihydropyrazoles on radioligand binding affinity and binding site availability were studied using Scatchard analysis (Fig. 5.2). Scatchard plots were linear and the B_{max} and K_d data for [^{3}H]nitrendipine radioligand alone show excellent agreement with data published by others (Turner *et al.*, 1985; Gould *et al.*, 1982). Under these conditions of assay RH-3421 is more effective than RH-5529 in reducing the number of binding sites available to radioligand as demonstrated by a lowering of B_{max} values by 44% (RH-3421) and by 22% (RH-

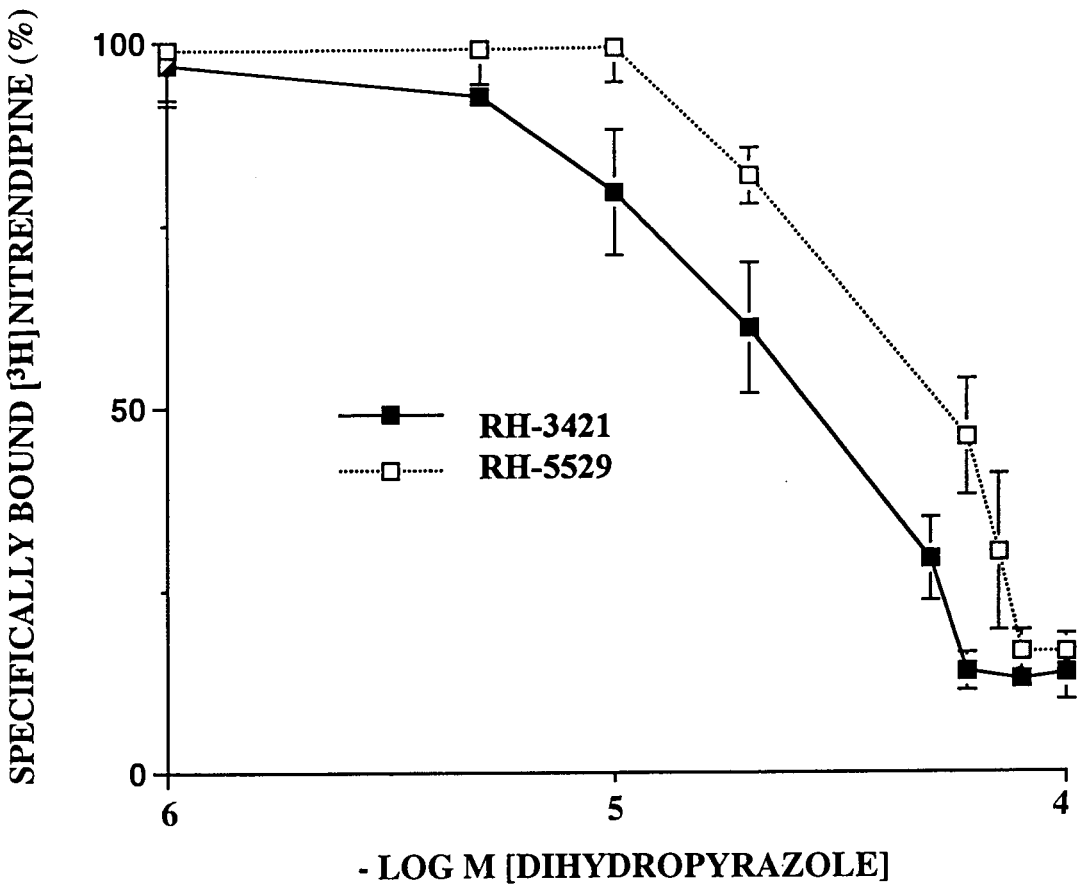


Fig. 5.1 Displacement of specific binding of [^{3}H]nitrendipine by RH-3421 and RH-5529 in mouse brain synaptosomal preparations. Values show means ± SD of three to five independentexperiments.

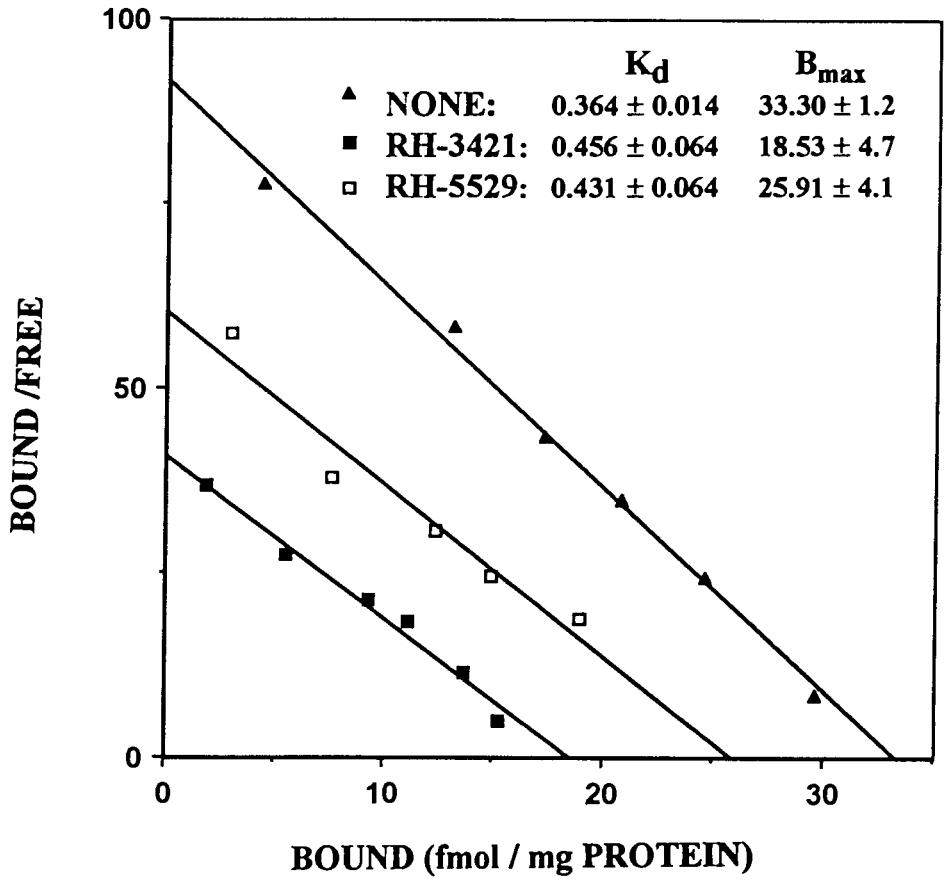


Fig. 5.2 Scatchard plots of [^{3}H]nitrendipine binding in the absence and presence of RH-3421 (30 μM) or RH-5529 (48 μM). Data points show the mean of three to five separate experiments.

5529). In contrast, the affinity of [^{3}H]nitrendipine for its binding site was not greatly changed by either of the two dihydropyrazoles (K_d of the control = 0.364 ± 0.014 nM; K_d with RH-3421 =0.456 ± 0.043 nM; K_d with RH-5529 = 0.431 ± 0.064 nM).

5.3.4 Association and Dissociation

Kinetic experiments were undertaken to help define more clearly the mechanism of binding inhibition by dihydropyrazoles. Simultaneous incubation of [^{3}H]nitrendipine with RH-3421 or RH-5529 at insecticide concentrations close to IC_{50} values had no discernable effect on the rate of association (Fig. 5.3). However, nifedipine, a competitive displacer of [^{3}H]nitrendipine binding, dramatically decreased the rate of [^{3}H]nitrendipine association under similar circumstances (Fig. 5.3).

Likewise the timecourse of radioligand dissociation was not altered by either of the two dihydropyrazoles when included in the assay at concentrations close to their IC_{50} values (Fig. 5.4) and saturating concentrations (90 μM; see Fig. 5.l) (Fig. 5.5). In contrast, the competitive antagonist of [^{3}H]nitrendipine binding diltiazem, (which was included as a positive control) slowed the rate of dissociation, an effect consistent with its documented action as an enhancer of specific binding of [^{3}H]nitrendipine (Fig. 5.4).

5.4 Discussion

During our consideration of the most appropriate calcium channel type to focus on for radioligand binding studies, the observation that K^+-evoked release of L-glutamate is blocked by nifedipine and verapamil, but not ω-conotoxin in mousebrain synaptosomes, provided an important clue that brain L-type, but not N-type Ca^{++} channels may be involved in mediating the actions of dihydropyra-

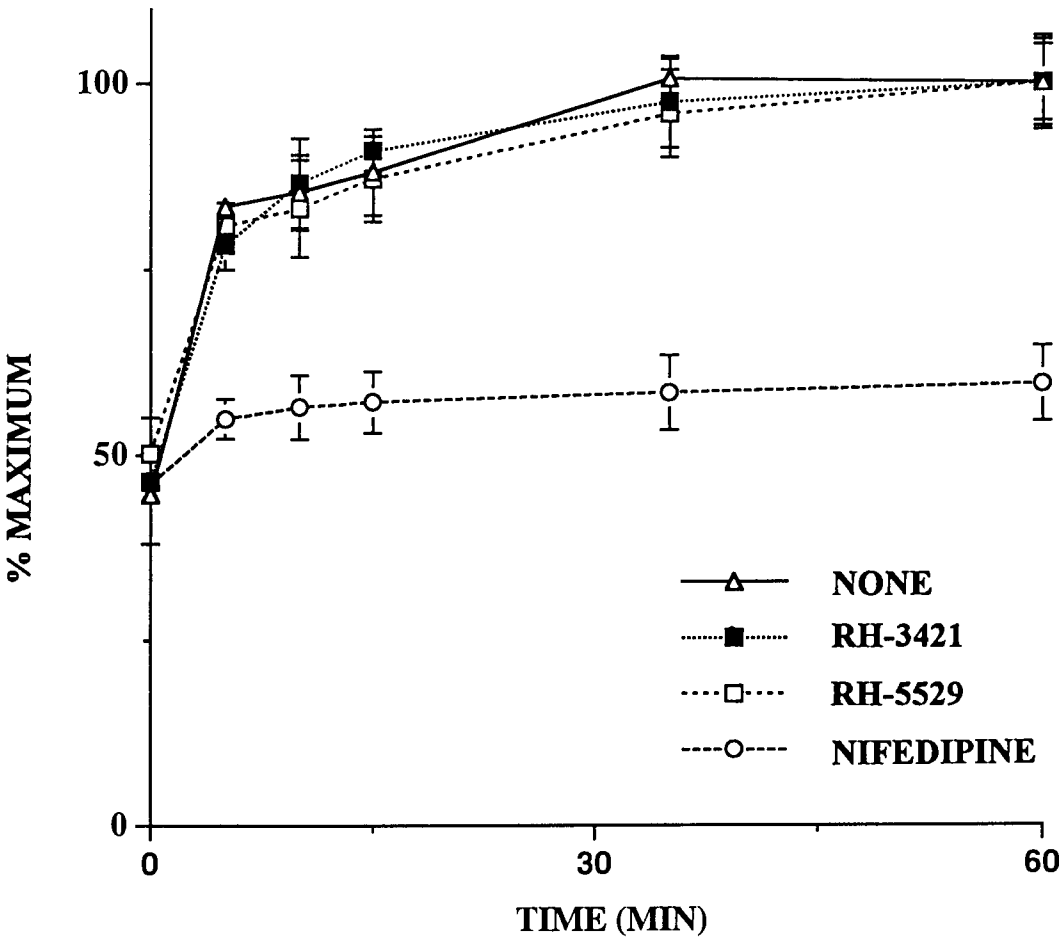


Fig. 5.3 Timecourse for association of [^{3}H]nitrendipine in the absence and presence of RH-3421 (30 μM), RH-5529 (48 μM) and nifedipine (5 μM). Values are means ± SD of three to five independent experiments.

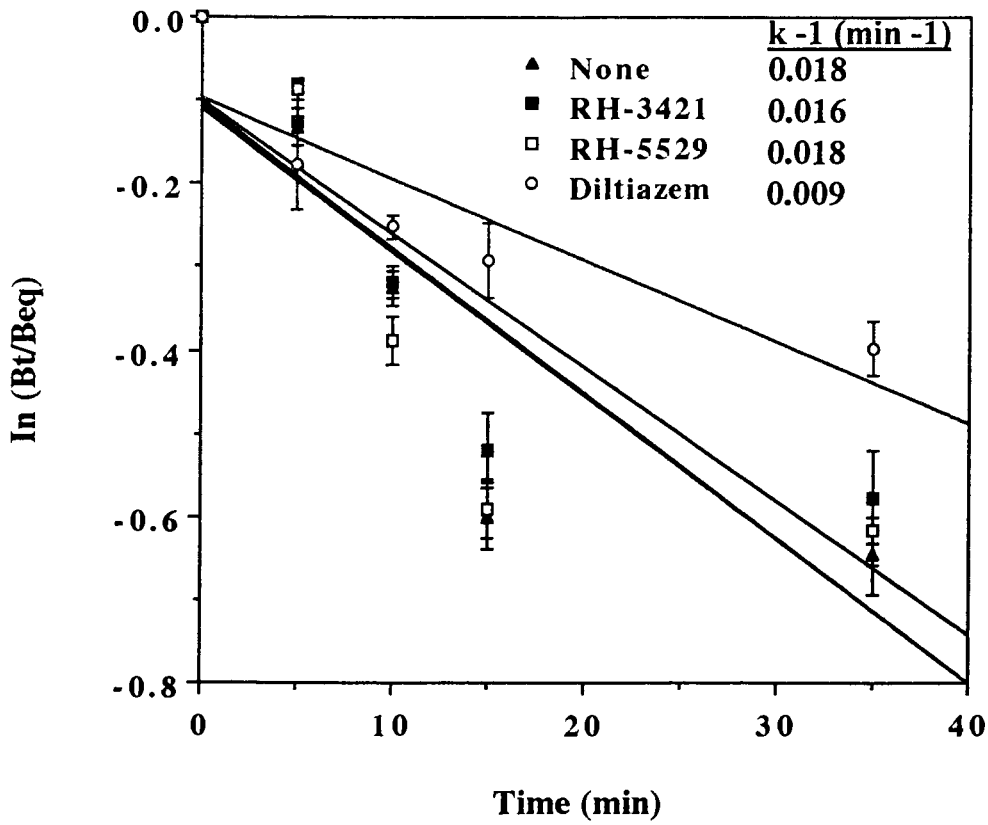


Fig. 5.4 Timecourses for dissociation of [^{3}H]nitrendipine from synaptosomal receptors in the presence and absence of RH-3421 (30 μM), RH-5529 (48 μM) or diltiazem (5 μM). Values show means ± SD three to five independent experiments.

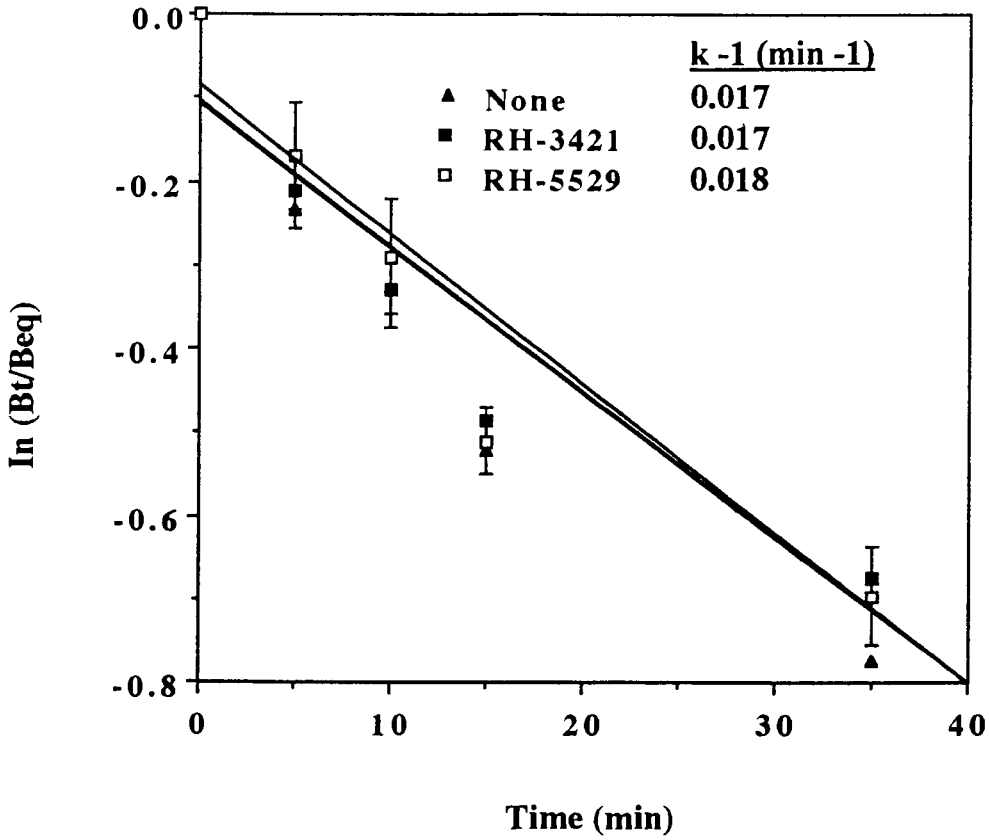


Fig. 5.5 Timecourses for dissociation of [^{3}H]nitrendipine from synaptosomal receptors in the presence and absence of RH-3421 or RH-5529 under saturating concentration (90 μM). Values show means ± SD three to five independent experiments.

zoles. The report by Mintz *et al.* (1990) that ω-conotoxin does not block K^+-stimulated radiocalcium entry into synaptosomes prepared from rodent brain, lends support to the idea that these insecticides may not be affecting presynaptic N-type calcium channels. While there is some controversy over the function of L-type Ca^{++} channels in the presynaptic terminals, it is evident that several reports exist in the literature which implicate dihydropyridine-sensitive (L-type) calcium channels in the release of neurotransmitters (Nowycky *et al.*, 1985; Bean, 1989; Hess, 1990), and demonstrate high affinity binding sites for dihydropyridine in synaptosomal preparations (Dunn, 1988; McClesky *et al.*, 1987). The only two radioligand classes for L-type Ca^{++} channels that are commercially available are dihydropyridines and phenylalkylamines. The decision was taken not to conduct the binding studies with [^{3}H]verapamil (a phenylalkylamine). Verapamil increases the dissociation of [^{3}H]nitrendipine (a dihydropyridine) by binding to a site that is separate, but coupled to the dihydropyridine binding site (Dopico *et al.*, 1996). To date the displacement of [^{3}H]verapamil binding by dihydropyridines has not been demonstrated. It was therefore argued that if insecticidal dihydropyrazoles interacted with either the dihydropyridine or phenylalkylamine binding site it would most likely be detected by a change in the binding parameters of [^{3}H]nitrendipine.

Scatchard analysis revealed that dihydropyrazoles interfere with binding of [^{3}H]nitrendipine through a mechanism which reduces the number of available radioligand binding sites (B_{max}) without markedly altering the affinity (K_d) for remaining sites (Fig. 5.2). This clearly identifies the dihydropyrazoles as non-competitive inhibitors of [^{3}H]nitrendipine binding. This idea is strengthened by kinetic data which show that the association rate of the radioligand is not altered when these study compounds are included in the assay. Reductions in radioligand binding site density under these conditions have been explained by irreversible (or

slowly reversible) binding of dihydropyrazoles to either the radioligand recognition site or an allosterically coupled site.

An allosteric involvement in the inhibition of [^{3}H]nitrendipine binding by dihydropyrazoles is not supported by the kinetic observations which show that at IC_{50} concentrations RH-3421 and RH-5529 are unable to affect the rate of [^{3}H]nitrendipine dissociation (Fig. 5.4) and, more convincingly, fail to increase the rate of dissociation at saturating concentrations (90 μM) (Fig. 5.5). This strongly suggests that dihydropyrazoles are not interacting with dihydropy-ridine-bound calcium channels, indicating that the dihydropyrazole-ion channel-radioligand ternary model proposed by Deecher and Soderlund (1991b) for inhibition of [^{3}H]batrachotoxinin A 20-α-benzoate binding to sodium channels by RH-3421 does not apply to the action of these compounds at L-type calcium channels. The pharmacology of dihydropyrazole binding at L-type calcium channels is also mechanistically distinct from that of other synthetic calcium antagonist drugs such as the phenylalkylamines and diphenylalkyamines, which allosterically enhance the dissociation of [^{3}H]nitrendipine (Murphy *et al.*, 1983; Ehlert *et al.*, 1982) and benzothiazepines which slow dihydropyridine dissociation (Yamamura *et al.*, 1982).

Although the present binding study does not clarify whether or not dihydropyrazoles act as irreversible inhibitors of [^{3}H]nitrendipine binding, the possibility that dihydropyrazoles may inhibit radioligand binding by interacting indirectly with the calcium channel is a mechanistic scenario also supported by the data. In this respect, the effects of dihydropyrazoles appear similar to those of endogenous brain factors, which also act non-competitively to reduce the number of binding sites for [^{3}H]nitrendipine (Sanna and Hanbauer, 1987; Ebersole *et al.*, 1988). It has been proposed that these brain-specific modulatory substances may modify the density of functional [^{3}H]nitrendipine binding sites through guanine nucleotide

binding proteins which have been implicated in the regulation of neuronal calcium channels (Hescheler *et al.*, 1987), or by release of enzymes from other cellular locations which interact with and degrade the dihydropyridine receptor (Ebersole *et al.*, 1988).

In the binding experiments RH-3421 is slightly more potent than RH-5529 as a displacer of [^{3}H]nitrendipine and IC_{50} values show good agreement with those found for radiocalcium uptake experiments in which presynaptic calcium channels are directly activated by K^+ depolarization (see Chapter 2). The concentration of RH-3421 necessary to inhibit these calcium channel-specific perturbations are higher than those needed to inhibit the alternative sodium channel target based on data from radiosodium uptake and [^{3}H]BTX binding experiments with mouse brain fractions (Deecher and Soderlund, 1991a; Deecher *et al.*, 1991b), confirming a great sensitivity of sodium channels in mammalian nervous system. The potencies of RH-3421 and RH-5529, however, compare well with that of diltiazem (a benzothiazepine) (Yamamura *et al.*, 1982), but are substantially lower than the commonly encountered phenylalkyamine and diphenylalkyamine drugs (Ehlert *et al.*, 1982).

Chapter 6. Alteration of Synaptic Plasma Membrane Properties by Insecticidal Dihydropyrazoles

6.1 Introduction

The data from the binding experiments with [^{3}H]nitrendipine (Chapter 5) provide compelling evidence that dihydropyrazoles interfere with the conformational integrity of voltage-gated calcium channels in the nerve membrane. The scatchard and kinetic analysis in particular support the idea that dihydropyrazole effects on the dihydropyridine receptor are non-competitive. The fact that there is no detectable allosteric component to dihydropyrazole action indicates that these insecticides may block Ca^{++} channels without binding to discrete sites on the channel complex.

It has been proposed that the physiological actions of some lipophilic drugs arise at least in part, by changes to the physical state of lipid biomolecules within synaptic membranes (Harris and Schroeder, 1982). Moreover, it is suspected that the effects observed on lipid bilayer components with ethanol, anesthetics and anticonvulsants may be associated with dysfunction of neuronal cation channels (Harris and Schroeder, 1982; Harris and Bruno, 1985). The thrust of this phase of the investigation therefore was to determine sensitivity of presynaptic plasmamembrane lipids to the effects of dihydropyrazoles. It was felt that this would help clarify the mechanisms by which these compounds influence calcium channel operation. In this study, two region-specific fluoroprobes [1,6-diphyenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethyammonium phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) were employed. It has been established that probes such as DPH exhibit an anisotropic motion. This property can be studied by measuring steady state fluorescence polarization and such measurements are the basis for estimation of the probes dynamic contribution (r_f), which is related to the fluidity

of the lipid microenvironment (Van Blitterswijk *et al.*, 1981). In addition, the static contribution, which facilitates calculation of the order (S) of fluoroprobe motion, describes the molecular packing of bilayer lipid components (Van Blitterswijk *et al.*, 1981). As previously alluded to (Chapters 1 and 4) dihydropyrazoles are highly lipophilic chemicals and as such are prime canadidates for intercalation with lipid molecules in biological membranes. This phase of the investigation sought to establish the extent to which actual changes in membrane fluidity and /or lipid order were induced by dihydropyrazoles and whether these such changes were likely contributors to the action of these insecticides at calcium channels.

6.2 Materials and Methods

6.2.1 Chemicals

Percoll and 1, 6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma (St. Louis, MO). 1-(4-Trimethylammonium phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) was obtained from Molecular Probes Inc. (Eugene, OR). Dihydropyrazoles RH-3421 and RH-5529 were gifts from Dr. Vincent L. Salgado, Rohm and Haas Co. (Spring House, PA).

6.2.2 Preparation of Synaptic Membranes

Synaptosomal membranes were prepared essentially using the method of Meyer and Cooper (1981). In this procedure, synaptosomes isolated from one CD1 male mouse brain were resuspended in 0.45 ml sucrose (0.32 M). The purified synaptosomes were lysed by adding a mixture of standard saline and distilled water (1:20) in a total volume of 30 ml and kept on ice for 20 min. The suspension was then centrifuged at 10,000g for 10 min (Beckman J2-HS). The resulting membrane pellet was then resuspended in 0.32 M sucrose (4.5 mg of protein/ml) and held on ice prior to fluorescence analysis.

6.2.3 Fluorescence Measurements

Each fluorescence probe was introduced into standard buffered assay saline by rapid injection in tetrahydrofuran (DPH) or equal parts tetrahydrofuran and water (TMA-DPH) followed by vigorous shaking. The resulting concentration of tetrahydrofuran in assay saline did not exceed 0.05%. Synaptosomal membranes (300 µl) were then added to 2.7 ml of standard saline containing DPH or TMA-DPH (final concentration = 0.5 µM for both probes). Fluorescence probes were incubated with synaptic membranes in darkness for one hour at room temperature to achieve equilibration prior to assay. The association of each probe with lipid is associated with a large increase in fluorescence intensity. Cuvettes containing probe-labeled membranes were placed in a waterbath at 35 ^{o}C and incubated with RH-342 and RH-5529 for ten minutes before initiating fluorescence polarization scans. All fluorescence measurements were conducted at 35 ^{o}C on continuously stirred membrane samples using a Perkin-Elmer LS 50 fluorescence spectrophotometer equipped with polarizers on the excitation and emission sides of the cuvette. The excitation wavelength was set to 365 nm (slit width 5 nm) and the emission signal was sampled between 435 and 450 nm (slit width 5 nm). Data processing was carried out using fluorescence data management software (Perkin-Elmer) and an IBM 50Z computer. After smoothing using Savitsky-Golay routines, absolute values for fluorescence polarization (P) were calculated according to the formula:

$$P = (I_{VV} - I_{VH})/(I_{VV} + I_{VH})$$

where VV = fluorescence intensity measured with the axis of the excitation and emission polarizer oriented vertically and V_H = fluorescence intensity measured

with the excitation polarizer oriented vertically and the emission polarizer oriented horizontally.

6.2.4 Calculation of the Physical Parameters of Synaptosomal Membranes

The fluorescence anisotropy (r), dynamic anisotropy component (r_f) and lipid order parameter (S) were calculated according to published methods (Blitterswijk *et al.*, 1981; Shinitzky and Barenholz, 1978). The steady-state fluorescence anisotropy (r_s) can be estimated from the fluorescence polarization (P) according to the following formula:

$$r_s = 2P/(3 - P) \qquad \text{(Shinitzky and Barenholz, 1978)}$$

The empirical relationship between limiting (r_∞) and steady-state (r_s) anisotropy can be expressed as:

$$r_\infty = 1.33\, r_s - 0.10 \qquad \text{(Van Blitterswijk } et\ al.\text{, 1981)}$$

The order parameter (S) can most conveniently be estimated by the following formula:

$$S^2 = r_\infty / r_0 \qquad \text{(Van Blitterswijk } et\ al.\text{, 1981)}$$

where r_0 is the maximal fluorescence anisotropy value in the absence of any rotational motion of the fluorophore. In these calculations r_0 values of 0.362 for DPH (Shinitzky and Barenholz, 1978) and 0.390 for TMA-DPH (Prendergast *et al.*, 1981) were employed.

6.3 Results

6.3.1 Dihydropyrazole-Induced Changes to the Polarization of Synaptic Membranes

In Table 6.1, the changes in fluorescence polarization (P) produced by dihydropyrazoles are displayed as a function of drug concentration. Both RH-3421 and RH-5529 reduced the fluorescence polarization of DPH and TMA-DPH as concentrations were increased from 33 to 100 micromolar.

RH-5529 was slightly more effective than RH-3421 in reducing the polarization when DPH was used as the probe. In contrast, the two compounds produced similar changes to fluorescence polarization when synaptosomal membranes were probed with TMA-DPH (Table 6.1).

6.3.2 Effects of Insecticidal Dihydropyrazoles on the Dynamic Component (r_f) and Lipid Order Parameter (S)

RH-3421 and RH-5529 were of similar efficacy in increasing the dynamic component (r_f) when TMA-DPH was used as the probe, while RH-3421-related changes in r_f were less pronounced in regions of the membrane probed by DPH (Table 6.2).

RH-5529 was also more effective than RH-3421 in reducing the order parameter with DPH (S_{DPH}) as insecticide concentration was increased (Fig. 6.1). However, when TMA-DPH was employed as the probe potency differences between RH-3421 and RH-5529 were minimal (Fig. 6.2).

6.4 Discussion

The demonstration that micromolar concentrations of RH-3421 and RH-5529 reduce the fluorescence polarization of mouse brain synaptosomal membranes probed with DPH and TMA-DPH (Table 6.1), provides evidence that these agents

Table 6.1 Effects of dihydropyrazoles on the fluorescence polarization (P) of TMA-DPH and DPH in mouse brain synaptic membranes.

Treatments	P [a] TMA-DPH	% Change	DPH	% Change
Control	0.334 ± 0.003		0.254 ± 0.004	
RH-3421 (33 μM)	0.306 ± 0.001*	-8.4	0.245 ± 0.004	-3.5
RH-3421 (66 μM)	0.284 ± 0.001*	-15.0	0.233 ± 0.002*	-8.3
RH-3421 (100 μM)	0.266 ± 0.004*	-20.4	0.219 ± 0.002*	-13.8
RH-5529 (33 μM)	0.316 ± 0.002*	-5.4	0.242 ± 0.002*	-4.7
RH-5529 (66 μM)	0.284 ± 0.002*	-15	0.218 ± 0.001*	-14.2
RH-5529 (100 μM)	0.263 ± 0.003*	-21.3	0.182 ± 0.006*	-28.4

* Denotes significant effect of dihydropyrazoles, $P < 0.01$.

[a] Values are the mean ± SD of three membrane preparations in all cases.

Table 6.2 Effects of dihydropyrazoles on the dynamic components (r_f) of fluorescence anisotropy measured with fluoroprobes TMA-DPH and DPH in mouse brain synaptic membranes.

Treatments	r_f [a] TMA-DPH	% Change	DPH	% Change
Control	0.017 ± 0.002		0.039 ± 0.001	
RH-3421 (33 μM)	0.024 ± 0.001*	41.2	0.041 ± 0.001	5.1
RH-3421 (66 μM)	0.031 ± 0.001*	82.4	0.045 ± 0.001*	15.4
RH-3421 (100 μM)	0.036 ± 0.001*	111.8	0.048 ± 0.002*	23.1
RH-5529 (33 μM)	0.022 ± 0.001*	29.4	0.042 ± 0.001	7.7
RH-5529 (66 μM)	0.031 ± 0.001*	82.4	0.048 ± 0.001*	23.1
RH-5529 (100 μM)	0.037 ± 0.001*	117.6	0. 057 ± 0.001*	46.2

[a] Values are the means ± SD of three membrane preparations in all cases.

* Significant effect of dihydropyrazoles, $P < 0.01$.

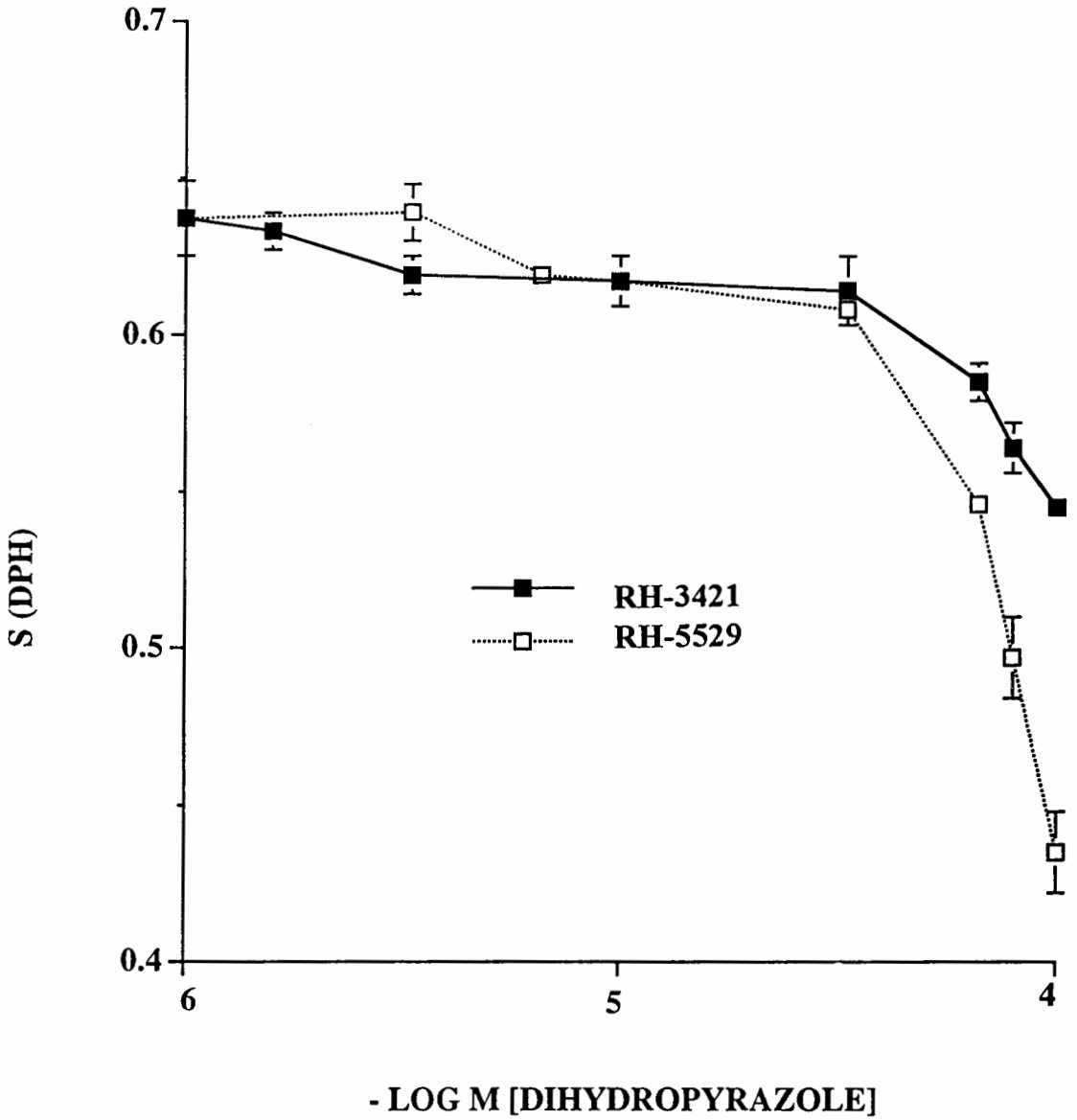


Fig. 6.1 The effect of RH-3421 and RH-5529 on the lipid order (S) in regions probed by DPH. Values show means ± SD of three independent experiments.

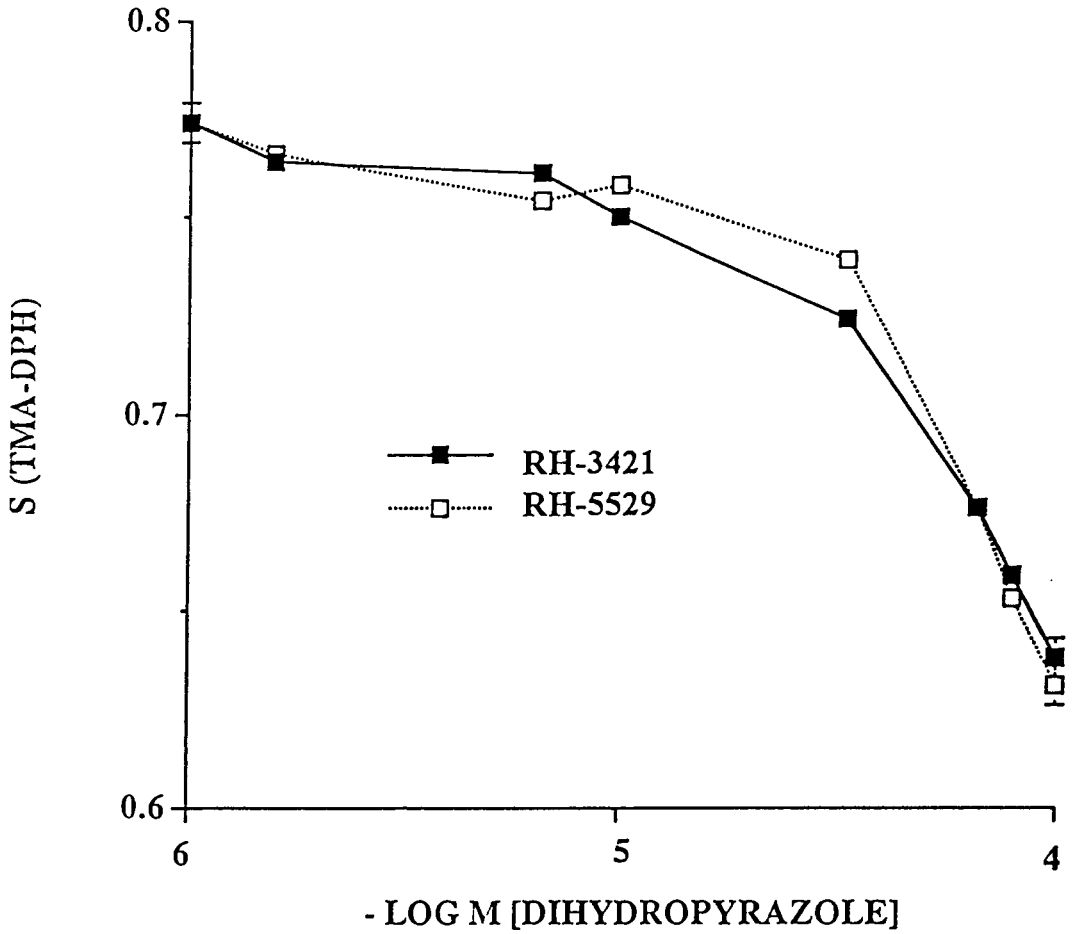


Fig. 6.2 The effect of RH-3421 and RH-5529 on the lipid order (S) in regions probed by TMA-DPH. Values show means ± SD of three independent experiments.

act as lipid perturbants at concentrations relevant to inhibition of $^{45}Ca^{++}$ influx and [^{3}H]nitrendipine binding. The lipid perturbing actions of dihydropyrazoles are similar in many respects to previous findings with anesthetic drugs which decrease the polarization of both fluoroprobes in mammalian brain synaptosomal membranes (Harris and Bruno, 1985). RH-3421 and RH-5529 increase the dynamic component (r_f) and decrease the order parameter (S), clearly demonstrating that these insecticides have the potential to induce gross fluidizing and lipid disordering effects on the membrane. The lipid disordering and fluidizing effects of RH-5529 in core regions of the membrane (accessed by DPH) are greater than in the surface regions (probed by TMA-DPH), see Fig. 6.1 and 6.2 and Table 6.2. Harris and Bruno (1985) came to similar conclusions in their studies on the membrane-perturbing actions of anesthetic drugs. Taken together, these observations support the proposal (Seelig and Seelig, 1980) that the lipid core of the membrane is very sensitive to physical modification by xenobiotics.

Compared with RH-3421, the effects on core regions of the membrane clearly discriminate in favor of RH-5529. However, in the binding study, the inhibitory potencies are higher and of reverse ranking, indicating a poor correlation with physical disturbances in the synaptosomal membrane core. A more favorable relationship exists between inhibition of [^{3}H]nitrendipine binding by dihydropyrazoles and both fluidity and lipid disorder increases in surface regions of the bilayer as determined by changes in TMA-DPH fluorescence (Figs. 4.1 and 6.2; Table 6.2). The studies presented in Chapter 5 have already demonstrated that dihydropyrazoles affect L-type (dihydropyridine-sensitive) calcium channels in mouse brain synaptosomes. In this respect, the dihydropyridine receptor, which resides on the α1 subunit on the extracellular surface of the calcium channel (Kass *et al*, 1991; Striessnig *et al.*, 1991), is appropriately located for induction of

conformational change as a result of dihydropyrazole-induced modification to the physical properties of surface lipids.

The physical disturbances to neuronal membranes identified in this study may also contribute to the delayed onset neurotoxicity reported in rodents receiving dietary RH-3421, where build up of significant quantities of this lipophilic compound in the nerve membrane would be anticipated.

Chapter 7. Summary, General Discussion and Future Prospects

Dihydropyrazoles have been found to exhibit a wide range of insecticidal activity and are particularly effective against a variety of coleopteran and lepidopteran pests (Mulder *et al.*, 1975; Jacobson, 1989). Approximately twenty years have passed since the discovery of the insecticidal properties of these compounds (Mulder *et al.*, 1975; Wellinga *et al.*, 1977; Van Hes *et al.*, 1978). At present, certain undesirable characteristics, for example, soil persistence and photoaromatization have essentially been eliminated by structural manipul-ation. However, the cumulative neurotoxic syndrome in mammals and relatively high toxicity to fish remain major obstacles to the successful registration and commercialization of this chemical series. Nevertheless, it is also clear that because dihydropyrazoles have a molecular action on the nervous system that is virtually unique amongst chemical pesticides, their introduction would strengthen our control of resistant insects that are otherwise difficult to manage with established insecticides.

In common with many of the synthetic insecticides discovered to date, the dihydropyrazoles disrupt the function of the nervous system . Poor coordination and tremor are the first signs of poisoning to develop in insects. This gives way to a period of quasi paralysis, during which it is possible to induce violent tremors and convulsions by mechanical stimulation (Salgado, 1990). Electrophysiological investigations on the dihydropyrazoles in invertebrates show quite clearly that they reduce the electrical activity of the central and peripheral nervous system significantly, an action which has been attributed to voltage-dependent block of sodium currents (Salgado, 1990; Salgado, 1992).

In support of the nervous system impairment observed in mammals during sub-chronic exposure, RH-3421 has been shown to inhibit the component of $^{22}Na^{+}$

uptake into mouse brain microvesicles that accompanies sodium channel activation (Deecher *et al.*, 1991a). Moreover, the binding of the sodium channel-specific radioligand, BTX-B to mouse brain membranes is also blocked by RH-3421 at low concentrations (Deecher *et al.*, 1991b). Therefore at this point in time, by far the majority of evidence supported the concept that dihydropyrazoles act exclusively on voltage-sensitive sodium channel in invertebrate and mammalian nervous system.

Studies in this laboratory using a GABA release assay, provided the first indication that dihydropyrazoles could also interfere with the functional properties of voltage-sensitive calcium channels in mammalian brain (Nicholson and Merletti, 1990). It is significant therefore that my results identify RH-3421 and RH-5529 as blockers of K^+- and veratridine-dependent increases in intraterminal free [Ca^{++}], $^{45}Ca^{++}$ uptake, synapsins Ia and Ib phosphorylation and L-glutamate release. Inhibition of [K^+] activated responses by dihydropyrazoles even occurs in the presence of high concentrations of TTX. The results of this thesis therefore provide strong evidence to support the hypothesis that voltage-sensitive calcium channels are sensitive to inhibition by dihydropyrazoles and accord with current theory that an important contribution to the increase in intraterminal free [Ca^{++}] in synaptosomes arises from depolarization-coupled entry of Ca^{++} from the extracellular compartment through voltage-sensitive calcium channels (Catterall, 1980; Rane *et al.*, 1987). My binding studies using [^{3}H]nitrendipine have further shown that calcium channels of the L-type are sensitive molecular targets of RH-3421 and RH-5529. Scatchard and kinetic analysis suggest that these compounds act as non-competitive blockers without allosteric involvement. In the light of present evidence, I cannot say whether or not the dihydropyrazoles bind irreversibly (or in a slowly reversible manner) to the dihydropyridine binding site or whether these compounds act via regulatory biomolecules in the environment of the L-type

calcium channel. The question of reversibility of dihydropyrazole binding could be approached either by preincubating synaptosomal preparations with dihydropyrazoles, followed by varying degrees of washing the preparation before conducting the binding assay (Deecher *et al.*, 1991b) or possibly by incubating the synaptosomes sequentially in two different concentrations of the dihydropyrazoles to determine whether the final level of binding inhibition was caused by the first or second treatment (Sheldon *et al.*, 1987). Dihydropyrazoles may of course act by a mechanism that has been proposed for endogenous brain factors (Ebersole *et al.*, 1988; Sanna and Hanbauer, 1987), perhaps by affecting guanine nucleotide binding proteins or causing the release of an enzyme which cleaves an essential fragment from the dihydropyridine receptor. Preliminary evidence for such actions could be derived by examining the effects of guanine nucleotide binding protein modulators and proteolytic enzyme inhibitors on dihydropyrazole action both in binding and functional assays.

In some situations, the regulation of voltage-dependent calcium channels can occur through participation of receptor-coupled G-proteins (Rosenthal and Schultz, 1987). Pertussis toxin is now widely used as a selective probe for G-proteins because it selectively catalyzes ADP-ribosylation of a G-protein involved in adenylate cyclase inhibition, rendering the G-protein unable to block the activity of adenylate cyclase (Ui, 1990). The adenosine analogue (-)phenylisopropyladenosine (PIA), which is proposed to stimulate an inhibitory G-protein and reduce adenylate cyclase activity, failed to inhibit veratridine- and K^+-stimulated [^{3}H]glutamate release from cerebellar neurons pretreated with pertussis toxin, suggesting that cAMP levels, which can modulate the release of [^{3}H]glutamate in this situation, are increased by pertussis toxin (Dolphin and Prestwich, 1985). In this context, it would be very interesting to determine whether the dihydropyrazole-induced inhibition of L-glutamate release and [^{3}H]nitrendipine binding can still be

observed in synaptosomes pretreated with pertussis toxin. This approach could provide important clues as to whether dihydropyrazoles interact with G-protein regulation of calcium channels in mammalian brain.

Many proteolytic enzyme inhibitors have been reported such as leupeptin (Aoyagi *et al.*, 1969; Aoyagi *et al.*, 1972), antipain (Umezawa, 1972), chymostatin (Umezawa, 1972) and elastatinal (Umezawa and Aoyagi, 1977; Umezawa and Aoyagi, 1983). All these inhibitors have an α-amino aldehyde group on the C-terminal moiety of the peptide chains which is concerned with specific binding to enzymes via the hydroxyl (or thiol) group of serine (or thiol) proteinases (Umezawa and Aoyagi, 1983). It may be informative to apply these proteolytic enzyme inhibitors to mammalian synaptosomal preparations and examine their ability to affect the dihydropyrazole-induced reduction in the density of [^{3}H]nitrendipine binding sites. However, the demonstration that the lipid bilayer of mouse brain synaptosomal membranes is disrupted by RH-3421 and RH-5529 suggests that the inhibition of [^{3}H]nitrendipine binding to calcium channels as a result of general physico-structural modification to surface regions of the bilayer is also an area of investigation that should be pursued.

The reason for the extremely high acute toxicity of RH-3421 to fish is not understood. Detoxification of this compound is slow in mammals, and it is of course feasible that this compound is degraded even more slowly in fish. An additional possibility is that neuronal sodium and/or calcium channels in fish brain have a high sensitivity to these compounds. Examination of RH-3421 action in fish brain synaptosome assays along similar lines to those employed in the present research, would enable direct comparisons of the target site sensitivities of dihydropyrazoles in a fish and mammal. Thus, conclusions could be reached on whether the high toxicity in fish has a pharmacodynamic basis.

In analysing potential correlations between *in vitro* results and *in vivo* toxicological data, detailed analysis of a group of structural analogs with diverse biological activities can yield useful information on the toxicological significance of a particular *in vitro* action, as has been found for certain insecticidal neurotoxicants (Casida and Lawrence, 1985; Lawrence and Casida, 1984) and therapeutic drugs (Sheldon *et al.* 1987). Further support for the relevance of an *in vitro* effect can be derived by examining the relationship between a compound's IC_{50} (or EC_{50}) in an *in vitro* assay and its concentration in blood (Sheldon *et al*, 1989) or target organ after *in vivo* administration, or, alternatively, by exploring electrophysiological correlates of poisoning (Salgado, 1990). Unfortunately no *in vivo* data has been published on dihydropyrazoles that would allow correlations of this type in mammals. However, because the majority of *in vitro* data suggest voltage-sensitive sodium channels are more sensitive to RH-3421 than voltage-sensitive calcium channels, a greater susceptibility of sodium channels to this compound during poisoning would be logically predicted. Nevertheless, the delayed onset neurotoxicity, as induced by dihydropyrazoles, involves a variety of symptoms, making it difficult to attribute these to interference with a single cellular target. Furthermore, because the symptoms of poisoning during delayed onset neurotoxicity can last for several days before death occurs and dihydropyrazoles are highly lipophilic compounds, high concentrations of dihydropyrazoles may accumulate in lipid rich tissues such as the brain. Therefore, despite the relatively high concentrations of dihydropyrazoles I used, inhibition of neuronal calcium channels and associated perturbations to membrane lipids may well play a role in the development of the neurotoxic syndrome observed in rodents exposed to dihydropyrazoles.

As far as pesticide registration is concerned, several aspects of the toxicology of RH-3421 (e.g. a low acute oral and dermal toxicity in mammals, a negative

Ames test result and a negative teratogenicity result etc.) can be considered favorable, but the delayed neurotoxicity observed in mammals under sub-chronic exposure regimes presents an obvious problem. High lipophilicity, combined with a relatively slow rate of degradation in non-target organisms were major factors responsible for the unacceptable bioaccumulative and chronic neurotoxicological problems with DDT and a variety of polychlorocycloalkanes including heptachlor, dieldrin and mirex. A highly lipophilic insecticide does not necessarily accumulate and cause chronic toxicity if biodegradation occurs rapidly. A classic example of this is seen with the synthetic pyrethroid insecticides, which are at least, if not more lipophilic, than DDT and the polychlorocycloalkanes, but fail to elicit neurotoxicity in mammals over a wide range of doses because biodegradation and elimination of parent compound proceeds very efficiently (Elliott *et al.*, 1972; Casida *et al.*, 1971; Casida, 1973). It will be of particular significance in the future therefore, to explore different ways of introducing biodegradeable substituents into the dihydropyrazole molecule to reduce *in vivo* stability in vertebrate systems, yet retain a good insecticidal and environmental profiles. Since insects can oxidize many xenobiotics, the introduction into the dihydropyrazole structure of an ester function (ester cleavage is generally less efficient in insects) may impart a rate of detoxification and elimination in mammals that is sufficiently rapid to avoid cumulative toxicity.

In closing, my experiments provide several independent lines of evidence to support the conclusion that insecticidal dihydropyrazoles block L-type voltage-sensitive calcium channels in nerve endings isolated from mammalian brain. I do not know how sensitive other voltage-gated calcium channels (e g. N and P types) are to these compounds, although a logical extension of this work would be to investigate how dihydropyrazoles might interfere with the binding of radioligands selective for other types of calcium channel.

References

Adam, K. R., Schmidt, H., Stampfli, R., Weiss, C. (1966) Effect of scorpion venom on single myelinated nerve fibers of the frog. Br. J. Pharmacol., **26**: 666-677.

Ahlijanian, M. K., Striessnig, J. and Catterall, W. A. (1991) Phosphorylation of an α1-like subunit of an ω-conotoxin-sensitive brain calcium channel by cAMP-dependent protein kinase and protein kinase C. J. Biol. Chem., **266**: 20192-20197.

Albuquerque, E. X., Daly, J. W. (1976) Batrachotoxin, a selective probe for channels modulating sodium conductances in electrogenic membranes. In *The Specificity and Action of Animal, Bacterial and Plant Toxins.* (P. Cuatrecasas, Ed.), pp297-338, Chapman & Hall, London.

Anderson, N. G., Maller, J. L., Tonks, N. K. and Sturgill, T. W. (1990) Requirement for integration of signals from two distinct phosphorylation pathways for action of MAP kinase. Nature, **343**: 651-653.

Angelides, K. J., Nutter, T. J., Elmer, L. W. and Kempner, E. S. (1985) Functional unit size of the neurotoxin receptors on the voltage-dependent sodium channel. J. Biol. Chem. , **260**: 3431-3439.

Aoyagi, T., Miyata, S., Nanbo, M., Kojima, F., Matsuzaki, M., Ishiznka, M., Takeuchi, T. and Umezawa, H. (1969) Biological activities of leupeptins. J. Antibiot., **22**: 558-582.

Aoyagi, T. and Umezawa, H. (1975) Structures and activities of protease inhibitors of microbial origin. In *Proteases and Biological Control.* (E. Reich, D. B. Riffkin and E. Shaw, Eds.), pp429-466, Cold Spring Harbor Laboratory, New York.

Armstrong, C. M. and Matteson, D. R. (1985) Two distinct populations of calcium channels in a clonal line of pituitary cells. Science, **227**: 65-67.

Armstrong, D. L., Rossier, M. F., Scherbatko, A. D. and White, R. E. (1991) Enzymatic gating of voltage-activated calcium channels. In *Calcium Entry and Action at the Presynaptic Nerve Terminal.* (E. F. Staneley, M. C. Nowycky and D. J. Triggle, Eds.), Ann. N. Y. Acad. Sci., **635**: 26-34.

Atlas, D. and Adler, M. (1981) α-Adrenergic antagonists as possible calcium channel inhibitors. Proc. Natl. Acad. Sci. USA.,**78**: 1237-1241.

Barnola, F. V., Villegas, R., Camejo, G. (1973) Tetrodotoxin receptors in plasma membranes isolated from lobster nerve fibers. Biochem. Biophys. Acta., **298**: 84-94.

Bean, B. P. (1989) Classes of calcium channels in vertebrate cells, Annu. Rev. Physiol., **51**: 367-384.

Bellemann, P. (1984) Binding properties of novel calcium channel activating dihydropyridine in monolayer cultures of beating myocytes. FEBS Lett., **167**: 88-92.

Benham, C. D. and Tsien, R. W. (1987) Calcium-permeable channels in vascular smooth muscle: voltage-gated, ligand-gated and receptor-operated channels. Soc. Gen. Physiol. Symp., **42**: 45-64.

Bennett, James P. Jr. (978) Methods in binding studies. In *Neurotransmitter Receptor Binding*. (H. I. Yamamura, S. J. Enna and M. J. Kuhar, Eds.), pp57-90, Raven Press, New York.

Bennett, M. K., Calakos, N. and Scheller, R. H. (1991) Gap junctions: new tools, new answers, new questions. Neuron, **6**: 305-320.

Bennett, M. K. and Scheller, R. H. (1993) The molecular machinery for secretion is conserved from yeast to neurons. Proc. Natl. Acad. Sci. USA, **90**: 2559-2563.

Berlin, J. R., Akera, T., Brody, T. M. and Matsumura, F. (1984) The inotropic effects of a synthetic pyrethroid decamethrin on isolated guinea pig atrial muscle. Eur. J. Pharmacol., **98**: 313-322.

Blaustein, M. P. and Goldring, J. M. (1975) Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescent probe: evidence that synaptosomes have potassium diffusion potentials. J. Physiol., **247**: 589-615.

Blaustein, M. P. (1975) Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals *in vitro*. J. Physiol., **247**: 617-655.

Bradford, H. F. (1975) Isolated nerve terminals *in vitro* preparation for the study of synapic aspects of transmitter metabolism and release, In *Hand book of Psychopharmacology; Section I: Basic Neuropharmacology.* (L. L. Iversen, S. D. Iversen, S. H. Snyder, Eds.), Vol. 1, pp191-252, Plenum Press; New York.

Brooks, M. W. and Clark, J. M. (1987) Enhancement of norepinephrine release from rat brain synaptosomes by alpha cyano pyrethroids. Pestic. Biochem. Physiol., **28**: 127-139.

Brown, G. B., Tieszen, S. C., Daly, J. W., Warnick, J. E., Albuquerque, E. X. (1981) Batrachotoxinin-A 20-α-benzoate: A new radioactive ligand for voltage-sensitive sodium channels. Cell Molec. Neurobiol., **1**: 19-40.

Brown, M. S., Mosher, H. S. (1963) Tarichatoxin: isolation and purification. Science, **140**: 295-296.

Brown, W., Yatan, A., VanDongen, M. J., Kirsch, G. E., Codina, J. and Birnbaumer, L. (1989) Network ionic channels by G proteins. In *G Protein and Signal Transduction.* (N. M. Nathanson and T. K. Harden, Eds.), Society of General Physiologists Series, **45**: 1-9.

Cahalan, M. D. (1975) Modification of sodium channel gating in frog myelinated nerve fibers by *Centruroides sculpturatus* scorpion venom. J. Physiol., **244**: 511-534.

Campbell, K. P., Leung, A. T. and Sharp, A. H. (1988) The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channels. TINS, **11**: 425-430.

Carafoli, E. (1987) Intracellular calcium homeostasis. Ann. Rev. Biochem., **56**: 395-433.

Carbone, E. and Lux, H. D. (1984) A low voltage activated, fully inactivating Ca channel in vertebrate sensory neurones. Nature, **310**: 501-511.

Casida, J. E., Kimmel, E. C., Elliott, M. and Janes, N. F. (1971) Oxidative metabolism of pyrethrins in mammals. Nature, **230**: 326-327.

Casida, J. E. (1973) Biochemistry of the pyrethrins. In *Pyrethrum, the Natural Insecticide.* (J. E. Casida, Ed.), pp101-116, Academic Press, New York.

Casida, J. E. and Lawrence, L. J. (1985) Structure-activity correlations for interactions of bicyclophosphorus esters and some polychlorocycloalkane and pyrethroid insecticides with the brain-specific *t*-butylbicyclophosphorothionate receptor. Env. Health Perspect, **61**: 123-132.

Casida, J. E., Nicholson, R. A. and Palmer, C. J. (1988) Trioxabicyclooctanes as probes for the convulsant site of the GABA-gated chloride channel in mammals and arthropods, In *Neurotox '88, Molecular Basis of Drug and Pesticide Action.* (G. G. Lunt, Ed.), pp125-144, Elsevier, Amsterdam.

Catterall, W. A. (1975) Activation of the action potential Na^+ ionophore of cultured neuroblastoma cells by veratridine and batrachotoxin. J. Biol. Chem., **250**: 4053-4059.

Catterall, W. A. (1977) Membrane potential-dependent binding of scorpion toxin to the action potential Na^+ ionophore: studies with a toxin derivative prepared by lactoperoxidase-catalyzed iodination. J. Biol. Chem., **252**: 8660-8668.

Catterall, W. A. (1980) Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu. Rev. Pharmacol. Toxicol., **20**: 15-43.

Catterall, W. A. and Gainer, M. (1985) Interaction of brevetoxin A with a new receptor site on the sodium channel. Toxicon **23**: 497-504.

Catterall, W. A. (1988). Structure and function of voltage-sensitive ion channels. Science, **242**: 50-61.

Catterall, W. A. (1992) Cellular and molecular biology of voltage-gated sodium channels. Physiol. Rev., **4**: S15-S40.

Catterall, W. A. (1993) Structure and modulation of Na^+ and Ca^{2+} channels. In *Molecular Basis of Ion Channels Receptors.* (H. Higashia, T. Yoshioka and K. Mikoshia, Eds.), Annals New York Acad. Sci., **707**: 1-19.

Catterall, W. A. (1995) Structure and function of voltage-gated ion channels. Annu. Rev. Biochem., **64**: 493-531.

Ceve, G. and Marsh, D. (1987) *Phospholipid biolayers: physical principles and models.* (E. Edward Bittar, Ed.), pp1-28, Wiley-Interscience Publication; John Wiley & Sons, New York.

Chapman, D. (1975) Lipid dynamics in cell membranes. In *Cell Membranes: Biochemistry, Cell Biology, and Pathology*. (G. Weissmann and R. Claiborne, eds), pp13-22, Hospital Practice.

Clark, J. M. and Matsumura, F. (1991) Enhancement of neurotransmitter release from invertebrate synaptosomes by pyrethroids during pulsed-depolarization: a functional assay for effects on repolarization. Pestic. Sci., **31**: 73-90.

Clark, J. M. (1995) Ion-channel diversity: Present and future roles in insecticide action. In *Molecular Action of Insecticides on Ion Channels*. (J. M. Clark, Ed.), ACS Symposium Series, **591**: 1-23.

Clark, J. M., Edman, S. J., Nagy, S. R., Canhoto, A.., Hecht, F. and Van Houten, J. (1995) Action of DDT and pyrethroids on calcium channels in *Paramecium tetraurelia*. In *Molecular Action of Insecticides on Ion Channels*. (J. M. Clark, Ed.), ACS Symposium Series, **591**: 173-190.

Cole, L. M., Nicholson, R. A. and Casida, J. E. (1993) Action of phenylpyrazole insecticides at the GABA-gated chloride channel. Pesti. Biochem. Physiol., **46**: 47-54.

Collingridge, G. L. and Bliss, T. V. P. (1987) NMDA receptors: their role in long-term potentiation. TINS, **10**: 288-293.

Corda, D., Pasternak, C. and Shinitzky, M. (1982) Increase in lipid microviscosity of unilamellar vesicles upon the creation of transmembrane potential. J. Membrane Biol., **65**: 235-242.

Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S., Smith, S. J. (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science, **247**: 470-473.

Couraud, F., Jover, E., Dubois, J. M. and Rochat, H. (1982) Two types of scorpion toxin receptor sites, one related to the activation, the other to the inactivation of the action potential sodium channel. Toxicon, **20**: 9-16.

Cruz, L. J. and Olivera, B. M. (1986) Calcium channel antagonist, ω-conotoxin defines a new high affinity site. J. Biol. Chem., **261**: 6230-6233.

Curtis, D. R. and Johnston, G. A. R. (1974) Amino acid transmitters in the mammalian central nervous system. Ergebn. Physiol., **69**: 97-188.

De Belleroche, J. S. and Bradford, H. F. (1973) The synaptosomes: an isolated, working, neuronal compartment. In *Progress in Neurobiology*. (G. A. Kerkut, and J. W. Phillis, Eds.), Vol. 1, pp275-298, Oxford, Pergamon.

Deecher, D. C. and Soderlund, D. M. (1991a) RH-3421, an insecticidal dihydropyrazole, inhibits sodium channel-dependent sodium uptake in mouse brain preparations. Pestic. Biochem. Physiol., **39**: 130-137.

Deecher, D. C., Payne, G. T. and Soderlund, D. M. (1991b) Inhibition of [^{3}H]batrachotoxinin A 20-α-benzoate binding to mouse brain sodium channels by the dihydropyrazole insecticide RH-3421. Pestic. Biochem. Physiol., **41**: 265-273.

Deng, Y. and Casida, J. E. (1992) House fly head GABA-gated chloride channel: toxicologically relevant binding site for avermectins coupled to site for ethynylbicycloorthobenzoate. Pestic. Biochem. Physiol., **43**: 116-122.

Dodd, P. R., Hardy, J. A., Oakles, A. E., Edwardson, J. A., Perry, E. K.and Delaunoy, J. P. (1981) A rapid method for preparing synaptosomes: comparison with alternative procedures. Brain Res., **226**: 107-118.

Dooley, D. J., Mahlmann, H., Brenner, O. and Osswald, H. (1987) Characterization of dihydropyridine binding sites of rat neocortical synaptosomes and microvessels. J. Neurochem., **49**: 900-904.

Dolphin, A. C. and Prestwich, S. A. (1985) Pertussis toxin reverses adenosine inhibition of neuronal glutamate release. Nature, **316**: 148-150.

Dopico, A. M., Lemos, J. R. and Triestman, S. N. (1996) Ethanol increases the activity of large conductance Ca^{++} activated K^+ channel in isolated neurohypophysial terminals, J. Mol. Pharmacol., **49**: 40-48.

Dunkley, P. R., Jarvie, P. E., Heath, J. W.M. Kidd, G. J. and Rostas, J. A. P. (1986) A rapid method for isolation of synaptosomes on Percoll gradients. Brain Res., **372**: 115-129.

Dunn, S. M. J. (1988) Multiple calcium channels in synaptosomes: voltage dependence of 1,4-dihydropyridine binding and effects on function. Biochemistry, **27**: 5275-5281.

Ebersole, B. L., Gajary, Z. L. and Molinoff, P. B. (1988) Endogenous modulators of binding of [^{3}H]nitrendipine in extracts of rat brain. J. Pharmacol. Exp. Ther., **244**: 971-976.

Ehlert, F. J., Roeske, W. R., Itoga, E. and Yamamura, H. I. (1982) The binding of [^{3}H]nitrendipine to receptors for calcium channel antagonists in the heart, cerebral cortex, and ileum of rats. Life Science, **30**: 2191-2202.

Elliott, M., Janes, N. F., Kimmel, E. C. and Casida, J. E. (1972) Metabolic fate of pyrethrin I, pyrethrin II, and allethrin administered orally to rats. J. Agr. Food Chem., **20**: 300-313.

Enan, E. and Matsumura, F. (1992) Specific inhibition of calcineurin by type II synthetic pyrethroid insecticides. Biochem. Pharmacol., **43**: 1777-1784.

Escueta, A. V. and Appel, S. H. (1969) Biochemical studies of synapses *in vitro*. II. Potassium transport. Biochemistry, **8**: 725-733.

Forn, J. and Greengard, P. (1978) Depolarizing agents and cyclic nucleotides regulate the phosphorylation of specific neuronal proteins in rat cerebral cortex slices. Proc. Natl. Acad. Sci. USA., **75**: 5195-5199.

Fuhr, F. Mittelstaedt, W. and Wieneke, J. (1980) Bilanzversuche mit ^{14}C-markiertem 1-(4-chlorophenylcarbamoyl)-3(4-chlorophenyl)-4-phenyl-2-pyrazolin (PH 60-42) nach boden- und spritz-applikation in freilandlysimetern. Chemosphere, **9** : 469-482.

Gammon, D. W., Brown, M. A. and Casida, J. E. (1981) Two classes of pyrethroid action in the cockroach. Pestic. Biochem. Physiol., **15**: 181-191.

Georgeauld, D. and Duclobier, A. (1978) Transient fluorescence signals from pyrene labelled pike nerves during action potential: possible implication for membrane labeled fluidity changes. Biochem. Biophys. Res. Commun., **85**: 1186-1191.

Gomez-Puertas, P., Martinez-Serrano, A., Blanco, P., Satrustegui, J. and Bogonez, E. (1991) Conditions restricting depolarization-dependent calcium influx in synaptosomes reveal a graded response of P96 dephosphorylation and a transient dephosphorylation of P65. J. Neurochem., **56**: 2039-2047.

Gould, R. J., Murphy, K. M. M. and Snyder, S. H. (1982) [^{3}H]nitrendipine-labelled calcium channels discriminate inorganic calcium agonists and antagonists, Proc. Natl. Acad. Sci. USA, **79**: 3657-3660.

Greengard, P., Valtorta, F., Czernik, A. J. and Benfenati, F. (1993) Synaptic vesicle phosphorylations and regulation of synaptic function. Science, **259**: 780-785.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem., 260: 3440-3450.

Haycock, J. W., Levy, W. B., Denner, L. A. and Cotman, C. W. (1978) Effects of elevated $[K^+]_o$ on the release of neurotransmitters from cortical synaptosomes: Efflux or secretion? J. Neurochem., **30**: 1113-1125.

Hardie, D. G. (1991) *Biochemical messengers: Hormones, neurotransmitters and growth factors*, Chapman & Hall press, London.

Harris, R. A. and Schroeder, F. (1982) Effects of barbiturates and ethanol on the physical properties of brain membranes. J. Pharmacol. Exp. Ther., **223**: 424-431.

Harris, R. A. and Bruno, P. (1985) Membrane disordering by anesthetic drugs: relationship to synaptosomal sodium and calcium fluxes. J. Neurochem., **44**: 1274-1280.

Heemskerk, F. M. J., Schrama, L. H., Ghijsen, W. E. J. M., De Graan, P. N. E., Lopes de Silva, F. H. and Gispen, W. H. (1991) Presynaptic mechanism of action of 4-aminopyridine: changes in intracellular free Ca^{2+} concentration and its relationship to B-50 (GAP-43) phosphorylation. J. Neurochem., **56**: 1827-1835.

Heinonen, E., Akerman, K. E. O., Kaila, K. and Scott, I. G. (1985) Dependence of cytoplasmic calcium transients on the membrane potential in isolated nerve endings of the guinea pig. Biochem. Biophys. Acta., **815**: 203-208.

Hescheler, J., Rosenthal, W., Trautwein, W. and Schultzi, G. (1987) The GTP-binding protein, G_o, regulates neuronal calcium channels. Nature, **325**: 445-446.

Hess, P. (1990) Calcium channels in vertebrate cells. Annu. Rev. Neurosci. **13**: 337-356.

Hille, B. (1992) *Ionic Channels of Excitable Membranes*, Sinauer Associates Sunderland, Mass.

Hitzemann, B. A., Hitzemann, R. J. and Harris, R. A. (1983) Further studies on developmental changes in synaptic membrane fluidity. Neurosci. Abst. Boston.

Hitzemann, R., Harris, R. A. and Loh, H. H. (1984) Synaptic membrane fluidity and function. In *Physiology of Membrane fluidity*. (M. Shinitzky, Ed.), Vol II, pp109-126, CRC Press, Inc.; Boca Raton, Florida.

Hodgkin, A. L. and Keynes, R. D. (1957) Movements of labelled calcium in squid giant axions. J. Physiol., **138**: 253-281.

Huang, J. M. C., Wu, C. H. and Baden, D. G. (1984) Depolarizing action of a red tide dinoflagellate brevetoxin on axonal membranes. J. Pharmacol. Exp. Ther., **229**: 615-621.

Jacobson, R. M. (1989) A new class of insecticidal dihydropyrazoles. In *Advances in the chemistry of insect control*, St. Catherines College, Oxford, England.

Jahnig, F. (1979) Structural order of lipid and proteins in membranes: evaluation of fluorescence anisotropy data. Proc. Natl. Acad. Sci. USA., **76**: 6361-6365.

Kandel, E. R. and Siegelbaum, S. (1985) Principles underlying electrical and chemical synaptic transmission. In *Principles of Neural Science*. 2nd edition, (E. R. Kandel and J. H. Schwartz, Eds.), pp89-107, Elsevier, New York.

Kanemoto, Y., Enan, E. E., Matsumura, F. and Miyazawa, M. (1992) Time-dependent changes in protein phosphorylation patterns in rat brain synaptosomes caused by deltamethrin. Pestic. Sci., **34**: 281-290.

Kao, C. Y. (1966) Tetrodotoxin, saxitoxin, and significance in the study of excitation phenomena. Pharmacol. Rev., **18**: 997-1049.

Kasai, H., Aosaki, T. and Fukuda, J. (1987) Presynaptic Ca-antagonist ω-conotoxin irreversibly blocks N-type Ca-channels in chick sensory nerons. Neurosci. Res., **4**: 228-235.

Kass, R. S., Arena, J. P. and Chin, S. (1991) Block of L-type calcium channels by charged dihydropyridines: sensitivity to side of application and calcium. J. Gen. Physiol., **98**: 63-75.

Kelly, R. B. (1988) The cell biology of the nerve terminal. Neuron, **1**: 431-438.

Kennedy, M. B. (1994). The biochemistry of synaptic regulation in the central nervous system. Annu. Rev. Biochem., **63**: 571-600.

Khodorov, B. I. (1978) Chemicals as tools to study nerve fiber sodium channels: Effects of batrachotoxin and some local anesthetics. In *Membrane Transport Processes*. (D. C. Tosteson, R. Orchininikov, R. Latorre, Eds.), Vol. II, pp153-174, Raven, New York.

Khodorov, B. I., Revenko, S. V. (1979) Further analyses of the mechanisms of action of batrachotoxin on the membrane of myelinated nerve. Neuroscience, **4**: 1315-1330.

Koch, W. J., Hui, A., Shull, G. E., Ellinor, P.and Schwartz, A. (1989) Characterization of cDNA clones encoding two putative isoforms of the α1 subunit of the dihydropyridine-sensitive voltage-dependent calcium channel isolated from rat brain and rat aorta. FEBS Lett., **250**: 386-388.

Komulainen, H. and Bondy, S. C. (1987) Increased free intrasynaptosomal Ca^{2+} by neurotoxic organometals: distinctive mechanisms. Toxicol. Appl. Pharmacol., **88**: 77-86.

Konno, Y. and Scott, J. G. (1991) Biochemistry and genetics of abamectin resistance in the house fly. Pestic. Biochem. Physiol. **41**: 21-28.

Krueger, B. K., Forn, J. and Greengard, P. (1977) Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx in rat brain synaptosomes. J. Biol. Chem., **252**: 2764-2773.

Kruepinski, J., Coussen, F., Bakalyar, H. A., Tang, W. J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R. and Gilman, A. G. (1989) Adenylyl cyclase

amino acid sequence: possible channel- or transporter-like structure. Science, **244**: 1558-1564.

Kuno, M. (1995) *The synapse: function, plasticity, and neurotrophism.* Oxford University Press Inc., New York.

Lansman, J. B., Hallam, T. J. and Rink, T. J. (1987) Single stretch-activated ion channels in vascular endothelial cells as mechanotransducer? Nature, **325**: 811-813.

Lawrence, L. J. and Casida, J. E. (1984) Interactions of lindane, toxaphene and cyclodienes with brain-specific t-butylbicyclophosphorothionate receptor, Life Sci., **35**: 171-178.

Leicht, R., Meves, H., Wellhoner, H. H. (1971) The effect of veratridine on *Helix pomatia* neurones. Pflugers Arch., **323**: 50-62.

Lemos, J. R. and Nowycky, M. C. (1989) Two types of calcium channels coexist in peptide-releasing vertebrate nerve terminals. Neuron, **2**: 1419-1426.

Ling, C. M. and Abdel-Latif, A. A. (1968) Studies on sodium transport in brain nerve-ending particles. J. Neurochem, **15**: 721-730.

Littleton, J. T. and Bellen, H. J. (1995) Synaptotagmin controls and modulates synaptic-vesicle fusion in a Ca^{2+}-dependent manner. TINS, **8**: 177-183.

Llinas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. and Greengard, P. (1985) Intraterminal injection of synapsin I or calcium/calmodulin dependent protein kinase II alters neurotransmitters release at the squid giant synapse. Proc. Natl. Acad. Sci. USA., **82**: 3035-3039.

Llinas, R., Sugimori, M., Lin, J. M., Cherksey, B. (1989) Blocking and isolation of a calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. Proc. Natl. Acad. Sci. USA., **86**: 1689-1693.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S. L., Matsudaira, P. and Darnell, J. (1995) *Molecular Cell Biology*; 3rd edition; Scientific American Books, W. H. Freeman and Company, New York.

Loscher, W., Bohme, G., Muller, F. and Pagliusi, S. (1985) Improved method for isolating synaptosomes from 11 regions of one rat brain: electron

microscopic and biochemical characterization and use in the study of drug effects on nerve terminal γ-aminobutyric acid *in vitro*. J. Neurochem. **45**: 879-889.

Lund, A. F. and Narahashi, T. (1983) Kinetics of sodium channel modification as the basis for the variation in the nerve membrane effects of pyrethroids and DDT analogs. Pest. Biochem. Physiol., **20**: 203-216.

Luo, M. and Bodnaryk, P. (1987) Synaptosomes and synaptosome membrane vesicles from the brain of *Mamestra configurata*: application to voltage-dependent and ATP-dependent Ca^{++} ion transport studies. Insect Biochem., **17**: 911-918.

Marchbanks, R. M. (1967) The osmotically sensitive potassium and sodium compartments of synaptosomes. Biochem. J., **104**: 148-157.

Marchbanks, R. M. (1968) The uptake of [^{14}C]choline into synaptosomes *in vitro*. Biochem. J., **110**: 533-541.

Marchbanks, R. M., Campbell, C. W. B. (1976) Sodium and chloride fluxes in synaptosomes *in vitro*. J. Neurochem., **26**: 973-980.

Matsumura, F. (1987) Deltamethrin induced changes in synaptosomal transport of ^{3}H-epinephrine in the squid optic lobes. Comp. Biochem. Physiol., **87C**: 31-35.

Matsumura, F. and Clark, J. M. (1988) Effects of pyrethroids on neural protein kinases and phosphatases of the squid optic lobe. In *Neurotox '88, Molecular Basis of Drug and Pesticide Action*. (G. G. Lunt, Ed.), pp235-244. Elsevier, Amsterdam.

McBurney, R. N. and Neering, I. R. (1987) Neuronal calcium homeostasis. TINS, **10**: 164-169.

McClesky, E. W., Fox, A. P., Feldman, D. H., Cruz, L. J., Olivera, B. M., Tsien, R. W. and Yoshikami, D. (1987) ω-Conotoxin: direct and persistent blockade of specific types of calcium channel in neurons but not muscle, Proc. Natl. Acad. Sci. USA, **84**: 4327-4331.

Meldolesi, J. and Pozzan, T. (1987) Pathways of Ca^{++} influx at the plasma membrane: voltage-, receptor- and second messenger-operated channels. Exp. Cell Res., **171**: 271-283.

Mendelson, W. B., Scolnik, P., Martin, J. V., Luu, M. D. (1984) Diazepam-stimulated increases in the synaptosomal uptake of $^{45}Ca^{2+}$: reversal by dihydropyridine calcium channel antagonists. Eur. J. Pharmac., **104**: 181-183.

Meyer, E. M. and Cooper, J. R. (1981) Correlations between Na^{+}-K^{+} ATPase activity and acetylcholine release in rat cortical synaptosomes. J. Neurochem., **36**: 467-475.

Middlemiss, D. N. and Spedding, M. (1985) A functional correlate for the dihydropyridine binding site in rat brain. Nature, **314**: 94-96.

Miller, R. J. (1993) A tale of two toxins. Curr. Biol. **4**: 481-482.

Miledi, R. and Parker, I. (1980) Blocking of acetylcholine-induced channels by extracellular or intracellular application of D600. Proc. R. Soc. Lond [Biol], **211**: 143-150.

Mintz, I. M., Venema, V. J., Swiderek, K. M., Lee, T. D., Bean, B. P. and Adams, M. E. (1992) P-type calcium channels blocked by the spider toxin ω-Aga-IVA. Nature, **355**: 827-829.

Mori, Y., Niidome, T., Fugita, Y., Mynlieff, M., Dirksen, R. T., Beam, K. G., Iwabe, N., Miyata, T., Furutama, D., Furuichi, T. and Mikoshiba, K. (1993) Molecular diversity of voltage-dependent calcium channel. In *Molecular Basis of Ion Channels and Receptors*. (H. Higashia, T. Yoshioka, K. Mikoshiba, Eds.), Annals New York Acad. Sci., **707**: 87-108.

Mulder, R., Wellinga, K. and van Dalen, J. J. (1975) A new class of insecticides. Naturwissenschaften, **62**: 531-532.

Murphy, K. M. M., Gould, R. J., Largent, B. L. and Snyder, S. H. (1983) A unitary mechanism of calcium antagonist drug action. Proc. Natl. Acad. Sci. USA., **80**: 860-867.

Nachshen, D. A. and Blaustein, M. P. (1980) Some properties of potassium-stimulated calcium influx in presynaptic nerve endings. J. Gen. Physiol., **76**: 709-728

Nakamura, Y., Nakajima, S., Grundfest, H. (1965) The action of tetrodotoxin on electrogenic components of squid giant axons. J. Gen. Physiol., **48**: 985-996.

Narahashi, T. and Yamasaki, T. (1960) Mechanism of the after-potential production in the giant axons of the cockroach. J. Physiol., **151**: 75-88.

Narahashi, T. (1962) Effect of the insecticide allethrin on membrane potentials of cockroach gianat axons. J. Cell Comp. Physiol., **59**: 61-65.

Narahashi, T., Anderson, N. C., Moore, J. W. (1966) Tetrodotoxin does not block excitation from inside the nerve membrane. Science, **153**: 765-767.

Narahashi, T. (1971) Effects of insecticides on excitable tissues. In *Advances in Insect Physiology*. (J. W. L. Beament, J. E. Treherne, V. B. Wigglesworth, Eds.), Academic Press: New York, **8**: 1-93.

Narahashi, T. (1974) Chemicals as tools in the study of excitable membranes. Physiol. Rev., **54**: 813-89.

Narahashi, T. (1979) Nerve membrane ionic channels as the target site of insecticides. In *Neurotoxicology of Insecticides and Pheromones*. (T. Narahashi, Ed.), pp211-243, Plenum, New York.

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

Narahashi, T. (1992) Nerve membrane Na^+ channels as targets of insecticides. TIPS, **13**: 236-241.

Navone, F., Greengard, P. and De Camilli, P. (1984) Synapsin I in nerve terminals: selective association with small synaptic vesicles. Science, **226**: 1209-1211.

Nicholls, D. G., Sihra, T. S. and Sanchez-Prieto, J. (1987) Calcium-dependent and independent release of glutamate from synaptosomes monitored by continuous fluorimetry. J. Neurochem., **49**: 50-57.

Nicholson, R. A., Botham, R. P. and Collins, C. (1983) The use of [^{3}H] permethrin to investigate the mechanisms underlying its differential toxicity to adult and larval stages of the sheep blowfly *Lucilia sericata*. Pestic. Sci., **14**: 57-63.

Nicholson, R. A. and Merletti, E. L. (1990) The effect of dihydropyrazoles on the release of [^{3}H]GABA from nerve terminals isolated from mammalian cerebral cortex. Pestic. Biochem. Physiol., **37**: 30-40.

Nicholson, R. A. and Kumi, C. O. (1991) The effects of pesticides, brevetoxin B, and the cardiotonic drug DPI 201 106 on release of acetylcholine from insect central nerve terminals. Pestic. Biochem. Physiol., **40**: 86-97.

Nicholson, R. A. (1992) Insecticidal dihydropyrazoles antagonize the depolarizing action of veratridine in mammalian synaptosomes as measured with a voltage-sensitive dye. Pestic. Biochem. Physiol., **42**: 197-202.

Nowycky, M. C., Fox, A. P. and Tsien, R. W. (1985) Three types of neuronal calcium channel with different calcium agonist sensitivity, Nature, **316**: 443-440.

Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., River, J., Santos, V., D. and Cruz, L. J. (1985) Peptide neurotoxins from fish-hunting cone snails. Science, **230**: 1338-1343.

Olivera, B. M., Miljanich, G. P., Ramachandran, J. and Adams, M. E. (1994) Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. Annu. Rev. Biochem., **63**: 823-867.

Orchard, I. and Osborne, M. P. (1979) The action of insecticides on neuro-secretory neurons in the stick insect, *Carausius morosus*. Pestic. Biochem. Physiol. **10**: 197-202.

Ottea, J. A., Payne, G. T., Bloomquist, J. R. and Soderlund, D. M. (1989) Action of sodium channels and inhibition of [^{3}H]batrachotoxinin A-20-α-benzoate binding by an N-alkylamide neurotoxin. Mol. Pharmacol., **36**: 280-284.

Pallen, C. J., Sharma, R. K. and Wang, J. H. (1985) Modulation of cAMP effects by Ca^{++}/calmodulin. BioEssays, **2**: 113-117.

Payne, G. T. and Soderlund, D. M. (1989) Allosteric enhancement by DDT of the binding of [^{3}H]batrachotoxinin A-20-α-benzoate to sodium channels. Pestic. Biochem. Physiol., **33**: 276-282.

Peterson, G. L. (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal. Biochem., **83**: 346-356.

Poli, M. A., Mende, T. J. and Baden, D. G. (1986) Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol. Pharmacol., **30**: 129-135.

Prendergast, F. G., Haugland R. P. and Callahan, P. J. (1981) 1-[4-(Trimethylamino) phenyl]-6-phenylhexa-1,3,5-triene: synthesis, fluorescence properties, and use as a fluorescence probe of lipid bilayers. Biochemistry, **20**: 7333-7338.

Randall, A. and Tsien, R. W. (1995) Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. J. Neurosci. **15**: 2995-3012.

Rane, S. G., Holz, G. G. and Dunlap, K. (1987) Dihydropyridine inhibition of neuronal calcium currents and substance P release. Pflugers Arch., **409**: 361-366.

Richards, C. D., Martin, K., Gregory, S., Keightley, C. A., Hesketh, T. R., Smith, G. A., Warren, G. B. and Metcalfe, J. C. (1978) Degenerate perturbations of protein structure as the mechanism of anesthetic action. Nature, **276**: 775-779.

Robinson, P. J. and Dunkley, P. R. (1983) Depolarization-dependent protein phosphorylation in rat cortical synaptosomes: Factors influencing magnitude of response. J. Neurochem., **41**: 909-918.

Robinson, P. J. and Dunkley, P. R. (1985) Depolarization-dependent protein phosphorylation and dephosphorylation in rat cortical synaptosomes is modulated by calcium. J. Neurochem., **44**: 338-348.

Rochat, H., Rochat, C., Kupeyan, C., Miranda, F., Lissitzky, S. and Edman, P. (1970) Scorpion neurotoxins: a family of homologous proteins. FEBS Lett., **10**: 349-351.

Roeder, K. D. and Weiant, E. A. (1946) The site of action of DDT in the cockroach. Science, **130**: 304-306.

Rosenthal, W. and Schultz, G. (1987) Modulation of voltage-dependent ion channels by extracellular signals. Trends Pharmacol. Sci. **8**: 351-374.

Sabria, J., Pastor, C., Clos, M. V., Garcia, A. and Badia, A. (1995) Involvement of different types of voltage-sensitive calcium channels in the presynaptic regulation of noradrenaline release in rat brain cortex and hippocampus. J. Neurochem., **64**: 2567-2571.

Sakamoto, J. and Campbell, K. P. (1991) A monoclonal antibody to the β subunit of the skeletal muscle dihydropyridine receptor immunoprecipitates the brain ω-conotoxin GVIA receptor. J. Biol. Chem., **266**: 18914-18919.

Salgado, V. L. (1990) Mode of action of insecticidal dihydropyrazoles: selective block of impulse generation in sensory nerves. Pestic. Sci., **28**: 389-411.

Salgado, V. L. (1992) Slow voltage dependent block of sodium channels in crayfish nerve by dihydropyrazole insecticides. Mol. Pharmacol., **41**: 120-126.

Sanchez-Prieto, J., Sihra, T. S. and Nicholls, D. G. (1987) Characterization of the exocytotic release of glutamate from guinea-pig cerebral cortical synaptosomes. J. Neurochem., **49**: 58-64.

Sanna, E. and Hanbauer, I. (1987) Isolation from rat brain tissue of an inhibition activity for dihydropyridine binding sites and voltage-dependent Ca^{++} uptake. Neuropharmacol., **26**: 1811-1819.

Schulman, H. and Greengard, P. (1978) Stimulation of brain membrane protein phosphorylation by calcium and an endogenous heat-stable protein. Nature, **271**: 478-479.

Scott, I. D. and Nicholls, D. G. (1980) Energy transduction in intact synaptosomes: Influence of plasma-membrane depolarization on the respiration and membrane potential of internal mitochondria determined *in vitro*. Biochem. J.,**186**: 21-33.

Seelig, J. and Seelig, A. (1980) Lipid conformation in model membranes and biological membranes. Ann. Rev. Biophs., **13**: 119-135.

Sheele, B. (1980) Untersuchung des photoabbaus von PH 60-42 auf maisblattern, glasplatten und bodendunnschichtplatten. Chemosphere **9**: 483-494.

Sheldon, R. S., Cannon, N. J. and Duff, H. J. (1987) A receptor for type I antiarrhythmic drugs associated with rat cardiac sodium channels. Circulation Research, **61**: 492-497.

Sheldon, R. S., Hill, R. J., Cannon. N. J. and Duff, H. J. (1989) Amiodarone: biochemical evidence for binding to a receptor for class I drugs associated with the rat cardiac sodium channel. Circulation Research, **65**: 477-482.

Shepherd, G. (1994) Synaptic potentials and synaptic integration. In *Neurobiology*. 3rd edition, (G. M. Shepherd, Ed.), pp132-150, Oxford University Press, New York.

Sher, E. and Clementi, F. (1991) Omega-conotoxin-sensitive voltage operated calcium channels in vertebrate cells, Neurosci., **42**: 301-307.

Shinitzky, M. and Barenholz, M. (1978) Fluidity parameters of lipid regions determined by fluorescence polarization. Biochem. Biophys. Acta., **515**: 367-394.

Shmeeda, H. R., Golden, E. B. and Barenholz, Y. (1984) Membrane lipids and aging. In *Biomembranes Structural and Functional Aspects*. (M. Shinitzky, Ed.), pp1-82, VCH Publishers Inc., New York.

Sikorski, A. F., Terlecki, G., Zagon, I. S. and Goodman, S. R. (1991) Synapsin I-mediated interaction of brain spectrin with synaptic vesicles. J. Cell. Biol., **114**: 313-318.

Singer, S. J. and Nicolson, D. (1972) The fluid mosaic model of the structure of cell membranes. Science, **175**: 720-731.

Stallcup, W. B. (1977) Comparative pharmacology of voltage-dependent sodium channels. Brain Res., **135**: 37-53.

Stefani, A., Calabresi, P., Pisani, A., Mericuri, N. B., Siniscalchi, A. and Bernardi, G. (1996) Felbamate inhibits dihydropyridine-sensitive calcium channels in central neurons. J. Pharmacol. Exp. Ther., **277**: 121-127.

Strichartz, G., Rando, T. and Wang, G. K. (1987) An integrated view of the molecular toxicology of sodium channel gating in excitable cells. Ann. Rev. Neurosci., **10**: 237-267.

Striessnig, J., Murphy, B. J. and Catterall, W. A. (1991) Dihydropyridine receptor of L-type calcium channels: identification of binding domains for [^{3}H]-(+)-PN200-110 and [^{3}H]azidopine within the α1 subunit. Proc. Natl. Acad. Sci. USA., **88**: 10769-10773.

Sudhof, T. C., De Camilli, P., Niemann, H. and Jahn, R. (1993) Membrane fusion machinery: insights from synaptic proteins. Cell, **75**: 1-4.

Sutro, J. B. (1986) Kinetics of veratridine action on Na channels of skeletal muscle. J. Gen. Physiol., **87**: 1-24.

Takacs, B. (1979). Electrophoresis of proteins in polyacrylamide slab gels. In *Immunological Methods*. (I. Lefkovits and B. Pernis, Eds.), pp81-104, Academic Press, New York.

Tanabe, T., Beam, K. G., Powell, J. A. and Numa, S. (1988) Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. Nature, **336**: 134-139.

Trimble, W. S., Linial, M. and Scheller, R. H. (1991) Cellular and molecular biology of the presynaptic nerve terminal. Ann. Rev. Neurosci., **14**: 93-122.

Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R. and Fox, A. P. (1988) Multiple types of neuronal calcium channels and their selective modulation. TINS, **11**: 431-438.

Tsien, R. W. and Tsien, R. Y. (1990) Calcium channels, stores, and oscillations. Ann. Rev. cell Biol., **6**: 715-760.

Tsien, R. W., Ellinor, P. T. and Horne, W. A. (1991) Molecular diversity of voltage-dependent calcium channels. Trends Pharmacol. Sci., **12**: 349-354.

Tsien, R. W., Lipscombe, D., Madison, D., Bley, K. R. and Fox, A. (1995) Reflections on Ca^{2+}-channel diversity, 1988-1994. Trends Neurosci., **18**: 52-54.

Tully, T. (1987) Drosophila learning and memory revised. Trends Neurosci., **10**: 330-335.

Turner, T. J. and Goldin, S. M. (1985) Calcium channels in rat brain synaptosomes: identification and pharmacological characterization. J. Neurosci., **5**: 841-849.

Ui, M. (1990) G proteins identified as pertussis toxin substrates. In *G Proteins and Calcium Signaling*. (P. H. Naccache, Ed.), pp3-18, CRC Press, Boca Raton.

Ulbricht, W. (1969) The effects of veratridine on excitable membranes in nerve and muscle. Ergeb. Physiol. Biol. Chem. Exp. Pharmakol., **61**: 17-71.

Umezama, H. (1972) *Enzyme Inhibitors of Microbial Origin*. (H. Umezama, Ed.), pp15-34, Univ. Tokyo Press, Tokyo.

Umezawa, H. and Agoyagi, T. (1977) Activities of proteinase inhibitors of microbial origin. In *Proteinases in Mammalin Cells and Tissues*. (A. J. Barrett, Ed.), pp637-665, North-Holland, Amosterdam.

Umezama, H. and Aoyagi, T. (1983) Trends in research of low molecular weight protease inhibitors of microbial origin. In *Proteinase Inhibiitors, Medical and Biological Aspects*. (N. Katunuma, H. Umezawa and H. Holzer, Eds.), pp3-15, Japan Scientific Societies Press, Tokyo.

Valtorta, F., Benfenati, F. and Greengard, P. (1992) Structure and function of the synapsins. J. Biol. Chem., **267**: 7195-7198.

Van Blitterswijk, W. J. , Van Hoeven, R. P. and Van der Meer, B. W. (1981) Lipid structural order parameters (reciprocal of fluidity) in biomembranes derived from steady-state fluorescence polarization measurements. Biochem. Biophys. Acta., **644**: 323-332.

Van Hes, R., Wellinga, K and Grosscurt, A. C. (1978) 1-Phenylcarbamoyl-2-pyrazolines, a new class of insecticides. 2. Synthesis and insecticidal properties of 3,5-diphenyl-1-phenylcarbamoyl-2-pyrazolines. J. Agric. Food Chem., **26**: 915-918.

Vijverberg, H. P. M., Van der Zalm, J. M., Van Kleek, R. G. D. M. and Van der Bercken, J. (1983) Temperature- and structure-dependent interaction of pyrethroids with the sodium channels of frog node of Ranvier. Biochem. Biophys. Acta., **728**: 73-82.

Walch-Solimena, C., Jahans, R. and Sudhof, T. C. (1993) Synaptic vesicle proteins in exocytosis: What do we know? Current Opin. Neurobiol., **3**: 329-336.

Weinstein, H., Varon, S., Muhleman, D. R. and Roberts, E. (1965) A carrier-mediated transfer mode for the accumulation of [^{14}C]γ-aminobutyric acid by subcellular brain particles. Biochem. Pharmacol., **14**: 273-288.

Wellinga, K., Grosscurt, A. C. and van Hes, R. (1977) 1-Phenylcarbamoyl-2-pyrazolines: a new class of insecticides. 1. Synthesis and insecticidal properties of 3-phenyl-1-phenylcarbamoyl-2-pyrazolines. J. Agric. Food Chem., **25**: 987-992.

Westenbroek, R. E., Sakurai, T., Elliott, E. M., Hell, J. W., Starr, T. V. B., Snutch, T. P. and Catterall, W. A. (1995) Immunochemical identification and subcellular distribution of the α_{1A} subunits of brain calcium channels. J. Neuorsci., **15**: 6403-6418.

Whittaker, V. P., Michaelson, I. A. and Kirkland, R. J. A. (1964) The separation of synaptic vesicles from nerve-ending particles ('synaptosomes'). Biochem. J., **90**: 293-303.

Wodtke, E. (1978) Lipid adaptation in liver mitochodrial membranes of carp acclimated different environmental temperatures: phospholipid composition, fatty acid pattern, and cholesterol content. Biochem. Biophys. Acta., **529**: 280-291.

Woodward, J. J. and Leslie, S. W. (1986) Bay K 8644 stimulation of calcium entry and endogenous dopamine release in rat striatal synaptosomes antagonized by nimodipine. Brain Res., **370**: 397-400.

Wouters, W. and Van den Bercken, J. (1978) Action of pyrethroids. Gen. Pharmacol., **9**: 387-398.

Yamamura, H. I., Schoemaker, H., Boles, R. G. and Roesko, W. R. (1982) Diltiazem enhancement of [^{3}H]nitrendipine binding to calcium associated drug receptor sites in rat brain synaptosomes. Biochem. Res. Commun., **108**: 640-646.

Zhang, A. and Nicholson, R. A. (1993) The dihydropyrazole RH-5529 blocks voltage-sensitive calcium channels in mammalian synaptosomes. Pestic. Biochem. Physiol., **45**: 242-247.

Zhang, J-F, Randall, A. D., Ellinor, P. T., Horne, W. A., Sather, W. A., Tanabe, T., Schwarz, T. C. and Tsien, R. W. (1993) Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalain CNS neurons. Neuropharmacology, **32**: 1075-1088.

Zimmermanna, H. (1993) *Synaptic Transmission: Cellular and Molecular Basis.* (H. Zimmermanna, Ed.), pp31-67, Oxiford University Press, New York.