SYNAPTOSOMAL CA^{++}+MG^{++}-ATPASE FROM THE BRAIN OF THE BERTHA ARMYWORM *MAMESTRA CONFIGURATA*: PREPARATION, PROPERTIES AND THE EFFECT OF INSECTICIDES

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Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

By

Ma Luo

In Partial Fulfillment of the

Requirements for the degree

of

Doctor of Philosophy

Department of Entomology

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BY

MA LUO

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Luo, Ma. Ph.D, The University of Manotiba, June, 1987. Synaptosomal Ca^{++}+Mg^{++}-ATPase from the Brain of Bertha Armyworm ***Mamestra configurata***: Preparation, Properties and the Effect of Insecticides. Major Professor: Robert P. Bodnaryk.

As calcium plays an important role in the action of hormones and neurotransmitters, the maintenance of calcium ion homeostasis is of special interest in insect biochemistry and insecticide toxicology. Disruption of the intracellular regulation of Ca^{++}, especially in the nervous system, may contribute in some as yet unexplained manner to the death of insects poisoned with certain insecticides. However, progress in understanding such complex interactions has long been retarded by a dearth of basic information on calcium ion transport and its regulation in insects, until recently, the lack of a good *in vitro* system such as synaptosomes and synaptosome membrane vesicles, to study the neurochemistry of insects.

Morphologically pure and functionally competent synaptosomes and synaptosome membrane vesicles were prepared from the brain of adult bertha armyworm *Mamestra configurata* using a modified Ficoll floatation technique. A Ca^{++}+Mg^{++}-ATPase, one of several important outward-directed calcium transport processes, was identified in both

synaptosomes and synaptosome membrane vesicles. The activities of Ca^{++}+Mg^{++}-ATPase and calcium transport displayed very high affinity for free calcium and were strictly dependent on ATP and magnesium, and little influenced by ouabain and potassium.

The effect of seven major groups of insecticides on synaptosomal Ca^{++}+Mg^{++}-ATPase was examined. Six of 18 insecticides examined inhibited enzyme's activity markedly at 10^{-4} M; four of these six are cyclodiene compounds. Dose response studies showed that these insecticides inhibited the activities of both Ca^{++}+Mg^{++}-ATPase and the calcium pump under identical conditions. *In vivo* insecticide treatment also significantly affected the activity of moth brain synaptosomal Ca^{++}+Mg^{++}-ATPase. The importance of the inhibition of Ca^{++}+Mg^{++}-ATPase and calcium pump by insecticides is discussed.

GENERAL INTRODUCTION

Many groups of modern insecticides act by disrupting nervous system function. Numerous electrophysiological studies have shown that many insecticides affect action potentials of the nerve membrane (Matsumura, 1975; Narahashi, 1976). The increased negative afterpotential, and prolonged repetitive afterdischarges caused by neurotoxic insecticides such as DDT, pyrethroids and cyclodienes suggested that the ion permeability of nerve membranes was affected (Matsumura, 1975; Narahashi, 1976). Electrophysiological experiments have indicated that DDT might attack the sodium inactivation mechanism of the nerve membrane which facilitates synaptic and neuromuscular transmission (Narahashi, 1978). Similarly, pyrethroids are thought to act by modifying sodium channels and keeping them in the open conformational state (Lund, 1984). Relatively few studies have been reported on the action of cyclodiene insecticides in relation to interference with axonic membrane excitation, cyclodiene compounds being regarded as mainly synaptic effectors (Doherty, 1984). Although electrophysiological studies have pinpointed possible sites of insecticide attack, the manner in which insecticides upset ion exchange across the nerve membrane, i.e. the biochemical mode of action of these neurotoxic insecticides, still remains poorly understood.

Calcium has a stabilizing effect on the nerve axon and high concentrations of calcium antagonize the excitatory effect of some neurotoxic insecticides (Matsumura and O'Brien, 1966;

Matsumura and Narahashi, 1971; Gammon, 1978a; 1980). Reducing the extracellular calcium concentration around arthropod nerve axons elicits repetitive discharges reminiscent of DDT (O'Brien, 1966). These observations suggested that calcium is also intimately associated with the toxic reaction between axon and insecticides, as DDT-induced repetitive discharges are difficult to explain solely in terms of sodium and potassium fluxes. Studies of the biochemical mode of action of insecticides found that a DDT-sensitive Ca^{++}-ATPase existed in an axonic nerve preparation from the lobster *Homarus americanus* (Ghiasuddin and Matsumura, 1979a,b; Matsumura *et al*, 1979). These observations led to the speculation that disruption of the intracellular regulation of Ca^{++}, especially in the nervous system, might contribute to the death of insects poisoned with certain insecticides (Beeman, 1982; Doherty, 1984). Moreover, it has been found that some insecticides act by provoking the release of insect neurohormones (Maddrell and Reynolds, 1972). Insecticides also profoundly disrupt the metabolism of cAMP and cGMP, the second messengers of hormones and neurotransmitters (Bodnaryk, 1976; 1977; 1982; Butler *et al*, 1977). These findings also suggest that the disruption of calcium regulation mechanisms could be a major cause of the excessive neurohormone release and of disrupted metabolism.

Calcium plays an important role in the nervous system. Calcium entering the nerve terminal during depolarization is essential for neurotransmitter release (Llinas, 1979). Calcium also acts as a second messenger of many hormones and

neurotransmitters (Rasmussen, 1970). A high concentration of calcium, however, is cytotoxic. The intracellular calcium level is kept at least 1000 times lower than that outside of the cell by several calcium regulating systems, such as a Na^{+}/Ca^{++} exchange mechanism and an ATP-dependent calcium pump, a Ca^{++}+Mg^{++}-ATPase, located in the cell membrane (Penniston, 1983; Reichardt and Kelly, 1983). Destruction of the calcium regulation mechanisms would disrupt calcium homeostasis, possibly resulting in excessive neurotransmitter and hormone release, and disruption of metabolic activities leading to death.

Several studies on biochemical and kinetic properties of the Ca^{++}+Mg^{++}-ATPase and ATP-dependent calcium pump in mammalian brain synaptosome preparations have demonstrated that the calcium pump and ATPase are functionally coupled (Javors *et al*, 1981; Ross and Cardenas, 1983; Garrett and Ross, 1985). These studies compared the biochemical and kinetic properties of the two systems using identically prepared neural tissue and identical substrate and reaction conditions, and provide the most useful data for understanding the relationship between calcium transport and ATP-hydrolysis. As yet, no studies have been reported on insect synaptosomal Ca^{++}+Mg^{++}-ATPase and the ATP-dependent calcium pump.

Ca^{++}-ATPase and Ca^{++}+Mg^{++}-ATPase of the nervous system of the lobster, *Homarus americanus* and vertebrates are sensitive to some insecticides, such as DDT, pyrethroids and some cyclodiene compounds (Doherty and Matsumura, 1975; Ghiasuddin and Matsumura, 1979a,b; 1981; Matsumura and Ghiasuddin, 1979;

Yamaguchi et al, 1979; Clark, 1981; Clark and Matsumura, 1982; Doherty et al, 1981). In addition, DDT and the pyrethroids were shown to affect ATP-dependent calcium exchange and calcium uptake by crude homogenate of crayfish nerve cord and lobster axon (Ghiasuddin and Matsumura, 1979a; Doherty et al, 1986). However, these studies did not demonstrate that the inhibition of Ca^{++}+Mg^{++}-ATPase activity would result in the disruption of its function. Furthermore, the biochemical identity of the DDT-sensitive 'Ca^{++}-ATPase' studied by Matsumura's group (Matsumura and Ghiasuddin, 1979) is open to question. The biochemical properties of the 'Ca^{++}-ATPase' have not been carefully studied. The only study to imply that DDT might attack Ca^{++}-ATPase of insects used the German cockroach, *Blattella germanica* L., as the experimental system (Ghiasuddin *et al*, 1981). The study found that brain Ca^{++}-ATPase of the DDT-resistant strains of the German cockroach *Blattella germanica* L. was less sensitive to DDT than that of susceptible strains. No studies have been reported on the effect of insecticides on insect synaptosomal Ca^{++}+Mg^{++}-ATPase. Thus, it is still not known if insecticides inhibit Ca^{++}+Mg^{++}-ATPase activity in the insect nervous system and disrupt its calcium transport function.

To study the effect of insecticides on insect neural Ca^{++}+Mg^{++}-ATPase activity and its function, two conditions are essential. First, a well-defined insect *in vitro* system, such as synaptosomes and synaptosome membrane vesicles, is needed to avoid complications caused by factors such as the variations in the degree of penetration through the cuticle and metabolic

activation or detoxification. Secondly, basic information about biochemical and kinetic properties of calcium ion transport and its regulation of the well-defined insect *in vitro* system is necessary. A dearth of basic information about calcium ion transport and its regulation in insects, as well as the lack of a well-defined in vitro system in insects until relatively recently (Donnellan et al, 1976; Breer and Jeserich, 1980; Gordon et al, 1982) have retarded progress in understanding the complex effects of insecticides on calcium homeostasis.

In this thesis I will explore the possibility that some neurotoxic insecticides act by disrupting one of the important calcium regulating mechanisms, Ca^{++}+Mg^{++}-ATPase, which is thought to be an ATP-dependent calcium pump extruding calcium from the neurone after depolarization. I first prepared morphologically pure and functionally competent synaptosomes and synaptosome membrane vesicles from the adult brains of the bertha armyworm *Mamestra configurata* WLK. (Chapter I). Secondly, I studied the biochemical properties of the Ca^{++}+Mg^{++}-ATPase and ATP-dependent calcium pump of these synaptosomes and synaptosome membrane vesicles (Chapter II). Finally, based on the results of my studies, I examined the effect of insecticides on synaptosomal Ca^{++}+Mg^{++}-ATPase activity and its function, ATP-dependent transport of calcium (Chapter III).

CHAPTER I

SYNAPTOSOME AND SYNAPTOSOME MEMBRANE VESICLE PREPARATION FROM THE MOTH BRAIN OF THE BERTHA ARMYWORM *MAMESTRA CONFIGURATA* WLK.

Introduction

Synaptosomes are pinched-off, resealed nerve endings which form during homogenization of nerve tissue. Since they display all the essential features of intact nerve endings, synaptosomes provide an invaluable system for studing neurochemical processes *in vitro*. Synaptosome preparations may be further manipulated by osmotic shock to generate synaptosome membrane vesicles. Synaptosome membrane vesicles devoid of sub-cellular organelles can be induced to form in "inside-out" or "right-side-out" configurations by using appropriate conditions. These membrane vesicles are useful for studing membrane transport phenomenon because transport can be studied in isolation from other confounding processes such as metabolism or sequestration into internal storage organelles (Gordon *et al.*, 1982).

Although synaptosomal preparations have long been a powerful and indispensible tool in neurochemical research in the vertebrate nervous system, functional synaptosomes from the nervous system of insects have not been prepared until

recently. Attempts to apply existing techniques developed for the vertebrate nervous system have been limited in number and scope (Telford and Matsumura, 1970) and have proved largely unsatisfactory. The use of Ficoll, rather than sucrose, to prepare density gradients has improved techniques for preparing synaptosomes from insects. Synaptosome-like structures have been obtained from crude fleshfly head homogenates (Donnellan *et al.*, 1976). Functional synaptosomes and membrane vesicles from osmotically shocked synaptosomes from insect nervous tissue have been prepared using a microscale floatation technique based on Ficoll (Breer and Jeserich, 1980; Gordon *et al*, 1982; Breer, 1983). However, these synaptosomal preparations were either derived from crude head homogenate instead of pure nerve tissue or were contaminated by synaptosomal mitochondria. Morphologically pure and functionally competent synaptosomal preparations derived from pure insect nerve tissue are required for the study of insect neurochemistry and the biochemical mode of action of neurotoxic insecticides.

This chapter describes the use of a modified microscale Ficoll floatation technique to obtain high yields of relatively pure, functionally competent synaptosomes and synatosome membrane vesicles from the brain of moths of *Mamestra configurata* WLK. Electron microscopy and marker enzyme assay have been used to examine the purity of the synaptic preparations. Two important neurochemical pheno-

mena: voltage-dependent and ATP-dependent calcium transport, have been studied to demonstrate the functional competence of the synaptosomal preparations.

Materials and Methods

Insects

The bertha armyworm, *Mamestra configurata* WLK., was reared in the laboratory on an artificial diet (Bucher and Bracken, 1976) at 20 ± 1 ℃, 60 ± 10% relative humidity and a 12h light:12h dark photoregime. Diapausing pupae were kept for one month at 20 ℃ and then stored at 0 ℃ for 6 to ten months to break diapause. Batches of about 200 pupae were returned from storage at 0 ℃ to 20 ℃ at regular intervals to generate adults for brain dissections.

Chemicals

Beta-NADH, NADPH and the calcium ionophore A23187 were purchased from Sigma (St. Louis, Mo.) and Ficoll was purchased from Pharmacia, Uppsala, Sweden. The $^{45}Ca^{++}$ was obtained from Amersham Corporation (Oakville, Ontario).

Preparation of Synaptosomes

Synaptosomes were prepared according to the method of Gordon *et al* (1982) with modifications as described below. Brains from 1 to 7-day-old moths were removed by dissection

in 0.25 M mannitol buffer, pH 7.4, containing 1.0 mM EDTA (buffer M). Pooled brains (typically, 150 brains, approx. 100 mg wet wt) were homogenized in 5 ml of ice-cold buffer M with 10 up and down strokes of a Potter-Elvehjem tissue grinder having a Wheaton '200' borosilicate glass tube and Teflon pestle with 0.125 mm clearance between parts. All the operations were done in an ice-bath and centrifugation was done at 0 to 4 °C. The homogenate was centrifuged for 10 minutes at 3250 rpm(1270g) in the SS34 rotor of a Sorvall RC-5B centrifuge. The supernatant was saved and the pellet resuspended by homogenizing in 5 ml buffer M and centrifuged as above. The supernatants were combined and centrifuged for 15 minutes at 15,000 rpm (27,100g). The resulting pellet (P2) was resuspended in a small volume (0.5-0.6 ml) of buffer M and 6 volumes of a 12% w/v Ficoll solution. To the resuspended P2, 6 volumes of Ficoll solution in buffer M were added to give a final Ficoll concentration of 10.3% (w/v). The suspension was thoroughly mixed in a narrow centrifuge tube (7.5 cm long X 0.9 cm i.d.) and centrifuged for 75 minutes at 10,000 rpm (12,000g). The resulting pellet (P2H) consists mainly of mitochondria and was discarded. The floating pellicle was collected and resuspended in buffer M, and centrifuged for 15 minutes at 15,000 rpm. The resulting pellet (P2L) represents the enriched synaptosome fraction.

Preparation of Synaptosome Membrane Vesicles

Synaptosome membrane vesicles were prepared from the enriched synaptosome fraction by osmotic shock. Pellet P2L was suspended in 0.5 ml of buffer M and added to 20 ml of 5 mM Tris-HCL buffer, pH 7.4, containing 1 mM EDTA. After slow stirring for 30 minutes in an ice-bath,the suspension was centrifuged for 15 minutes at 15,000 rpm. The resulting pellet was subjected to a second Ficoll fractionation as above to produce pellet P2H2 which consisted mainly of synaptosomal mitochondria and some undisrupted synaptosomes. The supernatant from the Ficoll fractionation was treated as above to produce pellet mvP2L which consisted of inside-out synaptosomal membrane vesicles.

Electron Microscopy

Fractions P2H, P2L, mvP2L and P2H2 were fixed as pellets in ice-cold primary fixative (2.5% gluteraldehyde, 0.1 M phosphate buffer, 0.1 M sucrose, and 2 mM $CaCl_2$, pH 7.2) for 1 h. The pellets were washed for 0.5 h with two changes of 0.1 M phosphate buffer containing 0.1 M sucrose (solution N), postfixed in 1% osmium tetroxide (in solution N) for 1 h in an ice-bath. After the second fixation, the pellets were washed for 1 h with several changes of solution N, dehydrated in graded series of ethanols and embedded in Spurr resin.

Ultrathin sections were cut by using Reichert OmU_2

ultramicrotome. The sections were stained in uranyl acetate for 30 minutes, washed in distilled water, and then in lead citrate for 5 minutes.

The stained sections were examined in an AE1 EM6B electron microscope or an AE1 EM801 electron microscope.

Marker Enzyme Assay

NADH- and NADPH-cytochrome C reductase (EC 1.6.99.3) were measured essentially as described by Duncan and Mackler (1966). The reaction mixture contained 0.01 M potassium phosphate buffer, pH 7.4, 1 mM KCN, 0.1 % NAD(P)H, 0.1% (w/v) cytochrome C, 5 μM rotenone plus sample. The rate of increase in A_{550} at 20 °C was measured spectrophotometrically.

Acetylcholinesterase (acetylcholine hydrolase, AChE; E.C. 3.1.1.7) was assayed basically according to the colorimetric method of Ellman *et al.* (1961). The 3.22 ml reaction mixture contained 3 ml 0.05 M potassium phosphate buffer (with 0.4 M NaCl), pH 7.4, 100 μl 0.03 mM DTNB, 20 μl 7.5×10^{-4} M AThchI, and the sample. The increase of A_{412} was measured spectrophotometrically.

Voltage-dependent Calcium Uptake into Synaptosomes

A method similar to that of Nachshen and Blaustein (1980) was employed. Synaptosomes prepared as above were suspended in a low-K^+ solution containing 145 mM NaCl, 5 mM

KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.5. The suspension was warmed for 30 min at 30 °C with gentle agitation. Aliquots (200 µl) of the warmed synaptosome suspension were added to equal volumes of either the low-K^+ solution with 0.5 µCi $^{45}Ca^{++}$ or high-K^+ solution composed of 73 mM NaCl, 77 mM KCl, 1 mM $MgCl_2$ and 10 mM glocose in 10 mM Hepes buffer, pH 7.5 with 0.5 µCi $^{45}Ca^{++}$. The entry of Ca^{++} into synaptosome was terminated by rapidly diluting the incubation media with 4.5 ml of ice-cold low-K^+ solution containing 2.0 mM $CaCl_2$. For brief (2 to 5 seconds) incubation times, the pipettings were timed with a metronome. After the entry of $^{45}Ca^{++}$ had been terminated, the diluted suspensions were filtered by suction through 0.45 micron millipore filters to collect synaptosomes. The filters were washed 3 times with 4.5 ml of cold low-K^+ solution, dried at 60 °C and placed in vials containing 10 ml of Scinti Verse II (Fisher Scientific) scintillation cocktail for radioactivity measurement in a Packard Tri-Carb 460 CD liquid scintilation counter.

ATP-dependent Calcium Uptake into Synaptosome Membrane Vesicles

Synaptosome membrane vesicles (10 µg protein) were incubated at 30 °C in 1.0 ml final volume of an assay medium containing 160 mM KCl, 30 mM imidazole buffer, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ouabain, 0.25 mM ATP, 1 µCi $^{45}Ca^{++}$

and various amounts of $CaCl_2$ to give 0 to 1.0 nM free calcium ion concentrations as computed by Perrin and Sayce (1967). Radioactive $^{45}Ca^{++}$ (1 µCi) was added to the medium 2 minutes prior to the addition of cold ATP to initiate calcium uptake. Uptake was allowed to proceed for 5 minutes and then terminated by adding 5 ml of stop solution containing 160 mM KCl, 10 mM Mg, 30 mM imidazole buffer, pH 7.4. The samples were filtered through 0.45-µM Millipore filters and the filters washed 3 times with the same stopping solution (5 ml each). The filters were dried at 60 °C and placed in vials containing 10 ml of Scinti Verse II scintillation cocktail for radioactivity measurement.

Protein Determination

Protein was measured by a protein-dye binding method (Bradford, 1976) using the Bio-Rad protein assay kit and bovine serum albumin as the standard.

Replication

Measurements of calcium uptake activity were made in duplicate for three separate experiments. Since the synaptosomal preparations vary in activity from preparation to preparation, data from representative experiments are usually given.

Results

Electron Microscopy

Electron microscopic examination of various Ficoll fractions of homogenized brains from *M. configurata* moths showed that fraction P2L consisted essentially of synaptosomes containing numerous synaptic vesicles and a few mitochondria (Fig. 1 and 2). Postsynaptic attachments were also seen. The P2L fraction also contained some synaptosome ghosts and a few free mitochondria. The P2H fraction contained almost entirely mitochondria (Fig.3). The mvP2L fraction obtained after osmotic shock of the P2L (synaptosome) fraction consisted mainly of synaptosome membrane vesicles and some unsealed membranes (Fig. 4). The vesicles were devoid of internal cellular organelles. The P2H2 fraction contained numerous twisted mitochondria resulting from osmotic shock, a few membrane vesicles and undisrupted synaptosomes (Fig. 5).

Marker Enzyme

NADPH-cytochrome C reductase was not detectable in either the enriched synaptosome fraction (P2L), the synaptosome membrane vesicle fraction (mvP2L) or the second mitochondron fraction (P2H2) but was readily detectable in the first mitochondron fraction (P2H) and the endoplasmic reticulum fraction (Table 1). The activity of

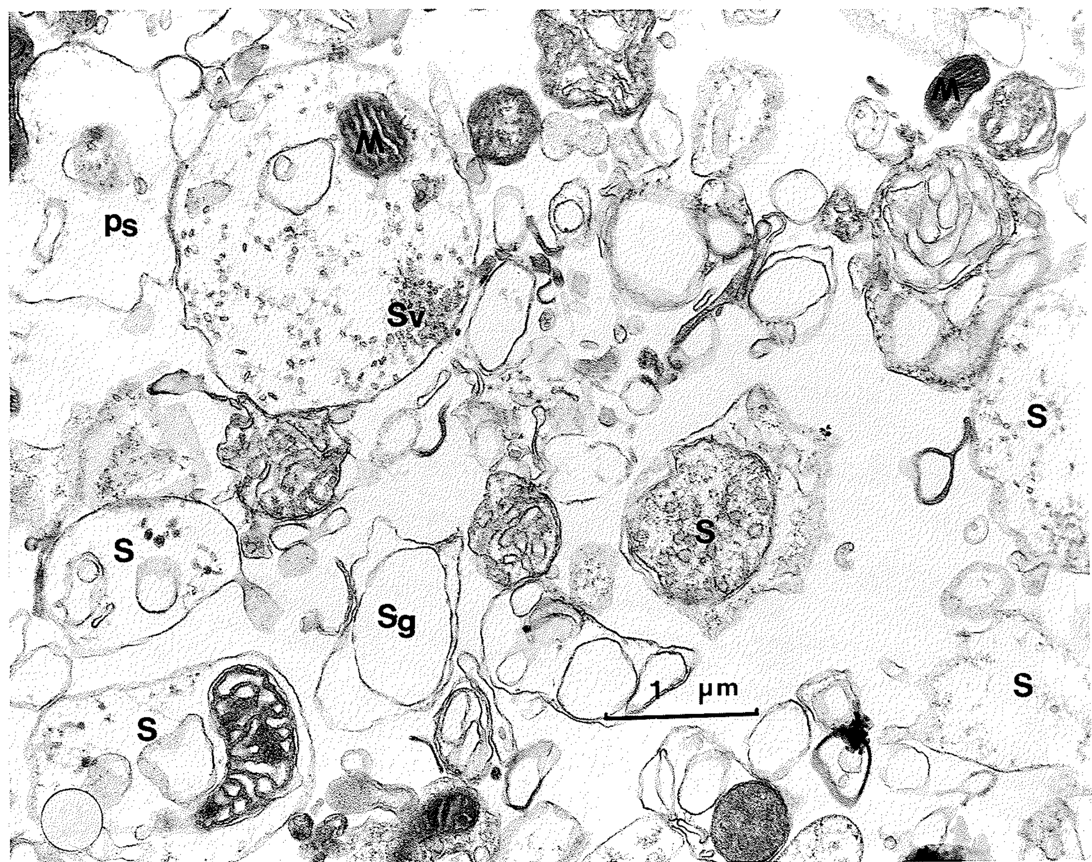

Figure 1. Electron micrograph of the enriched synaptosome fraction (P2L) of homogenized moth brains from *Mamestra configurata*.
Abbreviations: M: mitochondria; S: synaptosome; Sg: synaptosome ghost; Sv: synaptic vesicles; Ps, postsynaptic component

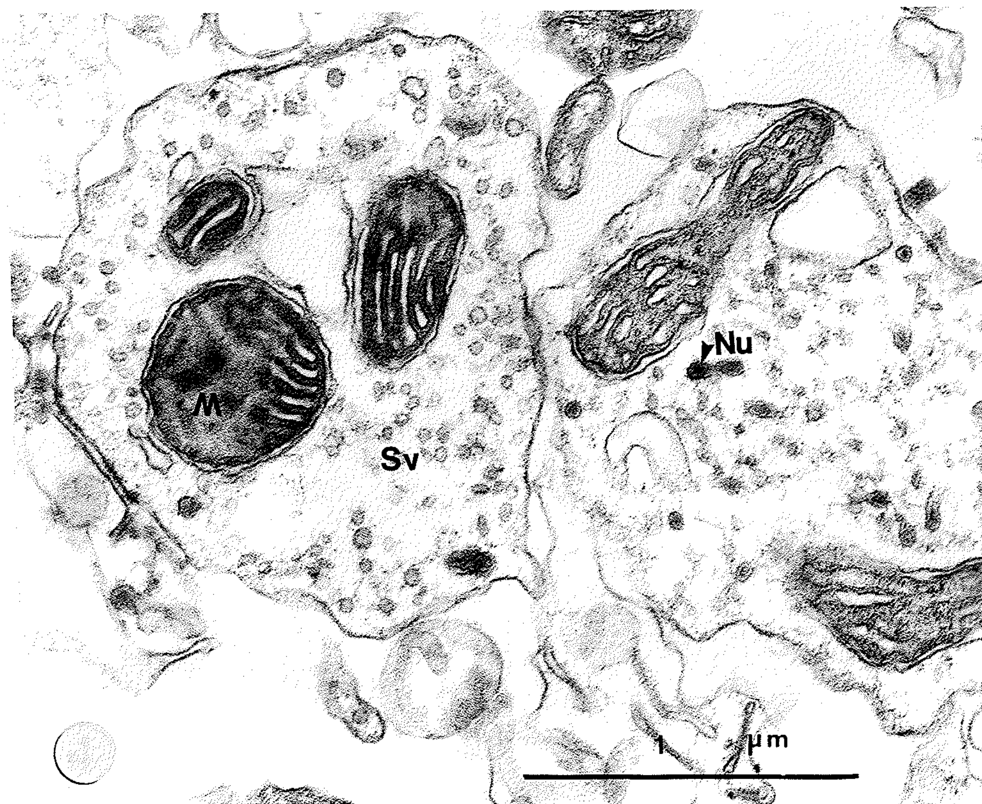

Figure 2. Electron micrograph of typical synaptosomes from the enriched synaptosome fraction (P2L).
Abbreviations: M: mitochondria; Sv: synaptic vesicles, Nu: neurosecretory granule.

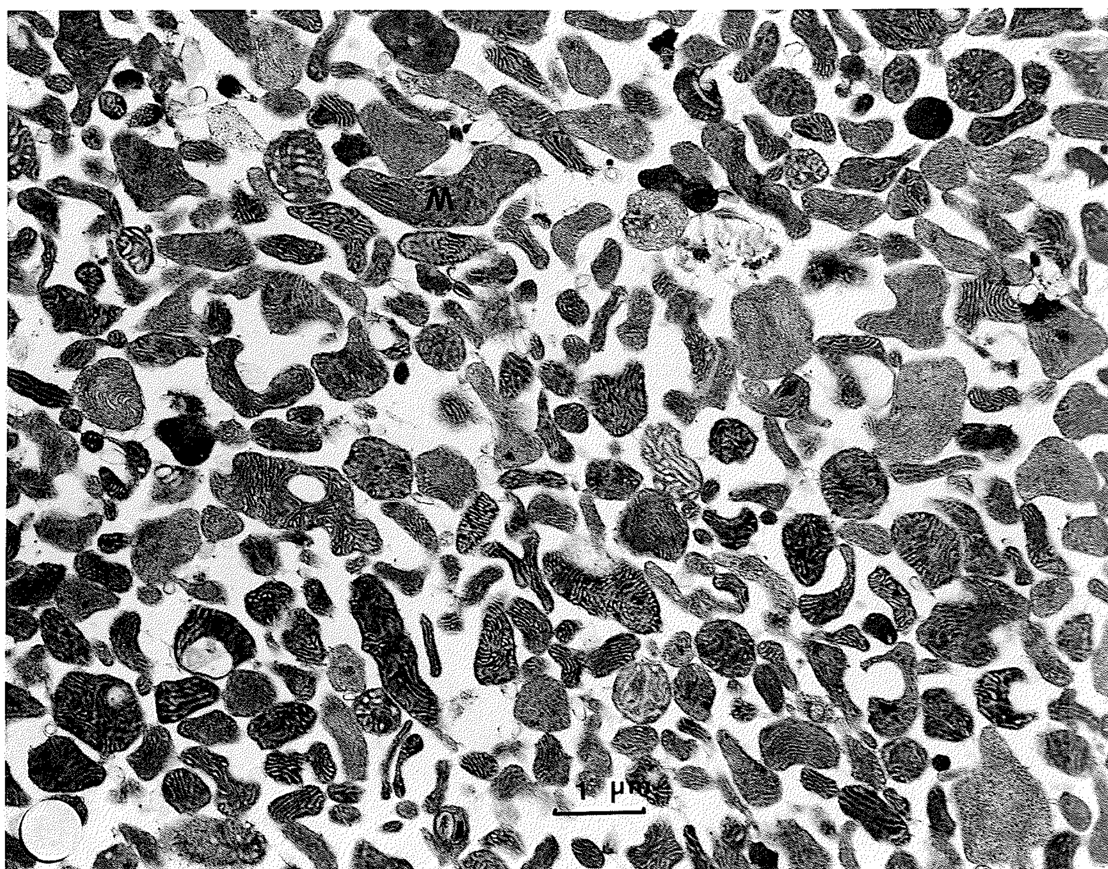

Figure 3. Electron micrograph of the first mitochondrial fraction (P2H).
Abbreviations: M: mitochondria.

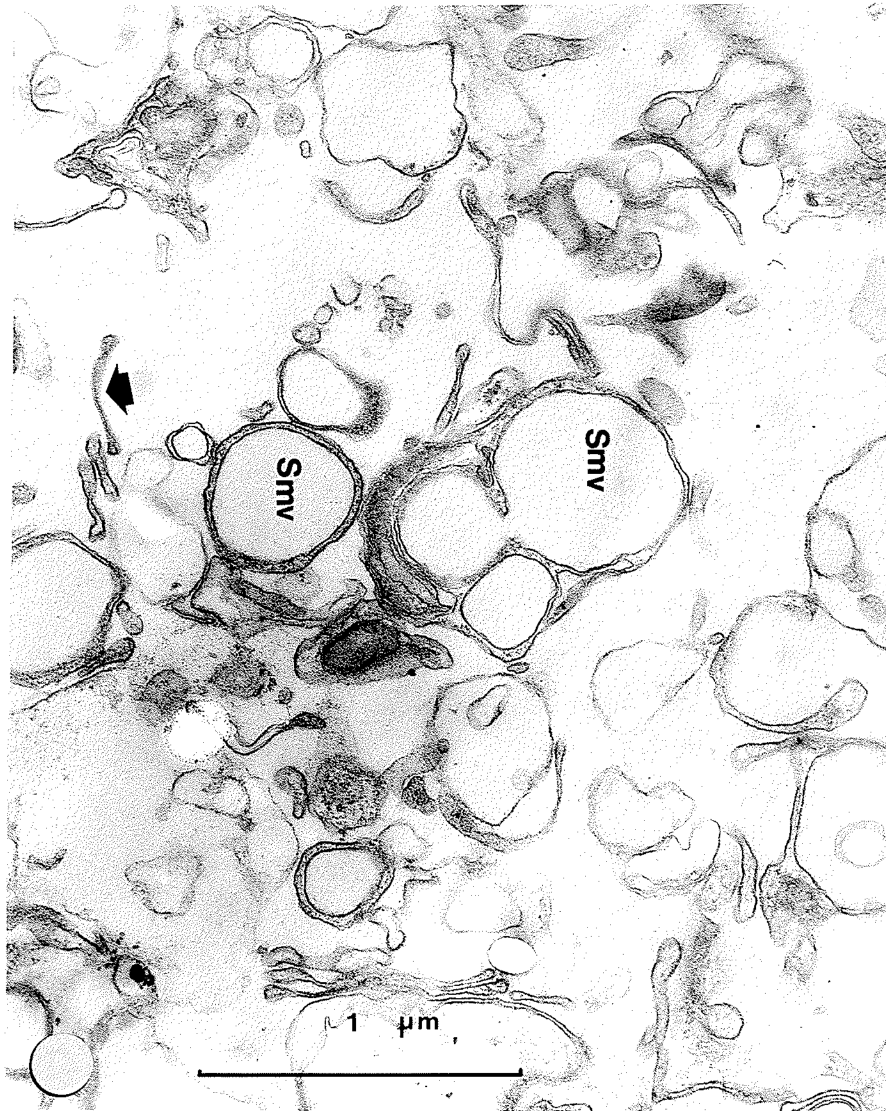

Figure 4. Electron micrograph of synaptosome membrane vesicle fraction (mvP2L). The micrograph shows membrane vesicular structures devoid of cellular organelles. There are also some unsealed membranes (arrow). Abbreviations: Smv: synaptosome membrane vesicles.

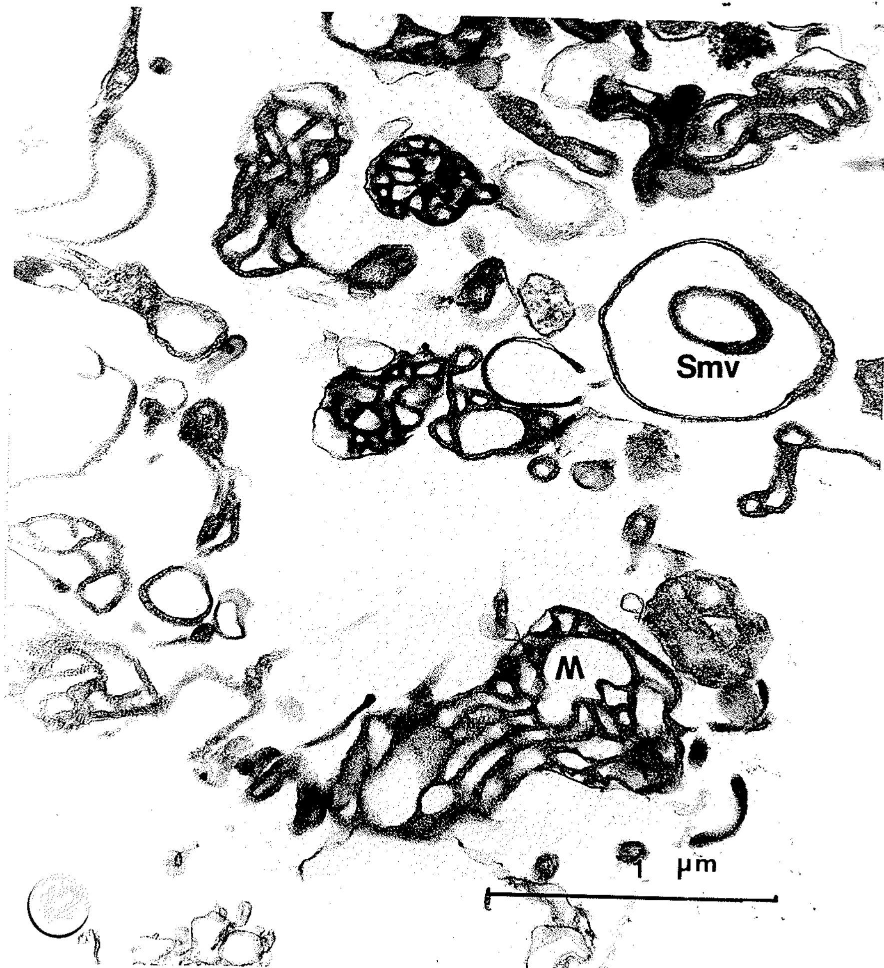

Figure 5. Electron micrograph of the second mitochondrial fraction (P2H2). The mitochondria are twisted because of osmotic shock. A few synaptosome membrane vesicles and disrupted synaptosomes can be seen in the micrograph.
Abbreviations: Smv: synaptosome membrane vesicles; M: mitochondria

TABLE 1. Distribution of three marker enzymes in various Ficoll fractions prepared from moth brains of *M. configurata* *

	NADPH-Cytochrome C Reductase (nmole/min/mg pro.)	NADH-Cytochrome C Reductase (nmole/min/mg pro.)	Acetylcholine Esterase (umole/min/mg pro.)
P2L	0	3.24 ± 1.93	121.28 ± 122.27
mvP2L	0	0	226.15 ± 143.18
P2H	13.45 ± 2.02	201.43 ± 37.48	115.87 ± 32.79
P2H2	0	89.28 ± 30.28	340.26 ± 109.16
ER	14.62 ± 1.52	5.71 ± 6.72	258.79 ± 76.33

P2L: Enriched synaptosome fraction
mvP2L: Synaptosome membrane vesicle fraction
P2H: First mitochondrial fraction
P2H2: Second mitochondrial fraction
ER: Endoplasmic reticulum fraction, the supernatant from P2
*: The results are means and standard deviation of four independent experiments.

NADH-cytochrome C reductase was very low in P2L and the endoplasmic reticulum fraction, undetectable in mvP2L but very high in P2H and P2H2 fractions. Acetylcholinesterase activity was found in all fractions.

Yields and Storage

Initial yields of synaptosomes using a continuous gradient with 10.0% Ficoll (w/v) were low. Quantities of material present in the floating pellicle derived from pellet P2 were small and the pellet P2H contained an unacceptably high content of synaptosomes (Fig. 6). This synaptosomal material, which sedimented with the mitochondria, was being lost from the preparation. Yields of synaptosomes were higher when a continuous gradient with 10.3% Ficoll (w/v) was used. The floating pellicle was visibly thicker and the pellet P2H contained little if any synaptosomal material (Fig.3).

Typical preparations from 150 moth brains (dissection time 5h) gave approximately 100 mg wet weight of brain tissue which yielded, after Ficoll fractionation, 1 mg of synaptosomal protein or 0.6 mg of synaptosome membrane vesicle protein. This was sufficient for 60 to 100 assays each of which requires 0.01 mg protein.

Synaptosomes and synaptosome membrane vesicles could be stored on ice for up to 10 days with little loss of activity. Storage in liquid nitrogen was less satisfactory. In this

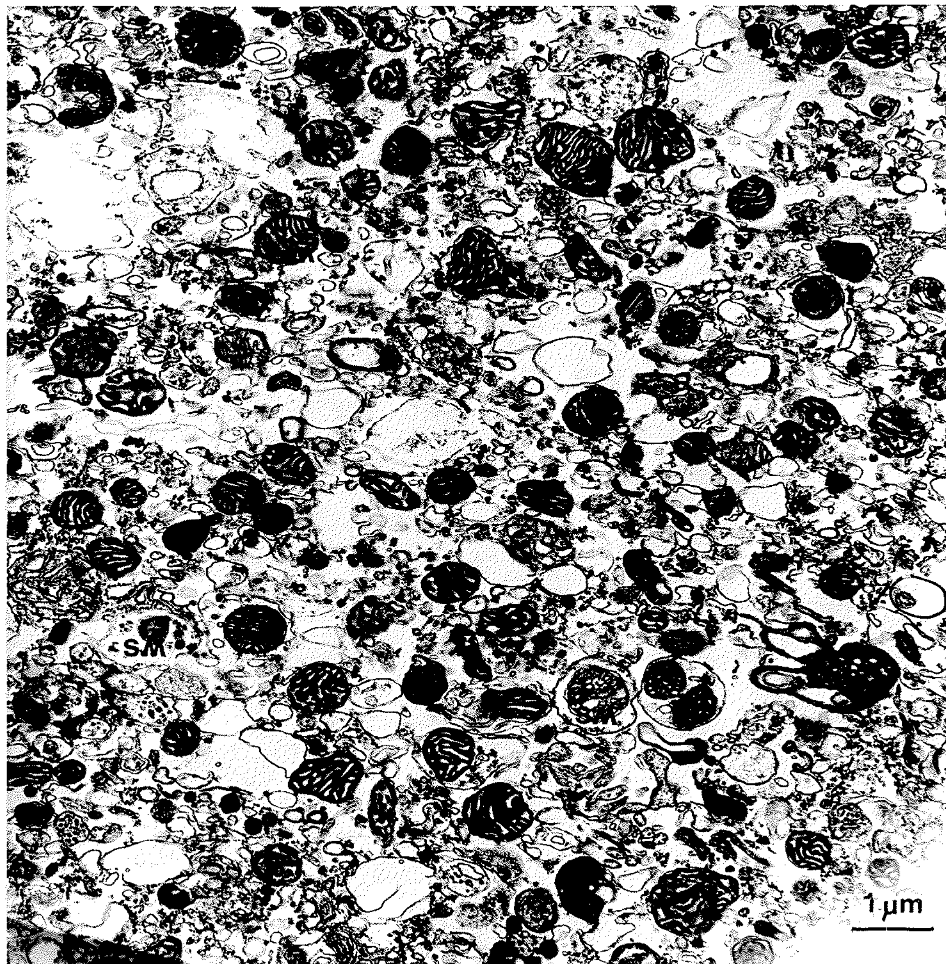

Figure 6. Electron micrograph of the first mitochondrial fraction (P2H) when using 10% Ficoll (w/v) continuous gradient to separate synaptosomes and mitochondra. A lot of synaptosomes can be seen in the fraction. Abbreviations: SM: synaptosome; M: mitochondria.

study, storage on ice was usually less than three days.

Voltage-dependent Calcium Uptake by Synaptosomes

Synaptosomes prepared from moth brains of *Mamestra configurata* when incubated in a low K^+ medium took up labelled calcium from the medium in a time-dependent manner (Fig. 7). The rate of uptake was greatly increased (up to 3-fold) when the synaptosomes were incubated in a depolarizing, high K^+ medium. Time-course studies of the net rate of uptake due to depolarization (solid line, Fig.7) indicate that the highest rate of uptake of Ca^{++} into the synaptosomes occurred within the first few seconds after depolarization. After about 10 seconds, the rate of uptake fell to low levels (Fig.7).

ATP-dependent Calcium Transport by Synaptosome Membrane Vesicles

Inside-out synaptosome membrane vesicles prepared by osmotic shock of moth brain synaptosomes, when incubated in a medium containing ATP, rapidly take up $^{45}Ca^{++}$ from the medium (Fig.8). Time course experiments showed that most of the uptake occurred within the first few minutes of incubation (Fig.8). It is possible that back fluxes of Ca^{++} occur during prolonged incubation of the vesicles, giving rise to erratic patterns of calcium uptake apparent after 15 minutes of incubation in Fig.8.

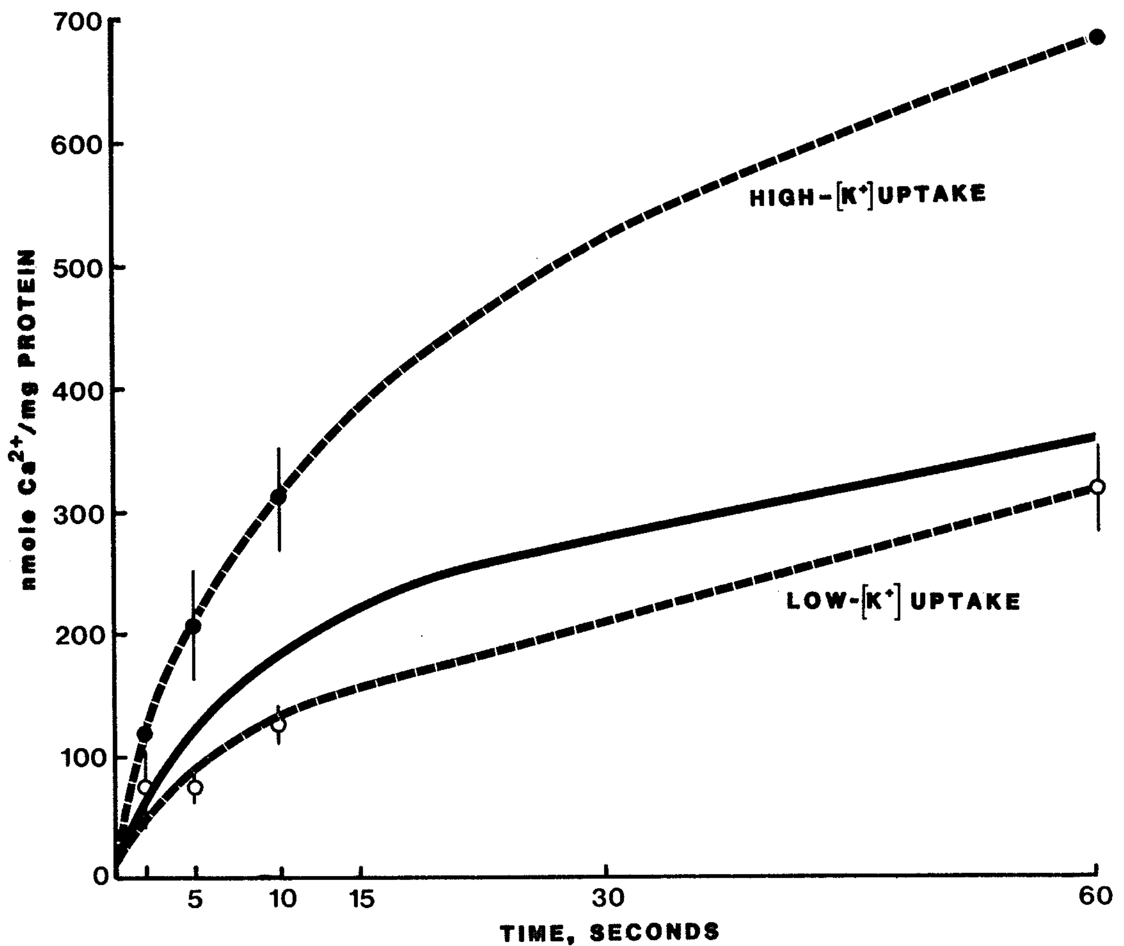

Figure 7. Voltage-dependent calcium uptake by brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK. The solid line is the difference between high-K^+-Ca^{++}-uptake (close circles) and Low-K^+-Ca^{++}-uptake (open circles). Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

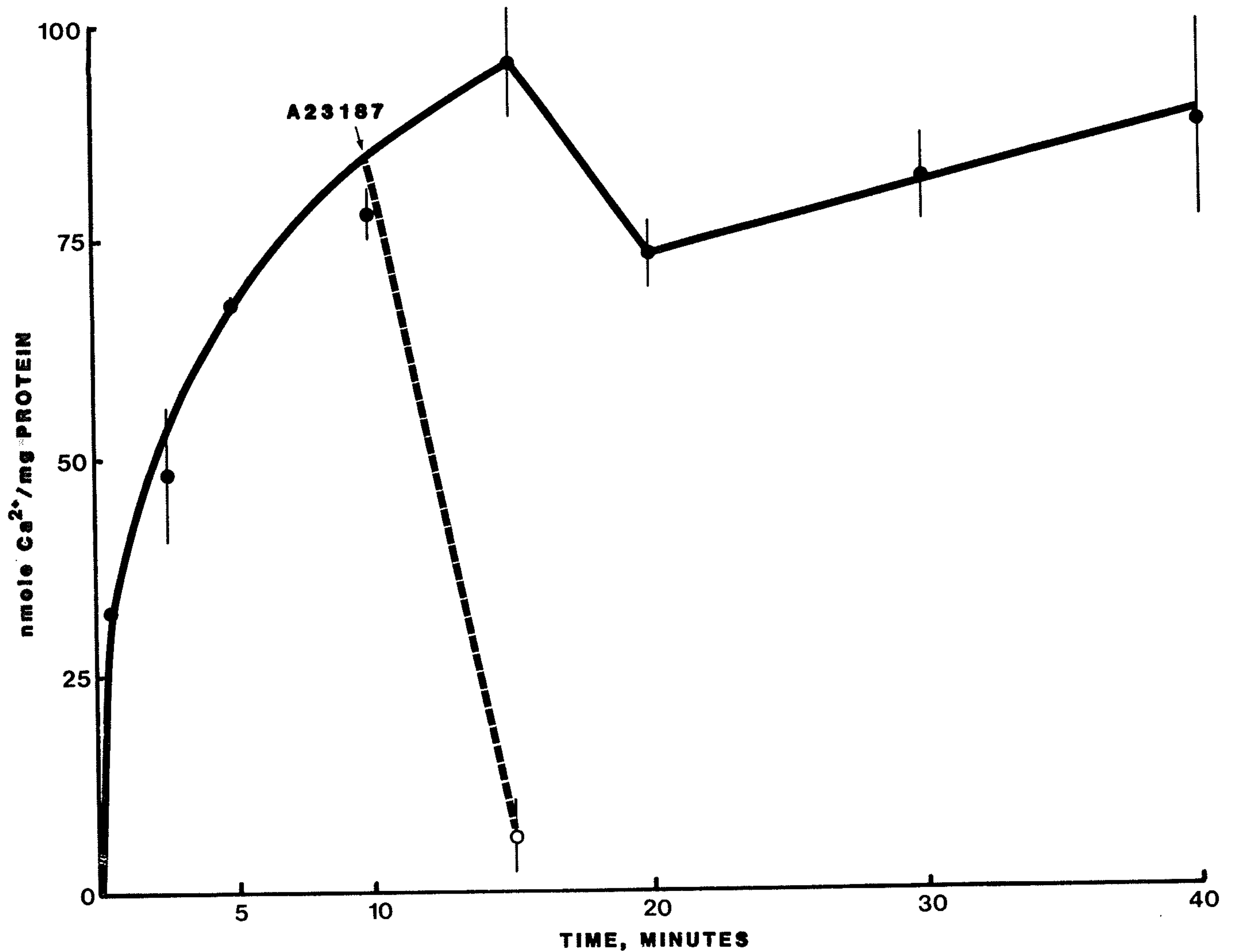

Figure 8. The time course of ATP-dependent calcium uptake by synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK. The arrow marks the addition of the calcium ionophore A23187 to the incubation medium. The solid line indicates the time course of ATP-dependent calcium uptake and the dotted line indicates the ATP-dependent calcium uptake after the addition of A23187. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

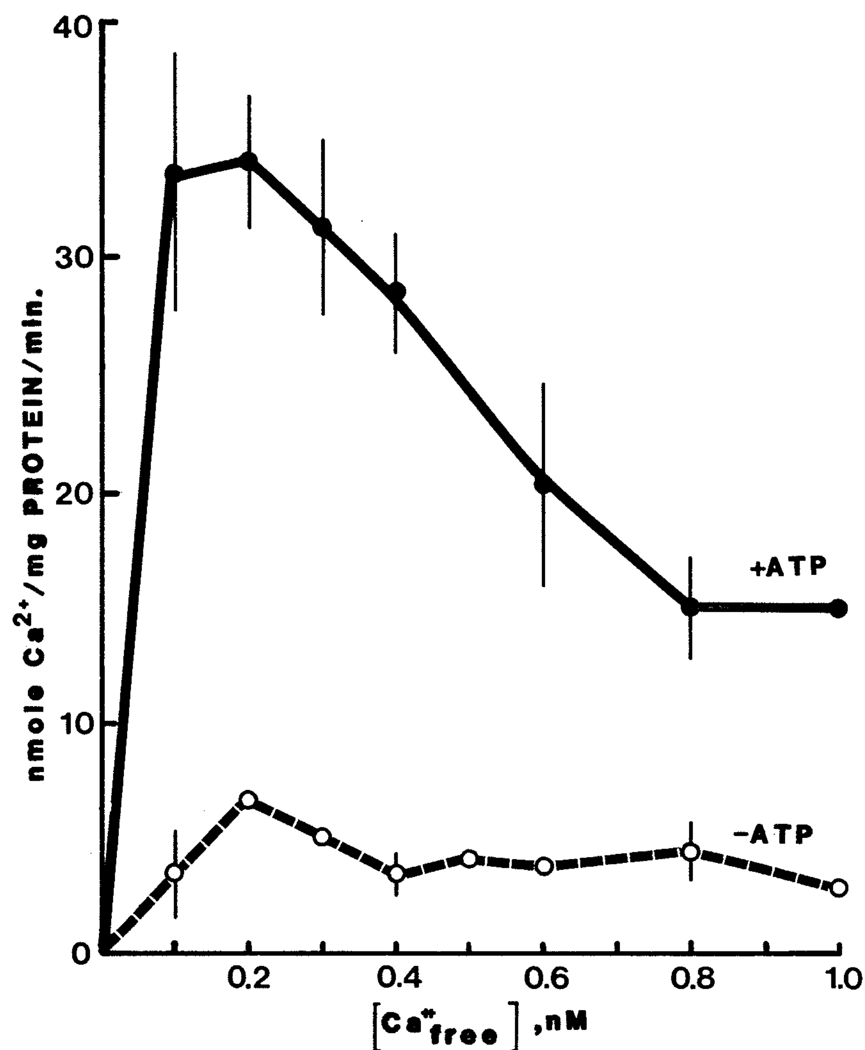

Figure 9. Effect of free calcium concentration and ATP on ATP-dependent calcium uptake by synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK. The solid line represents calcium uptake in the presence of ATP and the dotted line represents calcium uptake without ATP. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

Addition of the Calcium ionphore A23187 to the incubation medium after 10 minutes of incubation released most of the radioactivity of $^{45}Ca^{++}$ that had been taken up by the vesicles (Fig.8, arrow).

Uptake of calcium into synaptosome membrane vesicles was strongly dependent on ATP. When ATP was omitted from the incubation medium, the uptake of calcium into the vesicle was five to ten times lower than in the presence of 0.25 mM ATP (Fig.9).

Uptake of calcium into synaptosome membrane vesicles was also influenced by the concentration of free calcium in the medium. In the experiments shown in Fig.9, the concentration of free calcium in the incubation medium was controlled by a Ca^{++}-EGTA buffer system and computed by an iterative method (Perrin and Sayce, 1967). Maximal uptake of Ca^{++} into synaptosome membrane vesicles occurred when the free calcium concentration was 0.1 to 0.2 nM (Fig.9).

Discussion

This research demonstrates that high yields of relatively pure, morphologically well preserved, functionally competent synaptosomes and synaptosome membrane vesicles can be prepared from brains of moths of *Mamestra configurata* using a modification of the Ficoll floatation technique of Gordon *et al* (1982). Modifications to the Ficoll floatation

technique improved the purity and yields of synaptosomes and synaptosome membrane vesicles from the brains of *Mamestra configurata* moths in two ways. First, by increasing the Ficoll density from 10% to 10.3%, it was possible to obtain an improved separation of mitochondria and synaptosomes. The density of insect synaptosomes apparently varies from species to species, thus requiring adjustment of the gradient medium to obtain optimal separation between mitochondria and synaptosomes (Breer, 1981). Secondly, the addition of a Ficoll centrifugation step after osmotic shock of the synaptosome fraction further separated the synaptosomal mitochondria and other sub-cellular organelles from the synaptosome membrane vesicles. This additional purification step, which is usually not part of established protocols for preparing synaptosome membrane vesicles from neural tissues of other insects (Breer, 1981), should be included in the preparation of pure synaptosome membrane vesicles otherwise mitochondria and other sub-cellular organelles released from synaptosomes during osmotic shock contaminate the preparation.

Typical preparations from *Mamestra configurata* yielded 10 mg of synaptosomal protein or 6 mg of synaptosome membrane vesicle protein per gram of moth brains. This compares well with the best yields of synaptosomes from mammalian brain, which vary from 11 mg of synaptosomal protein per gram of cerebellum to 36 mg protein per gram of hypothalamus (Loscher *et al.*, 1985).

The moth brain synaptosomes and synaptosome membrane vesicles are virtually free of endoplasmic reticulum and mitochondrion contaminants as judged from marker enzyme studies. The activity of NADPH-cytochrome C reductase, an enzyme specific to the endoplasmic reticulum (Gurd *et al.*, 1974), is undetectable both in the enriched synaptosome fraction and in the synaptosome membrane vesicle fraction. The activity of NADH-cytochrome C reductase, an enzyme located in the outer mitochondrion membrane and the endoplasmic reticulum (Beattie, 1968; Gurd *et al.*, 1974) is low in the enriched synaptosome fraction and undetectable in the synaptosome membrane vesicle fraction. These results, taken together with the results of electron microscope studies of the various Ficoll fractions, leave little doubt that the moth brain synaptosomes and synaptosome membrane vesicles are relatively pure. The electron micrographs also show that the enriched synaptosome fraction consists of morphologically well-preserved synaptosomes and that the synaptic membrane vesicles derived from the synaptosomes by osmotic shock are free of intracellular organelles and synaptosomal mitochondrial contamination.

Moth brain synaptic preparations produced by this modified Ficoll floatation technique are neurochemically active and functionally competent. This is the first study of two aspects of calcium ion transport in insect brain using synaptic preparations, namely the inward movement of calcium

ions into presynaptic terminals through voltage-sensitive channels and the extrusion of calcium ions from the neurone by an ATP-dependent calcium transport mechanism. These processes are an integral part of synaptic transmission, since the release of neurotransmitters is dependent on the entry of calcium through voltage-sensitive channels (Kaze and Miledi, 1967; 1969; 1970; Llinas and Nicholson, 1975). Extrusion of calcium from the neurone after the action potential is also essential to maintaining low intracellular levels of this highly cytotoxic ion.

The inward movement of calcium ions into moth brain synaptosomes after chemically-induced depolarization by high K^+ concentration has an early (<2 sec) fast phase and a late (>10 sec) slow phase in which the rate of inward movement is only a small fraction of the rate of the fast phase. The intervening interval (>2<10 seconds) may consist of a mixture of fast-phase and slow-phase processes. These phases appear to correspond to the phases of uptake of calcium ions into rat brain synaptosomes (Nachshen and Blaustein, 1980; Gripenberg *et al.*, 1980; Leslie *et al.*, 1983). Little is known about the relationship of these phases to the physiology of nerve transmission other than that the fast phase encompasses the whole process of calcium entry and neurotransmitter release, a process which may occur in only a few hundred microseconds *in vivo*(Parsegian, 1977). Inspite of inherent problems of accurate measurement of such short

time intervals, which can only be partially resolved using a metronome and precise manual technique, it should be possible using brain synaptosomes to obtain much useful information on the effects of neurochemically active substances, including insecticides, on voltage-sensitive calcium channels in the insect central nervous system.

The function of ATP-dependent calcium transport systems is to extrude calcium ions from the cell against a steep concentration gradient at the expense of metabolic energy. ATP-dependent calcium transport can be studied using synaptosome membrane vesicles because a certain proportion (undetermined in this study of moth brain) of the vesicles form in an inside-out configuration when lysed in the osmotic shock solution in the absence of magnesium. The Ca^{++}-ATPase reponsible for transport (Dipolo, 1977; 1978; Dipolo and Beauge, 1979; Coutinho *et al.*, 1983) thus faces the incubation medium and calcium transport can be assayed as inward movement of calcium ions in response to various incubation conditions (Gill *et al.*, 1981). The movement of calcium ions into the interior of the vesicle, as opposed to binding to the surfaces of membranes can be tested readily using a calcium ionophore. Moth brain synaptosome membrane vesicles, after incubation in a medium containing ATP and radioactive $^{45}Ca^{++}$, rapidly lost their accumulated radioactivity when the calcium ionophore A23187 was added to the medium, leaving little doubt that the $^{45}Ca^{++}$ had been

accumulated in the interior of the vesicles.

A salient finding of the study of ATP-dependent calcium transport in moth brain synaptosome membrane vesicles is that maximal calcium transport occurs in the presence of extremely low concentrations of free calcium, indicating that moth brain $Ca^{++}+Mg^{++}$-ATPase has a very high affinity for calcium ions compared with that from mammals. Synaptosome membrane vesicles from mammals have similar rates of transport of calcium but at much higher concentrations of free calcium. For example, pituitary plasma membrane vesicles from rat anterior pituitary cells take up Ca^{++} at a maximum velocity of 3.4 nmoles of Ca^{++}/min/mg protein and display an apparent Km of 0.7 µM for free calcium. Various estimates of the Km for rat brain synaptosomal Ca^{++}-ATPase vary from 0.06 to 0.26 µM (Lin and Way, 1982; 1984). By comparison, moth synaptosome membrane vesicles take up Ca^{++} at a maximal velocity of 10.8 nmole/min/mg protein but at a free calcium concentration that is nearly a thousand times lower (0.1 to 0.2 nM). It is generally assumed that the ATPase responsible for calcium transport should be activated by a calcium concentration approximating its intracellular level, which in mammals is thought to be about 0.1 µM or lower (Kretsinger, 1979). Evidently, calcium transport processes in the brain of *Mamestra configurata* are maximally activated by much lower concentrations of free calcium which might suggest lower free intracellular calcium concentration in insect synaptosomes.

It is therefore of interest to determine whether moth brain synaptosomal Ca^{++}+Mg^{++}-ATPase is also maximally activated by such low levels of free calcium.

CHAPTER II

PROPERTIES OF A $CA^{++}+MG^{++}$-DEPENDENT ATPASE AND AN ATP-DEPENDENT CA^{++}-PUMP OF SYNAPTOSOMES AND SYNAPTOSOME MEMBRANE VESICLES FROM THE MOTH BRAIN OF THE BERTHA ARMYWORM *MAMESTRA CONFIGURATA* WLK

Introduction

The intracellular concentration of calcium in neurones and other cells is maintained at low levels (0.1 µM or less) by the extrusion of calcium to the exterior of the cell against steep concentration gradients. The outward-directed, ATP-dependent calcium transport process, which may maintain a 100 to 10,000-fold concentration gradient between the cytosol and extracellular space, is thought to be mediated by Na^{+}/Ca^{++} exchange and by a high affinity calcium-stimulated magnesium-dependent ATPase located in the cell membrane (Penniston, 1983; Reichardt and Kelly, 1983). Several independent studies have shown that the kinetic properties of the ATP-dependent calcium pump and $Ca^{++}+Mg^{++}$-ATPase in mamalian brain synaptosome preparations are similar, indicating a molecular relationship between the calcium pump and ATPase activity (Penniston, 1983). Many of these studies, however, have measured either ATP-dependent uptake of Ca^{++} into inside-out synaptosome membrane vesicles or the activity of $Ca^{++}+Mg^{++}$-ATPase in synaptosomes or in synaptosome membrane vesicles but not both. Comparisons of the biochemical and

kinetic properties of the two systems using identically prepared neural tissue and identical substrate and reaction conditions provide the most useful data for understanding the relationship between calcium transport and ATP-hydrolysis, but unfortunately such comparisons have been rare (Javors *et al*, 1981; Ross and Cardenas, 1983; Garrett and Ross, 1985).

The maintenance of calcium ion homeostasis in the insect neural system is of special interest to insect biochemistry and toxicology because it has been speculated that disruption of the intracellular regulation of calcium may contribute in some as yet unexplained manner to the death of insects poisoned with certain neurotoxic insecticides (Doherty, 1984). However, progress in understanding such complex interactions has long been retarded by the lack of a well-defined *in vitro* system, such as synaptosomes and synaptosome membrane vesicles, to carry out such studies and a dearth of basic information on calcium ion transport and its regulation in insects.

In Chapter I I described the preparation of morphologically pure and functionally competent synaptosomes and synaptosome membrane vesicles from the moth brain of the bertha armyworm *Mamestra configurata* WLK. I also identified an ATP-dependent calcium ion transport system in inside-out synaptosome membrane vesicles. The calcium transport system took up Ca^{++} from the incubation medium into the interior of the vesicles, was strictly dependent on ATP and had a very

high affinity for free calcium. In this Chapter, I will describe work leading to an understanding biochemical properties of calcium transport in the central nervous system of insects. I examined the biochemical and kinetic properties of a high affinity Ca^{++}-stimulated Mg^{++}-dependent ATPase of moth brain synaptosomes, which is considered to be an ATP-dependent calcium pump. Then, I explored the biochemical and kinetic properties of the ATP-dependent calcium pump and the Ca^{++}+Mg^{++}-ATPase in moth brain synaptosome membrane vesicles under identical substrate and reaction conditions in order to elucidate the molecular relationship between the calcium pump and Ca^{++}+Mg^{++}-ATPase.

Materials and Methods

Insects

Bertha armyworm, *Mamestra configurata* WLK. was reared in the laboratory on an artificial diet (Bucher and Bracken, 1976). The conditions for rearing and generating adults for brain dissections were the same as described in Chapter I.

Preparation of Synaptosomes

Synaptosomes were routinely prepared from brains of one- to seven-day-old moths by a Ficoll floatation technique (Gordon *et al*, 1982) modified as described in chapter I.

Preparation of Synaptosome Membrane Vesicles

Synaptosome membrane vesicles were obtained from adult brain synaptosomes by a Ficoll floatation technique (Breer and Jeserich, 1980; Gordon *et al*, 1982) modified to include a second Ficoll fractionation step (Luo and Bodnaryk, 1987). The preparation, yield, purity and functional status of the synaptosome membrane vesicles were essentially the same as described in Chapter I.

Ca^{++}+Mg^{++}-ATPase Assay

The activity of Ca^{++}+Mg^{++}-ATPase was measured by the formation of Pi from ATP, using a modified Fiske and Subbarow (1925) procedure. Synaptosomes or synaptosome membrane vesicles (10 uM protein) were preincubated at 30 °C for 30 minutes in 1.0 ml final volume of an assay medium containing 160 mM KCl, 30 mM imidazole buffer, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ouabain, 0.25 mM ATP, and various amounts of $CaCl_2$ to give 0.1 nM to 0.1 mM free calcium concentrations as computed by Perrin and Sayce (1967). The reaction mixture excluding added calcium contained less than 0.06 µM total calcium as measured by atomic absorption spectroscopy. Concentrations of ATP were 250 µM for most of the assays. The reaction was initiated by the addition of ATP, and was allowed to proceed at 30 °C for 2h. The ATPase activity was linear with the amount of added protein in a range of 1 to 25 µg and with

time for at least two hours. The reaction was terminated by rapidly cooling the assay tubes to 0 °C and adding 100 µl 35% perchloric acid. After 20 minutes in an ice-bath, 2 ml of ice-cold distilled water was added and samples were centrifuged at 0 to 4 °C at 4,000 rpm for 15 minutes to remove precipitated protein. The supernatant was transfered to test tubes each containing 357 µl of 2.5% ammonium molybdate in 3 N sulfuric acid at room temperature and mixed thoroughly. To each sample 143 µl of 10% Fiske and Subbarow reducer was added. The absorbance at 700 nm was recorded after 20 minutes. Two replicate determinations of at least two separate experiments were performed.

ATP-dependent Calcium Uptake

The assay of ATP-dependent calcium uptake by synaptosome membrane vesicles was essentially the same as described in Chapter I.

Protein Determination

Protein was measured by a protein-dye binding method (Bradford, 1976) as described in Chapter I.

Replication

Measurements of calcium uptake and Ca^{++}+Mg^{++}-ATPase activity were made in duplicate for each of three separate experiments. Since synaptosome membrane vesicle preparations

vary in activity from preparation to preparation, data from representative experiments are usually given.

Results

ATP Concentration and Ca^{++}+Mg^{++}-ATPase Activity of Synaptosomes

Moth brain synaptosome Ca^{++}+Mg^{++}-ATPase activity was strictly dependent on ATP (Fig. 10). The Km for ATP of the enzyme, derived from the double reciprocal plot (Fig. 10, insert), was about 122 µM ATP and the Vmax of the reaction was about 9.63 µM Pi/mg protein/hour.

Free Calcium Concentration and Ca^{++}+Mg^{++}-ATPase Activity of Synaptososmes

A Ca^{++}-EGTA buffering system was employed to examine the activity of Ca^{++}+Mg^{++}-ATPase over a wide range of free calcium concentrations from 10^{-10} to 10^{-4} M. The activity of the enzyme was highest at 10^{-10} M free calcium, fell by 50% to a plateau between 10^{-8} and 10^{-6} M free calcium and decreased further at higher concentrations of free calcium (Fig. 11). A double reciprocal plot of reaction velocity versus free calcium concentration revealed an unusual substrate inhibition pattern (Fig. 11, insert).

Since the activity of Ca^{++}+Mg^{++}-ATPase was highest at sub-micromolar concentrations of free calcium, additional

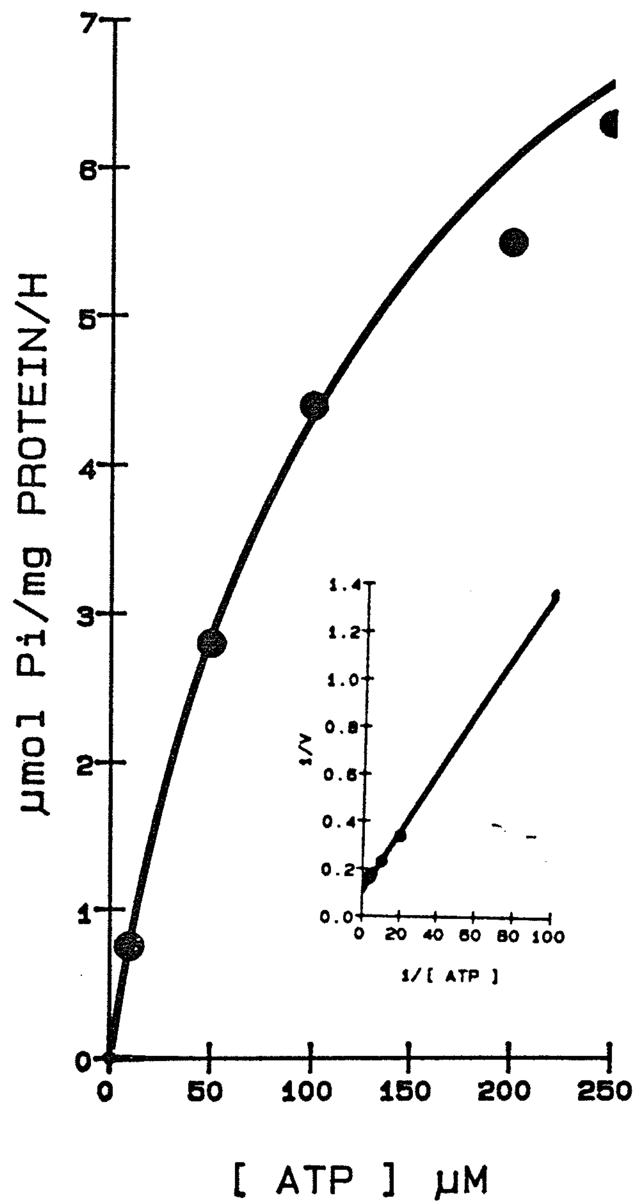

Figure 10. Effect of ATP concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosomes. Insert: Double reciprocal analysis. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

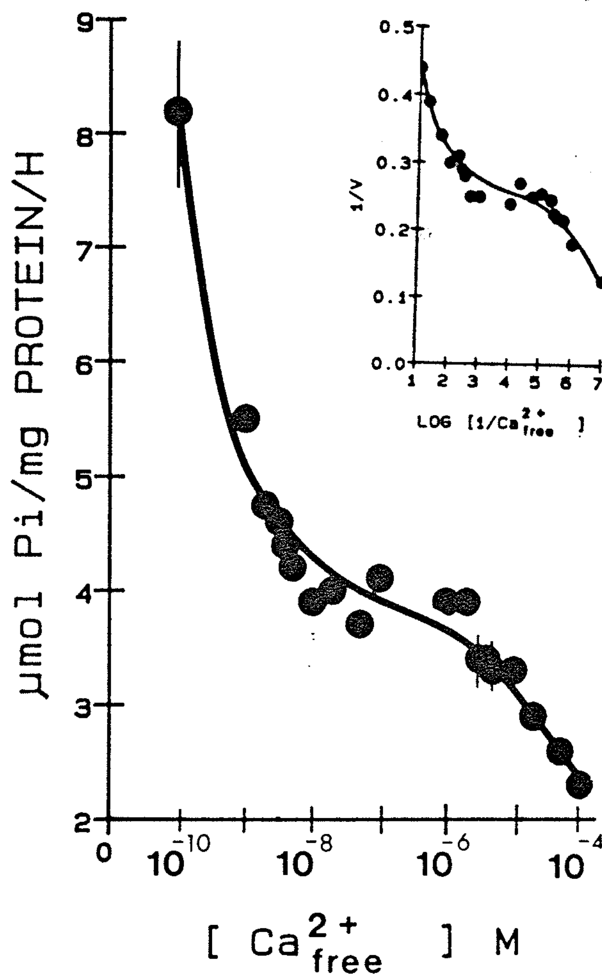

Figure 11. Effect of free calcium concentration (10^{-10}-10^{-4} M) on Ca^{++}+Mg^{++}-ATPase activity of synaptosomes. Insert: Double reciprocal analysis. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

measurements of enzyme activity were made in the range of 0 to 1.0 nM free calcium. In this range, the enzyme displayed a biphasic pattern of activity. Free calcium up to 0.2 nM stimulated activity but higher concentrations of free calcium inhibited activity (Fig. 12). Double reciprocal plot analysis revealed a typical substrate inhibition curve with an approximate Km=0.11 nM for free calcium and an approximate Vmax=10.3 μM Pi/h/mg protein (Fig. 12, insert).

EGTA Concentration and Ca^{++}+Mg^{++}-ATPase Activity of Synaptosomes

The effect of the EGTA component of the Ca^{++}-EGTA buffering system on enzyme activity was determined by keeping the concentration of free calcium constant at 0.3 nM and varying the concentration of EGTA from 10 to 250 μM. Concentrations of EGTA up to 50 μM had little effect on the activity of the enzyme. Higher concentrations gradually inhibited the enzyme (Fig. 13). A concentration of 100 μM EGTA provided an adequate level of Ca^{++} buffering without having much effect on enzyme activity and was therefore used in routine measurements.

Magnesium Concentration and Ca^{++}+Mg^{++}-ATPase Activity of Synaptosomes

The effect of magnesium (as total magnesium ion) on Ca^{++}+Mg^{++}-ATPase activity was investigated over a range from

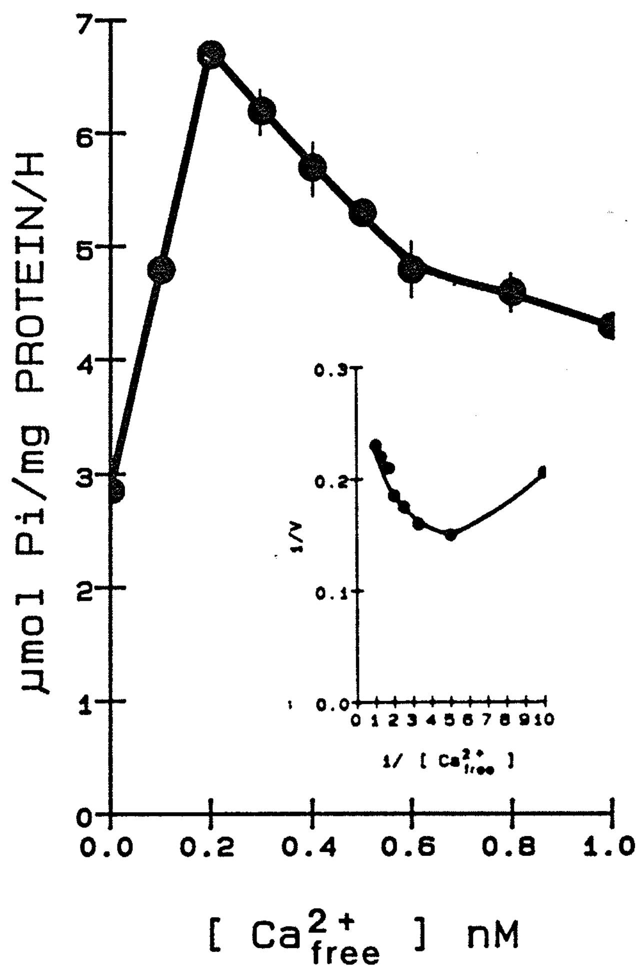

Figure 12. Effect of free calcium concentration (sub-nanomolar) on Ca^{++}+Mg^{++}-ATPase activity of synaptosomes. Insert: Double reciprocal analysis. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

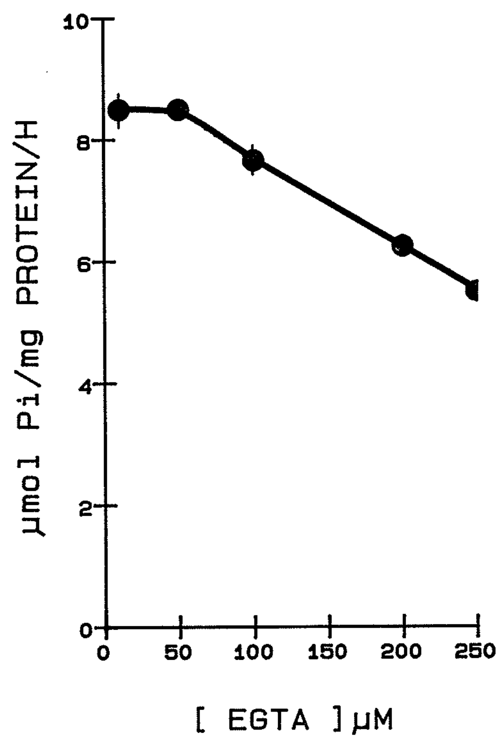

Figure 13. Effect of EGTA concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosomes. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

0.1 to 20 mM while keeping the free calcium constant at 0.3 nM. The enzyme displayed a biphasic response to Mg^{++}. Concentrations of magnesium up to 2 mM stimulated the enzyme activity while higher concentrations inhibited activity (Fig. 14). Double reciprocal plot analysis revealed a substrate inhibition type curve (Fig. 14, insert). It appeared that the Ca^{++}+Mg^{++}-ATPase has two binding sites for Mg^{++}, one with a high affinity for Mg^{++} with Km=85 µM, Vmax=3.90 µM Pi/mg protein/h and the other with a lower affinity for Mg^{++} with an approx. Km=1 mM and approx. Vmax=10.29 µM Pi/mg protein/h.

Potassium and Ouabain and Ca^{++}+Mg^{++}-ATPase Activity of Synaptosomes

Ca^{++}+Mg^{++}-ATPase activity was stimulated by increasing concentrations of K^+ in reaction mixtures. Maximum activity (30% above controls having no added K^+) occurred at about 40 mM K^+. Activity returned to control levels at higher concentrations (320 mM) of K^+ (Fig. 15).

Ouabain from 0 to 0.25 mM had little effect on the Ca^{++}+Mg^{++}-ATPase activity (Fig. 16) but was routinely added to the reaction mixture at 0.1 mM as a precautionary measure to inhibit Na^++K^+-ATPase.

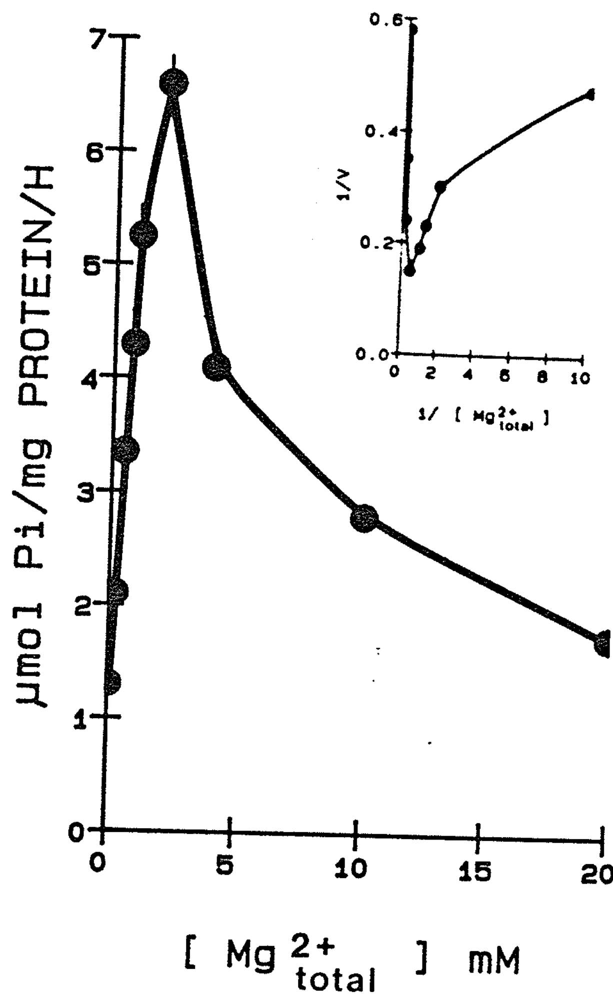

Figure 14. Effect of Mg++ concentration on Ca++ +Mg++-ATPase activity of synaptosomes. Insert: Double reciprocal analysis. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

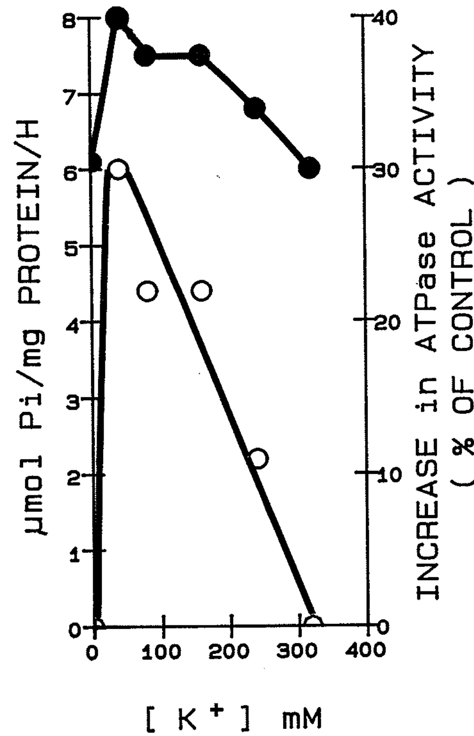

Figure 15. Effect of potassium concentration on $Ca^{++}+Mg^{++}$-ATPase of synaptosomes. The open circles represent the change in ATPase activity and the close circles represent $Ca^{++}+Mg^{++}$-ATPase activity. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

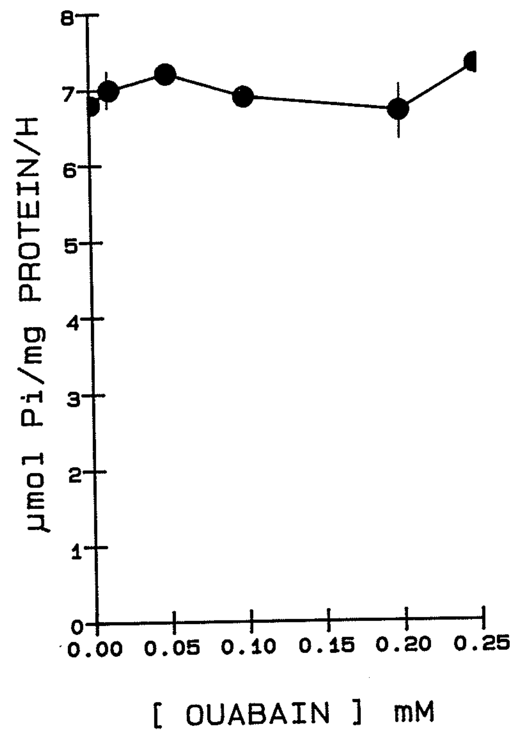

Figure 16. Effect of ouabain concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosomes. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

Effects of Protein Concentration on ATP-dependent Calcium Uptake and Ca^{++}-dependent ATP Hydrolysis by Synaptosome Membrane Vesicles

ATP-dependent calcium uptake and Ca^{++}+Mg^{++}-ATPase activity were linear with amount of added synaptosome membrane vesicle protein in the range of 1 to 25 µg protein per ml of incubation medium (Fig. 17A,B). A protein concentration of 10 µg/ml of incubation medium was used for standard assay conditions.

The Time Courses of ATP-dependent Calcium Uptake and Ca^{++}-dependent ATP Hydrolysis by Synaptosome Membrane Vesicles

Synaptosome membrane vesicles rapidly took up calcium from the incubation medium during the first few minutes of incubation. Uptake was linear for incubation times of <1 min (Fig. 18A). Thereafter the rate of uptake levelled off, and after about 15 minutes became erratic, possibly due to back fluxes of calcium. The data for calcium uptake during the first 15 minutes of incubation were fitted to a power curve ($y=58.01x^{0.286}$., $R^2=0.996$, Fig. 18A) and first derivatives ($y = abx^{b-1}$) were computed at various times of incubation to give instaneous uptake rates. At 1.0 minute, the rate of uptake was 16.6 nmol Ca^{++}/mg protein/min but at 10 minutes

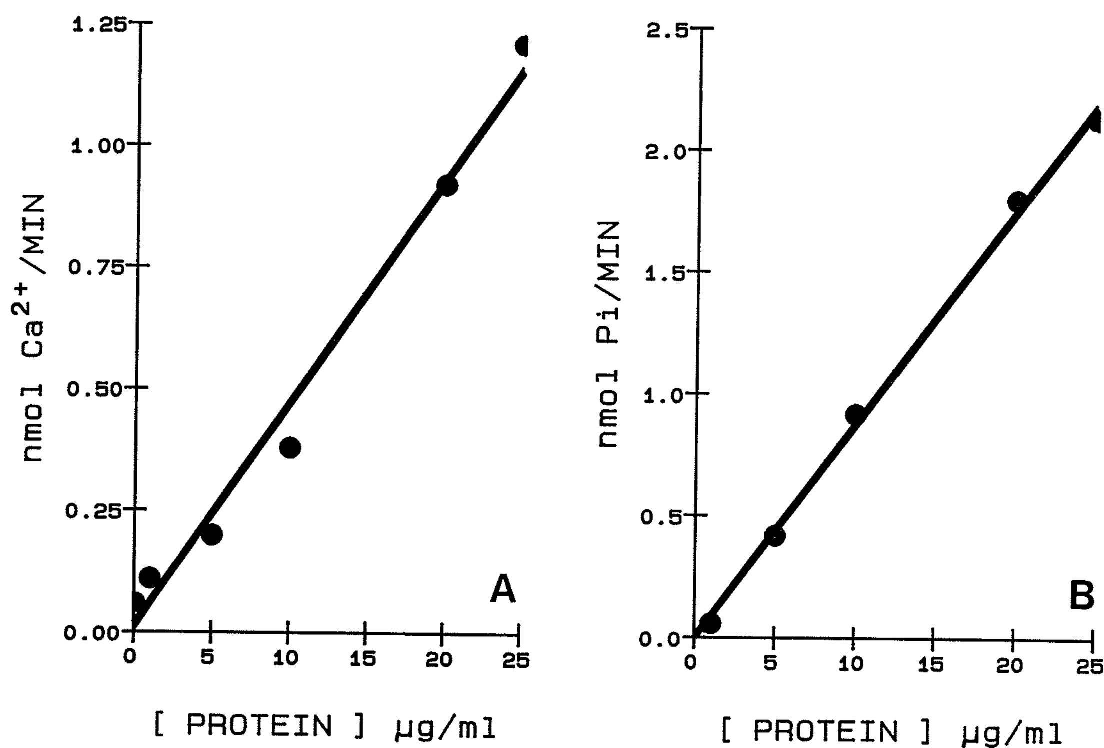

Figure 17 A. Effect of protein concentration on ATP-dependent calcium uptake activity of synaptosome membrane vesicles.

B. Effect of protein concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles.

Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

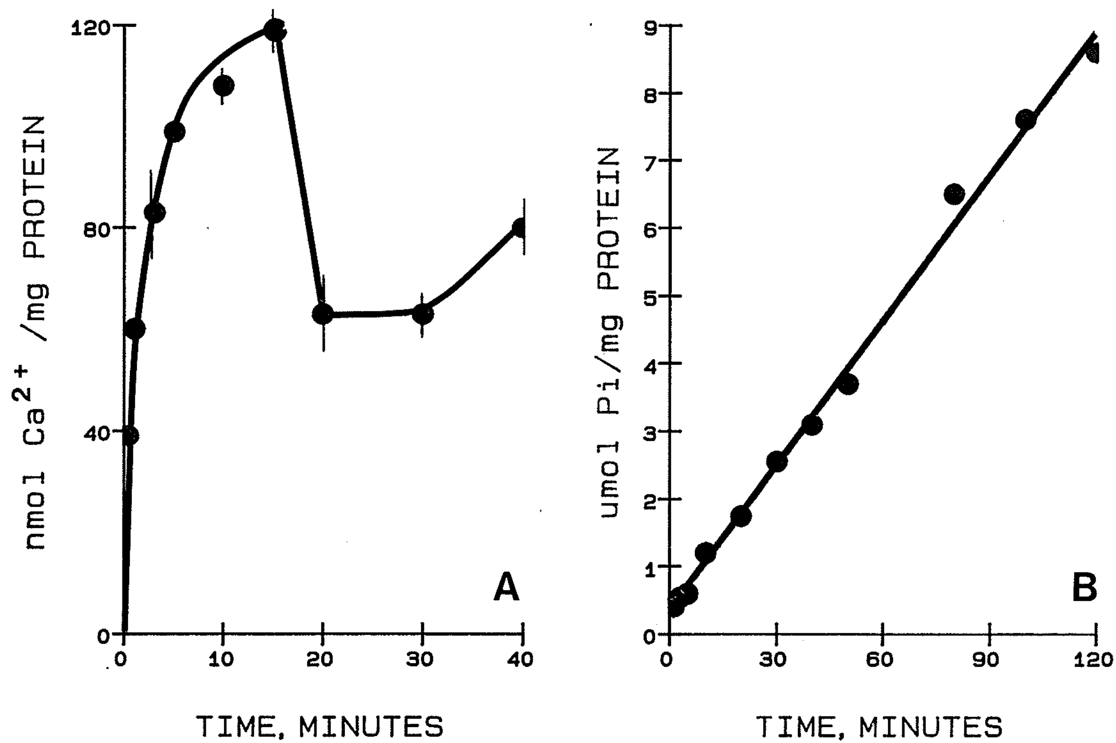

Figure 18 A. Time course of ATP-dependent calcium uptake by synaptosome membrane vesicles.
B. Time course of Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

the rate was only 3.21 nmol Ca^{++}/mg protein/min. All values for calcium uptake in this study are calculated rates at 1.0 min.

The activity of Ca^{++}+Mg^{++}-ATPase in the synaptosome membrane vesicles was linear with time over the range of 1 to 120 minutes (Fig. 18B).

Effect of ATP Concentration on Ca^{++}+Mg^{++}-ATPase Activity of and ATP-dependent Calcium Uptake by Synaptosome Membrane Vesicles

Both the uptake of calcium into synaptosome membrane vesicles and Ca^{++}+Mg^{++}-ATPase activity were dependent on ATP concentration and followed Michaelis-Menten kinetics in a concentration range of 0 to 250 µM (Figs.19A,B). Double reciprocal plot analysis revealed that both ATP-dependent calcium pump and Ca^{++}+Mg^{++}-ATPase have a single ATP binding site (Fig.19A,B, inserts). The Ca^{++}+Mg^{++}-ATPase of synaptosome membrane vesicles has a Km=116 µM for ATP and a Vmax=0.22 µmol pi/mg protein/min, similar to the synaptosome Ca^{++}+Mg^{++}-ATPase. The ATP-dependent calcium pump has a higher affinity for ATP with a Km=14 µM and a Vmax=0.047 µmol Ca^{++}/mg protein/minute.

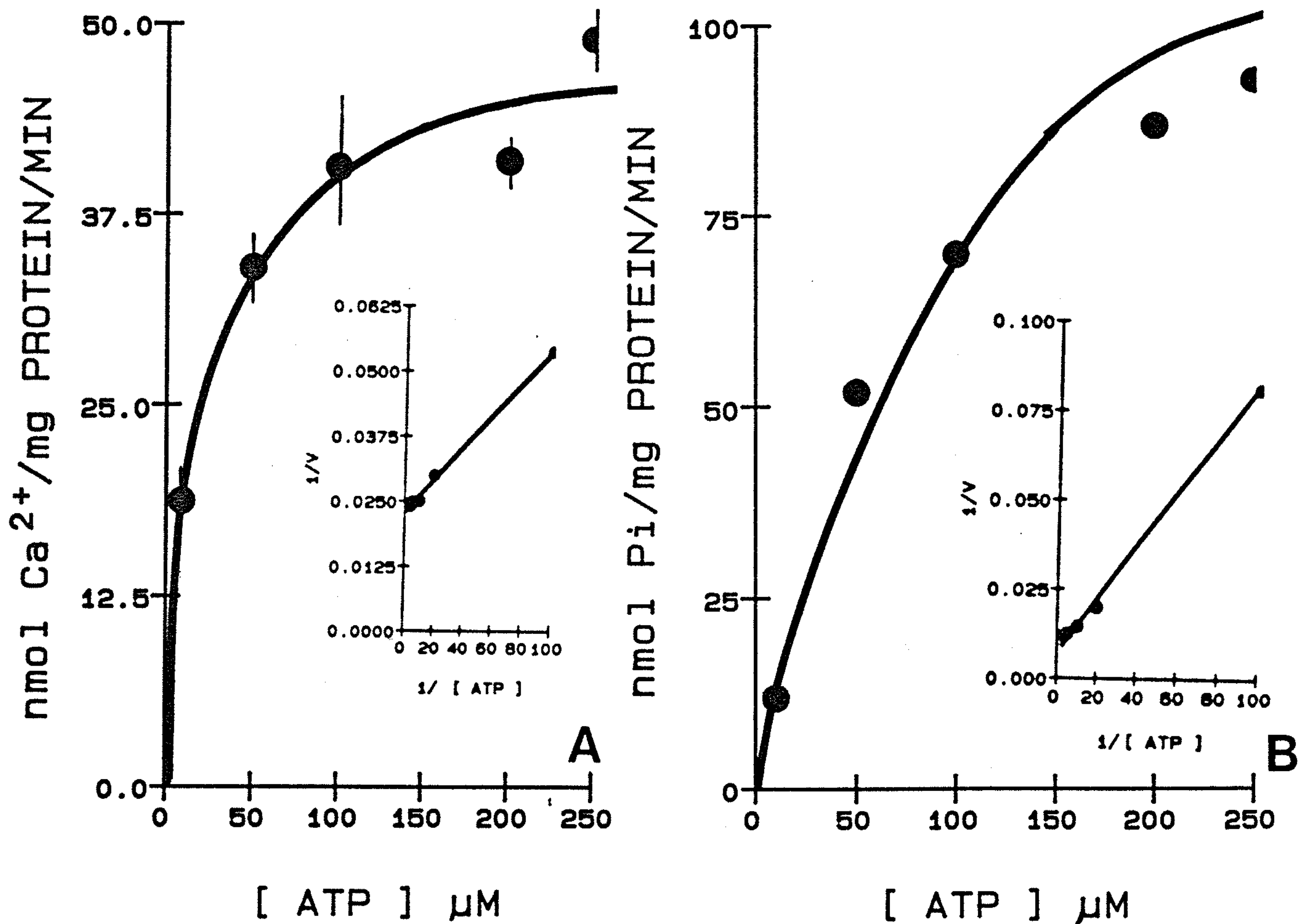

Figure 19 A. Effect of ATP concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles. Insert: Double reciprocal analysis.

B. Effect of ATP concentration on ATP-dependent calcium uptake by synaptosome membrane vesicles. Insert: Double reciprocal analysis.

Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

The Influence of Free Calcium Concentration on Ca^{++}+Mg^{++}-ATPase Activity and ATP-dependent Calcium Uptake by Synaptosome Membrane Vesicles

ATP-dependent calcium uptake was studied over a free calcium concentration range from 0.022 to 50 nM. The highest rate of uptake occurred at about 0.1 to 0.2 nM free calcium. At higher (>2 nM) concentrations of free calcium, the rate of uptake was only about a tenth of the maximal rate (Fig. 20). Double reciprocal plot analysis revealed a typical substrate inhibition type of curve with a Km=0.13 nmol for free calcium and a Vmax=110 nmol Ca^{++}/mg protein/minute (Fig. 20, insert).

Ca^{++}+Mg^{++}-ATPase activity was measured over a wide range of free calcium concentration from 0.1 nM to 0.1 mM (Fig. 21A,B). Maximal enzyme activity occurred at about 0.2 nM free calcium. Double reciprocal plot analysis in the range of 0.1 nM to 1.0 nM free calcium revealed a typical substrate inhibition type curve with a Km=0.072 nM for free calcium and a Vmax=0.17 µmol Pi/mg protein/min (Fig. 21A, insert). At higher concentrations of free calcium, the activity of Ca^{++}+Mg^{++}-ATPase declined by about half and remained relatively constant to form a plateau of activity over a range of 10^{-8} to 10^{-5} M free calcium. The activity of the enzyme then declined slightly from 10^{-5} to 10^{-4} M free calcium (Fig. 21B). Double reciprocal analysis revealed a biphasic inhibition curve (Fig. 21B, insert).

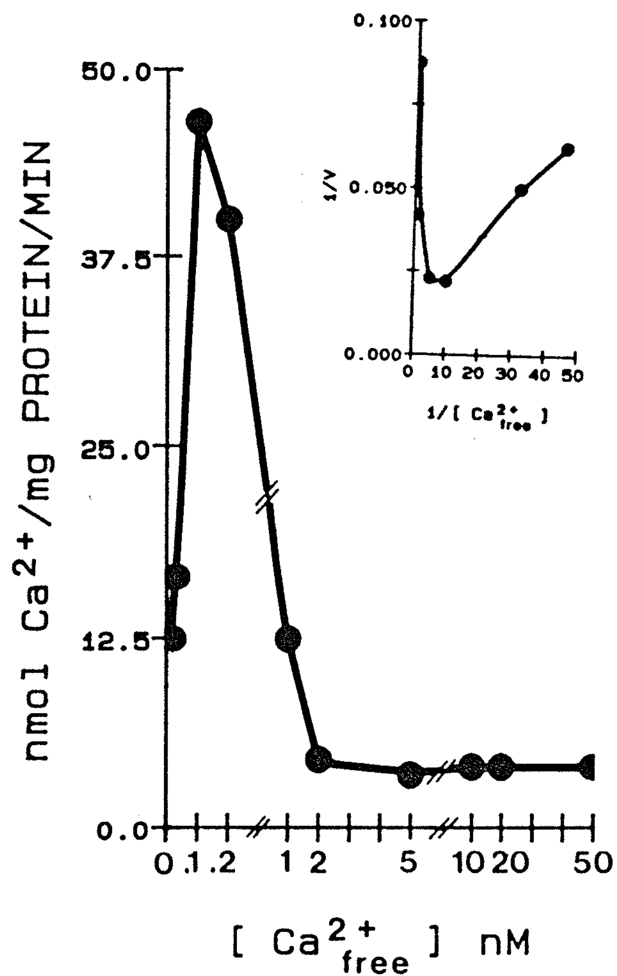

Figure 20. Effect of free calcium concentration on ATP-dependent calcium uptake by synaptosome membrane vesicles. Insert: Double reciprocal analysis. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

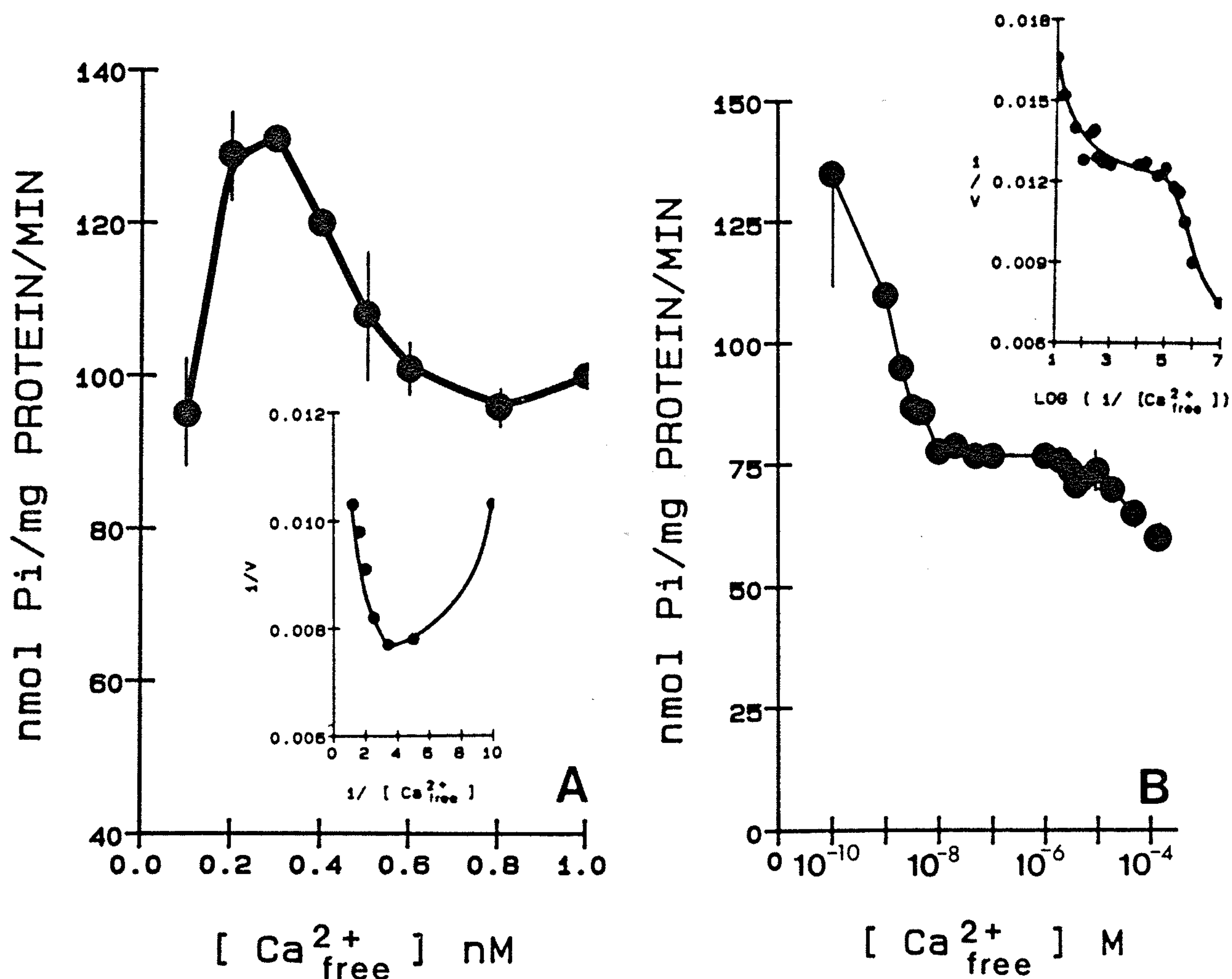

Figure 21 A. Effect of free calcium concentration (subnanomolar) on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles. Insert: Double reciprocal analysis.

B. Effect of free calcium concentration (10^{-10}-10^{-4} M) on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles. Insert: Double reciprocal analysis.

Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

The Effect of Magnesium Concentration on Ca^{++}+Mg^{++}-ATPase Activity of and ATP-dependent Calcium Uptake by Synaptosome Membrane Vesicles

ATP-dependent calcium uptake was maximal at a magnesium concentration of 2 mM. When magnesium was omitted from the incubation medium, the rate of uptake of calcium was only a small fraction (about one twentieth) of the maximal rate. Uptake was greatly reduced by higher (>5 mM) concentrations of magnesium (Fig. 22A). A double reciprocal plot revealed a complex inhibition curve. It appeared that the ATP-dependent calcium pump has two binding sites for Mg^{++}, one with a high affinity for Mg^{++} (Km=1.12 mM and a Vmax=48.4 nmol Ca^{++} /mg protein/min) and the other with a much lower affinity for Mg^{++} (approx. Km=3.78 mM and a Vmax=110 nmol Ca^{++}/mg protein/minute) (Fig. 22A, inset).

Ca^{++}+Mg^{++}-ATPase was maximally active at a magnesium concentration of 1 mM. When magnesium was omitted from the reaction mixture, the enzyme retained about one third of its maximal activity. Enzyme activity was partially inhibited by higher (>5 mM) concentrations of magnesium (Fig. 22B). A double reciprocal plot revealed a complex inhibition curve similar in general shape to the inhibition curve for ATP-dependent Calcium uptake. It appeared that the enzyme has two binding sites for Mg^{++}, one with a higher affinity for Mg^{++} with a Km=0.07 mM, Vmax=0.080 µmol Pi/mg protein/min) and

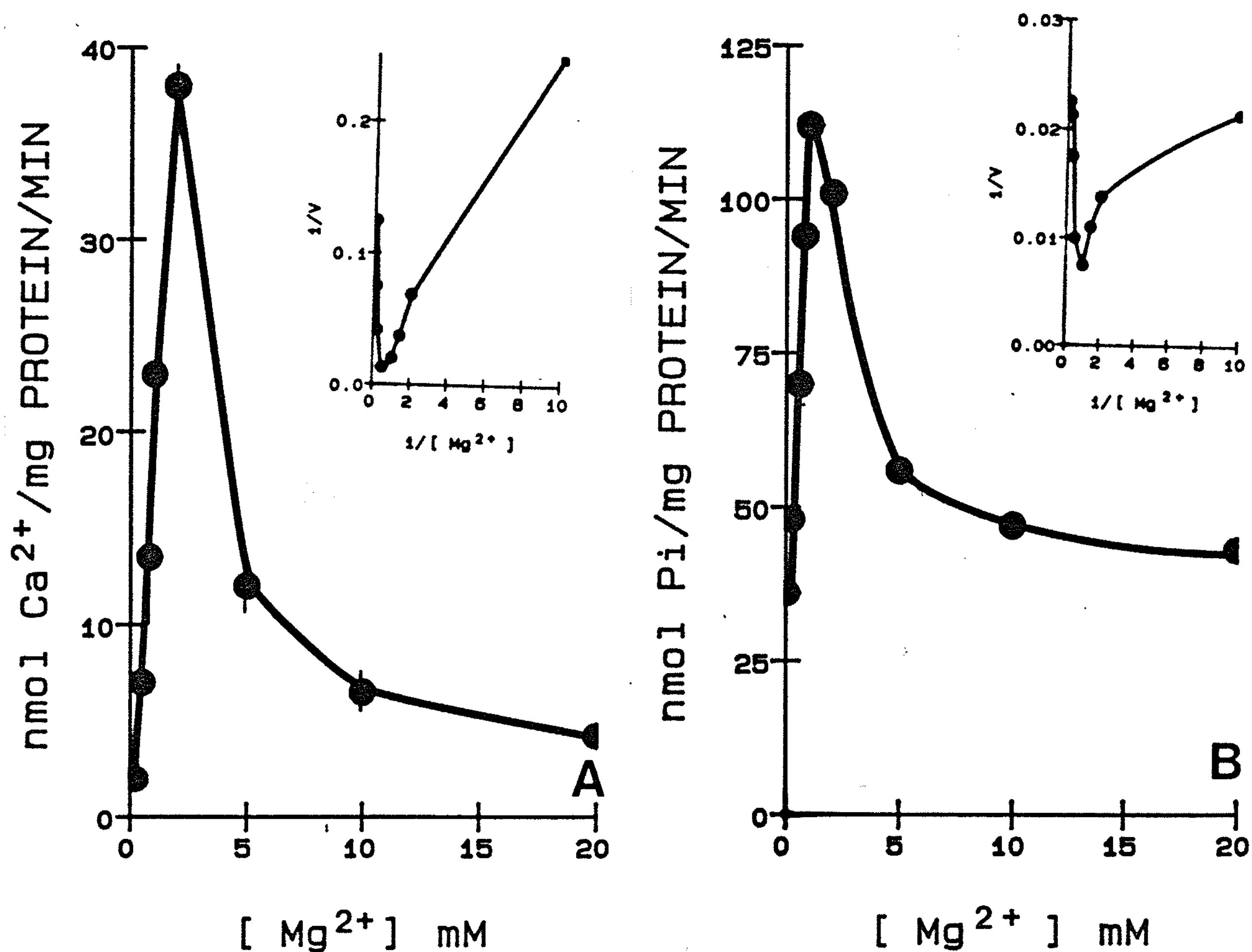

Figure 22 A. Effect of magnesium concentration on ATP-dependent calcium uptake by synaptosome membrane vesicles. Insert: Double reciprocal analysis.

B. Effect of magnesium concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles. Insert: Double reciprocal analysis.

Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

the other with a much lower affinity for Mg^{++} (approx. Km=1 mM and a Vmax=0.3 µmol Pi/mg protein/min) (Fig.22B, inset).

Effect of EGTA Concentration on ATP-dependent Calcium Uptake by and Ca^{++}+Mg^{++}-ATPase Activity of Synaptosome Membrane Vesicles

The effect of the EGTA component of the Ca^{++}-EGTA buffering system on calcium uptake and Ca^{++}+Mg^{++}-ATPase activity was determined by varying the concentration of EGTA while keeping the concentration of free calcium constant at 0.3 nM by addition of appropriate amounts of $CaCl_2$.

EGTA inhibited ATP-dependent calcium uptake in an exponential manner (Fig. 23A). At the lowest concentration (0.01 mM) of EGTA tested, the variability of calcium uptake measurements was high, possibly due to the low buffering capacity of the EGTA-Ca^{++} system at this dilution, pipetting error or both. EGTA inhibited Ca^{++}+Mg^{++}-ATPase activity in a linear manner and inhibition was much less pronounced than inhibition of Ca^{++}-uptake (Fig. 23A,B). A concentration of 0.1 mM EGTA was used in routine measurements as a compromise between adequate calcium ion buffering, reliability of measurement and activity of the system being studied.

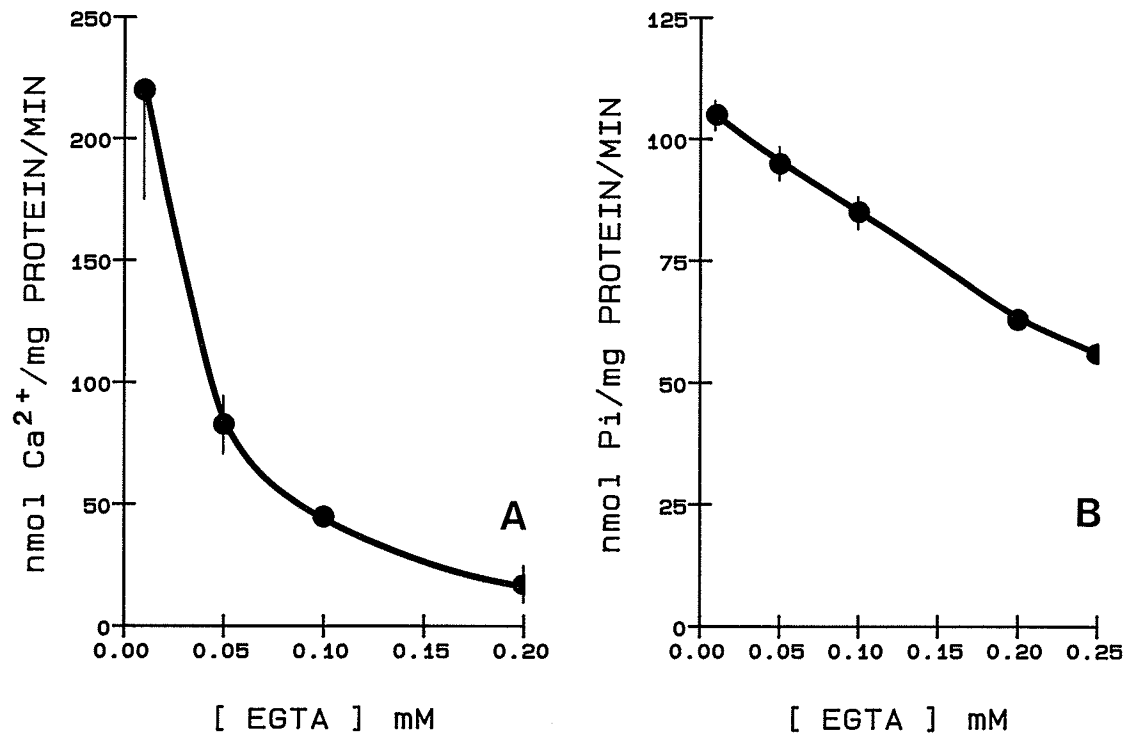

Figure 23 A. Effect of EGTA concentration on ATP-dependent calcium uptake by synaptosome membrane vesicles.
B. Effect of EGTA concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

Effect of Potassium and Ouabain on Ca^{++}+Mg^{++}-ATPase Activity of and ATP-dependent Calcium Uptake by Synaptosome Membrane Vesicles

Potassium ions over a concentration range of 0 to 320 mM had little effect on either ATP-dependent calcium uptake or Ca^{++}+Mg^{++}-ATPase activity (Fig.24A,B).

Ouabain, over 0 to 0.25 mM had little effect on calcium uptake or Ca^{++}+Mg^{++}-ATPase activity (Fig.25A,B).

Discussion

The biochemical and kinetic properties of the ATPase in brain synaptosomes from *M. configurata* are consistent with those of a high affinity Ca^{++}-stimulated Mg^{++}-dependent ATPase. The responses of the enzyme to ATP, calcium, magnesium, potassium and ouabain are characteristic of Ca^{++}+Mg^{++}-ATPase and distinguishable from other ATPases likely present in the synaptic membrane.

Ca^{++}+Mg^{++}-ATPase in the moth brain synaptosomes was strictly dependent on ATP and had a single, high affinity binding site for ATP (Km=122 µM). The affinity of the moth brain enzyme for ATP was lower than that of Ca^{++}+Mg^{++}-ATPase from mouse brain (Km=28 µM, Javors *et al.*, 1981) or rat brain (Km=18.9 µM, Michaelis *et al.*, 1983).

Moth brain synaptosome Ca^{++}+Mg^{++}-ATPase was stimulated by very low concentrations of calcium ion. Although maximal

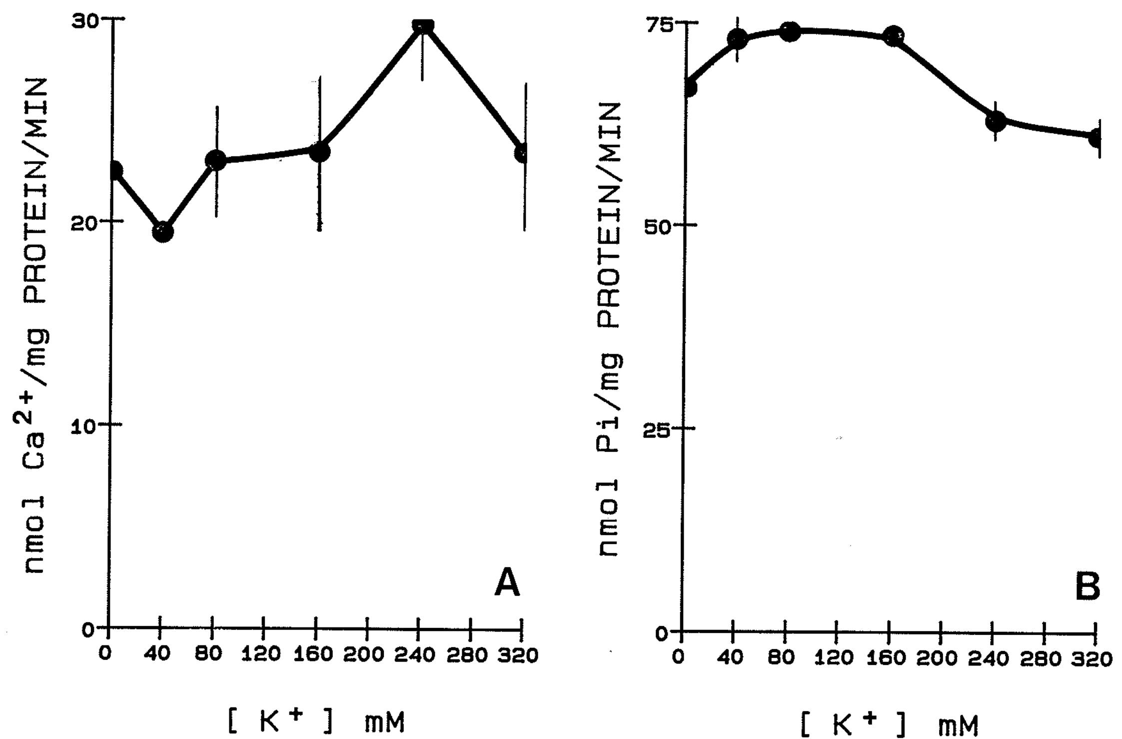

Figure 24 A. Effect of potassium concentration on ATP-dependent calcium uptake by synaptosome membrane vesicles.

B. Effect of potassium concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles.

Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

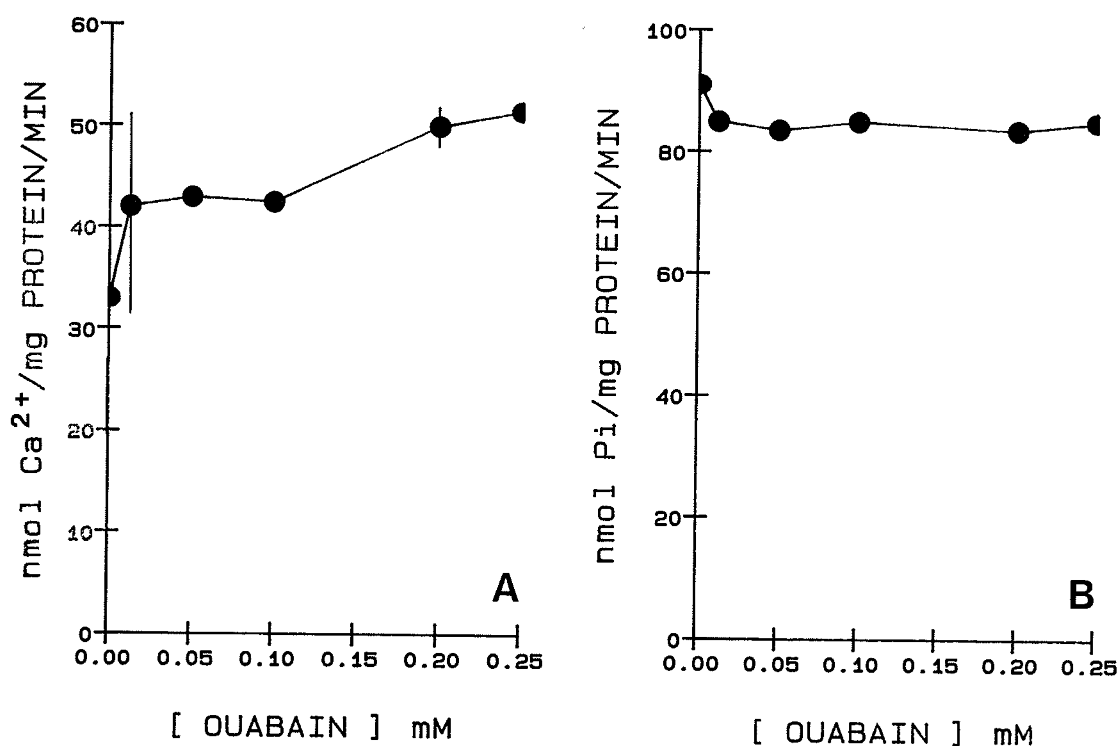

Figure 25A. Effect of ouabain concentration on ATP-dependent calcium uptake by synaptosome membrane vesicles.
B. Effect of ouabain concentration on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

stimulation occurred at about 0.2 nM free calcium, about half of the maximal activity of the enzyme was retained as a broad plateau of activity over a wide range (10^{-8} to 10^{-5} M) of free calcium concentrations. The data indicate that the enzyme has two kinetically distinct binding sites for Ca^{++}, one with a very high affinity for free calcium in the nanomolar range (Km=0.11 nM) and the other with a lower affinity for free calcium in the micromolar range. Binding sites for Ca^{++} at subnanomolar concentrations have not been reported and may represent an unique class of high affinity binding sites for calcium in insect brain synaptosomal Ca^{++}+Mg^{++}-ATPase. The lower affinity calcium binding site of moth brain enzyme appears to correspond to calcium binding sites of mammalian brain synaptosomal Ca^{++}+Mg^{++}-ATPase which typically have Km values in the range of 0.23 to 0.80 µM (Lin and Way, 1982; Michaelis *et al.*, 1983; Ross and Cardenas, 1983).

I also investigated the effect on Ca^{++}+Mg^{++}-ATPase of EGTA, a specific Ca^{++} chelator, that was used to control the concentration of free calcium in the reaction mixtures. In the presence of a constant concentration of free calcium, the enzyme was inhibited by EGTA *per se* but its effect was not pronounced (<10% inhibititon at 0.1 mM EGTA). An earlier report of the inhibitory effect of EGTA on the Ca^{++}+Mg^{++}-ATPase in the retinal nerve of the squid, *Lologo pealei* (Matsumura and Clark, 1980) is difficult to interpret

since the concentration of free calcium was not kept constant in the experiments. In that case inhibition of enzyme activity, therefore, could be attributable in part to reduction in the concentration of free calcium with increasing concentrations of EGTA in the reaction mixture.

Ca^{++}+Mg^{++}-ATPase from moth brain synaptosomes was stimulated by magnesium. The enzyme was maximally active at a magnesium concentration of 2 mM. When magnesium was omitted from the reaction mixture, the Ca^{++}+Mg^{++}-ATPase retained about 20% of its maximal activity in the presence of 0.3 nM free calcium. The enzyme exhibited a relatively high affinity for magnesium (Km=85 µM). Rat brain synaptosome Ca^{++}+Mg^{++}-ATPase exhibit a higher affinity for magnesium (Km=6.0 µM, Michaelis *et al*., 1983) than the moth brain synaptosome enzyme.

Ca^{++}+Mg^{++}-ATPase activity from moth brain synaptosomes was stimulated by potassium. The degree of stimulation (about 30%) and the optimal potassium concentration (40 mM) are similar to those observed from mammalian brain synaptosome Ca^{++}+Mg^{++}-ATPase (Blaustein, 1975; Lin and Way, 1982).

Finally, the moth brain synaptosome enzyme was insensitive to ouabain, an inhibitor of Na^{+}+K^{+}-ATPase (Michaelis *et al*., 1983), leaving little doubt that the enzyme is a Ca^{++}+Mg^{++}-ATPase.

The biochemical and kinetic properties of the moth brain synaptosome ATPase thus indicate that it is a typical

synaptic membrane $Ca^{++}+Mg^{++}$-ATPase presumably of similar function to other brain synaptic $Ca^{++}+Mg^{++}$-ATPases. The unusually high affinity of the enzyme for Ca^{++}, however, appears to distinguish the moth brain synaptosome enzyme from mammalian brain synaptosome $Ca^{++}+Mg^{++}$-ATPase.

The results in chapter I showed that ATP-dependent calcium transport in brain synaptosome membrane vesicles from *Mamestra configurata* also displays a very high affinity for calcium ion. Maximal transport of $^{45}Ca^{++}$ into the membrane vesicles occurred at a free calcium concentration of 0.1 to 0.2 nM, a value that is remarkably similar to the Km=0.11 nM for the high affinity of Ca^{++} binding site of the moth brain synaptosome $Ca^{++}+Mg^{++}$-ATPase. The kinetic properties of ATP-dependent calcium transport and $Ca^{++}+Mg^{++}$-ATPase thus suggest that the two processes are functionally coupled in the synaptic membrane and contribute to the maintenance of calcium homeostasis in the brain of *Mamestra configurata*. However, such studies cannot provide convincing evidence to demonstrate that ATP-dependent Ca^{++} transport and $Ca^{++}+Mg^{++}$-ATPase are functionally coupled unless both of them are studied using identical synaptosomal preparations and under identical substrate and reaction conditions. Therefore, in order to demonstrate the molecular relationship between $Ca^{++}+Mg^{++}$-ATPase and ATP-dependent calcium pump, the biochemical and kinetic properties of both ATP-dependent calcium pump and $Ca^{++}+Mg^{++}$-ATPase were studied using prepared

synaptosome membrane vesicles under indentical substrate and reaction conditions.

The results showed that under such strictly defined experimental conditions ATP-dependent Ca^{++}-transport and Ca^{++}+Mg^{++}-ATPase displayed many similarities, including responses to ATP, free calcium, magnesium, EGTA, potassium and ouabain, thereby indicating that the ATP-dependent calcium pump and Ca^{++}+Mg^{++}-ATPase are functionally coupled in synaptic membranes in the brain of *Mamestra configurata*.

Both ATP-dependent calcium pump and Ca^{++}+Mg^{++}-ATPase in moth brain synaptosome membrane vesicles were strictly dependent on ATP and had a single high affinity binding site for ATP. The calcium pump had a higher affinity for ATP and lower maximal reaction velocity (Km=14 µM; Vmax=0.047 µmol Ca^{++}/mg protein/min) than the ATPase (Km=116 µM; Vmax=0.22 µmol Pi/mg protein/min). The variability of various estimates of the Km for ATP for the ATP-dependent calcium pump in mammalian brain synaptosome membrane vesicles (16.3 µM (Javors *et al*, 1981); 40 µM (Gill *et al*, 1981); 125 µM (Barros and Kaczorowski, 1984)) may reflect differences in the preparation of synaptic vesicles used in Ca^{++} uptake measurements. Estimates of the Km for ATP for mammalian brain synaptic preparations (17 µM (Garrett and Ross, 1985); 18.9 µM (Michaelis *et al*, 1983); 28 µM (Javors *et al*, 1981)) lie in a narrower range and are considerably lower than the Km=116 µM for the moth brain ATPase. In study on calcium

transport in the brain synaptic membranes of mice in which both systems were studied under identical substrate and reaction conditions, the Km for ATP for the calcium pump and Ca^{++}+Mg^{++}-ATPase were 16.3 and 28 µM, respectively, providing support for the concept that calcium transport and ATP hydrolysis are common functions of the same membrane protein (Javors *et al*, 1981).

Both ATP-dependent calcium pump and Ca^{++}+Mg^{++}-ATPase in moth brain synaptosome membrane vesicles exhibited the same high affinity for free calcium. The Km for free calcium for the calcium pump was only 0.13 nM and the Km for free calcium for Ca^{++}+Mg^{++}-ATPase was only 0.072 nM. These values provide strong supporting evidence that the calcium pump and Ca^{++}+Mg^{++}-ATPase are functionally coupled in synaptic membranes in the brain of *Mamestra configurata*. In mammalian brain, the Km for free calcium of the ATP-dependent calcium pump is considerably higher, with a range from 0.11 to 12 µM (Gill *et al*, 1981; Gill *et al*, 1984; Hincke and Demaille, 1984) and the Km for free calcium of Ca^{++}+Mg^{++}-ATPase for various mammalian synaptic preparations lies in a range of 0.23 to 0.8 µM (Ross and Cardenas, 1983; Lin and Way, 1982). Evidently, the ATP-dependent calcium pump in synaptic membranes in the brain of *Mamestra configurata* has a much greater affinity for calcium than the calcium pump in the brain of mammals, but the physiological significance of this difference remains unknown. Moth brain Ca^{++}+Mg^{++}-ATPase

retains a significant proportion (at least half) of its maximal activity at micromolar concentrations of free calcium, indicating either the enzyme has two binding sites for calcium (a high affinity site at nanomolar free calcium and a low affinity site at micromolar free calcium) or that there are two enzymes with high and low affinity for calcium. Under the same substrate and reaction conditions, the moth brain ATP-dependent calcium pump displays relatively little activity (about 3% of the maximal) at micromolar concentrations of free calcium. However, the activity of the pump is more severely effected by EGTA in the reaction mixture than the enzyme (Fig.23A,B), possibly due to uncoupling of calcium transport from ATP hydrolysis by EGTA. The differential effect of EGTA on the two experimental systems leads to an underestimate of ATP-dependent calcium pump activity relative to Ca^{++}+Mg^{++}-ATPase activity and likely accounts for the apparent low activity of the calcium pump at micromolar concentrations of free calcium.

Both the ATP-dependent calcium pump and Ca^{++}+Mg^{++}-ATPase in moth brain synaptosome membrane vesicles were stimulated by magnesium and had similar kinetic properties. The Km values for magnesium for the high affinity Mg^{++} binding site for the calcium pump and ATPase were 1.1 and 0.07 mM, respectively, providing further evidence for the functional identity of the two systems. As well, both systems appear to have a second, low affinity binding site for Mg^{++}, with

approximate Km values for magnesium of 4 mM for the calcium pump and 1 mM for the ATPase.

Some experimental differences exist between the two systems on the degree of dependency on magnesium. The calcium pump shows a greater dependency on magnesium and retains only one-twentieth of its maximal transport rate in the absence of added magnesium. The $Ca^{++}+Mg^{++}$-ATPase is less dependent on magnesium for maximal activity and retains one-third of its activity in the absence of added magnesium. The possible significance of this difference for calcium transport has not been explored.

Both the ATP-dependent Ca^{++}-pump and $Ca^{++}+Mg^{++}$-ATPase were little affected by K^{+} in the reaction medium. K^{+} had a slight stimulatory effect on $Ca^{++}+Mg^{++}$-ATPase in moth brain synaptosomes and mammalian synaptic preparations (Lin and Way, 1982; Blaustein, 1975). The effect, however, was not obtained using moth brain synaptosome membrane vesicles, possibly due to the "inside-out" configuration of the vesicles. Finally, both the ATP-dependent calcium pump and $Ca^{++}+Mg^{++}$-ATPase were insensitive to ouabain, an inhibitor of $Na^{+}+K^{+}$-ATPase.

These results establish the identity and functional coupling of the two systems for calcium transport in synaptic membranes from the brain of *Mamestra configurata*.

CHAPTER III

THE EFFECT OF INSECTICIDES ON SYNAPTOSOMAL $Ca^{++}+Mg^{++}$-ATPase AND ATP-DEPENDENT CALCIUM PUMP FROM THE MOTH BRAIN OF THE BERTHA ARMYWORM *MAMESTRA CONFIGURATA* WLK.

Introduction

Although it is well known that some modern insecticides act by disrupting nervous system function, the biochemical mode of action of many classes of insecticides is still not clear. Several possibilities have been proposed concerning the mode of action of these insecticides. The discovery that some insecticides induce hormone release in insects as they cause paralysis (Maddrell and Reynolds, 1972), suggested that they might disrupt the central nervous system by provoking the release of insect neurohormones. Later, it was found that in the treated insects the metabolism of cAMP and cGMP, second messengers of hormones and neurotransmitters, was also disrupted by insecticides (Bodnaryk 1976; 1977; 1982; Butler and Drowder, 1977). It has also been speculated that the disruption of calcium ion homeostasis in the nervous system might be one of the important causes of poisoning by certain insecticides. This hypothesis is based on: i) the importance of calcium in the action of numerous hormones and neurotransmitters and its importance in the control of neurotransmitter and neurohormone release; ii) the finding

that a high concentration of calcium antagonized the effect of some neurotoxic insecticides (Matsumura and O'Brien, 1966; Matsumura and Narahashi, 1971; Gammon, 1978a; 1980). During the past few years, more and more evidence has appeared to support this hypothesis. Numerous studies showed that Ca^{++}-ATPase and Ca^{++}+Mg^{++}-ATPase of the lobster, *Homarus americanus* and vertebrate nervous system are very sensitive to some insecticides, such as DDT, pyrethroids and some cyclodiene compounds (Doherty and Matsumura, 1975; Ghiasuddin and Matsumura, 1979a,b; 1981; Matsumura and Ghiasuddin, 1979; Yamaguchi et al, 1979; Clark, 1981; Clark and Matsumura, 1982; Doherty et al, 1981). In addition, DDT and the pyrethroids were shown to affect ATP-dependent calcium exchange and calcium uptake by crude homogenate of crayfish nerve cord and lobster axon (Ghiasuddin and Matsumura, 1979a; Doherty et al, 1986). However, none of these experiments demonstrated that the inhibition of Ca^{++}+Mg^{++}-ATPase activity would result in the disruption of its function, i.e. extruding calcium at the expense of ATP. Furthermore, none of these studies used insect nerve preparations. Therefore, it remained to be proved that insecticides inhibit Ca^{++}+Mg^{++}-ATPase activity in the insect nervous system and disrupt its calcium transport function. Until recently, the lack of a good in vitro system, such as synaptosomes and synaptosome membrane vesicles, has been a major reason that we knew so little about basic insect

neurochemistry, including calcium ion transport and its regulation in insects. This, in turn, retarded progress in understanding the mode of actions of many neurotoxic insecticides.

In Chapters I and II I described the preparation of relatively pure and functionally competent synaptosomes and synaptosome membrane vesicles from the moth brain of *Mamestra configurata* WLK., identified a Ca^{++}+Mg^{++}-ATPase and demonstrated that the enzyme was functionally coupled to an ATP-dependent calcium pump in these synaptic preparations. In this Chapter I have examined, first, the effect of seven different groups of neurotoxic insecticides on the Ca^{++}+Mg^{++}-ATPase of moth synaptosomes and synaptosome membrane vesicles. Then, I have explored whether the inhibition of synaptosomal Ca^{++}+Mg^{++}-ATPase would affect its calcium transport function; and whether the inhibition of brain synaptosomal Ca^{++}+Mg^{++}-ATPase also occurs in living insects poisoned with insecticides.

Materials and Methods

Insects

The bertha armyworm, *Mamestra configurata* WLK. was reared in the laboratory on an artificial diet (Bucher and Bracken, 1976). The conditions of rearing, storage and generating adults for brain dissection were the same as

described in Chapter I.

Chemicals

Insecticides were obtained from Chemical Service Inc. West Chester, PA.

Preparation of Synaptosomes

Synaptosomes were routinely prepared from the pooled brains of about 150 one- to seven-day-old moths by a Ficoll floatation technique (Breer and Jeserich, 1980; Gordon, et al, 1982), modified as described in Chapter I.

Preparation of Synaptosome Membrane Vesicles

Synaptosome membrane vesicles were obtained from adult brain synaptosomes by a Ficoll floatation technique as described in Chapter I.

Effect of Insecticides on Ca^{++}+Mg^{++}-ATPase

Synaptosomes or synaptosome membrane vesicles (10 µg protein) were incubated at 30 °C in 1.0 ml final volume of an assay medium containing 160 mM KCl, 30 mM imidazole buffer, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ouabain, 0.25 mM ATP and $CaCl_2$ to give 0.3 nM free calcium ion concentrations as computed by Perrin and Sayce (1967). Insecticides were added 10 minutes before starting the reaction in an aliquot of 50 µl ethanol-water or dimethylsulfoxide-water solution (0.05%),

while an equivalent amount of ethanol or dimethylsulfoxide was added to the control. The reaction was initiated by the addition of ATP, and was allowed to proceed for 2 hours at 30 °C, followed by the Pi assay procedure as described in Chapter II. Two replicate determinations of at least two separate experiments were performed.

Effect of Insecticides on ATP-dependent Calcium Uptake

Synaptosome membrane vesicles (10 µg protein) were incubated at 30 °C in 1.0 ml final volume of an assay medium containing 160 mM KCl, 30 mM imidazole buffer, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM ouabain, 0.25 mM ATP, 1 µCi $^{45}Ca^{++}$ and $CaCl_2$ to give 0.3 nM free calcium ion concentrations as computed by Perrin and Sayce (1967). Insecticides were added 10 minutes before starting the reaction in an aliquot of 50 µl ethanol-water or dimethylsulfoxide-water solution (0.05%), while an equivalent amount of ethanol or dimethylsulfoxide was added to the control. Radioactive $^{45}Ca^{++}$ was added 2 minutes before starting the reaction. Other procedures were the same as described in Chapter I.

***In vivo* Insecticide Treatment**

Three insecticides were chosen for the *in vivo* poisoning experiments, DDT, allethrin and chlordane. In the *in vivo* insecticide treatments, one- to seven-day-old moths were collected and randomly separated into two groups. The

experimental group was treated topically with insecticides (5 μmol/moth) in acetone around the neck. The control group was topically treated with the same amount of acetone. Brains were dissected from moths at an early stage of poisoning after the appearance of mild symptoms (agitation, hyperactivity, uncoodinated movements and tremor) or at a late stage of poisoning when severe symptoms had developed (paralysis, prostration). Optic lobes were separated from brain during dissection and synaptosomes were separately prepared from brains and optic lobes by a Ficoll floatation technique described in Chapter I.

Effect of Temperature

The effect of temparature on insecticide inhibition of synaptomal Ca^{++}+Mg^{++}-ATPase was studied using synaptosome membrane vesicles. Synaptosome membrane vesicles were incubated at 20 °C or 30 °C, in the incubation medium described above for the Ca^{++}+Mg^{++}-ATPase assay. Insecticides were added 10 minutes before starting the reaction. The Ca^{++}+Mg^{++}-ATPase activity assay procedure was the same as described above.

Replication

Measurements of the effect of insecticides on ATP-dependent calcium uptake and Ca^{++}+Mg^{++}-ATPase activity were made in duplicate for each of at least two separate experiments.

Since synaptosome membrane vesicle preparations vary in activity from preparation to preparation, data from representative experiments are usually given. For the *in vivo* experiment each datum represents the result of four independent measurements.

Results

Preliminary Screening

The effect of 18 compounds from seven major groups of insecticides on synaptosomal Ca^{++}+Mg^{++}-ATPase was investigated at a high concentration of 10^{-4} M (Fig. 26 and 27). Both synaptosomes and synaptosome membrane vesicles were used in the experiments to examine whether the effect of insecticides was influenced by the orientation of the synaptosome membrane. The most potent inhibitors of Ca^{++}+Mg^{++}-ATPase activity were four cyclodienes (chlordane, heptachlor, aldrin and toxaphene which, strictly speaking, is not a cyclodiene compound). The cyclodienes endrin and dieldrin, however, were weak inhibitors of the enzyme. Other chlorinated hydrocarbons, notably DDT, showed some inhibition but methoxychlor and lindane were weak inhibitors. Among the pyrethroids tested, only allethrin showed appreciable inhibition. Carbamates, organophosphates and formamidines showed little or no inhibition (Fig. 26,27). The orientation of the synaptosome membrane had little effect on the inhibition except in the case of allethrin and DDT which

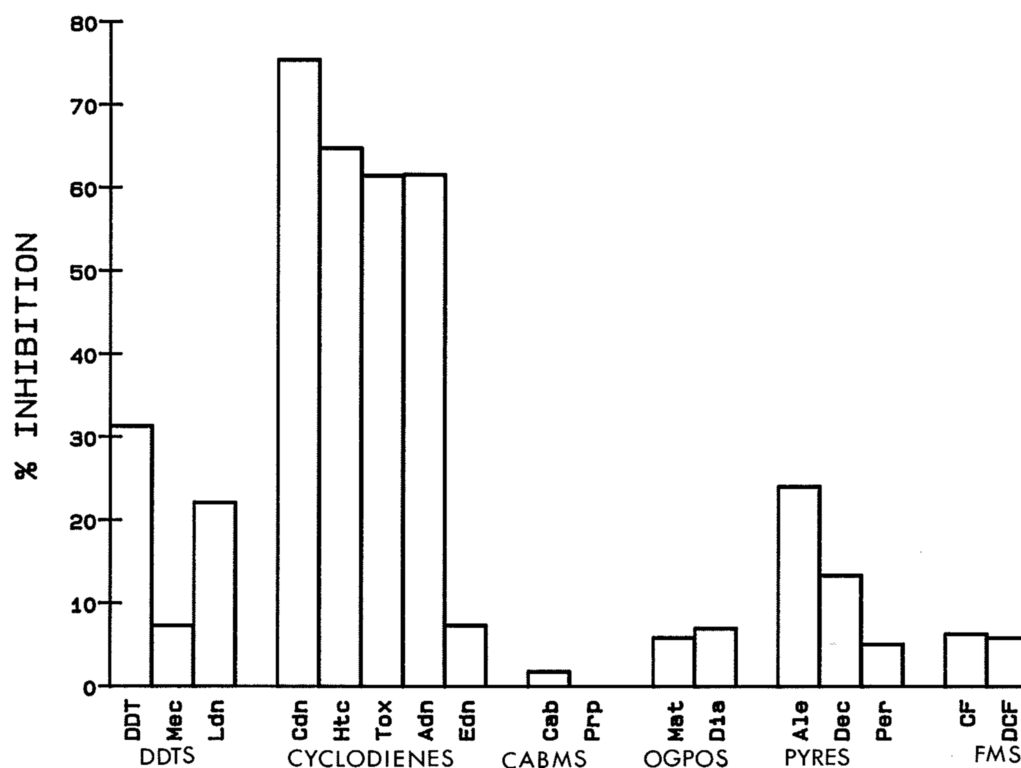

Figure 26. Effect of seven major groups of insecticides on $Ca^{++}+Mg^{++}$-ATPase activity of brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK. Abbreviations are given in the appendix.

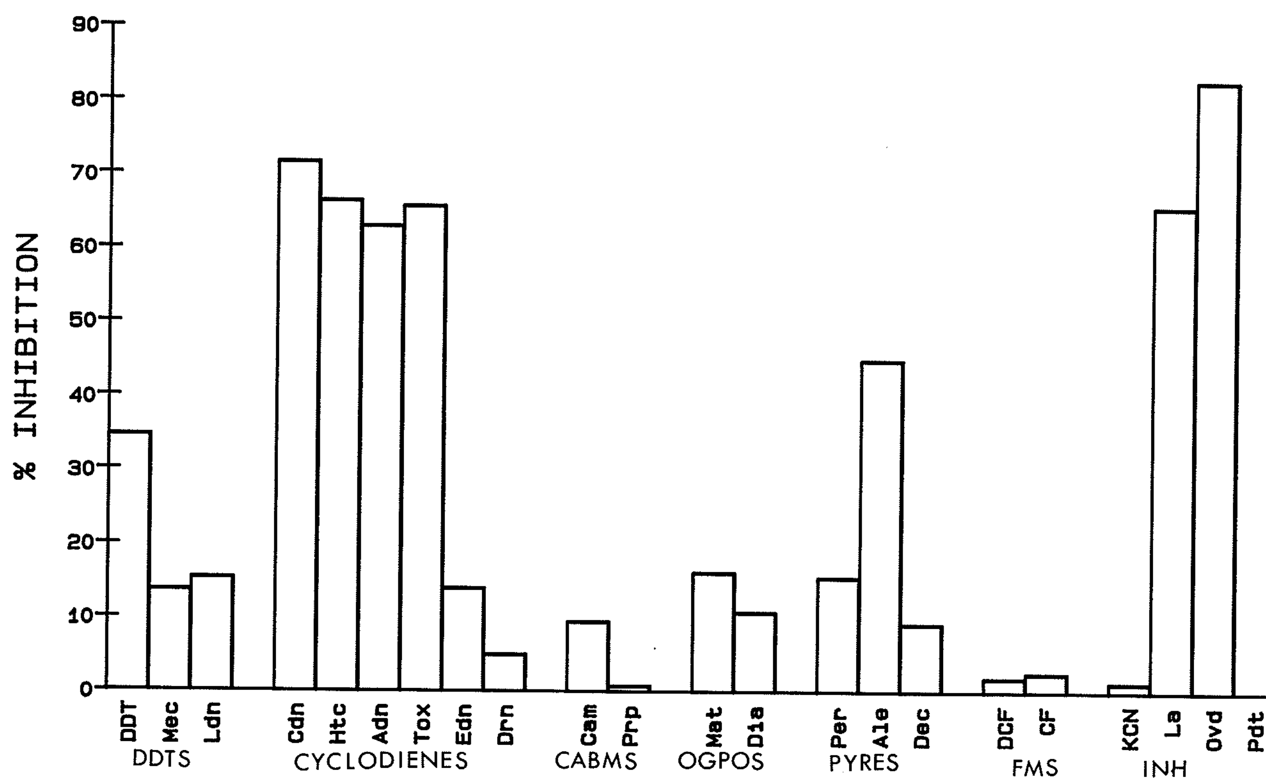

Figure 27. Effect of seven major groups of insecticides on Ca^{++}+Mg^{++}-ATPase activity of synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata WLK*.
Abbreviations are given in the appendix.

displayed higher inhibition on Ca^{++}+Mg^{++}-ATPase from synaptosome membrane vesicles.

The mitochondrial respiratory inhibitor potassium cyanate showed little inhibition of Ca^{++}+Mg^{++}-ATPase of synaptosome membrane vesicles, while the Ca^{++}+Mg^{++}-ATPase inhibitors lanthanium and orthovanadate potently inhibited the enzyme's activity. The gamma-aminobutyric acid (GABA) receptor inhibitor picrotoxinin had no effect on the activity of Ca^{++}+Mg^{++}-ATPase (Fig. 27).

Dose Reponse Studies of the Effect of Insecticides on Synaptosome Ca^{++}+Mg^{++}-ATPase

Insecticides which showed the highest inhibition of Ca^{++}+Mg^{++}-ATPase in the preliminary screening were further studied for their effect on Ca^{++}+Mg^{++}-ATPase activity of synaptosomes at different concentrations from 10^{-9} to 10^{-4} M. Five of these insecticides, chlordane, heptachlor, aldrin, toxaphene and DDT, greatly inhibited Ca^{++}+Mg^{++}-ATPase activity at concentrations between 10^{-6} to 10^{-4} M (Fig. 28 to 32). Allethrin was inhibitory only at concentrations between 10^{-5} to 10^{-4} M (Fig. 33).

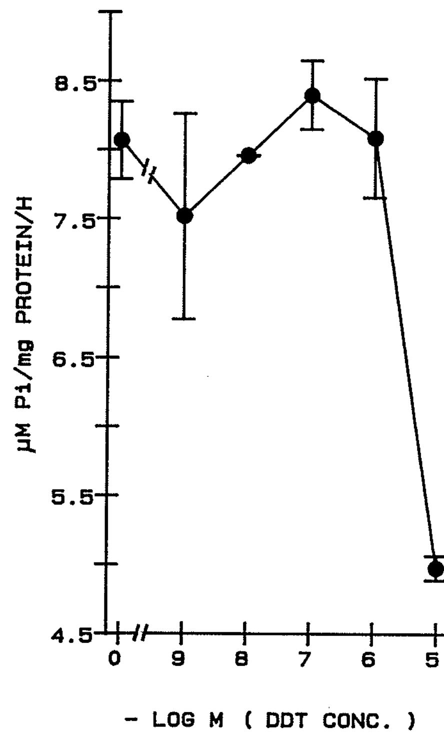

Figure 28. Effect of DDT on Ca^{++}+Mg^{++}-ATPase activity of brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

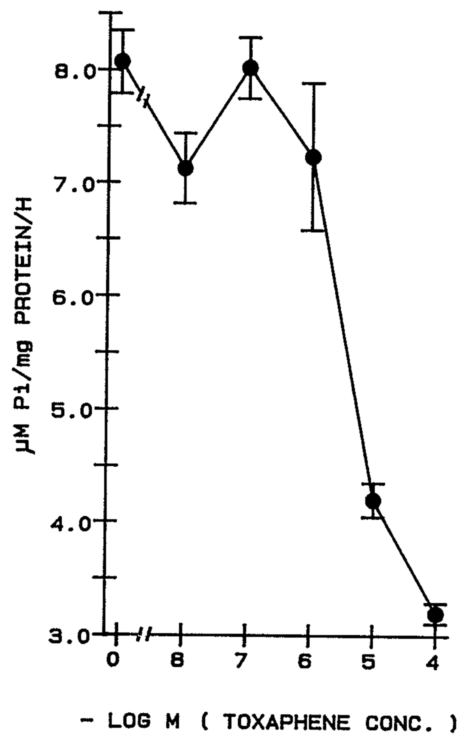

Figure 29. Effect of toxaphene on Ca^{++}+Mg^{++}-ATPase activity of brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

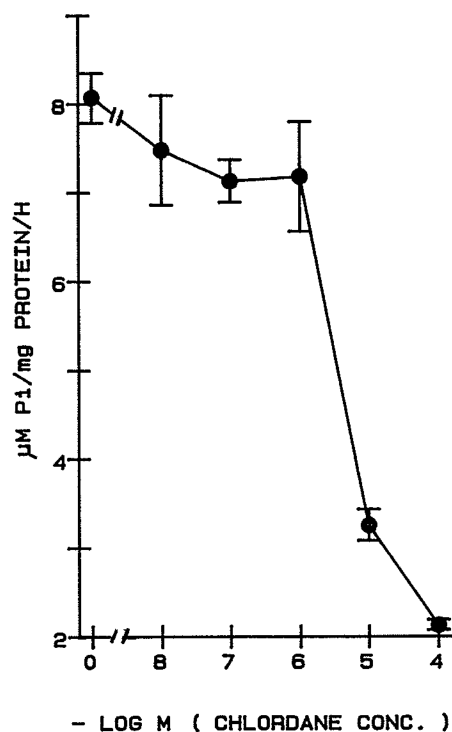

Figure 30. Effect of chlordane on Ca^{++}+Mg^{++}-ATPase activity of brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

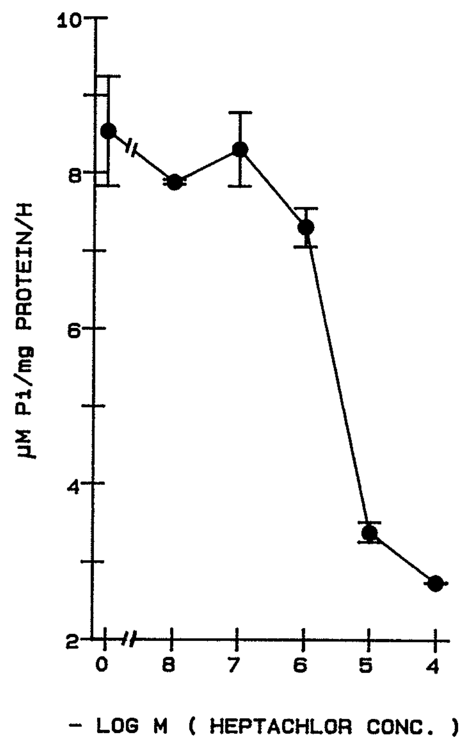

Figure 31. Effect of heptachlor on Ca^{++}+Mg^{++}-ATPase activity of brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

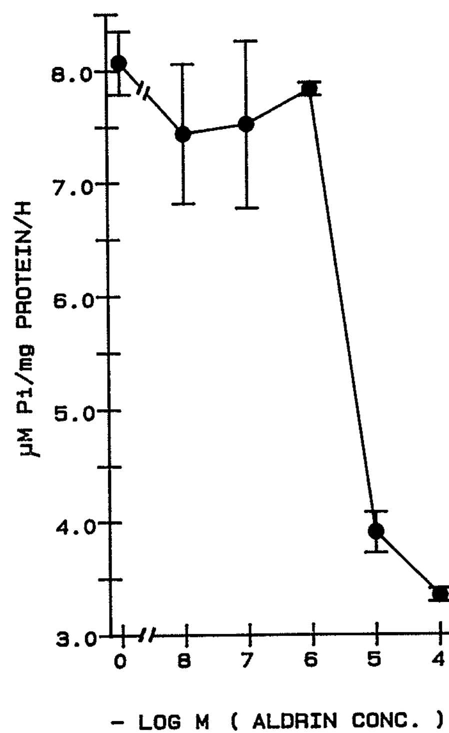

Figure 32. Effect of aldrin on Ca^{++}+Mg^{++}-ATPase activity of brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

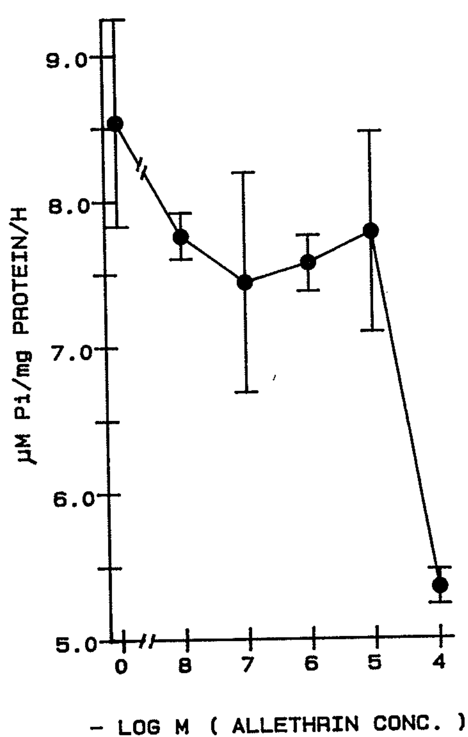

Figure 33. Effect of allethrin on Ca^{++}+Mg^{++}-ATPase activity of brain synaptosomes of the bertha armyworm *Mamestra configurata* WLK.
Experimental variability is reported as standard deviation. Error bars is omitted when they are within the circle.

Dose Response Studies of the Effect of Insecticides on Ca^{++}+Mg^{++}-ATPase and ATP-dependent Ca^{++}-pump of Synaptosome Membrane Vesicles

Since ATP-dependent Ca^{++}-transport activity can be measured easily using inside-out synaptosome membrane vesicles, I also studied the effect of insecticides on calcium transport for comparisons with Ca^{++}+Mg^{++}-ATPase inhibition. Insecticides which showed potent inhibition on synaptosomal Ca^{++} +Mg^{++}-ATPase in the preliminary screening were chosen for study. Both Ca^{++}+Mg^{++}-ATPase and ATP-dependent calcium pump activities of synaptosome membrane vesicles were measured under identical conditions against different concentrations of insecticides from 10^{-8} to 10^{-4} M.

Toxaphene, chlordane, aldrin, heptachlor and allethrin inhibited the activities of both Ca^{++} +Mg^{++} -ATPase and ATP-dependent calcium uptake in the synaptosome membrane vesicles (Fig.34-38). Chlordane, aldrin and heptachlor start to show inhibition of Ca^{++}+Mg^{++}-ATPase activity when their concentrations exceed 10^{-7} M and pronounced inhibition occurred when the concentration was over 10^{-6} M. The activity of ATP-dependent calcium transport was potently inhibited by aldrin and heptachlor when their concentration was over 10^{-6} M; and chlordane, over 10^{-7} M, affected the ATP-dependent calcium transport activity. Toxaphene inhibited the activities of both Ca^{++}+Mg^{++}-ATPase and ATP-dependent calcium pump when its concentration was greater than 10^{-7} M.

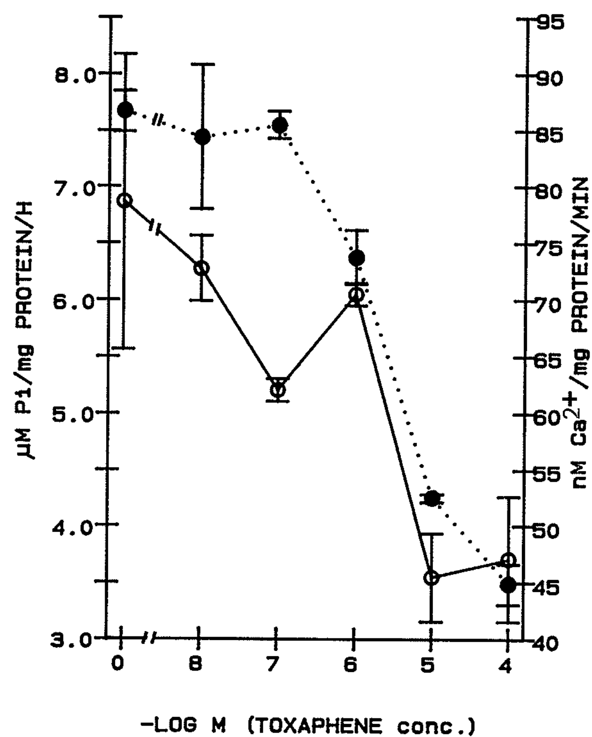

Figure 34. Effect of Toxaphene on Ca^{++}+Mg^{++}-ATPase (dotted line) and ATP-dependent Calcium uptake (solid line) activities of brain synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

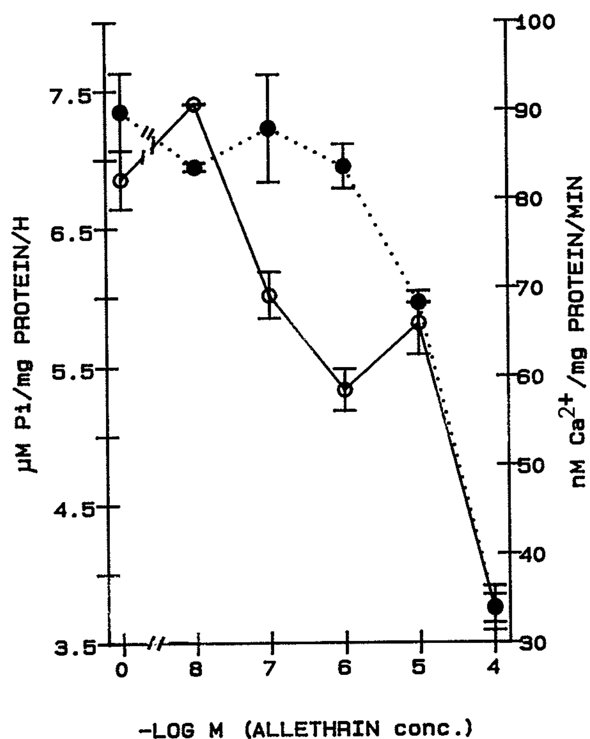

Figure 35. Effect of allethrin on Ca^{++}+Mg^{++}-ATPase (dotted line) and ATP-dependent Calcium uptake (solid line) activities of brain synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

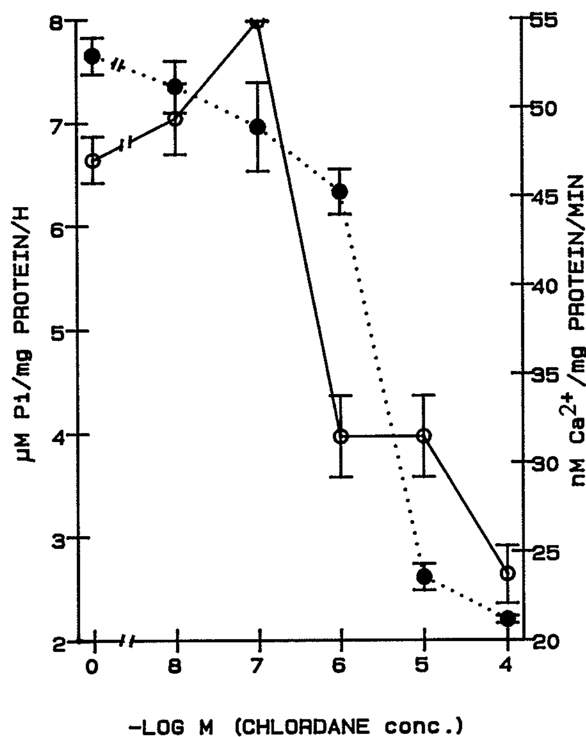

Figure 36. Effect of chlordane on Ca^{++}+Mg^{++}-ATPase (dotted line) and ATP-dependent Calcium uptake (solid line) activities of brain synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

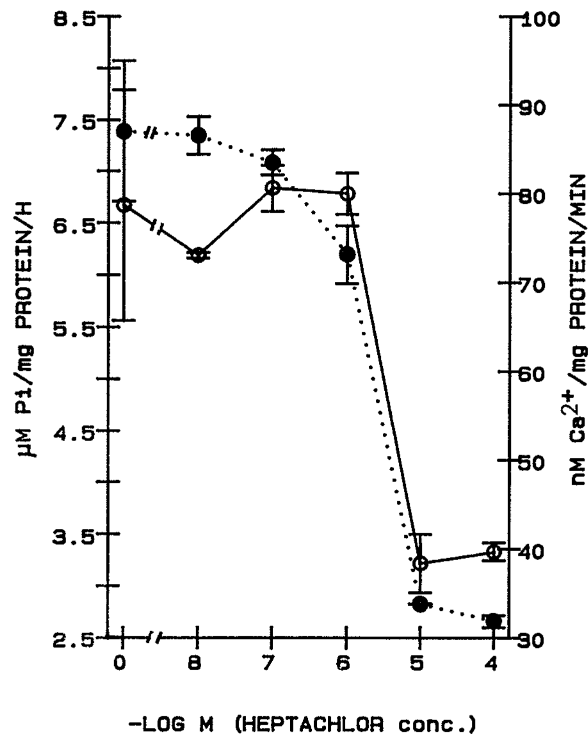

Figure 37. Effect of heptachlor on Ca^{++}+Mg^{++}-ATPase (dotted line) and ATP-dependent Calcium uptake (solid line) activities of brain synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

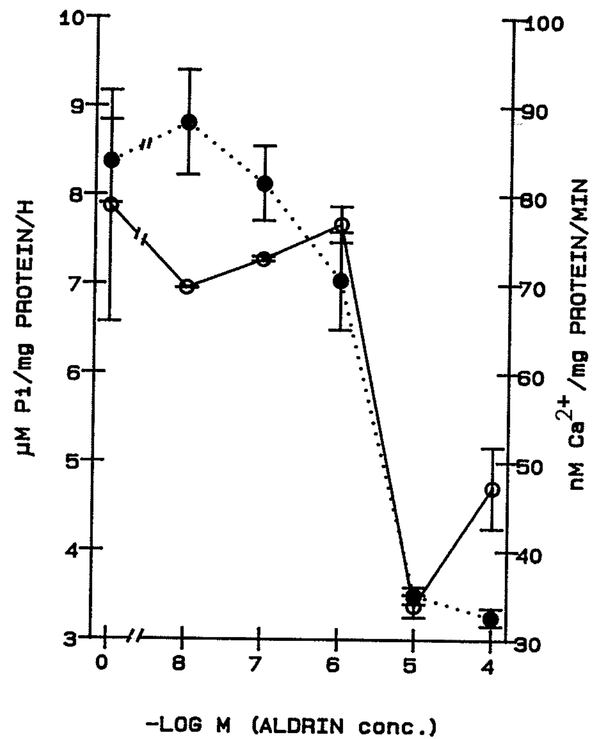

Figure 38. Effect of aldrin on Ca^{++}+Mg^{++}-ATPase (dotted line) and ATP-dependent Calcium uptake (solid line) activities of brain synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

Allethrin gradually inhibited Ca^{++}+Mg^{++}-ATPase activity when its concentration was increased from 10^{-7} to 10^{-4} and greatly affected ATP-dependent calcium transport activity when its concentration exceeded 10^{-8} M.

The effect of DDT on Ca^{++} +Mg^{++} -ATPase and the ATP-dependent calcium pump, however, displayed a different picture (Fig. 39). At concentrations between 10^{-7} to 10^{-4} M DDT gradually inhibited Ca^{++}+Mg^{++}-ATPase activity, but showed no effect on ATP-dependent calcium transport until its concentration reached 10^{-5} M. When DDT concentration was greater than 10^{-5} M it potently stimulated ATP-dependent calcium transport activity.

Since a previous study (Hayashi and Matsumura, 1966) found that dieldrin affected the rate of exchange of calcium ion in the abdominal nerve cord of the American cockroach *Periplaneta americana*, and since there is a superficial resemblance between dieldrin and DDT poisoning, the effect of dieldrin on ATP-dependent calcium transport was also examined at concentrations from 10^{-8} to 10^{-4} M. The effect of dieldrin on ATP-dependent calcium transport was very similar to that of DDT (Fig.40). Up to 10^{-5} M dieldrin showed little effect on the ATP-dependent calcium pump activity. When its concentration was greater than 10^{-5} M dieldrin stimulated the ATP-dependent calcium transport activity.

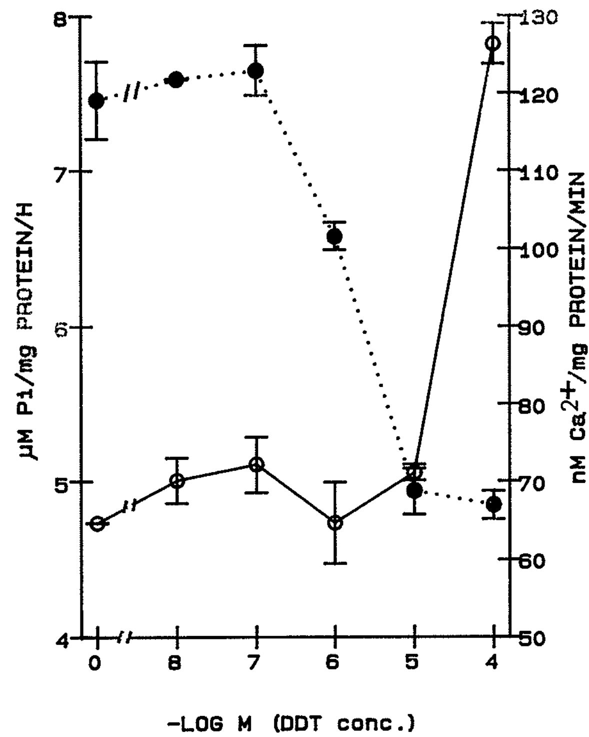

Figure 39. Effect of DDT on Ca^{++}+Mg^{++}-ATPase (dotted line) and ATP-dependent calcium uptake (solid line) activities of brain synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK.
Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

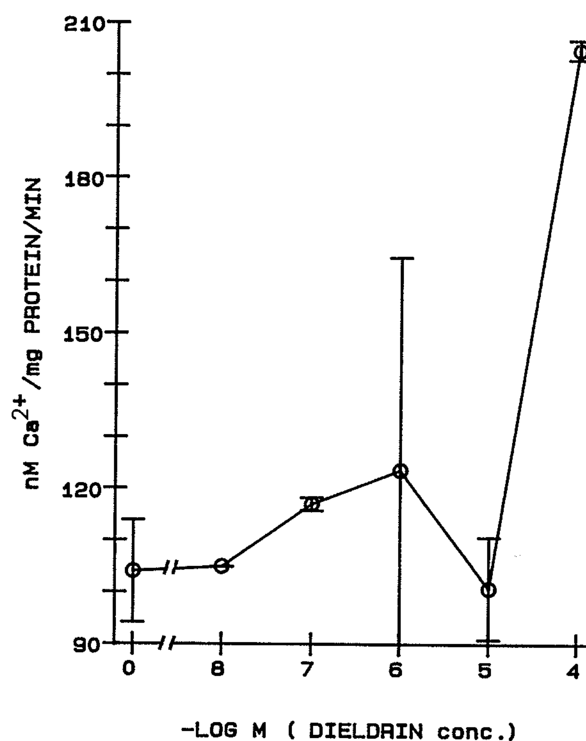

Figure 40. Effect of dieldrin on ATP-dependent calcium uptake by brain synaptosome membrane vesicles of the bertha armyworm *Mamestra configurata* WLK. Experimental variability is reported as standard deviation. Error bars are omitted when they are within the circle.

In vivo Poisoning Experiments

Moths were treated with various insecticides and after the onset of symptoms of poisoning their brains were removed for synaptosome preparation and measurement of the activity of Ca^{++}+Mg^{++}-ATPase. Since preliminary studies revealed regional differences in the activity of Ca^{++}+Mg^{++}-ATPase during poisoning, the brain hemispheres were separated from the optic lobes and each region was studied separately.

The effect of *in vivo* insecticide treatment on synaptosomal Ca^{++}+Mg^{++}-ATPase depended on the severity of poisoning. At an early stage of allethrin poisoning, the Ca^{++}+Mg^{++}-ATPase activity of optic lobe was inhibited significantly, but the synaptosome Ca^{++}+Mg^{++}-ATPase of brain was little affected (Fig. 41). When the insects were dissected at a late stage of allethrin poisoning, the synaptosome Ca^{++}+Mg^{++}-ATPase activities of both brain and optic lobe were inhibited (Fig. 42). In an early stage of DDT poisoning, brain Ca^{++}+Mg^{++}-ATPase activity was stimulated and the activity of synaptosome Ca^{++}+Mg^{++}-ATPase of optic lobe was inhibited (Fig. 43). At a late stage of DDT poisoning, the activities of synaptosome Ca^{++}+Mg^{++}-ATPase from both brain and optic lobe were inhibited (Fig. 44). The situation with respect to chlordane poisoning was somewhat different. At an early stage of poisoning, the synaptosome Ca^{++}+Mg^{++}-ATPase activity of optic lobe was stimulated significantly, whereas

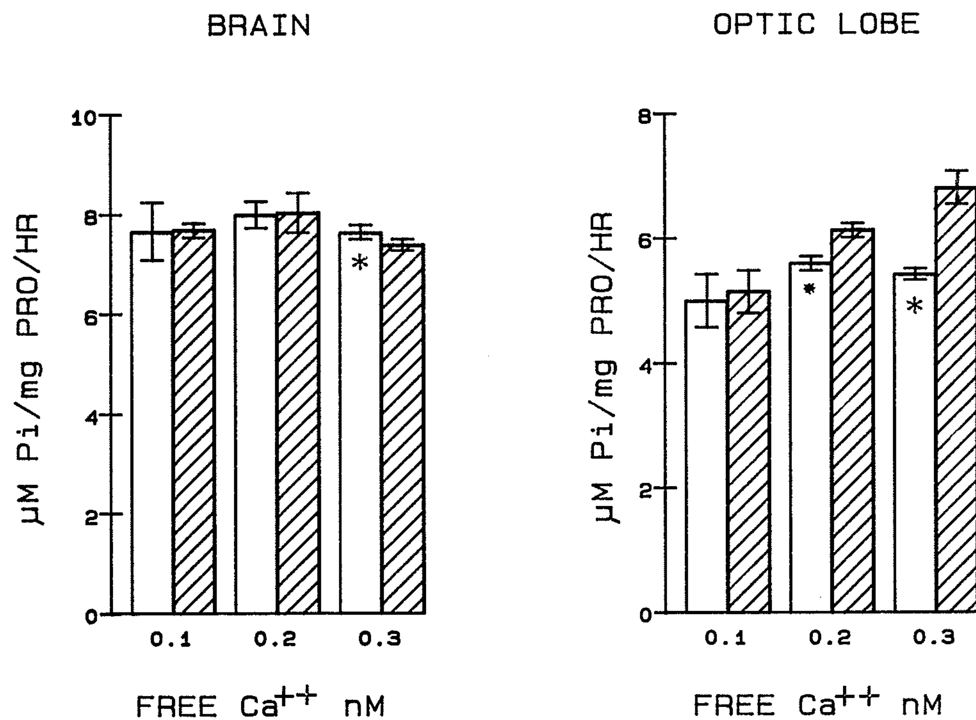

Figure 41. Effect of *In vivo* allethrin treatment on brain synaptosome Ca^{++} +Mg^{++}-ATPase activity of the bertha armyworm *Mamestra configurata* WLK. as a function of free calcium in the incubation medium. Brains were dissected when insects were at an early stage of poisoning. The error bars are standard deviation of four independent measurements.

[open box] insecticide treatment
[hatched box] control
* Significant by student "t" test at $P < 0.001$
* Significant by student "t" test at $P < 0.01$

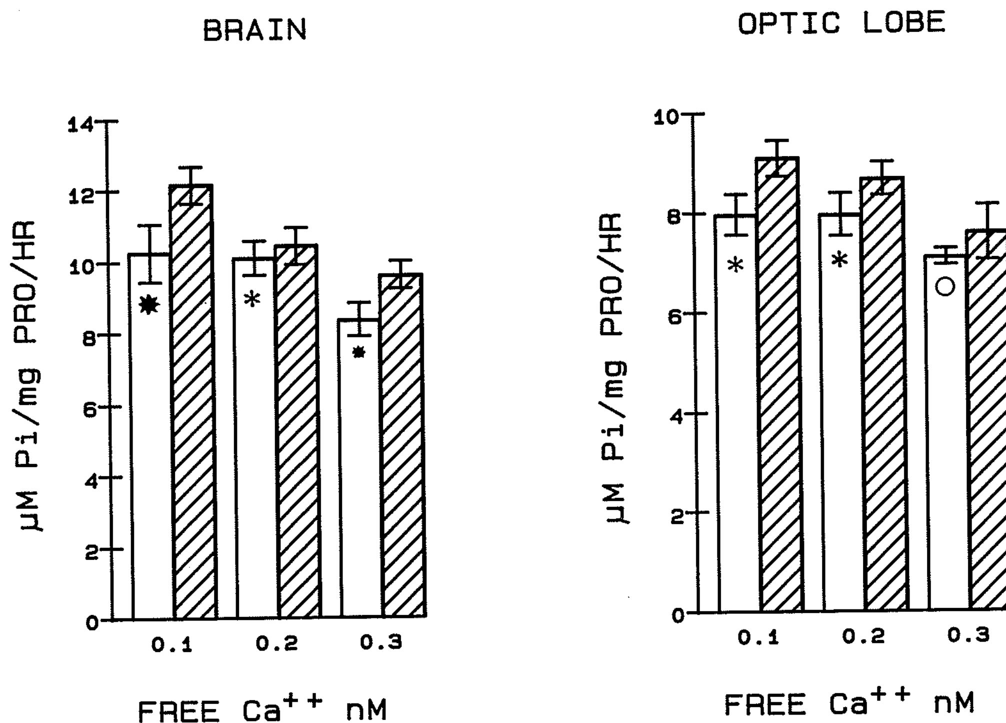

Figure 42. Effect of *In vivo* allethrin treatment on brain synaptosome $Ca^{++}+Mg^{++}$-ATPase activity of the bertha armyworm *Mamestra configurata* WLK. as a function of free calcium in the incubation medium. Brains were dissected when insects were at a late stage of poisoning. The error bars are standard deviation of four independent measurements.

- ▭ insecticide treatment
- ▨ control
- * Significant by student "t" test at $P < 0.001$
- ✱ Significant by student "t" test at $P < 0.005$
- * Significant by student "t" test at $P < 0.01$
- ○ Significant by student "t" test at $P < 0.05$

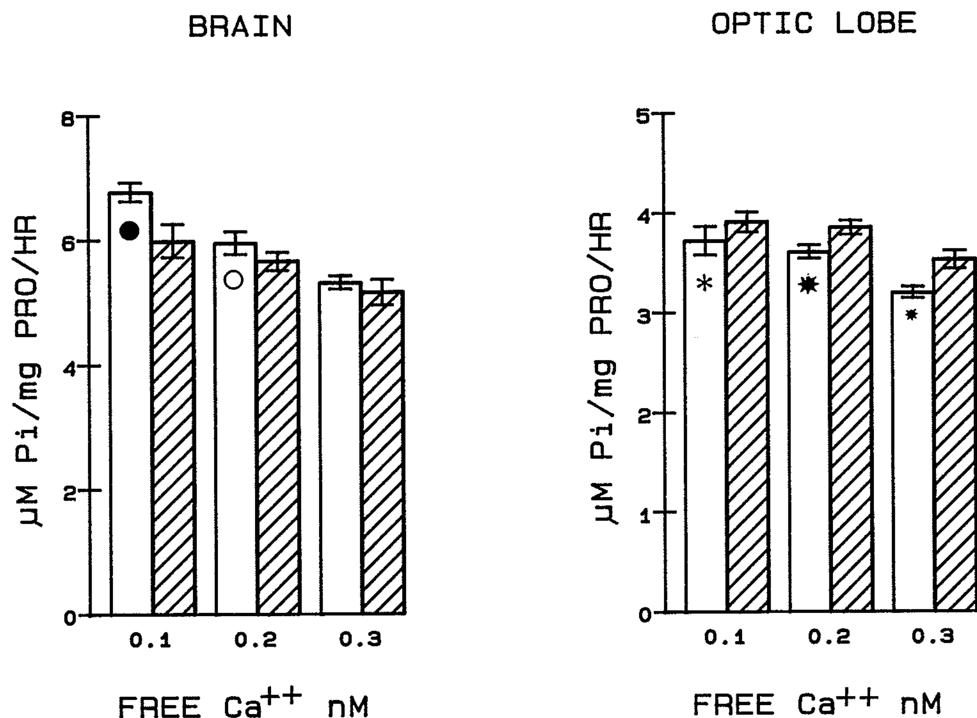

Figure 43. Effect of *In vivo* DDT treatment on brain synaptosome Ca^{++} $+Mg^{++}$ -ATPase activity of the bertha armyworm *Mamestra configurata* WLK. as a function of free calcium in the incubation medium. Brains were dissected when insects were at an early stage of poisoning. The error bars are standard deviation of four independent measurements.

- ▭ insecticide treatment
- ▨ control
- * Significant by student "t" test at $P < 0.001$
- ✸ Significant by student "t" test at $P < 0.005$
- * Significant by student "t" test at $P < 0.01$
- ● Significant by student "t" test at $P < 0.025$
- ○ Significant by student "t" test at $P < 0.05$

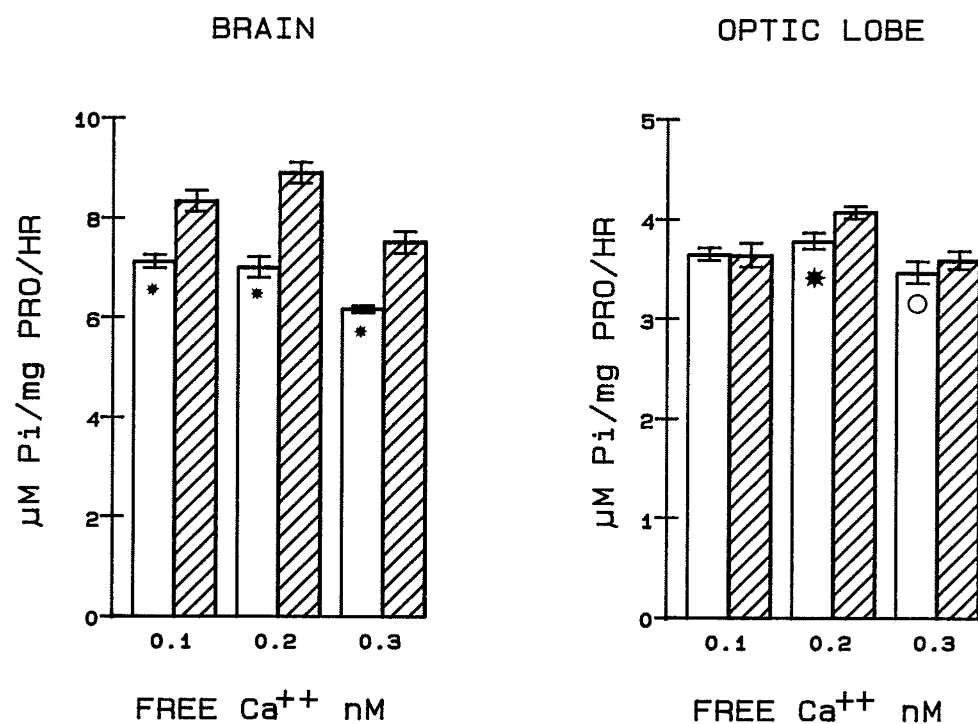

Figure 44. Effect of *In vivo* DDT treatment on brain synaptosome $Ca^{++}+Mg^{++}$-ATPase activity of the bertha armyworm *Mamestra configurata* WLK. as a function of free calcium in the incubation medium. Brains were dissected when insects were at a late stage of poisoning. The error bars are standard deviation of four independent measurements.

insecticide treatment (open bar)

control (hatched bar)

* Significant by student "t" test at $P < 0.001$

✸ Significant by student "t" test at $P < 0.005$

○ Significant by student "t" test at $P < 0.05$

the brain Ca^{++}+Mg^{++}-ATPase activity was inhibited (Fig. 45). As symptoms of poisoning became more severe, the brain synaptosome Ca^{++}+Mg^{++}-ATPase was stimulated and the optic lobe synaptosome Ca^{++}+Mg^{++}-ATPase was inhibited (Fig.46). At a late stage of chlordane poisoning, the brain synaptosome Ca^{++}+Mg^{++}-ATPase activity was inhibited, whereas the activity of optic lobe synaptosome Ca^{++}+Mg^{++}-ATPase was not different from that of the control (Fig. 47).

When different free calcium concentrations were used in the experiment, the pattern of the effect of *in vivo* insecticide treatment on Ca^{++}+Mg^{++}-ATPase varies. It appears that the *in vivo* insecticide treatment inhibited the part of Ca^{++}+Mg^{++}-ATPase activity which is sensitive to the change of free calcium concentration.

Effect of Temperature

Since temperature influences the action of many insecticides in different ways, I examined the effect of temperature on the inhibition of Ca^{++}+Mg^{++}-ATPase activity by five insecticides, a calmodulin inhibitor trifluoperazine and their mixture. Although the Ca^{++}+Mg^{++}-ATPase activity is much higher at 30 ℃ than that at 20 ℃ (Table 2.), the difference in percent inhibition of Ca^{++}+Mg^{++}-ATPase activity was not large. Permethrin, allethrin and decamethrin only exerted a slightly higher inhibition on Ca^{++}+Mg^{++}-ATPase at 20 ℃ than at 30 ℃, and so did the mixture of allethrin or DDT

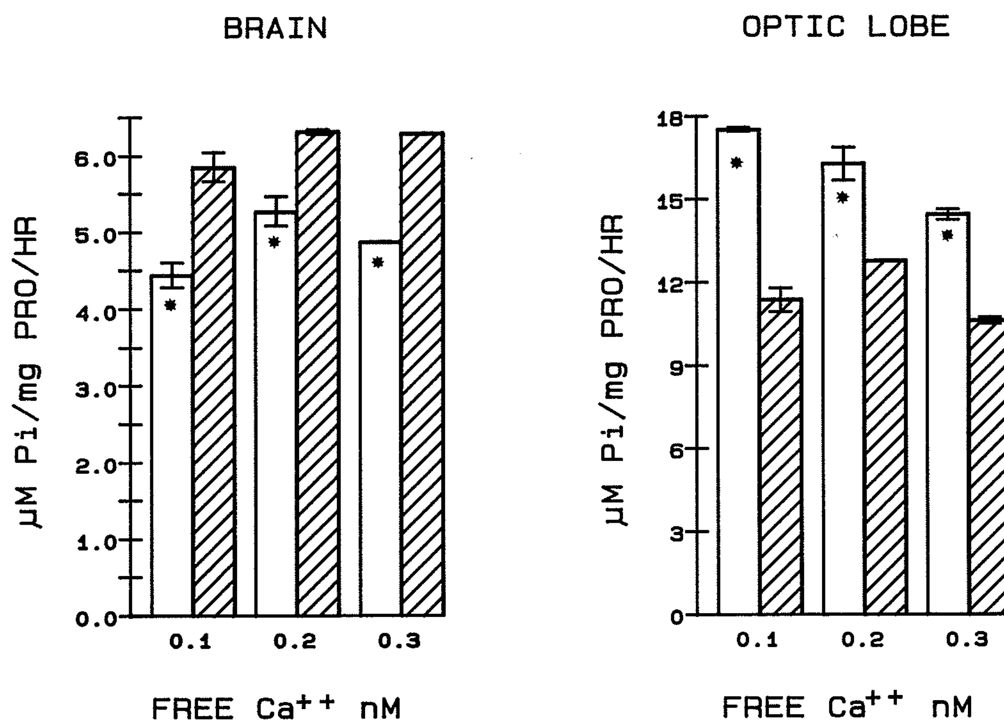

Figure 45. Effect of *In vivo* chlordane treatment on brain synaptosome Ca^{++}+Mg^{++}-ATPase activity of the bertha armyworm *Mamestra configurata* WLK. as a function of free calcium in the incubation medium. Brains were dissected when insects were at an early stage of poisoning. The error bars are standard deviation of four independent measurements.

▭ insecticide treatment

▨ control

* Significant by student "t" test at $P < 0.001$

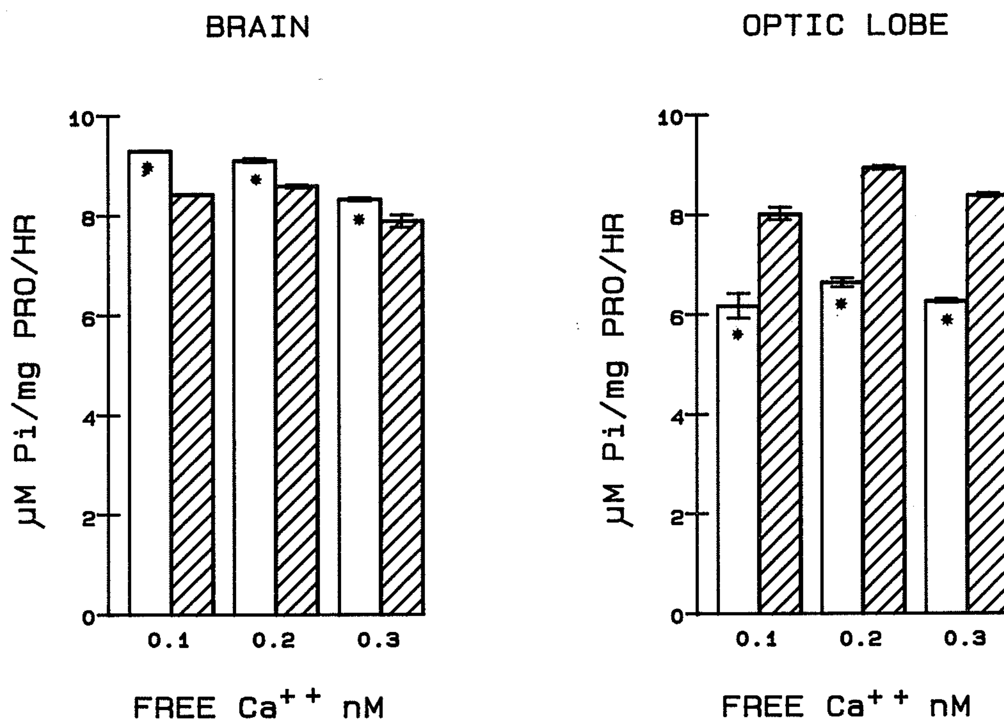

Figure 46. Effect of *In vivo* chlordane treatment on brain synaptosome Ca^{++}+Mg^{++}-ATPase activity of the bertha armyworm *Mamestra configurata* WLK. as a function of free calcium in the incubation medium. Brains were dissected when the poisoning symptom of insects was further developed. The error bars are standard deviation of four independent measurements.

☐ insecticide treatment

▨ control

* Significant by student "t" test at $P < 0.001$

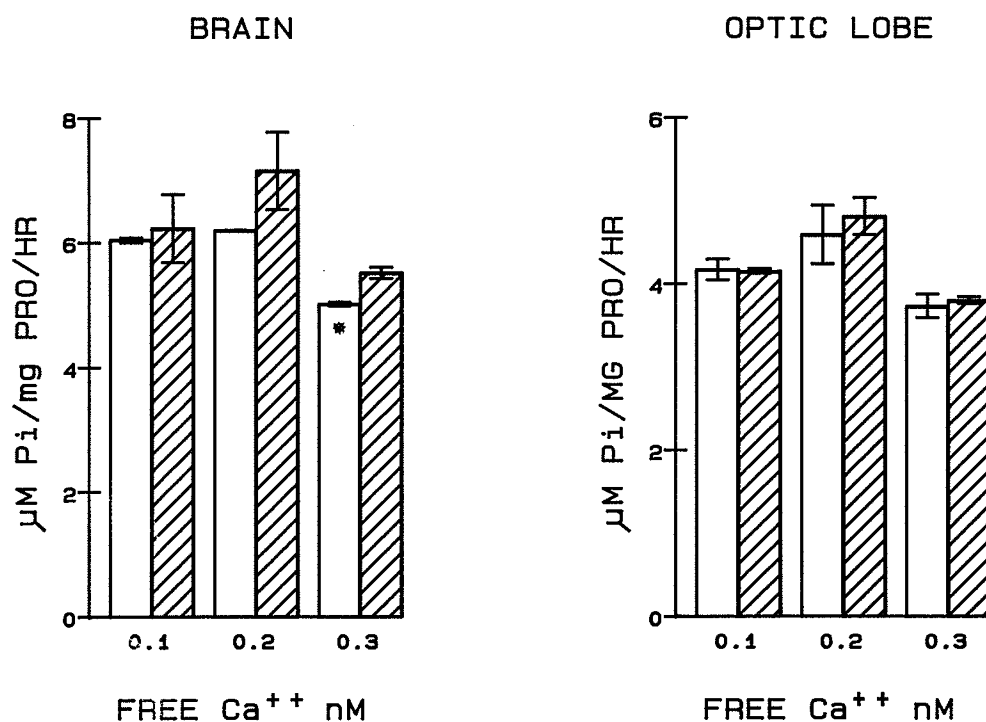

Figure 47. Effect of *In vivo* chlordane treatment on brain synaptosome Ca^{++}+Mg^{++}-ATPase activity of the bertha armyworm *Mamestra configurata* WLK. as a function of free calcium in the incubation medium. Brains were dissected when insects were at a late stage of poisoning.

▭ insecticide treatment

▨ control

* Significant by student "t" test at $P < 0.001$

TABLE 2. Effect of temperature on insecticide inhibition of moth brain synaptosomal Ca^{++}+Mg^{++}-ATPase of *Mamestra configurata* WLK.

	average inhibition (%)		enzyme activity * uM Pi/mg protein/h		remaining activity + (%)	
	20 °C	30 °C	20 °C	30 °C	20 °C	30 °C
control	0.0	0.0	3.80 ± 1.13	6.23 ± 1.73	61.00	100.00
permethrin	8.43	9.07	3.50 ± 1.12	5.61 ± 1.59	56.18	90.05
allethrin	21.47	29.07	3.06 ± 1.22	4.43 ± 1.57	49.06	71.11
decamethrin	8.00	5.17	3.51 ± 1.10	5.84 ± 1.59	56.29	93.69
DDT	27.20	33.57	2.79 ± 0.92	4.08 ± 1.10	44.78	65.54
toxaphene	56.90	70.17	1.68 ± 0.71	1.87 ± 0.71	26.91	30.02
trifluoperazine	11.27	20.87	3.19 ± 0.79	4.92 ± 1.60	51.26	78.92
permethrin + T	14.97	29.63	3.21 ± 0.84	4.39 ± 1.53	51.52	70.52
allethrin + T	45.87	51.83	2.16 ± 1.10	3.04 ± 1.26	34.62	48.85
decamethrin + T	22.20	31.70	2.96 ± 0.88	4.43 ± 1.32	47.51	71.05
DDT + T	41.37	49.80	2.26 ± 0.81	3.04 ± 0.62	36.28	48.80
toxaphene + T	67.43	78.37	1.30 ± 0.69	1.37 ± 0.61	20.87	21.94

T: trifluoperazine
*: Results are means and standard deviation of three independent experiments.
+: Activity of control at 30 °C taken as standard for both temperatures.

with trifluoperazine. In contrast, DDT, toxaphene, trifluoperazine and other mixtures all showed slightly higher inhibition of the activity of Ca^{++}+Mg^{++}-ATPase at 30 °C. However, upon further analysis of the results (Table 2.), it is clear that although there is little difference in the percent inhibition of Ca^{++}+Mg^{++}-ATPase at different temperatures, the total enzyme's activity at 20 ℃ was much lower than that at 30 ℃.

Because it is thought that Ca^{++}+Mg^{++}-ATPase is regulated by calmodulin (Chueng, 1980), a calmodulin inhibitor trifluoperazine was used in the experiment to examine if the inhibition of insecticides on Ca^{++}+Mg^{++}-ATPase is *via* the disruption of calmodulin regulation system. At 30 ℃ when trifluoperazine was added to the insecticide treatment, the effect were additive except in the case of toxaphene. The effect of toxaphene and trifluoperazine on Ca^{++}+Mg^{++}-ATPase was non-additive. However, at 20 ℃ the effect of trifluoperazine was non-additive with the effect of permethrin, was enhanced when mixed with allethrin and was additive to the effect of the rest of insecticides.

Discussion

Among 18 compounds from seven major groups of insecticides only six insecticides showed potent inhibition of Ca^{++}+Mg^{++}-ATPase from moth brain synaptosomes and synaptosome membrane vesicles. Four of these six insecticides

were cyclodiene compounds. Three of the cyclodiene compounds which showed the most potent inhibition are cyclodiene compounds containing no oxygen. Other cyclodiene compounds, such as endrin and dieldrin which displayed weak inhibition of Ca^{++}+Mg^{++}-ATPase, are cyclodiene compounds containing oxygen. Therefore, it appears that Ca^{++}+Mg^{++}-ATPase in the moth brain nerve endings is a target of some cyclodiene compounds, especially cyclodiene compounds containing no oxygen. The inhibition of Ca^{++}+Mg^{++}-ATPase which I have demonstated to be a calcium pump, would increase the concentration of free calcium in the presynaptic terminal, and therefore prolong neurotransmitter release. This provides a biochemical basis for early observations that cyclodiene insecticides facilitated transmission across central synapses in insects (Wang et al, 1971) and induced neurotransmitter release (Beeman, 1982).

In addition to these cyclodiene compounds, DDT also inhibited Ca^{++}+Mg^{++}-ATPase activity of moth brain synaptosomes. High concentrations of external calcium antagonize DDT- or pyrethroid-induced nerve excitation (Matsumura and Narahashi, 1971; Gammon, 1978a). Previous studies also showed that DDT is a potent inhibitor of a Ca^{++}-dependent ATPase in lobster peripheral axons (Ghiasuddin and Matsumura, 1979a,b,c). However, whether DDT would inhibit Ca^{++}+Mg^{++}-ATPase activity of insect nervous system had not been demonstrated. Using synaptosomal preparations from the

brain of bertha armyworm, I demonstrated for the first time that DDT inhibited the activity of Ca^{++}+Mg^{++}-ATPase in the insect nervous system.

The effect of pyrethroids on Ca^{++}+Mg^{++}-ATPase of moth brain synaptosomal preparations appears to be different from their effect on Ca^{++}+Mg^{++}-ATPase of squid axons (Clark and Matsumura, 1982). Permethrin, allethrin and decamethrin greatly inhibited Ca^{++}+Mg^{++}-ATPase of squid axons (Clark and Matsumura, 1982). However, among these three pyrethroids only allethrin showed inhibition of Ca^{++}+Mg^{++}-ATPase of the moth brain synaptosomes and synaptosome membrane vesicles. The allethrin inhibition of Ca^{++}+Mg^{++}-ATPase was higher when using synaptosome membrane vesicles. Because the synaptosome membrane vesicles consisted of inside-out membrane vesicles, rightside-out membrane vesicles and some unsealed membranes, it appears that the exposure of inner membrane surface to the insecticide increased the effectiveness of allethrin.

Since Ca^{++}+Mg^{++}-ATPase and the calcium pump are functionally coupled in the synaptic membrane, it has been assumed in the literature that insecticide inhibition of the ATPase would abolish calcium transport. This assumption has never been tested experimentally. Therefore, I compared the effect of insecticides on both Ca^{++}+Mg^{++}-ATPase and the ATP-dependent Ca^{++}-pump in moth brain synaptosome membrane vesicles under identical conditions. Five of the seven insecticides tested inhibited both the ATPase and calcium

transport. However, DDT and dieldrin inhibited Ca^{++}+Mg^{++}-ATPase activity but stimulated ATP-dependent calcium transport! Thus, it is erroneous to assume that insecticides which inhibit Ca^{++}+Mg^{++}-ATPase also inhibit calcium transport in the neurone. The mechanism by which DDT and dieldrin stimulated calcium transport is unresolved but differs from that of other insecticides. The stimulation of calcium transport in moth brain synaptosome membrane vesicles is consistent with the effect of these insecticides on calcium movement in the nervous system of other insects. For example, when isolated nerve cords of cockroaches were incubated with 10^{-5} M DDT, the efflux of $^{45}Ca^{++}$was higher than in the control (Matsumura and O'Brien, 1966). Dieldrin also activated calcium efflux from the nerve cord of the American cockroach and from isolated brain tissues of the German cockroach (Hayashi and Matsumura, 1966). Both of these observations are now explained by the fact that DDT and dieldrin stimulate Ca^{++}-transport in the synaptic membrane.

Cyclodiene insecticides may act by facilitating synaptic transmission and Ca^{++}-dependent neurotransmitter release (Wang et al, 1971; Schroeder et al, 1977; Beeman, 1982) through a disruption of calcium regulation in the nerve terminal (Yamagushi *et al.*, 1979, 1980). My study has provided the first direct evidence that some cyclodiene compounds, especially those cyclodienes containing no oxygen, inhibit the insect synaptosomal ATP-dependent calcium pump.

Inhibition of the ATP-dependent calcium pump will tend to increase free calcium concentration in the nerve terminal and accelerate neurotransmitter release, providing strong evidence that synaptosome Ca^{++}+Mg^{++}-ATPase is a target of certain cyclodiene insecticides. Cyclodienes containing oxygen, such as dieldrin and endrin, displayed very little inhibition of moth brain synaptosomal Ca^{++}+Mg^{++}-ATPase but nevertheless are insecticidal. This observation can be understood from the fact that dieldrin (and possibly endrin) has to be activated metabolically in order to be effective as an insecticide (Wang et al, 1971; Schroeder et al, 1977; Beeman, 1982). Evidently, synaptosomes and empty synaptosome membrane vesicles lack the cellular machinery to activate dieldrin which is a weak inhibitor of Ca^{++}+Mg^{++}-ATPase if it is not metabolically activated.

Of the three pyrethroids examined, permethrin, allethrin and decamethrin, only allethrin significantly inhibited the Ca^{++}+Mg^{++}-ATPase and ATP-dependent calcium transport activities of moth brain synaptosome membrane vesicles. These results differ from those on vertebrate and other arthropod neural Ca^{++}+Mg^{++}-ATPase (Clark, 1981; Clark and Matsumura, 1982). In those studies all three pyrethroids potently inhibited Ca^{++}+Mg^{++}-ATPase activity of squid *Loligo pealei* nervous system. The difference might be due to the difference in the nervous system between insects and other animals, or due to different neural preparations and assay

systems. *In vivo* allethrin poisoning also significantly inhibited moth brain synaptosomal Ca^{++}+Mg^{++}-ATPase, indicating that the inhibition of Ca^{++}+Mg^{++}-ATPase and ATP-dependent Ca^{++}-transport of moth brain nervous system may contribute to allethrin poisoning in insects.

Temperature influences the effectiveness of many insecticides in different ways. In some cases, high external temperature enhances the toxicity of insecticides, as in the case of cyclodiene insecticide poisoning. In other cases, the toxicity of insecticides is enhanced by low external temperature, such as in the case of DDT and the pyrethroids (Eaton and Sternbury, 1967; Wang *et al.*, 1972; Gammon, 1978a,b; Scott and Georghiou, 1984). The latter are said to have a negative temperature coefficient. Among factors which might be related to the negative temperature coefficient, the sensitivity of the target site appears to be an important factor. Electrophysiological studies suggested that the negative temperature coefficient of DDT action is the direct result of disrupting synaptic transmission (Gammon, 1978a). However, until now there has been no satisfactory explanation for the biochemical basis of the negative temperature coefficient. My results provide an explanation. First, the inhibition of synaptosomal Ca^{++}+Mg^{++}-ATPase, the ATP-dependent calcium pump, would disrupt the calcium regulation system and eventually lead to disrupted synaptic transmission. Second, Ca^{++}+Mg^{++}-ATPase activity at 20 ℃ is

40% lower than that at 30 ℃. This means that the calcium regulation system is more vulnerable to insecticide disruption at lower temperature. The same degree inhibition of Ca^{++}+Mg^{++}-ATPase would increase the disruption to a weakened calcium regulation system. Third, raising the temperature would strengthen a weakened calcium regulation system because of the higher Ca^{++}+Mg^{++}-ATPase activity at higher temperature. This could explain the observation that symptoms of DDT poisoning are reversible upon changing the temperature. The positive temperature coefficient of the toxaphene could also be explained by this result. Toxaphene inhibited 70% Ca^{++}+Mg^{++}-ATPase activity at 30 ℃ and at 20 ℃ only inhibited 56.9% Ca^{++}+Mg^{++}-ATPase activity. There is little difference between remaining activity of Ca^{++}+Mg^{++}-ATPase at 30 ℃ and that at 20 ℃.

SUMMARY AND CONCLUSION

Morphologically pure and functionally competent synaptosomes and synaptosome membrane vesicles were prepared from the moth brain of the bertha armyworm *Mamestra configurata* WLK.

A Ca^{++}+Mg^{++}-ATPase and an ATP-dependent calcium pump were identified in insect synaptosomal preparations and their biochemical and kinetic properties were studied under identical experimental conditions. The results showed that both Ca^{++}+Mg^{++}-ATPase and the ATP-dependent calcium pump had much higher affinity for free calcium when compared with that from vertebrates, displayed similar response to ATP, magnesium, EGTA, potassium, ouabain, and were functionally coupled in the synaptosome membrane.

Of eighteen compounds from seven major groups of insecticides, only six (DDT, allethrin, chlordane, aldrin, heptachlor and toxaphene) displayed potent inhibition of the synaptosomal Ca^{++}+Mg^{++}-ATPase. Three of these six insecticides (chlordane, aldrin and heptachlor) were cyclodiene compounds contained no oxygen. Dose response studies showed that five of these six insecticides, (chlordane, heptachlor, aldrin, toxaphene and allethrin), inhibited the activities of both Ca^{++}+Mg^{++}-ATPase and the calcium pump of moth bain synaptosomes under identical experimental conditions. DDT inhibited Ca^{++}+Mg^{++}-ATPase activity, but stimulated the

ATP-dependent calcium uptake aberrantly. *In vivo* insecticide treatment also significantly inhibited moth brain synaptosomal Ca^{++}+Mg^{++}-ATPase activity.

From these findings, it can be concluded that:

a) a Ca^{++}+Mg^{++}-ATPase which is an ATP-dependent calcium pump, exists in moth brain synaptosome membranes;

b) the very high affinity for free calcium, lower affinity for ATP and magnesium of the moth synaptosomal Ca^{++}+Mg^{++}-ATPase differentiate it from the Ca^{++}+Mg^{++}-ATPase of vertebrates;

c) the synaptosomal Ca^{++}+Mg^{++}-ATPase appears to be the target of some cyclodiene insecticides especially those containing no oxygen. The inhibition of the synaptosomal Ca^{++}+Mg^{++}-ATPase activity and its function also appears to be an important aspect of DDT and allethrin poisoning.

d) the inhibition of brain synaptosomal Ca^{++}+Mg^{++}-ATPase by insectides may provide an explanation for the negative temperature coefficient of some insecticides.

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APPENDIX I. LIST OF ABBREVIATIONS

Adn: aldrin

Ale: allethrin

AThch I: acetylthiocholine iodide

CABMS: carbamates

Cam: carbaryl

Cdn: chlordane

CF: chlordimeform

DCF: dimethylchlordimeform

Dec: decamethrin

Dia: diazinon

Drn: dieldrin

DTNB: 5,5′-dithio-*bis*-2-nitrobenzoic acid

Edn: endrin

EDTA: ethylenediaminetetraacetic acid

EGTA: ethyleneglycol-bis (beta-amino-ethyl ether) -N,N′-tetraacetic acid

FMS: formamidines

Hepes: N-2-hydroxyethylpiperazine-N′-2-ethanesulonic acid

Htc: heptachlor

INH: inhibitors

KCN: potassium cynate

La: lanthanum

Ldn: benzene hexachloride (lindane)

Mat: malathion

Mec: methoxylchlor

NADH: beta -nicotinamide adenine dinucleotide, reduced form

NADPH: beta -nicotinamide adenine dinucleotide phosphate, reduced form

OGPOS: organophosphates

Ovd: orthovanadate

Per: permethrin

Pdt: picrotoxinin

Prp: propoxur

PYRES: pyrethroids

T: trifluoperazine

Tox: toxaphene

Tri: trifluoperazine