## INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

# UNIVERSITY OF CINCINNATI

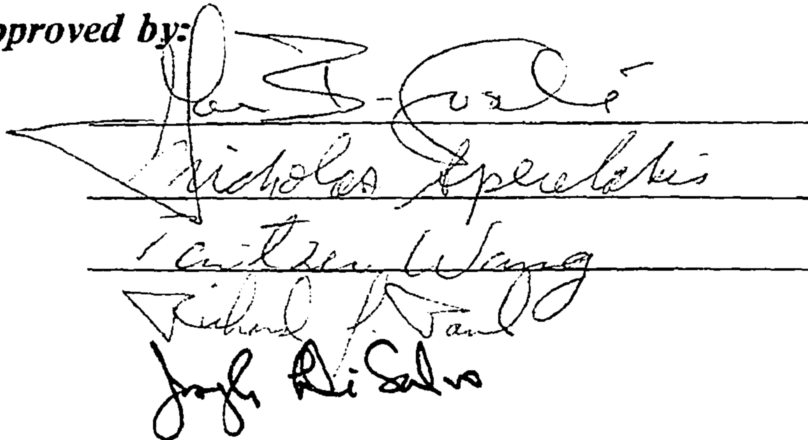
January 16 19 87

*I hereby recommend that the thesis prepared under my supervision by* Michael E. O'Leary

*entitled* Characterization of the Voltage-Sensitive Calcium Channels of Presynaptic Nerve Terminals

*be accepted as fulfilling this part of the requirements for the degree of* Doctor of Philosophy

*Approved by:*



Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

# Characterization Of The Voltage-Sensitive Calcium Channels Of Nerve Terminals

A dissertation submitted to the

Division of Graduate Studies

of the

University of Cincinnati

in partial fulfillment of the

requirements for the degree of

**Doctor of Philosophy**

in the Department of Physiology and Biophysics

of the College of Medicine

1987

by

**Michael Edward O'Leary**

B.S., University of Connecticut 1978

M.S., University of Connecticut 1982

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

UMI Number: DP16183

UMI®

UMI Microform DP16183

Copyright 2009 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

**Acknowledgments**

I would like to extend my thanks to my advisor, Dr. J.B. Suszkiw. The lessons in defining scientific questions and focusing research efforts I have learned under his guidance will follow me into the future.

To my thesis committee, Dr. Nicholas Sperelakis, Dr. Richard Paul, Dr. Joseph Di Salvo and Dr. Tiatzer Wang, I am indebted for the critical reviews which helped shape my thesis.

I would like to thank Dr. Kline, the faculty, students and employees of the department with whom I have favorably interacted during the course of my graduate education.

I would like especially to acknowledge my friend Michael K. Murawsky. His contributions have assisted me through many personal and scientific trials.

I am greatful for the love and patience of Ms. Randa Sifri. Her support has helped throughout the preparation of this dissertation.

This thesis is dedicated to my parents, James and Mary Ann O'Leary. Though not always familiar with the details of my endeavors, their unwavering faith has been a great source of strength for me.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## Abstract

Depolarization-dependent calcium influx is biphasic in synaptosomes (Nachshen and Blaustein, 1980, 1982). The "fast" and "slow" components of $^{45}Ca^{++}$ influx are proposed to occur through separate calcium channels. In this report the fast and slow components of depolarization-dependent $^{45}Ca^{++}$ influx are resolved. The fast component is inactivated by predepolarization, inhibited by low concentrations of lanthanum and closely correlates with the rapid release of $^{3}H$-norepinephrine from synaptosomes. The properties of the fast component of influx suggest that it is mediated by calcium channels which inactivate during the first second of depolarization. The slow component is not sensitive of predepolarization and is partially dependent on the internal $Na^{+}$ content of the synaptosomes. It is proposed that during depolarization a portion of the slow component of $Ca^{++}$ influx in synaptosomes is mediated by a $Na_i/Ca_o$ exchange mechansim.

The influx mediated by calcium channels is not sensitive to Bay K 8644 (0.01-1 mM), nitrendipine (0.1-10 mM) or nifedipine (0.01-10 mM). Calcium channels of synaptosomes are best classified as N-type (Nowycky et al. 1985).

Preincubation of synaptosomes with 1 mM 8-bromo cAMP, 50 $\mu$M forskolin and 10 $\mu$M diacetate phorbol ester did not alter depolarization-dependent $^{45}Ca^{++}$ influx. Under similar preincubation conditions forskolin and diacetate phorbol ester significantly increased the $^{32}P_i$ incorporation into several synaptosomal proteins ($M_r$ 55, 76, 81 kD).

Recent evidence indicates that the nitrendipine receptor of skeletal muscle calcium channels is a substrate for cAMP dependent protein kinase (Curtis and Catterall, 1985). These results suggest that the regulation of calcium channels by phosphorylation/dephosphorylation may be exerted via the dihydropyridine receptor. Calcium channels of synaptosomes are not sensitive to dihydropyridines and are not

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

regulated by either cAMP or protein kinase C dependent phosphorylation. The results demonstrate that calcium channels in brain nerve terminals are pharmacologically distinct from calcium channels of other systems.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## Table Of Contents


| | | Page |
|---|---|---|
| 1. | **Title Page** | 1 |
| 1. | **Acknowledgments** | 2 |
| 2. | **Abstract** | 3 |
| 4. | **Table of Contents** | 5 |
| 3. | **List of Figures** | 8 |
| 4. | **List of Tables** | 9 |
| 5. | **Introduction** | |
| | A. Overview | 10 |
| | B. The "Calcium Hypothesis" | 11 |
| | C. Cellular Metabolism Of Calcium | 13 |
| | D. Morphological and Metabolic Characteristics of Synaptosomes | 15 |
| | E. Ion Transport by Synaptosomes | 16 |
| | F. Metabolism of Neurotransmitter by Synaptosomes | |
| | 1. Uptake of Precursors and Neurotransmitters | 19 |
| | 2. Release of Neurotransmitter | 21 |
| | G. Calcium Transport by Synaptosomes | |
| | 1. Calcium Efflux | 22 |
| | 2. Calcium Influx | 23 |
| | H. Effects of Dihydropyridine Drugs on Calcium Channels | 25 |
| | I. Regulation of Calcium Channels by Phosphorylation | 27 |
| | J. Regulation of Calcium Channels by Inactivation | 28 |


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

| | | |
|---|---|---|
| | K. Summary | 30 |
| 6. | **Specific Objectives of this Research** | 32 |
| 7. | **Methods** | |
| | A. Hepes-buffered Krebs Ringer Solution | 33 |
| | B. Preparation of Synaptosomes | 33 |
| | 1. Hajos Preparation | 33 |
| | 2. Gray and Whittaker Preparation | 34 |
| | C. Measurement of Intrasynaptosomal Volume | 34 |
| | D. Measurements of Synaptosomal Sodium and Potassium Concentrations | 35 |
| | E. Calcium Influx Measurements | 36 |
| | 1. Dihydropyridine Drugs | 37 |
| | 2. cAMP, Forskolin, Phorbol Esters | 37 |
| | F. Inactivation of Calcium Influx | 37 |
| | G. Reactivation of Calcium Influx | 38 |
| | H. Voltage Dependency of Inactivation | 38 |
| | I. Lanthanum Blockade of Calcium Influx | 39 |
| | J. Measurement of the Slow Component of Calcium Influx | 39 |
| | K. Norepinephrine Release | 40 |
| | L. Preparation of Synaptosomes for Phosphorylation Experiments | 40 |
| | M. Polyacrylamide Gel Electrophoresis | 41 |
| | N. Incubation of Synaptosomes with Drugs | 41 |
| | O. Determination of Free Drug Concentrations | 42 |
| | P. Statistical Methods | 42 |


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

8. **Results and Discussion**

A. Intrasynaptosomal Concentrations of Sodium and Potassium Ions 43

B. Time Course of Depolarization-Dependent Calcium Influx 47

C. Inactivation of Calcium Influx 51

D. Reactivation of Calcium Influx 55

E. Voltage Dependency of Inactivation 57

F. Blockade of Calcium Influx by Lanthanum 59

G. Characterization of the Slow Component of Calcium Influx 62

H. Relationship of Norepinephrine Release to Calcium Influx 68

I. Effects of Dihydropyridine Drugs on Calcium Influx 73

J. Regulation of Calcium Influx by Protein Phosphorylation 81

9. **Conclusions** 88

10. **References** 92


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## List of Figures


1. Time Course of Depolarization-Dependent Calcium Influx 50
2. Voltage-dependent Inactivation and Reactivation 54
3. Voltage Dependency of the Inactivation Process 58
4. Blockade of Calcium Influx by Lanthanum 61
5. Characterization of the Slow Component of Calcium Influx 66
6. Contribution of Slow Component Influx during the First Second of Depolarization 67
7. Depolarization-Dependent Release of Norepinephrine 71
8. Comparison of Depolarization-Dependent Calcium Influx and Norepinephrine Release 72
9. Determination of Sub-Maximal Depolarization Conditions 77
10. Comparison of Calcium Influx from Several Types of Synaptosomes 78
11. Effects of Bay K 8644 on Depolarization-Dependent Calcium Influx 79
12. Effects of Dihydropyridine Antagonists on Depolarization-Dependent Calcium Influx 80
13. Effects of cAMP, Forskolin and Phorbol Esters on Depolarization-Dependent Calcium Influx 84
14. Phosphorylation Pattern of Synaptosomal Proteins 85
15. Autoradiogram of Synaptosomal Phosphorylation 86


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

**List of Tables**

1. Determination of Sodium and Potassium Concentrations of Synaptosomes 46

2. Phosphorylation of Peak I and Peak II 87

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

# Introduction

## A. Overview

Most cells generate a small electrical potential across their membranes. What distinguishes nerve and muscle from non-excitable tissue is that the electrical energy stored across the membrane as voltage can be selectively dissipated in order to produce current. The transfer of information throughout a given neuron is accomplished by the conductance of electrical signals along the cell membranes. The most important mode of conduction is the action potential. Action potentials are transient voltage changes produced across the membrane which are generated by time-dependent alterations of membrane permeability to $Na^+$ and $K^+$ (Hodgkin and Huxley, 1952). These ions traverse the hydrophobic lipid membrane through integral membrane proteins which form aqueous filled channels. The voltage sensitivity and transient nature of the ion fluxes are imparted by gating mechanisms intrinsic to the channel. Action potentials are generated by the dissapation of the membrane voltage due to ion flow through the channels. These potentials spread vectorially along membranes by affecting similar conductance changes in the channels of the adjacent patches of membrane. In this fashion action potentials of equivalent voltage amplitude are sequently produced along the entire neuronal membrane. This regenerative feature makes the action potential ideal for intracellular communication between the dendrites, cell body and nerve terminal, distances which are frequently measured in millimeters.

The role of neurons in information transfer required the co-ordinated interaction of individual cells. Coping with the task of intercellular communication is an obstacle which spurred further specialization of the neuron. Bridging the extracellular gap between neurons is accomplished by the release of neurotransmitters from specialized

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

presynaptic terminals. These chemicals diffuse across the synaptic cleft to the postsynaptic neuron where they bind to specific receptors for that particular chemical. This in turn may generate a response in the postsynaptic cell and the process of action potential conduction and transmitter release is repeated in the postsynaptic neuron. The role of the presynaptic terminal is to convert the electrical signal of the presynaptic cell to a corresponding release of neurotransmitter. The relationship between presynaptic ionic fluxes and the release of neurotransmitter at mammalian nerve terminals has not been accurately described.

B. The "Calcium Hypothesis"

Katz and Miledi established the frog neuromuscular junction as a model of synaptic function. Action potentials (AP) in the nerve fibers leading to the muscle were evoked by extracellular stimulation of the nerve while the post synaptic potentials (PSP) were recorded from the muscle. Tetrodotoxin (TTX) inhibits voltage-sensitive sodium channels and blocked both the nerve fiber AP and the subsequent muscle PSP (Katz and Miledi, 1968). It appeared that propagation of the action potential into the terminal region is required to initiate the release process. However, if the stimulus were localized so as to produce non-regenerative electrotonic potentials in the terminals, a PSP could be elicited in the presence of TTX or in the absence of $Na_0$. These results demonstrated that the requirement of $Na_0$ in the release process is limited to it's role in generating the action potential and that the release is the result of terminal depolarization and not directly due to $Na^+$ influx (Katz and Miledi, 1967b). It had been previously observed that neither action potentials (Katz and Miledi, 1965) nor electrotonic potentials (Katz and Miledi, 1967a) evoked a PSP when calcium was omitted from the media. In both instances the depolarizing stimulus invaded the terminal region but failed to stimulate

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

release. Utilizing localized ionotophoretic $Ca^{++}$ injections it was determined that $Ca^{++}$ must be present precisely during the terminal depolarization in order to affect transmitter release (Katz and Miledi, 1967a). This suggested that the influx of $Ca^{++}$ is the link between terminal depolarization and transmitter release.

The small size of the neuromuscular synapse makes the direct measurement of presynaptic activity difficult and postsynaptic recordings are employed as indicators of presynaptic transmitter release. To overcome these limitations Katz and Miledi adopted the squid giant synapse, a preparation in which both pre- and postsynaptic elements of the synapse can be simultaneously impaled with microelectrodes. As in the neuromuscular preparation, TTX blocked both the presynaptic action potential and the PSP (Katz and Miledi, 1967c). Direct intracellular stimulation of the presynaptic terminal produced an electrotonic potential which elicited a PSP. The magnitude of the PSP versus presynaptic depolarization is a steep function indicating that the PSP has a voltage threshold. Voltage-sensitive $Na^{+}$ and $K^{+}$ currents can be blocked by tetrodotoxin and tetraethylammonium respectively. In these preparations a small regenerative calcium current can be measured which is restricted to the presynaptic terminal (Katz and Miledi, 1969a, 1969b). It was clear that the previously observed threshold behavior of the PSP is a manifestation of the voltage dependency of the presynaptic calcium channels. These results corroborated their previous observations in the neuromuscular junction. In addition it was observed that electrotonic potentials of equivalent magnitude as action potentials are as effective in triggering transmitter release and eliciting post synaptic potentials (Katz and Miledi, 1967c). Increasing $[Ca^{+}]_{o}$ augments the PSP by increasing $Ca^{++}$ influx and hence facilitating transmitter release (Katz and Miledi, 1968, 1970). The role of $Ca^{++}$ in the release process was confirmed when Miledi (1973) observed that injections of $Ca^{++}$ directly into the presynaptic terminal resulted in post synaptic responses in the absence of appreciable depolarization

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of the presynaptic membrane. These results dissociated the depolarization of the presynaptic terminal from the release of transmitter and established $Ca^{++}$ influx as the link between the two processes. Further evidence supporting this hypothesis was provided by Llinas et al. (1972) and Llinas and Nicolson, (1975) by loading the squid synapse with the bioluminescent $Ca^{++}$ probe aequorin. The results demonstrated that the build up of ionized $Ca^{++}$ in the presynaptic terminal closely correlates with the release of transmitter and the generation of post synaptic responses. These experiments led Katz and Miledi to propose the "Calcium Hypothesis" (Katz and Miledi, 1969a). They envisioned that the invasion of the action potential into the nerve terminal causes a depolarization of the membrane resulting in the opening of voltage-sensitive calcium channels. The build up of ionized $Ca^{++}$ within the cytoplasm of the terminal triggers the release of neurotransmitter.

C. Cellular Metabolism of Calcium

The pivotal role played by calcium in the exocytosis process requires that the intracellular concentration of this ion be efficiently constrained within narrow limits. The maintenance of intracellular ionized $Ca^{++}$ at low levels is the result of interplay between the fluxes across the membrane, buffering and energy-dependent sequestering (Requena, 1983). In regulating internal $Ca^{++}$ the transmembrane $Na^{+}$ and electrical gradients are important considerations in a number of systems (Requena, 1983; Di Polo and Beauge, 1983). $[Ca]_i$ of the squid giant axon is 110 nM (DiPolo et al., 1982) despite the concentration of 4 mM in hemolymph (Blaustein, 1974). The squid axon is particularly well suited for the study of ionic fluxes because the interior of the axon is sufficiently large to allow dialysis and hence control of the internal solutions. It is proposed that the low concentration in axoplasm is maintained by an outwardly directed

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

calcium pump and the $Na_o/Ca_i$ exchange (NaCaX) (Requena, 1983). A large portion of the $Ca^{++}$ efflux from squid axon was found to be dependent on the $Na^+$ gradient (Blaustein and Hodgkin, 1969). Reduction $Na_i$ dramatically stimulates the rate of $Ca^{++}$ efflux which persists despite reduced ATP concentrations (Blaustein and Hodgkin, 1969; Blaustein and Russell, 1975; Blaustein, 1977). These results imply that the energy required to extrude $Ca^{++}$ from the axoplasm is supplied by the inward movement of $Na^+$ down it's electrochemical gradient. From energetic considerations the coupling of two or more $Na^+$ would be required to exchange for a single $Ca^{++}$. Depolarization induced by electrical stimulation or elevated $K_o$ was found to reduce $Ca^{++}$ efflux from dialyzed axons (Brinley and Mullins, 1974; Mullins and Brinley, 1975; Blaustein et al., 1974). The observed voltage sensitivity confirms that the process is electrogenic, translocating at least three $Na^+$ in exchange for each $Ca^{++}$.

Coupling of $Ca^{++}$ influx to the ouabain-insensitive $Na^+$ efflux is also observed in squid axon (Baker et al., 1969). $Ca^{++}$ influx is strongly dependent on the resting membrane potential and $Na_i$. Depolarization induced by elevated $K_o$ substantially increased $Ca^{++}$ influx indicating that the transport mechanism is electrogenic (DiPolo et al., 1982). Influx through this system is not inhibited when the membrane potential is held at the $Ca^{++}$ reversal potential (Requena et al., 1983). During depolarization the influx of $Ca^{++}$ into the axon is 70% via a TTX-sensitive pathway (ie. sodium channel), about 20% by a $Na_i$-dependent (NaCaX) and the remaining penetrates through a route which is insensitive to concentration changes of $Na^+$, $Ca^{++}$, ATP or TTX (DiPolo et al., 1982). It is proposed that the $Ca^{++}$ fluxes dependent on $Na^+$ (ie. influx and efflux) are manifestations of a single system that runs according to the direction of the $Na^+$ electrochemical gradient (Mullins and Brinley, 1975). The bi-directional nature of this exchange underscores it's importance in regulating the intracellular concentration of ionized $Ca^{++}$ (Requena, 1983; Di Polo and Beauge, 1983).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

D. Morphological and Metabolic Characteristics of Synaptosomes

Despite the abundance of evidence supporting the "Calcium Hypothesis" in the invertebrate system, the role of calcium in the release process of the mammalian nervous system remains uncertain because the small size and inaccessibility of the terminals eliminates direct electrical recording techniques. The majority of evidence supporting the "Calcium Hypothesis" in the mammalian central nervous system is derived from a preparation of pinched off nerve-endings termed "synaptosomes" (Whittaker, et al., 1964). Synaptosomes are isolated from the bulk of brain tissue by homogenization and sucrose density centrifugation (Gray and Whittaker, 1962; DeRobertis et al., 1962). During the disruption process the presynaptic terminal and the postsynaptic density to which it is attached are torn away from the axon and post-synaptic cell, respectively. The axon at its attachment to the synaptic terminal is slender, typically measuring one fifth the diameter of the synapse. During homogenization this juncture is severed and the synaptic membrane reseals. This resealing process occurs rapidly since the soluble enzymes and metabolites endogenous to the nerve terminal remain trapped within the synaptosome (Johnson and Whittaker, 1963; Magan and Whittaker, 1966; Bradford, 1969; Bradford and Thomas, 1969). Inspection via electron microscopy reveals oval structures approximately 0.5 $\mu$m in diameter with continuous delimiting membranes containing numerous organelles and an occasional small mitochondria (Whittaker, 1969). Typically contained within synaptosomes are round organelles approximately 42 nm in diameter which are believed to represent synaptic vesicles. Dense and coated vesicles are also seen in appropriately stained preparations.

The most important criteria for defining the integrity of the synaptosome preparation is that it retain the functional activity characteristic of nerve terminals from

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

intact tissue. The synaptosomes should demonstrate basic competency regarding the utilization of substrate to generate metabolic intermediates and synthesize energy storage compounds. Only if these criteria are fulfilled can the preparation be expected to accurately reflect the normal physiology of a synaptic terminal.

Synaptosomes suspended in ringer solution actively respire utilizing glucose as a substrate (Bradford, 1969). Incubation with glucose increases oxygen consumption and stimulates the synthesis of ATP and phosphocreatine. Glucose stimulated oxygen consumption and synthesis of energy storage phosphates are inhibited by metabolic poisons which block glycolysis or oxidative phosphorylation (Bradford, 1969). Under conditions which enhance oxidative phosphorylation, synaptosomes convert $^{14}$C-glucose into a variety of metabolic intermediates (Bradford and Thomas, 1969). Following incubation, $^{14}$C label was found in $CO_2$, lactate and amino acids. Glucose stimulated metabolism is also linked to $^{32}$P-phosphate incorporation into phospholipids (Hokin and Hokin, 1955; Hokin and Hokin, 1958; Abdel-Latif 1966). The diverse variety of metabolic activities demonstrated by synaptosomes suggests the presence of the enzymes and cofactors known to be involved in these processes (Bradford and Thomas, 1969).

E. Ion Transport by Synaptosomes

The ability of neuronal membranes to generate and maintain ion gradients is of paramount importance to the normal functioning of the synapse. Synaptosomes confine $Na^+$, $K^+$ and the cytoplasmic soluble enzyme lactate dehydrogenase in an osmotically sensitive compartment (Marchbanks, 1967). By examining the differential distribution of these synaptosomal constituents under iso- and hyperosmotic conditions it was shown that this osomotically sensitive compartment represents the intrasynaptosomal volume. Synaptosomes maintain a high $[K]_i$ and reduced $[Na]_i$ within this space (Marchbanks and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Campbell, 1976; Campbell, 1976). This differential distribution of ions is sensitive to the metabolic inhibitors cyanide and iodoacetate (Bradford, 1969). These results imply that $Na^+$ and $K^+$ are actively pumped against their respective concentration gradients. The transmembrane movement of $Na^+$ can be monitored by the flux of radiolabeled $^{22}Na^+$. The accumulation of the tracer $^{22}Na^+$ within synaptosomes is stimulated by cyanide and iodoacetate (Ling and Abdel-Latif, 1968). In the presence of metabolic inhibitors $Na^+$ efflux was reduced owing to the shortage of metabolic energy, consequently the synaptosomes became loaded with $Na^+$ by passive inward diffusion. Under similar conditions the efflux of $Na^+$ is inhibited by ouabain (Ling and Abdel-Latif, 1968). These results suggest that the efflux of $Na^+$ from synaptosomes is an energy dependent process and is mediated by NaK-ATPase. The synaptosomal $K_i$ content can be measured by atomic absorption or radiolabeled $^{42}K^+$ accumulation (Escueta and Appel, 1969). The $[K]_i$ increases from 71 mM immediately following isolation to 103 mM after incubation at 37° C. Since the $[K]_o$ in these experiments was 10 mM these values represent a significant accumulation against a concentration gradient. This uptake is inhibited by the metabolic poisons cyanide and 2,4-dinitrophenol as well as by the NaK-ATPase inhibitor ouabain. The accumulation of $K^+$ by synaptosomes also appears to be an energy dependent process (Escueta and Appel, 1969). Isolated plasma membranes which display NaK-ATPase activity can be prepared by sub-fractionation of synaptosomes (Hosie, 1965). $Na^+$, $K^+$ and $Mg^{++}$ each individually stimulate the membrane associated ATPase and act synergistically in combination (Hosie, 1965). This ATPase activity is inhibited by ouabain. ATPase activity with similar cation requirements and ouabain sensitivity is localized in plasma membranes of intact synaptosomes (Kurokawa and Kato, 1965; Festoff and Appel, 1968). The activity of the NaK-ATPase and $K^+$ uptake both vary with the extracellular $[Na^+]_o$ (Festoff and Appel, 1968; Escueta and Appel, 1968). This $Na^+$-dependency is consistent

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

with the properties of NaK-ATPase previously reported in nerve (Skou, 1957). These results support the hypothesis that the synaptosomal membrane acts as a permeability barrier to diffusing ions and is capable of generating asymmetric ion distributions by energy-dependent transport processes.

Utilizing a light scattering technique for measurement of osmotic swelling (Koch, 1961; Keen and White, 1970) Keen and White (1971) determined the relative permeabilities of $Na^+$, $K^+$ and $Cl^-$ (1:19:12). Similar permeability ratios have been reported (Blaustein and Goldring, 1975). Synaptosomes have membrane permeability characteristics similar to intact neurons and may generate resting membrane potentials close to the $K^+$ diffusion potential. The resting membrane potential for these preparations can be approximated using the Constant Field Equation provided the major determinants of membrane potential are $Na^+$, $K^+$ and $Cl^-$ (Goldman,1943; Hodgkin and Katz, 1949). Utilizing this method the resting membrane potential of synaptosomes has been estimated to be -27 mV (Campbell, 1976). A more direct assessment of the membrane potential is obtained by the use of potential sensitive fluorescent probes (Waggoner, 1979). These agents are permeant to the cell membrane and work by potential dependent redistribution between the bathing media and the interior of the synaptosome. The fluorescent probe 3,3'-diphenyl 2,2'- oxacarbocyanine was used by Blaustein and Goldring (1975) to measure the resting and stimulated membrane potentials of synaptosomes. The results demonstrated that the $[K^+]_i$ is approximately 100 mM, thus corroborating previous estimates while further supporting the conclusion that the synaptosomal resting potential is primarily determined by the equilibration distribution of potassium.

F. Metabolism Of Neurotransmitter By Synaptosomes

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1. Uptake of Precursors and Neurotransmitters

Synaptosomes are capable of the synthesis and storage of a number of neurotransmitters. The metabolic precursors required for the synthesis of neurotransmitters are specifically transported across synaptosomal membranes by high affinity systems. Such systems are known to transport the neurotransmitter precursors choline (Haga and Noda, 1973; Yamamura and Snyder, 1973) and trytophan (Belin et al., 1974; Hamon et al., 1974). Acetylcholine (ACh) is formed through the acetylation of choline by a reaction requiring acetylcoenzyme A and the enzyme choline acetyltransferase. An efficient mechanism for the accumulation of choline is important to neurons because nervous tissue is incapable of synthesizing choline _de novo_ (Bremer and Greenberg, 1961; Ansell and Spanner, 1967; Browning and Schulman, 1968). Between 50 and 80 percent of the choline entering the synaptosomes by the high affinity transport system ($K_M$=2$\mu$M) is converted to acetylcholine (Jope, 1979). This suggests a tight coupling of choline uptake to transmitter synthesis (Barker and Mittag, 1973; Jope and Jenden, 1978). High affinity choline transport (HAChT) is proposed to occur through a carrier mediated system whose energy is derived from the inwardly directed sodium gradient. HAChT is inhibited by incubation with metabolic poisons, ouabain and $K^+$ free media (Jope, 1979). These treatments inhibit the NaK-ATPase and decrease the inwardly directed $Na^+$ gradient (Abdel-Latif, 1969).

Tryptophan is an essential amino acid and a direct precursor of the neurotransmitter serotonin. Tryptophan uptake is mediated by a high affinity mechanism (Hamon et al., 1974) which segregates the precursor to be utilized for transmitter synthesis into a specific intra-synaptosomal pool (Grahame-Smith, 1971; Shields and Eccleston, 1972). Tryptophan uptake is via a carrier mediated $Na^+$-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

dependent mechanism similar to that described for choline uptake (Grahame-Smith and Parfitt, 1970).

Synaptosomes also have high affinity uptake mechanisms for many neurotransmitters. This mechanism is best exemplified by the uptake of norepinephrine (NE) into synaptosomes (*Colburn et al., 1968*). The accumulation of NE by synaptosomes is non-competitively inhibited in $K^+$ free medium or by inclusion of ouabain or metabolic inhibitors (Bogdanski, 1968; White and Keen, 1970). These treatments are known to reduce the inwardly directed $Na^+$ gradient by compromising the activity of the membrane associated NaK-ATPase (Abdel-Latif, 1969). The inhibition of NE uptake by these treatments appears to be secondary to the build up of intracellular $Na^+$. These results are consistent with a carrier which co-transports norepinephrine and $Na^+$. The energy expended to accumulate NE within the nerve terminals is derived from the sodium electrochemical gradient. Incubation of synaptosomes in $Na^+$ free media increases the carrier mediated efflux of NE indicating the mechanism is reversible depending on the direction of the $Na^+$ gradient (Bogdanski, 1973). Both the uptake and $Ca^{++}$ independent efflux of NE are equally inhibited by desipramine (Raiteri et al., 1977). Similar mechanisms for the uptake of norepinephrine, dopamine, serotonin and several putative amino acid transmitters have been demonstrated in synaptosomes (Iverson, 1970; Abdel-Latif, 1973; Snyder et al., 1973).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2. Release of Neurotransmitter

The release of neurotransmitter from nerve terminals must satisfy several criteria in order to be recognized as a stimulus-induced secretion. The release should be 1) rapidly induced by depolarization, 2) be $Ca^{++}$ dependent and 3) blocked by $Mg^{++}$ and other antagonistic divalent ions. $Ca^{++}$-dependent, $K_o$-evoked release of norepinephrine (Blaustein et al., 1972; Mulder et al., 1975; Haycock et al., 1978), dopamine (Drapeau and Blaustein, 1983; Leslie et al., 1985), gamma-aminobutyric acid (Haycock et al., 1978; Arias and Tapia, 1986), serotonin (Mulder et al., 1975), acetylcholine (Suszkiw and O'Leary, 1982, 1983; Adam-Vizi and Ligeti, 1984) and several amino acid transmitters (Snyder et al., 1973) has been demonstrated in synaptosomes. The release of these transmitters is stimulated by elevated $K_o$ (Blaustein et al., 1972; Drapeau and Blaustein, 1983), veratridine (Blaustein, 1975; Patrick and Barchas, 1976; Schoffelmeer and Mulder, 1983) and incubation with ouabain (Adam-Vizi and Ligeti, 1984). These treatments depolarize the synaptosomes and increase the intracellular free $Ca^{++}$ via it's entry through voltage-stimulated channels (Nachshen and Blaustein, 1980, 1982) and exchange mechanisms (Blaustein and Oborn, 1975; Blaustein and Ecktor, 1976). The depolarization-dependent release of neurotransmitters, activation of $Ca^{++}$ influx (Blaustein, 1975) and the build up of free cytosolic $Ca^{++}$ (Heinonen et al., 1985) have similar voltage threshold characteristics in synaptosomes. It appears that the threshold behavior of the release process is a manifestation of the voltage-dependency of $Ca^{++}$ entry. Veratridine is known to depolarize excitable cells (Ulbricht, 1969) by irreversibly opening $Na^{+}$ channels (Ohta et al., 1973). With channels locked in the open conformation $Na^{+}$ moves into the cells causing depolarization. Synaptosomes incubated with veratridine become depolarized (Blaustein and Goldring, 1975) resulting in the activation of voltage-dependent $Ca^{++}$ entry (Blaustein, 1975) and increased cytosolic free

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

$Ca^{++}$ (Heinonen et al., 1985). Veratridine-stimulated $Ca^{++}$ entry and transmitter release are inhibited in $Na^{+}$ free medium or in the presence of tetrodotoxin (Blaustein, 1975). $Ca^{++}$-dependent transmitter release from peripheral nerve terminals is competitively inhibited by $Mg^{++}$ (Meiri and Rahamimoff, 1972; Krnjevic, 1974). Elevated $Mg^{++}$ inhibits the depolarization-dependent $Ca^{++}$ influx (Drapeau and Nachshen, 1984) and release of transmitter (Cotman et al., 1976; Drapeau and Nachshen, 1984) in synaptosomes. The inorganic calcium channel antagonists barium and strontium (Hagiwara and Byerly, 1981) also inhibit $Ca^{++}$ influx and release in synaptosomes (Cotman et al., 1976; Drapeau and Nachshen, 1984). Depolarization in the absence of $Ca_o$ is insufficient to directly induce transmitter release from this preparation. However release can be stimulated without depolarization by treatment of the synaptosomes with the $Ca^{++}$ ionophore A23187 (Holz, 1975; Redburn et al., 1975; Shira et al., 1984). Incubation with ionophore stimulates $Ca^{++}$ entry and transmitter release which is independent of any changes in the membrane potential. These results support the "Calcium Hypothesis", and indicate that the mechanism of transmitter release at central synapses is similar to that at peripheral synapses.

## G. Calcium Transport By Synaptosomes

### 1. Calcium Efflux

$Na^{+}/Ca^{++}$ exchange (NaCaX) with properties similar to those of squid axon has been described for synaptosomes (Blaustein and Oborn, 1975; Blaustein and Ecktor, 1976; Coutinho et al., 1984) and isolated plasma membranes (Edreich et al 1983). NaCaX mediated $Ca^{++}$ efflux is reduced by lowered $Na_o$ and reversed (ie. calcium influx) by total replacement of $Na_o$ (Blaustein and Weismann, 1970). These results suggest that

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

NaCaX is a reversible system which transports $Ca^{++}$ depending on the direction of the $Na^{+}$ gradient. Synaptosomes loaded with $Na^{+}$ by veratridine or ouabain treatment also have a higher background level of free cytosolic $Ca^{++}$ measured with quin2 (Nachshen, 1985). The increase is attributed to the decreased efflux resulting from a reduction in the inwardly directed $Na^{+}$ gradient. $Ca^{++}$ efflux mediated by this exchange system is the prime determinant of intracellular free $Ca^{++}$ levels in synaptosomes (Nachshen, 1985).

2. Calcium Influx

Depolarization-dependent $Ca^{++}$ influx is biphasic in synaptosomes (Nachshen and Blaustein, 1980, 1982) and is mediated by kinetically distinct "fast" and "slow" calcium channels. The $Ca^{++}$ influx via the "fast" component is complete within the initial 2 seconds of depolarization. This phase is eliminated in synaptosomes preincubated under depolarizing conditions suggesting that this pathway undergoes voltage-dependent inactivation. $Ca^{++}$ influx during the fast phase is competitively inhibited by barium and strontium and blocked by low concentrations of lanthanum. The transient nature and permeability characteristics of the fast influx is consistent with a calcium channel which inactivates within several seconds following depolarization (Nachshen and Blaustein, 1980, 1982). This channel does not conduct $Na^{+}$ (Krueger and Nachshen, 1980) and is not blocked by tetrodotoxin (Nachshen and Blaustein, 1980). The $Ca^{++}$ influx through the channel is blocked by $Mg^{++}$, $Sr^{++}$, $Ba^{++}$, $Ni^{++}$, $Cd^{++}$, and $La^{+3}$ in a dose-dependent fashion (Nachshen, 1984). Influx inhibition can be overcome by increasing the concentration of $Ca_o$ in a manner suggesting that the metal ions compete with $Ca^{++}$ for a binding site within the channel. The sequence of cation binding to the channel (Ca>Sr>Ba>Mg) and pH sensitivity (Nachshen and Blaustein, 1979) are consistent with

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

anionic sites inside the channel to which cations must bind while crossing the membrane (Diamond and Wright, 1969; Nachshen, 1984). The "slow" phase of $Ca^{++}$ influx is resistant to prolonged depolarization indicating that this pathway does not inactivate. This pathway conducts barium and strontium to a lesser extent than the fast phase and is blocked by relatively high concentrations of lanthanum. It is not certain whether the slow phase of $Ca^{++}$ influx occurs through a non-inactivating channel (Nachshen and Blaustein, 1980, 1982) or a carrier mediated system (Blaustein and Oborn, 1975; Blaustein and Ecktor, 1976; Coutinho et al., 1984). Recent experiments have examined the relationship of "fast" $Ca^{++}$ influx to transmitter release (Redburn et al., 1975; Floor, 1983; Drapeau and Blaustein, 1983; Suszkiw and O'Leary, 1983; Leslie et al., 1985; Daniell and Leslie, 1985). Biphasic transmitter release from synaptosomes during the initial 5 seconds of depolarization, appears to correlate with the two phases of $Ca^{++}$ influx (Floor, 1983; Drapeau and Blaustein, 1983; Leslie et al., 1985; Daniell and Leslie, 1985).

In addition to the channel mediated influx, a second phase of $Ca^{++}$ influx is observed which is dependent on the $Na^+$ gradient and membrane potential (Blaustein and Oborn, 1875; Coutinho et al., 1984). This $Ca^{++}$ influx is powered by $Na_i$ content of synaptosomes (Blaustein and Oborn, 1975; Coutinho et al., 1984). Increasing $[K^+]_o$, depolarizes the synaptosomes due to the relatively high permeability of the resting membrane to this ion (Keen and White, 1971; Blaustein and Goldring, 1975). Depolarization increases the outwardly directed electrical gradient realized by $Na^+$ ions. The outward chemical gradient for $Na^+$ is exacerbated by the iso-osmotic replacement of $Na^+$ with $K^+$. During elevated $K_o$-depolarization both the chemical and electrical gradients for $Na^+$ are altered so as to promote $Na^+$ efflux. Coupling of $Ca^{++}$ influx to this augmented $Na^+$ efflux is predicted (Coutinho et al., 1984) but not clearly demonstrated. $Ca^{++}$ entry in synaptosomes is proposed to occur through three distinct

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

pathways. "Fast" and "slow" calcium channels (Nachshen and Blaustein, 1980, 1982) and the $Na_i/Ca_o$ exchange (Blaustein and Oborn, 1975; Coutinho et al., 1984). At present the separation of these individual pathways has not been adequately achieved.

H. Effects of Dihydropyridine Drugs on Calcium Channels

Despite compelling evidence indicating that the fast component of synaptosomal $Ca^{++}$ influx is channel mediated, little information exists regarding the modulation of these channels. One mode of regulating $Ca^{++}$ flux is by ligand binding to the channel proteins. Dihydropyridine (DHP) drug binding to smooth muscle preparations is to a single class of saturable, high affinity sites ($K_D$ 0.1-0.5 nM). There is good correlation between the affinity of DHP binding and the inhibition of depolarization induced muscle contraction in ileum (Bolger et al., 1983). Similar to smooth muscle, DHP binding to cardiac muscle is associated with high affinity sites ($K_D$ 0.1-0.5 nM) (Miller, 1984). However, the pharmacological blockade of $Ca^{++}$ influx by dihydropyridine drugs occurs at 2-3 orders of magnitude higher concentrations than their affinities for the binding sites (Lee and Tsien, 1983; Kass 1983). The reason for the discrepancy between binding and effect in cardiac muscle is as yet unclear. It has been postulated that the affinity of the channel for these drugs is regulated by an intrinsic modification of the channel which is not expressed in isolated preparations (Miller, 1984). Alternatively this may reflect a voltage-dependent drug/channel interaction (Bean, 1984; Sanguinetti and Kass, 1984). The T-tubular system of skeletal muscle contains calcium channels which are inhibited by DHP channel antagonists (Sanchez and Stefani, 1978; Eisenberg et al., 1983). These channels are of the low affinity type and are only blocked by high concentrations of drugs (Gonzales-Serratos et al., 1982). Despite some discrepancies most studies of muscle preparations are consistent with the idea that the dihydropyridine

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

binding sites represent voltage sensitive calcium channels (Bolger et al., 1983; Lee and Tsein, 1983; Kass et al., 1983; Miller, 1984).

The hypothesis that dihydropyridine drugs alter calcium channel activity in neuronal tissue is more complicated (Miller, 1984). High affinity DHP binding sites in synaptosomes have similar properties to those of cardiac and smooth muscle (Murphy and Synder, 1982; Gould et al., 1982; Ehlert et al., 1982; Belleman et al., 1983; Triggle and Janis, 1983). However the correlation between binding and pharmacological effect is exceptionally poor in synaptosomes where dihydropyridines are usually ineffective in blocking $Ca^{++}$ fluxes at concentrations of $10^{-5}$ M (Nachshen and Blaustein, 1979; Daniell et al., 1983; Rampe et al., 1984; Creba and Karobath, 1986; Wei and Chiang, 1986). The hypothesis that these binding sites are associated with synaptosomal calcium channels remains speculative. Some evidence indicates that the DHP receptors measured in synaptosome preparations may be localized in the postsynaptic elements which are attached to the synaptosomes (Siekewitz et al., 1985). Dihydropyridine binding to these receptors would not alter $Ca^{++}$ influx into synaptosomes and give the impression that the DHP receptors are inactive.

Three types of calcium channels coexist in chicken dorsal root ganglion cells (Nowycky et al., 1985). These channels can be distinguished by differences in voltage sensitivity, kinetics, unitary conductances and sensitivity to cadmium. T-type channels produce small currents which rapidly inactivate and are not sensitive to blockade by cadmium. L-type channels require strong depolarizations for activation, do not inactivate and are sensitive to low concentrations of cadmium. N-type channels undergo inactivation like T-type channels but are sensitive to cadmium like L-type channels. Only the L-type channels are sensitive to dihydropyridines. Incubation of dorsal root ganglion neurons with Bay K 8644 strongly enhances the L-type channel currents. L- and T-type channels have also been observed in cultured heart cells (Nilius et al., 1985).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Voltage-sensitive calcium channels in neuronal cell cultures are sensitive to dihydropyridine drugs (Freedman et al., 1984; Nowycky et al., 1985; Creba and Karobath, 1986). However the relevance of these calcium channels to the release of transmitter is not known. Recent reports indicate that in neurons where L- and N-type channels coexist, the N-type channels predominate at the neurotransmitter release sites (Thayer et al., 1986). Cultured hippocampal neurons were shown to have both dihydropyridine sensitive and insensitive calcium channels. The majority of voltage-stimulated $Ca^{++}$ influx in these neurons is blocked by nitrendipine. In contrast the release of transmitter from these neurons is inhibited by cadmium but unaffected by dihydropyridines. The results suggest that only calcium channels which are not sensitive to dihydropyridine drugs (ie. N-type) generate the $Ca^{++}$ fluxes relevant to the release of neurotransmitter. The function of the DHP sensitive $Ca^{++}$ fluxes in this preparation is not known. Similar results have been observed in cultured rat superior cervical ganglion cells (Hirning et al., 1986). It appears that calcium channels in somatic membrane are pharmacologically distinct from those of the nerve terminals (Miller, 1985; Reynolds et al., 1986; Miller, 1987).

I. Regulation of Calcium Influx by Phosphorylation

Slow channels in cardiac muscle appear to be regulated by a cAMP-dependent phosphorylating mechanism. Procedures which increase the intracellular levels of cAMP (Josephson and Sperelakis, 1978; Vogel and Sperelakis, 1981; Li and Sperelakis, 1983) or reduce intracellular ATP (Schneider and Sperelakis, 1974, 1975; Sperekalis et al., 1979) selectively modulate the activity of slow channels without having appreciable effect on voltage-sensitive sodium or potassium channels. The results suggest that only phosphorylated slow channels are available to become activated upon depolarization.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Prolonged intracellular perfusion of snail neurons results in a loss of $Ca^{++}$ currents which is attributed to the inactivation of the channels (Kostyuk et al., 1979). This decline in channel activity can be reversed by intracellular perfusion with cAMP, ATP and $Mg^{++}$ (Kostyuk, 1981). It is proposed that due to intracellular dialysis, factors important to the normal functioning of the calcium channels are removed from the cytoplasm. The reversal of this phenomena by perfusion with cAMP and ATP indicates that the activity of the calcium channels is regulated by a phosphorylation-dependent mechanism. Recent data suggests a second class of calcium channels in this preparation which are not sensitive to dialysis and not modulated by cAMP (Kostyuk et al., 1985). These results corroborate the recent observations that several pharmacologically distinct types of calcium channels can coexist within an individual neuron (Nowycky et al., 1985). Phosphoryaltion-dependent mechanisms are proposed to regulate the receptor operated (Sieglebaum et al., 1982; Shuster et al., 1985) and calcium-dependent (Ewald et al., 1985) potassium channels of mollusc.

The mechanisms which modulate the activity of synaptosomal calcium channels are not known. The possibility that the $Ca^{++}$ influx through these channels could be regulated by a phosphorylation-dependent mechanism would have important implications in the release of neurotransmitter. The role of protein phosphorylation in regulating the influx of $Ca^{++}$ in synaptosomes is one of the objectives of this research.

## J. Regulation of Calcium Channels by Inactivation

Inactivation of voltage-stimulated $Ca^{++}$ entry is observed in a number of preparations (Hagiwara and Byerly, 1981; Tsien, 1983). A simple voltage-dependent process similar to that described for the $Na^{+}$ current of the squid axon (Hodgkin and Huxley, 1952) has been described for calcium channels of marine egg membranes (Hagiwara et al., 1975;

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Fox, 1981) and cultured heart cells (Lee and Tsien, 1982). In these preparations the rate of inactivation proceeds at identical rates regardless of whether the current is carried by calcium, strontium or barium and is unaffected by previous loading of the cells with calcium. Neither $Ca^{++}$ influx nor elevated $Ca_i$ alter the channel activity. In contrast the inactivation of $Ca^{++}$ currents in molluscan neurons (Tillotson, 1979; Eckert and Tillotson, 1981; Plant and Standen, 1981), Paramecium (Brehm et al., 1980), insect muscle (Ashcroft and Stanfield, 1982) and frog heart (Mentrard et al., 1984) appear to be mediated by the entry of $Ca^{++}$. In Aplysia neurons 1) depression of the peak amplitude of the $Ca^{++}$ current depends on the extent of $Ca^{++}$ entry and not the level of depolarization, 2) depolarization to the $Ca^{++}$ equilibrium potential (+140 mV) results in minimal development of inactivation, and 3) barium and strontium currents display less inactivation than calcium for any given depolarization (Tillotson, 1979). These results cannot be explained by voltage-dependent inactivation. It is proposed that increased ionized $Ca^{++}$ near the inside of the channel promotes inactivation. This effect is specific for $Ca^{++}$ since other cations which permeate the membrane through the calcium channel do not support inactivation (Tillotson, 1979). The exact mechanism of this type of inactivation is not known. Intracellular injection of EGTA into Aplysia neurons only partially inhibits the inactivation process (Brown et al., 1981). It appears that both $Ca^{++}$ and voltage-dependent inactivation processes are operative in mollusks.

Depolarization-dependent $Ca^{++}$ influx occurs at two rates in synaptosomes (Nachshen and Blaustein, 1980, 1982). Non-linear influx as previously demonstrated, could be explained if a portion of the $Ca^{++}$ influx were to inactivate. Inactivation of $Ca^{++}$ influx has been shown in synaptosomes (Nachshen and Blaustein, 1980, 1982; Suszkiw and O'Leary, 1983; Nachshen, 1985). The data suggest that voltage-dependent inactivation is a regulatory mechanism of the synaptosomal calcium channels. The role

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of inactivation in determining the influx of $Ca^{++}$ into synaptosomes is an objective of this research.

K. Summary

Synaptosomes have many of the metabolic characteristics of nervous tissue <u>in vivo</u>. High energy phosphates generated from the metabolism of glucose by mitochondria are utilized to support a plethora of basic cellular activities. The membrane of synaptosomes effectively isolates the intracellular compartment of synaptosomes from the external environment. The permeability properties of this membrane in conjunction with the active transport processes promote transmembrane cation gradients and electrical potentials characteristic of more intact nervous tissue. Synaptosomes are capable of the specific transport of precursors and utilize these metabolites to synthesize neurotransmitter. These transmitters are stored by synaptosomes and are released in response to depolarization in the presence of $Ca^{++}$. In general, these observations attest to the competency of the synaptosome preparation as a suitable model for nerve terminals <u>in vivo</u>.

With this established, synaptosomes can be used to test the applicability of the "Calcium Hypothesis" to the mammalian central nervous system. I have used this preparation to probe the nature of the membrane permeability changes associated with depolarization. The voltage and time-dependency of channel mediated $Ca^{++}$ influx is described and two modes of non-channel influx are identified. Modulation of presynaptic calcium channels is particularly important due to their relevance to neurotransmitter release. Modulation of the channels by the dihydropyridine class of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

organic channels blockers is examined. I have also tested the hypothesis that the channels may be regulated by a phosphorylation-dependent mechanism similar to that described for cardiac slow channels.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## Objectives of this Research

1. Measure the depolarization-dependent $Ca^{++}$ influx during the first 5 seconds of depolarization. Quantitatively describe the contribution of calcium channels to this influx.
2. Describe the properties of these channels.
3. Isolate and identify alternate pathways of $Ca^{++}$ influx (ie. non-channel) during the initial 5 seconds of depolarization.
4. Correlate the depolarization-dependent $Ca^{++}$ influx with the release of neurotransmitter.
5. Determine if calcium channels in synaptosomes are affected by dihydropyridine drugs.
6. Determine if calcium channels in synaptosomes are regulated by phosphorylation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## Methods

### A. Hepes-buffered Krebs Ringer Solutions

Hepes-buffered Krebs Ringer (Na-HKR) contains: 140 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 10 mM Hepes buffer pH 7.4, and 10 mM glucose. High potassium solutions were prepared by the iso-osmotic replacement of NaCl with KCl. The non-depolarizing HKR (5 mM $K^+$ HKR) corresponding to the elevated $K_0$ solutions were made by iso-osmotic replacement of sodium with choline (Ch-HKR). Under these conditions the $Na^+$ concentration in both the elevated and normal $K^+$ solutions is equivalent. The $Ca^{++}$ concentrations of these solutions varied from 0.02 to 1.0 mM depending on the requirements of the experiment. In some experiments $Na^+$ was omitted from the preincubation solutions. These solutions were made by replacement of $Na^+$ with 140 mM choline (Ch-HKR). Osmolarity of the solutions were 320 mOsmoles/liter.

### B. Preparation of Synaptosomes

Adult white rats were decapitated, and the isolated cortex dispersed into 0.32 M sucrose using a glass-teflon homogenizer. Cellular debris was removed by low speed centrifugation (3000 x G/10 mins). The resulting supernatant was centrifuged 10,500 x G/20 minutes. This pellet represents a crude synaptosome preparation ($P_2$) and can be directly used for some types of experiments. Further purification of the synaptosomes can be performed by the methods of Hajos (1975) or Gray and Whittaker (1962).

#### 1. Hajos Preparation

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

$P_2$ synaptosomes were resuspended in 0.32 M sucrose and layered onto 0.8 M sucrose. This gradient was centrifuged at 9,000 x G/20 minutes. During this time the synaptosomes move into the 0.8 M sucrose layer but do not pellet due to the relatively low gravitational forces. This 0.8 M sucrose phase was collected and slowly diluted with distilled water in order to bring the osmolarity to 320 mOsm. Synaptosomes were then pelleted and resuspended in Hepes-buffered Krebs Ringer (HKR).

2. Gray and Whittaker Preparation

$P_2$ synaptosomes suspended in 0.32 M sucrose were layered over a discontinuous gradient of 0.8 M and 1.2 M sucrose and centrifuged at 105,000 x G/75 minutes. Synaptosomes form a band at the 0.8-1.2 M sucrose interface. The synaptosomes were harvested and slowly diluted with HKR to adjust the osmolarity to 320 mOsm. This enriched synaptosomal preparation is pelleted and resuspended in HKR.

$P_2$, Gray/Whittaker and Hajos synaptosomes were utilized in the course of this study. In addition, synaptosomes prepared from cortex (ie. brainstem removed) and whole brain (ie. cortex and brain stem) were compared as indicated in the figure legends.

C. Measurement of Intrasynaptosomal Volume

The volume of the synaptosomes is evaluated by the differential distribution of the volume marker $^3H\text{-}H_2O$ and the extracellular marker $^{14}C$ sucrose. 100 $\mu$l of synaptosomes was mixed with 100 $\mu$l of HKR containing both markers. The synaptosomes are incubated for 10-30 minutes before pelleting by centrifugation. Both markers appeared to equilibrate with their appropriate spaces within 10 minutes of incubation. The

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

to equilibrate with their appropriate spaces within 10 minutes of incubation. The supernatant was separated from the pellet and sampled for specific activity of $^3H$ and $^{14}C$ (DPM/$\mu$l). The pellet was dissolved in 1% Triton X-100 in 0.1 N HCl. An aliquot of the dissolved pellet was counted in aqueous fluor (DPM/pellet). The volume associated with each marker is calculated by dividing the number of DPM in the pellet (DPM/pellet) by the specific activity of the incubation solution (DPM/$\mu$l). The volume calculated for $^3H$-$H_2O$ is the total pellet volume ($\mu$l/pellet). The volume calculated for $^{14}C$-sucrose is the extra-cellular volume ($\mu$l/pellet) associated with the pellet. The differences between these volumes (ie. total - extracellular) is the intra-synaptosomal volume ($\mu$l/pellet). The calculated volumes are expressed as a ratio of the synaptosomal protein (mgP/pellet). The calculated synaptosomal volumes were also corrected for contamination by non-synaptosomal structures. Approximately 20% of the measured volume was not associated with synaptosomes based on electronmicroscopic criteria (Suszkiw et al., 1986). The measured volumes of synaptosomes were reduced 20% to account for this contamination. Intrasynaptosomal volumes ($\mu$l/mgP) were determined for synaptosomes preincubated in either Na-HKR or Ch-HKR.

D. Measurement of Synaptosomal Sodium and Potassium Concentrations

The content of $Na^+$ and $K^+$ ions in synaptosomes was determined by filtering 250 $\mu$l of synaptosomes onto 0.65 $\mu$m filters and extensively washing away extrasynaptosomal sodium and potassium by 25 ml washing with 145 mM choline HKR. The washed filters were placed into 1 ml of 0.1 N HCl. The HCl solution was assayed for $Na^+$ and $K^+$ content by Atomic Absorption. Samples were burned in a air-acetylene flame and the emissions recorded at 589 nm ($Na^+$) and 766.5 nm ($K^+$). Sodium and potassium standards were prepared in 0.1 N HCl and ranged from 12.5-250 mM. Both standard

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

HKR were analyzed by this method. The content of ions were expressed as $\mu$mol/mg protein. The ion content of synaptosomes was corrected for 20% non-synaptosomal content (Suszkiw et al., 1986). It was assumed that the contaminating structures were metabolically inert and therefore equilibrated with $Na^+$ and $K^+$. Therefore 0.2 x 140 mM $Na^+$ or 0.2 x 5 mM $K^+$ was subtracted from the respective total ion contents. The corrected values represent the ionic content of intact synaptosomes.

E. Calcium Influx Measurements

Calcium influx was measured by mixing solutions with rapid syringe injections using a 1:1 synaptosomes:HKR mixing paradigm. Synaptosomes were routinely suspended in HKR containing no calcium. The influx of $^{45}Ca$ measured by mixing 250 $\mu$l synaptosomes with 250 $\mu$l of either 100 mM $K^+$ HKR or 35 mM $K^+$ HKR containing 2 mM $^{45}Ca^{++}$. The final incubation conditions were either 52.5 mM $K^+$ or 20 mM $K^+$ HKR containing 1 mM $^{45}Ca^{++}$. The corresponding low potassium conditions were 5 mM $K^+$ with either 47.5 mM or 15 mM choline in replacement of sodium. The $Na^+$ concentration and osmolarity in both elevated and normal $K^+$-HKR were constant. Uptake incubation was terminated by injection of 0.5 ml of HKR containing 20 mM EGTA. A sample (0.8 ml) of the synaptosomes was filtered onto 0.65 $\mu$m Millipore filters and washed three times with 5 ml of HKR. The calcium trapped within synaptosomes was released by placing the filter into 0.5 ml of 1% Triton X-100 in 0.1 N HCl. $^{45}Ca$ in the mixture was determined by liquid scintillation counting in aqueous fluor. The specific activity of the $^{45}Ca^{++}$ in the uptake solution was measured. The protein concentration of the solution was determined by the method of Lowry et al. (1951) and the calcium influx expressed as (nmoles $Ca^{++}$/mg protein/uptake interval). The calcium concentrations during uptake ranged from 0.02 to 1.0 mM depending on the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The calcium concentrations during uptake ranged from 0.02 to 1.0 mM depending on the requirements of the experiment. Tracer $^{45}Ca$ varied with the concentration of added $Ca^{++}$.

1. Dihydropyridine Drugs

Synaptosomes (1 mgP/ml) were preincubated with drugs 20 minutes prior to test of calcium influx. 250 $\mu$l of synaptosomes were mixed with 250 $\mu$l of either 35 mM $K^+$ or 5 mM $K^+$/30 mM choline HKR containing 2 mM $^{45}Ca^{++}$. Influx was quenched after 5 seconds by the addition of HKR/20 mM EGTA.

2. cAMP, Forskolin, Phorbol Esters

Synaptosomes (1 mgP/ml) were preincubated 20 minuted prior to test for calcium influx. 0.5 ml of synaptosomes were mixed with 0.5 ml of either 35 mM $K^+$ or 5 mM $K^+$/30 mM choline containing 2 mM $^{45}Ca^{++}$. Influx was quenched after 5 seconds by the addition of 0.5 ml of HKR/20 mM EGTA.

F. Inactivation of Calcium Influx

In these experiments 50 $\mu$l of synaptosomes were predepolarized by addition of 450 $\mu$l of 57.8 mM $K^+$ HKR containing no calcium. After the predepolariztion interval, 0.5 ml of 52.5 mM $K^+$ HKR containing 1 mM $^{45}Ca^{++}$ was rapidly injected and uptake was allowed to proceed for 1 second prior to addition of HKR containing 20 mM EGTA. Samples were filtered and treated as in calcium influx. Basal uptake of calcium is that in 5 mM $K^+$ HKR containing 47.5 mM choline substituted for sodium. Uptake values

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

are expressed as a percentage of calcium uptake in non-predepolarized synaptosomes exposed to 52.5 mM $K^+$ HKR $^{45}Ca^{++}$ for 1 second. The non-linear decrease in fractional uptake versus predepolarization interval represents the voltage dependent inactivation of the calcium influx.

G. Reactivation of Calcium Influx

25 $\mu$l of synaptosomes are mixed with an equal volume of 100 mM $K^+$ HKR and incubated 10 seconds, an interval which previous experiments have shown that the potassium-stimulated $^{45}Ca^{++}$ influx is 80% inactivated. Following preincubation 475 $\mu$l of HKR containing no potassium is added so that the extra-synaptosomal potassium concentration is restored to the control 5 mM level. The synaptosomes were allowed to recover from the depolarization for between 1 and 60 seconds and then mixed with 525 $\mu$l of 100 mM $K^+$ HKR or 5 mM $K^+$/95 mM choline HKR containing 1 mM $^{45}Ca^{++}$. Calcium uptake was allowed to proceed for 1 second prior to quench by addition of HKR containing 20 mM EGTA. Synaptosomes were filtered and assayed for $^{45}Ca$ content. Recovery is expressed as a fraction of the uptake prior to any predepolarization. A plot of the recovery interval versus calcium uptake during the 1 second uptake interval is a measure of the recovery of calcium influx from inactivation.

H. Voltage Dependency of Inactivation

50 $\mu$l of synaptosomes were mixed with 450 $\mu$l of 16, 27, 38, 58, and 83 mM $K^+$ HKR. After 2 seconds 1 ml of 71, 66, 61, 53, and 41 mM $K^+$ HKR/1.1 mM $^{45}Ca^{++}$ was added and incubated 1 second prior to quench. The first additions altered the predepolarizing conditions to various potassium levels while the second paired addition changed the $K^+$

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

$^{45}Ca^{++}$ influx interval. The influx during each influx interval is expressed as a fraction of influx in 52.5 mM $K^+$ HKR into synaptosomes which were not predepolarized.

I. Lanthanum Blockade of Calcium Influx

Non-predepolarized calcium influx was measured by mixing 125 $\mu$l of synaptosomes with 125 $\mu$l HKR and incubating 20 seconds before adding 250 $\mu$l of 100 mM $K^+$ HKR containing 40 $\mu$M $^{45}Ca^{++}$. After 1 second $^{45}Ca^{++}$ influx was quenched by addition of 0.5 ml HKR containing 20 mM EGTA. The corresponding non-predepolarized influx was measured by adding 5 mM $K^+$/95 mM choline HKR containing 1 mM $^{45}Ca^{++}$ during the influx interval. Predepolarized $^{45}Ca^{++}$ influx was measured by mixing 125 $\mu$l of synaptosomes with 125 $\mu$l 100 mM $K^+$ HKR and incubating for 20 seconds. $^{45}Ca^{++}$ influx was stimulated by adding 250 $\mu$l 52.5 mM $K^+$ HKR containing 2 mM $^{45}Ca^{++}$ and influx quenched after 1 second by the addition of 0.5 ml of HKR/20 mM EGTA. Paired samples were determined with 20 $\mu$M lanthanum present during the influx interval.

J. Measurement of the Slow Component of Calcium Influx

Synaptosomes were preincubated 20 minutes in either 140 mM $Na^+$ (Na-SYN) or 140 mM choline (Ch-SYN) HKR. 25 $\mu$l of synaptosomes were mixed with 25 $\mu$l of 100 mM $K^+$ HKR. Following 10 seconds incubation influx was stimulated by addition of 450 $\mu$l of 52.5 mM $K^+$ HKR containing 1.1 mM $^{45}Ca^{++}$. Influx was quenched by addition of 1.5 ml of HKR containing 20 mM EGTA. The corresponding non-stimulated influx was measured by addition of 25 $\mu$l of 5 mM $K^+$ HKR during the first mixing and 450 $\mu$l of 5 mM $K^+$/47.5 mM choline/1.1 mM $^{45}Ca^{++}$ during the influx interval. The same

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

5 mM $K^+$/47.5 mM choline/1.1 mM $^{45}Ca^{++}$ during the influx interval. The same experiments were repeated with Ch-SYN except $Na^+$ was not present in any of the HKR solutions.

K. Norepinephrine Release

Synaptosomes are loaded with $^3$H-norepinephrine by 30 minute incubation in the presence of 0.1 μM norepinephrine/$^3$H-norepinephrine. After incubation 20 μM desipramine was included in the HKR solutions to inhibit further norepinephrine incorporation. Release was stimulated by 1:1 mixing (ie. 250 μl:250 μl)of the loaded synaptosomes with 5 mM $K^+$ HKR or 100 mM $K^+$ HKR solutions containing 2 mM $Ca^{++}$. Release was quenched by addition of 0.5 ml HKR containing 20 mM EGTA. Synaptosomes were filered onto Whattman GF/F filters and the radioactivity released into the supernatant assayed. Voltage-dependent transmitter release is that in 52.5 mM $K^+$ HKR minus that in 5 mM $K^+$ HKR. The amount released is expressed as a percentage of the initial $^3$H-norepinephrine content of the synaptosomes.

L. Preparation of Synaptosomes for Phosphorylation Experiments

Synaptosomes suspendend in HKR (1 mgP/ml) were mixed with HKR containing $^{32}P_i$ and incubated under oxygen for 30 minutes at 30$^0$ C. Following preincubation 0.25 ml aliquots of synaptosomes were distributed into separate tubes and 5 μl of various drugs added. These samples were incubated another 20 minutes before the addition of 125 μl of 10% SDS and the mixture boiled 2 minutes. After the samples had cooled, 27 μl of the following mixture was added; 500 mM Tris/HCl pH 7.4, 25 mM B-mercaptoethanol, sucrose (0.5 gm/ml) and 10% bromophenol blue tracking dye.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

M. Polyacrylaminde Gel Electrophoresis

The Gel electrophoresis technique is a variant of the methods of Laemmli (1970). The resolving gel (11cm x 16cm x 1.5mm) was 7.5% acrylamide, 0.2% bisacrylamide, 0.38 M Tris/Cl pH 8.8, 0.1% SDS; the stacking gel (11cm x 2cm x 1.5 mm) was 3% acrylamide, 0.08% bisacrylamide, 0.12 M Tris/Cl pH 6.8, 0.1% SDS. Gels were polymerized with 0.05% N,N,N'-tetramethylenediamine and 0.1% ammonium persulfate. The gels were mounted into a Bio Rad eletrophoresis apparatus. Both upper and lower buffer was 25 mM Tris/Cl pH 8.3, 0.2 M glycine, 0.1% SDS. 50 $\mu$l of synaptosomes (75 $\mu$g protein) was loaded into each well. The gels were run at 25 mA until the tracking dye had moved 1 cm into the resolving gel and then run at 50 mA for 4 hours. The gel was stained in 0.25% R-250 commassie blue, 40% methanol and 10% acetic acid. Destain was 40% ethanol and 10% acetic acid. The gel was dried on Whatman No. 1 filter paper under vacuum and placed on Kodak X-Omat film for 72 hours. The optical density of the autoradiogram bands were assessed by scanning on a Biomed Instruments densitometer model SL-TRFF. The relative incorporation of $^{32}P_i$ into the protein bands is inferred from the area under the densitometric scans.

N. Incubation of Synaptosomes with Drugs

Stock solutions of Bay K 8644 and 8-Bromo cAMP were made in $H_2O$, nifedipine and nitrendipine in EtOH, and myristate and 12,13-diacetate phorbol esters in DMSO. Stock solutions were made 500X more concentrated than required by the experiments, resulting in the addition of 0.5% DMSO or EtOH to the samples. To control for effects of the drug vehicles, 0.5% of $H_2O$, EtOH or DMSO was added to control samples. These

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

concentrations of $H_2O$, DMSO and EtOH do not affect $^{45}Ca^{++}$ influx. Preincubation with drugs was 20 minutes unless otherwise stated. In order to maintain a constant drug:protein ratio, synaptosomes were resuspended in HKR to 1 mgP/ml prior to experiments.

O. Determination Of Free Bay K 8644 Concentration During Preincubation

Free drug concentration (ie. unbound) was measured in the HKR solutions after the 20 minute preincubation. The assay conditions for binding were exactly the same as for the preincubation prior to $^{45}Ca^{++}$ uptake experiments except for the inclusion of $^{3}H$-Bay K 8644. After incubation a sample of the synaptosome mixture was counted to determine the specificity of the $^{3}H$-Bay K. The remainder was pelleted by centrifugation and the $^{3}H$-Bay K DPM remaining in the supernatant assayed.

P. Statistical Methods

Pooled and Paired T-Tests were performed by EPISTATS. A stastical package written in basic for the IBM PC (Tracy, 1982). Nonlinear least squares curve fitting program is written in basic for the Apple IIe computer. The Marquardt method (Marquardt, 1962) is used in the fitting program.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## Discussion

### A. Intra-synaptosomal Concentrations of Sodium and Potassium Ions

One method of determining the resting membrane potential of synaptosomes is to measure the intracellular volume and content of diffusable ions. From these data the intrasynaptosomal concentrations of the permeable ions are calculated and the membrane potential estimated by the Constant Field Equation (Goldman, 1943; Hodgkin and Katz, 1949). In Table 1. the ionic content (μmole/mgP) of $Na^+$ and $K^+$ is shown for synaptosomes incubated in either 140 mM sodium (Na-SYN) or 140 mM choline (Ch-SYN) containing Krebs Ringer. The volumes of Na-SYN and Ch-SYN synaptosomes are 2.7 and 2.4 μl/mgP, respectively. By evoking the simplifing assumption that the ions are evenly distributed within the volume of the synaptosomes the average intracellular concentrations of $Na^+$ and $K^+$ can be calculated from the synaptosomal content (μmol/mgP) and volume (μl/mgP). The membrane potentials are calculated by a modified version of the Constant Field Equation (see Campbell, 1976). This formulation ingnores the contribution of $Cl^-$ diffusion to the resting membrane potential. Blaustein and Goldring (1975) found that replacement of $Cl_o$ with methylsulfate did not alter the membrane potentials measured by fluorescent dyes. These results indicate that $Cl^-$ is not an important determinant of the membrane potential of synaptosomes and suggests that the modified equation should yield a valid estimate of this parameter. The permeability ratios employed were determined by Keen and White (1971) and are identical to those determined by Blaustein and Goldring (1975).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

$$E_m = 60 \text{ Log } \frac{P_{Na}/P_K[Na]_o + [K]_o}{P_{Na}/P_K[Na]_i + [K]_i}$$

$$P_{Na}/P_K = 0.05$$

The calculated resting membrane potentials after 10 minutes preincubation are -51 mV for Na-SYN and -83 mV for Ch-SYN (Table 1.).

Synaptosomes incubated in 140 mM $Na^+$ have a high concentration of $Na_i$ (85 mM). However, this does not appear to reflect a deficit in $Na^+$ transport. Under similar incubation conditions NaK-ATPase is responsible for a significant portion of the ATP turnover in synaptosomes (Scott and Nicholls, 1980). Likewise, the elevated $Na_i$ does not appear to be due to an increase in the membrane permeability to $Na^+$ (Keen and White, 1971). The permeability ratio $P_{Na}/P_K$ of synaptosomes is similar to the resting permeability ratio of other neuronal membranes (Hodgkin and Katz, 1949; Gorman and Marmor, 1970). The observation that $Na_i$ is lower than $Na_o$, suggests that the $Na^+$ transport system and plasma membrane are acting in concert to exclude $Na^+$ from the interior of the synaptosomes. One explanation for the unusually high determination of $Na_i$, is that not all the $Na^+$ associated with the osmotically-sensitive compartment is confined within synaptosomes. The estimate that 80% of the vesiculated structures in these preparations are synaptosomes is based on morphological criteria (Suszkiw et al., 1986). Inferring the functional integrity of the plasma membranes by this approach is problematic. If a smaller percentage (ie. <80%) of the vesiculated particles in the preparation are "functionally" intact synaptosomes then the estimates of $Na_i$ based on these calculations would decrease as a function of the increased contamination (see methods). To firmly establish the functional volume of the synaptosomes in these

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

preparations would require a detailed analysis of the osmotically-sensitive compartment. Such experiments are beyond the scope of this research project. However, to reduce the calculated $Na_i$ of synaptosomes to 15 mM requires that at most 35% of the vesiculated structures in the preparation be intact synaptosomes. It is doubtful that the morphological data has overestimated the intact synaptosome population by greater than 50%. Despite these arguments it seems clear that the $Na_i$ concentrations of synaptosomes is greater than expected of intact neuronal tissue. The estimate of 85 mM for $Na_i$ after 10 minute preincubation in Na-HKR probably represents an upper limit for this value.

A consequence of an elevated $[Na^+]_i$ is a reduction of the inwardly directed $Na^+$ gradient. This would be expected to compromise the $Na_o/Ca_i$ exchange located in the synaptosomal plasma membranes (Blaustein and Oborn, 1975; Coutinho et al., 1984). Despite the reduced gradient, the $Na_o/Ca_i$ exchange mechanism operates at a sufficient level to maintain the resting $Ca_i$ concentration (100-200 nM) at low levels (Nachshen, 1985; Suszkiw et al., 1986).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

| | | Content (umole/mgP) | | Concentration (mM) | | Em (mV) |
|---|---|---|---|---|---|---|
| | | $Na^+$ | $K^+$ | $[Na^+]$ | $[K^+]$ | |
| Na-SYN | 0 | 0.31 ± 0.08 | 0.21 ± 0.06 | 77 ± 20 | 77 ± 21 | --- |
| | 10 | 0.33 ± 0.03 | 0.22 ± 0.03 | 85 ± 8 | 83 ± 9 | -51 |
| | 20 | 0.37 ± 0.05 | 0.23 ± 0.03 | 99 ± 13 | 87 ± 10 | -51 |
| | 30 | 0.38 ± 0.09 | 0.23 ± 0.04 | 104 ± 24 | 86 ± 14 | -51 |
| Ch-SYN | 0 | 0.01 ± 0.01 | 0.24 ± 0.05 | 6 ± 3 | 122 ± 22 | --- |
| | 10 | 0.01 ± 0.003 | 0.29 ± 0.06 | 4 ± 1 | 120 ± 27 | -83 |
| | 20 | 0.01 ± 0.01 | 0.26 ± 0.05 | 6 ± 2 | 109 ± 19 | -80 |
| | 30 | 0.01 ± 0.01 | 0.24 ± 0.04 | 5 ± 2 | 98 ± 15 | -78 |

**Volumes**

| | | |
|---|---|---|
| Na-SYN | 2.7 | ul/mg P |
| Ch-SYN | 2.4 | ul/mg P |

$$E_M = 60 \text{ Log } \frac{P_{Na}/P_K [Na+]_o + [K+]_o}{P_{Na}/P_K [Na^+]_i + [K+]_i}$$

$$P_{Na}/P_K = 0.05$$

Table 1. **Sodium and potassium concentrations of synaptosomes**

Synaptosomes were incubated for 30 minutes in either 140 mM $Na^+$ (Na-SYN) or 140 mM Choline (Ch-SYN) HKR. The volumes of Na-SYN and Ch-SYN synaptosomes are 2.7 and 2.4 $\mu$l/mgP respectively. The membrane potentials are calculated from a modified version of the Constant Field Equation (see text).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

B. Time Course of Depolarization-Dependent Calcium Influx

Raising the $K_o$ from 5 to 52.5 mM depolarizes synaptosomes and causes an increase in membrane permeability to $Ca^{++}$. To probe the nature of this permeability change, $^{45}Ca^{++}$ influx under depolarizing and non-depolarizing conditions was examined. Three distinct modes of $Ca^{++}$ influx can be discerned based on kinetic and voltage-dependent characteristics (fig. 1A).

The specific depolarization-dependent $^{45}Ca^{++}$ influx was calculated as that measured in 52.5 mM $K^+$ HKR (fig. 1A, triangles) minus that in 5 mM $K^+$ HKR (fig. 1A, squares). The specific total depolarization-dependent influx (fig. 1B, triangles) was calculated. The voltage-dependent influx is characterized by a fast initial phase which diminishes within several seconds to a slow phase. The biphasic curve suggests that $^{45}Ca^{++}$ influx is composed of two components. The total depolarization-dependent influx data was best described by the following equation:

$$J = P(1-e^{-k_1 t}) + k_2 t$$

$$P = 2.4 \pm 0.4 \text{ nmol/mgP}$$

$$k_1 = 2.0 \pm 0.8 \text{ sec}^{-1}$$

$$k_2 = 0.24 \pm 0.1 \text{ nmol/mgP/sec}$$

Where J is the $Ca^{++}$ influx in nmoles/mgP at any given time (t), P is the value of the influx at the plateau, $k_1$ is the rate coefficient of the fast phase and $k_2$ is the rate of the slow phase.

It was previously observed that the fast component is eliminated by preincubating synaptosomes under depolarizating conditions (52.5 mM $K^+$, 20 sec.) prior to testing for $^{45}Ca^{++}$ influx (Nachshen and Blaustein, 1980; Suszkiw and O'Leary, 1982). The data for

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the slow component of $^{45}Ca^{++}$ influx after the inactivation of the fast component (fig. 1B, circles) was best described by a linear model.

$$J = kt + b \qquad k = 0.25 \pm .003 \text{ nmol/mgP/sec}$$
$$b = 0.19 \pm .009 \text{ nmol/mgP}$$

Where k is the rate of the slow component and b the Y-intercept. The rate of the linear component of the total depolarization-dependent influx (fig. 1B, triangles) appears to correlate with that calculated for the direct linear fit of the isolated slow component influx (fig. 1B, squares), 0.24 and 0.25 nmol/mgP/sec. respectively. The similarity between the rates for the isolated slow component and linear phase of the total influx curve suggests that these $^{45}Ca^{++}$ influx may be mediated by the same pathway. It appears that the fast and slow components of $^{45}Ca^{++}$ influx are independent events which are simultaneously activated by depolarization. The total depolarization-dependent $^{45}Ca^{++}$ influx is the sum of both these components. In order to accurately determine the time course of the fast influx, the slow component (fig. 1A, squares) was subtracted from the total depolarization-dependent influx (fig. 1A, triangles). The isolated fast influx was fit by the following equation (fig. 1C).

$$J = P(1-e^{-kt}) \qquad P = 2.1 \pm .009 \text{ nmol/mgP}$$
$$k = 2.2 \pm 0.5 \text{ sec}^{-1}$$

Where P is the influx plateau and k is the rate coefficient of the fast component. The rate coefficients calculated for the fast component of total influx ($k_1$=2.0 $sec^{-1}$) (fig. 1B, triangles) is in good agreement with that calculated for the isolated fast component (k=2.1 $sec^{-1}$) (fig. 1C).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

These results demonstrate that voltage-dependent $Ca^{++}$ influx is mediated by two distinct pathways. The fast component of influx is transiently activated by depolarization and is eliminated by previous exposure to depolarizing conditions. The slow component of influx is relatively linear with respect to time and is not sensitive to predepolarizing conditions. Both the fast and slow components of $Ca^{++}$ influx are simultaneously activated by depolarization. In this instance the fast component of influx is superimposed on the slow influx component. Consequently the total depolarization-dependent $Ca^{++}$ influx is the sum of both the slow and fast processes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

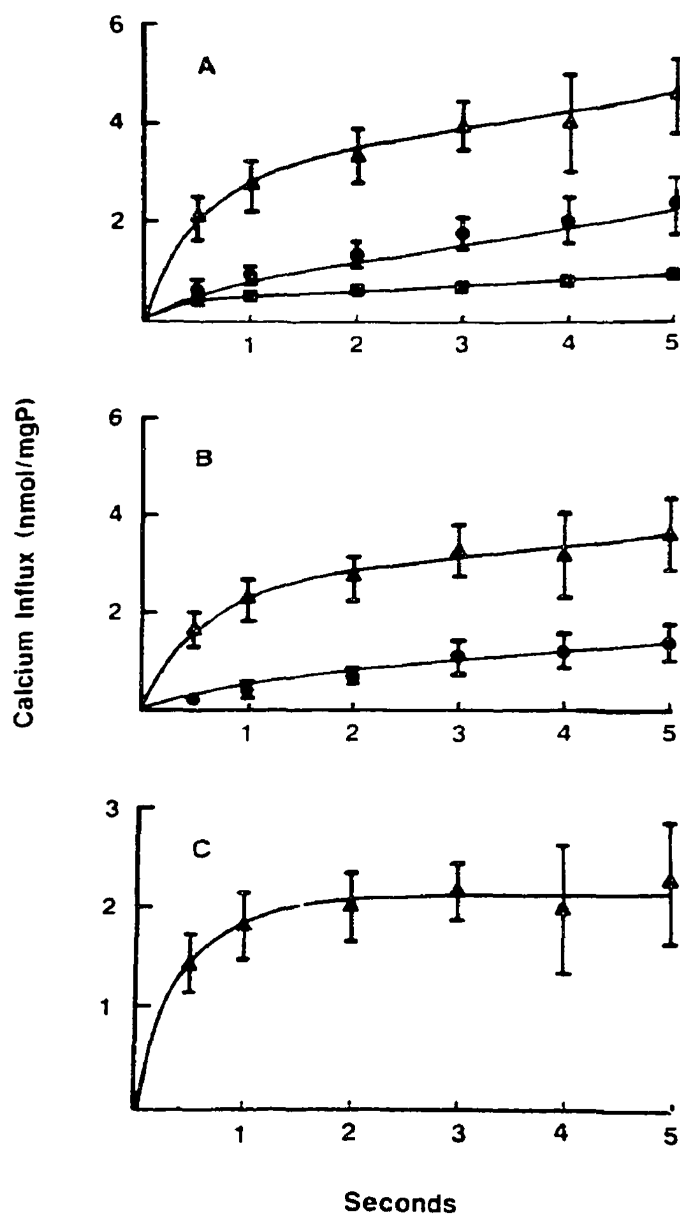



**Figure 1. Time course of depolarization-dependent calcium influx**

Calcium influx was determined by incubating synaptosomes in either 5 mM $K^+$ HKR or 52.5 mM $K^+$ HKR containing 1 mM $^{45}Ca^{++}$. The fast component of $Ca^{++}$ influx was inactivated by predepolarizing synaptosomes 20 seconds in 52.5 mM $K^+$ HKR prior to testing for $^{45}Ca^{++}$ influx. Data represent Means ± SD (N=6). Smooth curves were drawn to the non-linear least squares fit of the data (see text).

| | | |
|---|---|---|
| A. | Triangles | 52.5 mM $K^+$ HKR |
| | Circles | 52.5 mM $K^+$ HKR predepolarized |
| | Squares | 5 mM $K^+$ HKR |
| B. | Triangles | 52.5 mM $K^+$ HKR minus 5 mM $K^+$ HKR |

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

C. Inactivation of Calcium Influx

The fast component of $Ca^{++}$ influx is eliminated by preincubating synaptosomes under depolarizing conditions (fig. 1A, circles). The following experiments were designed to characterize the inactivation process of the fast $Ca^{++}$ influx component. The influx of $^{45}Ca^{++}$ per unit time is reduced the longer the synaptosomes are preincubated under depolarizing conditions (52.5 mM $K^+$ HKR). The observed reduction of $^{45}Ca^{++}$ entry per standard interval (ie. 1 sec. influx interval) represents the depolarization-dependent inactivation of the $Ca^{++}$ influx. The onset of inactivation is rapid during the initial 10 seconds of predepolarization and eventually declines to reveal a constant non-inactivating residual of $^{45}Ca^{++}$ influx (fig. 2). The inactivation data was best described by the the following equation:

$$Q = Pe^{-kt} + b \qquad P = 0.91 \pm 0.01$$

$$k = 0.29 \pm 0.04 \text{ sec}^{-1}$$

$$b = 0.09 \pm 0.01$$

Q is the fraction of inactivated channels at any given time (t), P is the fraction of influx undergoing inactivation, k is the rate coefficient for the inactivation process and b is the fraction of non-inactivating influx. This inactivation curve is the composite of synaptosomes preincubated in either 140 mM or 92.5 mM $Na^+$ HKR. The synaptosomes preincubated in reduced $Na_o$ are presumed to have lower $Na_i$. This did not change the calculated parameters for the inactivation curves and the results for both synaptosomal preincubations were combined for curve fitting purposes.

An alternative explanation for channel inactivation would be that the synaptosomes repolarized during the test interval. Repolarization would decrease $Ca^{++}$ influx by

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

causing de-activation rather than inactivation. The inward movement of $Cl^-$ could potentially result in the hyperpolarization (ie. repolarization) of the membrane potential. To eliminate its possible redistribution, the chloride in HKR solutions was replaced in some experiments with methysulfate. Methylsulfate is an impermeant anion which substitutes for $Cl^-$ in the extracellular solutions. Inactivation was unchanged by the absence of $Cl_o$ (fig. 2, open triangles). To futher test that repolarization was not responsible for the observed inactivation, the membrane potential of synaptosomes was measured during depolarization. Synaptosomes were equilibrated with the fluorescent dye 3,3'- dihexyloxacarbocyanine. This dye responds to changes in voltage by redistributing across membranes, which is associated with a change in the emission intensity (Sims et al., 1974; Waggoner, 1979). Elevation of the external potassium depolarized the synaptosomes for at least 5 minutes (Suszkiw et al., 1985). Depolarization-dependent inactivation cannot be explained by repolarization of the membrane potential and hence de-activation of the $Ca^{++}$ influx.

This inactivation was performed in the presence of no added calcium in the incubation media. Under these conditions only background concentrations of $Ca^{++}$ are present. It seems unlikely that this concentration of $Ca_o$ would generate sufficient $Ca^{++}$ influx to cause significant accumulation within the synaptosomes. This argument is supported by the observation that when synaptosomes are depolarized in $Ca^{++}$ free solutions there is no increase in the $Ca^{++}$ content of synaptosomes as measured by Quin-2 (Nachshen, 1985). This implies that during depolarization neither $Ca^{++}$ influx nor $Ca^{++}$ release from internal stores was stimulated. Therefore it appears that at least in reduced $Ca^{++}$ solutions (ie. the conditions of these experiments), channel inactivation is voltage-rather than calcium-dependent.

The biphasic inactivation curve is consistent with the previous observations that $Ca^{++}$ influx occurs at two distinct rates in synaptosomes (Nachshen and Blaustein, 1980).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

It is interesting to hypothesize that the voltage-dependent inactivation of channels is the major determinant of the time course of $Ca^{++}$ influx. In this instance the rapid and non-inactivating phases of inactivation (fig. 2) would correspond to the "fast" and "slow" phases of calcium influx (fig. 1B). This conclusion would be supported if the rate coefficients for the inactivation process equalled those for the decay of calcium influx. These results are difficult to compare due to differences in time resolution of the experimental protocols. The predepolarizing manipulations in the inactivation experiments limited the first available measurement of influx to 1 second. Previous results indicate that a significant portion, if not all of the "fast" influx is complete within this time interval (Nachshen and Blaustein, 1982; Nachshen, 1985; Suszkiw et al., 1986). This invalidates the direct quantitative comparison of these rate parameters. This does not exclude the possibility that voltage-dependent channel closure regulates the influx, however it does point out the requirement of experiments with better time resolution.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

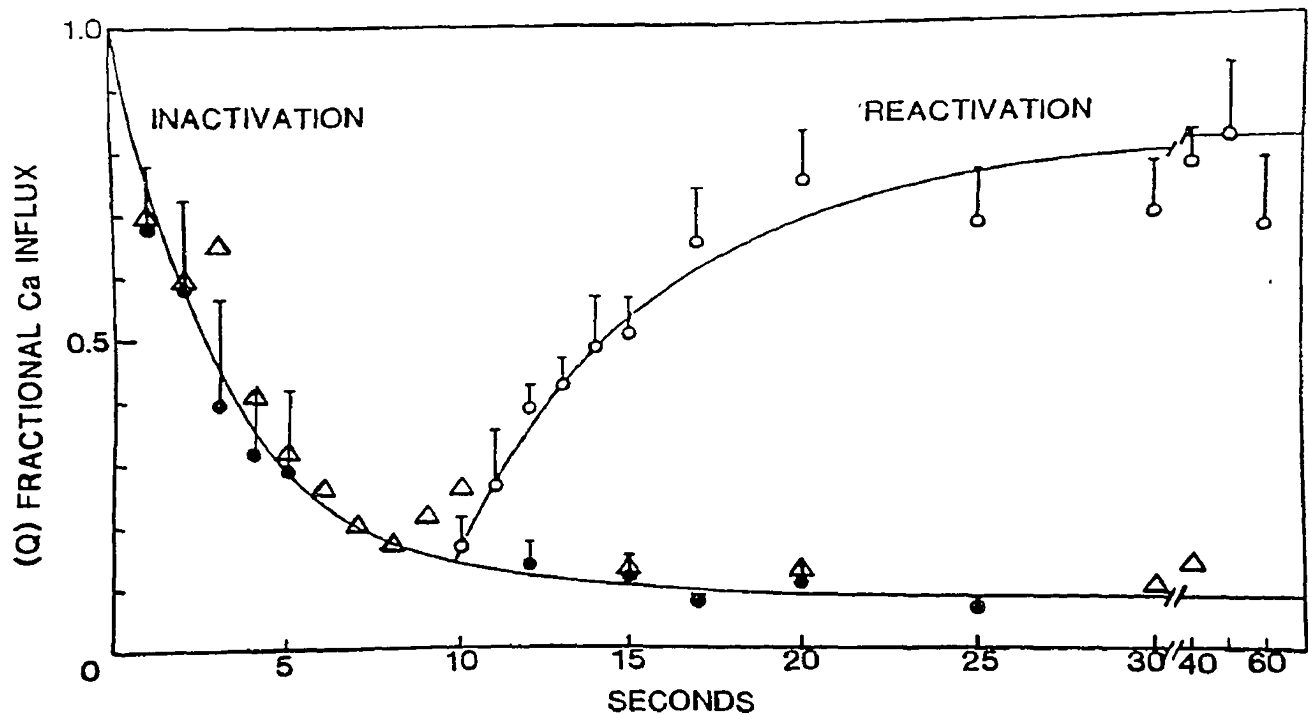



Figure 2. **Voltage-dependent inactivation and reactivation**

**Inactivation**: Synaptosomes were predepolarized in 52.5 mM $K^+$ HKR for various intervals prior to testing for $^{45}Ca^{++}$ influx. The influx measured after each predepolarization interval is expressed as percentage of the $^{45}Ca^{++}$ influx into non-predepolarized synaptosomes.

**Reactivation**: Synaptosomes were predepolarized 10 seconds in 52.5 mM $K^+$ HKR before addition of HKR so that the $[K^+]_o$ was returned to 5 mM. $^{45}Ca^{++}$ influx was measured as the synaptosomes recovered from inactivation. The influx is expressed as a percentage of the influx in non-predepolarized synaptosomes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

D. Reactivation of Calcium Influx

The following experiments were performed to test for the recovery of $Ca^{++}$ influx following voltage-dependent inactivation. Preincubation of synaptosomes for 10 seconds under depolarizing conditions inactivates the $^{45}Ca^{++}$ influx by 80% (fig. 2). Following inactivation, the recovery of $Ca^{++}$ influx is promoted by incubating the synaptosomes under non-depolarizing conditions. The recovery process (fig. 2) is described by the following equation:

$$Q_{rec} = q_1(1-e^{-kt}) + q_2 \qquad \begin{aligned} q_1 &= 0.63 \pm 0.06 \\ k &= 0.16 \pm 0.04 \text{ sec}^{-1} \\ q_2 &= 0.18 \pm 0.05 \end{aligned}$$

$Q_{rec}$ is the fraction of influx available to activate at any given time (t) during recovery, $q_1$ is the final level of recovery, k is the rate coefficient of recovery and $q_2$ is the residual non-inactivating fraction of influx. The depolarization-dependent $^{45}Ca^{++}$ influx recovers to 81% of its initial activity after 60 seconds incubation under resting conditions. Influx recovers to near control levels within three minutes (data not shown). The recovery of influx (0.16 $sec^{-1}$) is slower than the inactivation (0.28 $sec^{-1}$) (fig. 2).

Inactivation of the fast component of $Ca^{++}$ influx occurs in response to depolarization of the synaptosomes (fig. 2). The behavior of $Ca^{++}$ fluxes of other neuronal systems suggests that the inactivation should subside when synaptosomes are returned to their resting membrane potential (Hagiwara and Byerly, 1981). The voltage-dependent inactivation of $Ca^{++}$ influx is readily reversible under physiological

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

conditions. Voltage-dependent activation (fig. 1), inactivation (fig. 2) and recovery (fig. 2) are characteristic of a number of calcium channels (Hagiwara and Byerly, 1981; Tsien, 1983).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

E. Voltage Dependency of Inactivation

Inactivation is a function of the predepolarization interval (fig. 3). It was of interest to determine if the inactivation process is also a function of the predepolarization voltage. To test this hypothesis synaptosomes were predepolarized to different membrane potentials by varying the $K_o$ in the preincubation solutions. The $^{45}Ca^{++}$ influx after preincubation was determined and the fractional influx plotted versus $K_o$ (fig. 3A) and the calculated membrane potential (fig. 3B). It is clear that the larger the $K_o$ and hence the level of depolarization during preincubation the greater the resultant inactivation. The level of inactivation is directly proportional to the calculated membrane potential (slope=-1.026 $mV^{-1}$). This is consistent with the observation that the rate of inactivation in 52.5 mM $K^+$ HKR (k=0.28 $sec^{-1}$) is slower than that measured in 75 mM $K^+$ HKR (k=0.46 $sec^{-1}$). Calcium channel inactivation is a function of the predepolarizing voltage and time. $Ca^{++}$ influx (Nachshen and Blaustein, 1980, 1982) and transmitter release (Blaustein et al., 1972; Drapeau and Blaustein, 1983) display similar voltage-dependent characteristics.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

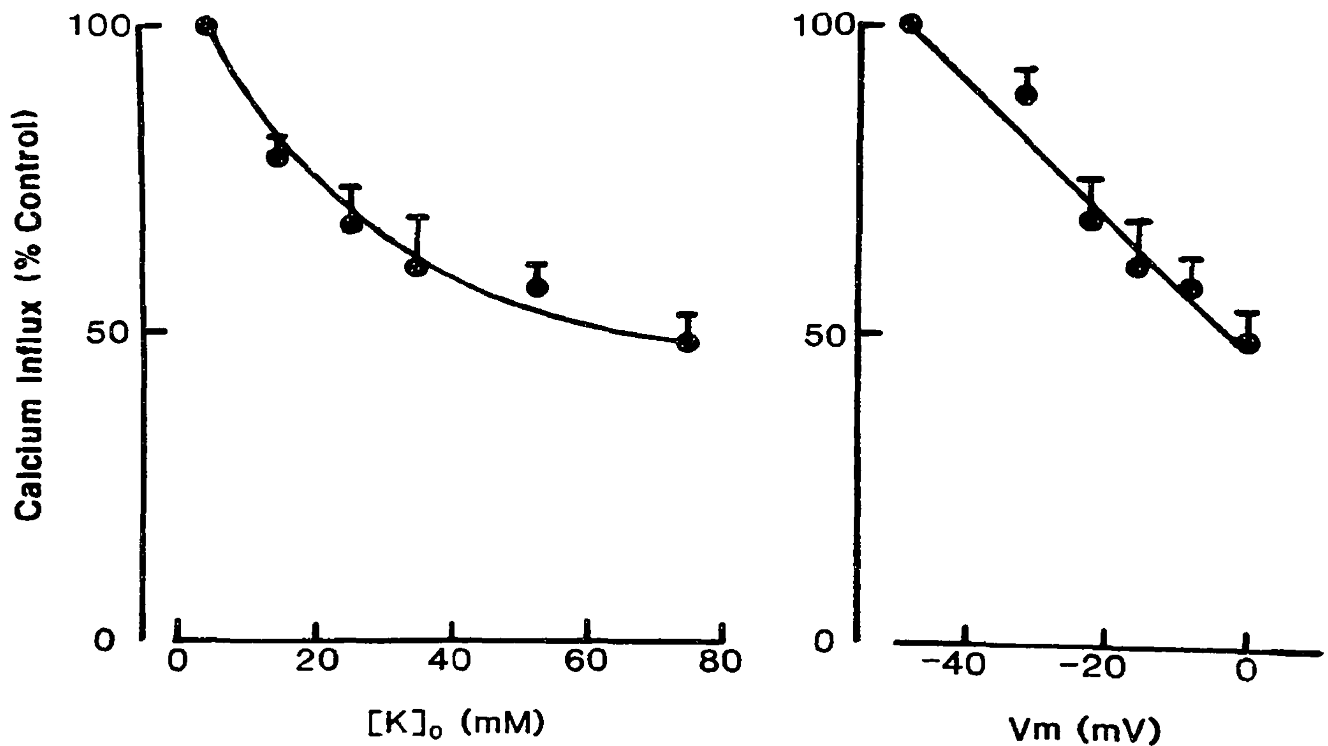



Figure 3. **Voltage dependency of the inactivation process**

**Left:** Synaptosomes were preincubated for 2 seconds by incubation in 15, 25, 35, 52.5 and 75 mM $K^+$ HKR. After preincubation the $K^+$ of each sample was adjusted to 52.5 mM $K^+$ containing 1 mM $^{45}Ca^{++}$. The influx during each interval is expressed as a percentage of the influx of non-predepolarized synaptosomes in 52.5 mM $K^+$ HKR.

**Right:** Inactivation is expressed as a function of the calculated predepolarization potential (Discussion A).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

F. Blockade of Calcium Influx by Lanthanum

Lanthanum has roughly the same ionic radius as calcium but has a valence of three (Takata et al., 1966). It inhibits the release of neurotransmitter at the neuromuscular junction (Heuser and Miledi, 1971) and the squid giant synapse (Miledi, 1971). The inhibitory effect of $La^{+3}$ in these preparations is attributed to its blockade of inward $Ca^{++}$ fluxes (Miledi, 1971). In accordance with these observations, $La^{+3}$ has been shown to be a potent inhibitor of synaptosomal $Ca^{++}$ influx (Nachshen and Blaustein, 1980) and transmitter release (Drapeau and Blaustein, 1983). The following experiments were designed to test the effect of $La^{+3}$ on the fast and slow components of $Ca^{++}$ influx (fig. 2).

The depolarization-dependent $^{45}Ca^{++}$ influx of synaptosomes was examined in the presence of 20 $\mu$M $La^{+3}$. $La^{+3}$ inhibited 85% of the $^{45}Ca^{++}$ influx into synaptosomes. The $^{45}Ca^{++}$ influx in this preparation is mediated by both the fast and slow pathways. In order to eliminate the fast component of $^{45}Ca^{++}$ influx, synaptosomes were first predepolarized 20 seconds prior to testing for $^{45}Ca^{++}$ influx. The slow component of $^{45}Ca^{++}$ influx was not significantly altered by the $La^{+3}$ treatment. The results suggest that the $^{45}Ca^{++}$ influx into non-predepolarized synaptosomes in the presence of $La^{+3}$ is equivalent to the slow component of influx in the absence of $La^{+3}$ (Paired T-Test, p=0.001). In effect, the fast component of $^{45}Ca^{++}$ influx was equally inhibited by either voltage-dependent inactivation or by incubation with $La^{+3}$ during the influx interval. The results suggest that $La^{+3}$ selectively inhibits the fast component of $Ca^{++}$ influx. These findings further support the conclusion that the transient and slow components of $Ca^{++}$ influx are mediated by distinct pathways. The voltage dependency and sensitivity to $La^{+3}$ are considered to be properties common to many calcium channels (Hagiwara

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

and Byerly, 1981; Tsien, 1983; Reuter, 1983). The results reported here are entirely consistent with the idea that the fast component of depolarization-dependent $Ca^{++}$ influx in synaptosomes is mediated by voltage-sensitive calcium channels.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

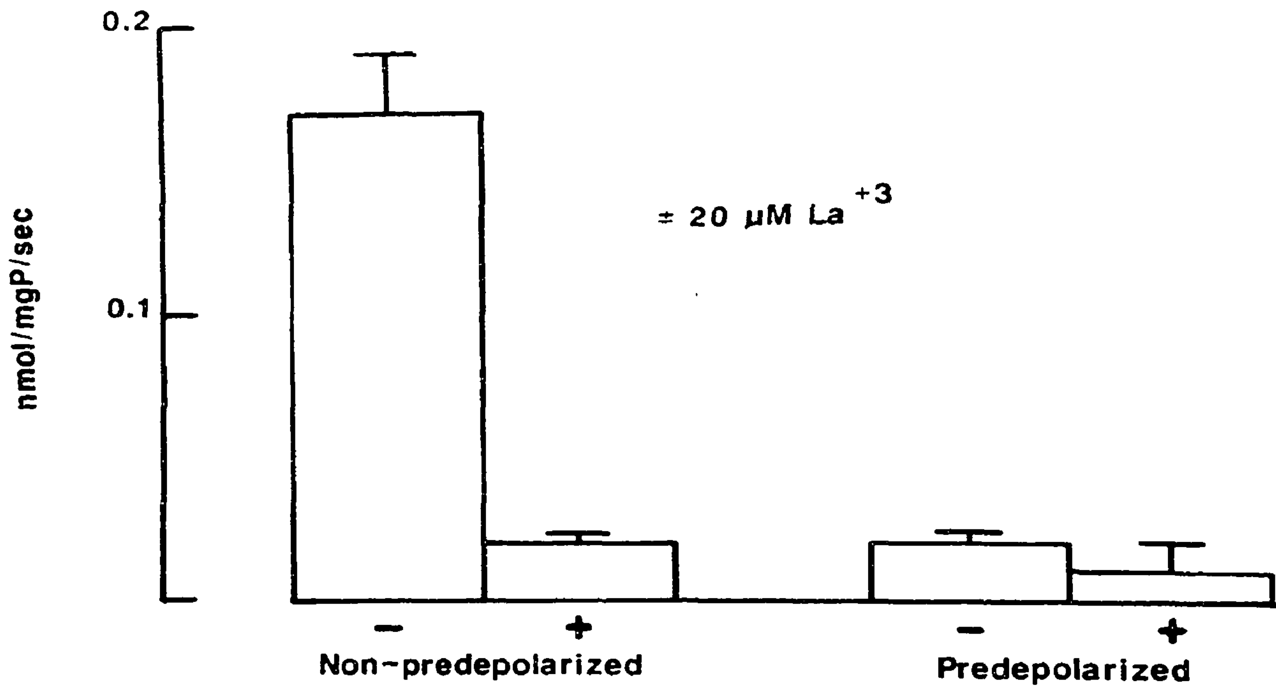



Figure 4. **Blockade of calcium influx by lanthanum**

$^{45}Ca^{++}$ influx (non-predepolarized) was stimulated by incubating synaptosomes in 52.5 mM $K^+$ HKR containing 20 mM $^{45}Ca^{++}$. Synaptosomes were incubated 20 seconds in 52.5 mM $K^+$ HKR (predepolarized) prior to the addition of 20 μM $^{45}Ca^{++}$. Paired samples were measured for both data in the presence (+) of 20 μM $La^{+3}$. Data are Means ± SD (n=3).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

G. Characterization of the Slow Component Calcium Influx

Synaptosomes incubated in 140 mM $Na^+$ HKR accumulate $Na^+$ (Table 1). At rest both the electrical and chemical gradients favor the inward flux of $Na^+$. Synaptosomes utilize the energy stored in the $Na^+$ gradient to power the efflux of $Ca^{++}$ (Blaustein and Oborn, 1975; Blaustein and Ecktor, 1976; Coutinho et al., 1984). Reduction in the $Na_o$ slows $Ca^{++}$ efflux while total $Na_o$ replacement promotes $Ca^{++}$ influx (Coutinho et al., 1984). These observations are consistent with the properties of the $Na^+/Ca^{++}$ exchange mechanism (NaCaX) described in other systems (Blaustein and Hodgkin, 1969; Barker et al., 1969). Raising the $K_o$ in the incubation media is a manipulation utilized to depolarize synaptosomes. In order to maintain the physiological osmolarity of these solutions, $K_o$ is iso-osmotically exchanged for $Na_o$. The result of increasing $K_o$ from 5 to 52.5 mM is a calculated depolarization of approximately 40 mV (Discussion A) and a reduction of the $Na_o$ from 140 to 92.5 mM. Under these conditions the electrical and concentration gradients may promote the efflux of $Na^+$ from synaptosomes (Coutinho et al., 1984). $Na^+$ efflux has been shown to be linked to the influx of $Ca^{++}$ in synaptosomes (Blaustein and Oborn, 1975; Coutinho et al., 1984). However most of these data were obtained utilizing $Ca^{++}$ influx intervals of minutes rather than seconds. The contribution of NaCaX to the $Ca^{++}$ influx during the initial seconds of depolarization (fig. 1) is not certain. The following experiments were designed to test what portion of the slow component of $Ca^{++}$ influx is mediated by the NaCaX.

Synaptosomes were incubated in either 140 mM choline (Ch-SYN) or 140 mM $Na^+$ HKR (Na-SYN). The $Na_i$ concentration of Na-SYN and Ch-SYN synaptosomes is approximately 83 and 4 mM respectively (Table 1). Na-SYN were preincubated under depolarizing conditions to inactivate the fast component of $^{45}Ca^{++}$ influx. The remaining influx is via the slow component. After preincubation $K^+$ HKR was added so

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

that $K_o$ was brought to 52.5 mM and the $Na_o$ reduced from 140 to 92.5 mM (fig. 4, top, triangles). Background $^{45}Ca^{++}$ influx was measured in 5 mM $K^+$/47.5 mM choline HKR (fig. 4, top, squares). The $Na_o$ of both depolarized and non-depolarized samples is equivalent in this paradigm so that the additional $^{45}Ca^{++}$ influx measured arises from the change in membrane potential and not from differences in the $Na^+$ gradient. The specific voltage-dependent slow component influx is shown (fig. 4, bottom, triangles). It is not clear whether the depolarization augments $^{45}Ca^{++}$ influx as a consequence of voltage-dependent changes in the $Na^+$ gradient or by a direct effect on the $Ca^{++}$ influx. To delineate the role of $Na^+$ in generation of the slow component a similar analysis for Ch-SYN influx was performed except that neither the 5 mM $K^+$ HKR (fig. 4, middle, squares) nor the 52.5 mM $K^+$ HKR (fig. 4, middle, circles) contained $Na_o$. Following preincubation, Ch-SYN have low levels of $Na_i$ (Table 1), and are incapable of supporting $Na_i/Ca_o$ exchange. The depolarization-dependent $Ca^{++}$ influx in predepolarized Ch-SYN is mediated by pathways other than the fast component of influx or the $Na_i/Ca_o$ exchange mechanism. The difference in the voltage-stimulated slow influx of Na-SYN (fig. 4, bottom, triangles) and Ch-SYN (fig. 4, bottom, circles) represents the depolarization-augmented, $Na_i$-dependent $Ca^{++}$ influx. The additional $Ca^{++}$ influx observed in Na-SYN is driven by the higher levels of $Na_i$ in this preparation. Approximately 50% of the depolarization-dependent slow influx of Na-SYN can be attributed to the $Na^+$ gradient. These results demonstrate that the $Na_i$-dependent $Ca^{++}$ influx makes a significant contribution to the total $Ca^{++}$ influx at short depolarization intervals and that this entry is sensitive to changes in membrane potential.

It appears that the slow phase of $Ca^{++}$ influx is mediated by two pathways. The first is dependent on the $Na^+$ gradient and the membrane potential and is probably conducted through a reversal of an electrogenic $Na_i/Ca_o$ exchange mechanism. Similar concentration and voltage dependencies are observed for the NaCaX mechanism of squid

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

axon (Mullins and Brinley, 1975; Mullins et al., 1981). The second component is clearly observed in Ch-SYN and is dependent on the membrane potential only and may represent a non-inactivating $Ca^{++}$ channel or a $Ca_i/Ca_o$ exchange mechanism. Two modes of slow influx similar to those described above have previously been reported in synaptosomes (Coutinho et al., 1984) and synaptic plasma membrane vesicles isolated from brain (Erdreich et al., 1983). However these studies examined the influx over relatively long intervals of depolarization (>30 sec.) and do not reflect the slow influx which occurs during channel activity.

These results indicate that the $Na_i/Ca_o$ exchange is capable of making a significant contribution to $^{45}Ca^{++}$ influx during the intervals in which $Ca^{++}$ channels are active (fig. 1). The following experiments were designed to quantify the contribution of the $Na_i$-dependent $Ca^{++}$ influx to the total depolarization-dependent $Ca^{++}$ influx measured in a preparation where both the $Ca^{++}$ channels and $Na_i/Ca_o$ are active. Na-SYN were depolarized in 52.5 mM $K_o$ containing 92.5 mM $Na_o$ or reduced 70 mM $Na_o$/22.5 mM choline. Both groups are Na-SYN, therefore the magnitude of the depolarization and the channel mediated influx is equivalent, however the $Na_o$ of the depolarizing solutions is altered by 22.5 mM. $^{45}Ca^{++}$ influx was not significantly different for synaptosomes incubated in 5 mM $K_o$ with either 92.5 or 70 mM $Na_o$ (fig. 5). The synaptosomes bathed in reduced $Na_o$ (ie. 70 mM) have an augmented outwardly directed [$Na^+$] gradient. This difference in $Na^+$ gradient does not stimulate additional $^{45}Ca^{++}$ accumulation. Under depolarizing conditions the synaptosomes incubated in 70 mM $Na_o$ accumulated significantly more $^{45}Ca^{++}$ than those incubated in 92.5 mM sodium. The specific uptake in the reduced $Na_o$ (70 mM) environment was 25% greater than that in control $Na_o$ (92.5 mM).

When the transmembrane [$Na^+$] gradient is small the enhancement of $^{45}Ca^{++}$ influx is only observed under depolarizing conditions. The influence of voltage on the exchange

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

appears to be more important than the alteration of the $Na^+$ chemical gradient. The results suggest that the depolarization directly stimulates the outwardly directed electrochemical gradient for $Na^+$ which indirectly increases $Ca^{++}$ influx through the stimulation of the $Na_i/Ca_o$ exchange. However it is important to emphasize that the electrochemical gradient for $Na^+$ is the primary determinant of direction and magnitude of the NaCaX. Though both the electrical and chemical components are sufficient to drive the exchange, these forces are more influential when acting in concert.

An additional factor which may contribute to the measured "calcium influx" is a reduction of $Ca^{++}$ efflux. Reduced $Na_o$ would be expected to decrease the inwardly directed $[Na^+]$ gradient and consequently the $Ca^{++}$ efflux through the NaCaX. Effects on $Ca^{++}$ efflux are not observed in synaptosomes until the $[Na^+]_o$ is reduced below 50 mM and the influx measured at relatively long (>30 sec) intervals (Blaustein and Oborn, 1975). Reduced efflux would not be expected to interfere with the measurement of $Ca^{++}$ influx at 1 second and 70 mM $[Na^+]_o$.

The results demonstrate that $Na^+$-dependent $Ca^{++}$ influx can make a significant contribution to the total $Ca^{++}$ influx at short depolarization intervals. In addition it emphasizes that depolarization-dependent $Ca^{++}$ influx is mediated by several different pathways. The total influx observed is a summation of these components. Isolation of the flux through an individual pathway requires the proper conditions to control for the remaining fluxes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

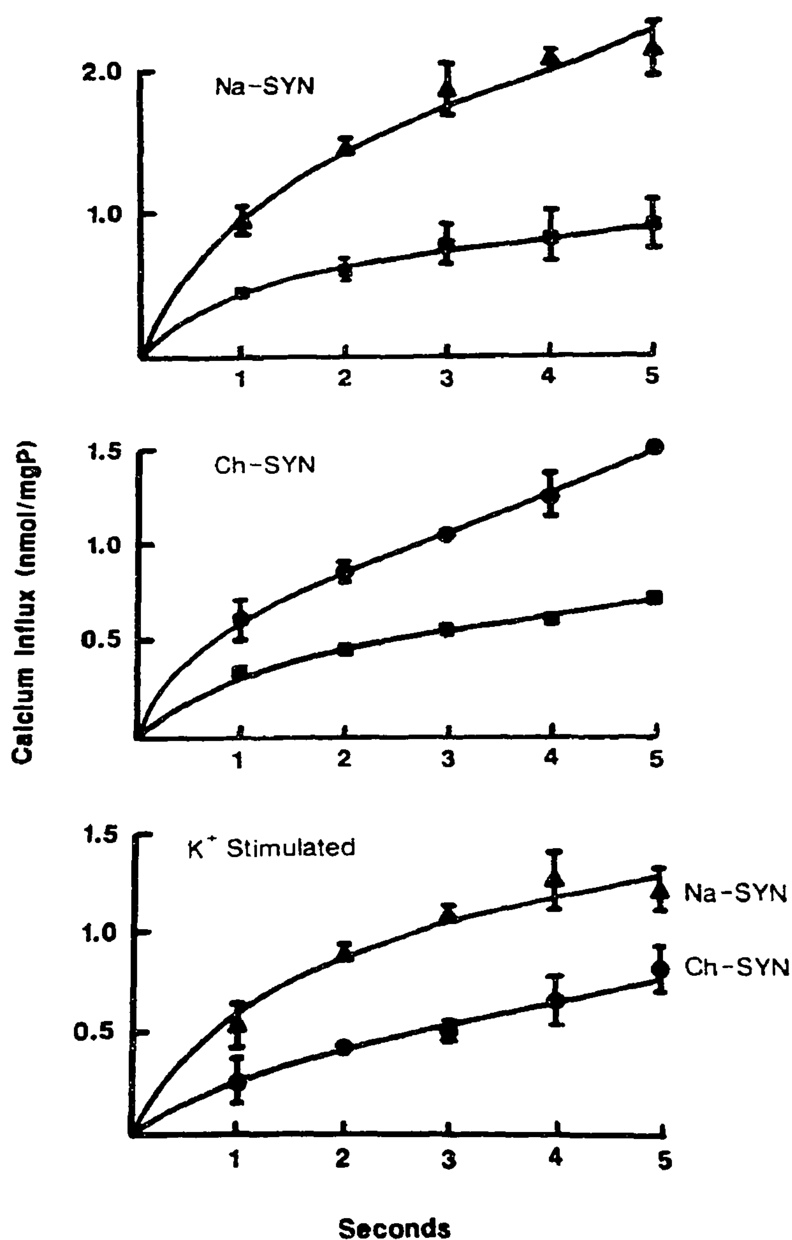



Figure 5. **Characterization of the slow component of calcium influx**

The slow component of $Ca^{++}$ influx was measured in either Na-SYN or Ch-SYN. The fast component was eliminated by preincubating synaptosomes 10 seconds in 52.5 mM $K^+$ HKR prior to adding 1 mM $^{45}Ca^{++}$. The $K^+$-stimulated influx is calculated as that in 52.5 mM $K^+$ minus that in 5 mM $K^+$ HKR.

| | | |
|---|---|---|
| Top | Triangles | Na-SYN in 52.5 mM $K^+$ HKR |
| | Squares | Na-SYN in 5 mM $K^+$HKR |
| Middle | Circles | Ch-SYN in 52.5 mM $K^+$ HKR |
| | Squares | Ch-SYN in 5 mM $K^+$ HKR |
| Bottom | Triangles | Na-SYN $K^+$-stimulated |

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

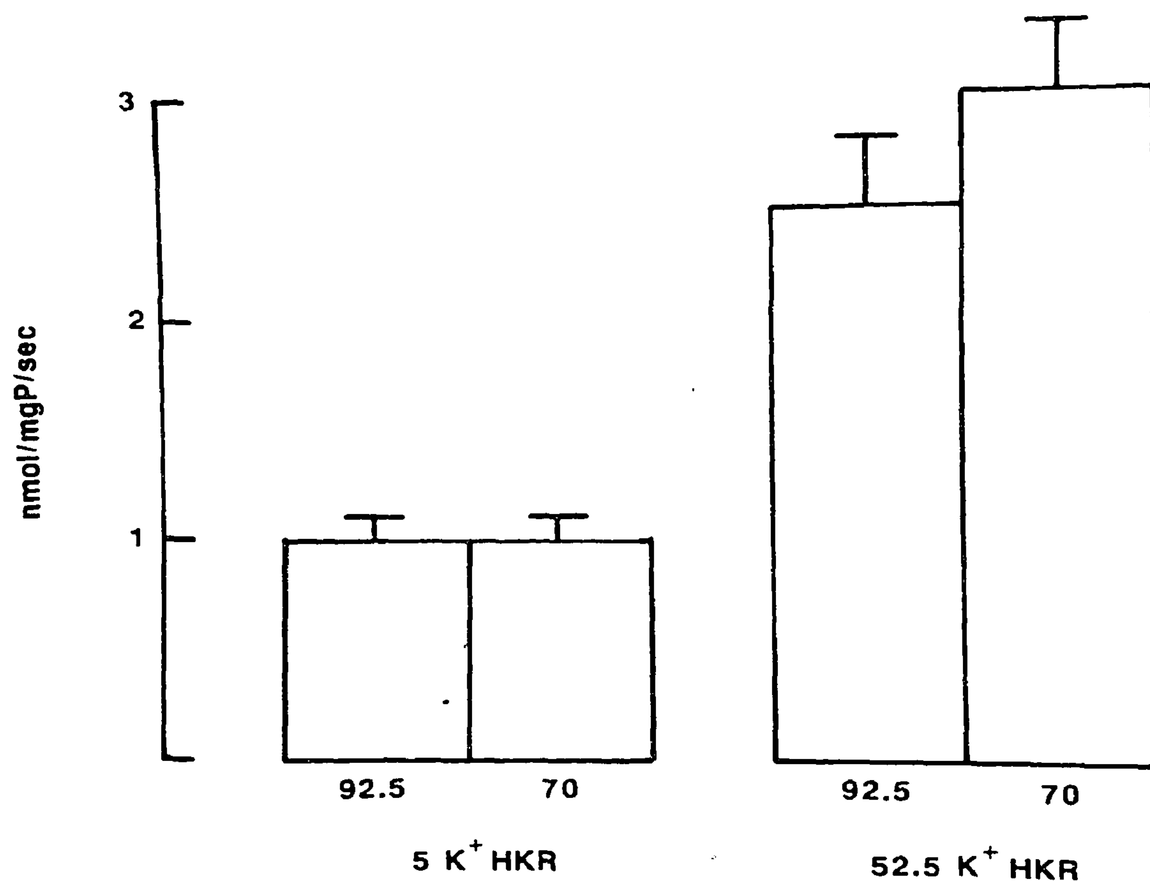



**Figure 6. Contribution of the slow component of calcium influx during the first second of depolarization**

$^{45}Ca^{++}$ influx in Na-SYN was measured at rest (5 mM $K^+$ HKR) and when depolarized (52.5 mM $K^+$ HKR). The $Na_o$ was varied (70 or 92.5 mM) by iso-osomotic replacement of $Na^+$ with choline. Depolarized influx in 70 mM $Na_o$ was significantly greater than that in 92.5 mM $Na_o$ based on paired T-Test (p=0.001, n=4).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

H. Relationship of Norepinephrine Release to Calcium Influx

In much of the early work the on the release of neurotransmitters from synaptosomes the preparations were depolarized for prolonged intervals (>30 sec.). It is now clear that the majority of $Ca^{++}$ entering during these treatments was not mediated by the voltage-dependent $Ca^{++}$ channels. More recent experiments have examined the relationship of $Ca^{++}$ influx to transmitter release during the initial 5 seconds of depolarization (Redburn et al., 1976; Floor, 1983; Drapeau and Blaustein, 1983; Suszkiw and O'Leary, 1983; Leslie et al., 1985; Daniell and Leslie, 1985). During this interval most of the $Ca^{++}$ which enters is via channels. The following group of experiments were performed in order to determine the relationship between channel mediated $Ca^{++}$ influx and neurotransmitter release.

Intra-synaptosomal norepinephrine (NE) pools were labeled by incubating synaptosomes with 0.1 $\mu$M $^3$H-NE. The uptake of NE at this external concentration is via the $Na_o$-dependent high affinity uptake mechanism ($K_M$=0.4 $\mu$M) which is specific to catecholaminergic synaptosomes (Colburn et al., 1968; Iversen, 1973). The uptake of norepinephrine by other types of synaptosomes and non-neuronal structures which lack the high affinity system is minimal under these conditions. The voltage-stimulated, $Ca_o$-dependent release of $^3$H-norepinephrine (NE) is biphasic (fig. 7). A rapid initial phase of release appears to terminate within the first 2 seconds while a slower tonic release persists through 5 seconds of depolarization. The profile of $^3$H-NE release is similar to the influx of $^{45}Ca^{++}$ measured under identical conditions (fig. 8). $^3$H-NE release and $^{45}Ca^{++}$ influx data were fit by the following equation:

$$J = P(1-e^{-k_1 t}) + k_2 t$$

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

J is the $Ca^{++}$ influx (nmol/mgP) or the $^3H$-NE release (% control), P is the plateau phase of each event, $k_1$ is the rate coefficient of the fast component and $k_2$ is the rate of the slow component. The parameters determined for these events are compared below.

| Release | Influx |
|---|---|
| $P = 3.2 \pm 0.8$ % | $P = 2.4 \pm 0.4$ nmol $mg^{-1}$ |
| $k_1 = 2.1 \pm 1.1$ $sec^{-1}$ | $k_1 = 2.0 \pm 0.8$ $sec^{-1}$ |
| $k_2 = 0.7 \pm 0.2$ % $sec^{-1}$ | $k_2 = 0.2 \pm 0.1$ nmol $mg^{-1}$ $sec^{-1}$ |

Both $Ca^{++}$ entry and $^3H$-NE release are biphasic through the first 5 seconds of depolarization. The rate coefficients of the fast components of influx and release are 2.0 and 2.1 $sec^{-1}$, respectively. The $k_1$ coefficient determined for $Ca^{++}$ influx is a measure of the time course of fast $Ca^{++}$ influx (ie. channel mediated). These results suggest that the $Ca^{++}$ influx mediated by the channel and the initial phase of transmitter release proceed at identical rates. This is expected if the $Ca^{++}$ influx is the trigger for transmitter release. The later component of $Ca^{++}$ influx has been ascribed to non-channel pathways (fig. 5). The slope of the linear portion of the transmitter release curve is 3.5 fold greater than corresponding portion of the $Ca^{++}$ influx curve. A release which exceeds $Ca^{++}$ entry would be predicted if the synaptoplasm became loaded with ionized $Ca^{++}$. Such a situation could occur if the capacity of the $Ca^{++}$ sequestering system were surpassed due to $Ca^{++}$ loading during prolonged depolarization (>2 sec). In addition, synaptosomes depolarized by elevated $K_o$ may have an outwardly directed $Na^+$ electrochemical gradient (fig. 6). This would inhibit the efflux of $Ca^{++}$ through the $Na_o/Ca_i$ exchanger and would be expected to further augment cytoplasmic ionized $Ca^{++}$ levels (Blaustein and Oborn, 1975; Blaustein and Ecktor, 1976). This exchanger is the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

main determinant of ionized $Ca^{++}$ under resting conditions and is the primary mechanism of reducing intracellular ionized $[Ca^{++}]$ following the loading of synaptosomes with this ion (Nachshen, 1985).

These results support the conclusion that transmitter release during the first seconds *of depolarization* is stimulated by $Ca^{++}$ entry through voltage-sensitive channels. The slow phase of $Ca^{++}$ influx qualitatively correlates with a reduced rate of transmitter release. It appears that coupling of transmitter release to $Ca^{++}$ influx mediated via either channel or exchange mechanism are equally effective in synaptosomes. Recent experiments suggest that $Ca^{++}$ influx mediated by the $Na_i/Ca_o$ exchange mechanism may participate in the regulation of evoked transmitter release (Meiri et al., 1986). Reduction of the $Na_o$ during electrical stimulation of the frog neuromuscular junction leads to an increase in the recorded end plate potential (Moiser et al., 1986). It is proposed that incubation in low $Na_o$ increases $Ca_i$ by abolishing its efflux and stimulating influx through the $Na_i/Ca_o$ exchange mechanism (Moore et al., 1986). A similar exchange mediated $Ca^{++}$ influx may play an important role in regulating transmitter release from synaptosomes.

Biphasic transmitter release has previously been reported within this time range. Dopamine release and $Ca^{++}$ influx exhibit a fast and slow component (Drapeau and Blaustein, 1983). Similar results have been observed for norepinephrine (Leslie et al., 1985; Daniell and Leslie, 1985) and Substance P (Floor, 1983). These reports emphasize that despite the continued presence of depolarizing-stimulus, the evoked transmitter release exhibited rapid temporal decay. It has been proposed that the abatement of the fast phase processes (ie. transmitter release and $Ca^{++}$ influx) results from the voltage-dependent inactivation of $Ca^{++}$ channels (Nachshen and Blaustein, 1980; Drapeau and Blaustein, 1983; Suszkiw and O'Leary, 1983).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

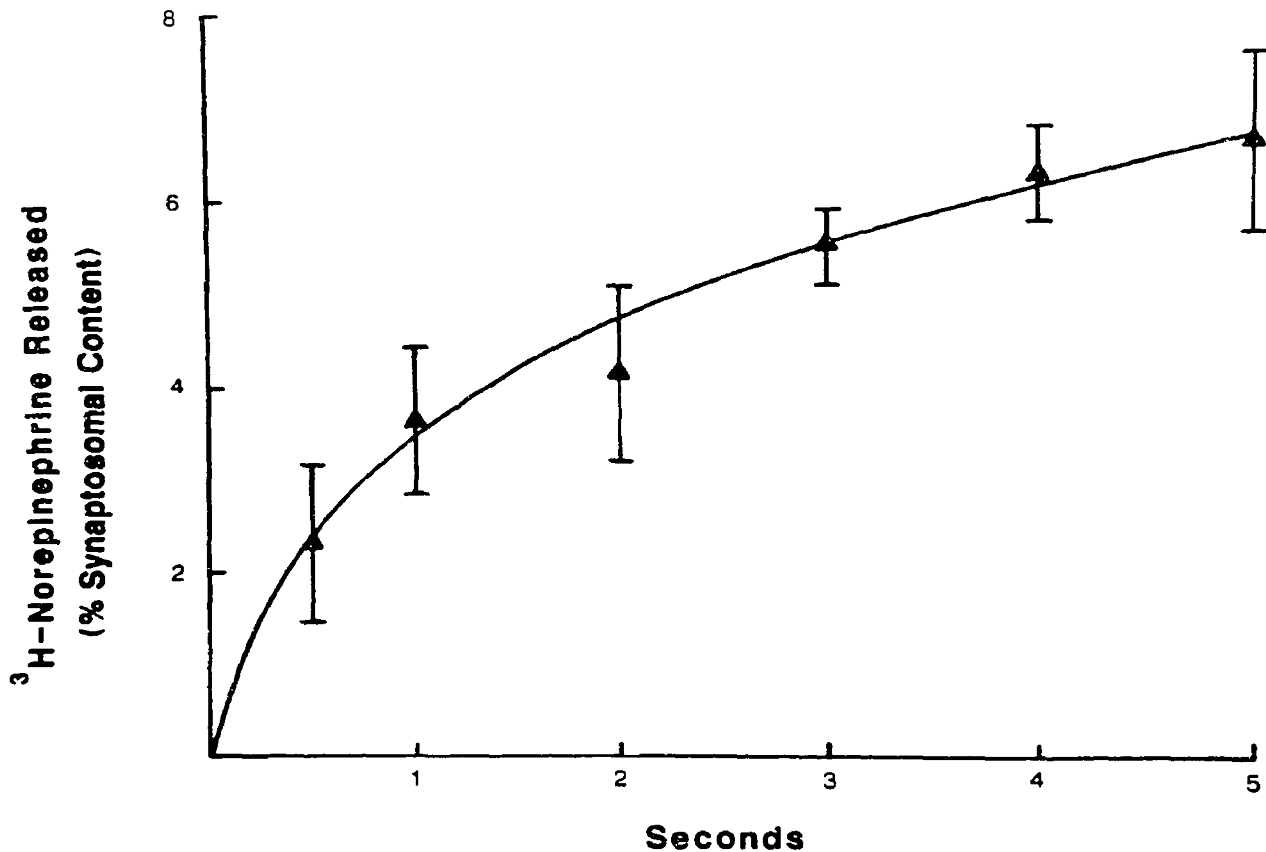



Figure 7. **Depolarization-dependent release of norepinephrine**

Synaptosomes were preloaded with $^3$H-norpeinephrine as described in the methods. Release of $^3$H-NE was stimulated by incubating the synaptosomes in 52.5 mM $K^+$ HKR containing 1 mM $Ca^{++}$. Background release was measured in 5 mM $K^+$ HKR. The specific release was calculated as the difference in release in 52.5 mM $K^+$ minus 5 mM $K^+$ HKR. The released $^3$H-NE is expressed as a percentage of the $^3$H-NE content of synaptosomes. The smooth curve were drawn to the non-linear least squares fit of the data.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

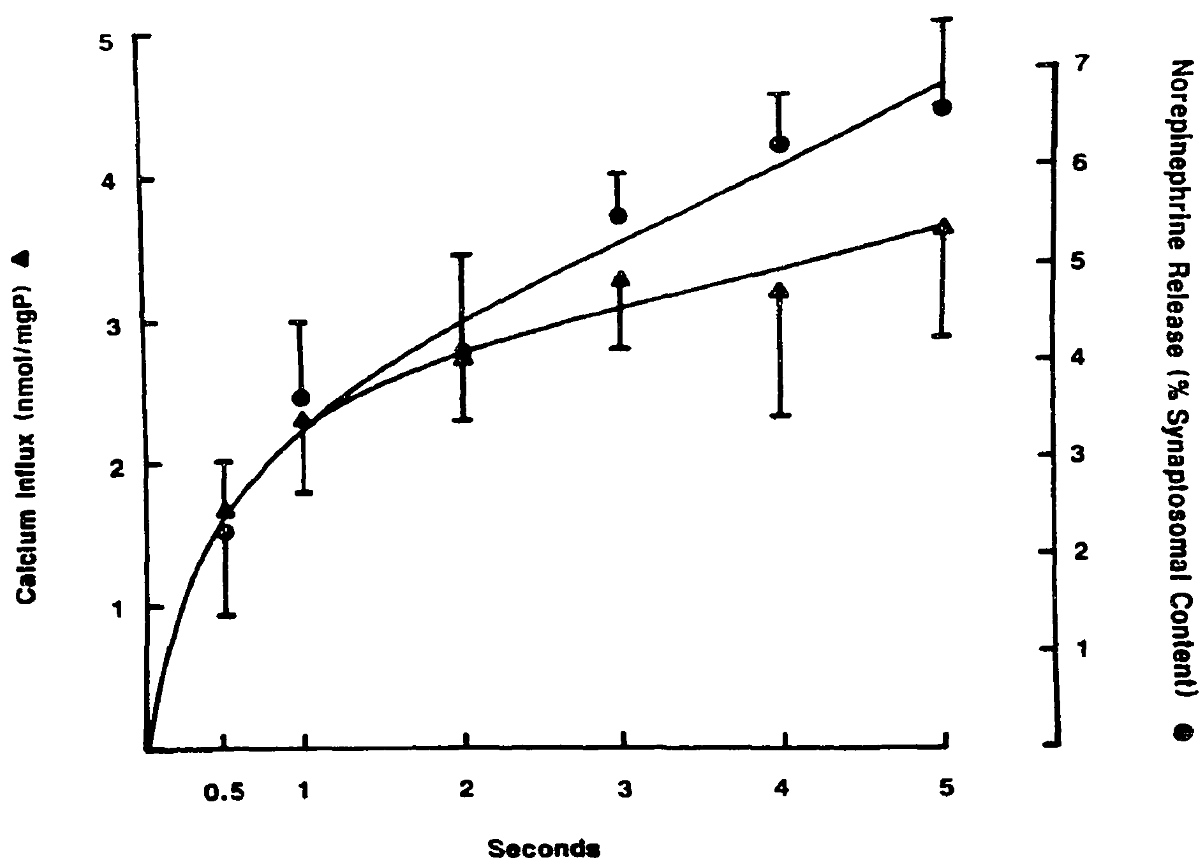



Figure 8. **Comparison of depolarization-dependent calcium influx and norepinephrine release**

The depolarization-dependent release of $^{3}H$-norepinephrine (% synaptosomal content) and $^{45}Ca^{++}$ influx (nmol/mgP) were compared. Both influx and release were measured under identical conditions (52.5 mM $K^{+}$ HKR/1 mM $Ca^{++}$). The smooth curves were drawn to the non-linear least squares fit of the data.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

I. Effects of Dihydropyridine Drugs on Calcium Influx

Synaptic membranes derived from synaptosome preparations have high affinity binding sites for dihydropyridine drugs ($K_D$ 0.1-1.0 nM) which are similar to the binding sites of other tissues (Murphy and Snyder, 1982; Gould et al., 1982; Ehlert et al., 1982; Bellemann et al., 1983; Janis and Triggle, 1983). Dihydropyridine drug binding to these receptors does not alter the voltage-dependent $Ca^{++}$ influx of synaptosomes (Nachshen and Blaustein, 1979; Daniell et al., 1983; Rampe et al., 1984; Creba and Karobath, 1986; Wei and Chiang, 1986). The idea that these binding sites are associated with functional calcium channels in synaptosomes remains questionable. Recently, a report showed an apparent block of $Ca^{++}$ influx by nitrendipine ($K_{APP}$=1.7 nM) which correlated with the dissociation constant ($K_D$=0.35 nM) for $^3$H-nitrendipine binding (Turner and Goldin, 1985). The results were in part attributed to the experimental conditions which proportedly minimize the contribution of the $Na_i/Ca_o$ exchange to the total $Ca^{++}$ influx. The following experiments were performed to investigate the effects of dihydropyridine channel agonists and antagonists on synaptosomal $Ca^{++}$ influx.

In performing these experiments care was taken to assure that the most favorable conditions were employed to promote the dihydropyridine affects. Synaptosomes were prepared from whole brain (ie. cortex and brainstem) or cortex only so that regional differences in dihydropyridine sensitivity would not be overlooked. In addition it was suggested that the differences in sensitivity to dihydropyridine drugs may vary with the method of synaptosome preparation (Turner and Goldin, 1985). To address this issue synaptosomes were prepared by two different methods and compared regarding drug sensitivity. Finally, several recent reports have suggested that the dihydropyridine drug sensitivity increases during submaximal stimulation (Middlemiss and Spedding, 1985;

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Rampe et al., 1984). In deference to this suggestion $Ca^{++}$ influx was studied under reduced depolarizing conditions.

The $Ca^{++}$ influx into crude ($P_2$) synaptosomes is linear to 10 seconds, indicating that voltage-dependent inactivation is minimal under these conditions (fig. 8). Based on this information $^{45}Ca^{++}$ influx measured in 20 mM $K_o$ for 5 seconds was used as the test for drug sensitivity. The depolarization-dependent $^{45}$ $Ca^{++}$ influx of $P_2$, Hajos whole brain and Hajos cortical synaptosomes are equivalent (fig. 9). Bay K 8644 is a proposed calcium channel agonist in smooth muscle (Schramm et al., 1983). The effect of Bay K 8644 on the depolarization-dependent $^{45}Ca^{++}$ influx in $P_2$ and Hajos synaptosome preparations is shown in figure 10. In all cases the drug treated $^{45}Ca^{++}$ influx did not significantly differ from the control values. It is concluded that neither the method of preparation nor the differences in the origin of the synaptosomes is important in regards to sensitivity to Bay K 8644. The depolarization-dependent $^{45}Ca^{++}$ influx in $P_2$ synaptosomes incubated with Bay K 8644, Nifedipine and Nitrendipine were not significantly different from control values (fig. 11). The synaptosomes incubated with Nifedipine were pre-equilibrated in 140 mM choline HKR (Ch-SYN). The potency of the dihydropyridines for blocking $Ca^{++}$ influx is maximized under reduced $Na_o$ conditions (Turner and Goldin, 1985).

The observations are in agreement with numerous reports that dihydropyridine drugs do not alter calcium channel function in synaptosomes (Nachshen and Blaustein, 1979; Daniell et al., 1983; Rampe et al., 1984; Creba and Karobath, 1986; Wei and Chiang, 1986; Miller and Freedman, 1984). Whether the lack of effect of dihydropyridine drugs represents insensitive channels or those that lack viable receptor/channel interaction cannot be acertained from these experiments. However these results are in direct conflict with recent published data (Turner and Goldin, 1985). The differences between this data and theirs cannot be explained by the differences in preparation, since at least

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

in part both studies utilized the Hajos method of synaptosome preparation. A partial explanation for this discrepancy is that these authors reported the free concentration of drugs rather than the total added drug concentration. They reasoned that only free drug is in equilibrium with the receptors and therefore represents the effective blocking concentration of the drug. This approach can lead to erroneous results when the synaptosomal protein concentration and hence the amount of non-specific binding of the drugs is high. In the experiments described above the protein concentration was held constant at 1 mg/ml throughout all drug incubation experiments. When the incubating concentration of Bay K 8644 was 10 nM or 1 μM, the measured free drug concentrations are 2.1 nM and 0.3 μM respectively. At this protein concentration the amount of free drug is significantly reduced. These unbound drug concentrations do not alter depolarization-dependent $^{45}Ca^{++}$ influx (fig. 10). The amount of non-specific drug binding is expected to increase with the synaptosomal protein concentration. When preincubating with high protein the free drug concentration is expected to be significantly lower than the total added drug concentration. The protein concentration in the Turner and Goldin experiments was 20 mgP/ml. It seems plausable that non-specific binding could lower the free drug concentration to the nanomolar range as reported. However the total amount of drug added to the synaptosomes would be significantly higher. The total added drug during preincubation is not reported by these authors and cannot be determined from the methodological description of the work. At 1 mgP/ml synaptosomal protein concentration (conditions of this work) the difference between total and free drug concentrations cannot account for the high affinity block of calcium channels described by these authors.

Synaptosomes have calcium channels which are not sensitive to dihydropyridine drugs. The activation and inactivation kinetics of the $Ca^{++}$ influx and its blockade by $La^{+3}$ suggests that synaptosomes have a single class of calcium channels whose properties

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

are similar to N-type channels (Nowycky et al., 1985). These channels mediate $Ca^{++}$ influx which supports neurotransmitter release (fig. 8). This is in agreement with the recent report that in neurons where several types of calcium channels coexist, the N-type channels predominate at the neurotransmitter release sites (Thayer et al., 1986).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

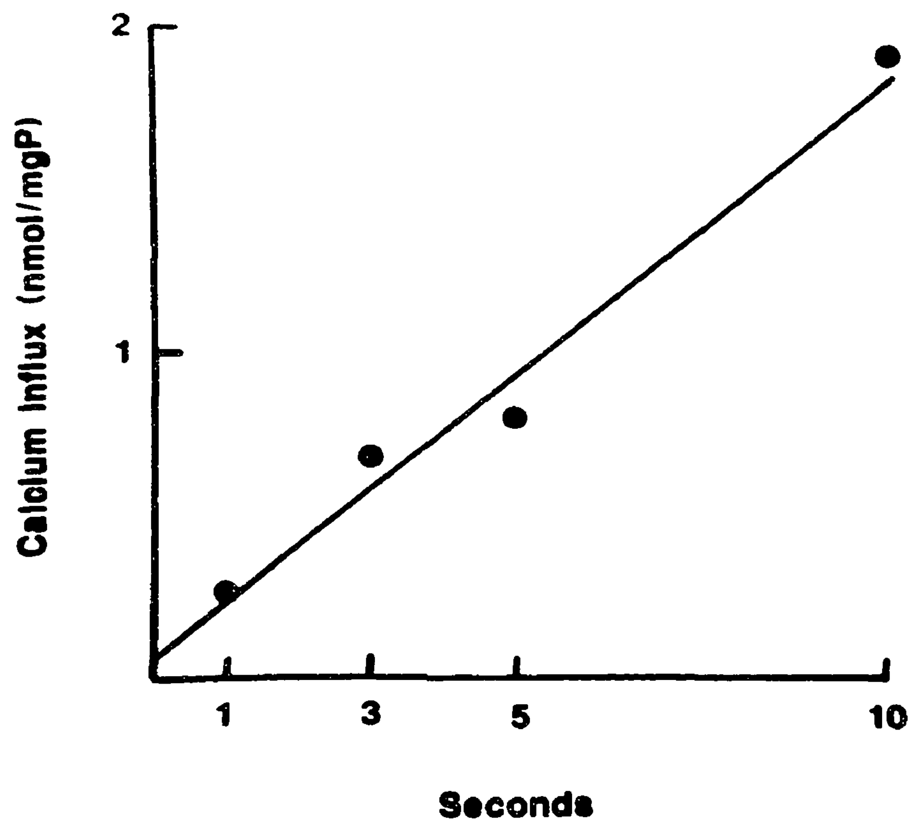



Figure 9. **Determination of sub-maximal depolarizing conditions**

Synaptosomes were depolarized in 20 mM $K^+$ HKR containing 1 mM $^{45}Ca^{++}$. The background influx was measured in 5 mM $K^+$/15 mM choline HKR. The specific $K^+$-stimulated influx is calculated as that in 20 mM $K^+$ minus that in 5 mM $K^+$ HKR.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

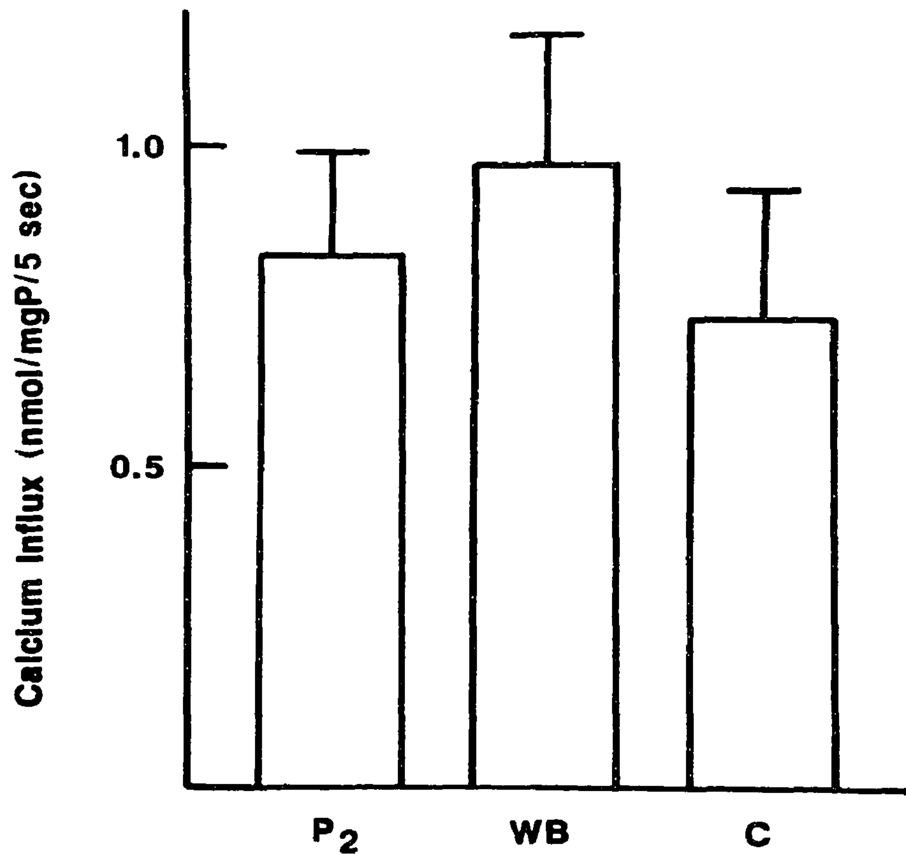



Figure 10. **Comparison of the calcium influx of several types of synaptosomes**

The $K^+$-dependent $^{45}Ca^{++}$ influx in crude ($P_2$), Hajos Whole Brain (WB) and Hajos Cortical (C) synaptosomes was determined under sub-maximal stimulating conditions (20 mM $K^+$ HKR/ 1 mM $^{45}Ca^{++}$/5 sec.). The influx in the various preparations was not significantly different.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

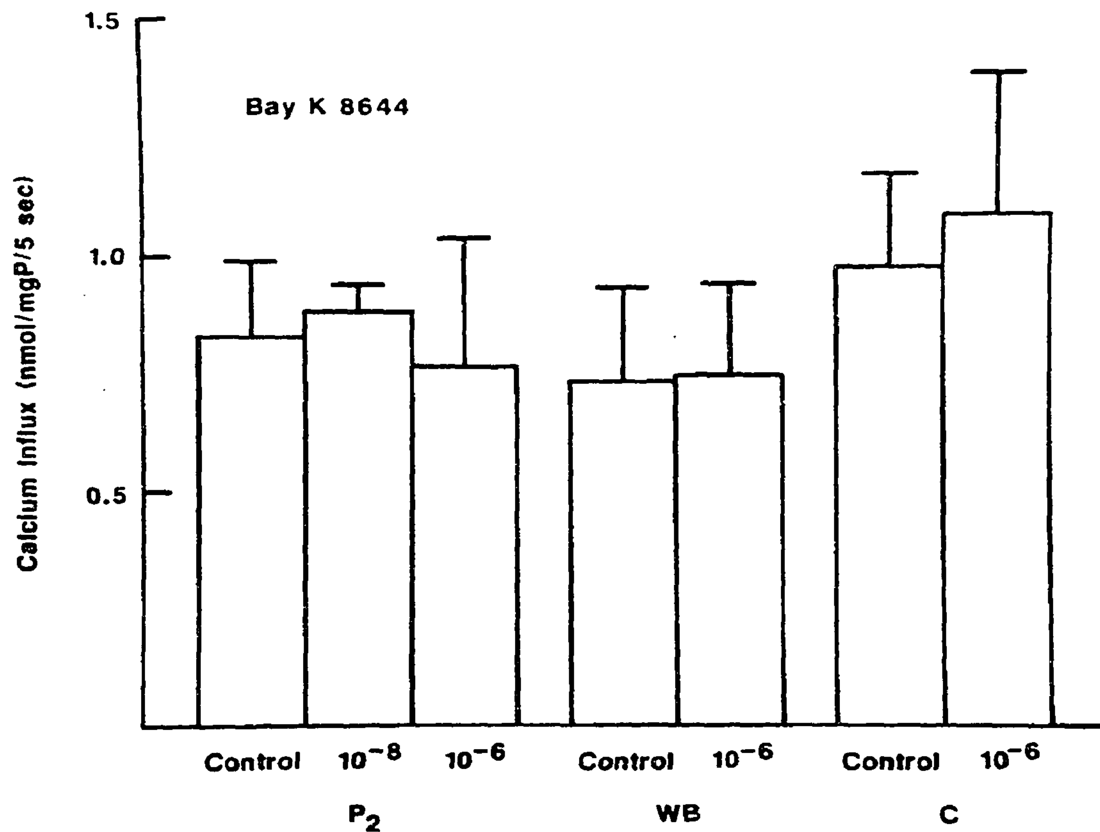



Figure 11. **Effects of Bay K 8644 on depolarization-dependent calcium influx**

Crude ($P_2$), Hajos Whole Brain (WB) and Hajos Cortical (C) synaptosomes were preincubated 20 minutes with Bay K 8644. $^{45}Ca^{++}$ influx was stimulated by incubation in 20 mM $K^+$ HKR containing 1 mM $^{45}Ca^{++}$. Bay K did not significantly alter $Ca^{++}$ influx in any of the preparations.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

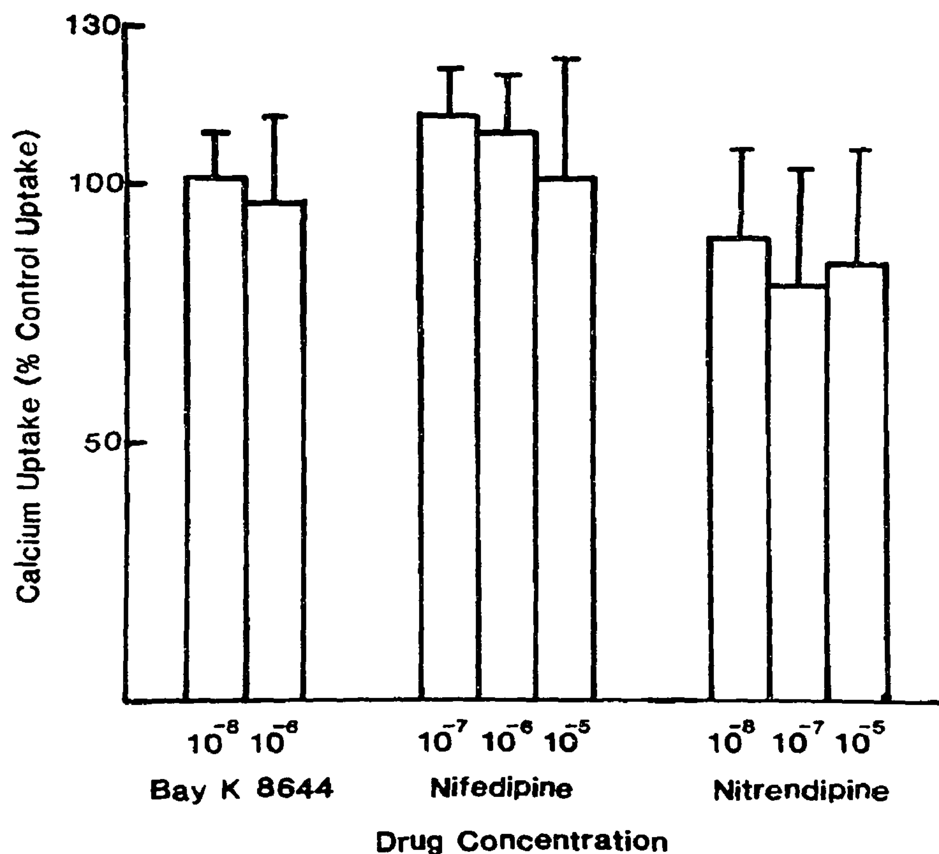



Figure 12: **Effects of dihydropyridine agonists and antagonists on depolarization-dependent calcium influx**

Crude ($P_2$) synaptosomes were preincubated 20 minutes with Bay K 8644, Nifedipine or Nitrendipine. The synaptosomes treated with Bay K and Nifedipine were Na-SYN while those treated with Nitrendipine were Ch-SYN. $^{45}Ca^{++}$ influx was stimulated by incubation in 20 mM $K^+$ HKR containing 1 mM $^{45}Ca^{++}$ for 5 seconds. None of the treatments significantly altered $Ca^{++}$ influx.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

J. Regulation of Calcium Influx by Phosphorylation

Voltage-sensitive calcium channels of snail neurons and the slow channels of cardiac muscle appear to be regulated by a phosphorylation-dependent mechanism (Kostyuk, 1981; Sperelakis, 1985). In these tissues, channels which are phosphorylated have a higher probability of opening upon depolarization then non-phosphorylated channels. The magnitude of the $Ca^{++}$ influx evoked by a given depolarization is dependent on the ratio of phosphorylated to non-phosphorylated channels. Regulation of presynaptic calcium channels could be an important strategy for modulating the release of neurotransmitter from nerve terminals. The following experiments were designed to test if protein phosphorylation is a regulatory mechanism of synaptosomal calcium channels.

Synaptosomes were preincubated with 8-bromo cAMP (1 mM), forskolin (50 $\mu$M) or 12,13-diacetate phorbol ester (10 $\mu$M) and the effects of these drugs on $^{45}Ca^{++}$ influx tested under sub-maximal stimulating conditions (fig. 12). None of the drug treatments altered the $^{45}Ca^{++}$ influx with respect to the control values. The results suggest that the voltage-sensitive calcium channels in synaptosomes are not regulated by a phosphorylation-dependent mechanism. In light of these results it is important to demonstrate that these drug treatments were stimulating phosphate incorperation into synaptosomal proteins. To test for effects on phosphorylation, synaptosomes were preincubated with $^{32}P_i$ prior to addition of drugs. Samples of these synaptosomes were prepared for gel electrophoresis and autoradiography (fig. 13). Three protein bands were choosen to illustrate the incorperation of $^{32}P_i$. Peak I is a single protein band ($M_r$ 55 kD) and Peak II is a doublet ($M_r$ 81 ,76 kD). The relative incorporation of $^{32}P_i$ into each protein band was inferred from the area under each autoradiogram peak (fig. 14). Forskolin reversibly activates adenylate cyclase by associating with the catalytic subunit (Seamon, 1985). Incubation of rat brain slices with 25 $\mu$M forskolin increases the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

cAMP levels by 35-fold (Seamon et al., 1981; Daly et al., 1982). Incubation of synaptosomes with forskolin significantly increased the $^{32}P_i$ incorporation into both Peaks I and II (Table 2). These results suggest that forskolin caused a sufficient rise in cAMP levels to stimulate protein kinase activity. In contrast 8-Br cAMP did not increase incorporation into any of the proteins examined. This is consistent with a previous report that neither 8-Br cAMP nor IBMX stimulate phosphorylation in intact synaptosomes (Krueger et al., 1977). Forskolin may be more effective in promoting cAMP-dependent phosphorylation because it stimulates the synthesis of cAMP in the intracellular space. This compartment may not be readily accessible to bath application of cAMP. Despite the increased $^{32}P_i$ incorporation stimulated by forskolin, the activity of calcium channels was not altered.

Protein kinase C is present in brain where a large portion is localized in synaptosomes (Nishizuka, 1983). Activation of this enzyme is dependent on diacylglycerol, $Ca^{++}$ and phospholipid. Phorbol esters substitute for diacylglycerol and directly activate protein kinase C (Nishizuka, 1983). Myristate and 12,13-diacetate phorbol esters significantly increased $^{32}P_i$ incorporation into both peaks (Table 2). This stimulation was not associated with an increase in depolarization-dependent $^{45}Ca^{++}$ influx. These results suggest that the protein phosphorylation mediated by a cAMP dependent mechanism and protein kinase C do not regulate the activity of synaptosomal calcium channels.

These results are in agreement with the recent observation that 12-deoxyphorbol 13-isobutyrate 20-acetate phorbol ester enhanced transmitter release from the squid giant synapse (Agustine et al., 1986). The augmented release resulted from an increase in the duration of the action potential rather than a direct effect on $Ca^{++}$ influx indicating that the activity of calcium channels was not altered by the drug. In PC12 and endocrine cells phorbol esters stimulate an increase in norepinephrine and prolactin release

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

respectively, without increasing the cytosolic free $Ca^{++}$ levels (Pozzan et al., 1984; Frey et al., 1986). Enhanced release in these preparations does not stem from increased $Ca^{++}$ influx. These results suggest that the phosphorylation mediated by protein kinase C is important in regulating exocytosis at a step after the entry of $Ca^{++}$ (Knight and Baker, 1983).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

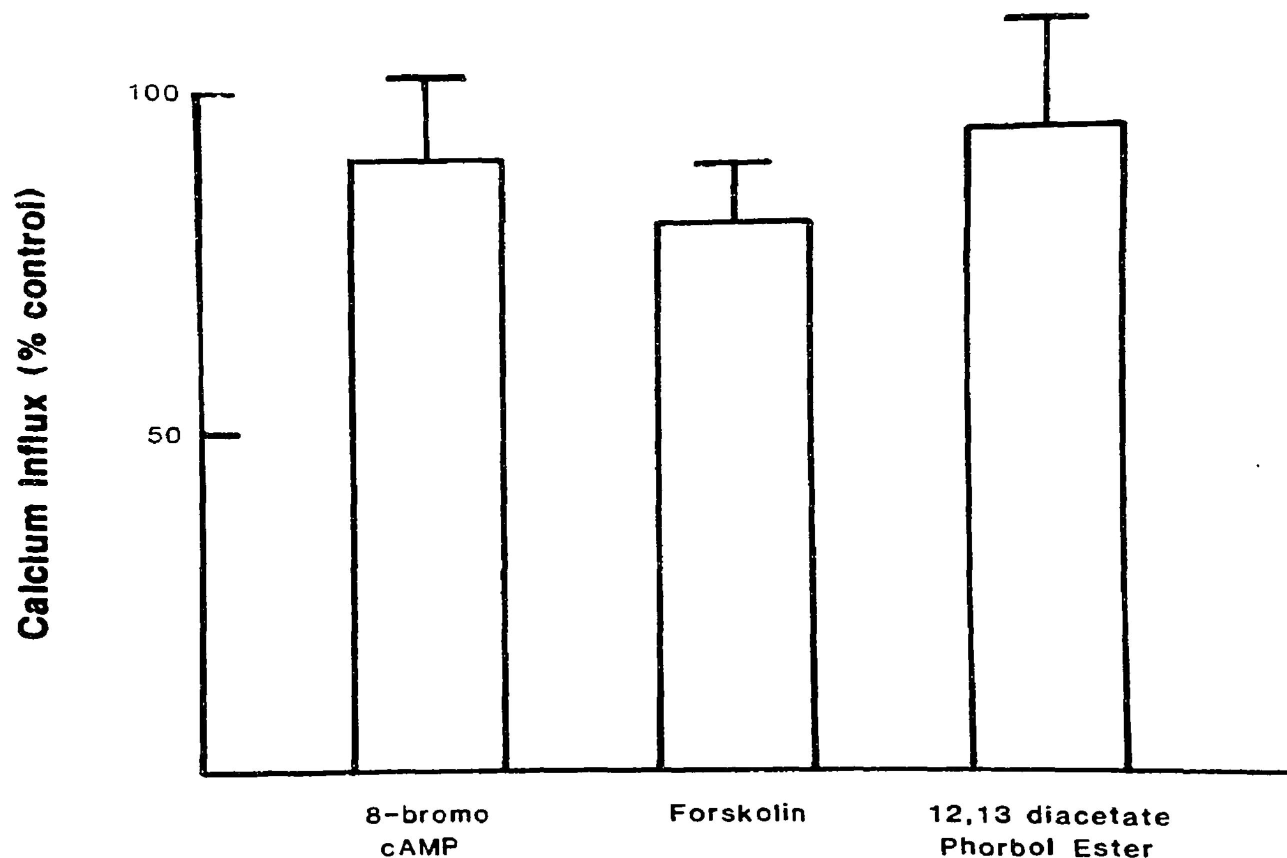



Figure 13. **Effects of cAMP, Forskolin and Diacetate Phorobol Ester on Depolarization-dependent calcium influx**

Synaptosomes were preincubated 20 minutes with 1 mM 8-Bromo cAMP, 50 $\mu$M Forskolin or 10 $\mu$M 12,13-Diacetate Phorbol Ester. $^{45}Ca^{++}$ influx was measured by incubating the synaptosomes in 20 mM $K^{+}$ HKR for 5 seconds. Influx is expressed as a percentage of similar non-treated controls. None of the treatments significantly altered $Ca^{++}$ influx.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

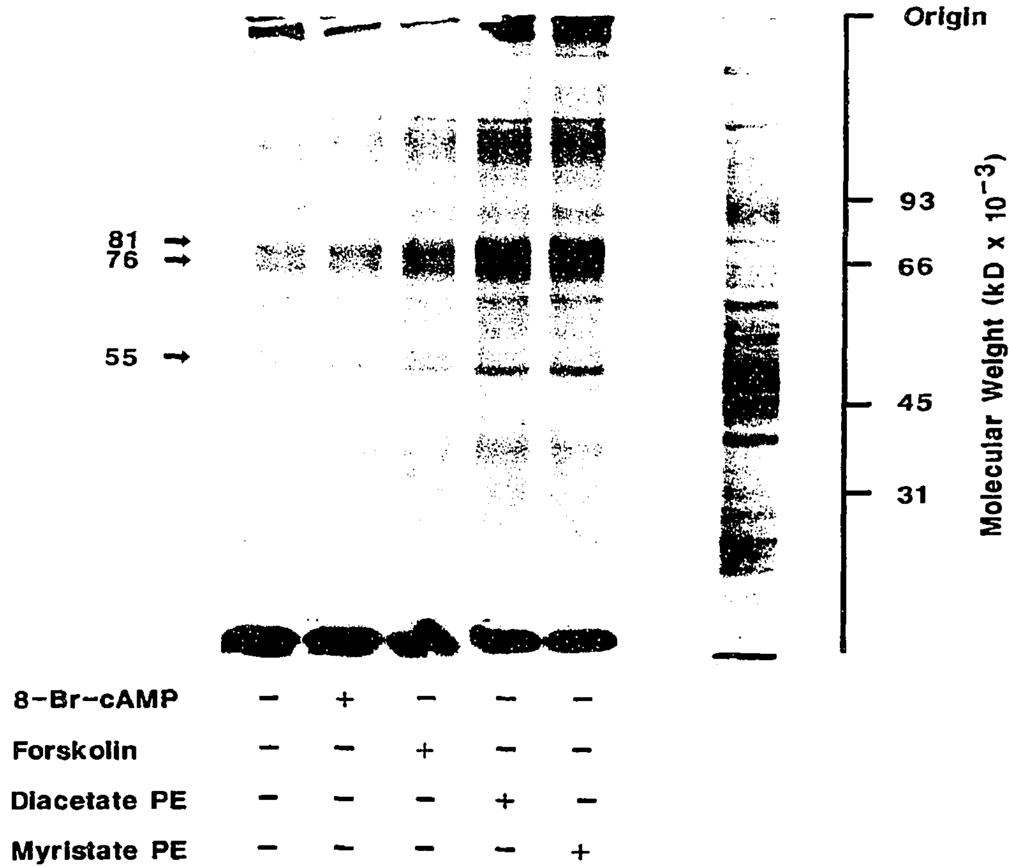



Figure 14. **Phosphorylation of synaptosomal proteins**

Synaptosomes were preincubated 30 minutes with $^{32}P_i$. 1 mM 8-Bromo cAMP, 50 $\mu$M Forskolin, 10 $\mu$M 12,13-Diacetate Phorbol Ester or 7 $\mu$M Myristate Phorbol Ester were added 20 minutes before samples were prepared for electrophoresis on 7.5% polyacrylamide gels. The first five lanes (left) are the autoradiograms for the various treatments. The right-most lane is the protein staining pattern.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

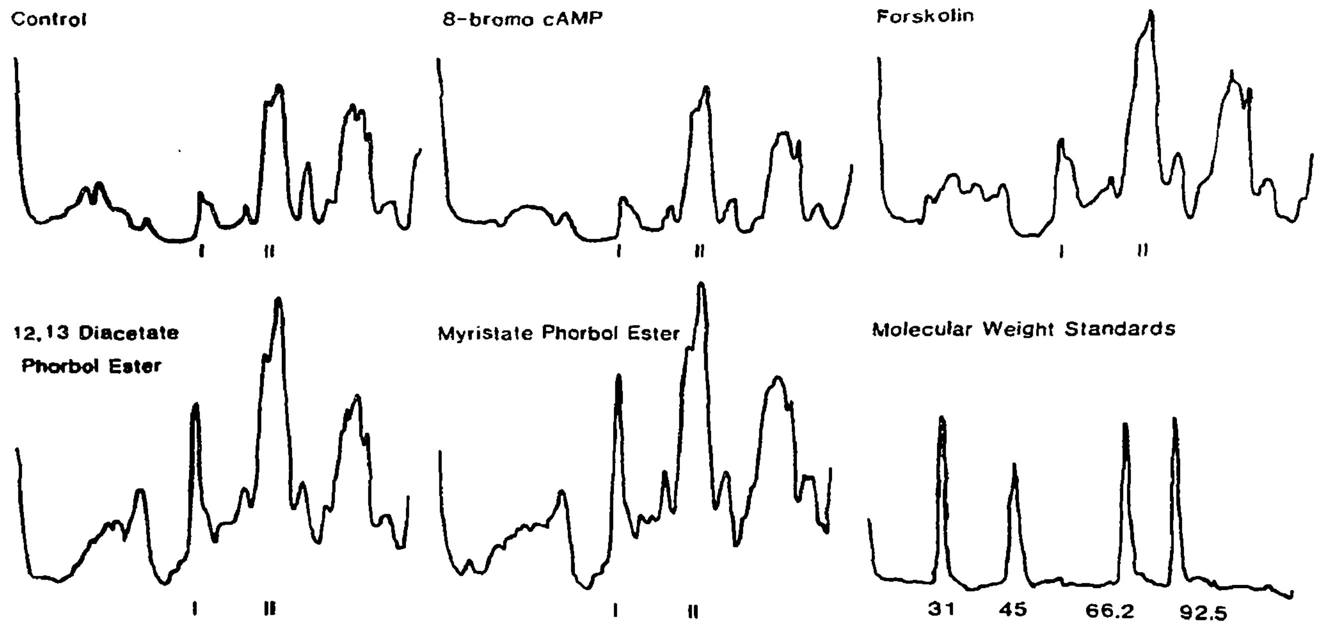



Figure 15. **Densitometric scan of the autoradiogram**

Autoradiograms of the gels (fig. 14) were developed for 72 hours. The densitometric scanning patterns were determined. Peak I is a single protein band ($M_r$ 55 kD) and Peak II is a doublet ($M_r$ 76,81 kD). Forskolin and both Phorbol Esters appeared to increase the phosphorylation of both Peak I and Peak II.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## Stimulation of Phosphorylation (Control = 1.0)

| | Peak I | | Peak II | |
|---|---|---|---|---|
| 8-bromo cAMP | 1.2 ± 0.2 | | 0.9 ± 0.1 | |
| Forskolin | 2.0 ± 0.5 | * | 1.3 ± 0.1 | * |
| 12,13 Diacetate Phorbol Ester | 3.1 ± 0.9 | * | 1.6 ± 0.3 | * |
| Myristate Phorbol Ester | 3.7 ± 1.2 | * | 1.6 ± 0.2 | * |

Table 2. **Quantification of the phosphorylation of Peaks I and II**

The relative incorporation of $^{32}P_i$ into each band is inferred from the area under the densitometric scan (fig. 15). The incorporation into each Peak is expressed as a fold increase over the non-treated control. 50 $\mu$M Forskolin, 10 $\mu$M Diacetate and 7 $\mu$M Myristate Phorbol Esters significantly increased the $^{32}P_i$ incorporation into both Peaks I and II.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

## Conclusions

The voltage-stimulated $Ca^{++}$ influx described in synaptosomes is mediated by at least three distinct pathways. The most prominent component is near maximally activated by incubation in 52.5 mM $K^{+}$ HKR and accounts for 80% of the $Ca^{++}$ influx measured during the initial second of depolarization. This component has been termed the fast $Ca^{++}$ influx. The rate of influx mediated by the fast component progressively decreases during depolarization so that after 1 second this component is completely abolished and no longer contributes to $Ca^{++}$ influx. Predepolarization of synaptosomes prior to testing for $Ca^{++}$ influx eliminates the fast component. It appears that the pathway which mediates this influx undergoes voltage-dependent inactivation. Stronger depolarizations prior to testing for $Ca^{++}$ influx further enhances the inactivation indicating that this process is voltage as well as time-dependent. Based on these observations it is hypothesized that the fast $Ca^{++}$ influx in synaptosomes is mediated by channels. The inactivation of the fast component results from the progressive voltage-dependent closure of the calcium channels.

The time-course of channel-mediated $Ca^{++}$ influx closely parallels the release of norepinephrine from synaptosomes. The correlation of these events attests to the physiological relevance of the $Ca^{++}$ influx. The channel is not modulated by dihydropyridine drugs but is blocked by low concentrations of the calcium channel antagonist lanthanum. The voltage-dependent inactivation and insensitivity to dihydropyridines tentatively classify this channel as an N-type calcium channel (Nowycky et al., 1985). This calcium channel is not regulated by a cAMP or Protein Kinase C dependent phosphorylation mechanisms.

Several recent reports have examined cultured neurons in which both dihydropyridine sensitive (L-type) and insensitive (N-type) calcium channels coexist

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

(Thayer et al., 1986; Hirning et al., 1986). In these preparations $Ca^{++}$ influx is blocked by dihydropyridine drugs but the corresponding transmitter release was unaffected. The channels which are blocked by dihydropyridine drugs mediate $Ca^{++}$ influx which is not associated with transmitter release. These observations re-enforce the developing view that the $Ca^{++}$ channels at nerve terminals are of the N-type. This conclusion is consistent with the results presented here which characterize synaptosomal calcium channels as N-type.

Phorbol esters have recently been linked to augmented secretion from a number of cell types. In the squid giant synapse phorbol esters enhanced transmitter release by inhibiting presynaptic $K^+$ currents (Augustine et al., 1986). This resulted in a broadening of the presynaptic action potential, prolonged depolarization and an increase in $Ca^{++}$ influx. The action of phorbol esters did not directly alter the activity of the presynaptic calcium channels. In PC-12 and cultured endocrine cells phorbol esters stimulate release without altering $Ca_i$ (Pozzan et al., 1984; Frey et al., 1986). Preincubation of synaptosomes with phorbol esters increases the depolarization-stimulated, $Ca^{++}$-dependent release of dopamine by 40% (Shu and Selmanoff, 1986). It appears that the activation of protein kinase C alters transmitter release at a step after the entry of $Ca^{++}$ (Knight and Baker, 1983). These results are consistent with the observation that phorbol ester treatments stimulate significant phosphorylation but do not regulate the activity of synaptosomal $Ca^{++}$ channels. The phorbol esters may enhance neurotransmitter release by modulating the release mechanism.

Recently the isolated dihydropyridine receptor (ie. $Ca^{++}$ channel) of the skeletal muscle T-tubule system was found to be a substrate for an endogenous phosphorylating mechanism (Curits and Catterall, 1985). It appears that the modulation by dihydropyridine drugs and regulation by phosphorylation occurs through the same subunits of the calcium channel. It can be inferred that dihydropyridine receptors and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

phosphorylation sites are linked characteristics of the calcium channels which have these particular subunits. Slow channels of cardiac muscle appear to fit this criteria (Reuter, 1983; Sperelakis, 1985). In contrast, the calcium channels of synaptosomes are not sensitive to dihydropyridine drugs and are not regulated by phosphorylating mechanisms. These results suggest that the calcium channels localized within the nerve terminals of mammalian brain are pharmacologically distinct from the calcium channels of skeletal and cardiac muscle.

Preincubation under depolarizing conditions abolishes the fast component of $Ca^{++}$ influx in synaptosomes. The remaining $Ca^{++}$ influx after channel inactivation constitutes a second component of depolarization-dependent $Ca^{++}$ influx which is termed the slow component. Slow $Ca^{++}$ influx is linear through the first 5 seconds of depolarization and conducts $Ca^{++}$ at 20% the rate of the fast component. This phase of influx is not altered by prolonged depolarization indicating that this component does not inactivate. In contrast to fast $Ca^{++}$ influx, the slow component is not blocked by lanthanum and is probably not mediated by channels. The slow component of $Ca^{++}$ influx can be further subdivided into two components based on sensitivities to the transmembrane $Na^+$ gradient. Synaptosomes which have a larger $[Na^+]_i$ generate 50% more slow component influx then those with low $[Na^+]_i$. The results indicate that depolarization, in conjunction with reduced $[Na^+]_o$ may cause a reversal of the $Na^+$ electrochemical gradient in those synaptosomes which have elevated $[Na^+]_i$. The depolarization-stimulated $Na^+$ efflux may be linked to the influx of $Ca^{++}$ through the $Na_i/Ca_o$ exchange mechanism (Coutinho et al., 1984). The slow component component of $Ca^{++}$ influx is both voltage- and $Na_i$-dependent. The voltage sensitivity indicates that the exchange mechanism is electrogenic, suggesting that the transport stoichiometry must be at least 3 $Na_i$ per $Ca_o$. The slow component of $Ca^{++}$ influx in synaptosomes with relatively low $[Na^+]_i$ (ie. Ch-SYN) probably represents the voltage-stimulated $Ca_i/Ca_o$

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

exchange or a non-inactivating channel. The slow components of $Ca^{++}$ influx appear to support a reduced rate of transmitter release. This observation suggests that the fast and slow $Ca^{++}$ entry pathways are located within relatively close proximity to the neurotransmitter release sites.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

**References**

Abdel-Latif A.A., (1966) Acetylcholine and the incorporation of $^{32}P$ into phospholipids of nerve endings of developing rat brain. Nature 211, 530.

Abdel-Latif A.A., (1973) Ion transport in the synaptosome and NaK-ATPase. Methods in Neurochemistry. 5, 147-188.

Abercrombie R.F. and R.A. Sjordin, (1977) Sodium Efflux in Myxicola giant axons. J. Gen. Physiol. 69, 765-778.

Adam-Vizi V. and E. Ligeti, (1984) Release of acetylcholine from rat brain synaptosomes by various agents in the absence of external calcium ions, J. Physiol. 353, 505-521.

Akerman K.E.O. and D.G. Nicholls, (1981) $Ca^{++}$ transport by intact synaptosomes: the voltage-dependent $Ca^{++}$ channel and re-evaluation of the role of sodium/calcium exchange. Eur. J. Biochem. 117, 491-497.

Akerman K.E.O. and D.G. Nicholls, (1981) Intrasynaptosomal compartmentation of calcium during depolarization-induced calcium uptake across the plasma membrane, Biochim. Biophys. Acta 645, 41-48.

Ansell G.B. and S. Spanner, (1967) The metabolism of labelled ethanolamine in the brain of the rat in vitro. J. Neurochem. 14, 873-885.

Arias C. and R. Tapia, (1986) Differential calcium dependence of aminobutyric acid and acetylcholine release in rat brain synaptosomes. J. Neurochem. 47, 396-404.

Ashcroft F.M. and P.R. Stanfield, (1982) Calcium and potassium currents in muscle fibers of an insect. J. Physiol. 323, 93-115.

Augustine G.J., L.R. Osses, S.R. Bary and M.P. Charlton, (1986) Presynaptic mechanism of kinase C activators at the squid giant synapse. Neurosci. Abst. 228.1.

Baker P.F. and D.E. Knight, (1979) The "leaky" adrenal medullary cell. Tred. neuro. Sci. Nov.

Barker P.F. and M.P. Blaustein, (1968) Sodium-dependent uptake of calcium by crab nerve. Biochem. Biophys. Acta. 150, 167-170.

Barker P.F., M.P. Blaustein, A.L. Hodgkin and R.A. Steinhardt. (1969) J. Physiol. 200, 431-458.

Barker L.A. and T.W. Mittag, (1973) Inhibition of synaptosomal choline uptake by naphthylvinylpyridiniums. FEBS Lett. 35, 141-144.

Bean B.P., (1984) Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. Proc. Natl. Acad. Sci. 81, 6388-6392.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Belin M.F., G. Chouvet and J.F. Pujol, (1974) Transport synaptosomal du tryptophan et de la tyrosine cerebrale; stimulation de la vitesse de capture apres reserpine ou inhibition de la monoamine oxydase. Biochem. Pharm. 23, 587-597.

Belleman P., A. Schade and R. Towart, (1983) Dihydropyridine receptor in rat brain labeled with $^{3}$H-nimodipine. Proc. Natl. Acad. Sci. 80, 2356-2360.

Blaustein M.P. and A.L. Hodgkin, (1969) The effect of cyanide on the calcium efflux from squid axon. J. Physiol. 200, 497-527.

Blaustein M.P. and Weisman, (1970) In: Drugs and Cholinergic Mechanisms in the CNS. eds. E. Heilbronn and A. Winter.

Blaustein M.P., E.M. Johnson and P. Needleman, (1972) Calcium-dependent norepinephrine release from presynaptic nerve endings In Vitro. Proc. Nat Acad. Sci. 64, 2237-2240.

Blaustein M.P., (1974) Interrelationship between sodium and calcium fluxes across the cell membrane, Rev. Physiol. Biochem. Pharm. 70, 33-82.

Blaustein M.P. and J.M. Russell, (1975) Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid giant axons. J. Membr. Biol. 22, 285-312.

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

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

Blaustein M.P. and C.J. Oborn, (1975) The influence of sodium on calcium fluxes in pinched-off nerve terminals in vitro. J. Physiol. 247, 657-686.

Blaustein M.P. and C. Ector, (1976) Carrier-mediated sodium-dependent and calcium-dependent calcium efflux from pinched-off presynaptic terminals (synaptosomes) in vitro. Biochim. Biophys. Acta 419, 295-308.

Blaustein M.P., (1977) Effects of internal and external cations and of ATP on sodium-calcium exchange in squid axon. Biophys. J. 20, 79-111.

Bogdanski D.F., (1973) Effects of electrolytes on amine transport and release mechanisms in nerve endings. In: Frontiers in Catecholamine Research, eds. E. Usdin and S.H. Snyder, Pergamon, Great Britain, pp. 427-429.

Bogdanski D.F., A. Tissari and B.B. Brodie, (1968) Role of Na, K, ouabain and reserpine in uptake, storage and metabolism of biogenic amines in synaptosomes. Life Sci. 7, 419.

Bolger G.T., P. Gengo, R. Klockowski, E. Luchowski, H. Siegel, R.A. Janis, A.M Triggle and D.J. Triggle, (1983) Characterization of binding of the $Ca^{++}$ channel

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

antagonist $^3$H-nitrendipine to guinea pig ileal smooth muscle. J. Pharmacol. Exp. Ther. 225, 291-309.

Bradford H.F., (1969) Respiration In vitro of synaptosomes from mammalian cerebral cortex. J. Neurochem. 16, 675-684.

Bradford H.F. and A.J. Thomas, (1969) Metabolism of glucose and glutamate by synaptosomes from the mammalian cerebral cortex. J. Neurochem. 16, 1495-1504.

Bradford P.G., G.V. Marinetti and L.G.Abood, (1983) Stimulation of phospholipase $A_2$ and secretion of catecholamines from brain synaptosomes by potassium and A23187. J. Neurochem. 41, 1684-1693.

Brehm P., R. Eckert and D. Tillotson, (1980) Calcium-mediated inactivation of calcium current in Paramecium, J. Physiol. 306, 193-203.

Bremer J. and D.M. Greenburg, (1961) Biochim. Biophys. Acta 46, 205-216.

Bremer J. and P.M. Greenberg, (1961) Methyltransfering system of lecithin (phosphatidylcholine). Biochim. Biophys. Acta 46, 205-216.

Brinley F.J. and L.J. Mullins, (1974) Effects of membrane potential on sodium and potassium fluxes in squid axon. Ann. NY Acad. Sci. 242, 406.

Brown A.M., K. Morimoto, Y. Tsuda and D.L. Wilson, (1981) Calcium current-dependent and voltage-dependent inactivation of calcium channels in Helix Aspersa. J. Physiol. 320, 193-218.

Browning E.T. and M.P. Schulman, (1968) $^{14}$C-Acetylcholine synthesis by cortex slices of rat brain. J. Neurochem. 15, 1391-1405.

Byerly L. and S. Hagiwara, (1982) Calcium currents in internally perfused nerve cell bodies of Limnea Stagnalis. J. Physiol. 322, 503-528.

Campbell L.B. and D.J. Jensen, (1970) Gas chromatographic evaluation of the influence of oxotremorine upon the regional distribution of acetylcholine in rat brain. J. Neurochem. 17, 1697-1699.

Campbell W.B.C., (1976) The $Na^+$, $K^+$, $Cl^-$ contents and derived membrane potentials of presynaptic nerve endings in vitro. Brain Res. 101, 594-599.

Colburn R.W., F.K. Goodwin, D.L. Murphy,W.E. Bunney and J.M. Davis, (1968) Quantitative studies of norepinephrine uptake by synaptosomes. Biochem. Pharm. 17, 957.

Cotman C.W., J.W. Haycock and W.F. White, (1976) Stimulus-secretion coupling processes in brain: Analysis of noradrenaline and gamma-aminobytyric acid release. J. Physiol. 254, 475-505.

Coutinho O.P., C.A.M. Carvalho and A.P. Carvalho, (1984) Calcium uptake related to K-depolarization and Na/Ca exchange in Sheep brain synaptosomes. Brain Res. 290, 261-271.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Creba J.A. and M. Karobath, (1986) The effects of dihydropyridine calcium agonists and antagonists on neuronal voltage sensitive calcium channels. Biochem. Biophys. Res. Comm. 134, 1038-1047.

Curtis B.M. and W.A. Catterrall, (1985) Phosphorylation of the calcium antagonist receptor of the voltage-sensitive calcium channel by cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. 82, 2528-2532.

Daly J.W., (1973) Regulation of cyclic AMP levels in brain, In: Frontiers In Catecholamine Research. Pergamon Press, pp. 301-306.

Daly J.W., W. Padgett and K.B. Seamon, (1982) Activation of cyclic AMP generating systems in brain membranes and slices by the diterpene forskolin: Augmentation of receptor mediated responses. J. Neurochem. 38, 532-544.

Daniell L.C., E.M. Barr and S.W. Leslie, (1983) $^{45}Ca^{++}$ Uptake into rat whole brain synaptosomes is unaltered by dihydropyridine calcium antagonists. J. Neurochem. 41, 1455-1459.

Daniell L.C. and S.W. Leslie, (1986) Correlation of rates of calcium entry and release of endogenous norepinephrine in rat brain region synaptosomes. J. Neurochem. In Press.

De Robertis E., A. Pellegrino de Iraldi, G. Rodrigeuz de Lores Arnaiz and L. Salganicoff, (1962) Cholinergic and non-cholinergic endings in rat brain. I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase. J. Neurochem. 9, 23-35.

Deutsch C. and U. Rafalowska, (1979) Transmembrane electrical potentials measurements in rat brain synaptosomes. FEBS Lett. 108, 274-278.

Deutsch C., C. Brown, U. Ralaowska and I.A. Sliver, (1981) Synaptosomes from rat brain: morphology, compartmentation and transmembrane pH and electrical gradients. J. Neurochem. 36, 2063-2072.

Diamond I. and R.A. Fishman, (1973) Development of Na-stimulated glucose oxidation in synaptosomes J. Neurochem. 21, 1043-1050.

Diamond J.M. and F.M. Wright, (1969) Biological membranes: the physical basis of ion and nonelectrolyte selectivity. Ann. Rev. Physiol. 31: 581-646.

Dipolo R., H. Rajos and L. Beague, (1982) Ca entry at rest and during prolonged depolarization in dialyzed squid axons. Cell Calcium 3, 19-41.

Dipolo R. and L. Beauge, (1983) The calcium pump and sodium calcium exchange in squid axon. Ann. Rev. Physiol. 45, 313-324.

Doroshenko P.A., P.G. Kostyuk, A.E. Martynyuk, M.D. Kursky and Z.D. Vorobeitz, Intracellular protein kinase and calcium inward currents in perfused neurons of the snail Helix Pomatia. Neurosci. 11, 263-267.

Drapeau P. and M.P. Blaustein, (1983) Initial release of $^{3}H$-dopamine release from striatal synaptosomes: correlation with calcium entry. J. Neurosci. 3, 703-713.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Drapeau P. and D.A. Nachshen, (1984) Manganese fluxes and manganese-dependent neurotransmitter release in presynaptic nerve endings isolated from rat brain. J. Physiol. 348, 493-510.

Eckert R. and D.L. Tillotson, (1981) Calcium-mediated inactivation of the calcium conductance in caesium-loaded giant neurons of Aplysia Californica. J. Physiol. 314, 265-280.

Ehlert F., E. Itoga, W.R. Roeske and H.I. Tamamura, (1982) The interaction of $^3$H-nitrendipine with receptors for calcium antagonists in the cerebral cortex and heart of rats. Biochem. Biophysic. Res. Comm. 104, 937-943.

Eisenberg R.S., R.T. McCarthy and R.L. Milton, (1983) Paralysis of frog skeletal muscle fibers by the calcium antagonist D-600. J. Physiol. 341, 495-505.

Erdreich A., R. Spanier and H. Rahamimoff, (1983) The inhibition of Na-dependent Ca uptake by verapamil in synaptic plasma membrane vesicles. Eur. J. Pharm. 90, 193-202.

Escuta A.V. and S.H. Appel, (1969) Biochemical studies of the synaptosome in vitro II. Potassium transport. Biochemistry 8, 725-733.

Ewald D.A., A. Willams and I.B. Levitan, (1985) Modulation of single $Ca^{++}$ dependent $K^+$ channel activity by protein phosphoryaltion. Nature 315, 503-506.

Festoff B.W. and S.H. Appel, (1968) Effect of diphenylhydantion on synaptosome NaK-ATPase. J. Clinical Invest. 47, 2752.

Floor E., (1983) Substance P release from K-depolarized rat brain synaptosomes at one second resolution. Brain Res. 279, 321-324.

Fox A.P., (1981) Voltage-dependent inactivation of a calcium channel. Proc. Natl. Acad. Sci. 78, 953-956.

Freedman S.B., G. Dawson, M.L. Villereal and R.J. Miller, (1984) Identification and characterization of voltage-sensitive calcium channels in neuronal clonal cell lines. J. Neurosci. 6, 1453-1467.

Frey E.A., J.W. Kebabian and S. Guild, (1986) Forskolin enhances calcium-evoked prolactin release from 7315c tumor cells without increasing the cytosolic calcium concentration. Mol. Pharm. 29, 461-466.

Goldman D.E., (1943) Potential impedence and rectification in membranes. J. Gen. Physiol. 27, 37-60.

Gonzales-Serratos H., R. Valle-Aquilera, D.A. Lathrop and M.C. Garcia, (1982) Nature 298, 292-294.

Gould R.J., K.M.M. Murphy and S.H. Synder, (1982) $^3$H-Nitrendipine labeled calcium channels discriminate inorganic calcium agonists and antagonists. Proc. Natl. Acad. Sci. 79, 3656-3660.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Grahame-Smith D.G., (1971) Studies "in vivo" on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. J. Neurochem. 18, 1053-1066.

Grahame-Smith D.G. and A.G. Parfitt, (1970) Tryptophan transport across the synaptosomal membrane. J. Neurochem. 17, 1339.

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

Grippenberg J., E. Heinonen and S.E. Jansson, (1980) Uptake of radiocalcium by nerve endings isolated from rat brain: Pharmacological studies. Br. J. Pharm. 71, 273-278.

Haga T. and H. Noda, (1973) Choline uptake systems of rat brain synaptosomes. Biochim. Biophys. Acta 291, 564-575.

Hagiwara S. , S. Ozawa and O. Sand, (1975) Voltage clamp analysis of two inward current mechanisms in the egg cell membrane of a starfish. J. Gen. Physiol. 65, 619-644.

Hagiwara and Byerly, (1981) Calcium channel Ann. Rev. Neurosci. 4, 69-125.

Hagiwara S. and L. Byerly, (1983) The calcium channel. Trends Neurosci. 2, 189-193.

Hajos F., (1983) An improved method for the preparation of synaptosomal fractions in high purity. Brain Res. 93, 485-489.

Hamon M., S. Bourgoin, Y. Morot-Gaudry, F. Hery and J. Glowinski, (1974) Role of active transport of tryptophan in the control of 5-hydroxytryptamine biosynthesis. Adv. Biochem. Psycopharm. 11, 152-162.

Haycock J.W., W.B. Levy, L.A. Donner and C.W. Cotman, (1978) Effects of elevated [K] on the release of neurotransmitter from cortical synaptosomes; Efflux or secretion? J. Neurochem. 30, 1113-1125.

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

Heuser J. and F.R.S. Miledi, (1971) Effect of lanthanum ions on function and structure of frog neuromuscular junction. Proc. R Soc. B 179, 247-260.

Hirning L.D., A.P. Fox, E.W. McCleskey, R.J. Miller, B.M. Olivera, S.A. Thayer and R.W. Tsien, (1986) Dominant role of N-type calcium channels in K-evoked release of norepinephrine from rat sympathetic neurons. Neurosci. Abst. 12.8.

Hodgkin A.L. and B. Katz, (1949) The effect of temperature on the electrical activity of the squid giant axon. J. Physiol. 109, 240-249.

Hodgkin A.L. and A.F. Huxley, (1952) A quantitative description of membrane current and it's application to conduction and excitation in nerve. J. Physiol. 117, 500-544.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Hokin L.E. and M.R. Hokin, (1955) Effects of acetylcholine on phosphate turnover in phospholipids of brain cortex in vitro. Biochem. Biophys. Acta 16, 229.

Hokin L.E. and M.R. Hokin, (1958) Acetylcholine and the exchange of inositol phosphate in brain phosphoinositol. J. Biol. Chem. 233, 822.

Holz, R.W., (1975) The release of dopamine from synaptosomes from rat striatum by the ionophores X537A and A23187. Biochim. Biopys. Acta 375, 138.

Hosie R.J., (1965) The localization of adenosine triphosphates in morphologically characterized subcellular fractions of Guinea-Pig Brain. Biochem. J. 96, 404-412.

Iverson L.L., (1970) Neuronal uptake processes for amines and amino acids. In: Biochemistry of Simple Neuronal Models. eds. E. Costa and E. Giacobini. Advances in Biochem. Pharm. 2, 109-132.

Iverson L.L., (1973) Neuronal and extraneuronal catecholamine uptake mechanisms. In: Frontiers in Catecholamine Research. eds. E. Usdin and S.H. Snyder, pp. 403-408. Pergamon, Great Britain.

Janis R.A. and D.J. Triggle, (1983) New developements in $Ca^{++}$ channel antagonsits. J. Med. Chem. 26, 775- 785.

Jansson S.-E., J.Grippenberg, M. Harfonen and P. Korpijoki, (1977) Methodological studies on the uptake of radiocalcium by nerve endings isolated from rat brain. Life Sci. 20, 1431-1440.

Johnson M.K. and V.P. Whittaker, (1963) Lactate dehydrogenase as a cytoplasmic marker in brain, Biochem. J. 88, 404.

Jope R.S., (1979) High affinity choline transport and acetylCoA production in brain and their roles in the regulation of acetylcholine synthesis. Brain Res. Reviews 1, 313-344.

Jope R.S. and D.J. Jenden, (1979) Choline and phospholipid metabolism and the synthesis of acetylcholine in rat brain. J. Neurosci. Res. 4, 69-82.

Josephson I. and N. Sperelakis, (1978) 5'-Guanylimidodiphosphate stimulation of slow $Ca^{++}$ current in myocardial cells. J. Mol. Cell Cardiol. 10, 1157-1166.

Kass R.S. and M.C. Sanguinetti, (1983) Calcium channel inactivation in the cardiac Purkinji fiber: Evidence for voltage-and calcium-mediated inactivation. J. Gen. Physiol.

Katz B. and R. Miledi, (1965) The effect of calcium on acetylcholine release from motor nerve terminal. Proc. R. Soc. London (B) 161, 496-503.

Katz B. and R. Miledi, (1967a) The timing of calcium action during neuromuscular transmission. J. Physiol. (London) 189, 535-544.

Katz B. and R. Miledi, (1976b) The release of acetylcholine from nerve endings by graded electrical pulses. Proc. R. Soc. (B) 167, 23-38.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Katz B. and R. Miledi, (1967c) A study of synaptic transmission in the absence of nerve impulses. J. Physiol. (London) 192, 407-436.

Katz B. and R. Miledi, (1968) The role of calcium in neuromuscular facilitation. J. Physiol. (London) 195, 481-91.

Katz B. and R. Miledi, (1969a) Tetrodotoxin-resistant electrical activity in presynaptic terminals. J. Physiol. (London) 203, 459-487.

Katz B. and R. Miledi, (1969b) The effects of divalent cations on transmission in the squid synapse. Pubbl. Stn. Zool. Napoli. 37, 303-310.

Katz B. and R. Miledi, (1970) Further study of the role of calcium in synaptic transmission. J. Physiol. (London) 207, 789-801.

Katz B. and R. Miledi, (1971) The effect of prolonged depolarization on synaptic transfer in the stellate ganglion of the squid synapse. J. Physiol. 216, 503-512.

Keen P. and T.D. White, (1970) A light scattering technique for the study of the permeability of rat brain synaptosomes in vitro. J. Neurochem. 17, 565-571.

Keen P. and T.D. White, (1971) The permeability of pinched-off nerve endings to sodium, potassium and chloride and the effects of gramacidin. J. Neurochem. 18, 1097-1103.

Knight D.E. and P.F. Baker, (1983) The phorbol ester TPA increases the affinity of exocytosis for calcium in "leaky" adrenal medullary cells. FEBS Lett. 160, 98-100.

Koch (1961) Some calculations on the turbidity of mitochondria and bacteria. Biochim. Biophys. Acta 51, 429-441.

Kostyuk P.G., (1979) Calcium ionic channels in electrically excitable membrane. Neurosci. 5, 945-959.

Kostyuk P.G., (1981) Calcium channels in the neuronal membrane. Biochim. Biophys. Acta 650, 128-150.

Kostyuk P.G., P.A. Doroshenko and A.E. Martynyuk, (1985) Fast decrease of the peak current carried by barium through calcium channels in the somatic membrane of mollusc neurons. Pflungers Arch 404, 88-90.

Krnjevic K., (1974) Chemical nature of synaptic transmission in vertebrates. Phys. Rev. 54, 418.

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

Krueger B.K. and D.A. Nachshen, (1980) Selectivity of $Na^+$ and $Ca^{++}$ channels in synaptosomes. Fed. Proc. Abst. 39, 2038.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Kuhar M.J., V.H. Sethy, R.H. Roth and G.K. Aghajanian, (1973) Choline: selective accumulation by central cholinergic neurons. J. Neurochem. 20, 581-593.

Kurokawa M., J. Sakamoto and M. Kato, (1965) Distribution of NaK-ATPase activity in isolated nerve-ending particles. Biochem. J. 97, 833.

Laemmli (1970) Cleavage and structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Langer S.Z., (1970) The metabolism of $^{3}$H-noradrenaline released by electrical stimulation from the isolated nictitating membrane of the cat and from the vas deferens of the rat. J. Physiol. 208, 515-546.

Lee K.S. and R.W. Tsien, (1982) Reversal of current through calcium channels in dialyzed single heart cells. Nature 297, 498-501.

Leslie S., E. Barr and L.J. Chandler, (1983) Comparison of voltage-dependent $^{45}$Ca uptake rates by synaptosomes isolated from rat brain regions. J. Neurochem. 41, 1602-1605.

Leslie S.W., J.J. Wooward and R.E. Wilcox, (1985) Correlation of rates of calcium entry and endogenous dopamine release in mouse striatal synaptosomes. Brain. Res. 325, 99-105.

Levi G., A. Coletti and M. Raiteri, (1976) Decrease of uptake and exchange of neurotransmitter amino acids after depletion of their synaptosomal pools. Brain Res. 103, 103-116.

Li T. and N. Sperelakis, (1983) Stimulation of slow action potentials in guinea pig papillary muscle cells by intracellular injection of cAMP, Gpp(NH)p, and cholera toxin. Circ. Res. 52, 111-117.

Ling C.M. and A.A. Abdel-Latif, (1968) Studies on sodium transport in rat brain nerve-ending particles. J. Neurochem. 15, 721-729.

Llinas R., J.R. Blinks and C. Nicholson, (1972) Calcium transient in presynaptic terminal of squid giant synapse: detection with aequorin. Science 176, 1127-1129.

Llinas R. and C. Nicholson, (1975) Calcium role in depolarization-secretion coupling: An aequorin study in squid giant synapse. Proc. Natl. Acad. Sci. 72, 187-190.

Llinas R. and J.R. Heuser, (1977) Depolarization-release coupling in neurons. Neurosci. Res. Prog. Bull. 276, 557-687.

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

Lowry O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall, (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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

Marchbanks R., (1969) The conversion of $^{14}C$-choline to $^{14}C$-acetylcholine in synaptosomes in vitro. Biochem. Pharm. 18, 1763-1766.

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

Maxwell R., P.D. Keenan, E. Chaplin, B. Roth, S. Batmanglidje and S.B. Eckhardt, (1969) Molecular features affecting the potency of tricyclic antidepressants and structurally related compounds as inhibitors of the uptake of tritiated norepinephrine by rabbit aortic strips J. Pharm. Exp. Ther. 166, 320-329.

Meiri H. and R. Ramimoff, (1972) Neuromuscular transmitter: Inhibition by manganese ions. Science 176, 308-309.

Meiri H., J. Zelingher and R. Ramimoff, (1986) A possible involvement of the NaCa exchanger in regulation of transmitter release at the frog neuromuscular junction. In: Calcium,Function and Transmitter Release. eds. R. Ramimoff and B. Katz, Nijoff.

Mendelson W.B., P. Skolnic, J.V. Martin, M.D. Lu, R. Wagner and S.M. Paul. (1984) Diazepam-stimulated increases in the synaptosomal uptake of $^{45}Ca^{++}$: Reversal by dihydropyridine calcium channel antagonists. Eur. J. Pharm. 104, 181-183.

Mentrard D., G. Vassort and R. Fischmeister, (1984) Calcium-mediated inactivation of the calcium conductance in cesium-loaded frog heart cells. J. Gen. Physiol. 83, 105-131.

Metlas R. and G. Nikezic, (1982) The effects of sodium depolarization-induced calcium uptake and acetylcholine release by synaptosomes. Biochim. Biopys. Acta 687, 340-342.

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

Miledi R., (1971) Lanthanum ions abolish the "calcium response" of the nerve terminals. Nature 229, 410-411.

Miledi R., (1973) Transmitter release induced by the injection of calcium ions into nerve terminals. Proc. R. Soc. (B) 183, 421-425.

Miledi R., P.C. Molenarr and R.L Polak, (1980) The effect of lanthanum ions on acetylcholine in frog muscle. J. Physiol. 309, 199-214.

Miller R.J. and S.B. Freedman, (1984) Are dihydropyridine binding sites voltage sensitive calcium channels? Life Sci. 34, 1205-1221.

Miller R.J., (1985) How many types of calcium channels exist in neurons. TINS 8, 45-47.

Moore J.W., H. Meiri and M.L. Hines, (1986) Does Na-Ca exchange participate in synaptic transmission. Neurosci. Abst. 12.11.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Mosier D.R., D.T. Lee, S. Molchan and J.E. Zengle, (1986) Roles of sodium and calcium in stimulation-induced changes in transmitter release. Neurosci. Abst. 12.10.

Mulder A.H., W.B. Van Der Berg and J.C. Stoof, (1975) Calcium-dependent release of radiolabeled catecholamines and serotonin from rat brain synaptosomes. Brain Res. 99, 419-424.

Mullins L.J. and F.J. Brinley, (1975) Sensitivity of calcium efflux from squid axons to changes in membrane potential. J. Gen. Physiol. 65, 135-152.

Mullins L.J., (1981) In: The Biophysical Approach to Excitable Systems. ed. W. Adelman, pp. 151-163. Plenum.

Murrin L.C., R.N. Dehaven and M.J. Kuhar, (1977) On the relationship between $^3$H-choline uptake and activation of $^3$H-ACh release. J. Neurochem. 29, 681-678.

Murphy K.M.M. and S.H. Snyder, (1982) Calcium antagonist receptor binding sites labeled with $^3$H-Nitrendipine. Eur. J. Pharm. 77, 201-202.

Nachshen D.A. and M.P. Blaustein, (1979) The effects of some organic "Calcium Antagonists" on calcium influx in presynaptic nerve terminals. Mol. Pharm. 16, 579-586.

Nachshen D.A. and M.P. Blaustein, (1979) Regulation of nerve terminal calcium channels selectivity by a weak acid site. Biophys. J. 26, 329-334.

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

Nachshen D.A. and M.P.Blaustein, (1982) Influx of calcium, strontium and barium in presynaptic nerve endings. J. Gen. Physiol. 79, 1065-1087.

Nachshen D.A. and P. Drapeau, (1982) A buffering model for calcium-dependent neurotransmitter release. Biophys. J. 38, 205-208.

Nachshen D.A., (1984) Selectivity of the Ca binding site in synaptosome Ca channel. J. Gen. Physiol. 83, 941-967.

Nachshen D.A., (1985) The early time course of potassium-stimulated calcium uptake in presynaptic nerve terminals isolated from rat brain. J. Physiol. 361, 251-268.

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

Nelson M.T., R.J. French and B.K. Krueger, (1984) Voltage-dependent calcium channels from brain incorporated into planer lipid bilayers. Nature 308, 77-80.

Nishizuka Y., (1983) Phospholipid degradation and signal translation for protein phosphorylation. Tred. Biol. Sci. Jan.

Nishizuka Y., (1984) Turnover of inositol phospholipids and signal transduction. Science 255, 1365-1370.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

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

Ohta M., T. Narahashi and R.F. Keeler, (1973) Effects of veratum alkaloids on membrane potential and conductance of squid and crayfish giant axons. J. Pharm. Exp. Ther. 184, 143-154.

Pozzan T., G. Gatti, N. Dozio, L.M. Vicentini and J. Meldolesi, (1984) $Ca^{++}$-dependent and independent release of neurotransmitter from PC12 cells: A role of protein kinase C activation. J. Cell Biol. 99, 628-638.

Raiteri M. and G. Levi, (1973) Depletion of synaptosomal neurotransmitter pools by sudden cooling. Nature 243, 180-182.

Raiteri M. and G. Levi, Antisynaptosomal antibodies affect synaptosomal permeability to neurotransmitters. (1973b) Nature 245 NB, 89-91.

Raiteri M., R. del Carmine, A. Bertollini and and G. Levi, (1977) Effects of desmethylimipramine on the release of $^{3}$H-norepinephrine induced by various agents in hypothalamic synaptosomes. Mol. Pharmacol. 13, 746-758.

Raiteri M., F. Cerrito, A.M. Cervoni, R. del Carmine, M.T. Ribera and G. Levi, (1978) Studies on dopamine uptake and release in synaptosomes. Adv. Biochem. Psycopharm. 19, 35-55.

Rampe D., R.A. Janis and D.J. Triggle, (1984) Bay K 8644, a 1,4-dihydropyridine $Ca^{++}$ channel activator: Dissociation of binding and functional effects in brain synaptosomes. J. Neurochem. 43, 1688-1691.

Redburn D.A., J.Bisla, D.L. Shelton and C.W. Cotman, (1975) Stimulus secretion coupling in vitro: A rapid perfusion apparatus for monitoring efflux of transmitter substances from tissue samples. Anal. Biochem. 67, 268-278.

Requena J., (1983) Calcium transport and regulation in nerve fibers. Ann. Rev. Bioeng. 12, 237-257.

Reuters H., (1979) Properties of two inward membrane currents in the heart. Ann. Rev. Neurosci. 41, 413-424.

Reuter H., (1983) Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 301, 569-574.

Reynolds L.J., J.A. Wagner, B.M. Olivera, R.J. Miller and S.H. Snyder, (1986) Omega-conotoxin blocks dihydropyridine-insensitive calcium uptake into synaptosomes. Soc. Neurosci. Abst. 49.11, p. 176.

Rodriguez de Lores Arnaiz G. and E. De Robertis, (1972) Properties of the isolated nerve endings. In: Current Topics in Membranes and Transport 3, 237-272.

Sanchez J.A. and E. Stefani, (1978) J. Physiol. 283, 197-207.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Sanguinetti M.C. and R.S. Kass, (1984) Voltage-dependent block of calcium channel current in the calf cardiac purkinje fiber by dihydropyridine calcium channel antagonists. Circ. Res. 55, 336-348.

Sano K., Y. Takai, J. Yamanishi and Y. Nishizuka, (1983) A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation. J. Biol. Chem. 258, 2010-2013.

Schmidt D.E., R.C. Speth, F. Welsch and M.J. Schmidt, (1972) The use of microwave radiation in the determination of acetylcholine in the rat brain. Brain Res. 38, 377-389.

Schneider J.A. and N. Sperelakis, (1974) The demonstration of energy dependence of the isoproterenol-induced transcellular $Ca^{++}$ current in isolated perfused guinea pig hearts- An explanation for mechanical failure in ischemic myocardium. J. Surg. Res. 16, 389-403.

Schneider J.A. and N. Sperelakis, (1974) Valinomycin blockade of slow channels in guinea pig hearts perfused with elevated $K^+$ and isoproterenol. Eur. J. Pharm. 27, 349-354.

Schramm M., G. Thomas, R. Thomas and Franckowiak, (1983) Novel dihydropyridines with positive inotropic action through activation of $Ca^{++}$ channels. Nature 303, 535-537.

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

Seamon K.B., W. Padgett and J. W. Daly, (1981) Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Natl. Acad. Sci. 6, 3363-3367.

Seamon K.B., (1985) Activation of hormone-sensitive adenylate cyclase by forskolin. Drug Develop. Res. 6, 181-192.

Seigelbaum S.A., J.S. Camardo and E.R. Kandel. (1982) Serotonin and cyclic AMP close $K^+$ channels in Aplysia sensory neurons. Nature 299, 413-417.

Shields P.J. and D. Eccleston, (1972) Effects of electrical stimulation of rat midbrain on 5-Hydroxtryptamine synthesis as determined by a sensitive radioisotope method. J. Neurochem. 19, 265-171.

Shira T.S., I.G. Scott and D.G. Nicholls, (1984) Ionophore A23187, verapamil, protonophores, and veratridine influence the release of gamma-aminobuytric acid from synaptosomes by modulation of the plasma membrane potential rather than the cytosolic calcium. J. Neurochem. 43, 1624-1630.

Shuster M.J., J.S. Camardo, S.A. Siegelbaum and E.R. Kandel, (1985) Cyclic AMP-dependent protein kinase closes the serotonin-sensitive $K^+$ channels of Aplysia sensory neurons in cell-free membrane patches. Nature 313, 392-395.

Sims P.J., A.S. Waggoner, C.-H.Wang and J.F. Hoffman, (1974) Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochem. 13, 3315-3330.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Shu C. and M. Selmanoff, (1986) Phorbol esters potentiate rapid dopamine release from median eminence and caudate nucleus synaptosomes. Neuro. Sci. Abst. 228.5

Skou J.C., (1957) The influence of some cations on an ATPase from peripheral nerves. Biochim. Biophys. Acta 23, 394.

Snyder S.H., A.B. Young, J.P. Bennett and A.H. Mulder, (1973) Synaptic biochemistry of amino acids. Fed. Proc. 32, 2039-2074.

Sperelakis N., L. Belardinelli and S.M. Vogel, (1979) Electrophysiology aspects during myocardial ischemia. In: Proc. VIII World Cong. Cardiol. Amsterdam, Excerpta Medica, ed. S. Hayase and S. Murao, pp. 229-236.

Sperelakis N., (1984) Hormonal and neurotransmitter regulation of $Ca^{++}$ Influx through voltage-dependent slow channels in cardiac muscle membrane. Memb. Biochem. 5, 131-166.

Sperelakis N., (1985) Phosphorylation hypothesis of the myocardial slow channels and control of $Ca^{++}$ influx. In: Cardiac Electrophysiology and Arrhythmias, Grune and Stratton.

Suszkiw J.B. and M.E. O'Leary, (1982) Differential labeling of depot and active acetylcholine pools in nondepolarized and potassium-depolarized rat brain synaptosomes. J. Neurochem. 38, 1668-1675.

Suszkiw J.B. and M.E. O'Leary, (1983) Temporal characteristics of potassium-stimulated acetylcholine release and inactivation of calcium influx in rat brain synaptosomes. J. Neurochem. 41, 868-873.

Suszkiw J.B., M.E. O'Leary and G.P. Toth, (1986) Relationship between calcium entry and ACH release in $K^+$-stimulated rat brain synaptosomes. In: Dynamics of Cholinergic Function. In Press. Plenum Press.

Swanson P.D., L. Anderson and W.L. Stahl, (1974) Uptake of calcium ions by synaptosomes from rat brain. Biochim. Biophys. Acta 356, 174-183.

Takata M., W.F. Pickard, J.Y. Lettvin and F.W. Moore, (1966) Ionic conductance changes in lobster axon membrane when lanthanum is substituted for calcium. J. Gen. Physiol. 50, 461-471.

Thayer S.A., K.M. Harris, S.N. Murphy and R.J. Miller, (1986) Dihydropyridine sensitive calcium channel channels in central neurons. Soc. Neurosci. Mtg. Abst. 49.6, p. 175.

Tilltoson D., (1979) Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. Proc. Natl. Acad. Sci. 79, 1497-1500.

Tsien R.W., (1983) Calcium channels in excitable cell membranes. Ann. Rev. Physiol. 45, 341-358.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Turner T.J. and S.M. Goldin, (1985) Calcium channels in rat brain synaptosomes: Identification and pharmacological characterization. High affinity blockade by organic $Ca^{++}$ channel blockers. J. Neurosci. 5, 841-849.

Ulbricht W. and W. Flacke, (1965) After potentials and large depolarizations of single Nodes of Ranvier treated with veratridine. J. Gen. Physiol. 48, 1035-1046.

Varon S., H. Weinstein, C.F. Baxter and E. Roberts, (1965) Uptake and metabolism of exogenous gamma-aminobutyric acid by subcellular particles in a sodium medium. Biochem. Pharm. 14, 1755-1764.

Vogel S. and N. Sperelakis, (1981) Induction of slow action potentials by microiontophoresis of cAMP into heart cells. J. Mol. Cell Card. 13, 51-64. Waggoner A.S., (1979) Dye indicators of membrane potential. Ann. Rev. Biophys. and Bioeng. 8, 47-68.

Wei J.-W. and D.-H. Chaing, (1986) Effects of calcium iantagonists on KCl-evoked calcium uptake by rat cortical synaptosomes. Gen. Pharm. 17, 261-265.

Weinstein H., S. Varon, D.R. Muhleman and E. Roberts, (1965) A carrier mediated transfer model for the accumulation of $^{14}C$ gamma-aminobutyric acid by subcellular particles. Biochem. Pharm. 14, 273-288.

White T.D. and P. Keen, (1970) The role of internal and external Na and K on the uptake of $^{3}H$-noradrenaline by synaptosomes prepared from rat brain. Biochim. Biophys. Acta 196, 285-295.

White T.D. and P. Keen, (1971) The permeability of pinched-off nerve endings to Na, K and Cl and the effects of gramicidin, Biochemistry 8, 725.

White T.D. and P.Keen, (1971) Effects of inhibitors of NaK-ATPase on the uptake of norepinephrine by synaptosomes. Mol. Pharm. 7, 40.

White T.D. and D.M. Paton, (1972) The effects of external Na and K on the initial rates of noradrenaline uptake by synaptosomes prepared from rat brain. Biochim. Biophys. Acta 266, 116-127.

White T.D. and J.T. Archibald, (1974) Rapid binding of newly accumulated noradrenaline within synaptosomes. Brain Res. 82, 360-364.

Whitaker V.P., I.A. Michelson and Kirkland, (1964) Biochem J. 90, 293.

Whittaker V.P. (1969) The synaptosome. In: Handbook of Neurochemistry Vol. II pp. 327-364, Abel Lajtha ed., Plenum Press, (1969).

Wilson C.-S., S.I. Walaas, A. Narin and P. Greengard, (1982) Calcium/phospholipid regulates phosphorylation of a $M_r$ "87k" substrate protein in brain synaptosomes. Proc. Natl. Acad. Sci. 79, 5249-5253.

Wu W., S. Ivar, A. Nairn, and P. Greengard, (1982) Calcium/phospholipid regulates phosphorylation of a $M_r$ "87 kD" substrate protein in brain synaptosomes. Proc. Natl. Acad. Sci. 79, 5249-5253.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Yamamura H.I. and S,H, Snyder, (1973) High affinity transport of choline into synaptosomes of rat brain. J. Neurochem. 21, 1355-1374.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.