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EFFECTS OF ANTIBIOTICS ON DEPOLARIZATION-INDUCED Ca^{2+} UPTAKE INTO SYNAPTOSOMES

By

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ABSTRACT

EFFECTS OF ANTIBIOTICS ON DEPOLARIZATION-INDUCED Ca^{2+} UPTAKE INTO SYNAPTOSOMES

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Antibiotics of four classes block neuromuscular transmission by a combination of pre- and postjunctional effects. The prejunctional effects may involve competitive block of Ca^{2+} entry into the nerve terminal. This has never been tested directly, however. The goal of the present study was to determine if antibiotics known to block neuromuscular transmission would impair depolarization-dependent uptake of calcium into isolated nerve terminals (synaptosomes). Drugs were applied in concentrations ranging from 1 - 1000 uM. Uptake of ^{45}Ca was determined during K^+-stimulated depolarization (K^+ = 77.5 mM). Both the fast and slow phases of Ca^{2+} influx, along with total influx were measured. Neomycin (500 and 1000 uM) decreased fast phase Ca^{2+} uptake. Oxytetracycline (1000 uM) decreased total and both phases of Ca^{2+} uptake. Polymyxin B (5 uM) increased fast phase uptake, while 500 and 1000 uM decreased total and slow phase Ca^{2+} uptake. The decrease in fast phase Ca^{2+} uptake caused by oxytetracycline and neomycin was reversed by increasing the

external calcium concentration. These results indicate that several antibiotics which cause neuromuscular block can alter depolarization-induced calcium uptake into synaptosomes at high concentrations.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

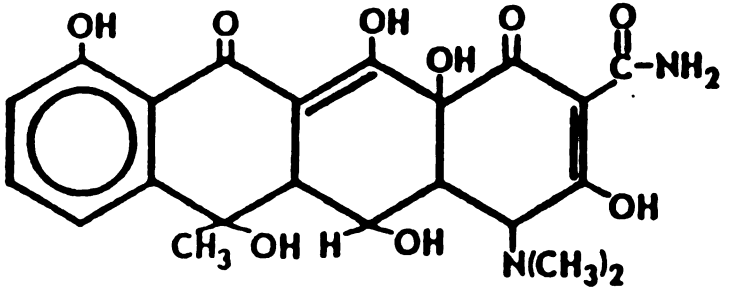
Antibiotic-Induced Neuromuscular Block

Neuromuscular block is a recognized clinical side effect when using some antibiotics (ABs) of the a) aminoglycoside, b) lincosamide, c) polymyxin and d) tetracycline classes (Pittinger et al, 1970; Pittinger and Adamson, 1972). Prolonged respiratory depression may occur when ABs are used in conjunction with general anesthetics (eg. enflurane, halothane, isoflurane, nitrous oxide) or neuromuscular blockers (eg. d-tubocurarine, pancuronium, succinylcholine) (Pittinger et al, 1970; Pittinger and Adamson, 1972; Fogdall and Miller, 1974), in patients with pre-existing neuromuscular (Pittinger et al, 1970) or renal disease, or in patients with electrolyte imbalance (eg. hypocalcemia). Reversal of AB-induced neuromuscular block clinically involves increasing the concentration of Ca^{2+} or giving neostigmine, but sometimes these regimens are not effective (Pittinger et al, 1970). Since it has been reported that the death rate is approximately 9% in patients experiencing respiratory problems related to AB-induced neuromuscular block (Pittinger et al, 1970), it is important to determine the mechanism by which ABs block neuromuscular

transmission, and thus find ways to prevent or counteract their life threatening actions.

Actions, Uses, and Side Effects of Antibiotics That Cause Neuromuscular Block

Although ABs of the four classes all cause neuromuscular block, their structures (figure 1) and antibacterial actions are different. The aminoglycosides and tetracyclines prevent bacterial growth by inhibition of protein synthesis. The aminoglycosides inhibit protein synthesis by preventing peptide initiation, by binding to the 30 S ribosomal subunit and they may also induce misreading of the genetic code (Luzzatto et al, 1969; Pestka, 1971; Hash 1972; Sanders and Sanders, 1979). The tetracyclines inhibit protein synthesis by interfering with binding of the aminoacyl-tRNA to the acceptor site, thus inhibiting elongation of the peptide chain (Pestka, 1971; Hash 1972; Sanders and Sanders, 1979). The lincosamides produce their antibacterial action by binding to the 50 S subunit of the bacterial ribosome, leading to inhibition of the peptidyl transferase reaction (Hash, 1972; Sanders and Sanders, 1979). Polymyxins produce their antibacterial actions by competitively displacing Mg^{2+} or Ca^{2+} from phosphate groups on membrane lipids, disrupting the normal packing of these lipids and thus changing the membrane permeability (Storm et al, 1977; Sanders and Sanders, 1979).



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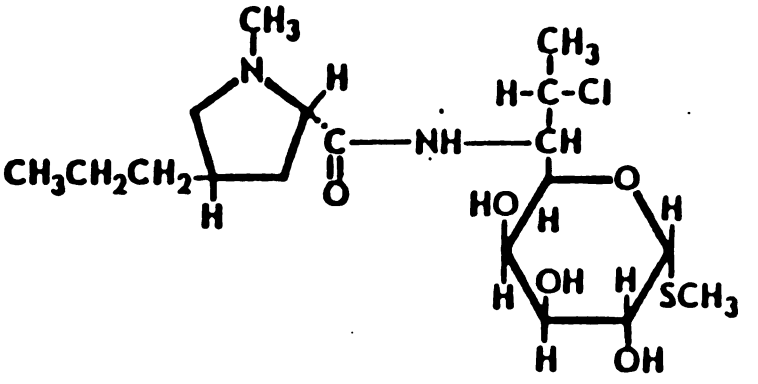

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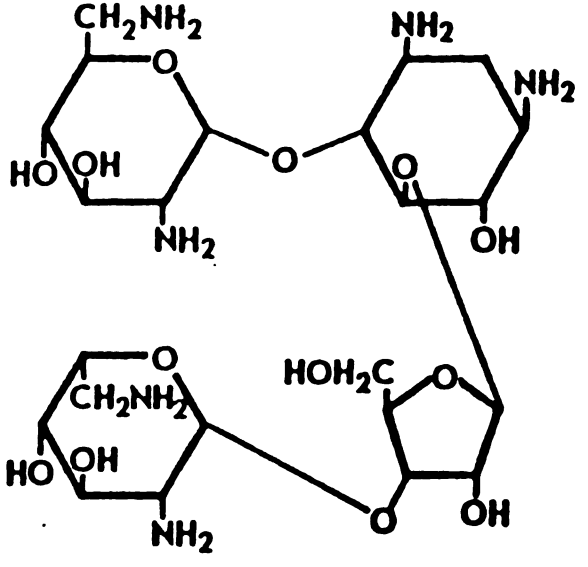

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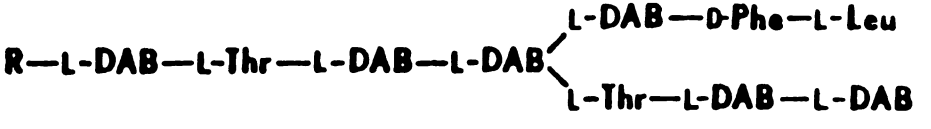


POLYMYXIN B
(DAC=DIAMINOBUTYRIC ACID)

Figure 1. Structures of four antibiotics that block neuromuscular transmission.

The four classes of ABs that cause neuromuscular block have widely divergent clinical uses (Gilman et al, 1985). For example, aminoglycosides are used primarily for treatment of infections due to gram-negative bacteria. Examples of these uses include topical application for burns and wounds, preparation of patients for bowel surgery and treatment of patients with tuberculosis. Lincosamides can be used for infections due to gram-positive cocci; examples of their uses are treatment of abdominal and lung abscesses and pneumonia. Polymyxins may be used for infections due to gram-negative bacteria; examples of their uses include urinary tract infections and topical use for infections of the skin, eyes and ears. Examples of the clinical uses of tetracycline include Rocky Mountain spotted fever, and urinary and ocular infections.

Since these ABs have different structures, mechanisms of action and uses, it is not surprising that they produce different side effects as well (Gilman et al, 1985). The primary side effects of the aminoglycosides include vestibular, cochlear and renal toxicity. The tetracyclines can cause hepatic and renal toxicity, and gastrointestinal irritation. In addition, tetracyclines have effects on calcified tissues (eg, deposition in teeth and bones). Clindamycin may cause development of pseudomembranous colitis, which may be lethal. Renal toxicity is the most significant side effect of polymyxin B. Other side effects of polymyxin B include facial flushing, dizziness, slurred

speech and blurred vision. Despite the differences in chemical structure, antibacterial mechanism, clinical uses and side effects, these ABs all have one feature in common: they block neuromuscular transmission.

Neuromuscular Transmission

Neuromuscular transmission is comprised of several steps beginning with synthesis of the neurotransmitter acetylcholine (ACh) and ending with its hydrolysis to stop its effects on the postjunctional membrane and thus terminate its action. ACh is synthesized in the nerve terminal from choline and acetyl CoA in a reaction catalyzed by the enzyme choline acetyltransferase (Dauterman and Mehrotra, 1963; Currier and Mautner, 1974). In the terminal, ACh is stored in synaptic vesicles, although there may be a nonvesicular fraction as well (De Robertis and Bennett, 1955; Collier and MacIntosh, 1969; Ritchie and Goldberg, 1970). Neurotransmitters, including ACh, are thought to be released in discrete amounts (quanta) (Del Castillo and Katz, 1954). After the depolarization of the action potential invades the presynaptic terminal, synaptic vesicles fuse with the membrane to release neurotransmitter by exocytosis (Heuser, 1977; Heuser, et al, 1974, 1979). The release of neurotransmitter is dependent on extracellular Ca^{2+} (Katz and Miledi 1965b; 1967; 1969; Dodge and Rahamimoff, 1967); somehow depolarization-induced Ca^{2+}

entry into the presynaptic nerve terminal leads to release of neurotransmitter (Llinas and Nicholson, 1975), but the exact mechanism is unknown. A proposed role of calcium is to open Ca^{2+}-activated k^{+} channels present in synaptic vesicles, thus causing osmotic changes that would lead to fussion of the vesicle with the membrane and exocytosis of the vesicle contents (Stanley and Ehrenstein, 1985). The estimated time required for calcium entry and neurotransmitter release is approximately 200-500 usec (Parsegian, 1977; Llinas et al, 1981b). After ACh is released from the presynaptic terminal, it crosses the synaptic cleft. When ACh reaches the postjunctional membrane it binds to the acceptor sites on a macromolecule known as the ACh receptor. This leads to a change in ion permeabilities in the postsynaptic membrane (Takeuchi and Takeuchi, 1960 Katz and Miledi, 1972). The changes in the postsynaptic membrane potential, in turn, cause generation of an action potential in the muscle, which leads to contraction of the muscle. The action of ACh is terminated by acetylcholinesterase which hydrolyzes the ACh to choline and acetate (Collier, 1977). Choline re-enters the nerve terminal by a carrier-mediated process and is reused to make ACh (Marchbanks, 1968, 1982; Diamond and Kennedy, 1969). The entire process of neurotransmitter release from depolarization to the appearance of the postsynaptic end-plate current occurs in milliseconds (msec) (Katz and Miledi, 1965a). The times of each individual synaptic delay

are not equal but fall in the range of 0 - 4 msec with a peak in the distribution at 0.75 msec (Katz and Miledi, 1965a).

Neuromuscular Block

ABs can theoretically block neuromuscular transmission at several sites these include: 1) conduction of the action potential into the nerve terminal, 2) synthesis, mobilization and release of neurotransmitter, 3) activation of the postsynaptic receptor, 4) generation and propagation of the action potential in the muscle membrane, and 5) excitation - contraction coupling in the muscle. The mechanism(s) by which ABs produce neuromuscular block is/are unknown even though numerous experimental studies have been undertaken in an attempt to study this effect (for example, Elmqvist and Josefsson, 1962; Brazil and Corrado, 1969; Wright and Collier, 1976a,b, 1977; Prado et al, 1978; Singh et al, 1978, 1979, 1982; Fiekers et al, 1979, 1983; Fiekers, 1981, 1983a,b). The techniques that have been used include; twitch tension measurements following electrical stimulation of motor axons or myofibers from different nerve/muscle preparations, (Becker and Miller, 1976; Wright and Collier, 1976a, 1977; Singh et al, 1978), intracellular recordings of postsynaptic potentials and action potentials from isolated nerve/muscle or nerve preparations, respectively (Singh et al, 1979, 1982; Caputy et al, 1981) and measurements of

endplate ionic currents using voltage clamp techniques (Fiekers et al, 1979, 1983; Fiekers, 1981, 1983a,b; Farley et al, 1982). These various studies have demonstrated two types of neuromuscular block caused by ABs: 1) postjunctional i.e. effects on the end-plate receptor or ionic channel (with d-tubocurarine as the prototype), and 2) prejunctional i.e. effects on the release of the neurotransmitter (with magnesium as the prototype). Postjunctional effects have been demonstrated as a decrease in the amplitude of miniature endplate potentials (MEPPs) or currents (MEPCs), a decrease in the amplitude of endplate potentials (EPPs) or currents (EPCs), or non-linearity in the endplate current-voltage relationship. Prejunctional actions of ABs have been demonstrated as a decrease in the amount of neurotransmitter released by a nerve impulse (mean quantal content, m). However for many ABs the effects cannot be classified as solely "curare - like" or "magnesium - like" (Singh et al, 1978); these ABs often produce a combination of effects.

A) Mechanisms of Antibiotic Induced Neuromuscular Block

The aminoglycosides are thought to possess a combination of pre- and postjunctional blocking actions, with prejunctional effects predominating (Elmqvist and Josefsson, 1962; Brazil and Corrado, 1969; Pittinger and Adamson 1972; Singh et al, 1979, 1982; Sokoll and Gergis,

1981; Farley et al, 1982; Fiekers, 1983a,b). This has been shown in combined studies of pre- and postjunctional effects of aminoglycosides using a two microelectrode voltage clamp of normal or transected twitch fibers of frog (Farley et al, 1982) or snake (Fiekers, 1983a,b), respectively. Neomycin and streptomycin both decreased m, as determined by the ratio of EPC/MEPC amplitude, at concentrations much lower than those which cause postjunctional reduction of MEPC amplitude or alteration of decay kinetics of the EPC. The effect of neomycin in particular was more prominent on prejunctional processes (Fiekers, 1983a).

The lincosamides, clindamycin and lincomycin, may cause neuromuscular block by a variety of mechanisms including a direct action on the muscle or by postjunctional block of receptor channels. In addition clindamycin may cause neuromuscular block by suppression of ACh release. Lincomycin and clindamycin cause a parallel decrease in muscle contractile response due to nerve stimulation and direct muscle stimulation (Wright and Collier, 1976a). This leads to the conclusion that a myogenic component of block exists. Lincomycin and clindamycin also decrease EPP or EPC amplitude and endplate sensitivity to exogenously applied ACh (Tang and Schroeder, 1968; Becker and Miller, 1976; Rubbo et al, 1977; Singh et al, 1979, 1982; Fiekers et al, 1983), leading to the conclusion that lincosamides act postjunctionally. Although there is much disparity in experimental data, there is evidence that clindamycin may

also have prejunctional effects. Clindamycin has variously been described to increase ACh release induced by nerve stimulation (Rubbo et al, 1977), decrease quantal content (Fiekers et al, 1983), and increase frequency of occurrence of spontaneous MEPPs (Rubbo et al, 1977).

Polymyxin B has both pre- and post-junctional effects, but postjunctional effects predominate. This is shown by reductions in quantal content similar to those caused by Mg^{2+} at high concentrations, and decreases in MEPP or MEPC amplitude at very low concentrations (Singh et al, 1979, 1982; Fiekers, 1981; Sokoll and Gergis, 1981).

The effects of tetracyclines on neuromuscular transmission are less well studied. Wright and Collier (1976b) showed that tetracyclines produced an initial augmentation and subsequent inhibition of muscle contraction in response to nerve stimulation. However, using a phrenic nerve-diaphram preparation they showed that the concentration required to block the response to ACh injection (via the inferior vena cava) was considerably less than the concentration required to block the response to nerve stimulation, suggesting that tetracycline causes neuromuscular block by acting postjunctionally. In opposition to this, Singh et al (1982) suggested that tetracycline may have predominantly prejunctional blocking activity along with actions that block muscle contractility. Singh et al (1982), used isolated frog sciatic nerve-sartorius muscle preparations to show that tetracycline,

like Mg^{2+}, did not decrease the MEPP amplitude, suggesting that tetracycline does not act postjunctionally. They also showed that tetracycline and oxytetracycline reduced both the maximum rate of rise and fall of action potentials in the muscle, suggesting again a direct myogenic action.

B) Postjunctional Neuromuscular Block

Despite the fact that ABs act at both pre- and postjunctional sites, the majority of experimental studies have focused on the postjunctional actions. ABs may cause one or more of the following postjunctional effects: block of receptor occupation by ACh, block of ion permeation through the receptor-activated ionic channels, inhibition of action potential propagation in the muscle, or disruption of muscle contractility. For example, lincomycin has been shown to interfere with muscle membrane excitation or muscle contraction (Wright and Collier, 1976a). Clindamycin and lincomycin each interact with the open state of the endplate receptor/channel complex in a noncompetitive manner (Fiekers et al, 1983). Streptomycin has mixed competitive and noncompetitive actions (Brown and Taylor, 1983), but the predominate postjunctional action is competitive block of the ACh receptor (Farley et al, 1982). Neomycin also exerts mixed competitive and noncompetitive actions, with the noncompetitive block of the ACh receptor predominating (Brown and Taylor, 1983). At higher concentrations,

neomycin reacts with the receptor channel when it is in an open configuration (Fiekers, 1983b). Polymyxin B interacts in a noncompetitive manner (Brown and Taylor, 1983) with the receptor/channel complex to produce a voltage-dependent block of the open channel (Fiekers, 1981).

C) Prejunctional Neuromuscular Block

Whereas the postjunctional blocking actions of ABs have been studied extensively, the prejunctional blocking actions of ABs have been studied incompletely. Drugs that have prejunctional effects can act on action potential propagation, neurotransmitter synthesis or release. More specifically ABs may ultimately block neurotransmission presynaptically by any of several mechanisms, such as 1) decreasing the calcium concentration in the external medium (by binding calcium, or altering the ionized to nonionized ratio), 2) blocking calcium from entering the nerve terminal (by blocking or inactivating the voltage-dependent calcium channel, 3) altering stimulus/secretion coupling, or 4) affecting neurotransmitter release (decreasing the size of a quantum, or altering the permeability of the membrane). There is evidence based on individual ABs for or against some of these possibilities. However, the effects of none of the ABs have been studied in sufficient detail to determine their mechanism of action, nor has a general mechanism responsible for prejunctional block been proven

for all ABs. For example, in the presence of streptomycin (Pittinger, 1970; Tamaki, 1983) or neomycin (Elmqvist and Josefsson, 1962; Wright and Collier, 1977) the concentration of the ionized form of calcium is not changed. These studies have excluded the possibility of calcium binding to the ABs as the mechanism of neuromuscular block. However, it has been postulated that tetracycline's mechanism of action is calcium chelation, with a resultant decrease in neurotransmitter release (Pittinger and Adamson, 1972). This does not seem likely since Wright and Collier (1976b) have shown that rolitetracycline did not decrease evoked release of ACh; a decrease would be expected if tetracycline acted by chelating calcium. Furthermore, Bowen and McMullan (1975) have shown (in horses) that calcium binding does not seem to contribute to the neuromuscular block produced by oxytetracycline. Clindamycin may have prejunctional effects due to its local anesthetic-like actions (Wright and Collier, 1976a). However, these only occur at very high concentrations and thus are unlikely to contribute significantly to the clinically observed effect.

Calcium Channels

One potential prejunctional mechanism by which ABs may act is to block Ca^{2+} entry through voltage-regulated calcium channels. Calcium channels are found in all excitable membranes, such as nerve and cardiac muscle membranes.

Calcium channels play a role in coupling membrane excitation to cellular responses, eg. secretion or contraction. Calcium channels are also essential for the rhythmic firing of nerve cells (Llinas and Sugimori, 1980). Calcium entry contributes to other neuronal functions such as neurite extension (Anglister et al, 1981), generation of dendritic calcium spikes (Llinas and Yarom, 1981) and calcium-dependent potassium conductances (Barrett and Barrett, 1976; Krnjevic et al, 1975, 1978; Llinas and Sugimori, 1980). In a variety of cells, depolarization of the membrane increases the membrane's permeability to calcium, i.e. these cells have voltage-dependent calcium channels.

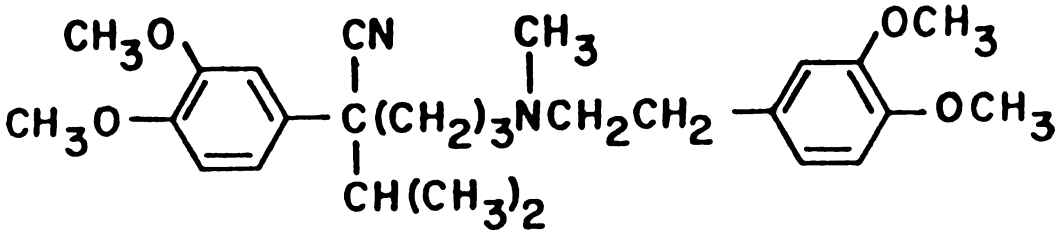
A) Multiple Types of Calcium Channels

The different functions of Ca^{2+} suggest different or multiple types of calcium channels may exist. Two distinct populations of calcium channels have been demonstrated in a clonal cell line derived from rat pituitaries (GH3 cells) based on their closing kinetics (Armstrong and Matteson, 1985). Two distinct types of voltage-activated calcium conductances have also been found in the unfertilized egg of Neanthes arenaceodentata, (Fox and Krasne, 1981), and Mediaster aequalis (Hagiwara et al, 1975), suggesting the presence of two calcium channels. Fishman and Specter (1981), working with neuroblastoma (N1E-115), have shown that the decay of the calcium currents exhibits two time

constants, which again suggests the existence of two calcium channels. Tsunoo et al (1984) have demonstrated two different types of calcium channels in neuroblastoma cells (N1E-115), based on difference in gating properties and insensitivity to cyclic AMP. Nowycky et al (1985), have demonstrated three types of calcium channels in dorsal root ganglion cells. Two types of calcium channels have been proposed to exist in synaptosomes (Nachshen and Blaustein, 1980). Recently, Penner and Dreyer (1986) have presented evidence suggesting the presence of multiple type of calcium channels in the motor nerve terminal at the mouse neuromuscular junction. Thus, results from many different preparations suggest the existence of multiple Ca^{2+} channels.

B) Calcium Channel Blockers

Calcium channels are blocked by a number of agents (figure 2). Note that these structures are markedly different from those of the ABs (figure 1). A peptide, isolated from Conus geographus and designated omega toxin, blocks calcium entry into the nerve terminal during the presynaptic action potential (Kerr and Yoshikami, 1984). Inorganic cations such as Ni^{2+}, Mn^{2+}, and Co^{2+} can block calcium influx in a competitive manner (Nachshen, 1984; Drapeau and Nachshen, 1984; and see reviews by Hagiwara and Byerly, 1981; Kostyuk, 1981; Edwards, 1982), while the



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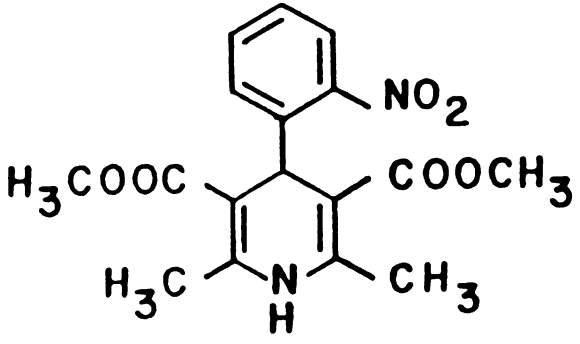

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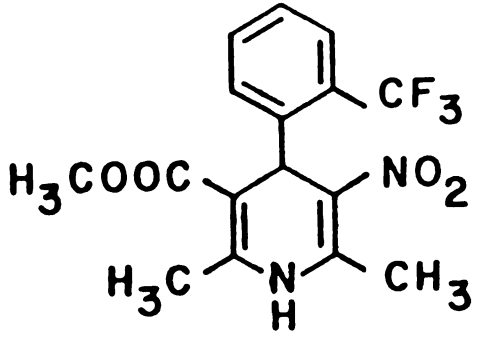

BAY K 8644

Figure 2. Structures of some agents that modify calcium channel activity.

organic cation, methylmercury, blocks calcium influx in an apparently noncompetitive fashion (Atchison et al, 1986). Other inorganic cations, La^{3+} (Nachshen and Blaustein, 1980, 1982) and Cd^{2+} (Nowycky et al, 1985; Narahashi et al, 1986) preferentially block some calcium channels. Organic calcium antagonists include verapamil and D-600; however, these antagonists may not be calcium specific but may also affect sodium channels (Van der Kloot and Kita, 1975; Nachshen and Blaustein, 1979). Other calcium antagonists, the dihydropyridines, have been shown to modify Ca^{2+} channel activity. For example, nifedipine and nitrendipine act as a calcium antagonists whereas Bay K 8644 acts as a calcium agonist (Hess et al, 1984). Calcium channels in neuronal tissue appear to be much less sensitive to verapamil, D-600 and dihydropyridines than are calcium channels in cardiac or vascular smooth muscle (Gotgil'f and Magazanik, 1977; Nachshen and Blaustein, 1979; Daniell et al, 1983; Glossman et al, 1984). This suggests that there may be differences between calcium channels in excitable membranes of various different tissues.

C) Calcium Channel Inactivation

Inward calcium currents are found in many cell membranes, and have been shown to relax with time (Standen, 1974; Hencek and Zachar, 1977; Kostyuk and Krishtal, 1977; Tillotson, 1979). In some cases the calcium currents relax

with time under a maintained depolarization (Tillotson, 1979; Eckert and Tillotson, 1981; Fox, 1981). Some calcium channels (for example, those in egg cell membrane of Neanthes, and in synaptosomes) have been shown to inactivate in a voltage-dependent manner (Fox, 1981; Nachshen, 1985a; Ashely, 1986). Other calcium channels (for example, neurons of Aplysia californica, rabbit sino-atrial node and calf Purkinje fibers) have been shown to inactivate in a calcium-dependent manner (Tillotson, 1979; Brown et al, 1981; Eckert and Tillotson, 1981; Marban and Tsien, 1981). It is also possible that some channels may inactivate in both a calcium- and voltage-dependent manner (possibly synaptosomes, Suszkiw et al, 1986). Therefore, flow of inward current through calcium channels in different tissues may be terminated by different mechanisms.

Competitive Antagonism -- A Possible Mechanism

Several lines of evidence suggest that ABs act as competitive antagonists of calcium at a common presynaptic site. ABs with neuromuscular blocking activity tend to be large, often charged molecules which suggests they should act outside the nerve terminal. The effects of some of the ABs occur rapidly, and are rapidly reversed by removing the drug, (Wright and Collier, 1977; Fiekers, 1983a; Prado et al, 1978; Tamaki, 1983), which also suggests they act outside the nerve terminal. Twitch tension measurements

obtained using the isolated phrenic nerve-diaphragm preparation of rats with bath application of aminoglycosides showed that increasing $CaCl_2$ in the bath caused a parallel shift to the right in the log dose response curve, thus demonstrating competitive antagonism (Prado et al, 1978).

Two lines of evidence are consistent with a general effect of ABs on calcium-dependent transmitter release. First, neomycin blocks release of norepinephrine and ACh (Wright and Collier, 1977) and streptomycin blocks release of L-glutamate (Washio, 1984), thus the effect of the ABs is not specific for cholinergic neurons. Second, increasing bath calcium has been shown to reverse or partially reverse the neuromuscular block caused by several ABs (Elmqvist and Josefsson, 1962; Pittinger, 1970; Prado et al, 1978; Singh et al, 1978, 1979, 1982; Fiekers, 1983a; Washio, 1984). Based on these findings coupled with the decrease in m, caused by aminoglycosides and other ABs, it has been proposed that ABs act as competitive inhibitors of calcium influx.

Although the available evidence is consistent with this notion, it is not sufficient to prove that blocking calcium influx is the cause of the neuromuscular block. AB-induced inhibition of calcium influx into the nerve terminal has not been tested directly. Since it has not been determined that the ABs block calcium influx, it is possible to explain the experimental data, showing that an increase in the external calcium concentration ($[Ca^{2+}]_o$) can reverse the AB-induced

neuromuscular block, based on functional antagonism. It is possible that the ABs have their effects intracellularly and that increasing the internal calcium concentration relieves the AB-induced effect by noncompetitive functional antagonism. For example agents such as 4-aminopyridine and tetraethylammonium (TEA) which prolong the presynaptic depolarization by blocking K^+ efflux (Armstrong and Binstock, 1965; Kusano et al, 1967; Yeh et al, 1976; Lundh, 1978) and thus increase the internal calcium concentration, can relieve the block of neuromuscular transmission caused by botulinum toxin (Cull-Candy et al, 1976; Lundh et al, 1977). Botulinum toxin does not block Ca^{2+} channels but is believed to affect ACh release by intracellular actions (Wonnacott et al, 1978; and see reviews by Narahashi, 1974; Howard and Gundersen, 1980). Moreover, the effects of myasthenia gravis, which is a postjunctionally-directed disease can also be relieved with aminopyridines (Kim and Sanders, 1980; Kim, 1982). Since aminopyridines and TEA cause functional antagonism by increasing the internal calcium concentration they can reverse the neuromuscular block caused by presynaptic intracellular events and/or postjunctional events. Therefore, in order to determine whether increasing $[Ca^{2+}]_o$ reverses the AB-induced neuromuscular block by competitive antagonism or by functional antagonism it is necessary to know if ABs block Ca^{2+} influx. The goal of this project is to determine directly whether ABs reduce depolarization-induced calcium

influx into the nerve terminal.

Synaptosomes

The small size of the presynaptic motor nerve terminal prohibits use of manipulations (eg, impalement with a recording micropipette, Ca^{2+} selective microelectrode, or injection of test agent) that would give direct information about calcium influx. Thus, in order to measure calcium influx another system must be used. Gray and Whittaker (1962) developed a procedure for preparing pinched off nerve terminals (synaptosomes) from mammalian brain tissue. Brain tissue is used as the source of synaptosomes, since isolation of nerve terminals from the neuromuscular junction is not feasible due to the small number of nerve terminals compared to the large quantity of muscle.

Synaptosomes provide a useful model for studying physiological and biochemical processes which occur at presynaptic nerve terminals (Blaustein, 1975; Blaustein *et al*, 1981). Synaptosomes retain many morphological and functional properties of intact neurons (Blaustein *et al*, 1977). For example, synaptosomes maintain a resting membrane potential that responds to depolarizing agents (Blaustein and Goldring, 1975). Synaptosomes retain a functional choline uptake system (Haga and Noda, 1973), and can synthesize and release ACh (Haga, 1971). Synaptosomes also retain functional glycolytic and oxidative metabolic

pathways. Synaptosomes accumulate potassium, extrude sodium (Blaustein et al, 1977), regulate Ca^{2+} influx (Blaustein, 1975; Nachshen and Blaustein, 1980) and release transmitter after a depolarization-induced calcium influx (Blaustein, 1975).

Despite these advantages there are several problems associated with the use of synaptosomal preparations (Blaustein et al, 1977). First, synaptosomal preparations may not contain only pure nerve terminals, but may also contain other membranes or subcellular organelles such as mitochondria. Second, damaged or nonfunctional nerve terminals may be present. Third, in most cases the terminals obtained from homogenates are heterogeneous with respect to the transmitter they contain. Fourth, and most important, the time resolution of radiolabel flux measurement into synaptosomes is much slower than the time course of synaptic transmission. Normal synaptic transmission is completed in milliseconds (Katz and Miledi, 1965a, 1967), whereas depolarization-evoked influx of Ca^{2+} into synaptosomes is measured over periods of seconds. Use of specialized rapid mixing techniques allow this measurement period to be shortened and permits a time resolution of 100 msec or less (Nachshen, 1985a; Suszkiw et al, 1986). Using these techniques, Nachshen (1985a) reported that results obtained during shorter time intervals corresponded reasonably well to those obtained at 1 sec, and that values obtained with the rapid mixer were similar to

values obtained by hand-pipetting at times of 1-10 sec.

Calcium Uptake Into Synaptosomes

Depolarization leads to an increase in calcium permeability of the prejunctional membrane (Baker et al, 1971; Llinas et al, 1981a). Since the extraterminal calcium concentration exceeds the intraterminal calcium concentration (Nachshen, 1985b), the increased permeability leads to calcium influx. This calcium influx is the trigger that couples nerve terminal excitation and transmitter release (Katz and Miledi, 1967, 1969). Calcium is known to be the trigger because axonal depolarization in the absence of calcium does not cause transmitter release (Katz and Miledi, 1965b; Miledi and Slater, 1966). The properties of calcium influx into synaptosomes have been studied extensively. Backflux of radioactive calcium is negligible during the first 10 seconds of incubation (Nachshen and Blaustein, 1980), thus the ^{45}Ca movement measured during this time represents influx, rather than net flux. Two phases of calcium entry into synaptosomes have been demonstrated (Gripenberg et al, 1980; Nachshen and Blaustein, 1980; Nachshen, 1985a): a fast phase mediated by a pathway that is inactivated after 1-2 seconds and is inhibited by low concentrations of La^{3+} (< 1 uM), and a slow phase, mediated by a pathway that is not inactivated during long-lasting depolarization (1-2 min) and is only blocked by

high concentrations of La^{3+} (> 0.1 mM) (Nachshen and Blaustein, 1980, 1982; Suszkiw and O'Leary, 1983; Nachshen, 1984). Based on the differential sensitivity to La^{3+}, the two phases of calcium entry have been proposed to correspond to two different calcium channels (Nachshen and Blaustein, 1980, 1982), although other interpretations have also been cited (Wang et al, 1985; Suszkiw et al, 1986). The fast pathway is of primary interest because it is associated with voltage- and calcium-dependent release of dopamine from striatal synaptosomes (Drapeau and Blaustein, 1983), voltage- and calcium-dependent release of substance P from lower brain synaptosomes (Floor, 1983), and voltage- and calcium-dependent release of ACh from forebrain synaptosomes (Suszkiw and O'Leary, 1983). Voltage-dependant norepinephrine release also paralleled Ca^{2+} uptake in a synaptosome preparation; both Ca^{2+} uptake and norepinephrine release have a fast and slow phase (Daniell and Leslie, 1986). The initial rate of dopamine release is close to the rate of release evoked by nerve stimulation of intact tissue (Drapeau and Blaustein, 1983) and the time course of dopamine release parallels calcium entry through the fast channel (Drapeau and Nachshen, 1984; Leslie et al, 1985). As suggested by Nachshen and Blaustein (1980) the slow phase of calcium uptake may be carrier mediated; Wang et al (1985) have shown that under certain conditions the slow phase may be mediated by a membrane potential-sensitive Na^{+}/Ca^{2+} exchange mechanism. Measurement of potassium-stimulated

calcium uptake by synaptosomes using rapid-mixing techniques has shown that the initial rate of calcium influx is faster than previously predicted, but that two phases of calcium influx are still apparent at the times previously tested (Nachshen, 1985a). These results imply that 1 and 10 second incubation times used in manual mixing experiments are useful time points for measure of the two phases of calcium influx.

The potassium-stimulated calcium uptake in a variety of preparations does not occur via the sodium channel as it is not blocked by the Na^+ channel blocker, tetrodotoxin (TTX) (Baker et al, 1973b; Blaustein, 1975; Nachshen and Blaustein, 1980) or by replacement of external sodium with choline (Blaustein, 1975). Both phases of calcium influx are insensitive to TTX (Nachshen and Blaustein, 1980), but can be blocked by Ni^{2+}, Mn^{2+}, Mg^{2+} and Co^{2+} in a competitive manner (Nachshen and Blaustein, 1980; Drapeau and Nachshen, 1984). Verapamil and D-600 also block calcium influx into synaptosomes, but with a much lower potency than divalent cations (Nachshen and Blaustein, 1979; Daniell et al, 1983). The slow phase of calcium influx into squid axons is not blocked by the K^+ channel blocker TEA (Baker et al, 1973a). There is a local electric response in squid neurons, in the presence of external TTX and internal TEA, when there is a high external calcium concentration. (Katz and Miledi, 1969). This evidence implied that the calcium does not enter the nerve terminal by either sodium or potassium

channels, so a calcium channel was proposed (Baker et al, 1973a). As previously described, it is possible that several calcium channels exist.

The increase in calcium permeability is depolarization-dependent; this implies that calcium uptake is voltage-dependent. Depolarization is normally caused by an electrical impulse, however potassium may be used to cause a depolarization and thus induce calcium influx (Blaustein, 1975). The potassium-induced change in calcium permeability is not caused by making the synaptosome irreversibly leaky to calcium since prior stimulation in a solution with a high concentration of potassium does not change the rate of calcium uptake in a solution with a low concentration of potassium (Blaustein, 1975). Nor is the potassium-induced increase in calcium permeability due to incorporation of bulk extracellular fluid by pinocytosis since the extracellular markers mannitol or inulin do not accompany the calcium as it is taken up by the synaptosomes (Blaustein, 1975).

Experimental Rationale

The studies cited herein were designed to clarify the mechanism by which ABs act prejunctionally. In particular I sought to determine whether block of depolarization-induced Ca^{2+} influx by ABs could be demonstrated in isolated nerve terminals. The experiments utilized a synaptosome-enriched

preparation that was depolarized by potassium to induce influx of calcium. Effects of ABs on calcium influx were studied during both the fast (1 sec) and slow (10 sec) phases, as well as for total influx over 10 sec. Several ABs: clindamycin, neomycin, oxytetracycline, and polymyxin B (one from each of the 4 groups known to block neuromuscular transmission) were tested for their effects on calcium uptake.

Previous experiments have not looked at calcium uptake directly; instead they have used measures (ACh release, EPP, MEPP, etc.) that are believed to be related to calcium influx. I determined calcium uptake into synaptosomes directly through the use of radioactive calcium. Based on previous experiments with isolated nerve/muscle preparations it was expected that the aminoglycoside and tetracycline would decrease calcium influx, clindamycin might increase calcium influx and polymyxin B might decrease calcium influx or have no effect. One possible mechanism by which ABs may decrease calcium influx is by competition with calcium for a common binding site. If the decrease in calcium influx was competitive and reversible, then increasing the $[Ca^{2+}]_o$ should reverse the effects of the AB. Inorder to determine if the ABs act as competitive antagonists to calcium entry, the ability of calcium to reverse AB-induced block of Ca^{2+} influx ($[Ca^{2+}]_o$ = 0.05mM - 1mM) was tested. The attempt at reversal was done using either the concentration of AB that caused 50 percent inhibition (IC_{50}) or, if the maximal

percent inhibition was less than 50%, a concentration of AB that caused maximal decrease of influx during the fast phase.

METHOD AND MATERIALS

Synaptosomes were isolated, and partially purified, from a homogenate of rat forebrain through the use of several steps of centrifugation. The partial purification was performed to isolate synaptosomes and attempt to remove other cellular (brain) contaminants such as mitochondria and axonal membranes. The mitochondria should be removed as they are capable of calcium uptake and thus could confound the measurements of tracer uptake. (However, there are data that imply that potassium-stimulated calcium uptake is probably associated with synaptosomes only and not with other organelles which may contaminate the synaptosome fraction (Blaustein, 1975)). Extra membranes and cellular components must be eliminated to obtain an accurate measurement of the amount of synaptosomal protein to standardize presentation of results.

Synaptosomes were prepared from homogenates of rat (Sprague-Dawley, 175-250 grams, male) forebrains, using a modification of the method of Gray and Whittaker (1962)(figure 3). A 10% (weight/volume) homogenate in 0.32 M sucrose was centrifuged at 1,000 x g for 10 min (Sorvall RC2B; Ivan Sorvall Inc., Norwalk, CT.). The supernatant was recentrifuged at 17,500 x g for 20 min (Sorvall). The

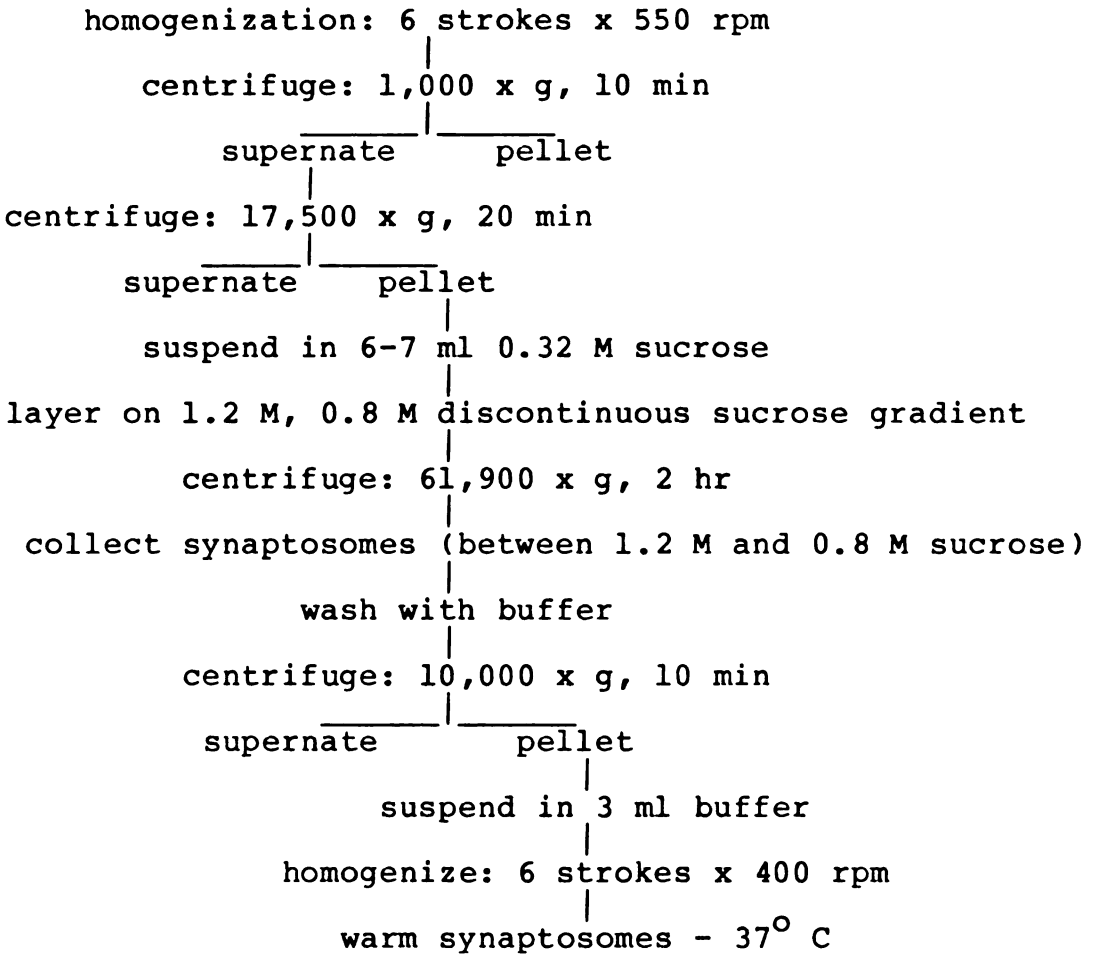

Figure 3. Preparation of synaptosomes. Flow chart shows the method used for preparation of synaptosomes from rat forebrain.

resulting pellet was resuspended in 6 - 7 ml 0.32 M sucrose, and layered onto two discontinuous sucrose gradients that were made with 10 ml of 1.2 M sucrose (bottom) and about 17 ml of 0.8 M sucrose (middle). The sucrose gradient was centrifuged (Beckman L8-55 ultracentrifuge; Beckman Instruments, Palo Alto, CA.) at 61,900 x g for 120 min using a SW-27 rotor. The synaptosome-rich fraction, between the 0.8 M and 1.2 M sucrose layers, was collected by centrifuging at 10,000 x g for 10 min and then suspended in Ca^{2+}-free buffer. After preparation, the synaptosomes were warmed to 37^{o} C, and were always used within 3 hr. Bradford (1975) has shown that synaptosomes remain viable for 3 - 4 hr, after preparation, at 37^{o} C.

Uptake of ^{45}Ca was measured by incubating 50 ul of synaptosomal suspension with 50 ul of "high-K^{+}" ($[K^{+}]$ = 77.5 mM) or "low-K^{+}" ($[K^{+}]$ = 5 mM) solution with tracer. The high-K^{+} buffer was used to cause a depolarization-induced Ca^{2+} influx and the low-K^{+} buffer was used to control for Ca^{2+} influx under nonstimulated conditions. ABs were added so as to give final concentrations ranging from 1 to 1000 uM. This range is larger than the dose range used clinically. ^{45}Ca uptake was terminated by rapidly diluting the incubation mixture with a quench buffer containing ethylene glycol bis-(beta-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA) and N-methylglucamine. EGTA was added to chelate the calcium making it unavailable to the synaptosomes. N-methylglucamine was included in the quench

buffer as a large ion that does not enter axonal sodium channels. Hille (1971) showed that sodium channels are impermeant to methylated ions. Because of this, N-methylglucamine prevents further depolarization. The incubation times were 1 sec (timed with a metronome) for the fast channel and 10 sec (timed with a stop watch) for total calcium uptake. Calcium influx during the slow phase was measured by first inactivating the fast channel by pre-depolarizing (10 sec) in high-K^+ buffer before the 10 sec incubation.

After ^{45}Ca uptake had been quenched, the reaction mixtures were filtered through 0.45 um Millipore filters (Millipore Corporation, Bedford, MA.). The filters were then washed twice with 5 ml aliquots of the quench buffer, and then placed in scintillation vials with solubilizer (1% Triton X-100 in 0.5 M HCL, weight/volume) for at least 10 min. Scintillation fluid, Formula 963 (New England Nuclear, Boston, MA) (10 ml) was then added and the sample counted in a liquid scintillation counter (Beckman LS 7000; Beckman Instruments, Fullerton, CA.). The amount of calcium influx was calculated as the difference between calcium uptake in solutions containing high-K^+ and low-K^+ concentrations. Appropriate blanks were run and their values were used to adjust the results for nonspecific binding of calcium to the filters. The results (calcium influx caused by depolarization) of initial studies used to determine whether the preparation was viable were expressed in terms of counts

per minute (cpm) per ug synaptosomal protein (Lowry et al, 1951). The results of the subsequent studies using ABs were expressed as femtomoles (fmoles; 1 fmole = 1 x 10^{-15} moles) of calcium influx per ug synaptosomal protein, or as percent of drug-free control.

Solutions and Chemicals

The buffer used to suspend the synaptosomes contained (mM): NaCl 145, KCl 5, $MgCl_2$ 1, d-glucose 10, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10. The high potassium buffer contained (mM): NaCl 72.5, KCl 77.5, $MgCl_2$ 1, $CaCl_2$ 0.04, d-glucose 10, and HEPES 10. The low potassium buffer contained (mM): NaCl 72.5, KCl 5, choline chloride 72.5, $MgCl_2$ 1, $CaCl_2$ 0.04, d-glucose 10, and HEPES 10. The quench buffer used to stop the reaction contained (mM): N-methylglucamine 145, KCl 5, $MgCl_2$ 1, EGTA 1, d-glucose 10, and HEPES 10. The solubilizer was 1% Triton X-100 in 0.5 M HCl (weight/volume). The scintillation fluid used was Formula 963 (New England Nuclear, Boston, MA). Sucrose concentrations (for discontinuous sucrose gradient) were 1.2 M, 0.8 M and 0.32 M. All solutions were made with deionized water (Milli-Q) and had an osmolality of 290-320 mOsm as determined by freezing point depression (Micro osmette, Precision Systems, Inc., Natick, MA). The pH of the solutions (except sucrose) was adjusted to 7.4 using glacial

acetic acid or NaOH. Drugs tested include clindamycin hydrochloride, neomycin sulfate, oxytetracycline dihydrate and polymyxin B sulfate (Sigma Chemical Co., St. Louis, MO), and lead acetate (J. T. Baker Chemical Co., Phillipsburg, NJ). The radioactive calcium was used in the form of $^{45}CaCl_2$ (ICN Pharmaceuticals, Irvine, CA).

Statistical Analysis

The results of studies of uptake through the fast and slow channel and of total uptake were analyzed using a randomized block analysis of variance (ANOVA). A randomized block ANOVA was performed for each drug and was used to determine if the blocks (different days the experiments were done on) were significantly different (Steel and Torrie, 1980). If the blocks were not significantly different, a completely random ANOVA was used (Steel and Torrie, 1980). In either case if the effects of AB treatment were significant, Dunnett's t-test (Steel and Torrie, 1980) was used to determine which concentrations caused significantly different effects compared to drug-free control. The results of the calcium reversal studies were analyzed using a blocked factorial ANOVA, followed by the least significant difference test (lsd) if significant differences were detected (Steel and Torrie, 1980). The criterion for significance was $p \leq 0.05$ for all studies.

RESULTS

Uptake of Ca^{2+} into Synaptosomes

Initial experiments were designed to test the effects of altering several different parameters of this system (under drug-free conditions) on calcium uptake, in order to assess the viability of the synaptosomal preparation.

The dependence of calcium uptake on the duration of potassium-induced depolarization was determined for periods of depolarization between 1 and 60 sec. The results do not extrapolate back to time zero, a result consistent with that of Nachshen and Blaustein (1980). This has been taken as evidence of multiple phases of uptake, a hypothesis which was substantiated in more elaborate studies using rapid quench, automated mixing techniques (Nachshen, 1985a; Suszkiw et al, 1986). As shown in figure 4, two apparent phases of uptake can be seen, a fast phase which appears to end by 2 sec followed by a slower phase which does not reach a plateau for at least 60 sec. This compares favorably with the results of others (Nachshen and Blaustein, 1980; Nachshen, 1985a), in which 1 and 10 sec incubations were used to study the fast and slow channels respectively.

The dependence of total calcium uptake on protein

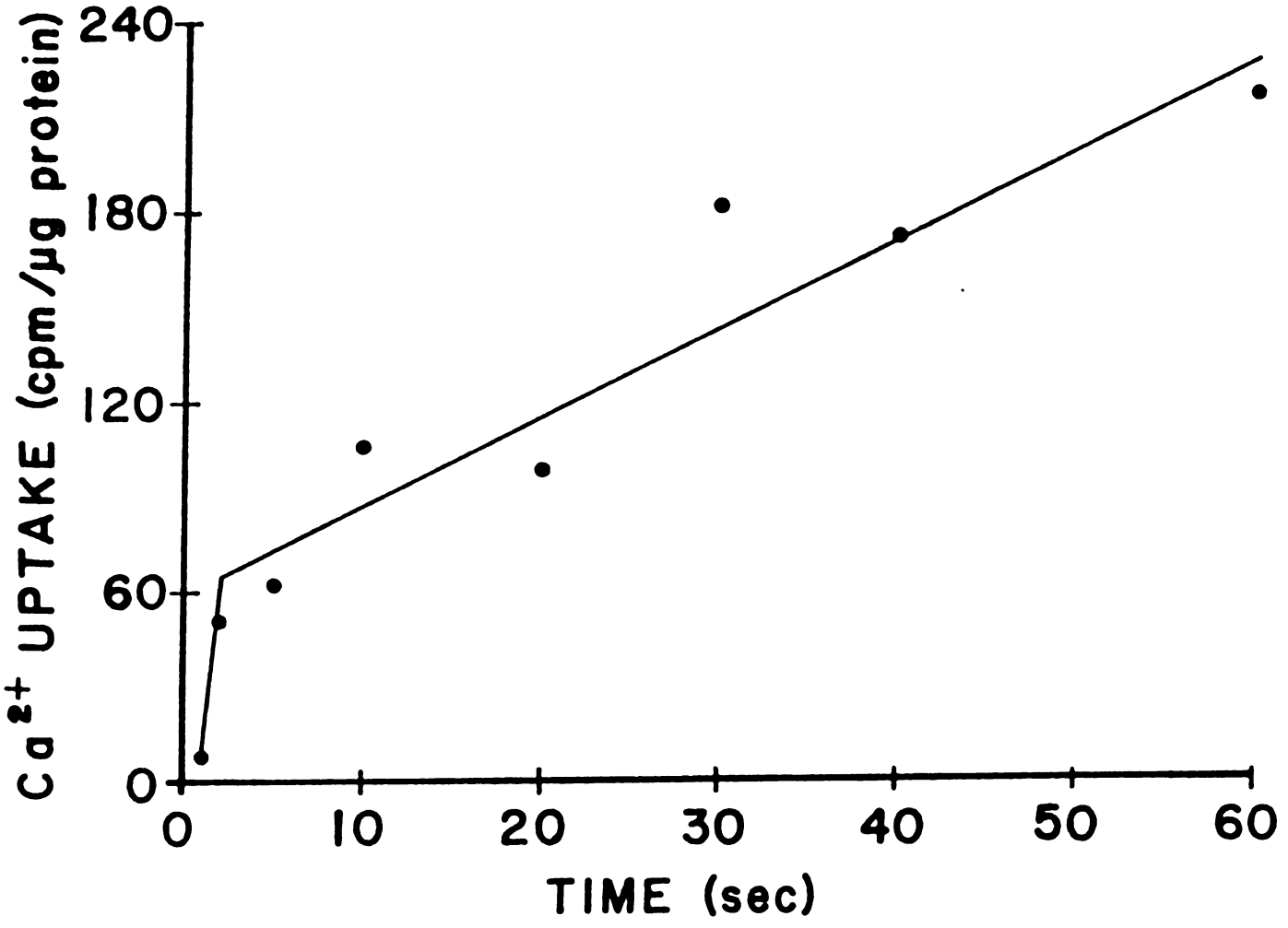

Figure 4. Dependence of Ca^{2+} uptake by depolarized synaptosomes on duration of depolarization. Synaptosomes were depolarized with a high potassium solution (77.5 mM) for incubation times ranging from 1 to 60 sec. Uptake of Ca^{2+} in the presence of low potassium (5 mM) was subtracted from Ca^{2+} uptake in the presence of high potassium to give net stimulated Ca^{2+} uptake. The values are from a single experiment done in triplicate.

concentration was also determined. Calcium uptake was measured using incubation mixtures that contained different amounts of synaptosomal protein. The concentration of protein ranged from approximately 100 - 500 ug. In order to be able to compare results from different synaptosomal preparations the data must be expressed per ug of synaptosomal protein. This is only possible if a linear relationship exists between the concentration of protein in the incubation mixture and calcium uptake. As shown in figure 5 the relationship between protein concentration and Ca^{2+} uptake was linear over the range tested. During subsequent experiments with ABs the concentration of synaptosomes used was maintained within this range.

Effects of increasing the $[Ca^{2+}]_o$ on depolarization-dependent Ca^{2+} uptake were determined (figure 6). The $[Ca^{2+}]_o$ was changed in a manner that kept the amount of $^{45}Ca^{2+}$ proportional to the total $[Ca^{2+}]_o$. The $[Ca^{2+}]_o$ ranged from 0.02 to 1.68 mM. Ca^{2+} uptake increased rapidly as the $[Ca^{2+}]_o$ was increased up to 0.42 mM; at this concentration the preparation began to exhibit saturation of depolarization-evoked uptake. In a viable preparation, Ca^{2+} uptake should increase initially due to the increased concentration driving force, however uptake should eventually saturate. The presence of saturation suggests the membrane is not leaky to calcium. Based on these results, subsequent experiments using ABs were performed

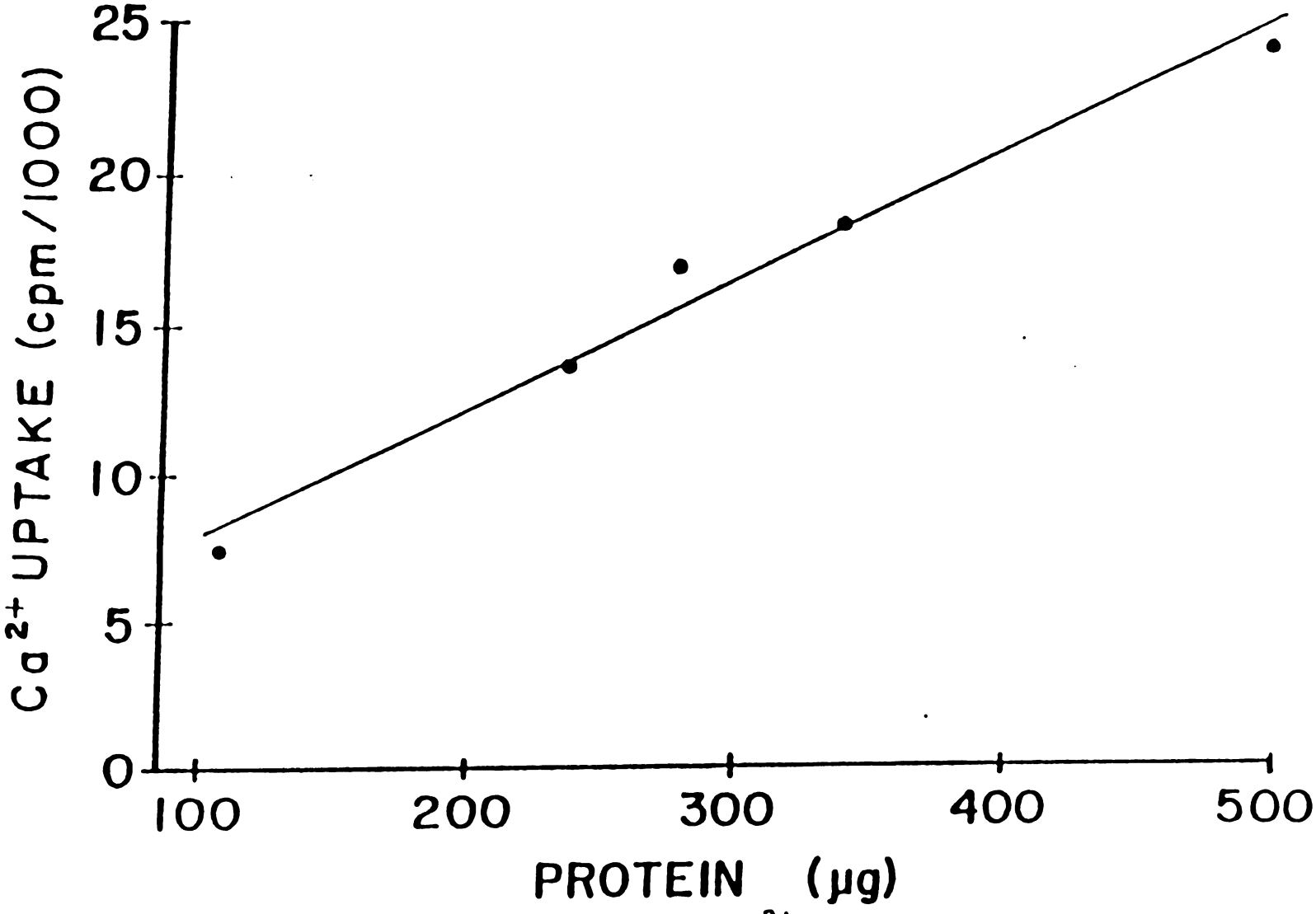

Figure 5. Potassium-stimulated (77.5 mM) Ca^{2+} uptake by different concentrations of synaptosomes (protein). Uptake of Ca^{2+} in the presence of low potassium (5 mM) was subtracted from Ca^{2+} uptake in the presence of high potassium to give net stimulated Ca^{2+} uptake. Shown are the results of a single experiment done in triplicate, with an incubation time of 10 sec.

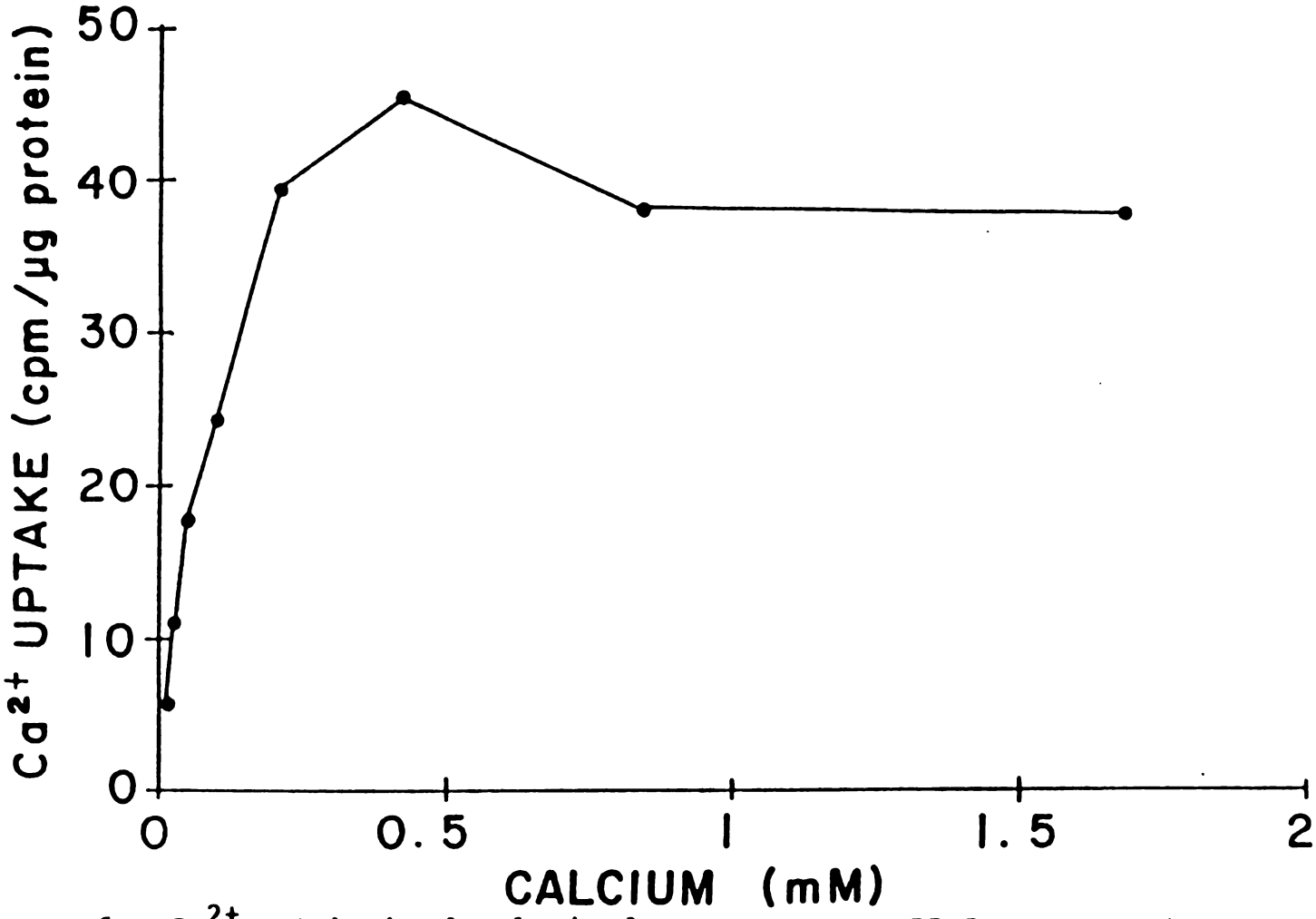

Figure 6. Ca^{2+} uptake by depolarized synaptosomes (77.5 mM potassium solution) as a function of $[Ca^{2+}]_o$. The different incubation mixtures contain the same ratio of ^{45}Ca to Ca, but at varying concentrations. Uptake of Ca^{2+} in the presence of low potassium (5 mM) was subtracted from Ca^{2+} uptake in the presence of high potassium to give net stimulated Ca^{2+} uptake. The values are from a single experiment done in triplicate, with an incubation time of 10 sec.

with a $[Ca^{2+}]_o$ that was equal to 0.05 mM. This concentration was in the rapidly-rising portion of the Ca^{2+} uptake curve.

The effect of lead was determined as a test of our ability to antagonize potassium-stimulated Ca^{2+} uptake. Lead is known to inhibit calcium uptake into synaptosomes (Nachshen, 1984; Suszkiw et al, 1984), and to block transmission at the vertebrate neuromuscular junction (Manalis and Cooper 1973; Atchison and Narahashi, 1984). Total Ca^{2+} uptake was not altered by lead at concentrations ranging from 0.1 - 10.0 uM. At a concentration of 100 uM lead caused a statistically significant decrease in total calcium uptake (figure 7). This decrease was at a higher concentration than that reported by other investigators (Suszkiw et al, 1984). Concentrations of 20 - 100 uM lead have been shown to cause a decrease in nerve-evoked release of ACh at the neuromuscular junction (Atchison and Narahashi, 1984), an effect that can be overcome by increasing extracellular calcium.

Based on the above results it appeared that the synaptosomal preparations were viable, were capable of taking up Ca^{2+} during depolarization and of having this effect blocked by a putative Ca^{2+} channel blocker that also caused neuromuscular block. Subsequent experiments were designed to test the effects of ABs on depolarization-dependent and -independent Ca^{2+} uptake.

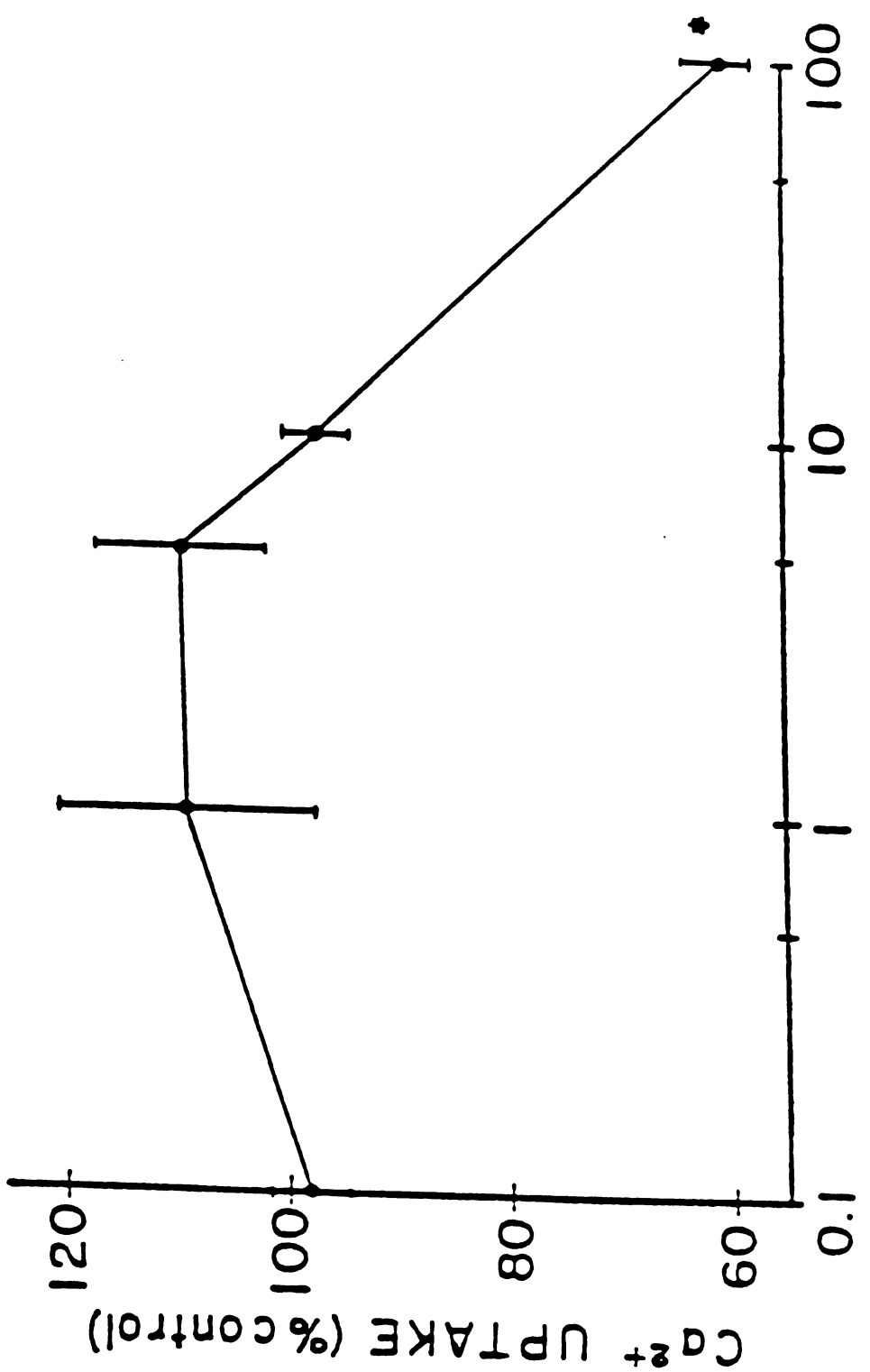

Figure 7. Ca^{2+} uptake by potassium-depolarized (77.5 mM) synaptosomes in the presence of various concentrations of lead. This graph shows the results of 3 experiments (each done in triplicate), expressed as percent of drug-free control (± standard error of the mean). The incubation time was 10 sec. An asterisk (*) indicates the value is significantly different from that of lead-free control.

Total Ca^{2+} Uptake

The first goal was to determine if the ABs had any effect on total uptake of Ca^{2+}. In subsequent experiments the two phases of Ca^{2+} uptake were examined separately to determine if ABs blocked one or the other phase preferentially. It should be noted that it is possible for ABs to alter Ca^{2+} uptake via the fast channel even in the absence of an apparent effect on total uptake.

Neither neomycin (n = 7), nor clindamycin (n = 9) had any effect on total uptake of Ca^{2+} over the entire concentration ranged tested (1 - 1000 uM). As shown in figure 8, neomycin may cause slight deviations from control but does not cause a statistically significant alteration of depolarization-evoked uptake. As shown in figure 9, clindamycin does not cause any significant deviations from its drug-free control value.

Oxytetracycline and polymyxin B each decreased total Ca^{2+} uptake. The results of seven experiments with oxytetracycline and eight experiments with polymyxin are shown in figures 10 and 11 respectively, and are expressed as percent of drug-free control. Oxytetracycline at concentrations of 1 - 100 uM caused a slight increase in Ca^{2+} uptake, but this effect was not statistically significant. The concentration of 500 uM caused a slight decrease in Ca^{2+} uptake, but again this effect was not statistically significant. At the highest concentration

Figure 8. Total Ca^{2+} uptake (10 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of neomycin (1 - 1000 uM). This graph shows the results of seven experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 74.14 $\pm$ 6.77 fmoles Ca^{2+} uptake/ug synaptosomal protein.

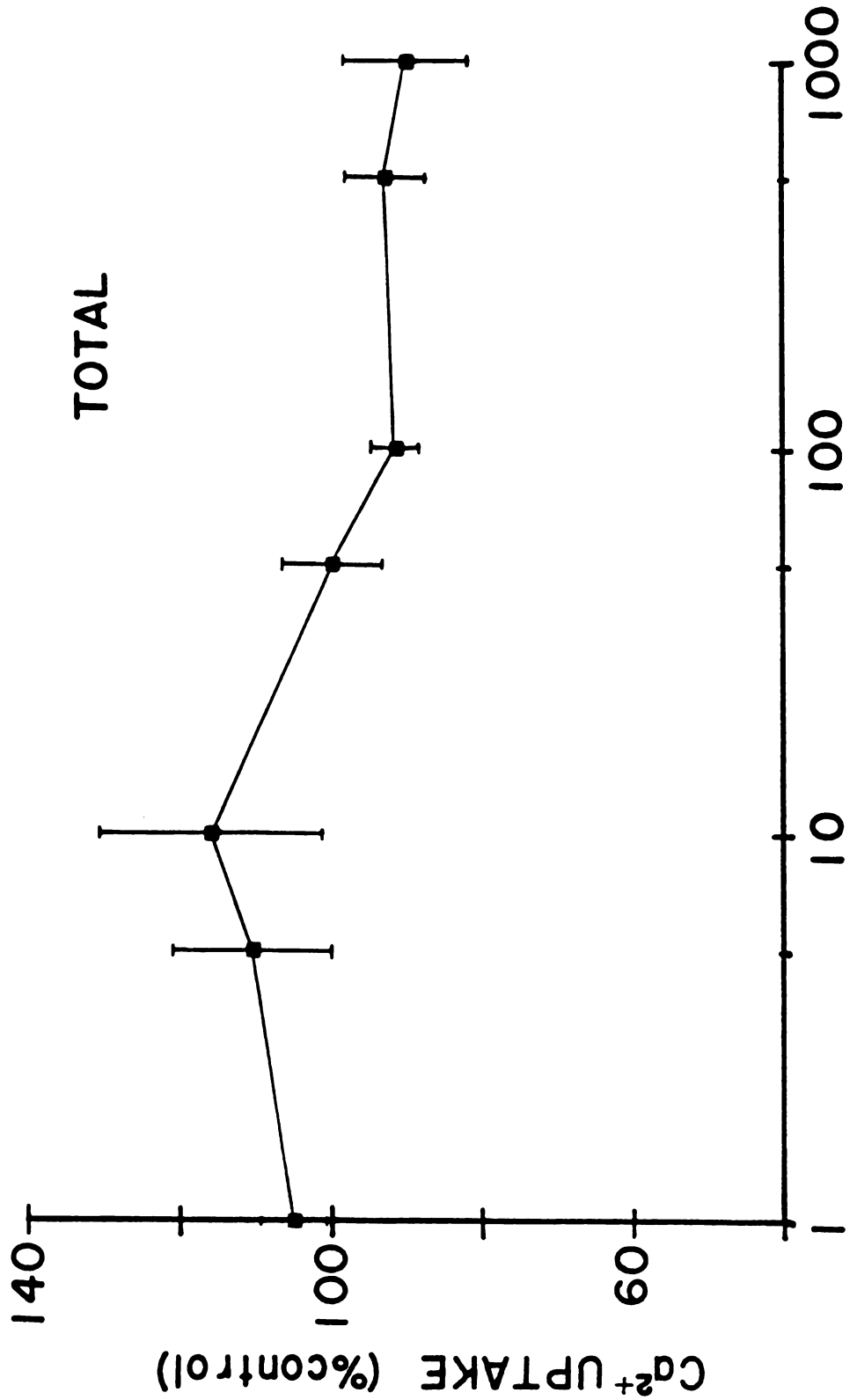

Figure 8

Figure 9. Total Ca^{2+} uptake (10 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of clindamycin (1 - 1000 uM). This graph shows the results of nine experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 194.78 $\pm$ 50.55 fmoles Ca^{2+} uptake/ug synaptosomal protein.

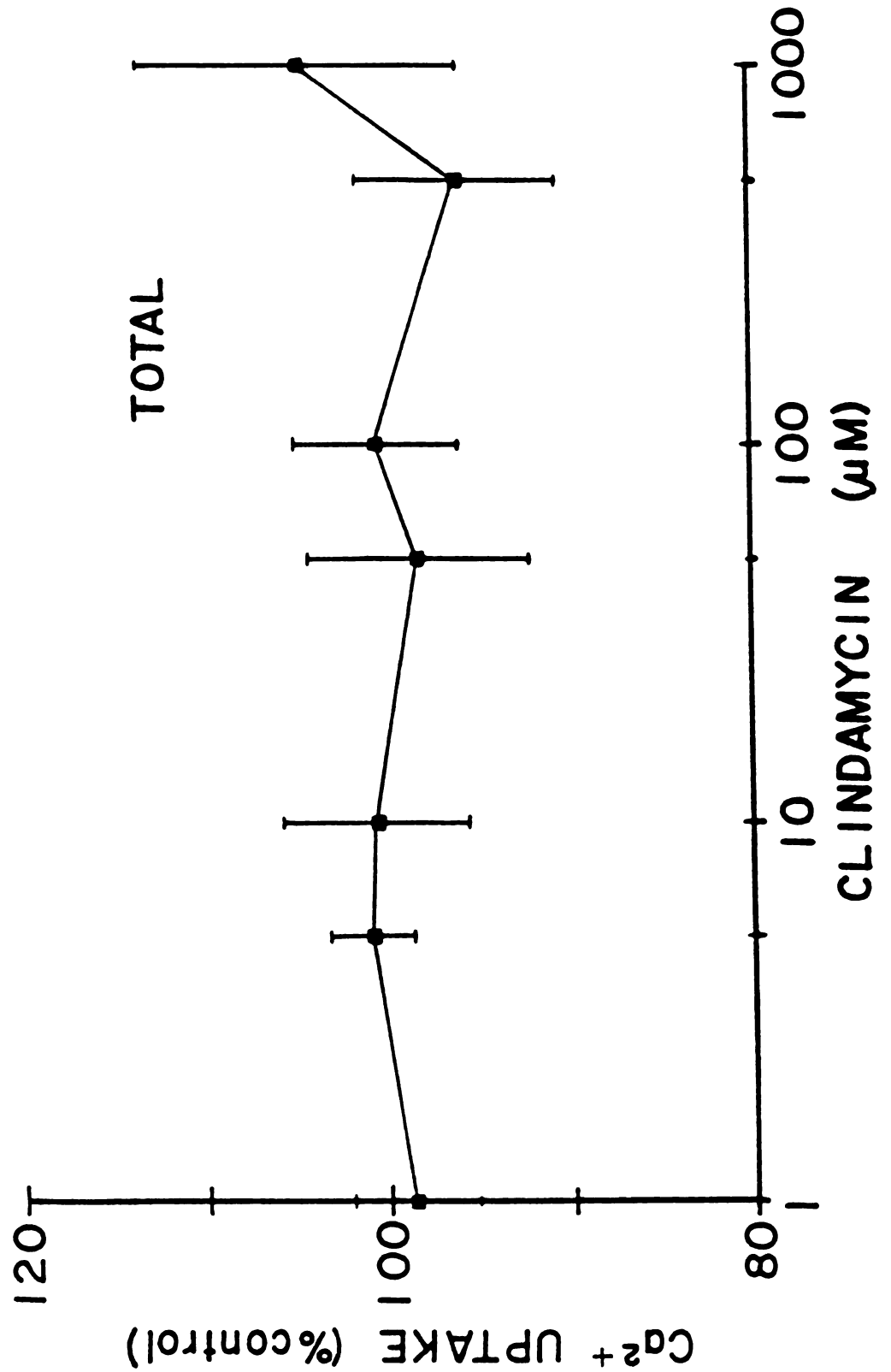
TOTAL
120
100
80
Ca^{2+} UPTAKE (%control)
1
10
100
1000
CLINDAMYCIN (μM)

Figure 9

Figure 10. Total Ca^{2+} uptake (10 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of oxytetracycline (1 - 1000 uM). This graph shows the results of seven experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 35.43 $\pm$ 8.72 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

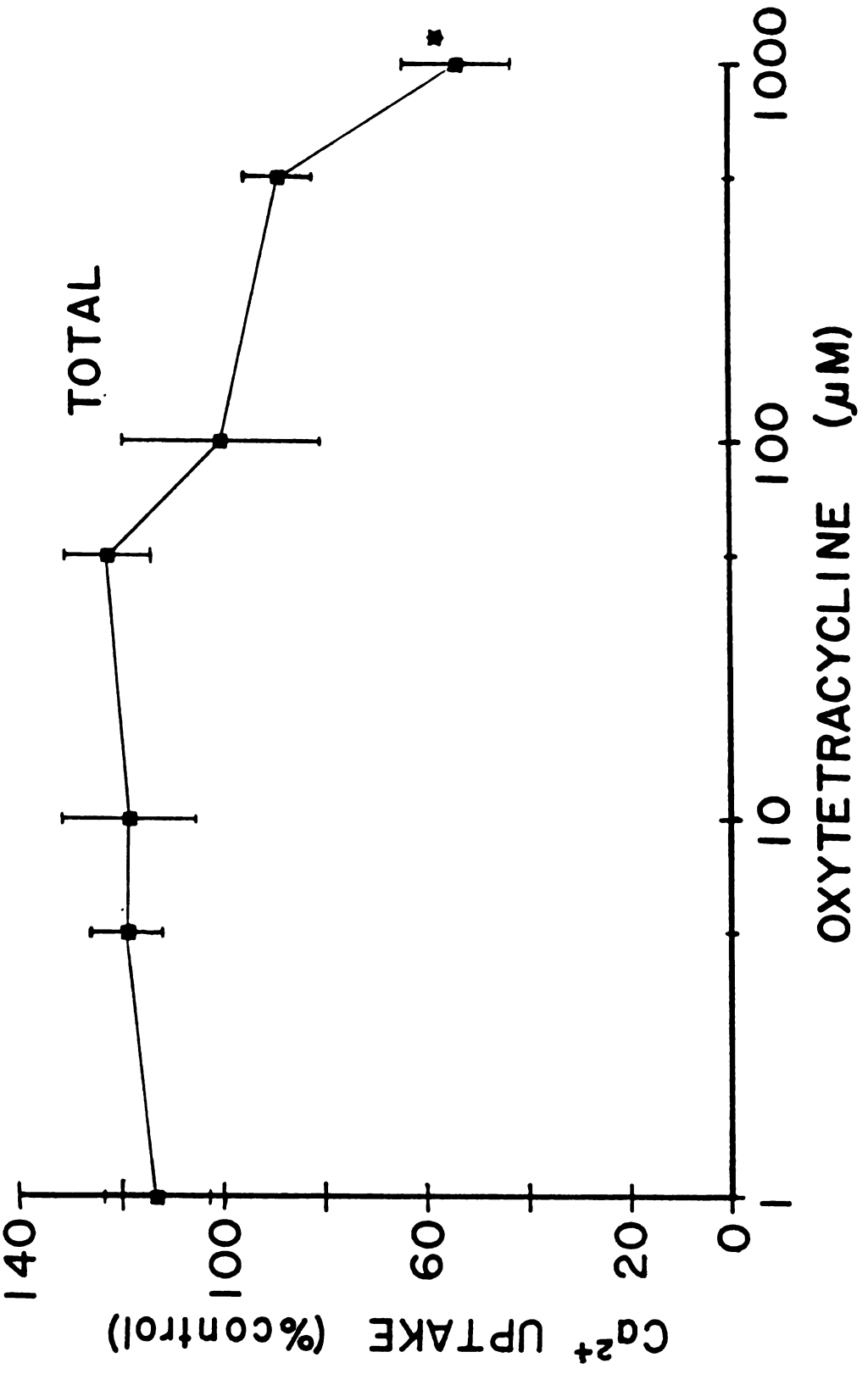
TOTAL
Ca^{2+} UPTAKE (%control)
140
100
60
20
0
1
10
100
1000
OXYTETRACYCLINE (μM)
*

Figure 10

Figure 11. Total Ca^{2+} uptake (10 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of polymyxin (1 - 1000 uM). This graph shows the results of eight experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 57.89 $\pm$ 3.87 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

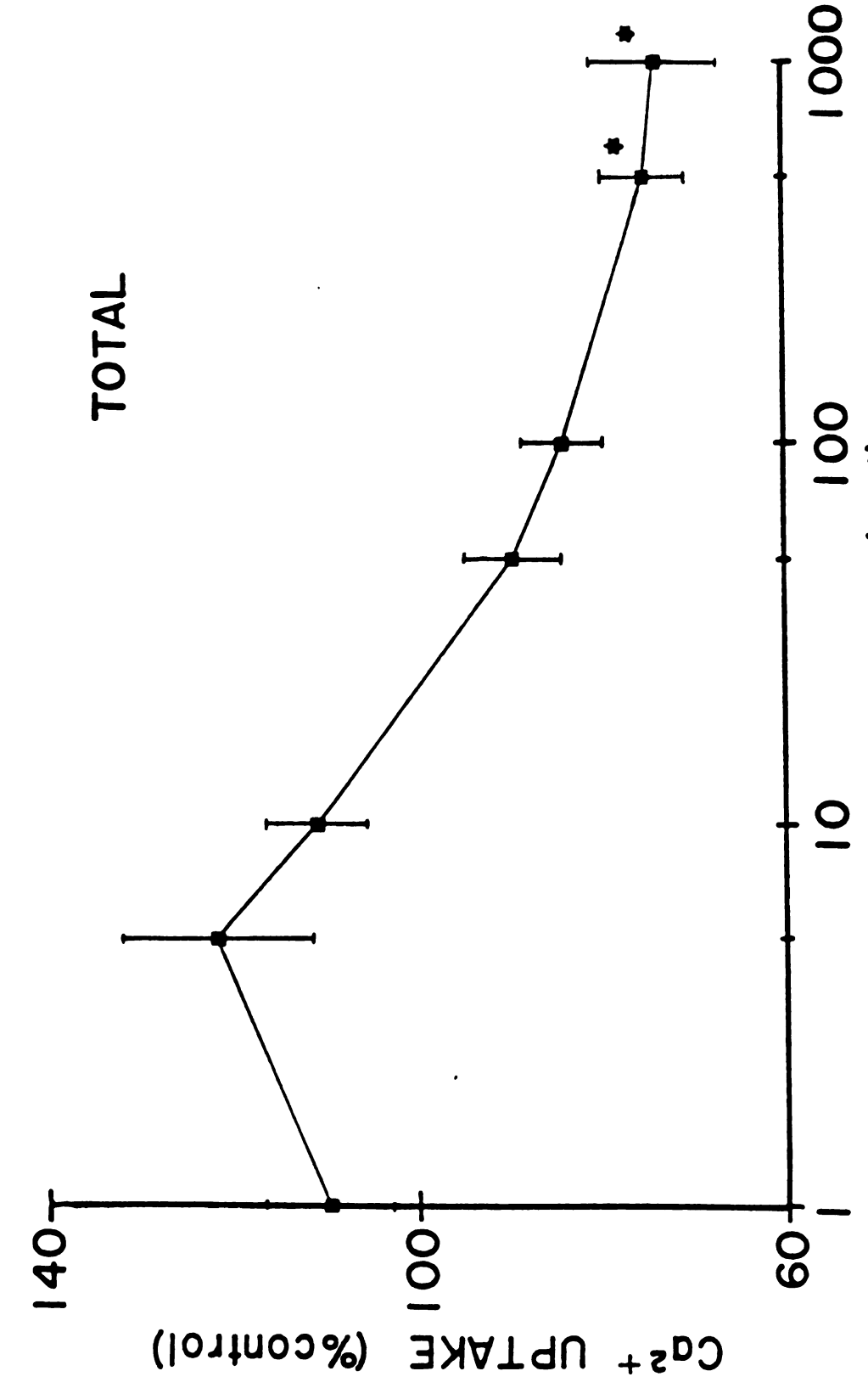
TOTAL
140
100
60
1
10
100
1000
POLYMYXIN (μM)
Ca2+ UPTAKE (%control)

Figure 11

(1000 uM) oxytetracycline reduced Ca^{2+} uptake to 63% of control. Similarly, polymyxin B at low concentrations (1 - 10 uM) caused a small, but statistically insignificant increase in Ca^{2+} uptake, and at concentrations of 50 and 100 uM caused a statistically insignificant decrease in Ca^{2+} uptake. At concentrations of 500 and 1000 uM, polymyxin B caused reductions to 75% and 74% of control respectively, in Ca^{2+} uptake. Both of these were significantly less than control.

Fast Phase of Ca^{2+} Uptake

The fast phase of Ca^{2+} uptake occurs via a putative calcium channel (Nachshen and Blaustein, 1980), and is thought to be associated with depolarization-induced release of neurotransmitter (Drapeau and Blaustein, 1983; Suszkiw and O'Leary, 1983; Drapeau and Nachshen, 1984; Leslie et al, 1985; Daniell and Leslie, 1986). Since the fast phase of Ca^{2+} uptake is associated with neurotransmitter release it is the phase that is of most interest to the neuromuscular block caused by ABs.

Neomycin (n = 7) caused a decrease in Ca^{2+} influx via the fast channel (figure 12). Ca^{2+} uptake was decreased by neomycin over the entire concentration range tested (1 - 1000 uM), however only the results at 500 and 1000 uM were significantly lower than control. These concentrations caused reductions to 66% and 54% of control respectively.

Figure 12. Fast phase Ca^{2+} uptake (1 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of neomycin (1 - 1000 uM). This graph shows the results of eight experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 20.57 $\pm$ 1.90 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

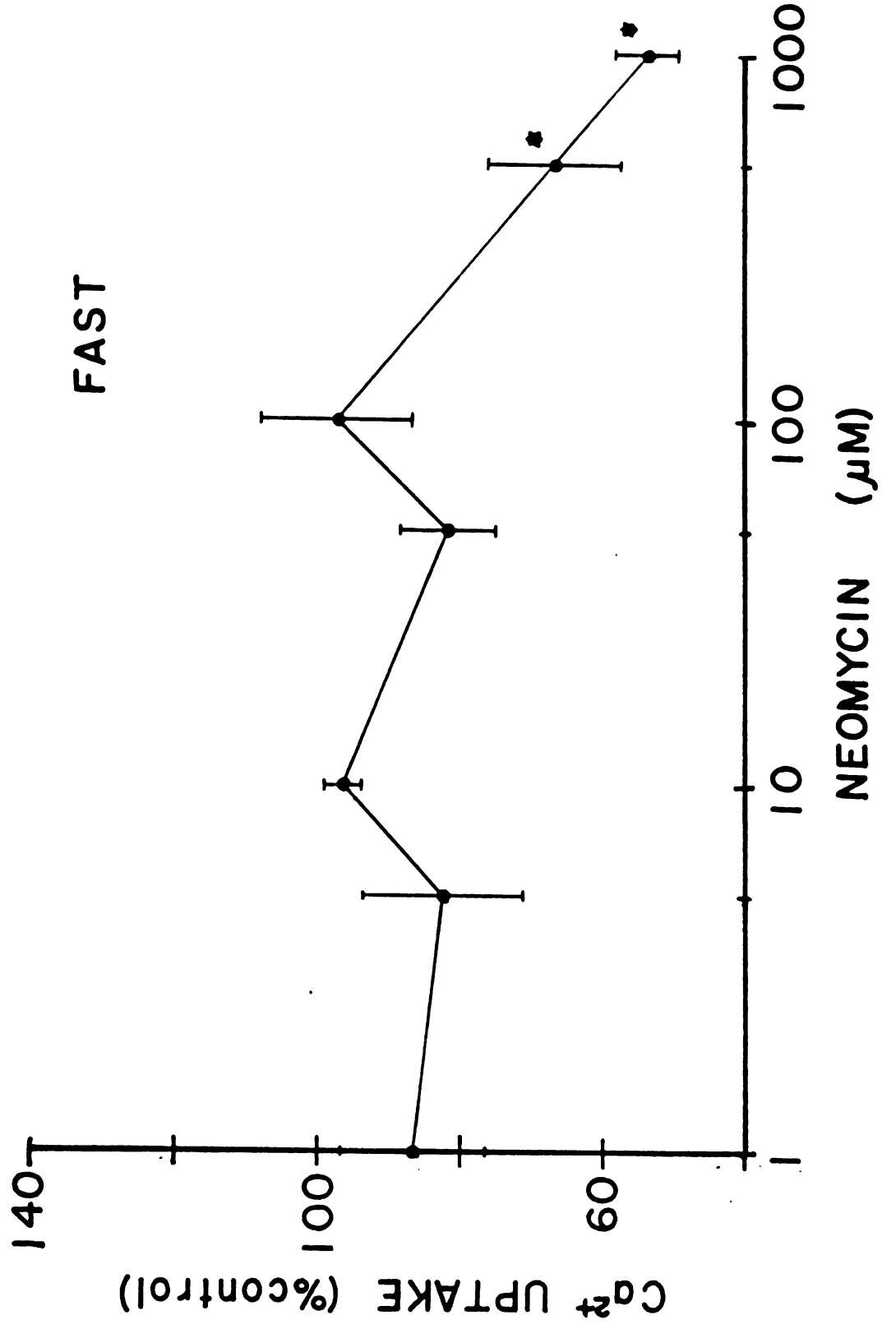

Figure 12

Figure 13. Fast phase Ca^{2+} uptake (1 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of oxytetracycline (1 - 1000 uM). This graph shows the results of seven experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 13.86 $\pm$ 2.90 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

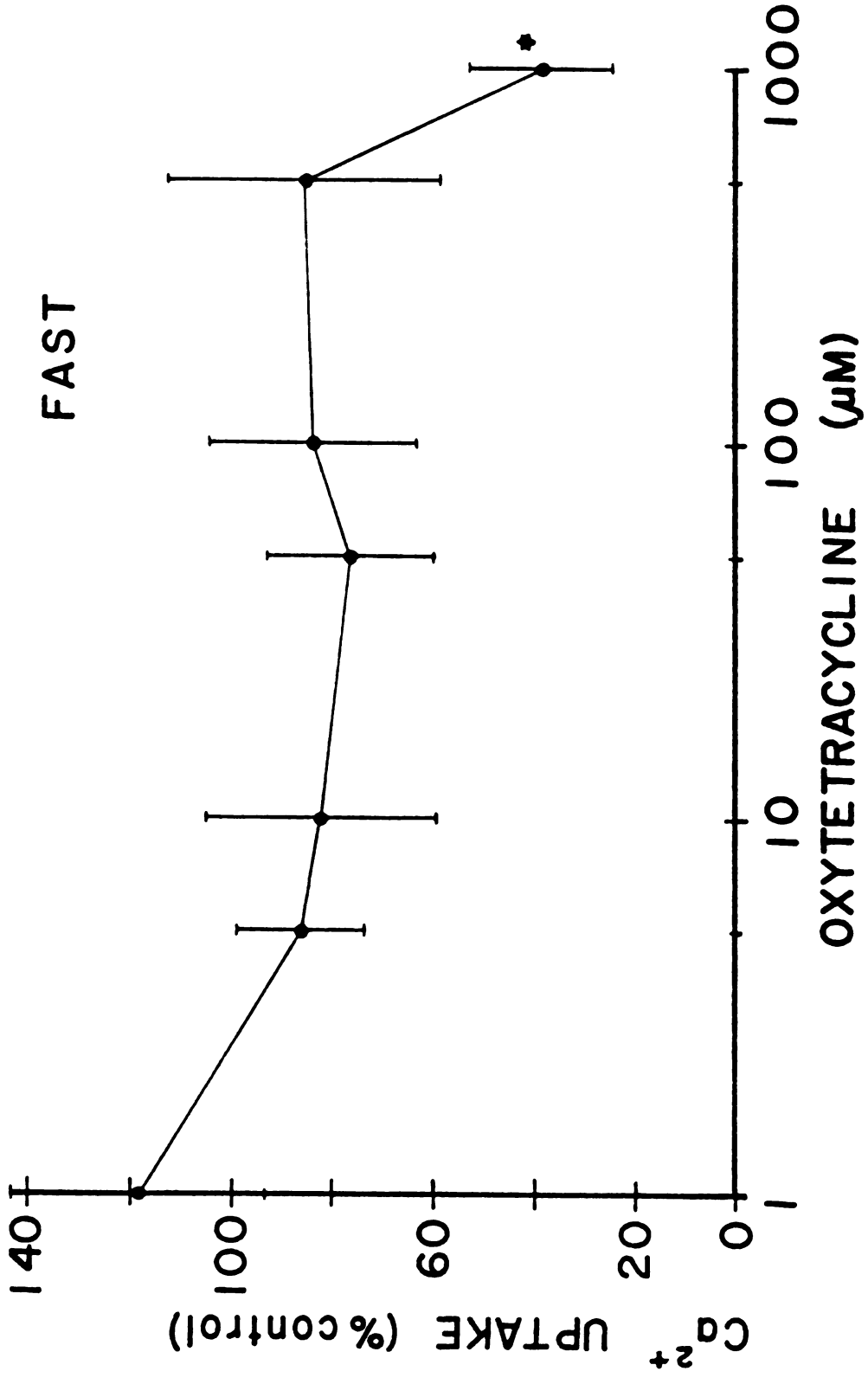
FAST
Ca^{2+} UPTAKE (% control)
140
100
60
20
0
1
10
100
1000
OXYTETRACYCLINE (μM)
*

Figure 13

Figure 13. Fast phase Ca^{2+} uptake (1 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of oxytetracycline (1 - 1000 uM). This graph shows the results of seven experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 13.86 $\pm$ 2.90 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

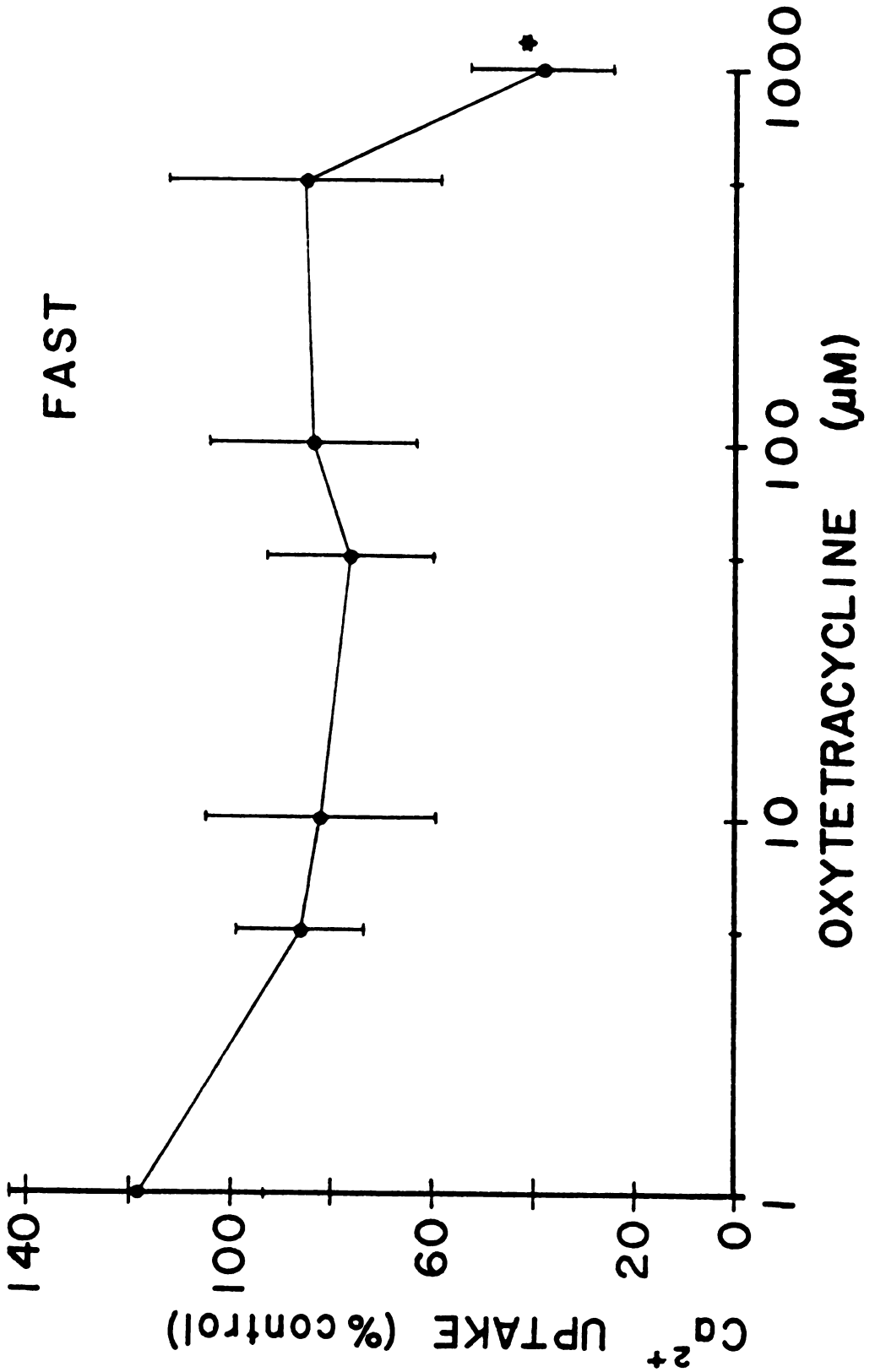
FAST
140
100
60
20
0
1
10
100
1000
OXYTETRACYCLINE (μM)
Ca^{2+} UPTAKE (% control)
*

Figure 13

Figure 14. Fast phase Ca^{2+} uptake (1 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of polymyxin (1 - 1000 uM). This graph shows the results of eight experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 9.56 $\pm$ 1.06 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

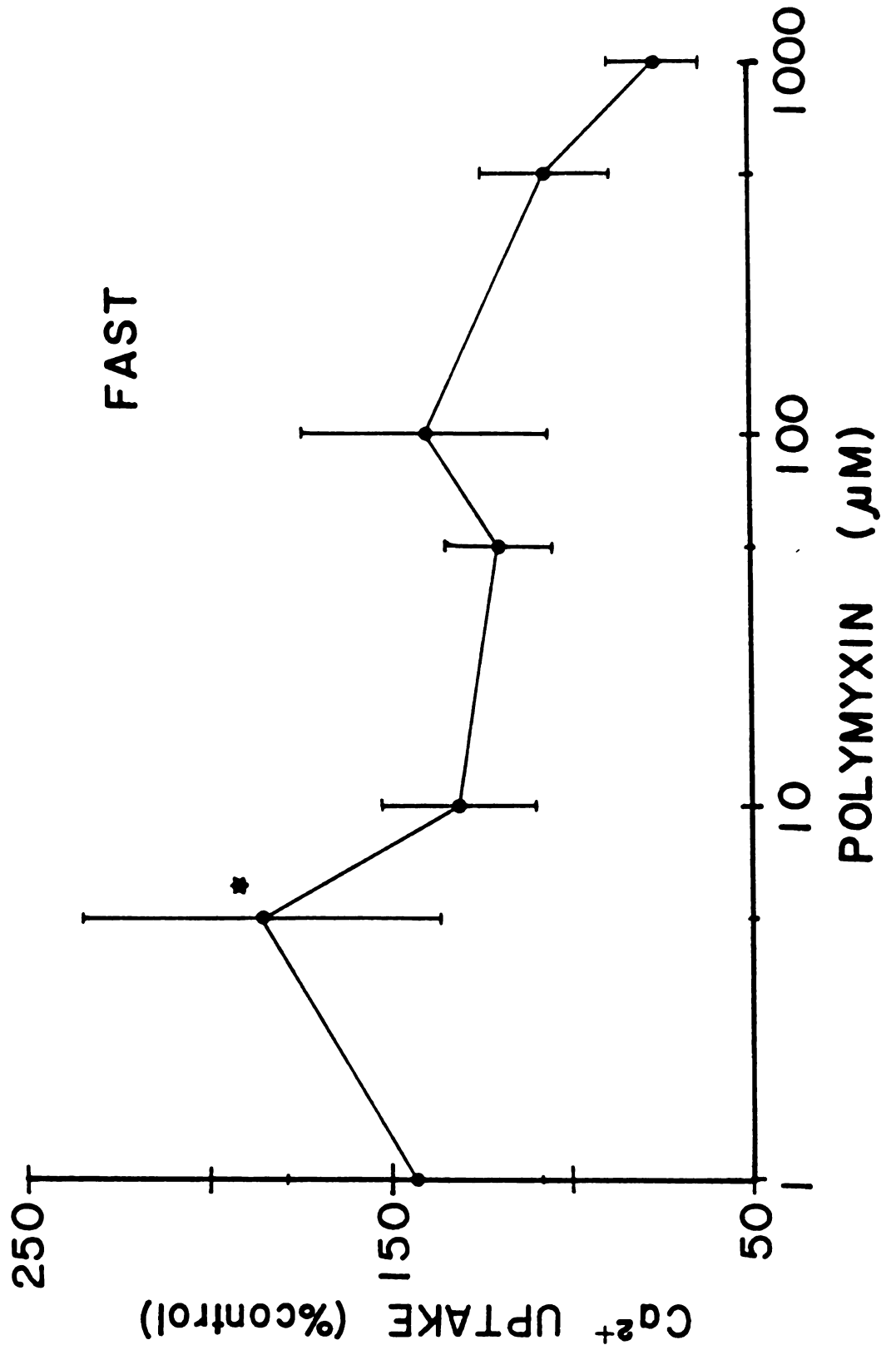

Figure 14

Figure 15. Fast phase Ca^{2+} uptake (1 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of clindamycin (1 - 1000 uM). This graph shows the results of nine experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 22.33 $\pm$ 8.69 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

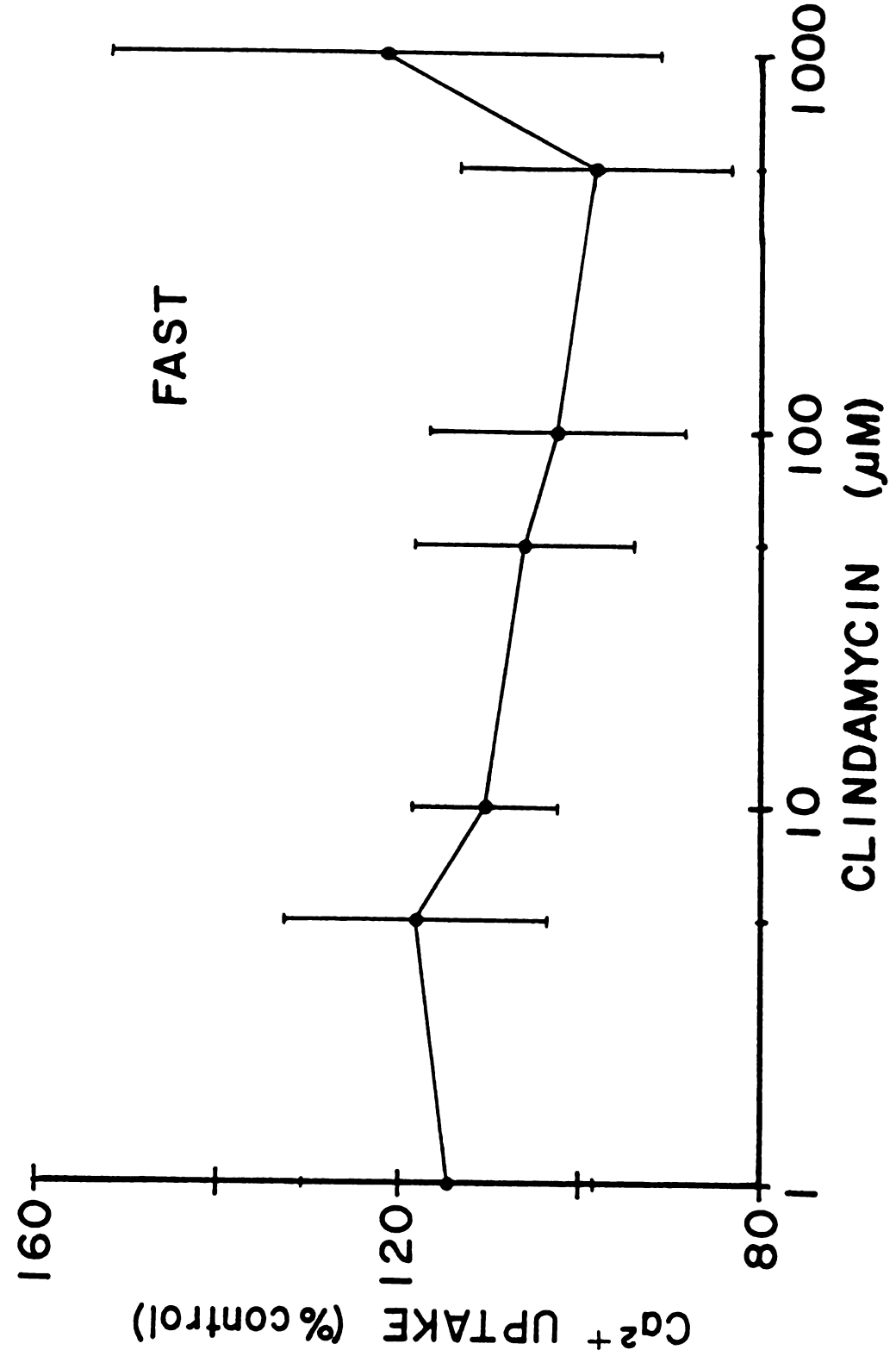
FAST
160
120
80
1
10
100
1000
CLINDAMYCIN (μM)
Ca2+ UPTAKE (% control)

Figure 15

Oxytetracycline (n = 7) caused a statistically significant decrease in Ca^{2+} uptake, to 35% of control, at a concentration of 1000 uM. The other concentrations ranging from 5 - 500 uM caused slight, though statistically insignificant decreases. The results of these experiments are shown in figure 13.

At concentrations ranging from 1 to 500 uM, polymyxin (n = 8) caused an increase in Ca^{2+} uptake (figure 14). However, only the results at the 5 uM concentration were significantly different from drug-free control. At 5 uM polymyxin caused an 85% increase in Ca^{2+} uptake via the fast phase. Polymyxin did not decrease fast phase Ca^{2+} uptake at any concentration tested.

Similarly, clindamycin (n = 9) does not decrease calcium uptake (figure 15). Rather, clindamycin causes a very small, yet non-significant, increase in Ca^{2+} uptake over the entire concentration range tested (1 - 1000 uM).

Slow Phase of Ca^{2+} Uptake

Neomycin (n = 6) does not cause a significant decrease in slow phase Ca^{2+} uptake. However, as depicted in figure 16, neomycin causes a slight decrease in slow phase Ca^{2+} uptake at the highest concentration (1000 uM).

Oxytetracycline (n = 6), at concentrations of 500 and 1000 uM, caused a significant decrease, to 70% and 36% of control respectively, in slow phase Ca^{2+} uptake (figure 17).

Figure 16. Slow phase Ca^{2+} uptake (10 sec incubation after predepolarization; K^{+} = 77.5 mM, no calcium) by potassium-depolarized (77.5 mM) synaptosomes in the presence of neomycin (1 - 1000 uM). This graph shows the results of six experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 71.00 $\pm$ 14.81 fmoles Ca^{2+} uptake/ug synaptosomal protein.

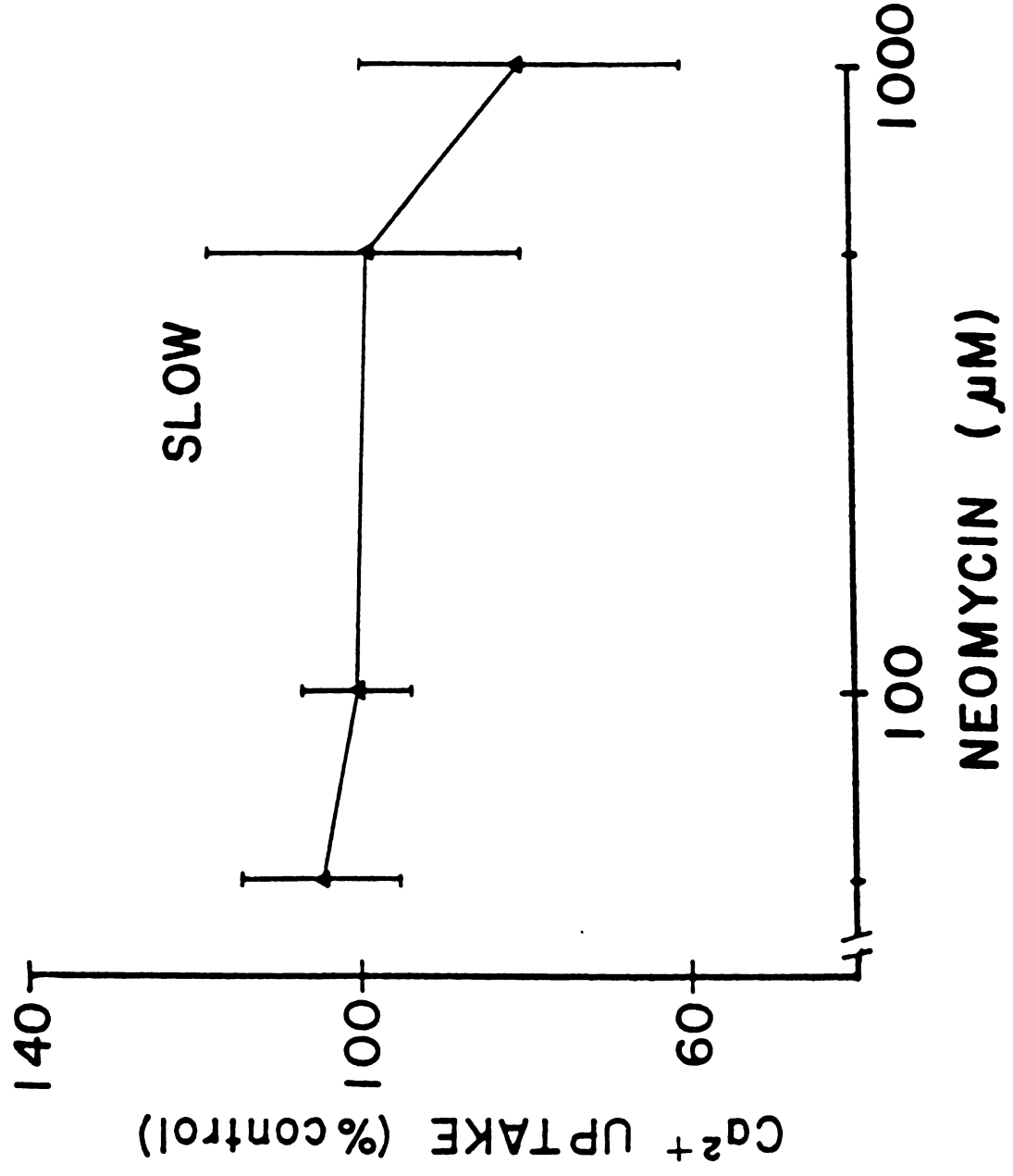

Figure 16

Figure 17. Slow phase Ca^{2+} uptake (10 sec incubation after predepolarization; K^{+} = 77.5 mM, no calcium) by potassium-depolarized (77.5 mM) synaptosomes in the presence of oxytetracycline (1 - 1000 uM). This graph shows the results of six experiments (each done in triplicate) expressed as percent of drug-free control ± SEM. The control equals 96.50 ± 8.39 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

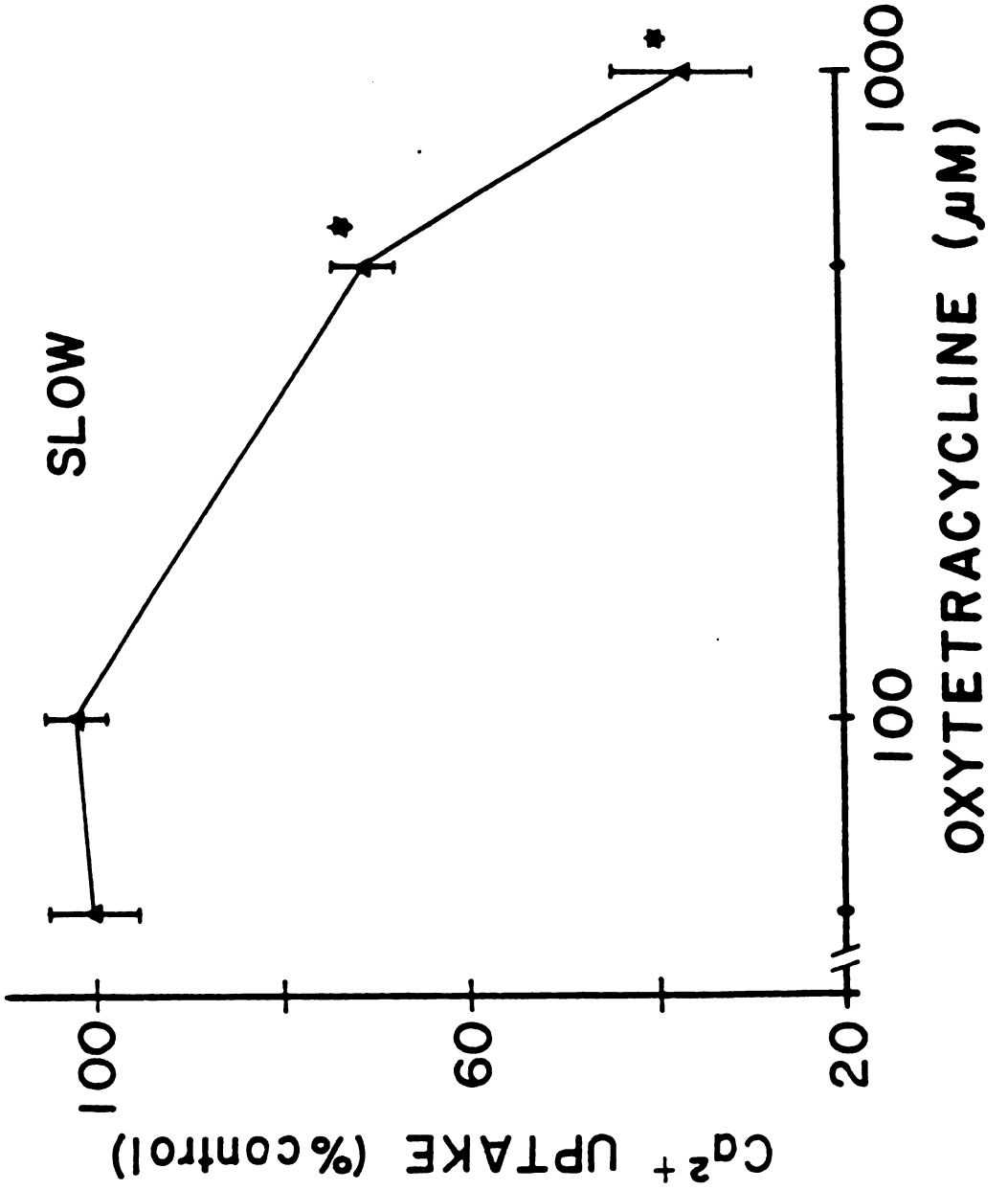
SLOW
100
60
20
100
1000
OXYTETRACYCLINE (μM)
Ca2+ UPTAKE (%control)

Figure 17

Figure 18. Slow phase Ca^{2+} uptake (10 sec incubation after predepolarization; K^{+} = 77.5 mM, no calcium) by potassium-depolarized (77.5 mM) synaptosomes in the presence of polymyxin (1 - 1000 uM). This graph shows the results of four experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 33.75 $\pm$ 1.80 fmoles Ca^{2+} uptake/ug synaptosomal protein. An asterisk (*) indicates the value is significantly different from that of drug-free control.

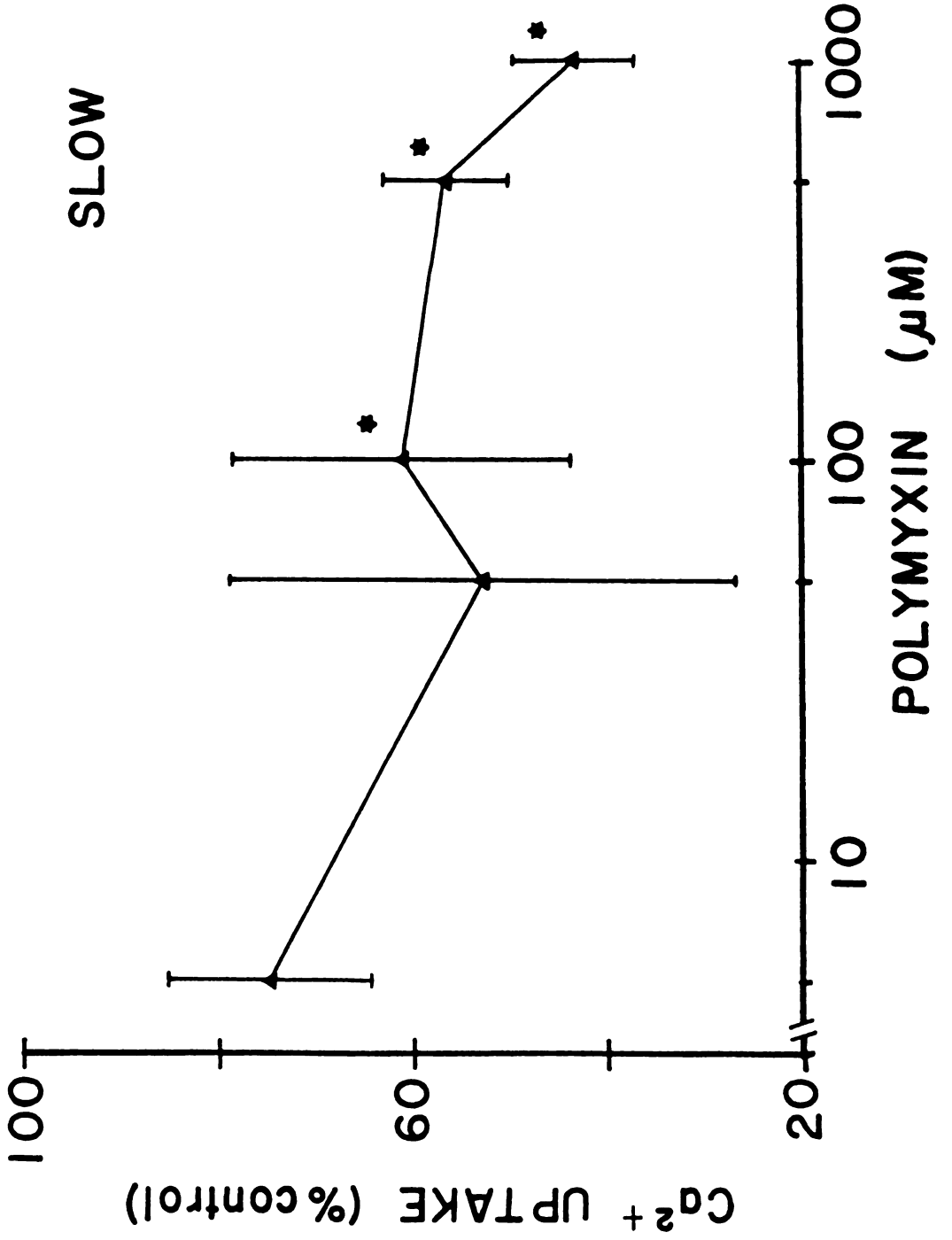

Figure 18

Figure 19. Slow phase Ca^{2+} uptake (10 sec incubation after predepolarization; K^{+} = 77.5 mM, no calcium) by potassium-depolarized (77.5 mM) synaptosomes in the presence of clindamycin (1 - 1000 uM). This graph shows the results of six experiments (each done in triplicate) expressed as percent of drug-free control $\pm$ SEM. The control equals 102.67 $\pm$ 20.61 fmoles Ca^{2+} uptake/ug synaptosomal protein.

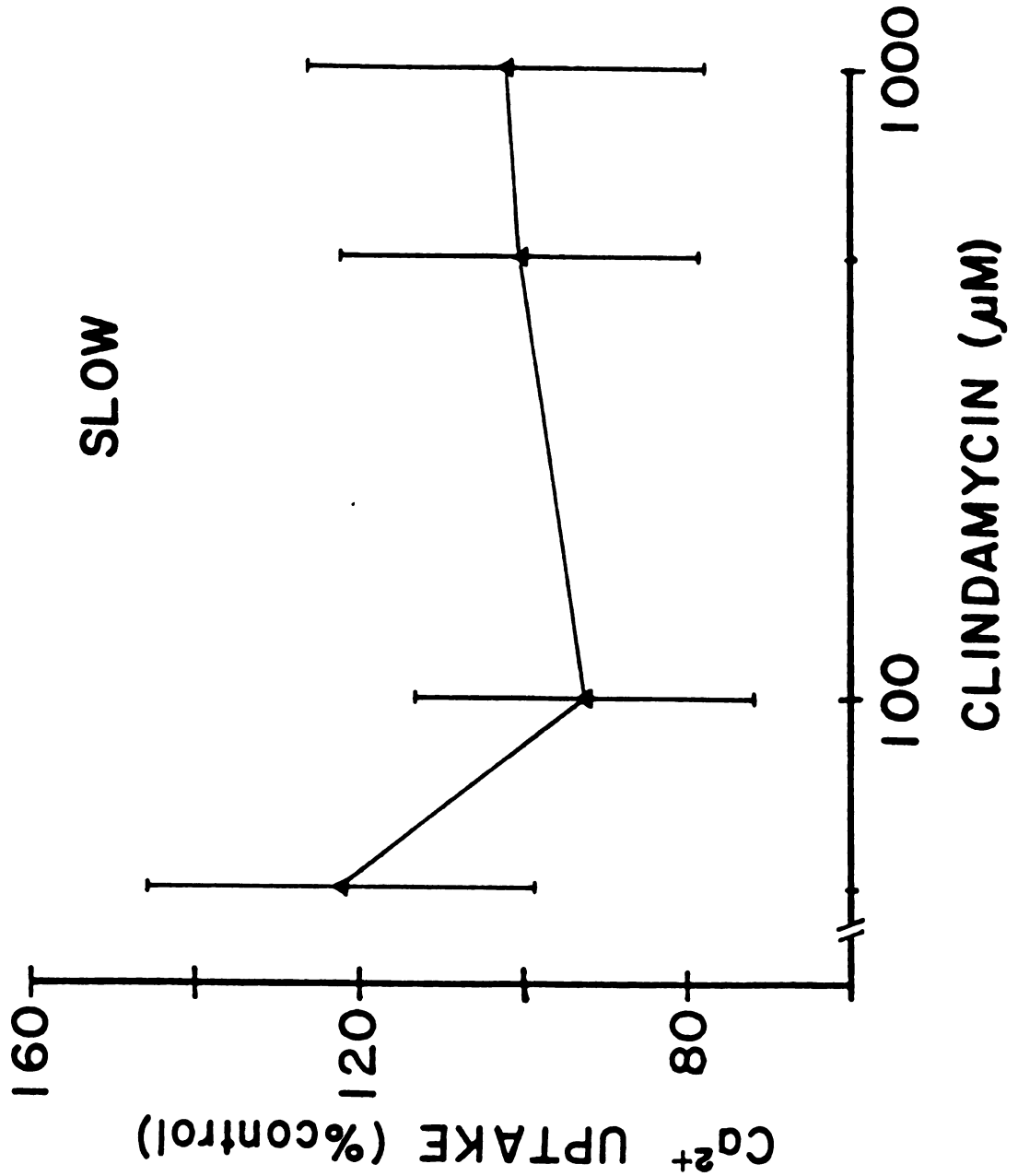

Figure 19

The lower concentrations did not decrease slow phase Ca^{2+} uptake.

Polymyxin B caused a decrease in total Ca^{2+} uptake but not in uptake via the fast channel; this would imply that polymyxin affects the slow phase of Ca^{2+} uptake. Polymyxin did cause a decrease in Ca^{2+} uptake via the slow phase over the entire concentration range tested (5 - 1000 uM). The results of four experiments with polymyxin (figure 18) showed a decrease from drug-free control, but the decrease was statistically significant only for concentrations of 100, 500 and 1000 uM. These concentrations caused reductions to 61%, 57% and 44% of control, respectively.

Clindamycin which had no effect on either total or fast channel Ca^{2+} uptake, also had no effect on the slow phase of calcium uptake (n = 6), as shown in figure 19.

The Effects of Antibiotics on Baseline (Low K^+) Ca^{2+} Uptake

Each of the ABs caused a statistically significant change in Ca^{2+} uptake under baseline conditions. Neomycin and polymyxin (5 uM) caused a statistically significant increase in Ca^{2+} uptake during 1 sec of incubation, under non-depolarized conditions. Neomycin, oxytetracycline and polymyxin, at high concentrations, each caused a statistically significant decrease in depolarization-independent Ca^{2+} uptake during 10 sec of incubation both in the absence and presence of predepolarization. Clindamycin,

at high concentrations, caused a statistically significant decrease in baseline Ca^{2+} uptake following predepolarization. The concentrations of ABs that caused changes in baseline Ca^{2+} uptake do not correspond exactly with the concentrations of ABs that caused changes in net stimulated Ca^{2+} uptake. Although baseline Ca^{2+} uptake was changed, the amount of Ca^{2+} uptake in the absence of stimulation is very small compared to that under K^{+} -stimulated conditions. Figures 20 - 22 depict the difference in baseline Ca^{2+} uptake and net stimulated Ca^{2+} uptake, for total Ca^{2+} uptake, fast and slow phase Ca^{2+} uptake, respectively. The values depicted are for drug-free control and the drug concentrations that cause a statistically significant difference in baseline Ca^{2+} uptake as compared to drug-free control. The baseline amounts of Ca^{2+} uptake is in fact smaller than the standard errors of the means of the net stimulated Ca^{2+} uptake.

Summary -- Concentration Response

Tables 1 and 2 summarize respectively the significant effects of the various ABs on total Ca^{2+} uptake, and Ca^{2+} uptake via the fast and slow phases. Neomycin and clindamycin had no effect on total Ca^{2+} uptake, while oxytetracycline (1000 uM) and polymyxin (500 and 1000 uM) both caused a decrease in total uptake of Ca^{2+}. Neomycin (500 and 1000 uM) and oxytetracycline (1000 uM) caused a

Figure 20. A comparison of baseline (K^+ = 5 mM) Ca^{2+} uptake during a 10 sec incubation (solid bars) and net K^+-stimulated (K^+ = 77.5 mM) total Ca^{2+} uptake (striped bars) into synaptosomes. Depicted are drug-free control and each AB concentration (uM) that caused a statistically significant change in baseline Ca^{2+} uptake. The abreviations stand for: NEO, neomycin; OXY, oxytetracycline; POLY, polymyxin B; CLIN, clindamycin.

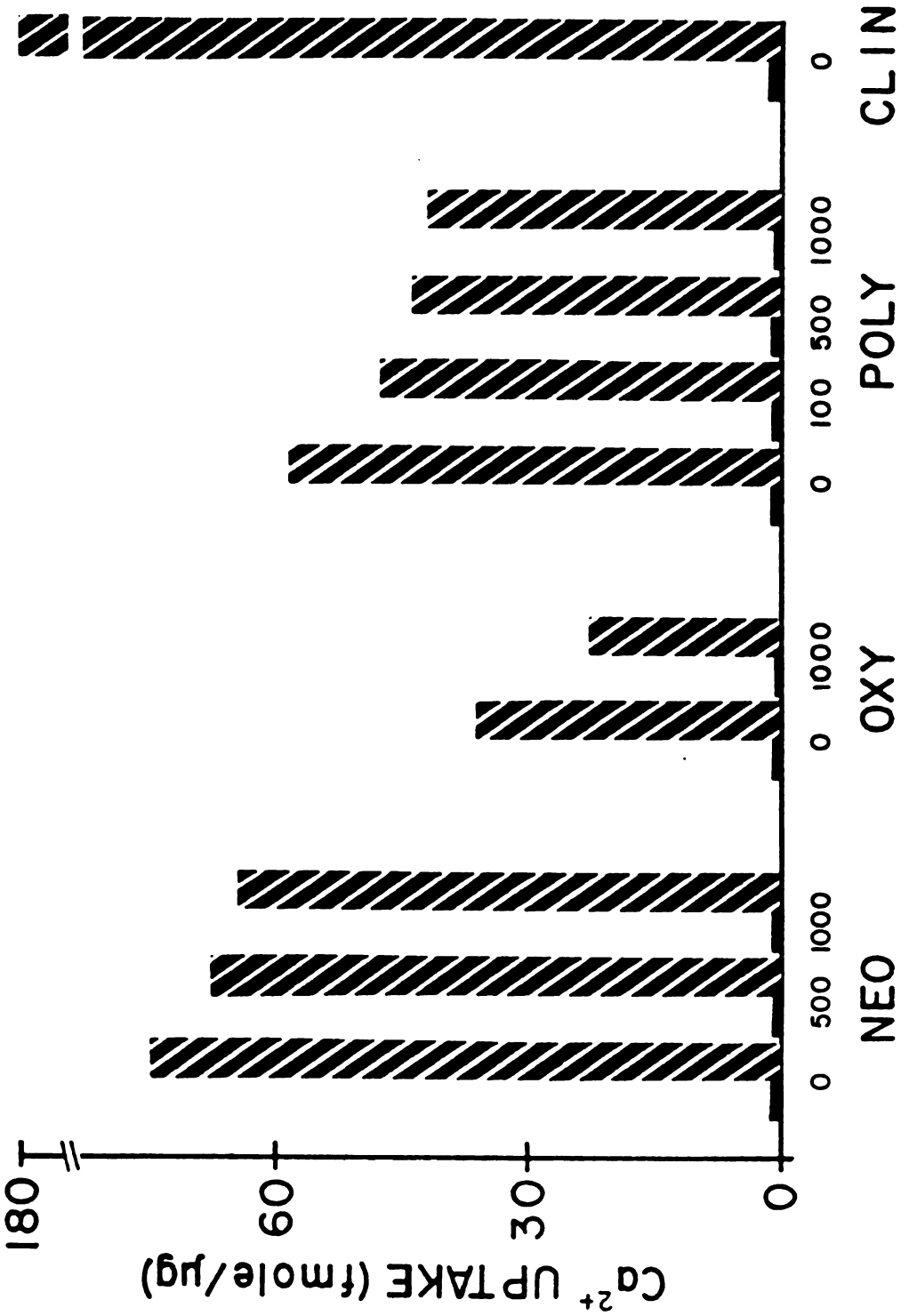
180
60
30
0
Ca^{2+} UPTAKE (fmole/μg)
0
500
1000
NEO
0
1000
OXY
0
100
500
1000
POLY
0
CLIN

Figure 20

Figure 21. A comparison of baseline (K^+ = 5 mM) Ca^{2+} uptake during a 1 sec incubation (solid bars) and net K^+-stimulated (K^+ = 77.5) fast phase Ca^{2+} uptake (striped bars) into synaptosomes. Depicted are drug-free control and each AB concentration (uM) that caused a statistically significant change in baseline Ca^{2+} uptake. The abreviations are the same as in figure 20.

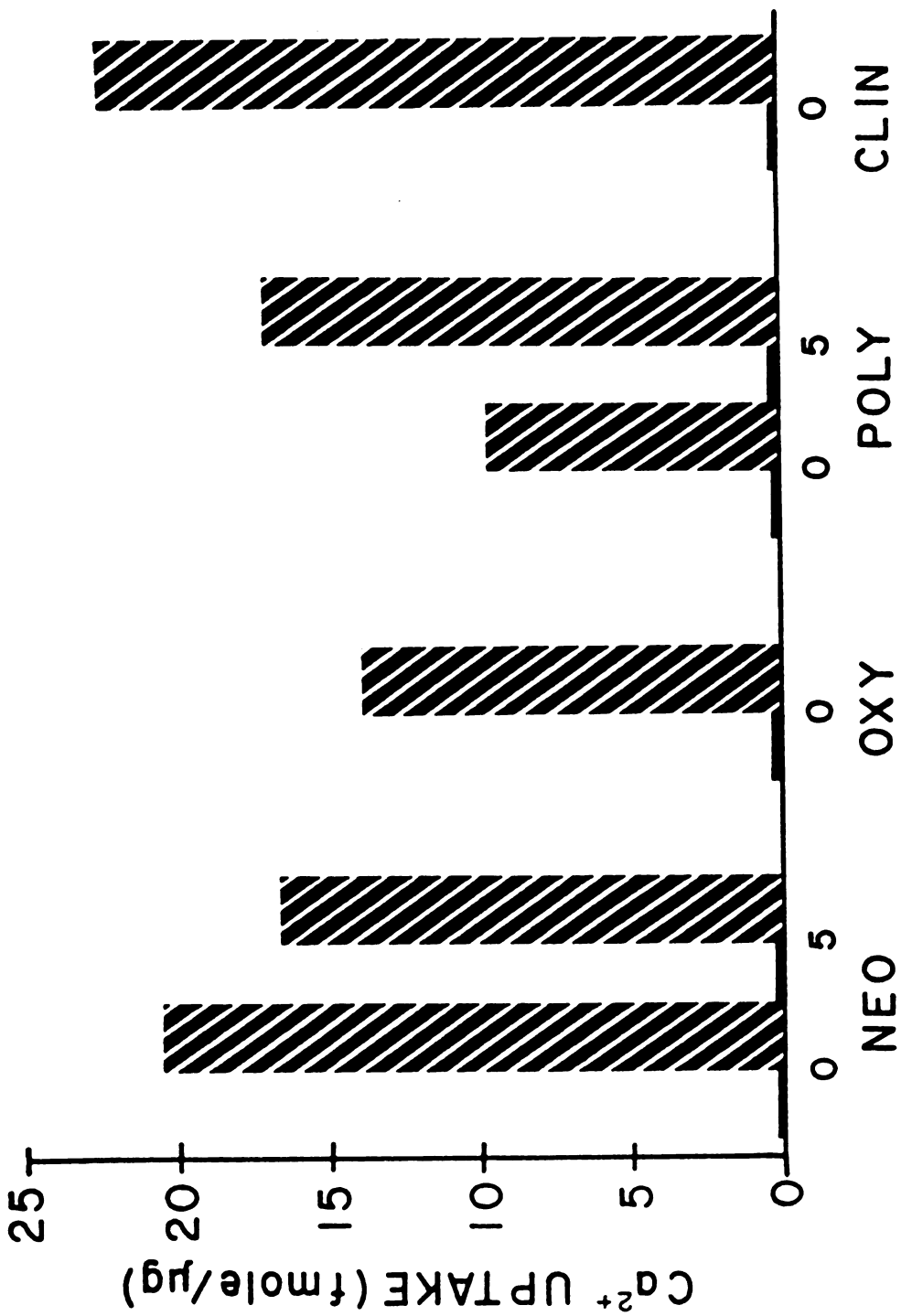

Figure 21

Figure 22. A comparison of baseline (K^+ = 5 mM) Ca^{2+} uptake during a 10 sec incubation following predepolarization (solid bars) and net K^+-stimulated (K^+ = 77.5 mM) slow phase Ca^{2+} uptake (striped bars) into synaptosomes. Depicted are drug-free control and each AB concentration (uM) that caused a statistically significant change in baseline Ca^{2+} uptake. The abreviations are the same as in figure 20.

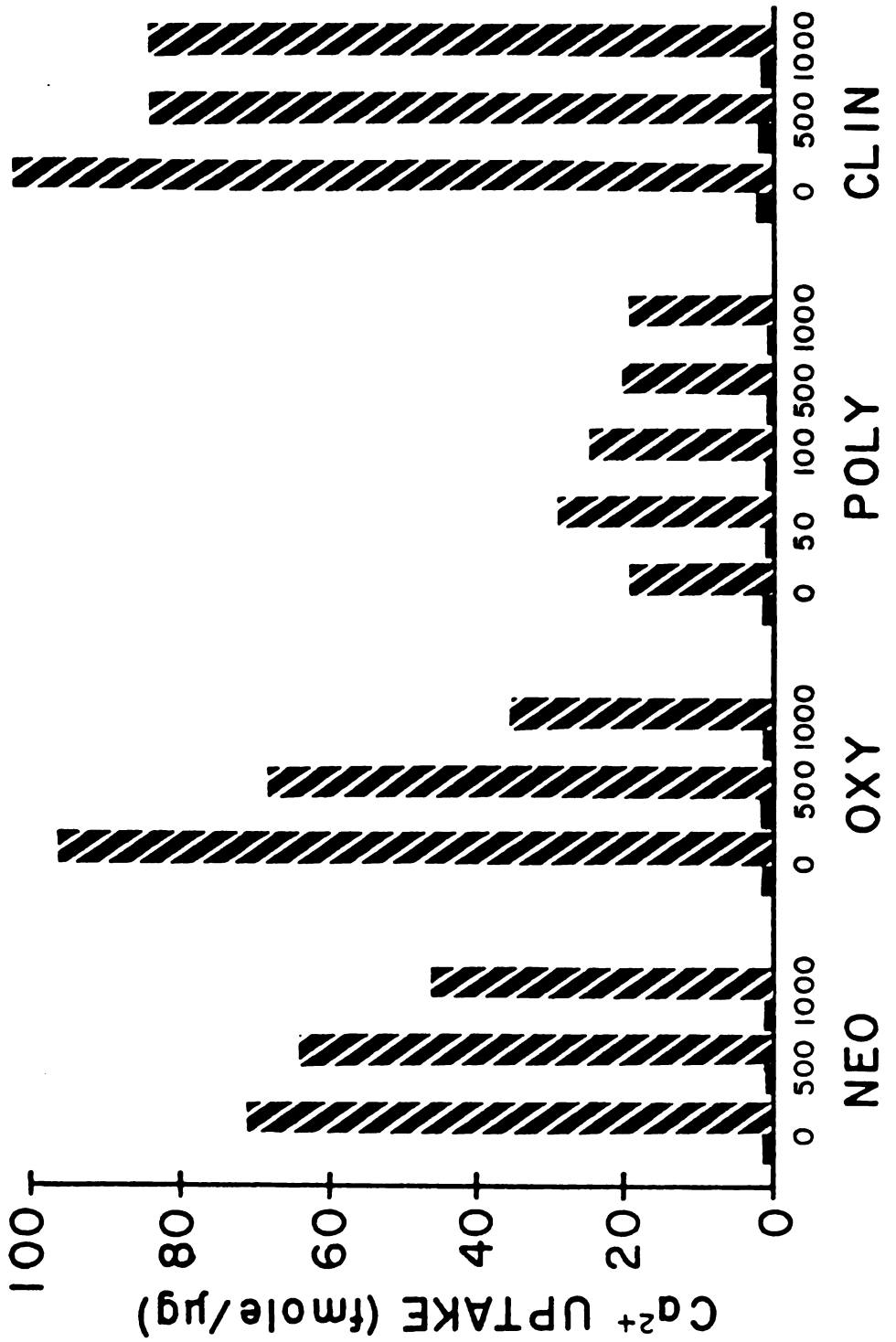
Ca^{2+} UPTAKE (fmole/μg)
100
80
60
40
20
0
0 500 1000
NEO
0 500 1000
OXY
0 50 100 500 1000
POLY
0 500 1000
CLIN

Figure 22

decrease in fast phase Ca^{2+} uptake, while polymyxin (5 uM) caused an increase in fast phase Ca^{2+} uptake and clindamycin had no effect. Neomycin and clindamycin had no effect on slow phase Ca^{2+} uptake, while oxytetracycline (500 and 1000 uM) and polymyxin (100, 500 and 1000 uM) caused a decrease in slow phase Ca^{2+} uptake.

Since the control values varied between experiments the control values are listed in Table 3 and Table 4, expressed as fmoles Ca^{2+}/ug synaptosomal protein. Table 3 (total uptake) and Table 4 (fast and slow phase uptake) also list the amount of Ca^{2+} uptake at all points that are significantly different from control. These results demonstrate that clindamycin has no effect on Ca^{2+} uptake into synaptosomes. Neomycin and oxytetracycline, the two ABs that act prejunctionally to cause neuromuscular block, decrease Ca^{2+} uptake during the fast phase of Ca^{2+} uptake (the Ca^{2+} uptake presumably related to release of neurotransmitter). Based on these results the possibility that ABs act prejunctionally to cause neuromuscular block by decreasing Ca^{2+} uptake has not been ruled out.

Calcium Reversal

Since ABs may have actions that block neuromuscular transmission by prejunctional competitive antagonism of Ca^{2+} entry into the terminal, I tested the hypothesis that increasing the $[Ca^{2+}]_o$ could reverse the neuromuscular block

Table 1. Significant effects of antibiotics on net K^+-stimulated (77.5 mM) total Ca^{2+} uptake (10 sec incubation) into synaptosomes.

ANTBIOTIC	n	TOTAL (% change)	DOSE (μM)
NEOMYCIN	7	No effect	
OXYTETRACYCLINE	7	-37	1000
POLYMYXIN	8	-25	500
		-26	1000
CLINDAMYCIN	9	No effect	

Table 2. Significant effects of antibiotics on net K^+-stimulated (77.5 mM) fast phase (1 sec incubation) and slow phase (10 sec incubation after predepolarization) Ca^{2+} uptake into synaptosomes.

ANTBIOTIC	n	FAST (% change)	DOSE (μM)	n	SLOW (% change)	DOSE (μM)
NEOMYCIN	7	-34	500	6	No effect	
		-46	1000			
OXYTETRACYCLINE	7	-65	1000	6	-30	500
					-64	1000
POLYMYXIN	8	+85	5	4	-39	100
					-43	500
					-56	1000
CLINDAMYCIN	9	No effect		6	No effect	

Table 3. Control values and values for each AB concentration that caused a statistically significant change in net K^+-stimulated (77.5 mM) total Ca^{2+} uptake (10 sec incubation) into synaptosomes. The values are expressed as fmoles Ca^{2+} uptake/ug protein ± SEM.

ANTIBIOTIC	n	TOTAL	DOSE (μM)
NEOMYCIN	7	74.14±6.77	0
OXYTETRACYCLINE	7	35.43±8.72	0
		22.14±5.36	1000
POLYMYXIN	8	57.89±3.87	0
		43.00±3.21	500
		41.56±4.24	1000
CLINDAMYCIN	9	194.78±50.55	0

Table 4. Control values and values for each AB concentration that caused a statisitcally significant change in net K^+-stimmulated (77.5 mM) fast phase (1 sec incubation) and slow phase (10 sec incubation after predepolarization) Ca^{2+} uptake into synaptosomes. The values are expressed as fmoles Ca^{2+} uptake/ug protein ± SEM.

ANTIBIOTIC	n	FAST	DOSE (μM)	n	SLOW	DOSE (μM)
NEOMYCIN	7	20.57±1.90	0	6	71.00±14.81	0
		13.29±2.03	500			
		11.14±1.06	1000			
OXYTETRACYCLINE	7	13.86±2.90	0	6	96.50±8.39	0
		4.91±1.45	1000			
POLYMYXIN	8	9.56±1.06	0	4	33.75±1.80	0
		17.00±2.53	5		25.00±4.24	100
					20.50±2.06	500
					19.50±3.12	1000
CLINDAMYCIN	9	22.33±8.69	0	6	102.67±20.61	0

caused by an AB. The ABs tested were neomycin and oxytetracycline, because they caused a decrease in Ca^{2+} uptake via the fast channel. The concentration of AB that was used was the one that caused the greatest decrease in Ca^{2+} influx.

Oxytetracycline (1000 uM) did not significantly alter Ca^{2+} uptake via the fast phase as compared to drug-free control, with $[Ca^{2+}]_o$ ranging from 0.05 - 1.0 mM. Figure 23 shows both the control and oxytetracycline results (n = 5), expressed as fmoles Ca^{2+} uptake/ug synaptosomal protein. When the $[Ca^{2+}]_o$ was 0.05 mM (the concentration used for the fast phase of Ca^{2+} uptake) oxytetracycline decreased Ca^{2+} uptake to 39.8 $\pm$ 7.4 percent of drug-free control. Even though oxytetracycline caused a slight reduction in Ca^{2+} uptake at all $[Ca^{2+}]_o$ the lines are not statistically different.

Neomycin (1000 uM) did not alter Ca^{2+} uptake via the fast phase as compared to drug-free control, with $[Ca^{2+}]_o$ ranging from 0.05 - 1.0 mM. Figure 24 compares the drug-free control values with Ca^{2+} uptake in the presence of neomycin (n = 5), these results are expressed as fmoles Ca^{2+} uptake/ug synaptosomal protein. When the $[Ca^{2+}]_o$ was 0.05 mM neomycin decreased Ca^{2+} uptake to 68.7 $\pm$ 7.4 percent of drug-free control.

The decrease in Ca^{2+} uptake produced by either oxytetracycline (1000 uM) or neomycin (1000 uM) was reversed by raising the $[Ca^{2+}]_o$. If these ABs cause competitive

Figure 23. Fast phase Ca^{2+} uptake (1 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of 1000 uM oxytetracycline (squares) or drug-free controls (circles), at various external calcium concentrations (0.05 - 1.0 mM). This graph shows the results of five experiments (each done in triplicate) expressed as fmoles Ca^{2+} uptake/ug synaptosomal protein $\pm$ SEM. When the SE bars are not indicated the SE was smaller than the width of the symbol. The lines are not significantly different.

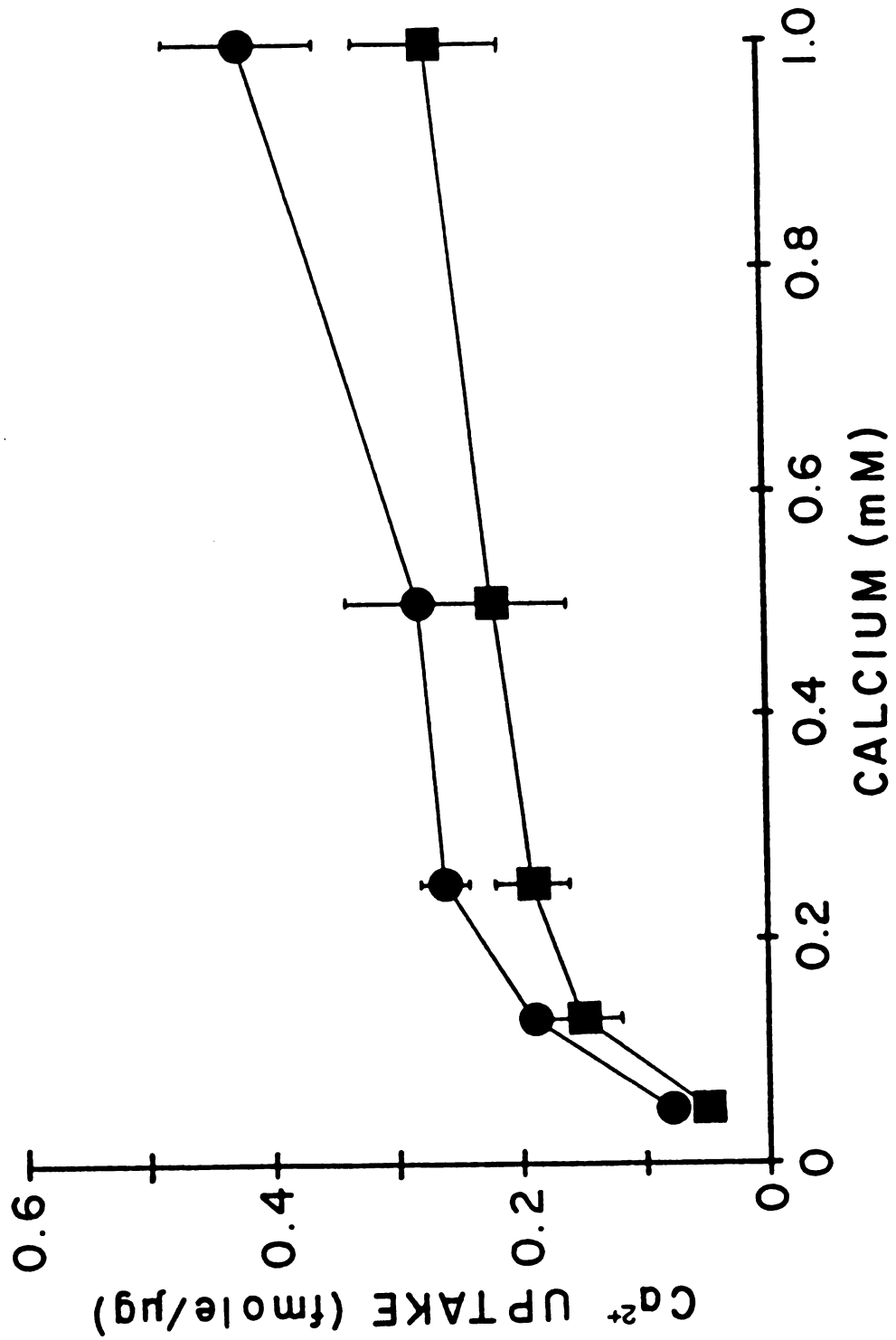

Figure 23

Figure 24. Fast phase Ca^{2+} uptake (1 sec incubation) by potassium-depolarized (77.5 mM) synaptosomes in the presence of 1000 uM neomycin (squares) or drug-free controls (circles), at various external calcium concentrations (0.05 - 1.0 mM). This graph shows the results of five experiments (each done in triplicate) expressed as fmoles Ca^{2+} uptake/ug synaptosomal protein $\pm$ SEM. When the SE bars are not indicated the SE was smaller than the width of the symbol. The lines are not significantly different.

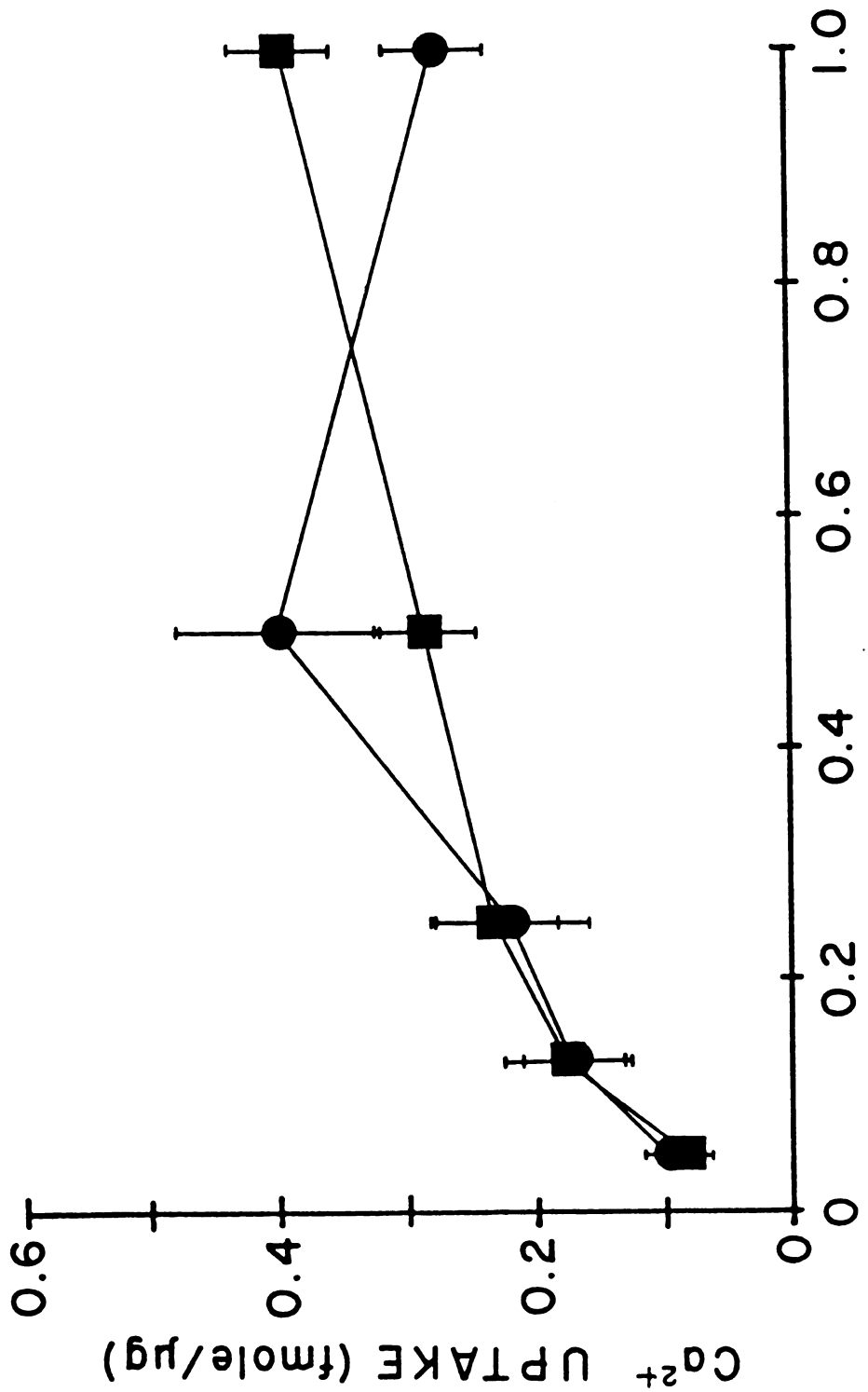

Figure 24

antagonism of Ca^{2+} influx raising the $[Ca^{2+}]_o$ should have reversed the AB-induced decrease in Ca^{2+} influx in a concentration dependent manner. Over the concentration range of $[Ca^{2+}]_o$ tested, reversal of AB-induced block of Ca^{2+} uptake did not appear to be strictly concentration dependent.

DISCUSSION

One of the proposed mechanisms for AB-induced neuromuscular block is competitive antagonism of Ca^{2+} influx into the presynaptic nerve terminal. Although many studies have been done in attempts to determine the mechanism by which ABs cause neuromuscular block, none of the experiments have examined calcium influx, directly. Therefore the experiments contained herein were designed to assess this problem. Because of methodological constraints limiting the use of the vertebrate neuromuscular junction, an alternate assay was used. The assay consisted of measuring Ca^{2+} uptake into synaptosomes in the presence of each of several ABs that cause neuromuscular block. Some of the ABs did cause a decrease in Ca^{2+} influx in this system. Since some of the ABs caused a decrease in Ca^{2+} influx via the fast phase it is feasible that this decreased calcium uptake can contribute to neuromuscular block.

The experiments contained herein used synaptosomes as a model for the neuromuscular junction. Some evidence for the accuracy of synaptosomes as a model for the neuromuscular junction, with respect to presumed calcium channels, was provided by Nachshen and Blaustein (1979). Both synaptosomes and the neuromuscular junction are relatively

insensitive to the calcium antagonists verapamil and D-600, whereas other tissues are more sensitive to these drugs. Nachshen (1984) demonstrated that a number of polyvalent cations block fast phase Ca^{2+} uptake into synaptosomes. These metal cations also block calcium-dependent transmitter release at the neuromuscular junction (Weakly, 1973; Forshaw 1977; Cooper and Manalis, 1983).

All of the ABs except clindamycin had the expected effects on Ca^{2+} uptake. Neomycin caused a decrease in Ca^{2+} uptake via the putative fast channel. This was expected since neomycin in particular and aminoglycosides in general have been reported to exert predominantly prejunctional neuromuscular blocking actions (Elmqvist and Josefsson, 1962; Brazil and Corrado, 1969; Pittinger and Adamson, 1972; Singh et al, 1979, 1982; Farley et al, 1982; Fiekers, 1983a). Oxytetracycline also caused a decrease in total and fast phase Ca^{2+} uptake. This decrease is in agreement with some of the proposed mechanisms of action for oxytetracycline (Singh et al, 1982). Polymyxin B caused a decrease in total Ca^{2+} uptake and in Ca^{2+} influx via the slow phase. In addition to the decrease in Ca^{2+} influx, low concentrations of polymyxin (5 uM) caused an increase in fast phase Ca^{2+} influx. Polymyxin is thought to cause neuromuscular block predominately by postjunctional actions (Singh et al, 1979, 1982; Fiekers, 1981), thus it was not expected to cause a decrease in Ca^{2+} influx. Although clindamycin has been shown to have prejunctional effects

(Rubbo et al, 1977; Fiekers et al, 1983), it did not cause any change in Ca^{2+} influx. The fast phase of Ca^{2+} uptake appears to be associated with neurotransmitter release (Drapeau and Blaustein, 1983; Suszkiw and O'Leary, 1983; Daniell and Leslie, 1986), thus it would be the component of concern if decreased Ca^{2+} influx was the mechanism of AB-induced neuromuscular block. From these results it can be seen that neomycin, which causes neuromuscular block by predominantly prejunctional actions, and oxytetracycline, whose mechanism of neuromuscular block is unknown, decrease Ca^{2+} influx during 1 sec of depolarization, whereas ABs (polymyxin and clindamycin) that produce neuromuscular block by predominantly postjunctional actions or for which Ca^{2+} does not provide effective reversal (Singh et al, 1982) do not decrease fast phase of Ca^{2+} uptake into synaptosomes.

Clindamycin did not have the predicted effect on calcium uptake; this may be due to the disparate nature of the data upon which the prediction was based. Rubbo et al (1977) and Singh et al (1982) have reported that clindamycin leads to an increase in spontaneous MEPP frequency and an increase in ACh release. Based on those results it was expected that clindamycin would cause an increase in Ca^{2+} uptake. However, Fiekers et al (1983) have shown that clindamycin altered EPC amplitude, increased EPC decay rate and decreased EPC quantal content. If the decrease in EPC quantal content is due to a decrease in the number of quanta released and not a decrease in the size of the quanta

released, then this would be consistent with the fact that clindamycin did not cause an increase in Ca^{2+} uptake into synaptosomes. Clindamycin did not alter Ca^{2+} uptake via the fast phase as would be expected (based on some results). This could have occurred if there is not a general mechanism for all ABs, but if more than one mechanism of prejunctional block exists. For example, neomycin and oxytetracycline could block by competitive antagonism of Ca^{2+} influx while clindamycin might have some other prejunctional effect. In order to determine if there is more than one mechanism of prejunctional block, other ABs that are known to block neuromuscular transmission by a prejunctional mechanism will have to be tested.

Polymyxin caused paradoxical apparent increase in fast phase Ca^{2+} uptake. Polymyxin has both pre- and post-junctional neuromuscular blocking actions (Singh et al, 1982) with the postjunctional actions predominating (Singh et al, 1979; Fiekers, 1981). Polymyxin-induced neuromuscular block is poorly reversed by calcium (Singh et al, 1978). Even though synaptic transmission is dependent on Ca^{2+} (Katz and Miledi, 1965b, 1967, 1969; Dodge and Rahamimoff, 1966; Miledi and Slater, 1966) it has been demonstrated that high levels of ionized calcium in the presynaptic terminal can act to block synaptic transmission (Miledi and Slater, 1966; Kusano, 1970; Adams et al, 1985). Perhaps an increase in the intracellular calcium concentration may contribute to neuromuscular block produced

by polymyxin.

The two ABs (oxytetracycline and neomycin) that decreased fast phase Ca^{2+} uptake did not alter fast phase Ca^{2+} uptake when the incubation was carried out in a solution containing a $[Ca^{2+}]_o$ ranging from 0.05 - 1.0 mM. Therefore the AB-induced block of Ca^{2+} uptake can be reversed. However this reversal did not appear to be concentration dependent over the range of concentrations tested. Perhaps lower $[Ca^{2+}]_o$ are needed to demonstrate a strict concentration-dependent competitive antagonism of Ca^{2+}. My results are comparable to the recent results of Yoshii et al (1986) in which they demonstrated that streptomycin (an aminoglycoside) can block Ba^{2+}-induced currents through Ca^{2+} channels into neuroblastoma N1E-115 cells and that increasing the external Ba^{2+} concentration overcame the AB-induced block of Ba^{2+} currents.

One problem with the results of the experiments contained herein is that the amount of Ca^{2+} uptake is about 33 - 35% of that obtained by others (Blaustein et al, 1977; Nachshen and Blaustein, 1980; Nachshen, 1984, 1985a,b; Leslie et al, 1983). This may be due to the fact that the other authors used different methods of synaptosome preparation, either the method of Hajos (1975) or the modification of Hajos's method by Krueger et al (1979). These other methods for preparing synaptosomes are reported to produce a purer synaptosomal fraction. Since the Ca^{2+} uptake results are expressed per ug of protein (the

assumption being all of the protein is synaptosomal) a purer preparation would give greater uptake values.

The physiological correlate for the slow phase of Ca^{2+} uptake is unknown. The slow phase of Ca^{2+} uptake may be associated with a channel (Nachshen and Blaustein, 1980) or Na^{+}/Ca^{2+} exchange (Wang et al, 1985; Suszkiw et al, 1986). Sheu et al (1986) have demonstrated, using rat ventricular myocytes, that both voltage-sensitive Ca^{2+} channels and voltage-sensitive Na^{+}/Ca^{2+} exchange contribute to the increase in the internal calcium concentration during membrane depolarization. If the slow phase of calcium uptake into synaptosomes is due to Na^{+}/Ca^{2+} exchange then there could be ion movements that would alter the membrane potential (an electrogenic pump). It is also possible that calcium flowing down its concentration gradient could provide energy to pump Na^{+} against its concentration gradient.

The intracellular calcium concentration is buffered by calcium binding or sequestration in mitochondria and other organelles (Lehninger, 1970; Alnaes and Rahamimoff, 1975; Blaustein et al, 1977; Kendrick et al, 1977; Scott et al, 1980). The internal calcium concentration is very low 104 ± 8 nM (based on experiments with quin-2, a Ca^{2+} specific fluorescent indicator) while the external calcium concentration is 1.2 mM (Richards et al, 1984). Depolarization causes a 2-fold increase in the internal calcium concentration (Richards et al, 1984). Thus the

internal calcium concentration remains well below the external calcium concentration. Calcium entry into synaptosomes is dependent on the ratio of the internal and external Na^+ concentrations (Coutinho et al, 1984; Nikezie and Metlas, 1985). Either increasing the internal Na^+ concentration or decreasing the external Na^+ concentration leads to greater Ca^{2+} uptake. The slow phase of Ca^{2+} uptake may be included in the mechanism by which the nerve terminal removes Na^+ that has entered during depolarization. Therefore the decrease in slow phase Ca^{2+} uptake probably does not contribute directly to the neuromuscular blocking actions of the ABs.

A wide range of concentrations for the ABs was used for the Ca^{2+} uptake experiments, yet in order to observe a decrease in Ca^{2+} influx high concentrations of the ABs were needed. The concentrations of ABs needed to cause a decrease in Ca^{2+} influx are considerably higher than the normal peak plasma levels of these ABs given clinically (neomycin, polymyxin and oxytetracycline < 10 uM; clindamycin < 40 uM). However, neuromuscular block usually does not occur at these plasma levels unless other agents are present (eg, general anesthetic or neuromuscular blockers) that compromise neuromuscular function. AB-induced neuromuscular block can also occur in patients with hepatic or renal dysfunction that cause an increase in the concentration of AB in the plasma.

The concentrations of ABs needed to cause a decrease in

Ca^{2+} influx are generally higher than those needed to cause neuromuscular block in isolated nerve-muscle preparations. Depending on what effect is measured, different concentrations of the ABs are needed to cause a significant effect on transmission. These effects occur at the following concentration ranges: neomycin 1-600 uM (Wright and Collier, 1977; Fiekers, 1983a), polymyxin 2 uM (Durant and Lambert, 1981), clindamycin 200-800 uM (Wright and Collier, 1976a; Fiekers et al, 1983), oxytetracycline 2,000-10,000 uM for $\geq$ 80% reduction (Singh et al, 1978, 1982). Thus the concentrations needed in synaptosomes are often, but not always higher than those needed in other systems. Several reasons could explain why higher concentrations are needed to see an effect in synaptosomes as compared to the isolated neuromuscular junction. First, synaptosomes are heterogeneous with respect to transmitter; this means that if the effects of ABs that cause neuromuscular block are specific for cholinergic nerve terminals (such as those found at the neuromuscular junction), the effects of the ABs may be obscured. For example the ABs might act only at certain types of Ca^{2+} channels. As described earlier there are multiple types of Ca^{2+} channels. If, as hypothesized by Yu and Nelson (1986), neurons that utilize different types of neurotransmitters have different Ca^{2+} channels, the ABs may not act the same at all types of neurons (eg. the ABs may have different affinities for or efficacy at different types of Ca^{2+} channels). Second, synaptosome preparations

are not pure nerve terminals; they contain free mitochondria, membrane fragments and other cellular debris. The mitochondria can also take up Ca^{2+} and thus confound the results. The Ca^{2+} uptake in these experiments was measured based on the amount of ^{45}Ca trapped by the filter. This means that any Ca^{2+} that entered mitochondria was also included in the measurement of Ca^{2+} influx. This problem was reduced by partial purification of the synaptosomes to remove mitochondria. Also the change in potassium concentration that caused an increase in Ca^{2+} uptake into the synaptosomes, does not alter Ca^{2+} uptake into mitochondria (Blaustein, 1975), so the experimental design should eliminate mitochondrial Ca^{2+} uptake as a potential variable from the final results (net stimulated Ca^{2+} uptake). Third, synaptosomes may not represent an accurate model of the neuromuscular junction. For example, different tissues have different types of Ca^{2+} channels, as shown by differences in the effects of verapamil, D-600 and dihydropyridines (Gotgil'f and Magazanik, 1977; Nachshen and Blaustein, 1979; Daniell et al, 1983; Glossman et al, 1984), thus synaptosomes may not have the same type of calcium channel as do the presynaptic motor nerve terminals. Even though evidence has been presented that shows synaptosomes and the neuromuscular junction respond the same way in the presence of organic calcium antagonists and multivalent cations (Nachshen and Blaustein, 1979; Nachshen, 1984), this does not mean that synaptosomes and the neuromuscular

junction will necessarily respond the same way in the presence of antibiotics. Fourth and finally, block of Ca^{2+} uptake may not be the only mechanism by which ABs cause neuromuscular block. Competitive antagonism of Ca^{2+} influx may be one of many factors involved in neuromuscular block, or it may not be involved at all. If ABs cause neuromuscular block by blocking the Ca^{2+} channel, it is not necessary that the ABs act as competitive inhibitors of Ca^{2+}. Also because of the multiple steps involved in synaptic transmission the ABs may also act at a step(s) other than Ca^{2+} influx to cause neuromuscular block.

Some experiments could be done in order to address some of these problems. The first problem could be avoided if the studies were repeated with a pure cholinergic preparation, such as the electric organ of Torpedo. If cholinergic synaptosomes could be used this would more closely resemble the neuromuscular junction than the transmitter-heterogenous synaptosomes which characterize those derived from the forebrain. The second problem can be addressed by using methods that isolate a synaptosome fraction of higher purity (Hajos, 1975; Krueger et al, 1979). The problem of determining if the calcium channels of the two tissues are identical is unavoidable, until more is known about the calcium channels of the respective preparations. The fourth issue could be addressed by testing ABs that do not cause neuromuscular block. If those ABs decrease Ca^{2+} influx then decreasing Ca^{2+} influx may not

cause neuromuscular block.

Some Ca^{2+} channel blockers may show preferential binding to inactivated Ca^{2+} channels. Nachshen (1985a) has demonstrated that inhibition of K^{+}-stimulated Ca^{2+} uptake by Ni^{2+}, La^{3+} and verapamil was enhanced in synaptosomes that were pre-depolarized for brief intervals in the presence of Ca^{2+} channel blockers. Pre-depolarization should cause Ca^{2+} channel inactivation in synaptosomes as the inactivation is voltage, and not Ca^{2+}-dependent (Nachshen, 1985a). This implies that some Ca^{2+} channel blockers may bind preferentially to inactivated Ca^{2+} channels. This possibility should also be tested for ABs, since this could help explain why ABs that cause neuromuscular block had no effect on Ca^{2+} uptake into synaptosomes. Synaptosomes should be depolarized in the presence of an AB before Ca^{2+} influx is measured. Experiments should also be done in which the synaptosomes are pre-incubated with the ABs, in case the ABs have their effect by inhibiting activation (opening) of the Ca^{2+} channel. Pre-incubation with ABs would resemble physiological conditions more closely than the conditions of the experiments contained herein. If the ABs had their effect by inhibiting opening of the Ca^{2+} channel or by binding to the inactivated Ca^{2+} channel the effects would not be seen in the experiments contained herein.

The results described herein do not disprove the idea that ABs can cause neuromuscular block by block of

depolarization-induced Ca^{2+} entry, but rather provide evidence which though weak, is at least consistent with the idea that some ABs may cause prejunctional neuromuscular block by decreasing Ca^{2+} uptake. Further study, especially with regards to the fast phase of Ca^{2+} uptake, is necessary to elucidate the mechanism(s) by which ABs cause neuromuscular block.

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LIST OF REFERENCES

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