Action of philanthotoxin on ion channels of arthropod muscle

by

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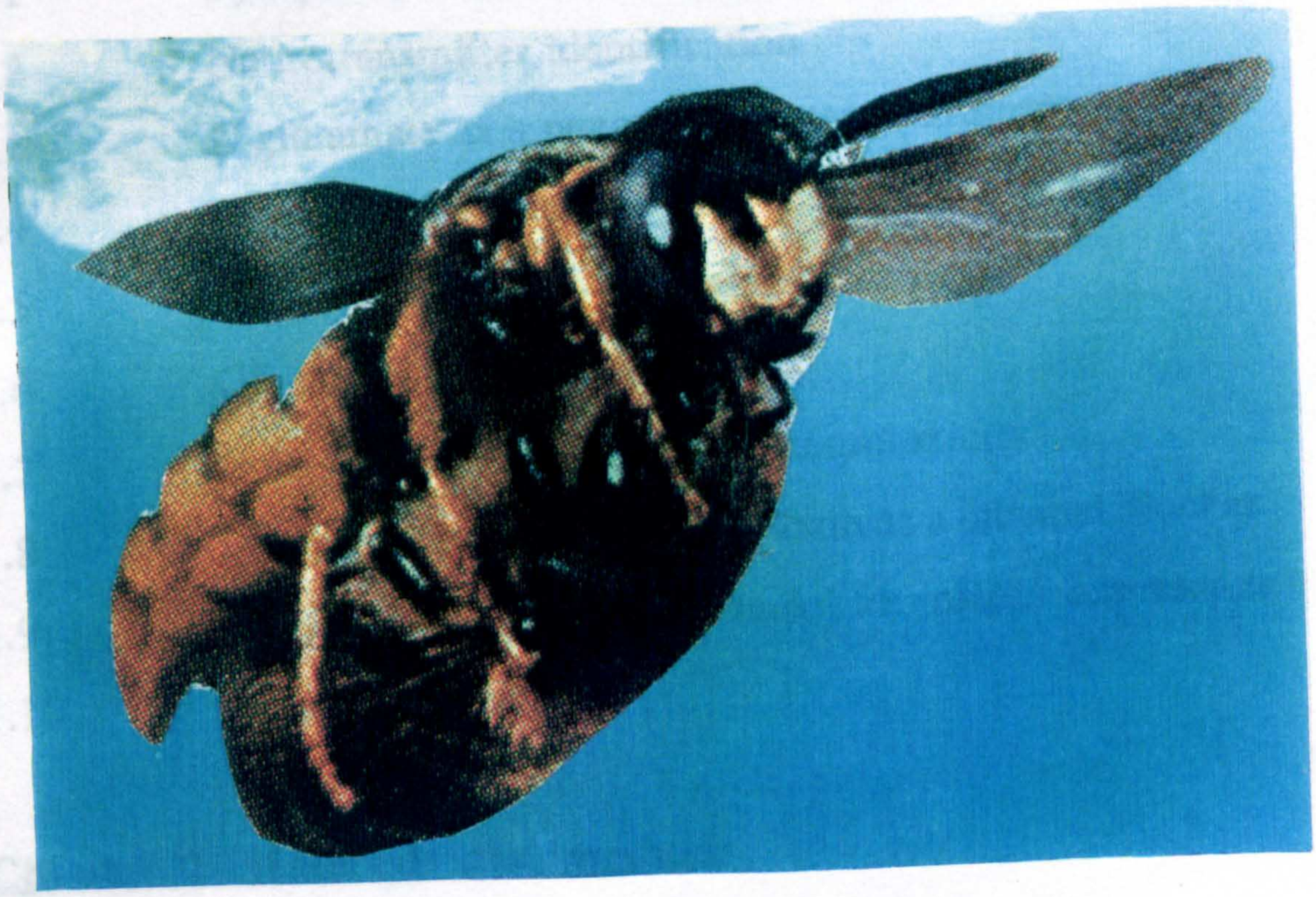

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Abbreviations

ACh	Acetylcholine receptor
AMPA	α–Amino-3-hydroxy-5-methyl-isoxazole-propionate
AP-5	D-(-)-2-amino-5-phosphopentanoic acid
CNS	Central nervous system
Con A	Concanavalin A
DEPC	Diethyl pyrocarbonate
DNP12-PhTX-343	Dinitro-phenyl12-PhTX-343
EPSP	Excitatory postsynaptic potential
GluR	Glutamate receptor
LGDEE	L-glutamate diethylester
mRNA	Messenger ribonucleic acid
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
PhTX-343	Philanthotoxin-343
PhTX-343-Arg	Philanthotoxin-343-Arginine
qGluR	L-quisqualate-sensitive glutamate receptor
RNA	Ribonucleic acid
Vm	Membrane potential
VSCC	Voltage-sensitive calcium channels

ABSTRACT

Calcium ions play an important role in many signalling pathways involved in normal cell metabolism. Pertrebations of normal Ca^{++} signalling may also play a pivotal role in the initiation of cell death. In these studies I have examined the influx of $^{45}Ca^{++}$ into the extensor tibiae muscle of the locust (*Schistocerca gregaria*).

$^{45}Ca^{++}$ entry could be stimulated by the addition of glutamate receptor agonists or by activation of voltage activated calcium channels. L-glutamate, L-quisqualate and NMDA stimulated the influx of $^{45}Ca^{++}$ while L-aspartate had only a small effect. DL-ibotenate, kainate, AMPA and glycine had no effect on $^{45}Ca^{++}$ uptake (all agonists were tested at concentrations up to (100μM). Glycine (1μM) enhanced the $^{45}Ca^{++}$ entry induced by NMDA and L-glutamate. Only the glycine potentiation of L-glu-stimulated responses was abolished in the presence of Mg^{++} (2mM) or AP5 (10μM) whereas the NMDA-stimulated response was completely abolished by these agents. These finding suggests that in the presence of glycine, L-glutamate may activate NMDA receptors and that in the absence of glycine L-glu-stimulated $^{45}Ca^{++}$ entry occurs via activation of the qGluR.

Depolarisation of the extensor tibiae muscles (50mM KCl) stimulated $^{45}Ca^{++}$ influx by activation of voltage-sensitive calcium channels. Philanthotoxin-343 (0.1μM) had no effect on depolarisation activated calcium entry, however, nifedipine (1μM) an L-type calcium channel antagonist inhibited this Ca^{++} influx. Nifedipine did not inhibit L-glu-stimulated Ca^{++} entry suggesting that in these muscles L-type Ca^{++} channels are not involved in the Ca^{++} influx pathway following GluR activation.

Philanthotoxin-433 (PhTX-433) and many of its synthetic analogues are potent inhibitors of locust GluR. In the future these analogues may prove as useful potential neuroprotective agents or as novel pesticides. Over 100 analogues of PhTX-433 have been synthesized with changes made in the four regions of the structure, the thermospermine moiety, the tyrosyl moiety, the butyryl moiety and the terminal amino moiety. The effects of different concentrations (10^{-4}M to 10^{-14}M) of synthetic analogues of PhTX-433 (PhTX-343, PhTX-343-Arg, PhTX-4) were investigated in the $^{45}Ca^{++}$ influx assay using locust extensor tibiae muscle. PhTX-343-Arg was more potent (IC_{50}= ~ $7x10^{-9}$) than PhTX-343 (IC_{50}= ~ 10^{-8}M) or PhTX-4 in blocking $^{45}Ca^{++}$ uptake. These findings were further supported by electrophysiological studies. The interaction of these synthetic analogues of philanthotoxin with GluR of locust muscle were further investigated by examining the effect of these compounds on evoked excitatory post synaptic potentials.

In recent years control of ticks have been very important issue because of the social and economical damage they cause. Neuromuscular transmission is a main target site for the chemical control of many pests. Philanthotoxin and its analogues block the glutamate receptors which are involved in arthropod neuromuscular transmission and thus may prove useful as novel pesticides. The action of synthetic analogues of philanthotoxin (C_7PhTX-343, DNP12-PhTX-343 and PhTX-343) were examined on evoked excitatory postsynaptic potential in tick coxal muscle. These compounds all antagonized the evoked EPSP. C_7PhTX-343 and DNP12-PhTX-343 exhibited same potency (IC_{50} = ~ 10^{-8}M) and both were more potent than PhTX-343 (IC_{50} =~$2X10^{-5}$M).

In recent years *Xenopus* oocyte has taken over a new role as a test tube for the study of the biogenesis, functional architecture and modulation of plasma membrane protein. Attempts were made to express mRNA from embryonic tissue of tick and locust leg muscle in to *Xenopus* oocyte for pharmacological studies. *Xenopus* oocytes failed to translate RNA faithfully and efficiently from these sources. Rat brain RNA injected oocytes used as control, expressed routinely.

CHAPTER ONE

INTRODUCTION

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INTRODUCTION

The crucial role played by ion channels in intracellular signalling make them obvious targets for pesticide and drug action. Studies of neurotransmitter receptors are therefore vitally important in both the agrochemical and pharmaceutical industries in their efforts to find novel sites for pesticide and drug action and especially, in the latter case, to develop potent drugs to combat certain neurological disorders.

It is now well-accepted that glutamate is a principal transmitter at excitatory synapses in mammalian central nervous system (CNS) (Curtis *et al.*, 1974; Fonnum, 1984; Nicholls *et al.*, 1990). Glutamate synapses are abundant in mammalian nervous system (Wroblewsk *et al.*, 1989) and play a central role in memory, learning and synaptic plasticity. To study the physiology of nervous tissue and to solve fundamental problems in neurobiology, invertebrates models offer some advantages over vertebrate models (Boden *et al.*, 1986; Leake and Walker, 1980). The main advantage is the comparatively simpler cellular organization of the nervous system of invertebrates and, the ready identification of some of neurones that they contain (Eldefrawi and Eldefrawi, 1988). Many workers have proved that glutamate is an important neurotransmitter in arthropods (Kravitz *et al.*, 1970; Usherwood and Grundfest, 1965). Robbins (1959), Takeuchi and Takeuchi (1965) and Usherwood (1969) amongst other reported that

glutamate mediates synaptic excitation at arthropod excitatory neuromuscular junctions. The regulation of insect skeletal muscle activity by glutamate give the receptors for this amino acid a special significance as a target for the chemical control of pest insects.

Two species of locust, *Schistocerca gregaria* and *Locusta migratoria* cause great economic loss (£30 million / year) by destroying crops in the field (Uvarov, 1951). *Schistocerca gregaria* occurs in the northern half of Africa and as far east as northern India. *Locusta migratoria* has a range extending from western and southern Europe through most of the African continent, to central and southern Asia to China and northern Australia (Fig. 1.1). Cramer (1967) reported that up to 14% of the destruction of agricultural production was caused by insects. Some arthropods also play an important role in transmitting diseases in human and domestic animals. Ticks are parasites of vertebrates and are most abundant on mammals and reptiles, though they are also common on birds and amphibia. Their food consists entirely of blood and lymph taken from their host. Smith and Kilbourne (1893) were the first to prove that the tick as a vector of Texas fever and red-water fever of cattle. Hoogstral (1951) presented the detail taxonomic classification of tick (Fig. 1.3). During the intervening years a large number of ticks have been shown to be the vectors of many serious diseases of man and other animals. Transmission of micro-organisms by blood sucking acarines (especially ticks) is rarely very specific, since a single species may transmit various pathogens and several pathogens are each spread by different species (Nunez *et al.*, 1985). The data presented by Nunez (1985) showed that in Argentina 20% of a cattle population of 60

million suffer from *Boophilus microplus;* and earlier, Primera Reunion Argentina de Ciencias Veterinarias (PRACIVE) (1972) reported that economic loss caused by this common cattle tick in Argentina was 112,000 US$/annum. Boophilus microplus are widely distributed in the area delineated (Fig. 1.2)

The serious economic consequences and social importance of damage caused by arthropod pests (especially in developing countries where the concurrent nutritional problems require urgent solutions) justifies attempts to control these organisms. Present chemical control methods are heavily based on the use of two groups of toxicants, the organophosphates and the pyrethroids which work mainly on acetylcholinesterase and on voltage-gated sodium channels respectively. Both of these classes of pesticide have been in use for a number of years and although they represent good methods for chemical control, organophosphates cause problems because of their potential toxicity to vertebrates, including the users, and resistance of pest organisms to both types of chemical is becoming endemic in many parts of the world. It is thus important to continue to search for new pesticides. In order to reduce the likelihood of resistance, it would be preferable to find compounds which work at different sites of action from the commercial pesticides.The glutamate receptor might provide such a site of action and it is, of particular interest to examine its pharmacology in arthropods.

1.1 Synapse and neuromuscular transmission

A synapse is a region where the membranes of two excitable cells are closely apposed. The transmission of information from one excitable cell to another occurs at this region. Transmission is either electrical or chemical. At electrical synapses, the current generated by the presynaptic cell directly affects the postsynaptic cell (Furshpan and Potter, 1959). Bennett (1974) demonstrated electrically-transmitting synapses in nervous systems of annelids, molluscs, and arthropods. Electrical synapses have also been reported in the spinal cord of frog (Grinnel, 1970), in the medulla of puffer fish (Bennett, 1973) and in the CNS of mammals (Llinas *et al.*, 1974). At chemically-transmitting synapses the axon of the presynaptic cell releases a chemical substance(s) or neurotransmitter into the synaptic cleft. When this neurotransmitter binds to receptors present in the synaptic membrane of the postsynaptic cell, it induces either excitation or inhibition through the activation of ionic currents

Loewi (1921) was the first to demonstrate chemical transmission by showing that the vagus nerve innervating a frog heart produces a chemical substance which slows the beating of another heart. This chemical was identified as acetylcholine. It was first called a neurotransmitter by Dale (1936). A neurotransmitter binds to the postsynaptic receptors of neurones, muscle fibres and gland cells where it causes changes in the conductance of the postsynaptic membrane either directly or indirectly. For example, activation of nicotinic acetylcholine receptors by acetylcholine causes the direct opening of

membrane channels leading to a change in membrane conductance. Indirect changes in membrane conductance are obtained by the activation of secondary messenger systems (e.g. adenylate cyclase or guanylate cyclase) followed by the opening of ion channels, as exemplified by the activation of acetylcholine on the muscarinic acetylcholine receptor (Dascal, 1987; Aidley, 1990). Transmission of information via a secondary messenger system is slower than via the direct opening of ion channels because of the need to activate biochemical reactions, which then lead to the final response (e.g. change in the membrane permeability). However in either case, the resultant movement of ions usually causes either a depolarisation or a hyperpolarisation of the postsynaptic membrane.

The region of contact between nerve and muscle is known as neuromuscular junction and the process of transmission of either excitation or inhibition is called neuromuscular transmission. There is considerable evidence that in arthropods glutamate mediates excitatory neuromuscular transmission. Stimulation of an excitatory axon innervating a locust excitatory input to a locust muscle fibre releases neurotransmitter L-glutamate from the motor axon terminals. This neurotransmitter causes an increased in the permeability of the postsynaptic membrane to potassium (K^+), sodium (Na^+) and, calcium (Ca^{++}) ions which leads to a change in the membrane potential of postsynaptic membrane, known as an excitatory postsynaptic potential (EPSP). Fatt and Katz were the first to explain the generation of EPSPs in vertebrate neuromuscular junction.

1.2 Neurotransmitter identification

For a substance to be considered an established transmitter it must meet several criteria (Paton, 1958).

1. The chemical, its precursors and the enzymes and proteins necessary for its synthesis and release must be present in the presynaptic neurone.

2. Stimulation of the presynaptic neurone must result in the release of neurotransmitter into the synaptic cleft.

3. When applied directly to the postsynaptic membrane the putative transmitter must elicit in the postsynaptic cell precisely the same physiological effects as does presynaptic stimulation.

4. Exogenously-applied putative neurotransmitter must interact with pharmacological agents (e.g. antagonists) in a manner similar to the neurally-released transmitter.

5. A specific receptor which binds the transmitter must be demonstrated to be present on the postsynaptic membrane.

These criteria, although logical, are often difficult to fulfil satisfactorily. Werman (1966) and Oreggo (1979) re-examined Paton's proposal and agreed that only criteria 3 and 4 really need to be satisfied. Bradford (1986) has emphasized the need to demonstrate the presence of specific receptors for putative neurotransmitters in the tissue containing the synapse.

1.3 Glutamate as a neurotransmitter in arthropods

Glutamic acid is an essential amino acid which is required for protein synthesis and intermediate metabolism. In the mammalian brain the glutamate concentration is higher than in any other organ and its concentration is higher than that of any other amino acid (Waelsch, 1951). In 1954 Hayashi observed that glutamate excited both crustacean muscle and mammalian brain. Robbins (1958, 1959), Van Harreveld (1960) and Van Harreveld and Mendelson (1959) suggested the possible role of glutamate as a synaptic transmitter. Robbins (1959) demonstrated that a low concentration (2×10^{-5}M) of glutamate potentiated the neurally-evoked contraction of the claw opener muscle of crayfish. A high concentration (10^{-4}M) of glutamate evoked a muscle contraction even when the claw opener nerve was not stimulated. In the same year, Van Harreveld (1959) reported that rabbit cerebral cortex extract caused a contraction of crayfish skeletal muscle. Chromatographic and electrophoretic results showed that in the extract, glutamate was the active compound. Similar results were obtained with other crustaceans (Van Harreveld and Mendelson, 1959). Even so, Van Harreveld (1959, 1960) suggested that it was unlikely that glutamate itself was the transmitter at the crustacean excitatory nerve-muscle synapse and that the naturally-occuring transmitter was most probably a complex structurally related to L-glutamate.

Many workers have since proved that glutamate plays an important role in arthropod neuromuscular transmission. Kravitz *et al.* (1963) showed that glutamate is the major excitatory compound in an extract of lobster

nervous system, but they did not relate the level of excitation with the concentration of glutamate. Usherwood and Grundfest (1965) found that locust skeletal muscle fibres respond with a depolarization to a low concentration of bath-applied L-glutamate. The first extensive studies of the effects of acidic amino acids on insect skeletal muscle preparation were made by Kerkut *et al.* (1965b). They showed that the twitch contractions of metathoracic leg of cockroach were reduced when L-glutamate was applied and that contractions of the leg muscle were evoked by low concentrations of this amino acid. In the same year Florey and Florey (1965) showed that body-wall muscle of the centipede contracted when challenged with glutamate. The work of Usherwood and Machilli (1966) with the isolated preparation of metathoracic retractor unguis muscle of the locust *in vitro*, contributed greatly to our understanding of the action of L-glutamate on locust muscle. These workers reported that application of glutamate at a concentration higher than 6.8×10^{-6}M caused contraction. Kerkut and Walker (1966, 1967) and McCann and Reece (1967) reported similar results for cockroach and *Sarcophaga bullata* muscles. Faeder and O'Brien (1970) also reported similar results for muscles of cockroach *Gromphadorhina portentosa.* A further study by Usherwood and Machili (1968) revealed that L-glutamate was the most active excitatory amino acid in evoking contractions of the locust retractor unguis muscle, D-glutamate was about 100 times less active, whereas L-aspartate, DL-cysteate and DL-homocysteate showed agonist activity only at very high concentrations.

The introduction of ionophoresis made possible an analysis of the site of action of glutamate on insect muscle fibre. With this technique pharmacological agents can be applied to very localized areas of a muscle fibre (Nastuk, 1953; Del Castillo and Katz; 1955, Curtis and Eccles, 1958). When glutamate was applied by this method, Takeuchi and Takeuchi (1963, 1964) reported depolarization in the opener muscle of the dactyl and walking leg of the cray-fish *Cambarus clarkii*. Takeuchi and Takeuchi (1964) concluded that the receptors which respond to L-glutamate are identical with the receptors present on the postsynaptic membrane of the muscle junction. Ozeki, Freeman and Grundfest (1966) described similar results with lobster muscle. Ionophoresis of L-glutamate to the surface of locust, cockroach, beetle, fly, moth and grasshopper muscles caused depolarizations (Beranek and Miller; 1968; Usherwood and Michili, 1968 and Usherwood 1969). Anwyl and Usherwood (1974) and Anwyl (1977) suggested that sodium and potassium are the principle ions responsible for the glutamate-evoked current of locust muscle, but calcium also plays some role. Anwyl (1977a, b, 1979) reported that the L-glutamate (and the natural transmitter) caused a similar increase in permeability to sodium and potassium, and that changes in the reversal potential of the glutamate-evoked current could be obtained by altering external potassium levels. Lea and Usherwood (1970) used ionophoresis to demonstrate for the first time the presence of extrajunctional amino acid receptors on locust muscle fibre. Working with the isoxazole DL-ibotenic acid they observed that this substance increased the Cl^- permeability of the locust coxal adductor muscle fibres without acting on the excitatory synaptic

membrane. The activation of non-synaptic membrane by ibotenic acid usually resulted in muscle hyperpolarization or less frequently, a small depolarization depending on the resting membrane potential of the fibre. These potentials were obtained when applying this isoxazole to any part of the membrane of these fibres, thus demonstrating the widespread distribution of sensitivity to this substance. Cull-Candy and Usherwood (1973) found that glutamate produced two types of response in locust muscle and they ascribed these to two types of glutamate receptor which they called D-(depolarising) and H-(hyperpolarising) receptors. Lea and Usherwood (1973b) and Usherwood *et al.* (1979) showed that the H-response is mediated by chloride ions. Application of L-glutamate to extrajunctional sites gave a biphasic effect due to the action of D-and H-receptors, but the application of DL-ibotenate produced only a hyperpolarization. This suggests that DL-ibotenate is a ligand only for the glutamate H-receptor.

The advent of single channel recording (Neher and Sakmann, 1976) has enabled kinetic analysis of channel gating mechanisms. The first single-channel studies of extrajunctional D-receptors of locust muscle called qGluR, because they are sensitive to L-quisqualic acid (Usherwood, 1981) were undertaken using a megaohm seal patch clamp technique (Patlak *et al.*, 1979; Gration *et al.*, 1981). Gating of the qGluR channel (125-150pS conductance) was studied at equilibrium with L-glutamate, with desensitization inhibited by concanavalin A (Con A) (Mathers and Usherwood, 1976; Patlak et al., 1979). Under these conditions, open and closed dwell-time probability density functions for glutamate at

concentrations extending over a 1000-fold range indicated that the qGluR channel has four open states and four closed states. In addition, correlations between successive channel dwell times suggested the presence of multiple channel isomerization pathways connecting these states (Kerry *et al.*, 1988). Bates *et al.* (1990) extended this to a ten-state Markovian model with five open states, five closed states. Openings of qGluR channel are rarely observed under equilibrium conditions in the absence of Con A due to desensitization (Gration *et al.*, 1980)

Glutamate receptors present at invertebrate synapses often desensitize when exposed to the native neurotransmitter. Many workers reported desensitization of D-and H-receptors by L-glutamate (Lea and Usherwood, 1973a; Cull-Candy and Usherwood, 1973; Usherwood and Cull-Candy, 1974; Cull-Candy, 1974, 1976, 1978; Clark *et al.*, 1979). Daoud and Usherwood (1978) studied the desensitization of glutamate receptors at the locust neuromuscular junction. The rate of desensitization during bath application of L-glutamate was accelerated by increasing the glutamate concentration. Increasing the extracellular calcium concentration was also found to accelerate desensitization, while removing calcium from the bathing medium produced the opposite effect. The rate of desensitization accelerated when L-glutamate was injected into the muscle fibre by ionophoresis from an intracellular microelectrode (Usherwood *et al.*, 1968; Beranek *et al.*, 1968). In 1978 Cull-Candy reported that D- and H-receptors at the same extrajunctional site on a locust muscle fibre showed no significant differences in desensitization. Clark *et al.*, (1979) studied the

desensitization kinetics of extrajunctional glutamate receptors on voltage-and current-clamped innervated or denervated locust muscle fibres. The desensitization of glutamate receptor of locust muscle is blocked when treated with Con A (10^{-6}M - 10^{-7}M) (Mathers and Usherwood, 1976, 1978, Anis *et al.*, 1979). They suggested that Con A might act by binding directly to junctional and extrajunctional D-receptor molecules, thereby preventing the conformational changes thought necessary for desensitization to occur, but leaving the initial molecular process associated with receptor activation and ionophore opening, unaffected. However, Mathers and Usherwood (1976) found that Con A blocked the desensitisation of glutamate D-receptors although it had no effect on the desensitization of H-receptors. Anis *et al.* (1979) reported that succinylated derivative of Con A (S-Con A) has no effect on desensitization of junctional glutamate receptor population of locust muscle.

1.4 Release of L-glutamate from nerve terminals of insect

Kerkut *et al.* (1965a,b) showed the release of glutamate from nerve-muscle preparations of shore crabs (*C. maenus*), cockroach (*P. americana*) and snail (*Helix aspersa*). By using one dimension thin layer chromatography they demonstrated the presence of a ninhydrin positive substance (NSP) in the perfusate after stimulation of these preparations. Only L-glutamate was detectable and the amount released was proportional to the number of stimuli applied. Usherwood *et al.* (1968) reported that the release of glutamate from isolated locust

retractor unguis muscle was modified by changing the stimulus frequency. By using an amino acid analyser they identified small quantities of alanine, glycine and aspartate as well as L-glutamate in perfusates obtained from these preparations. Usherwood *et al.* (1968) reported that the amount of glutamate release was increased by increasing the external calcium concentration and was inhibited by magnesium. Daoud and Miller (1976) found that stimulation of locust and crab leg muscles caused an increase in the release not only of the glutamate but also of many other amino acids.

In an alternative approach, stimulus-evoked release of neurotransmitter was monitored after allowing uptake of radiolabelled L-glutamate by a fly nerve-muscle preparation. In these studies Boden (1983) examined the release of [^{3}H] L-glutamate from body-wall muscle of larval *Lucilia sericata*. During stimulation of nerve, release of [^{3}H] was increased. This increase was abolished in the absence of Ca^{++}. Bates *et al.* (1985) used a similar method to that described by Boden to study the material released from insect neuromuscular junction. They revealed that elevation of extracellular K^+ was an effective way of inducing release of preloaded L-[^{3}H] glutamate from isolated extensor tibiae muscle of *Schistocerca gregaria*. A major advance was made in studies of L-glutamate release by the use of gas chromatograph-mass spectrometery (GCMS) (Takeuchi *et al.*, 1980). The use of this technique enabled the identification of neurally-released glutamate (Takeuchi *et al.*, 1980; Kawagoo *et al.*, 1981, 1982).

1.5 Pharmacology of glutamate receptors

Glutamate receptors (GluR) in the mammalian CNS are classified into ionotropic and metabotropic types. The ionotropic GluR are divided into N-methyl-D-aspartate (NMDA), α–amino-3-hydroxyl-5-methylisoxazole-propionic acid (AMPA) and kainate (KAIN) receptors. These receptors are named after the selective agonists which activate them (Monghan *et al.*, 1989; Watkin *et al.*, 1990). Seven subunits of AMPA/kainate receptors (GluRI - GluR7) have been cloned. Each has four transmembrane domains (Miller, 1991). Within the last few years four structurally different NMDAR have been cloned from rat brain and one from mouse brain (Mariyashi *et al.*, 1991; Meguro, 1992).

Many workers have reported that the malfunction of glutamatergic pathways can cause CNS disorders like epilepsy (Clifford *et al.*, 1990; Croucher and Bradford, 1990) and Parkinson's disease (Klokgether and Turski, 1989; and Graham *et al.*, 1990). It is possible that excitatory amino acid (EAA) receptor modulators will offer therapeutic opportunities.

Locust muscle D-receptors are very sensitive to L-quisqualate (Gration *et al.*, 1979)). In this respect they are similar to vertebrate AMPA receptors but unlike the later they are not sensitive to AMPA. LDGE, which antagonizes the quisqualate response of mammalian neurones (McLennan and Lodge, 1979; Hicks et al., 1976; Spencer, 1976) has no effect on locust muscle D-receptors (Boden, 1983). The ibotenate-

sensitive H-receptors of locust muscle do not appear to be equivalent to the ibotenate-sensitive mammalian NMDA receptors.

A subunit (DGluR-II) of a glutamate receptor in *Drosophila* embryonic muscle has been cloned and found to have 26-28% homology with the rat non-NMDA receptors (Schuster *et al.*, 1991). The cloned glutamate receptor from *Drosophila* showed low sensitivity to L-quisqualate, AMPA and kainate when expressed in *Xenopus* oocytes (Eldefrawi *et al.*, 1993).

1.6 Glutamate neurotoxicity

Lucas and Newhouse (1957) showed that high concentrations of glutamate damage retinal neurones. Injection of excitatory amino acids, particularly kainic acid, into the mammalian brain causes neuronal damage. This involves a loss of neurones, a phenomenon, called excitotoxicity (Olney, 1969, 1986). Kainic acid has been widely used by vertebrate neurobiologists to induce lesions in various parts of the central nervous system (McGeer *et al.*, 1978). This compound excites the cells of the brain and acts as a potent neurotoxin. McGeer *et al.* (1978) summarized three mechanisms which have been proposed to account for the neurotoxicity of kainate. Kainic acid metabolism in CNS might produce an unknown toxin that causes degeneration of neurones. Lakshamanan *et al.* (1974) suggested that kainic acid could release large quantities of L-glutamate from endings of glutamatergic neurones or inhibit the re-uptake of glutamate. The third mechanism

involves the action of kainate on the postsynaptic receptors. The CNS contains millimolar amounts of glutamate and aspartate, as well as other amino acids and peptides in lesser amounts, that can serve as agonists at glutamate receptors. Any process that disturbs cellular glutamate uptake or the integrity of cellular storage sites will increase extracellular glutamate. Choi *et al.* (1987) demonstrated that when cultured cortical neurones were exposed for 5 min to L-glutamate (100μM), a large proportion of them were destroyed. Such a brief, intense exposure can happen in several types of acute attacks, including hypoxia (Goldber *et al.*, 1987; Rothman *et al.*, 1984; Weiss *et al.*, 1986), ischemia (George *et al.*, 1988; Ozyurt *et al.*, 1988; Simon *et al.*, 1984), hypoglycaemia (Monyer *et al.*, 1989) and prolonged seizures (Ben-Ari *et al.*, 1985, Clifford *et al.*, 1989; Sloviter, 1983).

The channels opened by ionotropic glutamate receptors are cation channels being permeable to Na^+ and K^+. The channels opened by NMDA receptors and some AMPA/kainate receptors also permit transfer of calcium ions (MacDermott *et al.*, 1986). In locust glutamatergic system, when desensitization prevented the ionic imbalance due to influx of Ca^{++} and Na^+ through glutamate-activated channels this caused cellular damage (Duce *et al.*, 1982). The cortical neuronal injury caused by brief intense exposure of glutamate can be divided into two stages (Choi *et al.*, 1987). The first stage involves acute neuronal swelling and is dependent on the presence of extracellular sodium and chloride. It can be mimicked by depolarizing agents such as high potassium or veratidine (Choi *et al.*, 1986, Olney *et al.*, 1986; Rothman *et al.*, 1985). The activation of NMDA and kainate

receptors, in particular, is likely to be responsible for a large influx of extracellular sodium accompanied by passive chloride and water influx and resulting in cell volume expansion (Koh *et al.*, 1990). The second stage is marked by delayed neuronal disintegration and is dependent on the presence of extracellular calcium. It triggered by an influx of calcium (Choi *et al.*, 1987). Intracellular sodium may lead to calcium influx through several routes other than GluR channels, including voltage-gated calcium channels (Weiss *et al.*, 1990). Calcium may also enter via reverse operation of sodium-calcium exchanger (Nachsen *et al.*, 1986) and some cation channels activated by calcium (Partridge, 1988).

1.7 Role of calcium in glutamate neurotoxicity

It is well-established that Ca^{++} plays a central role in the regulation of a multitude of cellular functions. Calcium ions are essential in neuromuscular transmission (Fox *et al.*, 1987). An increase in intracellular Ca^{++} triggers numerous cellular events. Normally, the plasma membrane is poorly permeable to Ca^{++} ions. This ion (Ca^{++}) seems to play an important role in nerve cell physiology and patho-physiology (McBurney and Neering, 1987). Intra-cellular Ca^{++} acts as a second messenger and transduces electrical activity in neurones into biochemical events. Neuronal excitability can be directly controlled by the level of Ca^{++} ions because Ca^{++} ions gate some other ion channel such as Ca^{++} activated K^{+} channels, Ca^{++} activated monovalent cation

channels and Ca++ activated Cl^- channels (Blatz and Magleby, 1987; Owen *et al.*, 1984).

There are two basic types of mechanism used to produce an increase in Ca^{++}: (1) release from intracellular stores and (2) influx from the extracellular space. There are at least two ways by which Ca^{++} can enter the cytoplasm from intracellular stores. Agonist activation of a cell membrane receptor can activate a G-protein which in turn stimulates production of inositol 1,4,5 triphosphate (IP_3) via the phospholipase C (PLC) pathway. IP_3 then mobilizes Ca^{++} release from intracellular storage sites in the endoplasmic reticulum. Ca^{++} release from another distinct type of store is induced by cytoplasmic Ca^{++} itself. Another type of mechanism facilitates entry of Ca^{++} from the extracellular environment through (1) ligand-gated cationic channels which include the glutamate receptor/ ion channel complex and (2) voltage-activated Ca^{++} channels which can be regulated by receptor linked to G-protein.

Calcium channels that are activated by membrane depolarization, are voltage-sensitive. Voltage sensitive calcium channels (VSCCs) exist in a variety of cells including neurones, muscle, egg cells and endocrine cells. Calcium entering through VSCCs may act as an intracellular second messenger. There are several types of calcium channel. Until the mid 1980s it was generally believed that neuronal VSCCs were similar to those found in the cardiovascular system. Nowycky *et al.* (1985) demonstrated that sensory neurones, in addition to having a dihydropyridine-sensitive calcium channel similar to that found in the

heart, also have two other types of channel. Nowycky *et al.* (1985) named these channels the T- and N- types. The T type channel is insensitive to dihydopyridines. The other type of calcium channel (N-type), present in chick sensory neurones is very sensitive to blockade by cadmium.

N- type channels are sensitive to block by the peptide toxin ω–conotoxin GVIA (ω–CTX-GVIA). This toxin was isolated from the venom of the piscivorous mollusc, *Conus geogrophus* (Olivera *et al.*, 1985). Llinas *et al.* (1992) reported the distribution of P-type voltage-dependent, calcium channels in mammalian central nervous system and Mintz (1992) found that (ω–Aga-IVA), isolated from the funnel web spider , *Agelenopis aperta* is a specific antagonist for P-type channels.

1.8 Polyamine amides - philanthotoxin-343 (PhTX-343) as a channel blocker

Polyamines (putresine, cadaverine, spermidine and spermine) are present in both procaryotes and eucaryotes (Tabor and Tabor, 1964). Spermine and spermidine are present in mammalian tissue (including brain) at high concentrations (approximately 1mM). At physiological pH, polyamines have a highly charged polycationic structure in which the positive charges (protonated nitrogen atoms) are distributed along a flexible carbon chain. These positive charges mean that these polyamines can influence numerous cell processes involving negatively-charged molecules. Polyamines can bind to nucleic acids (Tabor and

Tabor, 1984), covalently bind with proteins (Park *et al.*, 1984), bind to phospholipid bilayers, promote membrane fusion (Hong *et al.*, 1983), and interact with the anionic sites of transmembrane ion channels (Koenig *et al.*, 1983a, 1983b, 1989a, 1989b). Polyamines satisfy many of the criteria for neurotransmitter status. For example, polyamine-synthesizing enzymes have been identified in nerve terminals (Schmidt and Cantoni, 1973). Polyamine release has also been demonstrated from the cerebral cortex following electrical stimulation (Russel *et al.*, 1979) and from necrotic neurones following cerebral ischaemia (Paschen *et al.*, 1992). In addition, the potentiating effects of spermine upon NMDAR (Brackley *et al.*, 1990; Rock and Macdonald, 1991) and the selective release of spermine and spermidine from the rat striatum following NMDAR activation (Carter *et al.*, 1991a) suggest that these compounds may also function as modulators of neurotransmitters (Carter *et al.*, 1991b).

The venom of some species of spiders and wasps contain cocktails of toxins co-existing with L-glutamate, acetylcholine and polyamines (Early and Michaelis, 1987; Zlotkin, 1988). Polyamine containing toxins are potentially useful pharmacological probes possessing a multitude of interesting properties that affect glutamatergic synaptic transmission. Recent rapid progress in the investigation of glutamatergic mechanisms is due, in part, to the discovery of specific chemical probes which act to affect postsynaptic responses to glutamate (Mayer *et al.*, 1987; Monaghan *et al.*, 1989). Some of these tools are natural toxins, produced by a variety of terrestrial and marine organisms. Neurotoxins of low molecular weight (<1KDa), such as

Joro spider toxin (JSTX) from the Joro spider (Kawai *et al.*, 1982; Miwa *et al.*, 1987), argiotoxin-636 from *Argiope trifasciata* (Jackson and Usherwood, 1988; Bateman *et al.*, 1985; Budd *et al.*, 1988; Kerry *et al.*, 1988; Grishin *et al.*, 1986,1989; Magazanik *et al.*, 1986; 1987; Antonov *et al.*, 1987, 1989) and a-agatoxin from *Agelenopsis aperta* (Adams *et al.*, 1989) venom are highly potent and selective antagonists of glutamate receptors. The venom of solitary digger wasp *Philanthus triangulum* contains a toxin which blocks glutamate receptors on locust muscle (Piek *et al.*, 1971). *Philanthus triangulum* attacks adult honeybees (*Apis mellifera*) and selectively injects the venom in the direction of the thoracic ganglia (Piek, 1982). This injected venom induces paralysis in the bee within a few seconds. The venom is effective in most species, only *Philanthus triangulum* itself and the mediterranean species of the digger wasp *Palerus variegatus* are immune to the poison (Rathmayer, 1962, 1978). This venom contains at least four neuroactive components known as α– β – γ and δ–philanthotoxins (PhTX) and one neuro-inactive component termed ε–PhTX (Spanjer *et al.*, 1982). Potency ratios as measured by the locust retractor unguis muscle contraction inhibition assay for β–, γ-, and δ– philanthotoxin were 1:30:100 (Spanjer *et al.*, 1982). Only β–PhTX (Karst *et al.*, 1990) and δ-PhTX (Piek *et al.*, 1988) have been chemically characterised. δ–PhTX has a butyryl/tyrosyl/spermine sequence and a molecular weight of 435. Natural δ–PhTX or PhTX-433 (numbers referring to the number of methylene groups between the amino groups of the polyamine moiety) are slightly more potent as antagonists of insect muscle glutamate D-receptors than the synthetic

analogue, PhTX-343. PhTX-433 and many of its synthetic analogues are efficient, non-competitive, reversible inhibitors of locust muscle qGluR (Eldefrawi et *al.*, 1988). Blagbrough (1990) and Piek and Nakajima (1989) have synthesized several analogues of PhTX-433 which are more active than PhTX-433. Over 100 analogues of PhTX-433 have been synthesized by Nakanishi and colleagues, with changes made in the four regions of the structure, the thermospermine (or polyamine) moiety, the tyrosyl moiety, the butyryl moiety , and the terminal amino moiety (Goodnow *et al.*, 1990; Nakanishi *et al.*, 1990; Nakanishi *et al.*, 1992). The most active analogues were characterised by a long polycation chain (polyamine), a hydrophobic region (butyryl group) and a bulky anchoring group with a moderate hydrophobicity. Reducing the polyamine chain length reduced the activity. The effect of these analogues of PhTX-433 on insect qGluR were investigated using the locust retractor-unguis muscle (Bruce *et al.*, 1990; Nakanishi *et al.*, 1992; Chiles *et al.*, 1992), locust extensor-tibiae muscle (Karts and Piek, 1991) and housefly larval muscle preparations (Benson *et al.*, 1992). All the pharmacological assays used indicate that shortening of the polyamine moiety (e.g. PhTX-433 to PhTX-43 to PhTX-4) produced a reduction in toxin potency (Bruce *et al.*, 1990; Benson *et al.*, 1992). Chemical modification of the terminal amino residue of PhTX-343 revealed that potency was increased when the terminal amino residue was replaced either by lysine (PhTX-343-Lys) or arginine (PhTX-343-Arg). The carboxylic acid groups of aspartic acid or glutamic acid that line the ion channel of qGluR provide an array of fixed negative charges (Usherwood and Blagbrough, 1989). The

increased potency obtained following the substitution of arginine for the terminal amino residue of PhTX-343 is postulated to arise from the delocalization of the positive charges of the guanidium group of arginine. This spreads the positive charge over a wider area than a primary amino terminal group and thus may be better able to accommodate the negative charges that may line the wall of the ion channel gated by qGluR (Bruce *et al.*, 1990; Nakanishi *et al.*, 1990; Chiles *et al.*, 1992).

The action of PhTX-433 on ionotropic receptors is not restricted to insect qGluR. The compound has also been found to be an antagonist of GluR of vertebrate CNS. This antagonism was studied by Schulter *et al.* (1992a, 1992b) and Brackley *et al.* (1990, 1991) on mammalian and chick brain GluR expressed in *Xenopus* oocytes. Low concentrations of PhTX-343 were reported to potentiate GluR of rat brain expressed in *Xenopus* oocytes (Brackley *et al.*, 1990, 1991; Federov *et al.*, 1993). In addition PhTX-433 and PhTX-343 have also been shown to interact with nicotinic acetylcholine receptors (nAChR) in insect CNS (Piek and Hue, 1989), and on frog muscle (Rozenthal *et al.*, 1989). Previously, analogues of PhTX-433 were demonstrated to reversibly antagonize nicotinic transmission in the CNS of cockroach (*Periplanata americana*) and locust (*Locusta migratoria*). However, philanthotoxin is approximately ten times less active at cholinergic synapses in the insect CNS than at insect glutamatergic neuromuscular synapses (Piek and Spanjer, 1986; Piek and Hue, 1989). Piek and Hue (1989) have confirmed that the length of polyamine chain is not only important in determining the potency of philanthotoxin block of insect qGluR but

also of insect nAChR. In patch-clamp studies of frog muscle, PhTX-433 was demonstrated to reduce the frequency of single channel openings induced by acetylcholine and increase the time the nAChR channels remained in the closed state (Rozenthal *et al.*, 1989). The ability of PhTX to interact with both nAChR and GluR suggest that there might be structural similarities between these receptors.

1,9 The *Xenopus* oocyte as a model for mRNA expression

Mature *Xenopus* oocytes have diameters of 1 to 1.2mm. They are surrounded by a noncellular, fibrous vitelline layer and by several layers of follicular cells (Dumont *et al.*, 1978). *Xenopus* oocytes have been extensively used to study biogenesis, functional architecture, and modulation of plasma membrane protein. In particular, the *Xenopus* oocyte is a standard tool for the expression of mRNAs encoding membrane receptors or ion channels. In 1971, Gurdon *et al.* reported that *Xenopus* oocytes translate foreign mRNA. *Xenopus* oocytes are capable of translation and post-translational modification (Ghysdael *et al.*, 1977), phosphorylation (Gedamu *et al.*, 1978) and glycosylation (Colman *et al.*, 1981; Lund *et al.*, 1986). A *Xenopus* oocyte can also assemble the subunits of a receptor and insert them into its surface membrane (Parker *et al.*, 1985) (Fig. 1.4).

Sumikawa *et al.* (1981) demonstrated the presence of binding sites for α–bungarotoxin when oocytes were injected with mRNA from the electric organ of *Torpedo marmorata*. Barnard *et al.* (1982) presented

the first electrophysiological evidence for the expression of functional nAChR in *Xenopus* oocytes. Expressed protein can be detected as long as two weeks after RNA injection (Lerma *et al.*, 1988).

The *Xenopus* oocyte technique has been extended to examine the properties of other receptors. Gunderson *et al.* (1983, 1984, 1986); Miledi *et al.* (1982); Sumikawa (1984); Parker *et al.* (1984, 1986) and, Sholfield *et al.* (1987) reported the successful expression of serotonin, dopamine, GABA and glycine receptors. Saito *et al.* (1987) reported for the first time that *Xenopus* oocytes successfully express glutamate receptors from lobster (*Palinurus japonicus*) abdominal muscle. L-glutamate responses in oocytes injected with lobster muscle RNA have been also reported by Kawai *et al.* (1989). Fraser *et al.* (1990) published that amino acid receptors expressed in *Xenopus* oocyte after microinjection of mRNA from the leg of locust *Schistocerca gregaria.* Marshal *et al.* (1990) found the complete sequence of a locust (*Schistocerca gregaria)* nervous system nAChR subunit (alpha-L1). RNA derived from alpha-L1 cDNA when injected alone in *Xenopus* oocyte resulted in the expression of functional nAChR. Arena *et al.* (1991) recently reported that *C. elegans* poly A^+ RNA injected into *Xenopus* oocytes expresses L-glutamate-sensitive chloride channels.

1.10 AIM

In the first section of this thesis the effects of synthetic analogues of PhTX-433 on glutamate-gated calcium entry into arthropod excitable tissue has been investigated to assess the role of polyamine amides as preventive excitotoxic agents. The second part of thesis deals with the effects of limited range of philanthotoxins on neuromuscular transmission in a locust and a tick. Finally, attempts have been made to express mRNA from arthropod excitable tissue in *Xenopus* oocyte in order to study the pharmacology of arthropod transmitter receptor proteins.

Fig. 1.1 Geographical distribution of the Desert Locust (above) and the African Migratory Locust (below). (Based on the information from The Centre for Overseas Research, Barrass, R. The Locust 2nd ed.).

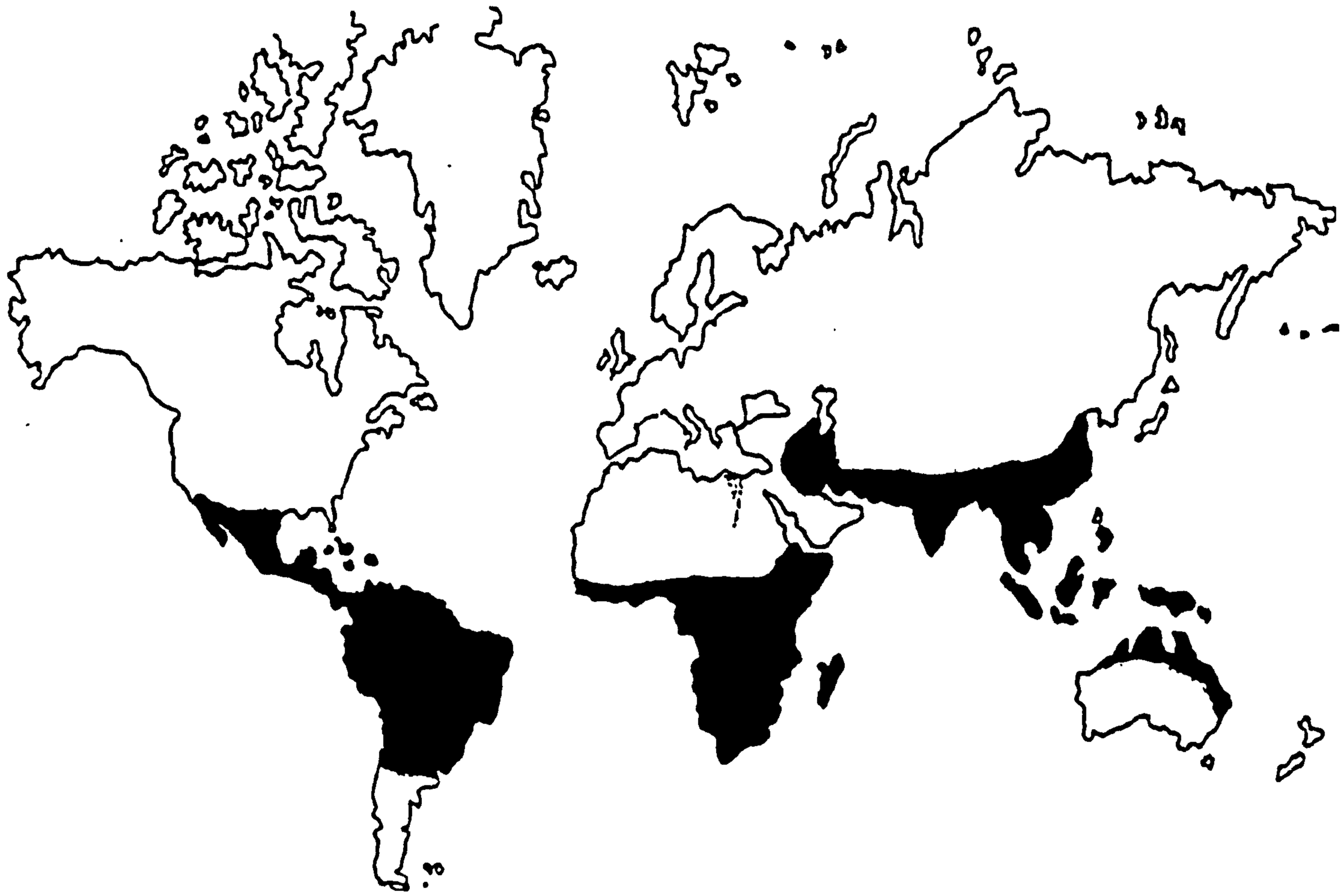


Fig. 1.2 Geographical distribution of *Boophilus microplus* (Nunez *et al.*, 1985).

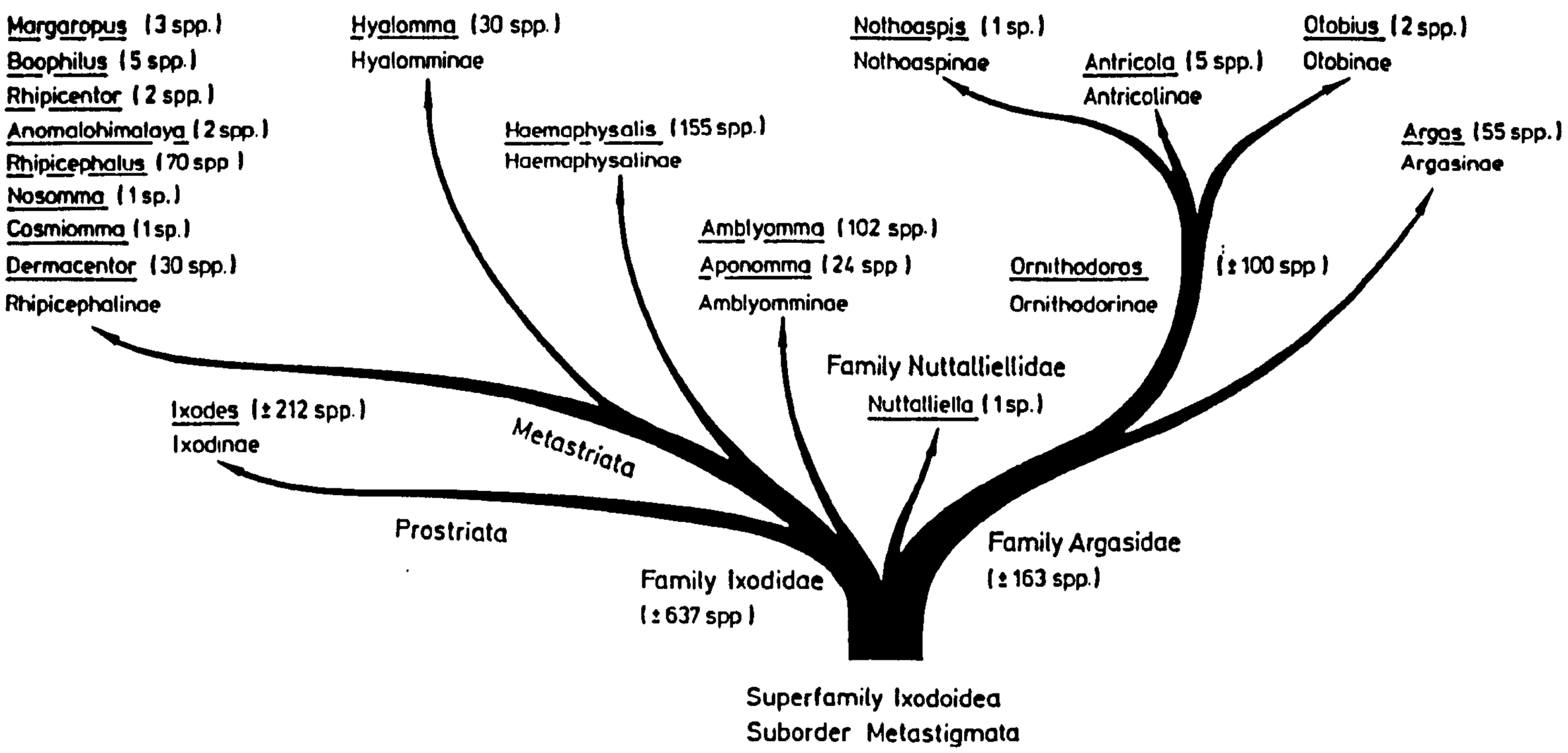


Fig. 1.3 Classification of the Acarina order (Hoogstrall, 1981)

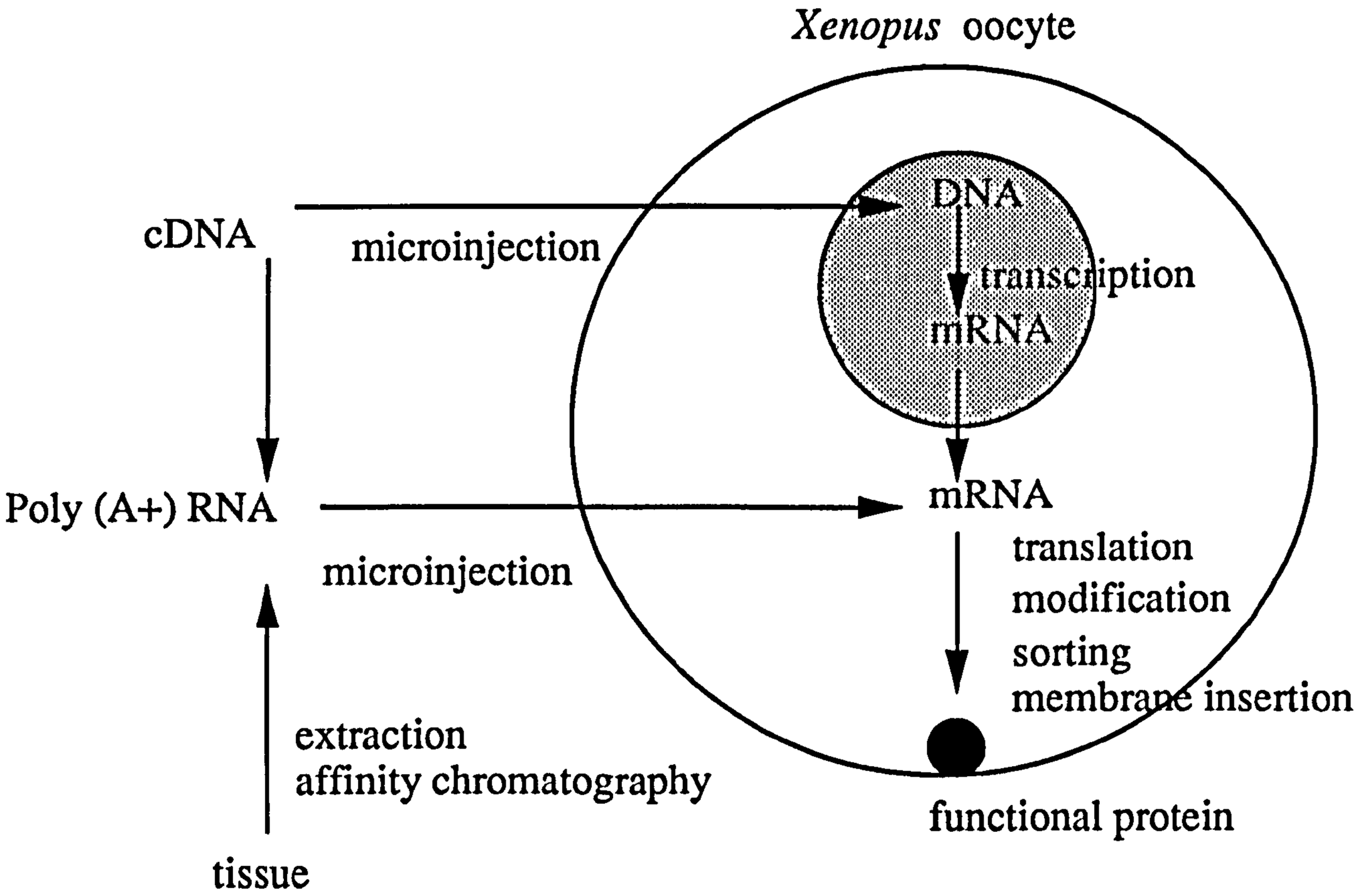


Fig. 1.4 Scheme illustrating functional plasma membrane protein expression in a *Xenopus* oocyte.

Fig. 1.5 A summary of the changes in toxin potency following structural modifications in the appropriate moieties of PhTX-433. (A: Spermine or polyamine moiety; B: tyrosyl moiety; C: butyryl moiety and; D: spermine amino terminal group.

CHAPTER TWO

MATERIALS & METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Locusts (*Schistocerca gregaria*) (Fig. 2.1) were reared in the insectary of the Life Science Department, Nottingham University. They were maintained between 28^0C- 30^0C at a relative humidity of 51-53% on a 12h light/dark cycle. They were fed on fresh-grown wheat supplemented with bran.

Adult male ticks (*Rhipicephalus appendiculatus*) (Fig. 2.3) and the eggs of *Boophilus microplus* were supplied by Schering Agrochemicals plc., Chesterford Park, U.K. Adults were maintained at 4^oC in the refrigerator and the eggs were routinely preserved in liquid nitrogen and kept in freezer at -80^oC prior to use.

Female *Xenopus laevis* (Fig. 2.9) were obtained from Blades Biological, Kent, and maintained in 20-gallon glass tanks of circulating, clean, chlorine-free water. The tanks were divided by perspex sheets to keep the animals separate, so that individuals could be identified. This was necessary because the quality of oocytes differed from animal to animal. The animals were exposed to a 12-hour light/dark cycle at 20^0C-22^0C and fed on small pieces of fresh beef heart, mealworms, maggots and locust hoppers.

Wasp Toxin

The analogues of philanthotoxin-433 (PhTX-343, PhTX-343-Arg, PhTX-4, DNP12-PhTX-343 and, C7PhTX-343) were synthesized by Professor Koji Nakanishi and his colleagues at Columbia University, New York (Fig. 2.13). Toxins were diluted in distilled water and the stock solutions were kept at -20°C prior to use.

Chemicals

Radiochemicals $[^{45}Ca]Cl_2$ was obtained from Amersham International and made up to 1000µl in nanopure water and kept in the refrigerator at 4°C. The specific activity of $^{45}Ca^{++}$ was between 0.37 - 1.5 GBq/mg calcium.

Biochemicals Unless otherwise stated, all biochemicals were obtained from Sigma chemical company, U.K.

Water All water used during this work was distilled and subsequently deionised, with a Barnstead "NANOpure II" system.

2.2 Methods

2.2.1 The study of $^{45}Ca^{++}$ uptake in extensor tibiae muscle of locust

Metathoracic legs (Figs. 2.2a, 2.2b) of female locusts (*Schistocerca gregaria*) were dissected in petri dishes, containing Sylgard (Dow Corning) resin. The extensor tibiae muscle was exposed by cutting along the ventrolateral edges of the femur and removing the overlying cuticle. The flexor tibiae muscle and the retractor unguis muscle, together with the two main tracheal trunks and the sheet of connective tissue which overlies the extensor tibiae muscle were removed. This procedure exposed the fine tracheal branches which run close to the bundles of extensor tibiae muscle. The extensor tibiae muscle fibres run diagonally across the axis of the leg and are attached to a central apodeme which extends through the femur and terminates in the cuticle of the tibia (Fig. 2.2c). Muscles were washed and incubated in standard locust saline (see Experimental Saline) for 10 min prior to experiment.

To study the effect of Con A on calcium uptake, muscles were treated with Con A (1μM) for 30 min before incubating in $^{45}Ca^{++}$. A solution (standard locust saline) containing $^{45}Ca^{++}$ was added to the dishes to give 0.037MBq/ml, final activity, and muscles were incubated for a further 3 min. The muscles were then treated with various concentrations of L-glutamate (10^{-2}M-10^{-10}M) for various times to study the glutamate-induced influx of Ca^{++}. Locust saline were added to control muscles. Control muscles were incubated in

$^{45}Ca^{++}$ for same time as L-glutamate. Analogues of L-glutamate were also applied in same fashion. To assess the effect of depolarising of the muscle on entry of Ca^{++} through voltage-gated calcium channels, the effect of elevating extracellular K^{+} to 50mM was tested. The effects of philanthotoxins or dihydropyridines were examined by incubating the muscles for 20 min in different concentrations of drugs following Con A treatment. The muscles were then depolarized with glutamate (10^{-4}M) or KCl (50mM) for 10 min to investigate the effect of these compounds on calcium uptake via glutamate-gated channels and voltage-gated calcium channels.

Incubation in $^{45}Ca^{++}$ was terminated by three 20s washes in cold (6^{o}C) locust saline and a final wash with ethanol. The ethanol was discarded and the muscles were detached from cuticle with a scalpel and left overnight to dry. Muscles were weighed using a Cahn 25 automatic electrobalance and then incubated in 350µl Triton X 100 (1% v/v) for 3-4h in a scintillation vial. After 3-4h, 4 ml of aqueous scintillation fluid (Packard "Emulsifier safe") was added and the radioactivity level was determined by liquid scintillation counting (Packard, 1900 TR). Results were corrected to cpm/mg dry muscle weight.

2.2.2 Recording of excitatory postsynaptic potentials from locust and tick leg muscles

2.2.2a Tick (*R. appendiculatus*) nerve muscle preparation

Un-engorged adult male tick (*R. appendiculatus*) were used for electro-physiological recordings. They were pinned ventral side down in a Sylgard petri dish and dissected in tick saline (see Experimental Saline). A cut was made along the posterior and lateral margins of the sclerotised scutal plate. The scutum was folded anteriorly and the gut, heart, and salivary glands were removed with the help of fine forceps to expose the synganglion and coxal muscles (Figs. 2.4 and 2.5). In common with most other arachnids the ganglia of tick nervous system is highly condensed into a peri-oesophageal mass or synganglion (Horridge, 1965) which is enclosed within a sinus of the circulatory system. The preparation was transferred into a perfusion chamber (0.5 ml) and perfused continuously with tick saline. Intracellular electrodes were inserted through the membrane of a single coxal muscle fibre and the cells were allowed to equilibrate for 10 min.

2.2.2b The locust (*Schistocerca gregaria*) nerve-muscle preparation

Electrophysiological experiments were performed on the metathoracic extensor tibiae muscle. The metathoracic extensor tibiae muscle was exposed by first removing the cuticle on the

ventral side of the femur, and then removing the flexor tibiae muscle (Fig. 2.2c). When exposed, the muscles were maintained in standard locust saline.

2.2.2c Electrophysiology

Glass microelectrodes (GC 150 TF-10, Clark Electromedical Instruments) were used for intracellular electrophysiological recordings from extensor tibiae muscles of locust (Fig. 2.6) and from coxal muscles of tick (Fig. 2.4). Microelectrodes were pulled using a moving coil microelectrode puller (753-Campden instrument) and then filled with 3M KCl. Electrodes (2-5 MΩ) were selected for use in order to obtain recordings with low noise levels. A silver chloride pellet reference electrode was connected to the bath saline. Preparations were perfused continuously at a fixed flow rate (4-5ml/min). The motor nerve supplying the muscle under study was taken up in a suction electrode which was connected to an isolated stimulator connected to a pulse generator. Pulses were normally delivered at 1 Hz (2-3 volts, 0.6 ms). A tight fit between the suction electrode and the cut end of the nerve was essential for stable stimulation. Suction electrodes were made from microelectrodes, the tips of which were broken under microscope to give a required diameter and the tips were then polished by heat. This procedure produced smooth tips, thus minimizing the possibility of nerve damage. Excitatory postsynaptic potentials (EPSPs) were recorded (Fig. 2.7) using a VCR (Sony, SL-F 25 UB PAL) and digital pulse control modulator (Sony, PCM 701 ES).

2.2.3 Expression of neurotransmitter receptors in *Xenopus* oocytes

2.2.3a Preparation of reagents for RNA Isolation

The following reagents (I-IV) were prepared for RNA extraction.

I. Water-saturated phenol

For the isolation of RNA standard phenol was purified in the following way. Phenol (BDH, Crystal) crystals were melted in a beaker in a water bath at 68°C. When melted, 0.1% 8-hydroxyquinoline was added. This compound is an antitoxidant, a partial inhibitor of RNAase and a weak chelator of metal ions. In addition, the yellow colour provides a convenient way to identify the phase. Approximately an equal volume of sodium acetate (50mM, pH 4) was added to the phenol, shaken for 15s and left to settle, with the aqueous phase on top and the phenol phase on the bottom. The aqueous phase was removed very carefully using a pipette and another volume of sodium acetate was added. This procedure was repeated several times until the aqueous phase reached pH 4.

II. Preparation of Solution D

Solution D (denaturing solution) consisted of 4M guanidium thiocyanate (Fulka), 25mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1M 2-mercaptoethanol. To reduce the hazardous effect of

guanidium thiocyanate the solution was prepared in the manufacturer's bottle. All of the procedures were undertaken in a waterbath (65°C) in a fume cupboard.

III. A sodium acetate (2M) solution at pH 4.5 (adjusted with glacial acetic acid) was prepared.

IV. A mixture of chloroform : isoamyl alcohol (49:1) was prepared.

2.2.3b Extraction of RNA

Total RNA was extracted from rat brain and from 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19-day eggs of *Boophilus microplus*. The method used was described by Chomczynski and Sacchi (1987) (Fig. 2.8). Boophilus eggs were rapidly frozen in liquid nitrogen and stored at -80°C. At the time of extraction the eggs were weighed and 1g of eggs were homogenised in 4M guanidium thiocyanate. This solution is an RNAase inhibitor. A phenol- chloroform extraction method was used to separate RNA from protein using reagents I -IV described above. A second precipitation step with isopropanol was undertaken and then the RNA was resuspended in 75% ethanol / 0.3M sodium acetate solution. There then followed a third precipitation step. The resultant pellet was washed in 95% ethanol and then desiccated before being re-suspended in autoclaved, DEPC-treated, sterile water. mRNA was purified by using Dynabead oligo-dt (deoxythymidine) column. The quality of RNA and mRNA was determined, (I) by checking optical density using a

spectrophotometer, (II) using a 1% agarose gel electrophoresis, and (III) by *in vitro* translation in rabbit reticulocyte lysate.

2 2.3c *In Vitro* translation. The mRNA and total RNA obtained from different ages of *Boophilus* embryos were translated *in vitro* using rabbit reticulocyte lysate (Amersham). L-[^{35}S]Methionine was used to label the newly-synthesized protein. A 2µl of sample of RNA was mixed with 12µl of rabbit reticulocyte lysate and 2µl L-[^{35}S]Methionine in 0.5 ml pre-cooled eppendorf tubes. The tubes were placed in water bath at 30^{0}C. After 10, 30 and 60 min 5µl of sample was added to 0.5ml of a 1M sodium hydroxide/ hydrogen peroxide mixture in 5ml glass tubes. The tubes were placed in a water bath for next 10 min to hydrolyse aminoacyl tRNA complexes. The tubes were then removed to an ice bath. 1ml of 25% trichloroacetic acid solution (containing caesin hydrolysate) was added, mixed by inversion and left for a minimum of 30 min. Finally, the contents of the tubes were passed through filter discs. Ice cold 8% trichloroacetic acid was used to wash the filter discs to remove unreacted labelled amino acid. The radioactivity of discs were counted by liquid scintillation counting.

2.2.4 Harvesting *Xenopus* oocyte and microinjection

Partial ovivectomy was performed to obtain oocytes for microinjection. Female *Xenopus* were anaesthetised by placing them in 500ml of 0.2% anaesthetic (3-aminobenzoic acid ethyl ester) for 30 min (Hamburger, 1960). To avoid the risk of

contamination, the oocytes were isolated in a lamina flow cabinet and sterilized cotton, disposable surgical gloves and petri dishes were used. Forceps and scissors were soaked in 75% Methcol and flame-sterilized prior to use.

Before an operation, the animal was rinsed thoroughly with distilled water and placed in the lamina flow cabinet on its back. An incision in the skin (about 1 cm) was made on either the left or right side of the lower part of abdomen to expose the abdominal wall. The abdominal wall was then cut carefully to expose the ovarian lobes. Oocyte lobes was removed and placed in petri dishes containing Barth's medium. The abdominal wall and outer skin were then stiched, using sterile silk suture (MERSILK).The animal was then rinsed and placed in a recovery tank containing distilled water before being transferred to the aquarium.

For microinjection, oocytes were separated mechanically by using a pair of forceps. The healthiest oocytes of stage 6 were selected and transferred to fresh Barth's medium (see Experimental Salines). These were about 1 mm in diameter, dark brown at the animal pole and bright yellow/green at the vegetal pole (Fig. 2.10). An equatorial band between animal pole and vegetal pole was a good indication of the quality of oocytes. After selection, the oocytes were maintained for 5-6 h before injection.

10µl Drummond glass microelectrodes (Drummond Scientific Corp.) were pulled and the tips were broken off by flame sterilized forceps to give a sharp tips of 15 µm diameter. The pipettes were filled with sterile-filtered liquid paraffin by using a syringe and

mounted on a Drummond microdispenser. RNA (1-2µl) was placed in a sterile petri dish and loaded in the pipette. An interface between liquid paraffin and aqueous RNA confirmed the successful loading. Each oocyte was transferred to a petri dish containing a well to support it during microinjection. Oocytes were injected very carefully by inserting the pipette tip (Fig. 2.11). Depending on the nature of experiment, 50-100 nl of RNA was injected per oocyte. Control oocytes were injected with sterile distilled water. After microinjection of RNA, oocytes were maintained at 18°C in Barth's modified media under aseptic conditions for 3-4 weeks. The saline was changed twice every day under sterile conditions.

To study expressed receptors, oocytes were defolliculated by removing the outer layers (Dumont and Brummet, 1978) either mechanically or enzymically (Colman, 1984), leaving the plasma membrane and the vitelline membrane intact (Fig. 2.10).

2.2.5 Electrophysiology

The microinjected oocytes were transferred to a perfusion bath containing experimental saline flowing at 3ml/min (Fig. 2.12). Two small pins were placed in the bath to hold the oocytes in position. The expression of neurotransmitter receptors was examined from 1 - 21 days post-injection using a two-electrode voltage clamp (Axoclamp 2A, Axon Instruments) Electrodes (GC 150TF-10, Clark Electromedical Instruments) were pulled to give a resistance between 0.8 MΩ -1 MΩ and were filled with 3 M KCl. Oocytes

were impaled at the vegetal pole, and after electrode penetration, 10-15 min were allowed for the cell to seal around the micropipette. Stable resting membrane potentials of -50mV to -70mV were obtained. The oocytes were usually clamped at -60mV (Fig. 2.12) and agonists were applied in ascending concentration to test the expression of neurotransmitter and currents were recorded using a digital pulse code Modulator (Sony, PCM 701 ES) and a VCR (Sony, SL-F 25 UB PAL).

Experimental salines

1. Standard locust saline

NaCl 180mM, KCl 10mM, $CaCl_2$ 1mM, HEPES 10mM, pH 6.8

2. Frog Ringer

NaCl 115mM, KCl 2.5mM, $CaCl_2$ 1.8mM, HEPES 10mM pH 7.4 with 2M NaOH.

3. Oocyte "deactivation" saline (OR_2 saline) :

This is a calcium-free saline of the following composition:

NaCl 82.5mM, KCl 2mM, $MgCl_2$ 1mM, HEPES 5mM, pH7.5 (Barnard and Bilbe, 1987). Double distilled water was used for this saline and the pH was adjusted with 2M NaOH. Saline was stored in autoclaved bottles after being pressure filtered through Whatman 0.2µm filters.

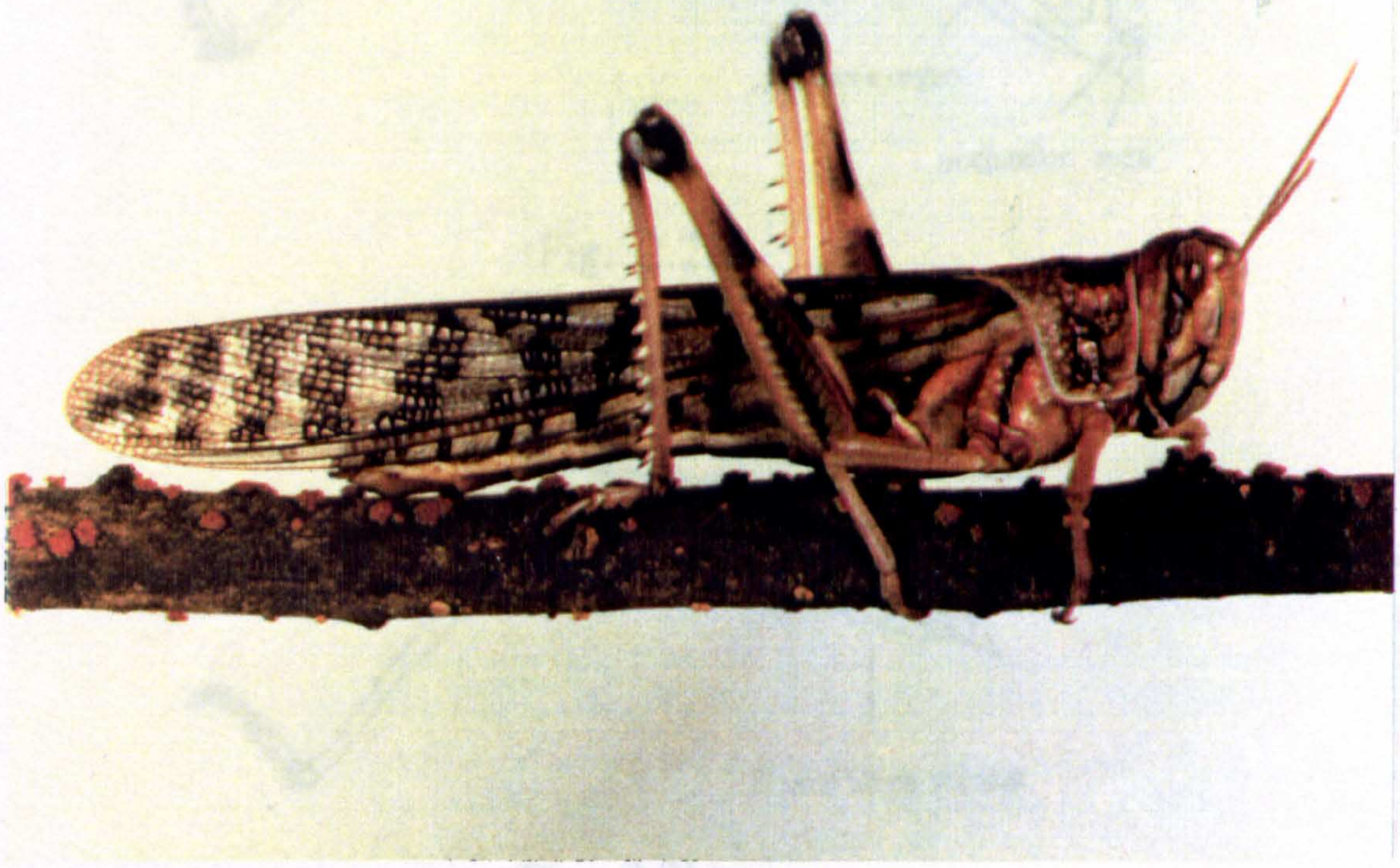
4. Incubation medium (Barth's modified medium):

Before and after microinjection, oocytes were maintained in autoclaved, Barth's modified medium of the following composition (Barnard and Bilbe, 1987) :

NaCl 88mM, KCl 1mM, $Ca(NO_3)_2.4H_2O$ 0.33mM, $NaHCO_3$ 2.4mM, $MgSO_4$ 0.82mM, $CaCl_2$ 0.41mM, HEPES buffer 15mM, Na pyruvate 1mM, gentamycin 20mg/lit, penicillin/streptomycin 4ml of 500unit/ 2000ml; pH 7.4.

5. **Tick saline:** Tick saline (Hart, 1982) was modified by reducing the $CaCl_2$ and $MgCl_2$ concentration from 20 mM to 5 mM and 1.5mM to 1 mM respectively. The saline had the following composition:

NaCl 200 mM, KCl 5mM, $MgCl_2$ 1mM, $CaCl_2$ 5mM, HEPES 2mM, pH 6.9.



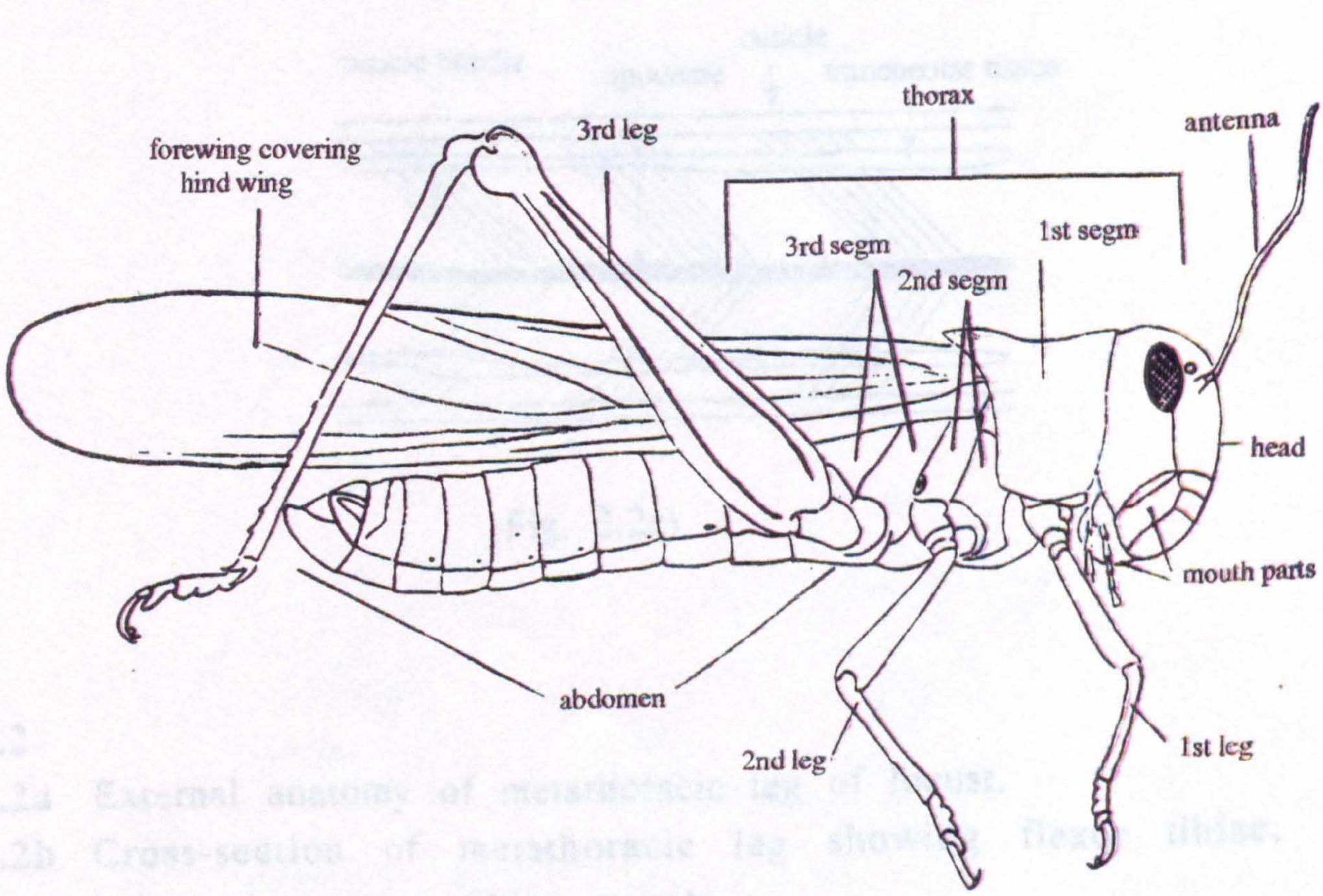
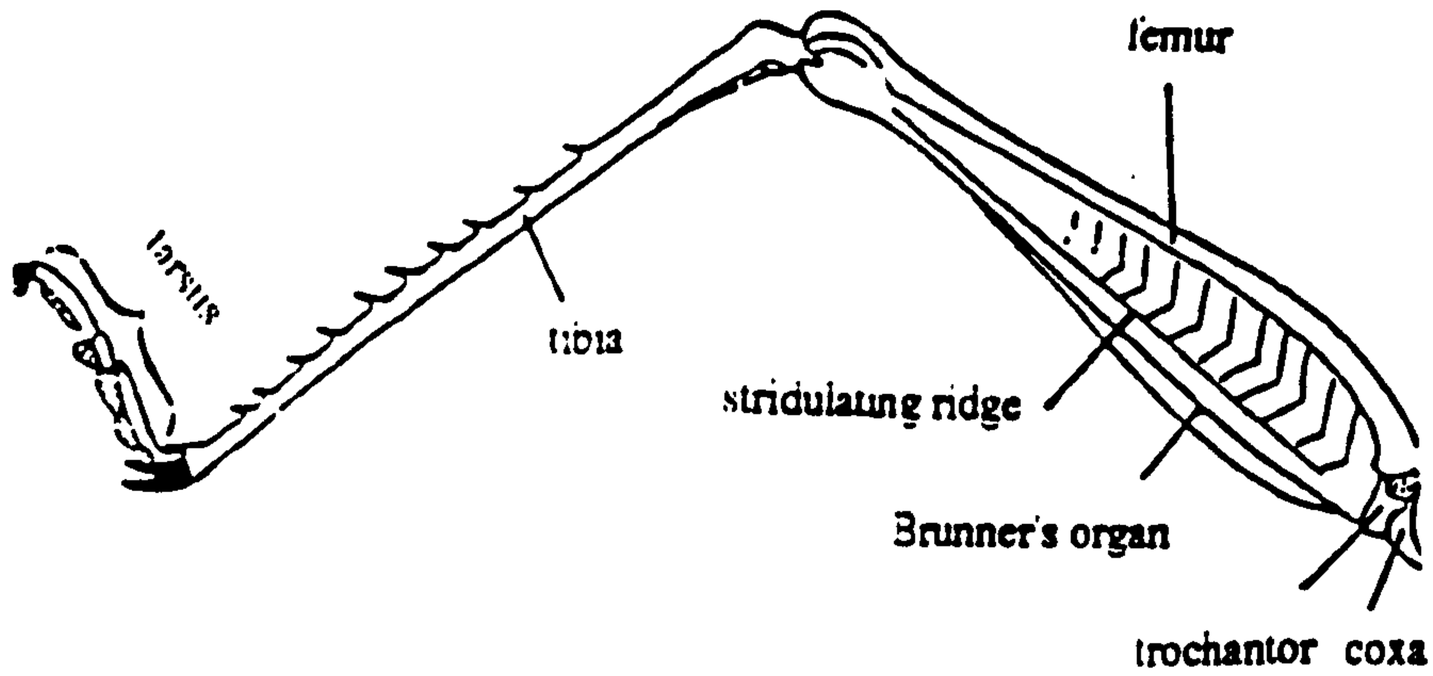
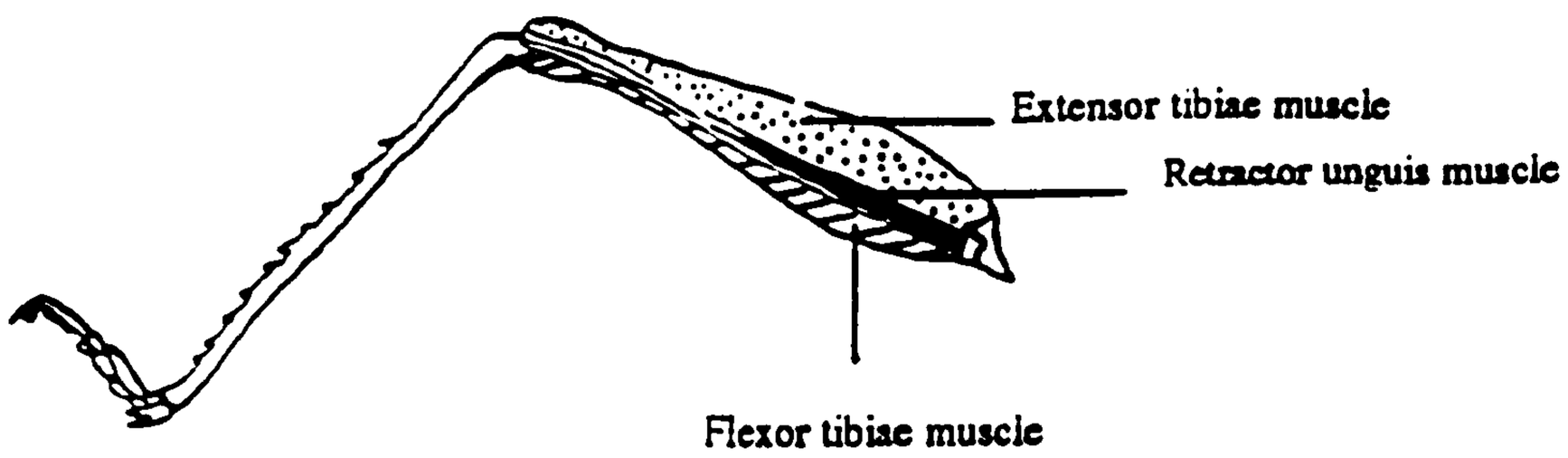


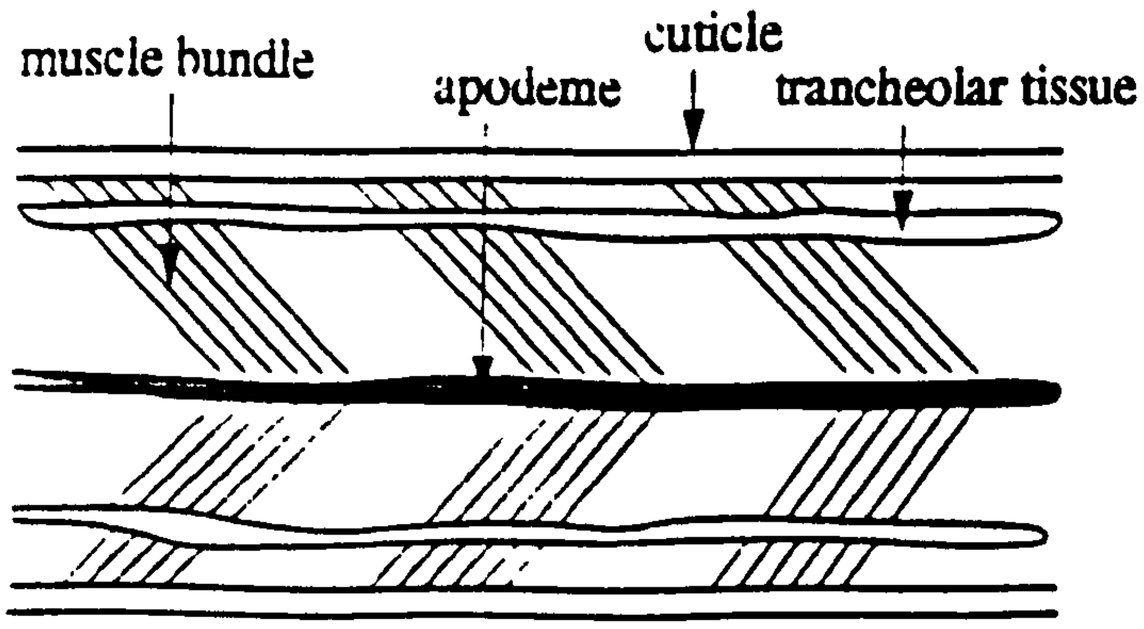
Fig. 2.1 Side view of an adult locust (*Schistocerca gregaria*).



(Fig. 2.2a)



(Fig. 2.2b)



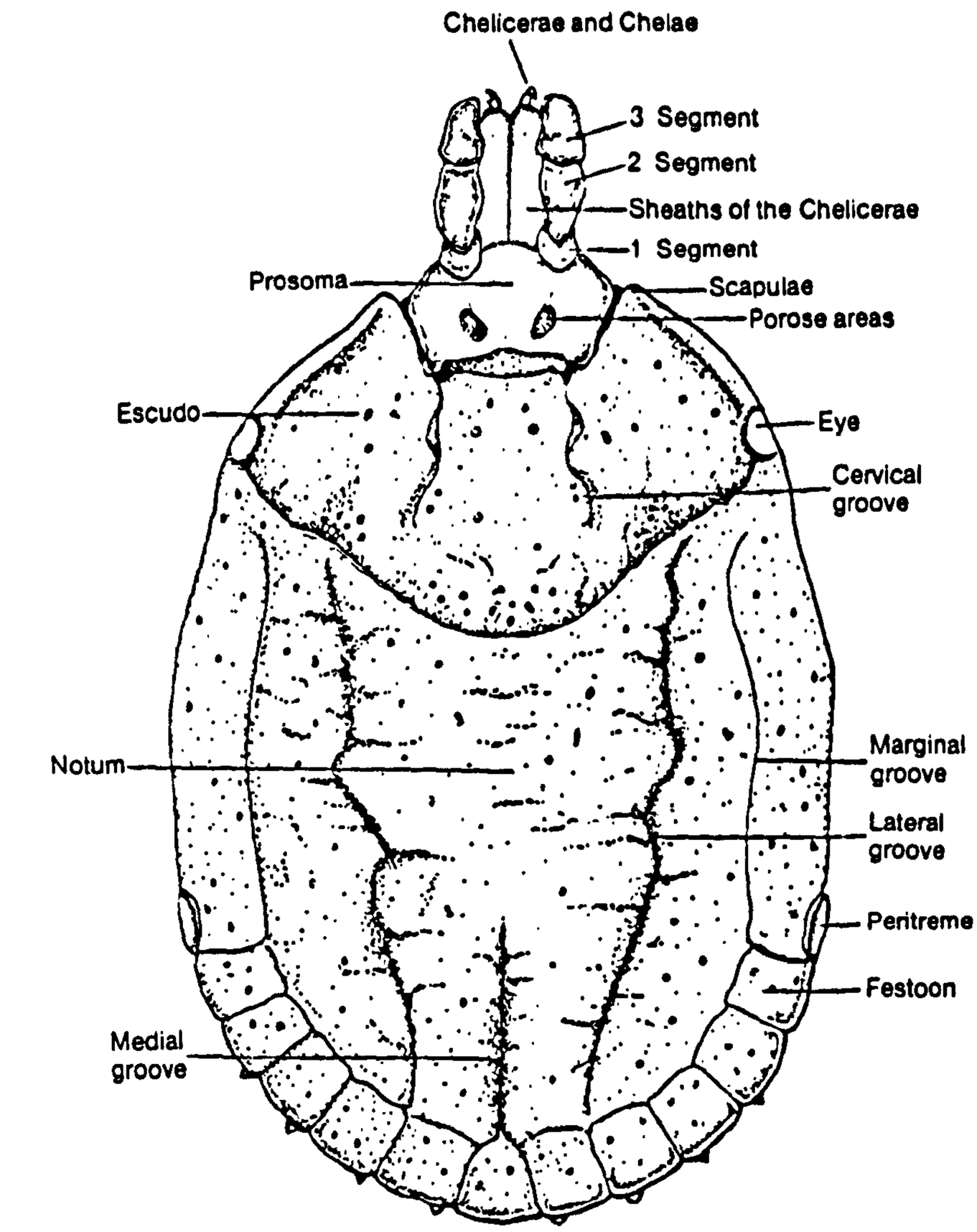
(Fig. 2.2c)

Fig. 2.2

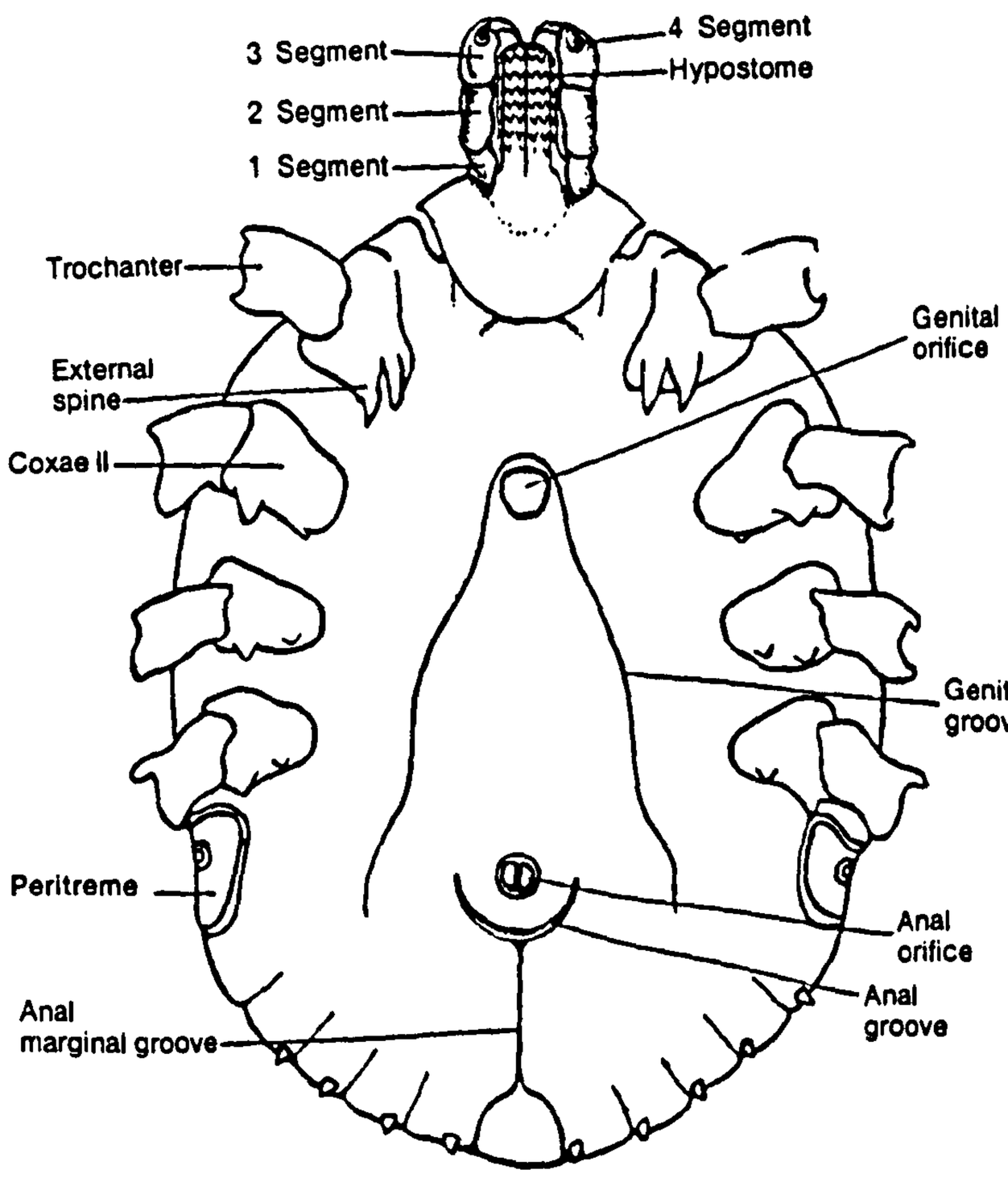
Fig. 2.2a External anatomy of metathoracic leg of locust.

Fig. 2.2b Cross-section of metathoracic leg showing flexor tibiae, retractor unguis and extensor tibiae muscle.

Fig. 2.2c A schematic diagram of the extensor-tibiae muscle preparation. Each muscle bundles contains approximately 10 to 20 individual muscle.



(Dorsal view)



(Ventral view)

Fig. 2.3 External morphological characteristic of the female Ixodidae.

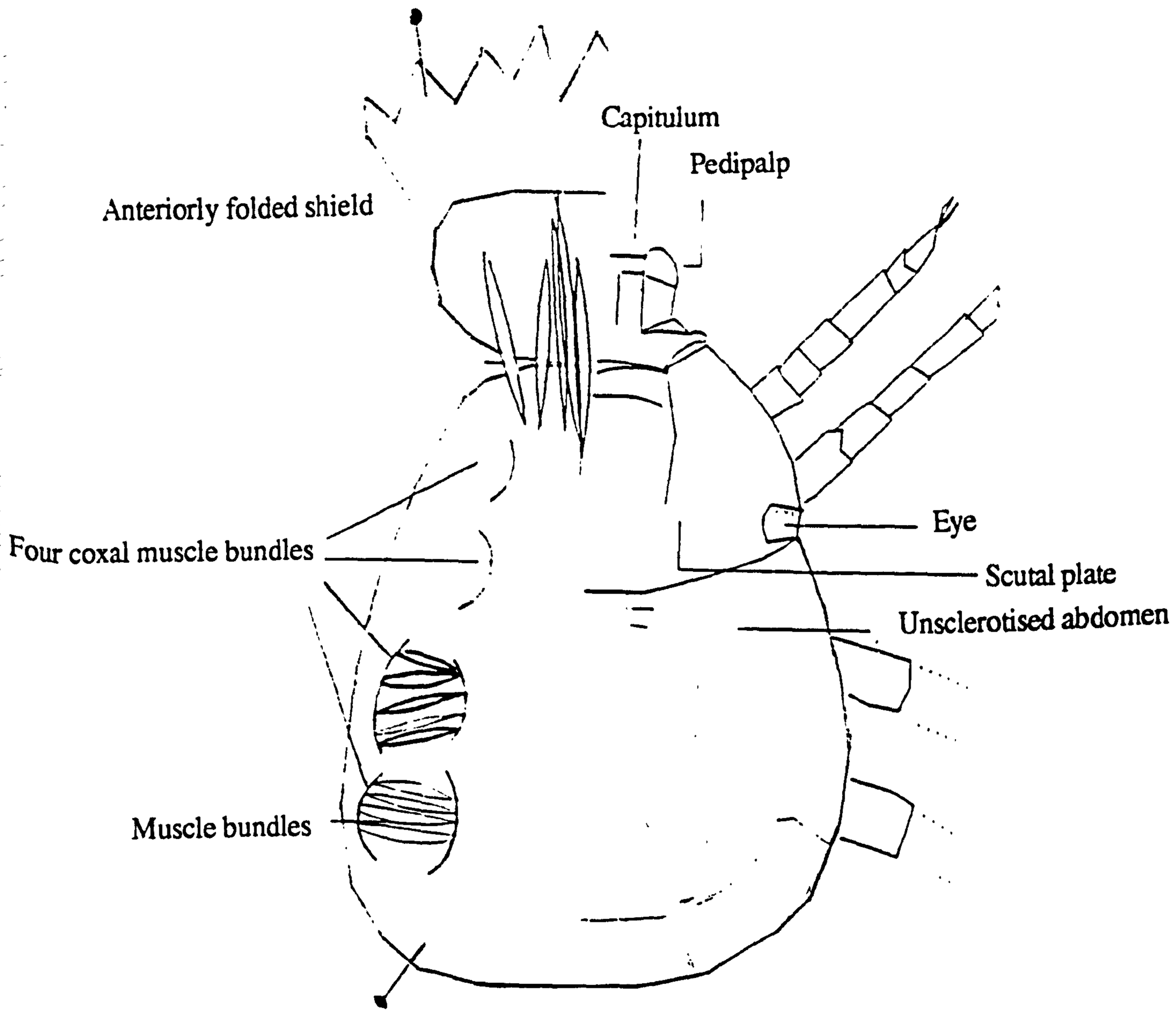


Fig. 2.4 A schematic diagram of a dissected female tick (*Rhipicephalus appendiculatus*). Left side shows the the bundles of exposed retractor muscles and the coxal muscles

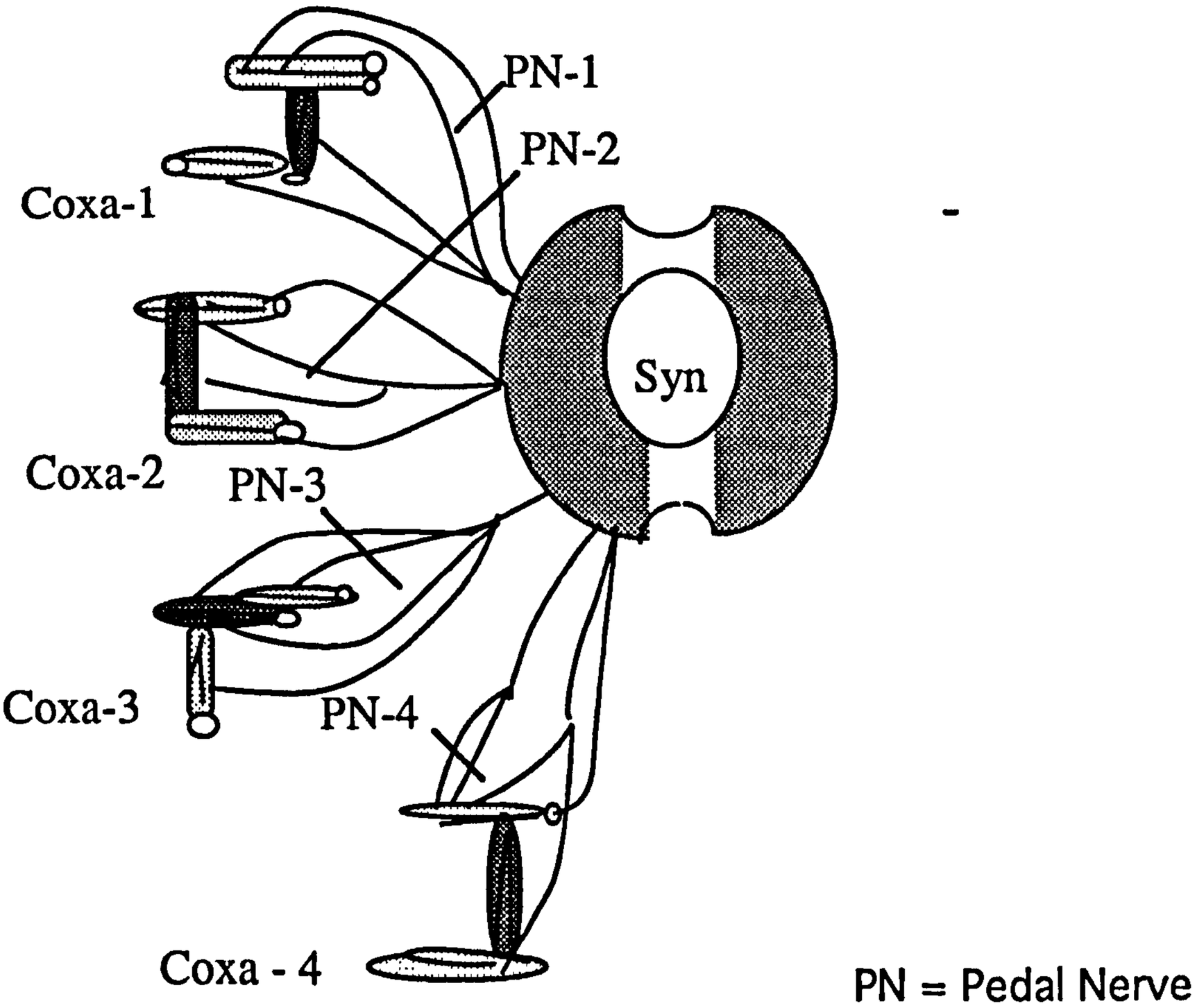


Fig. 2.5 Diagrammatic view of a tick's synganglion (Syn), coxae (1-4) and the nerves (PN-1-4) innervating in coxal muscle.

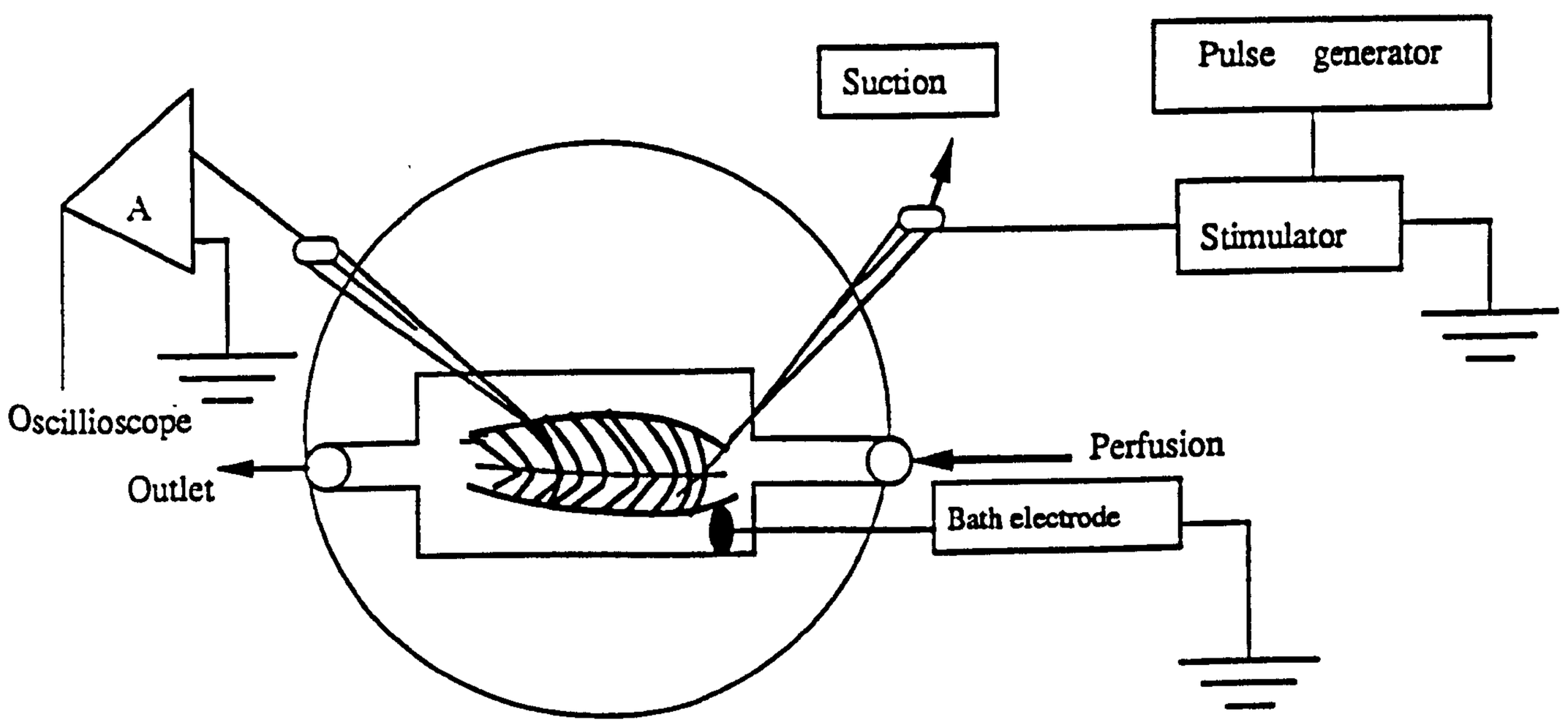


Fig. 2.6 Recording of EPSPs from locust extensor tibiae muscle. The system maintained a constant volume of saline (0.5 ml) in the perfusion chamber and a flow rate of 1-3 ml/min. The amplifier (A) measures the membrane potential (Vm) of muscle by recording the difference between the bath electrode and voltage electrode.

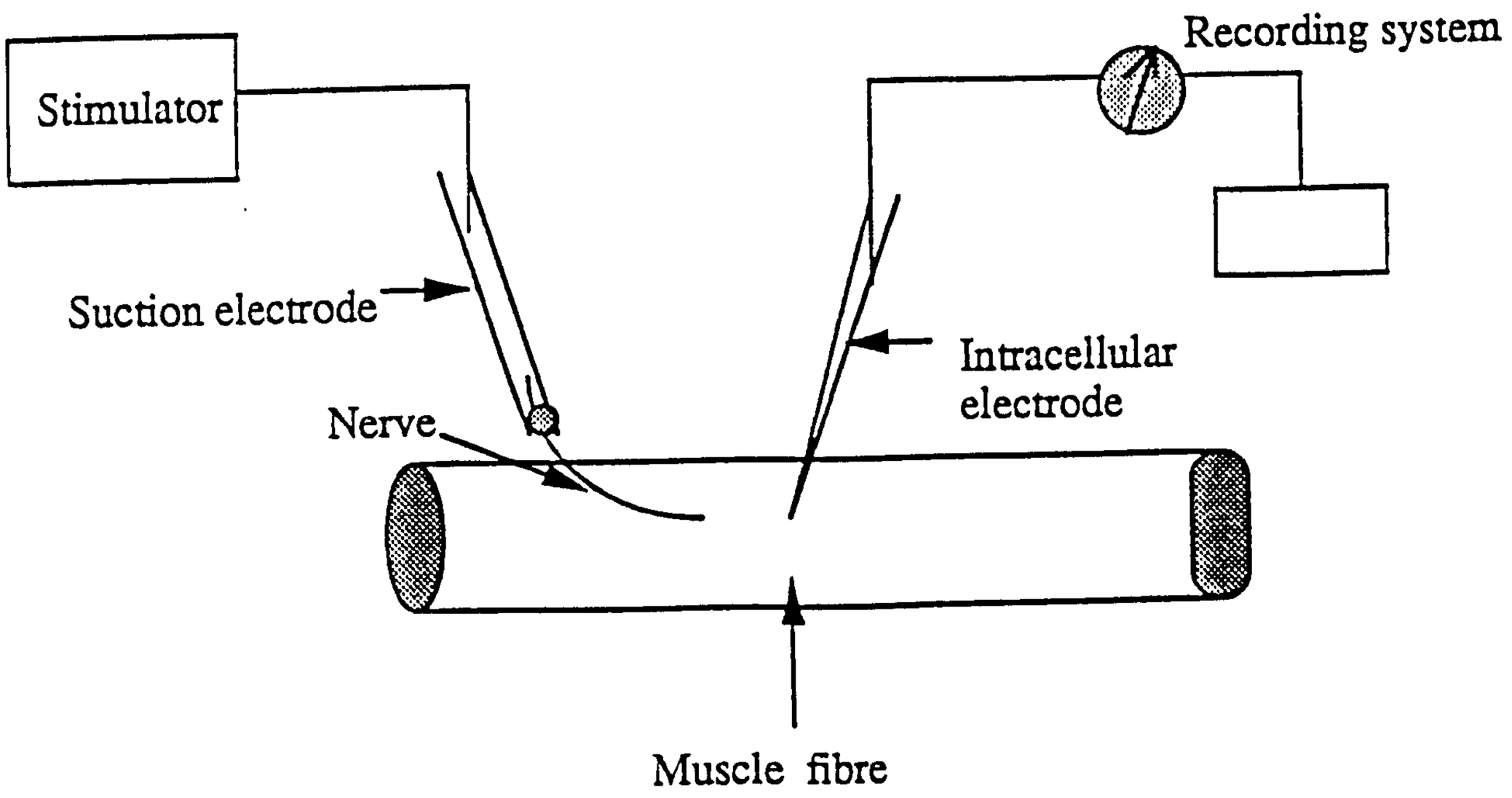


Fig. 2.7 Schematic diagram of intracellular recording from a single muscle fibre of locust, using a glass microelectrode filled with 3MKCl.

Tissue homogenised in 4M guanidium isothiocyanate (GITC) soln

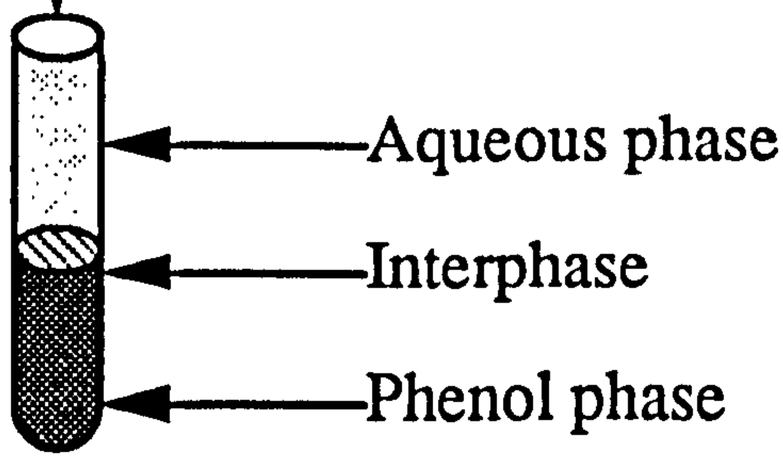
↓

1. Antifoam agent (200 µl)
2. 1ml of 2 M sodium acetate
3. 2ml chloroform/isoamyl alcohol(49:1)
4. 10 ml acid phenol

↓

Incubated in ice for 15 min and centrifuged
at 10,000g, at 4 C for 20 min.

↓



↓

Aqueous phase + same volume of isopropanol, centrifuged

↓

RNA pellet resuspended in GITC, re-precipitated

↓

RNA pellet washed with 75%, 95% ethanol and,
vacuum desiccated.

↓

RNA pellet re-suspended in DEPC-
treated distilled sterile water.

Fig. 2.8 A single step method for RNA extraction (Chomczynski and Sacchi, 1987)

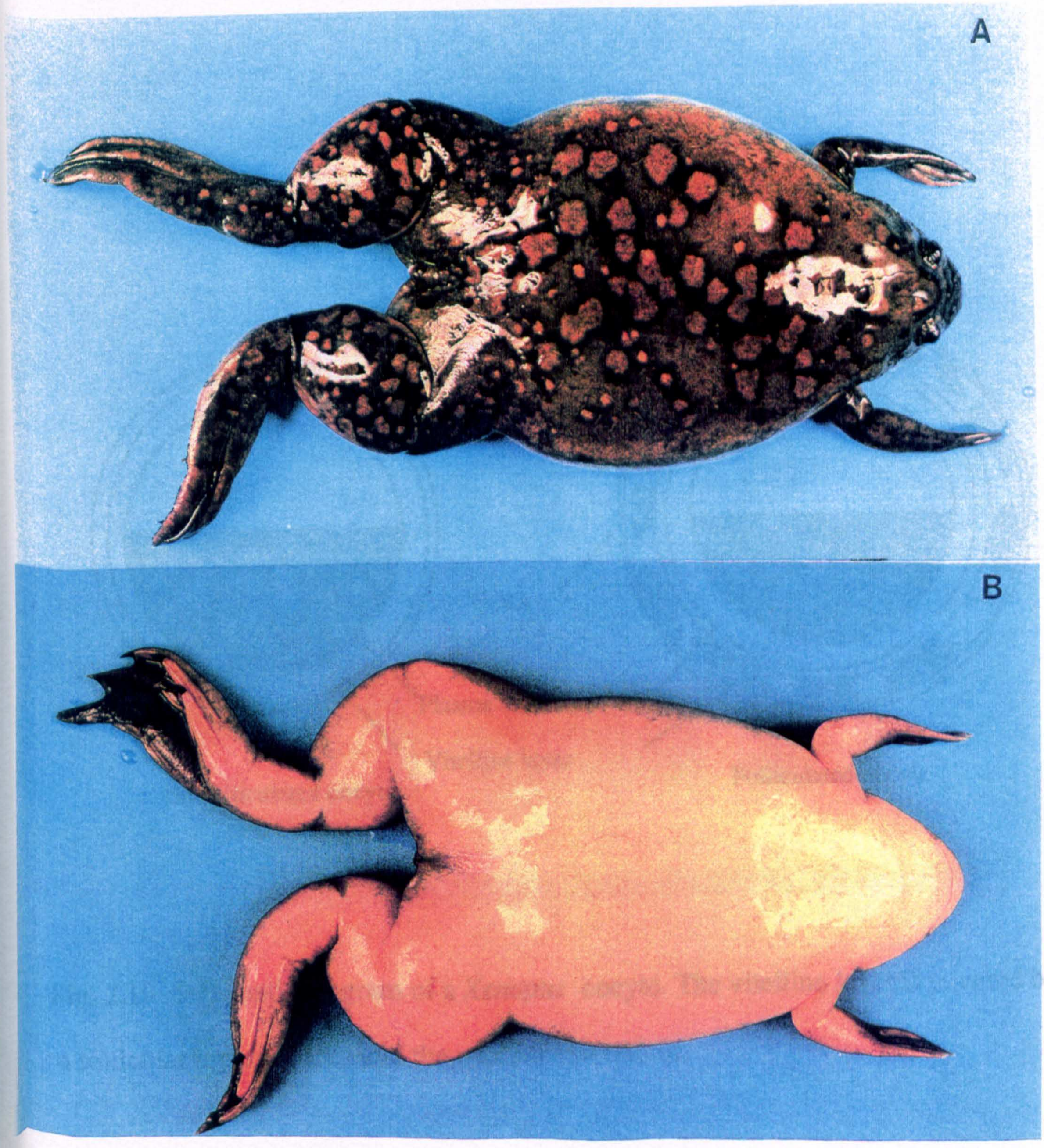


Fig. 2.9 Dorsal view (A) and Ventral view (B) of female *Xenopus laevis*.

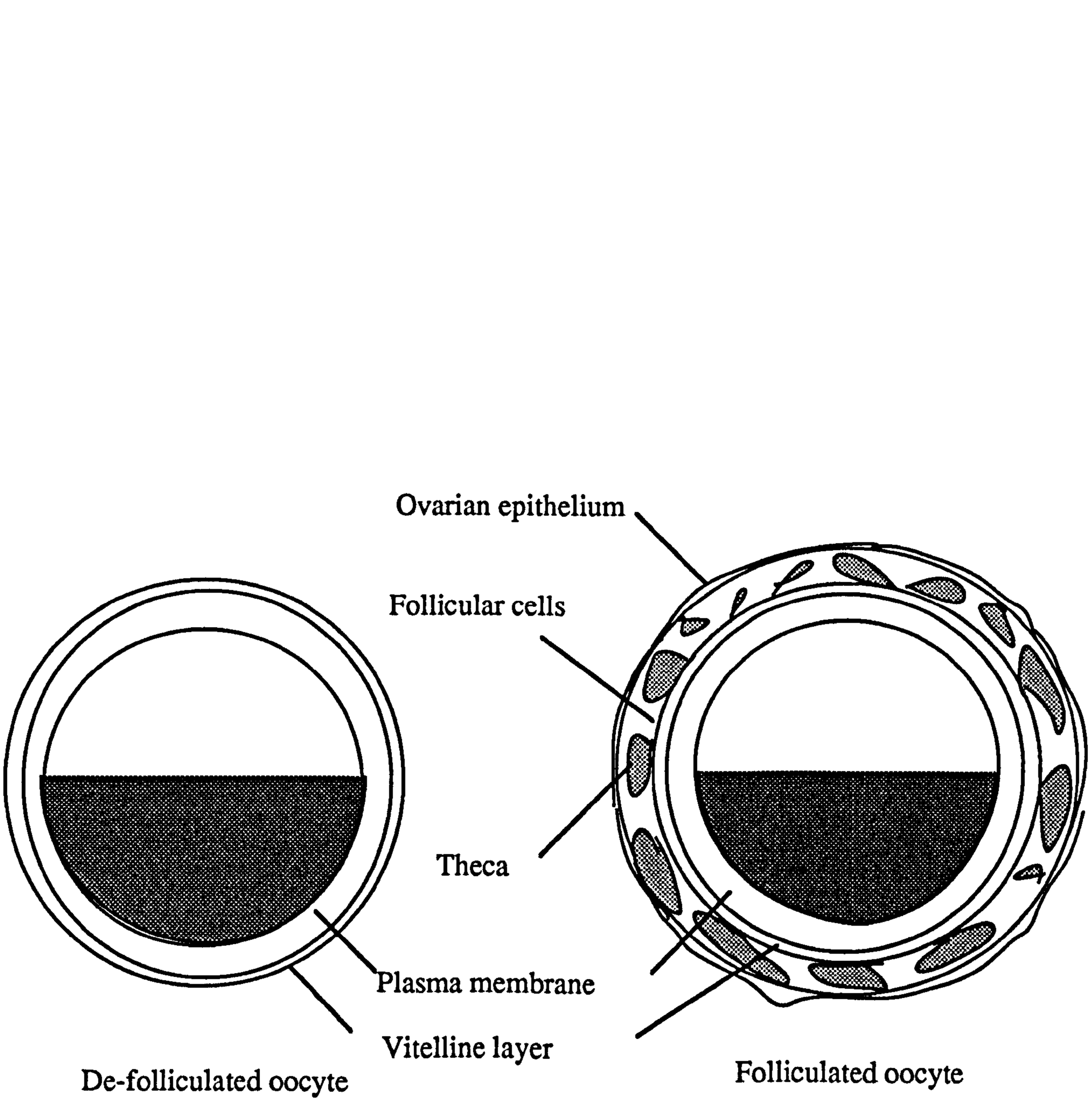


Fig. 2.10 Schematic diagram of a *Xenopus* oocyte. The vitelline layer is covered by a follicular layer, a theca and the ovarian epithelium.

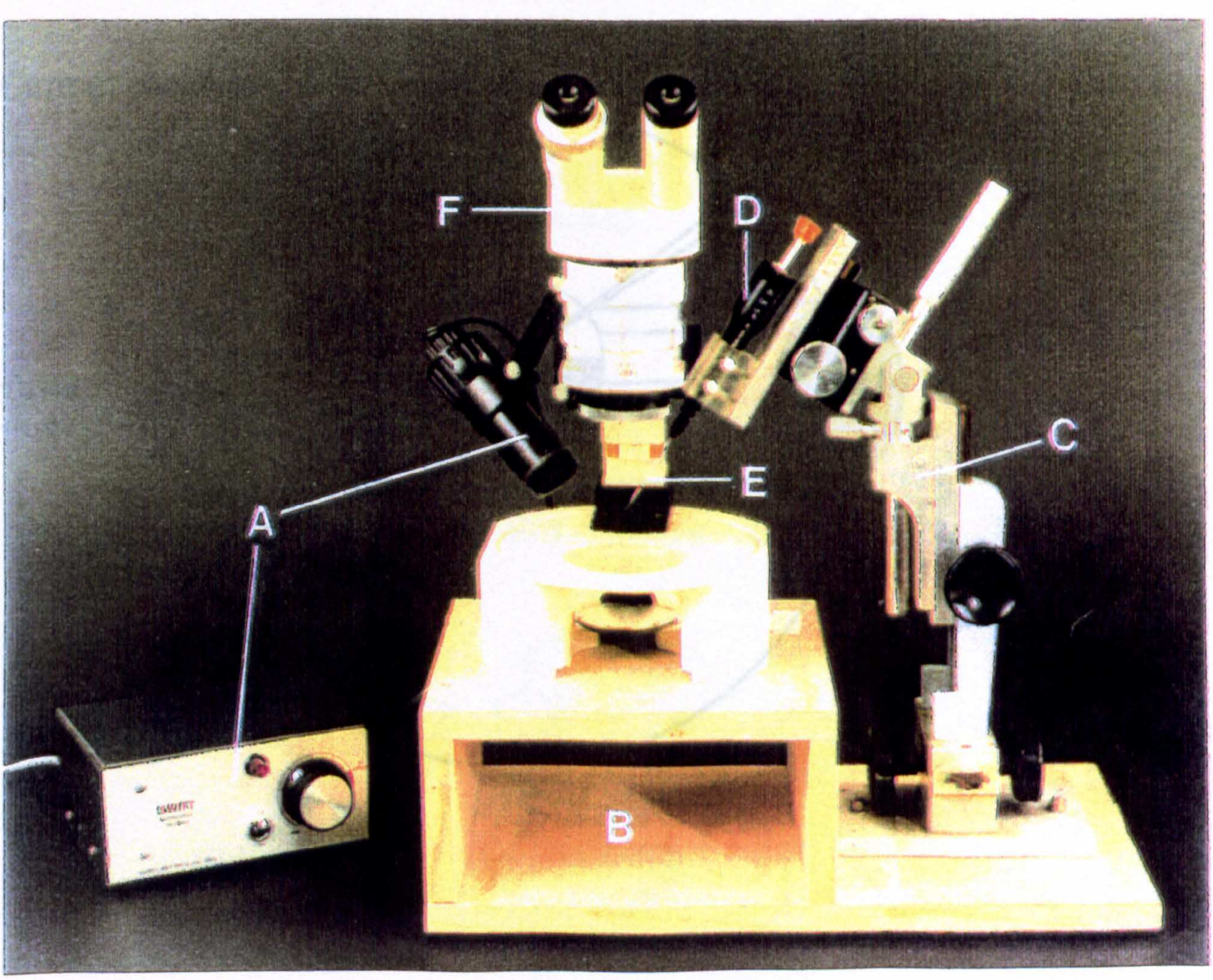


Fig. 2.11 Apparatus for microinjection of RNA.

A: Light Source
B: Base plateform
C: micromanipulator
D: Drummond microdispenser
E: dispensing pipette
F: Binocular microscope

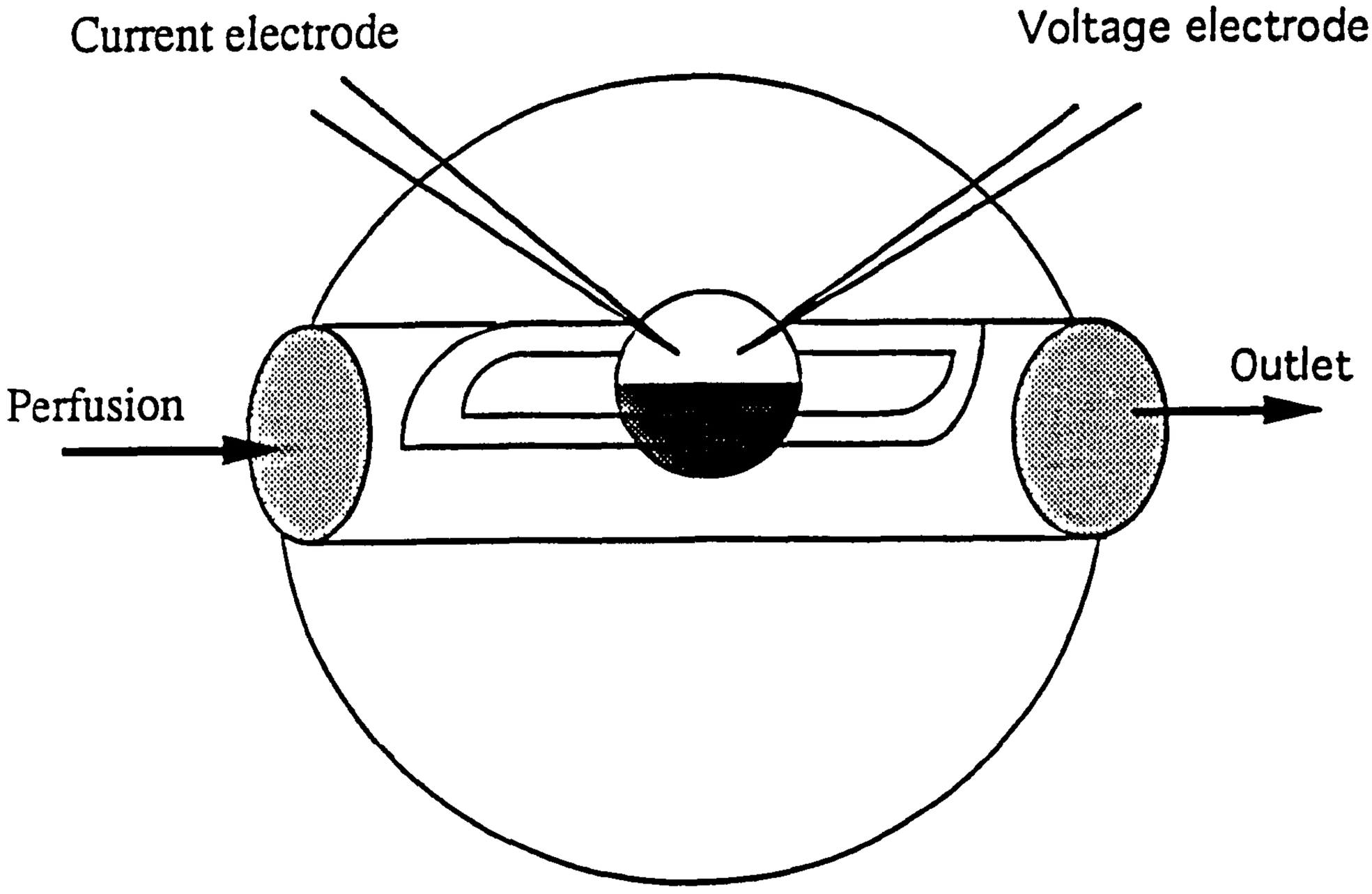
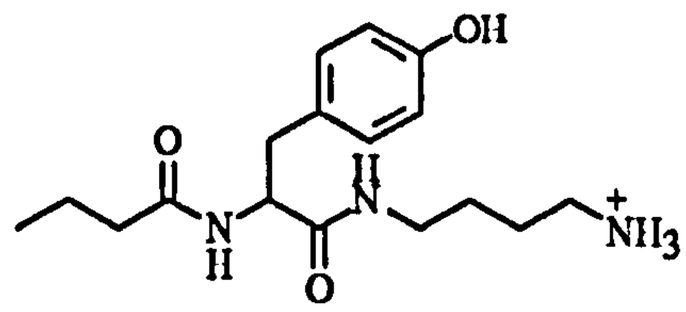
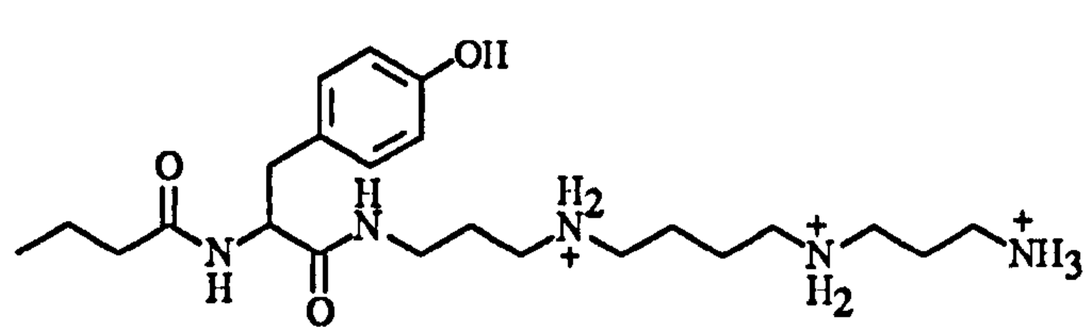


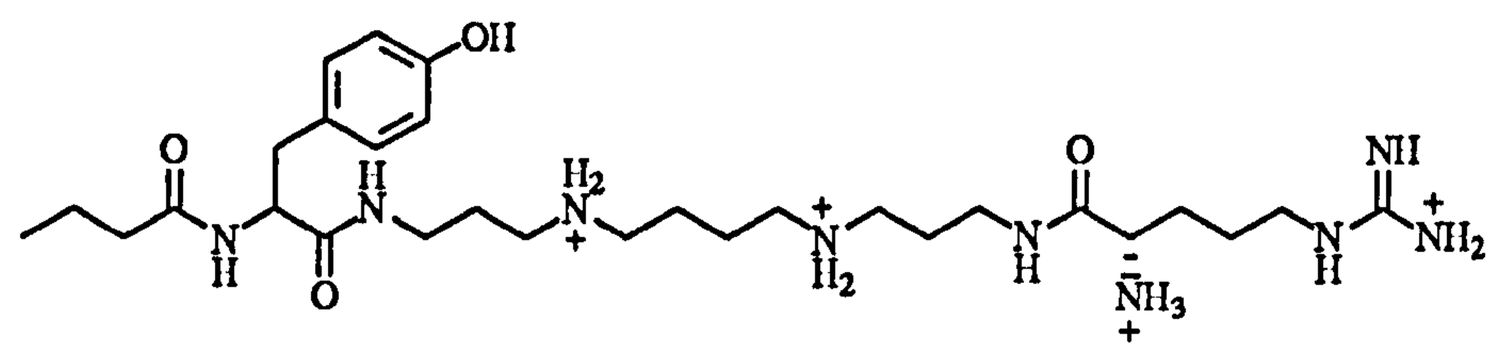
Fig. 2.12 Schematic diagram of a voltage clamped oocyte in a perfusing bath.



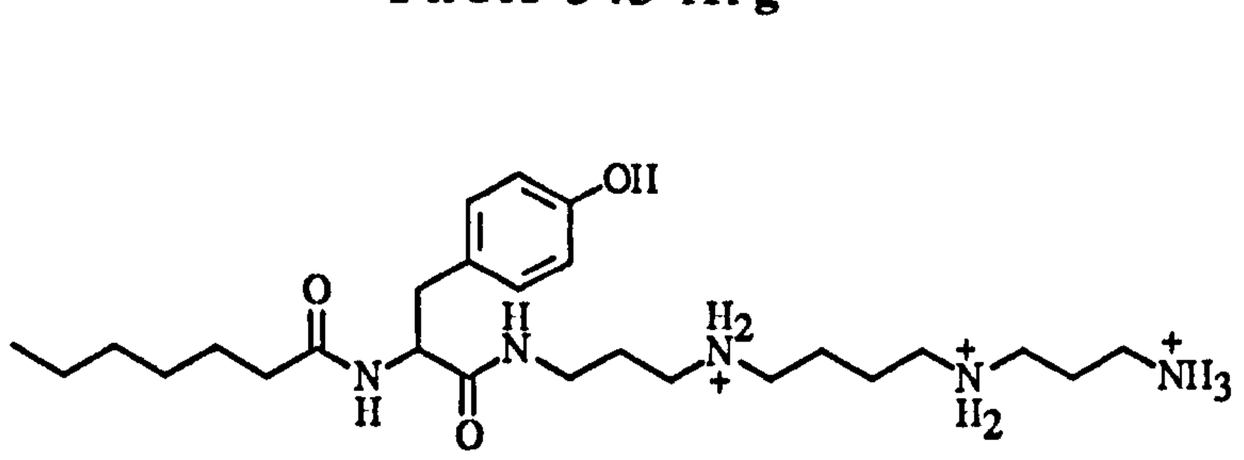
PhTX-4



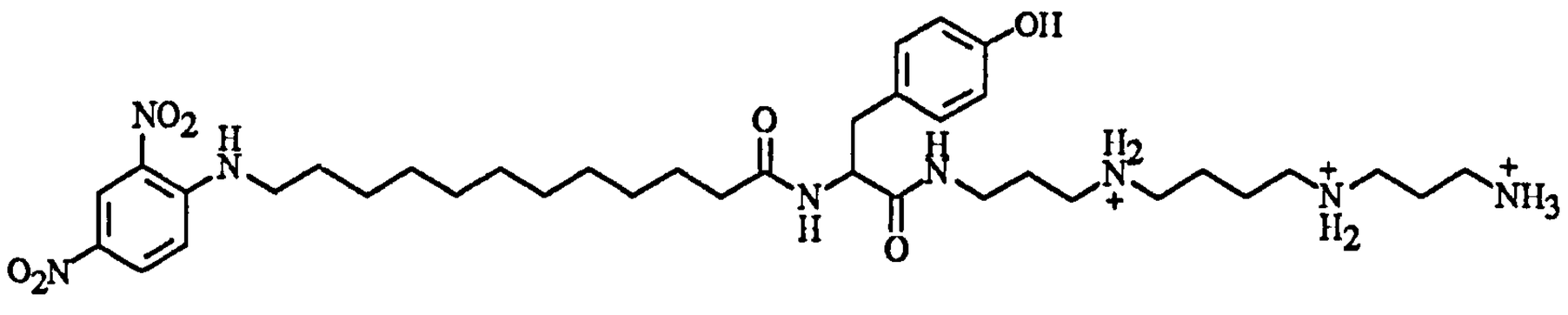
PhTX-343



PhTX-343-Arg



C_7-PhTX-343



DNP12-PhTX-343

Fig. 2.13. Structural comparison between analogues of PhTX-433

CHAPTER THREE

RESULTS

CHAPTER 3

RESULTS

3.1 Uptake of ^{45}Calcium in locust (*Schistocerca gregaria*) muscle

3.1.1 Action of L-glutamate on [$^{45}Ca^{++}$] uptake

Initially, a series of experiments were carried out to optimise the experimental conditions used. The length of time the muscles were preincubated in [$^{45}Ca^{++}$], the specific activity of [$^{45}Ca^{++}$] in the incubation medium and the length of time muscles were pretreated with Con A were each varied and the optimal conditions were determined. These were as follows:

1. 3 min incubation in $^{45}Ca^{++}$ (0.037 MBq/ml)

2. 10 min incubation in agonist (made up in standard locust saline containing 1mM Ca^{++})

3. 30 min incubation in Con A (10^{-6}M)

4. 20 min incubation in drug or toxin. In all control experiments (where no drug was added) the dishes had an equivalent volume of locust saline added.

L-glutamate causes a dose-dependent increase of $^{45}Ca^{++}$ into extenser tibiae muscles (from a control level of 1278 cpm/mg) of up to 40% at a concentration of 10^{-4}M (Fig. 3.1). L-glutamate receptors of locust muscle are known to desensitise in the presence of L-glutamate and their agonists. This desensitisation can be blocked by Con A, so the experiment was repeated following pre-incubation in Con A. The dose-dependent level of [$^{45}Ca^{++}$] uptake increased to a maximum of 85% on addition of 10^{-4}M L-glutamate (Fig. 3.2). At higher concentrations of L-glutamate a reduction of $^{45}Ca^{++}$ uptake was consistently seen. There was no difference in basal calcium influx between Con A-treated and untreated muscles (mean value of 1200±28cpm/mg and 1180±20cpm/mg respectively). There was also no difference in uptake of $^{45}Ca^{++}$ between male and female locusts muscle.

3.1.2 Time course of L-glutamate stimulated [$^{45}Ca^{++}$] uptake

In this experiment, the time after adding L-glutamate (10^{-4}M) until the end of the experiment was varied to determine the optimum time for maximum calcium uptake. The muscles were treated with L-glutamate for 3, 5, 7, 10, 15, 20 and 25 min. Each point had an untreated control. It can be seen from Fig. 3.3 that calcium influx in the muscle not treated with Con A increased with incubation

time up to 10min, and with longer incubations it declined, presumably because of desensitisation of glutamate receptors.

In the presence of Con A (1μM) for 30 min prior to stimulation with glutamate (10^{-4}M) the maximum influx of calcium occurred (Fig. 3.4) after 10min to 15min (186%±2). With longer incubation times there was only a small reduction in glutamate-stimulated $^{45}Ca^{++}$ accumulation. Thus, Con A enhances the glutamate-stimulated uptake of $^{45}Ca^{++}$ and prolongs its time course.

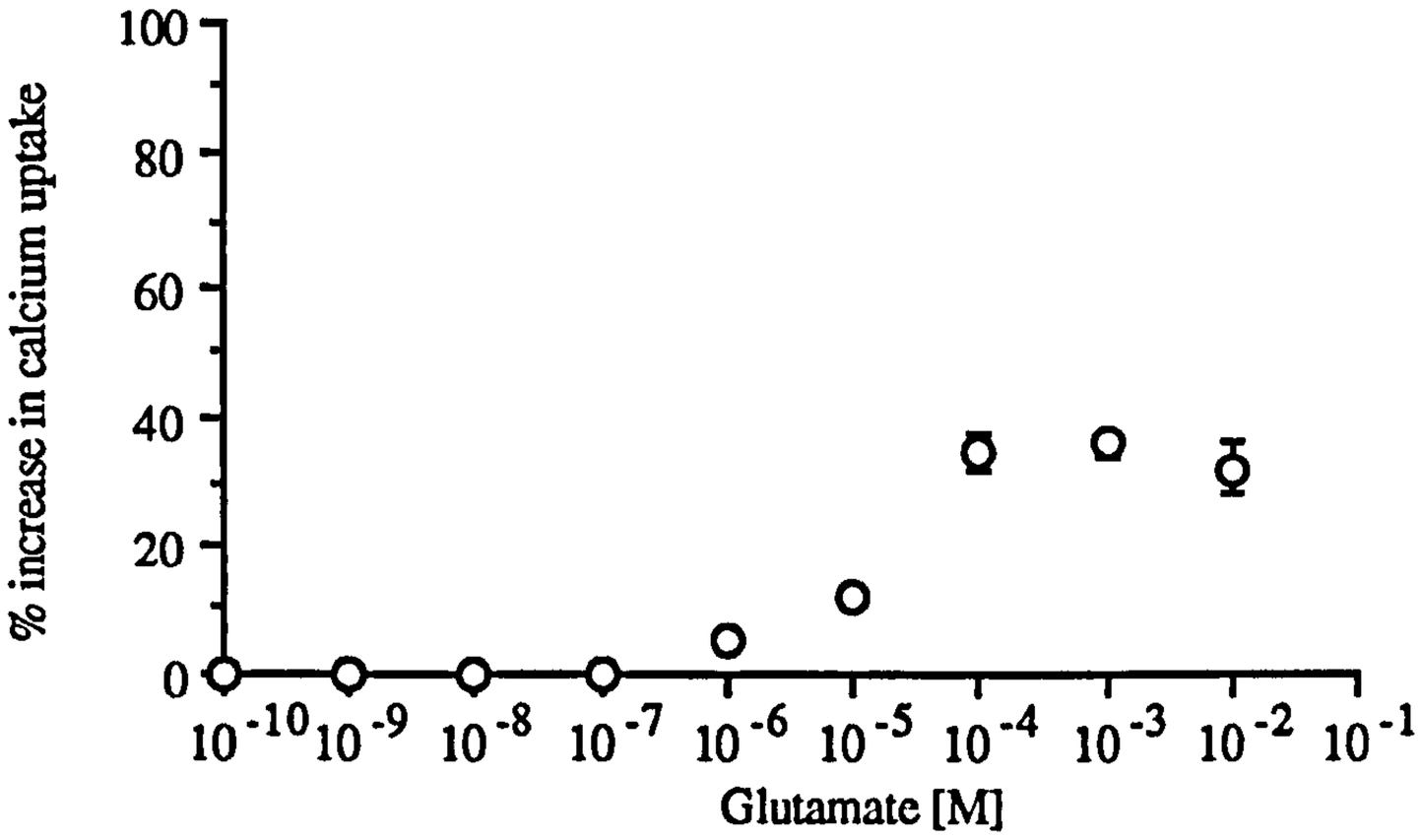


Fig. 3.1

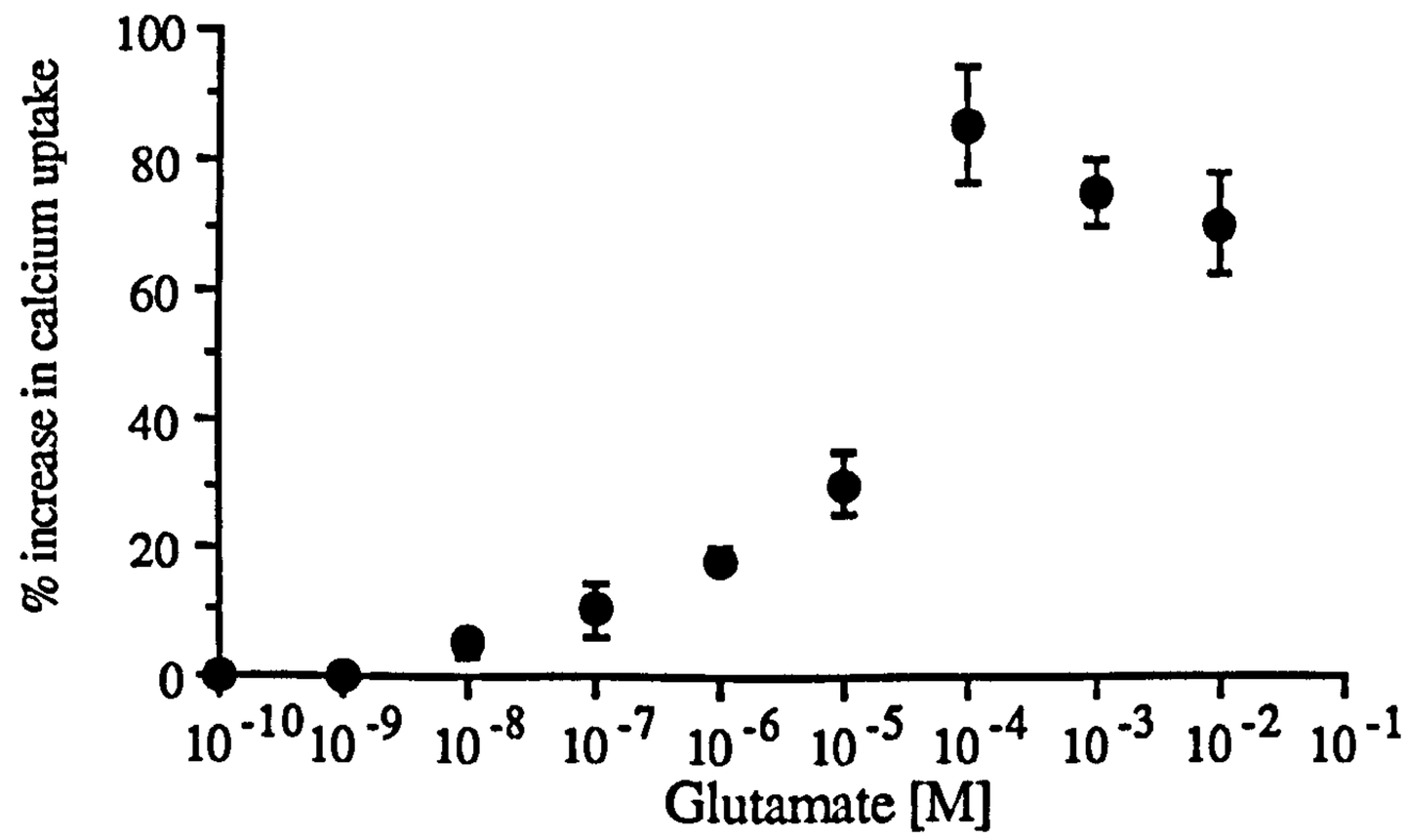


Fig. 3.2

Figs. 3.1 and 3.2. Increase (%) in uptake of $^{45}Ca^{++}$ into locust extensor tibiae muscle produced by addition of various concentrations of L-glutamate. Each point represent mean value ± S.D of 18 muscles.

Fig. 3.1 Pre-incubated in standard locust saline for 30 min.

Fig. 3.2 Pre-incubated in ConA (10^{-6}M) for 30min.

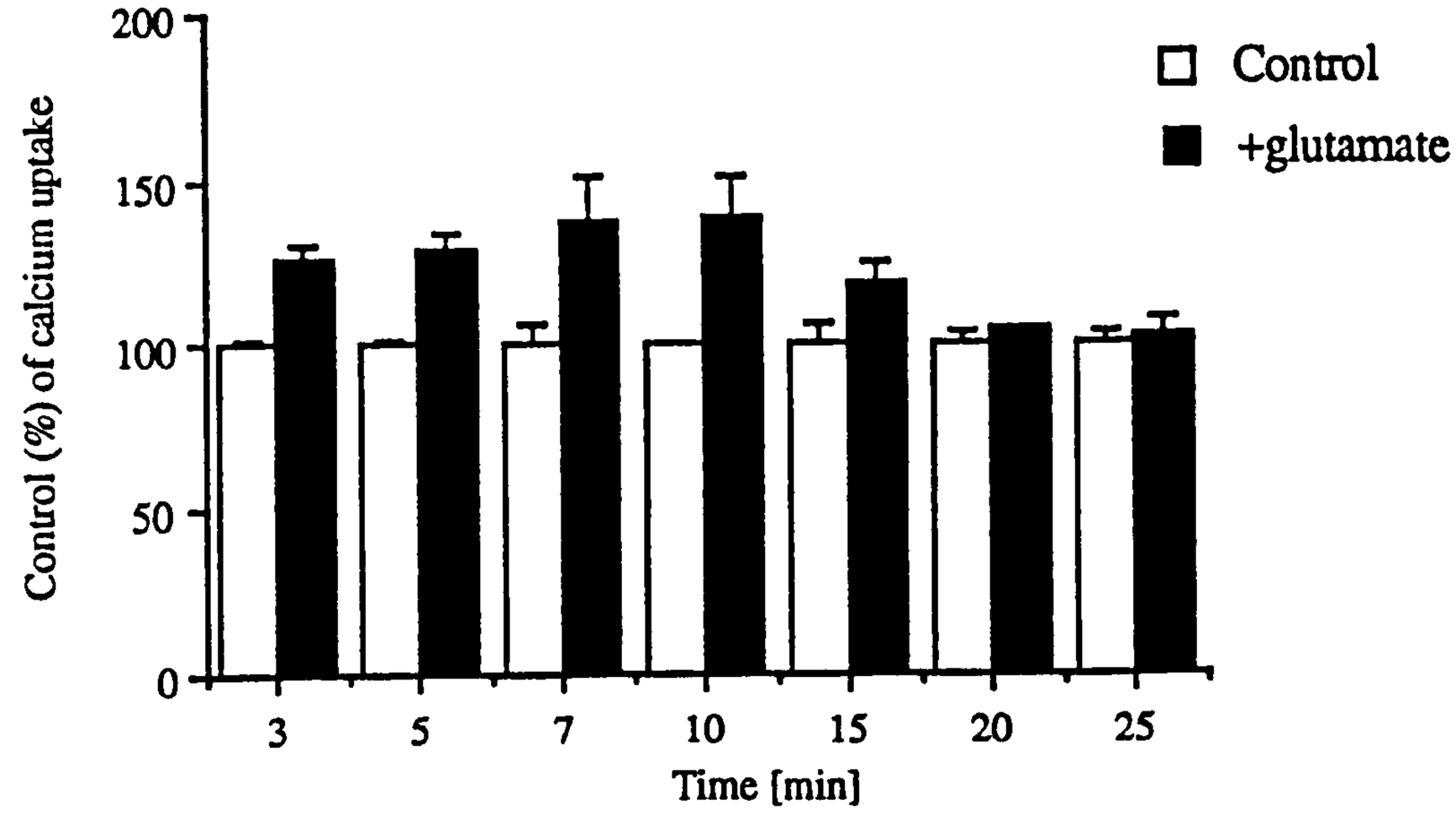


Fig. 3.3

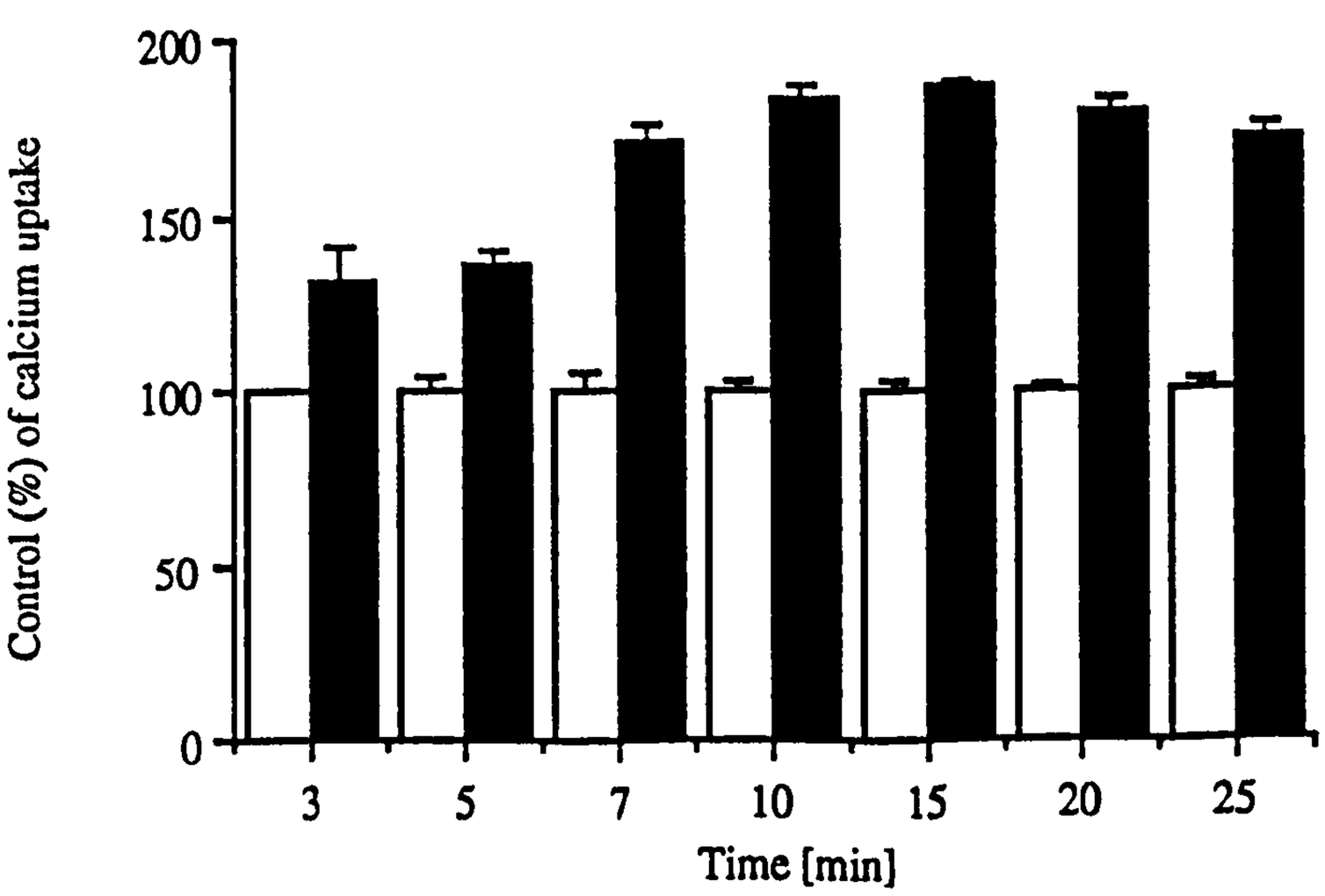


Fig. 3.4

Figs. 3.3 and 3.4. The time course of calcium accumulation following depolarisation of muscles with L-glutamate (10^{-4}M). In the absence of Con A (Fig. 3.3) the level of uptake peaks at about 40% above control level after 10min and then declines. Pre-tratment with Con A (10^{-6}M) for 30min resulted in a higher level of uptake (86%) which was largely maintained for up to 25min (Fig. 3.4)

3.1.3 Effect of philanthotoxin-343 (PhTX-343) on [$^{45}Ca^{++}$] uptake in locust (*Schistocerca gregaria*) muscle.

The effect of synthetic wasp toxin PhTX-343 on glutamate-activated calcium uptake was measured following treatment with Con A (1μM) for 30min. An experiment was carried out to determine the effect of time on the response to PhTX-343 (1μM). It was found that 20 min gave the maximum block by this toxin of $^{45}Ca^{++}$ uptake induced by L-glutamate (10^{-4}M). 20min of PhTX-343 incubation was used in all subsequent experiments (Fig. 3.5). PhTX-343 had no effect on resting calcium influx (Fig. 3.6), but when the muscles were depolarised with glutamate (10^{-4}M) the stimulated calcium influx was inhibited in a dose-dependent fashion (Figs. 3.6 and 3.7).

3.1.4 Effect of analogues of philanthotoxin on glutamate-stimulated [$^{45}Ca^{++}$] uptake,in locust (*Schistocerca gregaria*) muscle.

The main objective of this work was to investigate the action of PhTX-343-Arg and PhTX-4 on calcium uptake in locust muscle (Fig. 3.8). These toxins have been shown to be more active and less active respectively than PhTX-343 at blocking neurally-evoked contractions of locust retractor unguis muscle (Bruce *et al.*, 1990; Benson *et al.*, 1992). The action of PhTX-4 and PhTX-343-Arg

was studied by using the same methods as described for PhTX-343. PhTX-343-Arg was a more potent inhibitor of glutamate-activated calcium uptake (IC_{50} = ~ $7x10^{-9}$M) than PhTX-343 (IC_{50} = ~ 10^{-8}M) whereas PhTX-4 only produced 20% inhibition at 100nM. These data are consistent with the results of previous studies of these compounds (Bruce *et al.*, 1990). The time course of inhibition by both analogues of PhTX-343 mirrored that of PhTX-343 itself, reaching a maximum at 20 min (Fig. 3.9).

Brackley *et al.* (1990) reported the potentiation by low concentrations of PhTX-343 of kainate and NMDA receptors of rat brain expressed in *Xenopus* oocyte. To observe the effects of low concentrations of philanthotoxins, locust muscles were activated with L-glutamate after 3, 5, 10, 15 and 20 min incubation in 10^{-12}M and 10^{-14}M of PhTX-343, PhTX-343-Arg and PhTX-4. At these low concentrations these compounds did not affect calcium influx (Fig. 3.10).

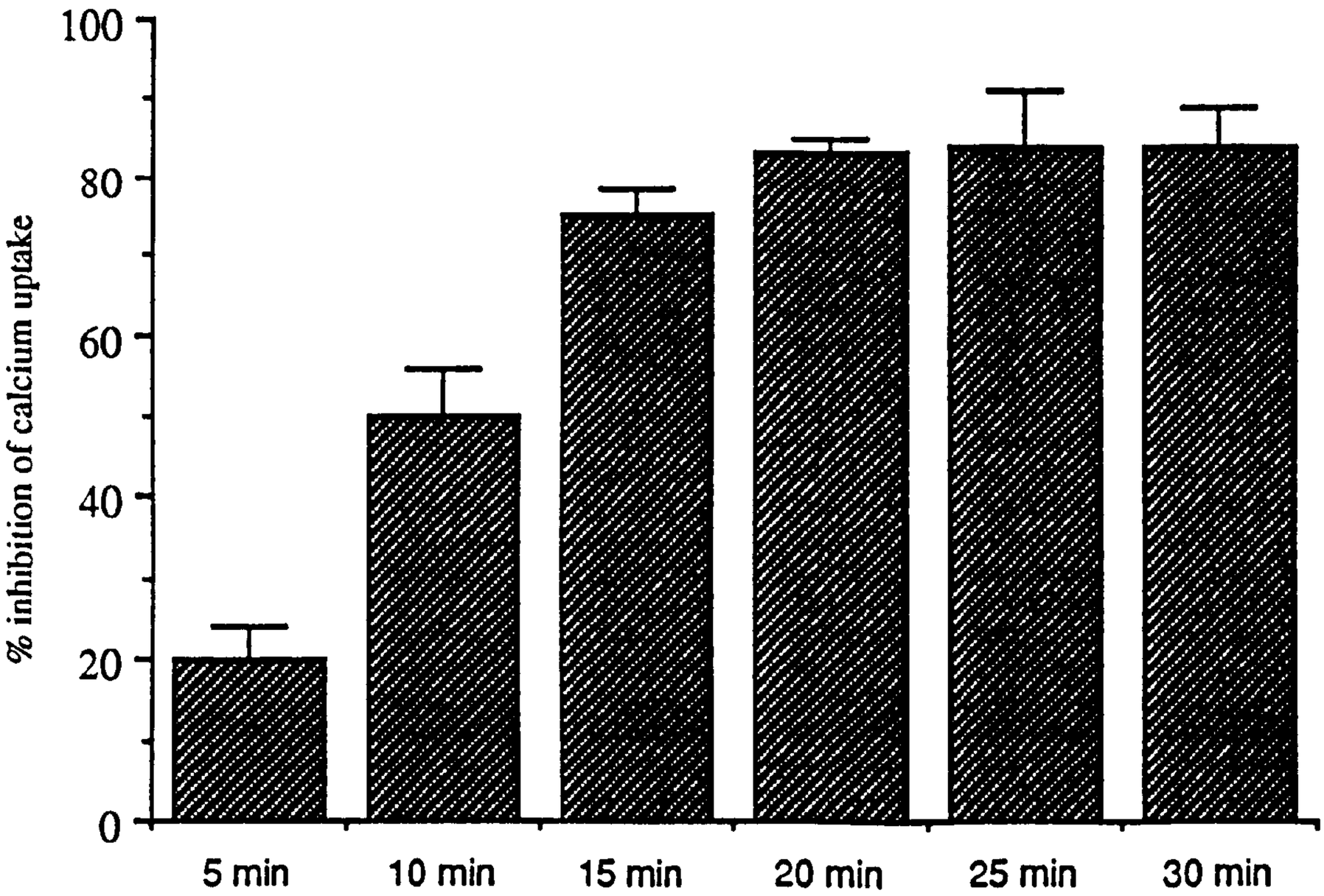
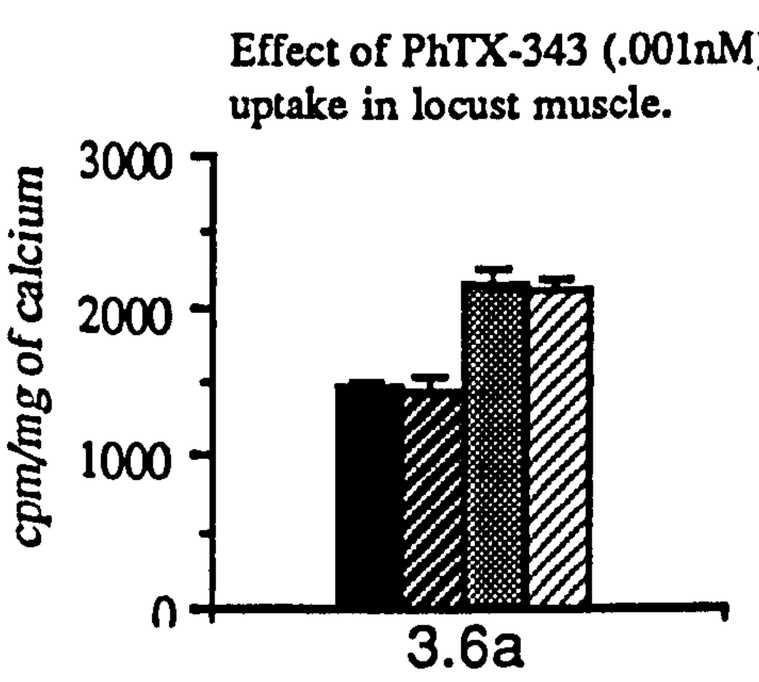
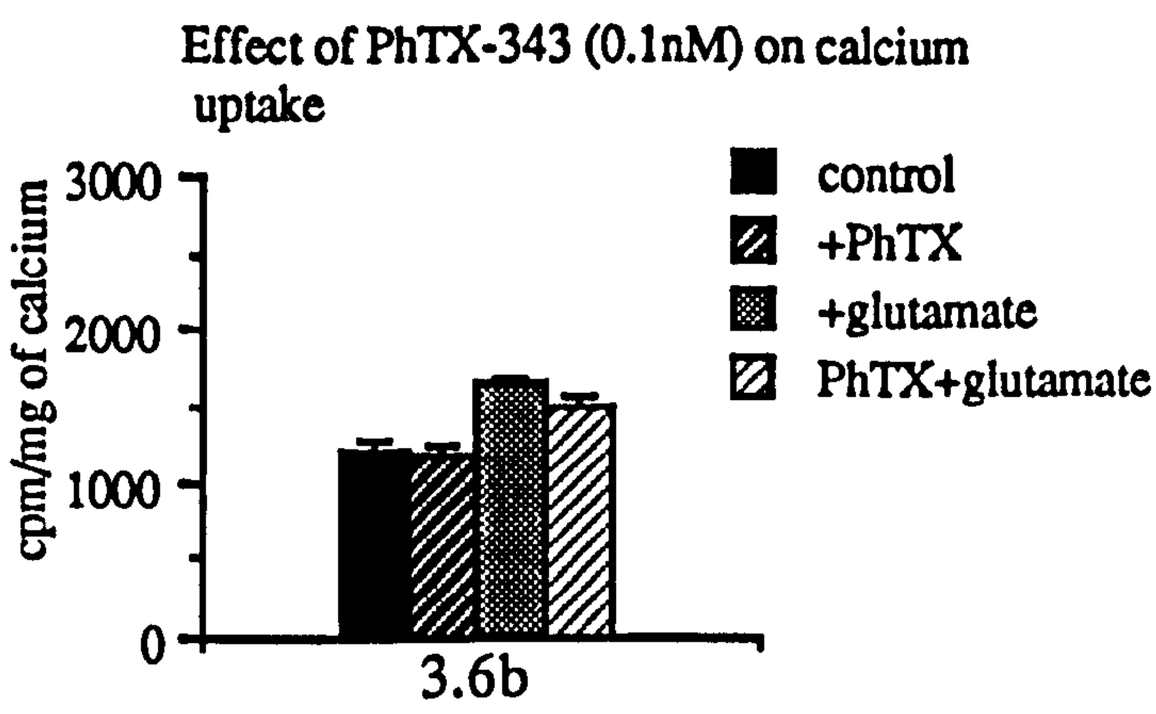
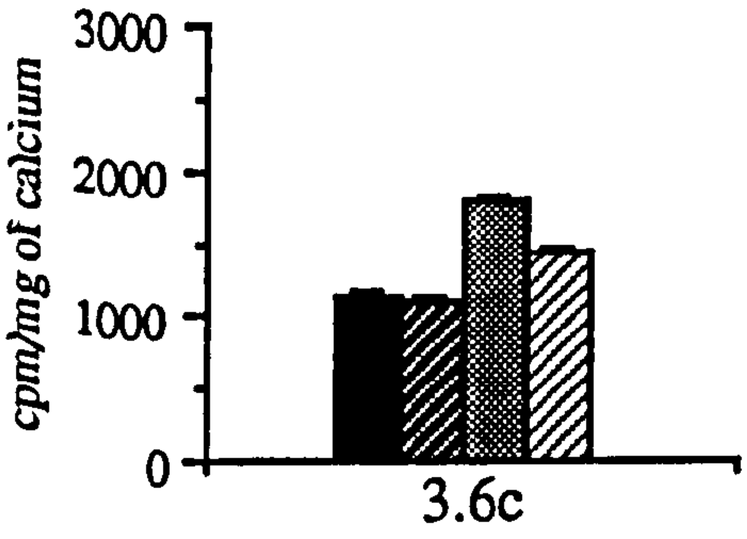
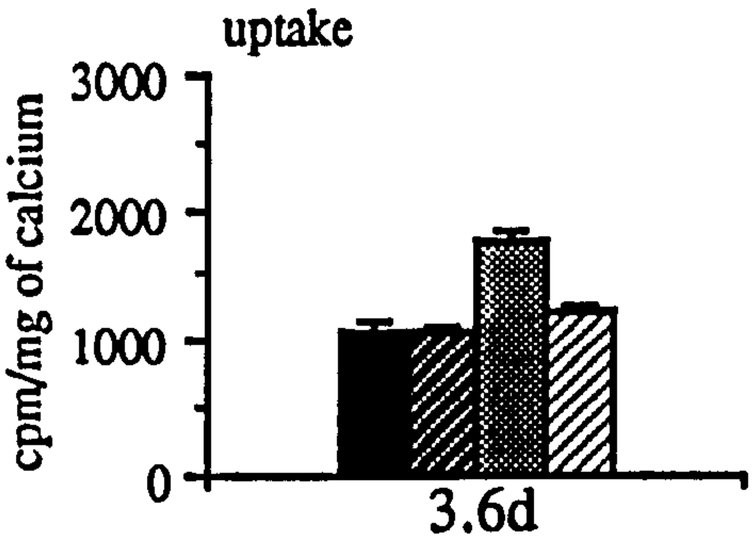


Fig. 3.5. PhTX-343 (1μM) was added to the incubation medium for various times before the muscles was depolarised with L-glutamate (10-4M). The inhibition of glutamate-stimulated Ca^{++} influx reaches a maximum after 20min.









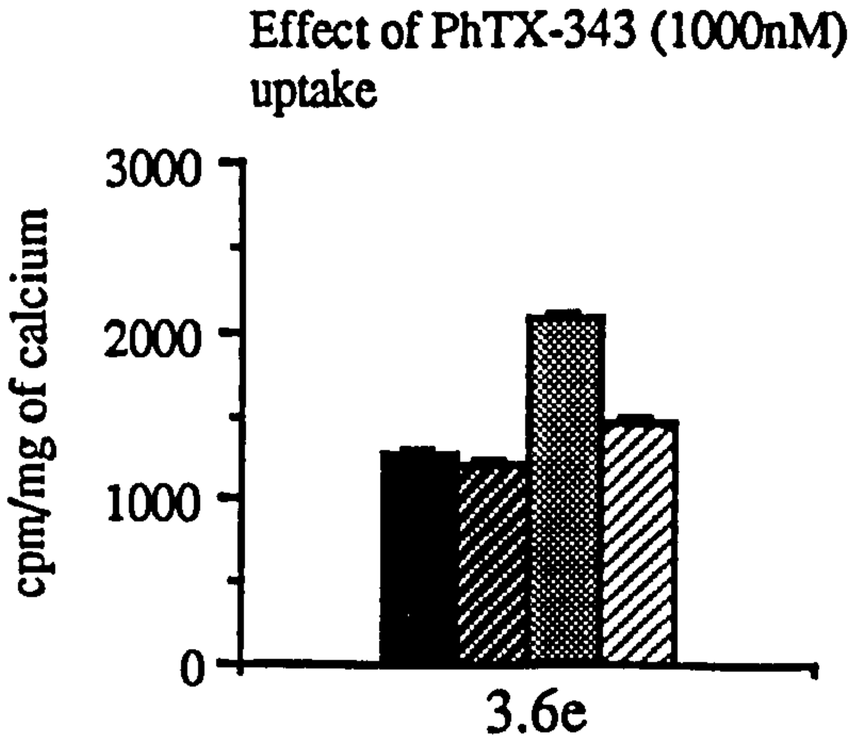


Fig. 3.6. Various concentrations of PhTX had no effect on basal [$^{45}Ca^{++}$] uptake into locust extensor tibiae muscles. However, the L-glutamate (10^{-4}M)-stimulated uptake of Ca^{++} was inhibited in a dose-dependent manner by PhTX-343.

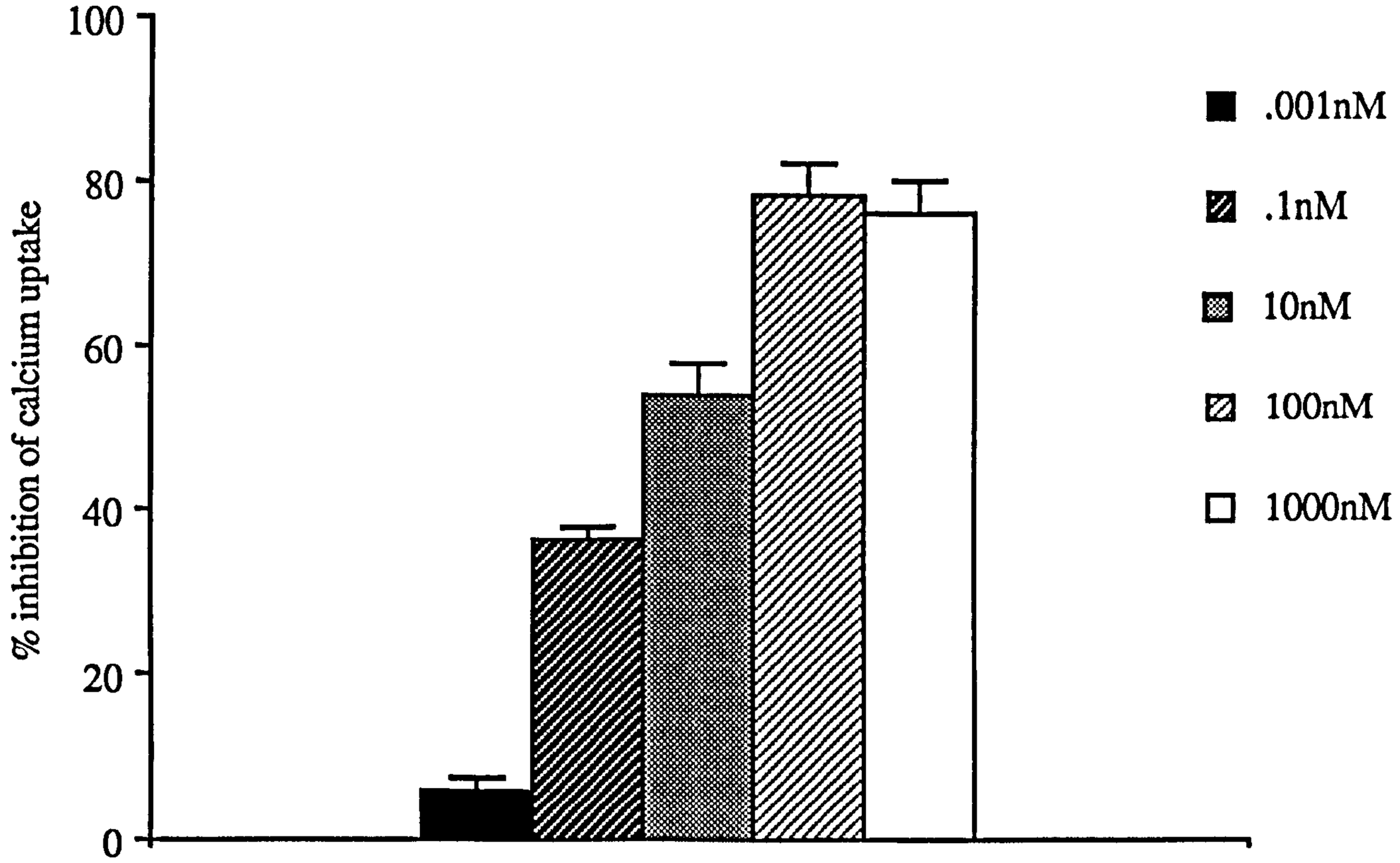


Fig. 3.7. Philanthotoxin-343 inhibited $^{45}Ca^{++}$ uptake induced by addition of L-glutamate (10^{-4}M). The IC_{50} value was approximately 10^{-8}M.

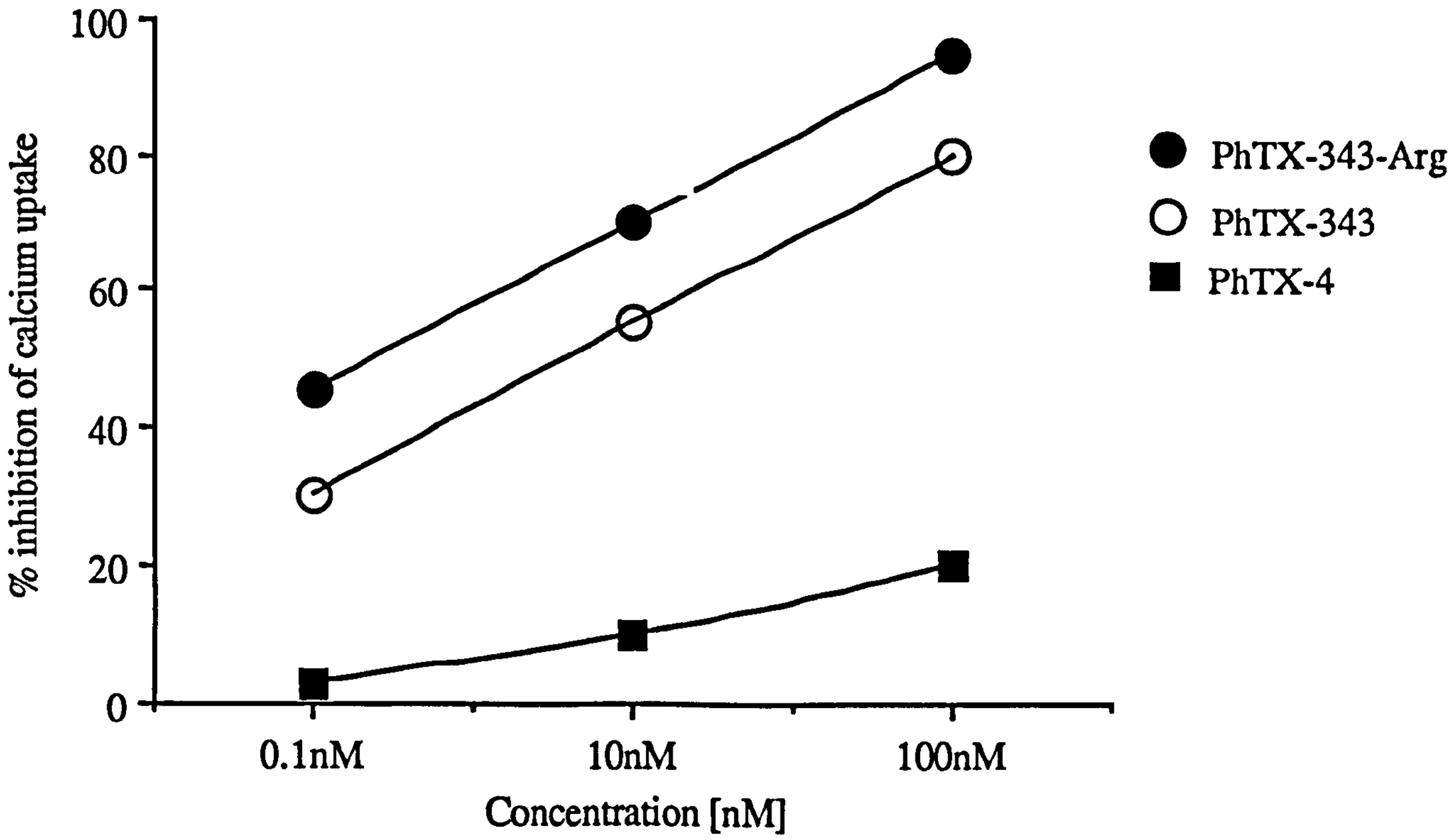


Fig. 3.8. Dose-inhibition relationship of PhTX-343 and two analogues, PhTX-4 and PhTX-343-Arg in blocking $^{45}Ca^{++}$ uptake induced by L-glutamate (10^{-4}M).

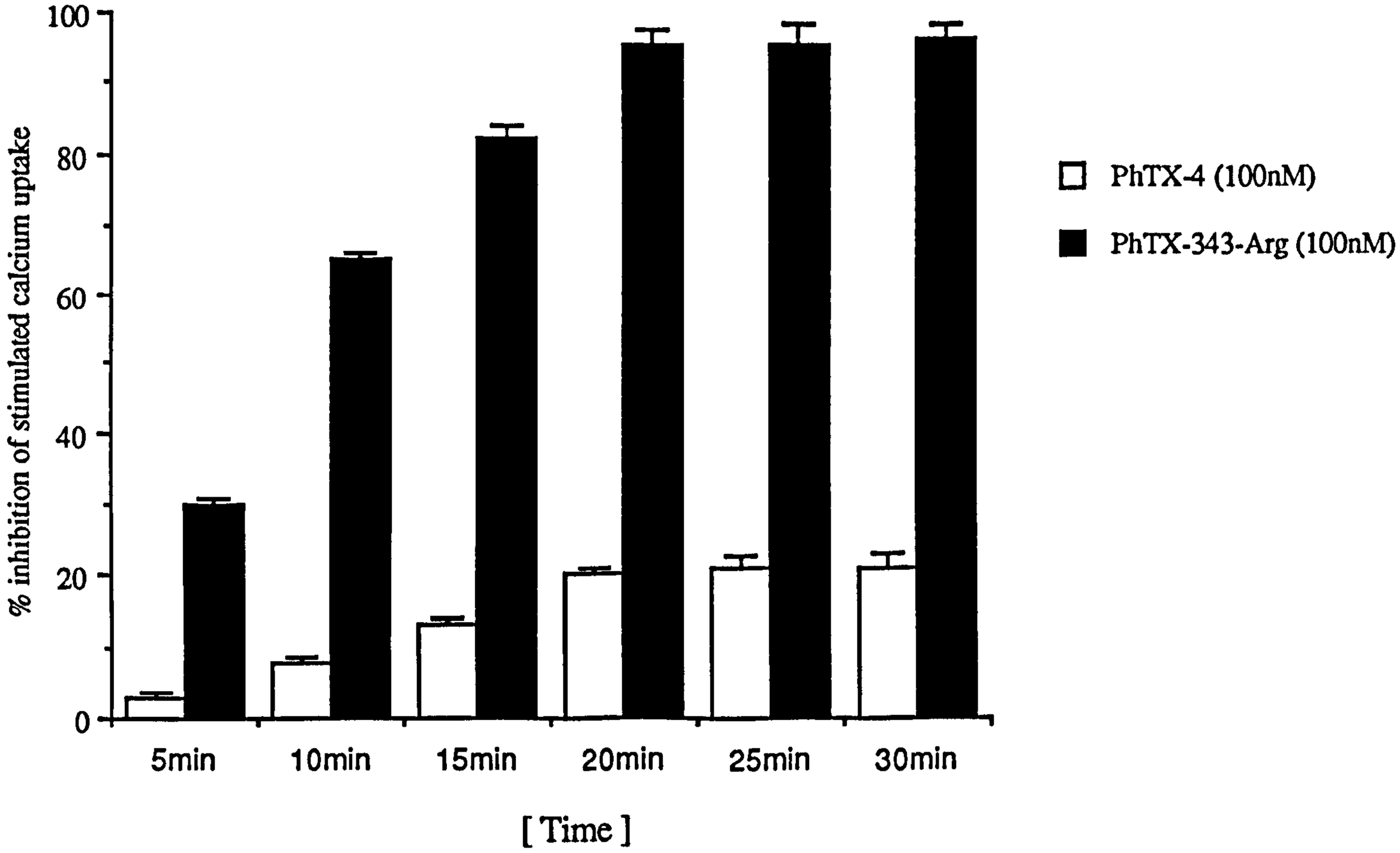


Fig: 3.9. The inhibition of 45Ca++ uptake induced by L-glutamate (10^{-4}M) was maximal after 20min for both PhTX-4 and PhTX-343-Arg.

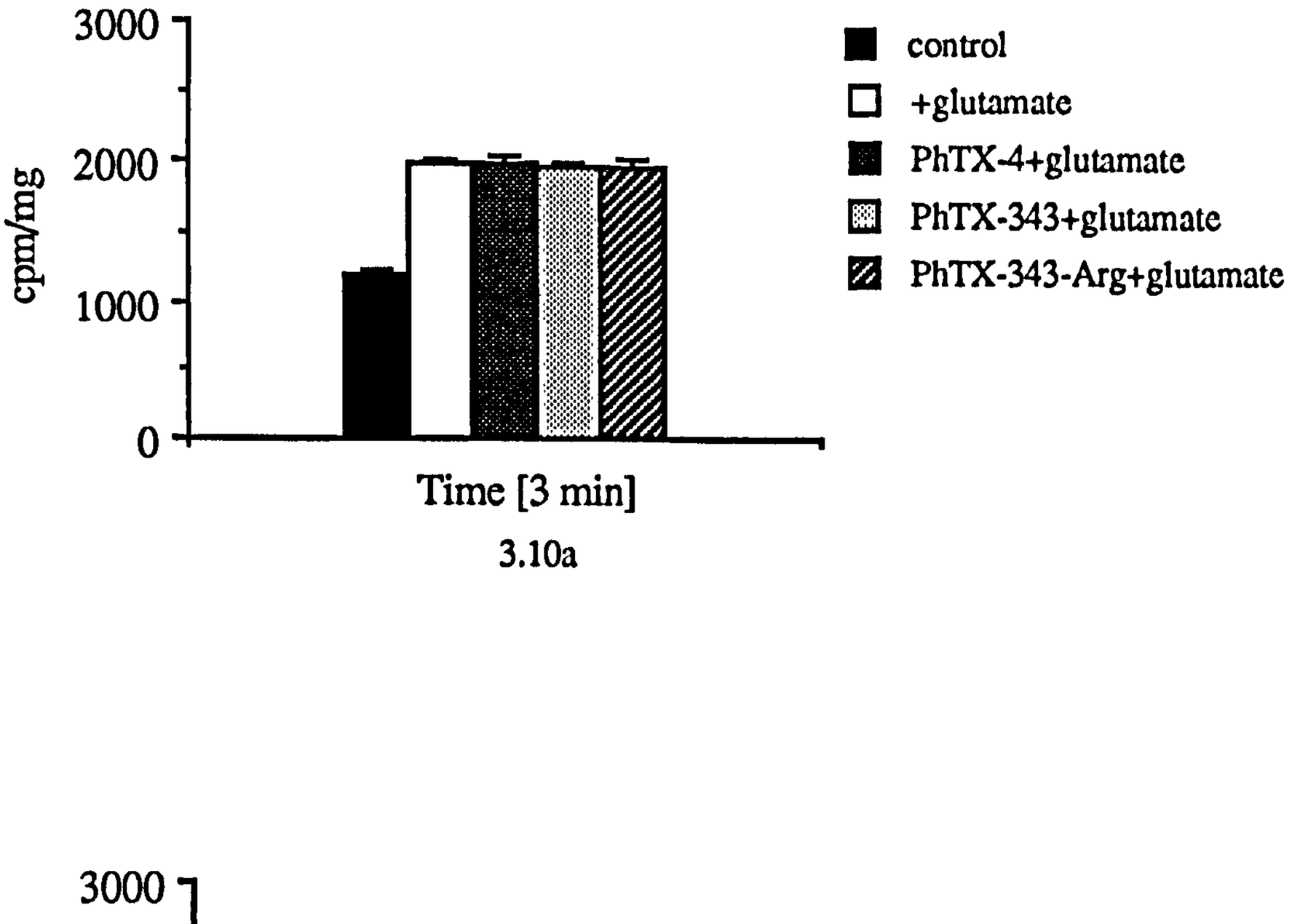


Fig:3.10. Low concentrations of PhTX-343, PhTX-4 and PhTX-343-Arg had no effect on L-glutamate stimulated uptake of $^{45}C^{a++}$.

3.10a. Muscles were incubated in PhTX (10^{-14}M) for 3min.

3.10b. Muscles were incubated in PhTX (10^{-14}M) for 30min.

3.1.5. Entry of [$^{45}Ca^{++}$] through voltage-activated Ca^{++} channels

L-glutamate acts directly on glutamate receptors in insect muscle which are known to gate Ca^{++} ions. However, depolarisation of muscle by glutamate may also activate voltage-gated Ca^{++} channels. A series of experiments were carried out to investigate the effect of depolarisation of the muscle with elevated extracellular potassium ions ($[K^+]_o$). The method used was similar to that described above for L-glutamate stimulated influx. Pre-incubation with Con A had no effect on the level of $^{45}Ca^{++}$ uptake induced by elevated $[K^+]_o$ (Fig. 3.11).

The entry of $^{45}Ca^{++}$ stimulated by $[K^+]_o$ possibly involves L-type Ca^{++} channels which are dihydropyridine-sensitive. The action of nifedipine on potassium-stimulated calcium uptake was studied, by using a similar experimental design for the experiments with philanthotoxin. The time course of nifedipine action in this system was similar to that of PhTX-343, reaching a maximum after 20 min (Fig. 3.12). Nifedipine blocked uptake of $^{45}Ca^{++}$ stimulated by $[K]_o$ in a dose-dependent manner with an IC_{50} about 50nM (Fig. 3.13).

The question then arose as to whether the $^{45}Ca^{++}$ entry via glutamate-activated channels can be selectively inhibited. Figs. 3.14 & 3.15 shows that nifedipine (1μM) blocked the entry of calcium (Fig. 3.14a) stimulated by elevated extracellular $[K]_o$ implying that

the main action of elevating extracellular $[K]_o$ involves gating of voltage-activated calcium channels. This is further confirmed by the observations that nifedipine does not inhibit glutamate-stimulated uptake (Fig. 3.14c). Conversely PhTX-343 (1μM) had very little effect on $^{45}Ca^{++}$ uptake stimulated by $[K+]_o$ (Fig. 3.14b) but effectively inhibited uptake stimulated by 10^{-4}M glutamate (Fig. 3.14d). Neither PhTX-343-Arg nor PhTX-4 had any effect on $^{45}Ca^{++}$ entry activated by $[K^+]_o$ (Fig. 3.16).

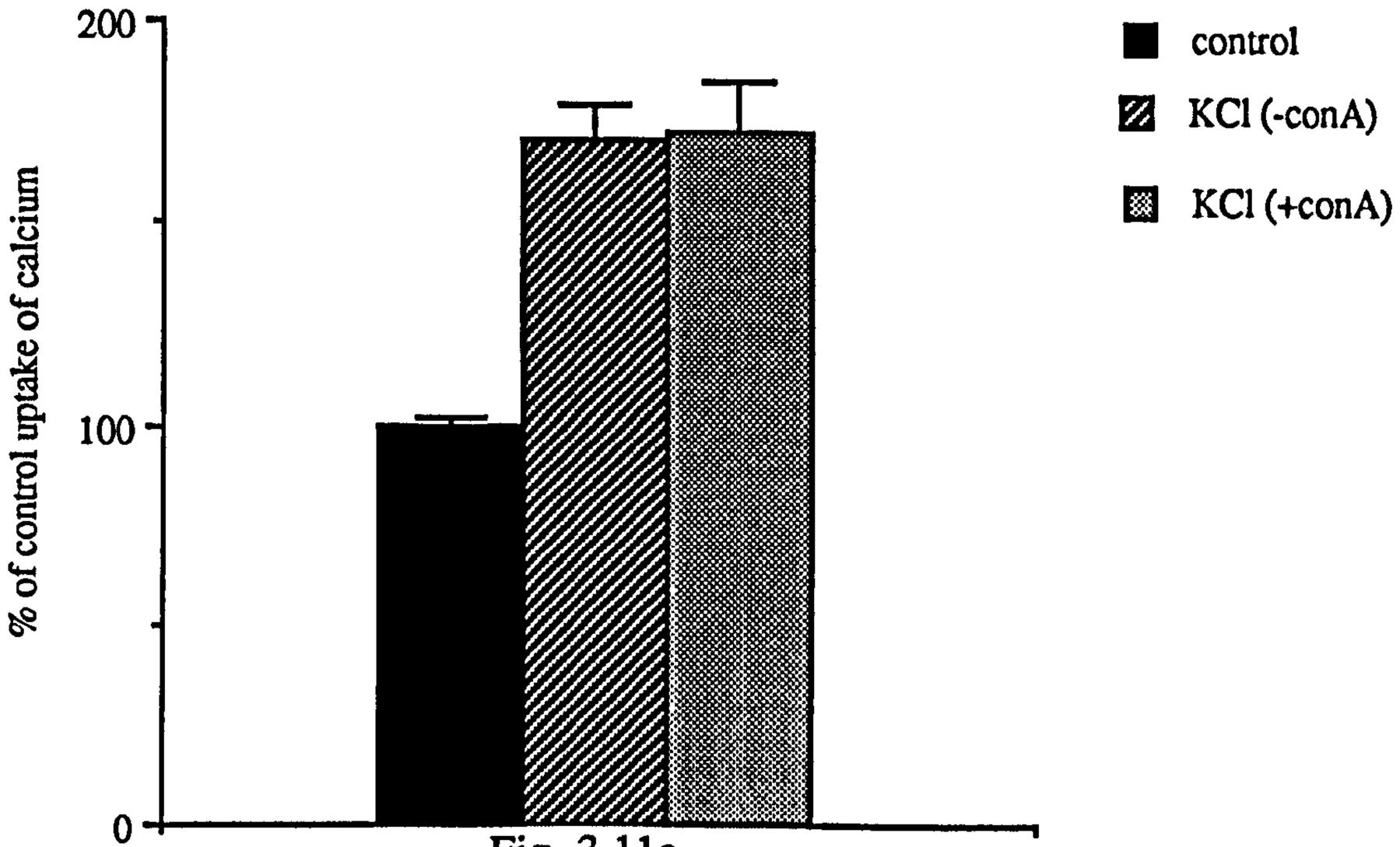


Fig. 3.11a

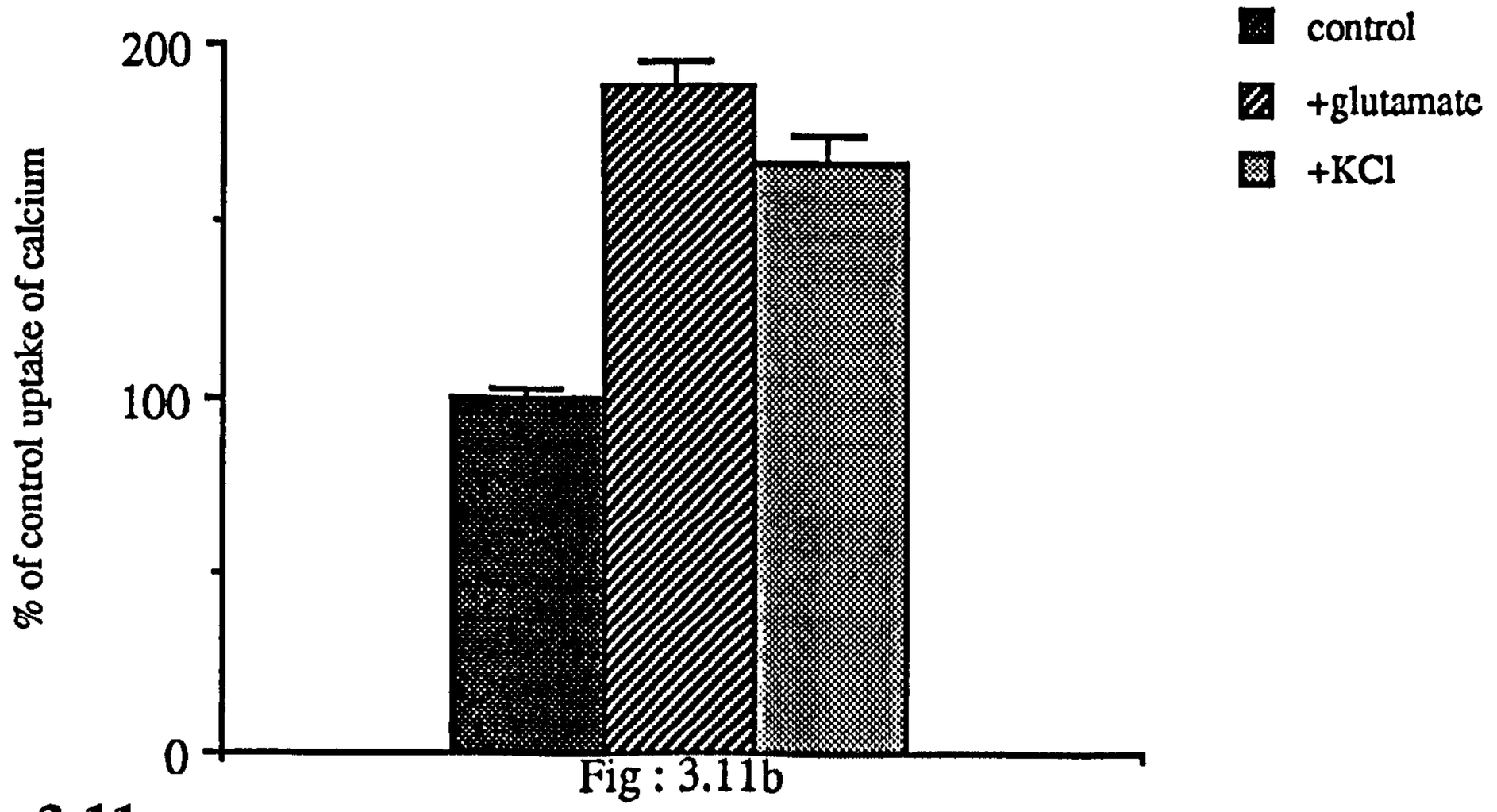


Fig : 3.11b

Fig. 3.11

Fig. 3.11a. Elevation of $[K+]_o$ to 50mM produced an increase in $^{45}Ca^{++}$ in the presence and absence of ConA (1μM).

Fig. 3.11b. Uptake of $^{45}Ca^{++}$ was elevated by both L-glutamate (10^{-4}M) and $[K]_o$ (50mM).

Effect of nifedipine (1μM) on KCl activated calcium uptake .

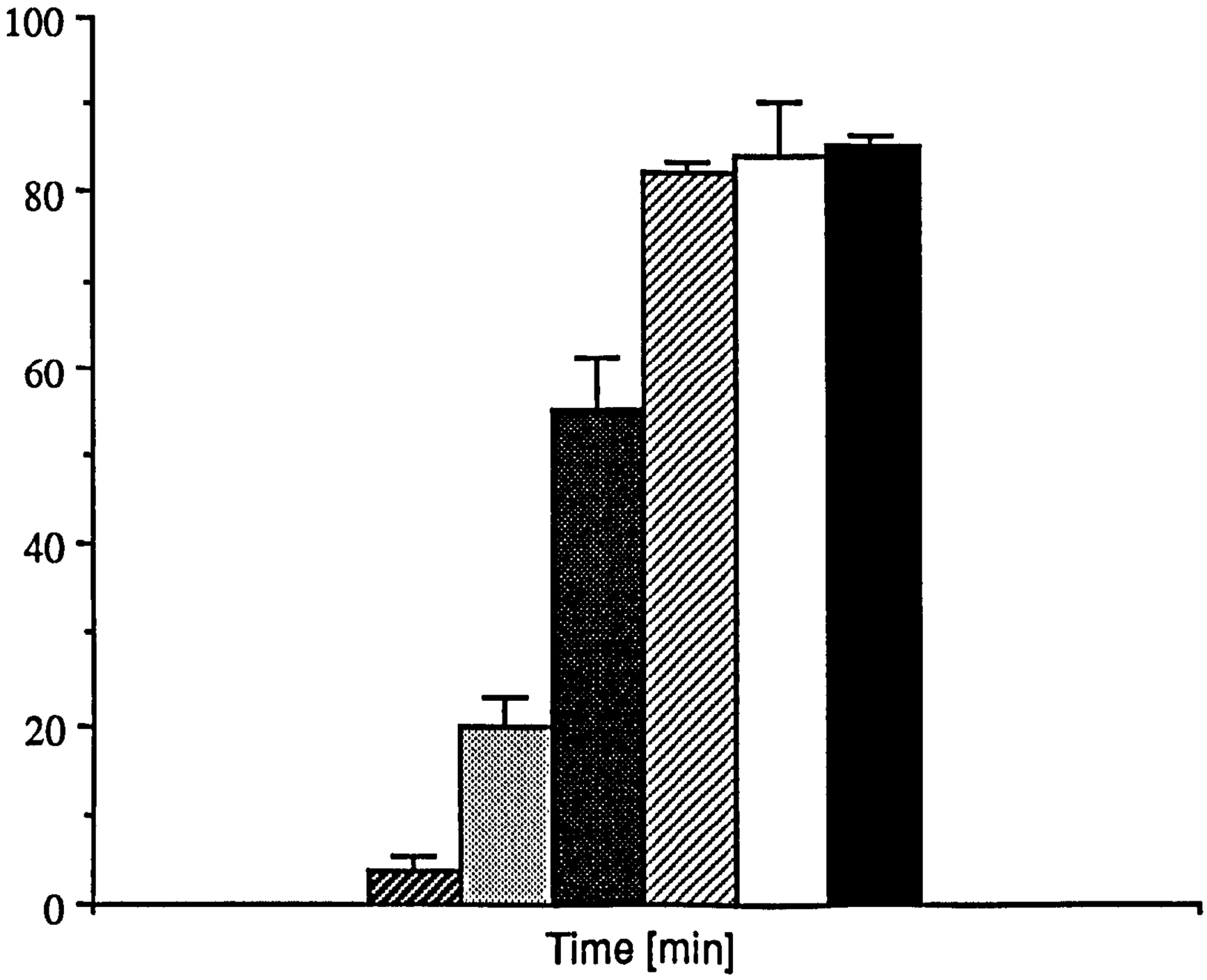
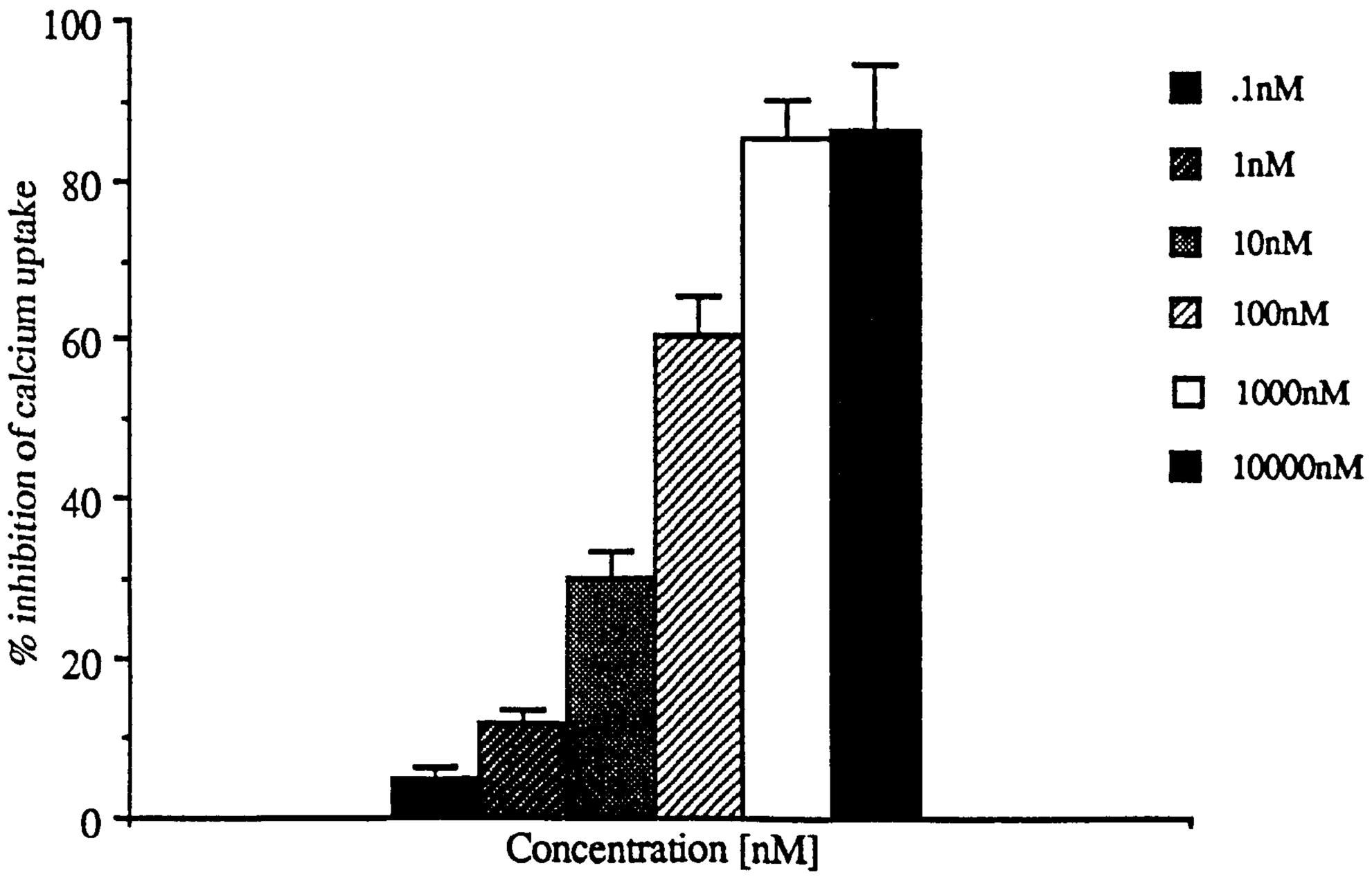
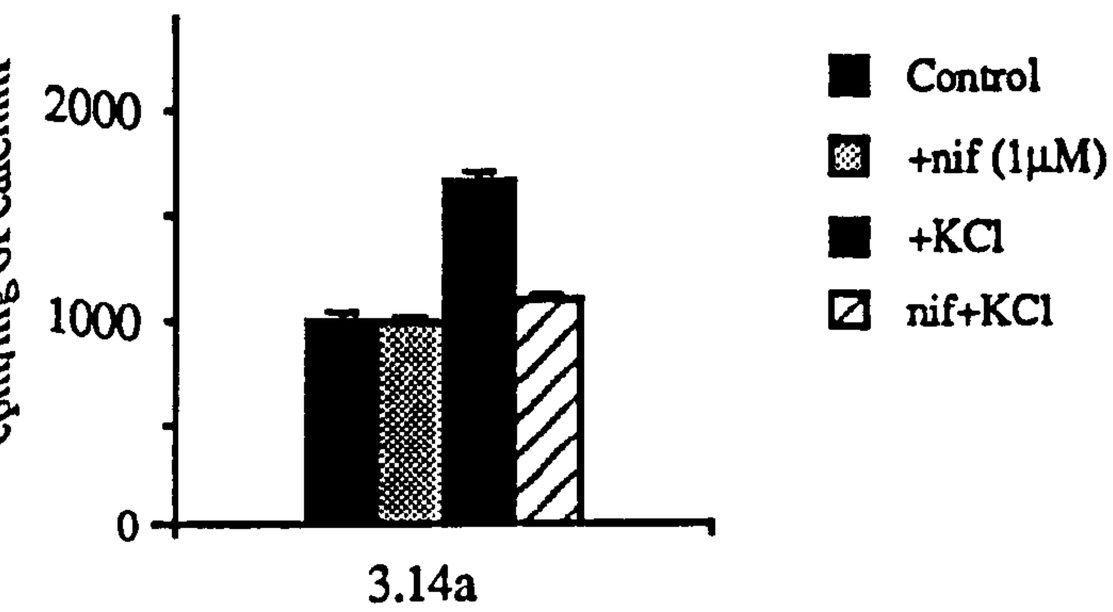


Fig. 3.12. Inhibition of $^{45}Ca^{++}$ uptake elevated by $[K^+]_o$ (50mM) reached a maximum after 20min incubation in nifedipine (1μM). This incubation time was used in subsequent experiments.

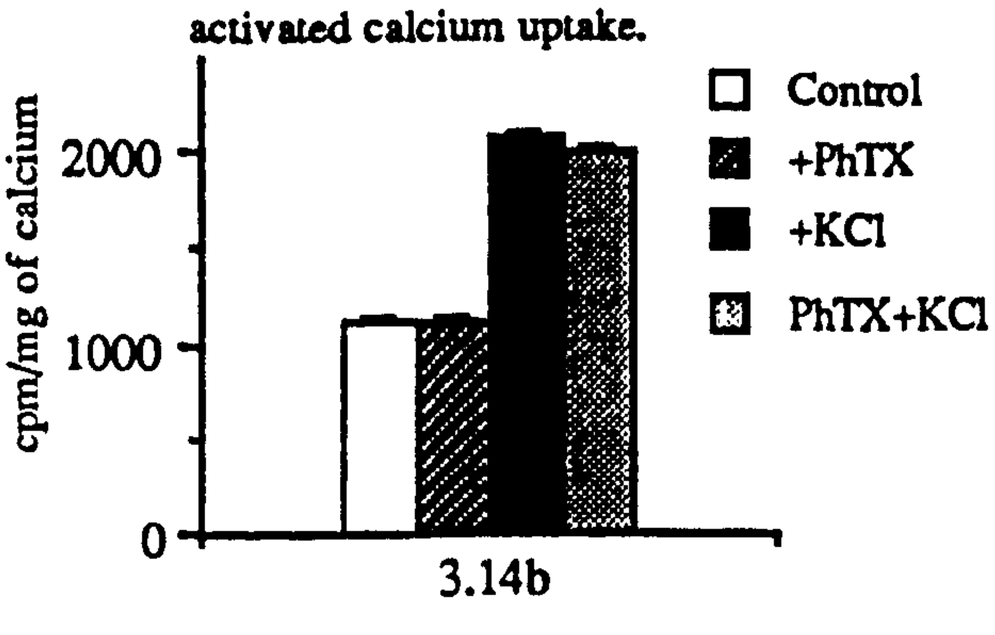
Effect of nifedipine on KCl (50mM) activated calcium uptake.



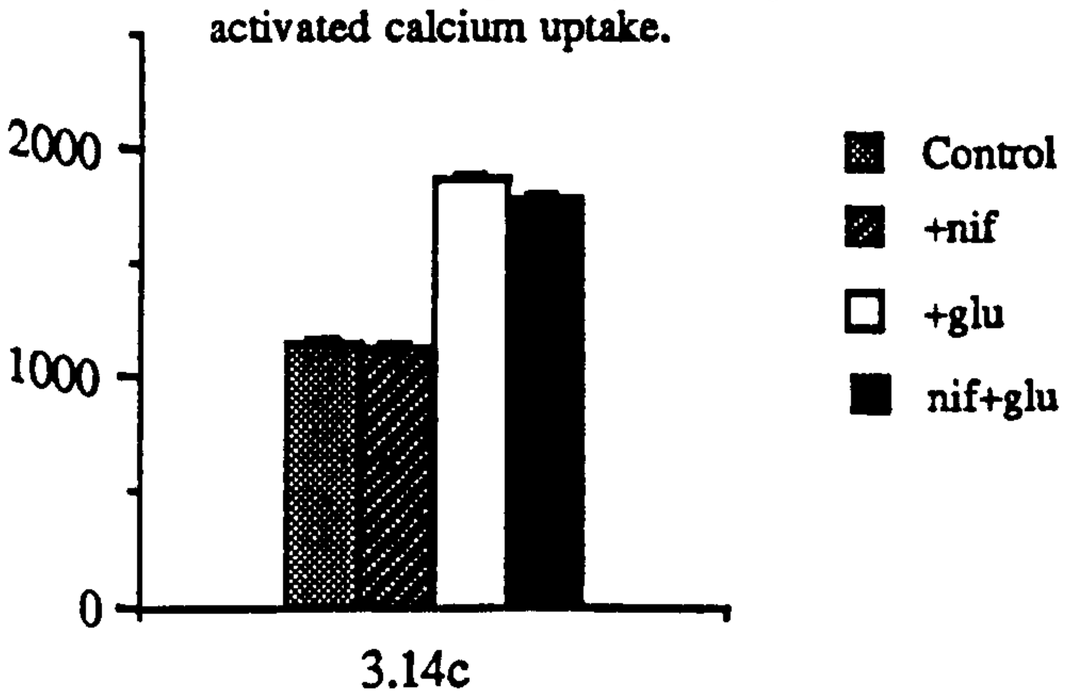
<u>Fig. 3.13.</u> Dose-response relationship for nifedipine inhibition of [45Ca^{++}] influx stimulated by 50mM $[K^+]_o$.



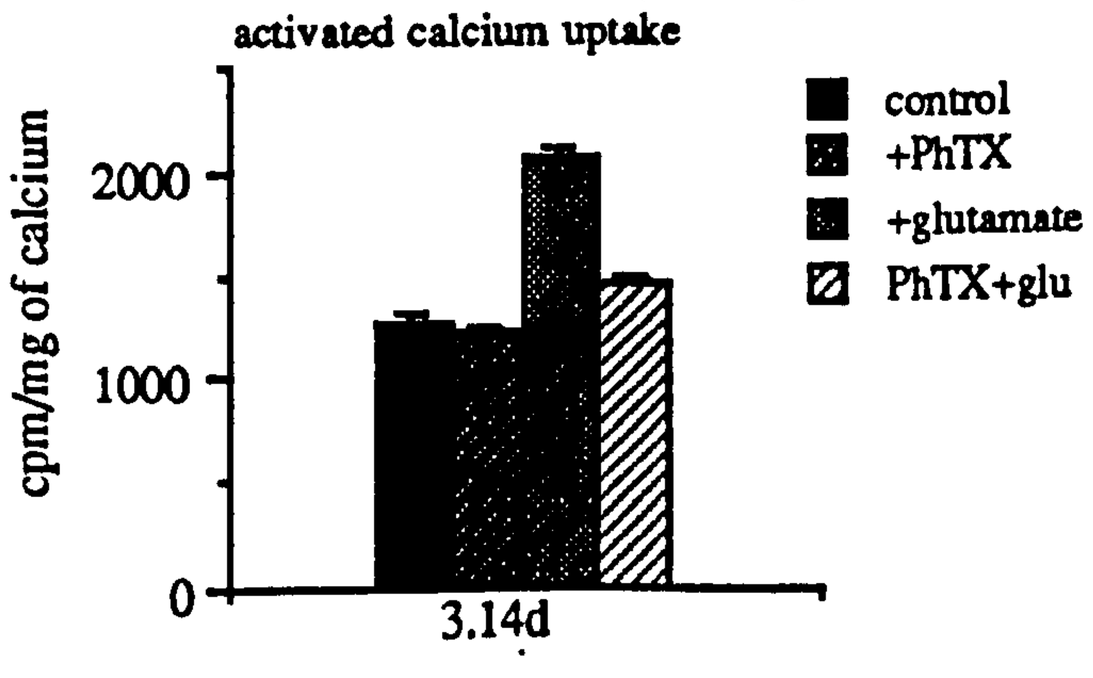
3.14a



3.14b



3.14c



3.14d

Fig. 3.14.

Nifedipine (1μM) reduced the uptake of [45Ca^{++}] stimulated by elevated $[K^+]_o$ (50mM) (Fig. 3.14a) whereas PhTX-343 at the same concentration (1μM) had very little effect on $[K+]_o$-induced 45Ca^{++} uptake (Fig. 3.14b). Nifedipine (1μM) failed to block L-glutamate-stimulated calcium uptake (Fig. 3.14c) whereas PhTX-343 (1μM) block glutamate-induced [45Ca++] entry (Fig. 3.14d).

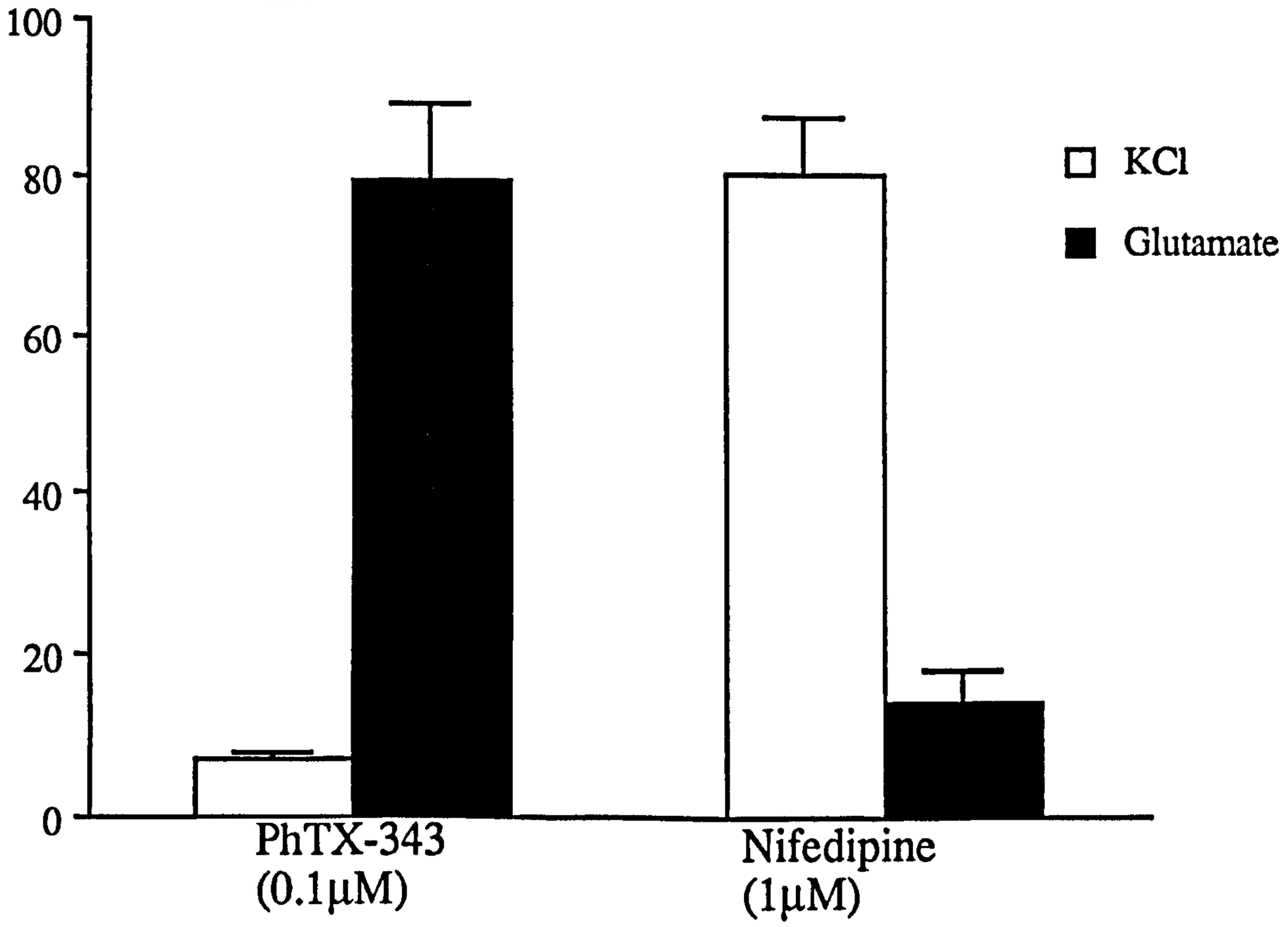


Fig. 3.15. PhTX-343 and nifedipine inhibited [$^{45}Ca^{++}$] entry induced by L-glutamate (10^{-4}M) and elevated $[K^+]_o$ (50mM) respectively.

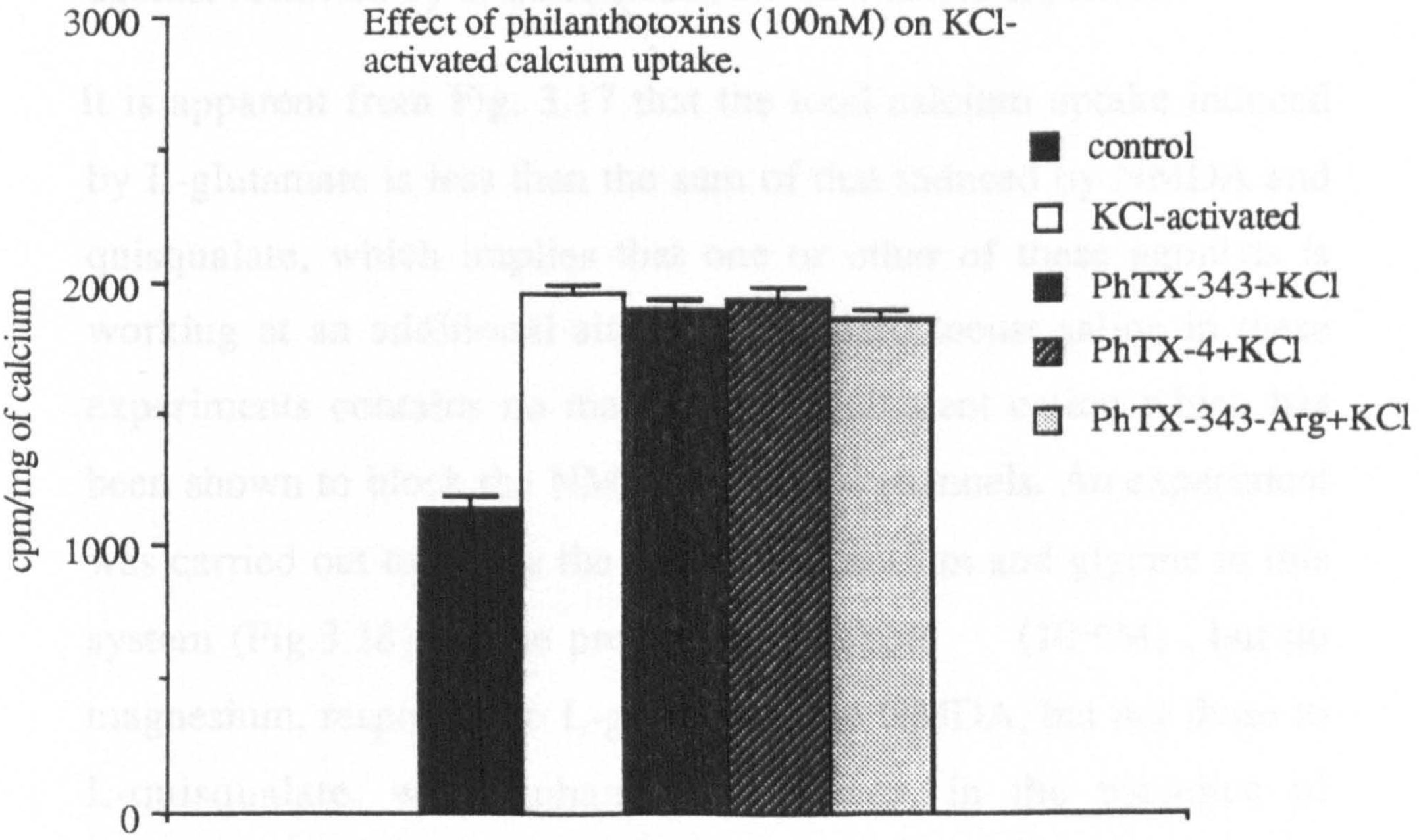


Fig. 3.16. Philanthotoxin analogues (100nM) did not inhibit uptake of [45Ca^{++}] activated by 50mM $[k^+]_o$

3.1.6. Effect of excitatory amino acids on [$^{45}Ca^{++}$] entry into locust muscle

DL-ibotenate, AMPA, kainate and glycine had no effect on [$^{45}Ca^{++}$] uptake (Fig. 3.17). L-glutamate was the most potent agonist followed by L-quisqualate, NMDA and L-aspartate.

It is apparent from Fig. 3.17 that the total calcium uptake induced by L-glutamate is less than the sum of that induced by NMDA and quisqualate, which implies that one or other of these agonists is working at an additional site. The standard locust saline in these experiments contains no magnesium, a divalent cation which has been shown to block the NMDA activated channels. An experiment was carried out to assess the role of magnesium and glycine in this system (Fig.3.18). In the presence of glycine ($10^{-6}M$) , but no magnesium, responses to L-glutamate and NMDA, but not those to L-quisqualate, were enhanced. However, in the presence of magnesium (2mM) the glycine-evoked potentiation and the action of NMDA was abolished. The action of L-quisqualate and the majority of the response to L-glutamate were unaffected by magnesium (Fig. 3.18).

These results are surprising and suggest that in the locust nerve-muscle system system L-glutamate and L-quisqualate act on the same population of receptors, but NMDA act at a different population which is only weakly stimulated by L-glutamate. Both populations appear to desensitise in a similar way and

desensitisation of both population can be blocked by Con A (Fig. 3.19).

The NMDA antagonist, 2-amino-phosphopentanoic acid (AP5) (10^{-5}M) applied for 20min before addition of excitatory amino acid abolished the action of NMDA and prevented glycine from potentiating the glutamate-induced Ca^{++} influx. However, AP5 (10^{-5}M) had no effect on L-quisqualate or L-aspartate responses (Fig. 3.20). The latter result may appear surprising and suggests that in this system aspartate is not an agonist at NMDA receptors, but is a weak agonist of the glutamate receptor which is quisqualate-sensitive (Watkin *et al.*, 1987)

It has already been shown that PhTX-343 can block the uptake of $^{45}Ca^{++}$ induced by glutamate in this system. As it now appears that there is a separate population of NMDA receptors at which glutamate is only weakly active the action of PhTX-343 on glutamate and NMDA responses was re-examined. Following pre-incubation of muscle in PhTX-343 (10^{-6}M), the $^{45}Ca^{++}$ uptake induced by both glutamate and NMDA was inhibited by about 80%. (Fig. 3.21). This may be analogous to the position in rat brain where PhTX-343 can block both NMDA and non-NMDA responses.

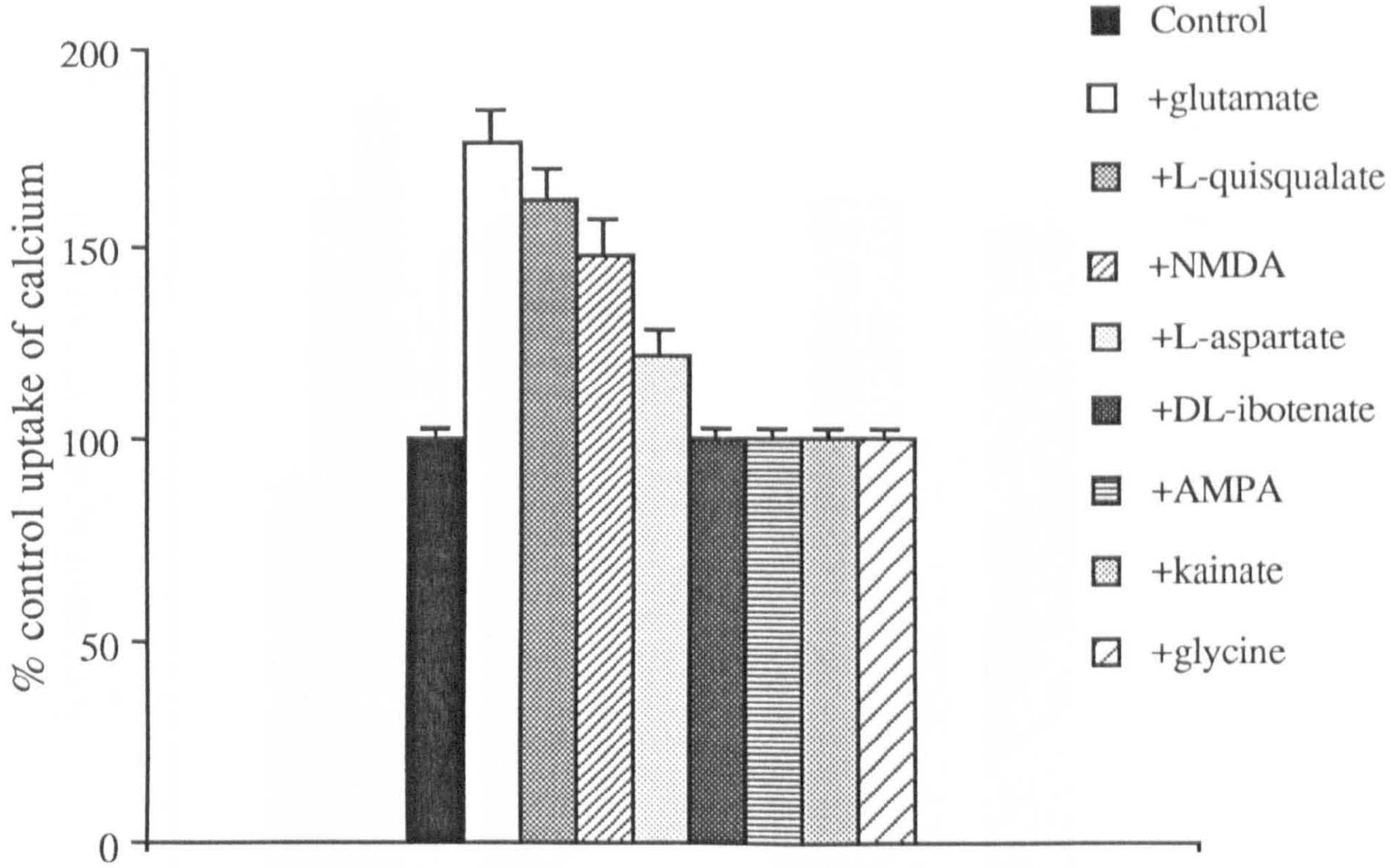


Fig. 3.17. Muscles were exposed for 10 min to an excitatory amino acid (10^{-4}M) following 30 min pre-incubation in ConA (10^{-6}M). [$^{45}Ca^{++}$] uptake was expressed as a percentage of the paired control muscle.

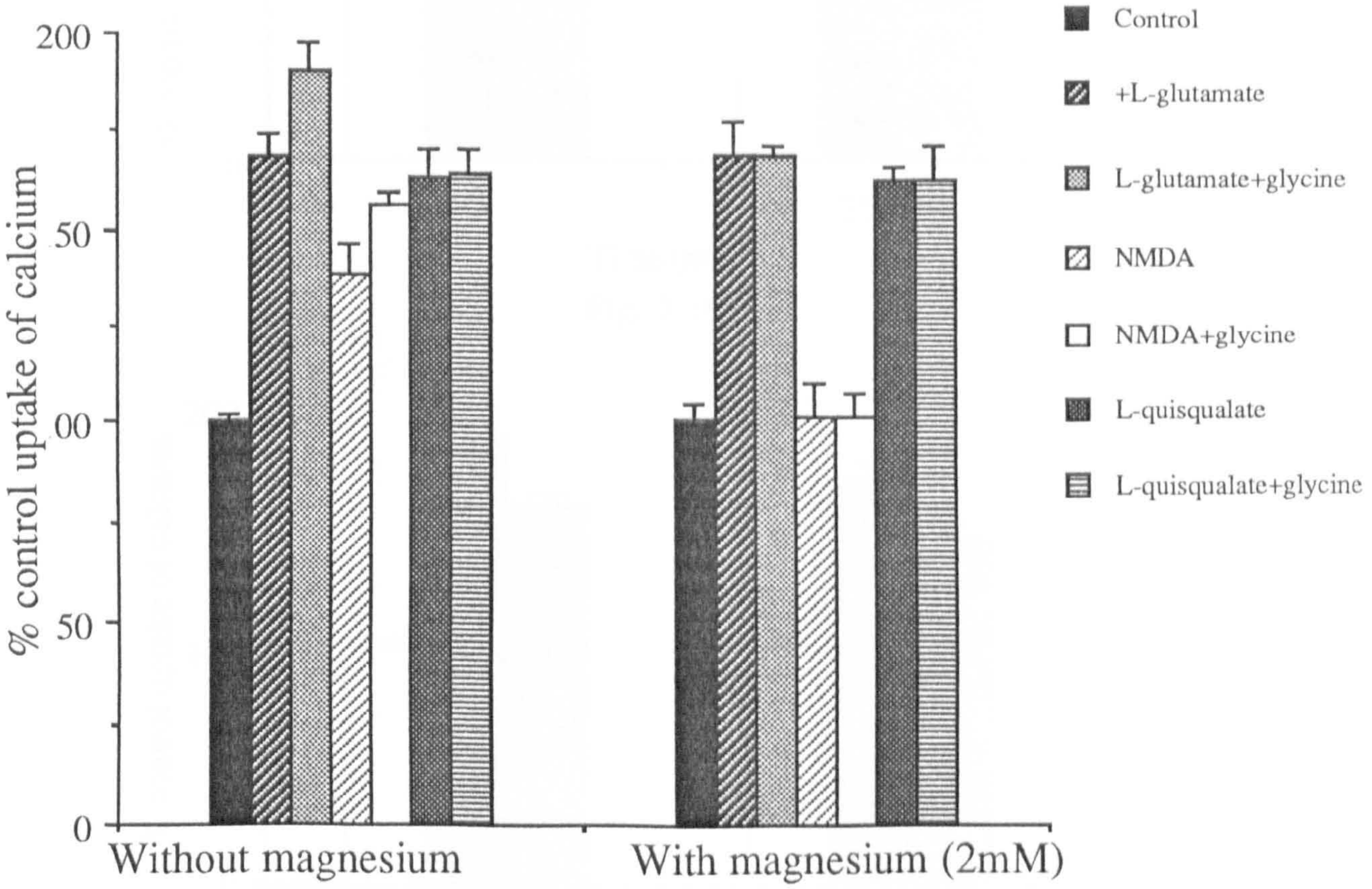


Fig. 3.18. In the absence of magnesium ions, glycine (10^{-6}M) potentiated the actions of L-glutamate (10^{-4}M) and NMDA (10^{-4}M), but not that of L-quisqualate. When magnesium (2mM) was present, the action of NMDA was abolished and the glycine stimulated L-glutamate response was blocked.

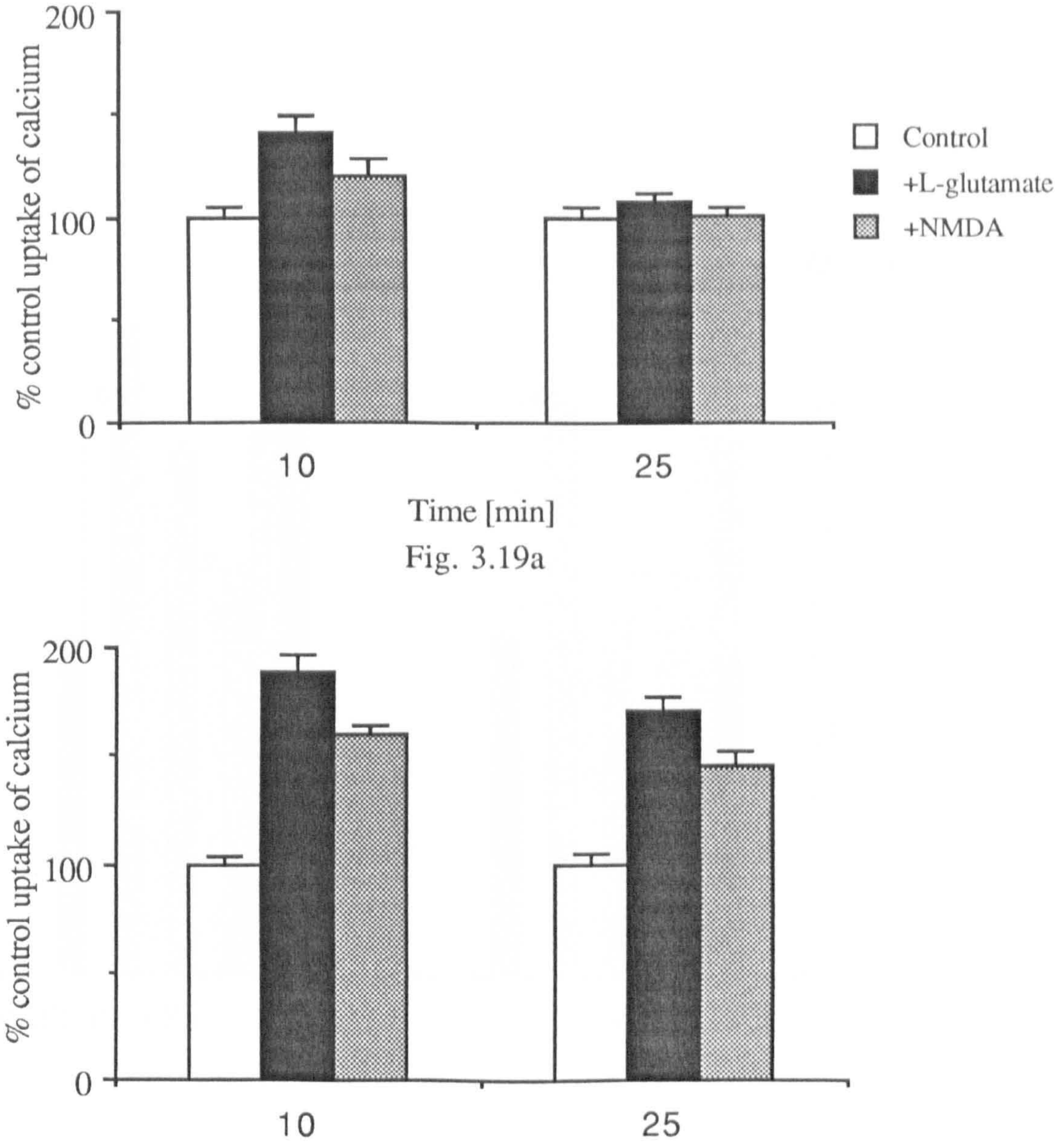


Fig. 3.19a

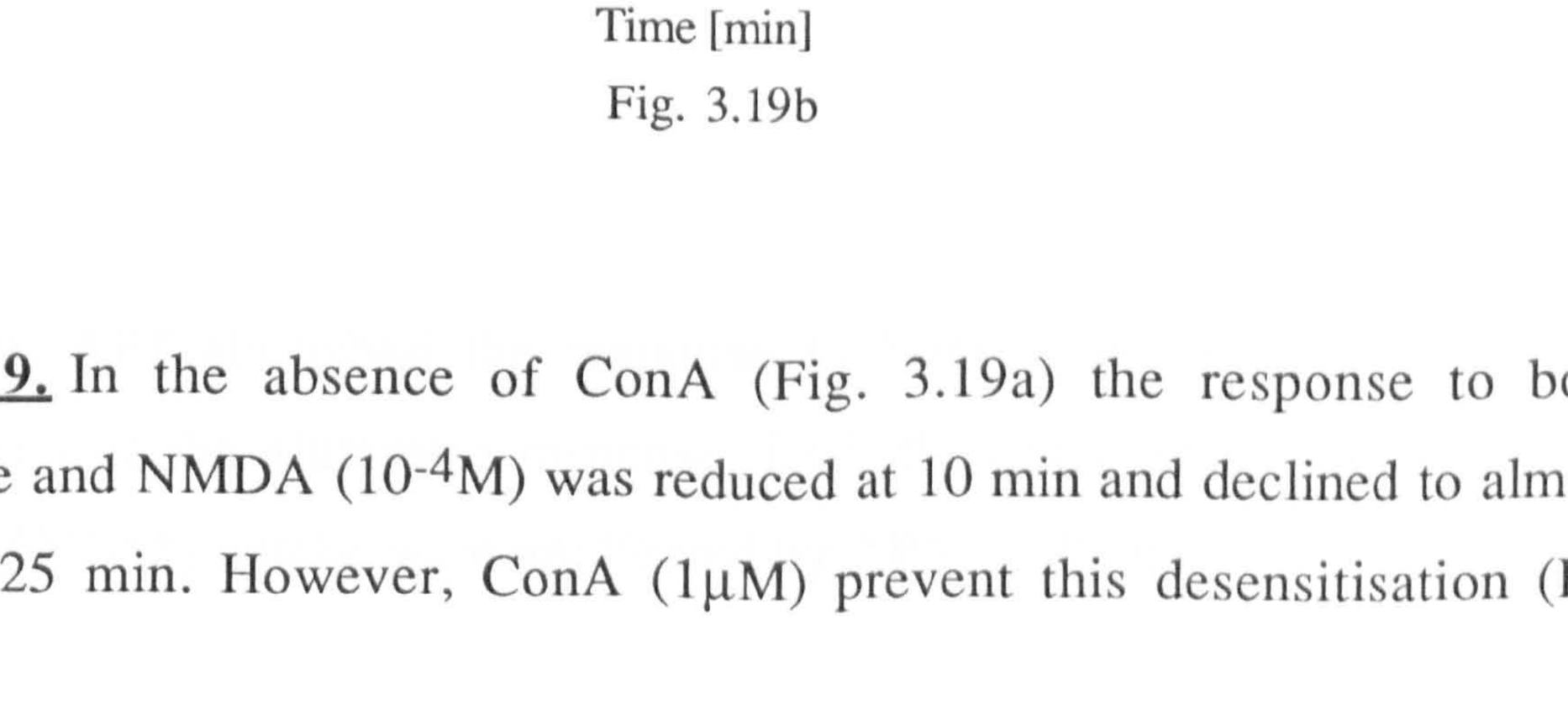


Fig. 3.19b

Fig. 3.19. In the absence of ConA (Fig. 3.19a) the response to both glutamate and NMDA (10^{-4}M) was reduced at 10 min and declined to almost zero by 25 min. However, ConA (1μM) prevent this desensitisation (Fg. 3.19b).

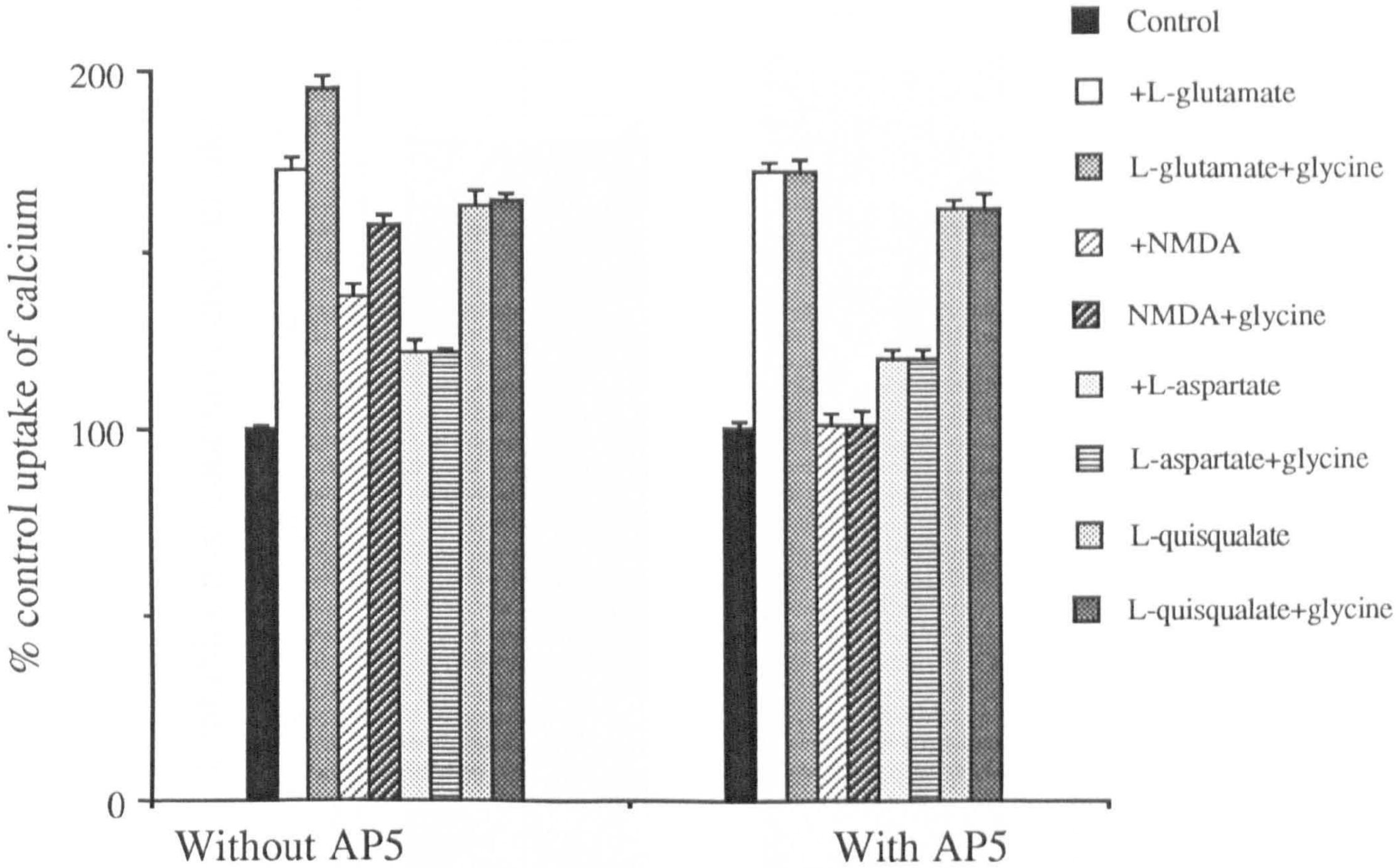


Fig. 3.20. AP5 abolished the response to NMDA and prevented glycine potentiation of the glutamate response. Both the quisqualate- and aspartate-induced [$^{45}Ca^{++}$] uptake were unaffected by AP5 or glycine.

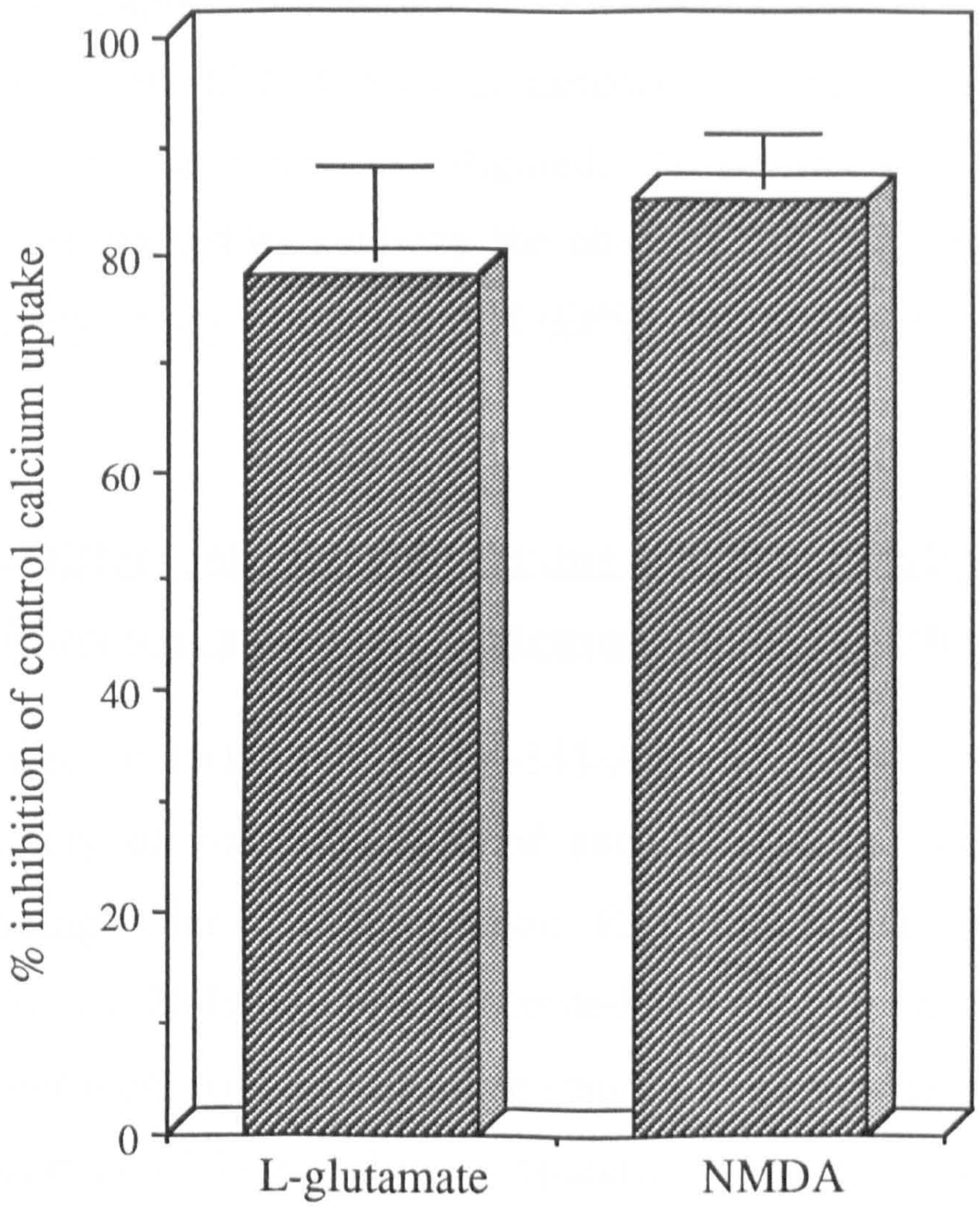


Fig:3.21. PhTX-343 (10^{-6}M) inhibited [$^{45}Ca^{++}$] uptake induced by L-glutamate (10^{-4}M) and NMDA (10^{-4}M) to similar extents.

3.2. Effect of PhTX-343 on excitatory postsynaptic potentials (EPSPs) of arthropod muscles.

In this chapter, the effect of PhTX-343 and other synthetic analogues of PhTX-433 on glutamatergic synapses of locust and tick muscle have been investigated. The potencies of these toxins were determined by studying the changes that they induced in the excitatory postsynaptic potential (EPSP) of the muscle.

3.2.1. Effect of philanthotoxins on the EPSPs of locust *(Schistocerca gregaria)* extensor tibiae muscle.

The effect of PhTX-343, PhTX-343-Arg and PhTX-4 were studied. A variety of concentrations of each compound was tested, in ascending order of concentration. Each experiment was repeated three times; 20 EPSPs being recorded at 3 min intervals throughout an experiment. Unless otherwise stated, the nerve was stimulated at a frequency of 1Hz and the preparation was perfused for 10 min with each concentration of toxin. The wash time between toxin application was 1h. Dose-inhibition relationships were derived from the data (Fig. 3.23). All three toxins reduced the amplitude of the EPSP (Figs. 3.24, 3.25, 3.26). PhTX-343-Arg was the most potent of the three toxins (IC_{50} = ~ $5x10^{-8}$M), whereas PhTX-4 was the least potent. PhTX-343-Arg (10^{-4}M) depressed the EPSPs by ~80% after 3 min application and the EPSPs were abolished

after 6 min (Fig. 3.28). PhTX-343 (10^{-4}M) abolished the EPSPs after 9 min, whereas the inhibition of the EPSPs by PhTX-4 plateaued at ~20% after 12 min (Fig. 3.28). The time taken for the appearance of steady-state inhibition by the three toxins was seemingly independent of the concentration of the toxin that were applied (Figs. 3.29 and 3.30). Complete reversal of the effects of low concentrations (10^{-8}M) of PhTX-343-Arg and PhTX-343 was observed following a 30 min washout of these toxins. With higher concentrations of toxin reversibility was either incomplete or absent (Figs. 3.24 and 3.26). The effect of PhTX-4 was fully reversible after 30 min washout, for all concentrations of this toxin that were studied (Fig. 3.25). The recovery was greater before started to decline when the Ca^{++} conncentration was raised to 3mM compared to normal physiological Ca^{++} concentration (1mM) (Table. 1 and 2). This decline in reversal amplitude was not seen when the Ca^{++} concentration was raised following 30min washout of toxin with standard locust saline (Fig. 3.27).

3.2.2. Effect of philanthotoxins on EPSPs evoked from tick (*R. appendiculatus*) coxal muscle

The resting potential of freshly dissected coxal muscle of *R. appendiculatus* varied between -50mV and -70mV. The amplitude of neurally evoked EPSPs obtained from coxal muscle varied between -20mV to -25mV. Miniature excitatory postsynaptic

potential (minEPSP) were frequently encountered in the muscles (Fig. 3.33) Bath applied L-glutamate 10^{-4}M eliminated both minEPSPs and neurally evoked EPSPs (Figs. 3.32 and 3.33). Low concentration (10^{-8}M) of bath applied L-glutamate increases the frequency of minEPSP. No effect was observed with octopamine, acetylcholine at same concentration on either spontaneous or evoked potentials.

Control EPSPs (n=20) were obtained from tick muscle in the absence of toxin and then the muscle preparation was incubated for 10 min with PhTX-343 (10^{-4}M- 10^{-6}M), in the absence of motor nerve stimulation. PhTX-343 was washed out for 30 min with toxin-free saline. The EPSPs recorded was not affected. When PhTX-343 was applied during motor nerve stimulation, the amplitude of EPSPs were significantly reduced (Table. 3). This observation supports the description of PhTX-343 as an open channel blocker of locust muscle qGluR (May and Piek, 1979; Piek and Spanjer, 1986; Piek *et al.*, 1986)

Dose-inhibition data were obtained for PhTX-343, C_7-PhTX-343 and DNP12-PhTX-343 (Fig. 3.34). C_7-PhTX-343 and DNP12-PhTX-343 were more potent (IC_{50} = ~ $5x10^{-8}$M) than PhTX 343 (IC_{50} = ~ $2x10^{-5}$M) (Tables. 4, 5, 6). The time taken for the appearance of steady-state inhibition of the EPSP was similar for all three toxins. The maximum effect was always observed after 12 min of application. (Fig. 3.37). The philanthotoxin antagonised EPSP in use dependent manner and the recovery of the EPSP

following removal of toxin depended on the concentration of toxin that was applied (Figs. 3.35 and 3.36).

The coxal motor nerve was stimulated between 1Hz and 8Hz and the effects of different concentrations of PhTX-343 on the EPSPs were studied. The data illustrated in Figs 3.38, 3.39, 3.40 show clearly that inhibition was increased when the stimulation frequency was raised. Reduction in EPSP amplitude were not seen when the muscle was stimulated at high frequencies in the absence of toxin.

2.2.3. Effect of Ca^{++} on EPSPs of locust and tick muscle

Locust and tick nerve muscle preparation were perfused with various concentrations of Ca^{++}. At each concentration, 20 EPSPs were recorded. Fig 3.22 shows that the maximum amplitude of the EPSP of locust muscle was obtained with 1mM to 2mM Ca^{++}, whereas the maximum amplitude of the EPSP of tick coxal muscle was obtained with 4mM to 7mM (Fig. 3.31).The locust EPSP started to decline when the muscle were perfused with a high concentration (>2mM) of Ca^{++} and was abolished at 10mM (Fig. 3.22). The EPSP of tick coxal muscle also declined when the Ca^{++} concentrations exceeded (>7mM) and was abolished when the saline contained 10-17mM Ca^{++}

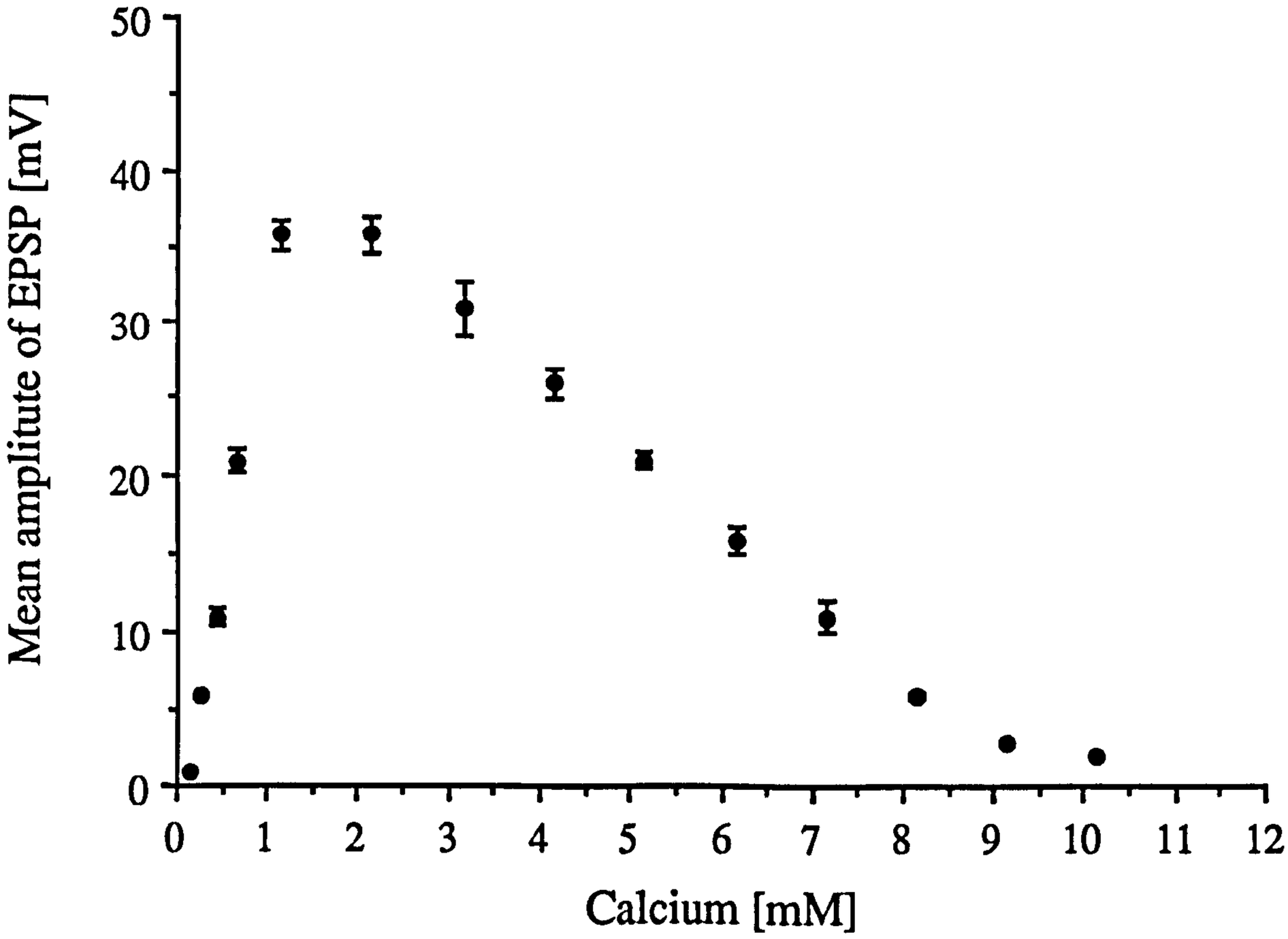


Fig. 3.22. The effect of different concentrations of Ca^{++} on the EPSP (n=20) of extensor tibiae muscle of locust (*Schistocerca gregaria*) during continuous stimulation of the motor-nerve.

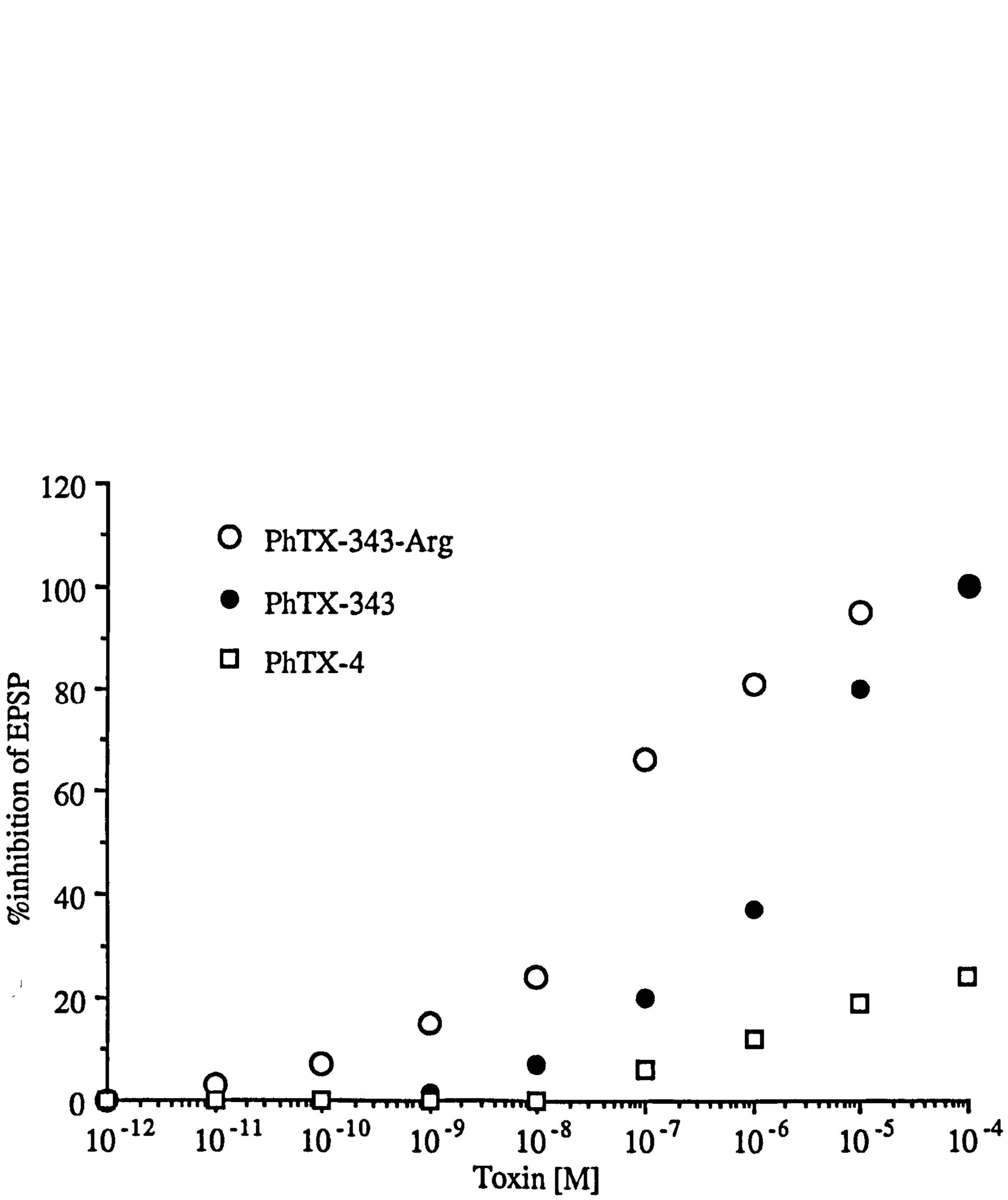
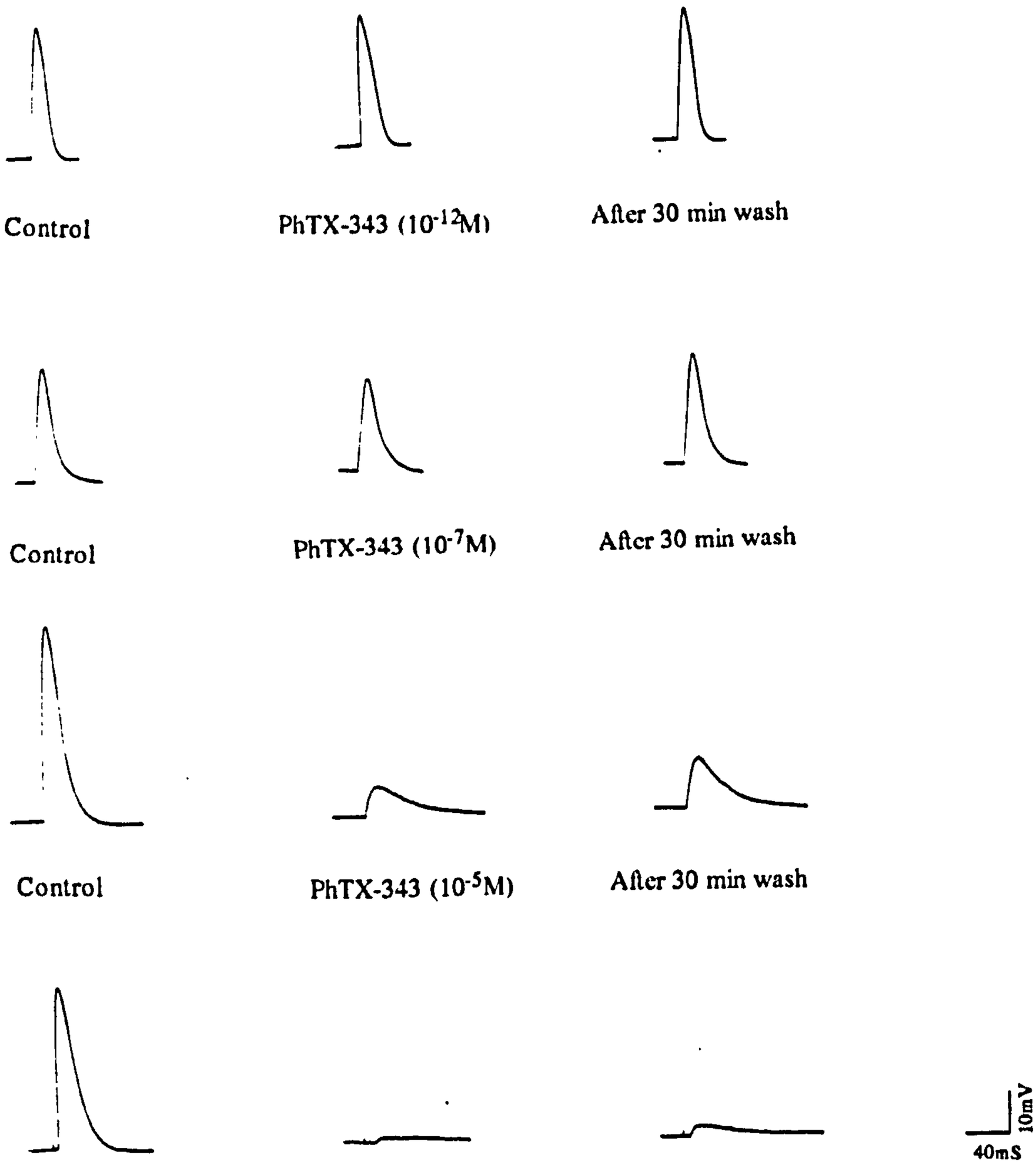


Fig. 3.23. Dose-inhibition relationship for antagonism of EPSP of locust extensor tibiae muscle by PhTX-343, PhTX-343-Arg and PhTX-4. PhTX-343-Arg was more potent (IC50=~5×10^{-8}M) than PhTX-343 (IC50=~5×10^{-6}M). PhTX-4 was much less effective than the other two toxins.



<u>Fig. 3.24.</u> Inhibition by PhTX-343 of EPSPs evoked from locust (*Schistocerca gregaria*) extensor tibiae muscle. Control EPSPs (n=20) were recorded from the muscle after a 10min perfusion with standard locust saline. A concentration-dependent inhibition was observed after 12min perfusion with toxin. Recovery of the EPSPs following removal of toxin (30min of washing with standard locust saline) depended on the concentration of toxin that was applied.

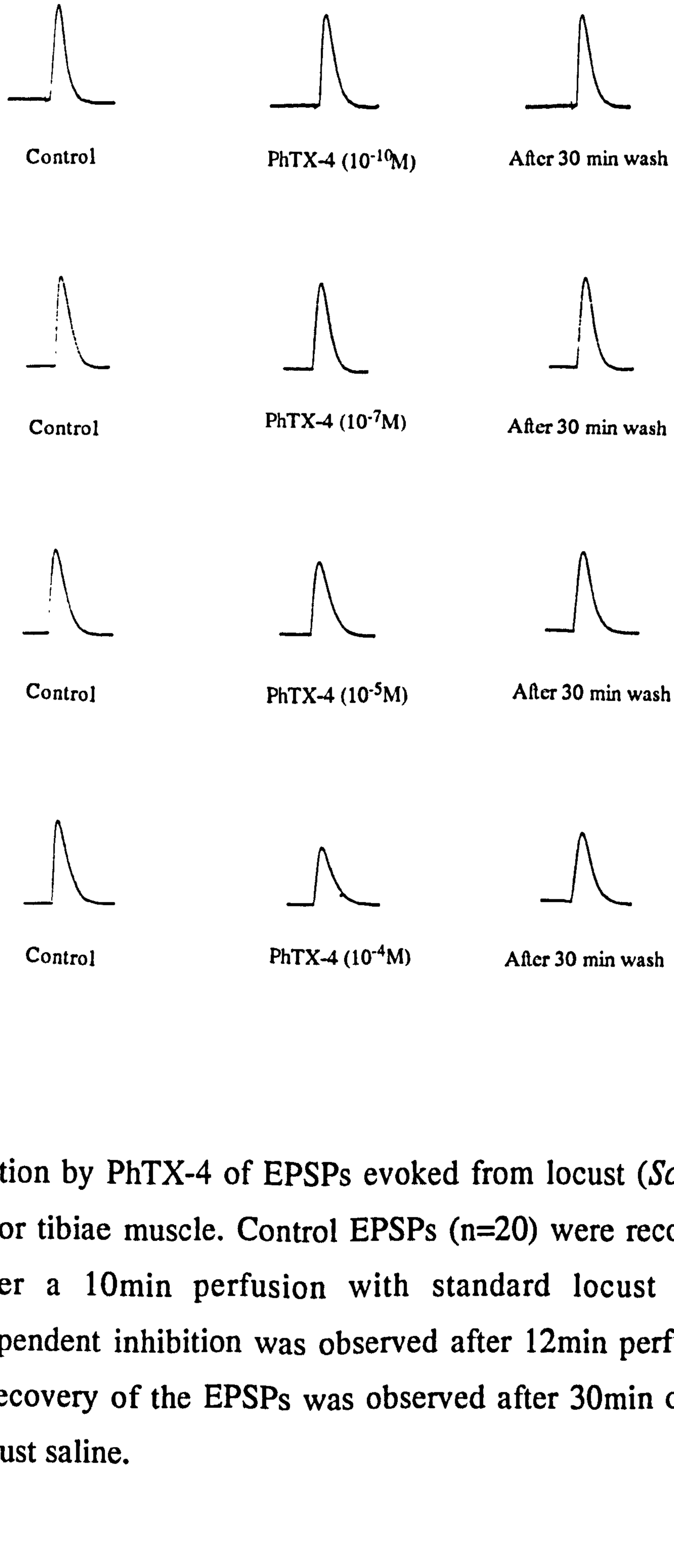


Fig. 3.25. Inhibition by PhTX-4 of EPSPs evoked from locust (*Schistocerca gregaria*) extensor tibiae muscle. Control EPSPs (n=20) were recorded from the muscle after a 10min perfusion with standard locust saline. A concentration-dependent inhibition was observed after 12min perfusion with toxin. The full recovery of the EPSPs was observed after 30min of washing with standard locust saline.

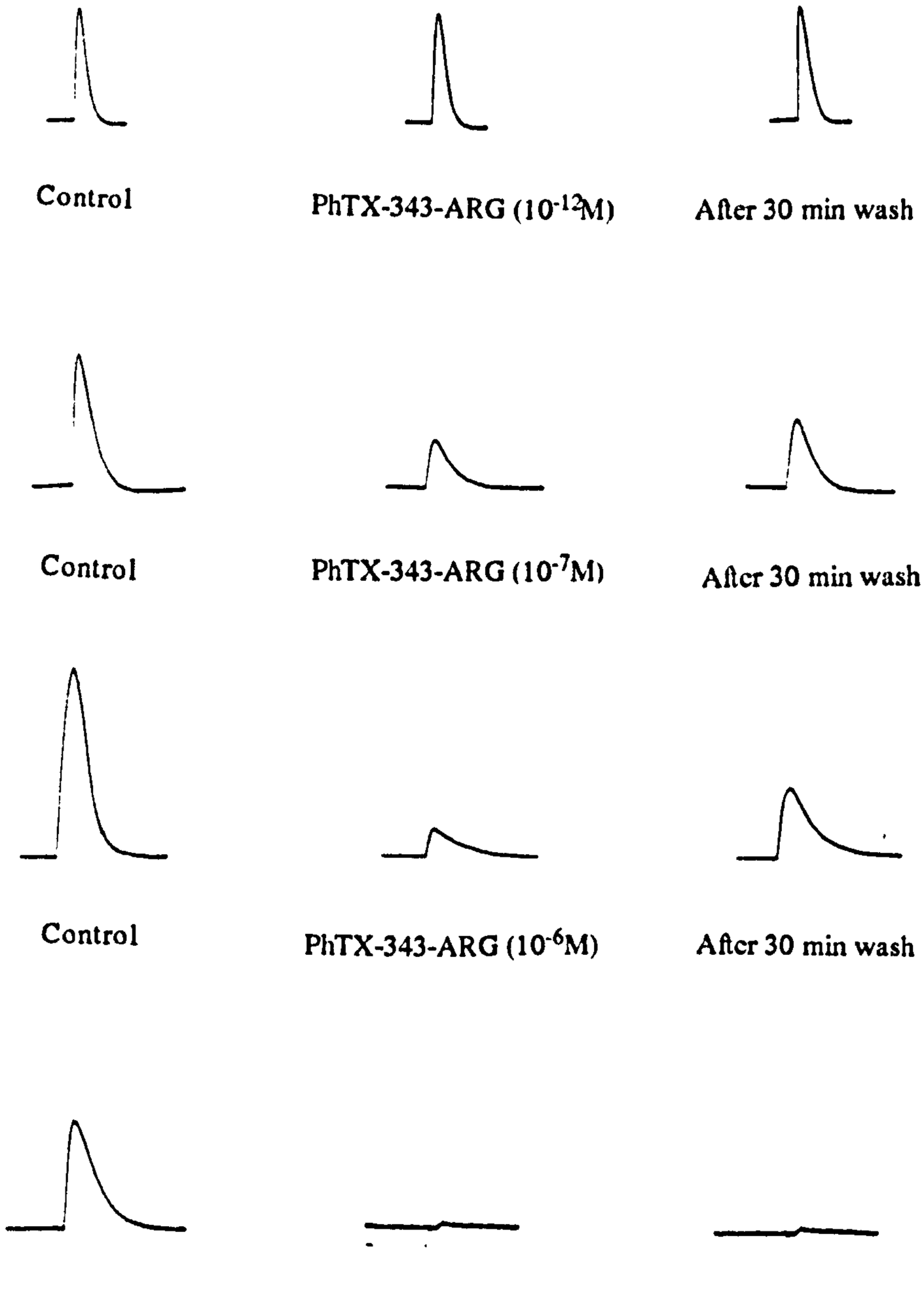


Fig. 3.26. Inhibition by PhTX-343-Arg of EPSPs evoked from locust (*Schistocerca gregaria*) extensor tibiae muscle. Control EPSPs (n=20) were recorded from the muscle after a 10min perfusion with standard locust saline. A concentration-dependent inhibition was observed after 12min perfusion with toxin. Recovery of the EPSPs following removal of toxin (30min of washing with standard locust saline) depended on the concentration of toxin that was applied.

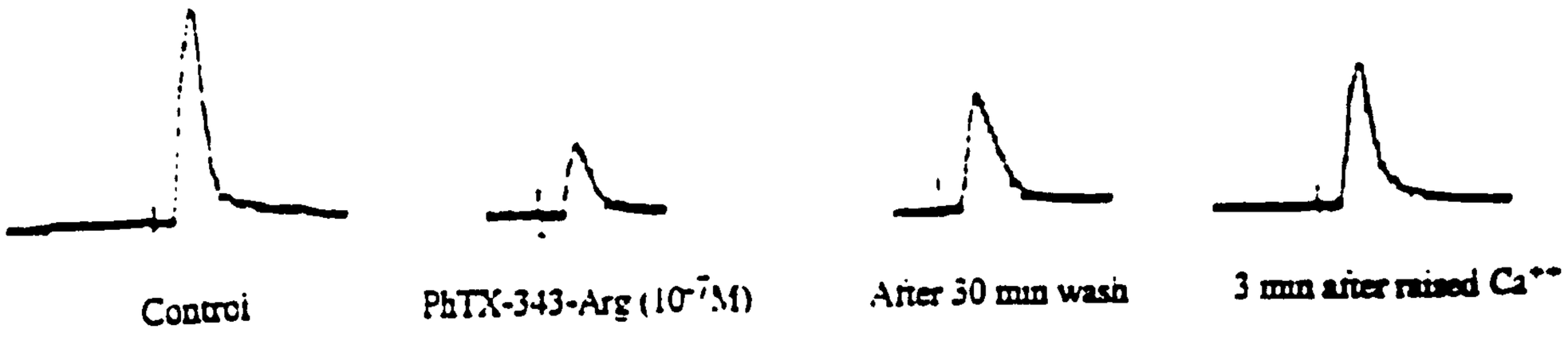
TABLE 1

Concentration	Inhibition (%)	% Recovery after 30 min wash-out with standard locust saline	% Recovery after 30 min wash-out with locust saline (3mM Ca^{++})
$10^{-12}M$	0%	0%	0%
$10^{-11}M$	3%±0.4	100%±0.2	100%±0.1
$10^{-10}M$	7%±0.8	97%±1	100%±o.6
$10^{-9}M$	15%±0.8	90%±0.8	100%±0.9
$10^{-8}M$	24%±0.5	89%±1.3	100%±1
$10^{-7}M$	66%±1.5	54%±1	75%±1.9
$10^{-6}M$	81%±1.8	30%±0.8	50%±2
$10^{-5}M$	95%±1.5	4%±0.1	10%±1
$10^{-4}M$	100%,1	0%	5%±0.5
$10^{-3}M$	100%±1.2	0%	0%

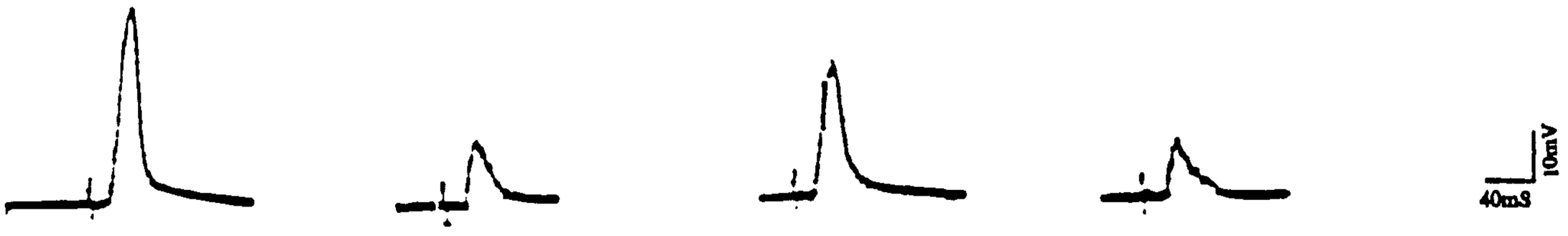
TABLE 2

Concentration	Inhibition (%)	% Recovery after 30 min wash-out with standard locust saline	% Recovery after 30 min wash-out with locust saline (3mM Ca^{++})
$10^{-12}M$	0%	0%	0%
$10^{-11}M$	0%±0.4	0%	0%±0.1
$10^{-10}M$	0%±0.8	0%	0%±o.6
$10^{-9}M$	1.5%±0.4	100%	100%±0.9
$10^{-8}M$	4%±0.5	100%±0.5	100%±0.7
$10^{-7}M$	20%±0.9	84%±1.5	100%±1
$10^{-6}M$	30%±1	80%±0.6	92%±1
$10^{-5}M$	80%±1.5	58%±0.1	65%±1.5
$10^{-4}M$	100%,1	5%	7%±1
$10^{-3}M$	100%±1.2	0%	0%

Tables. 1 and 2 Effect of different concentrations of PhTX-343-Arg (Table 1) and PhTX-343 (Table 2) on EPSPs evoked from locust extensor tibiae muscle. When toxin washed-out with locust saline containing 3mMCa^{++}, the recovery was always greater compared to normal physiological saline.



(Fig. 3.27a)



(Fig. 3.27b)

Fig. 3.27 Recovery of the amplitude of EPSPs evoked from locust leg muscle was greater when the Ca^{++} concentration was raised following a 30min washout of toxin with standard locust saline (Fig. 3.27a). When the Ca^{++} concentration was raised in the presence of toxin the reverse EPSP amplitude always decline (Fig. 3.27b).

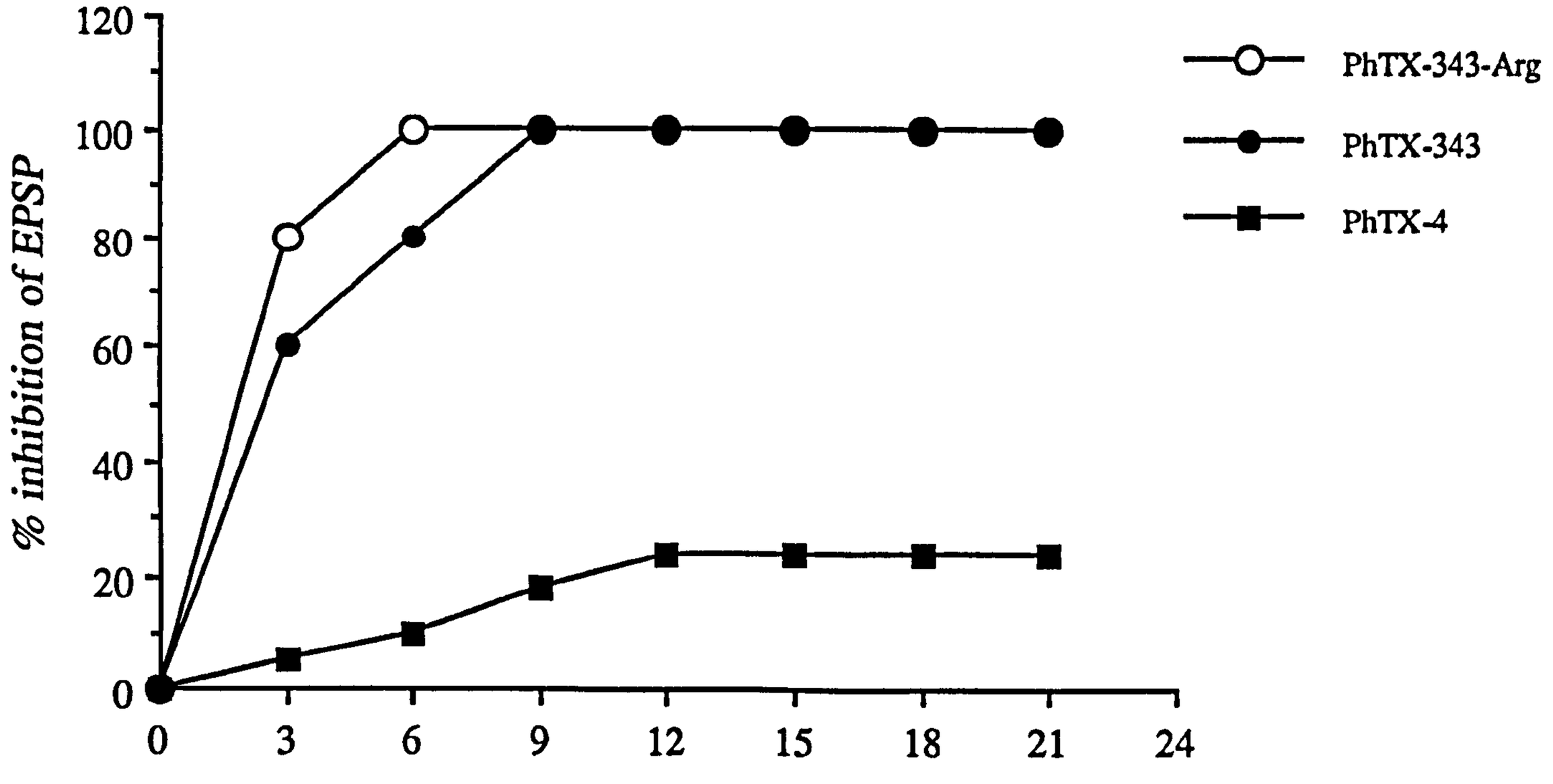


Fig. 3.28. Relationship between time of application of philanthotoxin and inhibition of EPSP of locust extensor tibiae muscle by 10^{-4}M PhTX-343-Arg, PhTX-343 and PhTX-4.

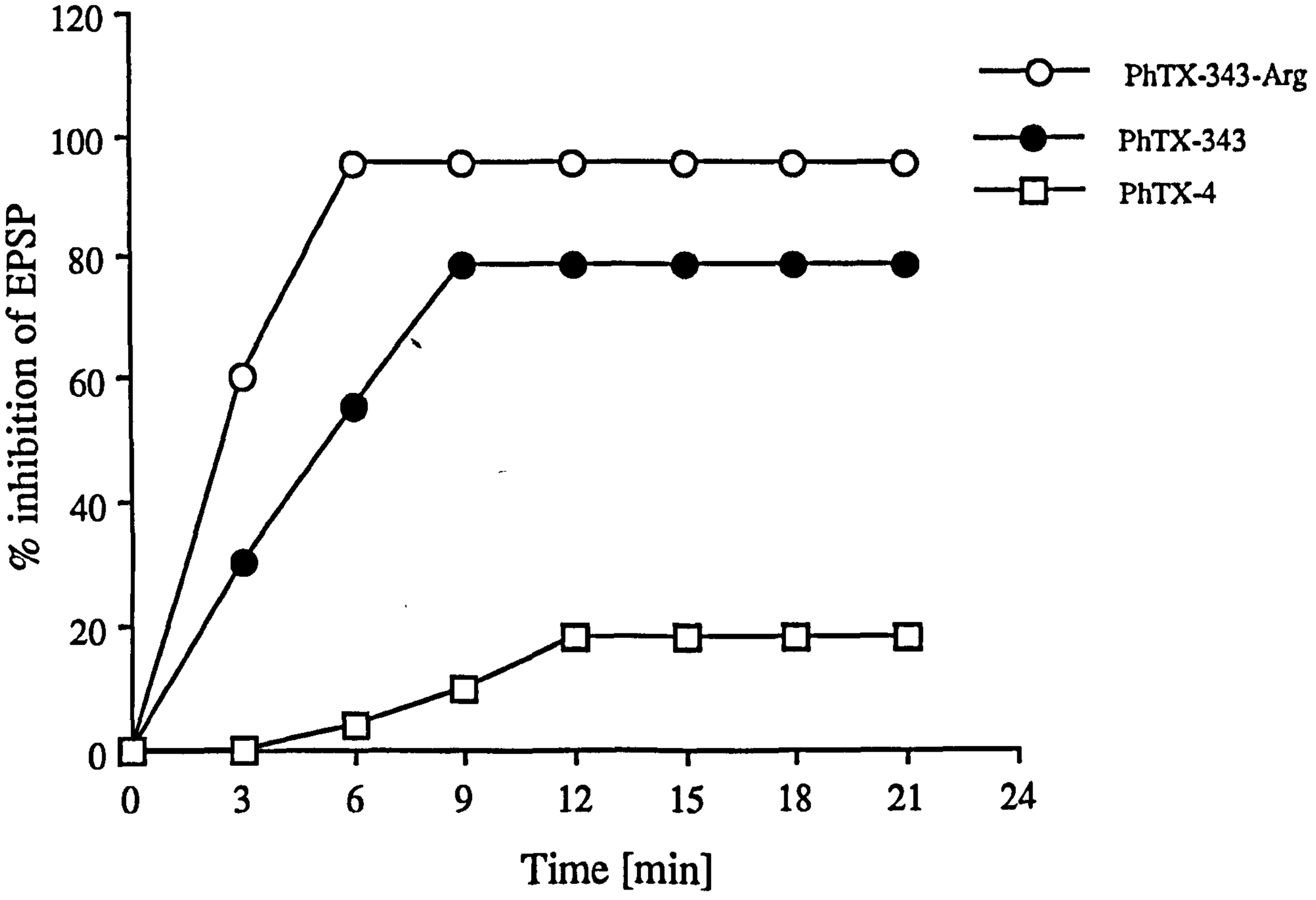


Fig. 3.29. Relationship between time of application of philanthotoxin and inhibition of EPSP of locust extensor tibiae muscle by 10^{-5}M PhTX-343-Arg, PhTX-343 and PhTX-4.

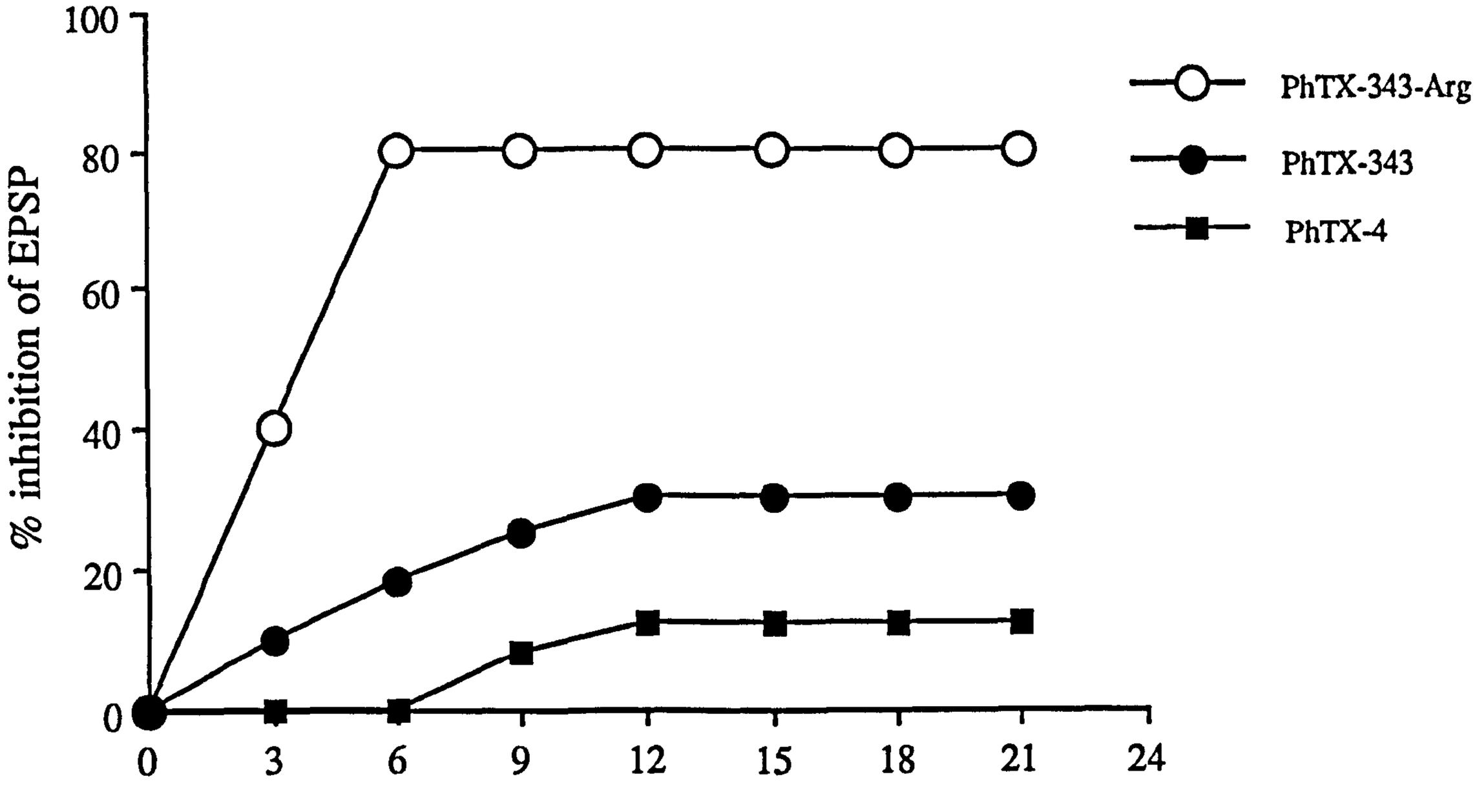


Fig. 3.30. Relationship between time of application of philanthotoxin and inhibition of EPSP of locust extensor tibiae muscle by 10^{-6}M PhTX-343-Arg, PhTX-343 and PhTX-4.

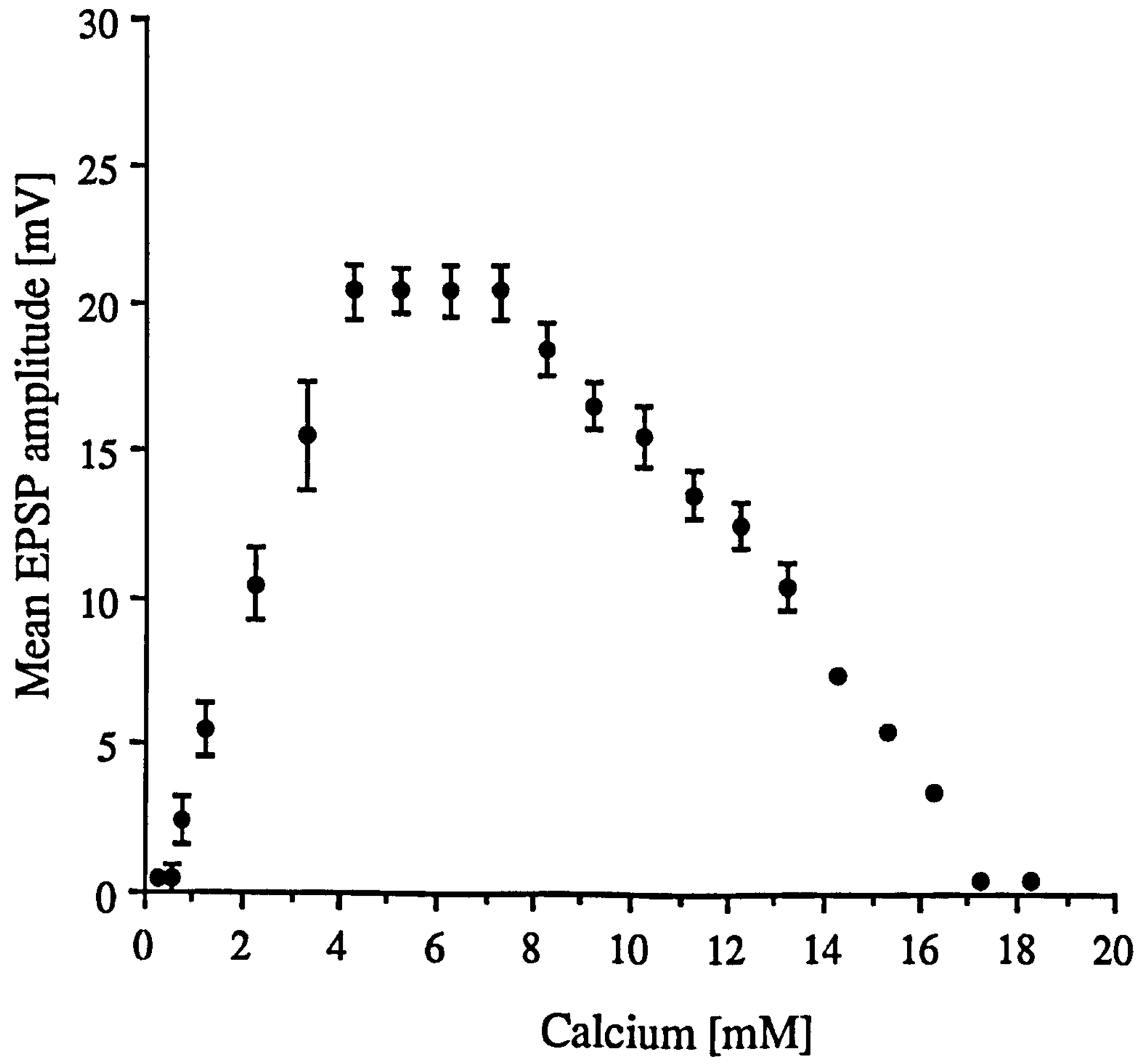
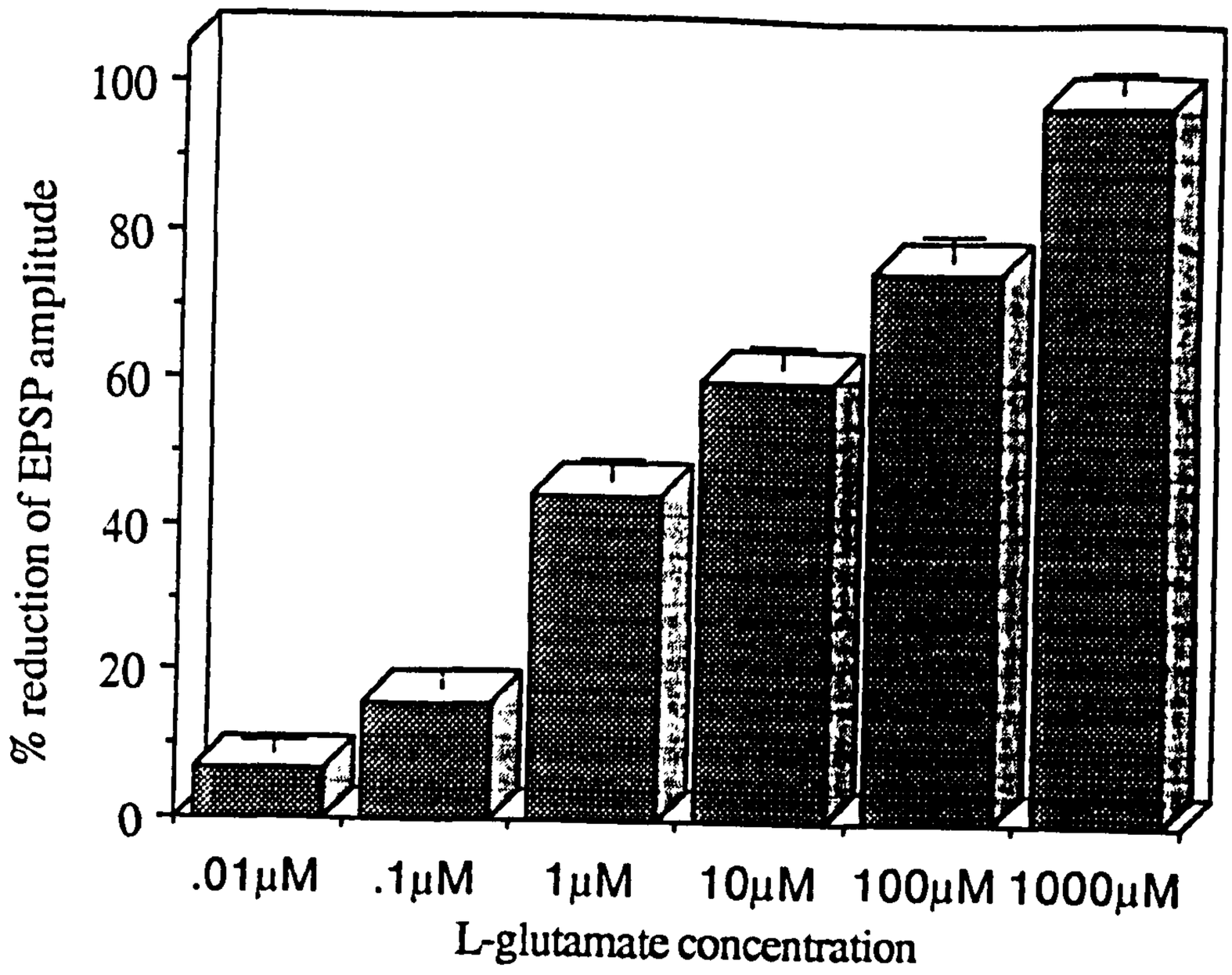
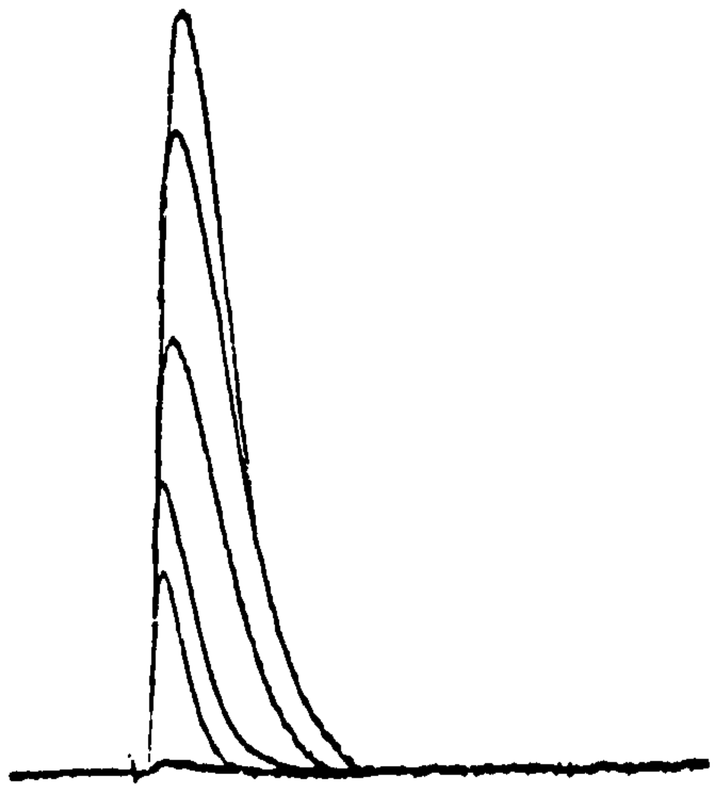


Fig. 3.31. Effect of different concentrations of Ca^{++} on the amplitude of the EPSP (n=20) of coxal muscle of tick (*R. appendiculatus*).



(Fig. 3.32a)



(Fig. 3.32b)

Fig. 3.32 EPSPs were evoked by stimulating nerves innervating coxal muscle fibres in the tick (pulses were normally delivered at 1Hz , 2-3 volts, 0.6mS). EPSPs were depressed in a dose-dependent manner by bath applied L-glutamate. The histogram (3.32a) shows a L-glutamate dose-dependent reduction in EPSPs, and this is further illustrated by the inset (3.32b) which shows the actual superimposed EPSPs.

Control

L-glutamate (10^{-8}M)

L-glutamate (10^{-5}M)

After 30 min wash

Fig. 3.33 Miniature EPSPs (min EPSPs) were recorded from tick coxal muscles. The low concentration of L-glutamate (10^{-8}M) increased the frequency after 1min of application. MinEPSPs abolished when L-glutamate was applied at high concentration (10^{-5}M)

TABLE. 3

PhTX-343 (Concentrations)	(A) EPSP (control)	(B) Mean evoked EPSP(after 30min wash of toxin)	(C) Mean evoked EPSP in the presence of toxin
10^{-6}M	22mV± 0.9	22mV±1	18mV±0.5
10^{-5}M	24mV±1.2	24mV±1	16mV±1.2
10^{-4}M	24mV±1.5	24mV±0.8	9mV±1.5

Table. 3 EPSPs (n=20) were recorded from tick (Rhipiciphalus appendiculatus) coxal muscles **(A).** The preparation was incubated in different concentration of philanthotoxin for 10 min in the absence of motor nerve stimulation. Toxin was washed out for 30 min with toxin free saline. The EPSP recorded was not affected **(B).** When PhTX-343 was applied during motor nerve stimulation, the amplitude of EPSP were significantly reduced **(C).**

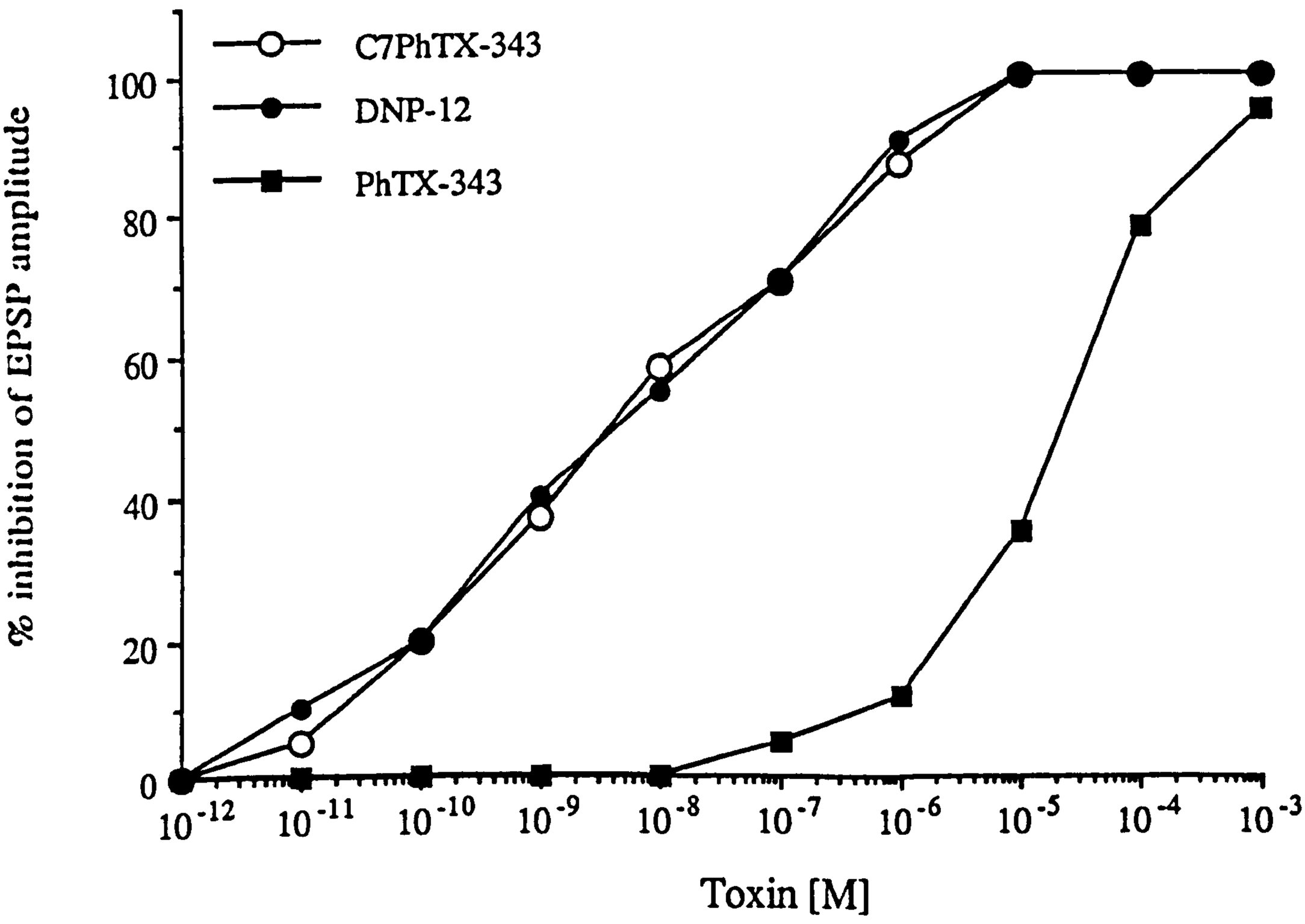


Fig. 3.34. Dose-inhibition relationship for antagonism of EPSP of tick coxal muscle by C_7PhTX-343, DNP12-PhTX-343 and PhTX-343. C_7PhTX-343 and DNP12-PhTX-343 were more potent (IC_{50}= ~ 10^{-8}M) than PhTX-343 (IC50= ~ $2x10^{-5}$M).

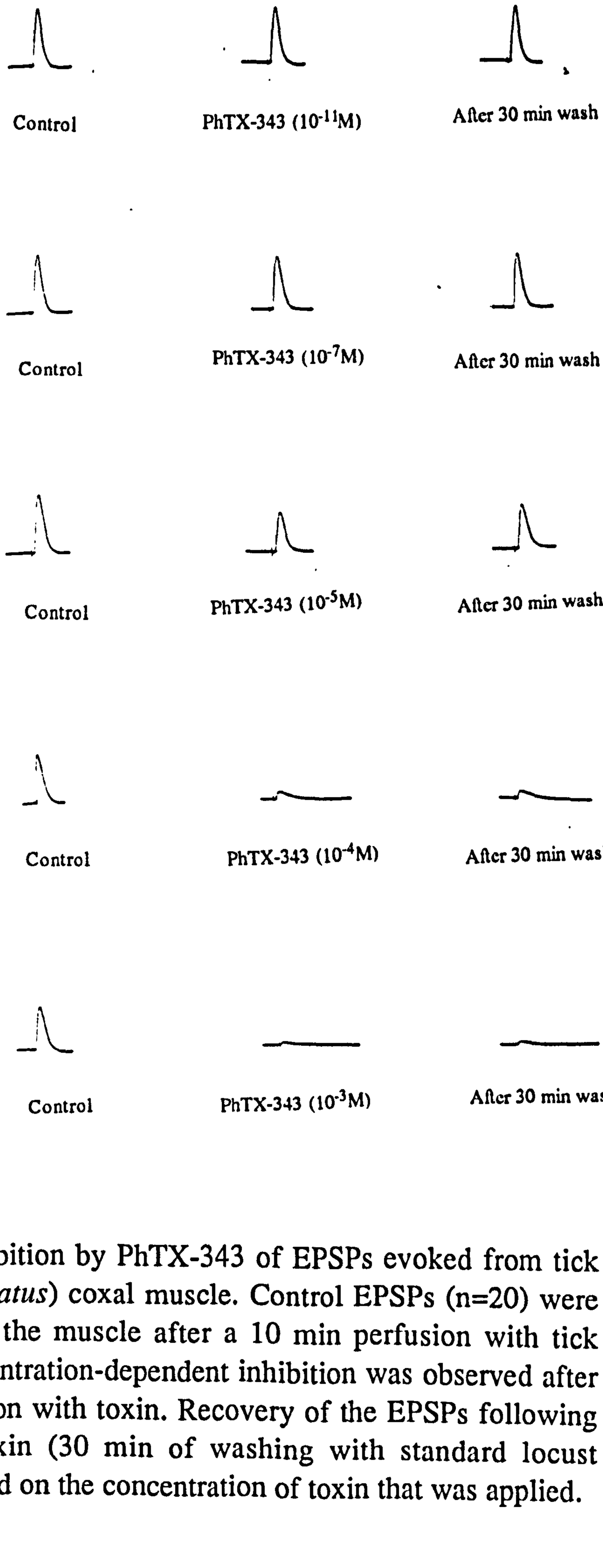


Fig. 3.35. Inhibition by PhTX-343 of EPSPs evoked from tick (*R. appendiculatus*) coxal muscle. Control EPSPs (n=20) were recorded from the muscle after a 10 min perfusion with tick saline. A concentration-dependent inhibition was observed after 12 min perfusion with toxin. Recovery of the EPSPs following removal of toxin (30 min of washing with standard locust saline) depended on the concentration of toxin that was applied.

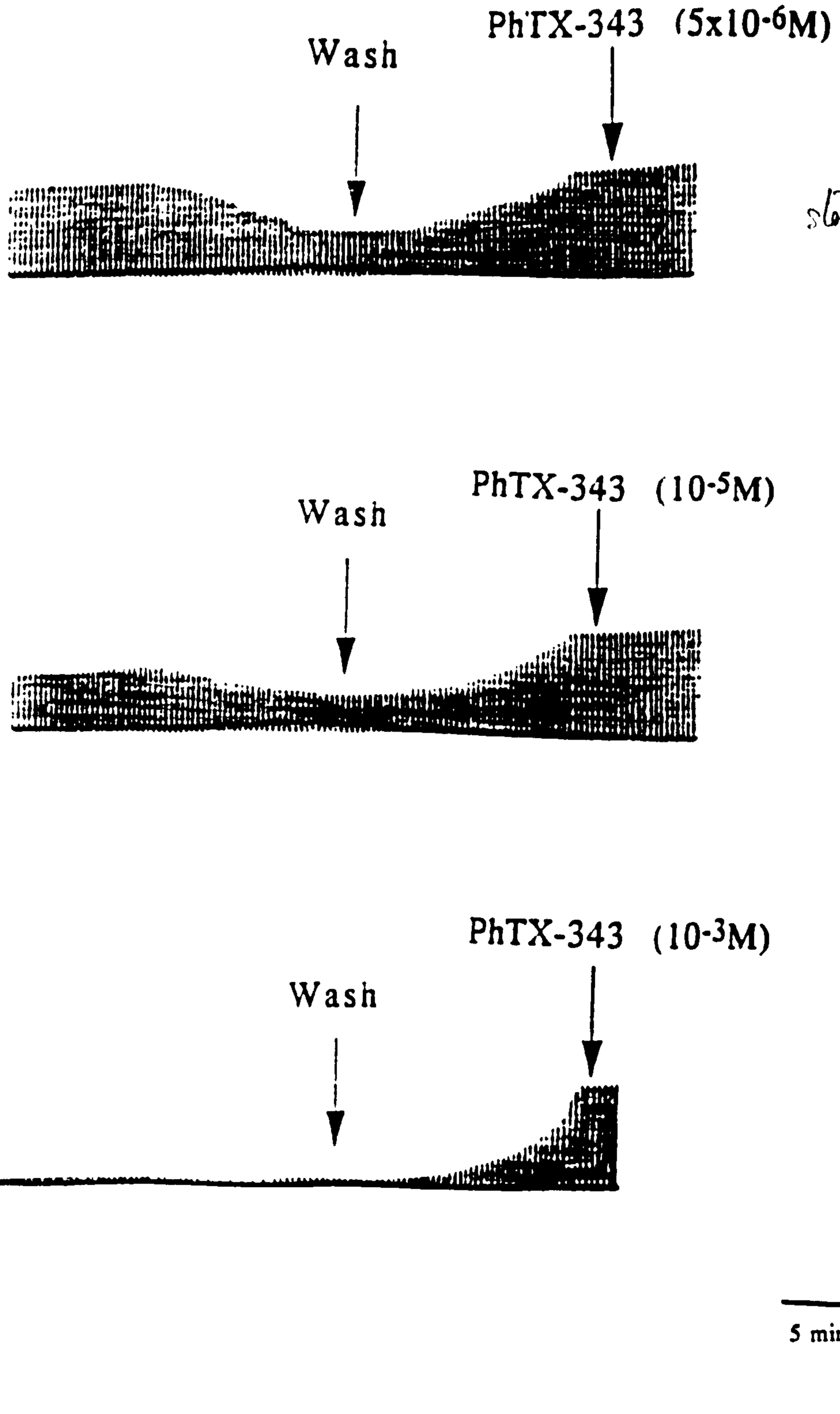


Fig. 3.36 Effect of different concentration of PhTX-343 on EPSPs evoked from tick coxal muscle. Recovery of the EPSP following removal of the toxin depended on the concentration of toxin that was applied.

TABLE 4

C7PhTX-343 (Concentrations)	Inhibition (%)	Recovery (%) after 30 min washout.
10^{-12}M	0%	0%
10^{-11}M	5%±0.2	100%±0.2
10^{-10}M	20%±0.9	80%±0.9
10^{-9}M	37%±1	85%±1
10^{-8}M	58%±1.4	45%±1.5
10^{-7}M	70%±1.8	40%±o.5
10^{-6}M	87%±1	25%±1
10^{-5}M	100%±0.5	0%
10^{-4}M	100%±0.7	0%
10^{-3}M	100%,0.2	0%

TABLE 5

DNP12 (Concentrations)	Inhibition (%)	Recovery (%) after 30 min washout.
10^{-12}M	0%	0%
10^{-11}M	10%±0.7	100%±0.7
10^{-10}M	20%±1	70%±0.8
10^{-9}M	40%±1.6	45%±1.7
10^{-8}M	55%±1	30%±1
10^{-7}M	70%±0.9	10%±0.5
10^{-6}M	90%±1.5	0%
10^{-5}M	100%±1	0%
10^{-4}M	100%±0.5	0%
10^{-3}M	100%±1.8	0%

TABLE 6

PhTX-343 (Concentrations)	Inhibition (%)	Recovery (%) after 30 min washout.
10^{-12}M	0%	0%
10^{-11}M	0%	0%
10^{-10}M	0%	0%
10^{-9}M	0%	0%
10^{-8}M	0%	0%
10^{-7}M	5%±0.5	100%±0.5
10^{-6}M	12%±1	100%±1
10^{-5}M	40%±1.5	70%±1.8
10^{-4}M	76%±1	0%
10^{-3}M	94%±1.2	0%

Tables. 4, 5 & 6 Effect of different concentrations of C_7PhTX-343 (Table 4), DNP12-PhTX-343 (Table 5) and PhTX-343 (Table 6) on EPSPs (n=20) of tick (*R. appendiculatus*) coxal muscle. The EPSP was recorded 30 min after washout of toxin. C_7PhTX-343 and DNP12-PhTX-343 was more potent than PhTX-343.

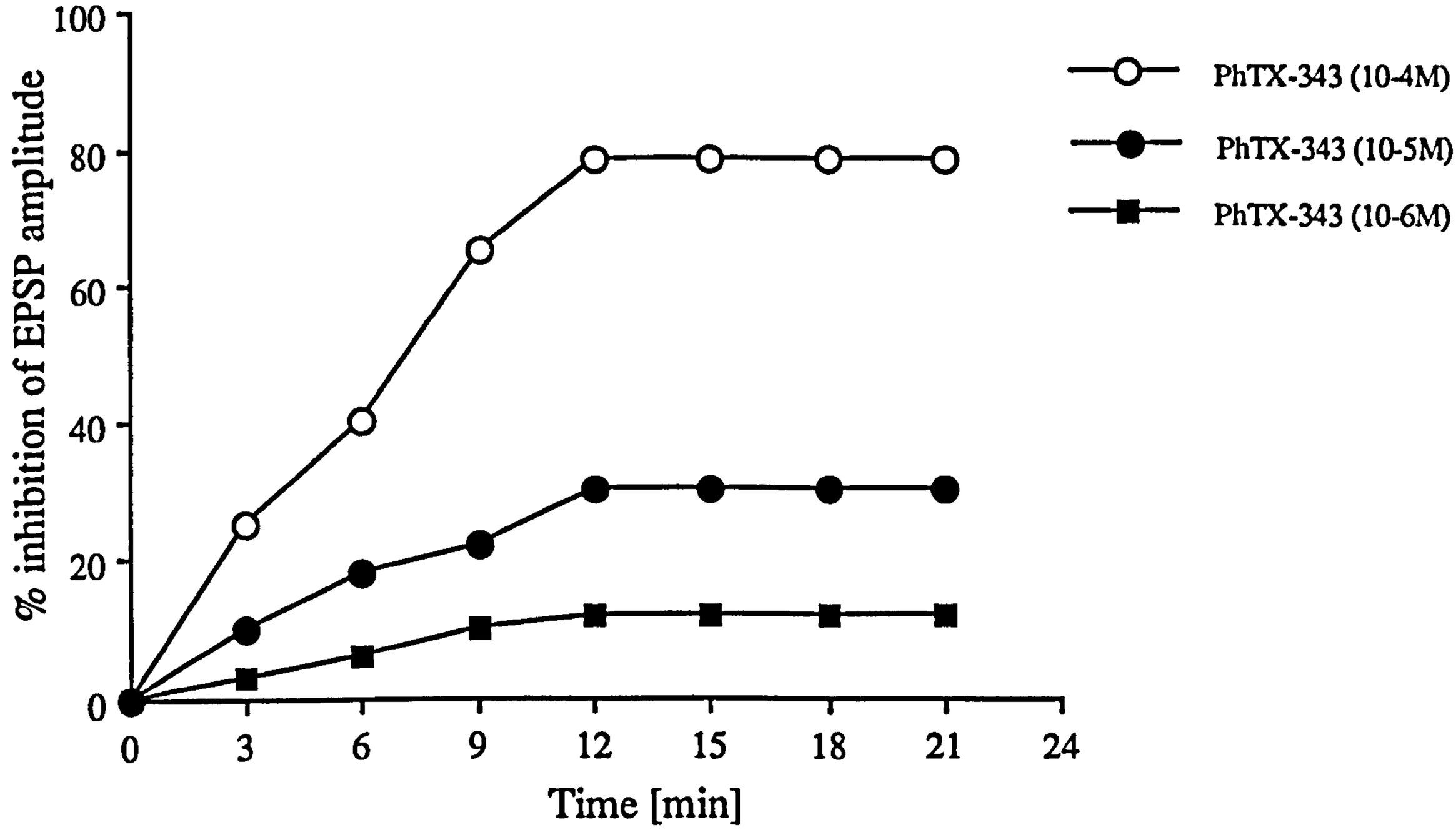
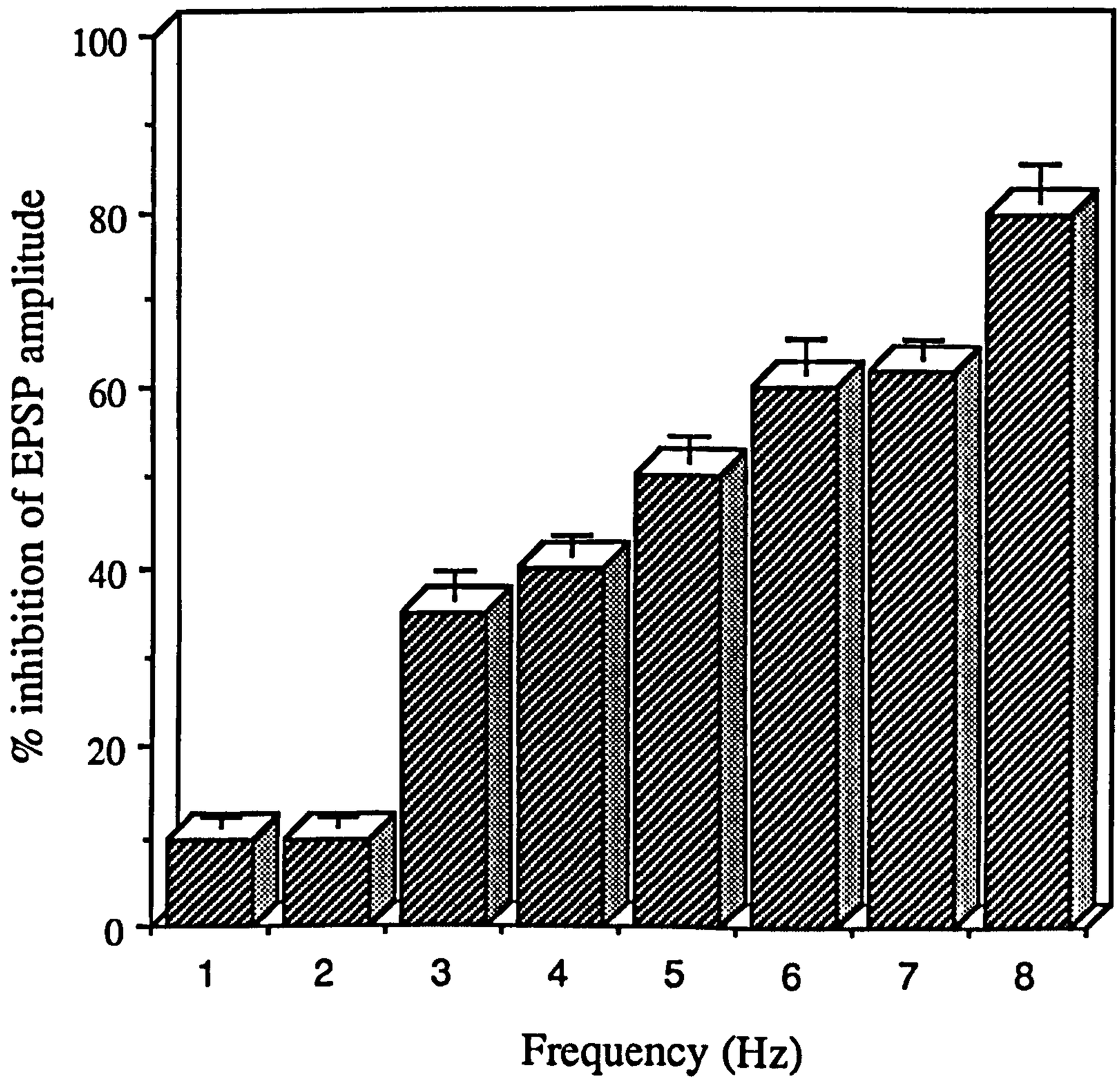


Fig. 3.37. Relationship between time of application of philanthotoxin and the concentration of philanthotoxin (PhTX-343) on the inhibition of the EPSP of coxal muscle of tick. With each concentration of toxin the maximal effect was always obtained after 12 min of application.

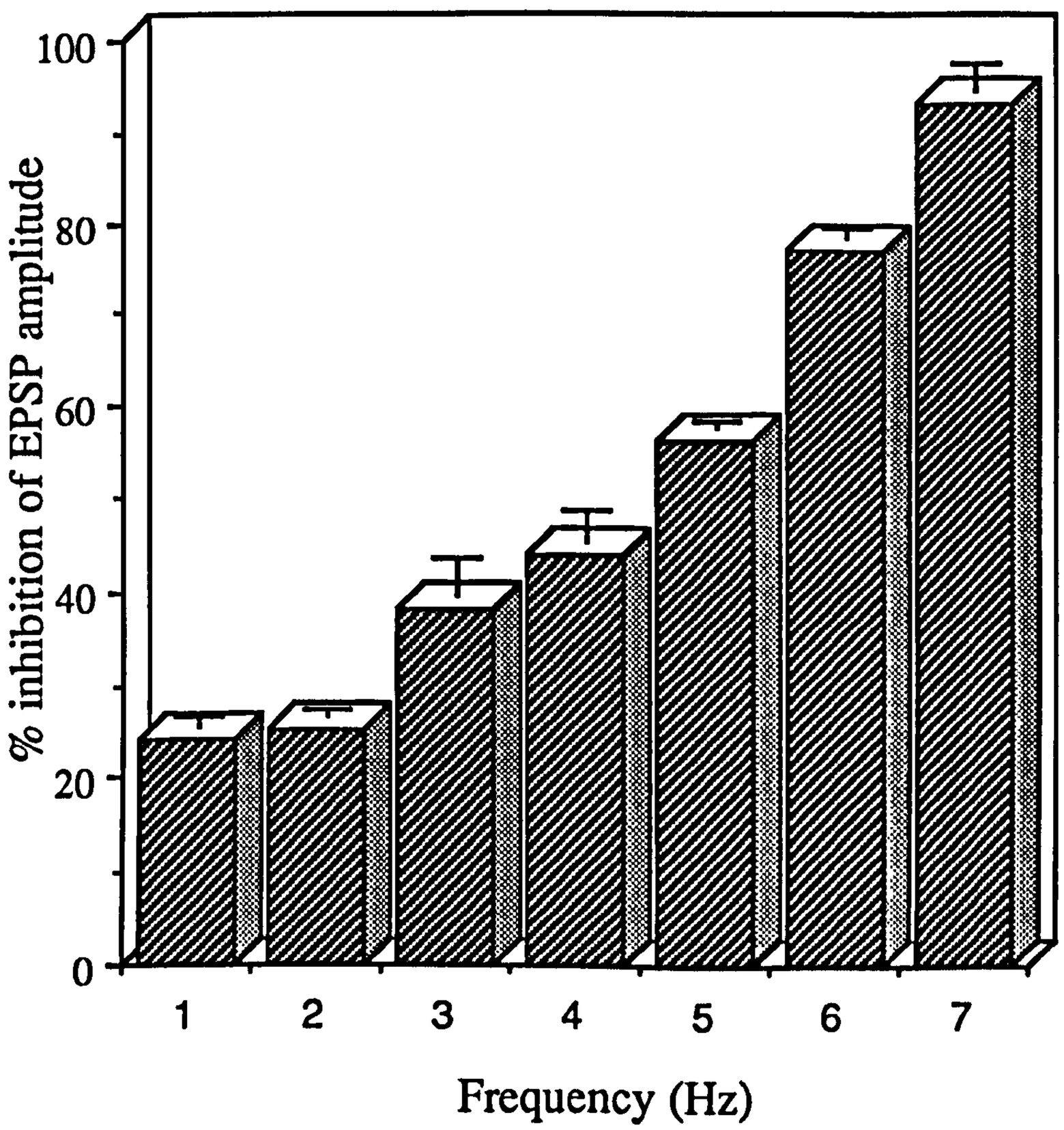
Effect of PhTX-343 (10-6M) on EPSP evoked from coxal muscle of tick.



Figs. 3.38

PhTX-343 (10^{-6}M) inhibits the EPSP of tick coxal muscle. The degree of inhibition increased when the nerve was stimulated on higher frequencies.

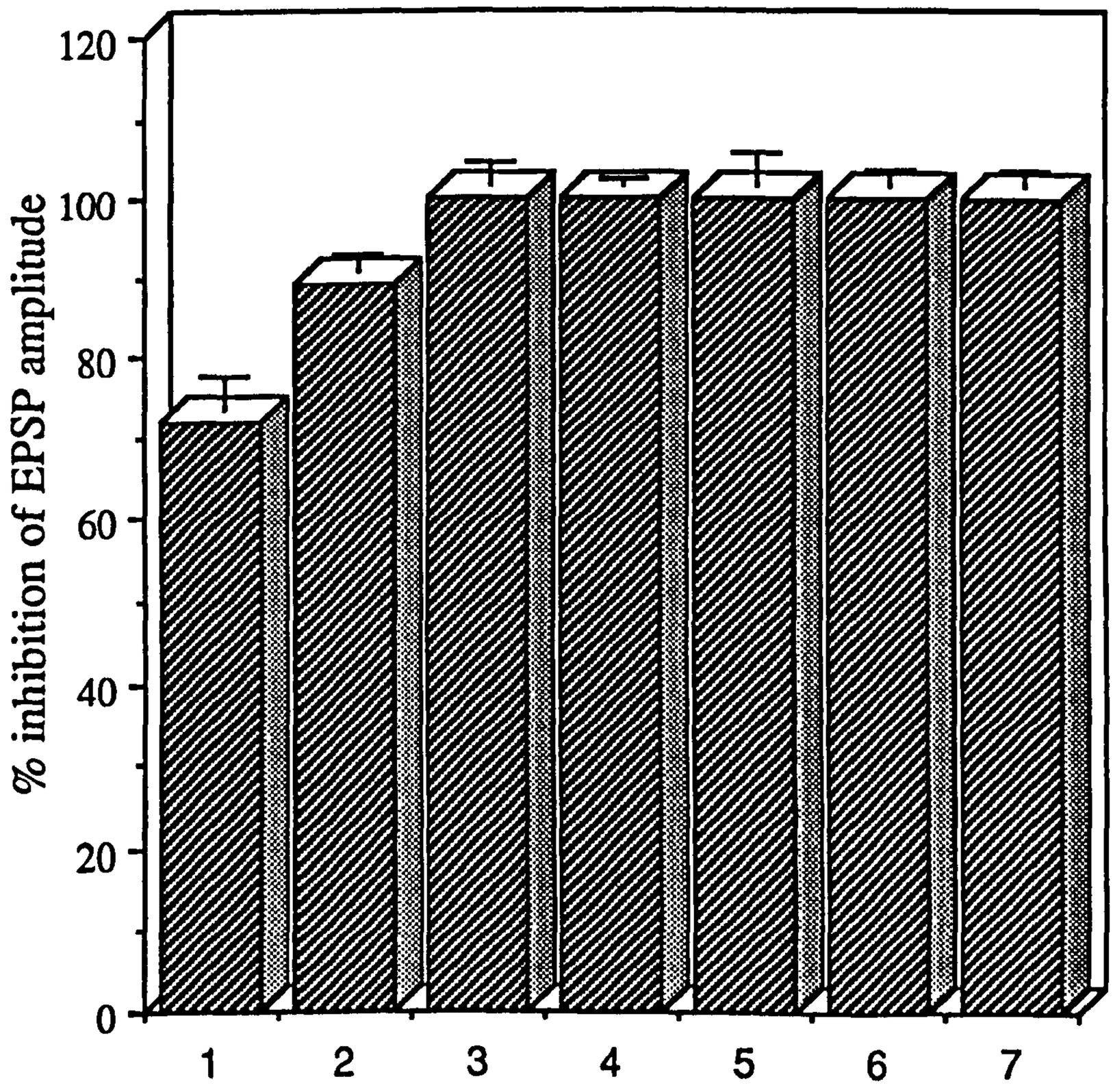
Effect of PhTX-343 (10-5M) on EPSP evoked from coxal muscle of tick.



Figs. 3.39

PhTX-343 (10^{-5}M) inhibits the EPSP of tick coxal muscle. The degree of inhibition increased when the nerve was stimulated on higher frequencies.

Effect of PhTX-343 (10-4M) on EPSP evoked from tick coxal muscle.



Figs. 3.40

PhTX-343 (10^{-4}M) inhibits the EPSP of tick coxal muscle. The degree of inhibition increased when the nerve was stimulated on higher frequencies.

3.3. The expression of mRNA coding for tick neurotransmitter receptor in *Xenopus* oocyte.

The objective of this part of project was to develop a system that would allow a study of the pharmacology of arthropod transmitter receptor proteins.

In the whole series of experiments, 40 batches of oocytes were injected and tested for receptor protein expression. Unless otherwise stated, ~150 oocytes were injected in each batch with total RNA or mRNA of tick and ~10 oocytes each with total RNA of locust leg and of rat brain. Oocytes were treated with Con A (10^{-6}M) for 30 min prior to test for receptor protein expression. L-glutamate, L-quisqualate, N-methyl-D-aspartate and DL-ibotenate were bath-applied to oocytes after 2 to 21 days post-injection with RNA.

Oocytes injected with distilled water failed to exhibit any response when tested with L-glutamate. However, oocytes injected with total RNA (50nl/oocyte) from rat showed expression (96% success rate) giving inward currents of 120nA - 220nA at a membrane potential of -60mV (Fig. 3.41). Oocytes injected with tick or locust RNA exhibited a very poor rate of expression (<0.2% and <0.8% respectively; Table: 7). In attempt to improve the expression rate, oocytes were de-folliculated and microinjected (50nl/oocytes) with RNA 3 times at 2-day intervals. Oocytes injected with tick embryo message failed to respond between 2-14 days post-injection. However, 7 oocytes 16 days after post-injection with total

RNA from18 day old embryos of tick exhibited small responses (~10nA) to L-glutamate or L-quisqualate (Fig. 3.41)

In another set of experiments, oocytes were co-injected with RNA obtained from 1, 3, 4, 7, 9, 11, 13, 15, 17, 19 days-old tick embryos but no improvement in expression was achieved. To exclude the possibility that antibiotics might be inhibiting synthesis of arthropod proteins, oocytes were maintained in Barth saline containing no antibiotics. The saline was changed twice every day under aseptic conditions and oocytes were successfully maintained for three weeks after microinjection. When tested electrophysiologically, the oocytes had resting potential of -50mV to -60mV. None of the oocytes (n=200) responded when tested with L-glutamate, N-methyl-D-aspartate, L-quisqualate or DL-ibotenate.

The genome of *Xenopus* contains two heat shock gene hsp 30, and hsp 70. However , synthesis of hsp 70 protein is not detected unless the temperature at which the oocyte is incubated is shifted from 18^0C to 37^0C (Bienz *et al.*, 1982). Although the function of *Xenopus* heat shock protein is not known, an interesting function has been ascribed to mammalian hsp 73 protein. This protein apparently has the ability to bind the poly(A) tail of mRNA and as consequence could regulate the stability or translatability, or both, of mRNA that have 3' poly(A) tails (Schonfelder *et al.*, 1985). Oocytes were injected with total RNA and mRNA from different ages of tick embryos and maintained at 18^0C for 24h. The oocytes were then transferred to an incubator at 37^0C for 2-5h. The oocytes were subsequently tested with a range of glutamate agonists, but none exhibited responses.Thus, it appears that failure to obtain good expression of tick embryos or locust muscle

RNA *in vitro* may be due to some unknown failure of the *Xenopus* oocyte to translate arthropod mRNA.

An *in vitro* (rabbit reticulocyte lysate) translation system was used to compare the RNA from rat brain with the invertebrate RNAs. The result of this study suggest that the capacity of translation of vertebrate and invertebrate message was almost the same (Fig. 3.42). Thus, it appears that failure to obtain good expression of RNA of tick embryos or locust brain RNA may be due to some unknown failure of *Xenopus* oocyte to translate arthropod mRNA.

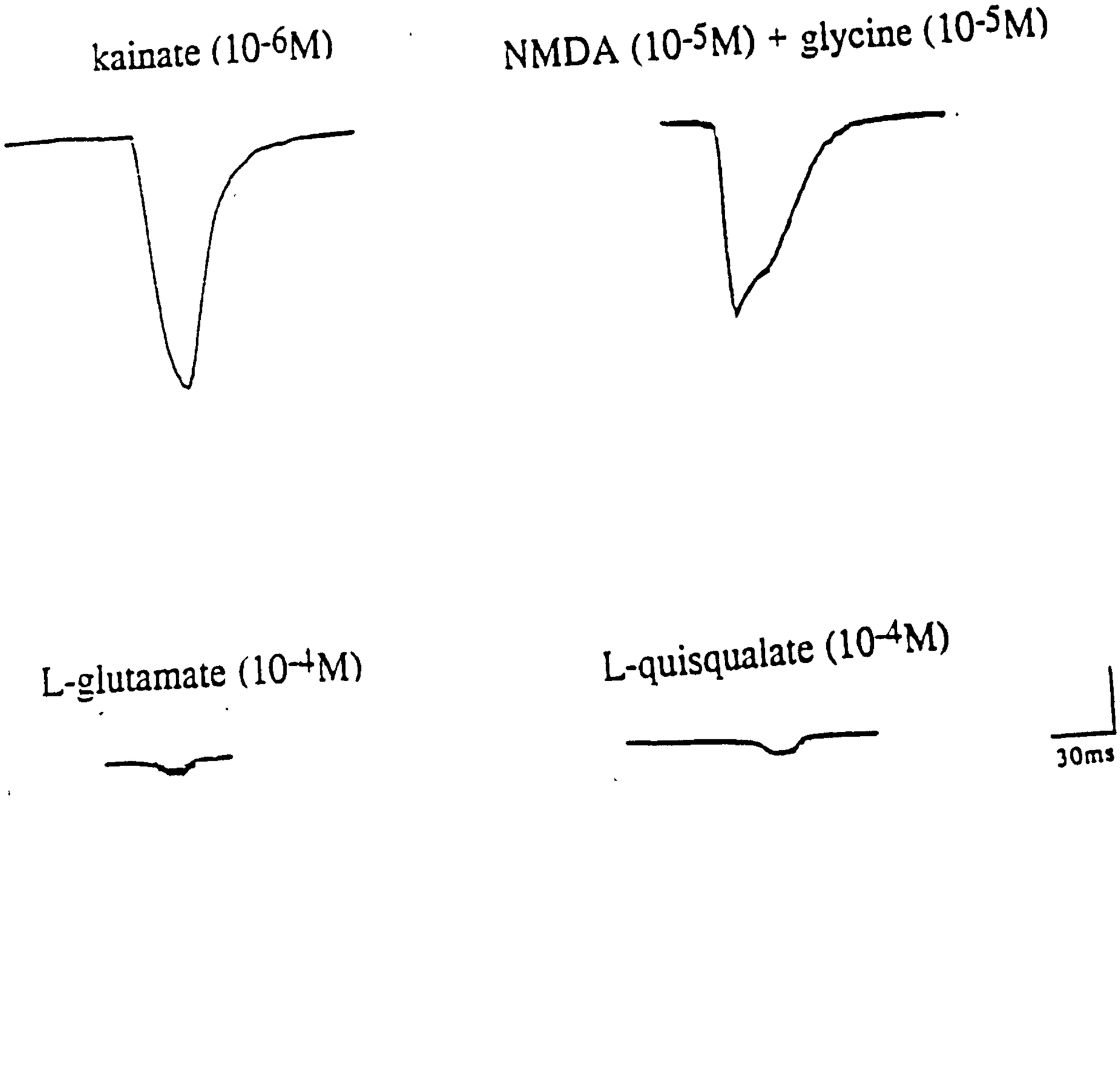


Fig. 3.41a

Fig. 3.41b

Fig. 3.41 Inward current (nA) produced after application of agonists from *Xenopus* oocytes injected with rat brain total RNA (Fig. 3.41a) and tick embryonic tissue total RNA (Fig. 3.41b).

TABLE 7

Source/type of injected RNA	Total number of oocytes tested	Oocytes showing expression of GluR	% of oocytes showing expression
Rat brain total RNA	400	388	97%
Locust leg muscle total RNA	400	3	0.7%
Tick embryonic tissue total/mRNA	6,000	7	0.11%

Table. 7 *Xenopus* oocytes injected with rat brain total RNA showed 97% expression when tested with L-glutamate. Oocytes injected with tick or locust RNA exhibited a very poor rate of expression.

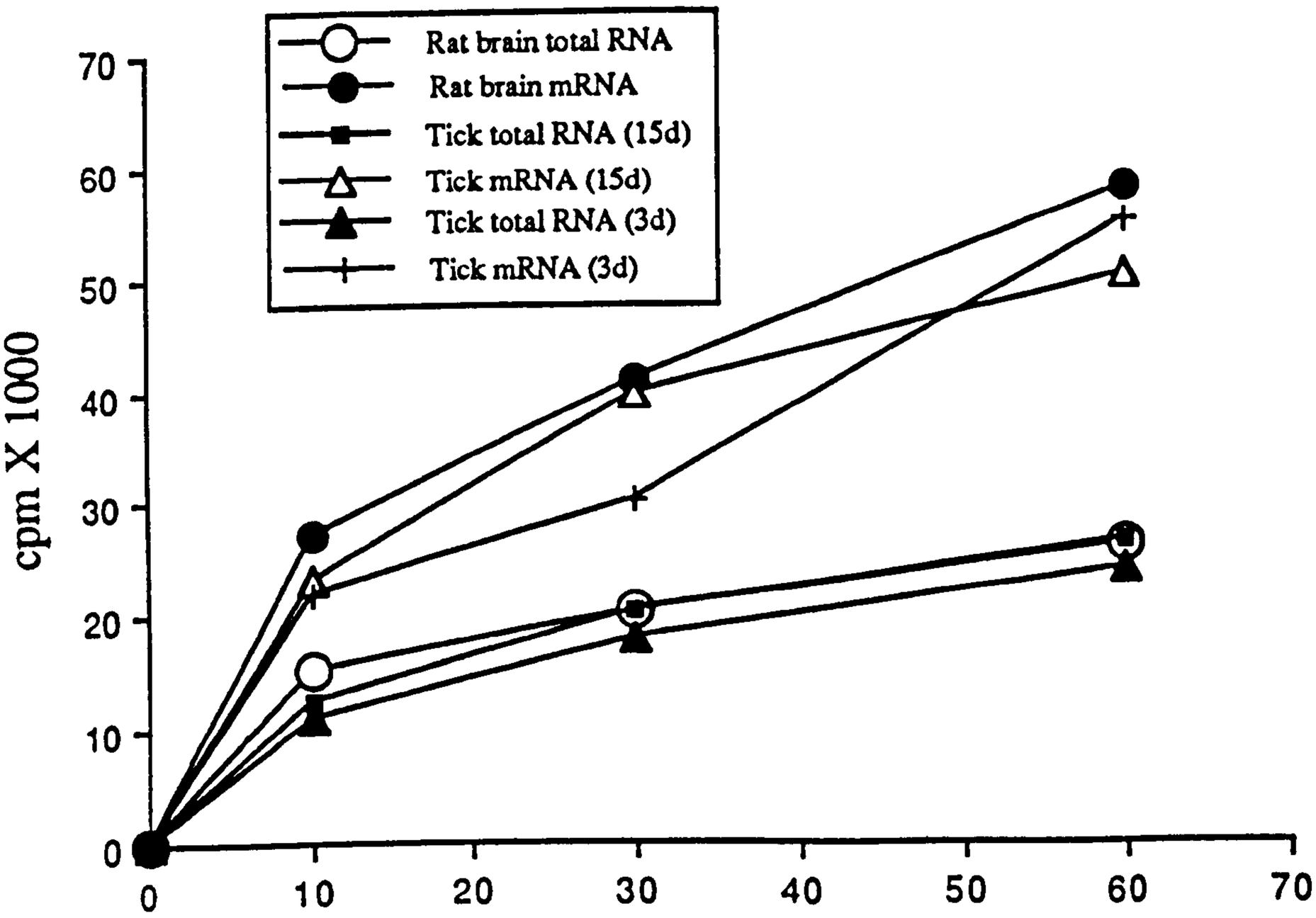


Fig. 3.42. Rat brain RNA and tick embryos RNA were translated *in vitro*. Newly synthesized proteins were labelled with L-[^{35}S] Methionine.

CHAPTER FOUR

DISCUSSION

Chapter 4

Discussion

Polyamine amide toxins are known to interact with several types of ion channel (Scott *et al.*, 1993). Their effects on cationic channels gated by excitant amino acids have received particular attention (Jackson and Usherwood, 1988). The ability of polyamine amides to regulate cell excitability in the vertebrate nervous system and the neuromuscular system of arthropods has potential for pharmaceutical and agrochemical discovery. Two aspects of polyamine toxins have been explored in this thesis:

1. Control of entry of calcium ions through glutamate-gated channels, which may relate to excitotoxic damage.

2. A limited structure-activity study of the inhibition of neuromuscular transmission in ticks and locusts by a range of philanthotoxin analogues.

4.1 Excitant amino acid cytotoxicity

Calcium ions play an important part in many pathways leading to cell death. It is likely therefore that entry of excessive amounts of calcium into cells will produce cellular injury. Persistent activation of excitant amino acid receptors in the mammalian brain has been suspected for many years to lead to calcium-mediated neuronal damage (Olney,

1969, 1986) resulting in a form of cellular damage known as excitotoxicity. Such conditions occur during epilepsy, hypoglycaemia, ischaemia and hypoxia when an increase in the local concentration of extracellular L-glutamate produces excessive activation of NMDA receptors and possibly also some types of AMPA receptors. These types of excitant amino acid receptors open ion channels which are permeable to calcium.

The elevated levels of L-glutamate thought to occur during these brain dysfunctions can be simulated by injection of excitatory amino acid into mammalian brain. Lesions were induced in various parts of CNS of rats by intracerebral injections of kainic acid (McGeer *et al.*, 1978). L-glutamate also caused degeneration of neurones in the inner layer of retina of neonatal rodent (Lucas and Newhouse, 1957).

Several properties of insect muscle suggest that it may be a suitable model system to study excitotoxicity. Duce *et al* (1982) showed that prolonged application of L-glutamate would lead to degeneration of locust muscle only when desensitisation of the L-glutamate receptors was blocked by application of ConA. Pharmacological analysis and manipulations of the ionic medium surrounding the muscles suggested that calcium entry through quisqualate sensitive L-glutamate gated ion channels was responsible for the degeneration. Direct examination of the entry of radiolabelled calcium confirmed these findings (Donaldson *et al.*, 1983).

4.1.1 Stimulated entry of $^{45}Ca^{++}$ in insect muscle

Data presented in this thesis support the finding that GluR present on the locust extensor tibiae muscle activate the entry of calcium [$^{45}Ca^{++}$] into the muscle. The influx of $^{45}Ca^{++}$ through qGluR channels appears to be transient in the continued presence of L-glutamate suggesting receptor desensitization. Prevention of this desensitization by ConA (Mather *et al.*, 1976; Anis *et al.*, 1979) enhances the glutamate stimulated traffic of calcium and prolongs its time course. These results confirm the findings of Duce *et al.* (1982) in the locust retractor unguis muscle and also relate to observations on mammalian muscle where calcium influx through acetylcholine receptor activated ion channels induced myopathy in mammalian muscle (Leonard *et al.*, 1979).

4.1.2 Action of philanthotoxins on calcium entry

Philanthotoxin (PhTX) produces open-channel block of glutamate receptors in locust muscle (Piek *et al.*, 1971; Clark *et al.*, 1982). Natural philanthotoxin (PhTX-433) is slightly more potent on insect muscle glutamate receptor than the isomer PhTX-343. In these studies PhTX-343 did not exhibit any effect on the basal influx of calcium. However, the influx of $^{45}Ca^{++}$ induced by L-glutamate was inhibited in a time and dose dependent fashion by PhTX-343.

It was reported (Bruce *et al.*, 1990; and Benson *et al.*,1992) that the potency of philanthotoxin is reduced by shortening of the polyamine moiety (PhTX-433 to PhTX-43 to PhTX-4). Karts *et al.* (1991) reported that PhTX-433 antagonized the locust qGluR by increasing the time the channel remained in the closed state. PhTX-43 and PhTX-33 were less active than PhTX-433 in causing an increase in the time-course of the closed state. In the present study the potency of PhTX-343 was compared with PhTX-4 in blocking calcium uptake and the reduced antagonism of the latter analogue supports the hypothesis that shortening of the polyamine tail reduces the potency of channel blocking.

Brackley *et al.* (1990) reported the potentiation of kainate and NMDA responses from *Xenopus* oocytes expressing vertebrate brain mRNA by low concentrations of PhTX-343. Concentrations of PhTX-343 lower than those needed to antagonize L-glutamate responses were reported to potentiate responses gated by qGluR in locust (*Schistocerca gregaria*), by increasing the time the channel remains in the open state (Brundell *et al.*, 1991). However, there is no evidence that the influx of calcium is similarly potentiated by low concentrations of PhTX-343, PhTX-4, or PhTX-343-Arg.

The potency of PhTX-343 was increased 1.8 fold when the terminal amino residue was replaced by lysine (PhTX-343-Lys) and a further increase by 3.7 fold was reported when it was replaced by arginine (PhTX-343-Arg) (Goodnow *et al.*, 1992). PhTX-343-Arg was also more potent than PhTX-343 in this system suggesting that the

terminal amino residue of PhTX-343-Arg interacts at a fixed location within the qGluR channel complex of locust. The increased potency obtained following the substitution of arginine for the terminal amino residue of PhTX-343 is postulated to arise from the delocalization of the positive charge of the guanidium group of arginine. This spreads the positive charge over a wider area than the primary amino group and thus is better able to accommodate the negative charges that lie on the wall of the ion channel gated by qGluR (Bruce *et al.*, 1990; Nakanishi *et al.*,1990; Chiles *et al.*, 1992). Polyamine spider toxins which are generally more potent than PhTX-343 often contain a terminal arginine (Adams, 1987; Jackson *et al.*, 1988).

4.1.3 Effect of philanthotoxin on volted-gated calcium channels

The predominent type of voltage dependent calcium channels in cardiac and skeletal muscle of vertebrates is the L-type calcium channels (Mc Cleskey *et al.*, 1986; Fosset *et al.*, 1983). These channels are sensitive to dihydropyridines (DHP), such as nifedipine. The number of DHP receptors is 50-100 fold higher in skeletal muscle than in cardiac muscle (Glossman *et al.*, 1983). In insect skeletal muscles calcium currents have been demonstrated across the non-junctional membrane (Washio, 1977; Fukuda *et al.*, 1977; Ashcroft, 1981), the channel types responsible for these currents have not been characterised, however evidence is available that they have a number

of characteristics typical of vertebrate L-type calcium channels, in particular they are sensitive to nifedipine.

Depolarisation of locust extensor tibiae muscle by elevating the external potassium concentration to 50mM, stimulated calcium influx presumably via voltage-sensitive calcium channels. Uptake was not affected by preincubation in ConA suggesting that high potassium was not gating entry via glutamate receptor activated channels, however the potassium-stimulated entry of calcium was blocked by addition of nifedipine. The hypothesis that elevated extracellular potassium was activating entry of calcium via voltage activated channels rather than those gated by glutamate receptors was supported by the observation that PhTX-343 did not exhibit any effect on potassium-stimulated uptake, at concentrations which blocked glutamate-activated calcium influx. Furthermore nifedipine had no effect on calcium entry induced by glutamate.

4.1.4 Pharmacology of L-glutamate activated calcium entry

Glutamate receptors in invertebrates do not precisely correspond to the widely accepted pharmacological sub-types described in vertebrates. In insect muscle the major depolarising receptor type is highly sensitive to quisqualate , but does not respond to kainate or AMPA. L-aspartate is a weak agonist at this site. The pharmacology of locust muscle glutamate receptors has been extensively reviewed, (Usherwood and Cull-Candy, 1975; Usherwood, 1978) and more

recently single channel studies have provided further insight into the actions of excitatory amino acids on locust muscle glutamate receptors (Usherwood, 1986; Sansom and Usherwood, 1990). In these studies NMDA receptors were not detected, however recent work by Magazanik and colleagues has pointed to the existence of a presynaptic NMDA receptor on motor nerve terminals of insect muscle (Antonov and Magazanik, 1993).

No entry of calcium was activated by application of: glycine, DL-ibotenate, AMPA, or kainate confirming earlier findings that these ligands do not activate cationic channels in locust muscle. L-quisqualate as expected produced a significant calcium influx, but in these experiments it was less effective than L-glutamate. This was unexpected as quisqualate is a more potent agonist than glutamate in experiments measuring muscle contraction (Clement et al, 1974), ionophoretic potentials (Usherwood and Cull-Candy, 1975), or single channel currents (Gration *et al.*,1981). However this result can be explained in part by the finding that NMDA also produced an increase in $^{45}Ca^{++}$ entry. The location of these NMDA receptors within the preparation is unknown, however the lack of any electrophysiological response of the muscle to NMDA and the recent findings of Magazanik and colleagues imply that the receptors may be located presynaptically (the preparation used is isolated from the CNS, but will posses intact peripheral motor innervation). This proposal could be tested by carrying out calcium uptake experiments on denervated muscles although even here the status of NMDA

receptors on the remaining glial cells and degenerating motor terminals is unknown.

In vertebrates NMDA receptor ion channels are voltage dependant and highly permeable to calcium (Mayer *et al.*, 1987). In addition two further features of NMDA receptors differentiate them from other ionotropic glutamate receptor: Mg^{++} blocks current flow through the open NMDA receptor channel and imparts a voltage dependence to channel activation (Davies *et al.*, 1977, Ascher and Nowak, 1987) and the activation of NMDA receptors by glycine (Johnson *et al.*, 1987). In these experiments the presence of glycine enhanced the uptake of calcium induced by NMDA and L-glutamate, however this response was abolished in the presence of 2mM Mg^{++}. The basal response of L-glutamate and the action of L-quisqualate was not affected by magnesium, suggesting that L-glutamate activates NMDA receptors in this preparation only in the presence of glycine.

D-α–aminoadipate was the first competitive antagonist discovered to exhibit a preference for the NMDA receptor (Davies *et al.*, 1979). Subsequently more potent blockers were developed in which a phosphonate group is substituted for the ω-carboxyl group and additional methylene groups extend the molecule length (Evans *et al.*, 1982) The most effective of these are 2-amino-5-phosphonopentanoate (APV or D-AP5) and 2-amino-7-phosphonopentanoate (D-AP7). D-AP5 blocked $^{45}Ca^{++}$ uptake into locust muscle induced by NMDA and the glycine stimulated component of the L-glutamate response. However the actions of L-

quisqualate and L-aspartate on calcium entry were unaffected, implying that in the absence of glycine, L-glutamate acts mainly at qGluR. The role of the NMDA receptors in this system is unknown, as is their location, however if they are presynaptically located it is possible that they act as autoreceptors involved in feedback regulation of neurotransmitter release.

Calcium influx activated by L-aspartate was unaffected when AP5 was applied suggesting that L-aspartate is not acting at NMDA receptors. Aspartate is known to act as a relatively weak ligand for vertebrate NMDA receptors with L-aspartate having a potency about 10 fold less than L-glutamate at displacing ^{3}H D-AP5 (Watkins and Olverman, 1988). It is therefore surprising that L-aspartate does not appear to act at the NMDA receptors identified in these studies. There are major pharmacological differences between excitant amino acid receptors in insects and vertebrates such as the lack of activity of AMPA at locust muscle quisqualate-sensitive receptors.

A further difference between the insect neuromuscular NMDA receptors seen here and those from rat brain mRNA expressed in *Xenopus* oocytes relates to the potentiation of the latter by low concentrations of PhTX-343 whereas in this study no potentiation of calcium influx was produced by low doses of PhTX-343. However PhTX-343 was equally potent at blocking both NMDA and quisqualate activated receptors desensitised in the presence of the agonist and in both cases the desensitisation could be blocked by ConA. These data suggest a close relationship between insect NMDA

and quisqualate receptors but until their molecular structures are determined these relationships will remain uncertain.

4.2 Neuromuscular transmission in arthropods.

Doyere (1840) was the first to observe the junction between a nerve and a muscle fibre in an invertebrate. He reported the branching of nerves over the muscle fibre. Invertebrate neuromuscular junctions often appear morphologically similar to interneuronal synapses because they lack the postsynaptic infoldings of vertebrate muscle. The terminals of axons innervating the muscles are normally covered by glia, but some terminals are uncovered and only separated from the haemolymph by the basal lamina. This type of innervation was found in the larvae of *Calliphora erythrocephala* (Hardie, 1976). In some junctions, the axon penetrates into the muscle, becoming completely surrounded by muscle cells or the axon terminal may lie superficially as in the case of *Drosophila melanogaster* flight muscle (Shafiq, 1964).

Wiersma (1961) presented the first report of polyneural innervation in crustacean muscle fibre which receives an inhibitory input as well as excitatory innervation. In crustacea some of the inhibitory axons innervate more than one muscle and the nerve is called the common inhibitor (Wiersma, 1961). Usherwood and Grundfest (1964) presented evidence for an

inhibitory innervation of insect skeletal muscle whilst Pearson and Bergman (1964) described a common inhibitor in the locust.

The innervation of insect skeletal muscle is very complicated. Iles and Pearson (1969) reported that some fibres of the anterior coxal depressor muscle of *P. americana* were innervated by three inhibitory axons. Philipps (1980) observed nine excitatory motor axons (3 fast, 3 slow and 3 intermediate) innervating the metathoracic flexor tibiae muscle of locust. In the stick insect *Carausius morosus* probably more than six motorneurones control the muscle in which at least one is inhibitory. The metathoracic extensor tibiae muscle of locust is the most intensively studied insect muscle. In *Locusta migratoria* this muscle contains about 3500 fibres (Hoyle, 1955). The muscles are arranged in bundles and insert diagonally on an apodeme running down the centre of the femur. Hoyle *et al.* (1980) divided the neurones which innervate the extensor tibiae muscle into four groups. They named them the fast extensor, slow extensor, common inhibitor and dorsal unpaired median unpaired neurone. Piek (1985) classified the neurones innervating the hind leg of locust into excitatory, inhibitory and neuromodulatory type.

The tick synganglion is similar to the ganglia of other arthropods. The general anatomy and histology of the tick central nervous system have been studied by Robinson and Davidson (1913), Douglas (1943) and Obenchain (1974). Chow *et al.* (1973) classified the neurones in the brain into two groups: the

ganglionic neurones and the motor neurones. They mapped a total of 153 motor neurones in the brain of tick. Saito (1960) provided an elegantly constructed scheme of the innervation of the coxal and dorso-ventral muscles in *Haemaphysalis flava.* Binnington (1981) described a similar pattern of coxal innervation in *Boophilus microplus.* Each pedal nerve trunk is contained within an arterial vessel. As it reaches the coxa, the vessel dilates and the pedal nerve branches, sending a major nerve through the coxa and into the leg,while other branches innervate the trochanteral and coxal muscle. Binnington (1981) reported that in *Boophilus microplus* the endings of these nerves were of a highly branched, simple, varicose type. Hart *et al* (1980) in an ultrastructural study on *Amblyomma variegatum* showed that the synaptic muscle receives a polyneural innervation. Although ultrastructural and electrophysiological studies of insect and crustacean are common, there have been very few such investigation of acarine muscle. Bhandal (1993) reported similarities of coxal muscle of tick *Amblyomma hebreaum* with other arthropods when studied ultrastructurally. He described the neuromuscular junction and observed two types of vesicles which are concentrated near to specialized region of nerve-terminal membrane which make synaptic contact with the adjacent muscle cell. The fine structure of skeletal muscle in *Boophilus decoloratus* (Beadle, 1973) and the structure of the neuromuscular junction of leg muscles and retractor muscles of *Boophilus microplus* and *Amblyomma variagatum* were studied

(Hart *et al.*, 1980; Booth *et al.*, 1985). These authors reported the retractor muscle of *A.Variagatum* and *B. microplus* resembles that of other skeletal muscles previously studied in the ixodid ticks. The same authors found that the neuromuscular junctions are similar to other tick leg muscles (Hart, 1980) and other arthropod skeletal muscles. The innervation of neurones were multisynaptic as in the leg muscle of locust (Usherwood, 1972, 1974). They also found that the synaptic vesicles are similar to those of insects and crustaceans (Atwood and Johnston, 1968; Osborne 1975).

Synapses are sites of rapid and precise information transfer between cells. Chemical synapses presumably are present in all animal phyla and in general characterized by vesicle-associated, paramembranous densities separated by a 15 to 30-nm-wide interacellular cleft. Information transfer occurs at these synapses as a result of release of a chemical by one neurone on to the surface of another neurone or effector cell. The ultrastructure of invertebrate synapses is poorly understood compared to that of vertebrate synapses. In both crayfish and lobster, excitatory neuromuscular junctions can be distinguished from inhibitory neuromuscular junctions on the basis of ultrastructure study. Excitatory junctions have an abundance of clear, round vesicles whereas inhibitory junctions contain fewer and less regular vesicles (Westfall, 1987). Stimulation of excitatory input to arthropod muscle fibre releases neurotransmitter from motor nerve terminal into the synaptic cleft. The released

neurotransmitters.bind to the postsynaptic GluR and produces an increase in the ionic permeability of the post synaptic membrane to K^+, Na^+ and Ca^{++} ions (Aildey, 1967; Anwyl and Usherwood, 1974. 1975; Anwyl, 1977). This ionic permeability causes changes in the membrane potential of the postsynaptic muscle and is referred to as an excitatory postsynaptic potential (EPSP). At the neuromuscular junction the spontaneous release of a quantum of neurotransmitter from a motor-nerve terminal is known as miniature excitatory postsynaptic potential (minEPSP). MinEPSPs were first recorded intracellularly from insect muscle by Usherwood (1961, 1963). In this study the frequency of min EPSP from tick coxal muscle increased when challenged with low concentration (10^{-8}M) of L-glutamate. This study support the previous findings of Kerkut and Walker (1966) and Usherwood (1967) that low concentration of L-glutamate cause an increase in the frequency of min EPSP.

A number of workers have shown that L-glutamate plays a significant role in arthropod neuromuscular transmission. Robbins (1958, 1959) and Van Harreveld and Mendelson (1959) reported the possible role of L-glutamate at excitatory synapses on crustacean muscle fibres. Later, Takeuchi and Takeuchi (1965), Ozeki and Grundfest (1967), Kravitz et al.(1970) and Dudel (1974, 1975) supported this possibility. Apart from skeletal muscle L-glutamate also activates crustacean visceral muscle (Jones, 1962). Early work on crustacean nerve-muscle preparation has been reviewed by Gerschenfeld (1973). Florey

and Florey (1965) reported that the body-wall muscle of centipede contracted when challenged with glutamate. The first extensive studies of the effect of acidic amino acid were made by Kerkut, Shapira and Walker (1965). They showed that twitch contraction of perfused metathoracic legs of the cockroach were reduced when L-glutamate was applied and that contraction of the leg muscle were evoked by low concentration of this amino acid. Usherwood and Machili (1966) reported that bath applied L-glutamate at low concentration caused contraction in the metathoracic retractor unguis muscle preparation of locust. Kerkut and Walker (1966, 1967) reported similar results for cockroach muscle. Recently Walker *et al.* (1992) studied the action of a series of glutamic acid analogues from identified Helix centrtal neurones sensitive to L-glutamate. They reported that out of a range of substituted glutamate analogues, only the L- and D-isomers of thio-glutamate possessed clear glutamate-like activity.

There is now substantial evidence that L-glutamate is the neurotransmitter at excitatory synapses on muscles of many insects (Usherwood and Cull-Candy, 1975; Anwyl, 1975; Usherwood, 1981; Piek, 1985). Irving *et al.* (1976) and Jan and Jan (1976) showed that L-glutamate is involved in transmission at synapses on blowfly and *Drosophila* larval muscle. Usherwood and Machilli (1966) showed that isolated skeletal muscle of locust contract when L-glutamate was applied extracellularly. They also described the depolarization of the membrane potential when L-glutamate was applied. Insect muscle is highly sensitive to L-

quisqualate and insensitive to kainate or AMPA (Usherwood and Cull-Candy, 1975; Usherwood, 1978, Sansom and Usherwood, 1990). The recent work of Magazanik (personal communication) showed the existence of a presynaptic NMDA receptor on motor nerve terminals of insect muscle. There are numerous reports of the action of aspartate on arthropod muscle (Crawford and Mc Burney, 1976, 1977a,b; Constanti and Nistri, 1978, 1979). Irving and Miller (1980) found evidence for two different receptor population in the body wall muscle of *Musca* larvae. They proposed that fast axon released glutamate and the slow axon , aspartate. Although the action of aspartate has been assumed to be due to effect on the L-glutamate receptor, it remains a possibility that separate aspartate receptor population exist on the muscle.

Electrophysiological investigations of the action of L-glutamate on the locust (*Schistocerca gregaria*) skeletal muscle suggest the existence of at least two populations of glutamate receptors (Cull-Candy and Usherwood, 1973; Usherwood and Cull-Candy 1974; Gration *et al.*, 1979). Junctional glutamate receptors and extra-junctional D-receptors for this amino acid gate cationic channels and are preferentially activated by L-quisqualic acid. These have been termed qGluR. Anwyl and Usherwood (1974) and Anwyl (1977) reported that the reversal potentials of currents generated by junctional and extra-junctional qGluR currents are similar. These currents are carried by sodium, potassium and calcium ions. There is also an extra-junctional population of glutamate receptors, the H-receptors, which gate chloride channels and

activated by DL-ibotenate (Lea and Usherwood, 1973; Clark, 1979) These are termed ibotenate sensitive glutamate receptors (iGluR). Although it was first concluded that ibotenate does not excite junctional glutamate receptors on locust muscle subsequent studies by Gration *et al.* (1979) showed that approximately 20% of the receptors at the junction on the locust metathoracic extensor tibiae muscle are depolarised by DL-ibotenate whilst an additional 16% are depolarised by L-aspartate.

The advent of the patch-clamp technique (Neher and Sakmann, 1976) allowed the measurment of single channel current in locust muscle membrane. The MΩ-recording technique was used extensively to investigate the channel-conductance and gating kinetics of extrajunctional qGluR from locust muscle fibre (reviewed by Sansom and Usherwood, 1990). Locust extensor tibiae and retractor unguis muscles, have been extensively studied with regard to their pharmacology (Usherwood, 1978; Gration et al 1979) and channel kinetics (Cull-Candy et al 1980; Gration, 1982; Kerry et al 1987). The L-glutamate-gated channel of locust metathoracic extensor tibiae muscle has been shown to be a rather non-selective cation channel (Latorre and Miller, 1983), normally conducting K^+ and Na^+. These results were extended by studying the effects of bath application of various salines on the excitatory postsynaptic currents and ionophoretically evoked excitatory junctional currents (Anwyl and Usherwood, 1975; Anwyl, 1977) and the role of K^+, Na^+ and Ca^{++} was described. The qGluR channel conductance was found to be relatively high (125-150 pS)

for monovalent ions with a selectivity sequence as follows (Rb^+> K^+ ~ Cs^+> Na^+>Li^+ (Kits and Usherwood, 1988). Ammonium was shown to permeate the channel, while guanidium, tetramethylammonium and choline ions were impermeable. Divalent cations did not contributed measurably to the ionic current and high concentration of these ions (Mg^{++} and Ca^{++}) appeared to block the channel.

Hart (1982) found that both aspartate and L-glutamate had similar effects on tick leg muscle causing depolarization and abolishing neurally evoked EPSP. Booth *et al.* (1985) reported that the retractor muscles of Gene's organ in *Amblyomma variegatum* are probably innervated by glutamatergic neurones. In the present study when L-glutamate was applied at low concentrations the frequency of minEPSP increased. Usherwood and Machilli (1966), Dowson and Usherwood (1972) and Kerkut and Walker (1966) previously reported that low concentration of L-glutamate increased the frequency of minEPSP of locust and cockroach muscle fibres. When L-glutamate was applied at high concentration, minEPSP of coxal muscle of *R. appendiculatus* were inhibited supporting previous findings of Hart (1982) and Booth *et al.* (1985). Hart (1982) detected both fast and slow potentials in tick leg muscle, which combine to form a compound EPSP. In this study on tick coxal muscle no evidence of fast and slow responses was found, since all potentials recorded having approximately the same time course which support the findings of Booth *et al.* (1985).

4.2.1 Effect of philanthotoxin on locust and tick neuromuscular transmission

Previous studies have shown that some wasp and spider venom contains polyamine toxins, that act on pre- and postsynaptic sites (reviewed in Usherwood, 1987, 1991). Polyamine toxins have been shown to act as antagonist of GluR from vertebrate CNS (Schulter *et al.*, 1992 and Brackley *et al.*, 1990, 1991). The venom of *Philanthus triangulum* contains four neuroactive compounds known as α, β, γ and δ–philanthotoxin. Natural δ–philanthotoxin or PhTX-433 and many of its synthetic analogues are efficient, non-competitive, reversible inhibitors of locust muscle qGluR (Eldefrawi *et al.*, 1988).

In this study when the tick and locust nerve-muscle preparations were incubated in PhTX-343 (10^{-5}M) for 10 min in the absence of motor-nerve stimulation and the toxin was then removed, the amplitude of the EPSP (n=20) recorded was not affected by the prior presence of PhTX-343. However, the application of PhTX-343, during motor-nerve stimulation significantly antagonized the EPSP supporting the hypothesis that philanthotoxin is an open channel blocker of GluR in arthropod muscle (Piek et al., 1971; Clark et al., 1982). The potency of PhTX-343 was greater (IC_{50} = ~ $5x10^{-6}$M) at locust muscle than tick coxal muscle (IC_{50} = ~ $2x10^{-5}$M). This study suggests that GluR present on the muscles

of these two different preparations have different sensitivities to PhTX-343. Alternatively another possibility may be that the difference in the arrangement of muscle fibres in locust and tick leg result in a change in accessability of the toxin to it's target receptor.

More than 100 analogues of PhTX-433 have been synthesized with changes made in the four region of the structure (Nakanishi *et al.*, 1990). Structure- activity studies have shown that the increased potency of the philanthotoxin arises from (I) the thermospermine moiety (II) tyrosyl residue and (III) butyryl side chain moiety (Anis *et al.*, 1990, Bruce *et al.*, 1990; Nakanishi *et al.*, 1990; Karst *et al.*, 1991; Karst and Piek, 1991).

4.2.2 Effect of philanthotoxin analogues on evoked EPSPs of locust (*Schistocerca gregaria*) leg muscle

The action of PhTX-343, PhTX-4 and PhTX-343-Arg on neurally evoked EPSPs of locust leg muscle were observed. PhTX-343-Arg (IC_{50} = ~ $5x10^{-8}$M) was more potent than PhTX-343 (IC_{50} = ~ $5x10^{-6}$M). Bruce *et al.* (1990) and Benson *et al.*, (1992) showed the modification of the terminal amino residue of PhTX-343 by arginine increased the potency. Usherwood (1989) reported that the carboxylic acid groups of aspartic acid or glutamic acid, that line the ion channel of qGluR provide an assay of fixed negative charges. The increased potency

of PhTX-343-Arg could be due to spreading of the positive charges or the toxin over a wider area than a primary amino terminal group and this may be better able to accommodate negative charges that may line the wall of the ion channel gated by qGluR (Bruce *et al.*, 1990; Nakanishi *et al.*, 1990; Chiles *et al.*, 1992).

In the present study PhTX-4 was less active than PhTX-343 suggesting that long polyamine chain is necessary and supporting the previous findings of Bruce *et al.* (1990) and Benson *et al.* (1992) that after curtailment of polyamine moiety (PhTX-433 to PhTX-43 to PhTX-4) the potency of PhTX reduced.

Antagonism of EPSPs by PhTX-343 and PhTX-343-Arg was transiently reversed (the amplitude of the EPSPs initially increased and then started to decline) when the Ca^{++} concentration was raised. According to Katz (1969) the exocytotic release of quantal neurotransmitter into synaptic cleft follows a transient influx of Ca^{++} ions through the channels located in the presynaptic membrane of the nerve terminal. Transmitter release from insect (*Calliphora vicina*) glutamatergic motor nerve terminal is Ca^{++} dependent and modulated by presynaptic NMDAR (Antov and Magazanik, 1993). The intracellular free Ca^{++} concentration is normally about 0.1μM (Baker 1977) whilst the concentration of extracellular free Ca^{++} in the locust saline in this study varied between 1mM to 3mM. Hence Ca^{++} will flow down the concentration gradient into the nerve terminal when the

presynaptic NMDAR are activated. The increase in intracellular Ca^{++} will result in enhanced release of neurotransmitter L-glutamate. This extra released glutamate may open additional qGluR channels resulting in an increased EPSPs.amplitude However, once the qGluR channels are open then the toxin will gain access and block them and the resultant block inhibit the EPSPs. The degree of EPSP antagonism was dependent on the exposure time of the preparation to the toxin and the recovery from the antagonism was Ca^{++} dependent. When the Ca^{++} concentration was raised to 3mM after 30 min wash out of toxin with standard locust saline the EPSP reversed more quickly. This study suggest that raised ($[Ca^{++}]_i$) may displace toxin from its binding site postsynaptically or enhance the presynaptic release of L-glutamate into synaptic cleft. This release of L-glutamate activate and open additional qGluR which have not previously exposed to this neurotransmitter and so increases EPSP amplitude.

4.2.3 Effect of philanthotoxin analogues on evoked EPSPs of tick (*Rhipicephalus appendiculatus*) coxal muscle

It has been previously shown that PhTX-343 and it's analogues block the open-channel of locust gated by qGluR (May and Piek, 1979; Clark *et al.*, 1982,; Piek, 1982; Piek and Spanjer, 1986; piek *et al.*, 1988).

The effect of analogues of philanthotoxin, (PhTX-343, C_7PhTX-343 and DNP12-PhTX-343 [Dinitro-phenyl-PhTX343]) was

studied on tick (*R. appendiculatus*) neuromuscular junction. Bruce *et al.* (1990) and Nakanishi *et al* (1990) reported successive increase in the potency of PhTX after increasing the length of chain of the butyryl group from 4 to 7 and then to 10. The potency of dinitro-phenyl analogue was similar to PhTX-343 when tested on neurally evoked twitch contraction of the locust, metathoracic extensor tibiae muscle (Bruce *et al.*, 1990). In this study the tick coxal muscle C7PhTX-343 and DNP12 both exhibited almost the same potency (IC_{50} = ~ 10^{-8}M) and both were more potent than PhTX-343 (IC_{50} = ~ $2x10^{-5}$M). This result suggests that sensitivity of GluR present on these two different preparation is different, however without further pharmacological characterisation the differences remain unknown.

The antagonism by philanthotoxin analogues was either reversible or semireversible and was dependent on the exposure time to toxin and the on the concentration of toxin. The amplitude of the partially antagonised EPSPs exhibited full recovery following a wash of the muscle with toxin free saline, whereas the reversibility was incomplete or partial from fully antagonised EPSPs. This study supports the previous findings that antagonism of EPSPs by philanthotoxin was use-dependent (Usherwood, 1991). When the motor nerve was stimulated at higher frequencies in the presence of PhTX-343 the degree of antagonism was greater. No reduction in EPSP amplitude was seen when the muscle was stimulated in the absence of PhTX-343.

Reversibility from this type of antagonism was difficult to achieve (<15%) even after 30 min washout with toxin free saline(<15%). This study suggests that binding of PhTX-343 with GluR depends on channel kinetics and supports the previous suggestion that PhTX-343 is an open channel blocker in arthropod neuromuscular system.

Some workers have reported the potentiation of excitatory amino acid responses by low concentration of spermine and philanthotoxin (Brackley *et al.*, 1990; McGurk *et al.*, 1990; Durand *et al.*, 1992). Kerry *et al.* (1993) and Brundell *et al.*(1991) have shown that low concentration of spermine increases the open time of qGluR single channel currents of locust muscle. In this study none of the analogues of philanthotoxin potentiated EPSPs of tick or locust muscle.

4.3 *Xenopus* oocyte as a model for the expression of invertebrate mRNA

Xenopus laevis oocytes have been introduced over the last 10 years as a model system for expression and pharmacological characterisation of the functional receptors following injection of exogenous RNA (Dascal *et al.*, 1987; Barnard *et al.*, 1987). Many workers have reported high success rate of expression of vertebrate message but few have reported expression of invertebrate mRNA (Fraser *et al.*, 1990). Injection of rat whole

brain total RNA into oocytes results in a high success rate of responses to L-glutamate and kainate in 3-6 days, with peak current of up to 500nA. In contrast expression of arthropods receptors was not seen until 14 days of post-injection and even then only small currents were elicited with very poor expression rate (0.2%). No increase in the amplitute was seen when the same oocyte was tested up to 20-21 days post injection. The poor expression of invertebrate exogenous RNA and relatively long time taken for expression has also been reported by Saito *et al.* (1987) and Kawai *et al.* (1989). The reason of this poor expression remains unclear. Fraser *et al.* (1990) suggested that the fraction of mRNA encoding the arthropod muscle receptor may be small relative to the fraction encoding other proteins present in the muscle or oocyte system may be somewhat less efficient at translating invertebrate mRNAs and/or post-translationally modifying the resultant polypeptides.

Attempts to increase the level of receptor expression by doubling the concentration of RNA (2μg/μl), by injecting three times in one week, by extraction of RNA from different developmental stages of embryos of tick, by removing antibiotics from media and by heat shock to initiate protein synthesis were unsuccessful. The pharmacological properties of expressed receptor remained unclear due to very poor expression rate and small currents.

This study suggests that *Xenopus* oocyte is a good model to study the pharmacology of vertebrate receptors but failed to translate

invertebrate mRNA very efficiently. The reason for this failure of functional expression of protein of interest may be due to low abundance of the specific mRNA(s) in the source tissue or stability of the mRNA into the oocyte cytoplasm after injection. Some polypeptides may be improperly modified, folded or assembled, and therefore not reach the plasma membrane.

4.4 Conclusion

Extracellular increases in the concentration of L-glutamate may contribute to the cell death observed in a number of neurodegenerative conditions. In this study selected analogues of philanthotoxin appeared to interact with the L-glutamate-stimulated calcium entry in the insect muscle fibre. Both NMDA, and L-quisqualate subtypes of receptor appear to be involved. Since calcium entry is an important factor involved in the initiation of cell death, in future philanthotoxin may be of value as a neuroprotective agent. Magnesium and AP5 do not interact with L-glutamate or L-quisqualate activated calcium entry, however blocked the NMDA activated calcium entry. Philanthotoxin however was equally potent at blocking both NMDA and L-quisqualate activated receptors. Further work is clearly needed to characterise these receptors pharmacologically and to assess the potential neuroprotective properties of polyamine amide toxins

The tick and locust neuromuscular junction has been shown to be accessible to a wide range of electrophysiological techniques. In

this study locust and tick nerve-muscle preparation has proved to be useful for providing as insight into the numerous and complex interactions that occur between philanthotoxin and glutamatergic neurotransmission. Philanthotoxin interacts with the open ion-channel of GluRs and is potentially useful for the isolation of EAARs from excitable tissues and for the development of novel pesticides.

The *Xenopus* oocyte expression system is a versatile and informative method for studying the pharmacological properties of EAARs. This model system faithfully translates foreign RNA from vertebrate sources, however it failed to translate invertebrate mRNA efficiently. The reason for this difference remains unclear.

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