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INVOLVEMENT OF LIPID METABOLISM IN THE ACTION OF PHOSPHOLIPASE A_2 NEUROTOXINS

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A number of potent presynaptically-acting neurotoxins (PSNTXs) from snake venom were examined as regards the role of phospholipase A_2 (PLA_2) activity in altering acetylcholine (ACh) release and choline (Ch) uptake. Also, the effects of PSNTXs and snake venom cardiotoxins (CTXs) on Ca^{2+} metabolism and activation of tissue lipolysis were examined. The inclusion of BSA in the incubation medium at the time of toxin expose has allowed a clear distinction to be made between nonPSNTX PLA_2s and the PSNTXs in synaptosome preparations. For those PSNTXs active on synaptosomes, PLA_2 activity (judged by fatty acid analysis) may play an important role in the enhancement of ACh release and inhibition of Ch uptake . CTXs, melittin and myotoxin a all cause Ca^{2+} release from the terminal cisternae of the sarcoplasmic reticulum through the Ca^{2+} release channel. β-Butx also causes a transient release of Ca^{2+} from the sarcoplasmic reticulum. Melittin and CTX both activate phospholipase C activity in cell cultures that appears to be specific for phosphatidylethanolamine over phosphatidylcholine and with no requirement for Ca^{2+}.

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FOREWORD

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INTRODUCTION

Abbreviations

ACh, acetylcholine; p-BPB, *p*-bromophenacyl bromide; BSA, bovine serum albumin; ß-Butx, ß-bungarotoxin; Ch, choline; CHE, cholesterol esters; CL, cardiolipin; CTX, cardiotoxin; DG, diacylglyceride; FA, fatty acid; FFA, free fatty acid; GC, gas chromatography; HSRF, heavy sarcoplasmic reticulum fraction; LPC, lysophosphatidylcholine; NL, neutral lipid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; PLA_2, phospholipase A_2; PLB, phospholipase B; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; PSNTX, presynaptically-acting PLA_2 neurotoxin; SM, sphingomyelin; TG, triacylglyceride; TCICR, threshold of Ca^{2+}-induced Ca^{2+} release; TLC, thin-layer chromatography.

Nature of the Problem

A number of potent (LD_{50} 1-50 µg/kg) presynaptically-acting neurotoxins (PSNTXs) have been isolated from snake venoms, all having phospholipase A_2 (PLA_2) activity. Very little is known about the mechanism(s) of action of these agents and the role of PLA_2 activity in altering acetylcholine (ACh) release and choline (Ch) uptake. In recent years it has become recognized that these toxins are potential biological warfare agents. Snake venoms also contain smaller molecular weight cardiotoxins (CTXs) that greatly potentiate phospholipid (PL) hydrolysis by PLA_2 enzymes, allowing penetration of these enzymes to formerly inaccessible substrates. Feasibly, the combination of a PSNTX with a CTX could result in effects on a wider variety of organ systems and a potency far greater than the sum of the two toxin effects. Understanding the modes of action of these toxins would aid the development of effective therapeutic and prophylactic approaches. The first half of this contract examines: 1) the action of PSNTXs and CTXs on ACh release and Ch uptake; 2) the role of PLA_2 activity in toxin action; 3) the action of PSNTXs and CTXs on Ca^{2+} regulation; and; 4) effects of the toxins on virtually all aspects of endogenous (tissue) lipid metabolism. The three hypotheses tested were: 1) the potency of the presynaptic neurotoxins is due to the expression of PLA_2 activity on the *inner* leaflet of the plasma membrane bilayer at a very crucial target site; 2) the CTXs activate endogenous PLA_2 activity; and 3) PSNTXs and CTXs alter Ca^{2+} regulation in cells, either as a direct action or secondary to alterations in lipid metabolism.

Background of Previous Work

PSNTXs from snake venoms vary considerably in size (ca. 13.5-80 kD; Howard, 1982; Chang, 1985; Harris, 1985). The site of action of snake venom PLA_2 neurotoxins was first identified to be the neuromuscular junction for ß-bungarotoxin (ß-Butx) - the prototypic model for this group of toxins (Chang and Lee, 1963). The cause of death following envenomation with these toxins is respiratory failure. The PLA_2 toxins cause a brief initial decrease in transmitter release, followed by greatly enhanced and, ultimately, completely abolished release (Howard, 1982; Chang, 1985; Harris, 1985).

The PSNTXs can be either single chain toxins or consist of two to four subunits (Chang, 1985; Harris, 1985). The majority of these toxins are basic, or have at least one basic subunit. The basic portion of the molecule appears to be the toxic unit and contains the PLA_2 activity (Harris, 1985). The

binding sites of the PSNTXs are most likely not identical (Chang and Su, 1980; Lambeau et al., 1989). The toxins do not appear to leave the plasma membrane once bound (Trivedi et al., 1989). However, it is not known whether they reach into the inner leaflet of the bilayer.

While the exact mechanism of action of PSNTXs is unknown, recent evidence has suggested that these toxins may block certain K^+ channels that are slowly activating (Dreyer and Penner, 1987) or voltage-dependent (Rowan and Harvey, 1988). Other consequences of the toxin action appears to be elevated cytoplasmic Ca^{2+} levels (Howard and Gundersen, 1980) and depolarization of nerve terminals (Sen and Cooper, 1978). The toxins also uncouple mitochondrial respiration (Howard, 1975; Ng and Howard, 1980; Rugolo et al., 1986). Either of these effects could be attributed to free fatty acids (FFAs). A differentiation between the action of ß-Butx on the plasma membrane and that on mitochondria has been observed that is dependent on the presence of bovine serum albumin (BSA) in the bathing medium (Rugolo et al., 1986).

The CTXs are, for the most part, small molecular weight (ca. 7 kD) polypeptides that induce arrhythmias and eventually cardiac arrest (Lee et al., 1968; Condrea, 1974; 1979). The CTXs are active on most membranes and can induce contractures in skeletal muscle, hemolysis of erythrocytes and lysis of cell cutures. These agents work in mutual synergy with PLA_2 enzymes, the PLA_2 activity greatly enhancing the hemolytic activity of CTX and the action of CTX greatly enhancing the penetrating ability, and consequently the hydrolytic activity, of the PLA_2 (Condrea, 1974; 1979). Melittin has many of the same characteristics as CTXs. We have recently demonstrated that melittin allows bee venom PLA_2 access to the inner leaflet of artificial membrane bilayers (Fletcher et al., 1990b). CTX has also been reported to activate endogenous PLA_2 enzyme activity (Shier, 1979).

All PSNTXs from snake venoms have PLA_2 activity. High levels of lipolysis will certainly alter neurotransmitter release in a nonspecific manner. Indeed, addition of a nonneurotoxic PLA_2 enzyme to synaptosomes does result in enhanced (Sen et al., 1976; Fletcher and Middlebrook, 1986) and, ultimately, inhibited (Fletcher and Middlebrook, 1986) transmitter release. The greater potency of ß-Butx relative to a less toxic PLA_2 enzyme in causing these effects (Fletcher and Middlebrook, 1986) may simply relate to a greater specific binding to a crucial site and PL hydrolysis on the *inner* leaflet of the bilayer. The toxin-induced inhibition of neurotransmitter release is likely due to inhibition of choline (Ch) uptake (Sen et al., 1976; Fletcher and Middlebrook, 1986). Low, barely detectable levels of hydrolysis are more relevant to upsetting a physiological mechanism of lipolysis associated with neurotransmitter release. In such a mechanism a highly localized change in the ratio of saturated to unsaturated FFAs may significantly alter neurotransmitter release. Unsaturated FFAs have effects on the uptake of Ch and amino acids and the release of ACh that mimic treatment with PLA_2 (Rhoads et al., 1983; Boksa et al., 1988). In contrast, saturated FFAs, which predominate in the membrane in the absence of lipolytic activity, have no effect on transmitter uptake or ACh release (Rhoads et al., 1983; Boksa et al., 1988).

Considerable controversy exists regarding the role of PLA_2 activity in the action of a number of toxin classes, including neurotoxic and cardiotoxic PLA_2 agents. The confusion regarding the role of PLA_2 activity in neurotoxin action is best expressed in a recent review (Harris, 1985). The PLA_2 activity of the PSNTXs is extremely low compared to other venom PLA_2 enzymes, and is

most often only observed on purified PL substrates in the presence of charged detergent substrates (Strong et al., 1976; Radvanyi et al., 1987). In general, most laboratories agree that there is a mechanism in addition to PLA_2 activity that plays a role in the toxic action of these agents. Certainly there is a binding component rendering specificity to these toxins that is active under conditions that (most likely) eliminate much of the enzyme activity. This binding action has been suggested to account for the initial depression of ACh release (Abe et al., 1977; Abe and Miledi, 1978; Livengood et al., 1978; Kelly et al., 1979; Caratsch et al., 1981; 1985; Halliwell et al., 1982).

Several laboratories have suggested that PLA_2 activity plays a crucial role in the latter two phases of toxin action (Strong et al., 1976; Abe et al., 1977; Chang et al., 1977; Abe and Miledi, 1978; Livengood et al., 1978; Kelly et al., 1979; Halliwell et al., 1982; Hawgood and Smith, 1989). While some evidence suggests that PLA_2 activity plays no role in the toxic action (Rosenberg et al., 1989), these studies are all inconclusive, as *no study to date has directly examined the* PLA_2 activity of these neurotoxins on the biological substrates affected. For example, replacement of Ca^{2+} by Sr^{2+} is presumed to eliminate PLA_2 activity; however, a surprisingly high level of PLA_2 activity is maintained under this condition (Fletcher et al., 1981; Ghassemi et al., 1988). Even if PLA_2 activity were determined on biological substrates, the methods employed by investigators in the field most likely would not be sensitive, specific or thorough enough to yield conclusive results.

Studies extrapolating results of PL hydrolysis on purified substrates to the action of toxins on biological substrates are meaningless. Chemical modification of a PLA_2 neurotoxin can greatly affect the enzymatic activity of the toxin, but often this only occurs toward one or a very few molecular species of PL. For example, the -COOH-C modification of ß-BuTX decreased enzymatic activity by 50% toward an egg yolk substrate, but had no effect on hydrolysis of a lecithin substrate (Rosenberg et al., 1989). Lethality was not affected. Interpreting the findings with egg yolk as dissociating enzymatic activity from pharmacological activity is obviously incorrect. In our current studies some CTX fractions completely devoid of PLA_2 activity on purified substrates exhibit very significant levels of PL hydrolysis on biological substrates (red blood cells), when gas chromatographic (GC) analysis of FFAs is employed.

Since different FFAs have different effects on transmitter release (Boksa et al., 1988), it is essential to employ a method such as GC, so that the individual FFAs can be quantitated and shifts in the ratio of saturated to unsaturated FFAs can be monitored. In the snake venom PLA_2 literature the emphasis has been on subclasses of PL (phosphatidylcholine, etc.) hydrolyzed, not molecular species as evidenced in a recent review (Harris, 1985). The existence of reacylating enzymes in tissues that "restore" the hydrolyzed PL by attaching another FFA on to it would mean PLs will appear *not* to have been hydrolyzed at levels of PLA_2 activity less than the tissue reacylation activity. Using GC analysis of FFAs will detect even these low levels of PLA_2 activity. Radiolabeling cell cultures allows examination of: 1) the glycerol moiety; 2) FFAs or esters, and; 3) the phosphorylated bases of PLs. Therefore we can distinguish acylation reactions from alterations of head groups.

Purpose of the Present Work

The present study tests three major hypotheses:

1. PSNTXs exert their effects on ACh release and Ch uptake by hydrolyzing specific PLs at crucial sites, perhaps on the inner leaflet of the plasma membrane bilayer
2. PSNTXs and CTXs activate endogenous processes of lipid metabolism
3. PSNTXs and CTXs alter Ca^{2+} regulation in cells

Methods of Approach

Seven objectives were proposed for the entire contract period. All seven objectives are listed below even though only numbers 1, 3, 4 and 5 were addressed in the first half of the contract.

Objective #1. Compare the potencies and efficacies of PSNTXs as enhancers and ultimately inhibitors of ACh release from synaptosomes and PC12 cells. The relative orders of potency of a series of PLA_2 neurotoxins on Ch uptake and ACh release were determined in mouse brain synaptosomes.

Our studies of PSNTXs have primarily been conducted with a highly purified synaptosomal preparation from mouse brain. We have examined:

1) The relative potencies of the toxins *in vivo*
2) Choline uptake ([^{14}C]choline)
   a) the time course of choline uptake
   b) the effects of temperature on choline uptake
   c) the kinetics (K_m, V_{max}) of choline uptake
   d) the dose-response relationships as regards inhibition of choline uptake by PSNTXs and a non PSNTX PLA_2
   e) the effects of coincubation with BSA on the action of the PSNTXs
3) Acetylcholine release ([^{14}C]choline)
   a) the use of a TLC method for separation of ACh and Ch
   b) tetraphenylboron/3-heptanone-based extraction for determination of ACh
   c) the dose-response relationships as regards stimulation of ACh release by PSNTXs and a non PSNTX PLA_2
   d) effects of coincubation with BSA on the action of PSNTXs
4) Effects of a synthetic peptide from *Trimeresurus wagleri* snake venom on the phrenic nerve-diaphragm preparation

Objective #2. Examine the interaction between snake venom PSNTXs and CTXs.

No studies have been conducted in relation to this objective during the first half of the contract.

Objective #3. Examine the role of PLA_2 activity in PSNTXs and CTX action. This is the first study to directly determine *all* substrates and products of PLA_2 activity (PLs, lysoPLs and FFAs) on the biological substrate affected by the toxins.

The role of PLA_2 activity in the action of the PSNTXs and CTXs has been examined in several ways:

1) Effects of *p*-BPB-treated CTXs (to abolish PLA_2 activity associated with any venom PLA_2 contamination) on lipid metabolism in cell culture systems
2) Effects of PSNTXs on lipid metabolism in cell culture systems

3) Effects of PSNTXs on FFA production in synaptosomes

Objective #4. Correlation of effects on ACh release and FFA release (lipid metabolism) with those on cytoplasmic Ca^{2+}. The effects of PSNTXs and CTXs were examined on Ca^{2+} release from terminal cisternae preparations.

The involvement of Ca^{2+} regulation in transmitter release (PSNTXs) or contracture induction (CTXs), either directly or as a consequence of altered lipid metabolism was examined in the following manner:

1) Effects of CTXs and related toxins (melittin and myotoxin a) on the threshold of Ca^{2+}-induced Ca^{2+} release (TCICR)
2) Effects of a PSNTX (β-Butx) on the threshold of Ca^{2+}-induced Ca^{2+} release (TCICR)

Objective #5. Examine the effects of PSNTXs and CTXs on lipid metabolism. The effects of the PSNTXs and CTXs were examined on skeletal muscle cultures in which the lipids are preradiolabeled on the head groups, glycerol backbone and fatty esters.

The effects of the PSNTXs and CTXs on lipid metabolism were examined in the following manner:

1) Identify aspects of lipid metabolism activated by CTXs and melittin in cells with radiolabeled FAs
2) Compare the effects of CTXs and melittin on lipid metabolism in cell cultures derived from normal and malignant hyperthermia susceptible patients since these are our primary sources of tissue
3) Radiolabeled head groups to identify whether phospholipase C (PLC) or phospholipase (PLD) are activated and whether specific substrates are hydrolyzed (phosphatidylcholine or phosphatidylethanolamine)
4) Examine the effects of pertussis and cholera toxin on CTX and melittin action to determine if there is the involvement of GTP-binding proteins sensitive to these agents
5) Examine the time course of activation of lipid metabolism to determine if this could be responsible for the effects on Ca^{2+} regulation
6) Examine whether myotoxin a also alters lipid metabolism
7) Determine the Ca^{2+} dependence of CTX and melittin-activated enzymes to examine whether the effects on lipid metabolism are the consequence of the effects on Ca^{2+} release
8) Determine the effects of β-Butx on lipid metabolism (see Objective #3)

Objective #6. Effects of altered lipid environment on PSNTX action.

No studies have been conducted in relation to this objective during the first half of the contract.

Objective #7. Study penetration to inner layer of bilayer.

No studies have been conducted in relation to this objective during the first half of the contract.

BODY

EXPERIMENTAL METHODS

Materials

Scutoxin, taipoxin and crotoxin were obtained from Ventoxin Laboratories, Inc. (Frederick, MD). The *Naja naja atra* PLA_2 was a gift from Dr. Leonard A. Smith (Fort Detrick, MD). CTX from *Naja naja kaouthia* venom (Lot# 125F-4007),

lipid standards, 4-(2-hydroxyetnyl)-1-piperazineethanesulfonic acid (HEPES), Percoll, FA-free BSA, β-bungarotoxin, *p*-bromophenacyl bromide (*p*-BPB), phosphocreatine, creatine phosphokinase, Mg-ATP, 3-([cholamidopropyl] dimethylammonio)-1-propanesulfonate (CHAPS), 1,4-piperazineethanesulfonic acid (PIPES), pepstatin, iodoacetamide, phenylmethylsulfonyl fluoride, leupeptin, benzamidine and arsenazo III were purchased from Sigma Chemical Company (St. Louis, MO). Morpholinopropane sulfonic acid (MOPS) was obtained from Calbiochem (San Diego, CA). Synthetic melittin from Peninsula Laboratories, Inc. (Belmont, CA). [^{3}H]Ryanodine (60 C_i/mmol) was purchased from New England Nuclear (Wilmington, DE). [^{14}C]Choline (50-60 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). In some cases the CTX fraction was treated with *p*-BPB to inactivate contaminating venom PLA_2 prior to use, as previously described (Jiang et al., 1989; Fletcher et al., 1991a).

I.V. LD_{50} Determinations

Male Swiss-Webster mice (20-25 g) were restrained and injected in the tail vein with about 0.2 ml of toxin in saline solution. Since the LD_{50} values have been published for all of these toxins, we focussed our analysis on those doses immediately above and below the published values. The number of animals surviving after 16 hr was used to estimate the LD_{50}.

Isolation of Synaptosomes

An enriched synaptosomal fraction was prepared from mouse brain (Dunkley et al., 1988). Briefly, whole brain from six Swiss Webster (Harlan Sprague Dawley; Indianapolis, IN) mice (20-30 g) was homogenized (3 g tissue per 9 ml) in sucrose (0.32 M), ethylenediaminetetraacetic acid (EDTA; 1 mM), dithiothreitol (0.25 mM), pH 7.4 (4°C). The homogenate was centrifuged (1,000 x g; 10 min) and the supernatant adjusted to 13 ml. The supernatant (4 mg protein/ml) was applied to a discontinuous Percoll gradient (2 ml each 23%, 15%, 10%, 3% Percoll; v/v; pH 7.4) and centrifuged at 32,500 x g for 15 min. Fractions 3 and 4 (Dunkley et al., 1988) were pooled for use in the ACh release and Ch uptake studies.

Choline Uptake

High-affinity choline uptake was determined by a modification of our previous study (Fletcher and Middlebrook, 1986), based on the original method of Yamamura and Snyder (Yamamura and Snyder, 1972). The pooled synaptosomal fractions were centrifuged (Beckman JA-20 rotor; 15,000 RPM; 15 min), the supernatant discarded and pellets resuspended in incubation buffer comprised of (in mM): HEPES 10, NaCl 137, KCl 2.7, $CaCl_2$ 1.7, $MgCl_2$ 0.7, D-glucose 20 and adjusted to pH 7.4. Aliquots of synaptosomes in 0.5 ml of incubation buffer were incubated with or without toxin and/or BSA 0.5% at 25°C (37°C when indicated) for 1 hr. The synaptosomes were centrifuged (Eppendorf 5414 microcentrifuge; 30 sec), the supernatant discarded and pellets washed, centrifuged and resuspended in incubation buffer. Aliquots of synaptosomes were added to tubes containing incubation buffer at 37°C and [^{14}C]choline (? μM). After a 4 min choline uptake period the incubates were filtered (Whatman GF/F filters) in a Hoefer (San Francisco, CA) FH224V filter holder and washed three times with 1 ml cold (4°C) incubation buffer. Scinti Verse™ II (Fisher Scientific Co.; Pittsburgh, PA) was used for liquid scintillation counting of radioactivity retained on the filters. In the case of Na^+-free uptake medium,

Tris-HCl (91 mM) was substituted for NaCl.

Acetylcholine Release

Acetylcholine release was determined by a tetraphenylboron and 3-heptanone extraction technique, as previously described (Fletcher and Middlebrook, 1986). In brief, synaptosomes were preloaded with [^{14}C]Ch to synthesize [^{14}C]ACh in an incubation buffer comprised of (in mM): HEPES 10, NaCl 137, KCl 2.7, $CaCl_2$ 1.7, $MgCl_2$ 0.7, D-glucose 20 and adjusted to pH 7.4. Aliquots of synaptosomes in 0.5 ml of incubation buffer were incubated with or without toxin and/or BSA 0.5% at 25°C (37°C when indicated) for 1 hr. A solution containing choline kinase was added to eliminate choline and to selectively extract ACh into tetraphenylboron and 3-heptanone. An aliquot of the organic phase was then evaporated and quantitated by liquid scintillation counting.

Phrenic Nerve-Diaphragm Preparation

The mouse phrenic nerve-diaphragm was isolated and mounted, essentially as previously described for the rat preparation (Fletcher et al., 1981). The preparation was bathed in a modified Krebs solution at 37°C (Fletcher and Rosenberg, 1985). Preparations were adjusted for optimum length for twitch tension and equilibrated 1 hr before drug or toxin addition. Preparations were then either exposed to synthetic peptide toxin (1 μM), or succinylcholine (50 μM). Preparations exposed to succinylcholine were washed an reequilibrated for 10 min before exposure to synthetic peptide (1 μM).

Radiolabeling and Analysis of Lipids in Cultured Cells

Primary cultures of human skeletal muscle were established from biopsies of vastus lateralis as previously described (Wieland et al., 1989). Ten to fourteen days (human and equine primary cultures), or 3-5 days (mouse C_2C_{12} cell line) following addition of fusion-promoting medium (Wieland et al., 1989), radiolabeled FA ([^{14}C]linoleic acid; 10 μM) and/or ethanolamine ([^{14}C]ethanolamine; 10 μM) was added to the culture medium for three days, as described previously for FAs in human and equine skeletal muscle cells (Fletcher et al., 1991a). Pertussis toxin (400 ng/ml) or cholera toxin (1000 ng/ml) were added to specified cultures 24 hrs prior to completion of labeling. Following incubation for 2 hrs with or without *p*-BPB-treated CTX or melittin at 37°C, the lipids were extracted and, for the FA studies, neutral and phospholipid components were separated by one-dimensional thin-layer chromatography (TLC) and the radioactivity associated with each phospholipid and neutral lipid component quantitated by a radioactivity scanner (Fletcher et al., 1990b; 1991a). For the ethanolamine studies, the same lipid extraction was performed. However, the aqueous phase of the two-phase extraction containing the cell-associated phospholipid polar head groups was lyophilized, resolvated with methanol and the radioactivity in ethanolamine and phosphoethanolamine was separated by one-dimensional TLC on Analtech (Newark, DE) AVICEL F cellulose TLC plates (20 x 20 cm) using a solvent system comprised of n-butanol:methanol:acetic acid:ethylacetate:H_2O [20:10:5:20:15 (Bluth et al., 1980)] and subsequent scanning of the TLC plate for radioactivity, as described above. The location of the radiolabel in phosphatidylethanolamine ($CHCl_3$ phase) was confirmed by one-dimensional TLC, using the same phospholipid solvent system as used with the FAs.

Extraction and Analysis of Lipids of Synaptosomes

Synaptosomes were prepared and incubated with or without toxin and/or BSA for 1 hr, as described above. The incubates were centrifuged (Eppendorf 5414 microcentrifuge; 30 sec) and the lipids were extracted separately from the supernatant and pellets by the methods of Marinetti et al. (Marinetti et al., 1959) and Folch et al. (Folch et al., 1957), as previously described for red blood cells (Fletcher et al., 1987) and muscle (Fletcher et al., 1982; 1990c). The neutral lipids were separated by one-dimensional thin-layer chromatography (Fletcher et al., 1987), the FFAs methylated (Morrison and Smith, 1964) and the FA methyl esters separated on a Shimadzu (Columbia, MD) GC-9A gas chromatograph and quantitated using Beckman (Allendale, NJ) System Gold software, as previously described (Fletcher et al., 1987; 1990c). Heptadecanoic acid was added to the extract as an internal standard (Fletcher et al., 1987; 1990c). Protein was determined by a modification (Markwell et al., 1978) of the method of Lowry et al. (Lowry et al., 1951).

Determination of Threshold of Ca^{2+}-Induced Ca^{2+} Release (TCICR)

Heavy sarcoplasmic reticulum fractions were prepared by differential centrifugation (8,000 - 12,000 x g; Nelson, 1983) of homogenates of equine semimembranosus, human vastus lateralis, or porcine gracilis muscle. The TCICR was determined with pyrophosphate to increase the sensitivity of the assay (Palade, 1987a) and arsenazo III to detect Ca^{2+}, as previously described (Fletcher et al., 1990c; 1991b; c). Ca^{2+} was added in 10 µM increments to 1.5 ml of a MOPS/KCl (pH 7.0) buffer containing 20-40 µg protein, ATP and an ATP regenerating system maintained at 37°C (Palade, 1987a). CTX (10 µM), melittin (0.1 µM) or myotoxin a (10 µM) was either added immediately after ATP-stimulated Ca^{2+} uptake had reached equilibrium, as previously described for the addition of FAs (Fletcher et al., 1990c), or after the indicated number of Ca^{2+} pulses (expressed as percent of the TCICR; Fletcher et al., 1991b).

Ryanodine Binding

The ryanodine receptor was isolated by CHAPS solubilization from porcine longissimus dorsi by a modification of a procedure for use with skeletal muscle frozen and stored in liquid N_2 (Valdivia et al., 1991b). The muscle was minced, thawed and then homogenized in a sucrose (0.3 M), HEPES-Tris buffer at pH 7.2 containing the following protease inhibitors: pepstatin, iodoacetamide, phenylmethylsulfonyl fluoride, leupeptin and benzamidine (Valdivia et al., 1991b). The supernatant from the first centrifugation step (4,000 x g_{max}; 20 min; Beckman JA-20 rotor) was transferred and centrifuged (16,000 x g_{max}; 20 min Beckman JA-20 rotor) a second time. The pellet from the second centrifugation step was resuspended in 4 ml of homogenizing buffer and diluted with 4 ml of CHAPS (1%) NaCl (1 M) and Tris maleate (40 mM) at pH 7.2. The preparations were incubated for 40 min at 4°C, centrifuged (60,000 x g_{max}; 40 min; Beckman Type 40 rotor) and the supernatant, containing solubilized ryanodine receptor, was used for binding studies. Competitive binding of the toxins for the ryanodine receptor was determined as described for a scorpion venom (Valdivia et al., 1991a). Ryanodine receptor preparations were incubated with a fixed concentration of [^{3}H]ryanodine (8 nM) and various concentrations (0.01-10 µM) of CTX or melittin for 90 min at 37°C by a 1:6.25 dilution of CHAPS-solubilized protein into incubation buffer containing 0.2 M KCl, 1 mM Na_2EGTA, 0.995 mM $CaCl_2$, 10 mM Na-PIPES adjusted to pH 7.2. The concentration of protein was 60 µg/ml and the concentration of free Ca^{2+} was calculated to

be 10 µM (Valdivia et al., 1991b). Incubates were filtered on Whatman GF/B, the filters washed with cold incubation buffer and the radioactivity associated with the filters determined using liquid scintillation techniques (Vita et al., 1991). Nonspecific binding was determined in the presence of 10 µM unlabeled ryanodine.

RESULTS

Relative Potencies of the Toxins In Vivo

To compare the relative potencies of the toxins *in vivo* to those *in vitro* we first established mouse i.v. LD_{50} values for several toxin fractions in our possession. These include: crotoxin (70 µg/kg); ß-Butx (40 µg/kg); taipoxin (< 7 µg/kg); *Naja naja atra* PLA_2 (8,600 µg/kg); and scutoxin (40 µg/kg). Pseudexin (ca. 1000 µg/kg) was tested and provided by Dr. John L. Middlebrook (USAMRIID). Interestingly, we initially examined i.p. LD_{50} values for ß-Butx and taipoxin and found the value for ß-Butx to be about the same as for i.v. administration, but the toxicity of taipoxin was reduced to below that of ß-Butx.

Choline Uptake

The choline uptake system was examined in a highly purified and rapidly isolated synaptosomal fraction (Bands 3 and 4; Dunkley et al., 1988). The time course of ^{14}C-choline (2 µM) uptake at 37°C was linear for about 5 min (Figure 1). Two separate synaptosomal preparations (filled and open circles) were used to examine the time course of uptake. A 4 min uptake period was used for all subsequent studies.

Varying the substrate (choline) concentration from 1 to 8 µM increased the uptake of radiolabel (Figure 2A) and this was then analyzed by a Lineweaver-Burk plot, yielding a single high affinity choline uptake process with a K_m of 4 µM (37°C, 4 min; Figure 2B). Although this K_m is slightly higher than the value of 1.2 ± 1.3 µM originally reported using similar methodology (Yamamura and Snyder, 1972), we use a higher temperature (37°C vs. 30°C) and mouse, not rat, synaptosomes. We previously reported a K_m value of 4 µM for guinea pig synaptosomes incubated at room temperature (Fletcher and Middlebrook, 1986).

The viability of the choline uptake process in synaptosomes is considerably greater at 25°C than at 37°C (Figure 3). Two separate synaptosomal preparations (filled and open circles) were used to examine the effects of incubation at each temperature. Therefore, all prolonged incubations were conducted at 25°C.

The dose-response relationships were examined as regards inhibition of choline uptake in the absence or presence of BSA for six PLA_2s or PSNTXs (Figure 4). There were 6-10 determinations at each concentration. While the choline uptake process may not be the primary target of some or all of the PSNTXs, it does appear to be an indicator of PSNTX action, especially in the cases of ß-Butx, scutoxin and pseudexin. Albumin, which is in blood, has complex effects on PLA_2s. BSA binds the FFAs generated by PLA_2 activity and this can enhance, inhibit, or have no effect on PLA_2 activity depending on the specific enzymes (Pluckthun and Dennis, 1985). In brief, BSA dramatically antagonizes a nonPSNTX PLA_2 from *Naja naja atra* venom and has little or no effect on the four PSNTXs (ß-BuTX, crotoxin, scutoxin, pseudexin) most active on mouse brain synaptosomes, as regards choline uptake (Figure 4). Taipoxin

has very little effect under the same conditions (Figure 4). We examined the effect of pseudexin on choline uptake that was not Na^+ dependent. The Na^+-independent (nonspecific) choline uptake process that is not coupled to ACh synthesis and release was greatly inhibited by pseudexin, even with BSA in the medium. This latter finding supports an action independent of nonspecific phospholipid hydrolysis.

Pseudexin was not included in the following detailed analysis as its effects on Ch uptake have only recently been examined. In the absence of BSA in tha incubation medium, four of the toxins (*Naja naja atra* PLA_2, β-bungarotoxin, crotoxin and scutoxin) caused a dose-dependent inhibition of high-affinity choline uptake over the concentration range of 1-100 nM (Figure 4). At a fixed concentration of toxin of 10 nM the only difference between the toxins, as determined by a one-way analysis of variance (ANOVA; $P<0.004$) and Sheffe test was the greater efficacy of β-bungarotoxin over taipoxin ($P<0.01$). At a concentration of 100 nM (one way ANOVA; $P<0.0001$) scutoxin was more efficacious (Sheffe test) than β-bungarotoxin ($P<0.0001$), crotoxin ($P<0.0001$) and taipoxin ($P<0.0001$). The *Naja naja atra* PLA_2 inhibited choline uptake to a greater extent than β-bungarotoxin ($P<0.008$), crotoxin ($P<0.003$) and taipoxin ($P<0.0001$). Both β-bungarotoxin ($P<0.03$) and crotoxin ($P<0.01$) were more effective than taipoxin. Therefore the overall efficacy at a 100 nM concentration of toxin in the absence of BSA was scutoxin = *Naja naja atra* PLA_2 > β-bungarotoxin = crotoxin > taipoxin.

BSA was added to the incubation medium to extract the more readily available membrane-bound synaptosomal FAs liberated from phospholipids by the toxins. Addition of BSA greatly antagonized ($P<0.007$; grouped two-tailed t-test) the inhibition of choline uptake by the 10 nM concentration of the *Naja naja atra* PLA_2, but had no significant effect ($P>0.27$ in all cases) on the other four toxins (Figure 4). At the 100 nM concentration of toxin, BSA significantly antagonized the action of the *Naja naja atra* PLA_2 ($P<0.0001$; grouped two-tailed t-test), β-bungarotoxin ($P<0.03$), crotoxin ($P<0.01$), taipoxin ($P<0.02$) and scutoxin ($P<0.0001$). This effect of BSA reduced the inhibition of choline uptake to about half that in the absence of BSA for the *Naja naja atra* PLA_2 and scutoxin (Figure 4), while the effects of BSA on the actions of β-bungarotoxin, crotoxin and taipoxin were considerably less. Inhibition of choline uptake in a BSA-containing medium was compared by a one-way ANOVA ($P<0.0002$) and Sheffe test at the 10 nM concentration of toxins. Crotoxin ($P<0.004$), scutoxin ($P<0.0001$), β-bungarotoxin ($P<0.0001$) and taipoxin ($P<0.01$) were all more efficacious than the *Naja naja atra* PLA_2. β-Bungarotoxin inhibited choline uptake to a greater extent than crotoxin ($P<0.02$) or taipoxin ($P<0.002$) and scutoxin inhibited choline uptake to a greater extent than taipoxin ($P<0.03$). Therefore, the order of efficacy at a 10 nM concentration of toxin in the presence of BSA was β-bungarotoxin = scutoxin > crotoxin = taipoxin > *Naja naja atra* PLA_2. Inhibition of choline uptake in a BSA-containing medium was compared by a one-way ANOVA ($P<0.0001$) and Sheffe test at the 100 nM concentration of toxins. β-Bungarotoxin ($P<0.0001$) and scutoxin ($P<0.0001$) were more efficacious than either the *Naja naja atra* PLA_2 or taipoxin. Also β-bungarotoxin ($P<0.005$) and scutoxin ($P<0.0006$) were more efficacious than crotoxin and crotoxin was more efficacious than taipoxin ($P<0.008$). Therefore, the order of efficacy for the 100 nM concentration of toxin in a BSA-containing medium was scutoxin = β-bungarotoxin > crotoxin > *Naja naja atra* PLA_2 = taipoxin.

Acetylcholine Release

The dose-response relationships were examined as regards stimulation of ACh release. Numerous problems with a TLC-based analysis of ACh and Ch originally proposed for use in the contract have caused us to use a previously described (Fletcher and Middlebrook, 1986) tetraphenyl- boron/3-heptanone-based extraction technique. Six toxins have, at least preliminarily (larger sample sizes will be acquired), been examined with regard to dose-response in BSA-containing or BSA deficient media (Figure 5). We found that BSA completely antagonizes the stimulation of ACh release by the *Naja naja atra* PLA_2, presumedly by removing the products of PLA_2 activity accessible to BSA at the outer leaflet of the membrane bilayer (Figure 5A). However, albumin has no effect, or even enhances the stimulatory effect of three of the PSNTXs (β-Butx, scutoxin, pseudexin) on ACh release (Figure 5B, 5E and 5F, respectively). Since the PLA_2 activity of these three toxins was unaffected by the BSA treatment (see below), this suggests that these toxins have an action on ACh release unrelated to gross PLA_2 activity and that could relate to site-directed (intracellular leaflet of plasma membrane bilayer) PLA_2 activity, or an action independent of PLA_2 activity altogether. Two PSNTXs (taipoxin and crotoxin) have very little effect on ACh release (110% and 115% of control, respectively) in the presence of albumin, even at 100 nM concentrations (Figure 5C and 5D), suggesting that these toxins have virtually no specific interaction with the ACh release mechanism in mouse brain synaptosomes.

We have begun to use the phrenic nerve-diaphragm preparation to examine toxin action, as previously described (Fletcher et al., 1981; Storella et al., 1992). We first examined a synthetic homologue of a toxin from *Trimeresurus wagleri* snake venom (Weinstein et al., 1991). By accident we noticed that pretreatment with succinylcholine (50 μM) unmasked a specific presynaptic effect of the toxin (Table 1).

Effects of p-BPB Treatment on CTX-Induced Lipid Metabolism in Cell Culture Systems

To examine if PLA_2 contamination could play a major role in the effects of the CTX fractions on lipid metabolism in primary cultures, as was observed with venom-derived melittin (Fletcher et al., 1990b), we tested the relatively more highly contaminated CTX fraction from *N. n. atra* venom (Table 2). Treatment with this CTX exhibited extensive PLA_2 activity that could be greatly reduced by treating the fraction with p-BPB prior to incubation with the cells. The patterns of hydrolysis by obvious PLA_2 contamination in the native fraction (Table 2) do not match those produced by the more highly purified CTX from *N. n. kaouthia* venom (described below). Due to the extreme level of phospholipid hydrolysis, only one of the three culture dishes treated with the native *N. n. atra* CTX had sufficient radiolabel remaining to examine the phospholipid distribution. A major marker of the PLA_2 activity was elevated lysophosphatidylcholine in the native fraction (Table 2). However, production of radiolabeled lysophosphatidylcholine only accounts for about 5% of the hydrolyzed phosphatidylcholine. About 36% of the phosphatidylcholine was hydrolyzed by the p-BPB-treated CTX. If PLA_2 were the only enzyme activated by CTX, this would produce enough lysophosphatidylcholine to account for about 1.2% of the total phospholipid assuming no loss of radiolabel from phospholipid, and a much larger percentage when the loss of label from the phospholipid is accounted for. The methodology employed would detect lysophosphatidylcholine as low as about 0.6% of the phospholipid Therefore,

some fraction of the FFA production (substantially less than 50% of the hydrolysis of phosphatidylcholine) would not be detected by the presence of lysophospholipid and might still be attributed to PLA_2 activity.

By greatly reducing PLA_2 activity in the CTX fraction with p-BPB, we totally eliminate the production of FFAs by the *Naja naja kaouthia* CTX in a mouse cell line (Figure 6A). The native toxin causes significant production of LPC, which is associated with PLA_2 activity (Table 3). Interestingly, in the mouse cell line there was no significant increase in diacylglycerol, the major product of PLC activity (Figure 6B), suggesting that the PLC enzyme was not present in the cell line, at least at the time tested. The time course of PLA_2 (CTX fraction contamination) and PLC (tissue enzyme) activities were also examined in human primary cell cultures of skeletal muscle by comparing native and p-BPB-treated CTX fractions (Figure 7). The production of FAs was greatly reduced by *p*-BPB treatment and the production of DG, while significantly reduced, was still very evident, supporting the presence of a PLC activated by CTX.

Effects of PSNTXs on Lipid Metabolism in Skeletal Muscle Cell Culture Systems

The studies of PSNTXs in human skeletal muscle cultures are meant to test the effects of these toxins on postsynaptic lipid metabolism and to set the stage for future studies with the PC12 cells. Exposure of primary cultures of human skeletal muscle to ß-Butx for 2 hrs did not cause any significant release of radioactivity to the supernatant (data not shown). The distribution of radiolabel into the neutral lipids and phospholipids (data not shown) was similar to that previously reported for human skeletal muscle (Fletcher et al., 1991a). Free FAs and lysophosphatidylcholine, the two main products of PLA_2 activity were undetectable in untreated cells (Figure 8). Since linoleic acid primarily labels the #2 position of phospholipids, the release of FFAs is the most sensitive indicator of PLA_2 activity. However, since some radiolabel is attached to the #1 position of phospholipids, lysophosphatidylcholine production can be followed at higher levels of PLA_2 activity. The level of FFA was slightly (about 3% of the total lipid associated radiolabel), but significantly ($P<0.01$; two-tailed t-test) raised at a concentration of ß-bungarotoxin of 0.1 µM (Figure 8). The levels of PLA_2 activity as determined by the formation of FFAs and lysophospholipids were considerably greater at higher concentrations of toxin (Figure 8). There was no change in the levels of diacylglyceride or triglyceride upon treatment with the toxin (data not shown). This low level of PL hydrolysis induced by ß-Butx contrasts with much higher levels of hydrolysis produced by 2 nM bee venom PLA_2 (Fletcher et al., 1990b).

Effects of PSNTXs on Fatty Acid Production in Synaptosomes

The production of FAs by five PLA_2s, including four PSNTXs, was examined under the same conditions as Ch uptake and ACh release. These studies were designed to determine whether BSA truely extracts the FFAs from the synaptosomal preparations and whether the more toxic PLA_2 neurotoxins produce FFAs that are inaccessible to BSA, suggesting that they are produced in the inner layer of the membrane bilayer. In brief, the synaptosomes are incubated $\pm$ BSA and with either *Naja naja atra* PLA_2, a PSNTX or no toxin. The synaptosomes are pelleted by centrifugation and the supernatant ($\pm$ BSA) is removed. The supernatant and pellet are then extracted and the FFAs analyzed. Only a 10 nM concentration of each toxin has been completely examined.

In the absence of BSA, the release of FFAs into the incubation medium by any of the toxins (Table 4) was minimal relative to the FA generation in the synaptosomal pellet (Table 5). The release of FAs into the incubation medium (Table 4) also did not correlate well with the extent of PLA_2 activity, as judged by the amount of FFA in the synaptosomal pellet (Table 5). For example, the ratio of total FAs in the *Naja naja atra* PLA_2-treated preparations to the control preparations was 1.7 for the supernatant (Table 4) and 6.4 for the pellet (Table 5). Therefore, the FAs were primarily retained within the membrane bilayer and did not migrate into the supernatant in the absence of BSA.

The values for total FFAs in the control synaptosomal preparations not treated with BSA (Table 5) were in agreement with those of other investigators using a similar synaptosomal preparation (Rhoads et al., 1983). There was very little hydrolysis of 18:0 by any of the PLA_2s (Table 5). The most abundant fatty ester at the #2 position in synaptosomal phospholipids, as determined by PLA_2 hydrolysis, was 22:6, followed by 20:4, 16:0 and 18:1. The order of total FA production for the toxins in the absence of BSA and at a 10 nM concentration was scutoxin = β-bungarotoxin > *Naja naja atra* PLA_2 > crotoxin = taipoxin. Thus, there was no relationship between gross PLA_2 activity and the order of inhibition of choline uptake, which was similar for all the toxins.

BSA extracted a similar amount of FFA into the supernatant in all toxin-treated preparations (Table 6). The patterns of FFAs extracted were also similar, suggesting that BSA-extracted FFAs are derived from the same readily extractable pool, presumedly the outer leaflet of the membrane bilayer, regardless the PLA_2 employed.

The values for total FFAs in the control synaptosomal preparations treated with BSA (Table 7) were also in agreement with those of other investigators using BSA-extracted synaptosomal preparations (Rhoads et al., 1983). Two phospholipid fatty esters, 18:0 and 18:2, were not hydrolyzed to any extent by the toxins in the presence of BSA (Tables 6 and 7), in agreement with the low levels of hydrolysis in the absence of BSA (Table 5). All of the FAs produced by the *Naja naja atra* PLA_2, except for some 20:4 and 22:6, were extracted by BSA, supporting primarily hydrolysis of the outer leaflet of the plasma membrane bilayer by the nonneurotoxic enzyme. The hydrolysis of three fatty esters (16:0, 16:1, 18:1) retained in the synaptosomal pellet in a BSA-containing medium (Table 7) correlated with the effects of β-bungarotoxin and scutoxin on high-affinity choline uptake (Figure 4). The hydrolysis of 16:0 by β-bungarotoxin and scutoxin was much greater than observed with any of the other PLA_2s, in agreement with the extent of inhibition of choline uptake by these agents at a 10 nM concentration in the presence of BSA. Also, 18:1 and 16:1 were hydrolyzed to a greater extent by β-bungarotoxin and scutoxin than by the other toxins (Table 7). Scutoxin hydrolyzed 22:6 and 20:4 to about a two-fold greater extent than the next most active toxin (Table 7). Since scutoxin and β-bungarotoxin are equally efficacious at antagonizing choline uptake at a 10 nM concentration (Figure 4), it is not likely that 22:6 and 20:4 play a significant role in inhibition of choline uptake.

The total PLA_2 activity is the difference between enzyme treated preparations and control preparations in the pellet plus supernatant. Indeed, in the presence of BSA total FA release by the *Naja naja atra* PLA_2 was reduced ($P<0.03$; grouped two-tailed t-test) by 37% from 45 $\pm$ 4 (mean $\pm$ SEM) nmol/mg protein to 29 $\pm$ 4 nmol/mg protein. Total phospholipid hydrolysis was unaffected ($P>0.05$; grouped two-tailed t-test) by the presence of BSA for the

rest of the toxins. However, a far greater decrease (75%) was observed in the amount of FA generated specifically in the synaptosomal pellet in the presence of BSA, suggesting that the predominant effect of BSA is not to reduce PLA_2 activity, but to remove from the plasma membrane FAs produced by the *Naja naja atra* PLA_2.

A second study was conducted with β-Butx and the *Naja naja atra* PLA_2 to confirm the first series of experiments. In the absence of BSA, the release of FFAs into the incubation medium by either of the toxins (Table 8) was minimal relative to those retained in the synaptosomal pellet (Table 9). There was very little hydrolysis of 18:0 by either PLA_2 toxin (data not shown), consistent with previous studies with red blood cells (Fletcher et al., 1987; 1990a; 1991a). Also, hydrolysis of 16:1 and 18:2 was very low and these FAs were not included in Tables 8-11 to focus on the major FAs produced. The most abundant fatty ester at the #2 position in synaptosomal phospholipids, as determined by PLA_2 hydrolysis, was 22:6, followed by 20:4, 16:0 and 18:1. In the absence of BSA and at a 10 nM concentration of toxin β-Butx and *Naja naja atra* PLA_2 produced about the same amount of FFA (Table 9).

Most of the FAs produced by the *Naja naja atra* PLA_2 were extracted from the synaptosomes by BSA (Tables 10 and 11), supporting primarily hydrolysis of the outer leaflet of the plasma membrane bilayer by this enzyme. The hydrolysis of two fatty esters (16:0, 18:1) retained in the synaptosomal pellet in a BSA-containing medium (Table 11) correlated well with the effects of β-Butx and *Naja naja atra* PLA_2 on inhibition of choline uptake in the same medium.

Effects of Toxins on the Threshold of Ca^{2+}-induced Ca^{2+} Release (TCICR)

The effects of CTX on the TCICR in terminal cisternae preparations from skeletal muscle have been examined, using techniques previously published (Fletcher et al., 1990c; 1991b; c).

The CTX from *Naja naja kaouthia* venom was previously reported to decrease the threshold of Ca^{2+}-induced Ca^{2+} release in porcine terminal cisternae preparations when injected after ATP-stimulated Ca^{2+} uptake had reached equilibrium (Fletcher et al., 1991b). However, the threshold of Ca^{2+}-induced Ca^{2+} release was not decreased by CTX in equine preparations using the same paradigm (Table 12). The threshold of Ca^{2+}-induced Ca^{2+} release in human preparations was more sensitive ($P<0.0.005$; two-tailed grouped t-test) to CTX than that in equine preparations (Table 12). A 100-fold lower concentration of melittin (0.1 μM) was as efficacious as a 10 μM concentration of CTX in reducing the threshold of Ca^{2+}-induced Ca^{2+} release from human preparations. Higher concentrations of melittin (1 μM) immediately released extremely large amounts of Ca^{2+} (beyond the upper limits of the arsenazo III range) and this appeared to be due to lysis of the vesicles. Unlike the 0.1 μM concentration of melittin, the release of Ca^{2+} by these higher concentrations did not require prior Ca^{2+} additions (data not shown). In contrast to CTX, there was no significant difference ($P>0.05$) between the effects of melittin on human and equine muscle (Table 12). As indicated by the large standard error of the mean and the wide range of values, there was a large variation in the effects of CTX or melittin on the threshold of Ca^{2+}-induced Ca^{2+} release, depending on the individual horse or human examined. Since the species difference in the action of CTX was unexpected, we verified that CTX (10 μM) induced contractures in equine muscle fiber bundles (data not shown) and found that these contractures were similar in all respects to those in a Ca^{2+}-containing

medium previously reported for human fiber bundles (Fletcher and Lizzo, 1987).

To test whether CTX had *any* action on Ca^{2+} release from equine muscle, terminal cisternae preparations were preloaded to 29-92% of the threshold of Ca^{2+}-induced Ca^{2+} release and were then challenged with CTX (10 µM; Figure 9A). Eleven of the 14 equine preparations preloaded to greater than 65% of the threshold of Ca^{2+}-induced Ca^{2+} release immediately released Ca^{2+} upon subsequent CTX addition (Figures 9B and 10). Of the 6 equine preparations preloaded to 65% or less of the threshold of Ca^{2+}-induced Ca^{2+} release, none exhibited any indication of Ca^{2+} release in response to CTX (Figures 9C and 10). Similar results were obtained with human preparations. All eight human preparations preloaded to greater than 65% of the threshold of Ca^{2+}-induced Ca^{2+} release exhibited Ca^{2+} release when challenged with CTX (Figures 9A, 9D and 10). One of four human preparations preloaded to 65% or less of the threshold of Ca^{2+}-induced Ca^{2+} release exhibited Ca^{2+} release upon challenge with CTX (Figures 9E and 10).

Even trace contamination of the CTX fraction with the *Naja naja kaouthia* snake venom PLA_2 has been suggested to account for many of the actions of CTX (Fletcher et al., 1991a). Therefore, the CTX fraction was pretreated with *p*-BPB to considerably reduce the contribution of venom PLA_2 to the CTX action (Fletcher et al., 1991a). This treatment had no effect on CTX action (Figure 9A), indicating that trace contamination with venom PLA_2 activity is not involved in the rapid activation of Ca^{2+} release by the toxin. Synthetic melittin was used to avoid similar PLA_2 problems associated with venom-derived melittin (Fletcher et al., 1990b).

We verified that synthetic melittin induced contractures in skeletal muscle (equine; unpublished observations), as previously reported for venom-derived melittin (^^Lin Shiau et al., 1975). Melittin also elicited Ca^{2+} release from Ca^{2+} preloaded terminal cisternae-containing fractions from equine (Figure 11A) and human (Figure 11B) muscle. As observed with CTX, melittin did not induce Ca^{2+} release if the terminal cisternae preparations were insufficiently preloaded with Ca^{2+} (Figure 11B). In some preparations melittin caused a slight, but transient, release of Ca^{2+} at low Ca^{2+} preloads (Figure 11B).

We confirmed that in the absence of CTX the threshold of Ca^{2+}-induced Ca^{2+} release, as determined in the present study, is decreased in equine and human muscle by ryanodine (30 µM), which opens the Ca^{2+} release channel, and this action is antagonized by ruthenium red (10 µM) (unpublished observations), as reported for rabbit muscle by other investigators using similar methodology (Palade, 1987b; Zimanyi and Pessah, 1991). The action of CTX in human preparations was antagonized by ruthenium red (10 µM; Figure 9F) whether determined by the effects on the threshold of Ca^{2+}-induced Ca^{2+} release as in Table 12 (n = 3), or on preloaded preparations as in Figure 9 (n = 2). Melittin-induced Ca^{2+} release from human (n = 1) and equine (n = 3) preparations was antagonized by ruthenium red (Figures 11C and E). A slight transient release of Ca^{2+} by melittin was observed in some preparations even in the presence of ruthenium red (Figure 11C).

Since dantrolene partially antagonized contractures to CTX in rat and human skeletal muscle (Fletcher and Lizzo, 1987), we examined the effects of dantrolene on CTX action in terminal cisternae preparations. Dantrolene (10 µM) did not antagonize Ca^{2+} release by CTX in either equine or human preparations (data not shown).

Like melittin and CTX, myotoxin a also decreases the threshold of Ca^{2+}-

induced Ca^{2+} release (TCICR) in terminal cisternae preparations from skeletal muscle. We are examining the dose-response relationships for the reduction of the TCICR in human and equine skeletal muscle by myotoxin a (Figure 12). Unlike CTX, but rather similar to melittin, myotoxin a is about equipotent on equine and human skeletal muscle (Figure 12). Ca^{2+} release by myotoxin a (10 µM) is blocked by ruthenium red (10 µM; data not shown) and is unaffected by verapamil (1 µM; Table 13). Addition of myotoxin a after preloading the HSRFs results in an immediate release of Ca^{2+} that is blocked by ruthenium red.

Effects of a PSNTX (β-Butx) on the TCICR

We have also examined the effects of β-Butx on Ca^{2+} release from the sarcoplasmic reticulum. In agreement with other investigators (Lau et al., 1974), we have observed that β-Butx causes Ca^{2+} release from terminal cisternae preparations (Figure 13). β-Butx differs greatly from CTX in its mechanism of Ca^{2+} release, as no Ca^{2+} preload is required for the action of β-Butx (Figure 13), as is required for CTX (Figures 9 and 10).

Effects of CTX and Melittin on Ryanodine Binding to the Ca^{2+} Release Channel

Melittin and cardiotoxin were found to have dual effects on the binding of ryanodine to the Ca^{2+} release channel (ryanodine receptor) and these effects were dependent on the preparation of sarcoplasmic reticulum employed (Figure 14). In one case (Figure 14A) melittin at a low concentration (0.01 µM) and CTX at a higher concentration (10 µM) inhibited [^{3}H]ryanodine binding. In this case a terminal cisternae-containing fraction (4,000 - 12,000 x g) was isolated and this fraction did not contain significant amounts of cytosolic protein or light membrane vesicles. A fraction containing these cellular components (Figure 14B) manifests an enhancing action of melittin and CTX on ryanodine binding. However, at the concentrations used to induce Ca^{2+} release (0.1 µM for melittin and 10 µM for CTX) neither toxin had significant effects on ryanodine binding under either condition.

Effects of CTX on Lipid Metabolism in Cell Culture

As described above, we have been successful in eliminating activation of PLA_2 as a primary mechanism of action of the CTXs (Table 2) and the bee venom analogue, melittin (Fletcher et al., 1990b). To better identify the actions of CTX and melittin on endogenous lipolytic enzyme activity the FAs and fatty esters of skeletal muscle cell cultures were radiolabeled with [^{14}C]linoleic acid, as previously described (Fletcher et al., 1990b; 1991a).

About 4% of the [^{14}C]linoleic acid radiolabel is incorporated into the cells three days after adding the FA to the cultures (data not shown). In the absence of toxin, no significant changes occurred in the distribution of radiolabel in the neutral lipids or phospholipids over a 2 h incubation; however, about 1% of the total radiolabel is released from the cells over this period (Table 14). The effects of the *N. n. kaouthia* CTX were examined in primary cultures of equine skeletal muscle from four different biopsy specimens (Table 14). There was very little variation in toxin action between cultures from different biopsy specimens. CTX action produces FFAs and diacylglycerol. The effects of CTX appeared to be completely accounted for by hydrolysis of the phospholipid fraction. There was no obvious preference for hydrolysis of any one particular phospholipid by the CTXs (Table 14). Since phosphatidic acid and cardiolipin were not completely resolved in some of the phospholipid separations, they were integrated as a single peak for all

cultures examined in Table 14.

The effects of melittin were examined in primary cultures of equine skeletal muscle (Table 15). At a low concentration (2 μM) the effects of melittin on lipid metabolism seemed similar to the higher (10 μM) concentration of CTX (Table 15). That is, melittin increased the diacylglyceride and free fatty levels, as observed for CTX. However, at higher concentrations of melittin (10 μM) significant triglyceride breakdown was also evident (Table 15). Despite an increase in FA production by the higher concentration of melittin, the amount of radioactivity released into the medium was lower than that released by the 2 μM concentration (Table 15).

The effects of CTX and melittin were examined in human muscle to determine if substantial species differences exist in regard to the toxin action (Table 16). CTX and melittin had similar actions in human and equine skeletal muscle cultures. While there was no significant difference between CTX (10 μM) and melittin (10 μM) in amount of radioactivity released from human muscle cells to the supernatant ($P>.05$; two-tailed grouped t-test), melittin caused significantly ($P<.001$) greater diacylglyceride and FFA formation than CTX. Melittin (10 μM) was the only toxin causing significant triglyceride breakdown ($P<.0001$). Melittin (2 and 10 μM) and CTX (10 μM) caused significant ($P<.01$) phospholipid hydrolysis, as determined by the relative reduction of radiolabeled phospholipid. CTX (3 μM) caused greater diacylglyceride and FFA formation ($P<.01$) than observed in control cells. CTX at 10 μM produced more diacylglyceride ($P<.01$) and FFA ($P<.001$) than at a 3 μM concentration. Likewise melittin at the higher (10 μM) concentration caused greater diacylglyceride ($P<.0001$) and FFA ($P<.001$) formation than at the lower (2 μM) concentration.

The composition of the radioactivity released to the supernatant by 10 μM concentrations of melittin and CTX was examined in cultures of human skeletal muscle. Although the amount of radioactivity was relatively low, it was clear that the distribution was very similar to that retained by the cells. The bulk of the radiolabel was phospholipid (70-80%). Also released at detectable levels were FFA (5-15%), diacylglyceride (6-8%) and triglyceride (4-5%).

The primary species difference in melittin action was the greater triglyceride breakdown in human muscle (Table 16) than in equine muscle (Table 15) at high melittin concentrations. However, the levels of muscle triglyceride were greater in the human muscle in the absence of toxin. The primary species dependent difference in CTX action was an apparent greater sensitivity of equine muscle (Table 14) to the release of radiolabeled lipid.

Human muscle biopsies of the vastus lateralis were obtained from patients referred for *in vitro* halothane and caffeine contracture testing for malignant hyperthermia (MH) susceptibility by the North American Protocol (Larach, 1989; Allen et al., 1990). We next examined whether cultures from patients diagnosed as MH susceptible (MH+) were suitable for use in the toxin studies, since triglyceride metabolism has been reported as altered in humans and swine with this disorder (Fletcher et al., 1989; 1990c). There were no significant differences between the MH- and MH+ groups ($P>0.05$; two-tailed grouped t-test) in the uptake of radiolabeled linoleic acid into any of the neutral lipids or phospholipids (Table 17) in primary cultures of human skeletal muscle (control condition). In the MH- cultures CTX pretreated with *p*-BPB appeared primarily to activate PLC based on the increase in diacylglycerol and FFA with no significant decrease in triglyceride levels (Table 17). While the decrease in phospholipid was not significant (Table 17) due to the large variation in the

percent of phospholipid labeled in the absence of toxin, there was a decrease in the percentage of radiolabeled phospholipid observed for cells from every MH- subject (15, 5, 3 and 2% decrease). In general agreement with the results obtained with MH- cultures, there was an increase in diacylglycerol and FFAs in MH+ cultures treated with CTX (Table 17), supporting activation of PLC. CTX caused significant, but subtle and likely inconsequential, differences between MH- and MH+ cells in the neutral lipid and phospholipid distribution (Table 17), suggesting that the MH+ cells were suitable for use in these toxin studies.

Using [^{14}C]ethanolamine to radiolabel the phospholipid head groups resulted in greater than 90% of the incorporated radiolabel being associated with phosphatidylethanolamine and no more than 2% being associated with any other phospholipid (data not shown). There was also no detectable radiolabel in the neutral lipid fraction. In the absence of toxin there was no significant change in either the phosphoethanolamine or ethanolamine radiolabel in the aqueous phase of the cell extract over the 2 hr incubation (data not shown for time 0). Phosphoethanolamine, a product of PLC activity, was increased to about the same extent in MH- and MH+ cultures following *p*-BPB-treated CTX or *p*-BPB-treated melittin treatment (Table 18). Ethanolamine, which could either be a direct product of PLD activity or an indirect product of sequential PLC and phosphoethanolamine phosphatase activities, was elevated two- to three-fold more by melittin than by CTX in these MH- and MH+ primary cell cultures (Table 18).

Again using radiolabeled ethanolamine (Table 19), the levels of phosphoethanolamine release induced by CTX and melittin in the absence of pertussis toxin were comparable to those observed in Table 19 with cultures also from an MH- patient. In contrast, levels of ethanolamine release (Table 19) were about one-third to one-fourth the values of the previous study (Table 18). Incubation with pertussis toxin (400 ng/ml) for 24 hrs prior to CTX or melittin addition did not significantly alter the uptake of radiolabeled ethanolamine over that period (data not shown). Pertussis toxin did not antagonize the release of either phosphoethanolamine or ethanolamine induced by CTX or melittin (Table 19). Indeed, pertussis toxin slightly, but significantly ($P<0.05$; grouped two-tailed t-test), increased the amount of phosphoethanolamine and ethanolamine released by melittin, but not CTX (Table 19). There was no effect of pertussis toxin on the percentage of radiolabel released to the incubation medium by CTX or melittin (Table 19). Similar results were obtained in a second identical study (data not shown).

The levels of phosphoethanolamine release induced by CTX and melittin were examined in cells exposed to cholera toxin (1000 ng/ml) in cultures from an MH- patient (Table 20). Incubation with cholera toxin for 24 hrs prior to CTX or melittin addition did not significantly alter the uptake of radiolabeled ethanolamine over that period (data not shown). Cholera toxin did not antagonize the release of either phosphoethanolamine or ethanolamine induced by CTX or melittin (Table 20). Like pertussis toxin, cholera toxin slightly, but significantly ($P<0.05$; grouped two-tailed t-test), increased the amount of phosphoethanolamine released by melittin. However, cholera toxin also increased ($P<0.05$) the amount of phosphoethanolamine released by CTX. The amounts of ethanolamine released by either CTX or melittin were not significantly affected by cholera toxin. There was no effect of cholera toxin on the percentage of radiolabel released to the incubation medium by CTX, but there was a slight, but significant ($P<0.01$), decrease in the melittin-induced

release of radioactivity into the incubation medium (Table 20). Similar results were obtained in a second identical study (data not shown).

The cells used in the cholera toxin study were simultaneously labeled with [^{14}C]linoleic acid to confirm the production of FFAs and diacylglycerol (Table 20). Both diacylglycerol and FFA production were significantly increased by CTX and melittin. Diacylglycerol production by CTX ($P<0.05$) and melittin ($P<0.001$) was significantly increased by cholera toxin. The production of FFAs was not significantly ($P>0.05$) affected by cholera toxin for either CTX or melittin (Table 20). Therefore, the action of cholera toxin on phosphoethanolamine resembled that on diacylglycerol in these cells.

Our more recent studies have demonstrated that under conditions in which PLC activity was increased by melittin and CTX (monitoring release of FFA and diacylglycercl), there was no release of phosphocholine despite extensive labeling of phosphatidylcholine, suggesting that the activated PLC had a substrate specificity for phosphatidylethanolamine and does not readily hydrolyze phosphatidylcholine (data not shown).

Since the effects of melittin and CTX on Ca^{2+} release could be mediated through the production of FFAs, which have effects similar to those of the toxins (Fletcher et al., 1990c), we examined the time course of melittin action. The time course of CTX action has been published (Fletcher et al., 1991b). Two separate experiments with melittin (10 µM) are shown in Figure 15 involving release of radioactivity into the bathing medium. Different levels of total radioactivity released are common in our system, as are different amounts of FFA formation, diacylglycerol production and phosphoethanolamine release. In both studies in primary cultures of human skeletal muscle it was obvious that significant levels of radioactivity are release at early (1 min) time points, consistent with a rapid effect of these toxins on this yet-to-be-defined exocytotic (apparently) process (Figure 15). The release of diacylglycerol and FFA for each of these experiments was also monitored (Figures 16 and 17). In both studies significant levels of at least one of these indicators of PLC activity was elevated within one min, consistent with the rapid action of melittin on Ca^{2+} release. It should be remembered that the effects of melittin on Ca^{2+} release were monitored at a 0.1 µM concentration of melittin. Such low levels of toxin have not been examined with regard to PLC activity activation in cell culture. Higher concentrations of melittin (2 µM) do cause significant activation of PLC, but these effects are difficult to quantitate at such low concentrations of toxin. Further evidence for a rapid effect of melittin on PLC activity is proovided in the second study in which phosphoethanolamine release was also significantly elevated within one min of toxin exposure (Figure 18). Ethanol release [an indicator of phospholipase D (PLD) activity] was not elevated at early time points and was never a major component of toxin action, suggesting that this was not activation of a PLD, but rather resulted from phosphatase activity on phosphoethanolamine. In all, these studies are at least preliminary evidence that activation of PLC by toxins may indirectly lower the TCICR by an action of the products of lipolysis. Certainly, such a process cannot be ruled out, but will be tested more directly in the future.

Since myotoxin a caused Ca^{2+} release (Figure 12), we tested whether this toxin might alsc be acting through activation of PLC activity. In a mouse cell line we observed no evidence for release of radioactivity into the medium by myotoxin a, despite marked liberation of radiolabel by melittin and CTX (Figure 19). In the same culture diacylglycerol levels were increased by

melittin and CTX, while no increase was observed with myotoxin a (Figure 20). In marked contrast, myotoxin a caused a dramatic increase in FFAs (Figure 20), supporting specific activation of PLA_2 and not PLC by the toxin. Using a similar analysis, further support for a specific tissue PLA_2 activation by myotoxin a was obtained in primary cultures of human skeletal muscle (Figure 21). There was no association between activation of PLA_2 activity by myotoxin a and release of radioactivity to the incubation medium (Figures 19 and 21). Previously we had demonstrated that contamination of venom toxin fractions with venom-derived PLA_2 could lead to a false impression that tissue PLA_2 activity had been activated (Fletcher et al., 1991a). We do not believe that the activation of PLA_2 by myotoxin a is due to trace amounts of venom PLA_2 contaminating the toxin fraction, as two other cultures treated with the toxin did not exhibit FFA production, presumedly because the tissue enzyme was not expressed at the time the culture was challenged. If venom PLA_2 contamination were responsible for the FFAs, then FFAs would always be observed on treatment with toxin.

Since many aspects of lipid metabolism have been reported to be Ca^{2+} dependent, it is possible that a direct stimulation of Ca^{2+} release by the toxins could account for their effects on lipid metabolism. However, it is unlikely that melittin and CTX would specifically activate PLC and that myotoxin a would specifically activate PLA_2 activity indirectly through a common action on Ca^{2+} release. In addition, we treated human cell cultures with EDTA (10 mM) and a Ca^{2+}-free medium to eliminate Ca^{2+} influx and added ruthenium red to block Ca^{2+} release by the toxins. Under these conditions, melittin (10 μM) did not induce release of radioactivity into the medium above control levels (Figure 22). However, note that the Ca^{2+}-free conditions promoted release of radiolabel in control preparations. Since diacylglycerol production and FFA release (Figure 23) were unaffected by Ca^{2+}-free medium, this means that release of radiolabel into the medium is independent of PLA_2 and PLC activation and suggests an additional action of melittin. In contrast to the lack of effects of melittin on release of radiolabel into the incubation medium under Ca^{2+}-free conditions, the toxin increases both diacylglycerol and FFA (Figure 23). Therefore, melittin activates PLC activity through a Ca^{2+}-independent mechanism. Attempts to repeat these studies in the mouse cell line failed because the EDTA-containing medium lifted the cells off the wells (not completely unexpected). We will later conduct the same study in Ca^{2+}-free buffer, but without EDTA.

TABLES

TABLE 1. Effects succinylcholine (50 μM) pretreatment on the subsequent action of a synthetic peptide (1 μM) based on *Trimeresurus wagleri* snake venom toxin on the mouse phrenic nerve-diaphragm preparation. Preparations in some cases were exposed to succinylcholine for 2 min, washed, equilibrated for 10 min and then synthetic peptide added. The effects of toxin treatment on indirect (nerve) or direct (muscle) stimulation after 60 min (37°C) were compared to the initial twitch. All values were compared by a one-way ANOVA and Sheffe test.

	n	% of Initial Twitch Height (mean ± SEM) Direct Twitch	Indirect Twitch	Column P-value
No succinylcholine pretreatment	4	83 ± 5	82 ± 6	n.s.
Succinylcholine pretreatment	5	88 ± 3	57 ± 6	.003
Row P-value		n.s.	.023	

TABLE 2. Effects of *Naja naja atra* CTX on lipid metabolism in human primary skeletal muscle cell cultures. Confluent cell cultures were radiolabeled with linoleic acid (3 days). Cultures (35 mm dishes) were then incubated at 37°C for 2 h with or without native or p-BPB treated *Naja naja atra* CTX (10 μM). Values are mean ± SD for 3 determinations.

	Control (2 h)	Native CTX	p-BPB CTX
	Lipid Fraction (% Distribution)		
PL	70 ± 3	17 ± 1	50 ± 2
DG	0.0 ± 0.0	4.0 ± 0.3	4.4 ± 0.7
FFA	0.0 ± 0.0	55 ± 7	14 ± 1
TG	28 ± 3	22 ± 6	30 ± 4
CHE	1.7 ± 0.3	1.8 ± 1.6	1.8 ± 0.8
	Phospholipids (% PL Distribution)		
LPC	0.0 ± 0.0	13	0.0 ± 0.0
SM	2.8 ± 0.7	10	3.1 ± 0.8
PC	68 ± 6	32	61 ± 2
PI	3.5 ± 0.3	8.6	4.4 ± 0.4
PS	4.5 ± 0.7	6.6	5.7 ± 0.2
PE	12 ± 3	11	13 ± 1
PA	2.2 ± 0.2	0.0	2.2 ± 2.0
CL	7.2 ± 2.4	18	10.6 ± 0.6

Abbreviations: PL, phospholipid; DG, diacylglycerol; FFA, free fatty acid; TG, triglyceride; CHE, cholesterol ester; LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; CL, cardiolipin.

TABLE 3. Effects of phospholipase A_2 contamination in the native *N. n. kaouthia* CTX fraction on the phospholipid distribution in a mouse cell line (C_2C_{12}).

	Phospholipids (% distribution; mean ± SEM; n=8)						
	LPC	SM	PC	PI	PS	PE	PA/CL
Control	0.3 ± 0.2	1.1 ± 0.2	38 ± 1	9.7 ± 0.2	9.9 ± 0.5	24 ± 1	16 ± 1
CTX 10 µM	0.9 ± 0.1[b]	1.3 ± 0.2	37 ± 1	10 ± 0.2[a]	8.9 ± 0.3	21 ± 1[c]	20 ± 0[d]

The radiolabeled cells were incubated in 35 mm petri dishes with or without toxin for 2 hrs at 37°C before extraction of the cell-associated lipids. PA and CL were not always clearly separated by TLC and were thus integrated as a single peak of radioactivity.
Abbreviations: see Table 2.
[a]P<.02; [b]P<.01; [c]P<.005; [d]P<.0001

TABLE 4. Analysis of free fatty acids released into the incubation medium (supernatant) by a 10 nM concentration of various PLA_2s in the absence of BSA.

	Free Fatty Acids (nmol/mg prot.; mean ± SEM)							
	16:0	16:1	18:0	18:1	18:2	20:4	22:6	Total
Control	0.3±0.0 $1^{c}5^{a}$	n.d.	0.2±0.1	0.1±0.0 1^{a}	n.d.	0.1±0.0	0.4±0.3	1.2±0.2
N.n.a. PLA_2	0.4±0.1 1^{b}	n.d.	0.2±0.1	0.3±0.1	n.d.	0.2±0.1	0.6±0.2	2.0±0.3
β-Butx	0.8±0.0 $1^{b}2^{b}3^{c}4^{a}$	n.d.	0.3±0.0	0.4±0.0 1^{a}	n.d.	0.2±0.0	0.5±0.1	2.3±0.2
Taipoxin	0.6±0.0 5^{a}	n.d.	0.2±0.1	0.3±0.0	n.d.	0.1±0.0	0.1±0.0	1.5±0.1
Crotoxin	0.4±0.0 2^{b}	n.d.	0.1±0.1	0.3±0.0	n.d.	0.0±0.0 1^{a}	0.3±0.2	1.3±0.3
Scutoxin	0.5±0.0 4^{a}	n.d.	0.3±0.0	0.3±0.0	n.d.	0.3±0.0 1^{a}	0.7±0.2	2.1±0.2

A one-way ANOVA was conducted for the control and all toxins for each fatty acid. Conditions with the same number differed (Sheffe test) by: [a]P<0.05; [b]P<0.01; [c]P<0.001; [d]P<0.0001.
n.d. = not detectable. Abbreviations: β-Butx, β-bungarotoxin; *N.n.a.*, *Naja naja atra.*

TABLE 5. Analysis of free fatty acids maintained in synaptosomal membranes (pellet) during exposure to a 10 nM concentration of various various PLA_2s in the absence of BSA.

	Free Fatty Acids							
	16:0	16:1	18:0	18:1	18:2	20:4	22:6	Total
	(nmol/mg prot.; mean ± SEM)							
Control	2.3±0.1 $^{3d}5^{c}$	0.1±0.0 1^{b}	2.2±0.1 $1^{a}2^{a}3^{b}4^{a}$	1.3±0.1 $1^{d}4^{d}5^{b}7^{b}9^{d}$	0.1±0.0 $1^{d}2^{d}4^{b}5^{a}6^{d}$	1.3±0.2 $3^{d}5^{b}7^{a}9^{a}10^{d}$	0.8±0.2 $2^{d}5^{d}7^{c}9^{d}10^{b}$	8.3±0.6
	$3^{d}7^{d}8^{b}10^{b}12^{d}$							
N.n.a. PLA_2	6.0±0.3 1^{c}	0.4±0.0	2.7±0.0 1^{a}	7.7±0.7 1^{d}	0.4±0.0 1^{d}	11.6±1.3 $1^{a}2^{b}3^{d}4^{b}$	24.0±1.9 $1^{a}2^{d}3^{b}4^{b}$	53±4 $1^{a}2^{a}3^{d}4^{a}$
β-Butx	15±2 $1^{c}2^{d}3^{d}4^{d}$	0.6±0.1 1^{b}	2.8±0.2 2^{a}	9.9±1.3 $2^{b}3^{b}4^{d}$	0.4±0.1 $2^{d}3^{a}$	7.3±0.8 $1^{a}5^{b}6^{c}$	20.8±1.9 $5^{d}6^{c}$	57±6 $5^{a}6^{b}7^{d}$
Taipoxin	5.5±0.5 4^{d}	0.3±0.0	2.7±0.0 4^{a}	5.2±0.3 $3^{b}7^{b}8^{b}$	0.2±0.0 $3^{a}5^{a}7^{a}$	6.1±0.2 $4^{b}9^{a}11^{d}$	12.7±1.2 $4^{b}10^{b}11^{d}$	33±2 $2^{a}6^{b}10^{b}11^{d}$
Crotoxin	5.7±0.8 2^{d}	0.2±0.1	2.8±0.0 3^{b}	5.5±0.3 $2^{b}5^{b}6^{a}$	0.3±0.0 4^{b}	5.8±0.6 $2^{b}7^{a}8^{d}$	14.4±2.2 $1^{a}7^{c}8^{d}$	35±4 $1^{a}5^{a}8^{b}9^{d}$
Scutoxin	10±1 5^{c}	0.4±0.1	2.7±0.1 5^{a}	9.2±0.2 $6^{a}8^{b}9^{d}$	0.4±0.0 $6^{d}7^{a}$	14.7±1.0 $6^{c}8^{d}10^{d}11^{d}$	36.5±0.5 $3^{b}6^{c}8^{d}9^{d}11^{d}$	74±1 $4^{a}9^{d}11^{d}12^{d}$

A one-way ANOVA was conducted for the control and all toxins for each fatty acid separately for either BSA absent or present. Conditions with the same number differed (Sheffe test) by: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; $^{d}P<0.0001$.
n.d. = not detectable. Abbreviations: see Table 4.

TABLE 6. Analysis of free fatty acids released into the incubation medium (supernatant) by a 10 nM concentration of various PLA_2s in the presence of BSA.

	Free Fatty Acids							
	16:0	16:1	18:0	18:1	18:2	20:4	22:6	Total
	(nmol/mg prot.; mean ± SEM)							
Control	2.1±0.2 1^{b}	0.0±0.0 $3^{d}5^{b}6^{c}$	1.6±0.2	1.5±0.2 $1^{b}2^{b}$	0.1±0.0 $1^{b}2^{b}3^{b}$	1.6±0.2 1^{a}	0.5±0.0 $1^{b}2^{d}3^{b}4^{b}5^{d}$	7.9±0.6 $1^{b}2^{a}3^{b}$
N.n.a. PLA_2	3.2±0.5 2^{a}	0.2±0.0 1^{b}	1.5±0.2	3.6±0.4	0.2±0.0	6.8±1.0	10.2±0.7 1^{b}	26±3
β-Butx	8.6±1.2 $1^{b}2^{a}$	0.5±0.0 $1^{b}2^{a}3^{d}4^{a}$	1.4±0.3	6.9±0.8 1^{b}	0.3±0.0 1^{b}	6.8±1.0	15.3±1.2 2^{d}	40±5 1^{b}
Taipoxin	5.0±1.0	0.3±0.0 $4^{a}5^{b}$	1.4±0.2	4.9±0.7	0.3±0.0 2^{b}	6.9±1.3	11.4±1.4 4^{b}	31±5 2^{a}
Crotoxin	5.1±0.8	0.3±0.0 2^{a}	1.6±0.2	4.7±0.5	0.2±0.0	5.7±0.8	11.5±0.7 3^{b}	29±3
Scutoxin	5.9±1.1	0.4±0.1 6^{c}	1.3±0.2	6.3±1.1 2^{b}	0.3±0.0 3^{b}	8.0±1.6 1^{a}	16.6±2.4 5^{d}	39±6 3^{b}

A one-way ANOVA was conducted for the control and all toxins for each fatty acid. Conditions with the same number differed (Sheffe test) by: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; $^{d}P<0.0001$.

TABLE 7. Analysis of free fatty acids maintained in synaptosomal membranes (pellet) during exposure to a 10 nM concentration of various PLA_2s in the presence of BSA.

	Free Fatty Acids							
	16:0	16:1	18:0	18:1	18:2	20:4	22:6	Total
	(nmol/mg prot.; mean ± SEM)							
Control	1.7±0.4 $1^d 2^{\bar{d}}$	0.0±0.0 $5^d 7^a 9^b 10^d$	2.1±0.6	0.9±0.3 $5^d 7^a 9^b 10^d$	0.1±0.0	0.5±0.1 $1^a 3^{\bar{a}} 6^b 8^d$	0.4±0.1 $2^d 5^{\bar{d}} 8^d 10^d$	5.9±1.6 $1^a 2^{\bar{d}} 3^d 4^c 5^a$
N.n.a. PLA_2	2.5±0.2 $3^d 4^{\bar{d}}$	0.0±0.0 $1^d 2^a 3^d$	1.8±0.1	2.2±0.1 $1^d 2^{\bar{d}}$	0.2±0.0	2.7±0.5 $1^a 2^{\bar{d}}$	7.0±0.7 $1^d 2^{\bar{d}}$	17±2 $1^a 6^{\bar{d}} 7^d$
β-Butx	12±1 $1^d 3^{\bar{d}} 5^d 6^d$	0.2±0.0 $1^d 4^{\bar{d}} 5^d 6^d$	3.3±0.2	5.6±0.2 $1^d 3^{\bar{d}} 4^c 5^d$	0.3±0.0	2.7±0.2 $3^a 4^{\bar{d}}$	9.0±0.3 $3^c 4^d 5^d$	33±1 $3^d 7^d 8^{\bar{c}} 11^b 12^d$
Taipoxin	4.9±0.6 $6^d 7^{\bar{c}}$	0.1±0.0 $2^a 6^{\bar{d}} 9^b 11^d$	2.2±0.2	3.1±0.3 $4^c 8^{\bar{d}} 9^b$	0.3±0.0	3.2±0.4 $6^b 7^{\bar{c}}$	8.4±0.6 $6^b 9^{\bar{d}} 10^d$	22±2 $4^c 9^{\bar{d}} 11^b$
Crotoxin	4.1±0.4 $5^d 8^{\bar{d}}$	0.1±0.0 $4^d 7^a 8^d$	2.3±0.2	2.5±0.2 $3^d 6^{\bar{d}} 7^a$	0.2±0.0	1.7±0.2 $5^{\bar{d}}$	5.1±0.1 $3^c 6^{\bar{b}} 7^d 8^d$	16±1 $5^a 10^{\bar{d}} 12^d$
Scutoxin	11±1 $2^d 4^{\bar{d}} 7^c 8^d$	0.2±0.0 $3^d 8^{\bar{d}} 10^d 11^d$	3.3±0.3	6.6±0.4 $2^d 6^{\bar{d}} 8^d 10^d$	0.3±0.0	6.7±0.6 $2^d 4^{\bar{d}} 5^d 7^c 8^d$	21.6±0.3 $1^d 4^{\bar{d}} 7^d 9^d 11^d$	50±2 $2^d 6^d 8^c 9^d 10^d$

A one-way ANOVA was conducted for the control and all toxins for each fatty acid separately for either BSA absent or present. Conditions with the same number differed (Sheffe test) by: $^aP<0.05$; $^bP<0.01$; $^cP<0.001$; $^dP<0.0001$.
n.d. = not detectable. Abbreviations: see Table 4.

TABLE 8. Analysis of free fatty acids released into the incubation medium (supernatant) by a 10 nM concentration of β-Butx or *Naja naja atra* PLA_2 in the absence of BSA.

	Free Fatty Acids				
	16:0	18:1	20:4	22:6	Total
	(nmol/mg prot.; mean ± SEM)				
Control	1.7 ± 0.6	0.6 ± 0.2	0.1 ± 0.1	0.3 ± 0.3	4.2 ± 0.9
β-Butx	1.1 ± 0.3	0.6 ± 0.2	0.2 ± 0.1	0.5 ± 0.3	3.5 ± 1.0
N.n.a. PLA_2	2.0 ± 0.4	1.1 ± 0.1	1.0 ± 0.0	1.2 ± 0.0	7.3 ± 0.8
	n.s.	n.s.	CA^bBA^b	n.s.	n.s.

Each fatty acid analyzed by one-way ANOVA and Sheffe test ($^aP<0.05$; $^bP<0.01$; $^cP<0.001$; $^dP<0.0001$).
Abbreviations: C, control; B, β-Butx; A, *Naja naja atra* PLA_2.

TABLE 9. Analysis of free fatty acids retained in the synaptosomal pellet on treatment with a 10 nM concentration of β-Butx or *Naja naja atra* PLA_2 in the absence of BSA.

	Free Fatty Acids				
	16:0	18:1	20:4	22:6	Total
	(nmol/mg prot.; mean ± SEM)				
Control	3.9 ± 0.1	2.2 ± 0.1	2.5 ± 0.1	1.2 ± 0.1	14 ± 0
β-Butx	24.8 ± 0.9	15.3 ± 0.6	10.3 ± 0.4	27.0 ± 1.2	84 ± 3
N.n.a. PLA_2	14.1 ± 1.9	13.5 ± 1.4	22.3 ± 2.2	38.9 ± 4.6	98 ± 18
	$CB^dCA^{\bar{b}}AB^b$	$CB^dCA^{\bar{c}}$	$CB^aCA^{\bar{d}}BA^b$	$CB^bCA^{\bar{c}}$	$CB^{\bar{c}}CA^c$

TABLE 10. Analysis of free fatty acids released into the incubation medium (supernatant) by a 10 nM concentration of β-Butx or *Naja naja atra* PLA_2 in the presence of BSA.

	Free Fatty Acids				
	16:0	18:1	20:4	22:6	Total
	(nmol/mg prot.; mean ± SEM)				
Control	4.4 ± 0.1	2.7 ± 0.1	3.0 ± 0.0	1.2 ± 0.0	15 ± 0
β-Butx	12.2 ± 0.6	13.6 ± 1.2	14.2 ± 1.8	33.8 ± 4.9	79 ± 7
N.n.a. PLA_2	13.3 ± 1.7	8.4 ± 0.3	14.9 ± 0.6	20.8 ± 1.1	66 ± 3
	CB^bCA^b	$CB^dCA^bBA^b$	CB^bCA^c	CB^cCA^b	CB^cCA^c

Each fatty acid analyzed by one-way ANOVA and Sheffe test ($^aP<0.05$; $^bP<0.01$; $^cP<0.001$; $^dP<0.0001$).

TABLE 11. Analysis of free fatty acids retained in the synaptosomal pellet on treatment with a 10 nM concentration of β-Butx or *Naja naja atra* PLA_2 in the presence of BSA.

	Free Fatty Acids				
	16:0	18:1	20:4	22:6	Total
	(nmol/mg prot.; mean ± SEM)				
Control	0.8 ± 0.2	2.1 ± 0.9	1.0 ± 0.3	1.6 ± 0.7	9 ± 2
β-Butx	19.5 ± 1.6	9.3 ± 0.9	3.7 ± 0.4	13.3 ± 1.7	53 ± 5
N.n.a. PLA_2	4.2 ± 0.3	2.9 ± 0.0	4.0 ± 0.1	8.8 ± 0.3	24 ± 0
	CB^dBA^d	CB^bBA^b	CB^bCA^b	CB^cCA^b	$CB^cCA^aBA^b$

Each fatty acid analyzed by one-way ANOVA and Sheffe test ($^aP<0.05$; $^bP<0.01$; $^cP<0.001$; $^dP<0.0001$).

TABLE 12. Effects of *Naja naja kaouthia* CTX (10 μM) and bee venom melittin (0.1 μM) on the threshold of Ca^{2+}-induced Ca^{2+} release in terminal cisternae preparations from equine and human muscle.

	Threshold of Ca^{2+}-induced Ca^{2+} release (% control)			
Species	CTX		Melittin	
	mean ± SEM (n)	range	mean ± SEM (n)	range
Equine	101 ± 4 (8)	88-124	80 ± 7 (6)	59-100
Human	73 ± 8 (6)	42-100	64 ± 10 (6)	33-87
Porcine	65 ± 7 (4)		n.d.	

Note. CTX or melittin were added before any Ca^{2+} pulses. The threshold of Ca^{2+}-induced Ca^{2+} release in the presence of toxin is expressed a a percentage of that in the absence of toxin (control) for aliquots from the same terminal cisternae-containing suspension.

TABLE 13. Effects of verapamil (1 μM) on myotoxin a (10 μM)-induced reduction of the threshold of Ca^{2+}-induced Ca^{2+} release (TCICR). Mean ± SEM for three determinations.

	TCICR (% Control)	
Myotoxin a alone	56 ± 4	
Myotoxin a plus verapamil	61 ± 11	P=0.65

TABLE 14. Effects of *Naja naja kaouthia* venom CTX on lipid metabolism in primary cultures of equine skeletal muscle. Values are Mean ± SEM (n=4 biopsy specimens from separate animals). Identical superscripts in the same row differ from each other, as determined by a two-tailed paired t-test ($^{a}P<.05$; $^{b}P<.01$; $^{c}P<.001$; $^{d}P<.0001$). If more than one comparison differed by the same level of significance, then a superscripted number was also used to further identify the two different values.

	Control (0 h)	Control (2 h)	CTX (10 μM; 2 h)
	Radioactivity (CPM)		
Released to Supernatant	95 ± 9^{c}	626 ± 35^{b,c}	9,453 ± 1,256^{b}
Cell Associated	45,393 ± 1,703^{b}	43,515 ± 3,951	34,593 ± 4,913^{b}
	Lipid Fraction (% Distribution)		
PL	90 ± 0.4^{d1}	90 ± 1.0^{d2}	83 ± 2.3^{d1,d2}
DG	1.7 ± 0.2^{d1}	1.3 ± 0.1^{d2}	4.0 ± 0.2^{d1,d2}
FFA	0.2 ± 0.1^{d2}	0.2 ± 0.1^{d2}	3.1 ± 0.6^{d1,d2}
TG	7.5 ± 0.5	8.7 ± 1.0	9.0 ± 2.6
CHE	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.2
	Phospholipid Fraction (% PL Distribution)		
LPC	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
SM	0.9 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
PC	60 ± 2	58 ± 2	59 ± 2
PI	6.8 ± 0.4	6.4 ± 0.4	6.2 ± 0.3
PS	5.0 ± 0.7	6.1 ± 0.7	5.8 ± 0.7
PE	17 ± 1	18 ± 1	16 ± 1
PA + CL	11 ± 2	11 ± 1	12 ± 1

Abbreviations: See Table 2.

TABLE 15. Effects of bee venom melittin on lipid metabolism in primary cultures of equine skeletal muscle. All preparations were incubated for 2 h with or without toxin. Values are Mean ± SD (n = 6 determinations). Identical superscripts in the same row differ from each other, as determined by grouped two-tailed t-test ($^{a}P<.05$; $^{b}P<.01$; $^{c}P<.001$; $^{d}P<.0001$). If more than one comparison differed by the same level of significance, then a superscripted number was also used to further identify the two different values.

	Control (2 h)	Melittin (2 μM; 2 h)	Melittin (10 μM; 2 h)
Released to		Radioactivity (CPM)	
Supernatant	592 ± 50^{d1,d2}	7,434 ± 2,271^{a,d1}	4,738 ± 1,543^{a,d2}
Cell Associated	41,102 ± 3,565^{a1}	30,261 ± 10,902^{a1,a2}	42,036 ± 4,410^{a2}
		Lipid Fraction (% Distribution)	
PL	91 ± 1^{c,d}	85 ± 3^{c}	82 ± 2^{d}
DG	1.6 ± 0.3^{d1,d2}	4.8 ± 0.3^{b,d1}	6.2 ± 0.9^{b,d2}
FFA	0.1 ± 0.2^{d1,d2}	2.7 ± 0.8^{d1,d3}	7.3 ± 0.6^{d2,d3}
TG	6.9 ± 1.4^{b1}	7.6 ± 2.3^{b2}	4.3 ± 0.9^{b1,b2}
CHE	0.3 ± 0.3	0.2 ± 0.3	0.5 ± 0.2
		Phospholipid Fraction (% PL Distribution)	
LPC	0.1 ± 0.2	0.1 ± 0.2	0.3 ± 0.3
SM	1.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.2
PC	62 ± 5^{d}	56 ± 4^{d}	57 ± 6
PI	6.5 ± 0.9	7.3 ± 0.9	7.3 ± 0.8
PS	5.3 ± 0.5	5.3 ± 0.2	5.2 ± 0.3
PE	16 ± 3	19 ± 3	20 ± 5
PA + CL	8.3 ± 2.7	11 ± 4	9.4 ± 1.8

Abbreviations: See Table 2.

ABLE 16. Effects of CTX and bee venom melittin on lipid metabolism in primary cultures of human skeletal muscle. alues are Mean ± SD (n = 3 determinations). See Results for statistical analysis.

	Control (0 h)	Control (2 h)	CTX (3 μM; 2 h)	CTX (10 μM; 2 h)	Melittin (2 μM; 2 h)	Melittin (10 μM; 2 h)
			Radioactivity (CPM)			
eleased to upernatant	89 ± 28	433 ± 225	951 ± 232	2,278 ± 367	2,128 ± 549	2,088 ± 177
ell Associated	31,838 ± 1,042	23,303 ± 6,595	24,888 ± 4,412	19,710 ± 636	24,766 ± 8,228	28,756 ± 2,961
			Fraction Released			
eleased/Total	0.3 ± 0.1	2.1 ± 1.7	3.8 ± 0.5	7.4 ± 0.7	8.0 ± 1.3	8.8 ± 1.2
			Lipid Fraction (% Distribution)			
L	-	80 ± 1	79 ± 3	74 ± 2	78 ± 1	72 ± 3
G	-	0.8 ± 0.1	1.9 ± 0.4	3.5 ± 0.3	2.9 ± 0.1	6.9 ± 0.4
FA	-	0.5 ± 0.0	1.3 ± 0.2	5.8 ± 0.8	3.2 ± 0.4	15 ± 2
3	-	18 ± 0.3	17 ± 2.7	16 ± 1.0	16 ± 1.2	5.9 ± 0.9
IE	-	0.0 ± 0.0	1.3 ± 0.6	0.1 ± 0.3	1.7 ± 0.1	0.7 ± 0.1
			Phospholipid Fraction (% PL Distribution)			
C	-	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	0.1 ± 0.2	0.6 ± 0.1
4	-	1.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
:	-	67 ± 1	64 ± 3	67 ± 1	63 ± 1	65 ± 1
[	-	5.7 ± 0.3	5.2 ± 0.2	5.6 ± 0.4	5.6 ± 0.3	6.2 ± 1.2
]	-	4.4 ± 0.1	5.4 ± 0.5	5.2 ± 0.3	5.5 ± 0.5	5.3 ± 0.7
:	-	8.8 ± 0.9	9.8 ± 1.8	9.1 ± 0.2	11 ± 1.4	11 ± 1.2
\	-	2.5 ± 0.6	2.0 ± 0.6	1.8 ± 0.1	2.1 ± 0.2	2.0 ± 0.3
.	-	9.9 ± 0.9	12 ± 4.1	8.8 ± 0.3	12 ± 0.4	8.9 ± 0.9

bbreviations: See Table 2.

TABLE 17. Effects of p-BPB-treated *N. n. kaouthia* CTX on lipid metabolism in normal and malignant hyperthermia (MH) susceptible patients.

		Neutral Lipids (% Distribution; mean ± SEM)				
	n	PL	DG	FFA	TG	CHE
Control						
No toxin	4	80±2	0.05±0.05	0.18±0.12	18±2	2.1±0.6
p-BPB toxin	4	74±2	3.6±0.3[c]	6.1±1.1[b]	16±2	1.1±0.6[c]
MH susceptible						
No toxin	4	77±3	0.40±0.26	0.20±0.12	20±3	1.8±0.4
p-BPB toxin	4	74±3	3.4±0.3[c]	4.7±0.8[b]	17±2[a]	1.4±0.4

Naja naja kaouthia cardiotoxin (10 μM), 2 hr incubation at 37°C.
[a]P<.05; [b]<P<.01; [c]P<.005 by two-tailed paired t-test (no toxin vs. toxin).

	Phospholipids (% of Phospholipid Labeled; mean ± SEM)						
(n = 4)	LPC	SM	PC	PI	PS	PE	PA/CL
Control							
No toxin	0.0±0.0	2.6±0.1	64±2	4.0±0.2	4.6±0.4	15±2	11±1
p-BPB toxin	0.0±0.0	2.8±0.4	62±2	3.3±0.4	4.3±0.5	15±2	13±1[a]
MH susceptible							
No toxin	0.3±0.2	2.5±0.1	62±2	3.7±0.2	4.6±0.4	17±2	11±1
p-BPB toxin	0.2±0.2	2.6±0.2	60±2	3.8±0.6	5.6±0.4[c]	15±2[b]	12±1

Naja naja kaouthia cardiotoxin (10 μM), 2 hr incubation at 37°C.
[a]P<.05; [b]<P<.01; [c]P<.005 by two-tailed paired t-test (no toxin vs. toxin).

TABLE 18. Effects of *Naja naja kaouthia* CTX (10 μM) and melittin (10 μM) on phospholipid metabolism in primary cell cultures of human skeletal muscle

	Phosphoethanolamine (Counts per 20 min)	Ethanolamine (Counts per 20 min)
MH-		
Control	1542 ± 235	466 ± 23
CTX	3244 ± 627[a]	647 ± 67[a]
Melittin	3038 ± 133[b]	1955 ± 316[b]
MH+		
Control	3130 ± 369	636 ± 117
CTX	5726 ± 288[b]	1242 ± 47[b]
Melittin	8769 ± 195[c]	2762 ± 823[a]

Note. Cultures from one MH- or one MH+ patient were prelabeled with [^{14}C]ethanolamine were then incubated in the absence or presence of CTX or melittin for 2 hrs at 37°C and the radiolabel associated with phosphoethanolamine or ethanolamine in the aqueous phase of the cell extracts determined. The values are the mean ± SE for three determinations.
[a]P<0.05; [b]P<0.01; [c]P<0.001 greater than control by grouped two-tailed t-test.

TABLE 19. Effects of pretreatment for 24 hrs with pertussis toxin (400 ng/ml) on the subsequent actions of *Naja naja kaouthia* CTX (10 μM) and melittin (10 μM) in primary cell cultures of human skeletal muscle

	Phosphoethanolamine (Counts per 20 min)	Ethanolamine (Counts per 20 min)	Radioactivity Released (% of cell-associated)
No Pertussis Toxin			
Control	953 ± 92	70 ± 6	2.9 ± 0.4
CTX	2784 ± 414[a]	218 ± 14[b]	17 ± 2[b]
Melittin	4262 ± 307[b]	433 ± 19[c]	36 ± 3[c]
Pertussis Toxin			
Control	1157 ± 39	92 ± 6	3.2 ± 0.1
CTX	3505 ± 401[b]	279 ± 18[c]	20 ± 1[c]
Melittin	5459 ± 201[c]	547 ± 22[c]	36 ± 3[c]

Note. Cultures from one MH- patient were prelabeled with [^{14}C]ethanolamine and incubated with or without pertussis toxin for the last 24 hrs of radiolabeling. The cells were then incubated in the absence or presence of CTX or melittin for 2 hrs at 37°C and the radiolabel associated with phosphoethanolamine or ethanolamine in the aqueous phase of the cell-associated extracts determined. The values are the mean ± SE for three determinations. The percentage of radioactivity released is relative to the total incorporated radiolabel (released plus cell-associated).
[a]$P<0.05$ greater than control by grouped two-tailed t-test.
[b]$P<0.01$ greater than control by grouped two-tailed t-test.
[c]$P<0.001$ greater than control by grouped two-tailed t-test.

TABLE 20. Effects of pretreatment for 24 hrs with cholera toxin (1000 ng/ml) on the subsequent actions of *Naja naja kaouthia* CTX (10 μM) and melittin (10 μM) in primary cell cultures of human skeletal muscle

	Phosphoethanolamine (Counts per 20 min)	Ethanolamine (Counts per 20 min)	Radioactivity Released (% of cell-associated)
No Cholera Toxin			
Control	584 ± 102	167 ± 46	3.0 ± 0.3
CTX	1551 ± 76[b]	954 ± 245[a]	26 ± 4[b]
Melittin	2102 ± 190[b]	1099 ± 292[a]	37 ± 1[c]
Cholera Toxin			
Control	857 ± 64	288 ± 54	3.2 ± 0.1
CTX	3207 ± 543[a]	723 ± 84[a]	33 ± 1[c]
Melittin	3667 ± 274[c]	509 ± 23[a]	32 ± 0[c]

	DG (% of Total Radiolabel)	FFA (% of Total Radiolabel)
No Cholera Toxin		
Control	0.0 ± 0.0	0.0 ± 0.0
CTX	0.7 ± 0.2[b]	1.1 ± 0.3[a]
Melittin	1.4 ± 0.1[c]	2.9 ± 0.3[c]
Cholera Toxin		
Control	0.2 ± 0.0	0.2 ± 0.1
CTX	1.8 ± 0.2[c]	1.9 ± 0.2[b]
Melittin	2.6 ± 0.1[c]	2.3 ± 0.1[c]

TABLE 20 (cont.)

Note. Cultures from one MH- patient were prelabeled with [^{14}C]ethanolamine and [^{14}C]linoleic acid and ware then incubated with or without cholera toxin for the last 24 hrs of radiolabeling. The cells were then incubated in the absence or presence of CTX or melittin for 2 hrs at 37°C and the radiolabel associated with phosphoethanolamine or ethanolamine in the aqueous phase of the cell-associated extracts determined and the radiolabel associated with DG and FFA in the organic phase determined. The values are the mean $\pm$ SE for three determinations. The percentage of radioactivity released is relative to the total incorporated radiolabel (released plus cell-associated).

Abbreviations: see Table 18.

[a]$P<0.05$ greater than control by grouped two-tailed t-test.

[b]$P<0.01$ greater than control by grouped two-tailed t-test.

[c]$P<0.001$ greater than control by grouped two-tailed t-test.

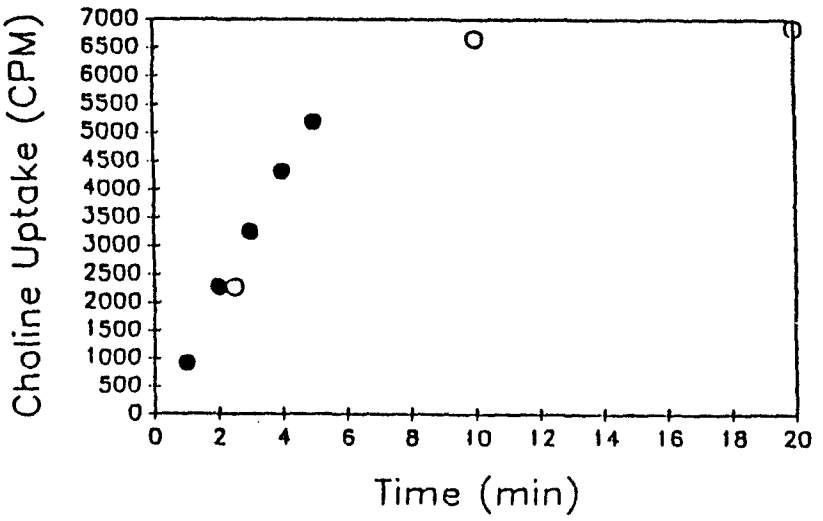


FIGURE 1. Time course of [^{14}C]choline uptake. Synaptosomes were incubated at 37°C for the indicated times and the uptake of choline determined by filtration and liquid scintillation counting of the radioactivity retained on the filters. Two separate experiments are shown (filled and open circles).

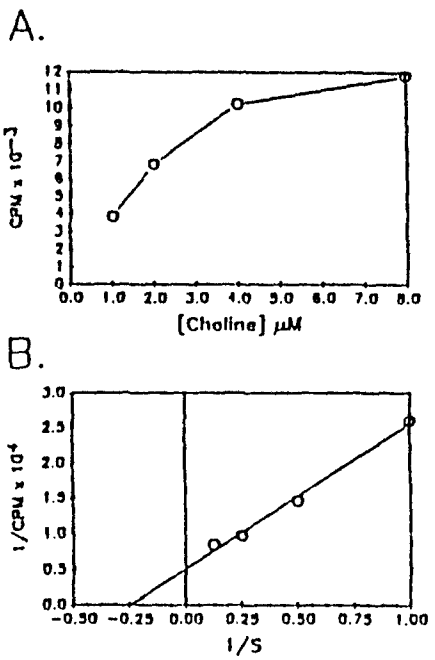


FIGURE 2. Lineweaver-Burke analysis of [^{14}C]choline uptake. Choline uptake was determined in the presence of various concentrations of radiolabeled choline. The CPM uptake is shown in Panel A and the Lineweaver-Burke plot is shown in Panel B.

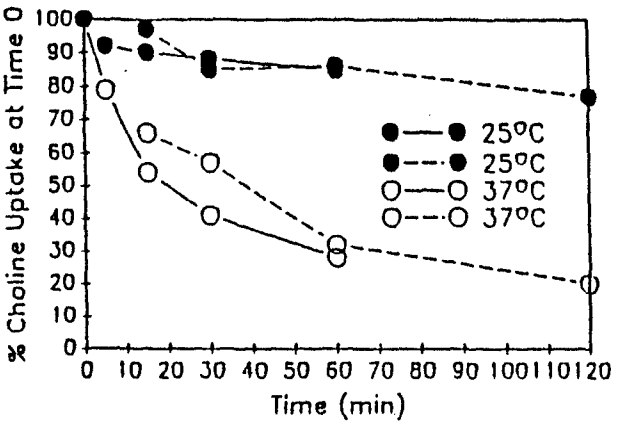


FIGURE 3. Stability of choline uptake process at 25 and 37°C. Synaptosomes were incubated for the indicated time at either 25 or 37°C and choline uptake determined (4 min) at 37°C. Each symbol is the average of duplicate determinations. Two separate experiments were run at each temperature.

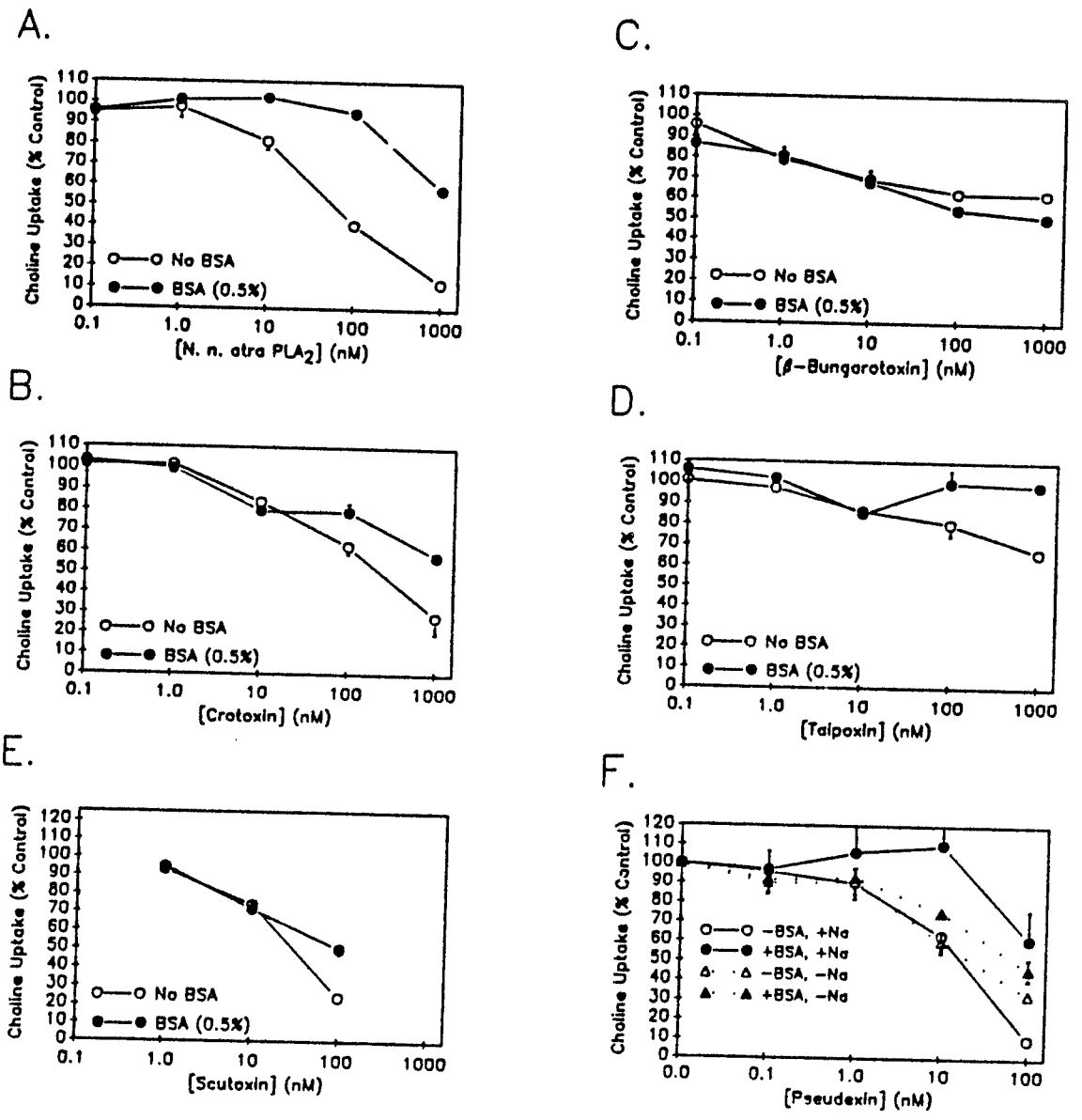


FIGURE 4. Effects of BSA on the inhibition of choline uptake into mouse brain synaptosomes. Choline uptake, expressed as percent of control, was examined after exposure to *Naja naja atra* PLA_2 (Panel A), crotoxin (Panel B), β-Butx (Panel C), taipoxin (Panel D) and scutoxin (Panel E) for 60 min at 25°C. The bars indicate the SEM for 6 to 10 determinations. In Panel F (pseudexin) choline uptake was determined in the presence (circles) or absence (triangles) of Na^+. BSA 0.5% was either absent (open symbols) or present (filled symbols) in the incubation medium.

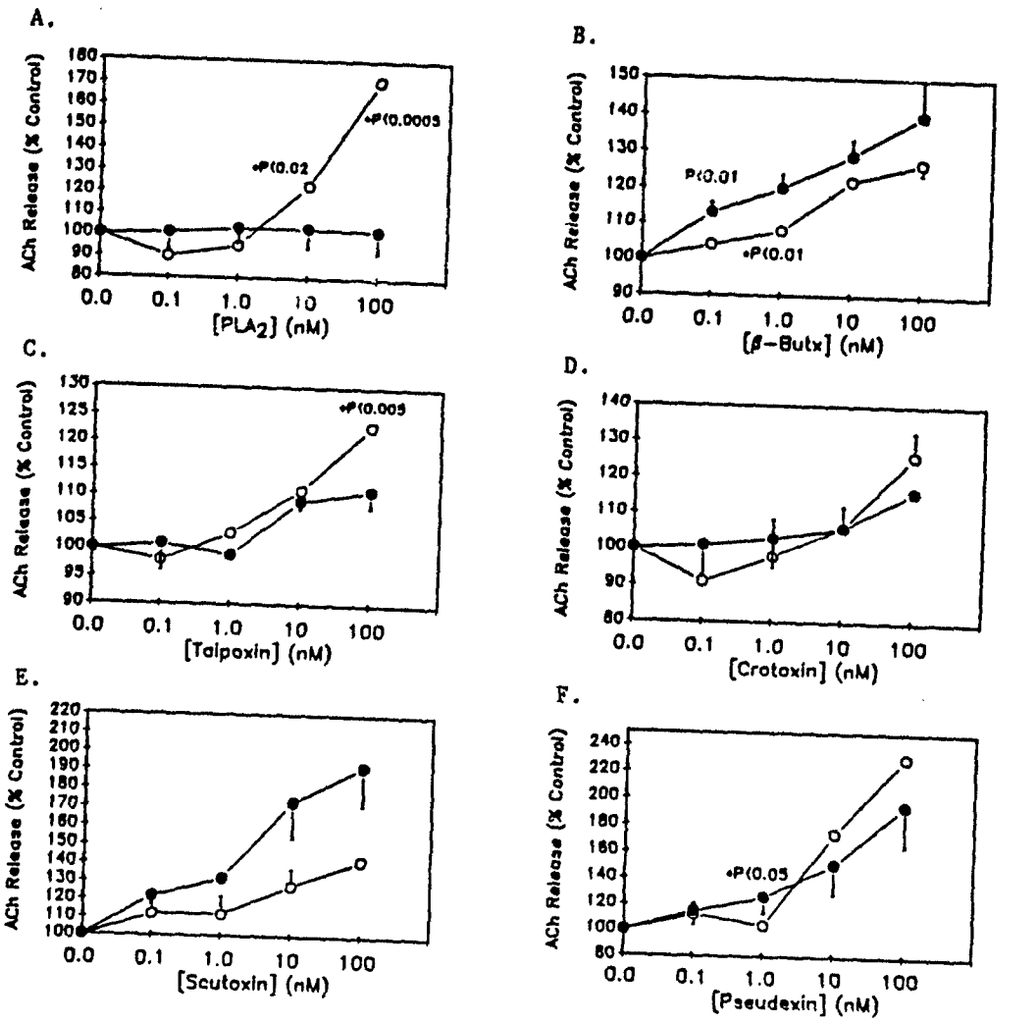
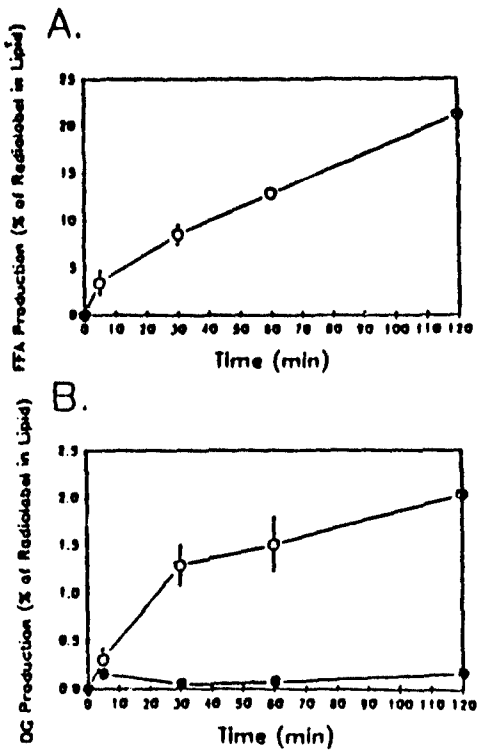


FIGURE 5. Effects of six toxins on acetylcholine release from mouse brain synaptosomes. Synaptosomes were preloaded with [^{14}C]choline (2 μM; 25°C; 30 min) and then incubated (± toxin) in the absence (open symbols) or presence (filled symbols) of BSA (0.5%) for 30 min at 37°C. The synaptosomal suspensions were centrifuged and the radioactivity in the supernatant associated with ACh selectively extracted and quantitated by liquid scintillation counting. Each data point is comprised of three determinations and includes the SD bar.



IRE 6. The effects of *N. n. kaouthia* CTX
'ree fatty acid and diacylglycerol
luction in the C_2C_{12} mouse muscle
ılast. The production of (A) free fatty
ls and (B) diacylglycerol was determined
n Fig. 4. The open circles are native CTX
6) and the filled circles are p-BPB-
ted fractions (n = 3). Standard error of
means is indicated when it exceeds the
of the symbol. There was no detectable
fatty acid production at any time point
p-BPB-treated CTX.

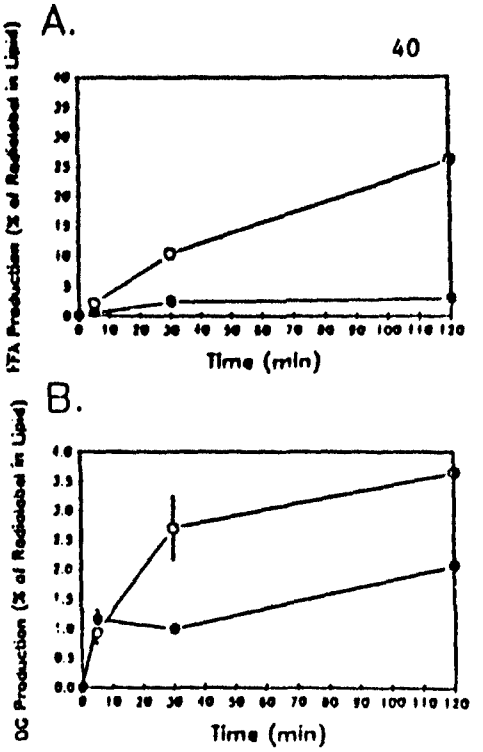


FIGURE 7. The effects of *N. n. kaouthia* CTX on free fatty acid and diacylglycerol production in primary cultures of human skeletal muscle. The production of (A) free fatty acids and (B) diacylglycerol was determined by extraction of the lipids contained within the cells and subsequent TLC and quantitation of radioactivity in each neutral lipid peak. The open circles are native CTX (n = 3) and the filled circles are p-BPB-treated fractions (n = 3). Standard error of the means is indicated when it exceeds the size of the symbol.

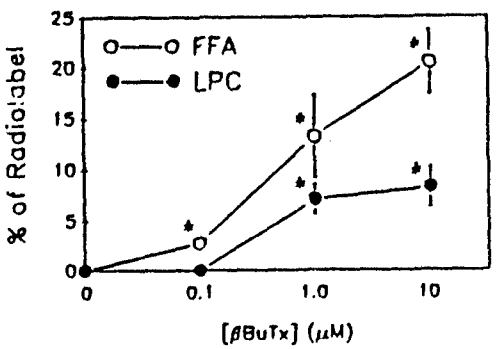


FIGURE 8. Hydrolysis of cellular phospholipids by β-bungarotoxin. Primary cultures of human skeletal muscle in which the fatty esters were radiolabeled were exposed to various concentrations of β-bungarotoxin (37°C; 2 hrs) and the lipids extracted and the radioactivity associated with the various lipid fractions subsequently determined. The radioactivity associated with free fatty acids (FFA) is expressed as percent of total radioactivity and that associated with lysophosphatidylcholine (LPC) is expressed as percent of lipid-associated radioactivity. The values are derived from three independent determinations and the SEM bars are shown when they exceed the size of the symbol.

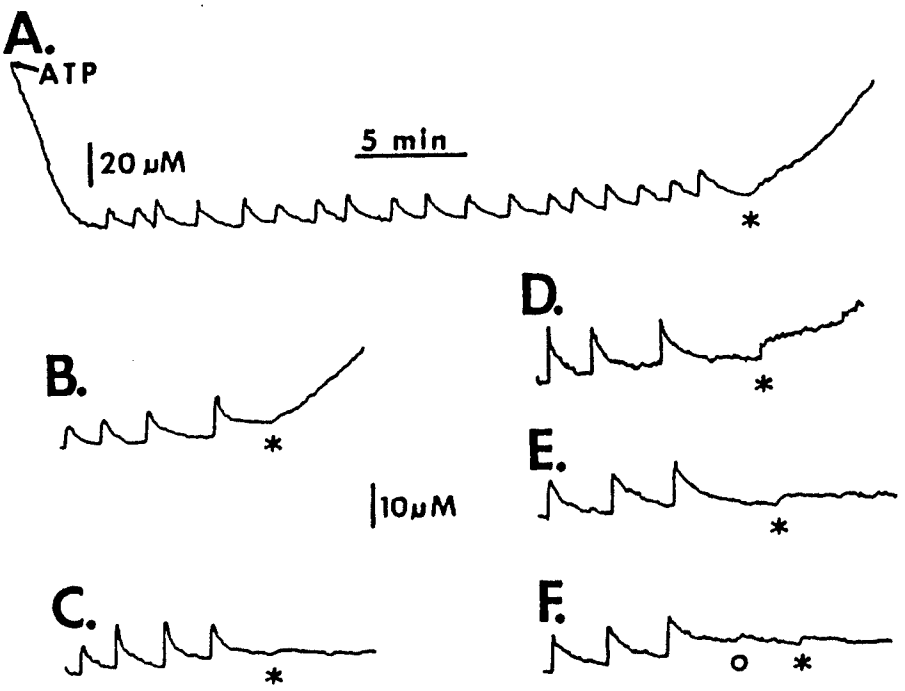


FIGURE 9. The dependence of *Naja naja kaouthia* CTX (10 µM)-induced Ca^{2+} release on the amount of Ca^{2+} preload and the effects of ruthenium red (10 µM). Panel A: A typical tracing in which *p*-BPB-treated CTX (10 µM) was added to human terminal cisternae preloaded to 78% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel B: CTX-induced Ca^{2+} release from equine terminal cisternae preloaded to 77% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel C: Failure of CTX to elicit Ca^{2+} release from equine terminal cisternae preloaded to 62% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel D: CTX-induced Ca^{2+} release from human terminal cisternae preloaded to 88% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel E: Failure of CTX to elicit Ca^{2+} release from human terminal cisternae preloaded to 62% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel F: Effects of ruthenium red (RR) on CTX-induced Ca^{2+} release from a human terminal cisternae preparation preloaded to 86% of the threshold for Ca^{2+}-induced Ca^{2+} release. In all cases CTX (10 µM) was added at the asterisk. RR (10 µM) was added at the circle.

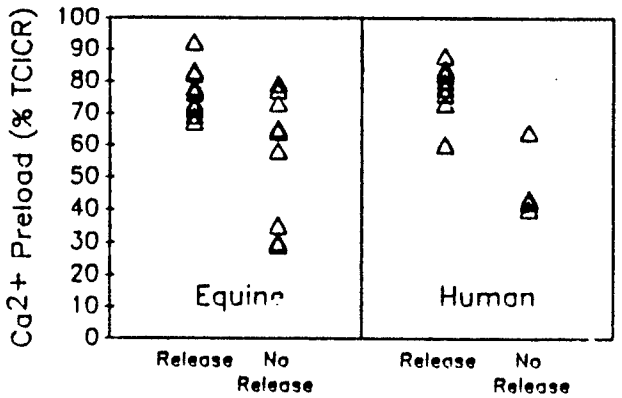


FIGURE 10. Scattergram of the dependence of the effects of *Naja naja kaouthia* CTX on Ca^{2+} release on the amount of Ca^{2+} preload. Equine or human preparations were preloaded with Ca^{2+} to various levels and then challenged with CTX (10 µM). The amount of preload is expressed in the ordinate as the percent of Ca^{2+} required to reach the threshold of Ca^{2+}-induced Ca^{2+} release (TCICR) in a separate aliquot from the same terminal cisternae-containing suspension. The preparations either exhibited a sustained release of Ca^{2+} in response to CTX (Release), or did not respond to CTX (No Release). Each triangle represents a different horse or human.

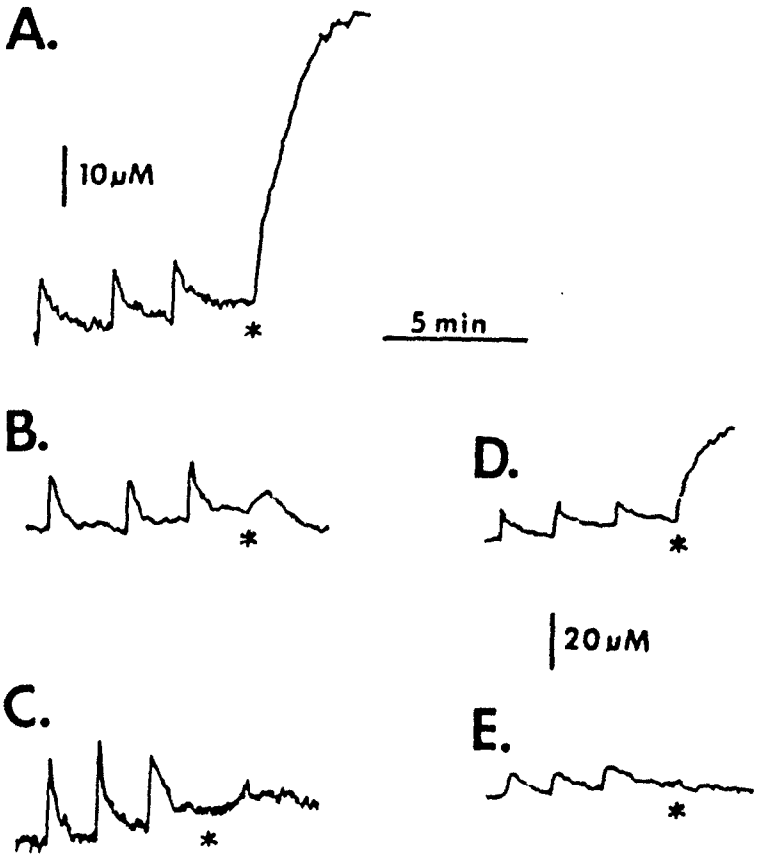


FIGURE 11. The dependence of melittin (0.1 μM)-induced Ca^{2+} release on the amount of Ca^{2+} preload and the effects of ruthenium red (10 μM). Panel A: melittin-induced Ca^{2+} release from equine terminal cisternae preloaded to 79% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel B: Failure of melittin to elicit Ca^{2+} release from equine terminal cisternae preloaded to 38% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel C: Effects of ruthenium red (RR) on melittin-induced Ca^{2+} release from an equine terminal cisternae preparation preloaded to 79% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel D: Melittin-induced Ca^{2+} release from human terminal cisternae preloaded to 80% of the threshold for Ca^{2+}-induced Ca^{2+} release. Panel E: Effects of ruthenium red on melittin-induced Ca^{2+} release from a human terminal cisternae preparation preloaded to 80% of the threshold for Ca^{2+}-induced Ca^{2+} release. In all cases melittin (0.1 μM) was added at the asterisk. Ruthenium red (10 μM) was added before any Ca^{2+} pulses.

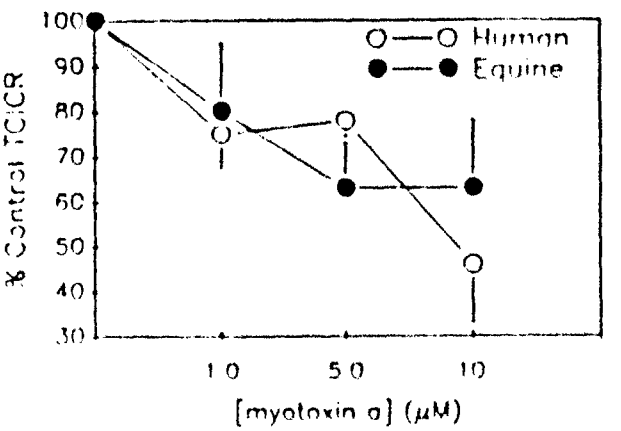


FIGURE 12. The effects of myotoxin a on the threshold for Ca^{2+}-induced Ca^{2+} release (TCICR). Myotoxin a was added after ATP and before any Ca^{2+} pulses, as previously described for FFAs (see Fletcher et al., 1990b). The TCICR is expressed as % control ± SEM for human (filled circles) and equine (open circles) preparations.

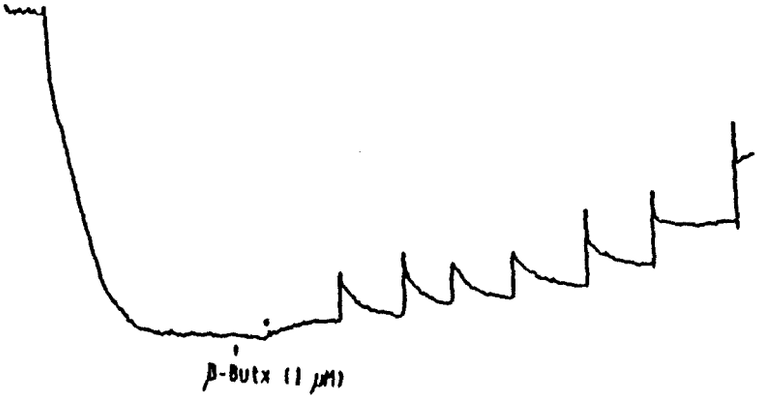


FIGURE 13. Effects of β-Butx on the threshold of Ca^{2+}-induced Ca^{2+} release (TCICR). β-Butx had no effect on the TCICR, but did cause Ca^{2+} release independent of preload.

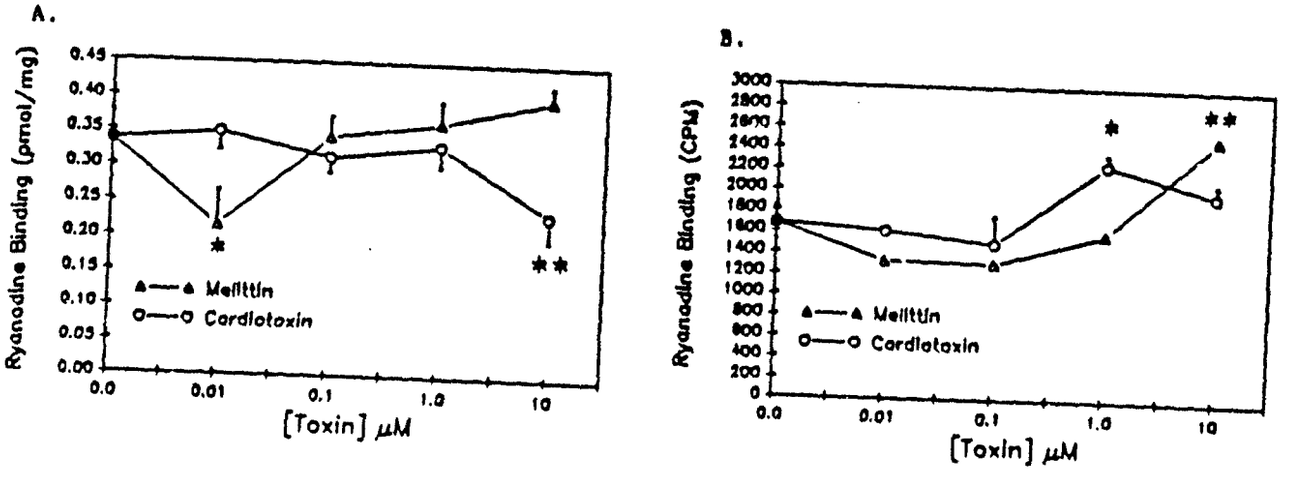
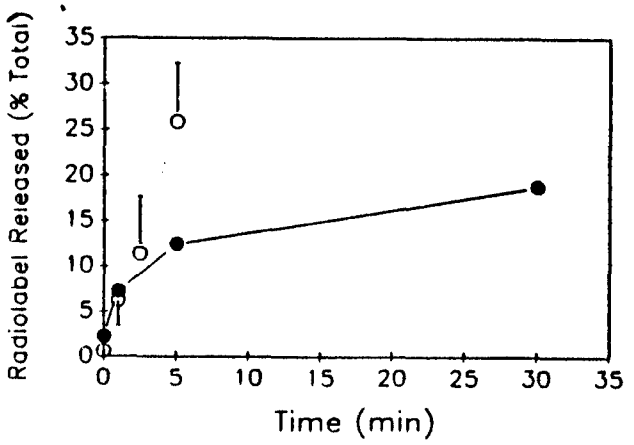


FIGURE 14. The effects of melittin and CTX on [^{3}H]ryanodine binding to the Ca^{2+} release channel. Terminal cisternae-containing preparations were prepared either by: (A) isolating an HSRF preparation (4,000-12,000 x g), or (B) by using the supernatant from a single 4,000 x g centrifugation step. Ryanodine binding was conducted in the presence of [^{3}H]ryanodine (8 nM) for total binding and in the presence of an additional 10 μM unlabeled ryanodine for nonspecific binding. Only specific binding is indicated. In panel A the radioactivity has been corrected for pmol ryanodine bound per mg protein.



:IGURE 15. Time course of radioactivity release into supernatant (expressed as % total radiolabel incorporated into the cells) from primary cultures of human skeletal muscle treated with melittin (10 μM). Two separate experiments on different cultures from different patients are shown. One time course was 0-5 min and the other was 0-30 min. Cells were preradiolabeled with [^{14}C]linoleic acid (10 μM, 3 days) and in one case (0-30 min) also with [^{14}C]ethanolamine (10 μM, 3 days) to radiolabel phosphatidylethanolamine.

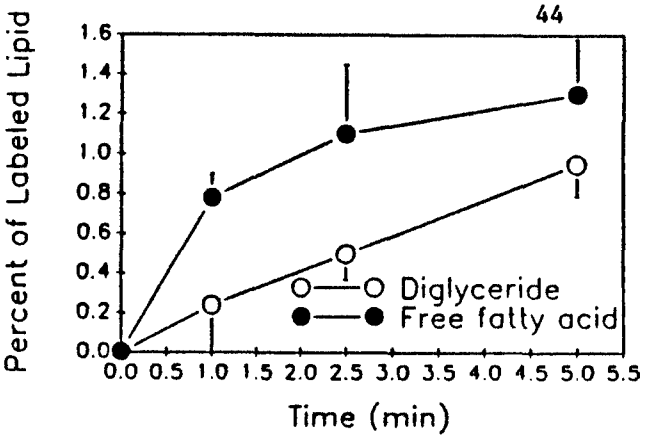
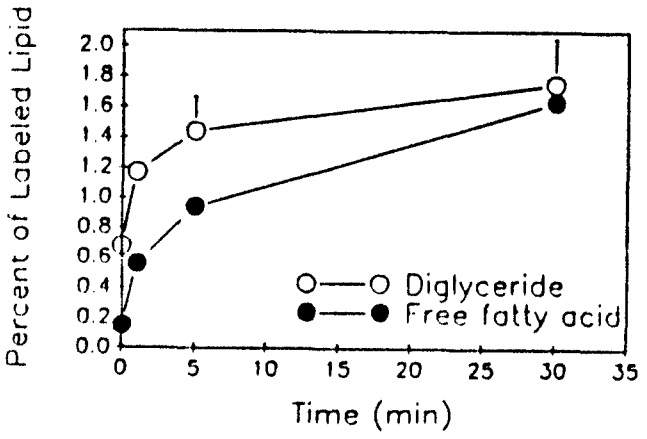


FIGURE 16. Time course of diglyceride and FFA production in the same primary cultures of human skeletal muscle treated with melittin (10 μM) in Figure 15 (0-5 min experiment). Cells were preradiolabeled with [^{14}C]linoleic acid (10 μM, 3 days).



IGURE 17. Time course of diglyceride and FFA roduction in the same primary cultures of uman skeletal muscle treated with melittin 10 μM) in Figure 15 (0-30 min experiment). ells were preradiolabeled with [^{14}C]linoleic cid (10 μM, 3 days) and with ^{14}C]ethanolamine (10 μM, 3 days) to adiolabel phosphatidylethanolamine.

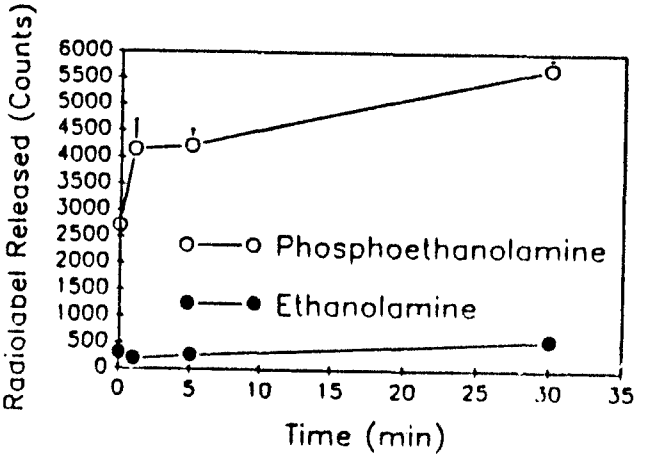


FIGURE 18. Time course of phosphoethanolamine and ethanolamine release from primary cultures of human skeletal muscle treated 0-30 min with melittin (10 μM). Cells were preradiolabeled with [^{14}C]linoleic acid (10 μM, 3 days) and with [^{14}C]ethanolamine (10 μM, 3 days) to radiolabel phosphatidylethanolamine.

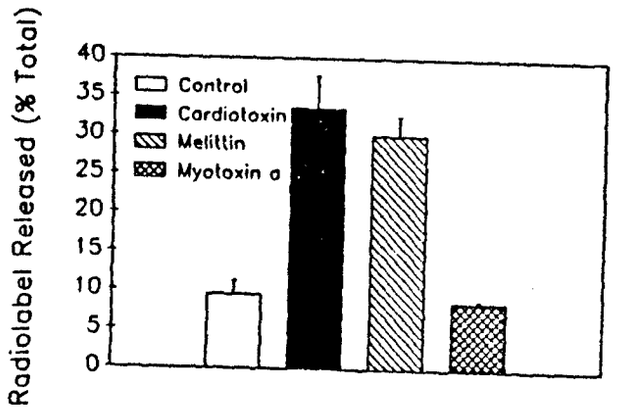


FIGURE 19. Comparison of the effects of cardiotoxin, melittin and myotoxin a (all at 10 μM concentrations) on radioactivity release into supernatant (expressed as % total radiolabel incorporated into the cells) from a mouse C_2C_{12} cell line. The cells were prelabeled with linoleic acid (10 μM, 3 days) and incubated for 2 hrs with toxin.

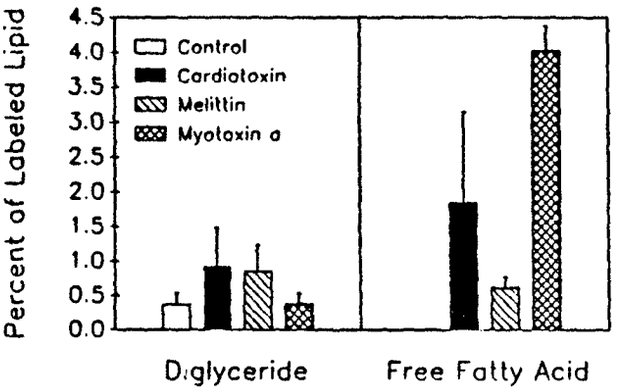


FIGURE 20. Comparison of the effects of cardiotoxin, melittin and myotoxin a (all at 10 μM concentrations) on diglyceride and FFA production in a mouse C_2C_{12} cell line. The cells were prelabeled with linoleic acid (10 μM, 3 days) and incubated for 2 hrs with toxin.

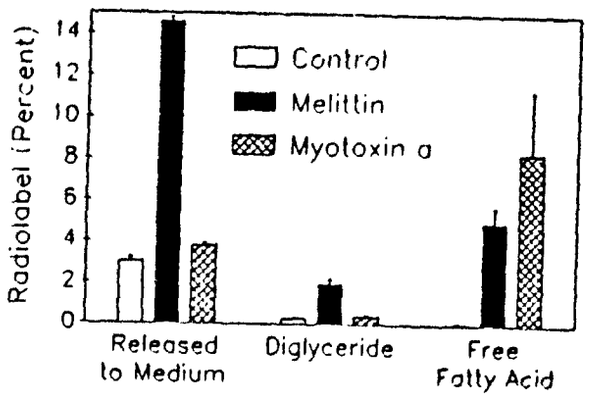


FIGURE 21. Comparison of the effects of melittin and myotoxin a (at 10 μM concentrations) on radioactivity release into supernatant from (expressed as % total radiolabel incorporated into the cells) and diglyceride and FFA production in primary cultures of human skeletal muscle. The cells were prelabeled with linoleic acid (10 μM, 3 days) and incubated for 2 hrs with toxin.

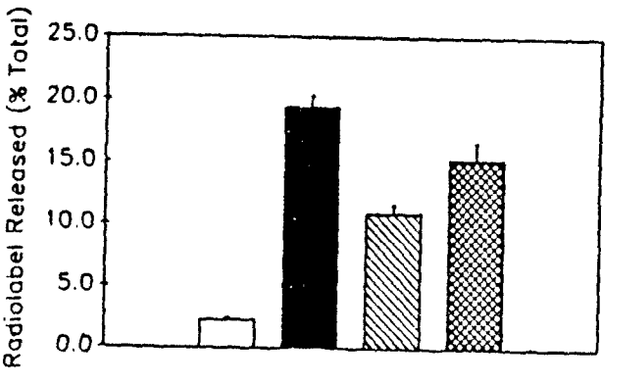


FIGURE 22. Role of Ca^{2+} in the release of radiolabel into the incubation medium by melittin (10 μM). Primary cultures of human skeletal muscle were prelabeled with linoleic acid (10 μM, 3 days) and incubated in HEPES-buffered Krebs in the absence (open bar) or presence (filled bar) of melittin for 2 hrs. Alternatively the cell were incubated in a Ca^{2+}-free buffer containing EDTA (10 mM) and ruthenium red (10 μM). These latter cells were then incubated in the absence (diagonal bar) or presence (crosshatch bar) of melittin for 2 hrs.

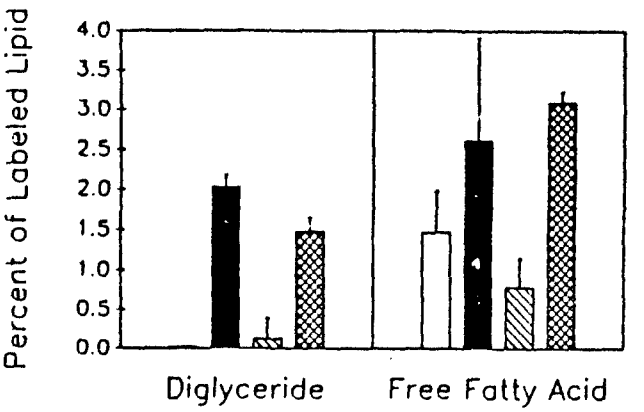


FIGURE 23. Role of Ca^{2+} in the production of diglyceride and FFA by melittin (10 μM). Primary cultures of human skeletal muscle were prelabeled with linoleic acid (10 μM, 3 days) and incubated in HEPES-buffered Krebs in the absence (open bar) or presence (filled bar) of melittin for 2 hrs. Alternatively the cell were incubated in a Ca^{2+}-free buffer containing EDTA (10 mM) and ruthenium red (10 μM). These latter cells were then incubated in the absence (diagonal bar) or presence (crosshatch bar) of melittin for 2 hrs.

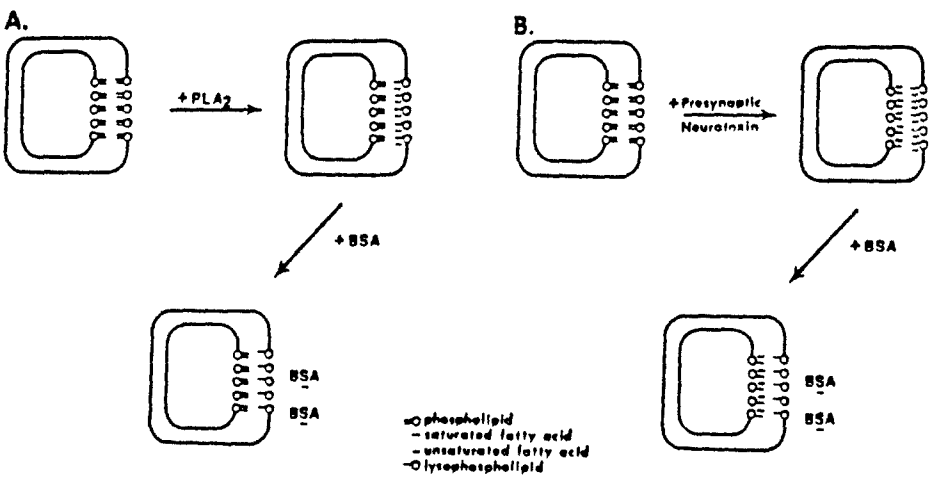
CONCLUSIONS

Effects of Presynaptic Neurotoxins on Ch Uptake and ACh Release in Mouse Brain Synaptosomes

Several important findings have been made. First, the inclusion of BSA in the incubation medium at the time of toxin expose has allowed, for the first time, a clear distinction to be made between nonPSNTX PLA_2s (e.g., *Naja naja atra* PLA_2) and the PSNTXs. Second, not all of the PSNTXs are active on the synaptosome preparation, based on their effects at a concentration of 100 nM on ACh release in a BSA-containing medium. The inactive PSNTXs include crotoxin (115% increase of ACh) and taipoxin (112% increase of ACh). These actions are barely above control levels. In contrast, β-Butx (140%), scutoxin (195%) and pseudexin (195%) are much more active. Third, pseudexin appears unusual among the PSNTXs active on ACh release, since its action on Ch uptake at a 10 nM concentration is antagonized to a much greater extent by BSA than that of β-Butx or scutoxin.

Role of PLA_2 Activity in the Action of PSNTXs and CTXs

We have found that PLA_2 activity may actually play an important role in the enhancement of ACh release. This finding stands in contrast to those of other investigators for one simple reason. By using BSA, we have distinguished between gross PLA_2 activity and that PLA_2 activity resulting from a penetrating action of the toxins. We believe that the nonPSNTXs only hydrolyze the outer leaflet of the membrane bilayer (see Panel A in Figure below), whereas the PSNTXs hydrolyze the inner leaflet (see Panel B in Figure below). Notice that the PSNTXs produce free fatty acids on the inner leaflet, which have potent effects on a variety of ion channels, including K^+. To reach the inner leaflet most likely requires a protein binding site and this site is absent in synaptosomes for crotoxin and taipoxin. All PLA_2 activity associated with CTXs and melittin were the result of contamination of these toxin fractions with venom PLA_2. There is not role for PLA_2 in the action of truely purified CTX or mellitin.



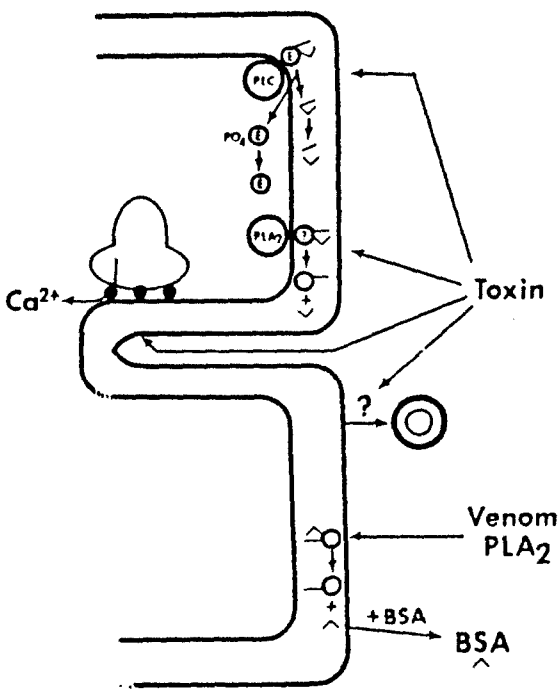
Action of CTXs, Melittin, Myotoxin a and β-Butx on Ca^{2+} Release

CTXs, melittin and myotoxin a all cause Ca^{2+} release from the terminal cisternae of the sarcoplasmic reticulum through the Ca^{2+} release channel. The actions of the toxins appear to be somewhat different from one another. Neither CTX nor melittin appear to act at the site of the classical Ca^{2+} release channel toxin, ryanodine. β-Butx also causes a transient release of Ca^{2+} from the sarcoplasmic reticulum. However, this action needs to be examined in much greater detail using our studies on the CTXs, melittin and myotoxin a as a guide. At this time we are unsure whether the effects of the toxins on Ca^{2+} regulation are direct, or a consequence of their effects on lipid metabolism (see below).

Effects of the PSNTXs and CTXs on Tissue Lipid Metabolism

β-Butx had no detectable effect on tissue lipid metabolism in primary cell cultures of skeletal muscle. We will examine the effects of β-Butx on nerve cell lines in the near future. Melittin and CTX both activate PLC activity and have no detectable effect on PLA_2 activity. In contrast, myotoxin a activates what appears to be PLA_2 activity and has no effect on PLC. The actions of CTX and melittin on PLC appear to be specific for phosphatidylethanolamine over phosphatidylcholine as the substrate, they have no requirement for Ca^{2+} and are independent of cholera or pertussis toxin sensitive G proteins.

Overall, CTX, melittin and myotoxin a have four possible actions, as summarized in the figure below. The actions can all be dissociated from one another by the large series of studies in the Body of the report.



	Melittin	CTX	Myotoxin a
PLC	++	+	-
PLA_2	-	-	+++
Ca^{2+} Release	++++	+	++
Membrane Release	+++	++	-

Recommended Changes or Future Work to Better Address the Problem

Since some of the PSNTXs are not active on the synaptosome preparation, we will focus only on those that are (pseudexin, ß-Butx and scutoxin) and test the inactive PSNTXs (crotoxin, taipoxin) on the PC12 nerve cell line. Presently our studies need to be expanded in numbers of determinations for ACh release. We are expanding our investigation of the role of PLA_2 activity in the enhancement of ACh release by examining the 100 nM concentration of toxins.

Since CTXs, melittin and myotoxin a all cause Ca^{2+} release from the terminal cisternae of the sarcoplasmic reticulum through the Ca^{2+} release channel we wish to examine whether this is the consequence of the effects of these toxins on lipid metabolism. The action of ß-Butx and other PSNTXs on release of Ca^{2+} from the sarcoplasmic reticulum will be examined in greater detail.

Although ß-Butx had no detectable effect on tissue lipid metabolism in primary cell cultures of skeletal muscle, we must examine the effects of ß-Butx and other PSNTXs on nerve cell lines in the near future.

The emphasis on the CTXs in the first half of the contract have laid a foundation for similar studies to be conducted on the PSNTXs during the second half of the contract. Overall, there will now be a shift in emphasis toward the PSNTXs and the nerve cell line.

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