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THE EFFECTS OF PYRIDOSTIGMINE AND PHYSOSTIGMINE ON THE CHOLINERGIC SYNAPSE

ANNUAL REPORT

C. SUE HUDSON

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Diaphragms from male albino, Charles River or Edgewood rats were assessed for morphological alterations following acute or subacute exposures to pyridostigmine bromide. Low acute doses (threshold 0.001 LD50) seemed to affect the presynaptic area with no apparent effects of the muscle cell. At higher doses (0.01 - 1.0 LD50) both pre- and postsynaptic elements became involved. A 14-day subacute exposure to 20 mg of pyridostigmine produced pre- and post-synaptic alterations which were more extensive than those seen following exposure to a 3 mg 14 day exposure. However, no subacute dose created damage equivalent to that seen 30 minutes after a single LD50 dose. Presynaptic alterations included withdrawal of the terminal from the junctional folds, invasion of the synaptic cleft with Schwann cell processes, and disruption of axon terminal organelles. Postsynaptic alterations included subjunctional supercontraction; disruption of

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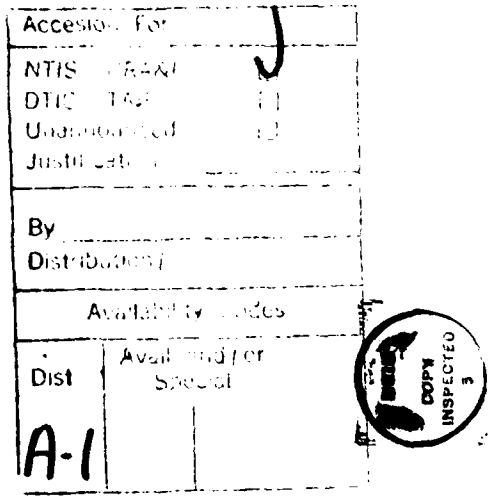
myofibrillar apparatus with the Z-lines apparently being the most sensitive element, and disruption of subjunctional mitochondria. Evidence of recovery from the drug-induced effects included reinnervation of junctional areas and the replacement or repair of subcellular elements. In conclusion, the data indicates a dose-dependent effect of pyridostigmine on muscle ultrastructure with subsequent recovery.



SUMMARY

The quaternary carbamate, pyridostigmine bromide, is widely used in treatment of mysathenia gravis and has been suggested for use in prophylaxis against intoxication with irreversible cholinesterase inhibitors. Since there are virtually no anatomical data concerning the neuromuscular toxicity of the drug, this study was undertaken to evaluate the effects of acute and subacute doses of pyridostigmine on the ultrastructure of rat diaphragm neuromuscular junctions (NMJs) and muscle fibers. In this regard, the completion of one milestone was required by this contract (DAMD-17-83-C-3126) for contract year 1 (April 1, 1983 - March 31, 1984). Specifically, a thin-section electron microscope analysis of the dose-response effects of acute exposure to pyridostigmine on the rat diaphragm was to be accomplished. This milestone has been completed. The procedures, data and analyses for the study are detailed respectively in the Methods, Results, and Discussion sections of this report.

Additional work originally scheduled for completion in contract year 2 has also been finished. This work includes a completed thin-section analysis of the subacute dose response effects of pyridostigmine on the rat diaphragm NMJs and muscle fibers as well as preliminary data concerning NMJ recovery from acute and subacute exposure to pyridostigmine. Detailed explanations for these invesigations are also included in the Methods, Results, and Discussion sections of this report.

The completed contract milestones included the following experiments and analyses. Diaphragms from male albino, Charles River or Edgewood rats were assessed for morphological alterations following acute or subacute exposures to pyridostigmine bromide. Acute doses of pyridostigmine bromide in Mestinon -equivalent buffer were administered by single subcutaneous injection in doses that ranged from 0.0036 to 3.6 mg/kg (0.001 - 1.0 LD_{50}). Tissues were analyzed in the acute experiments 10 - 30 minutes, 24 hrs., and one week postinjection. Subacute exposures were administered by subcutaneously implanted osmotic minipumps (Alzet® 2ML2 pumps; 2 ml volume; continuous infusion rate of 5.93 µl/hr for up to 14 days) that contained either 3.0 or 20 mg pyridostigmine in Mestinon®-equivalent buffer. Tissues were analyzed after subacute exposures of 3, 7, and 14 days and with post-exposure recovery times of 7. 14, and 23 days. In select animals, whole blood cholinesterase activity was measured by radiometric assay.

The morphological effects of pyridostigmine were localized predominantly at the neuromuscular junction with both pre- and postsynaptic regions involved. The data indicated that there was a variation in the extent of the damage between different muscle fibers and between different areas of individual neuromuscular junctions. With acute doses, there was a dose dependent response which was manifest in terms of the location of the pathology. Low doses (threshold 0.001 - 0.01 LD_{50}) seemed to affect the presynaptic area with no apparent effects on the muscle cell. At higher doses

(0.1 - 1.0 LD_{50}), both pre- and postsynaptic elements became involved. The subacute doses examined also created dose-dependent lesions in the diaphragm. At 14 days, the 20 mg dose group exhibited pre- and postsynaptic alterations which were more extensive than those seen in the 3 mg group although no subacute dose created damage equivalent to that seen 30 minutes after a single LD_{50} dose. Presynaptic alterations included withdrawal of the terminal from the junctional folds, invasion of the synaptic cleft with Schwann cell processes, and disruption of axon terminal organelles. Postsynaptic alterations included subjuctional supercontraction, disruption of myofibrillar apparatus with the Z-lines apparently being the most sensitive element, and disruption of subjunctional mitochondria.

Ultrastructural recovery of the diaphragm after acute or subacute exposure to pyridostigmine varied. The evidence suggests that subacute exposure had the greatest effect within the first week of exposure with the damage being sustained for the duration of exposure but not increasing in severity. In contrast, diaphragms subjected to high acute doses (e.g., 0.72 LD_{50}) exhibited a progression in the degenerative process for at least one week post-exposure. During this time, junctional fold breakdown was evident. This phenomenon was not present under subacute exposure conditions. Together, with the progression of morphological alterations, was evidence of recovey from the drug-induced effects. Reinnervation of junctional areas appeared to be in progress and the subcellular elements were being replaced and/or repaired.

In conclusion, the data suggest a dose-dependent effect of pyridostigmine on muscle ultrastructure with the threshold for presynaptic alterations being near 0.001 LD_{50} (approx. 10% whole blood ChE depression). The threshold for morphological alteration of postsynaptic elements was near 0.01 LD_{50} (approx. 20% whole blood ChE depression). The data also indicate that, morphologically, the diaphragm does recover from the deleterious effects of pyridostigmine. The drug-induced abnormalities in neuromuscular morphology and the time course of morphological recovery may not coincide with abnormalities in and recovery of normal neuromuscular transmission. Prediction of an endpoint to the recovery process vis-a-vis muscle function is beyond the means of morphological studies. Thus, full recovery from the pyridostigmine-induced damage should additionally be assessed by physiological and/or behavioral studies. Furthermore, the studies described herein were performed with rats under rest conditions which may reveal only minimal morphological and functional alterations. It is recommended that a more accurate evaluation of the systemic effects of pyridostigmine should be performed with stressed (i.e. exercised) animals. Experiments of this type might allow a more realistic assessment of possible drug effects on military personnel under stress conditions.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)

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STATEMENT OF PROBLEM

In a variety of experimental animals, pyridostigmine has been shown to be an effective prophylactic against systemic exposure to irreversible cholinesterase inhibitors (e.g., Berry and Davies, 1970; Gordon et al., 1978; Dirnhuber et al., 1979). The clinical use of pyridostigmine in humans has been largely limited to patients with neuromuscular disorders, such as myasthenia gravis, or to patients under curaraform neuromuscular blockade. While there is a dirth of clinical literature on the toxicology of pyridostigmine, it has been known for at least 17 years that acute and subacute intoxication with cholinesterase inhibitors (e.g., Preusser, 1967; Ariens, 1969; Engel et al., 1973; Renichel et al., 1972, 1974; Laskowski, et al., 1975, Wecker and Dettbarn, 1976; Wecker et al., 1979; Salpeter et al., 1979, 1982) including the tertiary carbamate physostigmine (Wecker et al., 1976) and the quaternary carbamate neostigmine (Engel and Santa, 1973; Kawabuchi et al., 1975; Ward et al., 1975; Hudson et al., 1978) causes myopathies and/or neuropathies and produces abnormal physiology at the mammalian neuromuscular junction (NMJ).

Previous morphological studies have placed major emphasis on the postsynaptic myopathic alterations which appear more severe than the alterations to the presynaptic apparatus. However, presynaptic changes have been demonstrated following acute, subacute, and/or chronic exposure to cholinesterase inhibitors. Morphologically, increased numbers of coated vesicles, swollen mitochondria (Laskowski, et al., 1975; Hudson et al., 1978), decreased nerve terminal size (Engel et al., 1973) and reduced numbers of nerve terminal endings (Glazer et al., 1978) have been noted. The functional correlates of either the pre- or postsynaptic damage are not clear. Reports of abnormal miniature endplate potential amplitude and frequency (Tiedt et. al., 1978; Laskowski and Dettbarn, 1975) and antidromic activity of the motorneuron (Laskowski and Dettbarn, 1975) might be related, in part, to the drug-induced presynaptic alterations. The postsynaptic damage could be a contributing factor to the muscle weakness reported for pyridostigmine treated rats (Gillies and Allen; 1977).

The anatomical studies of anticholinesterase agent-induced neuromuscular pathology have concentrated primarily on the irreversible inhibiting drugs. Thus, there are virtually no anatomical data and little physiological data concerning the quaternary carbamate pyridostigmine which has been suggested as a possible prophylactic agent against intoxication with irreversible cholinesterase inhibitors (Gordon et al., 1978). Therefore, a series of experiments were planned to assess the potential neuro- and myotoxicity of the drug. Specifically, a thin-section electron microscope study was designed to evaluate the pre- and postsynaptic ultrastructural alterations associated with the dose-response and recovery of the rat diaphragm NMJ region following acute and subacute exposure to pyridostigmine bromide. Preliminary results of these studies have been presented in abstract form (Foster and Hudson, 1983)

BACKGROUND

Alterations of endplate fine structure of the rat neuromuscular junction have been reported after exposure to a variety of anticholinesterase agents including neostigmine, physostigmine, pyridostigmine, paraoxon, parathion, diisopropylfluorophosphate, soman and tabun. In all cases, the mechanism for producing the degenerative alterations have been attributed primarily to the increase in endogenous acetylcholine (ACh) concentration within the synaptic cleft resulting from inactivation of acetylcholinesterase (Ariens et al., 1969; Renichel et al., 1972, 1974; Kawabuchi et al., 1976; Laskowski et al., 1975; Preusser, 1967; Ward et al., 1975).

The above anticholinesterase (AChE) agents are ferquently categorized as reversible or irreversible cholinesterase inhibitors. The latter group is composed of organophosphorus inhibitors and includes soman, tabun, and sarin, the extremely potent toxins which were developed as chemical warfare agents. Most of the organophosphorus inhibitors combine with AChE to form a phosphorylated complex which is extremely stable to significant regeneration of the enzyme by hydrolysis. With some of these potent inhibitors, return of AChE activity may depend on synthesis of new AChE which may take days or months (Taylor, 1980).

Treatment with sublethal doses of some of the irreversible organophosphorus compounds [tabun, paraoxon and diisopropylfluorophosphate (DFP)] produces a progressive myopathy with characteristic alterations (Laskowski and Dettbarn, 1975; Laskowski et al., 1975; Ariens et al., 1969; Renichel et al., 1972, 1974). These changes include loss of organization of the subjunctional myofibrillar apparatus, an increase in presynaptic coated vesicles and the presence of swollen presynaptic mitochondria. These changes are similar in nature to those observed after treatment with reversible anticholinesterase agents (see below). With sublethal doses, the alterations are reversible (Ariens et al., 1969).

In an attempt to develop antidotes to the organophosphorus agents, considerable research has been directed toward the use of oximes alone or in combination with other drugs to speed the reactivation of AChE (Smith et al, 1981; Wolthius et al., 1981). While certain oximes have been demonstrated as effective antidotes against sarin and tabun poisoning, they are at best of limited value or without effect against soman poisoning (Smith et al., 1981; Wolthius et al., 1981). Thus, other avenues for prophylactic chemical protection of animals from soman and soman-like poisons must be appraised.

To this end a number of studies have begun to test the concept of utilizing various of the reversible anticholinesterase agents as pretreatment drugs to protect against the effects of the organophosphorus agents. This line of investigation is based on the assumption that reversible cholinesterases such as pyridostigmine, physostigmine, and neostigmine will

temporarily inhibit acetylcholinesterase and prevent an irreversible agent from permanently inhibiting the enzyme.

Recent investigations designed to test the efficacy of pretreatment with pyridostigmine, physostigmine,, and neostigmine have been successful in protecting animal models from lethal doses of organophosphorus compounds (Harris et al., 1980). Possibly most encouraging is work demonstrating survival of soman poisoned rats and rabbits which were pretreated with mecamylamine and atropine and physostigmine or atropine and neostigmine (Harris et al., 1980). The importance of these results are magnified since the degree of chemical protection against soman is species-related with the rat being among the least protected animals (Harris et al., 1980; Gordon et al., 1978). The chemical structure and reactions of the reversible AChE agents are therefore of prime interest to investigators in this field.

Compounds such as physostigmine, neostigmine and pyridostigmine possess a tertiary amino or quarternary ammonium group as well as a carbamyl ester linkage or urethan structure and are classified as reversible cholinesterase inhibitors. In reality, only a negligible amount of "reversible" inhibitor is released from acetylcholinesterase reversibly. The drugs are actually hydrolyzed by the enzyme at a rate that is exceedingly slow (many minutes) compared to the similar hydrolysis of acetylcholine (microseconds). These "reversible" AChE inhibitors can be divided into subclasses on the basis of their structure and physiologic effect. Some, such as physostigmine possess a tertiary amine group, cross the blood brain barrier easily and are absorbed readily from the gastrointestinal tract (Taylor, 1980). Other AChE inhibitors such as pyridostigmine and neostigmine possess a quarternary ammonium, do not cross the blood brain barrier or the gastrointestinal mucosa easily. Both groups have a number of additional effects on structure and function which increase in severity with increasing dose and duration of treatment (Taylor, 1980).

A number of the studies on the alterations induced by reversible AChE inhibitors have been conducted. The effects of neostigmine have probably been most thoroughly examined due to its widespread use as a therapeutic agent in the management of myasthenia gravis. Short term treatment (up to 7 days) with neostigmine caused decreased miniature endplate potential amplitude and frequency, endplate potential amplitude and junctional ACh sensitivity (Tiedt et al., 1978; Roberts and Thesleff, 1969; Engel et al., 1973). Ultrastructurally, the depletion of synaptic vesicles and the presence of numerous coated vesicles indicated continuing nerve terminal hyperactivitiy of at least some terminals (Hudson et al., 1978). Many endplates possessed degenerating junctional folds (Engel et al., 1973; Ward et al., 1975; Hudson et al., 1978) but partial recovery was observed with chronic treatment (Hudson et al., 1978; Teidt et al., 1978). Clearly, the rate of transmitter release decreased (Chang et al., 1973) as did the number of ACh receptors (Chang et al., 1973; Engel et al., 1973; Hudson et al., 1978). No similar ultrastructural studies concerning the effects pyridostigmine or physostigmine

are available. The experiments described in this document were undertaken to determine the morphological effects of pyridostigmine on mammalian NMJs and muscle fibers. These results should aid in the evaluation of the use of pyridostigmine as a prophylactic drug against nerve agents.

MATERIALS AND METHODS

Male albino rats (Edgewood or Charles River strains) weighing 180 to 250 gms received subcutaneous, acute and subacute exposures to pyridostigmine bromide. All acute exposures were by single syringe injections under the skin of the midback region and all subacute exposures (maximum 14 days) were via osmotic minipump (Alzet® 2ML2 minipump; ALZA Corp., Palo Alto. Ca.) implanted under the skin of the midback. Pyridostigmine bromide was administered in a Mestinon® -equivalent diluent composed of 1.30 mg/ml citric acid monohydrate, 4.10 mg/ml sodium citrate dihydrate, 0.50 mg/ml methyl paraben, 0.05 mg/ml propyl paraben and 7.40 mg/ml sodium chloride in sterile water at pH 5.1.

Acute Drug Exposure. Acute doses of pyridostigmine ranged from 0.0036 mg/kg to 3.6 mg/kg (1 LD_{50} subcutaneous injection = 3.6 mg/kg determined by Probit analysis). Animals in the acute control group received a single injection of the Mestinon® -equivalent diluent. A minimum of 3 experimental and 2 control animals were prepared for each dose level analyzed. These animals were sacrificed under deep barbiturate anesthesia by transcardiac vascular perfusion 10 - 30 minutes, 24 hours and 1 week postinjection.

Subacute Drug Exposure. Subacutely treated animals had 2 ml osmotic minipumps implantd subcutaneously to provide continuous infusion of pyridostigmine. The rationale for using continuous infusion of the drug rather than multiple acute doses (e.g., Hudson et al., 1978) hinges on an attempt to hold constant the cholinesterase (ChE) depression and the tissue concentration of the drug. Since pyridostigmine is a reversible inhibitor of ChE, maintaining a constant depression of ChE via subcutaneous injections would have required approximately 4-10 injections per day for up to 14 days. Presumably, each injection would have produced a drastic decrease (enzyme carbamylation) then increase (enzyme decarbamylation) of ChE level with the attendant single-dose acute effets. It was also important to maintain a constant tissue concentration of the drug during long term exposure without the variables of metabolism inherent in a multiple injection paradigm. Use of osmotic minipumps allowed continuous relase of the drug which presumably maintained both tissue-ChE and tissue-drug levels as constant as possible.

Subacute doses were studied using minipumps loaded with 1.5 or 10.0 mg/ml pyridostigmine bromide in the Mestinon®-equivalent buffer. Control animals were implanted with minipumps containing the Mestinon® - equivalent buffer. The morphological effects of chronic infusion of pyridostigmine were evaluated after periods of 2, 7 and 14 days exposure. A minimum of 3 experimental and 2 control animals were prepared and analyzed for each dose at each exposure

period. Since the Alzet® pumps utilized in this study released their contents at approximately 5.9 µl/hr, the low dosage group animals (pumps containing 1.5 mg/ml pyridostigmine) were exposed to an approximate total of .43, 1.5 and 3.0 mg of drug on 2, 7 and 14 days respectively. Animals in the high dosage group (pumps containing 10 mg/ml pyridostigmine) had total exposures of 2.8, 10.0 and 20 mg of drug after 2, 7 and 14 days, respectively. Blood ChE levels of each drug-treated and control animal were analyzed (Siakotos et al., 1969, see below) periodically (at least twice) during the course of the experiment.

Preparation for Electron Microscopy. All animals were prepared by whole body perfusion through the left ventricle using an initial perfusate of oxygenated, rat Ringer's solution (millimolar concentrations were NaCl, 135.0; KCl, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 2.0; $NaHCO_3$, 15.0; and Na_2HPO_4, 1.0) containing 10 units/ml of heparin followed by the fixation perfusate containing 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4). The perfusates were maintained at room temperature. The diaphragm of each animal was immediately removed and fixed for an additional hour in cold 2.5% glutaraldehyde. NMJs were identified by staining for ChE in a solution of 5 mg acetylthiocholine iodide, 6.5 ml 0.1 M sodium cacodylate buffer (pH 7.4) with 0.2 M sucrose, 0.5 ml 0.1 M sodium citrate, 1.0 ml 30 mM copper sulfate and 1.0 ml ddH_2O (modified from Karnovsky and Roots, 1964). Endplate regions and tissue remote from the endplates were removed by careful dissection, postfixed in 1% OsO_4, stained en bloc in aqueous uranyl acetate or 2% uranyl acetate in 70% ethyl alcohol embedded in an Epon-Araldite mixture (10% Polybed 812, 20% Araldite 6005, 70% dodecenyl succinic anhydride with 1.5% DMP-30) and polymerized at 70 C for 24 hours. Ultrathin sections were cut with a diamond knife using an LKB III or IV ultramicrotome, poststained with lead citrate (Venable and Coggeshall, 1965) and aqueous or methanolic uranyl acetate and examined with a JEOL 100 B or 100 CX electron microscope.

Cholinesterase Assay. The radiometric method of Siakotos et al. (1969) using ^{14}C-acetylcholine as the ChE substrate was utilized throughout. The relative ChE depression in whole blood (i.e., percent carbamylation of the enzyme) produced by acute or subacute exposure to pyridostigmine was determined. Blood (100 µl) was drawn from the tail into heparinized capillary tubes and processed for an immediate assay or frozen in liquid nitrogen for subsequent assay. In order to test for the effects of freezing whole blood on the assay results, the 100 µl blood was divided into two 50 µl aliquots, one used for immediate assay and the other frozen for later assay. Freezing prior to assay resulted in a variation of approximately 5% of the ChE depression established by immediate assay. This was within an acceptable range for the purposes of the present study.

The enzyme assay was performed in the following manner. Up to 100 µl usually 5 - 50 µl) of blood sample was added to 100 µl 0.1M sodium phosphate (pH 7.4), 10 µl $MgCl_2$ and 100 µl ^{14}C-acetylcholine. The solution was mixed immediately and incubated for 5 minutes at 37 C (with shaking). The reaction was stopped by adding sufficient resin-dioxane mixture (20g Amberlite CG - 120

x 8 and 100 ml dioxane) to increase the total volume to 5 ml. The enzyme-resin-dioxane volume was then brought to 10 ml with dioxane, mixed by inversion (3 times) and centrifuged at 900g for 3 minutes. Then 1.0 ml of supernatant was combined with 10 ml of cocktail and the radioactivity assayed in a Beckman LS-7800 liquid scintillation counter. A reagent blank was run simultaneously using water in place of sample.

Since it was not practical to gather the ChE values for all of the acutely-treated animals, a parallel study (using a group of rats not utilized in the morphological study) established a dose-response curve of enzyme carbamylation at the time point of 30 minutes post-injection (single subcutaneous). The dose range utilized in this determination was 0.005 - 3.2 mg/kg. A minimum of three drug-treated and one control animal was used for the determination of total whole blood ChE depression at each dose.

For the animals which were in the subacute exposure groups, ChE levels were assayed before and periodically after the implantation of the osmotic minipump. The assay was performed on these animals (a) to confirm that each minipump was releasing the drug and (b) to establish the general dose/ChE depression relationship for each dose and each animal.

RESULTS

General Observations

Considerable individual variation was observed in the behavioral response of rats during the 30 minutes following a single injection of 3.2 or 3.6 mg/kg (0.9 or 1.0 LD50) pyridostigmine. Initially, all animals exhibited increased oral and ocular secretions simultaneous with rapid fasciculations and tremor of superficial muscle groups. An increased sensitivity to sounds (startle to hand claps) was also noted. As intoxication progressed, some aniamls displayed no additional behavioral responses (usually the survivors). Other animals (usually those which died before 30 minutes post-injection) exhibited whole body piloerection, periods of ballistic kicking movements of the fore- and hind limbs, continued gross muscle fasciculations tremor and breathing difficulties.

Animals which received 2.6 mg/kg (0.72 LD50) pyridostigmine generally displayed fewer and less intense reactions although several animals did not survive 30 minutes. No deaths resulted from any of the lower acute doses. Rats given single injections of 0.36 mg/kg (0.1 LD50) pyridostigmine or less and those injected with diluent (control group) displayed no outward signs of anticholinesterase intoxication.

Similarly animals implanted with the osmotic pumps containing 20 mg or 3 mg of pyridostigmine or diluent exhibited no obvious behavioral signs of pyridostigmine intoxication over a 14-day period of observation with the

exception of four animals. These 4 of the 24 animals that received high dose Alzet® pumps exhibited increased ocular secretions (chromodacryorrhea) within the initial several hours of pyridostigmine exposure, but displayed no other typical sign of anticholinesterase intoxication. The chromodacryorrhea cleared within the first 24 hours and did not reappear. These animals as well as the rest of the experimental animals remained symptom-free for the duration of the experiment.

Since irreversible ChE inhibitors have been reported to effect weight gain in rats under subacute exposure conditions, the question arose as to the effect of a reversible inhibitor on weight gain. All rats in the 7 and 14 day subacute exposure groups were weighed at least three and six times, respectively, during the drug-exposure period. No consistent effect of drug exposure on weight gain was detected.

Whole Blood ChE Levels

Acute Exposures to Pyridostigmine. Animals were exposed to various doses of pyridostigmine to determine the average inhibition (enzyme carbamylation) of whole blood ChE activity after 30 minutes exposure. The data plotted as the percent of ChE activity inhibited vs. log dose resulted in a S-shaped curve with a steep log-linear midregion between doses of 0.01 mg/kg (22±1.9% inhibition) and 1.0 mg/kg (71±1.0% inhibition). With regard to the doses used in the present study, the following whole blood ChE depression (inhibition) values were determined: 0.0036 mg/kg (0.001 LD50) - 10% depression; 0.036 mg/kg (0.01 LD50) - 25% depression; 0.36 mg/kg (0.1 LD50) - 55% depression; 2.6 mg/kg (0.72 LD50) - 70% depression; 3.24 mg/kg (0.9 LD50) - 78% depression; and 3.6 mg/kg (1.0 LD50) - 80% depression.

Subacute Exposures to Pyridostigmine. The whole blood ChE levels of subacutely treated animals were also monitored. At the low and high ends of the dosage spectrum, animals exposed to 1.5 mg/ml pyridostigmine were maintained at an average ChE depression of 23.4±4.0% while rats in the 10 mg/ml group had a much larger, 65.9±3.5% depression of ChE. An observation worth noting is that ChE activity levels in some of the low dose animals began returning toward control values by day 14 of exposure. Whether this result indicates osmotic pump failure or development of drug tolerance is unresolved. However, those low dose subacute animals which demonstrated this phenomenon have been excluded from our description.

Ultrastructural Observations.

Neuromuscular Junction Morphology of Control Animals. Animals which received a single injection of diluent or which were implanted with Alzet® pumps containing diluent served as controls for the acute and subacute experimental groups. Ultrastructural morphology of NMJs from rats treated by the two methods appeared similar (figs. 1 & 2). Nerve terminal plasma membranes were separated from the crests of the postsynaptic junctional folds

by a 50 nm wide primary cleft with basement membrane evenly interposed between the pre- and postsynaptic elements. The nerve terminals contained various numbers of mitochondria, numerous clear synaptic vesicles, a few coated vesicles and occasional smooth endoplasmic reticulum cisternae and coated pits. In fortuitous sections, nerve terminals could be observed in continuity with the innervating myelinated nerve (fig. 1). The nerve terminals were overlain by Schwann cells with processes that extended to the margins of the primary cleft but not into the cleft (see arrowhead, fig. 1 & 2). Thus, the NMJs of the control animals presented no sign of any untoward effect of the Mestinon® -equivalent buffer alone.

Presynaptic Effects of Acute Exposure to Pyridostigmine. All acute doses employed resulted in morphological alterations ranging from slight to pronounced in all of the diaphragmatic NMJs analyzed. Nonetheless, it is important to keep in mind that 1) different NMJs from the same animal were affected to different degrees and 2) that different presynaptic areas within the same NMJ frequently were affected to varying extent (figs. 4 & 5). In general, the alterations were observed in mitochondria, vesicles or in the spatial relationship of the nerve terminal both to its overlying Schwann cell and to the junctional folds of the innervated muscle cell.

Changes in presynaptic organelle ultrastructure were dose dependent. Nerve terminals exposed to the lowest acute doses (0.0036 and 0.036 mg/kg) possessed mitochondria with small rarefied areas (figs. 3 & 4) in the matrix. At higher doses, mitochondrial alterations varied from small rarefactions in the matrix (fig. 5) to complete disruption of the cristae or absence of the matrix altogether (fig. 6). The latter, more extreme and probably irreversible alterations were not always observed in terminals even at the highest doses (3.6 mg/kg; fig. 8). Vesicles two or more times the average diameter of synaptic vesicles were present as were occasional vesicles with denser than normal contents (figs. 4, 5 & 6). Whether these large vesicles represent abnormal synaptic vesicles or originate from another source is unresolved.

The shape and spatial geometry of nerve terminals were also altered following pyridostigmine exposures. In all experimental groups, all NMJs sampled possessed at least small areas of terminal membrane which were no longer closely apposed to the basement membrane. In these regions, the width of the primary cleft was highly variable and frequently exceeded twice the cleft width observed in control preparations. Within the primary synaptic clefts, finger-like processes (apparently from the Schwann cells) were commonly seen separating areas of the nerve terminal from postsynaptic folds (figs. 3, 5 & 6). The Schwann cell overlying the nerve terminal remained in close apposition to the nonsynaptic terminal membrane surface (figs. 3,4,6 - 8) but sometimes invaded the nerve terminal with processes (figs. 4, 7 & 8). Figure 8 illustrates how some invading Schwann processes interdigitate with and sequester portions of the nerve terminal. Profiles of both Schwann cell and nerve terminal projections appeared finger-like in longitudinal section

(figs. 4 & 8). In cross section, the nerve terminal projections appeared as separate vesicle-containing, membrane bound structures within the nerve terminal or within Schwann cell processes (figs. 4 & 7). No evidence indicated that the nerve terminal projections were physically separated from the body of the nerve terminal.

Presynaptic effects of Subacute Exposure to Pyridostigmine. Neuromuscular junctions of animals implanted with Alzet® pumps containing 1.5 mg/ml or 10 mg/ml pyridostigmine were assessed for damage 2, 7 and 14 days following implantation of the pumps. Both dose levels induced alterations at all time intervals analyzed. However, the extent of the damage varied with the duration of exposure. In general, the alterations from subactue dosages (figs. 9 - 12) were qualitatively similar to the observations in acutely treated rats (figs. 3 - 8), i.e., nerve terminal organelles were altered and the spatial geometry of the NMJ was affected. Reminiscent of acute exposures, the extent of nerve terminal damage (figs. 9 - 12) from any subacute exposure varied within a single muscle and varied within individual NMJs.

Figures 9 - 12 illustrate organelle alterations which resulted from subactue pyridostigmine exposure. Several presynaptic mitochondria possessed rarefied areas in their matrices and nerve terminal vesicles exhibited an unusual range of diameters. The organelle alterations following subacute exposure never progressed to the degree of severity observed following acute exposures. Complete disruption of mitochondria was not observed and the numbers of abnormal vesicles were lower.

Alteration of the spatial relationship of the nerve terminal and muscle cell was the most obvious effect of subacute drug exposure. The most subtle, observable changes occurred following a low dose exposure to pyridostigmine for 2 days. This treatment resulted in regional separation of the nerve terminal from the junctional fold crests (fig. 9 & 10). This phenomenon was present in every NMJ analyzed following 21 or more days of low or high dose exposure. In some NMJs, processes of overlying Schwann cells were interposed between the nerve terminal and the junctional fold crests (fig. 10 & 11) which also resulted in separation of presynaptic transmitting surface from the postsynaptic receptor-containing junctional fold crests. Following 7 and 14 days of subacute exposure, similar alterations were observed. Although quantitation of the severity of changes between low and high dose exposures was not performed, animals exposed to the higher dose of pyridostigmine appeared to possess more obvious and extensive alterations. In general, longer exposure resulted in continued invasion of primary synaptic clefts by Schwann cells (fig. 11), regional withdrawal of some nerve terminal membranes (fig. 12), and complete absence (withdrawal) of some nerve terminal portions as evidenced by vacant positions adjacent to postsynaptic folds (fig. 11). Membrane fragments near the crests of folds indicated degeneration of the missing segments of nerve terminal. Finally, NMJs with many terminal branches that appeared morphologically similar to those from control preparations sometimes possessed a single branch that exhibited drug-induced alterations (fig. 11).

Postsynaptic Effects of Acute and Subacute Exposure to Pyridostigmine. At low acute doses (0.0036 and 0.036 mg/kg) minimal alteration of postsynaptic organelles and myofibrils was detected (figs. 13 - 15). Occasional fibers possessed some swollen mitochondria and sarcoplasmic reticulum elements (fig. 13). No other changes were observed. All higher acute doses consistently produced subjunctional damage in all fibers analyzed. Following an acute injection of 0.36 mg/kg, mitochondria displayed alterations ranging from matrix rarefactions to matrix dissolution (figs. 16 & 17). Subjunctional endoplasmic reticulum and nuclei were also affected. These organelles displayed swelling of cisternae and the nuclear envelope, respectively (fig. 17). At this dose, sarcomeres often lacked distinct striation patterns and the z-lines frequently lacked typical uniformity (fig. 16). These abnormalities were pronounced in the immediate subjunctional region but decreased in severity and finally disappeared with increasing distance from the NMJ.

Acute doses of 2.6 mg/kg or greater resulted in dramatic changes in end-plate morphology of many fibers. The most severe postsynaptic damage included complete disruption of mitochondria and other membranous organelles and extreme supercontration of the sarcomeres (fig. 18). In cases of extreme supercontraction, synaptic components were pushed out of the normal muscle contour, z-line morphology was obliterated and the organized orientation of thick and thin filaments was lost (figs. 18 & 19). These changes to sarcomere structure, as well as those to membranous organelles, were graded with distance from the junction. At the margins of the muscle cell damage, disrupted myofibrillar components blended into normal sarcomeres (fig. 19).

In comparison, even high dose subacute exposure to the drug produced less severe postsynaptic changes. Swelling of subjunctional membranous organelles was minimal when present (fig. 20), but was generally absent even following exposure to 20 mg of pyridostigmine over a 14-day period (fig. 21). Disruption of myofibrillar components was observed in a few fibers (fig. 20). When present, the sarcomere damage was limited to a small subjunctional region (fig. 20) or to small, randomly restricted regions of non-subjunctional sarcomeres in contrast to the extensive areas affected by high acute doses. Although myofibrillar disruption by subacute dosage was not widespread or frequent, drug effects on sarcomere length were still obvious (fig. 21). Subjunctional sarcomeres were often shorter in length (arrowheads, fig. 21) than sarcomeres in control muscles or sarcomeres distal to the affected NMJ (fig. 21, lower right) or sarcomeres from adjacent muscle fibers (fig. 21, left). Thus, even fibers which appeared minimally altered revealed some pyridostigmine-induced effects with the most notable changes being localized to the immediate subjunctional region.

Recovery from Acute and Subacute Pyridostigmine Exposure. A limited number of NMJs at two recovery intervals have been analyzed in a preliminary assessment of the recovery of pre- and postsynaptic NMJ components following acute and subacute drug exposures. Seven days following a single injection of

2.6 mg/kg pyridostigmine, NMJ morphology was variable. Some NMJs demonstrated degenerative phenomena (fig. 22) not observed in tissues gathered 30 minutes after a single injection. Specifically, abundant debris was present in the primary and secondary synaptic clefts which indicated degeneration of junctional folds and/or presynaptic elements (fig.22). In addition, profuse Schwann cell processes were present and some nerve terminal portions were widely separated from the junctional folds. Pre- and postsynaptic membranous organelles appeared minimally affected. In contrast, other NMJs subjected to identical exposures reflected minimal damage to postsynaptic myofibrillar components and minimally alterd pre- and postsynaptic spatial relationships.

Seven days of recovery from a 14-day subacute exposure to 20 mg of pyridostigmine revealed NMJs with no additional and many fewer residual drug effects than acute dosing. NMJS recovering from subacute exposure had few abnormal pre- or postsynaptic membranous organelles (figs. 24 & 25). However, Schwann cell processes remained abundant between some nerve terminal branches and remained in the primary synaptic clefts of some NMJs (fig. 25). Other NMJs and potions of NMJs were free of structural alterations in the synaptic region (figs. 24 & 25).

Twenty three days of recovery from a 14-day subacute exposure to 20 mg of pyridostigmine revealed only a few NMJs with a structural abnormality (fig. 27) and many with no apparent residual affects of the drug (fig. 26). The pre- and postsynaptic cellular compartments of all NMJs analyzed were normal in appearance. Only in the spatial relationship of the pre- and postsynaptic elements were residual drug affects noted. Thus, even with 23 days recovery, some NMJs retained Schwann cell processes in the primary synaptic cleft (fig. 27). Results from other recovery intervals following acute and subacute drug exposure are being analyzed.

DISCUSSION

Administration of pyridostigmine, in quantities capable of producing a short- or long-term whole blood ChE depressions of approximately 10% or more resulted in morpological alterations to rat diaphragm NMJs. Both low and high doses of acute and subacute exposure induced changes in NMJ organelle ultrastructure and/or alterations in the spatial relationships of the presynaptic nerve terminal to the overlying Schwann cell and to the postsynaptic folds. It was clear that the severity of both types of ultrastructural modifications were dose dependent within a given experimental regimen (i.e., acute exposure via injection or subacute exposure via osmotic pump). Comparison of NMJ morphology following injections of 0.0036 mg/kg pyridostigmine (10% ChE depression: figs. 3, 16, 17) and 2.6 mg/kg pyridostigmine (70% ChE depression: figs. 6, 18, 19) revealed greater damage following the larger acute dose. Similarly, comparison of low and high subacute doses of drug revealed less extensive alterations following exposure

to 1.5 mg/ml pyridostigmine (25% ChE depression: figs. 9, 10) than exposure to 10 mg/kg of drug (65% ChE depression: figs. 11, 12, 20, 21).

Similar ChE depressions produced at low doses by different methods of administration (i.e., acute injection versus subacute infusion) resulted in alterations of the same type. An acute injection of 0.036 mg/kg and subacute exposure of 1.5 mg/ml both produced ChE depressions of approximately 25% with correspondingly similar ultrastructural modifications (compare figs. 5, 14, 15 to 9, 10 respectively). In contrast, high acute doses had a significant impact on pre- and postsynaptic membranous organelles while high subacute exposures resulted in more prominent alterations to presynaptic geometry. For example, comparison of NMJs exposed to an acute dose of of 2.6 mg/kg (figs. 6, 18, 19) and a subacute dose of 10 mg/ml (0.4 LD50/day; figs. 11, 12, 20, 21) revealed more striking damage to pre- and postsynaptic organelles and myofibrillar apparatus following the acute exposure. On the other hand, changes in the geometric relationships of the nerve terminals with the adjacent Schwann and muscle cells were more notable after 7 or 14 days of subacute exposure to pyridostigmine or 7 days following the single acute exposure (fig. 22).

These observed differences in pathology resulting from acute or subacute dosing require us to consider what intrinsic differences in drug dosage regimen would be responsible since the resultant ChE depressions are similar. Inherent in the two modes of drug administration (i.e., single injection and osmotic minipump) are markedly different times to maximum enzyme carbamylation. Acute drug injection results in abrupt ChE inhibition which is maximum during the first 30 minutes following injection (L. Harris, personal communication). This is followed by normal decarbamylation of the enzyme during the period of recovery of ChE levels. In contrast, subacute exposure by minipump results in slow release of drug (5.9 µl/hr) and consequent slow rate of ChE carbamylation. Maximum depression occurs only after several hours with sustained depression for up to 14 days. Therefore, the severity of organelle damage following acute ChE depression may be related to the rate of ChE inhibition and attendant rapid physiological responses rather than the level of ChE depression.

With regard to organelle damage, the most notable change was expressed in the mitochondria as a rarefaction or disruption of the matrix. In addition, presynaptic vesicle profiles of unusual diameters and densities were present. These abnormalities could reflect an unusual level of mitochondrial activity and/or an irregularity in the membrane recycling/vesicle formation system. An alternative cause could be related to swelling due to shifts in ion concentrations. Such ionic imbalances could be influenced by direct action of the drug on the presynaptic membrane or by unusually high levels of acetylcholine (ACh) in the primary cleft. Either condition could presumably alter presynaptic structure and function. A definitive answer can not be formulated from these data. However, it is important to note that other studies have shown that some anticholinesterase agents can alter function by inducing presynaptic activation.

Dramatic changes in geometric relationships of the cells, such as elimination of nerve terminal branches apparently require more than 30 minutes to be accomplished. Thus extensive changes of this nature were observed only after 7-14 days of subacute exposure to pyridostigmine or after several days following an acute exposure (fig. 22). Indeed, analysis of acutely treated animals in an extended time frame (7-14 days postinjection, i.e., recovery) also reflected extensive alterations in cellular geometric relationships (fig. 22). This observation supports the suggestion that the severity of altered spatial relationships may be a function of time.

For purposes of discussion, several possible reasons for the two types of drug-induced morphological presynaptic alterations (i.e., organelle versus spatial relationship) can be proposed. An initial possibility is that pyridostigmine may have direct chemical actions on the terminal membranes. We cannot presently contribute to the discussion of this idea. A second possibility is that pyridostigmine generates terminal activation and antidromic axonal firing akin to the effects of paraoxon (Laskowski and Dettbarn, 1975). This drug action has been reported for other ChE inhibitors. In a study of the effects of ChE inhibition on the mammalian NMJ, paraoxon induced presynaptic activation which was demonstrable with physiological and morphological techniques. Paraoxon-induced presynaptic effects are manifest electrophysiologically as antidromic activation and increased miniature endplate potential frequencies and amplitudes (Laskowski and Dettbarn, 1975). Morphologically the effects of paraoxon are demonstrated by decreased numbers of synaptic vesicles and increased amounts of clathrin coat material as well as increased numbers of coated pits, coated vesicles and elongated cisternae (Laskowski et al., 1975). Since, in the present study only occasional NMJs exhibited characteristics associated with nerve hyperactivity, no substantive morphological evidence suggests presynaptic activation by pyridostigmine. Since both paraoxon and pyridostigmine inhibit junctional ChE, the differences in their presynaptic action at the morphological level is unresolved.

As a third possibility, nerve terminals may withdraw as a result of attempted neuronal autoregulation of agonist levels which is followed by appropriate Schwann cell reaction. It is obvious that carbamylation of acetylcholinesteras (AChE) by pyridostigmine can create levels of acetylcholine in the cleft of the NMJ which are greater than normal and possibly greater than that which can be induced by high frequency nerve stimulation. The question arises, does the nerve terminal attempt to autoregulate the levels of junctional agonist by altering normal junctional morphology? Clearly, prolonged nerve stimulation produces little alteration of the normal nerve-muscle cell contact region (Heuser and Reese, 1973). However, junctional ACh levels in stimulation experiments might not be as high as those resulting from AChE inhibition. While it is beyond the scope of the present observations, the hypothesis of autoregulation can be tested morphologically bu utilizing nerve-muscle preparations exposed to high levels of ACh with and without AChE inhibition.

A fourth idea concerning presynaptic alteration deals with the possible loss of trophic relations (Brimijoin, 1983; Guth, 1968; Massoulie and Bon, 1982) between junctional AChE and the nerve terminal; i.e., normal levels of junctional AChE may be required to maintain the normal cell-to-cell contact. Perturbation of the trophic relationship would be followed by nerve terminal withdrawal coincident with or followed by a reaction of the Schwann cell. A corollary to this fourth idea is that the Schwann cell initiates the alteration due to its reaction to inhibition of the AChE inserted in the junctional basal lamina, a structure which the Schwann cell shares with the muscle cell. An argument against this notion is the observation that there are regions of normal nerve terminal apposition and regions of terminal withdrawal devoid of intervening Schwann cell fingers.

To complete the discussion of possible reasons for pyridostigmine-induced neuromuscular pathology, the postsynaptic effects need to be considered. Since all ChE inhibitors studied to date can cause myopathy, commonality in the etiology of the pathology may be inferred. Indeed, Salpeter and co-workers (1982) have proposed an attractive hypothesis for the cause of ChE inhibitor-induced myopathies. They argue that the myopathy may be an agonist-induced phenomenon. Thus, excess agonist (ACh) caused by enzyme (AChE) inhibition causes calcium ion imbalances in the subjectional muscle cell which in turn trigger calcium-activated proteases. These proteases, then, would be responsible for the disruption of the z-lines and myofibrils that is typical of the drug-induced myopathy. While there is evidence to support the Salpeter hypothesis, the experimental evidence is indirect and not altogether compelling. Studies have been initiated to examine calcium ion distribution in NMJs from pyridostigmine treated animals utilizing electron probe analysis.

As is the nature of ultrastructural analyses, we can not approximate the percentage of muscle fibers in the diaphragm that were affected. However, all acute and subacute doses employed induced alterations in every NMJ analyzed. This suggests that the neuro- and myopathies were common under the drug conditions studied. On the other hand, a consistent feature of the damage was the variation in severity which was present within a NMJ and between NMJs from a single muscle. This is not a unique phenomenon since such variation is commonly observed in mammalian NMJs affectd by myasthenia gravis (Engel and Santa, 1973), interrupted axoplasmic transport (Hudson et al., 1984), or NMJs undergoing normal synaptic turnover (Cotman et al, 1981; Mark, 1980). Assuming that sampling bias caused by electron microscopic examination of only a small portion (several thin sections) of each NMJ from a limited number of fibers is not the reason for the observed variability, the mechanism underlying variable effects could be attributed to a number of factors including localized differences in drug concentration, differences in the distribution of AChE, differences in the innervating axon, and/or differences in muscle fiber type. While variability in the drug-induced effects exist, at every dose, under both drug administration conditions, the overall effect was that the percentage of nerve terminal membrane in close apposition with junctional fold creasts decreased contact with junctional folds decreased.

RECOMMENDATION

While the precise reason underlying the variation in structural modification is not within the realm of this study, the presence of the variable morphological alteration is of great importance if one attempts to predict the pathophysiological effects of pyridostigmine in relation to dose and duration of exposure. It is not altogether clear that morphological effects of pyridostigmine could or should be manifest in results from standard electrophysiological studies (e.g., Tiedt et al., 1978) of drug treated muscles or muscle fibers. Since Gillies and Allen (1977) have reported pyridostigmine-induced muscle weakness in behaving rats, it is tempting to predict that the structural effects of the drug would be most apparent in exercising animals or in muscles from physically stressed subjects.

Thus, it is recommended that a series of experiemtns (similar to these described herein) be designed to assess the acute and subacute effects of pyridostigmine on excersized animals. A study of this nature could reveal functional and/or behavioral alterations not manifest in rested animals. Such data would provide a more substantial base for predicting pyridostigmine effects on military personnel functioning under conditions of stress.

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Appendix

Figure 1. Preterminal myelinated axon and neuromuscular junction from an acute control rat diaphragm. This electron micrograph illustrates the relationship between an innervating myelinated axon (ax) and the junctional folds (jf) of a muscle cell from an animal injected with Mestinon-equivalent buffer 30 minutes prior to fixation. The myelin sheath ends with typical paranodal loops (pn) bounding the terminal heminode. The nerve terminal (nt) of this axon possesses mitochondria with normal matrices and numerous clear synaptic vesicles of uniform diameter. The 50 nm primary synaptic cleft (bounded by arrowheads) has an evenly distributed basal lamina. The nonsynaptic surface of the nerve terminal is overlain by Schwann cell (sc) processes which terminate at the margins (arrowheads) of the primary synaptic cleft.

Figure 2. Neuromuscular junction from a control rat subacutely exposed to Mestinon-equivalent buffer for 14 days prior to fixation. The relationship of the nerve terminal (nt), Schwann cell (sc) and postsynaptic junctional folds (jf) appears normal and comparable to the acute control junction illustrated in fig. 1. This electron micrograph illustrates several cellular features of the subjunctional region. The muscle cell nucleus (n), sarcomere myofibrillar thick and thin filaments (mf), dense z-line (z) and subjunctional folds (jf) appear normal. The nerve terminal is evenly apposed to the junctional folds and separated by the typical 50 nm primary cleft (bounded by arrowheads) which contains a typical basal lamina. Note that the overlying Schwann cell processes (sc) do not enter the primary cleft (arrowheads).


1
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nt
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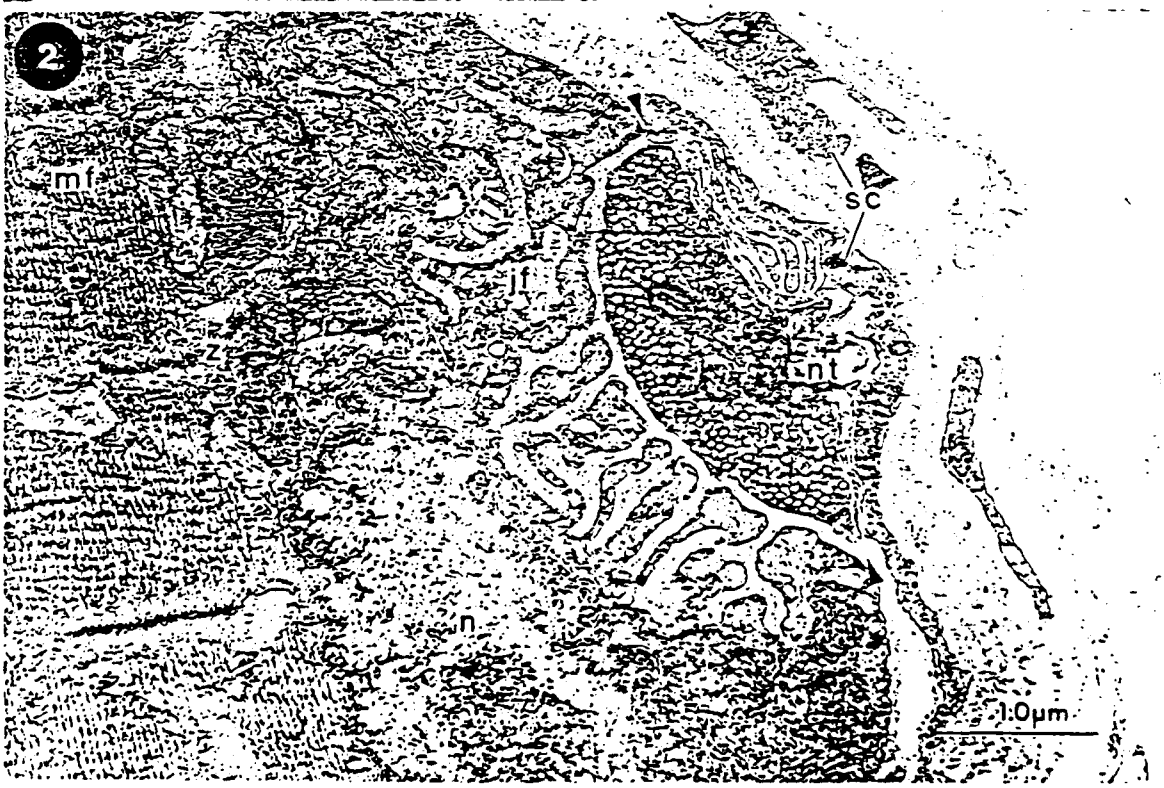
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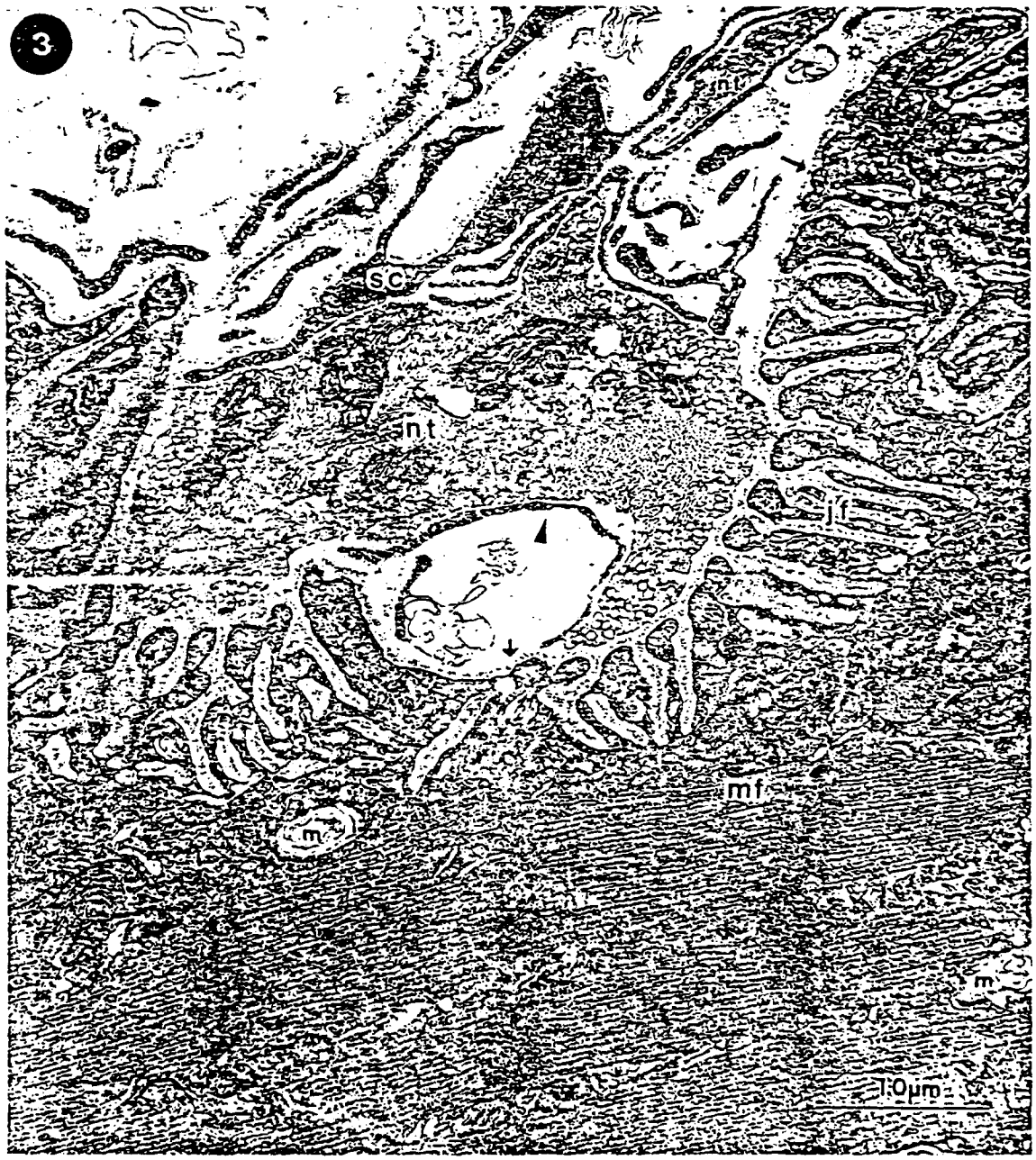
Figure 3. Acute dose-response: Presynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (0.0036 mg/kg; 0.001 LD50; 10% whole blood ChE depression). This electron micrograph illustrates the effects of the lowest drug dose used in the study. Even at this dose, the nerve terminal morphology and spatial relationship of the nerve terminal (nt) to the junctional folds (jf) are altered. Within the nerve terminal, only the mitochondria appear effected with rarefied areas in the matrix of some. The other organelles of the terminal appear normal including the synaptic vesicles which remain clear and normally sized. Regional withdrawal of the nerve terminal from the junctional folds is evident in this electron micrograph (between arrowhead & small arrow). At upper right (between asterisk & open asterisk), an area of junctional folds is devoid of nerve terminal and invaded by Schwann cell processes (sc). Also at upper right, a portion of nerve terminal (nt) is completely sequestered by a Schwann cell process. Where the nerve terminal has withdrawn, normal basal lamina remains (small arrows). Postsynaptically, the myofibrillar apparatus (mf) of the muscle cell appears mostly normal although some mitochondria (m) have been effected in this cell. This electron micrograph represents the greatest damage seen at the lowest drug dose.


3
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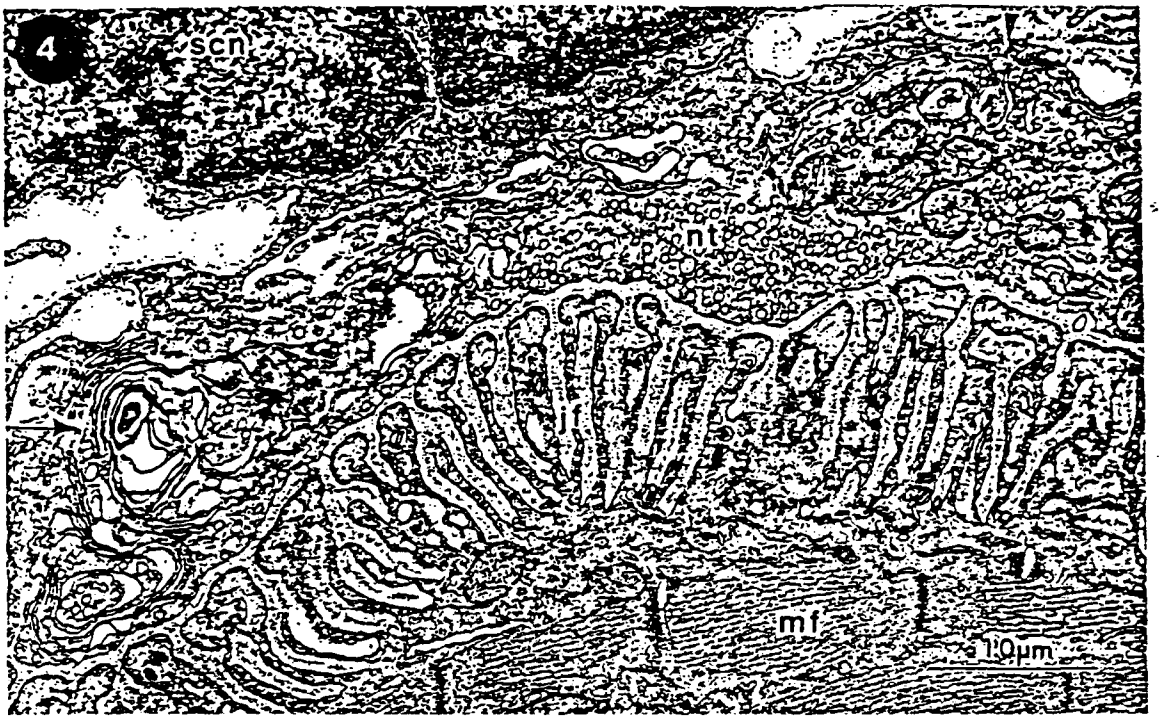
1.0μm

Figure 4. Acute dose-response: Presynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (0.036 mg/kg; 0.01 LD50; 25% whole blood ChE depression). While the postsynaptic morphology in this electron micrograph exhibits normal junctional folds (jf) and myofibrillar apparatus (mf), the presynaptic ultrastructure is altered. The nerve terminal (nt) is normally apposed to the junctional folds over much of this field. At the asterisk, however, there is evidence of terminal withdrawal from the junctional folds. Damage appears in the form of multiple membranous layers (arrow) some of which sequester vesicle containing nerve terminal portions (see right of arrows). Abnormally large diameter vesicles are evident in the nerve terminal. The arrowhead points to a Schwann cell process which has invaded the nerve terminal from the nonsynaptic side.

Figure 5. Acute dose-response: Presynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (0.36 mg/kg; 0.1 LD50; 55% whole blood ChE depression). All compartments in this neuromuscular junction are obviously effected at this dose. The nerve terminal (nt) contains altered mitochondria (m) and abnormal vesicular inclusions. The nerve terminal is withdrawn from various regions (asterisks) of the junctional folds while in other areas normal synaptic morphology is preserved. In regions of withdrawal vesicular debris, Schwann cell processes and small nerve terminal profiles are evident. Postsynaptically, the mitochondria are most obviously effected.


4
scn
nt
jf
mf
1.0μm

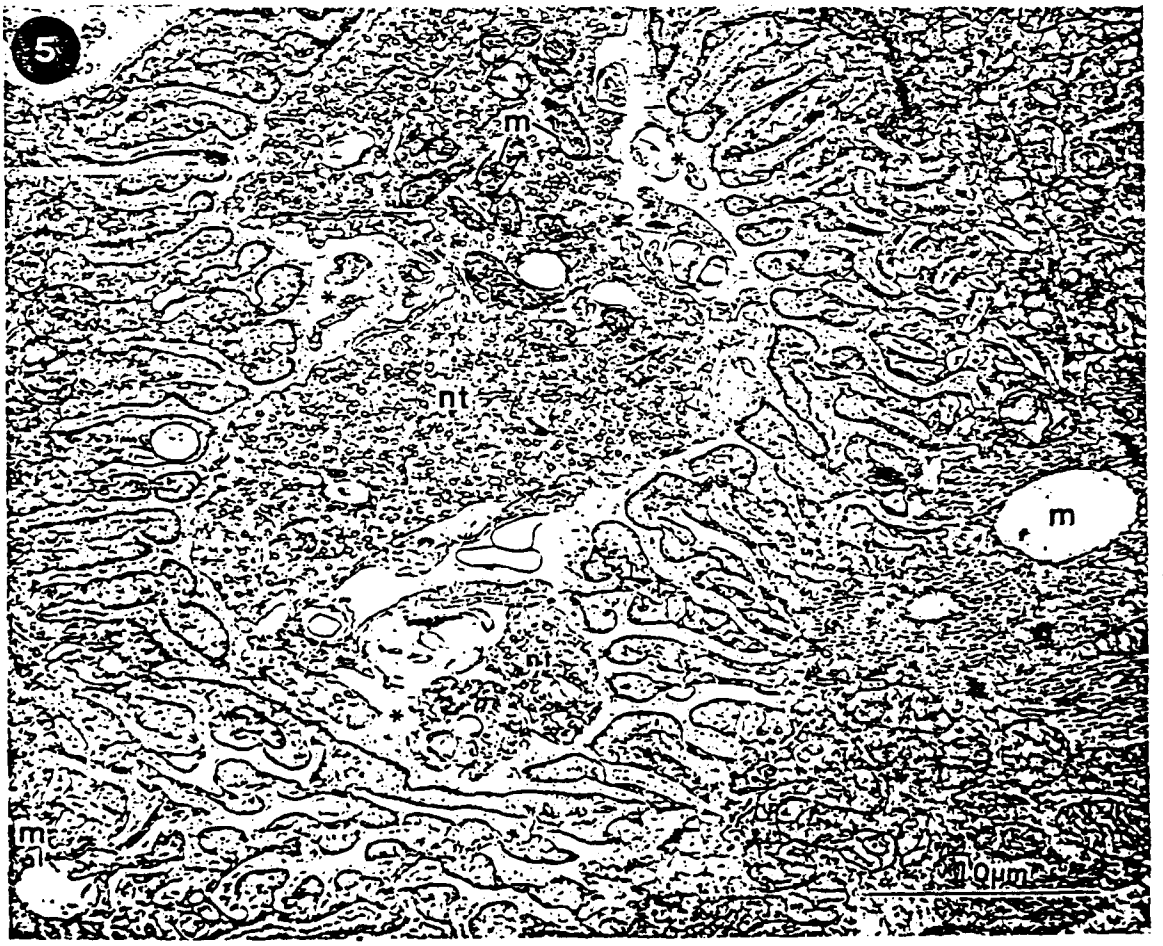

5
m
nt
m
m
1.0μm

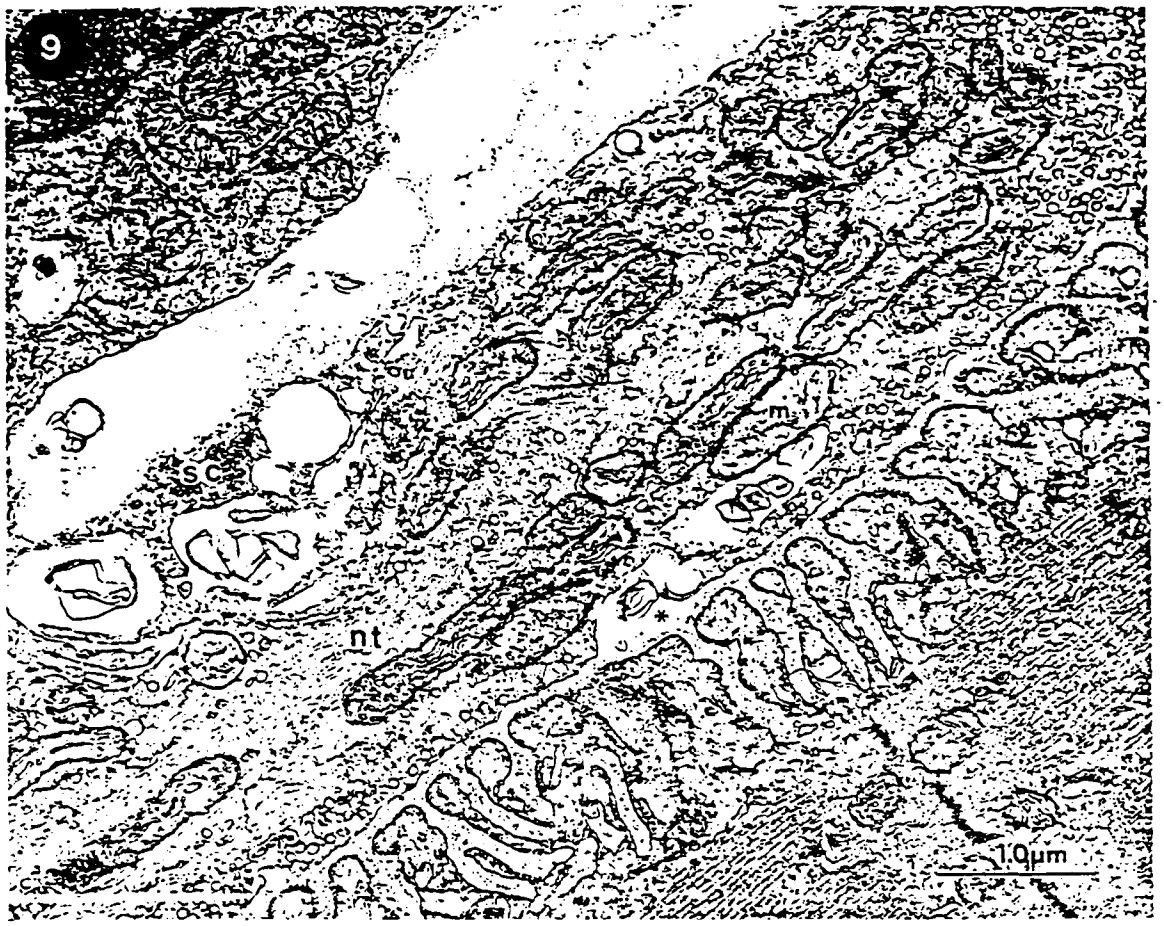
Figure 6. Acute dose-response: Presynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (2.6 mg/kg; 0.72 LD50; 70% whole blood ChE depression). A high magnification electron micrograph illustrates altered presynaptic mitochondria (m) and a Schwann cell process (asterisk) separating the pre- and postsynaptic membranes. An occasional membrane bound inclusion (arrowhead) is present in the junctional folds.

Figure 7. Acute dose-response: Presynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (0.036 mg/kg; 0.01 LD50; 25% whole blood ChE depression). An electron micrograph to illustrate the sequestration of nerve terminal (nt) by Schwann cell processes (arrowheads). These abnormally sequestered vesicle-containing profiles appear different as a function of plane of section (e.g., see dotted line in fig 8).

Figure 8. Acute dose-response: Presynaptic effects of pyridostigmine on rat diaphram neuromuscular junction with fixation 17 minutes after a single, subcutaneous injection of drug (3.6 mg/kg; 1.0 LD50; 30% whole blood ChE depression). This figure illustrates 1) alterations similar to those in figs. 3-7 occur relatively rapidly after drug administration; 2) that there is not always more damage with higher dose; and 3) the Schwann cell sequestration of nerve terminal portions is a consistent feature of the drug-induced alterations. In this electron micrograph, a Schwann cell process has invaded the primary synaptic cleft (two arrowheads at bottom). The nerve terminal (nt) contains abnormal vesicular profiles and has withdrawn from the junctional folds (asterisk). The nonsynaptic side of the nerve terminal has been invaded by a Schwann cell process (three arrowheads). The dotted line indicates a plane of section which would demonstrate a nerve terminal profile sequestered within a Schwann cell process similar to those seen in figs. 4 and 7.

6
E
1.0μm
7
nt
0.5μm
8
nt
0.5μm

Figures 9 and 10. Subacute dose-response: Presynaptic effects of a 2-day exposure to pyridostigmine on rat diaphragm neuromuscular junction (0.43 mg total drug exposure; sustained 24% whole blood ChE depression). Both electron micrographs illustrate the predominance of the drug-induced presynaptic alterations regardless of dosage method. Regional withdrawal (asterisks) of the nerve terminal (nt) from the junctional folds is apparent. Schwann cell processes (sc) have invaded both the nonsynaptic regions of the nerve terminal and the primary cleft (arrowheads, fig. 10). Presynaptic organelle alteration is variable as illustrated in fig. 9 by the difference in the matrices of the various mitochondria (m).


9
sc
nt
m
1.0μm

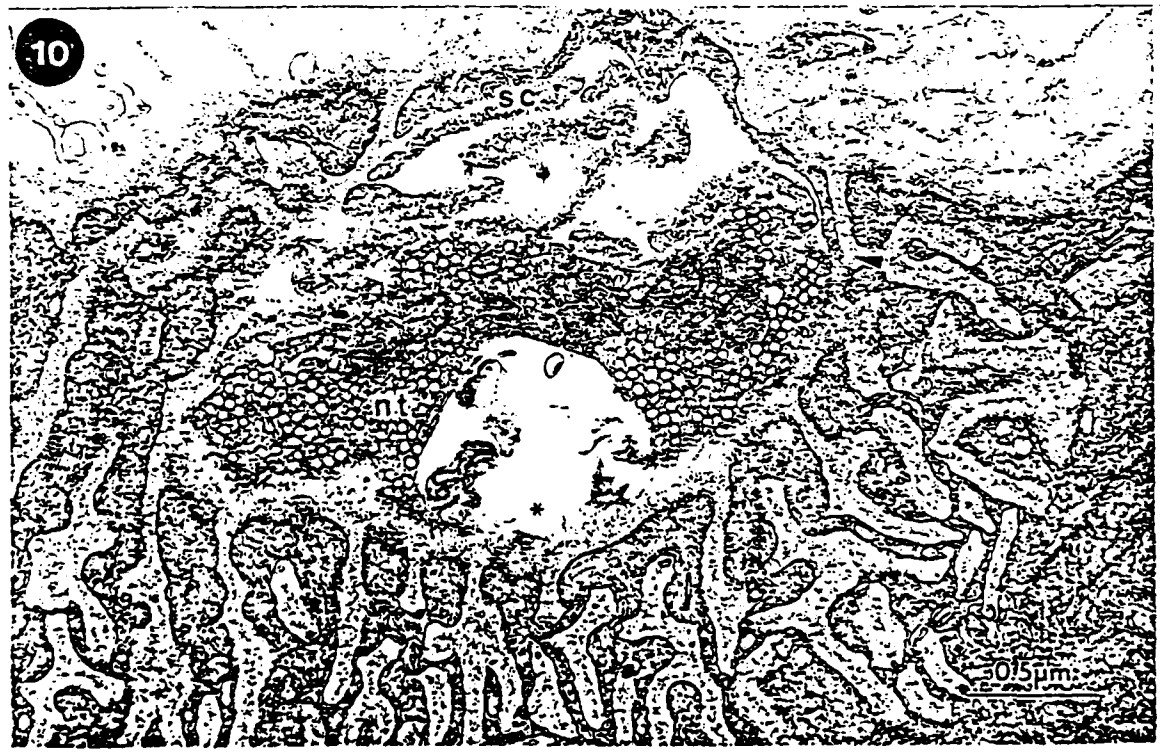

10
sc
nt
0.5μm

Figure 11. Subacute dose-response: Presynaptic effects of a 7-day exposure to pyridostigmine on rat diaphragm neuromuscular junction (10 mg total drug exposure; sustained 65% whole blood ChE depression). Both pre- and postsynaptic alterations are evident in this electron micrograph. A portion of the neuromuscular junction (between arrows) is devoid of nerve terminal while Schwann cell processes (arrowheads) have invaded another portion. The nerve terminal has abnormal vesicular debris and abnormal mitochondria. The subjunctional myofibrillar apparatus (mf) is disrupted to the point of complete disorganization in contrast to the junctional folds which appear relatively normal.

Figure 12. Subacute dose-response: Presynaptic effects of a 14-day exposure to pyridostigmine on rat diaphragm neuromuscular junction (20 mg total drug exposure; sustained 65% whole blood ChE depression). This electron micrograph illustrates gross presynaptic alterations with minor postsynaptic involvement. Schwann cell (sc) fingers have invaded the primary cleft and the nerve terminal has withdrawn (asterisks) from the junctional folds. Some nerve terminal mitochondria (m) are abnormal. The subjunctional myofibrillar apparatus (mf) is abnormally contracted. In this field, the muscle cell nucleus (n) appears unaffected.

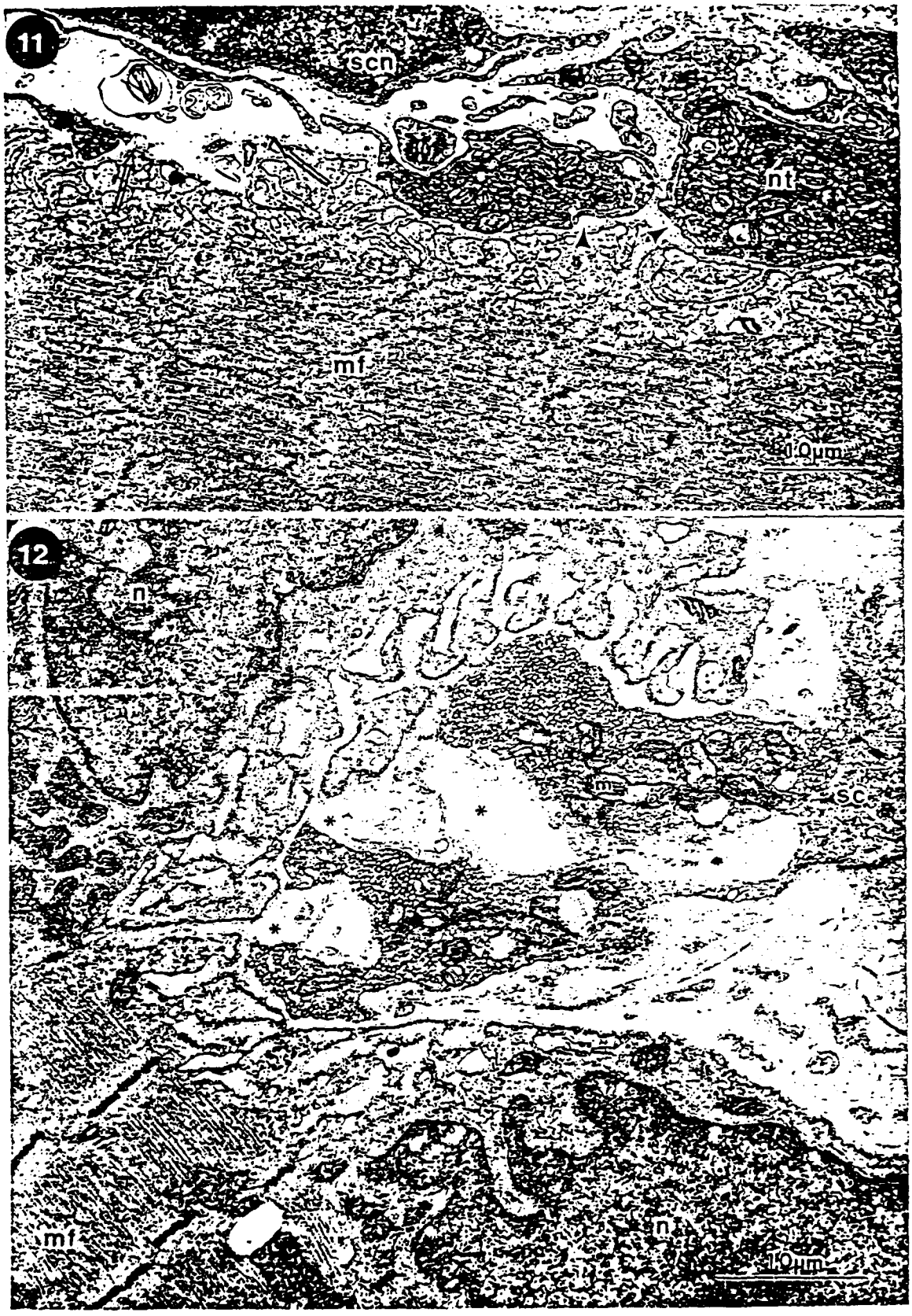
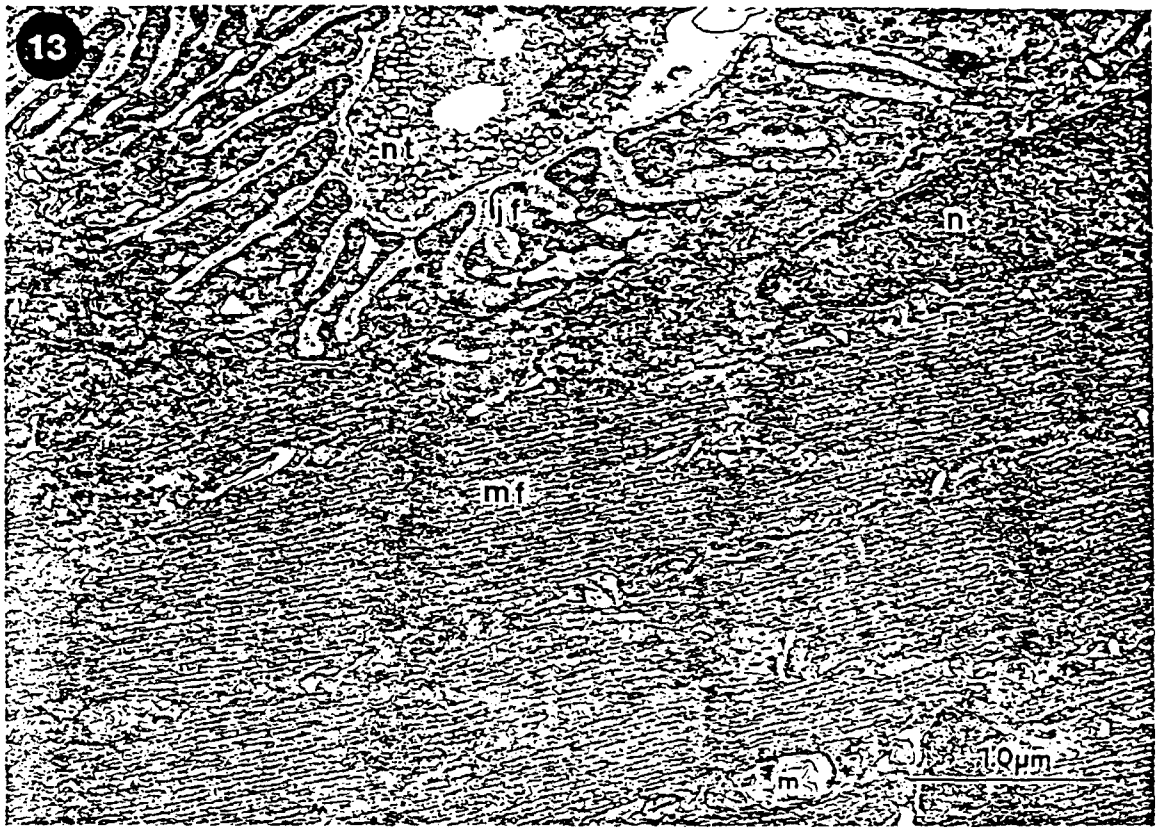
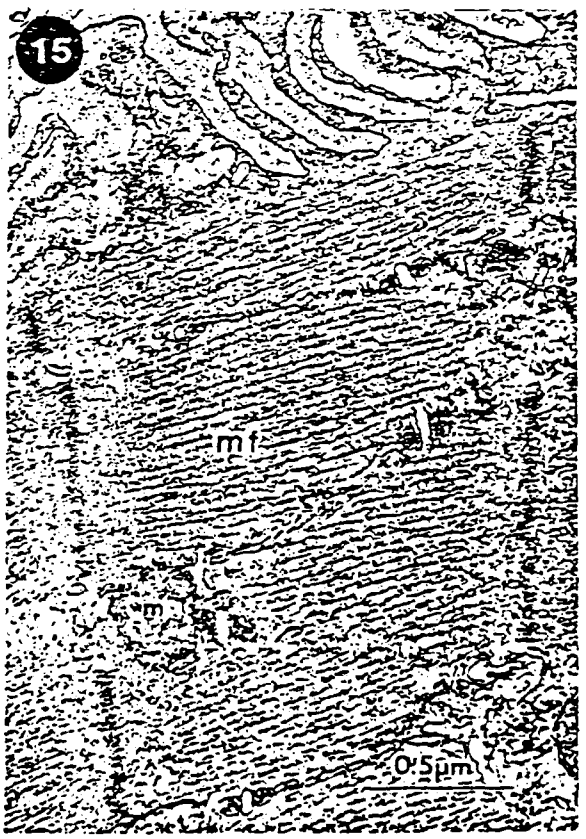

11
scn
nt
mf
1.0μm
12
n
sc
mf
n
1.0μm

Figure 13. Acute dose-response: Postsynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (0.0036 mg/kg; 0.001 LD50; 10% whole blood ChE depression). The postsynaptic effects at this dose are minimal and usually manifest as mitochondrial (m) alterations. As in fig. 3, nerve terminal (nt) withdrawal (asterisk) is present. The postsynaptic myofibrillar apparatus (mf) appears normal as does the muscle cell nucleus (n).

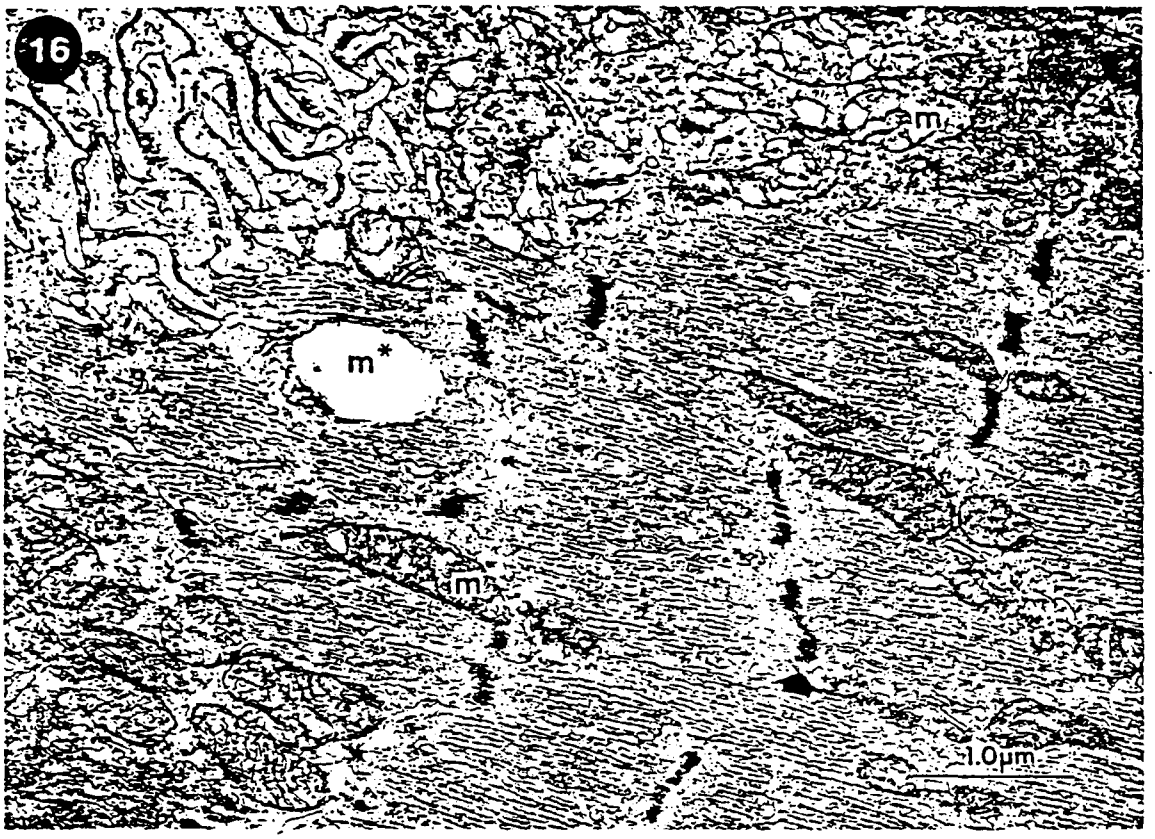
Figures 14 and 15. Acute dose-response: Postsynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (0.036 mg/kg; 0.01 LD50; 25% whole blood ChE depression). Low and high magnification electron micrographs illustrate relatively normal muscle cell morphology at a dose higher than that in fig. 13. A mitochondrion (m) in fig. 14 is labelled for orienting references to the same organelle in fig. 15. While some muscle cell mitochondria exhibit rarefied matrices, the sarcomere length and myofibrillar apparatus (mf) appear normal. As in fig. 4 the presynaptic compartment is altered with nerve terminal withdrawal (asterisk, fig. 14) and Schwann cell invasion of the primary cleft (arrowhead, fig. 14).

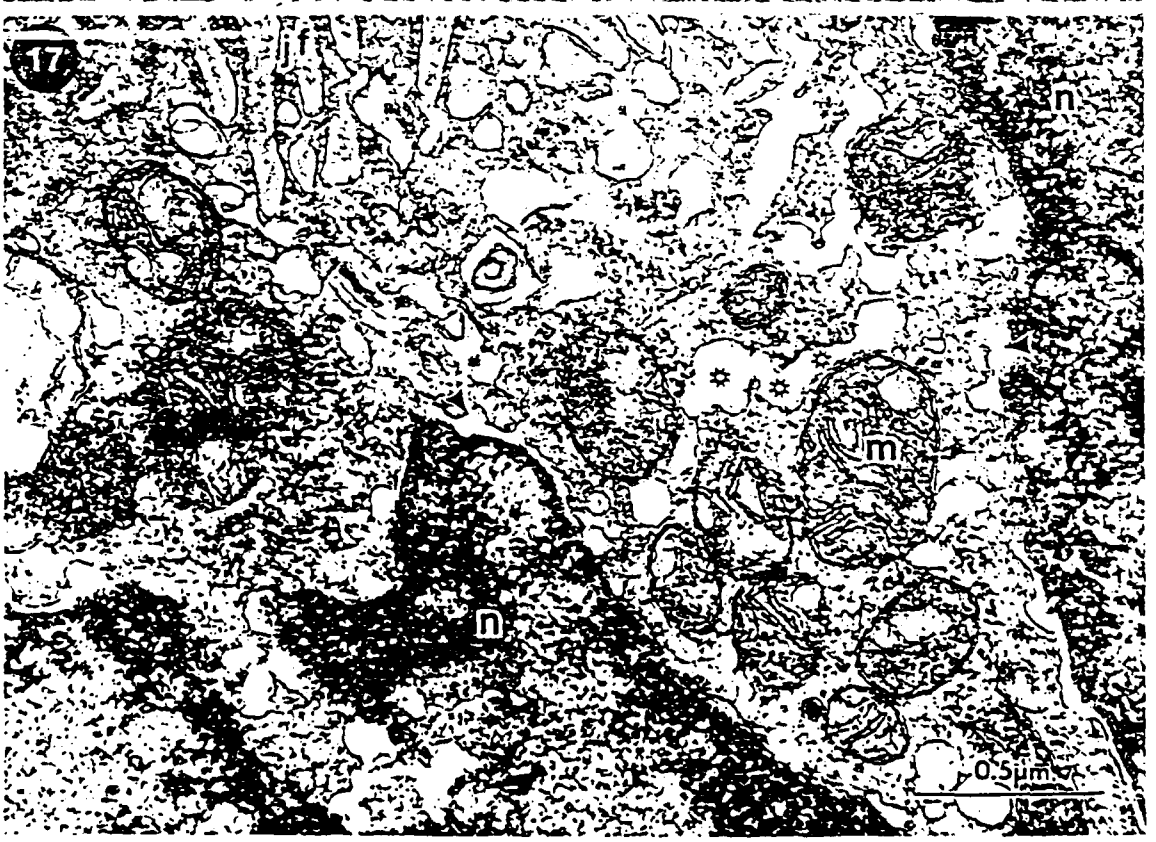

13
nt
f
n
mf
m
1·0μm


14
mf
1·0μm

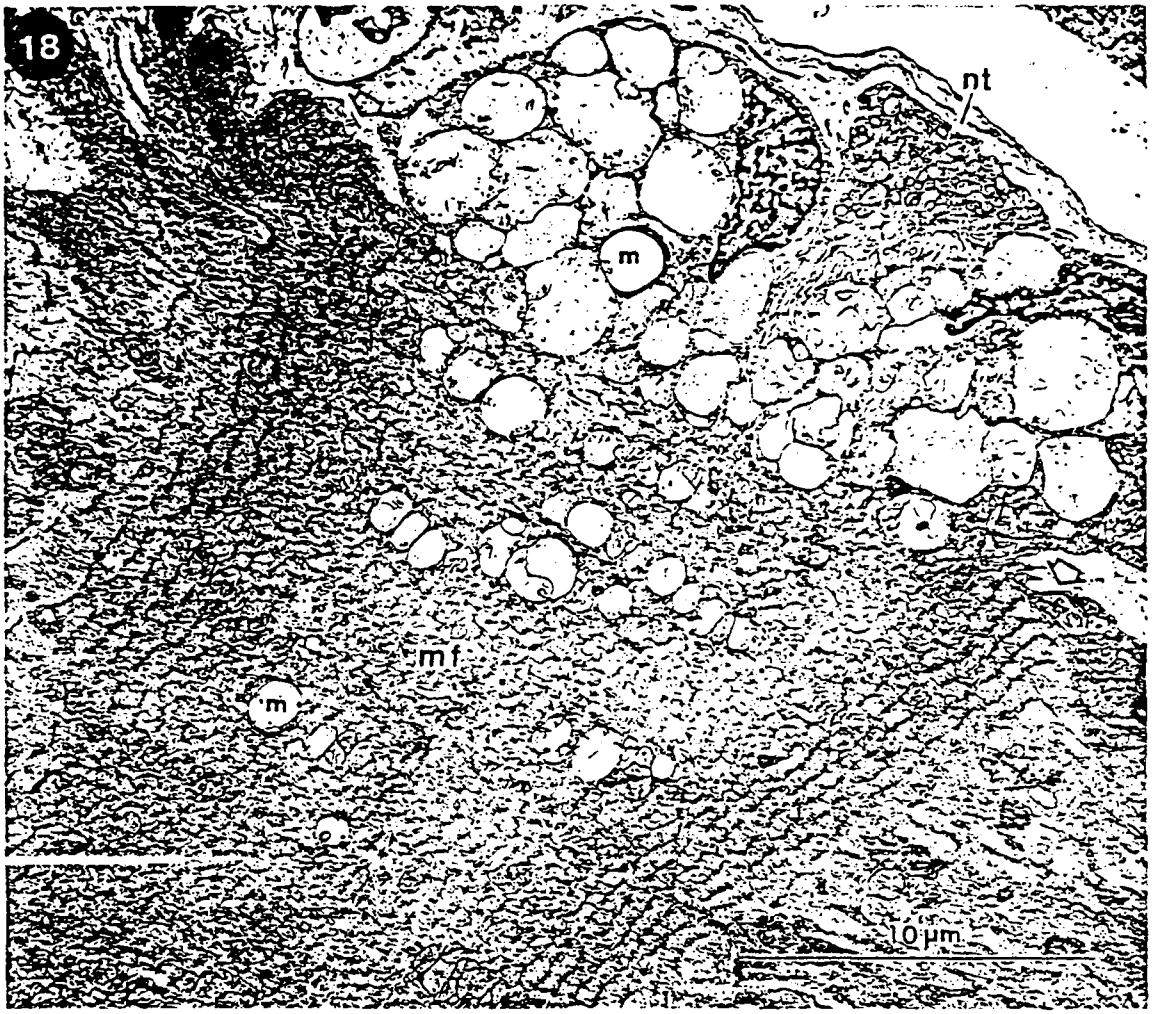

15
mf
m
0·5μm

Figures 16 and 17. Acute dose-response: Postsynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (0.36 mg/kg; 0.1 LD50; 55% whole blood ChE depression). Low and high magnification electron micrographs illustrating some of the most severe effects of this acute dose. Fig. 16 illustrates mitochondrial damage which varies from rarefaction of the matrix (m) to complete disruption (m). The subjunctional (note junctional folds, jf) z-lines begin to look abnormally diffuse (large arrow) at this dose. At higher magnification, fig. 17 illustrates alterations near the muscle cell nucleus (n). Abnormal mitochondria (m) and swollen endoplasmic reticulum (open asterisks) are evident. Note the swelling of the nuclear envelope (arrowhead) in this field.


16
m
m*
m
1.0μm


17
n
m
n
0.5μm

Figures 18 and 19. Acute dose-response: Postsynaptic effects of pyridostigmine on rat diaphragm neuromuscular junction with fixation 30 minutes after a single, subcutaneous injection of drug (02.6 mg/kg; 0.72 LD50; 70% whole blood ChE depression). The most severe postsynaptic alterations were seen with doses between 0.1 and 1.0 LD50. At 0.72 LD50, fig. 18 illustrates supercontraction (between open arrows) of the subjunctional sarcomeres with attendant gross mitochondrila (m) and myofibrillar (mf) damage. The nerve terminal (nt) and its subjunctional area have been pushed out of position in this field. The myofibrillar damage is graded with distance from the junction area. Fig. 19 illustrates gradation of the sarcomere damage in a non-subjunctional area. The mitochondria (m) in this field are relatively normal. From upper right to lower left, the myofibrillar apparatus (mf) and sarcomere (arrowheads) morphology ranges from completely abnormal to normal. Note the graded effect on z-line (z) ultrastructure.


18
nt
m
mf
m
1.0 µm

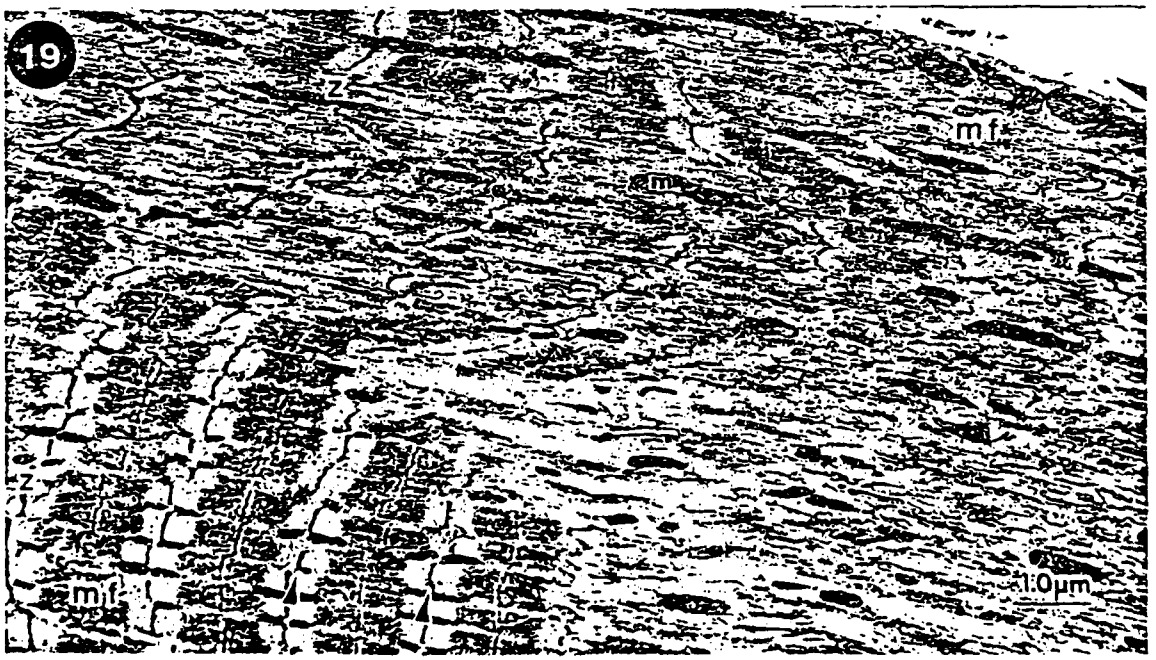

19
z
mf
m
z
mf
1.0µm

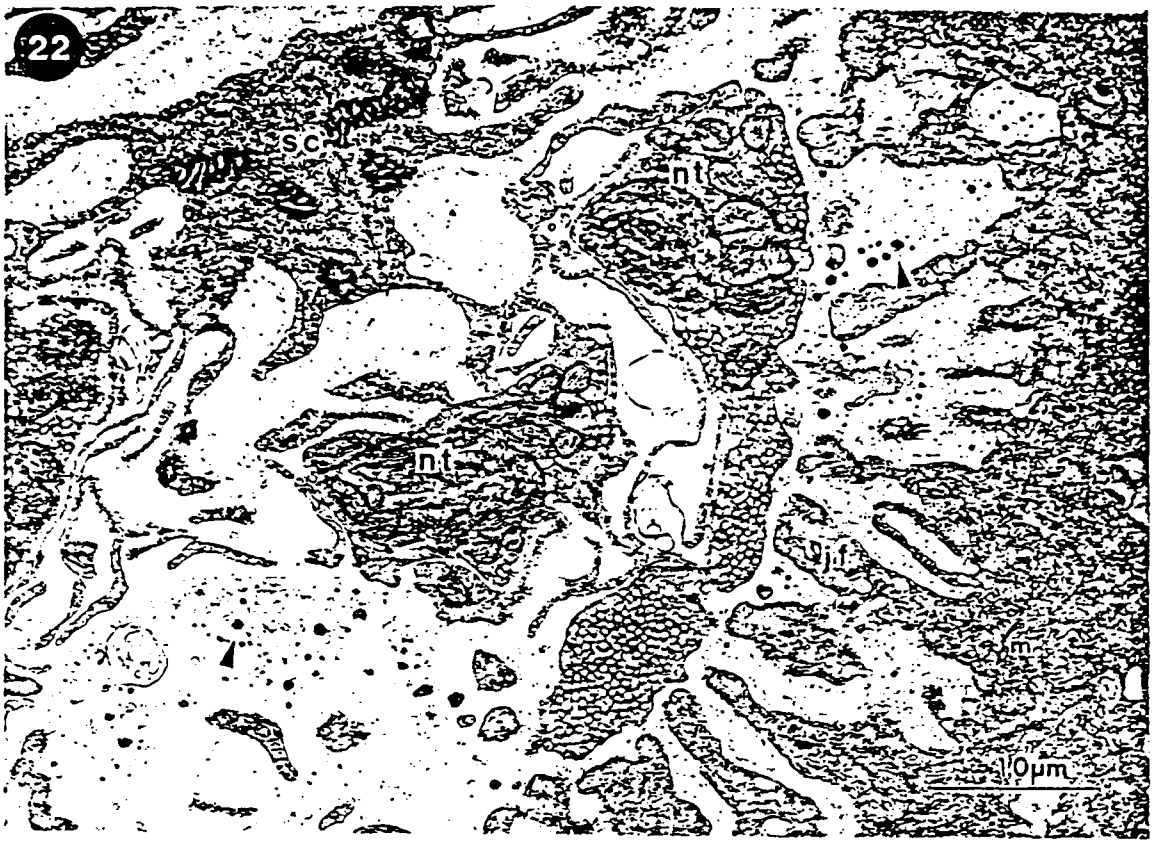
Figure 20. Subacute dose-response: Postsynaptic effects of a 7-day exposure to pyridostigmine on rat diaphragm neuromuscular junction (10 mg total drug exposure; sustained 65% whole blood ChE depression). This electron micrograph illustrates the most severe postsynaptic damage encountered with subacute dosing. The open arrows mark the boundary of myofibrillar (mf) damage which is manifest as disruption of sarcomere structure. Subjunctional sarcoplasmic reticulum appears slightly swollen (arrowhead). While the junctional folds (jf) appear normal, the relationship of the nerve terminal (nt) to the junctional folds is altered as described in fig. 11. This electron micrograph also illustrates that the drug induced damage is graded with distance from the junctional area.

Figure 21. Subacute dose-response: Presynaptic effects of a 14 day exposure to pyridostigmine on rat diaphragm neuromuscular junction (20 mg total drug exposure; sustained 65% whole blood ChE depression). This electron micrograph illustrates the variability in the nature and extent of drug induced damage when compared to fig. 20. While the two muscle cells in this field are relatively unaffected, note the typical nerve terminal (nt)/presynaptic alterations (see also fig. 12). Postsynaptically, the drug effect is manifest in subjunctional shortening of sarcomere length -- compare sarcomeres a, b & c (length = a-subjunctional, arrowheads < b- non-subjunctional, arrows at lower right < c- another cell, arrows at left).

20
nf
jf
mf
1.0μm

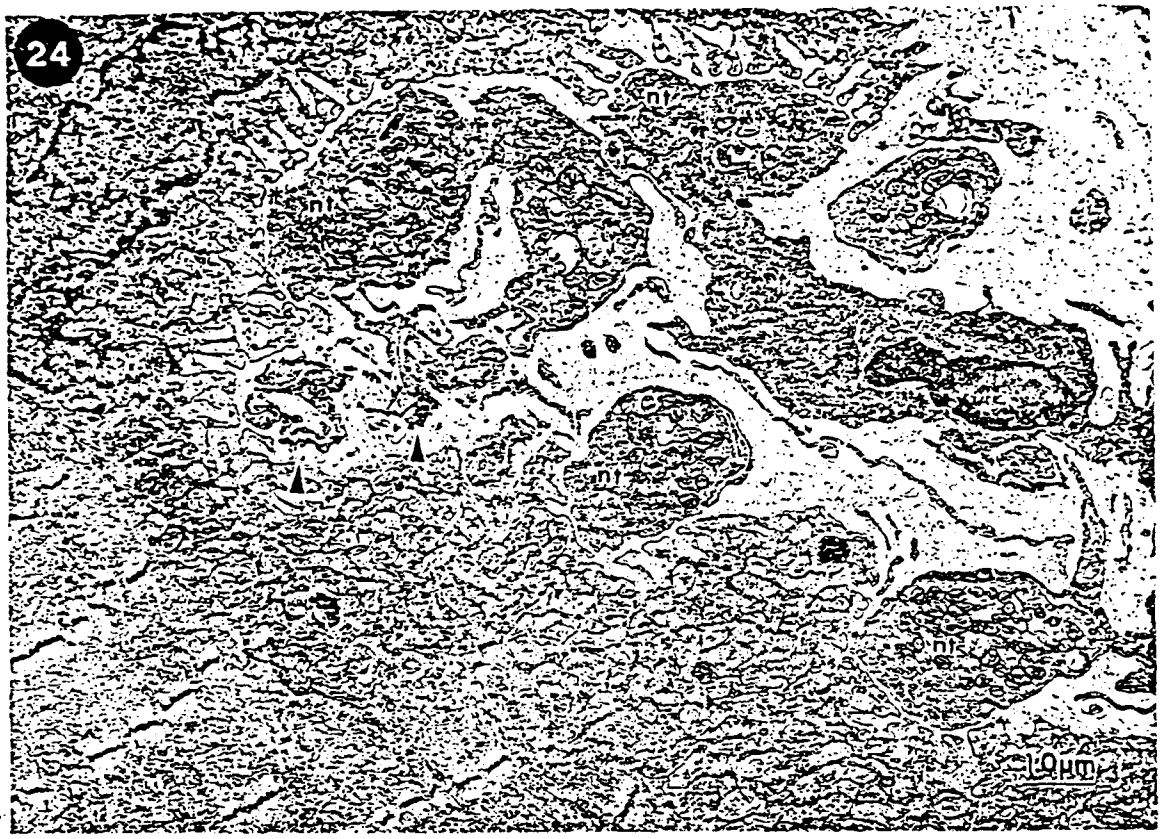
21
c
1.0μm

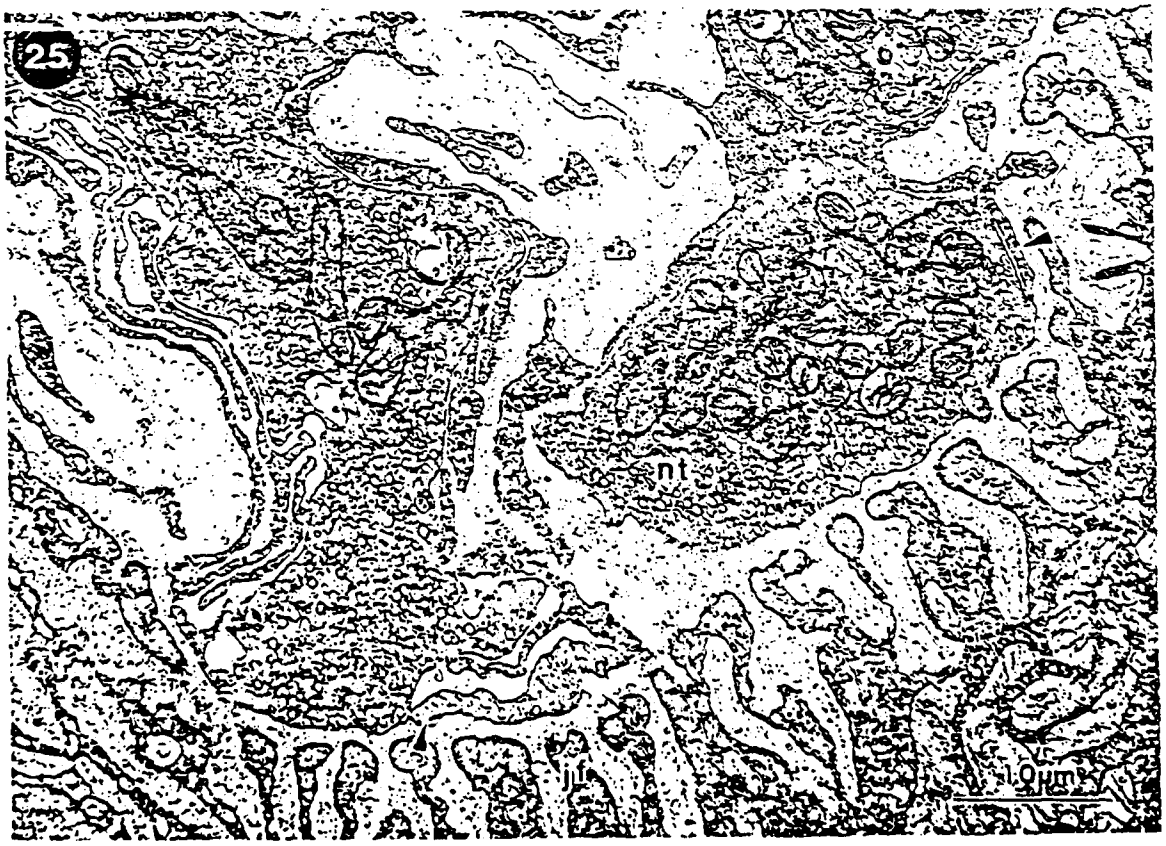
Figures 22 and 23. Acute dose recovery: Recovery of rat diaphragm neuromuscular junction from a single dose of pyridostigmine (2.65 mg/kg; 0.72 LD50; 70% whole blood ChE depression) administered 7 days prior to fixation. These electron micrographs illustrate the variability in the process of recovery following a single drug exposure. Unlike the 30 minute image in fig. 6, the drug effect shown in fig. 22 has progressed to include junctional fold (jf) breakdown, further withdrawal of the nerve terminal (nt) profile, profuse Schwann cell processes (sc), and abnormal debris (arrowheads) in the primary and secondary clefts. The remaining mitochondria are normal in appearance. This field is reminiscent of a neuromuscular junction from a human patient with myasthenia gravis. In contrast, fig. 23 illustrates a neuromuscular junction which is minimally abnormal under the same recovery conditions. While a Schwann cell process (arrowhead) can be seen in the primary cleft, the junctional folds (jf) appear normal (compare to fig. 22). The subjunctional myofibrillar apparatus (mf) is minimally altered with the z-lines being slightly diffuse.


22
sc
nt
nt
jf
1.0μm


23
nt
jf
mf
1.0μm

Figures 24 and 25. Subacute dose recovery: Seven days recovery of rat diaphragm neuromuscular junction from a 14-day exposure to pyridostigmine (20 mg total exposure; sustained 65% whole blood ChE depression). At low magnification, fig. 24, parts of the nerve terminal (nt) appear in normal apposition to the junctional folds while other parts are simply separated (asterisk) or completely altered (arrowhead). The subjunctional muscle cell has little alteration. Thus, seven days of recovery are not sufficient to completely reverse the presynaptic effects of subacute drug exposure. At a higher magnification, fig. 25, illustrates the lack of recovery of the presynaptic alterations. Schwann cell processes (arrowheads) are present in the primary cleft. While the nerve terminal is normally a continuous structure overlying a contiguous portion of junctional fold, the drug effects include partitioning of the terminal into smaller bouton-like endings separated by abnormal Schwann cell processes.


24
nt
nt
nt
nt
1.0μm


25
nt
1.0μm

Figures 26 and 27. Subacute dose recovery: Twenty three days recovery of rat diaphragm neuromuscular junction from a 14-day exposure to pyridostigmine (20 mg total exposure; sustained 65% whole blood ChE depression). The diaphragm neuromuscular junction in fig. 26 exhibits relatively normal junctional fold (jf) and myofibrillar apparatus (mf) ultrastructure. The overlying Schwann cell is in normal apposition to the nerve terminal (nt) in the nonsynaptic region with the exception of a region (arrowhead) between two nerve terminal profiles. Figure 27 illustrates the variability of recovery from subacute dosing. While the neuromuscular junction is not as fully recovered as that in fig. 26, the only remarkable alteration remaining is the interposition of Schwann cell processes (arrowhead) in the primary cleft. As in fig. 6, the myofibrillar apparatus is unaffected.


26
nt
nt
mf
1.0μm


27
nt
SC
nt
mf
1.0μm