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THE PHARMACOLOGY OF EXPERIMENTAL MYOPATHIES

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A large group of disorders affect striated muscle in man. The direct approach to the study of these diseases presents major difficulties. Biopsies can be useful in diagnosing a muscle disease and in confirming its progress. However, at a time when muscle weakness and other neurologic abnormalities become obvious, the myopathy is usually well advanced. It is also impossible to provide adequate controls to follow the development of the myopathy in man and its reversibility with experimental techniques. Consequently, a wide variety of animal models of human muscle diseases have been developed. These models include myopathies occurring spontaneously in highly inbred strains of mice, hamsters, goats, and other mammals. However, a new and rapidly expanding area of inquiry involves the pharmacologically induced myopathies developed with a wide variety of drugs. This review is concerned with this second area of research, that of pharmacologically induced experimental myopathies.

A survey of the literature of the past ten years reveals many attempts to reproduce pharmacologically human myopathies in animals. Three areas have been particularly active. These include experimental myotonia, experimental Duchenne's muscular dystrophy, and general neuromuscular disorders.

PHARMACOLOGICALLY INDUCED MYOTONIA

Of all the pharmacologic myopathies that have been attempted, experimental myotonia bears the closest resemblance to its human counterpart. When rats are injected with 2,4-dichlorophenoxyacetic acid or fed a diet containing 20,25-diazocholesterol, muscle fibers develop abnormal membrane parameters closely resembling those in patients with myotonia. A similar abnormality is observed in the myotonic goat.

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Similarities Between Human and Drug-Induced Forms of Myotonia

In man, myotonia is characterized by sustained contraction of muscle produced either by a voluntary contraction, percussion with an instrument, or electrical stimulation of the muscle (1, 2). The irritability of the muscle is markedly increased after insertion of a needle electrode. Electromyography reveals the typical "dive-bomber" bursting of muscle action potentials, but the myotonic effect is reduced by repeated contractions ("warm up period"). Myotonia is particularly evident in myotonia congenita which is an autosomal inherited disease (2).

Myotonic effects are produced experimentally by a wide variety of compounds including the monocarboxylic acids (3), cholesterol derivatives (4), veratrum alkaloids (5), acridine (6), several amines (7), and indoleacetic acid (8). Closer examination at the membrane level reveals that only a few of these agents, monocarboxylic acids and cholesterol derivatives, produce effects approximating those seen in myotonia congenita (3).

The effects of two drugs in particular have been examined in detail. These are 2,4-dichlorophenoxyacetic acid (2,4-D) and 20,25-diazocholesterol (20,25-D).

The Myotonic Effects of 2,4-Dichlorophenoxyacetic Acid

2,4-D is a monocarboxylic aromatic acid which was also a widely used weed killer (9). Accidental ingestion of this drug by animals and man has led to symptoms of myotonia (10). When given to rats (200 mg/kg i.p.), 2,4-D produces a myotonic electromyogram (EMG) pattern within 30 min (11). Similar effects are observed when normal diaphragm muscles are perfused in vitro with 2.5 mM 2,4-D (4, 12). These studies have revealed several major changes in muscle contraction produced by 2,4-D: (*a*) Within the first few minutes of exposure to the drug, the peak amplitude of a single contraction is increased severalfold. Shortly thereafter there is a ten to twenty fold increase in the duration of contraction. (*b*) These effects are augmented when a tetanic burst of stimuli, instead of a single pulse, is given to the muscle. (*c*) A conditioning or "warm up" stimulus reduces the myotonic effect of a subsequent stimulus. (*d*) The prolonged contraction is associated with a prolonged series of spontaneous repetitive muscle action potentials (12). These major observations produced in vitro by 2,4-D closely parallel the abnormal muscle contractions observed in human myotonia (2) and in the goat (13).

The Myotonic Effects of 20,25-Diazocholesterol

The second drug that induces a myotonic-like condition in rats is 20,25-diazocholesterol (20,25-D). This drug inhibits the enzyme desmosterol reductase (14), resulting in decreased plasma cholesterol and increased plasma desmosterol, a precursor of cholesterol (15). The myotonia-inducing side effects of this drug were first observed in patients being treated for hypercholesterolemia (16, 17). Muscle cramping and spasm, together with EMG patterns similar to myotonia, were reported. In the experimental animal model, the drug is given in the diet or through an esophageal tube for 5 to 8 weeks (4, 18). Several other cholesterol analogues including 25-azocholesterol can be injected s.c. for a similar period to produce the same effect (19–21). Like 2,4-dichlorophenoxyacetic acid, the 20,25-D animal model closely

parallels human myotonia with respect to abnormal contractile properties, as well as changes in electromyography.

Membrane Abnormalities in Experimental Myotonia

The underlying membrane abnormalities in both inherited and experimental myotonia are similar. Myotonic fibers of both man and the goat have an increased membrane resistance (3). In addition, the mean resting chloride conductance is significantly less than in normal fibers, while the potassium conductance is increased. However, muscle resting membrane potential was unaffected (3, 22). The myotonia produced experimentally in the rat with 2,4-D or 20,25-D displays similar changes in membrane cable properties (4, 23). There is an increase in specific membrane resistance and a fall in chloride conductance. Like inherited myotonia, muscle fiber resting membrane potential is normal or somewhat higher than normal (3, 4, 23–26). However, unlike human and goat myotonia, the myotonia produced by 20,25-D does not display increased potassium conductance (23). We have found no report on the effect of 2,4-D on potassium conductance. The effect on potassium conductance may depend on the type of myotonia-inducing drug employed since several monocarboxylic aromatic acids other than 2,4-D increase potassium conductance (3).

An abnormally low resting chloride conductance by itself could account for both increased membrane resistance and the spontaneous firing of action potentials (27, 28). When untreated rat diaphragm muscles were exposed to low chloride solutions, the muscles fibers became myotonic within 5 min (23). Membrane resistance increased with no change in potassium conductance. Spontaneous firing and the warm-up phenomenon (reduced myotonia with exercise) occurred during the first half hour. A short burst of stimulating pulses produced an augmentation and a prolongation of tension. Unlike the resting membrane potential in nerves, a major component of membrane conductance in skeletal muscle is chloride conductance. In frog muscles that do not display myotonia (3), chloride provides approximately 70% of the resting membrane conductance (29). Chloride is an even greater factor in mammalian muscle where it accounts for up to 85% of resting membrane conductance (30, 31).

Sites of Action of Myotonia-Inducing Drugs

The foregoing evidence indicates that the primary defect in inherited myotonia is an abnormally low membrane conductance to chloride ions, and that many of the properties of myotonic fibers can be reproduced in vitro with low chloride solutions or with various drugs. It appears that a wide variety of chemical agents with little structural similarity are all capable of producing a very specific effect, namely the reduction of chloride conductance. What is needed is a careful examination of the altered cable properties produced by each drug and a comparison of these effects with abnormal membrane characteristics in inherited myotonia. As previously mentioned, the monocarboxylic acids in general and 2,4-D in particular are capable of reproducing the myotonia-like condition in animals (3). On the basis of potency in blocking chloride conductance, Bryant & Morales-Aguilera (3) have determined the chemical requirements for agents that block the chloride channel and have sug-

gested a steric block of the channel. Much less is known about the binding potency of cholesterol derivatives other than that 20,25-diazocholesterol also produces a reduced chloride conductance and increased membrane resistance (23). Most studies of membrane biophysics during experimental myotonia have been devoted to only 2,4-D and other monocarboxylic aromatic acids. On the other hand, biochemical studies have considered almost exclusively the inhibition of cholesterol biosynthesis, primarily 20,25-D. Consequently, it is difficult to make meaningful comparisons of their respective sites of action on muscle membranes. While the simplicity of a common mechanism of action for both groups of drugs is appealing, it appears that 2,4-D and 20,25-D may act at different membrane sites.

In sarcolemmal membranes isolated from 20,25-diazocholesterol–treated skeletal and cardiac muscle, the amount of cholesterol is reduced and desmosterol is increased (32). The myotonic effect is highly dependent on membrane cholesterol, since including cholesterol in the diets of 20,25-D–treated rats reverses the myotonia (19). Membrane cholesterol levels have not been reported after 2,4-D or other monocarboxylic acids.

Most reports show that Na^+-, K^+–stimulated ATPase activity is increased in 20,25-D–treated rats (15, 18, 32, 33), with one exception which demonstrates a decrease in activity (33). Sarcolemmal Ca-stimulated ATPase activity was also increased after 20,25-D treatment (18, 32). In contrast to the action of 20,25-D, 2,4-dichlorophenoxyacetic acid had no effect on Na^+, K^+-stimulated ATPase but increased the activity of basic *p*-nitrophenylphosphatase (*p*-NPPase) activity (34). There is evidence that this sarcolemmal enzyme may regulate the gate for passive flux of K ions (34). Increased activity of *p*-NPPase increases K^+ efflux from leukocytes (35). This may explain the increased K conductance observed after some monocarboxylic acids (3). The activity of *p*-NPPase in human or myotonic goat muscle has not been described, nor has the effect of 20,25-diazocholesterol been evaluated with respect to *p*-NPPase activity. Until this area is further explored, no unifying hypothesis can be put forward. Clarification of this point will be essential in determining the importance of altered potassium conductance.

A logical step in analyzing the mechanism of myotonia is to look for morphological abnormalities. Unlike human myotonic dystrophy, myotonia congenita is not associated with any generalized structural abnormalities. In the goat one minor change was an increased density of T-tubules (37). Similarly, rats fed 20,25-diazocholesterol did not show abnormal morphological correlates of altered function (38). However, a nonspecific toxic side effect of 20,25-D was observed, possibly due to the drug's effect on muscle sterols.

The Relationship of the Nerve to Drug-Induced Myotonia

The myotonic response does not involve the neuromuscular junction (39). Myotonia can still be induced pharmacologically in the presence of curare (11, 40). Rats treated with 25-azocholesterol demonstrate a tendency toward neuromuscular failure which may contribute to muscle weakness, but this is separate from the typical myotonic effect seen clinically and in vitro (21). However, an intact nerve supply may be required for the development of experimental myotonia (41–43).

Once established, the myotonia produced by 2,4-D was not affected by subsequent nerve section. However, section of the sciatic nerve 7 days prior to administration of 2,4-D prevented the typical repetitive discharges and the waxing and waning of activity (41). Caccia et al (43) have observed a similar dependence on innervation for the myotonia produced by 20,25-D. However, when treatment with 20,25-D was extended for several weeks after denervation, EMG and muscle contractile behavior closely approximated myotonia (44). A difference is that typical myotonic potentials were rare.

The alteration in membrane cable properties following denervation is highly complex. Muscle membrane depolarization begins within a few hours after nerve section, followed after 3 to 4 days by increases in membrane resistance, capacitance, and time constant (45, 46). The increase in membrane resistance has been attributed to a reduction in potassium conductance (47–49). It is during this period of increased membrane resistance that 2,4-D no longer produces myotonia in diaphragm muscles (42). An explanation for this may lie in the observation that untreated muscles in chloride-free media display typical myotonia activity (27, 23). This spontaneous activity is abolished in high K^+ solutions where K conductance would be lower (28). Thus the relative insensitivity of denervated muscles to 2,4-D may be due to decreased K^+ conductance. However, much more work is required with both 2,4-D and 20,25-D treated animals before the subtleties of neural regulation of myotonia can be defined.

The pharmacologic model of myotonia congenita closely parallels its human inheritable form. While a wide variety of unrelated drugs produce myotonia symptoms in rats, those few drugs that have been closely studied show a common action on muscle membrane involving altered chloride conductance and possibly potassium as well. A more thorough examination of membrane transport proteins may also reveal a common biochemical change in the sarcolemma.

Myotonic changes in man precede the histologic changes in dystrophia myotonica (2). The slight histopathologic changes which have been reported in myotonia congenita are very similar to early changes observed in dystrophia myotonica (36). Recent studies show that chronic treatment with a myotonic drug, 2,4-D, began to reproduce some of the histopathologic abnormalities of dystrophia myotonica (50). The significance of the myotonia model may lie in the long-term effects of these drugs and the possibility of providing an animal model for the more severe disease, dystrophia myotonica.

PHARMACOLOGIC MODELS OF DUCHENNE'S MUSCULAR DYSTROPHY

The development of experimental models for progressive muscular dystrophies, of which the most debilitating is Duchenne's, has been much more difficult to achieve than the model for myotonia congenita. Myotonia is primarily a membrane-related dysfunction with minimal histopathologic abnormalities. Duchenne's muscular dystrophy is far more complex in both its etiology and its pathology. At the present time no animal model, either genetic or pharmacologic, accurately reproduces all

aspects of Duchenne's muscular dystrophy. However, a first approximation to the disease has been achieved with several drug-induced myopathies.

For an animal model to reproduce accurately the pathology of Duchenne's dystrophy, several minimal criteria must be met: (*a*) Initial histopathologic changes in biopsies of Duchenne's patients include grouped fiber necrosis surrounded by normal muscle fibers (51). (*b*) As the disease progresses there is a wide variation in fiber diameter and an infiltration of endomysial connective tissue and fat (51, 53). (*c*) There is a fluctuating increase in serum levels of many enzymes including creatine phosphokinase (CPK), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and lactic dehydrogenase (LDH) (52). (*d*) The proximal muscles of the pelvis and lower extremities are the first affected (53).

Some of these criteria are met by the genetically developed dystrophies in a wide variety of animals, of which the mouse and chicken have been studied most extensively (54). An alternative approach to simulating Duchenne's dystrophy in a genetic model has been the recent development of the pharmacologically induced experimental myopathies. Two general approaches have been followed. The first was based upon the initial studies of Hathaway et al (55), which involved circulatory obstruction with or without treatment with various monoamines. A second approach has been strictly pharmacologic. Either pharmacologic model meets several of the above listed criteria for Duchenne's dystrophy. In addition, they offer greater control in analyzing the basic mechanisms that produce the myopathy.

The Vascular Model of Duchenne's Dystrophy

A unique and diagnostic characteristic of a muscle biopsy from a patient in an early stage of Duchenne's dystrophy is the characteristic grouped fiber necrosis (51). This has led to the suggestion that the circumscribed area of necrotic fibers surrounded by normal muscle may represent the field of perfusion by a dysfunctioning terminal blood vessel (55). This vascular hypothesis is supported by histopathologic abnormalities in blood vessels of some patients in the early stage of the disease (55, 56), but others have not been able to confirm this (57).

Complete ligation of the femoral artery and vein yields no histologic abnormalities in the muscles of the lower extremity of rabbits (55). However, the relative ischemia produced by injection of 20μ–80μ dextran particles has led to a nearly complete reproduction of the histopathology of Duchenne's muscular dystrophy (55). Two weeks after an injection of the particles, early changes such as grouped fiber necrosis and phagocytosis occurred, and areas of regeneration were surrounded by fibers of normal appearance. The muscles of animals sacrificed 3 months after the initial treatment showed characteristic middle- and end-stage lesions such as proliferation of endomysial connective tissue, fatty infiltration, and a wide range of muscle fiber diameters. Occasional thickened walls of arterioles and some occluded vessels were also noted. These observations suggested that the histopathologic picture of Duchenne's muscular dystrophy could be reproduced experimentally by producing a relative ischemic condition in muscle.

A similar development of necrosis and phagocytosis at the light microscopic level was reproduced by aortic ligation by itself in rats (58). However, a thorough exami-

nation of the myopathy at electron microscopic level revealed significant differences from the ultrastructural changes seen in human Duchenne's dystrophy. The authors also noted that aortic ligation was selectively destructive to soleus muscle when compared to gastrocnemius muscle. This selectivity was presumably due to the higher oxidative metabolic demand of soleus muscles, since increasing the work load by contralateral denervation or tenotomy exacerbated the myopathy. The experimental methods of producing the partial ischemia differ greatly between the two studies. Consequently, until the ultrastructure of muscles treated with the microsphere approach is examined, the "relative ischemia" hypothesis cannot be fully accepted.

Ligation Plus Serotonin Model

Because very few abnormalities are seen in arteriolar walls of Duchenne's muscle biopsies, a new approach was developed combining aortic ligation plus the vasoactive agent serotonin (5-HT) (59). The rationale to using this combined approach has been given added significance in view of the observation that in Duchenne's patients, the platelet uptake of serotonin is greatly diminished (60). Serotonin itself has been suggested as a causative agent in carcinoid myopathy (61). Contrary to earlier observations (59), rats given 5-HT alone display some histopathologic abnormalities (62). In the rat, normal circulating levels of serotonin are too low to contract vascular smooth muscle (63). However, when the isolated rat hind limb was perfused with 2 to 50 mg/liter serotonin (5-HT creatinine sulfate) a decrease in blood flow in the femoral artery occurred (59). The combination of aortic ligation plus 20–75 mg/kg 5-HT, was given to rats for 5 days to 3 months either acutely or chronically. Either method alone produced no muscle necrosis, but in combination, a histopatholic picture reminiscent of Duchenne's dystrophy was produced (59). No structural abnormality was observed in blood vessel walls, leading the authors to suggest that a functional inadequacy rather than a structural lesion is responsible for the relative ischemia. Aortic ligation in the rat previously has been reported to render skeletal muscle more susceptible to vasoactive agents (64). The same characteristic pattern of muscle lesions has been reproduced with aortic ligation and another vasoactive agent, 3 mg/kg norepinephrine (59, 65).

Additional evidence for the "functional ischemic" model of Duchenne's dystrophy was obtained by examining plasma enzyme levels in rats given serotonin after aortic ligation (65). Aortic ligation itself produced some increase in plasma enzymes which returned to control levels within 72 hr. Administration of serotonin produced a marked increase in creatine phosphokinase (CPK), glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and lactic dehydrogenase (LDH). Enzyme levels returned to normal within 48–72 hr, but repeated injections produced a return to elevated enzyme levels (65). These data suggested that a second requirement for the animal model for Duchenne's dystrophy was achieved, that of increased levels of muscle enzymes. Damaged liver as a source for these enzymes was ruled out because there was no increase in alkaline phosphatase. Ligation plus norepinephrine was also effective in increasing enzyme levels (66). Pretreatment with phenoxybenzamine and chlorpromazine, both α-adrenergic blockers, pre-

vented the elevated enzyme levels. Pretreatment with imipramine which blocks the preterminal amine uptake pump (67) had a paradoxical effect. Low doses (1–2 mg/kg) maintained low plasma enzyme levels whether noradrenaline or serotonin was studied. However, high doses of imipramine (20 mg/kg) actually increased the plasma enzyme levels elevated by norepinephrine (66). This interaction between imipramine and norepinephrine must be explored adequately before firm conclusions can be made with respect to overstimulation of α-receptors. In apparent conflict with these results, Melmed & Karpati (68) observed that phenoxybenzamine plus aortic ligation significantly increased the ischemic myopathy. It is quite clear that further studies are required to explain the fundamental difference between the myopathic effects of aortic ligation between the two groups. It is likely a difference in ligating techniques, but the question must be resolved before detailed pharmacology is explored. In addition, it will also be necessary to provide a more complete pharmacologic analysis in the prevention or exacerbation of histopathologic changes. Depletion of amines with reserpine, blockage of synthesis with *p*-chlorophenylalanine, and destruction of aminergic nerve terminals with 6-hydroxydopamine all must be thoroughly tested.

It has been emphasized recently that increased serum enzyme levels actually precede significant muscle necrosis in Duchenne's dystrophy (69). Significant abnormalities have been observed in dystrophic muscle and erythrocyte membrane composition and transport. It is conceivable that an expressed genetic error in membranes may be the earliest stage in the pathogenesis of Duchenne's dystrophy (69, 82). It should be noted that peak increases in enzyme levels occurred 12 hr after 5-HT, that is, well before the first lesions were apparent (66). Much work needs to be done to clarify early abnormalities in membrane transport and composition during the initial hours after drug treatment and before the development of lesions.

Vasoactive Amines and the Development of Myopathies

Two more specific pharmacologic approaches have been attempted. The first combined imipramine and serotonin. The second studied the myopathic effects of the monoamine oxidase (MAO) inhibitor, pargyline. Imipramine is a tricyclic antidepressant which blocks the uptake of primarily serotonin but also other amines into nerve terminals (67). Parker & Mendell (70) have succeeded in reproducing the characteristic histopathology of Duchenne's dystrophy by pretreating rats for three days with imipramine (10 mg/kg). On the fourth day serotonin was given (100 mg/kg). This 4-day procedure was repeated each week for eleven weeks. The combination of drugs closely reproduced the early and midstage lesions of Duchenne's dystrophy with the addition that proximal muscles were selectively affected. This procedure also raises plasma enzyme levels of CPK (71). When untreated muscle was perfused in vitro with imipramine and 5-HT, ^{14}C-labeled serotonin was taken up 3 times faster than control. However, oxygen uptake and the amino acid analogue γ-aminoisobutyric acid were both significantly reduced (71).

When rats were injected with the MAO inhibitor pargyline, soleus muscles contained a flurorescent material generally characterized by the Falck-Hillarp technique as catecholamines (72). Duchenne's dystrophy is the only human myopathy

known to contain catecholamine-fluorescent fibers (73). It is not known, however, whether the ligation-plus-5-HT or imipramine-plus-5-HT myopathies contain fluorescence. The pargyline myopathy displayed grouped fiber necrosis and, with seven-day treatment, significant connective tissue formation in the endomysium (72). It is very much unlike Duchenne's dystrophy in that only the soleus is affected with no lesions produced in the gastrocnemius (72), or diaphragm, or quadriceps muscles (M. B. Laskowski and W. D. Dettbarn, unpublished observations). Denervation reduced the myopathy, which was assumed to be "neurogenic." This approach is questionable since both motor, sensory, and sympathetic innervation are nonselectively removed and, of course, muscle activity and metabolic requirements are significantly reduced. In none of the above-mentioned experimental myopathies has any significant ultrastructural examination of the muscle, blood vessels, or nerve been reported. It is clear that before the 5-HT model can be accepted as a replica of Duchenne's dystrophy a thorough point-by-point comparison must be made with biopsied dystrophic muscle at the fine structural level.

Recent reports have suggested that an abnormal axoplasmic flow in motor nerves may be responsible for several of the myopathies produced by vasoactive amines (74, 75). Two MAO inhibitors, pargyline and phenelzine, were reported to increase the rate of fast axoplasmic transport (75). The increased flow rate was partially prevented by pretreatment with α-methylparatyrosine. Aortic ligation with or without 5-HT produced a similar increase in axoplasmic transport (74). However, Komiya & Austin (76) found that ligation of the iliac artery plus 5-HT did not alter flow rate although the myopathy was fully developed in the gastrocnemius muscle. It should be noted that in the reports of increased fast transport mentioned above, confirmatory histologic examination of muscle lesions was not described. Resolution of the controversy will add valuable information as to whether or not a trophic material from the motor nerve might be augmenting or reducing the myopathy.

Recently a "neurovascular hypothesis" has been proposed to explain the mechanism of the myopathies produced by vasoactive amines (77). A single genetic deficit was proposed, that of reduced effectiveness of sympathetic vasoconstriction. According to this hypothesis, the normal reflex vasoconstriction during exercise is impaired, resulting in an overperfusion of unused muscles (78). Reduced blood flow to exercising muscle in Duchenne's patients has been observed (79, 80). Such patients also display reduced oxygen tension in exercising muscles (81). While the hypothesis is simple and attractive, much more work is required at the pharmacologic and ultrastructural levels before it can be accepted. Sympathetic innervation of biopsies of Duchenne's patients must be examined at the electron microscopic level. In addition, more thorough pharmacologic analyses must be made of the "functional ischemia" experimental myopathies. What is required at this point is very basic and thorough work to help clarify the origin of ischemic lesions and the way these are enhanced with vasoactive amines. Such studies will provide pharmacologic models that will be an even closer approximation to Duchenne's dystrophy. This approach will certainly provide a potentially productive area for drug development and testing.

GENERAL NEUROMUSCULAR DISEASES

Investigation of the involvement of the neuromuscular junction in muscle disease had been active before the development of electron microscopic techniques. Based upon preliminary work at the light microscopic level, ultrastructural investigations have revealed a frequent involvement of the motor end-plate in a wide variety of muscle diseases (82). Zacks (83) has written a thorough review of this topic. In a majority of muscle diseases, motor end-plate degeneration is secondary either to a primary myopathy or to motor nerve degeneration. Myasthenia gravis is a prominent exception (83). Less frequently occurring diseases that specifically affect the neuromuscular junction are carcinomatous neuromyopathy (84–86), amyotonia congenita syndrome (87), Isaacs' syndrome (88), Coxsackie B virus (89), and a variety of neurotoxins (83).

Experimental myasthenia gravis is being pursued in several laboratories. Such studies involve the myasthenic dog (90, 91) and rats made chronically autoimmune to ACh receptors (92). In recent years we have been actively pursuing studies of end-plate–mediated myopathies using a variety of cholinesterase inhibitors and cholinomimetic drugs (93–97). Rather than developing a model for a specific disease entity, this approach permits an examination of the delicate nerve-muscle relationship and explains how altering this relationship can initiate muscle lesions.

Neural Control of Muscle Properties

The now classic work of Buller, Eccles & Eccles (98, 99) revealed the significant influence that motor nerves have over the structural, functional, and biochemical properties of muscles they innervate. The trophic function of nerves was originally defined as a function that is not mediated by nerve impulses (100). However, only a few of these trophic actions are independent of nerve impulse transmission and muscle contraction (101, 102). The level of muscular activity influences contractile properties, content of specific enzymes, its sensitivity to ACh, and its ability to accept further innervation. The structural and functional integrity of skeletal muscle depends on the presence and normal function of neuromuscular transmission. Problems may arise from pharmacologic manipulation of neuromuscular transmission and may lead to changes in the development, maintenance, and integrity of the end-plate and muscle fiber. The physiology of neuromuscular transmission can be affected in several ways. The amount of the transmitter acetylcholine which is released may be increased or reduced by a wide variety of drugs (103–108). Postsynaptic sensitivity may be changed by altering the number of ACh receptors. Pharmacologic manipulation of the input resistance of the muscle fiber membrane will alter the threshold for generation of muscle action potentials. Variation in one of these factors or in combination with one another can be contributing causes to neuromuscular degeneration and can be altered experimentally (109–113).

Some of the changes that are seen after denervation may be due to disuse rather than loss of the trophic function of nerve. Other experimental procedures will interrupt various aspects of nerve-muscle interaction, while leaving the neuromuscular junction and its nerve structurally intact. Some of these approaches such as the

use of vinblastine and colchicine block axoplasmic transport without interfering with the synthesis and release of ACh or the propagation and transmission of impulses (114). The effects of these compounds are almost similar to those of denervation. Total disuse of the neuromuscular junction can be induced by chemical application of local anesthetics (102) inducing block of conduction and transmission without changing structure and function of the neuromuscular junction. Such treatment also induces phenomena seen after denervation.

Cholinesterase Inhibition and Transmitter Release

Altered acetylcholine release or a reduction of its hydrolysis by loss in cholinesterase activity is observed as early pathophysiologic changes in murine dystrophy (115, 116), chicken dystrophy (117), atrophy (118), some experimental myopathies (93), and some forms of human muscle disease (119, 120). There has been considerable debate as to whether the nerve terminal or the postsynaptic region of muscle is the primary site of action of anti-ChE drugs (121), and whether either or both of these sites are affected by neuromuscular disease processes, such as muscular dystrophy and myasthenia gravis (112).

Few reports have been published on the effects of anticholinesterases, especially organophosphorus agents, on miniature end-plate potential (MEPP) frequency and end-plate potential (EPP) quantum content. Neostigmine increased MEPP frequency over a narrow concentration range but at higher doses decreased frequency (122, 123). Edrophonium was found to have similar effects on MEPP frequency (123, 124). These drugs, however, contain a quaternary ammonium ion which by itself is capable of increasing MEPP frequency (125). Indirect estimates of quantum content during neostigmine, edrophonium, and ambenonium, drugs that facilitate neuromuscular transmission, indicate that quantum content is elevated (123). Recent work has shown, however, that the method of paralyzing the muscle for intracellular recording affects the estimate of quantum content in edrophonium (124, 126).

Paraoxon, an irreversible organophosphorus inhibitor of ChE activity when injected into rats, causes (*a*) an augmentation of spontaneous transmitter release, (*b*) a reduction in quantum content, and (*c*) spontaneous and evoked antidromic nerve action potentials (94). Similar effects were observed when paraoxon was applied in vitro (94, 127).

A critical question is whether paraoxon produces these effects directly or acts indirectly through its inhibition of cholinesterase. The strongest evidence indicating that paraoxon acts through ChE inhibition comes from the experiments with pyridine-2-aldoxime methiodide (2-PAM). Reactivation of inhibited ChE with 2-PAM reduces the paraoxon-augmented MEPP frequency and eliminates antidromic nerve activity. Another organophosphorus compound diisopropylfluorophosphate (DFP) was without effect on MEPP frequency (128). However, these experiments were conducted while basal MEPP frequency was artifically accelerated with 10 mM K ion. Paraoxon depolarized muscle membranes by approximately 15 mV (127). This depolarizing effect was partly reversed with 10^{-6} M tetrodotoxin (TTX) and totally abolished in combination with α-bungarotoxin. The depolarizing effects of pa-

raoxon are due to the combined depolarization of ACh receptors and extrajunctional receptors controlling Na-conductance channels. Paraoxon has no effect on the K-conductance channels (127).

Tetrodotoxin reduced the paraoxon-accelerated MEPP frequency but did not alter spontaneous release in control preparations. It has been reported previously that TTX blocks nerve and muscle action potentials while leaving the depolarization-release mechanism intact (129). Consequently, an intact action potential generating system must be present for paraoxon to yield its full effect. Whether the faster MEPP frequency is produced by reinvasion of nerve terminals with spontaneous nerve impulses or some other mechanism remains to be determined.

Acute Cholinesterase Inhibition and Muscle Fiber Degeneration

Irreversible inhibitors of the ChE activity such as paraoxon, DFP, tabun, sarin, soman, and parathion, as well as reversible inhibitors such as physostigmine and neostigmine, inhibit AChE activity at the neuromuscular junction. This loss of enzyme activity blocks the hydrolysis of ACh and leads to increased activity and stimulation of the skeletal muscle fiber. Reports from several laboratories have shown that all of the above-mentioned ChE inhibitors are myopathic (130–135).

The earliest evidence relating ChE inhibitors to necrotic lesions of skeletal muscle was in a report by Carey (130). Since then this observation has been repeated by several investigators; however, not all of them were aware of previous investigations (93, 131–134). Injection of paraoxon produces a progressive myopathy in the rat diaphragm, soleus, gastrocnemius, and quadriceps muscles. The diaphragm is the most severely affected of these muscles (133, 135), followed by the soleus and the gastrocnemius muscles. The earliest lesions noted were focal areas of abnormality close to the surface of the muscle fiber (93, 133). On H-E stain this area appeared to be more basophilic. The trichrome stain demonstrated an area of red-staining, and the normal basic pattern of mitochrondria, usually identified with LDH and NADH reactions, was disrupted by clumping of highly reactive material. These focal changes progressed to a generalized breakdown of fiber architecture, characterized by a loss of staining quality followed finally by phagocytosis. Longitudinal sections indicated that the early changes in a focal necrosis affected only a small segment of fiber lengths. The later stages affected progressively greater lengths of muscle fibers (95).

Following i.p. application of soman (methylpinacolyloxyphosphoryl fluoride), local necrotic lesions are seen scattered throughout the diaphragm muscle. Some fibers displayed severe lesions characterized by loss of cytoarchitecture and phagocytosis (131).

Within 2 hr of an injection of DFP or tabun the earliest light miscroscopic changes are seen characterized by localized eosinophilia, swelling of the sarcoplasm, and loss of striations in several muscle fibers. Approximately 12 hr after the i.v. injection a complete but localized necrosis has developed in the affected fibers (133). A delayed neuropathy beginning at the nerve terminals develops within three weeks after exposure to DFP (136, 137). At this time muscle contractile strength is returning toward normal.

Ultrastructural Changes Produced by Cholinesterase Inhibitors

Motor nerve terminals showed varying degrees of changes within 30 min to 2 hr after injection of soman and paraoxon (131, 138). Some nerve terminals appear relatively normal with the exception of slightly swollen mitochondria. These nerve terminal abnormalities are more severe after soman than after paraoxon. More obvious changes are seen in the subsynaptic area and the surrounding muscle fiber. More severely affected nerve terminals display myelin figures, membrane enclosures, and an increase in the number of large coated vesicles. Soman, paraoxon, and neostigmine initiate the formation of vesicular structure in the primary and secondary subsynaptic cleft (95, 131, 139). Occasionally some of these are seen in the sarcoplasm. Many of the cleft vesicles are similar in density and size to synaptic vesicles but with considerable variations in diameter. The severity of lesions in the subsynaptic folds varies even within the same muscle. Normal subsynaptic clefts with few cleft vesicles are seen side by side with subsynaptic clefts with many cleft vesicles and a widening of the cleft itself (95, 131).

The ChE inhibitors, in addition to the changes seen in the region of the end-plate, cause changes in the muscle fiber itself. Muscle surrounding the motor end-plate shows a disruption of cytoarchitectural organization. Initially the first changes are in the mitochrondria which show swelling leading to lysis of the central cristae. Myelin figures beneath the end-plate are frequently observed while the region more distal to the end-plate is less affected. The nucleoli of the muscle cell nucleus are enlarged and move to the periphery of the nucleus. There is an increase in the sarcoplasmic ribosomes, the sarcoplasmic reticulum becomes dilated followed by a loss of striation of the myofibrils, and later total destruction of the myofilaments and fragmentation of Z bands occurs (95, 131).

Muscle Lesions Developing During Chronic Cholinesterase Inhibition

Treatment of rats with paraoxon over a period of seven consecutive days produces a progressive myopathy (93, 95, 135). In the diaphragm a maximal effect is seen after 3 days of treatment. All stages of the myopathic process were maximal at this point and 6.5% of all fibers were found to be affected. By day 7 the muscle appeared to be relatively normal. In soleus muscle the maximum number of lesions was seen at day 3, but only 0.7% of the total fiber population was affected. The gastrocnemius muscle, unlike the diaphragm and the soleus, exhibited maximal lesions on days 5 to 7. The total number of fibers involved at this time was 0.8%. In all muscles studied, maximal loss of ChE activity occurred during the first 30 min after the initial paraoxon treatment. Within 24 hr of each injection of paraoxon, the enzyme activity in diaphragm and soleus muscle had recovered to about 50% of control activity, while in gastrocnemius muscle the ChE activity had returned to 67% of control.

There is a marked difference in the susceptibility of muscle type to the paraoxon action. The muscles tested are distinct from each other as to predominant fiber type, rate of firing, speed of contraction, and inherent metabolism. The myopathy is much more severe in the diaphragm, which is predominantly a slow contracting, tonic or

red muscle, than it is in soleus, which consists of a majority of intermediate fibers, or in the gastrocnemius, which is a fast contracting phasic or white muscle (97, 135).

Modification of the Myopathy

In animals undergoing right sciatic or left phrenic nerve transection 4–7 days prior to the application of the irreversible inhibitor, the innervated muscles showed a slight increase in the number of lesions, while the denervated muscles were protected against the myopathic process and instead underwent typical denervation atrophy (93, 97, 133). Animals that were given hemicholinium, an inhibitor of cholineacetyltransferase, did not demonstrate any histologic abnormalities in the muscles to paraoxon. The maximum number of lesions was significantly reduced as compared with animals receiving a similar dose of paraoxon without the hemicholinium (93). Internal skeletal fixation by pinning the right ankle and knee three days before treatment with paraoxon protected the soleus from the paraoxon-induced myopathy and potentiated the myopathy in the soleus of the unfixed limb, which had assumed a greater weight-bearing function.

A normal, untreated diaphragm is capable of sustaining a 10-sec tetanic contraction at indirect stimulation frequencies of 25, 50, 100, and 200 per second. A diaphragm removed from a DFP-treated rat 2 to 4 hr after the injection has lost the ability to sustain a contraction at 200 stimuli per second, and performs subnormally at 100 stimuli per second. Unilateral stimulation of the sciatic nerve with 5 stimuli per second during 6 hr in anesthetized DFP-treated rats produced extensive necrosis on the stimulated side only (133). Curare prevented the necrosis when given every 2 hr during the 6 hr indirect stimulation period. Necrosis developed, however, when the curare concentration was reduced. The muscles were still insensitive to indirect stimulation, but apparently sensitive to the necrotic effects of ACh (133).

Reactivation of Phosphorylated ChE

When given at a concentration of 0.23 mg/kg s.c. to rats, paraoxon produced an 85% inhibition of neuromuscular ChE of the diaphragm, and the enzyme remained at this level of inhibition for the next 2 hr. Administration of 2-PAM (20–60 mg/kg i.p.), a reactivator of phosphorylated ChE, at various time intervals after the paraoxon injection (10–120 min) increased ChE activity to 75% of control. When administered between 10–30 min after paraoxon, 2-PAM totally prevented the development of the paraoxon myopathy. At longer intervals between paraoxon and PAM there was a time related increase in muscle necrosis. If ChE inhibition proceeded uninterrupted for 2 hr prior to PAM administration, the muscle necrosis occurred in 4.2% of the fibers. The myopathic process depends upon the degree and duration of ChE inhibition (134, 135).

Rats treated with 2-PAM within 2 hr after DFP or soman poisoning showed no necrosis; if the PAM injection was postponed, the necrosis developed as usual (133, 134).

Reversible Cholinesterase Inhibition and Myopathies

Long-term treatment of rats with prostigmine sulfate for 42 to 150 days showed degeneration of postsynaptic folds, mainly in red muscle fiber and less so in white

muscle (139, 140). The postsynaptic membrane profile concentration was decreased by 29% in red muscle fibers and by 10% in white muscle fibers. The mean miniature end-plate potential amplitude was decreased by 29%. Frequency, quantum content, and muscle resting membrane potential were not affected by neostigmine (139).

In acute experiments, prostigmine as well as physostigmine in concentrations between 0.2 to 0.6 mg/kg cause muscle fiber necrosis, not unlike that seen with the irreversible inhibitor of ChE. The number of necrotic fibers rises with increasing inhibitor concentration. The total number of necrotic fibers, however, is less than that caused by paraoxon (M. B. Laskowski and W-D. Dettbarn, unpublished observations). Signs of cholinergic intoxication, such as salivation, diarrhea, as well as body tremor and pronounced muscle fasiculation, are seen only for about 30 min after reversible inhibition of ChE. The same symptoms can be observed for over 2 hr after irreversible inhibition of the enzyme. Repeated application of the reversible inhibitor, i.e. 3 times during a given 1.5 hr period, leads to an increasing number of lesions.

Mechanisms for the Development of Myopathies

The observations reported above indicate the reversible or irreversible inhibition of ChE at the neuromuscular junction produces a progressive myopathy, primarily associated with the motor end-plate region. Possible mechanisms for this myopathy include the following. (*a*) The myopathy is the result of the abnormal functioning of the myoneural junction when ChE activity is reduced as a consequence of the presence of increased amounts of ACh. (*b*) Increased circulating catecholamines may cause local ischemia. (*c*) The inhibitors may act directly on the postsynaptic membrane or other components of the muscle cell. (*d*) The inhibitors may exert nonspecific toxicity. Besides diffusion, the enzyme AChE is responsible for the rapid removal of ACh from end-plate receptors. Thus, inhibition of this enzyme allows ACh to accumulate, to react longer, and interact with a larger area of the motor end-plate. The end result is an increase in amplitude and duration of the MEPPs. Formation of these leads to action potentials and spontaneous twitching of muscle fibers. Similar effects on end-plate potentials may be observed. In addition, as is the case with paraoxon, the frequency of MEPPS is drastically increased (94). The effect of the ChE inhibitors leads to increased excitability of the nerve terminal and therefore increased antidromic activity (141–147). The back response to a single nerve stimulus and the repetitive muscle action potentials are prolonged and intensified. The myopathy-inducing action of guanidine (96), which does not inhibit ChE but causes an increased release of ACh per impulse and produces spontaneous muscle twitching by inducing spontaneous multiquantal end-plate potentials, again supports the role of ACh in the genesis of this myopathy. By inhibiting antidromic activity, curare prevents the increased MEPP frequency, blocks neuromuscular transmission and spontaneous muscle twitching, and thus prevents the ChE inhibitor–induced myopathy. Additional support for the role of ACh as the myopathy-inducing agent comes from the experiments with 2-PAM, a reactivator of phosphorylated ChE. 2-PAM when given within 10–30 min after the organophosphorus ChE inhibitor not only reactivates the enzyme, but also greatly reduces

antidromic firing and the MEPP frequency. It also completely prevents the myopathy, unless given after a critical time, usually between 60–90 min (133–135). Hemicholinium has been shown to reduce the amount of ACh in the quantum released and reduces significantly the severity of the myopathy (93).

By inhibiting ChE in vitro, cholinergic as well as adrenergic stimulation is achieved. All preganglionic nerve fibers, as well as motor nerves, are cholinergic and the preganglionic transmitter is ACh. Under conditions when ChE is inhibited, simultaneous stimulation of postganglionic cholinergic and adrenergic and motor nerves will occur. This results in an increased release of ACh or norepinephrine from postganglionic fibers. Under these conditions not enough blood may be supplied to the most active muscle fibers and thus relative ischemia might result. This could lead to ischemic changes, leakage of muscle enzymes, and eventual necrosis. The effects of prolonged cholinergic vasodilation in combination with reduced adrenergic vasoconstrictor tone during the periods of increased ChE inhibitor-induced muscle activity could lead to functional ischemia in certain hyperactive muscle fibers and not in others. Less active muscle may have normal oxygen requirements, but may receive the same blood supply. The functional ischemia could appear during the period of the ACh-induced muscle fiber activity. This could also explain the prevention of necrosis by denervation due to immobilization of the denervated muscle.

It is generally accepted that two groups on the enzyme are essential for hydrolysis, the esteratic and anionic site. The anionic site interacts by ionic binding with the cationic head of the ACh molecule. There is good evidence that part of the chemical forces binding ACh to the enzyme are more or less similar to those between ACh and its receptor. It is therefore not surprising that drugs that inhibit ChE may, to some extent, also react with the ACh receptor. DFP, for instance, in higher concentrations than needed to inhibit enzyme activity has a curare-like action which may block neuromuscular transmission (128). In contrast to ChE inhibition, this latter effect is reversible on washing. Furthermore, it has not been shown as yet whether this higher concentration is due to direct action on the receptor or on the ionophore which controls the permeability once the receptor has been activated, an effect not unlike that of local anesthetic. The myopathic effects of the ChE inhibitors can be explained entirely on the basis of their inhibition of the enzyme and the increased action of ACh on pre- and postganglionic receptor sites. Other effects that have been described occur at a much higher concentration of the inhibitor.

Besides their inhibition of ChE, or interaction with the ACh receptor and or the ionic conductance modulator, organophosphates may have direct effects on the muscle. We have observed that paraoxon directly depolarizes muscle membranes (127), and subsarcolemmal abnormalities of muscle fine structure are occasionally seen not associated with an end-plate (95). This unspecific action as a cause of the myopathy could not explain the selectivity of the myopathy, since only 6–8% of the fibers are affected.

The importance of studying the myopathies produced by cholinesterase inhibitors derives not from the accurate reproduction of a specific human disease but rather from the insight they provide into the myopathic effects of altering the delicate

nerve-muscle relationship. With this approach it is possible to follow a highly controllable disease process from its origin at the end-plate within 30 min after the drug, to its completion with necrosis and phagocytosis after 24 hr. The crucial questions may lie with the mechanisms which spare most fibers, rather than the disease process itself.

CONCLUSIONS

Within the last ten years there have been major developments in the field of experimental models of human muscle disease. Recently models of Duchenne's muscular dystrophy have been pursued most actively because of the particularly sinister nature of the disease. It is essential now to define carefully the mechanisms underlying the generation of these myopathies and to clarify major areas of controversy. This will require a multidisciplinary approach combining physiology, ultrastructure, and biochemistry, using the same drug under the same experimental conditions. Duchenne's dystrophy is a multisystem disease. The significance of pursuing the pharmacologic models rests with the development of drugs important to combating muscle degeneration.

The models for myotonia present a different problem. The pharmacology of these experimental myopathies has been examined in considerable detail. A review of the literature demonstrates that membrane physiology has been examined thoroughly in one model and biochemistry in another. Again, what is required is a multidisciplinary investigation of each model toward the end of defining whether a single common defective mechanism is responsible for the myotonic state.

Finally the myopathies induced by experimental alteration of nerve-muscle relationships have been developed to analyze the basic pathophysiology of muscle degeneration. The most actively studied of these have been the experimental myopathies induced by cholinesterase inhibitors. Muscles respond to insult with a limited arsenal of physiological and histological alterations. In-depth multidisciplinary analyses of the very earliest of these myopathic processes can provide insight into some of the basic mechanisms underlying muscle fiber degeneration.

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