DOES PB PRODUCE NEUROMUSCULAR JUNCTION CHANGES?

Acetylcholinesterase (AChE) inhibitors, including PB, produce acute destructive changes at the neuromuscular junction, the site of connection between nerve cells and muscle fibers, at which nerve cells signal muscles to contract. Because studies of the neuromuscular junction require microscopic inspection of muscle tissue and tests of electrical and chemical properties of the neuromuscular junction at a fine level, studies investigating these alterations have not been performed in living humans but primarily in muscle preparations from rats and frogs exposed to PB or other AChE inhibitors (also called “anticholinesterases”). These studies have examined the effects of PB and other “carbamate” AChE inhibitors, sarin and other OP nerve-agent AChE inhibitors, and nonnerve-agent OP (e.g., pesticide) AChE inhibitors on the muscle and neuromuscular junction.

These observed alterations fall into several classes.

“Histopathological” and “ultrastructural” changes are changes in structure and fine structure of the muscle and nerve muscle junction observed by light and electron microscopy. Some of the muscle changes are referred to as “AChE inhibitor myopathy.”

“Prejunctional” changes refer to changes that occur on the nerve or signaling side of the neuromuscular junction. Prejunctional or “presynaptic” changes include changes in production and release of the neurotransmitter acetylcholine (ACh); or withdrawal of nerve branches from the motor endplate (muscle side).

“Postjunctional” or “postsynaptic” changes refer to changes that occur on the muscle or signal-receiving side of the neuromuscular junction. Since the signal is “received” by binding of ACh to receptors on the postjunctional side, changes in receptor sensitivity or density are examples of postjunctional changes. These changes are reflected in alterations in currents and voltages produced, for
instance, by binding the ACh to the receptor; and changes in time constants of electrical response measured at the “motor endplate.”

Clinical or behavioral changes are changes in muscle function or strength believed to result from alterations at the neuromuscular junction.

Many of the alterations observed are similar regardless of which AChE inhibitor is used. However, specific drugs differ to some degree in their effect even within a class of anticholinesterase chemicals (for instance, different carbamates, or different organophosphorus nerve agents may produce effects that differ in detail) (Albuquerque, 1986). For example, differences may occur in how extensive certain changes are, in which specific muscles are affected and to what degree, in the time-course of the effect, and in the response to a change in dosage. Differences are not only quantitative but may also be qualitative, and a drug that produces a more profound change than another when examined by one technique (for instance, by examining receptor blocking) may produce a less pronounced change when viewed through the lens of another technique (for instance, AChE inhibition, or “MEPP rise time”).

The existence of marked, and in some instances long-lasting, changes at the neuromuscular junction is unequivocal. (These findings derive primarily from animal studies using high doses of AChE inhibitors.) The clinical significance these changes may have is less clear. Some have suggested that they may be responsible for a putative reduction in the rate of cure of myasthenics with thymectomy since the onset of treatment with PB (Rockefeller, 1997; Phillips and Torner, 1996). Others have suggested that these alterations may be responsible for symptoms in ill PGW veterans (Tiedt, 1994). In fact, current information is sufficient neither to confirm nor exclude long-lasting structural and/or functional effects at the motor endplate in humans with the doses and durations of PB treatment used in PGW veterans. Current information is also insufficient to confirm or exclude a role for changes in symptoms, such as fatigue or joint pain, reported by PGW veterans. (“Joint” pain might or might not be produced by abnormal regulation of signals to opposing muscle groups during rest or activity. Abnormal strength or timing of signals could result in alterations in the pattern of highly coordinated contraction of some muscles with relaxation of others that occurs during normal activity and at rest.) Concomitant exposure to other AChE-inhibiting drugs, such as sarin or organophosphorus pesticides, could conceivably cause, modify, or amplify effects on the motor endplate. Again, while existing evidence does not exclude long-lasting biological and clinical effects on the motor endplate with PB use, neither is there persuasive evidence favoring such effects. Data to address the clinical significance and time-course of such changes as those seen in animal studies remain inadequate.
This chapter does not purport to contain a wholly comprehensive review of the literature regarding AChE inhibitors and motor endplate alterations. Rather, a sample of available information is provided to impart the nature of changes seen, the similarity and variation of alterations observed with different types of AChE inhibitors, and some of what is known regarding the time-course of these changes. Effects not only of PB but of other AChE inhibitors are reviewed. Because not all measures have been performed with PB, including assessments of long-term effects, it is useful to describe effects both for PB and for other AChE inhibitors.

**STUDIES WITH AChE INHIBITORS**

**Animal Studies**

Animal studies (largely using muscle from rats given AChE inhibitors, such as PB; occasionally from frogs and other species treated with cholinesterase inhibitors) constitute the bulk of the evidence indicating changes in the neuromuscular junction that occur with use of PB and other anticholinesterases.

**Histopathological and Ultrastructural Changes: Postsynaptic and Myopathic Changes.** PB has been shown to lead to light and electron microscopic changes in rats. These changes are primarily postsynaptic (causing alterations at the muscle side of the nerve-muscle junction), and myopathic (destructive to muscle tissue). Some investigators find the presynaptic region (the nerve side of the nerve-muscle junction) to be less affected. The nature of the changes in the muscle of rats given high-dose PB (98 mg/kg/day orally in feed—76 times the Gulf War daily dose and 228 times the per-dose Gulf War dose) include alteration in the striped appearance of muscle filaments and changes in the appearance of subcellular structures like mitochondria and sarcoplasmic reticulum. Some evidence of regeneration appears by 15 days, partially reversing the destructive changes.1

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1In rats given high-dose PB (98 mg/kg/day administered orally in feed), unlike control animals, degenerative changes in muscle are seen with light and electron microscopy. Under the light microscope, approximately 1 percent of muscle fibers (or “myofibers”) from the diaphragm were damaged. (Such damage is made evident by loss of “striation” or stripe pattern of “myofilament” architecture and presence of many centralized, highly convoluted cell nuclei in the muscle cells (Bowman, Schuschereba, et al., 1989).) On electron microscopy, myofibers were seen to contain swollen mitochondria (mitochondria are energy-producing “organelles” or subcellular structures) with accumulation of internal inclusions and numerous inflammatory cells (Bowman, Schuschereba, et al., 1989).

By two days of PB administration, degenerative changes were seen in the postsynaptic region of the neuromuscular junction. By seven days, myofibers had additional changes. Myofibers were still damaged, with centralized nuclei, dilated sarcoplasmic reticulum, and disruption of “Z bands”; and myofibers had increased glycogen. By 15 days, damaged fibers appeared to be in the process of regeneration, evidenced by large nuclei with dispersed chromatin and prominent nucleoli with
It has been suggested that some adaptive mechanism may be responsible for the partial reversal of early myopathy even in the presence of continued treatment. Seventy-four percent or more inhibition of AChE was observed throughout the study (Bowman, Schuschereba, et al., 1989), so that the reversal of myopathy cannot be the result of lessened cholinesterase inhibition, for instance from heightened metabolism of PB.

A second study of rats with somewhat lower doses of PB (20 and 40 mg/kg—47 and 93 PGW dose-equivalents, respectively) but still greater than 50 percent AChE inhibition confirms the presence of changes on light microscopy. Changes were unexpectedly more severe in the group with the lower dose of PB, for reasons that are unclear. The earliest changes could be detected immediately after the two-hour period required for complete inactivation (Gebbers, Lotscher, et al., 1986).

In a third study on rat diaphragm, PB producing 78 percent reduction in AChE activity was again seen to induce marked changes, most directly adjacent to the synapse, with damage greatly reduced a few microns away and some normally appearing muscle filaments 12–14 microns away. Stimulated muscles (in which the nerve leading to the muscle was stimulated to produce a signal) showed the same pattern of organelle damage and myofibril disorganization, but the damage was more severe and the affected area more extensive. Damage was well developed 30 minutes after PB administration and was maximal at two hours. Myopathic or destructive changes can involve the endplate region of nearly all muscle fibers, but light microscopy revealed necrosis in only about 10 percent of fibers sampled, even after nearly complete ACh inhibition. It is unclear why some nerve fibers degenerate but most are preserved in spite of marked abnormalities in the fine structure of the fibers ("ultrastructural"

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2Changes included acute focal necrosis (cell death) of disseminated single fibers and groups of fibers, mixed leukocyte infiltrates, and marked changes in motor endplates in skeletal muscle; changes were greater in diaphragm than in quadriceps muscle in this study (Gebbers, Lotscher, et al., 1986) (though another study found similar changes in three tested muscle types differing in the amount of fast- and slow-twitch muscle fibers, namely diaphragm, soleus, and extensor digitorum longus (Hudson, Foster, et al., 1985)).

3Changes included supercontracted sarcomeres near the endplate region, subjunctional areas marked by disorganization of the myofibrillar apparatus, streaming of the Z-bands, and mitochondrial swelling. The most severely affected mitochondria were observed proximal to the junctional folds with progressively less severe alterations away from the junction. Regions closest to the synaptic cleft had the most severely damaged organelles. Essentially all mitochondria immediately adjacent to the neuromuscular junction exhibited marked intracristal swelling and some mitochondria showed an apparent absence of matrix. Contractile filaments were supercontracted with only remnants of Z-line material visible. The ultrastructural damage was greatly reduced within a few microns from this region, and by 12–14 microns from the subjunctional membrane it was possible to detect precisely aligned sarcomeres and "normal" intracellular organelles (Adler, Hinman, et al., 1992).
The incidence of necrotic fibers is dose-dependent, suggesting that some muscle fibers may be especially sensitive to AChE inhibitors, perhaps related to the threshold or firing pattern of the muscle cell (or “motor unit”) (Adler, Hinman, et al., 1992).

Importantly, PB administration in rats produced marked postsynaptic ultrastructural changes, even when no animals displayed outward signs of anticholinesterase intoxication (Hudson, Foster, et al., 1985).

**Presynaptic Changes.** Some neuromuscular junctions of all three muscles tested in rats underwent partial denervation (withdrawal of nerve terminal branches from the muscle) following either acute injection of PB (1 mg/kg—or 2.3 times the Gulf War dose—by single injection) or subchronic delivery of PB by osmotic minipump (with constant 70 percent inhibition throughout a 14-day period) (Hudson, Foster, et al., 1985). The three tested muscles were the diaphragm, the soleus (a posterior lower leg muscle), and the extensor digitorum longus (which extends the toes). Together these muscles include fast-twitch and slow-twitch fiber types.

Withdrawal of nerve branches constitutes a presynaptic change, because the nerve branches are from the signaling side. Although recovery was “in process” and in some cases reportedly complete at the termination of the follow-up 60 days after cessation of treatment, some changes were still present at this time (Hudson, 1985), primarily histological changes in muscle. (This is the longest identified period of follow-up after cessation of PB treatment in these studies.

A related study using PB (0.36 mg/kg by single subcutaneous injection, or subacute PB by osmotic minipump—0.84 of a Gulf War dose) noted that intrusion of processes from non–nerve supporting or “glial” cells termed “Schwann cells” in the synaptic region (the synaptic “cleft”) and partial withdrawal of nerve terminals acted as a type of denervation, effectively reducing the amount of synapse surface available for optimal functioning (Hudson, Foster, et al., 1985).

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4 Postsynaptic changes with acute PB treatment (0.36 mg/kg by single subcutaneous injection) included rarefied areas in mitochondria, and occasional mitochondria were associated with membranous lamellar structures. Subjunctional myofibrillar structure was disorganized in occasional neuromuscular junctions. Subacute treatment with PB (for two days) led to highly variable changes, with swelling or rarefaction of the mitochondrial matrix, some grossly altered mitochondria with multilayered membrane structures in subjunctional regions, and similar variability within each of three tested muscle types. There was loss of myofibrillar organization in some neuromuscular junctions, with more subtle changes in Z-lines, myosin, and actin filament organization in others.

5 Synaptic vesicles (vesicles or inpouchings of membrane that contain neurotransmitters) were so densely packed that there was no space to accommodate cytoplasm in the nerve terminal. With acute treatment, mitochondria presynaptically were either similar to the controls’ or were characterized by swollen regions in neuromuscular junctions of all three muscles (Hudson, Foster, et al., 1985). With subacute treatment, there was local separation of pre- and postsynaptic components. In some regions, crests of several junctional folds lacked the typical apposing nerve terminal, and
Studies in rats, using neostigmine (a carbamate compound related to pyridostigmine), have also noted significant presynaptic alterations\(^6\) including depletion of neurotransmitter-containing vesicles (Hudson, Rash, et al., 1978).

It has been shown that destructive ultrastructural changes such as those described require that ACh interact with its receptor. These changes were eliminated if the nerve synapsing on the neuromuscular junction was cut, if ACh receptors at the endplate were chemically inactivated (by the agent “alpha bungarotoxin”), if oxime treatment was administered promptly to pull the AChE inhibitor off the AChE, or if a receptor blocker (namely d-tubocurarine, a paralytic agent) was simultaneously administered, thus preventing spontaneous fasciculations of the muscle (Salpeter, Kasprzak, et al., 1979; Gebbers, Lotscher, et al., 1986; Adler, Hinman, et al., 1992; Hudson, Rash, et al., 1978). Moreover, damage is promoted by nerve stimulation (Adler, Hinman, et al., 1992). These findings indicate that the destructive changes may result from increased synaptic activity because of heightened availability of ACh and that they depend on successful interaction of ACh with its receptor. It has been suggested that the diaphragm may be particularly vulnerable because of its constant activity (Gebbers, Lotscher, et al., 1986; Dettbarn, 1984).

The condition may be more pronounced in slow-twitch rather than fast-twitch muscle (Wecker and Dettbarn, 1976), or this finding may simply reflect differences in muscle activity. Myopathy in in vitro muscles occurs both in the absence and presence of nerve stimulation. Although nerve stimulation facilitates this effect, so evidently does spontaneous muscle twitching or “fasciculation” (Adler, Hinman, et al., 1992), which may result when spontaneously released or leaked neurotransmitter binds to the receptor. While the detailed mechanism of the muscle damage or “myopathy” that results when AChE inhibitors (such as PB) are given is not well understood, this damage is known to be mediated through calcium (Yamaguchi, Robson, et al., 1983; Toth, Karscu, et al., 1983; Dettbarn, 1984; Kawabuchi, 1982; Salpeter, Kasprzak, et al., 1979; Leonard and Salpeter, 1979). Several possible mechanisms have been postulated.\(^7\)

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Schwann cells frequently occupied the position normally occupied by the nerve terminal (Hudson, Foster, et al., 1985).

\(^6\)Presynaptic changes, in addition to synaptic vesicle depletion, included appearance of numerous coated vesicles and membrane cisternae indicating continued nerve terminal hyperactivity (Hudson, Rash, et al., 1978); degeneration and partial recovery of the nerve axon were observed with chronic treatment (Hudson, Rash, et al., 1978).

\(^7\)For instance, membrane phospholipase may be activated by calcium, directly or through calmodulin or phosphatidylinositol; the latter is split into diacylglycerol and inositol triphosphate, with the latter activating calcium, and calcium in turn activating protein kinases. Calcium in muscle fibers may activate an endogenous neutral protease that can catalyze degradation of myofibrils and Z-lines. Excess depolarization of endplate receptors may enhance calcium influx. PB may also directly influence neuromuscular transmission, acting as a weak agonist, thus further increasing
Other AChE Inhibitors. Similar patterns of damage to skeletal muscle have been reported in experiments with other carbamates (e.g., neostigmine) (Osame, Kawabuchi, 1975; Kawabuchi, Osame, et al., 1976; Kawabuchi, 1982; Engel, Lambert, and Santa, 1973), with OPs (Gebbers, Lotscher, et al., 1986; Salmeter, Kasperzak, et al., 1979; Preussler, 1967; Laskowski, Olson, et al., 1975; Laskowski, Olson, et al., 1977; Fischer, 1968; Gebbers, Lotscher, et al., 1986; Dettbarn, 1984; Feng, Rogers, et al., 1973; Fenichel, Kibler, et al., 1972; Fenichel, Kibler, et al., 1974; Ariens, Meeter, et al., 1969) like paraoxon (Laskowski, Olson, et al., 1975; Laskowski, Olson, et al., 1977; Fenichel, Kibler, et al., 1974); DFP (Feng, Rogers, et al., 1973), and with the nerve agents tabun and soman (Gebbers, Lotscher, et al., 1986; Preussler, 1967). For example, one study found that paraoxon, as well as OP cholinesterase inhibitors, produced progressive myopathy in skeletal muscle (Wecker and Dettbarn, 1976).8

Partial reversibility of these muscle damaging effects has been commonly reported. For instance, in rats exposed to DFP for two weeks, by day 14 there was loss of sensitivity to the necrotizing actions in all the muscles tested (diaphragm, soleus, extensor digitorum longus) (Gupta, Patterson, et al., 1986). Typically, some reversal of the damage occurs even when AChE-inhibiting agents continue to be given.

As noted previously, AChE-inhibiting agents that differ substantially from one another chemically produce similar effects, supporting presence of a mechanism involving heightened acetylcholinergic activity. Moreover, direct ACh enhancement produces the same effect (Fenichel, Kibler, et al., 1974). Nonetheless, neostigmine was shown to have direct action on motor nerve endings9 in addition to inhibiting AChE, suggesting that some mechanisms idiosyncratic to individual chemicals may also be operative (Braga, Rowan, et al., 1993).

Variability of the Effect. Some studies report differences from one muscle type to another in the “ultrastructural” (pertaining to the fine structure of tissue, seen with an electron microscope) pre- and postsynaptic alterations of the neuromuscular junction. These differences have been attributed to different amounts of fast- versus slow-twitch (red versus white) muscle fibers or to differences in muscle activity that occur with the different types of muscles.

8Myopathic changes included presence of splitting fibers, then enlargement of central nuclei, more-intense splitting and breakdown of fiber architecture, and finally total fiber necrosis and phagocytosis (Wecker and Dettbarn, 1976). There was significant recovery of enzyme activity within 24 hours. It was suggested that there may be a critical period of cholinesterase activity to initiate the myopathic process.

9Direct actions included blocking delayed rectifier potassium channels and enhancing transmitter release (Braga, Rowan, et al., 1993).
In one study, alterations of the neuromuscular junction occurred in diaphragm, soleus, and extensor digitorum longus with acute PB (0.36 mg/kg sc—or 0.84 Gulf War doses) or subacute PB (two days, 10 mg/ml PB by subcutaneously implanted osmotic minipump); both doses produced 60–70 percent whole blood cholinesterase depression). Changes occurred in each muscle of every animal examined, but there was considerable variation in the extent of damage even within individual fibers. The severity varied from fiber to fiber, but variability appeared “random” and not strongly related to the specific fiber type or to the dosage regimen (though effects in diaphragm muscle may have been greater) (Hudson, Foster, et al., 1985). No information was given to allow determination of the effect of muscle fiber activity on these changes.

**Mitochondrial Effects.** Increased cholinergic activity induced by PB (perhaps through increased intracellular free calcium) has been reported to cause deterioration in mitochondrial function resulting in heart and muscle damage. One report suggests that this may be mediated by reductions in the mitochondrial-bound enzyme “hexokinase,” which is closely linked to “oxidative metabolism” in the mitochondria. PB led to a reduction in mitochondrial hexokinase in rats’ tibialis anterior muscle (a muscle in the anterior lower leg) but not in heart muscle. (30 PGW doses led to 60 percent reduction of hexokinase in tibialis anterior muscle, a reduction that was statistically significant (p < .005). Hexokinase was reduced by only 10 percent in the heart, a difference that was not significant (Glass-Marmor and Beitner, 1996).)

**Prejunctional Effects: Changes in Neurotransmitter Production.** Choline acetyltransferase is the enzyme that “catalyzes” or facilitates the final step in ACh production (Albuquerque, Boyne, et al., 1983). Choline acetyltransferase activity was increased in muscle (in intramuscular nerves) following administration of the OP DFP (Gupta, Patterson, et al., 1986) and following nerve agent administration (Albuquerque, Boyne, et al., 1983); this increased activity should lead to increased production of ACh, an effect that could potentiate the AChE-inhibiting effects of these agents. No data were supplied on the effect of PB on choline acetyltransferase activity. The relative time-course of this effect, and the relation to the time-course of multiple potentially downregulatory effects, was not elucidated.

**Prejunctional Effects: Changes in Neurotransmitter Release.** Most studies evaluating changes at the motor endplate employ high doses of PB, leading to at least 50 percent AChE inhibition. Data are limited on effects of lower doses of PB, doses more comparable to those employed with PGW veterans.

Studies have found marked but reversible reduction in the amount of ACh released by each nerve impulse with five to seven days of (1 mg/kg) neostigmine given to rats (Roberts and Thesleff, 1969). Also, reduction in quantal
Content of nerve-stimulated endplate potentials has been shown (endplate potentials are changes in voltage on the postjunctional side produced by release of multiple “quanta,” or vesicles of ACh, at the presynaptic side following a signal by the postsynaptic neuron) in rat extensor digitorum muscle (Tiedt, Albuquerque, et al., 1978). It has been suggested that chronic use of carbamates in myasthenia gravis patients may depress neuromuscular transmission in part because of a low endplate potential quantal content (Roberts and Thesleff, 1969), a form of “downregulation” of the acetylcholine system in response to excessive ACh activity; however, reduced transmitter release in one study had returned almost to normal after 22–25 days of continued treatment (Tiedt, Albuquerque, et al., 1978).

Postjunctional Effects: Electrophysiological and in Receptor Changes. ACh binds to receptors at the postjunctional side of the neuromuscular junction, inducing electrical currents and changes in voltage in the motor endplate (postjunctional membrane). If these changes are adequate, they result in contraction of the muscle fiber.

A single vesicle of ACh may be randomly released; one vesicle contains one “quantum” of ACh, which comprises several thousand ACh molecules, representing the smallest amount of ACh released (Kuffler and Yoshikami, 1975). If a nerve cell is excited and “fires,” producing an “action potential” to signal to the muscle, about 200 quanta are released into the synapse or neuromuscular junction (Kuffler, Nicholls, et al., 1984).

Release of a single quantum by the prejunctional nerve leads to relatively small changes in current at the motor endplate. (The current is produced when ACh binds to receptors at the motor endplate, leading ions to traverse specialized ion channels in the membrane.) This change is termed a miniature endplate current (MEPC), and the size of the current is determined by fixing the voltage at the endplate. Alternatively, the endplate current can be held constant to determine the change in voltage, or miniature endplate potential (MEPP). If a nerve cell “fires” (generates an action potential), the complement of about 200 quanta produces larger changes in current and voltage termed the endplate current and endplate potential, respectively.

Nerves that signal muscle cells may be artificially stimulated to fire, and endplate currents and potentials can then be measured. MEPCs and MEPPs may be measured when random quanta are released. Moreover, ACh or other cholinergic agonists can be directly applied to the endplate to determine receptor “sensitivity,” by measuring changes in currents or voltages. Information from MEPCs and from endplate currents differs slightly; for instance, a MEPC may be reduced if there are fewer ACh molecules in a quantum, or if there is reduced sensitivity of the receptor to ACh. An endplate current may be reduced
if there are fewer ACh molecules in a quantum, fewer quanta for each action potential, or reduced receptor sensitivity. Moreover, amplitude is not the only property of currents and potentials—they also have temporal properties. Studies have looked both at the electrophysiological measures with spontaneous or induced signaling of nerve to muscle and at receptor sensitivity.

In several studies of rat diaphragm, soleus, and extensor digitorum muscle, PB led to prolongation of MEPC decay (threefold to fourfold slowing), with small (23 percent) increases in MEPC amplitude (Adler, Hinman, et al., 1992); and MEPP with a slow rise-time (>1 ms) and low frequency (Meshul, Boyne, et al., 1985). Levels of AChE inhibition were higher than in PB-treated PGW veterans, e.g., 78 percent reduction in AChE activity (Adler, Hinman, et al., 1992). This effect was seen not only with PB but with other cholinesterase inhibitors, including sarin and soman; the effect was more pronounced with sarin and PB than with soman (Meshul, Boyne, et al., 1985). Moreover, PB affected both the extensor muscle and the soleus, whereas sarin affected endplates primarily of the soleus, and soman affected neither (Meshul, Boyne, et al., 1985). (This suggests that testing of only one or two muscle groups may be inadequate to exclude an effect, and effects identified may not generalize with fidelity to other muscle groups.)

In rat extensor digitorum longus muscle, treatment with neostigmine produced decreased MEPP amplitude and frequency and decreased endplate potential amplitude (accompanied by decreased junctional ACh sensitivity and decreased quantal content of nerve-evoked endplate potentials). Although by 22–25 days of continued treatment the reduced rate of transmitter release had returned almost to normal, the alterations in the postsynaptic membrane lasted for the full 106 days of continued treatment (Tiedt, Albuquerque, et al., 1978). In rat diaphragm muscle in vitro, low concentrations of neostigmine (.0001 mmol/L) led to decrement then full recuperation of compound muscle action potential (CMAP) amplitudes on repetitive stimulation of the phrenic nerve (the nerve that supplies the diaphragm), increased MEPP amplitudes, and prolonged decay time for the MEPC. Higher concentrations led to unimodal reduction in the CMAP, endplate potential, and MEPP amplitudes and a double exponential time-course of MEPC decay (Maselli and Leung, 1993a and 1993b). It was concluded that low concentrations impair neuromuscular transmission by transient depolarization of the endplate, while higher concentrations induce desensitization and direct blockade of the endplate receptor channel, probably in its open configuration.

Studies with nerve agents have actually shown opposite effects on endplate currents with low- and high-dose treatment. Except for tabun, the other OP nerve agents (soman, sarin, and VX) at low concentrations (<1 µM) facilitated, while high doses (>10 µM) of all four depressed, the endplate current peak ampli-
tude, with VX producing the greatest depression (Albuquerque, 1986). Moreover, the time constant of endplate current decay $t_{EPC}$ was prolonged, and a maximum increase was achieved with 1 µM in the case of VX, sarin, and tabun, whereas soman produced a maximal increase at 0.1 µM concentration. Doses of >1 µM of all OPs shortened $t_{EPC}$ from an enhanced level achieved by a low dose. At the 1 µM concentration, all four OPs produced near-maximal enhancement (approximately three times greater than control) of $t_{EPC}$, while at doses of 10 µM or greater, the $t_{EPC}$ values appeared lower than with the 1 µM dose; a greater reduction was observed with VX (Albuquerque, 1986). The enhancement of $t_{EPC}$ can be attributed to inhibition of AChE, while reduction with higher doses appears to be due to their action on the ACh receptor ion channel complex, or on the associated ionic channels (Albuquerque, 1986).

PB also facilitates receptor desensitization (a functional form of downregulation) (Meshul, Boyne, et al., 1985); indeed, it has been suggested that PB may have its therapeutic effects against OP compounds partly because it produces a “desensitized” state of the ACh receptor (Albuquerque, Boyne, et al., 1983). Receptors desensitized by PB would be insensitive to the anticholinesterase effects of the OP compounds.

Desensitizing effects of PB on the nicotinic ACh receptor have been shown in investigations of macroscopic as well as the microscopic events. Using “fluctuation analysis” as well as the “patch clamp” technique (in which the voltage of the membrane is held constant and electrical currents, resulting from movement of ions across channels, are measured), the agent has been shown to decrease single-channel conductance (conductance of ions across a single ACh receptor following binding of two molecules). PB decreases the endplate current and MEPC peak amplitudes, and, after an initial prolongation of these events presumably due to anticholinesterase effects (that is, due to increased availability of ACh), brings the time-constant of decay back to control levels (Albuquerque, Boyne, et al., 1983).

The desensitization of the ACh receptor induced by PB has been demonstrated by ACh iontophoresis experiments (direct application of ACh onto receptors) but can also be demonstrated by a patch clamp technique.10

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10By patch clamp one can measure the characteristics only of open channels. There are multiple closed states with different receptor configuration; specific sequences of closed states must be gone through prior to a channel’s opening, but this technique does not allow distinction among them. It has been possible to demonstrate the existence of an intermediate state of channel behavior by such agents as PB that enhance desensitization. The channels in the presence of PB, for example, reveal intense “flickering,” and the channel openings appear in a bursting pattern, uncharacteristic of the channels in control condition. These are the channels that are presumably about to become desensitized and fail to open at all or open with a markedly decreased conductance. Following the appearance of many channels with much flickering and successive “waves” of bursting activity,
Receptor desensitization produced by PB may contribute to reduction in benefit of PB to myasthenics over time. (Throughout this section, other mechanisms are identified that may potentially be involved in functional cholinergic downregulation and could also contribute to reduction in the benefit of PB to myasthenics with continued treatment.) Neuromuscular adaptation has also been demonstrated following administration of the OP DFP in the rat; this adaptation was found to be caused by the recovery of AChE activity due to de novo synthesis of AChE, and reduction in the number of nicotinic ACh receptors (Gupta, Patterson, et al., 1986).

PB’s ability to facilitate receptor desensitization does not prevent the development of endplate myopathy (Meshul, Boyne, et al., 1985), though one could speculate that it may contribute to the failure of endplate myopathy to progress, and the eventual partial regression of this process. More careful characterization of the time-course of the two is needed. Desensitization rates vary for different carbamate anticholinesterases acting on the ACh receptor ion channel complex, at least in rats. The effect of neostigmine exceeds that of physostigmine, which in turn exceeds pyridostigmine (Meshul, Boyne, et al., 1985). These effects differ from certain other downregulating effects, which require continued AChE inhibition and therefore are weaker (or absent) for physostigmine than for PB, since physostigmine has a shorter time-course.

Failure of neuromuscular transmission—loss of ability of a signal from a nerve cell to produce contraction in the muscle fiber to which it connects—has been reported with anticholinesterases, but the reasons for this failure are incompletely understood. When a nerve signals to a muscle, the membrane voltage becomes less negative, a process called “depolarization,” which occurs as ions (such as sodium or potassium) move through channels in the membrane. Adequate depolarization leads the muscle fiber to generate an action potential (a self-propagating electrochemical signal that produces contraction of the fiber). However, prolonged depolarization inactivates the sodium channels so that effective signaling cannot take place.

According to a study using DFP in rat diaphragm muscle, two patterns of stimulation-induced endplate depolarization have been described that may account for the impairment in neuromuscular transmission reported with anticholinesterases. One pattern exhibited progressive “staircase summation” of long decay endplate potentials resulting in maximal depolarization at the end of the stimulation period. The other had maximal endplate depolarization at the start of the stimulation period followed by progressive return to the prestimulation levels (Maselli and Leung, 1993a). Transient endplate depolarization decreases channel opening frequency drops drastically and channel conductance decreases (Albuquerque, Boyne, et al., 1985).
ization was proposed to underlie the decremental response followed by re-
cuperation of CMAP amplitudes in humans at early stages of toxicity with OP
compounds (Maselli and Leung, 1993a). Transient endplate depolarization was
suggested to account for failure of neuromuscular transmission induced by low
concentrations of anticholinesterases; while high concentrations induce
additional desensitization and direct blockade of the endplate receptor, which
becomes the dominant mechanism for failure of neuromuscular transmission
(Maselli and Leung, 1993a and 1993b). (These are mechanisms of possible
cholinergic downregulation with AChE inhibitors.)

With 14 days of DFP administration in rats, postsynaptic nicotinic receptor
density (Bmax) was reduced to 44 percent without a change in the affinity con-
stant (Gupta, Patterson, et al., 1986), another potential mechanism for at least
temporary downregulation.

**Changes in AChE.** With DFP administration in rats, though AChE activity was
initially reduced to 20 to 24 percent of control, no further inhibition of AChE
occurred with continued DFP for 14 days, but there was recovery of enzyme
activity, especially certain forms (the 4SD and 10S forms) (Gupta, Patterson, et
al., 1986). Whether this recovery results from increased AChE production,
reduced sensitivity of AChE to DFP, or other effects was not elucidated.
Depending on the mechanism involved, this recovery could conceivably
produce another form of downregulation. However, other studies employing
PB have found persistent inhibition of AChE with continued treatment.

The effect on AChE inhibition and on ACh release following discontinuation of
PB has not been well defined, and the relative contributions and time-courses
of agonism and downregulation remain to be determined.

**Clinical Changes.** Despite marked ultrastructural damage to muscle fibers,
postsynaptic elements, and perhaps presynaptic elements, some reports indi-
cate that muscle function (in in vitro tests) is not compromised, at least accord-
ing to certain tests. Diaphragm muscles underwent no significant reduction in
twitch or tetanic tension during or after a two-week exposure to PB, which was
proposed to be due to the confined area of ultrastructural damage. Synaptic
transmission and action potential generation, based on in vitro findings, were
“expected to remain functional,” with decrements in tension due to abnormal
properties of the myopathic regions presumably too small to detect (Adler,
Hinman, et al., 1992). Other studies (using neostigmine) have shown a reduc-
tion in indirectly and directly elicited muscle contraction with three days of
treatment (Tiedt, Albuquerque, et al., 1978). In another study of rats exposed to
DFP for 14 days, there was initial appearance followed by disappearance of
fasciculations in all muscles tested (diaphragm, soleus, extensor digitorum
longus) (Gupta, Patterson, et al., 1986).
It is not known whether ultrastructural changes such as those observed in animal studies could produce apparently normal function in selected muscle function challenge tests but also lead to symptoms of subjective fatigue (perhaps corresponding to abnormalities that these particular tests do not capture).

In studies of rats demonstrating ultrastructural and electrophysiological changes, animals expressing these changes demonstrated behavior varying from no identified findings to death, with fasciculations, prostration, salivation, urination, defecation, and rhinorrhea seen (Adler, Hinman, et al., 1992). Many of these changes represent activation of the muscarinic system. Of greater interest would be careful studies of muscle function and animal activity during and after acute and chronic cessation of the agent.

**Human Studies with PB: Comparison of Myasthenic Changes in Humans to PB-Induced Changes in Animals**

**Ultrastructural and Electrophysiological.** It has been speculated that carboxylicates used to treat myasthenia gravis, such as PB and neostigmine, may contribute in part to the neuromuscular alterations observed in myasthenia gravis. The changes produced by PB are similar to but not identical with those seen in rabbit and human myasthenia (Tiedt, Albuquerque, et al., 1978). Similarities with myasthenia gravis include reduced MEPP amplitude and frequency, reduced junctional ACh sensitivity (see “Downregulation”), alterations in neuromuscular geometry, and disruption of synaptic folds. Dissimilarities include the frequency distribution of MEPP amplitude, variation of neuromuscular junction ACh receptor sensitivity and ACh potential time-course, muscle membrane cable properties, and state of contraction of the endplate sarcoplasm (a membrane within the neuron that contains calcium) (Tiedt, Albuquerque, et al., 1978). One study concluded that “it appears likely that neostigmine treatment in patients with myasthenia gravis does not have an important role in the pathogenesis of the disease, but that it may contribute to the observed alterations.” (Tiedt, Albuquerque, et al., 1978.) Others voice concern that the ultrastructural appearance of myasthenic endplates is similar to the ion accumulation myopathy caused by PB (Meshul, Boyne, et al., 1985); they note that administration of a “therapeutic” agent that increases the desensitizing effects of ACh and can still lead to excess ion accumulation (with ion leak into muscle cytoplasm as a possible proximal cause of myasthenic myopathy) may have “complex and possibly counterproductive consequences that have not been anticipated.” (Meshul, Boyne, et al., 1985.)

**Clinical.** Some studies suggest that clinical effects on muscle function do not occur subacutely with PB use, while others have shown modest effects. For
example, muscle strength and endurance were not significantly affected by
eight days of PB treatment leading to 20–30 percent cholinesterase inhibition in
a group of 35 healthy male 18–20-year-old volunteers in a double-blind placebo
controlled trial that tested isometric handgrip, isokinetic elbow flexor and
extensor strength, and knee flexor and extensor strength (Glickson, Achiron, et
al., 1991). Other studies in smaller samples failed to find differences in grip
strength after one or several 30 mg tablets of PB (n = 7 and n = 5; Levine, Kolka,
et al., 1991; Forster, Barber, et al., 1994) (one or several Gulf War doses). Of
these, one also examined and failed to show a difference in 60 percent peak
hand-grip endurance time, or peak torque for leg extension (Levine, Kolka, et
al., 1991). In contrast, one small (n = 7) double-blind, placebo-controlled cross-
over study did demonstrate a modest (~3 percent, p < .05) reduction in grip
strength in those receiving pyridostigmine (Cook, Kolka, et al., 1992). (Subjects
received PB for one week and placebo for one week and received daily focused
examinations.) The possibility cannot be excluded that modest effects, delayed
effects, or effects with different motor measures occurred.

One study performed evaluations of neuromuscular function in 20 PGW vet-
erans with severe muscle fatigue, weakness, or myalgia (muscular pain) inter-
fering with their daily activities (Amato, McVey, et al., 1997). Tests included
nerve conduction, effects of repetitive nerve stimulation, quantitative single
fiber electromyography, and muscle biopsies. Mild increases in CK (an enzyme
released by the muscle with acute muscle damage) were seen in six of 20
patients (range 223–768 IU/l); one patient who reportedly had not received PB
had mildly increased jitter on single-fiber EMG, and muscle biopsies showed
abnormalities in 5 of 20 patients. Both patients with tubular aggregates
reported having received PB. The abnormal findings “were not believed to be
clinically significant.” However this conclusion is at best problematic in the
face of marked clinical symptoms in these subjects, because no testing was
performed in matched controls to determine the frequencies of these abnor-
malities in asymptomatic individuals of similar age and training. (The authors
performed no power calculations; neither did they define at the outset how
many abnormal biopsies or CKs would be expected if the group were “normal.”)
Manually tested muscle strength was measured and was reportedly normal in
all instances. Unfortunately, this is an extremely crude measure (it may supply
the same score for a world champion weight lifter and a non-physically-active
grandmother; it is not sensitive to even substantial differences within this
“normal” range). No sensitive quantitative measures of muscle strength, and
comparisons to healthy non-PGW deployed individuals of similar age and
training, were performed.

In summary, clinical findings with PB on motor outcomes have used short-term
trials evaluating acute effects, without long-term follow-up after discontini-

uation, in small samples, using limited tests of motor function. Some studies but not others have shown significant but subtle effects on measured functions. One study performed biopsies, muscle enzyme testing, and limited neuromuscular testing in symptomatic PGW veterans with muscular complaints but failed to employ a control group. A set of observed abnormalities was construed as not clinically significant and unrelated to possible toxin exposures, although no cogent justification for this position was provided.

**Duration of Changes**

The changes that take place pre- and postsynaptically appear to be partially or mostly reversible, even with continued treatment with anticholinesterases, including PB, with much of the reversal occurring early. However there are provisos to this reversibility. First, the reversal may not be altogether complete. One study stated that recovery of "twitch tensions" following cessation of PB was "essentially complete" by one day after cessation (Adler, Maxwell, et al., 1984), but in fact although the value was indeed stable from one to 15 days following cessation, recovery to the original baseline never occurred. Second, at least some changes appear to be long-lasting. Studies in rats (extensor digitorum longus muscle) have shown that some changes at the neuromuscular junction (alterations of the postsynaptic membrane) persisted for as long as 106 days, the duration of continued treatment (with neostigmine) (Tiedt, Albuquerque, et al., 1978). More directly relevant to the issue of PGW veterans is the question of whether changes persist beyond the cessation of treatment. The study with the longest identified follow-up after cessation of PB delivery—60 days—found that while recovery was reportedly complete 60 days after cessation of PB treatment in some rats (following a single injection of PB or 14 days of PB delivery by osmotic minipump), in other animals changes were still present 60 days after discontinuing PB, perhaps more so in specific muscles such as the soleus. Sixty days is relatively chronic in the life of a rat. (One rat week is said for some purposes to correspond to one human year. Using this crude guide, extrapolation to humans of data from the rat study would suggest continued tissue damage in some individuals extending to roughly eight years, with no further testing thereafter.) Thus, current data do not exclude the possibility that some animals never fully recover the pre-PB appearance of their muscle tissue. The clinical correlation of this finding, if any, remains to be defined.

In general, each of the changes—histological and ultrastructural, electrical and chemical, and presynaptic and postsynaptic; changes in receptor density and sensitivity, withdrawal of nerve terminals, reductions in quantal content and quantal release, alterations in AChE production, or susceptibility to inhibition—needs to be evaluated more carefully for time-course of reversal following PB
discontinuation, for interactions with other changes, and for individual differences in extent and in time-course.

CONCLUSIONS

Current evidence from studies in animals suggests that toxic effects result from cholinergic excitation at the neuromuscular junction in reaction to high doses of AChE inhibitors including PB. PB and other AChE inhibitors lead to alterations in function of the neuromuscular junction as well as physical destruction. These effects are partially reversible even with continued PB administration, but they may not be completely reversed even long after PB discontinuation. AChE inhibitors lead to changes in ACh production, ACh release, receptor response to ACh administration, muscle fiber organization, and clinical symptoms. These effects appear to result at least in part from excitation at the neuromuscular junction and the interaction of ACh with its receptor (with a concomitant influx of calcium). For this reason, tonically, persistently, or highly “active” muscles or muscle sites may be more affected. Some effects vary with the individual AChE inhibitor chosen or the dose employed (effects may even be opposite in direction with low and high dose of cholinesterase-inhibiting agents) (Albuquerque, Boyne, et al., 1983; Albuquerque, 1986), although the effects of dose for the many reported changes in receptor function, ultrastructure, and electrophysiological properties have not been well characterized. Many changes—in quantal release of ACh, in current and voltage changes in response to ACh at the motor endplate, and in the sensitivity to postjunctional ACh receptors—appear to reduce cholinergic function. This reduction in function may occur in response to, and may partially offset, the heightened ACh delivery at the synapse during the period of pharmacological AChE inhibition.

Although the duration of most effects (on ACh production and release, receptor sensitivity, myopathic changes, etc.) have not been well characterized, some effects have been shown to be quite long-lasting following discontinuation of the AChE inhibitor. It is not known whether all effects eventually reverse in all or most cases. The time-course of different effects has not been well characterized, neither have the doses at which the several effects are first seen. The effects have not been well studied across species or in primates. Moreover, the clinical sequelae of these changes, if any, are not well understood. It could be postulated that one consequence of these changes—namely functional cholinergic downregulation attended by reduced cholinergic activity—may lead to subjective fatigability in humans (conceivably leading to symptoms suggestive of “chronic fatigue”), but no data directly support or refute this proposition. This hypothesis would be weakly supported if treatment with cholinergic drugs led to subjective improvement in PGW veterans who report fatigue. However,
by this same hypothesis such treatment could produce undesired consequences.

**LIMITATIONS IN PRESENT EVIDENCE**

As noted above, the present data have several limitations. Different studies do not all evaluate or report findings in a similar fashion—that is, they do not necessarily employ similar doses, muscle groups, or observations, so the extent of agreement across studies within and between AChE-inhibiting drugs (including PB) is difficult to characterize. Observations of different types, such as ultrastructural changes in ACh production and release, receptor sensitivity and density, and electrophysiological change, are not well described as a function of dose of drug (especially of PB), of duration of treatment, of specific drug used, or of animal species observed. The influences of potentially important covariates also have not been identified, including age at exposure, prior (or subsequent or contemporaneous) chemical exposures, or baseline AChE and BChE status. Importantly, the duration of these effects at the motor endplate following cessation of PB or other AChE inhibitory treatment is also not well characterized.

**SCIENTIFIC RECOMMENDATIONS**

1. Data should be obtained on ACh production and release, AChE production, endplate current and potential amplitude and time-course, receptor sensitivity and density, and ultrastructural changes using low doses of PB, with dose and route of administration more comparable to that experienced by PGW veterans.

2. Data as per first recommendation should be compared when AChE inhibition occurs with muscles at rest and with stimulation or exercise, because some data suggest that active muscles may be more susceptible to destructive effects.

3. Extended recovery studies should be performed, measuring data as above (first recommendation) following low-dose PB in one animal model, such as the rat.

4. Work should be extended to other mammals to evaluate the robustness of the effects across species. If doubts remain regarding extrapolation of data to primates (such as humans), consideration could be given to evaluating these effects in nonhuman primates.

5. The time-course of different effects (such as those noted in the first recommendation) should be characterized and compared, evaluating effects of
dose and AChE inhibitor combinations, in different muscle groups and different animals.

6. More careful correlation of the above effects to clinical sequelae (such as quantitative muscle strength and spontaneous activity of the animal) should be performed.

7. Effects on the neuromuscular junction (peripheral nicotinic effects) should be compared to and correlated with corresponding peripheral muscarinic effects and central nicotinic or muscarinic effects (determined through in vitro studies and/or by giving PB to rats under stress, or physostigmine to rats without stress). Effects of interactions of PB with DEET and other putative interactants should also be compared in these preparations.

8. Studies of muscle function in ill PGW veterans with muscular complaints should include matched controls, preferably of similar pre–Gulf War physical ability; sensitive quantitative measures of muscle function (including strength and perhaps latency); and power calculations with adequate sample size to allow determination of whether abnormalities in muscle enzyme tests or in muscle biopsies (performed blinded and preferably in several muscle groups) occur at increased rates in ill PGW veterans.

9. Studies of the effect of PB on muscle function in healthy volunteers should include an adequate placebo control group, sensitive measures of muscle function, and extended follow-up after PB discontinuation. Consideration should be given to performing (blinded) muscle biopsies, recalling that effects may not show up in all muscle groups.

SUMMARY ANALYSIS

Evidence of Compatible Exposure

An estimated 250,000 PGW veterans were exposed to PB (Brake, 1997). Neuromuscular junction abnormalities in animals have been demonstrated with PB. However, studies have not been performed at low doses, such as those experienced by PGW veterans.

Evidence of Compatible Symptoms

Subjective fatigue and musculoskeletal complaints have been reported by many veterans. For example, in one evaluation of 263 veterans referred for complaints felt referable to their PGW service, 86 percent complained of fatigue, 84 percent of arthralgias, and 60 percent of muscular weakness (Amato, McVey, et al., 1997). These may or may not constitute symptoms “compatible” with neuromuscular junction effects.
Evidence of Connection Between Exposure and Symptoms

According to one study, the factor-analysis-derived syndrome of “arthro-myoneuropathy” in ill PGW veterans, in which musculoskeletal symptoms are prominent (including joint and muscle pains, muscle fatigue, and difficulty lifting) (Haley, Kurt, and Hom, 1997), is significantly associated with self-reported adverse acute response to administration of PB in the PGW, as well as to amount of insect repellent applied (Haley and Kurt, 1997). No other evidence has been identified that addresses a connection between musculoskeletal symptoms and use of, or response to, PB. A recent report regarding British veterans finds a link between self-report of use of PB in the PGW and in Bosnia and subsequent “Gulf War Illness” by CDC factor-analysis derived criteria (Unwin, Blatchley, et al., 1999); musculoskeletal symptoms represent one of the three major domains, in two of which symptoms must be present for the diagnosis. (PB was not the only exposure that was associated with increased illness. This study is addressed in greater detail below.)