THE USE OF NEUROPOISONS IN THE STUDY OF CHOLINERGIC TRANSMISSION

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INTRODUCTION

Poisons are useful investigational tools for the obvious reason that a truly poisonous substance must be active in minuscule quantities and therefore highly selective in its site of action. Furthermore, that site must be crucial to the function of an isolated tissue or to an entire organism. Poisons, by their very nature, are substances whose sites and mechanisms of action are highly specific.

Poisons are valuable investigational tools because they modify their target organs in ways that are both reliable and predictable. Hence, poisons have been used to analyze innumerable physiological processes. This review will focus on the mechanism of cholinergic transmission. There is probably no area of research that has relied more upon poisons than has the study of synaptic and neuromuscular transmission. In deference to their longstanding importance (and existence), poisons of biological origin are the main topics of discussion in this review.

ACETYLCHOLINE AND AXONAL TRANSMISSION

Acetylcholine (ACh) is believed to be a transmitter at numerous central and peripheral synapses. Another hypothesis, but one that has few advocates, is that ACh is involved in axonal transmission. The major advocate of this hypothesis has been Nachmansohn (1, 2). According to this investigator, ACh is stored in an inactive form (bound or sequestered) within conducting membranes. Following nerve stimulation, ACh is released and diffuses to a receptor, which is also within membranes. The interaction between ACh and its receptor leads to conformational changes, and these in turn initiate the sequence of events that culminate in a propagated action potential. The role of ACh at synaptic and neuromuscular junctions is hypothesized to be analogous to that in conducting membranes.

To be accepted as an essential component of axonal transmission, ACh must satisfy certain minimal criteria. In all likelihood, the criteria used to identify putative transmitter substances (3–6) are equally germane in identifying putative agents that regulate axonal transmission. In brief, it is expected that a substance be present and releasable in sufficient amounts to exert its supposed action, and that there be means both for generating the substance and for removing it from its site of action. In addition, an exogenous substance of known identity must mimic the action of the putative endogenous substance. All known components of the cholinergic system have been studied with these criteria in mind. If nerves of the lobster walking leg are deemed representative, ACh is present in axons (7, 8). Axonal and nerve terminal ACh are both released into perfusing medium which contains an esterase inhibitor, and that release is affected by local concentrations of calcium (9). Axons and nerve terminals are also similar in that choline acetylase (ChA) is primarily a cytoplasmic enzyme (8, 10), and acetylcholinesterase (AChE) is partially membrane bound (8).

While it is true that axons contain components of the cholinergic system, it is not certain that their presence is related to electrical excitability. A more widely held belief is that these components are merely in transit from the soma to the nerve terminal. Because of the differing opinions on the reasons for the presence of the cholinergic system in axons, many workers have studied the action of ACh applied exogenously to axons. Early studies demonstrated that ACh could depolarize the nerve trunk (11). However, the amount of ACh needed to produce effects on axons was far greater than that needed to elicit effects at junctional regions. There was, and is, a valid explanation. There are permeability barriers around the axon that limit diffusion of ACh and other drugs to potentially reactive sites. Any test of whether ACh is equiactive at junctional and axonal sites must contend with differences in diffusion rates.

The claim that axons are protected by permeability barriers was substantiated by the use of snake venoms (for review and tabulation of data, see 12). An acid-boiled fraction of cottonmouth venom substantially decreased permeability barriers to ACh and other quarternary agonists and antagonists (13). Thus, axons treated with venom became more sensitive to exogenous ACh. The mechanism by which venoms increase permeability is not clear, because venoms contain so many pharmacologically active substances (14). An earlier notion that phospholipase A is the active component that sensitizes axons appears untenable (15, 16). Yet, regardless of the mechanism by which venoms decrease barriers to penetration, they do not render axons as sensitive to ACh as are junctional regions and isolated electroplax (12).

Research utilizing snake venoms has not generated compelling evidence that axons require ACh for conduction. However, the work has renewed interest in axonal ChA and AChE. That interest has resulted in two noteworthy studies. The first (10) demonstrated that 80% inhibition of squid giant axon ChA did not alter nerve excitability. The other (17) showed that 98–99% inhibition of squid axon AChE did not affect electrical activity. Neither report encourages the belief that cholinergic activity and electrical excitability are interrelated.

TRANSMITTER STORES

According to theory, ACh is stored inside synaptic vesicles. During synaptic transmission, vesicles discharge their contents into the cleft. This theory, although widely accepted, is based mainly on indirect evidence. Several attempts have been made to provide direct evidence that vesicles are essential to transmission. The main approach has been to stimulate neuromuscular preparations repetitively in an effort to deplete nerve terminals of their synaptic vesicles. Early work along these lines was not particularly successful (18). However, more recent studies have demonstrated that intense stimulation can influence vesicle turnover (19–23).

Almost simultaneously with the stimulation research, a new technique for studying vesicles and transmission was developed. The technique is based on the finding that a variety of pharmacological agents will deplete nerve terminals of their vesicles. Among these agents are ammonium (24), lanthanum (25), β-bungarotoxin (26, but see 27), and black widow spider venom (28, 29). Black widow spider venom has been most widely used, so it can be considered a prototype for the group.

Most spiders are not venomous. Notable exceptions are members of the genus *Latrodectus. Latrodectus mactans,* more commonly known as the black widow spider, is of particular interest. When frightened or perturbed, it will bite and envenomate man. Of a variety of pathophysiological effects that result from envenomation, the more prominent are spreading pain, hypertension, and spasmodic muscular contractions. The toxic effects of the venom can be reversed by appropriate antibodies. Death from envenomation is rare.

Initial studies (30) on the cellular pharmacology of black widow spider venom indicated that the substance does not block nerve propagation or muscle contracture, whereas it does block synaptic and neuromuscular transmission. Detailed studies (28, 29) on the venom have shown that it produces two profound effects. Electrophysiologically, the venom evokes an extraordinary increase in the rate of spontaneous miniature endplate potentials. The increase in rate is not antagonized by an absence of calcium or an excess of magnesium. Apparently the venom changes the properties of the nerve terminal in such a way as to promote explosive release of ACh. Morphologically, the substance causes the disappearance of synaptic vesicles. Concomitantly with the loss of vesicles, there is an increase in the mass of presynaptic membrane (31). It may be that venom-induced disappearance of vesicles is due to coalescence of synaptic membrane with nerve terminal membrane. It has been reported that morphological changes in the frog are reversible (31). In contrast, it has been reported that mammalian motor nerve terminals are destroyed (32). The venom acts both peripherally and centrally on cholinergic terminals (33). Also, the substance acts to deplete catecholamine containing nerve fibers of their transmitter stores (34).

Black widow spider venom has aided in several aspects of the study of cholinergic transmission. First, it has provided reasonable evidence that vesicles and transmission are interrelated. This conclusion follows from the observation that venom-induced vesicle depletion stops both transmission and spontaneous ACh release (29,

31). Second, the venom has provided a means for quantitating the number of transmitter packets in nerve terminals (28). During the period of explosive ACh release, the number of spontaneous potentials can be monitored. The number of quanta that were present originally may be determined by calculation of the number of spontaneous potentials. Such calculations have produced estimates of the number of vesicles that are comparable to estimates made by other techniques (35). Finally, the venom has provided a means for determining the residual stores of transmitter in terminals that have been subjected to other forms of experimental treatment (36). For example, venom will evoke release of ACh from nerves that have been tetanically stimulated. Quantitation of venom-induced ACh release permits estimation of residual stores of transmitter that withstood tetanization.

RELEASE OF TRANSMITTER

We know little about the mechanism by which nerve terminals discharge their stores of transmitter substance (37). Nevertheless, it has been established that calcium plays an essential role. Although preceded by other studies, the work of Harvey & MacIntosh (38) was instrumental in linking calcium to ACh release. They demonstrated that sympathetic ganglia of the cat did not release ACh when perfused with calcium-free medium. During the years since that report, there have been hundreds of studies dealing with calcium and exitation-secretion coupling (39).

Several research strategies can be used to study calcium and cholinergic transmission. The simplest is to modify the concentration of calcium in the perfusate bathing isolated tissues. Such studies have indicated a quantitative relationship between transmitter release and extracellular calcium concentration (40–42). In addition, there is cooperativity in the release phenomenon; that is, more than one calcium ion is necessary to evoke quantal release of transmitter (43–45, but see 46). A more sophisticated strategy is to omit calcium from the bathing medium and then apply it iontophoretically to suspected sites of action. A representative study of this nature was conducted by Katz & Miledi (47). They demonstrated that calcium promotes ACh release by acting at the junctional region, and not remotely on the axon. In a similar type study, Miledi & Slater (48) showed that calcium must cross the nerve membrane to be active, because direct injection of calcium into the nerve terminal does not cause transmitter release.

Because other ions are known to interact or compete with calcium, it would be useful to minimize their effects. Sodium is one of the ions whose presence complicates interpretation of calcium studies. The most direct of several approaches that have been used to diminish the effects of sodium has been to suspend tissues in medium in which sodium is replaced by calcium (49, 50). Tissues bathed in high calcium release ACh spontaneously, and depolarizing currents will evoke additional transmitter release. A limitation of this approach is that prolonged exposure to high calcium will cause morphological changes and irreversible losses of function (50). It would be more desirable to have all ions present at physiological concentrations, under conditions in which calcium, but not sodium, can affect excitation-secretion coupling. Tetrodotoxin makes this possible.

Tetrodotoxin is a biological substance that can be extracted from the tissues of two unrelated creatures: the puffer fish and the newt. The active substance is an amino perhydroquinazoline ($C_{11}H_{17}N_3O_8$). The structure of tetrodotoxin is known (51), but factors such as relative insolubility in water, nonvolatility, and instability at high or low pH, made structural studies laborious. Intuitive leads were hampered by the fact that tetrodotoxin was unrelated to any other biological product known at the time. The molecule is a highly polar zwitterion, and it contains a guanidinium group. Most investigators agree that the guanidinium group contributes to the neurotoxicity of the substance (52, 53). In view of the similarity in the dimensions of guanidinium and sodium, the proposal seems reasonable. However, the guanidinium group alone does not account for neurotoxicity because slight alterations of the molecule that leave the guanidinium group intact cause marked losses in activity (51, 54).

Tetrodotoxin is neurotoxic by virtue of its ability to block sodium permeability associated with action potentials (55–58). It does this without altering the resting membrane potential or the flux of other ions. Understandably, tetrodotoxin has been used in many experimental settings to study the relation between sodium (or sodium channels) and tissue excitability. Nearly a decade ago the substance had been used in so many studies that it was the subject of an excellent review (59). Besides its obvious usefulness for investigating propagated potentials, tetrodotoxin has also been invaluable to the study of cholinergic transmission because it does not block the action of endogenous or exogenous ACh. Moreover, tetrodotoxin-treated preparations release ACh in response to locally applied depolarization (60, 61). Therefore, tetrodotoxin can be used to block propagated potentials and sodium flux, without affecting synaptic and neuromuscular transmission. (Needless to say, the existence of a drug that differentially affects axonal transmission and synaptic transmission does not support the Nachmansohn model.)

Tetrodotoxin has been used to great advantage in studying the link between calcium and excitation-secretion coupling. Space does not permit consideration of the many fine studies that have appeared. As an alternative, two representative reports will be described. In one report, Katz & Miledi (62) used tetrodotoxin to study the timing of calcium action during neuromuscular transmission. Double-barreled micropipettes were inserted into the junctional region of tetrodotoxin-treated sartorius muscle. One barrel was used to apply depolarizing pulses, and the other was used to inject calcium. The interval between depolarizing pulses and iontophoretic discharge of calcium was varied. The data indicated that calcium was most effective in promoting transmitter release when it was present immediately prior to depolarization. Injection of calcium during synaptic delay did not facilitate ACh release. It was concluded that movement of calcium into nerve terminals is only the first step in a sequential process leading to transmitter release.

In another report, Weinreich (63) studied post-tetanic potentiation (PTP) in control and in tetrodotoxin-treated neuromuscular preparations. Previous work (64) had suggested that PTP might be caused by an intracellular accumulation of sodium. Tetrodotoxin was used to abolish sodium flux during repetitive stimulation associated with tetanus. It was found that PTP developed in tetrodotoxin-treated

preparations just as it did in control preparations. In addition, PTP developed in muscles bathed in isotonic calcium chloride. It was concluded that PTP is dependent upon the movement of calcium rather than sodium into nerve terminals.

ACETYLCHOLINE RECEPTOR

Considerable progress has been made toward isolation and characterization of the ACh receptor. Much of this progress stems from research using α-bungarotoxin. This substance is one of several neurotoxins that can be isolated from elapid venoms. Alpha bungarotoxin is a polypeptide with 74 amino acid residues in a single chain (65). There is a remarkable degree of similarity between the structures of sea snake neurotoxins, cobra neurotoxins, and α-bungarotoxin (see references 14 and 15 for comparisons of amino acid sequence). All of these substances produce postsynaptic blockade of cholinergic transmission. This action is due to an irreversible, or only slowly reversible, binding of the toxins to the ACh receptor (66–69). The binding of α-bungarotoxin is least reversible, perhaps due to its relatively greater number of hydrophobic amino acids (14).

Generally speaking, there are two techniques that have been used in attempts to isolate the ACh receptor (70). One technique relies upon reversible agonists and antagonists, and the other relies upon irreversible antagonists. The rationale underlying the two approaches is somewhat different. With a reversible agent, an investigator can examine binding and dissociation of several drugs at each step during purification. With an irreversible agent, an investigator expedites receptor isolation. He need only monitor the whereabouts of a receptor ligand (radiolabeled) as purification proceeds.

Several biological products have been used as reversible agonists or antagonists in isolating the receptor, including ACh itself, atropine, muscarone, *d*-tubocurarine, and nicotine (70). There are two classes of irreversible antagonists. One class, the synthetic affinity label, has been used by Karlin and his associates (71, 72). Their technique involves the formation of a covalent bond between site-directed (affinity) compounds and the ACh receptor (73, 74). The second class of antagonists are the snake neurotoxins, of which α-bungarotoxin is the favored substance. The various methodologies are not necessarily incompatible. For example, one group has used reversible agents during isolation of the receptor and then used α-bungarotoxin to aid in confirming identity of the endproduct (75). Another group has used reversible agents as ligands in affinity chromatography, and then used α-bungarotoxin to assess purification of the chromatographically isolated receptor (76). Finally, a group has studied the interaction between snake neurotoxins and irreversible affinity labels (77). It was concluded that "neurotoxin and . . . affinity reagents have overlapping although not identical sites or modes of attachment to the receptor."

Localization of the Receptor

Because of the high specificity of α-bungarotoxin for the ACh receptor, it has proved valuable in both histochemical and biochemical research. Radiolabeled bungarotoxin has been used to localize ACh receptors autoradiographically at mouse

(78) and rat (79) neuromuscular junctions. Estimates of the number of receptors per endplate vary modestly, but the average is about 4×10^7. There is a one-to-one ratio between receptors per endplate and cholinesterase active sites per endplate (78). Similar findings have been noted for electric organs (68, 69). Comparative autoradiographs for the receptor (^{3}H–α-bungarotoxin label) and for cholinesterase (^{3}H-diisopropylfluorophosphate label) indicate that the two molecules are different (78).

Isolation of the Receptor

Changeux et al (68) were the first to demonstrate binding of bungarotoxin to membrane fragments rich in cholinergic receptor protein. Their report was followed by studies on electric organs (69, 80–83), striated muscle (84, 85), and brain (82, 86). As might be predicted, the amount of bungarotoxin-binding protein varies from tissue to tissue, depending upon the richness of cholinergic innervation. There is also some variability in the reported molecular weight of the receptor protein, with most estimates being reasonably close to 30,000–50,000. Variability probably reflects both species differences and differences in isolation techniques.

Two surprising findings have emerged from the intense research on the ACh receptor: one deals with methodology, and the other with results. Levinson & Keynes (87) have published a report on the use of organic extraction procedures for isolation of receptor-ligand complexes. Their work was prompted by that of DeRobertis and associates, who routinely use chloroform-methanol extraction followed by column chromatography (see 88 for review). Levinson & Keynes found that ligands could be eluted from columns in a manner that suggested specific binding to a receptor, whereas binding was not in fact occurring. They concluded that earlier findings by the Argentine group (see 24) may have been artifactual.

O'Brien and associates have reported the surprising finding that the internal (89, 90) surface of axonal membranes from lobster nerves contains macromolecules that bind cholinergic drugs. Among the substances tested was α-bungarotoxin. The axonal "receptor" exhibited several properties characteristic of postsynaptic receptors. However, axonal receptors had a lower affinity than junctional receptors for ACh. It was proposed that the axonal protein that binds ACh may be a component of sodium and potassium gates. This is an important proposal, and one that should be further explored. Along this line, Raftery et al (81) did not detect significant binding of α-bungarotoxin to axons of either *Torpedo* or gar fish.

TROPHIC EFFECTS ON MUSCLE

Efferent nerves exert a variety of trophic effects on muscle (91). Among those aspects of muscle physiology that are regulated by nerve, the most important are degree of innervation, sensitivity to ACh, distribution of cholinesterases, speed of contracture, concentration of enzymes, and rate of metabolism. Typically, studies of trophic effects on muscle have involved sectioning of nerve trunks. Denervated muscles undergo profound changes, most of which are reversible following reinnervation. In such studies, muscle should preferably be pharmacologically disconnected without the trauma of nerve section. Botulinum toxin is ideally suited to this purpose.

Botulinum toxin is produced by *Clostridium botulinum,* an organism that is nearly ubiquitous in soil. The toxin is produced in several immunologically distinct forms designated types A, B, C_α and C_β, D, E, and F. The organisms are not totipotential in their ability to produce toxins; each strain produces but one type, this being true also for C_α and C_β. Type A botulinum toxin is the most potent of the group, and it has been the main subject of research. Type A toxin was originally crystallized by investigators at Camp Detrick (92, 93). The crystalline molecule has a molecular weight of approximately 1,000,000, and it is composed of at least two biologically active subunits. One of the subunits, referred to as botulinum hemagglutinin, causes agglutination of red blood cells. The other, known as botulinum neurotoxin, causes paralysis of cholinergic transmission. The two active components of type A botulinum toxin have been separated chromatographically (94). It appears that the neurotoxic fragment has a molecular weight of about 150,000 (94, 95). A claim (96) that the neurotoxin has a molecular weight in the range of 10,000 has not been confirmed (97–99).

The precise mechanism by which botulinum toxin blocks cholinergic transmission is not understood. It has been known for many years that the toxin acts at nerve terminals to prevent both nerve impulse transmission and spontaneous release of ACh (100, 101). The toxin does not act like magnesium, i.e. it is not a competitive antagonist of calcium-evoked transmitter release (102). The mechanism of action of the toxin appears to be much more complex. Recent studies (103, 104) indicate that the toxin binds rapidly and irreversibly to the neuromuscular junction, and that this binding occurs independently of transmitter release. Following binding, there is a second step that is dependent on transmitter release. In the absence of calcium, or in the presence of high magnesium concentrations, botulinum activity is greatly retarded. It may be that transmitter release exposes reactive sites in the nerve that are not otherwise available for interaction with toxin. It is interesting that another biological poison, β-bungarotoxin, has a similar dependence on transmitter release (27). It has been suggested that the active sites in botulinum toxin may be free amino groups (105, 106). This could account for the finding that botulinum toxin is inactivated by the sialic acid groups of gangliosides (107, 108). Gangliosides have frequently been suggested as important components of the transmitter releasing system (109, 110). The precise mechanism by which transmitter release is blocked by botulinum toxin is not known. Nevertheless, the certainty with which the substance paralyzes cholinergic transmission, plus the absence of any other known effects, make the toxin a highly suitable agent for functionally disconnecting nerve and muscle.

Degree of Innervation

In mammals there exists a one-to-one relationship between muscle fibers and the nerves that innervate them. Furthermore, innervated muscles will not develop additional neuromuscular contacts, even when accessory nerves are mechanically implanted. Only when a muscle has been denervated will it accept and make functional contacts with other nerves. Such findings suggest that intact nerves govern the receptivity of muscle to endplate formation. A likely candidate for the governing

role is ACh. It has been demonstrated that botulinum-poisoned gastrocnemius muscle will accept implants of peroneal nerves (111). It has also been shown that new sprouts will generate from the terminal arborizations of poisoned nerves, and that these sprouts will develop contact with underlying muscle (112). The development of nerve sprouts and of muscle endplates proceeds more rapidly in slow muscle (soleus) than in fast muscle (gastrocnemius; 113, 114).

Because of the known action of botulinum toxin in blocking ACh release, the findings described above have been interpreted to mean that ACh release controls neurotization. While this is probably a valid conclusion, there is at least one limitation worth considering. It appears that a variety of substances are released from cholinergic nerves, including protein (115, 116), prostaglandin (117, 118), and ATP (119). The ability of botulinum toxin to block release of these substances has not been determined. Therefore, it is possible that an unidentified substance, by itself or in concert with ACh, regulates formation of neuromuscular contacts.

Sensitivity to Acetylcholine

The endplate region of normal muscle is highly sensitive to iontophoretically applied ACh. Sensitivity to the transmitter diminishes with distance from the junctional region. There is reason to believe that the nerve itself acts to restrict the area of maximum sensitivity. For example, following denervation there is a gradual spread of increased sensitivity (denervation supersensitivity) beyond the endplate region (120). Conversely, reinnervation causes a gradual loss of sensitivity in extra-junctional regions (121). It has been reported that supersensitivity will develop in skeletal muscle poisoned with botulinum toxin (122). In addition, spread of sensitivity appears greatest in fibers that have the lowest spontaneous release of ACh.

While it may be true that ACh release acts to restrict the chemosensitive zone, how it does this is unresolved. Spontaneous release of ACh alone is not sufficient to restrict development of denervation supersensitivity (123, 124). In fact, it may be that sensitivity is not even regulated by ACh per se, but instead by the consequence of ACh release, i.e. muscle activity. In an interesting report, Drachman & Witzke (125) studied ACh sensitivity in muscles that were electrically stimulated to mimic normal patterns of activity. They found that electrical stimulation greatly diminished the spread of supersensitivity in denervated diaphragms. The authors concluded that "muscle activity may account for neurotrophic regulation of the acetylcholine sensitivity."

Distribution of Cholinesterases

There is a complex relation between denervation and muscle cholinesterase activity. Factors such as species, duration of denervation, and muscle group under study, all contribute effects (91). Moreover, cholinesterases are both neural and muscular in origin. Thus, assays of denervated neuromuscular preparations must distinguish neural from muscular changes. Under the circumstances, the use of botulinum toxin is highly preferable to severing nerves. In studies utilizing the toxin, it has been shown that muscle cholinesterase does not depend upon ACh release. This has been demonstrated both biochemically (126) and histochemically (113). As there is evi-

dence that muscle cholinesterase is partially under neural regulation (91), the regulatory factor must be something whose release or activity is not impaired by botulinum toxin.

Atrophy

Atrophy is a term that signifies gradual loss of metabolic and mechanical activity. It implies a deteriorative change that involves nearly the whole of muscle physiology. There is convincing evidence that ACh is the trophic factor that supports muscle integrity. The most important evidence is the repeated observation that botulinum toxin-induced neuromuscular blockade will produce atrophy (127–130). The possibility that some substance other than ACh may be implicated has been diminished by the work of Drachman (131). He has demonstrated that atrophy occurs in muscles treated with hemicholinium, *d*-tubocurarine, or botulinum toxin. These three drugs block ACh activity at three different sites. It is difficult to envision how they could have comparable effects on a substance other than ACh.

CONCLUDING REMARKS

The number of poisons that have been used to study cholinergic transmission, and the number of contexts in which they have been used, far exceeds the bounds of a single review. Suffice it to say, poisons are indispensable to the study of synaptic and neuromuscular transmission. Biological products that have been used range from the commonplace to the esoteric. Among the more commonly used substances, *d*-tubocurarine has become nearly an essential ingredient to research on postsynaptic potentials. Among those substances of unknown value are antibodies directed against specific protein and lipid components of pre- and postsynaptic membranes.

It is likely that the importance of poisons in neurobiology will increase. As our understanding of neural phenomena approaches molecular level, our need for drugs with well-defined mechanisms and sites of action increases. And thus poisons, whether biologic or synthetic in origin, will continue in a paramount role.

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