MICROPHARMACOLOGY OF VERTEBRATE NEUROMUSCULAR TRANSMISSION

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Neuromuscular physiology is now being explored at the subcellular level so that the term 'microphysiology' seems appropriate to describe present research endeavors. Micropharmacology seems the logical description of the search for drugs with predominant action on one of the subsystems involved in neuromuscular transmission.

This review is designed to pinpoint these subsystems and briefly outline the signs that such a system is being affected by a drug, mentioning particularly drugs whose action has been worked out, and giving some attention also to the inevitable overlap of drug action between systems. Antibiotic actions on neuromuscular transmission have recently been reviewed (1) and have therefore been omitted from this account. Foldes (2) has recently reviewed presynaptic aspects of neuromuscular transmission while Nastuk (3) and Cookson & Paton (4) have reviewed mechanisms of neuromuscular blockade.

Figure 1 illustrates the present conception of neuromuscular transmission (5). Acetylcholine (ACh) is manufactured in motor nerve terminal cytoplasm from acetyl coenzyme A (acetyl CoA) of mitochondrial origin, and choline derived from extracellular fluid by an active membrane mechanism. A cytoplasmic enzyme, choline-o-acetyltransferase (ChAc, EC. 2.3.1.6) accelerates synthesis and unknown mechanisms load the ACh into synaptic vesicles. The vesicles, like the mitochondria and ChAc molecules, are derived from the cell body by transport along the motor nerve. As Figure 1 shows, the vesicles fuse with the terminal membrane and empty their contents into the synaptic cleft. The vesicle membrane is then incorporated into the terminal membrane and later recovered as a complex vesicle, which in turn sheds its coat to reveal a synaptic vesicle, which is refilled with ACh and recycled.

Superimposed on these mechanisms is the nerve impulse, enormously increasing the rate of release by a mechanism of which only the first two steps are known. These are the depolarization of nerve terminals, which increases their calcium conductance, and the entry of calcium ions (Ca^{2+}). This process will be termed 'depolarization secretion coupling'.

Soluble acidic proteins (6) and ATP (7) are released with ACh, as is prostaglandin (8). The protein and ATP may play some role in ACh binding in vesicles. Prostaglandin E_1 has no effect on neuromuscular transmission (9) and its function is unknown. The possibility that other choline esters might be released together

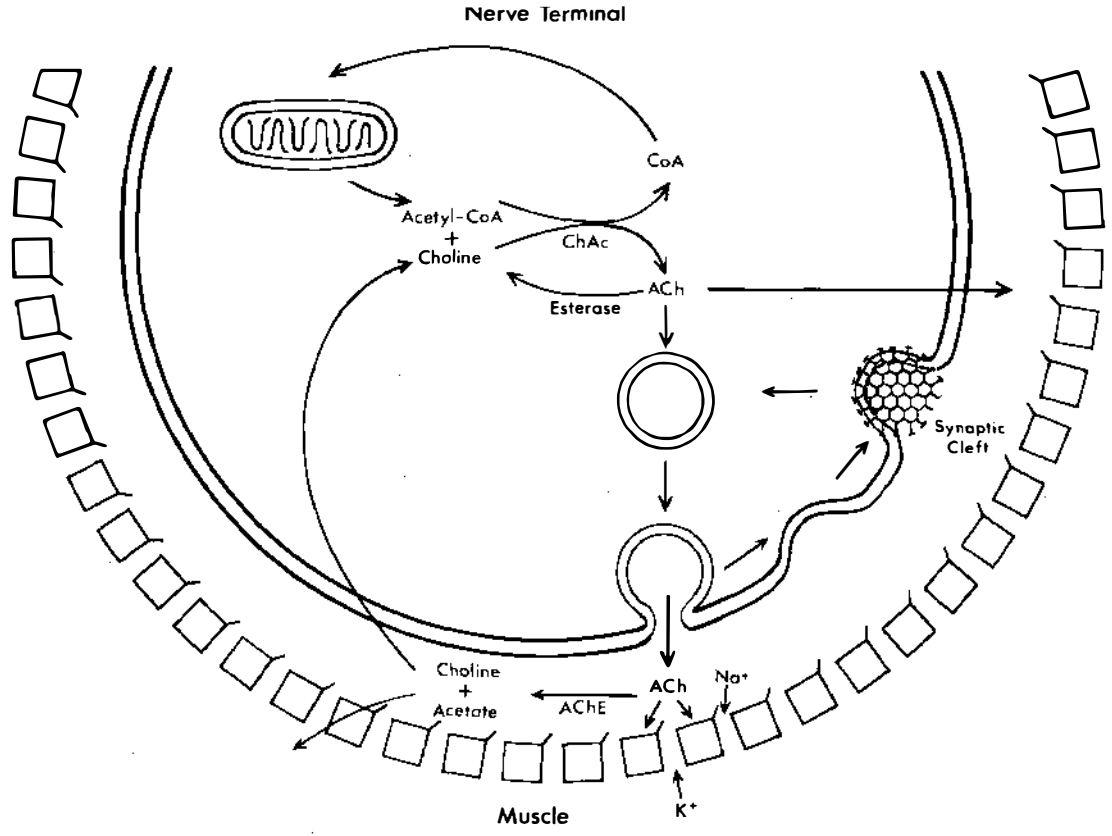

FIG. 1. Neuromuscular transmission at the subcellular level. Note the mitochondrion and the synaptic vesicles in the nerve terminals and in the terminal cytoplasm the biosynthesis of ACh. In the synaptic cleft note the release and degradation of ACh and the uptake of choline by the nerve terminal. ACh receptors line the muscle surface (squares) with ionic gates activated by combination with ACh. Note that vesicles fuse with the terminal membrane as complex vesicles. Complex vesicles in turn shed their coats to appear as synaptic vesicles.

with or instead of ACh (10, 11) appears remote. The results of gas-chromatographic and biological assay experiments are the same. ACh is the only choline ester found in perfusates of nerve-muscle preparations after nerve stimulation (12, 13). Acetyl-1-carnityl choline and carnityl choline have recently been much investigated by Hosein and his group (e.g., 14) but it appears that these compounds are 10,000 times less active than ACh when assayed on the frog rectus preparation (15).

Released ACh may diffuse away from nerve terminals or be broken down by an enzyme, acetylcholine acetyl hydrolase (acetylcholinesterase, AChE, EC. 3.1.1.7), superficially located in relation to the folded muscle membrane. As Figure 1 indicates, following the hydrolysis some 50% of choline molecules liberated into the synaptic cleft are taken up by nerve terminals. Before diffusing away or being hydrolyzed, however, an ACh molecule may combine with specific macromolecules in the subsynaptic membrane. These molecules, the ACh receptors, are probably tetrameric lipoproteins (16). The result of ACh combination is an increased muscle membrane conductance for sodium and potassium ions, possible through separate channels, as Figure 1 shows. It is unclear at the present time whether the ACh receptor is the molecule whose rearrangement leads to the increase in membrane conductance or whether the ACh receptor allosterically affects another molecule—an ionophore—which then provides the ionic channels. In any event, at frog junctions each receptor ACh interaction appears to effect the net passage of some 50,000 cations, thus generating a 0.2 μV depolarization (17, 18). The result of a large number of such elementary events in parallel is a rapidly increasing membrane depolarization—the endplate potential (EPP) which reaches its peak as ACh action ceases. A passive recharging of the depolarized muscle membrane follows, so that the EPP decays with the time constant of the muscle membrane.

Neuromuscular transmission, like other physiological processes, is regulated by hormonal action. At vertebrate neuromuscular junctions, known hormonal actions include the facilitating effects of epinephrine and norepinephrine release by the sympathetic nervous system and the adrenal cortex (19–21). A depressant effect of thymic factors has recently been reported (reviewed in 22). Hormonal effects on neuromuscular structure that could affect ACh release are also known. Sufferers from Myasthenia Gravis may have their symptoms alleviated after 10 days by large daily doses (100 units) of corticotropin. A structural basis for this effect may be inferred from the finding (23) that the muscles of rabbits treated for 28 days with 15 units/kg/day of corticotropin had endplates about 30% longer than controls, with branching of preterminal fibers increased some 50%. Kuno, Turkanis & Weekly (24) have positively correlated length of endplates in frog muscle with ability to release transmitter.

SUPPLY

ChAc, mitochondria, and vesicles are supplied to nerve terminals from the parent cell body (reviewed in 25). The role of microtubules and neurofilaments in transport is still unclear but disruption of either element or the denial of ATP

to any axon segment will stop transport (see e.g., 26). Studies of transport inhibiting drugs upon neuromuscular function are in their infancy. Colchicine, which disrupts neurotubules, does produce a presynaptic block of neuromuscular transmission (27). The blocking effect, however, is too rapid to be attributed to effects on supply (Peters & Hubbard, unpublished observations).

SYNTHESIS

As Figure 1 indicates, ChAc catalyses the formation of ACh from choline and acetyl-CoA. Specific inhibitors of this enzyme have long been sought. In a test tube the styrylpyridine analogs described by Smith, Cavallito & Foldes (28) are the most potent, competitive, and reversible inhibitors (29). However, inhibitors have to cross the nerve terminal membrane to act in vivo. Lipid soluble compounds such as 4-(1-naphthylvinyl) pyridine (NVP) have been extensively tested. NVP and other analogs do not, however, appear to be ChAc inhibitors in vivo. Even when intra-arterially infused into the femoral artery of cats, NVP did not affect indirectly evoked contractions of the gastrocnemius muscle. Binding of NVP by plasma components was demonstrated in these experiments and this may have reduced the NVP concentration below an effective dose (30). In experiments on a wider range of preparations, Hemsworth & Foldes (31) detected no certain signs of ChAc inhibition in vivo using NVP or in vitro using NVP and eleven other styrylpyridine analogs. NVP did induce a frequency dependent block of neuromuscular transmission, but similar effects occurred on direct stimulation of muscles, so the site of NVP action was uncertain. Furthermore, the block of neuromuscular transmission took hours of washing to reverse whereas in vitro reversal of ChAc inhibition by NVP is rapid.

CHOLINE TRANSPORT

While choline uptake is not energy dependent, presumably a specific carrier molecule is involved since uptake may be competitively inhibited by drugs, is saturable and temperature dependent and requires Na^+ in the external medium. Drugs affecting choline transport have recently been reviewed by Bowman & Marshall (32).

The compound α,α'-dimethylethanolamino 4,4′-biacetophenone (hemicholinium-3, HC-3) is the best known inhibitor of choline transport. It reduces the ACh content of and ACh release from nerve terminals (33–35), and is dramatically antagonized by choline (34–36). Characteristically it produces a block of neuromuscular transmission dependent on the frequency of stimulation and thus the rate of ACh depletion. The hemiacetal structure of the drug is responsible for its activity (37). Analogs with similar activity include the N-methylpiperidine (38), N-allyl and the 3 and 4 methylpyridinium derivatives (39–41), and the bisected hemicholinium molecule (42).

Linear choline analogs are known that are essentially similar to HC-3 in their action. Best known is triethyl (2-hydroxyethyl) ammonium, commonly known as

triethylcholine, which is actually transported into nerve terminals and acetylated to form acetyltriethylcholine (43–45). This compound is devoid of ACh-like actions so the block of neuromuscular transmission is due to absence of ACh synthesis, not the release of a false transmitter (46).

A wide range of monoquaternary choline analogs was studied by Bowman and his colleagues (47, 48). The two ends of the range (small onium substituents, e.g., trimethyl or large substituents such as propyl) have postjunctional blocking actions. Compounds with intermediate size substituents have HC-3 like activity. Bretylium tosylate also resembles HC-3 in its action on rat diaphragm (48–50). The tetraethylammonium ion (TEA) in addition to its known effect on nerve conduction (blocking potassium channels, 51) also inhibits choline uptake (52).

Bisquaternary compounds are also known that have HC-3 like activity. Of a series of polymethylene bis (hydroxethyl) dimethylammonium salts the decamethylene compound (C-10, dicholine) was potent in reducing ACh output from stimulated nerve muscle preparations. The C-5 and C-6 compounds had weaker activity (53). αα-dimethyl and α-ethyl and NN-triethyl derivatives of succinyldicholine had similar effects (48, 54).

Trimethoxybenzoic acid esters also inhibit choline uptake. The most potent are troxonium tosylate and troxypyrrolium tosylate (reviewed in 32). The latter indeed is more potent than HC-3 or triethylcholine on chick biventer cervicis muscle (55).

No drug is presently known that has an action solely on choline transport. The most effective inhibitors (HC-3, triethylcholine, troxonium, and troxypyrrolium tosylate) are in addition postjunctional blocking agents of the nondepolarizing type (34, 56, 57). Triethylcholine produces an initial increase in ACh release before transmission fails (58). The bisquaternary choline uptake inhibitors are postjunctional blocking agents of the depolarizing type (53). Conversely it may be said that weak inhibition of choline uptake is a feature of the action of a number of agents well known as depolarizing or nondepolarizing postjunctional blocking agents or anticholinesterases (anti-ChEs). ACh and its analogs, methylcholine and carbamylcholine, compete with choline. Studies on rat motor nerve terminals indicate that ACh can only be taken up in the presence of physostigmine (35). The presence of physostigmine presumably allows the competition between ACh and choline uptake to shift in favor of ACh. Tubocurarine (dtC), hexamethonium, atropine, decamethonium, physostigmine, and neostigmine all inhibit choline uptake into synaptosomes (59) and must be suspected of a similar action on motor nerve terminals.

ACh Release

Normally, quantal release of transmitter at the neuromuscular junction occurs in two forms: (*a*) spontaneous random discharge at a rate of about 1/sec recorded as miniature endplate potentials (MEPPS); (*b*) simultaneous discharge of as many as several hundred quanta, in response to a presynaptic action potential. The postsynaptic response to these units represents the EPP.

It is now clear that the release of transmitter by a nerve action potential is a very brief but enormous acceleration of spontaneous discharge (reviewed in 5). In the presence of low [Ca] or in high [Mg], the secretory response to presynaptic polarization and to presynaptic nerve impulses are inhibited in parallel; thus there appears to be a distinct mechanism sensitive to [Ca] and [Mg] by which transmitter secretion is linked to nerve terminal depolarization.

Direct Effects on Secretion

A direct effect of an agent on transmitter release is one that occurs independently of the level of presynaptic membrane potential or of the extracellular [Ca^{2+}]. Such an effect is exerted by ethanol, which acts to multiply spontaneous transmitter release, the quantal content of EPPS ,and the transmitter release evoked by presynaptic depolarization to the same extent (60, 61). Moreover, the multiplication of MEPP frequency by any given increment of ethanol concentration is independent of how much ethanol is present, i.e., the response-dose is a simple exponential function (61). This behavior can be accounted for by an ethanol-induced reduction, in graded fashion, of an energy barrier that normally limits release of transmitter packages.

A Ca^{2+}-independent action on transmitter release, similar to that of ethanol, is exerted by other alcohols, many drugs, e.g., chloral hydrate, pentobarbital, urethane, paraldehyde, chloroform, ether, chlorpromazine, and theophylline (61), the fluorescent probe 1-aniline-8-naphthalene sulphonate (62), the thiol oxidizing agent, diamide (63), and certain toxins (64, 65). For some of these agents (61) there is a close correlation between relative potency in multiplying MEPP frequency and 'stabilization' of erythrocyte membranes [as defined by Seeman (66)]. This suggests that their action on transmitter secretion may be simply secondary to membrane expansion, which could alter distribution of charges on the inner face of the membrane. Many of these drugs resemble an osmotic gradient (67) in that they exert an inhibitory action on depolarization-secretion coupling as well as their accelerating effect on spontaneous release. The result is that the net action on transmitter release varies with the degree of nerve terminal depolarization and the concentrations of Ca^{2+} and Mg^{2+} in the bathing medium (68).

Effects on vesicles.—While the events by which vesicles and nerve terminal fuse are obscure, it has been suggested that the process is one of Van der Waal's binding. The mutual repulsion produced by the positive charge of vesicle and terminal membrane is thought to be overcome by masking of charges by entering Ca^{2+} (69). Drugs increasing intracellular calcium would then increase the number of potential releasing events. A number of drugs that are uncouplers of oxidative phosphorylation and induce mitochondria in vitro to release Ca^{2+}, do in fact increase spontaneous release of ACh from neuromuscular junctions in vitro in the absence of extracellular Ca^{2+} (70).

Drug actions on the cycle of events by which vesicles are filled with ACh, later empty their contents, become part of the terminal membrane, and are reformed and refilled, also seem highly possible. Drugs that blocked vesicle filling would produce a prejunctional block of neuromuscular transmission due to reduction of ACh release, be not well antagonized by choline, and have no effect on ChAc. The tertiary base 2-(4-phenyl piperidino) cyclohexanol (AH 5183) is such a drug (71) and this role has been suggested (72). The prototype action here is that of botulinum toxin. This neurotoxin of *Clostridium Welchii* is produced in several immunogenically distinct forms—A, B, Cα, Cβ, D, E, and F (73). Recent investigations suggest that neither the A nor the D form completely blocks spontaneous ACh release as had previously been thought (74, 75). Simpson (76) has shown that paralysis is linked to ACh secretion rather than to effects on depolarization secretion coupling and has suggested that release of ACh exposes reactive sites to the toxin. It is known that the toxin binds strongly to gangliosides (77), perhaps in the walls of synaptic vesicles. Speculatively, it may be that the effect of the toxin is to prevent the uptake of ACh by synaptic vesicles.

The effects of β-bungarotoxin and black widow spider venom on nerve terminal structures (65, 78) indicate an effect on the vesicle cycle. After the great but transient increase in MEPP frequency produced by these agents ceases, electron microscopy reveals a terminal devoid of vesicles. The nerve terminal membrane is, however, expanded (78). Speculatively, the toxins act on sites where vesicles are normally reformed, blocking the process.

Tetanus toxin also causes a presynaptic block of neuromuscular transmission by unknown mechanisms (79, 80). It is of interest in that with sublethal doses a preferential blockade of slow twitch muscles can be produced (81). Less marked indications of differential effects of botulinum toxin on fast and slow muscle have also been found (82).

Effects on Depolarization-Release Coupling

The action potential.—The relationship between the magnitude of nerve terminal depolarization and the rate of transmitter release is a very steep power function. Similarly, the duration of depolarization and the rate of release are strongly related. Very small increases in action potential amplitude or duration will thus cause increases in transmitter release without necessarily altering the spontaneous release rate. Tetraethylammonium (TEA), which prolongs the action potential by inhibiting delayed rectification (51), has exactly this action (83, 84).

Nerve terminal membrane potential.—Depolarization of nerve terminals as well as provoking spontaneous release (MEPP frequency increase) will reduce the amplitude of action potentials and thus reduce the magnitude of evoked quantal release. Compounds with such actions include a variety of metabolic inhibitors (85–88) and the steroidal alkaloid, bactrachatoxin (89). Anoxia of the preparation will mimic these effects (90). ACh itself, and its agonists, apparently depolarize

nerve terminals (91–93). The effect is so small there is no change in spontaneous release (91 92, 94, 95) but delicate tests still show a fall in evoked release (91, 96, 97).

Tubocurarine apparently has two possible actions on nerve terminals. It may block ACh action and it may itself depolarize (91). A similar dual action is known in the cortex (98). Evidence is accumulating that ACh release may be affected by dtC. A reduction of the ability to maintain release during repetitive stimulation (99–101), and a small reduction of release by single impulses, have been reported by some (100) and denied by others (102, 103). The effects could be mediated by dtC receptors in nerve terminals but alternative mechanisms, e.g., interference with choline uptake, have not been excluded. Dretchen and his group (104, 105) have recently reported reduction of ACh release in the presence of a variety of nondepolarizing and depolarizing blocking agents.

Block of conduction in fine nerve terminals in nerve muscle preparations in vitro is commonly the result of their depolarization due to anoxia (90, 106). It seems likely that the conduction block found in vitro following exposure of nerve muscle preparations to ACh analogs and anesthetics (107–109) may be due to a similar depolarizing action, particularly in the stretched preparation (108, 109).

Repetitive activity.—Anti-ChEs such as prostigmine set up presynaptic action potentials with backfiring (110, 111). The mechanism is obscure but involves some terminal depolarization (91). The resultant twitch potentiation (112) presumably accounts in part for the success of such compounds in the treatment of Myasthenia Gravis. It may be noted that the fasculations of skeletal muscle that follow close arterial injection of ACh and agonists are reflex in origin (113). Contractures of the intrafusal muscles cause spindle activity.

A better worked out mechanism of repetitive nerve terminal activity is exhibited by guanidine. This drug is thought to prevent the inactivation of sodium entry, a normal event during action potential generation (114). Similar mechanisms of action for veratrine and germine mono- and germine-diacetate, which have similar actions (115, 116), may be suggested. These drugs improve neuromuscular transmission by other mechanisms too, which presumably contribute to their successful use in treatment of botulinum poisoned patients (117, -guanidine and germine mono- and diacetate) and some cases of myasthenia gravis (118, -germine mono- and diacetate). Guanidine, for example, produces spontaneous twitching in muscle (119) which is related to the appearance of giant multi-quantal MEPPS. It also acts like TEA to increase the quantity of transmitter released per impulse (120). This action is not shared by germine mono- and diacetate (121, 122) but these latter compounds have a direct potentiating effect on muscle contraction (115, 116).

Effects on Ca conductance.—The entry of Ca^{2+} is the link between terminal depolarization and release. Manipulation of the amount of calcium entering or the amount of (presumably) intracellular Ca is thus a potent way of controlling release. Ouabain, and other cardiac glycosides block active transport of Na^{+} out of terminals, thus causing an accumulation of intracellular Ca, for Ca and Na

removal are linked. Plausibly, this is the explanation for the increased evoked release in the presence of these compounds (123, 124). Proponents of a role for cyclic AMP in ACh release will take no comfort from the finding that adenosine, which should cause accumulation of cyclic AMP, apparently depresses spontaneous and evoked release, possibly by reducing Ca entry (125).

Effects on other steps in coupling.—A number of agents alter transmitter release by interacting with the coupling mechanism in unknown ways. Phenol, for instance, increases the quantal content of EPPS (126, 127), while having little action on spontaneous MEPP frequency in similar concentrations (Hackett & Quastel, unpublished). Norepinephrine and epinephrine have a similar action (128) evidently mediated via an α receptor (129). In contrast, uroporphyrins and amino acids depress the increased spontaneous release evoked by terminal depolarization (130, 131). The neuromuscular blockade, sometimes associated with bronchial carcinoma and known as the myasthenic syndrome, is characterized by a low quantum content of EPPS (132). The block in vitro is relatively insensitive to raised $[Ca^{2+}]$ so it appears that there has been interference with some earlier stage in the coupling mechanism.

Post-tetanic potentiation.—The pharmacology of the increased quantal release evoked by a testing nerve impulse at selected intervals after tetanic stimulation, known as 'post-tetanic potentiation' (long intervals), or facilitation (short intervals), has not been much explored. Gage & Hubbard (86) found that metabolic inhibitors selectively inhibited potentiation without affecting facilitation. This might indicate separation of the two processes. Alternatively, if sodium transport was blocked, tetanic nerve stimulation might increase terminal depolarization and thus obscure underlying continued potentiation.

The ACh Receptor

The ability partially to isolate and to measure the number of receptors has developed from the finding that the venom of some Crotalid and Elapid snakes contains true postsynaptic blocking substances resembling dtC. They differ from dtC in that the block is slower in onset and apparently irreversible (reviewed in 133). The α toxin of *Bungaris multicinctus* (α-Bgt) for instance, binds irreversibly to the ACh receptor but does not bind to AChE. α-Bgt can be radioactively labeled and thus is an excellent tool for quantitative studies of receptor membrane density. Such studies (134, 135) indicate that at rat and mouse endplates there are $2\cdot-4 \times 10^7$ binding sites which, if one α-Bgt molecule is bound per receptor, may be equated with the number of receptor molecules. Receptor density on this basis is between $1-2 \times 10^4$ μm^2 at the endplate and only about 5×10^2 per μm^2 elsewhere.

Receptor type.—In terms of Dale's classification, there is good evidence that all ACh receptors of striated muscle, whatever the animal, are nicotinic (136) and that actions of muscarinic substances exerted on striated muscle are exerted by combination with the same nicotinic receptors (137). The problem of what makes

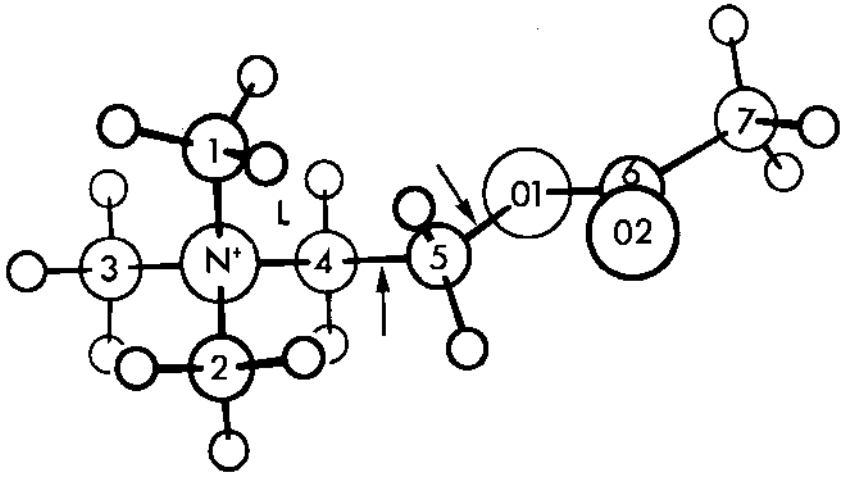

FIG. 2. A representation of a ball and stick model of ACh in the conformation thought to interact with AChE, kindly supplied by Dr. P. Pauling. The two largest circles (01 and 02) represent 0 atoms. The changed N atom is so marked and the smallest circles (unnumbered) represent H atoms. The C atoms, numbered 1 through 7, are represented by circles intermediate in size between the N atoms and the H atoms. Arrows indicate bonds around which rotation is possible. Further description in text.

a receptor nicotinic or muscarinic appears to have been recently solved by crystallographic analysis of ACh and its agonists (Fig. 2). The difference appears to lie in the conformation of ACh with which the receptor combines. ACh is a flexible molecule. It contains two groups, each in their own planes (C3-N^+-C4-C5; 01-C6-(02, C7)). As the arrows in Figure 2 indicate, rotation is possible around the bonds between C5 and 01 and between C4 and C5. The other possible sites of rotation (01-C6, N^+-C4) are fixed by stereochemical principles (138, 139). Muscarinic and nicotinic molecules differ in the degree of rotation at the two sites of torsion. The conformation of ACh relative to nicotinic receptors, for instance, has the torsion angle C4-C5-01-C6 = 180° (antiplanar) and the angle N^+-C4-C5-01 = 75° (positive synclinal). As Figure 2 shows, in this conformation the molecule has two sides, termed by Chothia (139) the *methyl* side, defined by a line close to C1, 01, and C7, and the *carbonyl* side, defined by a line close to C2, C5, and 02. Analysis of the crystal structure of potent nicotinic agonists with little muscarinic acitvity indicates that in every case when compared with ACh, the carbonyl side is preserved and the methyl side blocked (139). The reverse is true of muscarinic agonists (muscarine itself does not even contain a carbonyl group). The methyl side of nicotinic agonists is thus only part of a supporting matrix holding the three dimensional arrangement of the carbonyl side in the correct conformation.

Iso-receptors.—All nicotinic receptors are not the same. There are isoreceptors (140). Differences between nicotinic receptors of the same muscle in different species (141) and of different muscles within the same species are well documented. The nondepolarizing blocking agent, 2β,16β-dipiperidino-5α-androstane-3α,17β-dial diacetate dimethobromide (pancuronium bromide) for instance, is a better blocker of ACh action in cat white than cat red muscle (142) and is much more active on cat muscle than it is upon rat muscle (143). There is reason to think that some differences in the effectiveness of the same drug on different myoneural junctions are due to differences in the experimental variables, particularly temperature, in the two situations rather than receptor differences (144–146).

Within the same muscle, receptors at the endplate (intrinsic) and elsewhere (extrinsic) may have different pharmacologies. For instance, in the rat diaphragm, dtC combines with only half as many sites as does α-Bgt. As ACh can reduce α-Bgt binding further (to about 81%), dtC cannot be binding to all ACh receptors (135). It is known that binding of radioactive dtC does not increase much after denervation (147) and further that the sensitivity of the extrajunctional sites to dtC after denervation is not increased as much as would be expected. It is tempting to suppose that intrinsic receptors bind ACh, α-Bgt, and dtC while extrinsic bind only ACh and α-Bgt. Receptor differences are also indicated by the finding that hexafluorenium is less effective than dtC in producing neuromuscular block at the frog endplate, whereas it is much more effective than dtC in reducing or, by pretreatment, preventing the depolarization produced by carbamylcholine (148).

Nature of combining groups.—Much pharmacological evidence derived from studies of the relative effectiveness of homologous series of ACh agonists and antagonists complements the crystallographic suggestion that ACh receptors bind ACh at two points. The principal bond is ionic (149), between an anionic site on the receptor and the cationic quaternary head of the ACh molecule, indeed, nitrogen-free isosteres of ACh that lack an onium group do not react with ACh receptors (150). Pharmacological studies suggest that the second active site may be 'esterophilic' as it has an affinity for the highly polarized ACh ester group (136). The claim that an ACh-like action could be produced by combination with the esterophilic site (151) only, has not been substantiated (152).

Receptor binding, nondepolarizing blocking drugs.—Studies with dtC and gallamine (153, 154) demonstrate that in the rat diaphragm preparation the onset and offset of neuromuscular blockade are diffusion controlled. Apparently the increased rate of onset of block seen with successive applications of the same concentration of dtC can be accounted for by a progressive decrease in the tortuosity factor as the preparation ages.

The search for a nondepolarizing muscle relaxant without undesirable side effects has achieved some success with the development of pancuronium bromide (155), which lacks histamine releasing properties, ganglion blocking activity, and atropine-like activity (156). Studies in vitro in frog and rat preparations confirm its dtC-like action but 5 – 10-fold greater potency (155, 156).

Pancuronium bromide is not ideal however, in that its duration of action is prolonged. A so-far unsuccessful search for shorter acting substitutes has included tests of a new group of mono- and diquaternary N-substituted choline esters of carboxylic acids (157, 158). The monoquaternary compounds had considerable nicotinic stimulant activity and appeared unsuitable for clinical use. Some of the diquaternary compounds did have a reasonable potency, nondepolarizing blocking activity, and short duration of action in vivo, but anti-ChE activity was an inherent property of their structure and may limit their usefulness. A series of α-ω-bis(3, 3 dialkyl-3,4 dihydroisoquinolium) alkanes have also been investigated (159). All had a more rapidly reversible blocking action than dtC but showed anti-ChE and vagal blocking actions as well.

A number of other drugs of clinical or research interest have recently been shown to be nondepolarizing blocking drugs in vitro. These include (frog endplate): chlorpromazine (160), 5-hydroxytryptamine (161), lobeline (162); (rat endplate): pyridine-2-aldoxime methochloride (PAM) in concentrations above 6 mM (163), bretylium tosylate (164). Bretylium activity was complicated by an anti-ChE action that was predominant at concentrations less than 0.25 mM.

Depolarizing drugs.—The change in membrane conductance brought about by the ACh receptor combination has recently been brought under scrutiny at the molecular level. Katz & Miledi (17, 18) have shown that iontophoretically applied ACh produces an increase in membrane voltage noise, thought to be generated by variations in and summation of the activity of ACh operated 'gates' in the membrane. When the power spectrum of the noise is analyzed it develops that the average gate is open for about a msec.

It appears that ACh is effectively removed (by AChE and diffusion) well before the gate action is complete (reviewed in 5). One line of evidence is that the ACh agonist, carbamylcholine, and the depolarizing blocking agent, decamethonium, will also produce membrane noise but analysis indicates the membrane gates are only open for an average of 0.4 msec(18). It appears that other types of drugs may also bind to the ACh receptor complex and alter the resulting conductance change. Xylocaine, better known as a local anesthetic (165), and neostigmine (166), well known as an anti-ChE, apparently exert such actions at the levels normally used to demonstrate the alleged primary actions.

The potential complexity of action of some blocking drugs is well exemplified by a recent study of the effects of the adrenergic β-receptor blocker, propranolol (167). In low doses this drug had a curariform action with a slowly developing and slowly reversible block of neuromuscular transmission. In higher doses it showed depolarizing activity with a rapid block of transmission. A postulated depression of quantal release (168), based on indirect considerations, was not confirmed upon direct examination (167).

Inactivation.—When ACh or any other depolarizing compound is applied to the endplate region of muscle it is found that the depolarization produced is not maintained and does not parallel the drug concentration. Instead it fades from its

peak value at a rate that is a function of the amount of depolarization and the nature of the depolarizing drug.

The apparent conversion of ACh receptors to an inactive form during maintained depolarizing drug action has been recently termed 'receptor inactivation'. The mechanism is still obscure but there is much support for the hypothesis that prolonged endplate depolarization and the consequent increase in Ca^{2+} conductance, normally not important because of the small number of Ca^{2+} present, leads to transmembrane movement of Ca^{2+} and their combination with anionic sites controlling ionic channels for sodium and potassium, thus reducing membrane depolarization (169). In general, a postjunctional blocking agent that depolarizes will increase the rate of inactivation. Nondepolarizing blocking agents (dtC, atropine) are generally without effect. TEA however does bring about marked inactivation, perhaps by calcium displacement from anionic sites (170).

A variety of drugs are known to affect the conductance changes produced by ACh and to have a lesser or no effect upon the ACh receptor, as judged from competition experiments (see e.g., 171). Some of these drugs have been shown to affect the rate and magnitude of inactivation. For instance, the anesthetics, diethylether (172) and halothane (173), and the diquaternary drug, hexafluorenium (174), increase the rate of inactivation. The antibiotic, nystatin (175) and the catecholamines, norepinephrine and epinephrine, reduce inactivation and this effect is blocked by α blocking drugs such as phentolamine, but not by propranolol (176, 177). It may be relevant to the finding that decamethonium is a potent inactivating drug, that it is taken up and by muscle fibers in the endplate region (178), possibly by an ion exchange mechanism (179).

The Removal of ACh

It is well established that ACh action ceases during the rising phase of the EPP with the termination of the underlying conductance change. More recent investigations suggest indeed that ACh is removed (by diffusion and enzymatic action) before the conductance changes are over (reviewed in 5).

A range of esterases are now known to be present at neuromuscular junctions including AChE (EC. 3.1.1.7), acetylcholine acylhydrolase (BuChE, EC. 3.1.1.8), and nonspecific eserine resistant carboxylic esterases (nsE, EC. 3.1.1.1). The three groups of esterases can be distinguished by pharmacological criteria. Di-isopropyl phosphofluoridate (DFP) inhibits all of them, but AChE and BuChE can be specifically protected from DFP by eserine, or reactivated by specific drugs after DFP action (180).

From a physiological standpoint, AChE is clearly the important enzyme. BuChE and nsE can be inactivated without affecting neuromuscular transmission (181). BuChE is present at high concentrations at some vertebrate neuromuscular junctions and has a high affinity for ACh. Some investigators consider it may play a minor role in ACh splitting (182).

The classical concept of ACh hydrolysis involves an esteratic site, acetylated during reactivation with ACh, and a nearby anionic site binding ACh before acetylation occurs by Coulombic forces. A more complex binding site is indicated

by recent research. For instance, compounds have been found that inhibit the enzyme as far as ACh hydrolysis is concerned by combining irreversibly with it, but leave it 100% active in the hydrolysis of other substrates such as indophenyl acetate (183, 184). These compounds are thought to alkylate the anionic site, leaving the esteratic site untouched. Investigation of enzyme activity in the alkylated state, however, shows that alkylation affects V_{max}, and the effects of different substrates are due to varying effects upon the acetylation step of the hydrolysis. Substrates have the rate constant for acetylation either increased or decreased by previous alkylation. The simplest, but not the only explanation for this action, is that these substrates bind to one of two sites distinct from the already occupied anionic site (185).

Drugs inhibiting AChE and other esterases in vivo and in vitro have recently been reviewed (186). Such drugs are traditionally assayed either directly by their anti-ChE activity or indirectly by their ability to prolong the time course of EPPS or their ability to reverse the neuromuscular blocking action of dtC. In view of the strong evidence that some anti-ChEs exert their EPP prolonging activity by a direct effect on the ACh receptor complex in addition to their undoubted anti-ChE activity, it is hazardous to extrapolate from indirect effects directly to effects on AChE.

Investigators of anti-ChEs, besides specifying which esterase(s) their drug inhibits and how it acts, must make a further distinction on the question of whether the drug can penetrate nerve membranes. Eserine, for instance, which inhibits AChE and BuChE, penetrates nerve terminals and presumably inhibits cytoplasmic esterases, for there is a large increase in (presumably) cytoplasmic ACh in nerve terminals that can be detected in assay experiments (35).

There has been some interest in long continued AChE inhibition. Twice daily injections of the reversible anti-ChE, neostigmine, produced neuromuscular weakness in rats. In vitro studies of diaphragm muscles from affected rats showed a fall in quantal content of EPPS, perhaps due to interference with choline uptake (187). After a single sublethal intramuscular injection of one of the so-called irreversible cholinesterase inhibitors (DFP, Soman), there was necrosis of muscle, beginning in the endplate area and taking five days to clear. The damage was correlated with ACh release and accumulation by a number of tests (188, 189).

Recovery from poisoning by DFP and other organo-phosphate anti-ChEs is of obvious clinical importance and has been studied in great detail (190). In vivo it involves a regeneration of the inhibited enzyme by spontaneous hydrolysis of the enzyme inhibition complex and synthesis of new enzymes. Chemical reactivation in vitro, e.g., by PAM, is also possible. Chemical and spontaneous reactivation are of limited effectiveness and fail after a period of exposure to the inhibitor due to a change (aging) in the substituents of the phosphoryl group that makes the P-O bond insensitive to the reactivation.

Careful analysis of the extent and mechanism of reversibility, using neuromuscular preparations in vitro, has only just begun. Welsch & Dettbarn (191) for instance, report that rat diaphragm phrenic nerve preparations, after treatment with 10^{-4} M DFP for 30 min followed by washing for an hour in Lockes' solution,

showed only about a 10% recovery of total ChE activity. Seven percent of AChE appeared reactivated and only about 3% of BuChE. After pretreatment with 5×10^{-5} M paraoxon, the recovery of total ChE activity upon washing was somewhat larger but the esterase reactivated was largely BuChE. Unfortunately inhibitors of protein synthesis such as chloramphenicol and cycloheximide (5-500 μg/ml) had an anti-ChE action in control preparations, so that the role of enzyme synthesis in recovery could not be assessed.

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