PHARMACOLOGICAL REVIEWS Vol. 29, No. 3
 Printed in U.S.A.

The Actions of Cholinergic Drugs on Motor Nerve Terminals*

MICHAEL D. MIYAMOTO†

Department of Pharmacology, Schools of Medicine and Dental Medicine, University of Connecticut, Farmington, Connecticut

* This work was supported by the U.S. Public Health Service (NS 12270) and the Epilepsy Foundation of America.

† Present address: Department of Pharmacology, College of Medicine, East Tennessee State University, Johnson City, Tenn. 37601.

I. Introduction

THE existence of a functional discontinuity between nerve and muscle was postulated as early as the 1850's when Claude Bernard (20) noted that the site of curare blockade is neither nerve nor muscle but somewhere in between (181, 184). Since these early studies, many attempts have been made to investigate and characterize the physiology and pharmacology of synaptic transmission. While there is agreement that acetylcholine (ACh) mediates transmission at the neuromuscular junction and at sympathetic ganglia, the site and mechanism of action of certain ACh-like or cholinomimetic drugs have been under some dispute. From his observations, Bernard first thought the primary site of curare action might be the nerve terminal. However, there is compelling evidence that *d*-tubocurarine (dTC) blocks at the postjunctional site.

The question arises whether the postjunctional membrane is the only site of action. Evidence from differing sources has indicated a prejunctional cholinergic action, in addition to or in place of the postjunctional one. As shown in a variety of biologic systems, ACh can act on any membrane which is unprotected by a myelin sheath, *e.g.,* ACh causes depolarization of sensory fibers (70, 102), nonmyelinated vagal C-fibers (4), lobster nerve fibers (68), sympathetic fibers (85), in addition to preganglionic (98, 99, 137, 180) and motor nerve terminals (114). In accord with these findings, a number of hypotheses have been proposed which incorporate into the transmission process a cholinoceptive action on nerve terminals (45, 134, 201). In view of this, the controversy can be stated: 1) Are there prejunctional cholinoceptors? 2) If so, what are their actions? and 3) Are these actions significant?

Although a large part of the controversy stems from interpretation of results, part also arises from differences in approach, *i.e.,* microscopic localization of ACh receptors (AChR), measurement of ACh release and recording of antidromic nerve activity and synaptic potentials. A brief description is therefore given of the methods and limitations for each experimental approach, and critical analyses are provided when warranted. For finer details, reliance is placed on a number of recent reviews (36, 56, 89, 92, 95, 100, 105, 108, 112, 151, 153, 200, 215).

II. Present Concepts

A. The Release Process

1. Morphology and ultrastructure. The neuromuscular junction consists of the axon impinging onto a specialized area of the muscle known as the end-plate (fig. 1). It is a one-to-one type of synapse, easily accessible to drugs and microelectrode studies. The axon is covered with a myelin sheath containing nodes of Ranvier but is bare at the ending. There is a predominance of mitochondria (21) and synaptic vesicles (183) within the terminal. The vesicles are clustered near narrow transverse ridges or "active zones," which lie opposite the junctional folds (71). Upon nerve stimulation, the ridges are surrounded by small dimples. Because these dimples do not appear when Ca^{++} is absent (185) or when Mg^{++} is present (107), they are believed to represent "vesicle attachment sites." The nerve terminal is separated from the end-plate by a gap of 200 Å. The subsynaptic membrane of the end-plate contains the AChR, the ion conducting channels, and the acetylcholinesterase (AChE).

2. Synaptic potentials and synaptic vesicles. Synaptic activity at the junction consists of neurally evoked end-plate potentials

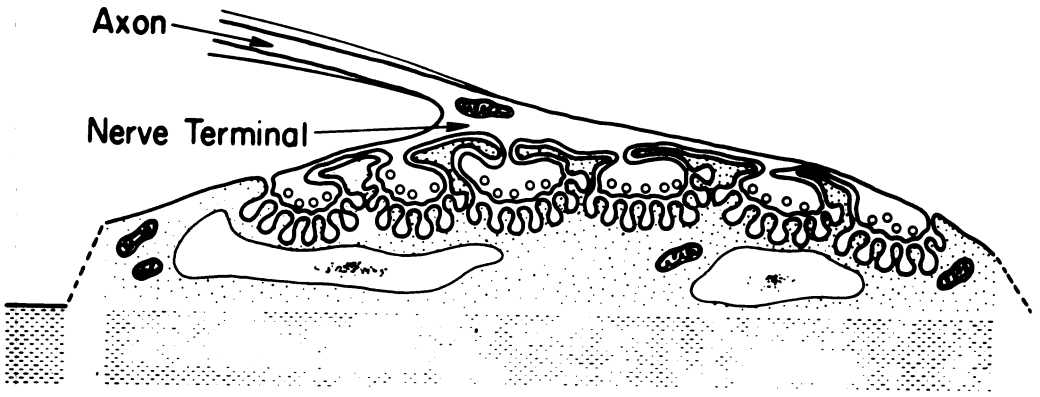

FIG. 1. Semidiagrammatic representation of the neuromuscular junction at a fast twitch fiber (extensor digitorum longus) of the rat. The axon is covered with a myelin sheath but is bare at the actual end-plate region. The bulblike nerve terminal expansions are apposed by regular and deep junctional folds. (Reproduced with permission from Ellisman *et al.*: Studies of excitable membranes. II. A comparison of specializations at neuromuscular junctions and nonjunctional sarcolemmas of mammalian fast and slow twitch muscle fibers. J. Cell Biol. **68**: 752–774, 1976.)

(EPPs) (73, 101) and spontaneously occurring miniature end-plate potentials (MEPPs) (83). The link between the two can be demonstrated by applying Mg^{++}, which decreases the EPP in increments which are multiples of the MEPP (39, 64). The resulting idea that MEPPs represent the basic unit of transmission is known as the "quantum hypothesis" (64). The concept that synaptic vesicles are the morphologic correlates of the MEPPs is known as the "vesicular hypothesis" (66) [see 100, 111, 112].

3. Events at the nerve terminal membrane. Action potentials propagating to the terminal cause depolarization and lead to transmitter release. Release can still occur in the presence of agents which block Na^{+} (tetrodotoxin, TTX) and K^{+} (tetraethylammonium, TEA) currents if the terminal is focally depolarized (32, 125) (see 5, 179, 221). Therefore, depolarization of the membrane, rather than the actual movement of Na or K ions, is what is required for transmitter release. Presumably depolarization causes an increase in membrane conductance to Ca^{++} or possibly to some Ca^{++} carrier in the membrane (126, 127). The amount of Ca^{++} entering is determined by the Ca^{++} gradient and the voltage-dependent Ca^{++} conductance. Internal Ca^{++} may normally be kept low by a Ca^{++} pump and sequestering by mitochondria (8).

4. Subcellular mechanisms. How Ca^{++} brings about release is not known. One suggestion (31) is that vesicular and nerve terminal membranes are negatively charged, so that there is normally electrostatic repulsion. Ca^{++} may neutralize this effect. Accordingly, injection of Ca^{++} into the terminal causes the release of some transmitter (167). The electrostatic repulsion may also be neutralized by depolarization of the terminal membrane. To explain the inhibitory effect of Mg^{++} (167), however, one must assume that Mg^{++} binds to the Ca^{++} conductance and/or vesicular binding sites (188) but is ineffective in promoting release.

ACh synthesis occurs at mitochondria by reaction of acetyl coenzyme A with choline and is catalyzed by choline acetyl transferase. Choline acetyl transferase and AChE are synthesized at the cell body and transported to the terminal by axoplasmic flow. Choline, but not ACh, is extracted from the extracellular fluid by high affinity uptake (234). Synthesized ACh is protected from cytoplasmic AChE by storage in vesicles. There is an available and a reserve pool of ACh (23), and depletion of the available pool causes mobilization of ACh from the reserve. However, newly synthesized ACh

is preferentially released (54, 192). "Surplus ACh" forms on treatment with a membrane penetrating anti-AChE (55). This ACh is not releasable by nerve stimulation and may represent "free ACh" not yet packaged and subject to hydrolysis by intracellular AChE [see (105, 151)].

B. Postjunctional Events

1. Activation of ionic currents. Transmitter released from the vesicles diffuses across the synaptic gap. Estimates of the amount of ACh per vesicle range from 10^5 (166) to 2,000 molecules (9). The released ACh may either 1) diffuse away into the systemic circulation, 2) combine with subsynaptic AChR, or 3) bind to AChE and be hydrolyzed. The reaction of ACh with AChR probably involves an electrostatic binding and a conformational change in the AChR (76, 121, 196). This is linked to the ionic channel(s) which allow the flow of Na^+, K^+ and Ca^{++} to generate the end-plate current (219).

2. Single channel conductance. The molecular mechanism of the ionic channel causes an increased voltage fluctuation or "synaptic noise" on application of agonists (129). By use of Fourier analysis of the component frequencies in the noise, it is possible to study conductance changes of single channels, in response to differing agonists and physiologic conditions (2) (see 92, 215).

III. Electrophysiologic Investigations

A. Muscle Contraction

The classic method of quantifying neuromuscular function is measurement of muscle twitch to nerve (indirect) and muscle (direct) stimulation. A decrease in response to nerve but not muscle stimulation indicates an inhibition of transmission. If responses to both nerve and muscle stimulation are diminished, then the effect can be on the nerve, the muscle, or both. Clearly, while this approach is useful for screening effects on transmission, it is impossible to differentiate whether such effects are pre- or postjunctional (153).

Some have improved this technique by varying the rate of stimulation, the implication being that the depression observed with high frequencies reflects a prejunctional effect (30, 59, 194, 204, 227, 233). Depression with frequency can be enhanced by dTC, ether or nicotine (227). This effect occurs on transmission, since both nerve and muscle conduction are unaffected. Any decrease in response occurring with high frequencies is known as "Wedensky inhibition."

1. Effect of antagonists. Wedensky inhibition has been used to deduce the site of action of dTC. In anesthetized or decerebrate cats, Hutter (117) notes that the initial depression of twitch (tibialis) is similar to the decline of EPPs in dTC-treated preparations. Because of this similarity in frequency-dependency between dTC-treated (EPPs) and untreated (twitches) preparations, he suggests that dTC may act solely to reduce postjunctional sensitivity.

With hind limbs of rabbits, Naess (177) has found that increasing stimulus frequency from 2 to 300 Hz produces an increase in overall twitch which is maintained at that level. With dTC, the twitch increases with frequency but is *not* maintained. This effect is not reversed with neostigmine, which indicates that the inhibition cannot be explained solely by postjunctional sensitivity. The effect with dTC occurs only on prolonged exposure to the drug, whereas the postjunctional action (reduction of overall twitch) appears in a few minutes. Naess (177) attributes this to the differences in the ability of dTC to "penetrate the active surfaces of nerve and muscle," so that the order of inhibition is muscle first and nerve second.

Naess (176) has also found that post-tetanic facilitation of the twitch (presumably a prejunctional phenomenon (117)) is more pronounced during initial administration of rather than prolonged exposure to dTC. This indicates that 1) dTC reduces a pre-

junctional phenomenon, and 2) this effect is slow in onset. Despite these findings, Naess (176) considers that the reduced sensitivity to ACh seen with single stimuli (117) indicates the effect of dTC is predominantly postjunctional.

In experiments with frog and rat, Blackman (30) has examined whether the dependence on stimulus frequency with dTC is due to a postjunctional action. To maintain a constant degree of neuromuscular block, increases in dTC are titrated with increases in neostigmine. Despite the increase in dTC, there is no change in the frequency-dependency, which indicates that the effect is not due to an action on the postjunctional AChR. Blackman (30) concedes, however, that desensitization of postjunctional AChR may explain tetanic fade to depolarization blockers such as succinylcholine (SCh).

The possibility of a prejunctional AChR sensitive to hexamethonium (C-6) has been suggested (223). In indirectly stimulated (0.1 Hz) tibialis muscles of anesthetized cats, edrophonium produces a facilitation of contraction which can be antagonized by the ganglionic blockers, C-6 and trimethaphan. This occurs at drug concentrations having no effect on the unfacilitated twitch. Although the techniques used provide no information as to the site or mechanism of action, Volle (223) has suggested that a block at the prejunctional site would be consistent with known effects of C-6 on ACh-induced stimulation of sensory receptors (70), nerve endings and autonomic ganglia.

More recently, Bowman and Webb (37) have studied the comparative effects of cholinergic antagonists on the tetanic fade of transmission. Soleus muscle contractions to indirect stimuli are monitored in anesthetized cats and tetanic fade produced by 5 sec of 100 Hz stimulation. C-6 produces a rapid and complete waning of tetanic tension, whereas peak twitch to single stimuli is not affected. Pancuronium, by contrast, produces only a slight decrease in tetanic response, whereas peak twitch is significantly reduced. The effects of dTC fall between those of C-6 and pancuronium. These results show first that effects on peak twitch can be separated from those on tetanic fade. Second, if peak twitch reflects a postsynaptic action (117) and tetanic fade a presynaptic effect (227), then C-6 has a presynaptic action, pancuronium a postsynaptic action and dTC an action at both pre- and postsynaptic sites. In view of the above results, it may be more appropriate to use C-6 to investigate the actions of AChR at the motor nerve terminal.

B. Antidromic Backfiring

Another method used to examine neuromuscular function is based on antidromic spike discharging at the motor nerve (84, 161). Active drugs such as neostigmine cause bursts of spikes at the ventral root, coincident with muscle fasciculation (synchronous contraction of fiber bundles). This indicates that drugs initiate backfiring at or near the nerve terminal.

This technique has been refined to give the "matched pair preparation" (197, 198, 202, 211, 213, 230), which means that pre- and postjunctional events can be monitored simultaneously. The ventral root is cut close to the spinal cord and a small nerve branch teased out. The stimulating and a first pair of recording electrodes are placed on this branch. A second pair of recording electrodes is placed on the muscle near the endplates, and the muscle attached to a transducer. Drugs are administered by close i.a. injection.

Antidromic activity is recorded under two basic conditions, 1) on close i.a. injection of active drugs (drug-induced activity, DIA), and 2) after orthodromic conditioning stimulation (stimulus-bound repetition, SBR). The first applies to activity initiated solely by administration of drugs, whereas the second refers to that which requires a conditioning stimulus. SBR includes conditioning by single (postdrug repetition, PDR) or tetanic stimuli (post-tetanic repe-

tition, PTR), in the presence or absence of facilitatory drugs (89, 199). Riker (199) suggests placing depolarizating drugs into the DIA category. Facilitatory drugs which do not directly depolarize are then placed into the SBR classification.

1. Origin of antidromic activity. Severing the ventral root means that antidromic firing originates at the nerve periphery, presumably the nerve terminals (161). This interpretation has been complicated by muscle spiking which is able to excite the terminals by ephaptic (reversed synaptic) transmission (74, 149). However, analyses of the time course of the two phenomena (230, 231) have shown that the muscle discharge is separate from the neural discharge. Furthermore, antidromic activity can be initiated after muscles are cut and prevented from discharging (11), which indicates that the firing is at least in part due to a *direct* action at the terminals.

Another possibility is that end-plate depolarization by agonists causes an efflux of K^+ (69, 124), so that K^+—rather than a prejunctional ACh action—is the initiator of antidromic firing. This explanation has been discounted for two reasons. First, if K^+ is able to initiate antidromic activity, then injection of KCl should elicit discharges, but none has been found (84). Second, if ACh or anti-AChEs cause a significant efflux of K^+, there should be an increase in spontaneous transmitter release (146), but none has been observed (83, 114, 145). Therefore, the most satisfactory explanation appears to be the existence of AChR at pre- as well as postjunctional sites (72, 110) (see also 36, 108).

2. Effect of facilitatory drugs. A facilitatory drug, as used in the context of this review, is one which enhances muscle contraction to indirect supramaximal stimulation. There is evidence in mammals that facilitation, as well as fasciculation, is a result of repetitive muscle firing. This, in turn, is due to antidromic firing along the axon reflex, so that there is recruitment of the entire motor unit (200, 230). Facilitation is associated with nerve stimulation and thus SBR, whereas fasciculation is a result of DIA in the absence of stimulation. This distinction is important for deducing the mechanism of action of facilitatory drugs.

Facilitatory drugs injected in the absence of nerve stimulation (DIA) produce spontaneous muscle fasciculation. The drugs include: neostigmine (161, 226), physostigmine (28, 74), ambenonium (28, 226), methoxyambenonium (28), diisopropylfluorophosphate (DFP) (203, 222) and anilinium compounds (201), including edrophonium (226). Comparison of the actions of edrophonium, neostigmine and ambenonium in anesthesized cats shows that edrophonium is the least effective and most transient initiator of DIA, whereas neostigmine is the most effective and persistent (226). Actual recordings of DIA have been made for neostigmine (161, 226), DFP (222) and edrophonium (25, 226), so this effect is obtained with carbamates, organophosphates and anilinium ions, respectively.

Initiation of DIA at the nerve ending must involve either 1) a direct depolarizing action, or 2) an anti-AChE action which allows endogenous ACh to depolarize the terminals. It is unlikely that DFP acts by depolarization, since the compound has no onium group and cannot bind to the anionic site of the AChR (*cf.* 76, 121, 196). Similarly, DFP binds to the esteratic rather than the anionic site on the AChE molecule (136). Therefore, the simplest explanation is that DFP (and possibly other facilitatory drugs) works by an anti-AChE action, the common property of the three drug types mentioned.

Facilitatory drugs also promote antidromic firing following conditioning stimuli (SBR). This effect has been found for neostigmine, physostigmine, DFP (11) and edrophonium (25). In cut muscles of rat diaphragm, there is a pronounced SBR after neostigmine but a less pronounced effect after DFP (11). Low doses of ACh cause a significant increase in the DFP-induced activity (11), whereas high doses produce blockade (203). These results are consistent with an anti-AChE action of DFP, which,

in conjunction with low or high doses of ACh, produces depolarization or desensitization, respectively.

DFP also produces facilitation of muscle twitch (due to initiation of SBR) (222). On prolonged incubation with DFP, this effect becomes biphasic, *i.e.*, the facilitation returns to control levels. Repeating the experiment after washout of DFP shows that the effect is irreversible. These findings can also be explained by an anti-AChE action of DFP, whereby cumulated ACh causes depolarization (facilitation) followed by desensitization (return to control).

SBR initiated with neostigmine in anesthetized cats is abolished by the ACh antagonists, C-6, dTC, gallamine and pancuronium (226). C-6 is the most effective and gallamine and pancuronium the least effective blockers. Accordingly, Webb and Bowman (226) suggest that neostigmine prolongs the action of endogenous ACh on the terminal, but because of the differential sensitivity to C-6, this action is more nearly like that in ganglia; if, however, neostigmine works by a direct effect [*cf.* Riker (199)], it must be very similar to a direct cholinoceptive action.

The noncompetitive AChE inhibitor, ecothiopate, causes SBR in rat diaphragm with a dose-dependent rate of onset (174). Because SBR is generated after ecothiopate is washed out, Morrison (174) has concluded that a direct drug action on the terminals is unlikely. He instead proposes that ecothiopate works by an anti-AChE action, to *both* prolong the EPP (and thereby cause repetitive muscle firing) and enhance the action of ACh at the terminal.

3. Effect of agonists. Injection of ACh also initiates DIA, but the resulting spikes do not occur in bursts as with neostigmine (161). In fact, DIA is generated in only 20% of the nerve fibers (198). Similarly, the effectiveness of ACh in promoting SBR is much less than that of neostigmine, generating at most 5 spikes to 20 with neostigmine. Riker (198) has concluded that ACh has three actions at the terminal: 1) a depolarization, 2) a definite but limited facilitation and 3) a long lasting depression. He also suggests that the sensitivity of the junction to ACh is due to an effect at the terminal, but because ACh is less effective than neostigmine and because high doses block antidromic firing, ACh cannot have a physiologic role in transmission (*cf.* Koelle (134)).

Standaert and Adams (214) have shown that SCh, like other onium compounds, affects the nerve terminal. SCh promotes antidromic activity after single stimuli (PDR), but this effect is biphasic since there is depression at higher concentrations of the drug. A biphasic response with SCh is also noted in the absence of stimulation (DIA). Because these effects occur at doses lower than those blocking the twitch, Standaert and Adams (214) conclude that the primary site of action is the nerve terminal.

In the anterior tibialis of the cat, SCh or decamethonium (C-10) promotes antidromic firing in the presence (PDR) or absence (DIA) of stimulation (28). In higher doses, these drugs have no effect, in agreement with other studies showing a biphasic excitatory-inhibitory response (214). The explanation for drugs of this type appears to be an initial excitation due to activation of prejunctional AChR, followed by inhibition at higher doses of agonists due to desensitization of AChR (133).

4. Effect of antagonists. Blockade of antidromic firing by dTC has been found by a number of investigators. Masland and Wigton (161) indicate that dTC blocks neostigmine-induced discharging in doses which do not abolish the indirect excitability of the muscle. Similarly, Werner (229) finds that dTC suppresses PDR in doses below those affecting transmission. In higher doses, dTC abolishes the short latency back response, which represents activity generated at the muscle. Again, Standaert (212) notes that dTC blocks PTR, whereas inhibition of the muscle twitch requires a 25-fold higher concentration. Identical results have been obtained by Chang *et al.* (47).

The effect of gallamine is variable. Wer-

ner (229) finds that gallamine blocks antidromic activity initiated with 3-hydroxyphenyltetraethylammonium (3-OH PTEA). On the other hand, Sokoll *et al.* (209), recording in frogs, note that low concentrations of gallamine initiate antidromic activity, in accord with earlier observations in the cat (203). With higher concentrations, PTR and post-tetanic potentiation of the twitch are abolished.

5. Threshold for antidromic firing. One means of quantitating nerve terminal excitability is by frequency and duration of antidromic spiking (197). A second means is to measure the threshold for antidromic backfiring (114). Extracellular microelectrodes can be focused at the nerve ending by recording extracellular EPPs or MEPPs. Pulses are applied through the electrode, so that spikes are generated and recorded at the cut end of the nerve. By noting the current needed to trigger activity, an index of terminal excitability is obtained. A lowering of threshold presumably indicates membrane depolarization (114).

With the above technique, Hubbard *et al.* (114) have examined the effects of agonists and antagonists on terminal excitability. Application of high concentrations of ACh causes a rapid decrease in nerve terminal threshold in Mg-paralyzed rat diaphragm. With continued application, there is a spontaneous reversal in effect, suggesting desensitization of the AChR (133). Perfusion with a lower concentration of ACh causes a maintained decrease in threshold, and this effect is antagonized by dTC.

Curiously, dTC itself reduces the threshold for nerve stimulation, which suggests that it depolarizes the membrane (114). Although unexpected at the neuromuscular junction, depolarization by dTC has been seen in the central nervous system. This is also consistent with observations (208) of an increase in transmitter release by low doses of dTC.

Depolarizing the terminals with K^+ (7.5 mM) also reduces the threshold. Higher concentrations (11–23 mM) cause a reversal in effect, presumably due to loss of membrane excitability (Na inactivation). Because K^+ acts like ACh to lower threshold, the question arises whether the observed effects of ACh are due to end-plate K^+ (124). This appears unlikely, however, since a reduction in nerve terminal threshold by ACh has been observed at a time when there is no end-plate depolarization (114).

6. Nerve terminal afterpotentials. The "excitability cycle" of the terminal membrane after conditioning stimulation has been examined, to see whether effects on the threshold are produced by *endogenously* released ACh (110, 114). The assumption is that changes in the cycle are due to ACh released by the conditioning stimulus. The arrangement is as described above, except for the use of a *supra*threshold conditioning pulse, followed by a threshold measuring pulse. Plots of threshold (excitability) *vs.* time after conditioning show a sequence of 1) relative refractory period (1.5–4 msec), 2) supernormal period (10–20 msec) and 3) subnormal period (50–90 msec). Neostigmine prolongs and dTC shortens the refractory and supernormal periods.

Changes in the supernormal period can be attributed to changes in the negative afterpotential at the nerve terminal (97). Presumably, the observed changes (114) consist of a depolarization by endogenous ACh superimposed on the negative afterpotential (110). The opposing actions of neostigmine and dTC in altering the supernormal period indicate, therefore, either enhancement or reduction of the action of endogenous ACh. On the assumption that this interpretation is true, these findings provide further evidence for a direct effect of ACh at the motor nerve terminal, by a means subject to pharmacologic manipulation.

C. Intracellular Microelectrode Studies

1. MEPP frequency. MEPPs are usually recorded by penetrating the end-plate with a microelectrode. MEPP amplitude de-

pends on the size of the quantal packet, the AChE activity, the postjunctional AChR sensitivity, and the properties of the muscle membrane (123, 132). MEPP frequency, however, can only reflect a prejunctional event (124), since quanta are either released or not released. Depolarizing the nerve terminal with focal electrodes increases MEPP frequency (65), whereas depolarizing the muscle affects only MEPP size. MEPP frequency is thus a sensitive and unequivocal index of nerve terminal activity.

a. Effect of facilitatory drugs. Although the question at hand should be answered by monitoring MEPP frequency, such results have been equivocal. Fatt and Katz (83) indicate that neostigmine has little effect on MEPP frequency in frogs but show in one figure a striking increase. Similarly, in rat diaphragm, Liley (145) finds no effect of neostigmine on MEPP frequency and suggests that fibrillation (asynchronous twitching of individual fibers) can be explained by increased end-plate sensitivity. However, he does not account for the concomitant fasciculation, which is indicative of nerve activity. Finally, Blaber and Christ (27) imply that neostigmine, edrophonium, ambenonium and methoxyambenonium have no effect on MEPP frequency in cat tenuissimus. However, inspection of their data reveals that these drugs all produce significant increases in MEPP frequency. In contrast to these studies, Boyd and Martin (38) find in cat tenuissimus a *consistent* increase in MEPP frequency with neostigmine.

b. Effect of agonists. In rat diaphragm, neither bath nor iontophoretically applied ACh causes an increase in MEPP frequency (114). Focal recording at the nerve terminals, however, shows a decrease in threshold by ACh, which presumably indicates depolarization (114). Because depolarization is known to increase MEPP frequency (146), the failure of ACh to increase frequency is difficult to explain. Hubbard *et al.* (114) have thus proposed that ACh acts at the first node of Ranvier rather than at the terminus. Depolarization of the node would then be measurable but would be insufficient to electrotonically cause an increase in MEPP frequency at the ending.

In cat tenuissimus, Blaber and his colleagues (27) find a small but significant increase in MEPP frequency with SCh, C-10 and ACh. However, because the increase in frequency translates to a membrane depolarization of only 1 to 3 mV (28), they indicate that these compounds have little direct excitatory effect on the nerve terminal.

By contrast, a more substantial effect has been noted by Galindo (94). In rat diaphragm, ACh or C-10 causes a rapid but transient increase in MEPP frequency during the first 3 to 5 min after application.

The reasons for this variability in effect can presently be explained (173). If nerve terminals are partially depolarized with K^+, a small additional depolarization of the terminal results in a large increase in MEPP frequency. This is due to a shift to the steeper part of the exponential "depolarization-MEPP frequency" curve (146). In rat diaphragm treated with K^+, carbamylcholine (CCh) causes a consistent increase in MEPP frequency (fig. 2). This effect is not altered by TTX but is antagonized by dTC (10–60 nM). Spike generation at the nodes (231) is not involved, since the increase in frequency occurs in the presence of TTX. Local depolarization at the nodes (*cf.* 114) is also unlikely, because it would be insufficient to release transmitter (128), due to electrotonic decrement with distance. Therefore, for the effect to be seen, depolarization by CCh must be directly at the terminal rather than at the first node.

The discrepancy in the above studies, therefore, may be due to differences in the state of nerve terminal polarization. Katz and Miledi (129) find that ACh increases MEPP frequency at some junctions and relate the variability to the initial level of the terminal membrane potential (E_m). If ACh releases transmitter by depolarizing

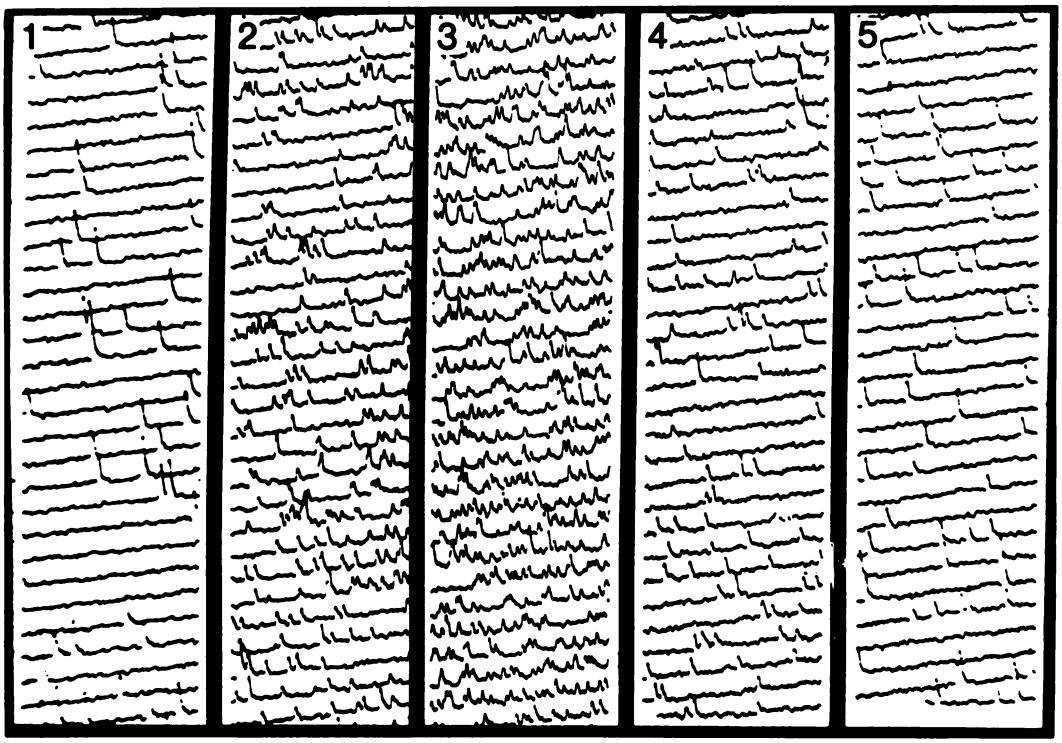

FIG. 2. Effect of CCh on spontaneous transmitter release in partially depolarized (13 mM K^+) rat diaphragm. Sample records show a reduction in MEPP amplitude and concomitant increase in frequency with 10 μM CCh. 1, control, frequency = 31/sec, amplitude = 1 mV. 2, 5.5 min after application of CCh, frequency = 137/sec. 3, 1 min after washout of drug, frequency too high and amplitude too low to measure. 4, 5.3 min after start of wash, frequency = 57/sec. 5, 12 min after start of wash, frequency = 32/sec. The increase in MEPP frequency is not illusory due to an increased noise, because the frequency increase is apparent at a time when basal noise level is unchanged (panel 2). (Reproduced with permission from Miyamoto and Volle (173).)

the terminal, then this action must be greatest in terminals partially depolarized, or in the comparable physiologic state, during the active phase of the nerve terminal spike.

An alternative explanation for the increased MEPP frequency with CCh (173) is that K^+ released from the end-plate acts to depolarize the nerve terminal (124). One way of eliminating this possibility is to repeat these experiments with voltage-clamp techniques: nerve terminals are depolarized with K^+ and the potassium equilibrium potential (E_K) is calculated with the Nernst equation. If end-plates are clamped at the new E_K, then any depolarization will consist only of inward Na^+ current. An increase in MEPP frequency with CCh under these conditions should rule out any K^+ "feedback."

c. Effect of antagonists. Since cholinergic agonists appear to depolarize nerve terminals and release transmitter, blocking drugs would be expected to have little effect on MEPP frequency. However, with exceedingly low concentrations of dTC (1–10 pg/l), Sokoll *et al.* (208) found an increase in MEPP frequency in isolated frog muscle. This suggests that dTC may act as an agonist to promote transmitter release and accords with the finding that dTC depolarizes nerve terminals (114). Similar results are seen with gallamine (209).

2. Neurally evoked transmitter release (EPPs). Although less direct than measuring MEPP frequency, analysis of EPPs allows examination of the release mechanism under physiologic conditions, *i.e.,* during activation of the nerve terminal by the spike. This requires, however, the elimina-

tion of muscle action potentials and contraction. This can be effected by 1) reducing transmitter release with Mg^{++} (63), 2) reducing postjunctional sensitivity with blocking drugs, 3) transverse cutting and depolarization of muscle fibers (11, 12) or 4) pretreatment with glycerol-Ringer's solution to disrupt excitation-contraction coupling (93, 171).

A change in EPP size may reflect a pre- or postjunctional action or both. It is necessary, therefore, to calculate the "quantum content" or number of quanta released (m) per nerve impulse. The amount released (m) is the product of the number of quanta available (n) and the mean probability of release (p) (64). Methods of calculating m include: 1) the direct method, 2) the failures method, 3) the variance method and 4) the combined variance and rundown method (see pp. 130–141 in Ref. 113). Methods 1 and 2 apply to Mg^{++}-paralyzed junctions, where it is possible to record MEPPs. Method 3 is used for muscles paralyzed with cholinergic blocking drugs, where it is not possible to record MEPPs. Measurements are instead made of amplitude fluctuations in a train of EPPs, and m is calculated as $1/(CV)^2$ (coefficient of variance) (157). Method 4 takes this one step further; in the absence of Mg^{++} block, rapid stimulation causes a "rundown" of EPPs followed by a "plateau." The rundown represents depletion of the available store (n) (220), and the plateau represents mobilization of transmitter from accessory stores (109). The variance method is used to calculate m from the plateau. By extrapolating the rundown to zero and summating the quanta released (77), estimates are then made of n. Finally, dividing m by n gives the probability of release (p) (pp. 150–152 in Ref. 113). It should be noted, however, that each method has been subject to criticism as to its accuracy and as to whether or not it reflects the release mechanism under normal conditions (96).

a. Effect of agonists. An inhibitory action of ACh has been found for neurally evoked transmitter release. In Mg^{++}-paralyzed frog muscles, ACh produces a reduction in both twitch and m (variance method). Because the decrease in twitch occurs at the same ACh concentration which reduces m, the inhibition of contraction appears to be in part due to a direct action on the nerve terminal (52). Similarly, in Mg^{++}-paralyzed rat diaphragm and in the presence of neostigmine, ACh produces a consistent decrease in m (failures method). However, neostigmine by itself has no effect (114).

Other agonists are also inhibitory in Mg^{++}-blocked frog muscle. SCh or nicotine causes a decrease in m (failures or variance method), when stimulus frequency is low (75, 216). However, with repetitive stimulation, no effect on m is seen with SCh or C-10 (variance method), at drug concentrations which depolarize the end-plate (35).

On the other hand, there is good evidence for a facilitatory effect (28, 29). In cut tenuissimus muscles of the cat, C-10 causes an increase in the m of the first EPP, in n, and in the mobilization of transmitter (variance and rundown method; 200 Hz) (24). These effects are antagonized by dTC. According to Blaber (24), there are prejunctional AChR which can promote transmitter release. He suggests, however, that these AChR are nonfunctional to endogenous transmitter, because of the rapid removal of ACh from the junctional area.

A facilitatory effect in the absence of dTC or Mg^{++} is also found by Galindo (94). In rat diaphragms, muscle spikes are abolished by massive nerve stimulation which depolarizes end-plates by 15 to 30 mV. SCh or C-10 causes an increase in both contraction and EPP size but with higher concentrations, there is the expected diminution in effect. These results are interpreted as supporting a presynaptic depolarizing action, which when sufficiently large, causes reduction or block of the nerve spike. The failure of other investigators to note facilitation may therefore be due to the use of dTC or high Mg^{++} which obscures the effect (24).

b. Effect of antagonists. In Mg^{++}-paralyzed *frog* muscles, neither dTC nor lobeline causes any change in m with 1 Hz stimulation (216). In one experiment in

which dTC was used but MEPPs could still be measured (156), dTC had no effect on *m* (direct method), when stimulus rate was low (0.33 Hz). There was also no effect on *m* or the pattern of repetitive EPPs when stimulus rate was high (40 Hz) (35).

No effect of dTC on transmitter release is seen in *mammalian* muscles paralyzed with Mg^{++}. In rat diaphragm and with low frequency stimulation, dTC has no effect on *m* in concentrations which decrease the EPP by 50% (19). To determine whether stimulus rate is a factor, Bauer and Kruckenberg (13, 14) have examined transmitter release from hamster diaphragm. Alterations in dTC concentration or stimulus rate (5–100 Hz) produced no changes in either *m* or the pattern of EPPs. However, these results may be inconclusive, because transmitter release was not studied in the absence of dTC.

On the opposite side are a number of studies showing a reduction in transmitter release by dTC. In rat diaphragm, graded application of dTC causes an increased rate of EPP rundown and a decreased plateau (50 Hz) (147). This implies a reduction of transmitter output, since end-plate sensitivity is unchanged during the EPP rundown (117, 182). Similar results are obtained from cut muscles of rat diaphragm (116). At 100 Hz, dTC causes a decrease in *m*, in *n* and in the rate of transmitter mobilization (variance and rundown method). The postsynaptic effect (decreased quantum size) occurs before the decrease in *m*, which suggests a slower onset of the presynaptic effect. A similar inhibition of transmitter release is found in frogs (116, 154).

In rat diaphragm *not* blocked with dTC or Mg^{++}, Galindo (96) finds a decrease in *m* with dTC but not with pancuronium. However, both drugs cause an increase in *p*, indicating an initial stimulatory effect. A similar increase in *p*, but no change in *m*, is noted in cut tenuissimus muscles of the cat (24, 26). From comparisons of the structure-activity relationships of lidocaine, benzoquinonium, pancuronium, and dTC, Blaber (26) concludes that the phenolic group acts to increase *p*, whereas the onium group acts to decrease transmitter mobilization.

Auerbach and Betz (6) have indicated that the reported effect of dTC may be due to technical reasons. To circumvent these problems, they selectively crushed frog nerves to prevent twitching and voltage-clamped end-plates to avoid nonlinear correction (156). Low doses of dTC caused no change in end-plate current/MEPP at 1 Hz stimulation. In glycerol-treated muscles, dTC did increase CV by 22%, which was equivalent to a decrease in *m* from 236 (control) to 158 quanta (dTC). However, this was interpreted as an artifact of the decreased signal/noise, on application of dTC (6).

Auerbach and Betz (6) also note that, if recordings are nonfocal, attenuation of the EPP with distance results in an overestimate of *m*. With dTC, the overestimate is reduced and gives an *apparent* decrease in *m*. However, their calculations are based on Martin's correction for nonlinearity (156) which is an overestimate (158, 172, 217), so their argument needs to be reconsidered. Using a combination of the direct and variance methods (159), they find in rat diaphragm a 10% reduction in *m* with dTC and conclude that the action on transmitter release, if any, is small.

The above objections have been re-examined by Hubbard and Wilson (115), in studies in which the electrode is localized within 100 μm of the end-plate. Application of dTC causes a significant lowering of *m* and *n* but an increase in *p*. They conclude that the discrepancy with other studies is based on differences in stimulation rate, temperature, or dTC concentration and not to the considerations proposed by Auerbach and Betz (6).

3. Preganglionic terminals. The only report of an intracellularly measured effect of ACh on the nerve terminal is that by Pilar (186, 187) with chick ciliary ganglion. At the presynaptic membrane, ACh causes depolarization of 5 to 10 mV as well as a decrease in membrane resistance, presyn-

aptic spike and evoked quantum content (m). Repeated application of ACh (2–3 min intervals) results in no further changes, which indicates possible desensitization of the AChR.

According to Pilar (187), the reduction in m by ACh does not preclude an *increase* in release, *e.g.,* after repetitive stimulation, postsynaptic potential size and miniature potential frequency are increased (160). He notes, however, that a "facilitatory mechanism, if present, would not increase transmitter release indefinitely, because after 60 sec the inhibitory effect of ACh on transmitter release would be activated" (187). This assumes that the effect of endogenous ACh is the same as that of exogenous ACh.

IV. Measurement of ACh Release

The conventional method for quantitatively measuring ACh is the use of "sensitized" animal tissues (49, 61, 152). More recent developments include detection with radiotracers (55, 192), enzyme fluorometry (43) and gas chromatography-mass spectroscopy/ fragmentography (119). Details and precautions of these techniques can be found in Ref. 103.

A. Localization and Origin of ACh

Support for a *neural* localization of ACh is provided by studies in which denervation of the salivary gland causes the disappearance of half its ACh content within 24 hr (49). Moreover, in cat, dog or frog tissues, stimulation of either nerve or muscle causes the appearance of ACh, whereas stimulation of denervated muscle releases no ACh (61). If nerves are stimulated to exhaustion (as monitored by muscle twitch), ACh is no longer released (61).

Evidence for a *non-neural* origin of ACh comes from studies which show no difference in the *resting* release of ACh, from acutely denervated and 7-day chronically denervated muscle (218). This resting release (7–8 ng/20 min) is too great to be explained by spontaneous quantal release (0.5 ng/20 min) or leakage from the cut nerve (0.6 ng/20 min), and indicates that the source of ACh may be the muscle (218). Added support is provided by the demonstration that the resting release persists in the presence of botulinum toxin (44), which prevents the release of cholinergic transmitter (41). Finally, examination of the amount of ACh released upon direct *stimulation* appears to show no difference between innervated and 10-day chronically denervated rat diaphragms (104).

A repetition of these experiments, however, indicates that resting release from denervated diaphragm is in fact 50% lower than that from innervated controls (139, 170). Similarly, a reexamination of the study on evoked ACh release shows a large difference between innervated and 3 to 4 week denervated diaphragm (12 *vs.* 2.3 pmol/min, respectively). Krnjević and Straughan (139) suggest that the failure of others (104) to note this difference may be due to the relative insensitivity of their assay. They also conclude that the bulk of ACh released during direct muscle stimulation comes in fact from the nerve.

If innervated muscle is instead depolarized with K^+ (30 mM), there is only a 3-fold increase in ACh release, much less than the expected increase in MEPP frequency (200- to 300-fold) (170). This indicates that MEPPs represent only a small fraction (1–3%) of the total ACh release. One explanation is that much of the measured ACh comes from the preterminal region of the nerve and is not involved in synaptic activity (81, 88). A second explanation is the existence of a steady leakage of ACh from the nerve terminal cytoplasm (131, 170). Accordingly, cytoplasmic ACh may constitute the bulk of resting release but be distinct from quantal ACh.

The evidence on the precise origin of ACh is equivocal. The argument for a non-neural ACh source, *i.e.,* that resting release occurs in the presence of botulinum toxin, must be tempered by the recent finding that the toxin does not completely block spontaneous transmitter release (60, 210). Failure to block ACh release with the toxin then, does not preclude a possible neural

origin of ACh. On the other hand, there is no good explanation for the relatively large (50%) resting release of ACh from *de*nervated muscle. Possibilities include 1) the presence of residual nerve fragments which are capable of releasing ACh (170), or 2) the existence of Schwann cells which engulf junctional debris as well as ACh (22) and subsequently release the captured ACh (67, 118, 165).

B. Effect of dTC on ACh Release

The issue of whether dTC affects the release of ACh upon nerve stimulation has also produced some divergence of opinion. Evidence against a prejunctional action of dTC has been obtained by Dale *et al.* (61) who note 1) that nerve stimulation releases ACh from muscles perfused with Locke's solution, and 2) that dTC, in concentrations blocking transmission, has no effect on the amount of ACh released. A similar lack of effect of dTC on ACh release has been found in cat muscles perfused with blood (78).

Further evidence against a prejunctional dTC action has been provided by Cheymol *et al.* (51). They find with low (0.1 Hz) and moderate (25 Hz) rates of stimulation that dTC does not decrease the amount of ACh liberated, whereas hemicholinium-3 does. On the assumption that dTC may interfere with the bioassay, Fletcher and Forrester (88) have separated the dTC from the assayable ACh using gel filtration. They also find that paralytic doses of dTC fail to alter ACh output on nerve stimulation (1 Hz, 60 min).

The only report of a measurable effect on ACh release is by Beani *et al.* (15), who indicate that dTC reduces ACh release, under *some* conditions. Their protocol includes 1) the use of DFP, which is applied in high doses and then washed out, and 2) the combined study of temperature and stimulus frequency. At 28 and 33°C and low frequency stimulation, dTC produces a 50% reduction in ACh output. At 38°C and low frequency stimulation, however, dTC has no effect on ACh release, in agreement with the experimental conditions and findings of Dale *et al.* (61). A decrease in output is seen at 38°C only when frequency is raised to 50 Hz.

Chang *et al.* (47), however, have repeated these experiments and reached the opposite conclusion. At 50 Hz stimulation and with a variety of anti-AChEs including DFP, dTC causes no reduction in ACh release, whereas hemicholinium-3 does. These conditions are similar to those under which Beani *et al.* (15) observed a large decrease in ACh release.

The present evidence indicates that dTC has little (15) or no effect on stimulus-evoked ACh release (47, 51, 61, 78, 88, 138). The explanation most compatible with electrophysiologic studies which show an effect by dTC is that the *total* ACh measured by assay consists of a large leakage of cytoplasmic ACh (131, 218) but only a small amount of quantal ACh (170, 218). Accordingly, any effect by dTC on quantal or synaptically functional ACh may be masked by the large excess of cytoplasmic ACh. However, because electrophysiologic methods measure primarily quantal and not cytoplasmic ACh release (*cf.* 131), a significant effect by dTC would be detectable with this approach.

C. Effect of Agonists on Preganglionic Terminals

The question of whether cholinomimetics promote the release of ACh has been examined at preganglionic terminals (42, 55). "Depot ACh" in superior cervical ganglia is first labeled with [^{3}H]choline. Subsequent nerve stimulation causes the release of labeled ACh, but injection of ACh, CCh (42, 55), nicotine or methacholine (42) does not. When "surplus ACh" in resting ganglia is labeled with [^{3}H]choline (see Section II), nerve stimulation does not release labeled material, but ACh or CCh does. This means that cholinomimetics can release "surplus ACh" but not the "depot ACh" which is directly involved in synaptic transmission.

According to Koelle (135), the failure of

ACh to release "depot ACh" may be due to desensitization of the presynaptic AChR by the high doses of drug. If this is so and if ACh release is "regenerative" (134), then desensitization of presynaptic AChR should block ACh feedback and reduce ACh output (151). However, application of ACh during nerve stimulation does *not* block ACh release at doses which block the ganglionic response (55). This makes it unlikely that release of "depot ACh" is due to a regenerative mechanism.

V. Microanatomic Localization of AChR

A. Postjunctional AChR

The question of whether cholinergic drugs act presynaptically is, of course, dependent on whether AChR exist at the presynaptic membrane. Quantitative studies were first attempted by injecting mice with [^{14}C]dTC and using autoradiography (225). Waser (224) counted about 4×10^6 dTC-binding sites per end-plate and estimated that only 1% of the end-plate need be covered by dTC to cause paralysis. However, this was a severe underestimate, because of crude estimates of the surface area and because much of the [^{14}C]dTC was washed away.

Studies on the AChR have since been facilitated by the snake venom toxin, α-bungarotoxin (α-BuTX) (48, 141, 142). The toxin is a basic polypeptide of about 8,000 MW and is highly specific for nicotinic AChR of the end-plate. Distinction must be made between AChR of the end-plate and those of ganglia, since α-BuTX does not block ganglionic transmission (46). The bond between the toxin and the AChR can be disrupted by concentrated salts or sodium dodecyl sulfate (193) but under normal conditions is irreversible (169). By labeling the toxin, it is possible to study the localization and numbers of AChR and to undertake its chemical isolation (40, 168). The assumption that one toxin molecule binds to one AChR is implicit but not yet proved. To this point, Miledi and Potter (169) have found 4×10^7 toxin binding sites per end-plate for rat diaphragm, an estimate 10-fold higher than that made by Waser with dTC (224). Part of the discrepancy is explained by the washing away of [^{14}C]dTC in Waser's (224) study, but part must be due to the bis-quaternary nature of dTC, *i.e.,* one dTC molecule binding to two AChR. This explanation is supported by Changeux *et al.* (50) who find in eel electroplax twice as many binding sites for α-BuTX as for C-10. α-BuTX apparently binds at the receptor, because pretreatment with dTC reduces but does not eliminate toxin binding. Colquhoun (56) suggests that this failure of dTC to fully protect against α-BuTX can be explained by their different rates of binding.

Another approach to localizing AChR is to label α-BuTX with fluorescent dyes (fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate) (3). Muscles are exposed to the conjugate for 15 to 60 min, fixed and examined with fluorescence microscopy. The conjugate is less potent than native toxin but retains its pharmacologic properties, *i.e.,* its actions are blocked by dTC or CCh but not by atropine or neostigmine. The fluorescence appears as a localized pattern at the end-plate region. Staining for AChE (122) produces reaction product around the edges of the junction, and the toxin fluorescence is found confined within the borders of the AChE stain (3).

When applying the toxin-conjugate to frog or mouse skeletal muscle, Anderson and Cohen (3) find no difference in the intensity of fluorescence before and after denervation. This suggests that the AChR is associated with the subsynaptic and *not* nerve terminal membrane. However, by this method, AChR is also seen localized in the junctional folds, a finding not supported by other higher resolution studies (see below). Therefore, while this technique may be useful for studying AChR distributions, it is limited by its low sensitivity (*cf.* immunofluorescence by Bourgeois *et al.* (34)).

Comparison of the relative numbers of AChR with AChE molecules has been

made by Barnard *et al.* (10) using autoradiography. AChR and AChE are estimated with α-[^{3}H]BuTX and [^{3}H]DFP binding, respectively. The number of AChR range from 3 to 9 $\times$ 10^7 per end-plate, and the ratio of toxin to DFP binding sites is 1:1. Since the number of toxin binding sites is not changed by pretreatment with DFP and *vice versa,* it appears that the two sites are separate and independent.

α-[^{3}H]BuTX and autoradiography have also been used for electron microscopic analysis (1, 189–191). Porter *et al.* (191) indicate that AChR sites are distributed homogenously at the subsynaptic membrane, in a density of 8,500/μm^2. However, using α-[^{125}I]BuTX Fertuck and Salpeter (86) find that the binding sites are *not* evenly distributed but are concentrated at the "peaks" of the junctional folds. Little labeling is seen in the folds themselves. The possibility that the toxin did not reach the folds is unlikely, since incubation with toxin was halted only after muscle paralysis occurred. If AChR are present in the folds, they must then be nonfunctional to released transmitter. Accordingly, the density of AChR can be revised upward to about 30,000/μm^2 (87). These findings were confirmed by Porter and Barnard (189, 190) who revised their estimate upward to 20–25,000/μm^2 (bat and mouse diaphragm).

Location of the AChR at the "peaks" of the junctional folds poses a problem for transmitter-receptor contact, since transmitter release sites are situated opposite the folds rather than "peaks." It has been suggested (189) that vesicles are arranged at the edges of the active zones (107) and expel their contents diagonally onto the "peaks," thereby ensuring maximal transmitter-AChR interaction.

To examine the distribution of AChR after denervation, Porter and Barnard (190) have compared the grain density distribution of α-[^{3}H]BuTX in innervated and 5-day denervated mouse diaphragm. No change is found in the distribution of label or in the total amount of postjunctional membrane as revealed by morphometric measurements. This indicates that the total AChR population and the absolute binding site densities are unchanged by the absence of the nerve terminal. According to Porter and Barnard (190), this provides strong evidence for the absence of nicotonic AChR at the nerve terminal.

B. Prejunctional AChR

Although Porter and Barnard (190) claim an absence of presynaptic AChR, they indicate an error in their density distribution of about 10%. Fertuck and Salpeter (86, 87) point out, however, that the resolution in the method of Porter and Barnard (190) 1) is limited to 500 Å, a distance greater than the synaptic cleft, and 2) is not able to detect even a 20% difference in total radioactivity (87, 207). In any case, a 10% error can represent a presynaptic AChR density of up to 25% of that at the postjunctional side, so the reported absence of presynaptic AChR is inconclusive. These arguments can be aimed at similar claims by Bourgeois *et al.* (33) who used eel electroplax.

The issue regarding presynaptic AChR has been intensified with the development of high resolution techniques involving conjugation of horseradish peroxidase (HRP) with α-BuTX (7, 178). One technique consists of a staining for an α-BuTX-immunoperoxidase complex (16, 17, 62). Tissues are incubated in 0.5 nM α-BuTX for 1.5 to 2 hr to establish binding to AChR, then washed, fixed with 2% periodate-lysine-paraformaldehyde (163) and quick frozen. For electron microscopy, muscles are treated at room temperature with 1) 1 hr incubation in rabbit anti-α-BuTX, 2) washing for 30 min and 3) 1 hr incubation in a HRP conjugate of the IgG fraction from goat antiserum to rabbit IgG. Antibody-treated preparations are then stained with 3,3′-diaminobenzadine (DAB).

Treating frog and mouse neuromuscular junctions by this method shows the expected staining of the end-plate region (62). Pretreatment with dTC, CCh, nicotine or C-10 markedly reduces the staining. The heaviest stain is found at the "peaks" of the

synaptic folds, with little or none in the valleys (*cf.* 86, 190). There is a less intense but notable staining of the presynaptic membrane, which is prevented by treatment with C-10. Daniels and Vogel (62) have suggested that the presynaptic staining may be due to translocation to apposing structures of either the antigen-antibody complex or the DAB reaction product. They note in support of this that basement membrane and Schwann cell processes are also stained.

Staining of the presynaptic membrane for AChR (fig. 3) can be found in rat and human muscle (16). Little stain occurs in the extrajunctional region 5 μm outside the subneural area, and only a slight amount is found in the cleft. No stain is seen in either myofibrils, mitochondria, transverse tubules or sarcoplasmic reticulum.

Another high resolution technique for localizing AChR involves the *direct* conjugation of α-BuTX to HRP (178). Muscles are incubated for 0.5 to 2 hr with the conjugate, fixed with 3% glutaraldehyde for 1 hr and incubated in DAB and H_2O_2 (143, 144). The conjugate has a reduced pharmacologic activity but still blocks MEPPs and EPPs. The advantage of this method is in its fine, dense precipitate which affords a high resolution and its relative simplicity compared to immunohistochemical techniques.

Neuromuscular junctions of newt, frog and rat have been examined with this technique by Lentz *et al.* (144). Staining is completely prevented by preincubation

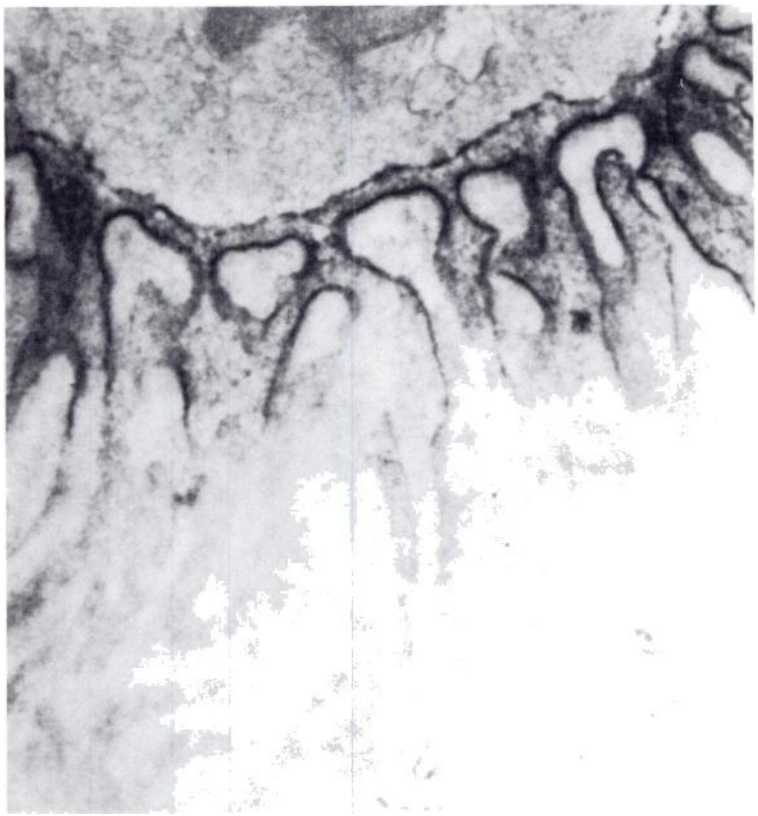

FIG. 3. Electron micrograph of a neuromuscular junction in normal human muscle. The electron dense areas represent DAB staining for an immunoperoxidase-α-BuTX conjugate bound to membrane AChR. Intense staining at the peaks, but not valleys, of the junctional folds can be seen. A less dense but precise staining of the presynaptic membrane is also seen, indicating the presence of nicotinic AChR. Magnification ×25,000. (Reproduced with permission from Bender, A. N. *et al.*: Immunoperoxidase localization of alpha bungarotoxin: A new approach to myasthenia gravis. Ann. N.Y. Acad. Sci. **274**:20–30, 1976.)

with native α-BuTX but is only partially blocked by dTC. In accord with other studies (86, 190), reaction product is found primarily at the "peaks" of the postjunctional folds, with little staining in the folds themselves. A consistent finding is the staining of the presynaptic membrane, particularly of the axolemma overlying the active zones. Although less intense and less frequent, staining also occurs at the *non*synaptic areas of the nerve terminal membrane. This is consistent with recent findings of a tight α-[^{125}I]BuTX binding to peripheral nerve (155).

In contrast to Daniels and Vogel (62), Lentz *et al.* (144) state that the presynaptic localization is not a result of diffusion, since 1) reaction product is found on the axolemma but never on the Schwann cell fingers interposed between nerve and muscle—in some cases, the axolemma behind the Schwann cell is stained, 2) after physical removal of nerve terminals from the end-plates with collagenase or protease, staining is still found on the axolemma (fig. 4) and 3) staining is found on embryonic neuronal material.

Acceptance of a prejunctional cholinoceptive action has been hampered by early studies showing an apparent absence of both AChE (58, 79) and AChR (190, 224) at the nerve terminal. However, recent studies with [^{3}H]DFP show that, although 85% of the total AChE resides at the postjunctional membrane, as much as 10 to 15% may be located presynaptically (205, 206). Because the relative surface area of pre- to postjunctional membrane is roughly 1:10 and because DFP binding sites are evenly distributed over the postjunctional folds

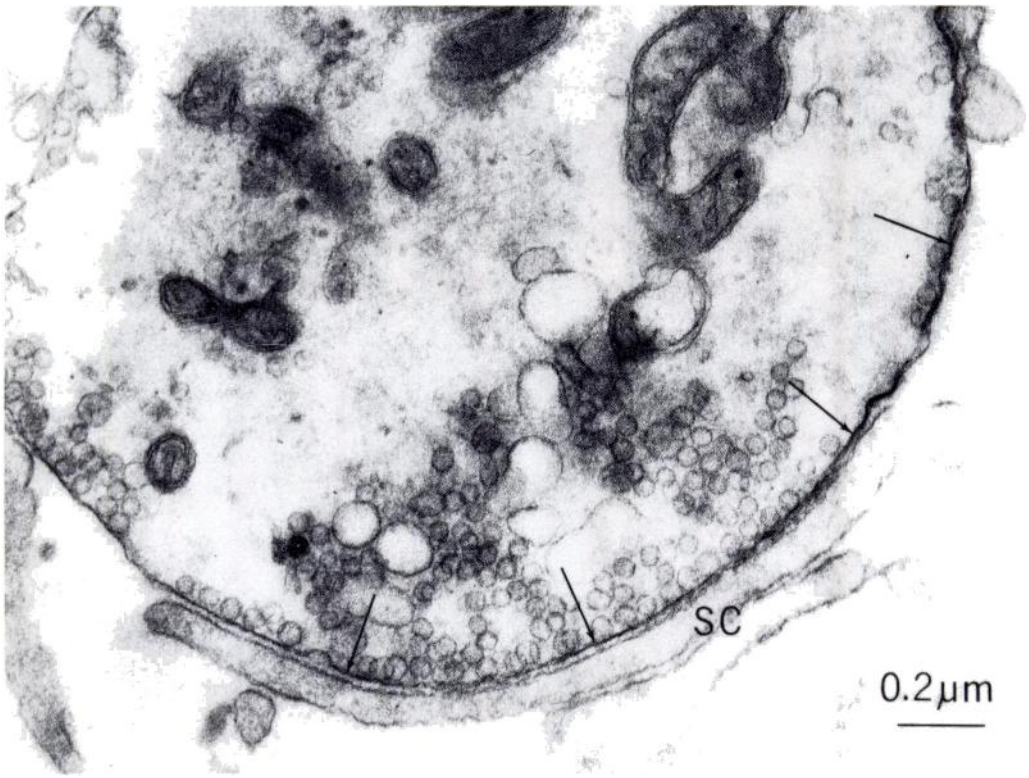

FIG. 4. Electron micrograph of a frog motor nerve terminal separated from the end-plate by treatment with collagenase and protease. The electron opaque areas represent binding to AChR of HRP-α-BuTX conjugate. Heavy activity occurs at areas devoid of the Schwann cell (SC) covering, but activity is also found on the axolemma behind the unreactive SC. Magnification ×56,000. (Reproduced with permission from Lentz *et al.* (144).)

(189), it appears that the absolute densities of AChE at pre- and postsynaptic sites are comparable. Similarly, recent demonstrations of a staining for α-BuTX at the prejunctional membrane have provided direct evidence for the presence of AChR at that site. There appears, therefore, to be little reason to question whether cholinergic drugs can act presynaptically.

VI. Conclusions

Prejunctional drug actions: direct or K^+-mediated? The existence of prejunctional AChR has been implied by pharmacologic experiments. The argument for a direct drug action rather than one mediated by K^+ is based on differences in effects of ACh and K^+ at the terminals (72, 110). Some differences are expected, because ACh acts on AChR and changes only membrane conductance, whereas K^+ is nonspecific and alters membrane E_m, E_K and threshold.

Recent findings, however, suggest that the differences in ACh and K^+ effects may *not* be so clear cut, *i.e.,* 1) K^+, like ACh, causes antidromic firing (53), 2) K^+, like ACh, can cause a decrease in evoked transmitter release (80) (*cf.* 110), and 3) cholinomimetics, like K^+, can increase MEPP frequency (129, 173). Furthermore, an effect of postsynaptic K^+ on presynaptic function has been implicated in cortical neurons (91) and at squid giant synapse (228).

Nonetheless, the weight of evidence points to a direct drug action, since it is highly unlikely that these effects would occur differently from those at other systems, *e.g.,* vagal C-fibers, where a role of K^+ release from other sites is untenable. However, further study is needed to exclude a K^+ mechanism *unequivocally.*

Generation of antidromic firing in the absence of nerve stimulation (DIA). There is good evidence that drug activation of prejunctional AChR results in depolarization of the nerve terminal membrane (70, 99, 114, 187). To explain the initiation of DIA by facilitatory compounds, one must therefore postulate either 1) a direct depolarizing action, or 2) an anti-AChE effect which prolongs the depolarizing action of endogenous ACh. Anilinium ions may act by direct depolarization, because they have little anti-AChE activity but produce marked DIA (199). However, an anti-AChE mechanism is also a possibility, since DFP does not depolarize but does initiate DIA (222).

Depolarization of the terminal, relative to the first node, may thereby initiate antidromic firing. This is analogous to the "generator potential" at sensory nerve endings, which respond to a variety of drugs/conditions including ACh (175). The proposal that ACh depolarizes *only* at the node (114) must be superseded by the idea that cholinomimetics can depolarize the ending (173). There is in fact no reason to believe that the membrane at the node is any different from that at the terminal (200). A depolarization at the terminal rather than the node also eliminates the problem of how sufficient ACh in the cleft can act at the distantly removed first node (*cf.* fig. 1).

Once initiated, DIA travels to adjacent terminal arborizations and recruits the entire motor unit (230, 232). The result is a synchronous contraction of the muscle bundle (fasciculation), as if the nerve branch were stimulated normally.

Antidromic firing after conditioning stimulation (SBR). Generation of SBR involves an augmentation of the negative afterpotential of the conditioning spike (110), such that the nerve terminal membrane is hyperexcitable for a protracted period. This is supported by the finding that the period of SBR augmentation corresponds to the duration of the negative afterpotential (230).

Facilitatory drugs may initiate SBR by 1) a direct depolarization, or 2) an indirect anti-AChE action, both of which result in superposition of a depolarization on the negative afterpotential of the conditioning spike (72, 114). Methonium compounds may work by one or both of these mechanisms (200). A third means of generating

SBR may involve drug-induced alterations in membrane conductance to ions. *Decreasing* K^+ conductance with ethonium ions or 4-aminopyridine (18, 148, 150) prolongs the negative afterpotential by inhibiting spike *re*polarization (see 5, 179, 221). Because this action is necessarily linked to the conditioning spike, antidromic activity can be generated only as SBR and not DIA, *i.e.,* there should be no activity in the absence of nerve stimulation. On the other hand, guanidine and veratridine may prolong the negative afterpotential by *increasing* ion conductances, possibly to Ca^{++} (150, 162) and Na^+ (179), respectively.

The actions of anilinium ions and neostigmine remain unsettled. The weak anti-AChE properties of anilinium drugs suggest they must have a direct depolarizing action (199, 203). However, it has been shown that too *great* an anti-AChE action may cause a *decrease* in facilitation (222), presumably due to AChR desensitization by high levels of endogenous ACh. Therefore, the possibility that a weak or moderate anti-AChE action may account for facilitation cannot be completely excluded. The difficulty with neostigmine, on the other hand, may arise from its structure. Because it possesses depolarizing (onium head), anti-AChE (carbamate bond), and anilinium properties, its marked facilitatory action may be a mixture of all three factors. Evidence for an anti-AChE action comes from the finding that SBR is blocked by high Mg^{++} but enhanced by high Ca^{++} (195). Since these cations have similar effects in stabilizing membranes but opposite effects on transmitter release, the enhancement of SBR is a function of released transmitter. Accordingly, an anti-AChE effect of neostigmine would prolong the life of released transmitter and thereby promote SBR.

Relationship of antidromic firing to enhancement of transmitter release. Because antidromic firing is highly sensitive to cholinergic drugs, it has been postulated that the nerve terminal is the primary site of drug action (198, 212, 214). By the same token, however, the finding that dTC blocks antidromic firing before affecting transmission (161, 212, 229) argues against may major role of these AChR in transmission. The questions arise, whether activation of prejunctional AChR can occur *without* initiating antidromic firing and whether initiation of firing is important for transmitter release *at that same ending.*

One approach to these questions would employ the difference in response between amphibian and mammalian preparations. Amphibian motor nerve terminals do not generate antidromic activity to physostigmine, edrophonium, ambenonium, neostigmine or methonium compounds but do respond to ethonium compounds (TEA, 3-OH PTEA, PTEA), guanidine, and veratrum alkaloids (200). A notable distinction between the two species is the extracellular K^+ and thus resting E_m. In frog muscle with an E_m of 90 mV (2.5 mM K), an EPP of 40-50 mV is needed to trigger spikes (82, 120), whereas in mammalian muscle with an E_m of 65 mV (5 mM K), only 10 to 20 mV EPPs are needed (39, 145). Assuming that nerve endings are similar to muscles, it is about three times more difficult to initiate spike activity in frogs than mammals.

It should be possible to simulate amphibian muscle conditions by perfusing mammalian muscles with a high Ca^{++}, low K^+ Krebs' solution. This should, respectively, inhibit antidromic firing (increased threshold) but enhance evoked transmitter release (increased Ca^{++} gradient and larger terminal spike). It is probable that initiation of DIA and SBR with methonium drugs (*i.e., de*polarization) will be three times more difficult, as in frogs. However, with ethonium compounds which act on *re*polarization, SBR should be initiated and enhanced because of the larger E_m and thus larger nerve terminal spike. The experiments with methonium compounds should demonstrate in mammals an activation of prejunctional AChR, which does not result in antidromic activity. They should also demonstrate an increase in transmitter release which is not associated with antidromic activity at the same ending. It is

possible, however, that antidromic firing may increase transmitter release at another ending of the axon reflex, by initiating repetitive EPPs (25).

Cholinergic drugs and transmitter release. Cholinergic agonists cause a decrease in evoked transmitter release when muscles are paralyzed with Mg^{++} (52, 114). It is noteworthy that studies claiming an increase in release have been carried out with otherwise drug-free preparations (24, 94, 173). Similarly, no effect of dTC on transmitter release is found with Mg^{++}- or dTC-blocked muscles, whereas a measurable decrease is found with drug-free preparations.

The explanation most compatible with this evidence is that exogenous or endogenous ACh can increase transmitter release (*cf.* Koelle (134)), that sufficient endogenous ACh is present, either from previous release or from leakage of cytoplasmic ACh (131), and that dTC can decrease transmitter release by blocking the effect of endogenous ACh.

Synaptic transmission: primary or secondary release of ACh? Although the above effects are partially consistent with Koelle's (134) hypothesis, the stipulation by Koelle that the major component of transmission is the secondary release of ACh is *not* supported by the existing evidence. As mentioned, high concentrations of ACh which block the presynaptic AChR have *no* effect on the amount of ACh released (55), so release cannot be dependent on these AChR being functional.

An important consideration is whether cholinergic agonists can promote ACh release at all, since agonists fail to effect release from ACh depots in ganglia (42). One explanation is that the large drug doses cause desensitization of presynaptic AChR before significant amounts of ACh are released (135). Evidence for desensitization is provided by the transient depolarization of preganglionic (99, 187) and motor nerve terminals (114). A second explanation is that nerve terminals must be partially depolarized to show an increase in ACh release, since the sensitivity is an exponential function of nerve terminal E_m (146). A third possibility is that exogenous chemical application can never duplicate the conditions of nerve stimulation.

Significance of prejunctional cholinoceptive action. The summarized evidence does not argue for a new mechanism of transmission but remains compatible with present concepts (see Section II). The primary release of ACh leads to activation of postjunctional AChR and generation of EPPs. In addition to this release which is quantal, there is a resting release which can lead to an [ACh] in the synaptic cleft of 10^{-8} M (131). The low ratio of AChE/AChR at the peaks of the postjunctional folds (87, 189) may allow some of this ACh to remain intact. However, prejunctional AChE (205, 206) may normally hydrolyze ACh near the terminal.

With anti-AChE but no stimulation, junctional ACh may accumulate and trigger DIA. However, with both anti-AChE and nerve stimulation, junctional ACh and/or neurally released ACh may depolarize the terminal, add to the negative afterpotential of the subsequent spike and lead to SBR. In anti-AChE-treated muscles, tetanic stimulation leads to a cumulative depolarization lasting several seconds (130). Katz and Miledi (130) have shown by noise analysis that this is predominantly due to residual ACh in the junction. In view of this and the large resting release of ACh (88, 131, 170, 218), there appears to be little question that there can be sufficient endogenous ACh to initiate prejunctional actions.

It is possible that a small depolarization of the terminal by ACh increases Ca^{++} conductance (57) and thereby increases transmitter release. Larger depolarizations by ACh may instead reduce the incoming nerve spike and lead to a decrease in release (94). Accordingly, it may be possible to obtain either an increase or decrease in evoked release.

The final question is, what is the physiologic significance of the cholinergic action? The EPP is several times larger than

needed to trigger muscle action potentials (140), and supramaximal nerve stimulation always produces a maximal twitch. Therefore, if presynaptic AChR are a means of modifying transmitter release, it cannot be important under normal circumstances. However, this mechanism may be important in stressful situations, as *e.g.*, in fatigue after repetitive stimulation, in systems with abnormal AChE activity, or in systems with alterated plasma K^+ or Ca^{++}.

Because of the large safety factor in transmission, facilitation of the twitch is possible only by 1) prolonging the muscle spike as with TEA, so that the Ca^{++} influx per impulse is increased (106), or by 2) repetitive muscle firing, so that there is summation of the "active state" and development of tetanic tension. Repetitive muscle firing, in turn, may be due to either recurrent nerve firing (200, 232) or prolonged depolarization by the EPP (74, 164, 174) (see, however, 25, 90). The involvement of repetitive nerve firing then, is certainly of importance for twitch facilitation in mammals.

Note added in proof: Support for a prejunctional action of ACh has recently been provided by:

FULTON, B. P. AND USHERWOOD, P. N. R.: Presynaptic acetylcholine action at the locust neuromuscular junction. Neuropharmacology **16:** 877–880, 1977.

MILEDI, R., MOLENAAR, P. AND POLAK, R.: α-Bungarotoxin enhances transmitter 'released' at the neuromuscular junction. Nature (London) **272:** 641–642, 1978.

Acknowledgments. The author thanks R. A. Previti and J. W. Miyamoto for help with references, and F. DiBattista for typing. Figures 3 and 4 were furnished by Drs. A. N. Bender and T. L. Lentz, respectively.

REFERENCES

1. ALBUQUERQUE, E. X., BARNARD, E. A., PORTER, C. W. AND WARNICK, J. E.: The density of acetylcholine receptors and their sensitivity in the post-synaptic membrane of muscle endplates. Proc. Nat. Acad. Sci. U.S.A. **71:** 2818–2822, 1974.
2. ANDERSON, C. R. AND STEVENS, C. F.: Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. J. Physiol. (London) **235:** 655–691, 1973.
3. ANDERSON, M. J. AND COHEN, M. W.: Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. J. Physiol. (London) **237:** 385–400, 1974.
4. ARMETT, C. J. AND RITCHIE, J. M.: The action of acetylcholine and some related substances on conduction in mammalian non-myelinated nerve fibres. J. Physiol. (London) **155:** 372–384, 1961.
5. ARMSTRONG, C. M.: Ionic pores, gates, and gating current. Q. Rev. Biophys. **7:** 179–210, 1975.
6. AUERBACH, A. AND BETZ, W.: Does curare affect transmitter release? J. Physiol. (London) **213:** 691–705, 1971.
7. AVRAEMEAS, S. AND TERNYNCK, T.: Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry **8:** 1175–1179, 1971.
8. BAKER, P. F., HODGKIN, A. L. AND RIDGEWAY, E. B.: Depolarization and calcium entry in squid giant axon. J. Physiol. (London) **218:** 709–755, 1971.
9. BARKER, L. A., DOWDALL, M. J., ESSMAN, W. B. AND WHITTAKER, V. P.: *In* Drugs and Cholinergic Mechanisms in the CNS, ed. by E. Heilbronn and A. Winter, pp. 193–223, Institute of National Defence, Stockholm, 1970.
10. BARNARD, E. A., WIECKOWSKI, J. AND CHIU, T. H.: Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions. Nature (London) **234:** 207–210, 1971.
11. BARSTAD, J. A. B.: Presynaptic effect of the neuro-muscular transmitter. Experientia (Basel) **18:** 579–581, 1962.
12. BARSTAD, J. A. B. AND LILLEHEIL, G.: Transversely cut diaphragm preparation from rat. Arch. Int. Pharmacodyn. Thér. **175:** 373–390, 1968.
13. BAUER, H.: Die Freisetzung von Acetylcholin an der motorischen Nervenendigung unter dem Einfluß von d-Tubocurarin. Pflügers Arch. Gesamte Physiol. Menschen Tiere **326:** 162–183, 1971.
14. BAUER, H. AND KRUCKENBERG, P.: Die präsynaptische Wirkung von d-Tubocurarin and der motorischen Endplatte. Pflügers Arch. Gesamte Physiol. Menschen Tiere **297:** R23–24, 1967.
15. BEANI, L., BIANCHI, C. AND LEDDA, F.: The effect of tubocurarine on acetylcholine release from motor nerve terminals. J. Physiol. (London) **174:** 172–183, 1964.
16. BENDER, A. N., RINGEL, S. P. AND ENGEL, W. K.: The acetylcholine receptor in normal and pathologic states. Neurology **26:** 477–483, 1976.
17. BENDER, A. N., RINGEL, S. P., ENGEL, W. K., VOGEL, Z. AND DANIELS, M. P.: Denervated skeletal muscle fibers identifiable by a new technique, localization of alpha-bungarotoxin binding. Neurology **25:** 373, 1975.
18. BENOIT, P. R. AND MAMBRINI, J.: Modification of transmitter release by ions which prolong the presynaptic action potential. J. Physiol. (London) **210:** 681–695, 1970.
19. BERÁNEK, R. AND VYSKOČIL, F.: The action of tubocurarine and atropine on the normal and denervated rat diaphragm. J. Physiol. (London) **188:** 53–66, 1967.
20. BERNARD, C.: Analyse physiologique des propriétés des systèms musculaire et nerveux au moyen du curare. C.R. Nebd. Séances Acad. Paris **43:** 825–829, 1856.
21. BIRKS, R., HUXLEY, H. E. AND KATZ, B.: The fine structure of the neuromuscular junction of the frog. J. Physiol. (London) **150:** 134–144, 1960.
22. BIRKS, R., KATZ, B. AND MILEDI, R.: Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. J. Physiol. (London) **150:** 145–168, 1960.
23. BIRKS, R. AND MACINTOSH, F. C.: Acetylcholine metabolism of a sympathetic ganglion. Can. J. Biochem. Physiol. **39:** 787–827, 1961.
24. BLABER, L. C.: The effect of facilitatory concentrations of decamethonium on the storage and release of transmitter at the neuromuscular junction of the cat. J. Pharmacol. Exp. Ther. **175:** 664–672, 1970.
25. BLABER, L. C.: The mechanisms of the facilitatory action of edrophonium in cat skeletal muscle. Brit. J. Pharmacol. **46:** 498–507, 1972.
26. BLABER, L. C.: The prejunctional actions of some non-depolarizing blocking drugs. Brit. J. Pharmacol. **47:** 109–116, 1973.
27. BLABER, L. C. AND CHRIST, D. D.: The action of facilitatory drugs on the isolated tenuissimus muscle of the

cat. Int. J. Neuropharmacol. **6:** 473–484, 1967.

28. BLABER, L. C. AND GOODE, J. W.: A comparison of the action of facilitatory and depolarizing drugs at the mammalian motor nerve terminal. Int. J. Neuropharmacol. **7:** 429–440, 1968.
29. BLABER, L. C. AND KARCZMAR, A. G.: Interaction between facilitating and depolarizing drugs at the neuromyal junction of the cat. J. Pharmacol. Exp. Ther. **156:** 55–62, 1967.
30. BLACKMAN, J. G.: Stimulus frequency and neuromuscular block. Brit. J. Pharmacol. Chemother. **20:** 5–16, 1963.
31. BLIOCH, Z. L., GLAGOLEVA, I. M., LIBERMAN, E. A. AND NENASHEV, V. A.: A study of the mechanism of quantal transmitter release at a chemical synapse. J. Physiol. (London) **199:** 11–35, 1968.
32. BLOEDEL, J., GAGE, P. W., LLINÁS, R. AND QUASTEL, D. M. J.: Transmitter release at the squid giant synapse in the presence of tetrodotoxin. Nature (London) **212:** 49–50, 1966.
33. BOURGEOIS, J. P., RYTER, A., MENEZ, A., FROMAGEOT, P., BOQUET, P. AND CHANGEUX, J. P.: Localization of the cholinergic receptor protein in Electrophorus electroplax by high resolution autoradiography. FEBS Letters **25:** 127–133, 1972.
34. BOURGEOIS, J. P., TSUJI, S., BOQUET, P., PILLOT, J., RYTER, A. AND CHANGEUX, J. P.: Localization of the cholinergic receptor protein by immunofluorescence in eel electroplax. FEBS Letters **16:** 92–94, 1971.
35. BOWEN, J. M. AND MERRY, E. H.: Influence of *d*-tubocurarine, decamethonium and succinylcholine on repetitively evoked end-plate potentials. J. Pharmacol. Exp. Ther. **167:** 334–343, 1969.
36. BOWMAN, W. C. AND WEBB, S. N.: Acetylcholine and anticholinesterase drugs. *In* International Encyclopedia of Pharmacology and Therapeutics, ed. by J. Cheymol, pp. 427–502, Pergamon Press, Oxford, 1972.
37. BOWMAN, W. C. AND WEBB, S. N.: Tetanic fade during partial transmission failure produced by non-depolarizing neuromuscular blocking drugs in the cat. Clin. Exp. Pharmacol. Physiol. **3:** 545–555, 1976.
38. BOYD, I. A. AND MARTIN, A. R.: Spontaneous subthreshold activity at mammalian neuromuscular junctions. J. Physiol. (London) **132:** 61–73, 1956.
39. BOYD, I. A. AND MARTIN, A. R.: The end-plate potential in mammalian muscle. J. Physiol. (London) **132:** 74–91, 1956.
40. BROCKES, J. P. AND HALL, Z. W.: Synthesis of acetylcholine receptor by denervated rat diaphragm muscle. Proc. Nat. Acad. Sci. U.S.A. **72:** 1368–1372, 1975.
41. BROOKS, V. B.: An intracellular study of the action of repetitive nerve volleys and of botulinum toxin on miniature end-plate potentials. J. Physiol. (London) **134:** 264–277, 1956.
42. BROWN, D. A., JONES, K. B., HALLIWELL, J. V. AND QUILLIAM, J. P.: Evidence against a presynaptic action of acetylcholine during ganglionic transmission. Nature (London) **226:** 958–959, 1970.
43. BROWNING, E. T.: Fluorometric enzyme assay for choline and acetylcholine. Anal. Biochem. **46:** 624–638, 1972.
44. BURGEN, A. S. V., DICKENS, F. AND ZATMAN, L. J.: The action of botulinum toxin on the neuro-muscular junction. J. Physiol. (London) **109:** 10–24, 1949.
45. BURN, J. H. AND RAND, M. J.: Acetylcholine in adrenergic transmission. Annu. Rev. Pharmacol. **5:** 163–182, 1965.
46. BURSZTAJN, S. AND GERSHON, M. D.: Discrimination between nicotinic receptors in vertebrate ganglia and skeletal muscle by alpha-bungarotoxin and cobra venoms. J. Physiol. (London) **269:** 17–31, 1977.
47. CHANG, C. C., CHENG, H. C. AND CHEN, T. F.: Does *d*-tubocurarine inhibit the release of acetylcholine from motor nerve endings? Jap. J. Physiol. **17:** 505–515, 1967.
48. CHANG, C. C. AND LEE, C. Y.: Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. Arch. Int. Pharmacodyn. Thér. **144:** 241–257. 1963.
49. CHANG, H. C. AND GADDUM, J. H.: Choline esters in tissue extracts. J. Physiol. (London) **79:** 255–285, 1933.
50. CHANGEUX, J. P., KASAI, M. AND LEE, C. Y.: Use of snake venom toxin to characterize the cholinergic receptor protein. Proc. Nat. Acad. Sci. U.S.A. **67:** 1241–1247, 1970.
51. CHEYMOL, J., BOURILLET, F. AND OGURA, Y.: Action de quelques paralysants neuromusculaires sur la libération de l'acétylcholine au niveau des terminaisons nerveuses motrices. Arch. Int. Pharmacodyn. Thér. **139:** 187–197, 1962.
52. CIANI, S. AND EDWARDS, C.: The effect of acetylcholine on neuromuscular transmission in the frog. J. Pharmacol. Exp. Ther. **142:** 21–23, 1963.
53. CITRIN, G.: Actions of KCl on the neuromuscular junction. Fed. Proc. **26:** 512, 1967.
54. COLLIER, B.: The preferential release of newly synthesized transmitter by a sympathetic ganglion. J. Physiol. (London) **205:** 341–352, 1969.
55. COLLIER, B. AND KATZ, H. S.: The release of acetylcholine in the cat's superior cervical ganglion. Brit. J. Pharmacol. **39:** 428–438, 1970.
56. COLQUHOUN, D.: Mechanisms of drug action at the voluntary muscle end-plate. Annu. Rev. Pharmacol. **15:** 307–325, 1975.
57. COOKE, J. D. AND QUASTEL, D. M. J.: The specific effect of potassium on transmitter release by motor nerve terminals and its inhibition by calcium. J. Physiol. (London) **228:** 435–458, 1973.
58. COUTEAUX, R. AND TAXI, J.: Recherches histochimiques sur la distribution des activités cholinestérasiques au niveau de la synapse myoneurale. Arch. Anat. Micro. Morph. Exp. **41:** 353–392, 1952.
59. COWAN, S. L.: The initiation of all-or-none responses in muscle by acetylcholine. J. Physiol. (London) **88:** 3P, 1936.
60. CULL-CANDY, S. G., LUNDH, H. AND THESLEFF, S.: Effect of botulinum toxin on neuromuscular transmission in the rat. J. Physiol. (London) **260:** 177–203, 1976.
61. DALE, H. H., FELDBERG, W. AND VOGT, M.: Release of acetylcholine at voluntary motor nerve endings. J. Physiol. (London) **86:** 353–380, 1936.
62. DANIELS, M. P. AND VOGEL, Z.: Acetylcholine receptor staining: Immunoperoxidase visualization of α-bungarotoxin binding sites in muscle endplates. Nature (London) **253:** 339–341, 1975.
63. DEL CASTILLO, J. AND ENGBAEK, L.: The nature of the neuromuscular block produced by magnesium. J. Physiol. (London) **124:** 370–384, 1954.
64. DEL CASTILLO, J. AND KATZ, B.: Quantal components of the end-plate potential. J. Physiol. (London) **124:** 560–573, 1954.
65. DEL CASTILLO, J. AND KATZ, B.: Changes in end-plate activity produced by presynaptic polarization. J. Physiol. (London) **124:** 586–604, 1954.
66. DEL CASTILLO, J. AND KATZ, B.: Local activity at a depolarized nerve-muscle junction. J. Physiol. (London) **128:** 396–411, 1955.
67. DENNIS, M. J. AND MILEDI, R.: Electrically induced release of acetylcholine from denervated Schwann cells. J. Physiol. (London) **237:** 431–452, 1974.
68. DETTBARN, W. D. AND DAVIS, F. A.: Effects of acetylcholine on axonal conduction of lobster nerve. Biochim. Biophys. Acta **66:** 397–405, 1963.
69. DIAMOND, J.: The effect of injecting acetylcholine into normal and regenerating nerves. J. Physiol. (London) **145:** 611–629, 1959.
70. DOUGLAS, W. W. AND GRAY, J. A. B.: The excitant action of acetylcholine and other substances on cutaneous sensory pathways and its prevention by hexamethonium and *d*-tubocurarine. J. Physiol. (London) **119:** 118–128, 1953.

71. DREYER, F., PEPER, K., AKERT, K., SANDRI, C. AND MOOR, H.: Ultrastructure of the "active zone" in frog neuromuscular junction. Brain Res. **62:** 373–380, 1973.
72. ECCLES, J. C.: The Physiology of Synapses, Springer-Verlag, Berlin, 1964.
73. ECCLES, J. C., KATZ, B. AND KUFFLER, S. W.: Nature of the 'end-plate potential' in curarized muscle. J. Neurophysiol. **4:** 362–387, 1941.
74. ECCLES, J. C., KATZ, B. AND KUFFLER, S. W.: Effect of eserine on neuromuscular transmission. J. Neurophysiol. **5:** 211–230, 1942.
75. EDWARDS, C. AND IKEDA, K.: Effects of 2-PAM and succinylcholine on neuromuscular transmission in the frog. J. Pharmacol. Exp. Ter. **138:** 322–328, 1962.
76. ELDEFRAWI, M. E.: Neuromuscular transmission—The transmitter-receptor combination. *In* The Peripheral Nervous System, ed. by J. I. Hubbard, pp. 181–200, Plenum Press, New York, 1974.
77. ELMQVIST, D. AND QUASTEL, D. M. J.: A quantitative study of end-plate potentials in isolated human muscle. J. Physiol. (London) **178:** 505–529, 1965.
78. EMMELIN, N. G. AND MACINTOSH, F. C.: The release of acetylcholine from perfused sympathetic ganglia and skeletal muscles. J. Physiol. (London) **131:** 477–496, 1956.
79. ERANKÖ, O. AND TERÄVÄINEN, H.: Distribution of esterases in the myoneural junction of the striated muscle of the rat. J. Histochem. Cytochem. **15:** 399–403, 1967.
80. ERULKAR, S. D. AND WEIGHT, F. F.: Extracellular potassium and transmitter release at the giant synapse of squid. J. Physiol. (London) **266:** 209–218, 1977.
81. EVANS, C. A. N. AND SAUNDERS, N. R.: An outflow of acetylcholine from normal and regenerating ventral roots of the cat. J. Physiol. (London) **240:** 15–32, 1974.
82. FATT, P. AND KATZ, B.: An analysis of the end-plate potential recorded with an intracellular electrode. J. Physiol. (London) **115:** 320–370, 1951.
83. FATT, P. AND KATZ, B.: Spontaneous subthreshold activity at motor nerve endings. J. Physiol. (London) **117:** 109–128, 1952.
84. FENG, T. P. AND LI, T. H.: Studies on the neuromuscular junction. XXIII. A new aspect of the phenomena of eserine potentiation and post-tetanic facilitation in mammalian muscles. Chin. J. Physiol. **16:** 37–50, 1941.
85. FERRY, C. B.: The sympathomimetic effect of acetylcholine on the spleen of the cat. J. Physiol. (London) **167:** 487–504, 1963.
86. FERTUCK, H. C. AND SALPETER, M. M.: Localization of acetylcholine receptor by ^{125}I-labeled α-bungarotoxin binding at mouse motor endplates. Proc. Nat. Acad. Sci. U.S.A. **71:** 1376–1378, 1974.
87. FERTUCK, H. C. AND SALPETER, M. M.: Quantitation of junctional and extrajunctional acetylcholine receptors by electron microscope autoradiography after ^{125}I-α-bungarotoxin binding at mouse neuromuscular junctions. J. Cell Biol. **69:** 144–158, 1976.
88. FLETCHER, P. AND FORRESTER, T.: The effect of curare on the release of acetylcholine from mammalian motor nerve terminals and an estimate of quantum content. J. Physiol. (London) **251:** 131–144, 1975.
89. FOLDES, F. F.: Presynaptic aspects of neuromuscular transmission and block. Anaesthesist **20:** 6–19, 1971.
90. FREEMAN, S. E. AND TURNER, R. J.: Facilitatory drug action on the isolated phrenic nerve-diaphragm preparation of the rat. J . Pharmacol. Exp. Ther. **174:** 550–559, 1970.
91. FRITZ, L. C. AND GARDNER-MEDWIN, A. R.: The effect of synaptic activation on the extracellular potassium concentration in the hippocampal area, in vitro. Brain Res. **112:** 183–187, 1976.
92. GAGE, P. W.: Generation of end-plate potentials. Physiol. Rev. **56:** 177–247, 1976.
93. GAGE, P. W. AND EISENBERG, R. S.: Action potentials without contraction in frog skeletal muscle fibers with disrupted transverse tubules. Science (Washington) **158:** 1702–1703, 1967.
94. GALINDO, A.: Depolarizing neuromuscular block. J. Pharmacol. Exp. Ther. **178:** 339–349, 1971.
95. GALINDO, A.: The role of prejunctional effects in myoneural transmission. Anesthesiology **36:** 598–608, 1972.
96. GALINDO, A.: Curare and pancuronium compared: Effects on previously undepressed mammalian myoneural junctions. Science (Washington) **178:** 753–755, 1972.
97. GASSER, H. S. AND ERLANGER, J.: The ending of the axon potential and its relation to other events in nerve activity. Amer. J. Physiol. **94:** 247–277, 1930.
98. GINSBORG, B. L.: On the presynaptic acetylcholine receptor in sympathetic ganglia of the frog. J. Physiol. (London) **216:** 237–246, 1971.
99. GINSBORG, B. L. AND GUERRERO, S.: On the action of depolarizing drugs on sympathetic ganglion cells of the frog. J. Physiol. (London) **172:** 189–206, 1964.
100. GINSBORG, B. L. AND JENKINSON, D. H.: Transmission of impulses from nerve to muscle. *In* Handbook of Experimental Pharmacology, vol. 42: Neuromuscular Junction, ed. by E. Zaimis, pp. 229–364, Springer-Verlag, New York, 1976.
101. GÖPFERT, H. AND SCHAEFER, H.: Über den direkt und indirekt erregten Aktionsstrom und die Funktion der motorischen Endplatte. Pflügers Arch. Gesamte Physiol. Menschen Tiere **239:** 597–619, 1938.
102. GRAY, J. A. B. AND DIAMOND, J.: Pharmacological properties of sensory receptors and their relation to those of the autonomic nervous system. Brit. Med. Bull. **13:** 185–188, 1957.
103. HANIN, H. C.: Choline and Acetylcholine: Handbook of Chemical Assay Methods, Raven Press, New York., 1974.
104. HAYES, A. H. AND RIKER, W. F., JR.: Acetylcholine release at the neuromuscular junction. J. Pharmacol. Exp. Ther. **142:** 200–205, 1963.
105. HEBB, C.: Biosynthesis of acetylcholine in nervous tissue. Physiol. Rev. **52:** 918–957, 1972.
106. HENDERSON, E. G. AND VOLLE, R. L.: Twitch potentiation, contractures and alterations in calcium fluxes produced by tetraalkylammonium ions. J. Pharmacol. Exp. Ther. **178:** 631–647, 1971.
107. HEUSER, J. E., REESE, T. S. AND LANDIS, D. M. D.: Functional changes in frog neuromuscular junctions studied with freeze-fracture. Neurocytology **3:** 109–131, 1974.
108. HOBBIGER, F.: Pharmacology of anticholinesterase drugs. *In* Handbook of Experimental Pharmacology, vol. 42, Neuromuscular Junction, ed. by E. Zaimis, pp. 487–581, Springer-Verlag, New York, 1976.
109. HUBBARD, J. I.: Repetitive stimulation at the mammalian neuromuscular junction and the mobilization of transmitter. J. Physiol. (London) **169:** 641–662, 1963.
110. HUBBARD, J. I.: The origin and significance of antidromic activity in motor nerves. *In* Studies in Physiology, ed. by D. R. Curtis and A. K. McIntyre, pp. 85–92, Springer-Verlag, Berlin, 1965.
111. HUBBARD, J. I.: Physiological and biochemical aspects of the normal neuromuscular junction. *In* International Encyclopedia of Pharmacology and Therapeutics, ed. by J. Cheymol, pp. 57–118, Pergamon Press, Oxford, 1972.
112. HUBBARD, J. I.: Microphysiology of vertebrate neuromuscular transmission. Physiol. Rev. **53:** 674–723, 1973.
113. HUBBARD, J. I., LLINÁS, R. AND QUASTEL, D. M. J.: Electrophysiological Analysis of Synaptic Transmission, Edward Arnold, London, 1969.
114. HUBBARD, J. I., SCHMIDT, R. F. AND YOKOTO, T.: The effect of acetylcholine upon mammalian motor nerve terminals. J. Physiol.(London) **181:** 810–829, 1965.
115. HUBBARD, J. I. AND WILSON, D. F.: Neuromuscular transmission in a mammalian preparation in the absence of blocking drugs and the effect of *d*-tubocura-

rine. J. Physiol. (London) **228:** 307–325, 1973.
116. HUBBARD, J. I., WILSON, D. F. AND MIYAMOTO, M.: Reduction of transmitter release by d-tubocurarine. Nature (London) **223:** 531–533, 1969.
117. HUTTER, O. F.: Post-tetanic restoration of neuromuscular transmission blocked by d-tubocurarine. J. Physiol. (London) **118:** 216–227, 1952.
118. ITO, Y. AND MILEDI, R.: The effect of calcium-ionophores on acetylcholine release from Schwann cells. Proc. Roy. Soc. London B **196:** 51–58, 1977.
119. ITO, Y., MILEDI, R., MOLENNAR, P. C., VINCENT, A., POLAK, R. L., VAN GELDER, M. AND DAVIS, J. N.: Acetylcholine in human muscle. Proc. Roy. Soc. London B **192:** 475–480, 1976.
120. JENERICK, H. P. AND GERARD, W.: Membrane potential and threshold of single muscle fibres. J. Cell. Comp. Physiol. **42:** 79–95, 1953.
121. KARLIN, A.: Molecular interactions of the acetylcholine receptor. Fed. Proc. **32:** 1847–1853, 1973.
122. KARNOVSKY, M. J.: The localization of cholinesterase activity in the rat cardiac muscle by electron microscopy. J. Cell Biol. **23:** 217–232, 1964.
123. KATZ, B.: Microphysiology of the neuro-muscular junction. A physiological 'quantum of action' at the myoneural junction. Bull. Johns Hopkins Hosp. **102:** 275–295, 1958.
124. KATZ, B.: The transmission of impulses from nerve to muscle, and the subcellular unit of synaptic action. Proc. Roy. Soc. Lond B **155:** 455–477, 1962.
125. KATZ, B. AND MILEDI, R.: Release of acetylcholine from a nerve terminal by electric pulses of variable strength and duration. Nature (London) **207:** 1097–1098, 1965.
126. KATZ, B. AND MILEDI, R.: The timing of calcium action during neuromuscular transmission. J. Physiol. (London) **189:** 535–544, 1967.
127. KATZ, B. AND MILEDI, R.: The role of calcium in neuromuscular facilitation. J. Physiol. (London) **195:** 481–492, 1968.
128. KATZ, B. AND MILEDI, R.: The effect of local blockage of motor nerve terminals. J. Physiol. (London) **199:** 729–743, 1968.
129. KATZ, B. AND MILEDI, R.: The statistical nature of the acetylcholine potential and its molecular components. J. Physiol. (London) **224:** 665–669, 1972.
130. KATZ, B. AND MILEDI, R.: The nature of the prolonged endplate depolarization in anti-esterase treated muscle. Proc. Roy. Soc. London B **192:** 27–38, 1975.
131. KATZ, B. AND MILEDI, R.: Transmitter leakage from motor nerve endings. Proc. Roy. Soc. London B **196:** 59–72, 1977.
132. KATZ, B. AND THESLEFF, S.: On the factors which determine the amplitude of the 'miniature end-plate potential.' J. Physiol. (London) **137:** 267–278, 1957.
133. KATZ, B. AND THESLEFF, S.: A study of the "desensitization" produced by acetylcholine at the motor endplate. J. Physiol. (London) **138:** 63–80, 1957.
134. KOELLE, G. B.: A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. J. Pharm. Pharmacol. **14:** 65–90, 1962.
135. KOELLE, G. B.: Current concepts of synaptic structure and function. Ann. N.Y. Acad. Sci. **183:** 5–20, 1971.
136. KOELLE, G. B.: Anticholinesterase agents. *In* The Pharmacological Basis of Therapeutics, 5th ed., ed. by L. S. Goodman, A. Gilman, A. G. Gilman and G. B. Koelle, pp. 445–466, The Macmillan Company, New York, 1975.
137. KOKETSU, K. AND NISHI, S.: Cholinergic receptors at sympathetic preganglionic nerve terminals. J. Physiol. (London) **196:** 293–310, 1968.
138. KRNJEVIĆ, K. AND MITCHELL, J. F.: The release of acetylcholine in the isolated rat diaphragm. J. Physiol. (London) **155:** 246–262, 1961.
139. KRNJEVIĆ, K. AND STRAUGHAN, D. W.: The release of acetylcholine from the denervated rat diaphragm. J. Physiol. (London) **170:** 371–378, 1964.
140. KUFFLER, S. W.: Electric potential changes at an isolated nerve-muscle junction. J. Neurophysiol. **5:** 18–26, 1942.
141. LEE, C. Y.: Chemistry and pharmacology of polypeptide toxins in snake venoms. Annu. Rev. Pharmacol. **12:** 265–286, 1972.
142. LEE, C. Y., TSENG, L. F. AND CHIU, T. H.: Influence of denervation on localization of neurotoxins from Elapid venom in rat diaphragm. Nature (London) **215:** 1177–1178, 1967.
143. LENTZ, T. L. AND CHESTER, J.: Localization of acetylcholine receptors in central synapses. J. Cell Biol. **75:** 258–267, 1977.
144. LENTZ, T. L., MAZURKIEWICZ, J. E. AND ROSENTHAL, J.: Cytochemical localization of acetylcholine receptors at the neuromuscular junction by means of horseradish peroxidase-labeled α-bungarotoxin. Brain Res. **132:** 423–442, 1977.
145. LILEY, A. W.: An investigation of spontaneous activity at the neuromuscular junction of the rat. J. Physiol. (London) **132:** 650–666, 1956.
146. LILEY, A. W.: The effect of presynaptic polarization on spontaneous activity at the mammalian neuromuscular junction. J. Physiol. (London) **134:** 427–443, 1956.
147. LILLEHEIL, G. AND NAESS, K.: A presynaptic effect of *d*-tubocurarine in the neuromuscular junction. Acta Physiol. Scand. **52:** 120–136, 1961.
148. LLINÁS, R., WALTON, K. AND BOHR, V.: Synaptic transmission in squid giant synapse after potassium conductance blockage with external 3- and 4-aminopyridine. Biophys. J. **16:** 83–86, 1976.
149. LLOYD, D. P. C.: Stimulation of peripheral nerve termination by active muscle. J. Neurophysiol. **5:** 153–165, 1942.
150. LUNDH, H. AND THESLEFF, S.: The mode of action of 4-aminopyridine and guanidine on transmitter release from motor nerve terminals. Eur. J. Pharmacol. **42:** 411–412, 1977.
151. MACINTOSH, F. C. AND COLLIER, B.: Neurochemistry of cholinergic terminals. *In* Handbook of Experimental Pharmacology, vol. 42, Neuromuscular Junction, ed. by E. Zaimis, pp. 99–228, Springer-Verlag, New York, 1976.
152. MACINTOSH, F. C. AND PERRY, W. L. M.: Biological estimation of acetylcholine. Methods Med. Res. **3:** 78–92, 1950.
153. MACLAGAN, J.: Competitive neuromuscular blocking drugs. *In* Handbook of Experimental Pharmacology, vol 42, Neuromuscular Junction, ed. by E. Zaimis, pp. 425–432, Springer-Verlag, New York, 1976.
154. MAENO, T. AND NOBE, S.: Analysis of presynaptic effect of *d*-tubocurarine on the neuromuscular transmission. Proc. Jap. Acad. **46:** 750–754, 1970.
155. MARQUIS, J. K., HILT, D. C. AND MAUTNER, H. G.: Direct binding studies of ^{125}I-α-bungarotoxin and ^{3}H-quinuclidinyl benzilate interaction with axon plasma membrane fragments. Evidence for nicotinic and muscarinic binding sites. Biochem. Biophys. Res. Commun. **78:** 476–482, 1977.
156. MARTIN, A. R.: A further study of the statistical composition of the end-plate potential. J. Physiol. (London) **130:** 114–122, 1955.
157. MARTIN, A. R.: Quantal nature of synaptic transmission. Physiol. Rev. **46:** 51–66, 1966.
158. MARTIN, A. R.: The effect of membrane capacitance on non-linear summation of synaptic potentials. J. Theor. Biol. **59:** 179–187, 1976.
159. MARTIN, A. R. AND PILAR, G.: Quantal components of the synaptic potential in the ciliary ganglion of the chick. J. Physiol. (London) **175:** 1–16, 1964.
160. MARTIN, A. R. AND PILAR, G.: Presynaptic and postsynaptic events during post-tetanic potentiation and facilitation in the avian ciliary ganglion. J. Physiol. (London) **175:** 17–30, 1964.
161. MASLAND, R. L. AND WIGTON, R. S.: Nerve activity accompanying fasciculation produced by prostigmin. J.

Neurophysiol. 3: 269–275, 1940.
162. MATTHEWS, G. AND WICKELGREN, W. O.: Effects of guanidine on transmitter release and neuronal excitability. J. Physiol. (London) 266: 69–89, 1977.
163. MCLEAN, I. W. AND NAKANE, P. K.: Periodate-lysine-paraformaldehyde fixative, a new fixative for immunoelectron microscopy. J. Histochem. Cytochem. 22: 1077–1083, 1974.
164. MEETER, E.: The relation between end-plate depolarization and the repetitive response elicited in the isolated rat phrenic nerve-diaphragm preparation by DFP. J. Physiol. (London) 144: 38–51, 1958.
165. MILEDI, R.: The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. J. Physiol. (London) 151: 1–23, 1960.
166. MILEDI, R.: From nerve to muscle. Discovery 22: 442–450, 1961.
167. MILEDI, R.: Transmitter release induced by injection of calcium ions into nerve terminals. Proc. Roy Soc London B 183: 421–425, 1973.
168. MILEDI, R., MOLINOFF, P. AND POTTER, L. T.: Isolation of the cholinergic receptor protein of Torpedo electric tissue. Nature (London) 229: 554–557, 1971.
169. MILEDI, R. AND POTTER, L. T.: Acetylcholine receptors in muscle fibres. Nature (London) 233: 599–603, 1971.
170. MITCHELL, J. F. AND SILVER, A.: The spontaneous release of acetylcholine from the denervated hemidiaphragm of the rat. J. Physiol. (London) 165: 117–129, 1963.
171. MIYAMOTO, M. D.: Binomial analysis of quantal transmitter release at glycerol treated frog neuromuscular junctions. J. Physiol. (London) 250: 121–142, 1975.
172. MIYAMOTO, M. D.: Estimates on magnitude of non-linear summation of evoked potentials at motor endplate. J. Neurophysiol. 41: 589–599, 1978.
173. MIYAMOTO, M. D. AND VOLLE, R. L.: Enhancement by carbachol of transmitter release from motor nerve terminals. Proc. Nat. Acad. Sci. U.S.A. 71: 1489–1492, 1974.
174. MORRISON, J. D.: The generation of nerve and muscle repetitive activity in the rat phrenic nerve-diaphragm preparation following inhibition of cholinesterase by ecothiopate. Brit. J. Pharmacol. 60: 45–53, 1977.
175. MOUNTCASTLE, V. B.: Physiology of sensory receptors: Introduction to sensory processes. *In* Medical Physiology, 12th ed., ed. by V. B. Mountcastle, pp. 1345–1356, The C. V. Mosby Co., St. Louis, 1968.
176. NAESS, K.: The mechanism of action of curare. Acta Pharmacol. Toxicol. 8: 149–163, 1952.
177. NAESS, K.: Effects of brief and protracted curarization. Acta Pharmacol. Toxicol. 8: 400–408, 1952.
178. NAKANE, P. K. AND KAWAOI, A.: Peroxidase-labelled antibody. A new method of conjugation. J. Histochem. Cytochem. 22: 1084–1091, 1974.
179. NARAHASHI, T.: Chemicals as tools in the study of excitable membranes. Physiol. Rev. 54: 813–889, 1974.
180. NISHI, S.: Cholinergic and adrenergic receptors at sympathetic preganglionic nerve terminals. Fed. Proc. 29: 1957–1965, 1970.
181. OLMSTED, J. M. D. AND OLMSTED, E. H.: Claude Bernard and the Experimental Method in Medicine, Schuman, New York, 1952.
182. OTSUKA, M. AND ENDO, M.: Presynaptic nature of neuromuscular depression in the frog. Nature (London) 188: 501–502, 1960.
183. PALADE, G. E. AND PALAY, S. L.: Electron microscope observation of interneuronal and neuromuscular synapses. Anat. Rec. 118: 335, 1954.
184. PATON, W. D. M.: An experiment of Claude Bernard on curare: The origins of the Physiological Society. J. Physiol. (London) 263: 26P–29P, 1976.
185. PFENNINGER, K. H. AND ROVAINEN, C. M.: Stimulation- and calcium-dependence of vesicle attachment sites in the presynaptic membrane; a freeze-cleave study on the lamprey spinal cord. Brain Res. 72: 1–23, 1974.
186. PILAR, G.: Effect of ACh on pre- and postsynaptic elements of avian ciliary ganglion synapses. Fed. Proc. 28: 670, 1969.
187. PILAR, G.: The physiology of preganglionic terminals. *In* Morphological, Biochemical, and Physiological Studies of Acetylcholine Release at Synapses, pp. 46–61, Nato Advanced Study Institute, 1969.
188. POLITOFF, A. L., ROSE, S. AND PAPPAS, G. D.: The calcium binding sites of synaptic vesicles of the frog sartorius neuromuscular junction. J. Cell Biol. 61: 818–823, 1974.
189. PORTER, C. W. AND BARNARD, E. A.: The density of cholinergic receptors at the endplate postsynaptic membrane: Ultrastructural studies in two mammalian species. J. Membrane Biol. 20: 31–49, 1975.
190. PORTER, C. W. AND BARNARD, E. A.: Distribution and density of cholinergic receptors at the motor endplates of a denervated mouse muscle. Expl. Neurol. 48: 542–556, 1975.
191. PORTER, C. W., BARNARD, E. A. AND CHIU, T. H.: The ultrastructural localization and quantitation of cholinergic receptors at the mouse motor endplate. J. Memb. Biol. 14: 383–402, 1973.
192. POTTER, L. T.: Synthesis, storage and release of [^{14}C] acetylcholine in isolated rat diaphragm muscles. J. Physiol. (London) 206: 145–166, 1970.
193. POTTER, L. T.: Acetylcholine receptors in vertebrate skeletal muscles and electric tissue. *In* Drug Receptors, ed. by H. P. Rang, pp. 295–312, Macmillan, London, 1973.
194. PRESTON, J. B. AND VAN MAANEN, E. F.: Effect of frequency of stimulation on the paralyzing dose of neuromuscular blocking agents. J. Pharmacol. Exp. Ther. 107: 165–171, 1953.
195. RANDIC, M. AND STRAUGHAN, D. W.: Antidromic activity in the rat phrenic nerve-diaphragm preparation. J. Physiol. (London) 173: 130–148, 1964.
196. RANG, H. P.: Acetylcholine receptors. Q. Rev. Biophys. 7: 283–399, 1975.
197. RIKER, W. F., JR.: Pharmacologic considerations in a reevaluation of the neuromuscular synapse. Arch. Neurol. 3: 488–499, 1960.
198. RIKER, W. F., JR.: Actions of acetycholine on mammalian motor nerve terminals. J. Pharmacol. Exp. Ther. 152: 397–416, 1966.
199. RIKER, W. F.: Pre-junctional effects of neuromuscular blocking and facilitatory drugs. *In* Muscle Relaxants, ed. by R. L. Katz, pp. 59–102, North-Holland Publishing Co., Amsterdam, 1975.
200. RIKER, W. F., JR. AND OKAMOTO, M.: Pharmacology of motor nerve termimals. Annu. Rev. Pharmacol. 9: 173–208, 1969.
201. RIKER, W. F., JR., ROBERTS, J., STANDAERT, F. G. AND FUJIMORI, H.: The motor nerve terminal as the primary focus for drug-induced facilitation of neuromuscular transmission. J. Pharmacol. Exp. Ther. 121: 286–312, 1957.
202. RIKER, W. F., JR., WERNER, G., ROBERTS, J. AND KUPERMAN, A.: Pharmacologic evidence for the existence of a presynaptic event in neuromuscular transmission. J. Pharmacol. Exp. Ther. 125: 150–158, 1959.
203. RIKER, W. F., JR. AND WESCOE, W. C.: The direct action of prostigmine on skeletal muscle; its relationship to the choline esters. J. Pharmacol. Exp. Ther. 88: 58–66, 1946.
204. ROSENBLUETH, A. AND MORISON, R. S.: Curarization, fatigue and Wedensky inhibition. Amer. J. Physiol. 119: 236–256, 1937.
205. SALPETER, M. M.: Electron microscope radioautography as a quantitative tool in enzyme cytochemistry. I. The distribution of acetylcholinesterase at motor endplates of a vertebrate twitch muscle. J. Cell Biol. 32: 379–389, 1967.
206. SALPETER, M. M.: Electron microscope radioautography as a quantitative tool in enzyme cytochemistry. II. The

distribution of DFP-reactive sites at motor endplates of a vertebrate twitch muscle. J. Cell Biol. **42:** 122–134, 1969.

207. SALPETER, M. M., MCHENRY, F. A. AND SALPETER, E. E.: Resolution in electron microscope autoradiography. IV. Application to analysis of autoradiographs. J. Cell Biol. **76:** 127–145, 1978.
208. SOKOLL, M. D., DRETCHEN, K. L., GERGIS, S. D. AND LONG, J. P.: *d*-Tubocurarine effects on nerve-terminal and neuromuscular conduction. Anesthesiology **36:** 592–597, 1972.
209. SOKOLL, M. D., DRETCHEN, K. L., GERGIS, S. D. AND LONG, J. P.: The effects of gallamine on nerve terminals and endplates. Anesthesiology **38:** 157–165, 1973.
210. SPITZER, N.: Miniature end-plate potentials at mammalian neuromuscular junctions poisoned by botulinum toxin. Nature New Biol. **237:** 26–27, 1972.
211. STANDAERT, F. G.: Post tetanic repetitive activity in cat soleus nerve. Its origin, cause and mechanism of generation. J. Gen. Physiol. **47:** 53–70, 1963.
212. STANDAERT, F. G.: The action of *d*-tubocurarine on the motor nerve terminal. J. Pharmacol. Exp. Ther. **143:** 181–186, 1964.
213. STANDAERT, F. G.: The mechanism of post tetanic potentiation in cat soleus and gastrocnemius muscles. J. Gen. Physiol. **47:** 987–1001, 1964.
214. STANDAERT, F. G. AND ADAMS, J. E.: The actions of succinylcholine on the mammalian motor nerve terminal. J. Pharmacol. Exp. Ther. **149:** 113–123, 1965.
215. STEINBACH, J. H. AND STEVENS, C. F.: Neuromuscular transmission. *In* Frog Neurobiology, ed. by R. Llinás and W. Precht, pp. 33–92, Springer-Verlag, New York, 1976.
216. STEINBERG, M. I. AND VOLLE, R. L.: A comparison of lobeline and nicotine at the frog neuromuscular junction. Naunyn-Schmiedeberg's Arch. Pharmacol. **272:** 16–31, 1972.
217. STEVENS, C. F.: A comment on Martin's relation. Biophys. J. **16:** 891–895, 1976.
218. STRAUGHAN, D. W.: Release of acetylcholine from mammalian motor nerve endings. Brit. J. Pharmacol. Chemother. **15:** 417–424, 1960.
219. TAKEUCHI, A. AND TAKEUCHI, N.: On the permeability of the end-plate membrane during the action of transmitter. J. Physiol. (London) **154:** 52–67, 1960.
220. THIES, R. E.: Neuromuscular depression and apparent depletion of transmitter in mammalian muscle. J. Neurophysiol. **28:** 427–442, 1965.
221. ULBRICHT, W.: Ionic channels and gating currents in excitable membranes. Annu. Rev. Biophys. Bioeng. **6:** 7–31, 1977.
222. VAN DER MEER, C. AND MEETER, E.: The mechanisms of action of anticholinesterases. II. The effect of diisopropylfluorophosphonate (DFP) on the isolated rat phrenic nerve-diaphragm preparation. A. Irreversible effects. Acta Physiol. Neerl. **4:** 454–471, 1956.
223. VOLLE, R. L.: Blockade by hexamethonium of drug-induced neuromuscular facilitation. Arch. Int. Pharmacodyn. Thér. **167:** 1–9, 1967.
224. WASER, P. G.: Receptor localization by autoradiographic techniques. Ann. N.Y. Acad. Sci. **144:** 737–755, 1967.
225. WASER, P. G. AND LUZTHI, U.: Autoradiographische Lokalisation von ^{14}C-Calebassen-Curarin I und ^{14}C-Decamethonium in der motorischen Endplatte. Arch. Int. Pharmacodyn. Thér. **112:** 272–296, 1957.
226. WEBB, S. N. AND BOWMAN, W. C.: The role of pre- and post-junctional cholinoceptors in the action of neostigmine at the neuromuscular junction. Clin. Exp. Pharmacol. Physiol. **1:** 123–134, 1974.
227. WEDENSKY, N. E.: Die Erregung, Hemmung und Narkose. Pflügers Arch. Gesamte Physiol. Menschen Tiere **100:** 1–144, 1903.
228. WEIGHT, F. F. AND ERULKAR, S. D.: Modulation of synaptic transmitter release by repetitive postsynaptic action potentials. Science (Washington) **193:** 1023–1025, 1976.
229. WERNER, G.: The presynaptic action of *d*-tubocurarine and flaxedil at the neuromuscular junction. Fed. Proc. **18:** 458, 1959.
230. WERNER, G.: Neuromuscular facilitation and antidromic discharges in motor nerves: their relation to activity in motor nerve terminals. J. Neurophysiol. **23:** 171–187, 1960.
231. WERNER, G.: Generation of antidromic activity in motor nerves. J. Neurophysiol. **23:** 453–461, 1960.
232. WERNER, G.: Antidromic activity in motor nerves and its relation to a generator event in nerve terminals. J. Neurophysiol. **24:** 401–413, 1961.
233. WISLICKI, L.: Effects of rate of stimulation and of fatigue on the response to neuromuscular blocking agents. Brit. J. Pharmacol. Chemother. **13:** 138–143, 1958.
234. YAMAMURA, H. I. AND SNYDER, D. H.: Affinity transport of choline into synaptosomes of rat brain. J. Neurochem. **21:** 1355–1374, 1973.