MECHANISMS OF DRUG ACTION AT THE VOLUNTARY MUSCLE ENDPLATE[1]

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INTRODUCTION

Recent work on the mechanism of action of drugs on the postsynaptic membrane of vertebrate skeletal muscle is discussed in this review. Several recent reviews and symposia impinge on this topic (1–9).

Work on the location and number of ACh receptors is discussed first, followed by recent ideas concerning the mechanism of ion channel-opening by drugs, the effects of agonists on the postsynaptic membrane, the effects of antagonists, and of other drugs (local anesthetics, alcohols, etc), and finally a discussion of the special considerations that arise when the agonist is applied for a very brief time only, as endogenous ACh is.

THE LOCATION, NUMBER, AND BIOCHEMICAL PROPERTIES OF RECEPTORS

The Location of Receptors

Electrophysiological methods show the receptors to be on the outer surface of the muscle membrane (10). Detailed mapping with iontophoretically applied ACh shows that most of them are confined to the endplate (10–12). A zone of much lower sensitivity to ACh attributed to extrajunctional receptors, extends as far as 400 μm beyond the endplate (13–17). After denervation, ACh sensitivity spreads over the whole mammalian and frog muscle fiber (11, 15,18). At first separate patches of ACh sensitivity develop in frog muscle, but eventually the sensitivity becomes uniform (19).

[1]Abbreviations used throughout this review: ACh, acetylcholine; BuTX, α-bungarotoxin; TC, (+)-tubocurarine; E_{rev}, reversal potential; epp, endplate potential; epc, endplate current; mepp, miniature endplate potential; mepc, miniature endplate current.

Toxin Binding Studies

Almost all recent work has been based on the ability of the snake venom toxins, α-bungarotoxin and cobra (*Naja naja*) toxin, to bind nearly irreversibly and with (relative to most drugs) fairly high specificity to ACh receptors, while having little effect on presynaptic function (20–24). Labeled toxin is bound selectively at the endplate (25). It is not known how many BuTX binding sites correspond to one ion channel (possibly as many as four), so there is a corresponding uncertainty in calculations that assume the number of ion channels is the same as the number of BuTX binding sites.

Rat and mouse diaphragm have 1.4–4.7 × 10^7 BuTX binding sites per endplate (1.3–4 pmol/g tissue), whether determined by binding of iodine or ^{3}H-acetyl-labeled BuTX to intact (26–28) or homogenized (29) diaphragms, or by autoradiography (30–32). The binding site density is estimated to be 8,000–10,000/μm^2 (33, 34) or 13,000/μm^2 (30), the receptors occupying perhaps 20% or so of the area of the postsynaptic membrane if it is assumed that they are uniformly distributed over it (33, 34). Recent evidence that there may be no receptors in the postjunctional folds of mouse sternomastoid muscle would increase the estimates of receptor density four- to sixfold, implying a near monolayer of receptor molecules (35).

Fourteen to 20 days after denervation the total number of BuTX sites in rat muscle rises to a maximum of 44–70 pmol/g, i.e. a 20- or 30-fold increase (26, 27, 29). The density of extrajunctional receptors is low [less than 5/μm^2 (36)] in normal muscle, though there are probably enough to account for extrajunctional ACh sensitivity. After denervation the density rises to a maximum of 1700/μm^2 paralleling the increase in ACh sensitivity to a maximum (36). This is still a good deal lower density than at the endplate, though the sensitivity to iontophoretically applied ACh (in millivolts per nanocoulomb) reaches a value similar to that at the endplate (36), perhaps because the endplate receptors are less accessible than extrajunctional receptors (but see ref. 12), or because ACh is more effective on extrajunctional receptors [cf increased lifetime of ACh channel in denervated frog muscle (37)].

Denervation of rat muscle causes development of tetrodotoxin-resistant action potentials and a fall of tetrodotoxin binding by a factor of 2.8 (probably caused by reduction of binding capacity from 2.5 to 0.9 pmol/g), as well as an increase of about 40 pmol/g in BuTX binding sites (29). The inequality of these changes makes it unlikely that sodium channels are converted to ACh-operated channels after denervation.

TC has been reported to be surprisingly ineffective in preventing the blocking effect of cobra toxin (22, 23) and the binding of BuTX (26, 28, 32) in whole muscle. But, with a virtually irreversible agent like BuTX, it is necessary to measure the effect of the protecting agent on the *rate* rather than the extent of binding, because at equilibrium the irreversible ligand must prevail (29, 38). Measurements of the retardation of BuTX binding by TC and other drugs in denervated rat diaphragm, homogenized to prevent diffusion limitation of the rate, showed that most (90% or

so) of the binding was inhibitable by TC (29, 38). The equilibrium constant for TC was the same as is found by other methods. Agonists also reduce the blocking effect of cobra toxin (22, 23) and the binding of BuTX (26, 28, 38). There is evidence (22, 23, 26) that this is because the desensitized receptor they induce has a *low* affinity for BuTX.

Frog muscle has more BuTX sites than rat (26), but autoradiography has shown that in frog muscle, unlike rat muscle (30, 32, 36), many bound BuTX molecules are located on extrajunctional membrane and also intracellularly, especially if high BuTX concentrations are used (32). The number at the endplate itself is about 3×10^7 per endplate (32), similar to other species. Most of these extrajunctional BuTX sites are not ACh-sensitive ion channels, but it is not known with certainty whether BuTX binding to them is prevented by TC or by agonists, though the results of Miledi & Potter suggest some protection (26, 39).

Slow extrajunctional binding is also found in rat and mouse muscle when it is incubated with higher BuTX concentrations for longer times (40, 41). This means that it is difficult to saturate endplates at the center of whole muscles with BuTX, without producing extrajunctional binding in the outermost fibers.

Cholinesterase

The number of cholinesterase-like molecules at the endplate, measured by DFP binding, is very similar to the number of BuTX binding sites (31, 34), but only a fraction (varying with the species) of the DFP sites is acetylcholinesterase (40). Cholinesterase does not accompany the new receptors that appear after denervation, and it can be removed with collagenase from frog neuromuscular junction without affecting the ACh receptors (42–44).

Solubilization of Receptors

There have been far fewer reports on muscle than on electric tissue (1). The BuTX receptor complex in frog and rat muscle can be solubilized with 1% Triton X-100 (26, 28, 39, 41). Berg et al (28) report a single 9S component in rat muscle, whereas Potter (39) found mixtures of interconvertible 9.3, 12, 15, and 18S components in rat and frog muscle and in electric tissue, and postulates subunits (mol wt 42,000) polymerizing predominantly to a hexamer (mol wt 252,000). Gel filtration experiments with Triton extracts of mouse muscle suggested an apparent molecular weight of 550,000 for the junctional BuTX-receptor complex (which was greatly increased in amount after denervation), and of 200,000 for complexes with extrajunctional receptors (41).

The solubilized receptor from rat muscle will bind BuTX after extraction (28) and has apparent affinities for a number of protecting agents similar to those found in homogenized muscle (D. Colquhoun and H. P. Rang, unpublished).

A rather close similarity between the receptors of muscle and electric tissue is suggested by the neuromuscular block produced in rabbits by antibodies induced with purified *Torpedo* ACh receptor protein (45, 46).

MECHANISMS OF DRUG ACTION

Two-State Theories

Several reviews (1, 3, 5, 7) of the classical approach to drug-receptor interaction have appeared. It was implicit in the classical theory that occupation of a receptor by an agonist induced a conformation change that opened an ion channel. To explain the fact that drugs differed in efficacy it was supposed that different drugs produced different sorts of conformation change. Current interest has centered on models that explicitly distinguish between inactive (closed ion channel) and activated (open channel) conformations of the receptor. It would greatly simplify matters if the channel were to exist in only two states, i.e. if the open state were the same for all drugs. There is some indirect evidence that this is so (see refs. 1, 3, 5). Further suggestive evidence for the two-state hypothesis from studies on agonist-induced membrane noise, is discussed below.[2]

If there are in fact only two states, then drugs of low efficacy must be supposed to be able to open a small fraction of channels only. To explain the meaning of efficacy in molecular terms it is therefore necessary to abandon the idea that an occupied receptor is necessarily in the open (activated) conformation. This is just what would be expected from the simplest two-state receptor model (1, 3, 5, 47–49), i.e.

$$\begin{array}{ccc} & \overset{L}{\rightleftharpoons} & \\ A + T & & R + A \\ K_{AT} \Big\| \Big| & & \Big| \Big\| K_{AR} \\ & \underset{k_2}{\rightleftharpoons} & \\ AT & & AR \end{array} \qquad 1.$$

where A represents the drug, T represents the inactive (shut), and R the active (open) conformation of the receptor. The allosteric constant L is the equilibrium constant between the two conformations in the absence of drug, [T]/[R]. The microscopic dissociation constants for the interaction of the drug A with R and T are K_{AR} and K_{AT}. Their ratio, $M = K_{AR}/K_{AT}$ measures the selectivity of the ligand for the two conformations. The fraction of receptors occupied (the occupancy, or binding function) increases hyperbolically from 0 to 1 with concentration, with an effective equilibrium constant $K_{eff} = K_{AR}\ (L + 1)/(LM + 1)$. The fraction of receptors (occupied or otherwise) in the open conformation (the state function) also increases hyperbolically with concentration, with the same K_{eff}, from $1/(L + 1)$ at zero drug concentration, to $1/(LM + 1)$ at very high drug concentration.

[2]Note added in proof: Recently, Colquhoun, Dionne, Steinbach & Stevens (*Nature* 1975. 253:204–5) measured current fluctuations for four agonists, at the voltage-clamped frog end-plate. The drugs had mean channel lifetimes covering a sevenfold range. The single channel conductances were not identical but covered a twofold range, from 12.8 ± 1.1 to 28.6 ± 1.0 pmho. This suggests that the open state cannot be identical for all drugs.

For an agonist drug, M would be small, i.e. the ligand would have greater affinity for the R (open) conformation and so shift the $T \rightleftharpoons R$ equilibrium to the right. An antagonist drug would have $M \geq 1$ so it would not open, or would actually shut, channels. Not all occupied receptors are in the open conformation, the equilibrium constant for $AT \rightleftharpoons AR$ being $LM = k_{-2}/k_2 = [AT]/[AR]$. Drugs with low efficacy are not very selective for the open conformation. Thus LM will not be very small (L is a constant for a given tissue), so many occupied receptors will be in the shut conformation, and the maximum fraction of channels that can be opened at high drug concentrations, $1/(LM + 1)$, will be substantially less than one. The quantity $(1/M) - 1$ bears a close analogy to the efficacy (5, 50) as originally defined by Stephenson (51).[3]

Simplified Two-State Model

Several workers, e.g. Katz et al (37, 52, 53) and Stevens et al (54, 55), have used a simplified model which resembles the special case of model 1 when $L >> 1$. When L is large the results above show that occupancy will increase hyperbolically with concentration from 0 to 1 with $K_{eff} = K_{AR}\ L/(LM + 1) = K_{AT}\ LM/(LM + 1)$, and the state function will increase hyperbolically with concentration from near zero to $1/(LM + 1)$ with the same effective equilibrium constant. L must be quite large at the endplate, where few channels can be open in the absence of drug. The species R in model 1 has therefore been ignored, and the model written thus:

$$A + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AT \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AR \qquad 2.$$

The binding function increases hyperbolically with concentration from 0 to 1 with $K_{eff} = K_1\ K_2/(K_2 + 1)$ where the equilibrium constants are defined as $K_1 = k_{-1}/k_1$, $K_2 = k_{-2}/k_2$. The state function increases hyperbolically with concentration for 0 to $1/(K_2 + 1)$. Comparison with the results above shows that K_1 and K_2 in model 2 correspond with K_{AT} and LM, respectively, in model 1. Katz et al (e.g. 56) refer to $1/K_2$ as "efficacy." Since L is constant for a particular tissue this definition ($1/LM$) is closely related to that above, $(1/M) - 1$ (5, 50), and hence to Stephenson's (51) definition.

The simplification shown in model 2 is, however, not so satisfactory when the kinetics of the response are considered. Model 2 suggests that the mean lifetime of the R is $1/k_{-2}$ (see 57, 58). But the rate of $AR \rightleftharpoons A + R \rightleftharpoons A + T$, which is not included in model 2, cannot necessarily be neglected even if [R] is very small. Nevertheless it can be shown (A. G. Hawkes, M. J. Irish, and D. Colquhoun, unpublished) that the above lifetime is correct for model 1 also, *if* the drug concentration is sufficiently high.

[3]The abscissa of Figures 6*c* and 6*d* in Colquhoun (5) should be in concentration, not normalized concentration, units.

Cooperativity

The models described above show a hyperbolic relationship between the fraction of channels in the open (R) conformation, i.e. the state function, and drug concentration. There is, on the contrary, some evidence that the endplate conductance increase (assumed directly proportional to the state function) is related in a sigmoid, cooperative manner to drug concentration (1, 5, 16, 59).

Voltage responses (60–62) and voltage-clamped current responses (3, 49) of the frog endplate both show cooperativity with a Hill slope of about 2 for carbachol. The Hill slope of 1.5 found (49) for the partial agonist diethyldecamethonium was, as might be expected (see below), somewhat smaller than for powerful agonists. These results are compatible with there being up to roughly four subunits per ion channel (5).

The linear relation between ACh concentration and conductance at the frog endplate found in one study (63) could be an artifact (see ref. 1 for discussion).

The Interpretation of Cooperativity

Empirical models of the type that suggest n molecules of agonist combine with one receptor are still occasionally invoked (64–66) but have largely been abandoned because (*a*) they predict integer Hill slopes, which are not generally observed (49), (*b*) they do not allow unambiguous prediction of the effect of competitive antagonists, and (*c*) their physical basis is dubious. They have given way to models with clearer physical foundations.

The Monod-Wyman-Changeux (MWC) model (47, 48) postulates n interacting subunits (see refs. 1, 5, 47, 48, 50 for details). The cooperativity is conveniently measured by the slope n_H of the Hill plot. The maximum slope of the Hill plot, which cannot exceed the number of subunits n, is given (5) by $n_H(\max) = n\sum_0^{n-1} M^i/(\sum_0^{n-1} M^{i/2})^2$. It would thus be expected to be larger for full agonists (small M) than for partial agonists, as found experimentally in one case (above). Because there is no independent way to determine M, n cannot be determined from the Hill coefficient, which at best gives only a lower limit for n.

There is little evidence concerning the correctness of the MWC model (1, 5). The binding function for agonists, but not antagonists, is predicted to be sigmoid, but no sufficiently exact studies of agonist binding at the endplate have been published.

Of several two-state alternatives (3, 5, 49) to the MWC model for cooperativity, the simplest is that which postulates a group of n subunits of the model 1 type functioning *independently* of each other, and that all n have to be in the active (R) conformation for an ion channel to open. This model resembles that postulated by Hodgkin & Huxley for the control of the voltage-dependent sodium channel of axon membranes (see ref. 1). Efficacy can be interpreted in terms of the selectivity of the ligand for the R state (i.e. the parameter M) as before (5). The state function, i.e. number of open channels, is related to drug concentration in a sigmoid manner, as observed experimentally, but the predicted Hill plots differ only slightly from those of the MWC model (5). Unfortunately technical difficulties with voltage-clamping prevent determination of Hill plots for powerful agonists. The most obvious differ-

ence from the MWC model is that the *binding* of agonists, as well as antagonists, should be hyperbolic, not cooperative, according to this model, but adequate tests of this prediction have yet to be made.

Compatibility of Earlier Experimental Results with Cooperative Models

A large number of experiments appear to confirm the classical occupation theory of drug action (1, 3, 5, 7, 49). This theory was based on hyperbolic binding according to Langmuir's (1918) equation, first given by Hill (67) in 1909.

Because of the unknown relation between receptor occupancy and response, tests of this theory were necessarily based on the use of *null* methods, i.e. methods in which combinations of drugs producing *equal responses* were employed. Such methods have been worked out for determining the receptor affinities of antagonists and agonists, and also the relative efficacies of partial agonists (for summary and references see ref. 5). The results have almost always been as predicted by the classical theory. But it has been shown that virtually identical results are predicted *for null experiments* by the MWC model (5, 50), the independent model described above and any other two-state model belonging to a wide class (5). This is true regardless of the number of subunits involved.

When the results of null experiments are interpreted in terms of two-state models (5), the determinations of equilibrium constants yield an approximation to K_T, the equilibrium constant for the inactive conformation, and the ratio of efficacies for drugs A and B actually estimates $(1/M_A - 1)/(1/M_B - 1)$. The only real problem arises in the interpretation of the irreversible antagonist method for determining affinities for powerful agonists (5).

Other Models

Models based on ion exchange (68, 69) and on surface charge density changes (70) have been proposed.

EFFECT OF CHOLINOMIMETIC AGONISTS ON THE ENDPLATE

The Permeability Change Produced by Cholinomimetic Agonists

ACh increases the conductance of the frog endplate, driving the membrane potential towards $E_{rev} \approx -15$mV, the ACh reversal potential (71, 72). Tables of reversal potential measurements are given in (1, 4, 73). Voltage clamp studies showed that ACh increases the endplate permeability to Na^+ and K^+ but not to Cl^- (74, 75). Rather than following the Goldman equation (76, 77), which has a clear physical basis (78, 79), the effect of ion concentration on E_{rev} was described by a model that represents, formally, two separate Hodgkin-Huxley type (see refs. 4, 73) ohmic ion channels. The curious fact remains unexplained that the model fits only if the ratio of the ACh-induced conductance increases to Na^+ and K^+, $\Delta g_{Na}/\Delta g_K$, is assumed to be a constant (=1.29) which is little affected by Na^+ or K^+ concentrations (see refs. 1, 4 for discussion).

The E_{rev} for suberyldicholine at ordinary temperatures (80, 85) and for ACh at 2°C (86), are, unlike the usual case, insensitive to K^+ concentration as predicted by the Goldman equation (see ref. 1 for discussion). The E_{rev} for carbachol and decamethonium have been found to be the same as that for ACh (–16 mV) at the frog endplate (16, 17), which supports a two-state model. However, it was found (17) that extrajunctional receptors (those not directly under the nerve ending, and those at the myotendinous junction) had a more negative E_{rev} (–43 mV), again the same for all three drugs. The effects of varying $[K^+]_o$ and $[Na^+]_o$ suggested that $\Delta g_{Na}/\Delta g_K$ was reduced to 0.6 (again constant) for extrajunctional channels, which suggests an altered ionic selectivity.

Frog [but not cat (18) or mouse (80)] muscle after denervation develops an unusually negative E_{rev} (–42 mV) for ACh at junctional sites as well as on the rest of the muscle membrane (17, 81). In frog muscle E_{rev} changes steeply, from –15 mV to –50 mV, as pH is increased. In denervated muscle there is a similar relation, but shifted by about 2 pH units (81), cf block of axonal Na^+ channels (82, 83) and tetrodotoxin binding (84) by H^+ ions.

It is still not possible to answer the fundamental question: do Na^+ and K^+ go through the same or separate channels at the endplate? There is some evidence favoring separate channels (81, 87–90), but it is by no means conclusive (1, 4, 91–96, 127), and is certainly not as compelling as the evidence for separate Na^+ and K^+ channels in nerve membranes (97). The results of noise measurements (below) suggest a single channel (54, 55), as does the apparent interaction between Na^+ and K^+ movements (1). Furthermore, work on toxin binding is consistent with control of permeability by a single sort of ACh receptor (22, 23, 29, 38), as are the observations that reversal potential is independent of the agonist used (16, 17) and is unaffected by TC (75). If Na^+ and K^+ go through the same channel the separate Δg_{Na} and Δg_K values have no physical significance (98) and may not be estimable even if there were two channels (4, 99).

Inferences from Agonist-Induced "Noise"

Katz & Miledi (100, 101) observed that the "noise" resulting from random fluctuations in the number of channels opened by ACh could be interpreted in terms of the properties of single channels. Some of the theory has been reviewed by Stevens (102).

In the intracellular work of Katz & Miledi on frog muscle (37), results were corrected for the nonlinear summation of voltage (37, 103), but it was assumed (37) that (*a*) cooperativity could be ignored as in model 2, (*b*) a small fraction only of channels was open, so that conductance could be assumed directly proportional to ACh concentration, (*c*) the voltage change produced by the opening of a single channel could be described by randomly occurring exponential blips of amplitude a and time constant τ i.e. $f(t)=ae^{-t/\tau}$, (*d*) random variations of a could be neglected. Some deviations from this model were detected (37), in the theoretically expected direction. The model implies, after correction for nonlinear summation, that the depolarization (V) produced by a drug should be $V = n a \tau$ where n = frequency of channel openings, and that the variance of the voltage (E) fluctuations

should be $\bar{E}^2 = Va/2$. The former relationship suggests that $n = V/a\tau \approx 4$ million channel openings/sec produce a depolarization of $V = 10$ mV, if $a = 0.25$ μV and $\tau = 10$ msec (see below). The linear relation between $\bar{E}^2$ and V predicted by the latter relationship was confirmed, so the elementary depolarization a could be estimated. It was about 0.25 μV for ACh at 22°C (37). This is about 1000- to 2000-fold smaller than the mepp, which suggests that 1000–2000 channels are opened during a mepp. As the conductance change during a mepp is about 100 nmho (87, 104–106), the conductance of an open channel must be about 100 pmho (37). The elementary depolarizations, produced by other drugs at around 20°C in the frog, were found to be (37, 53) as follows, relative to ACh: ACh = 1, carbachol 0.3, decamethonium 0.2, acetylthiocholine 0.2, suberyldicholine 1.2. The rat diaphragm gave (53) $a = 0.7$ μV for ACh and 0.5 μV for decamethonium.

The different size of the elementary depolarization produced by different drugs is accounted for by the fact that they cause the channel to remain open for different lengths of time. The mean lifetime of an open channel, τ_o say, turns out (see below) to be a good deal shorter than the membrane time constant, τ_m, in frog muscle, so the rising phase of the exponential blip can be taken as approximately instantaneous (see above). Insofar as the membrane charges exponentially, the depolarization produced by the opening of a single channel for a time t_o would be $a = a_\infty[1 - \exp(t_o/\tau_m)]$ where a_∞ is the depolarization eventually attained if the channel is held open. Now on the simple assumption that t_o is exponentially distributed, with mean τ_o (see 55, 57, 58), it follows that a should be a random variable with probability density function, for $0 \leq a \leq a_\infty$

$$f(a) = \frac{\tau_m}{\tau_o a_\infty}\left(1 - \frac{a}{a_\infty}\right)^{\left(\frac{\tau_m}{\tau_o} + 1\right)} \qquad 3.$$

which has a mean value $\mu_a = a_\infty \tau_o / (\tau_o + \tau_m)$ and variance $\sigma_a^2 = \mu_a^2 \tau_m / (\tau_m + 2\tau_o)$ (D. Colquhoun, unpublished). Using the data of Katz & Miledi (37, 53), Rang (1), plotted a (assumed to be an estimate of μ_a) against $\tau_o[\tau_m/(\tau_o + \tau_m)]$, and found an approximately linear relationship. This is what is expected if a_∞, and hence the channel conductance, is the same for different drugs.[4] This result is consistent with the two-state hypothesis outlined above.

The time constant for the decay of the elementary voltage blip was found from the power spectrum of intracellularly recorded voltage fluctuations to be, as expected, close to the membrane time constant τ_m i.e. about 10 msec for frog muscle (37).

The spectral density of noise recorded with focal extracellular electrodes (37), which is not limited by the passive electrical properties of the membrane, showed that the lifetime of the open channel, τ_o, was about 1 msec for ACh, though the

[4]See, however, footnote to *Two State Theories* section.

estimate from the half-power frequency, f_c (i.e. $\tau_o = 1/2\pi f_c$) gave nearly twice the value found from the power at zero frequency. Channel lifetimes were estimated similarly (37, 53) to be as follows: carbachol 0.3–0.4, decamethonium 0.1, acetylthiocholine 0.12, and suberyldicholine 1.65 msec. The channel lifetimes do not necessarily correspond with the lifetime of the drug receptor complex, as the models already discussed make clear. These results suggest (37) that during the 1 msec or so for which ACh opens an ion channel, about 50,000 ions pass through (e.g. 75 mV × 100 pmho × 1 msec = 7.5×10^{-5} coulombs = 4.7×10^4 ions).

Anderson & Stevens (55) used model 2 as a basis for interpretation of current fluctuations under voltage clamp. They assumed (*a*) channels fluctuate between open and shut state according to a Poisson process, producing rectangular current pulses, (*b*) the ACh concentration c is low enough that the fraction of open channels is low, and is proportional to c^n (so some allowance is made for cooperativity). The spectral density for current fluctuations predicted by this model, which has the same form as that predicted by Katz & Miledi (37), fitted the observations on glycerol-treated frog muscle closely.

Using this model, the conductance of a single channel opened by ACh was inferred to be about 30 pmho in frog muscle (55), rather smaller than the more indirect estimate (37). This value did not depend on temperature or membrane potential. On the other hand, the lifetime of the open state produced by ACh increased when temperature was lowered ($Q_{10} \approx 3$ at resting potential) or when the membrane potential was made more negative (55). The lifetime of the channel decreases by a factor about 1.4 for each 50 mV that the membrane is depolarized. At −70 mV and 22–25°C the channel lifetime for ACh was similar to, or slightly longer than, the 1 msec found by Katz & Miledi (37). The characteristics of functioning channels were unchanged by partial desensitization (55).

It is interesting to compare the ACh-operated ion channels with the voltage-operated sodium channels of muscle and axon membranes. The sodium channels in nerve (84, 107) and muscle (29) are much sparser than endplate channels, and much more voltage sensitive, the rate constants changing about 15-fold for a 50 mV depolarization. On the other hand, sodium channels resemble endplate channels in having similar conductance when open (29, 97, 107) and similar temperature sensitivity of both the rate constants ($Q_{10} = 2$–3) (55, 108, 109) and of the open channel conductance ($Q_{10} = 1$–1.3) (55, 109).

Affinity of Agonists

There are far fewer studies of the affinity and efficacy of agonists in skeletal muscle, especially twitch muscle, than there are in smooth muscle.

It is possible to measure affinities of partial agonists for ACh receptors by four different null methods, and on the whole these methods give similar results (see refs. 5, 111). Barlow et al (110, 111) made such measurements for a larger number of quaternary ammonium compounds on the frog *rectus abdominis* muscle. They estimated (110), for example, that the dissociation constant for the partial agonist methyltriethylammonium was 2.75 or 4.07 mM when measured respectively, by comparison, or by interaction with a full agonist. Likewise, Rang using the same

methods on the voltage-clamped frog endplate, found equilibrium constants of 42 or 56 μM respectively for decamethonium (3), and 48 and 58 μM respectively (5) for decamethylene-bis (ethyldimethylammonium) (EC10).

Another approach is to measure the reduction in the rate constant for BuTX binding produced by the ligand of interest. Colquhoun & Rang (38) used this method on denervated rat diaphragm homogenized to minimize distortion of the BuTX binding rate by diffusion. They found an apparent dissociation constant of 2.0 μM for decamethonium, about 20-fold lower than the value quoted above. This difference could be (*a*) a reflection of the fact that the null methods should measure K_T (5) whereas inhibition of BuTX binding rate would, in the simplest case (independent, model 1-type subunits; no preference of BuTX for R state), measure $K_{eff} \approx K_T\ LM/(LM + 1)$ which must be less than K_T for agonists; (*b*) an indication that agonists protect by producing desensitized receptors that may have a lower affinity for BuTX than normal receptors (22, 23, 26, 39, and above); or (*c*) a species difference. It is not possible to decide between these alternatives at the moment.

Within the classical framework, it is also possible to measure the affinity of full agonists by a null method, but there are no published studies of this method at the endplate, and the results of using this method are not simply interpretable using current models (5). Reduction of BuTX binding rates gives apparent equilibrium constants of 1.3 μM for succinylcholine, 4 μM for carbachol (38), and 0.9 μM for acetylcholine (D. Colquhoun and H. P. Rang, unpublished). These values may be too low if agonists protect by desensitization rather than by receptor occupancy.

Efficacy of Agonists

Assuming 3×10^7 channels/frog endplate with a conductance of 30 pmho (55), only about 4% of channels would need to be open to produce 90% of the maximum possible depolarization,[5] so any agonist that could open 4% or so of channels would look like a full agonist, i.e. there are spare receptors (51) for the depolarization response and *a fortiori* for the contractile response. However, the response of primary interest is channel opening, i.e. membrane conductance. If all channels were open this would be roughly 1 mmho, so a current of 60 μA would be needed to clamp the endplate at –70 mV. Unfortunately it is technically impossible to pass much more than 100 nA through a microelectrode for any length of time, so it is impossible to measure maximum conductance responses for most drugs, even for decamethonium which is undoubtedly a partial agonist, as judged by its interaction with more powerful agonists (3, 37, 52). The only published value is for the weak partial agonist EC10, which produces a maximum current of about 35 nA in the frog endplate clamped at –90 mV (112), i.e. a conductance increase of 0.4 μmho, suggesting that EC10 is capable of opening roughly only 0.04% of channels.

As a consequence, it is still not known whether ACh or any other drug is a full rather than partial agonist, i.e. it is not known whether it can, in sufficient concentra-

[5]Number open, $n = [f/(1-f)]/R\gamma$ where f = fraction of maximum depolarization, R = input resistance taken as 0.25 MΩ, γ = single channel conductance (103).

tion, open most of the channels. Katz & Miledi (56) estimate indirectly that the fraction of channels opened is roughly only 10% of the fraction of receptors occupied by ACh, i.e. that ACh is only a partial agonist for channel opening (efficacy rather low, M large enough to make LM greater than 1 in model 1).

The various homologous series studied by Barlow et al (110, 111) confirm, on the whole, Stephenson's prediction (51) that drugs with high efficacy have low affinity and vice versa. Insofar as affinity measurements yield affinities for the T (shut) conformation (5, 50), this is exactly what is predicted by two-state models outlined earlier.

ANTAGONISTS

Recent work has confirmed the classical view that drugs like (+)-tubocurarine (TC) work mainly by occupying the same binding site as the agonist and so excluding it. Results predicted by cooperative models based on this assumption are very similar to the classical predictions (5), which experimental results fit well (e.g. 1, 3, 5, 60). TC does not alter the ACh reversal potential, i.e. $\Delta g_{Na}/\Delta g_K$ (75). Electrical noise measurements have shown, as predicted, that the nature of the channels opened by ACh is not changed, apart from a reduction in the number opened, by TC (37, 101) or by BuTX (113).

The affinity of TC for receptors is similar in many species. Null (dose ratio) measurements give dissociation constants as follows: frog sartorius, 0.73 μM (60); frog *ext. dig. long.* IV, 0.43 μM (60); guinea pig diaphragm and lumbrical muscle, 0.11 μM (62, 114); frog *rectus abdominis,* 0.32 μM (60) and 0.69 μM (111); chick *biventer cervicis,* 0.38 μM (115). Using a non-null method described below, Kruckenberg & Bauer (116) found a value of 0.39 μM at the golden hamster endplate. Reduction of BuTX binding rate to denervated rat diaphragm gave values of 0.3 (38) or 0.4 μM (29). This, in contrast with decamethonium, is much the same as the value found using null methods. This is as predicted by two-state models because for antagonists, unlike agonists, K_{eff} should be much the same as K_T.

Equilibrium constants for a number of other blockers, in various muscles, have been reported (38, 60, 62, 110, 111, 114, 115).

Some antagonists, in chick muscle, become more effective when applied in the presence of an agonist; the *metaphilic* effect (115, 117). This probably happens because these antagonists have a greater affinity (cf BuTX) for desensitized than for normal receptors.

Strong evidence for some presynaptic depression of the quantal content of endplate potentials in rat diaphragm by TC has been found by Hubbard & Wilson (118) who discuss recent work on this problem.

MISCELLANEOUS DRUGS

The response of the endplate to agonists is affected by local and general anesthetics, alcohols, barbiturates, and atropine. Unlike TC and BuTX (above), these drugs do not seem to act simply by exclusion of the agonist from the receptor.

Atropine (119, 120) and barbiturates (121, 122) make the frog epp or epc much briefer than normal. Atropine has this effect mainly because, unlike TC, it causes the channels to stay open for a shorter time (123). In addition this briefer molecular effect of ACh may reduce its binding and hence facilitate its removal (56). Ether has a similar effect (123). The observation that atropine changes the ACh reversal potential (124) suggests that the Na-K selectivity of the channel may also be altered.

Halothane inhibits depolarization by ACh and CCh in frog (125) and guinea pig (62) muscle. It flattens as well as shifts the response-log dose curve, but does not change the affinity of TC or pancuronium for the receptors (62), which suggests that it may act in the membrane lipid phase. This is comparable with the lack of effect of local anesthetics, batrachotoxin, and veratrine on the binding of tetrodotoxin and saxitoxin to axonal sodium channels (84, 107).

Aliphatic alcohols, on the other hand, increase the size of mepp's (see 126 for refs.). This is due primarily to lengthening of the decay of mepc's, without changing the usual exponential nature of the decay or its temperature and potential dependence (126). Noise analysis showed that the prolonged decay resulted from an equivalent prolongation by alcohols of the time for which channels are opened by ACh (126). The results could be accounted for by partition of the alcohols into the lipid membrane phase, changing its dielectric constant and hence changing the reaction rate of the dipoles involved in gating.

Local anesthetics, and higher concentrations of long chain alcohols (126) which have a similar membrane-buffer partition coefficient, have a more complex effect. The initial decay of the mepc or epc is faster than normal but is followed by a prolonged slow phase. The action of local anesthetics has been ascribed to separate effects on Na^+ and K^+ conductances (87–90) or to combination of the anesthetic with activated receptor, modifying its properties (94, 95) in a voltage-dependent manner (127). On balance, the evidence does not support the existence of separate Na^+ and K^+ channels (94–96, 126, 127), and the actions might result (*a*) from a change in the fluidity or dielectric constant of the lipid membrane without a direct action on the receptor (126) or (*b*) from insertion of the local anesthetic (94, 95, cf 128) or barbiturate (122) into the previously opened channel.

THE ACTION OF ENDOGENOUS TRANSMITTER

Normal Neuromuscular Transmission

Quanta of ACh contain roughly 12,000–50,000 ACh molecules (see ref. 118). Their spontaneous release causes a peak conductance increase of about 60–70 nmho in frog and rat (86, 104–106). This ACh opens 1000–2000 channels (e.g. 60 nmho/30 pmho per channel) for a millisecond or so. If two thirds of the ACh molecules are bound (56, and below), only about 10% of occupied receptors can correspond to open channels.

The rising phase of the mepc is little effected by temperature ($Q_{10} \approx 1.2$), membrane potential, or neostigmine (96). It is not certain what controls this rate; it may be diffusion across the cleft (96), though simple diffusion appears to be too fast to

do so (129). The rising phase of the epc is slower and is probably limited by the rate of transmitter release (130–133).

On the other hand, the falling phase of both mepc and epc is strictly exponential after the peak, and the time constant is sensitive to both temperature ($Q_{10} \approx 3$) and membrane potential (74, 91, 96, 106, 134). Anderson & Stevens (55) show that this time constant is the same, at any given potential, as the mean lifetime of individual channels, and it does not vary with the size of the epc (134). This is strong evidence for the view that the decay phase of epc and mepc represents the rate of closing of ion channels, i.e. the rate of the R $\longrightarrow$ T conformation change, rather than the time course of ACh concentration in the synaptic cleft. The dipole moment that the gating molecule would need to have to account for its voltage sensitivity is in the range commonly found in proteins (54).

Alternatively, the decay phase of the epc and mepc could be governed by a voltage-sensitive rate of dissociation of ACh (92, 93). It may be noted that according to certain models this explanation could be, but is not necessarily, almost the same as that above. If hyperpolarization, because of the dipole moment of the gating molecules, favors the open state, and ACh has a high affinity for the open conformation then hyperpolarization *might* delay dissociation of ACh.

It is likely that the ACh concentration in the synaptic cleft falls more rapidly than the epc (131, 133). Magleby & Stevens (54) present a detailed model in which the cleft ACh concentration and the occupancy of channels in the closed conformation reaches a peak in 200 μsec and falls with a time constant of only 200 μsec, so it has fallen to a low level by the peak of the epc.

The actual ACh concentration is not known, but a rough upper limit has been estimated (33). The calculations are, of course, crude because of uncertainties in the numbers and in the hypotheses outlined above and because of the likelihood that transmitter is not uniformly distributed throughout the cleft (refs. 56, 129, 135). The estimated cleft volume is 450 μm^3 in rat diaphragm (33), and taking the ACh release to be 3–6 $\times$ 10^6 molecules (136, 137), a mean concentration of 10–20 μM would be achieved if there were no loss by diffusion, hydrolysis, or uptake. If one assumes 4 $\times$ 10^7 receptors/endplate, and an effective equilibrium constant of about 1 μM then binding to the receptors alone could reduce the free concentration to approximately 0.1 μM, 99% of released ACh being bound (see below, however). The upper limit of the mean fraction of receptors occupied is thus about 10%. This may be compared with the number of open channels at the peak of the epp, which should be about 4 $\times$ 10^5, roughly 1% of all channels, i.e. at least 10% of the occupied channels. This value assumes a quantal content of a round 200 (118), each quantum opening about 2000 channels, causing a conductance increment of approximately 10 μmho, which is similar to the value in the frog (71, 72). It is possible, but not certain (56), that there is local receptor saturation during an mepp.

Diffusion of the Synaptic Cleft

Free diffusion should clear the synaptic cleft of ACh with a half-time of about 2 msec (129). With hydrolysis, the actual rate of clearance is probably faster (see above). But after anticholinesterase treatment, clearance of ACh may be slower than this,

probably because, as Katz & Miledi (56) point out, the binding of ACh to receptors will slow down its diffusion. TC and BuTX reduce the duration and size of the epp and mepp (particularly in the presence of anticholinesterases) (56), despite the fact that they do not affect channel lifetime (37, 101, 113). This shortening can be explained by the more rapid diffusion of ACh after some of its binding sites have been eliminated by the blocker (56, cf 138). If diffusion is assumed to be slowed by a factor of $1/(1-p)$ where p is the fraction of released ACh molecules that are bound (not the occupancy), the shortening of the mepp by TC implies that normally about two thirds of the quantal packet of ACh is rapidly bound to receptors, slowing its diffusion threefold (56).

The slowing factor $1/(1-p)$ is equal to $(M/KV) + 1$ (assuming low concentration and hyperbolic binding), where M equals number of receptors, with equilibrium constant K, contained in volume V. The factor for slowing of diffusion by binding in a single compartment is approximately M/KV (107, 139, 140). Taking $M = 3 \times 10^7$ sites, $K = 1\ \mu M$ and $V = 450\ \mu m^3$, this factor is about 150. It predicts a greater slowing of diffusion than is inferred by Katz and Miledi. However, the localized action of quanta probably means that ACh molecules never "see" many of the receptors, especially those released near the edge of the cleft, so the value of M used may be effectively too large. Also the value of K may be too small.

In any case it is clear that this delayed diffusion greatly complicates the interpretation of rate measurements, and the values of the rate constants for drug association and dissociation are not known for any drugs (see e.g. 7, 141), though there is some evidence that they may have an influence on the observed rate of action of a few drugs, e.g. decamethonium (53). The situation is further complicated by the fact that when access to receptors is limited by diffusion plus binding, the kinetics of onset and offset may closely mimic drug-receptor interaction kinetics, even to the extent that the ratio of two quite wrong rate constants may give the correct equilibrium constant (107, 140).

Antagonist Action

A substantial fraction of receptors (e.g. 0.76 in cat) must be blocked before contraction fails in any fibers at all (31, 114, 142). It appears that drugs like TC do not have time to equilibrate with released ACh, which acts very briefly, for either or both of the following reasons: (*a*) there is not time for TC to dissociate from receptors during the brief ACh action; (*b*) at moderate concentrations, a large fraction of the TC in the cleft is bound to receptors (M/KV is large) so a *small* reduction in the amount bound, produced by competing ACh, will produce a *large*, though transient, relative increase in the free TC concentration in the small volume of the cleft. Thus, the occupancy would still tend to be buffered at a constant value during a *brief* exposure to ACh, even if the TC could dissociate very rapidly (7, 138, 142). These effects make TC look rather like an irreversible antagonist when tested by means of a brief agonist application, e.g. endogenous ACh release. This assumption fits experimental results for moderate TC concentrations at the hamster endplate and leads to an equilibrium constant of 0.39 μM (116), as found by other methods.

The classical treatment of drug-receptor interaction rates predicts that when the responses to a briefly applied partial agonist are inhibited by a slowly dissociating antagonist, the subsequent addition of another antagonist may actually cause an *increase* in the response, despite the fact that either antagonist alone depresses it (143, 144). This can happen when the first antagonist dissociates slowly enough to appear irreversible during the brief agonist application (see above), but the second antagonist, which partly replaces the first one on the receptors, dissociates sufficiently rapidly that it can at least partially equilibrate with the agonist during even a brief application. This effect has been observed in smooth muscle (143, 144) and may be the basis of the recovery of TC-inhibited neuromuscular transmission produced by hexamethonium, which is itself a blocker (145). It is interesting that the same effect is predicted by a model that assumes that the drug-receptor equilibration is instantaneous (cf 138, 141). For an antagonist with high affinity (large M/KV), the ratio of bound to free drug in the synaptic cleft will be high, so the occupancy by this drug will be buffered and it will appear irreversible to a briefly applied agonist. But an antagonist with lower affinity (lower M/KV) will have a lower bound to free ratio and will equilibrate more rapidly with a briefly applied agonist, because displacement of the same *absolute* amount of antagonist from the receptors will cause a smaller relative increase in the concentration of free antagonist in the cleft.

Anticholinesterase Drugs

It is well known that the epp and epc are prolonged by anticholinesterase drugs. The prolongation is not great, however, being about two- or threefold in frog muscle (54, 71, 74, 92, 146) and about sixfold in rat muscle (147). Neostigmine does not alter the lifetime of individual channels, despite producing a four- to sixfold prolongation of the mepc (56). This suggests that in the presence of neostigmine, the decline of the mepc is no longer a reflection of the channel lifetime but depends on the diffusion (prolonged by binding) of ACh out of the cleft.

In experiments that appear to contradict this interpretation, it has been found that neostigmine changes neither the exponential shape nor the voltage sensitivity of the epc decay rate (134) and appears to prolong the inferred ACh concentration time course only slightly (54). This result would certainly not be expected if the rate normally was controlled by channel relaxation but by the time course of ACh concentration in the presence of neostigmine. Some additional direct effect of neostigmine on the conductance mechanism has been postulated (54, 134).

This apparent contradiction has not yet been resolved, though it may be noted that if ACh binding were voltage sensitive this should make the ACh diffusion rate voltage sensitive also.

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