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Topical Review

Acetylcholine Receptor Kinetics

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Introduction

The postsynaptic membrane of vertebrate endplates and fish electroplaques contains very high concentrations of a protein complex whose function is the transduction of a specific chemical signal into a permeability response. The physiological chemical signal is the presence of acetylcholine (ACh) at the outer surface of the membrane. ACh binding leads to opening of rather short lived channels permeable to all monovalent and divalent cations below about 8 Å in diameter (Dwyer, Adams & Hille, 1980). The details of this transduction process have been intensively studied in recent years, though we are still far from mechanistic understanding. Such understanding should come through reconstitution experiments, in which some success has recently been reported (Epstein & Racker, 1978; Changeux et al., 1979; Wu & Raftery, 1979). Since successful reconstitution depends on systematic restoration of all major functional properties of the receptor-channel complex, characterization of the properties of the native membranes using electrophysiological or biochemical techniques is clearly imperative. The major properties available for study at the present time are the kinetics of certain transitions of the receptor channel complex. Additionally, knowledge of the kinetics of the transitions may itself be mechanistically revealing.

Three major classes of transition are dealt with in this review: channel opening; channel blocking; desensitization. Channel opening refers to the conversion of resting unliganded receptor-channel complexes in a zero conductance state to active agonist-liganded complexes in an open state of approximately 25 pS conductance. Channel blocking refers to transitions to nonconducting states induced by binding of certain ligands to sites believed to be within the channel lumen itself. Desensitization refers to ligand-induced transitions from resting, open or blocked states to a refractory state which recovers only slowly to the resting state on removal of inducing ligands. The receptor complex bears at least three classes of binding sites. The agonist trigger sites are one such set. Binding of suitable agonists to these sites allows or induces rapid (millisecond) transitions from resting to active states, and slow (second) transitions to desensitized states. Another class of sites binds "channel blockers", mainly local anesthetic-type drugs, but also including some agonists. Binding to this type of site occurs upon active or open complexes, and shuts the channel. However, it may also occur upon resting or desensitized states. Finally, there may be a third class of sites to which rather nonspecific molecules bind and promote desensitization. It will be apparent that this system is very complex. This complexity is compounded by the possibility that there may be more than one site of any one type per complex, and by the overlapping specificity of various ligands for the different types of site. In the absence of definitive structural correlations for the sites or transitions, the main tool available at present for unravelling this complexity is kinetic analysis, which depends upon the binding reactions and the state transitions having distinct and characteristic rates, so that the various processes can be unfolded in time.

Although structural correlations are as yet scant, it may help the reader to end this introduction with a brief sketch of present structural knowledge of the receptor-channel complex. Most of this information comes from *Torpedo* membranes, though it appears that the muscle receptor shares many analogies (Lindstrom, Walter & Einarson, 1979). The protein complex can be directly visualized in rapid freeze fracture or fracture-etch material as particles at a density of about $10^4\,\mu m^{-2}$. These particles transverse the membrane and project out some 50 Å into the synaptic cleft. The particles are very roughly cylindrical with a

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diameter of 8 nm, though freeze fracture may overestimate the true particle diameter. The external surface of the particle shows a prominent hole. In quick frozen material the particles tend to form linear arrays. Some authors distinguish topographical detail within the particle in etched or negatively stained material. Similar conclusions are reached by conventional EM techniques, neutron scattering and X-ray diffraction (Rosenbluth, 1975; Allen, Baerwald & Potter, 1977; Ross et al., 1977; Cartaud et al., 1978; Karlin et al., 1978; Heuser & Salpeter, 1979; Klymkowski & Stroud, 1979; Wise, Karlin & Schoenborn, 1979). Purified membranes containing high concentrations of these particles, and with very high specific activity for α-neurotoxin binding (~ 5 nM mg^{-1}) contain only five major polypeptides of approximate apparent molecular weights 40(α), 43, 50(β), 60(γ) and 65(δ) K. The 43-K polypeptide can be removed without loss of function (Neubig et al., 1979). The remaining four chains are still present after detergent solubilization and further purification and are probably components of the ACh receptor-channel complex, properly speaking. These very pure preparations (specific activity ~ 10 nM α toxin per mg protein) sediment or migrate as one band after reduction of S-S bonds. The apparent molecular weight of this pure complex is $\sim 250{,}000$ K. Without reduction these complexes form dimers by disulfide bridges between the δ subunits (Chang & Bock, 1977; Hamilton, McLaughlin & Karlin, 1978). Possibly the dimers form artificially during receptor purification, though Chang and Bock (1977) find that the receptor is obtained mostly as dimer if disulfide interchange is minimized. *Electrophorus* receptor does not form covalent dimers. However, noncovalent association in the membrane is possible. It is not known whether dimerization is necessary for function, nor whether the particles seen in the electron microscope represent monomers or dimers, though the probable mass of the particle is quite close to 250,000 daltons. Each dimer appears to carry four alpha toxin binding sites and one or two local anesthetic sites (Krodel, Beckman & Cohen, 1979; Elliot & Raftery, 1979). It is not known with certainty upon which chain these compounds bind. However, at least some of the α toxin sites appear to be on the α chain, as judged by cross-linking experiments (Witzemann & Raftery, 1978; Nathanson & Hall, 1979; Hamilton, McLaughlin & Karlin, 1978), and by inhibition of binding of certain quaternary ammonium compounds to -SH groups on the alpha chain revealed by receptor reduction in intact cells or membrane fragments. Since one of these compounds, bromoacetylcholine, can act as an irreversible agonist (Silman & Karlin, 1969), the alpha chain is likely to bear at least one of the agonist binding "trigger" site.

The question of the stoichiometry of ACh and toxin binding has long been controversial. Perhaps the most careful recent measurements (Neubig & Cohen, 1979) suggest that there are two ACh binding sites corresponding to the two toxin binding sites per monomer. Though only one of these sites can be affinity labeled after disulfide reduction, both sites appear capable of triggering permeability and desensitization response (Delegeane & McNamee, 1980).

Channel Opening

Steady-State Measurements

Application of a suitable agonist to cholinergic membranes, either in intact cells or as vesicles, leads to channel opening, increased cation permeability, and ion flux. If the agonist treatment is prolonged, the permeability response spontaneously wanes. This fade is called desensitization and is discussed below. It can obscure the relationship of the flux amplitude to agonist concentration, since the response may be measured only after some desensitization has occurred. In the case of vesicle preparations, another problem is the nonlinear relation between the measured amount of label retained or released and the number of open channels (e.g., Moore, Hartig & Raftery, 1979). For the moment, flux measurements in vesicles only provide a qualitative measure of channel opening and the effects of blockers and desensitization (but *see* Miller et al., 1978; Neubig & Cohen, 1980; Cash & Hess, 1980). In whole cells the voltage clamp technique does provide reliable measures of numbers of open channels, often with excellent temporal resolution. In this technique the net ion flux across the membrane is automatically compensated by an injected electrical current. Unfortunately, this technique has only been applied to *Electrophorus* electroplaque and muscle endplates. At low agonist doses, for which the distorting effects of desensitization are negligible, the number of open channels varies roughly as the square of the agonist concentration (Adams, 1975a; Lester, Changeux & Sheridan, 1975; Dionne, Steinbach & Stevens, 1978; Adams & Sakmann[1]; but *cf.* Dreyer, Peper & Sterz, 1978). This observation has been widely taken to show that channels usually only open when the receptor is doubly liganded. For higher agonist doses, some indication of saturation of the response in usually seen. For certain agonists, called partial agonists, this saturation occurs when only a few of the channels appear to be open. One interpretation of this observation is that even when doubly liganded by such agonists, the

[1] Adams, P.R., Sakmann, B. Rates of channel opening and closing at frog end-plate membrane. (*in preparation*)

receptor is unlikely to reach the active state. A simple kinetic scheme which has been proposed on the basis of these results (Adams, 1975*a*, 1977*a*) is the following:

$$\underset{\text{(closed)}}{A+R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(closed)}}{AR} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{(closed)}}{A_2R} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \underset{\text{(open)}}{A_2R^*} \qquad (1)$$

α/β would be large for partial agonists, and from the shape of the dose response curve 4 $k_{-1}k_2 \sim k_1k_{-2}$ could be deduced. However, the premature saturation seen with partial agonists could reflect instead channel blocking by the agonist itself (Adams & Sakmann, 1978*a*), or occult desensitization.

Acetylcholine or carbachol, on the other hand, produce very large currents at high concentrations, and can probably open a significant number of channels. Reliable direct measurement of the maximum currents has never been achieved. Nor is it possible to determine very accurately from measurements of receptor density and single channel current what maximum current *would* be observed were all the channels open. Despite these difficulties, several authors have attempted to use data above the low concentration power law range to fit reaction schemes and to estimate binding and isomerization constants for full agonists (Adams, 1975*a*; Dionne et al., 1978; Sheridan & Lester, 1977; Dreyer et al., 1978). For carbachol the concentration estimated to elicit the half-maximum response is in the range 100–400 μM, and the percentage of channels open at saturation around 20 to 60%. Accurate estimation of two or especially more parameters from this type of data is probably unwarranted at this time.

Fortunately, there exist two alternative approaches to the problem of measuring fractions of channels opened by various agonist concentrations. The first method depends on the relationship between the rate of block (k) by a slowly-binding channel blocker such as quinacrine and the fraction of open channels y (Adams & Feltz, 1977, 1980*b*; Colquhoun, Dreyer & Sheridan, 1979):

$$k = yf^*c + b^* \qquad (2)$$

where f^* and b^* are the rate constants for channel block (*see* below), and c is the blocker concentration. Estimates of y obtained in this way are shown in Fig. 1.

The other method depends on the thermal fluctuations ("noise") in the number of open channels during a steady-state response. The relationship between the mean fraction of channels open, y, and the statistical variance of this mean, *var*, will be given by the binomial distribution, provided the channels have only two states, open and closed, and operate independently.

$$var = y(1-y) \qquad (3)$$

From measurements of the noise variance/mean current ratio, y can be determined (Fig. 2).

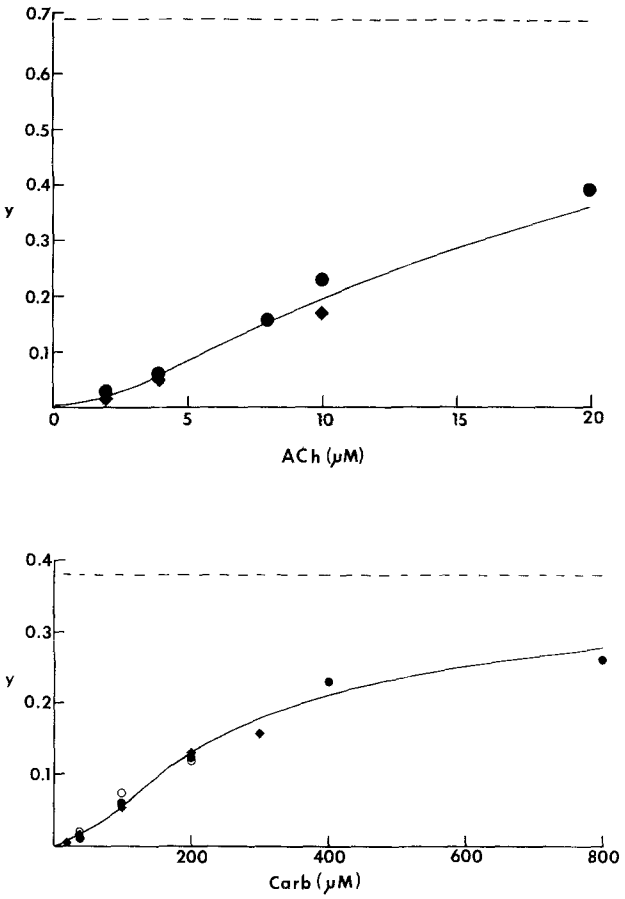

Fig. 1. Relation between fractions of available channels opened (y) and agonist concentration. y was calculated using text Eq. (2), where k is the rate constant for the slow quinacrine-induced voltage jump relaxation at −140 mV membrane potential (*see* Adams & Feltz, 1977, 1980*b*, for further details). *Rana esculenta* at 22 °C, with inactivation of esterase using an irreversible inhibitor for experiments with ACh. For both graphs $f^* = 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $b^* = 1 \text{ s}^{-1}$ were used. These values were independently determined (Adams & Feltz, 1980*a*). For the upper graph circles show mean data from 5 experiments using 0.5 μM quinacrine, and diamonds show mean data from 8 experiments using 1 μM quinacrine. The solid curve was calculated assuming $\gamma = \beta'/(\beta' + \alpha')$, where β', α' are given by text Eq. (5), and $k_{-1}/k_2 = 20$ μM and $\alpha/\beta = 0.44$. The dashed line shows the fraction of channels openable by saturating ACh concentrations. For the lower graph open circles show mean data from 5 experiments with 2 μM quinacrine, diamonds show data from 11 experiments with 1 μM quinacrine, and filled circles show data from 8 experiments with 0.5 μM quinacrine. The solid curve was calculated from text Eq. (6) with $k_{-1}/k_2 = 200$ μM and $\alpha/\beta = 1.64$. The dotted line shows the fraction of available channels openable by saturating carbachol concentrations. (Data of A. Feltz and P.R. Adams)

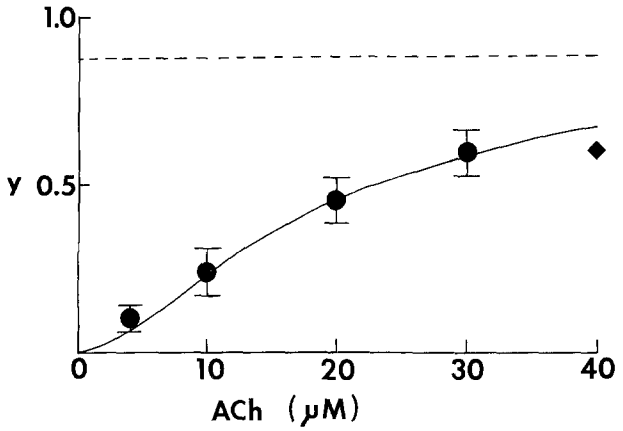

Fig. 2. Relationship between fraction of available channels opened (y) and ACh concentration. y was calculated from the ratio of the extracellular noise variance to the DC shift at a given ACh concentration, divided by the same ratio obtained using 1 μM ACh, without moving the focal recording pipette. This procedure assumes that a negligible fraction of the channels are opened by 1 μM ACh. Six experiments are shown for *R. pipiens* sartorius muscles at 10 °C and at −80 mV, after inactivation of esterases by MSF treatment. The triangle refers to a single observation. Error bars represent ± SEM. The solid curve was calculated assuming Scheme (1) in the text, with $\beta = 2.3\ ms^{-1}$, $\alpha = 0.3\ ms^{-1}$, $k_1 = 2k_2$, $k_{-2} = 2k_{-1}$ and $k_{-1}/k_2 = 40\ \mu M$. The dashed line shows the limiting fraction of channels that would be opened by very high ACh concentrations. (Data of B. Sakmann and P.R. Adams)

Both sets of data can in fact be fitted reasonably well by scheme (1) above, with values for the constants that are quite similar to those determined by dose-response curve analysis (*see* legends to Figs. 1 and 2). But again the data is not very accurate and could probably be fitted by other related kinetic schemes, especially ones with several constants.

Transient Measurements

In principle, a more direct way of determining a valid kinetic scheme for channel opening would be to perform kinetic measurements. Rapid mixing and temperature-jump measurements on functional membrane fragments have not yet revealed processes that can be unambiguously interpreted as channel opening, though much progress is being made (Bonner, Barrantes & Jovin, 1976; Grünhagen, Iwatsubo & Changeux, 1977; Cohen & Boyd, 1979; Heidmann & Changeux, 1979*a*, *b*; Quast, Schimerlik & Raftery, 1979; *see* discussion below). Therefore, at present, one has again to resort to electrophysiological techniques. Three such techniques are currently in use:

a) Agonist concentration jumps. This method is restricted to the photoisomerizable agonist Bis Q, which can be converted from the inactive *cis* isomer, and *vice-versa*, to the active *trans* isomer by a flash of light. (Lester & Chang, 1977; Nass, Lester & Krause, 1978). In a variant of this procedure a photoisomerizable agonist QBr is "tethered" to the receptor via a previously reduced disulfide bond. After washing, the free ligand concentration is zero, but the fraction of the tethered molecules in the active conformation can be modulated by light flashes (Lester et al., 1980).

b) Voltage jump relaxations. This technique exploits the fact that the fraction of channels opened by an agonist is dependent upon the transmembrane voltage (Dionne & Stevens, 1975; Adams, 1976*a*). If the membrane potential is stepped to a hyperpolarized level, an exponential increase in the voltage clamp current is recorded. (Adams, 1975*b*; Neher & Sakmann, 1975; Sheridan & Lester, 1975). The relative amplitude of this increase is itself concentration dependent, in a way that parallels the concentration dependence of the noise variance (Sakmann & Adams, 1976; Sheridan & Lester, 1977). Indeed, it may be shown that the relative amplitude is maximal for $\sqrt{y_\infty y_o} = 0.5$, whereas the variance is maximal for $y = 0.5$. This corresponds to the fact that a chemical equilibrium is most easily displaced (by external or internal forces) when the reactants are present in equal concentrations.

c) Noise analysis. This technique has already been mentioned. The kinetics of the spontaneously occurring fluctuations can be extracted by *autocorrelating* the noisy record. The resulting autocorrelation function is essentially equivalent to the relaxation that would be observed were *all* the channels suddenly opened and allowed to relax back to the equilibrium state. It is *not* equivalent to the relaxation observed in the limit of small perturbations. The amplitude of the autocorrelation function is simply the variance of the noisy current record.

Fortunately, all three of these techniques give essentially identical results. They all show that throughout the accessible concentration range only one major kinetic process contributes to channel opening. Thus, channel opening can be represented empirically as follows

$$\text{closed} \underset{\alpha'}{\overset{\beta'}{\rightleftharpoons}} \text{open}. \qquad (4)$$

The value of α' at any given temperature and membrane potential is characteristic of the agonist being used. For ACh, α' is about $1\ ms^{-1}$ at room temperature and the normal resting membrane potential. For the widely used ACh analog carbamylcholine (CCh), α' is about three times as large, whereas for the bisquaternary ester suberyldicholine (Sub) α' is 2 to 3 times smaller. α' is increased by depolarizing the fiber, by an e-fold amount for 80 mV potential change (Magleby & Stevens, 1972; Adams, 1975*b*). This voltage sensitivity, though much less than that of the voltage-controlled channels underlying the nerve impulse (Hodgkin & Huxley, 1952), indicates that during the molecular changes in the receptor to which α' applies, the equivalent of about 1/3 of an electronic charge moves across the membrane. This charge movement could be due to movement of the agonist to its binding site, to a change in the net dipole moment of the entire receptor protein, to a redistribution of ions between the channel and the external solutions (Marchais & Marty, 1979), or to an effect

on the lipid bilayer in which the receptor is embedded. α' is also increased by warming the muscle. Its apparent activation energy is around 18 kcal/mole (Gage & McBurney, 1975), though this may be dependent upon the agonist used (Nelson & Sachs, 1979; Lester et al., 1980). Finally, α' does not depend on the concentration of agonist used, at least for low concentrations (Adams, 1977*a*; Sakmann, Patlak & Neher, 1980; Adams & Sakmann, 1980[2]).

The properties of β' often contrast with those of α'. Most strikingly, β' does not appear to be detectably voltage sensitive (Neher & Sakmann, 1975; Adams, 1977*a*; Sheridan & Lester, 1977; Dionne & Stevens, 1975). It increases dramatically as the agonist concentration is raised (Sheridan & Lester, 1975, 1977; Sakmann & Adams, 1976, 1978*a, b*; Adams & Sakmann, 1978*b*[3]). The original work from Lester's laboratory suggested that the relation between β' and agonist concentration a was linear. However, neither very low nor very high agonist concentrations were tested. In the endplate work, a clear initial upward curvature in the β' *vs.* a relation could be demonstrated (Sakmann & Adams, 1978; *see* Fig. 3). This curvature corresponds to the requirement for two agonist molecules to open the channel previously mentioned. In the middle range of agonist concentrations, the endplate work confirmed the linear relation seen in electroplaques. In both cases the pseudofirst-order rate constants obtained from this slope were around $10^7\,M^{-1}s^{-1}$ for ACh and $10^6\,M^{-1}s^{-1}$ for CCh. Kinetic data for high ACh concentrations (>40 μM) could not be obtained, because (a) the desensitization produced by these concentrations is very extensive, and (b) because when most of the channels are open the noise variance becomes very small (*see* Eq. (3) above). Neither of these problems were as acute for CCh, and in the concentration range 400 μM–2 mM some indications of saturation of the rate constant were seen (Fig. 3). Such saturation is also suggested by the observation that covalently bound agonists elicit channel opening rates that appear to be limited by some intramolecular step (Cox et al., 1980; Lester et al., 1980).

These kinetic data have a double significance. Empirically, they quantitatively characterize the agonist dependence of channel opening, albeit under a rather restricted set of conditions. For example, the ACh concentrations for which the channel opening rate equals the channel closing rate is about 20 μM; this is the concentration which indeed opens half the available channels, judging from the variance data (Fig. 2; *see also* Sakmann et al., 1980). Because of the voltage dependence of α', the dose-response curve shifts with voltage (Lester, Koblin & Sheridan, 1978; Adams & Sakmann, 1978*c*). Theoretically, the data can be used to construct plausible – though not unique – reaction schemes, which might give physical significance to β' and α'. So far, the most successful of these schemes is scheme (1) above. It is assumed that the binding steps proceed independently ($k_1 = 2k_2$; $k_{-2} = 2k_{-1}$), and that the isomerization step is rate limiting ($\alpha + \beta) \ll (k_2 + k_{-1}$). The expressions for β' and α' which result:

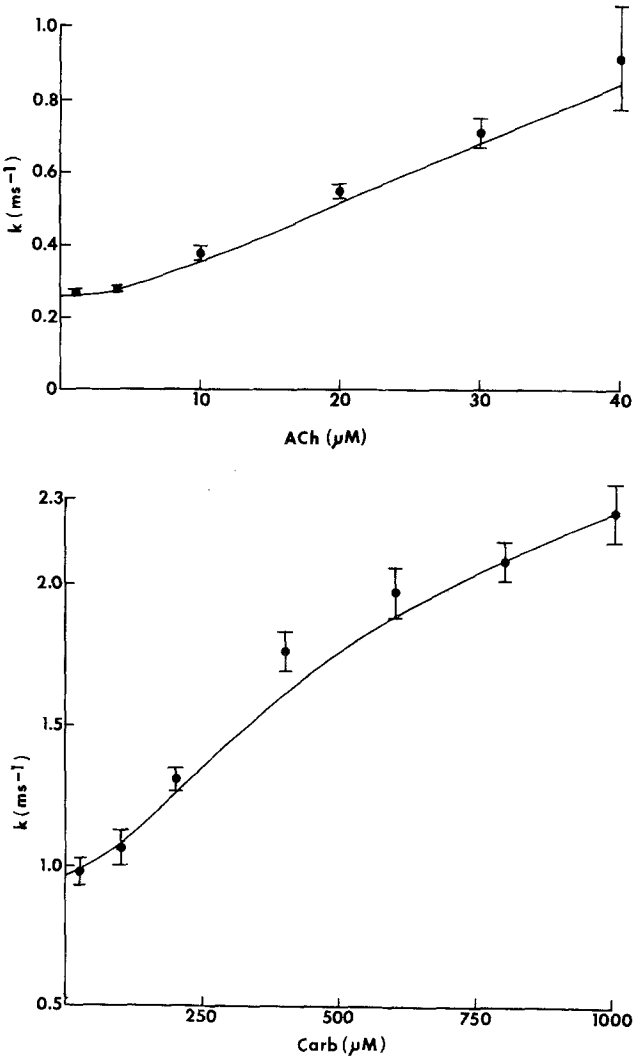

Fig. 3. Relation between reciprocal time constant of decay (k) of autocorrelation function of agonist-induced focally recorded noise, and agonist concentration. *R. pipiens* sartorius muscles at 12 °C and −80 mV holding potential. Upper graph shows mean (±SE) data from 6 experiments with ACh as agonist, in fibers pretreated with MSF. The solid curve was calculated assuming text Eq. (5) ($k = \beta' + \alpha'$) with $\alpha = 0.26\ ms^{-1}$, $\beta = 2.3\ ms^{-1}$ and $k_{-2}/k_1 = 40$ μM. Lower graph shows data from 8 experiments with carbachol as agonist. The solid curve was calculated assuming $\alpha = 0.97\ ms^{-1}$, $\beta = 2.5\ ms^{-1}$ and $k_{-2}/k_1 = 400$ μM. (Data of B. Sakmann and P.R. Adams)

[2] *See* footnote 1, p. 162.
[3] *See also* footnote 1, p. 162.

$$\beta' = \left(\frac{a k_2}{a k_2 + k_{-1}}\right)^2 \beta, \alpha' = \alpha \quad (5)$$

fit the kinetic data rather well (Fig. 3). The kinetic data for ACh also explain the size and shape of the postsynaptic response produced by natural quantal packets of ACh (Adams & Sakmann, 1980[4]; Adams, 1980).

A final advantage of this type of scheme should be mentioned. This is that voltage jumps which produce appreciable shifts in the fraction of biliganded channels that are open will only produce small shifts in the fraction of monoliganded receptors, since at least for low agonist concentration there are far more monoliganded receptors than biliganded ones. If this were not so, the amplitude and time course of relaxations produced by voltage jumps would be greatly distorted by depletion of agonist from the restricted volume of the synaptic cleft.

Single Channel Measurements

The above data were obtained using *macroscopic* kinetic techniques, in the sense that the behavior of populations of channels were studied. However, recently electrophysiological resolution has improved to the point that the transitions of single channels can be directly observed (Neher & Sakmann, 1976*a*; Neher, Sakmann & Steinbach, 1978; Sakmann & Heesemann, 1979).

Since individual molecular transitions are random, analysis of single channel data entails the painstaking evaluation of the statistical distributions of the measureable quantities. The outcome of such an analysis is a histogram. In the early experiments the histogram of the distribution of open channel times could be fitted quite well to a single exponential whose "time constant" equalled the expected value of $1/\alpha'$. This result strongly reinforced the macroscopic analysis. The histogram of the channel current amplitudes fitted a Poisson distribution, after allowance for instrumental noise and edge effects. This reflected the use of low agonist concentrations ($\beta' \ll \alpha$), and gave a single channel current that agreed beautifully with that obtained by noise analysis. Recently, this simple picture has been slightly complicated, by the appearance of extraneous lumps in the histograms, even at low agonist concentrations. These lumps seem to be due to minor heterogeneity in the populations of (extrajunctional) channels used to perform these studies. The additional channels in fact have all the hallmarks – slightly larger currents and considerably shorter lifetimes – of junctional channels (*see* Dreyer, Walther & Peper, 1976; Neher & Sakmann, 1976*b*; Sakmann & Neher, 1980).

Another type of deviation from the simplest possible picture has very recently been observed, when the distribution of waiting times between channel openings was examined. In some recordings an unusually short interval between successive opening events was observed more frequently than expected from the average opening frequency ("Nachschlag"; *see* Nelson & Sachs, 1979). This appears to be due to an open channel passing into a nonconducting state different from the normal closed state, from which it has a finite possibility of returning to the open state before finally closing. This temporarily nonconducting state might be a "blocked" state analogous to that induced by local anesthetics (*see* below). But, and more interestingly, it might correspond to a liganded closed state of the receptor, such as A_2R in Scheme (1) above (Colquhoun & Hawkes, 1977). For a simple two-step model:

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR^* \quad (6)$$

the probability that a Nachschlag would be observed would then be given by $\beta/(\beta + k_{-1})$, and the average duration of the silent interval approximately $1/\beta$. Macroscopic noise analysis suggested that the dissociation constant for binding to resting receptors (k_{-1}/k_2 in Scheme (5) above) is around 30 μM for ACh. If k_2 is assumed to be no greater than some reasonable upper limit for a diffusion-controlled reaction, say $10^9\ \mathrm{M^{-1}s^{-1}}$, an upper limit of $60\ \mathrm{ms^{-1}}$ for k_{-2} would result. This in turn predicts that more than 2% of opening will be followed by a Nachschlag. Nachschlag frequency will be much less for CCh, but uninfluenced by membrane potential or agonist concentration. For three-step models such as Scheme (1) above, channels can return to the conducting (A_2R^*) state via A_2R *and* AR so the Nachschlag frequency will be concentration dependent. It will be interesting to see whether these predictions will be borne out, and whether the effect can be seen in noise spectra.

Very recently, Sakmann, Patlak, and Neher (1980) have succeeded in measuring the concentration dependence of single channel opening. This was possible by using high agonist concentrations and allowing desensitization to proceed sufficiently that only one active channel was left in the patch being recorded from. Under these circumstances the distribution of *closed* times should be exponential with time constant equal to $1/\beta'$. β' determined in this way shows an increase with agonist concentration comparable to that discussed above. It will be important

[4] *See* footnote 1, p. 162.

to see if the suspected saturation behavior of β' can be confirmed using this powerful direct technique.

Channel Blocking Kinetics

The membrane response to a single quantal packet of ACh, the miniature endplate current (mepc), consists of a very brief (300 μsec) phase of channel opening followed by an exponential tail of channel closing. Since the time constant of the mepc decay is close to the average lifetime of single ACh-opened channels, it appears that the mepc decay reflects random channel closing uncontaminated by channel opening (Anderson & Stevens, 1973). Observation of mepc decays thus affords an accurate measurement of the closure of a small set of open channels according to the scheme

$$0 \xrightarrow{\alpha'} C.$$

It has been known since 1966 that in the presence of local anesthetics the mepc decay becomes complex. Voltage clamp studies (Steinbach, 1968; Kordaš, 1970; Deguchi & Narahashi, 1971; Maeno, Edwards & Hashimura, 1971) revealed that the drug-modified mepc decay was composed of two components, decaying according to the equation

$$I_{\text{tail}} = I_{\text{peak}}(A_f \exp -\lambda_f t + A_s \exp -\lambda_s t) \tag{7}$$

with $A_f + A_s = 1$. The values of λ_f, λ_s and A_f/A_s showed a complicated dependence upon local anesthetic concentration and membrane potential. In 1975 (Adams, 1975*c*) it was pointed out that these effects could be explained simply by assuming that the cationic local anesthetics could transiently enter, plug, and prevent the closure of the open endplate channels, rather along the lines suggested by C.M. Armstrong for alkyl TEA block of squid potassium channels (Armstrong, 1971). The kinetic scheme describing mepc decay would then become

$$\begin{array}{ccc} 0 & \xrightarrow{\alpha'} & C \\ {\scriptstyle f^*c}\downarrow\uparrow{\scriptstyle b^*} & & \\ B & & \end{array} \tag{8}$$

where f^* and b^* are the blocking and unblocking rate constants, and c is the anesthetic concentration. This scheme successfully predicts the reported relations between c and A_f/A_s, τ_f and τ_s. Furthermore, since movement into the channel will be aided by hyperpolarization, and egress retarded (Woodhull, 1973), varying the membrane potential will vary f^* and b^*, and hence A_f/A_s, λ_f and λ_s. The effects of potential changes predicted by this model agreed well with those reported in the literature. This hypothesis for local anesthetic-like actions has now been very widely accepted (Ruff, 1977; Katz & Miledi, 1978; Neher & Steinbach, 1978; Feltz, Large & Trautmann, 1977; Adler & Albuquerque, 1976; Adler, Albuquerque & Lebeda, 1978; Ascher, Marty & Neild, 1978; Marty, 1978; Koblin & Lester, 1979; Ascher, Large & Rang, 1979; Colquhoun et al., 1979; Tsai et al., 1978, 1979; Dryer et al., 1979; Pennefather & Quastel, 1980).

Although convenient, recording mepcs is not an ideal way to study quantitatively channel blocking effects. This is because mepc generation itself is complex and poorly understood (*see* Wathey, Nass & Lester, 1979; Adams, 1980). The most detailed studies have therefore used other techniques, principally relaxation and noise analysis. Before considering such studies, a simple technique closely related in principle to mepc analysis should be mentioned. This is the double-pulse iontophoretic method (Adams, 1976*a*; Katz & Miledi, 1978; Adams & Feltz, 1980*b*). The essential idea is that if the block affects *open* channels and is *slowly* reversible, it should show use dependence (*cf.* Strichartz, 1973). That is, if successive attempts are made to open a fixed set of channels for short periods of time, channels will gradually enter the blocked pool, and become unavailable. Conversely, if the blocked channels are allowed to recover without being challenged to open, then the number that can be opened by a challenge will gradually increase. The challenges used are short pulses of agonist applied from multibarrelled micropipettes positioned close to the subsynaptic membrane. Recovery of channels from a blocked state induced by quinacrine is shown in Fig. 4. The recovery is exponential, and highly potential dependent. The recovery process can be described by scheme (8) above. It depends on the quinacrine concentration, because channels that unblock are left momentarily in an open state, and can reblock rather than close. The average time a channel takes to return to the resting condition will therefore be greater than $1/b^*$, by a factor $(f^*c+\alpha+b^*)/\alpha$. The dependence of the recovery time constant on α provides a powerful test of the implicit assumption that when the channel is held in an open, albeit blocked, state by local anesthetic, agonist is retained on the receptor, and continues to determine the channel closing rate constant, even though more than a second may have elapsed since agonist was present in solution. Thus, if carbachol is used for the conditioning agonist challenge, the recovery proceeds faster, by an amount that corresponds to that expected were carbachol, rather than ACh, trapped on the blocked channel-receptor complex (Adams & Feltz, 1980*a*; *see also* Tsai et al., 1979). These results have two important con-

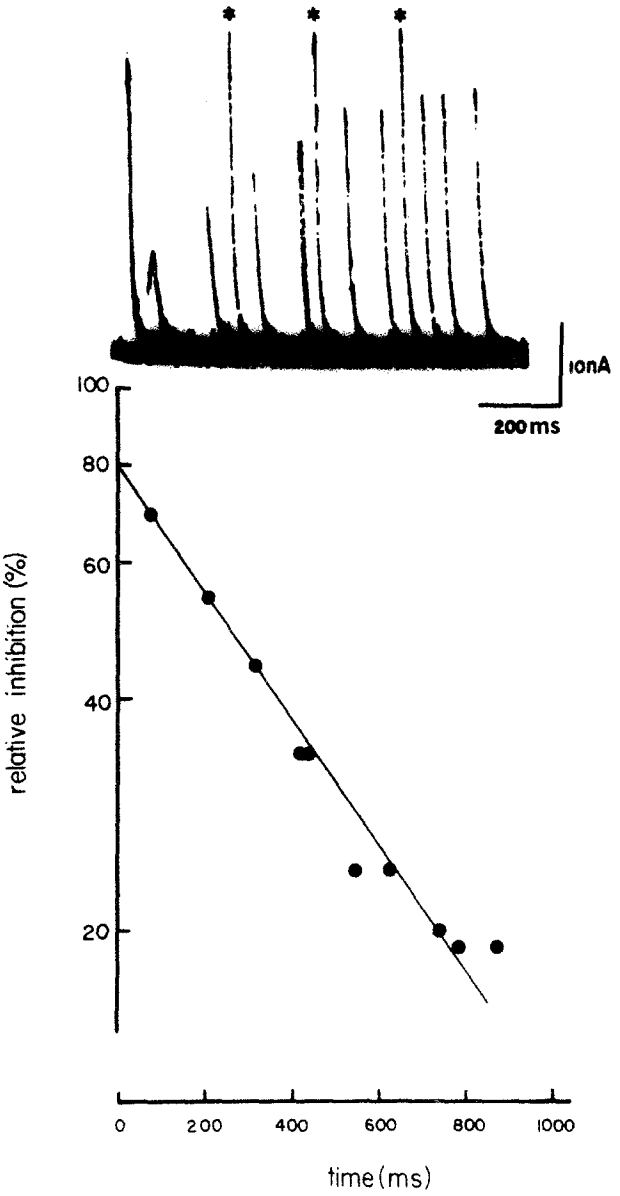

Fig. 4. Iontophoretic double pulse experiment in the presence of 2 μM quinacrine. Holding potential −80 mV. The records show *inward* currents produced by iontophoretic ACh applications. Thirteen traces were superimposed. In 10 of these traces a conditioning pulse (at time zero) was followed by test pulses at various times. In three of the traces the conditioning pulse was absent, and the responses to test pulses are marked with asterisks. Conditioning and test pulses were applied from separate barrels of the iontophoretic pipette. In the graph the amplitude of the test pulses preceded by conditioning pulses is plotted as a percentage of the amplitude of the unconditioned test pulse, as a function of the interpulse interval. (Reproduced, with permission, from Adams & Feltz, 1980*a*)

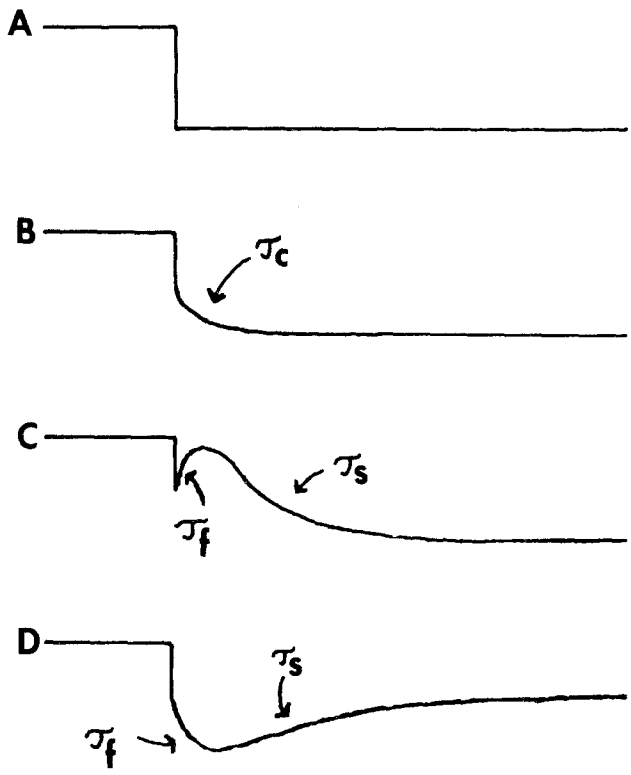

Fig. 5. Sketch of voltage-jump relaxation patterns exhibited in the presence of open-channel blockers. Row *A* shows the step hyperpolarization, and rows *B* – *D* the resulting current waveforms. Row *B* shows a control relaxation. Row *C* shows a relaxation in the presence of a fast voltage-dependent open-channel blocker, such as procaine or QX222. Row *D* shows the waveform in the presence of a slow open channel blocker such as quinacrine or curare. Note that for both conditions (*C* or *D*) $\tau_f < \tau_c < \tau_s$

sequences. (a) They reinforce the assumption previously made in analyzing kinetic data for agonists, that the agonist is very unlikely to dissociate from the receptor when the channel is in its open state (*see also* Nass et al., 1978). (b) They show that local anesthetics are capable of recruiting receptors into the high affinity "open" state.

Quinacrine has also been used in voltage-jump studies (Adams & Feltz, 1977; 1980*b*). In the presence of this drug the normal channel opening relaxation triggered by a hyperpolarizing voltage step is followed by a slow, exponential "inverse" relaxation of the current, reflecting a slow voltage-dependent blockage of open channels (Fig. 5*D*). Similar inverse relaxations are seen in the presence of curare (Ascher et al., 1978; Colquhoun et al., 1979; Ascher et al., 1979). The dependence of the rate constant of the slow relaxation, k, upon agonist and quinacrine concentration agrees rather well with the prediction of the channel blocking model Eq. (2) above. f^* and b^* could be independently determined by analysis of mepc decays and double pulse experiments. Because non-negligible agonist concentrations were used, the simple kinetic scheme used to describe mepc decays has to be extended to include explicitly the channel opening rate:

$$C \underset{\alpha'}{\overset{\beta'}{\rightleftharpoons}} 0 \underset{b^*}{\overset{f^*c}{\rightleftharpoons}} B. \qquad (9)$$

Provided the two time constants predicted by this scheme, τ_f and τ_s, are well separated ($\tau_s \geqq 10\tau_f$), as is usually the case in published studies, the following useful approximate expressions should hold (Adams & Feltz, 1977*b*).

$$1/\tau_f = \beta' + \alpha' + f^* c + b^*$$
$$1/\tau_s = \frac{\beta' b^* + \alpha' b^* + \beta' f^* c}{\beta' + \alpha' + f^* c + b^*}. \quad (10)$$

These formulae also hold for the qualitatively quite different relaxations observed in the presence of the local anesthetic procaine (Adams, 1977*b*). In this case the initial fast relaxation is the inverse one, and it is followed by a slow "channel opening" (Fig. 5*C*). The difference arises because the rate constant for procaine leaving the channel is three orders of magnitude faster than for quinacrine.

Procaine-like relaxation are also seen using the muscle relaxants decamethonium and gallamine (Adams & Sakmann, 1977; Colquhoun & Sheridan, 1979). Decamethonium is itself an agonist, although the kinetic analysis of this action is hampered by the concomitant local anesthetic effect. In cat muscle and eel electroplaques the agonist action can be seen at concentrations low enough that the local anesthetic effects are negligible (Sheridan & Lester, 1977; Wray, 1979).

Decamethonium action could also be studied by noise analysis. It was possible to show that the same two time constants were present in both the voltage-jump relaxation and in the noise autocorrelation function. However, the relative amplitude of the fast process is much greater when using noise analysis (*see also* Moore & Neher, 1976). Accurate evaluation of the time constants is ideally performed using both noise and relaxation techniques.

Further refinement of the channel blocking model has come with the advent of the single-channel recording technique (Neher & Steinbach, 1978). In the presence of QX222, a single channel opening shows "chatter", arising from repeated random plugging and unplugging of the open channel, and it is prolonged, since the channel is prevented from closing whenever it is blocked. The average channel open time and the average unblocked time show similar dependence on local anesthetic concentration as τ_s and τ_f above.

In the cases of procaine, piperocaine, and the arrow poison histrionicotoxin there is also evidence for voltage-dependent block of *closed* endplate channels (Albuquerque, Kuba & Daly, 1974; Masakawa & Alberquerque, 1978; Adams, 1977*b*; Tiedt et al., 1979). These authors thus favor a cyclic scheme:

$$\begin{array}{ccc} C & \underset{\alpha'}{\overset{\beta'}{\rightleftharpoons}} & O \\ {\scriptstyle fc}\downarrow\uparrow{\scriptstyle b} & & {\scriptstyle b^*}\downarrow\uparrow{\scriptstyle f^*c} \\ AC & \underset{\alpha''}{\overset{\beta''}{\rightleftharpoons}} & AO \end{array} \quad (11)$$

The blocked channels AC and AO seem to interconvert only very slowly.

Finally, it should be mentioned that neutral molecules such as barbiturates and benzocaine can also block open channels (Adams, 1976*b*; Ogden & Siegelbaum, 1980).

Physical Location of Local Anesthetic Site

Formal kinetic analysis is dumb as to the possible physical location of the anesthetic binding site. There are three circumstantial pieces of evidence that the site may indeed be within the lumen of the channel.

(i) Most of these compounds when applied inside axons will also block potassium or sodium channels, whose luminal regions would be candidates for similarity to ACh channels.

(ii) The binding of the cationic anesthetics is voltage dependent, whereas that of neutral anesthetics is not. The voltage dependence is such as would be generated by movement of a drug molecule down one half to three-fourths of the membrane potential. Such movement is likely to be along the channel.

(iii) Decamethonium can penetrate right through the channel (Creese & England, 1970; Case et al., 1977). The flux shows saturation, as expected were there a site within the channel to which decamethonium binds. Similarly, calcium and magnesium fluxes show saturation, and these ions will block *other* sodium fluxes (Lewis, 1979). Several further tests should be performed in this regard:

(a) Can local anesthetics reach the site from the cytoplasmic side? QX222 does not appear to do so (Horn, Brodwick & Dickey, 1979).

(b) Will permeant ions such as sodium, lithium, or calcium compete for the binding site?

(c) Will internal pH modulate the block by externally applied tertiary amines (Schwartz, Palade & Hille, 1977)?

Biochemical work has recently identified specific sites for local anesthetics at the *Torpedo* receptor (Eldefrawi et al., 1978; Elliot & Raftery, 1979; Krodel, Beckman & Cohen, 1979).

The compounds used were meproadifen and histrionicotoxin. The former shows significant binding only in the presence of agonist, whereas the latter binds to resting membranes. It is possible that meproadifen binds mainly to open channels, and histrionicotoxin to closed, in line with the electrophysiology. It is, however, possible that the observed binding relates to desensitization processes, since it has so far been studied only at equilibrium. It would be extremely useful to know whether agonist-enhancement of meproadifen binding occurs with a time scale resembling channel opening or desensitization.

Biochemical correlations with electrophysiology can also be attempted with spectroscopic methods. Relevant here is the detailed study of quinacrine fluorescence of membrane fragments by Grünhagen, Iwatsubo and Changeux (1977). Rapid mixing of agonist and membranes stained with quinacrine leads to a fluorescence increase. The time course of this increase, and its dependence on agonist concentration, closely paralleled that of the slow inverse relaxation seen in voltage-clamp experiments. If this identification can be sustained, it would establish remarkable similarities between the channel kinetics in *Torpedo* electroplaque and frog endplate.

Desensitization

If an endplate or electroplaque is exposed to agonist for any length of time, the permeability response is not maintained. This sag of the response is referred to as desensitization. Electrophysiological desensitization has been studied by applying the agonist either by iontophoresis or by perfusion. The first method is very fast, but the second has the great advantage that the agonist concentration is known. Using the perfusion approach, it has been generally found that the desensitization onsets roughly exponentially and at a rate that increases with agonist concentration (Adams, 1975*d*; Lester et al., 1975; Scubon-Mulieri & Parsons, 1978). Figure 6 presents a composite summary of some of the published rates using carbachol as agonist. The perfusion method has shown recovery to be exponential with a time constant around 3 min (Rang & Ritter, 1970; Scubon-Mulieri & Parsons, 1977). This is close to onset rates determined with very low agonist concentrations (Terrar, 1974). These data can be fitted with a model assuming a simple isomerization of the active receptor (Fig. 6), though this probably only reflects the inadequacy of the data.

Since during iontophoresis the local agonist concentrations are often unknown, or, worse, nonuniform, it is pointless to compare onset rates obtained by perfusion and iontophoresis. Offset rates should be comparable; however, they are not, recovery from iontophoretically induced desensitization occurring with a time constant of several *seconds* (Katz & Thesleff, 1957). A fast phase of recovery can also be seen following rapid bath applications (Adams, 1975*d*). It follows that "desensitization" is composed of at least two processes. Very little is known about the kinetics of the fast process; presumably it tends to occur mainly at high agonist concentrations. The slow process is prominent even for very low agonist concentrations, which only open small fractions of channels. The plateau response achieved after allowing the desensitization to procede to completion varies little with concentration above 50 μM carbachol (Fig. 6), indicating that the overall dissociation constant for carbachol binding in the desensitized state is quite small. This is in line with Katz and Thesleff's original suggestion that the desensitized receptor was in a high affinity state. It is not yet clear how the second and minute time scale processes detected by macroscopic techniques relate to the "burst" and "cluster" phenomena reported by Sakmann et al. (1980).

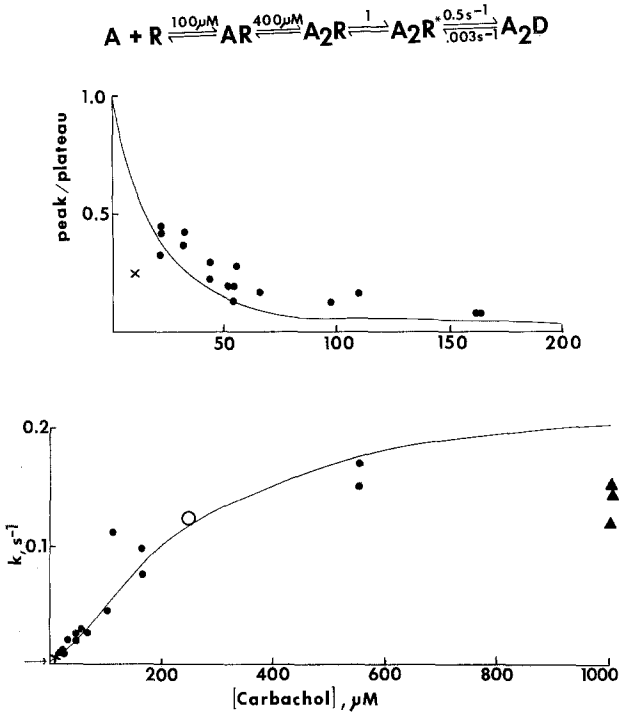

Fig. 6. Estimates of desensitization amplitude and rate as a function of carbachol concentration. Data taken from Adams, 1975*d* (●); Terrar, 1974 (×); Scubon-Mulieri and Parsons, 1977 (▲); and J. Lambert, R.M. Spannbauer and R.L. Parsons (*unpublished*) (○). The upper graph shows the ratio of the initial peak conductance response to the final plateau conductance level. The lower graph shows the rate constant for exponential decline from peak to plateau conductance. The solid curves were calculated assuming the scheme shown in the figure, where only A_2R^* is conducting. This is basically the scheme used to generate Fig. 1, save that the quinacrine blocking rates have been replaced by desensitization rate constants. The recovery rate constant was taken from the work of Scubon-Mulieri and Parsons and is marked with an arrow on the lower graph. The forward rate was taken from Katz and Thesleff (1957)

Two important factors which seem to affect desensitization rates are membrane potential (Magazanik & Vyskocil, 1970; Scubon-Mulieri & Parsons, 1978) and calcium concentration (Manthey, 1966; Parsons, Cochrane & Schnitzler, 1973). However, raising calcium concentration may only accelerate desensitization for *high* agonist doses (Terrar, 1973).

Recent biochemical work with *Torpedo* membrane fragments has thrown much light on the desensitization issue. Foremost here has been the correlation of desensitization of the permeability response, and increase of the agonist affinity, in Changeux's lab. The desensitization was shown by preincubating patent, radiolabeled-cation loaded membrane microsacs with low agonist doses, and challenging at various times with higher agonist doses (Sugiyama, Popot & Changeux, 1976). The affinity increase was demonstrated by monitoring the inhibition of the initial rate of binding of labeled neurotoxin by various concentrations of competing agonist, with or without preincubation with agonist (Weber, David-Pfeuty & Changeux, 1975). Both the affinity increase and the desensitization occurred with qualitatively the same time course. The identity of the time courses of the two phenomenon in muscle cell cultures has recently been reported by Sine and Taylor (1979).

Several groups have refined the measurements of toxin binding kinetics to the point of comparison with detailed molecular models, usually based on the idea of rapid binding of agonist to two interconverting receptor states, high affinity/desensitized (D) and low affinity/resting (Quast et al., 1978; Weiland et al., 1977; Wieland & Taylor, 1979; Barrantes, 1978).

$$\begin{array}{ccc} R & \underset{k_{-o}}{\overset{k_o}{\rightleftharpoons}} & D \\ {\scriptstyle k_f}\Big\uparrow\Big\downarrow{\scriptstyle k_b} & & {\scriptstyle k'_b}\Big\uparrow\Big\downarrow{\scriptstyle k'_f} \\ AR & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & AD \end{array} \qquad (12)$$

This scheme will certainly explain the protection curves seen with various lengths of agonist preincubation; most strikingly, it explains the biphasic protection curve seen with zero preincubations. The high affinity foot of the curve corresponds to the binding to pre-existing desensitized receptor, (of the order 20% of total), and the low affinity component to binding to the native form. After equilibration, the protection curve becomes monophasic, and is located between the two distinct affinities seen before incubation (Weiland & Taylor, 1979).

This analysis has now been taken much further in two brilliant studies using rapid mixing techniques. These two investigations, though quite different in approach, are remarkably concordant. In one study (Cohen & Boyd, 1979), the binding of radioactive ACh and CCh to membrane fragments was directly followed. In the other (Heidmann & Changeux, 1979*a*), the kinetics of the binding of the fluorescent agonist DNS-C6-Ch to similar fragments was monitored. In both of these studies a very rapid component of binding that behaved as a simple bimolecular process, to a fraction (20%) of the total sites was observed. The forward binding rates for ACh and DNS-C6-choline were about $10^8\,\mathrm{M^{-1}s^{-1}}$. The reverse rates could be obtained directly by measuring the dissociation of ligand with a cold chase (ACh; $0.15\,\mathrm{s^{-1}}$) or displacement (DNS-C6-Ch; $0.3\,\mathrm{s^{-1}}$). The concentration dependence of the amplitude of the fast process could be successfully predicted from the binding rate constants.

The rapid process could be assigned to agonist binding to spontaneously desensitized receptor D in scheme (2), since it corresponds to a high affinity binding, and could be increased in amplitude by preincubating the receptor with agonist, provided the agonist was diluted away following the incubation.

Both studies also identified a slow process which reflected the conformational transition from R to D states. The amplitude of this process was largest for intermediate agonist concentrations. Its rate constant moves between a low concentration limiting value and a high concentration limiting value. In the Cohen-Boyd work the low concentration limiting value (i.e., recovery rate) was about $0.01\,\mathrm{s^{-1}}$, while in the Heidmann-Changeux work it was about $0.045\,\mathrm{s^{-1}}$ (both determinations at room temperature). In principle these values should be identical ($k_o + k_{-o}$ in Scheme (12)). The Cohen-Boyd value is probably more reliable, as it was directly determined, and is quite close to the rate of recovery of the flux response. The upper concentration limiting value, which should yield $k_1 + k_{-1}$, was about $0.18\,\mathrm{s^{-1}}$ in both studies (but at different temperatures). An identical value is found for the limiting rate of the slow intrinsic fluorescence increase following rapid mixing with ACh (Bonner, Barrantes & Jovin, 1976).

Finally, in both studies an intermediate kinetic component was identified, which represents binding to the R state. The forward rate constants were only $3.5 \times 10^5\,\mathrm{M^{-1}s^{-1}}$ (DNS-C6-Ch) or $2 \times 10^6\,\mathrm{M^{-1}s^{-1}}$ (ACh). Both groups suggest that this process is itself an overall reaction that includes isomerizations to other states of the receptor, including the active state A, and a putative "pre-desensitized" state D'. Some independent evidence for this D' state has recently come from a comparison of the apparent affinity constants for carbachol binding to the R state ($\sim 20\,\mu\mathrm{M}$) and activation of Na flux ($\sim 600\,\mu\mathrm{M}$; Neubig & Cohen, 1980). It is intriguing that the rate of dissociation of agonist from this low affinity state is similar to the rate of recovery from iontophoretically induced desensitization (*see* above).

The overall picture that emerges from these stud-

ies is rather convincing, because the model parameters are not purely the result of curve fitting: they can often be measured in two quite separate ways and are remarkably consistent. Addition of very low agonist amounts to membrane fragments leads mainly to binding to a pre-existing high affinity desensitized state; addition of intermediate concentrations leads to a slow conversion of unliganded undesensitized receptor to the high affinity form, which immediately binds agonist; addition of high agonist concentrations also leads to a conversion; however, binding to the low affinity form can occur prior to this. When the free agonist is diluted away, the bound agonist dissociates from the equilibrium mixture of $R+D$ forms over several seconds; excess D receptor then slowly (minutes) reverts to the R state, until the resting R/D ratio is achieved. Thus, at short times agonist binding is heterogeneous (to R and D states); at long times the binding is single component, with a K_{app} that is a weighted average of the low and high affinity constants, the weights being determined by the resting R/D ratio. The fact that even the binding to the low affinity form is tighter than required for channel opening may indicate the existence of new conformations and transitions.

This picture has two further wrinkles. Firstly, it is probable that some of the states discussed are multiliganded. Secondly, local and general anesthetics can themselves shift the $R-D$ equilibrium, and change the isomerization rates, by an action at a site that is not the agonist binding site, and may not be the channel site (Boyd & Cohen, 1979; Heidmann & Changeux, 1979*b*). Both these studies find that histrionicotoxin (or its perhydro derivative) is poorer at promoting the $R \rightarrow D$ conversion than are conventional local anesthetics. This in turn implies that HTX has a relatively higher affinity for the R state, which would be in line with indications from direct binding studies (Elliott & Raftery, 1979; Eldefrawi, Mansour & Albuquerque, 1978; Krodel et al., 1979). It is particularly striking that the anesthetic-induced D state is functionally identical to that induced by agonists, and that HTX and anesthetics compete at the same site (Cohen, Boyd & Shera, 1980). It is tempting to suppose that the receptor states A_2R^* and D are rather similar, in that both have high agonist affinities (dissociation rates $<1\,s^{-1}$) and both are stabilized by certain anesthetics, though not by HTX or procaine.

Conclusions

Acetylcholine receptor kinetics is an explosive field of research in membrane biology today. The present review has only skimmed over much of the new data and is heavily tinged by the writer's own preoccupations, and probably misconceptions. Numerous important kinetic investigations have been neglected, in order to impose some, inevitably artificial, clarity. At last the link between binding and response (Colquhoun, 1979) is being bridged. Undoubtedly some of the major themes dealt with above will stand, and correlations with structural information will start to emerge. Hopefully, the 1980's will see the emergence of true understanding of the functioning of the acetylcholine receptor.

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