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Voltage Fluctuations at the Frog Sartorius Motor Endplate Produced by a Covalently Attached Activator

Robert N. Cox*, Masataka Kawai, Arthur Karlin, and Philip W. Brandt

Departments of Anatomy, Biochemistry, and Neurology, Columbia University, New York, New York 10032

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Summary. The depolarization that develops after covalent attachment of trimethylammonium benzoyl to the dithiothreitol-reduced frog sartorius acetylcholine receptor is accompanied by a small increase in voltage fluctuations. The amplitude of the elementary voltage event produced by the covalently attached activator is about 0.04 μV, almost an order of magnitude below the acetylcholine shot-effect amplitude in the control preparation, and about one-fourth the acetylcholine shot amplitude after disulfide-bond reduction. Spectral density plots of trimethylammonium-benzoyl noise can be analyzed in terms of two relaxation rates that bracket the single rate observed in response to acetylcholine.

The rate-limiting step in the interaction of drugs with the acetylcholine (ACh) receptor remains unidentified. Various possible combinations of voltage-dependent and rate-limiting steps have been discussed in detail (Adams, 1977*a*; Sheridan & Lester, 1977). Magleby and Stevens (1972*a, b*) have argued that the neurally-evoked postysynaptic current lasts on the average much longer than the time required for removal of Ach from the synaptic cleft. Similar arguments have been advanced by Gage and McBurney (1975) and Sheridan and Lester (1977). The two most obvious remaining candidates for the rate-limiting step in receptor activation are the binding and unbinding of agonist or isomerization of the drug-receptor complex. Several indirect considerations, most based on analogy with enzyme-substrate kinetics, have been educed in favor of rate-limiting conformational change (Magleby & Stevens, 1972*a, b*; Anderson & Stevens, 1973; Stevens, 1975).

A plot of apparent rate constant (opening rate+closing rate; measured, for example, by fluctuation analysis or by voltage-jump relaxation techniques) *vs.* agonist concentration will increase indefinitely if the

* *Address for reprints:* Department of Neurology, Columbia University, 630 West 168th St., New York, N.Y. 10032.

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slower of the two steps is agonist binding and unbinding, whereas the same plot will eventually level off if the rate-limiting step is isomerization. Sheridan and Lester (1977) have observed no leveling off of the reciprocal relaxation time *vs.* ACh curve for concentrations up to 100 μM; Adams (1977*a*) has similarly reported no saturation in the channel activation rate for concentrations of suberyldicholine up to 14 μM. Although this method of differentiating between the two models is hampered by the rapidly developing desensitization induced by high agonist concentrations, Adams and Sakmann (1978*a*) and Sakmann and Adams (1978) have recently succeeded in measuring a saturation in the carbamylcholine-induced receptor activation rate.

Because the quaternary ammonium moiety of a covalently bound activator is held always within a small distance of the receptor anionic subsite, the covalently attached group may produce, by default, the high concentration limit condition. We therefore undertook to investigate fluctuations in voltage at the frog motor endplate during depolarization induced by covalently attached trimethylammonium benzoyl (TMB). We report here our success in observing TMB-induced relaxation noise, and in a time range well within the limits of what is most commonly cited (ie., $10^2 - 10^4$ sec^{-1}; *cf.* Hammes, 1968*a, b*; Morawetz, 1972; Citri, 1973) for conformational transitions in macromolecular complexes. A preliminary account of these results has been presented (Cox, Karlin & Brandt, 1979*b*).

Materials and Methods

The experimental chamber, optics, and physiological salines were as described in the previous paper (Cox *et al.*, 1979*a*). Iontophoretic and pressure release capillaries were positioned above junctional regions, some 50 – 100 μm away, in order to minimize contamination noise arising from minute fluctuations in drug efflux rates.

Recorded membrane potential was applied to a low-noise FET-input operational amplifier (Analog Devices, model 43K) with a fixed gain of 20. The signal was then split into two parallel channels, one leading directly to a digital voltmeter for the measurement of average level of membrane potential, the other to a filtered (2nd order Butterworth filter with variable time constant, 40 db/decade rolloff) and capacitor-coupled (0.129 sec time constant) amplifier with variable gain. The amplified signal was led to a photon-coupled isolation unit and subsequently through low-capacitance cable to a Data General 12-bit analog-to-digital converter. Overall system gain was typically in the range 2000 to 20,000 and was adjusted so as to assure that the amplitude of the analog signal occupied as much of the available quantizing range as possible. Voltage fluctuations were monitored on the oscilloscope at the output of the AC-coupled amplifier. Resistance of recording electrodes was monitored via a second high-impedance FET-input amplifier. A silver-silver chloride coil inserted into the bath served as an indifferent electrode. System frequency

response was calibrated by applying computer-generated sine waves of known amplitude and frequency.

The microscope, micromanipulators, preamplifiers, and current clamp were enclosed in a Faraday cage of copper mesh. The 43K preamplifier and the constant current device were battery-powered to avoid introducing AC contamination into the cage. Artifacts resulting from mechanical vibrations in the walls and floor were eliminated by mounting the entire apparatus on a three-legged pneumatic isolation system (Lansing Research Corp., Ithaca, N.Y.).

For examination of intracellularly-recorded noise, records were digitized at intervals of 2.0 msec (bandwidth 1.5–200 Hz) for periods of 5–10 sec before, during, and after application of drugs. Several control and experimental records were routinely collected at each endplate. Reconstructed raw data traces were printed out on a Gould 5000 printer-plotter and screened for contamination by mechanical artifacts, low-frequency trends, and miniature endplate potentials. Variance, autocorrelation, and power spectral density (Fourier transform of correlation function; *cf.* Dixon, 1968) were calculated on a Data General Nova 1220 digital computer. Individual control and experimental records from a single preparation were then averaged together.

On the assumption that background noise and chemically-induced noise derive from uncorrelated processes, the power spectrum of background noise was routinely subtracted from that of agonist noise. Both individual and averaged experimental records from a single endplate were subtracted against the appropriate averaged control and plotted out on the Gould 5000. Finally, subtraction routine output files (with common frequency increment) could, if desired, be fed into a secondary averaging routine that normalized and averaged together records from many different experiments.

Results

ACH-Induced Noise

Before investigating noise accompanying activation by covalently attached TMB, we tested our system by examining ACh-induced fluctuations, before and after exposure of the endplate to dithiothreitol (DTT). These experiments were similar to, and their results in good agreement with, those previously reported (Katz & Miledi, 1970, 1971, 1972; Landau & Ben-Haim, 1974; Ben-Haim, Dreyer & Peper, 1975). The grand average value of 138 estimates of the control ACh shot effect amplitude "a" drawn from 40 experiments was 0.31 ± 0.01 μV (mean ± SE) (calculated according to the equations of Katz and Miledi, 1972, and employing their correction factor for nonlinear summation of depolarization).

An example of the spectral behavior of ACh noise is shown in Fig. 1. The fluctuations are restricted to a rather low frequency range, as is to be expected, given the filtering effect of the membrane capacitive time constant. The half-power point in this experiment was 25 Hz, corresponding to a time constant of 6.4 msec. The average of 104 estimations of the half-power frequency of ACh noise was 23.1 ± 0.7 Hz. Comparable

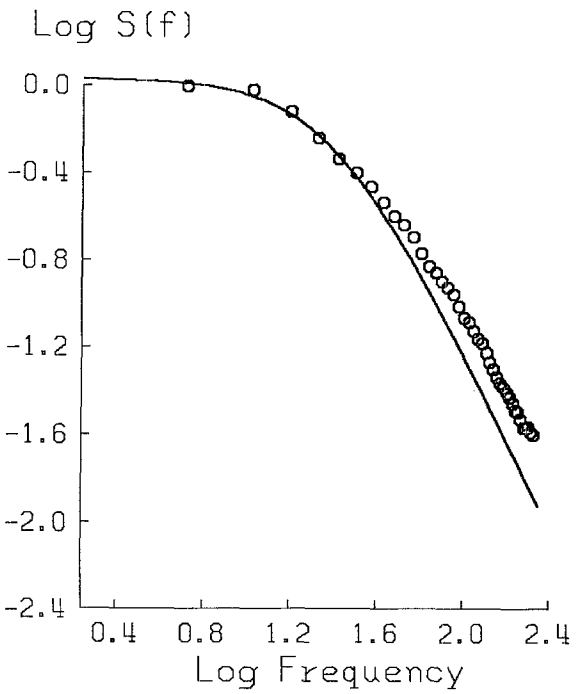

Fig. 1. Double-logarithmic plot of spectral density of ACh-induced voltage fluctuations, normalized to zero-frequency value of 1. Average of 8 records from a single endplate. The effect of the Butterworth filter has been corrected for. Solid line is Eq. (11) of Katz and Miledi, 1972, with $f_c = 25$ Hz. Note the discrepancy between the experimental points and the theoretical curve above 35 Hz. The relative attenuation in power density between 10 and 100 Hz is 10.87 for the experimental curve, 14.66 for the theoretical curve

values obtained by previous investigators are 19.5 Hz (Katz & Miledi, 1972) and 24.1 Hz (Landau & Ben-Haim, 1974). The average attenuation in power density between 10 and 100 Hz was 13.9 ± 1.1. The deviation in the higher frequencies between the experimental points and the theoretical curve in Fig. 2 (Eq. (11) of Katz & Miledi, 1972, with $f_c = 1/2\,\pi\tau =$ 25 Hz) was found also by Katz and Miledi and illustrates the well-known oversimplification of modeling membrane impedance as a parallel resistance and capacitance (*cf.* Falk & Fatt, 1963; Eisenberg & Gage, 1967; Freygang, Rapaport & Peachey, 1967; Gage & Eisenberg, 1969; Schneider, 1970; Eisenberg, 1971; Valdiosera, Clausen & Eisenberg, 1974*a*, *b*).

Statistical analyses of fluctuations in voltage (Landau & Ben-Haim, 1974) and current (Ben-Haim, *et al.*, 1975) at the frog neuromuscular junction have shown that DTT treatment results in an impaired transla-

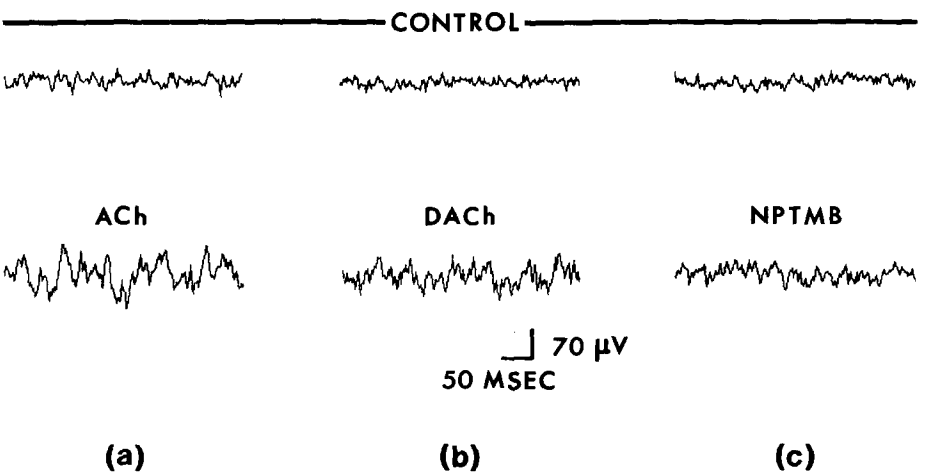

Fig. 2. Intracellular recordings from frog neuromuscular junction, before (upper traces) and during (lower traces) application of (*a*) ACh (control preparation), (*b*) ACh (DTT-treated preparation), (*c*) NPTMB. Reconstructed from the digitized data; sample interval, 2.0 msec; bandwidth, 1.5–200 Hz. (*a*): The mean ACh potential of 10.7 mV was accompanied by an increase in the rms value of the noise from 14.2 to 35.6 μV. The "difference" rms, representing ACh noise alone, was 32.6 μV before correction for nonlinear summation of potential, 43.8 μV after correction. (*b*): Mean ACh potential–7.01 mV. Rms value of the control noise was 11.9 μV, total noise–23.8 μV. The "difference" rms was 20.6 μV before correction, 24.6 μV after correction. (*c*): The mean TMB depolarization of 12.44 mV was accompanied by an increase in the rms value of the fluctuating potential from 10.9 to 15.9 μV. The net excess noise due to action of covalently attached TMB was 11.6 μV before correction, 15.9 μV after correction

tion of ACh binding into postsynaptic permeability increase. Both the amplitude, and, to a lesser extent, the mean duration of the elementary gating event are reduced. In addition, the affinity of ACh for the receptor binding site is reduced about 3.5-fold (Ben-Haim *et al.*, 1975). A similar increase in the apparent dissociation constant of the *Electrophorus* electroplax to carbamylcholine after disulfide bond cleavage has been reported (Karlin, 1969; Karlin & Bartels, 1966).

The marked reduction in single-channel conductance and the less dramatic decrease in mean channel open time after disulfide bond reduction are both reflected in a smaller single-channel voltage. The average of 89 estimates of "*a*", drawn from 40 preparations with 30-min exposures to DTT, was 0.18 ± 0.01 μV, a reduction to 58% of the control value, in good agreement with results reported in previous studies (Landau & Ben-Haim, 1974; Ben-Haim *et al.*, 1975). The corner frequency of ACh-induced voltage noise after disulfide bond reduction differs little from that of control ACh noise. The average half-power frequency after expo-

sure to DTT was 24.9 ± 0.7 Hz, an 8% increase over the control value. The attenuation in the higher frequency portion of the spectral density curves provides a more sensitive index of changes in mean channel open time. The average attenuation in power density between 10 and 100 Hz after treatment with DTT was 9.7 ± 0.6, a reduction to 70% of the control value.

TMB Noise

Depolarization of sartorius postjunctional membrane after exposure to the nitrophenyl ester *p*-carboxyphenyltrimethylammonium iodide (NPTMB) is accompanied by a small but regular noise increase (Fig. 2). When NPTMB is applied in small increments by pressure release, a slow and monotonic decrease in membrane potential can be achieved (*see* Cox *et al.*, 1979*a*). In this case, very little excess unreacted drug should be present at any given instant, so that a reasonable approximation to equilibrium should obtain at all levels of depolarization. The steady-state probability that a covalently bound channel is open should remain

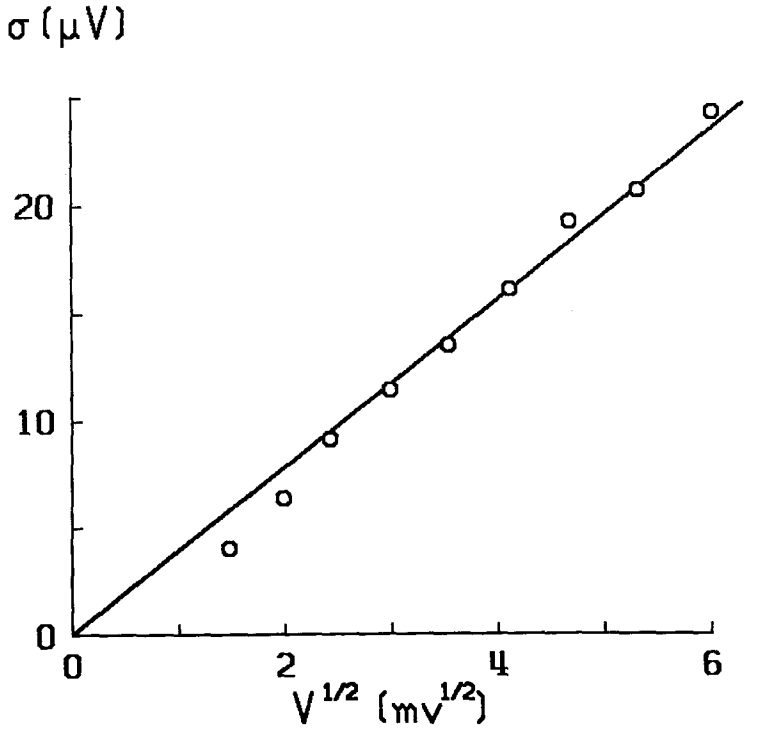

Fig. 3. Relation between TMB-induced noise and mean potential. From slope of eyedrawn line, average value of shot effect amplitude was 0.031 μV

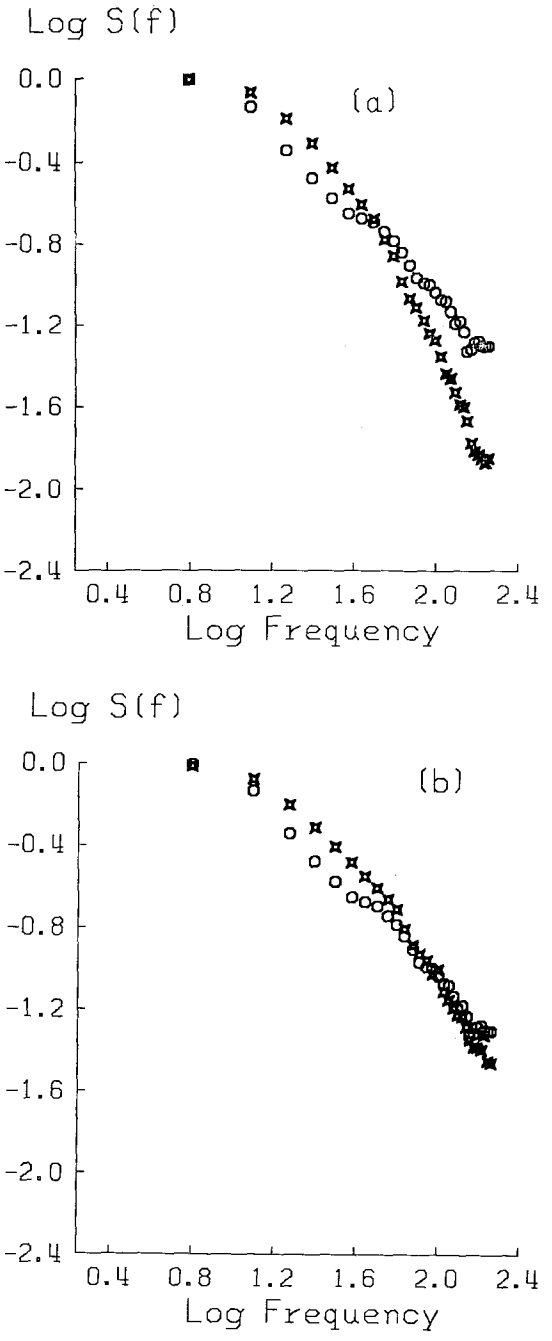

Fig. 4. Comparison between spectral distribution of TMB-induced noise and ACh-induced noise. (*a*): Control ACh noise (stars) *vs.* TMB noise (circles). The ACh curve is the average of 20 records from 9 experiments; the TMB curve is the average of 18 records drawn from 5 separate experiments. Corner frequencies: 23.13 Hz (ACh), 15.31 Hz (TMB, first component). (*b*): ACh noise after treatment with DTT (stars) *vs.* same TMB curve as in (*a*) (circles). The DTT ACh curve is the average of 51 records from 30 experiments. Corner frequency 25.00 Hz

effectively constant as the depolarization increases, and, even though this probability may not be small compared to 1, the relation between noise variance and mean depolarization should be linear.

Figure 3 shows the result of an experiment in which several records were obtained during a TMB depolarization that eventually reached 24 mV. In these and 14 similar experiments, a convincingly linear relationship between the mean and variance of the TMB-induced potential was obtained. The mean value of 129 estimates of the TMB shot effect amplitude drawn from 16 experiments in which V was allowed to decline slowly was 0.040 ± 0.002 μV, a reduction to 13.0% and 22.6% of the ACh shot amplitude in the control and DTT-treated conditions.

The very small variance increases that accompany TMB depolarizations complicate the evaluation of spectral density records. However, with averaging of data from several experiments (*see* Materials and Methods), reasonably smooth curves can be obtained. In Fig. 4, a TMB curve is compared with ACh curves before and after exposure to DTT. Most notable is the complex nature of the TMB curve. Up to about 30 Hz the rolloff is greater, and in the higher frequencies less, than in the ACh curves. A two-time-constant shape was observed in virtually all TMB records, the inflection occurring in the range 20 to 40 Hz. The average of 137 estimates of the half-power frequency of the first component was 18.7 ± 0.7 Hz.

To evaluate more quantitatively the apparently composite nature of

Table 1.

		10/30	50/100	10/100
ACh *vs.* DTT ACh	(df=75)	$z=2.096$ ($P<0.025$)	$z=3.298$ ($P<0.005$)	$z=3.636$ ($P<0.005$)
ACh *vs.* TMB	(df=40)	$z=1.873$ ($P<0.05$)	$z=6.250$ ($P<0.005$)	$z=3.564$ ($P<0.005$)
DTT ACh *vs.* TMB	(df=69)	$z=3.922$ ($P<0.005$)	$z=4.588$ ($P<0.005$)	$z=1.030$ ($P>0.1$)

Unpaired test for comparison of means (degrees of freedom $=n_1+n_2-2$). In most experiments, several records were obtained for each penetration. An averaged spectral density curve was then calculated, and attenuations were estimated from this averaged curve. For each parameter, the number of original estimates, the number of estimates after averaging (which is n for purposes of calculating degrees of freedom), and the number of experiments were:

ACh	104,	24,	17
DTT ACh	89,	53,	40
TMB	137,	18,	18

Table 2.

		10/30	50/100	10/100
DTT ACh *vs.* TMB	(df=17)	$t=2.515$ ($P<0.025$)	$t=3.920$ ($P<0.005$)	$t=0.248$ ($P>0.1$)

Paired test for comparisons of means (degrees of freedom $=n-1$). Number of original estimates, number of estimates after averaging, and number of experiments are the same as in Table 1.

the TMB spectra, the relative attenuations for all curves were subdivided into the ranges 10/30 and 50/100 Hz. Unpaired tests for comparison of means were performed on the attenuations in these ranges and between 10 and 100 Hz. Also, paired tests for comparison of means were performed between the DTT ACh and the TMB parameters. As shown in Tables 1 and 2, all differences proved statistically significant except the 10/100 attenuation in the DTT ACh *vs.* the TMB curves.

Discussion

The fluctuation increases that accompany the TMB-induced depolarization are small, but with care to reduce background noise, and with averaging of data, noise analysis is clearly a feasible experimental strategy. Repeated attempts to produce an increase in noise by depolarizing the endplate with a second, current-passing, electrode were unsuccessful. This result, and the linear relation between noise root mean square (rms) and mean TMB depolarization, support the interpretation that the excess noise observed after covalent attachment of TMB derives from the opening and closing of synaptic channels. TMB apparently classifies as a very weak (low efficacy) agonist, the amplitude of the elementary voltage event resulting from activation by the covalently bound moiety being about one-fourth that of the ACh-induced elementary event after DTT treatment, and nearly an order of magnitude below the ACh shot effect amplitude in the unmodified receptor. The diminished shot amplitude with TMB could result from a reduced open-channel conductance and/or mean open-channel lifetime, as well as from an equilibrium probability of the open state that is not greatly less than 1. A rough estimate of the TMB-induced single-channel conductance can be calculated by dividing the average value of "a" by V_oR, where $V_o=$maximum possible depolarization. Taking $R=200{,}000\Omega$ (Fatt & Katz, 1951), $V_o=75$ mV, and $a=0.04$ μV, we obtain $\gamma=2.67$ pS. Although this is at best a crude

estimate, it seems likely that a measure of γ under voltage-clamp conditions would be smaller than for any reversible activator yet tested (the present low is 12.8 pS for the trimethylammonium derivative PPTMA (Colquhoun *et al.*, 1975)).

There are several possible origins for the observed composite nature of the TMB spectral density curves. Among these are:

1) TMB may induce at least two open states with differing mean lifetimes.

2) Assuming there is only one open state, channel opening is likely to be the last of a complicated sequence of steps involving more than one closed state. Even in the simplest such case, a three-state scheme with one open state, a two-time-constant spectral density can result if the "binding" (the relation between conventional agonist binding and the behavior of a covalently labeled receptor is discussed in more detail below) and conformational change rates are not widely separated.

3) TMB may activate distinct populations of receptors (*cf.* Dreyer, Walther & Peper, 1976).

4) Two reversibly-binding trimethylammonium derivatives (HPTMA, PPTMA) that, like TMB, are weak activators and possess a benzene ring have been found to produce two-time-constant spectra (Colquhoun *et al.*, 1975). Ruff (1976, 1977; *cf. also* Adams, 1977*b*; Beam, 1976*a*, *b*; Neher & Steinbach, 1978) has provided evidence that the composite spectra obtained from ACh in the presence of local anesthetics derive from blocking of open channels by anesthetic molecules and has speculated that the complex shape of HPTMA and PPTMA spectra may arise from a similar, "self-blocking" action. Adams and Sakmann (1978*b*) have recently shown that in high concentrations the partial agonist decamethonium exerts both agonist and local anesthetic effects and have suggested that even full agonists, when applied in sufficient concentrations, may bind to and block open channels. It is possible that TMB exerts a local anesthetic-like action, although it is difficult to visualize how such an effect could be achieved by a molecule attached covalently near the receptor binding site.

5) Experimental artifact. The distortive effects of the membrane time constant are not to be underestimated, but it seems unlikely, considering the failure to observe composite spectra with ACh before or after DTT treatment, that membrane impedance alone could have given rise to the spectral shapes observed during receptor activation by covalently bound TMB. A second, and probably more serious, potential source of artifact derives from the low amplitude of TMB fluctuations and

consequent distortions introduced by the subtraction of background noise. The averaging procedures were developed to minimize this danger.

As an additional qualitative check on the difference between ACh and TMB spectra, some computer modeling was performed. The program could generate a two-time-constant function to simulate membrane impedance, a one- or two-time-constant Lorentzian ("input" spectrum), and the output that would result from passing the Lorentzian through the impedance. ACh spectra were found to be well fit by single-component Lorentzians of time constant $1-2$ msec, as detected at the output of the equivalent impedance, whereas TMB spectra could be approximated only by an input spectrum (applied to the same impedance) that was the sum of two single-component Lorentzians with rather widely spaced time constants, on the order of 7.5 and 0.35 msec. Although useful, such exercises are qualitative at best.

In general, current experimental results from fluctuation and relaxation measurements are consistent with any scheme in which the ACh-induced endplate conductance change is governed by a single rate-limiting first-order process. The meaning of the parameters estimated by fluctuation analysis, however, varies greatly depending on whether agonist dissociation or the subsequent isomerization of the drug-receptor complex is rate-limiting. An oversimplified but useful kinetic scheme for demonstrating the anticipated relations between observed rate constants and the actual rate-limiting step in receptor-agonist interaction is the following (*cf.* Del Castillo & Katz, 1957; Katz & Miledi, 1972; Anderson & Stevens, 1973; Neher & Sakmann, 1976):

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

where A is the drug, R the receptor, AR the inactive drug-receptor complex, and AR^* is the active conformation, reached from AR by a reversible molecular transformation.

If the vast majority of agonist-receptor associations resulted in rapid dissociation, and if those few associations that converted to the open conformation rarely did so more than one time for a given occupation ($k_{-1} >> \beta$), then the rate constant measured in the limit of low agonist concentration would be the channel relaxation rate (α), and the amplitude of the estimated elementary permeability change would be that of a single open channel. Differing single-channel conductances for different drugs would then imply that the channel can assume a range of agonist-dependent conformational states characterized by varying degrees of cation permeation.

If the conformational change is in rapid equilibrium relative to the binding and unbinding of agonist, the parameters obtained from noise analysis have a much different, and less satisfying, significance. The mean elementary event duration measured by noise analysis (Katz & Miledi, 1973; Colquhoun *et al.*, 1975; Dreyer *et al.*, 1976) and the relaxation time constants derived from voltage-jump experiments (Neher & Sakmann, 1975; Adams, 1974, 1977*a*) correlate rather well with the apparent affinity of agonist for receptor. This raises the possibility that the measured "duration" is that of the occupied state (binding is rate-limiting (Adams, 1977*a*)) and that the conformational change occurs with an as yet undetected rapidity. In this case, the observed rate constant, even in the limit of low drug concentration, provides only an estimate of the mean duration of the total occupied state (i.e., occupied but closed + occupied and open). Also, the actual conductance of a single open channel is underestimated for any agonist by the factor $(\alpha+\beta)/\beta$. This means that differing agonist efficacies could be explained solely on the basis of the equilibrium fraction $(\beta/(\alpha+\beta))$ of channels opened by a saturating concentration of the drug. Hence, there would still be only one "open" state available to the channel.

A conventional bimolecular encounter between agonist and receptor binding site is absent when TMB is bound covalently to a receptor sulfhydryl. This, combined with our observation that the relaxation rates for the TMB- and the ACh-activated channel are similar, might lead one to conclude that a conformational change of the sort envisaged by Magleby and Stevens must be rate-limiting in both cases. In our view, this conclusion is not automatic. Although the conventional binding/unbinding step has been eliminated, a new intramolecular transformation (rotation of covalently bound TMB into and out of the ACh binding site) has been introduced, and only when this new step is demonstrated to be in rapid equilibrium relative to the subsequent conformational change will the latter have been proved to be rate-limiting. We consider it probable, however, that the receptor anionic locus, being within a molecular distance of the point of covalent attachment of the TMB moiety, experiences an agonist "association" rate similar to what it would experience were the endplate exposed to very high concentrations of a reversible activator[1]. Bimolecular rate constants for the binding

[1] Assuming a TMB moiety is bound to a planar surface and can rotate in all directions, it would sweep out a hemisphere of volume $(2/3)\pi r^3 = 1527$ Å^3 (where we take $r = 9$ Å, the approximate maximum length of the trimethylammonium benzoyl group). One molecule per 1527 Å^3 is the equivalent of a free drug concentration of about 1 M.

of small ligands to proteins are commonly quite large, on the order of 10^6-10^8 M^{-1} sec^{-1} (Peller & Alberty, 1961; Bloomfield, Peller & Alberty, 1962; Eigen & Hammes, 1963). This is close to, but slightly below, the practical upper limit of about 10^9 M^{-1} sec^{-1} for a diffusion-controlled reaction (Burgen, 1966). Whether or not such rates apply to the rotational movement of TMB about its point of covalent attachment is presumably dependent on the degree of complementarity between covalently attached ligand and receptor binding site. The steric factors would have to be exceedingly unfavorable to shift this "association" rate into the range of relaxation times (i.e., $0.001-0.01$ sec) observed in this study. A more likely alternative is that the relaxation times observed here reflect conformational transitions in the drug-receptor complex.

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