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Voltage-Dependence of Drug-Induced Conductance in Frog Neuromuscular Junction

(acetylcholine/endplate/relaxation/noise analysis)

ERWIN NEHER AND BERT SAKMANN

Max-Planck-Institut für biophysik. Chemie, D 34 Göttingen, West Germany

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ABSTRACT **Membrane currents from voltage-clamped frog muscle fibers were recorded during iontophoretic application of steady doses of carbachol, acetylcholine, and suberyldicholine to the endplate region. In the presence of these drugs, an exponentially relaxing current was observed after step changes of membrane potential. The time constant of relaxation was found to be voltage-dependent. It was equal to the time constant obtained from the autocorrelation function of drug-induced conductance fluctuations measured under similar conditions. Analysis of instantaneous currents at the on- and offsets of voltage-clamp pulses showed that there is no shift in equilibrium potential during the pulses.**

Transmitter-induced current flow across subsynaptic membranes is generally assumed to consist of a superposition of many contributions from individual ionic channels (1). These channels can exist in open or closed conformations. For slowly varying concentrations of iontophoretically applied transmitter, an equilibrium is established between both states, which, in its simplest form, is described analogous to a molecular transformation:

$$\mathrm{TR} \underset{k_b}{\overset{k_f'}{\rightleftarrows}} \mathrm{TR}^*. \qquad [1]$$

This scheme denotes that a transmitter-receptor complex TR undergoes a transformation to an activated—or conducting—state TR* with a forward rate constant k_f' and a backward rate constant k_b. The prime in k_f' was added to indicate that this rate constant may be different from the true rate constant because of a preceding reaction such as association of transmitter and receptor. More complicated reaction schemes specifying these processes have been discussed in the literature (1–3).

In this scheme the experimentally observed current is given by

$$I = [\mathrm{TR}^*] \cdot \gamma \cdot (V - V_{\mathrm{equ}}) \qquad [2]$$

where V and V_{equ} are membrane potential and equilibrium potential, respectively, and γ is the conductance of the unit channel. The statistical superposition of the opening and closing of channels generates membrane noise which has been analyzed by Katz and Miledi (1) and Anderson and Stevens (4). Noise analysis by means of the power spectrum or the autocorrelation function yields the amplitude of the elementary conductance step γ and a time constant τ, which is given by (5):

$$\tau = \frac{1}{k_f' + k_b}. \qquad [3]$$

Anderson and Stevens (4) gave reasons to assume that in this expression, k_f' is small in comparison to k_b such that $\tau = 1/k_b$, which by definition of k_b is equal to the mean lifetime of the open channel. Relation [3] also holds for the time constant of the relaxation, which would be obtained when the equilibrium of the reaction [1] was changed by a steplike external perturbation (5–7).

There are two experimental facts indicating that the equilibrium between open and closed states at frog muscle endplates is voltage-dependent: (*a*) voltage clamp records of endplate currents show voltage-dependent time constants of current decay (8–10), and (*b*) analysis of the above mentioned voltage—or current—fluctuations reveal voltage-dependent time constants (4).

For hyperpolarized membranes these observations can be interpreted as either the result of an increased lifetime of the open ionic channel or equivalently as a decreased backward rate constant k_b in scheme [1]. With the application of a voltage step to a chemically excited membrane, an instantaneous increase in current should be seen, which is proportional to the number of open channels immediately preceding the step. Thereupon, the number of open channels should increase exponentially to a new equilibrium with a time constant τ given by [3]. Concomitantly, the current should increase exponentially with this time constant. According to the above outlined reaction scheme, the equilibrium between open and closed channels is described by

$$[\mathrm{TR}^*] = \frac{N}{1 + 1/K} = N \cdot \frac{k_f'}{k_f' + k_b} \qquad [4]$$

where N is the total number of available receptors and $K = k_f'/k_b$ is the equilibrium constant of the reaction. Since $1/k_b$ [or more accurately $1/(k_f' + k_b)$] changes with voltage, [TR*] should also change with voltage, except for the unlikely case that k_f' and k_b change exactly by the same relative amount. Similar reasoning led Adams to investigate conductance kinetics during pulse experiments by extracellular focal recording (11).

In the experiments reported, we have observed a relaxation of the membrane conductance with a time constant similar to the one found by noise analysis.

METHODS

Experiments were done at endplates of cutaneous pectoris muscle fibres of the frog *Rana esculenta* in standard Ringer's solution (12). Conventional voltage-clamp techniques em-

ploying two microelectrodes were applied (13). Current was measured by an operational amplifier which supplied a virtual ground to the Ringer's bath.

Since only the immediate neighborhood of the clamp electrodes is under space clamp condition, application of the drug has to be restricted to this area. This was achieved by iontophoresis (14). Iontophoresis current was adjusted manually to give drug-induced current plateaus of 20–80 nA amplitude and 20–40 sec duration.

In the case of voltage step experiments, charging of membrane capacitance at distant membrane regions led to slow current tails, prolonging the capacitive transients. In many cases, these transients were of the same order of magnitude and duration as the expected current changes. In order to correct for these effects, we gave identical pulses to the membrane immediately before, during, and after drug application. Current responses before application were subtracted from those during application, which eliminated contributions from leakage conductance and most of the capacitive transients (10). This procedure relies on the assumption that capacitive transients are not changed by the presence of the drug. In order to test this point, we determined the range of action of iontophoretically applied transmitter to ascertain that it only acts in the space-clamped region. It turned out that standard doses were ineffective when the iontophoresing electrode tip was more than 150 μm away from the muscle fiber. Secondly, we checked whether the voltage signal inside a muscle fiber, 200 μm away from the measuring electrode of the clamp, was affected by a standard iontophoresis experiment. Changes in voltage signal (pulse amplitude) during acetylcholine action, as measured by a third independent electrode, were 2 ± 2 percent (mean ± SD). This result justifies the use of the subtraction procedure, which especially reduced the low frequency components of the transients that originate at more distant parts of the muscle fiber. Since the above mentioned control experiments show that the voltage signal at remote membrane areas is not different in runs made during drug application and controls, it follows that the capacitive transients are very similar.

Difference curves were also calculated between control runs before and after drug application so that we could estimate the influence of irreversible and random changes during applications (changes in leakage conductance, pulse parameters, etc.). These difference traces were always small in amplitude compared to normal runs; capacitive transients were shorter than 0.3 msec, and any other contributions (difference in leakage) were strictly ohmic.

Differences between current traces were calculated by digitalization and point by point subtraction on a Nicolet, model 1074 Instrument computer. Normally four to eight sweeps were averaged.

We used a Saicor model SAI 42a auto- and crosscorrelation analyzer for noise analysis of current records in order to compare time constants from voltage step experiments with those obtained from noise analysis. Autocorrelation was preferred to spectrum analysis because time constants and noise variances can be obtained in a more convenient way (15).

Representative values for amplitude γ and life time τ of acetylcholine induced channels found by correlation analysis are (mean ± SE): γ = 23 ± 0.2 pmho (picosiemens), 28 experiments; τ = 0.95 ± 0.05 msec, 11 experiments at −80 mV, 20°. These compare favorably with values (τ = 1.04 msec) reported by Katz and Miledi (17) and Colquhoun *et al.** [γ = 22 pmho (picosiemens); τ = 1.2 msec].

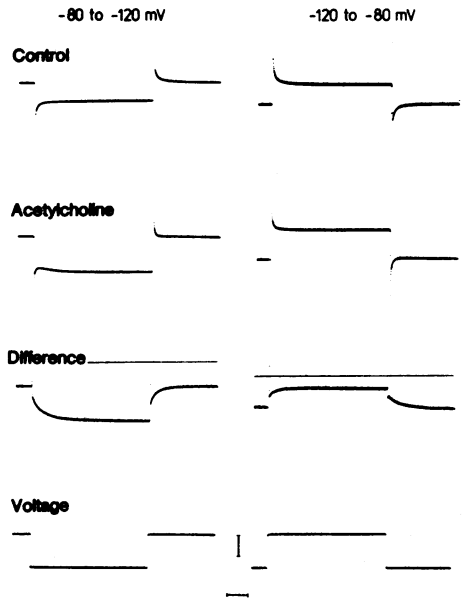

FIG. 1. Voltage clamp currents recorded during step changes of membrane potential at neuromuscular junction in the absence and presence of acetylcholine. *Membrane voltage* (lowest trace in both columns) was stepped from a holding potential of −80 mV to −120 mV in the left column. Depolarizing steps from −120 mV to −80 mV shown in the right column, are for same muscle fiber as in the left column. The membrane potential was kept for 60 msec at the new potential and then was stepped back. *Control*, before application of acetylcholine; *acetylcholine*, during acetylcholine action; *difference*, difference between voltage clamp currents in the presence and absence of acetylcholine (see *Methods*). The lines above the difference traces indicate zero level for acetylcholine-induced current. Horizontal bar: 10 msec; vertical bar: 25 mV for voltage trace, 50 nA for the upper traces in each column, and 25 nA for the traces marked *Difference*. Temperature was 8°. Time constants as determined from logarithmic plots were: left column: τ_{120} = 6.5 msec, τ_{80} = 3.5 msec; right column: τ_{120} = 6 msec, τ_{80} = 3.5 msec.

RESULTS

Current measured in response to a 60 msec voltage step from −80 mV to −120 mV membrane potential before and during application of acetylcholine is shown in Fig. 1 (left column). This figure also shows the difference between both traces. In a second experiment, on the same fiber, the membrane potential was stepped to −80 mV from a holding potential of −120 mV. The corresponding sequence of current traces is shown in the right column of Fig. 1. As expected, there was an instantaneous rise in difference current that was proportional to the conductance immediately before the pulse. Thereupon, current relaxed exponentially to a new steady state. In both experiments, the time constant of the relaxing current was slower at −120 mV as compared to −80 mV. Comparison of both difference traces in Fig. 1 shows that the time constants at −120 mV are the same for steps in the hyperpolarizing and depolarizing direction. The time constants from the relaxation experiment illustrated in Fig. 1

* Values from Colquhoun *et al.* (16) were recalculated to 20°.

TABLE 1. *Comparison of τ-values (mean ± SE)**

Agonist	Temp., °C	Membrane potential (−80 mV)		Membrane potential (−120 mV)	
		τ_{noise} (msec)	$\tau_{relax.}$ (msec)	τ_{noise} (msec)	$\tau_{relax.}$ (msec)
Carbachol	18	0.4 ± 0.2	<0.65	0.55 ± 0.2	<0.9
Acetylcholine	8	3.2 ± 0.2	3.6 ± 0.2	4.4 ± 1	5.3 ± 0.5
Acetylcholine	18	1.1 ± 0.1	1.4	1.4 ± 0.1	2.8
Suberyldicholine	8	5.3 ± 0.3	6.6 ± 0.6	10.9 ± 3	13 ± 1
Suberyldicholine	18	3.5 ± 0.2	3.1 ± 0.5	4 ± 0.7	5 ± 1
Suberyldicholine†	8	24 ± 11	23 ± 3	26 ± 8	40 ± 3

* Not all values shown in Fig. 3 are listed.
† Experiments on denervated, hypersensitive muscle fibers.

agree with those derived from noise analysis within 20%. In some fibers, however, additional slow processes were present, which made it necessary to use inclined base lines for the exponential curve fitting. For an example see Fig. 2, carbachol.

Pulse and noise experiments were done at different temperatures (8–9° and 18–19°) and different potentials (−80 and −120 mV), by applying the agonists acetylcholine, suberyldicholine, and carbachol. Finally experiments were performed both on normal endplates and at locations away from the endplates on muscles that had been denervated for more than 40 days. The observed relaxation time constants and the time constants obtained from noise analysis have the following properties in common: (*a*) temperature dependence corresponding to an activation energy of 1.7 kcal (7.1 kJ)/mol (4), (*b*) strong voltage dependence (4), (*c*) different time constants obtained for different agonists (16, 17), and (*d*) slower time constants are found in denervated muscle fibers than in normal fibers (1). Values of time constants under different conditions are given in Table 1. Representative examples for current relaxations are shown in Fig. 2.

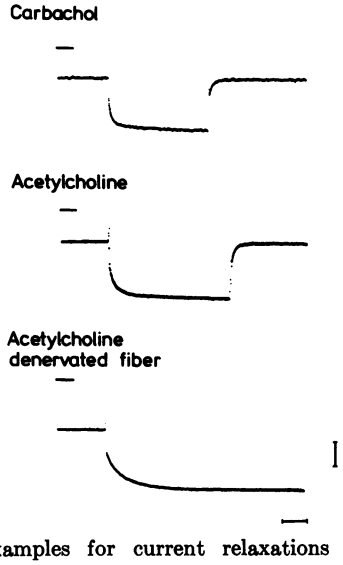

FIG. 2. Examples for current relaxations following step changes in membrane potential from −80 mV to −120 mV. Each curve was obtained by the subtraction procedure illustrated in Fig. 1. Zero level for agonist-induced current is indicated by a small horizontal bar above each trace. *Upper trace:* current relaxation during application of carbachol. In addition to the relaxing current, a slower inward component is present. *Middle trace:* current relaxation during acetylcholine application. Same muscle fiber as in upper trace. *Lower trace:* current relaxation during acetylcholine application to hypersensitive extrasynaptic membrane of a fiber that had been denervated for six weeks. Horizontal bar: 20 msec; vertical bar: 12.5 nA for carbachol; 25 nA for acetylcholine and acetylcholine—denervated fiber. Temp. was 8°.

It should be noted that the time constants under different conditions change over two orders of magnitude and that these changes occur in parallel for noise and pulse analysis. Time constants as derived from pulse experiment are plotted against time constants derived from noise analysis in Fig. 3. A strong correlation between both quantities is obvious. All values from denervated muscle are larger by a factor of 3 to 5 as compared with their corresponding endplate values (see also Table 1).

Besides the evaluation of time constants, valuable information about the voltage dependence of reaction [1] can be obtained when the relaxation amplitudes are analyzed.

At the instant of a steplike voltage change from voltage V_1 to voltage V_2, an instantaneous current I_{inst} will be measured:

$$I_{inst} = [TR^*]_1 \cdot \gamma \cdot (V_2 - V_{equ}) = N \cdot \gamma \cdot (V_2 - V_{equ}) \frac{k'_{f,1}}{k'_{f,1} + k_{b,1}}$$

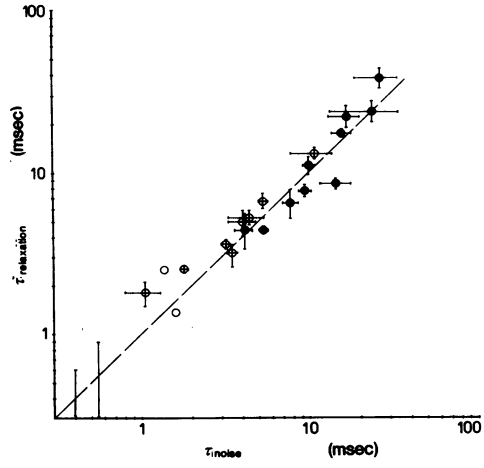

FIG. 3. τ-Values obtained by analysis of current fluctuations are plotted against corresponding values from current relaxation. Mean values found for different temperatures, different agonists, and membrane potentials in normal and denervated muscle fibers are shown. *Open circles* represent values obtained from measurements at the endplate region; *solid circles* are values found in denervated muscle fibers. Vertical and horizontal bars represent SE. Some of the data points are listed in Table 1.

where the subscript one refers to the number of open channels and to the rate constants immediately before the voltage step (at voltage V_1).

At equilibrium the current I_{equ} will be obtained which is:

$$I_{equ} = [TR^*]_2 \cdot \gamma \cdot (V_2 - V_{equ}) = N \cdot \gamma \cdot (V_2 - V_{equ}) \frac{k_{f,2}}{k'_{f,2} + k_{b,2}}. \quad [5]$$

The ratio of equilibrium and instantaneous currents is given by

$$\frac{I_{equ}}{I_{inst}} = \frac{k'_{f,2}}{k'_{f,1}} \cdot \frac{k'_{f,1} + k_{b,1}}{k'_{f,2} + k_{b,2}}. \quad [6]$$

The second part of this expression is known from the kinetic measurements since it is the ratio of time constants.

$$\frac{I_{equ}}{I_{inst}} = \frac{k'_{f,2}}{k'_{f,1}} \cdot \frac{\tau_2}{\tau_1}. \quad [7]$$

τ_1 and τ_2 are the time constants at voltage V_1 and V_2, respectively. Thus, the voltage-dependence of the forward rate constant (the quantity $k'_{f,2}/k'_{f,1}$) can be obtained by comparison of the quantities I_{inst}/I_{equ} and τ_1/τ_2, which are experimentally available. Quantities I_{inst}/I_{equ} were measured by extrapolation of observed time courses, back to the time of application of voltage steps. For steps -80 mV to -120 mV, this ratio was 1.59 ± 0.04 (mean $\pm$ SE) when averaged over all our data. There were no systematic differences when different temperatures, different agonists, or normal as well as denervated muscles were used. Similarly, the comparison of values derived from steps -80 to -120 mV with those from steps -120 to -80 mV showed no systematic differences.

The average ratio of time constants τ_2/τ_1 was 1.63 ± 0.05; again there were no systematic differences under different conditions. From this, the ratio of forward rate constants $k'_{f,2}/k'_{f,1}$ is calculated to be

$$k'_{f,2}/k'_{f,1} = \frac{I_{equ}/I_{inst}}{\tau_2/\tau_1} = 0.98 \pm 0.06. \quad [8]$$

This shows that the forward rate constant k_f' in scheme [1] is only negligibly voltage-dependent and that the observed voltage-dependence of the equilibrium originates predominantly from a voltage-dependent backward rate k_b.

Comparison of current values, before a step-like perturbation, with the instantaneous current after the step, yields the slope of the instantaneous current voltage relation. Assuming ohmic behaviour, the equilibrium potential for the transmitter-induced conductance can be obtained.

Equilibrium potentials calculated in this way scatter for different preparations, since small errors in the instantaneous current values, obtained by extrapolation to the time of step-change, result in large changes in V_{equ}. The mean value from 57 experiments with acetylcholine at 8° was $+2 \pm 12$ mV (mean $\pm$ SD). No systematic differences between measurements at the beginning and end of a pulse were observed.

DISCUSSION

The experiments show that application of voltage steps to a chemically excited membrane yields the same kinetic information as the analysis of transmitter-induced conductance fluctuations. This result, which is predicted by a simple model, is a further indication substantiating the assumption that the process leading to membrane conductance may be described in terms of a simple molecular transformation (2–4, 9).

The method of applying voltage steps for evaluation of time constants has several advantages over noise analysis. Time constants can be obtained from lower doses and shorter application times of agonist; 10–20 nA of agonist-induced current, flowing for 5–10 sec, were sufficient for the evaluation of time constants. This compares to 50–80 nA flowing for 20–40 sec, which were normally required for noise analysis. This increased sensitivity of the method is of particular importance when membranes of low receptor density, such as extrasynaptic regions of denervated muscles, are to be investigated. The disadvantage of the method is that fast time constants (<1 msec) cannot be resolved, since they are obscured by capacitive transients.

Analysis of the relaxation amplitudes reveals that the voltage dependence of the process is a property of the backward rate constant and that the forward rate constant is only little voltage-dependent. This result is not dependent on assumptions about the shape of the instantaneous I–V-relation, since the corresponding terms cancel by forming the ratio in [6]. The voltage-dependence of k_b is the dominant reason for a pronounced curvature demonstrated for the I–V-relation of iontophoretically-induced currents (18, 19).

When considering our value for the equilibrium potential, it is important to recognize that this value is obtained by linear extrapolation from two points of the instantaneous I–V-relation. Any curvature (e.g., a curvature of the I–V-relation of single channels) will change the true equilibrium potential. More importance is attributed to the fact that the equilibrium potential does not change significantly during changes in current. This ensures that the observed time courses are not modified or caused by local accumulations or depletions of ions.

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