Desensitization Contributes to theSynaptic Response of Gain-of-Function Mutants of the Muscle Nicotinic Receptor

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Although the muscle nicotinic receptor (AChR) desensitizes almost completely in the steady presence of high concentrations of acetylcholine (ACh), it is well established that AChRs do not accumulate in desensitized states under normal physiological conditions of neurotransmitter release and clearance. Quantitative considerations in the framework of plausible kinetic schemes, however, lead us to predict that mutations that speed up channel opening, slow down channel closure, and/or slow down the dissociation of neurotransmitter (i.e., gain-of-function mutations) increase the extent to which AChRs desensitize upon ACh removal. In this paper, we confirm this prediction by applying high-frequency trains of brief (~1 ms) ACh pulses to outside-out membrane patches expressing either lab-engineered or naturally occurring (disease-causing) gain-of-function mutants. Entry into desensitization was evident in our experiments as a frequency-dependent depression in the peak value of successive macroscopic current responses, in a manner that is remarkably consistent with the theoretical expectation. We conclude that the comparatively small depression of the macroscopic currents observed upon repetitive stimulation of the wild-type AChR is due, not to desensitization being exceedingly slow but, rather, to the particular balance between gating, entry into desensitization, and ACh dissociation rate constants. Disruption of this fine balance by, for example, mutations can lead to enhanced desensitization even if the kinetics of entry into, and recovery from, desensitization themselves are not affected. It follows that accounting for the (usually overlooked) desensitization phenomenon is essential for the correct interpretation of mutagenesis-driven structure–function relationships and for the understanding of pathological synaptic transmission at the vertebrate neuromuscular junction.

INTRODUCTION

As is the case for most other neurotransmitter-gated ion channels, the desensitized state of the muscle nicotinic acetylcholine receptor (AChR) is the most stable allosteric form for the fully liganded (i.e., diliganded) receptor (Katz and Thesleff, 1957; Karlin, 1967; Edelstein and Changeux, 1998). This “state” comprises a number of kinetically distinguishable protein conformations (Heidmann and Changeux, 1980; Neubig and Cohen, 1980; Reistetter et al., 1999; Elenes and Auerbach, 2002) in which the channel is ion impermeable (like in the closed state), and ACh is bound with high affinity (like in the open state). Indeed, when AChRs are exposed to a step change in ACh concentration from zero to saturating, the increase in the current is only transient, reflecting the initial channel opening followed by entry into the more stable, desensitized state (Katz and Thesleff, 1957; Magleby and Pallotta, 1981; Cachelin and Colquhoun, 1989; Dilger and Liu, 1992; Franke et al., 1993). Thus, under the steady presence of saturating concentrations of ACh, nearly all AChRs are desensitized (Sakmann et al., 1980). However, the particular time course of ACh in the synaptic cleft, combined with the particular kinetics of the wild-type AChR, results in the AChR not normally accumulating in desensitized states.

It is probably a reasonable approximation to assume that, during normal neuromuscular transmission, AChRs are alternatively exposed to millimolar and nearly zero levels of ACh as cycles of neurotransmitter release and removal take place. Typically, each “pulse” of millimolar ACh lasts only a few hundred microseconds (Magleby and Stevens, 1972; Wathey et al., 1979; Land et al., 1981; Dudel et al., 1999), whereas the duration of the intervening, interpulse intervals is ~10 ms or longer (from the notion that, in mammals, the firing frequency of fast motor units rarely exceeds ~100 Hz; Hennig and Lømo, 1985). Although each pulse of millimolar ACh is only a few hundred microseconds long, most AChRs are expected to be open and bound to two molecules of ACh by the end of the pulse. Because diliganded wild-type AChRs desensitize relatively slowly, though, entry into desensitization (and the accompanying current decay) is minimal during ACh pulses.

During interpulse intervals, however, the endplate current through wild-type AChRs decays completely,
following a nearly monoexponential time course (Magleby and Stevens, 1972). Inspection of the kinetic scheme in Fig. 1 suggests that the kinetics of this decay depend on the rate constants of interconversion among the different diliganded conformations, and on the rate constants of neurotransmitter dissociation from them (the concentration of neurotransmitter in the cleft becomes so low that its reassociation can be neglected). In the particular case of the muscle AChR, reopening of diliganded desensitized receptors (DA$_2$→OA$_2$ or DA$_2$→CA$_2$→OA$_2$; Fig. 1) is much slower than ACh dissociation from them (DA$_2$→DA; Franke et al., 1993).

As a result, diliganded receptors oscillate a few times between the open and closed conformations until the desensitized state is entered (most frequently through an OA$_2$→DA$_2$ transition; Auerbach and Akk, 1998) or ACh unbinds from the closed or the open states (followed by closure; Grosman and Auerbach, 2001). Indeed, using the scheme in Fig. 1, and under the condition that the probability of the channel being open while fluctuating between the OA$_2$ and CA$_2$ states is close to unity (which is the case for the wild-type and mutant AChRs studied here), it can be calculated that the time course of the current decay upon stepping the ACh concentration to zero is dominated by a single exponential component, in agreement with experimental observations (Fig. 2 A). Further, it can be shown that the time constant of this component (the exact value of which is given by one of the eigenvalues of the minus Q matrix corresponding to the scheme in Fig. 1) is very well approximated by an extremely simple analytical expression (Grosman and Auerbach, 2001; see red arrows in Fig. 1):

$$\tau_{\text{deactivation}} = \frac{1}{D_+ + 2j_- + \frac{\alpha_22k_-}{\beta_2 + 2k_-}}. \quad (1)$$

This time constant is usually referred to as the deactivation time constant and, in the particular context of the neuromuscular junction, is also referred to as the time constant of the endplate current decay. Interestingly, this macroscopic quantity can also be obtained from single-channel recordings performed at very low concentrations of neurotransmitter, as the time constant of the slowest component of the closed=→open burst length distribution (Fig. 2 B and Fig. 3; Grosman and Auerbach, 2001; for the underlying theory, see Colquhoun et al., 1997; Wyllie et al., 1998).

The relative contributions of entry into desensitization, ACh dissociation from the closed state, and ACh dissociation from the open state (followed by closure) to the current decay upon ACh removal depend, as expected, on the particular values of the rate constants in the pertinent kinetic scheme. In the framework of the model in Fig. 1, the probability of the AChR entering a desensitized conformation during deactivation can be calculated as

$$\text{Probability of desensitizing} = \frac{D_+}{D_+ + 2j_- + \frac{\alpha_22k_-}{\beta_2 + 2k_-}}. \quad (2)$$

Thus, using Eq. 2 and wild-type values for the adult form of the receptor (i.e., $D_+ = 25$ s$^{-1}$, $k_- = 20,000$ s$^{-1}$, $j_- = 12$ s$^{-1}$, $\beta_2 = 50,000$ s$^{-1}$, $\alpha_2 = 2,000$ s$^{-1}$), it can be calculated that the $\alpha_22k_-/(\beta_2 + 2k_-)$ term in Eq. 2 is so large ($\sim 900$ s$^{-1}$) that the extent of entry into desensitization during channel deactivation is small ($\sim 0.03$ after each individual ACh pulse). Similarly, the contribution of ACh dissociation from the open state to the deactivation time course can be calculated to be small in the wild-type AChR. This is entirely consistent with the well-established notion that, in normal endplates, it is the ACh dissociation from the closed state that terminates most bursts of diliganded closed=→open isomerizations and, therefore, that the deactivation time constant is, roughly, the reciprocal of $\alpha_22k_-/(\beta_2 + 2k_-)$ (Colquhoun and Hawkes, 1982).

However, the contribution of desensitization to channel deactivation is expected to increase as the $\alpha_22k_-/(\beta_2 + 2k_-)$ term in Eq. 2 is reduced by, say, mutations (Grosman and Auerbach, 2001). That is, not only changes in the kinetics of entry into desensitization, but also changes in the kinetics of gating and neurotransmitter dissociation are expected to affect the contribution of desensitization to the current decay that occurs upon stepping the ACh concentration to zero. Many mutations,
both lab-engineered and naturally occurring, decrease this term. These are usually referred to as gain-of-function mutations because they slow down the time course of deactivation (i.e., they increase τ_deactivation; see Eq. 1). Gratifyingly, the prediction of an increased extent of desensitization during deactivation, as a result of gain-of-function mutations, was fully borne out by our experimental observations.

MATERIALS AND METHODS

Heterologous Expression and Mutagenesis

HEK-293 cells were transiently transfected with mouse muscle AChR complementary DNA (provided by S.M. Sine, Mayo Clinic, Rochester, MN), using a calcium-phosphate precipitation method (Purohit and Grosman, 2006). Mutations were engineered with a QuikChange site-directed mutagenesis kit (Stratagene) and were confirmed by dideoxy sequencing. All mutant δ subunits were co-expressed with wild-type α, β, and ε AChR subunits. For all macroscopic current recording experiments, cells were grown on poly-l-lysine–coated coverslips, whereas, for single-channel recordings, cells were grown on either coated coverslips or directly onto 35-mm plastic culture dishes.

Solution Exchange and Patch-Clamp Recordings

Step changes in the ACh concentration applied to outside-out patches were achieved by the rapid switching of two solutions flowing from either barrel of a piece of theta-type capillary glass tubing (Hilgenberg), essentially as described by Jonas (Jonas, 1995). The theta tube was mounted on a piezo-electric device (Burleigh-LSS-3100; Exfo), the movement of which was controlled by a computer using a Digidata 1322A interface (Molecular Devices) and pClamp 9.0 software (Molecular Devices). To minimize distortions in the time course of the solution exchange, the computer-generated rectangular waveforms (brief pulses, long pulses, and trains of brief pulses) were low-pass filtered (f_c = 125–150 Hz) before being applied to the piezo-electric device. The recording chamber (designed in-house) contained two compartments that could be isolated from one another by varying the total volume of solution in the chamber. One compartment was used for placing the coverslip with cells, whereas the other one was used for placing the theta tube and the patch pipette during recordings. The latter compartment was continuously perfused using a gravity-fed system, whereas the solutions flowing through the theta tube were pressure driven (ALA BPS-8, ALA Scientific Instruments). To estimate the time course of the solution exchange, the change in liquid junction potential was periodically measured with an open-tip patch pipette (Fig. 4). During experiments, the same KCl-based solution was used in the patch pipette, in both barrels of the theta tube, and in the gravity-fed perfusion; its composition was (in mM) 142 KCl, 5.4 NaCl, 1.8 CaCl_2, 1.7 mM MgCl_2, 10 mM HEPES/KOH, pH 7.4. In addition, during macroscopic current recordings, the solution flowing through one of the theta tube barrels also contained 100 μM ACh. During (steady-state) single-channel recordings, both in the cell-attached and outside-out configurations, the solution bathing the extracellular aspect of the patch also contained 30–100 nM ACh. Both macroscopic and single-channel currents were recorded with an Axopatch 200B amplifier (Molecular Devices) at −80 mV and room temperature (≈22°C), and were digitized at 100 kHz. Series resistance compensation was used and set to 70–80% during all macroscopic recordings. The effective bandwidth before data analysis was DC-10 kHz for macroscopic currents, and DC-20 kHz for single-channel currents.

Data Analysis

Macroscopic currents were analyzed using a combination of pClamp 9.0 (Molecular Devices), SigmaPlot 7.101 (SPSS Inc.), and in-house developed programs, whereas single-channel currents were analyzed using the SKM-MIL combination in QuB software (Qin and Auerbach, 1996; Qin, 2004; www.qub.buffalo.edu).

The expressions describing the time courses of the different states in Fig. 1 were obtained by numerically computing the eigenvalues and eigenvectors of the corresponding Q-matrix (using Maple 6 software, Maple Waterloo, Inc.), following the methods described by Colquhoun and Hawkes (1995). On the other hand, the expressions describing the time courses of the different states in the simplified scheme in Fig. 9 A (used for Fig. 6, Fig. 9 B, and Fig. 10, A–D) were obtained by analytically solving the corresponding set of differential equations.

Figure 2. AChR deactivation. (A) The kinetics of deactivation were estimated by exposing outside-out patches (∼80 mV) to ∼1-ms, 100 μM ACh pulses. Each plotted trace is the average response of a patch to 10 such pulses applied as a ∼1-Hz train. For clarity, only the responses of some of the studied constructs are shown. All traces were aligned and normalized for easier comparison, and their decaying phases were fitted (least-squares method) from the peak of the current until the end of the transient with single exponential components. The deactivation time constants, averaged over a number of patches per construct, are listed in Table I.
The slowest time constant values of single-channel closed→open burst-length distributions (Fig. 2 B) were computed from the estimates of transition rates with approximate allowance for missed events (Qin et al., 1996). In turn, these transition rates were estimated by fitting the rate constants of uncoupled-type kinetic models (Rothberg and Magleby, 1998) to the idealized sequences of dwell times using full maximum-likelihood methods (Qin et al., 1996). Bursts were defined as series of (one or more) openings separated by shuttings shorter than a critical time ($t_{\text{crit}}$). A separate $t_{\text{crit}}$ value was determined for each patch, on the basis of the corresponding shut-time distribution, using the criterion proposed by Jackson et al. (1983) with only minor modifications (Purohit and Grosman, 2006). Particularly in the case of the gain-of-function mutants, not only brief (a few tens of microseconds), but also longer (hundreds of microseconds) shuttings appeared to separate openings within a burst. These longer shuttings, which are too long to be ascribed to sojourns in the closed diliganded state, have been consistently observed in recordings from gain-of-function AChR mutants (Grosman and Auerbach, 2001). Although their origin is unclear, these shuttings were considered to be part of a burst when calculating $t_{\text{on}}$ values.

The kinetics of entry into desensitization of the different constructs are expressed in terms of desensitization half-times (Table I). These, in turn, were calculated from the parameters of mono- or double-exponential fits to the decaying phase of the macroscopic current response to step changes in the ACh concentration from 0 to 100 µM (Fig. 7 and Fig. 11 B). The use of these phenomenological “half-times” was necessary, here, because we needed to somehow compare the time courses of desensitization of the analyzed constructs. And although the wild-type AChR and some of the mutants desensitize following a monoexponential time course, others do so with double-exponential kinetics. Of course, when a time course is best described by a double-exponential function, nothing exact can be done to compare it with the kinetics of a monoexponential time course. We found, however, that the half-decay times ($t_{\text{1/2}}/\ln 2$, more precisely), obtained from the parameters of the double-exponential fits, provide an excellent approximation to the desensitization time course during the first tens of milliseconds. In other words, when we plot the experimentally obtained double-exponential desensitization decays and the monoexponential decays calculated from the respective $t_{\text{1/2}}$ values, we find a very close agreement between both curves during the first tens of milliseconds. After these initial milliseconds, the time courses deviate from one another, but it is these initial tens of milliseconds that matter in the context of the 50-Hz trains (i.e., 20-ms interpulse intervals) applied here. Hence, mono- and double-exponential desensitization time courses are compared using their corresponding half-times.

**RESULTS**

**Gain-of-Function Mutants of the Muscle AChR Desensitize during Channel Deactivation**

Although previous single-channel data recorded from a series of gain-of-function AChRs were consistent with the existence of pathways other than just ACh dissociation from the closed state for the termination of endplate
currents (Grosman and Auerbach, 2001), the specific suggestion that desensitization contributes to deactivation was based on rather indirect observations (i.e., kinetic analysis of single-channel currents). To define this issue in an unequivocal manner, we applied trains of brief (1 ms) ACh pulses (100 μM) at different frequencies to outside-out patches expressing wild-type or gain-of-function AChRs. In these experiments, receptor desensitization is expected to be manifest as a frequency-dependent decrease in the peak value of successive macroscopic current responses, as the availability of activatable receptors diminishes along the train.

Fig. 5 shows example traces recorded from eight gain-of-function adult-type mutants (Thr, Cys, Glu, Asp, His, Val, Asn, or Gln substituting for the wild-type Ser at position 12 of the δ-subunit M2 segment) exposed to 1, 50-Hz trains. These mutations affect mostly the kinetics of gating, speeding up channel opening and slowing down channel closure (i.e., the mutations increase βδ and decrease αδ; Fig. 1; Grosman and Auerbach, 2000, 2001). Traces recorded from the adult wild-type AChR (i.e., containing the ε subunit), as well as from its fetal counterpart (containing the γ subunit, instead of ε), and the Ala and Gly substitutions at δ-M2 12’ (both with little effect on gating), are also shown in this figure. The plots are arranged, from δS268A to δS268Q, in increasing order of deactivation time constant (Table I). Fig. 6 shows the average response of patches expressing each of these constructs.

Together, these results lend support to the hypothesis that gain-of-function mutations increase the probability of the AChR entering a desensitized conformation.

**Table I**

<table>
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<tr>
<th>Construct</th>
<th>τ&lt;sub&gt;deactivation&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>τ&lt;sub&gt;slowest&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>τ&lt;sub&gt;desensitization&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>τ&lt;sub&gt;recovery&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
<th>τ&lt;sub&gt;recovery&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td>δS268A</td>
<td>0.72 ± 0.02 ms</td>
<td>0.95 ± 0.08 ms</td>
<td>34 ± 8 ms</td>
<td>ND</td>
<td>1,333 ms</td>
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<tr>
<td>wild-type/ε</td>
<td>0.99 ± 0.08 ms</td>
<td>1.20 ± 0.06 ms</td>
<td>26 ± 4 ms</td>
<td>306 ± 19 ms</td>
<td>500 (6)</td>
</tr>
<tr>
<td>δS268G</td>
<td>1.4 ± 0.1 ms</td>
<td>1.85 ± 0.09 ms</td>
<td>11 ± 4 ms</td>
<td>ND</td>
<td>286 (4)</td>
</tr>
<tr>
<td>wild-type/γ</td>
<td>4.6 ± 0.5 ms</td>
<td>5.7 ± 0.5</td>
<td>31 ± 12 ms</td>
<td>290 ± 33 ms</td>
<td>167 (4)</td>
</tr>
<tr>
<td>δS268T</td>
<td>7.5 ± 0.8 ms</td>
<td>11.6 ± 1.5 ms</td>
<td>91 ± 19 ms</td>
<td>ND</td>
<td>182 (5)</td>
</tr>
<tr>
<td>δS268C</td>
<td>8.0 ± 0.1 ms</td>
<td>10.33 ± 0.09 ms</td>
<td>72 ± 6 ms</td>
<td>478 ± 25 ms</td>
<td>444 (4)</td>
</tr>
<tr>
<td>δS268E</td>
<td>12.4 ± 0.5 ms</td>
<td>13.2 ± 0.2 ms</td>
<td>45 ± 10 ms</td>
<td>ND</td>
<td>500 (4)</td>
</tr>
<tr>
<td>δS268D</td>
<td>15.4 ± 0.1 ms</td>
<td>26 ± 2 ms</td>
<td>92 ± 25 ms</td>
<td>ND</td>
<td>1,000 (5)</td>
</tr>
<tr>
<td>δS268H</td>
<td>16 ± 2 ms</td>
<td>23 ± 3 ms</td>
<td>30 ± 6 ms</td>
<td>ND</td>
<td>364 (5)</td>
</tr>
<tr>
<td>δS268V</td>
<td>18.7 ± 0.3 ms</td>
<td>20.1 ± 0.6 ms</td>
<td>104 ± 19 ms</td>
<td>316 ± 16 ms</td>
<td>400 (2)</td>
</tr>
<tr>
<td>δS268N</td>
<td>24 ± 2 ms</td>
<td>44 ± 4 ms</td>
<td>70 ± 13 ms</td>
<td>497 ± 25 ms</td>
<td>500 (7)</td>
</tr>
<tr>
<td>δS268Q</td>
<td>28 ± 1 ms</td>
<td>36 ± 2 ms</td>
<td>42.1 ± 0.1 ms</td>
<td>ND</td>
<td>333 (2)</td>
</tr>
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ND, not determined.

<sup>a</sup>Deactivation time constants estimated from monoexponential fits. The numbers in parentheses indicate the number of <1-Hz trains of 1-ms, 100 μM ACh pulses analyzed. Each train consisted of at least 10 such pulses, the responses to which were averaged and fitted. Average values across trains are given as mean ± standard error.

<sup>b</sup>Time constants of the slowest component of single-channel burst length distributions. The numbers in parentheses indicate the number of patches (both cell-attached and outside-out) analyzed.

<sup>c</sup>Desensitization half-times. In some cases, the time course of entry into desensitization was best fitted with two (rather than one) exponential distributions. The numbers in parentheses indicate the number of patches analyzed to generate the plots in Fig. 8 B.

<sup>d</sup>Recovery-from-desensitization time constants estimated from the response to paired-pulse protocols. The numbers in parentheses indicate the number of patches analyzed to generate the plots in Fig. 8 B. Recovery-from-desensitization time constants estimated from the fitting of the mechanism in Fig. 9 A directly to plots of normalized peak current vs. pulse number (Fig. 6). The numbers in parentheses indicate the number of responses analyzed to generate these plots.

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**Figure 4.** Calibration of the solution-switching system. The different parameters of the solution-switching system (i.e., diameter of the theta tube openings, relative positioning of the theta tube and patch pipette, flow rate of solutions, bandwidth of the computer-generated waveform) were adjusted so as to optimize the time course of the solution exchange. The latter was estimated by measuring the liquid junction potential by alternatively exposing the theta tube openings, relative positioning of the theta tube and patch pipette, and flow rate of solutions, bandwidth of the computer-generated waveform. The calibration was based on rather indirect observations (i.e., kinetic analysis of single-channel currents). To define this issue in an unequivocal manner, we applied trains of brief (1 ms) ACh pulses (100 μM) at different frequencies to outside-out patches expressing wild-type or gain-of-function AChRs. In these experiments, receptor desensitization is expected to be manifest as a frequency-dependent decrease in the peak value of successive macroscopic current responses, as the availability of activatable receptors diminishes along the train.

The time constants were estimated from monoexponential fits. The numbers in parentheses indicate the number of patches (both cell-attached and outside-out) analyzed. In some cases, the time course of entry into desensitization was best fitted with two (rather than one) exponential distributions. The numbers in parentheses indicate the number of patches analyzed to generate the plots in Fig. 8 B. Recovery-from-desensitization time constants estimated from the response to paired-pulse protocols. The numbers in parentheses indicate the number of patches analyzed to generate the plots in Fig. 8 B. Recovery-from-desensitization time constants estimated from the fitting of the mechanism in Fig. 9 A directly to plots of normalized peak current vs. pulse number (Fig. 6). The numbers in parentheses indicate the number of responses analyzed to generate these plots.
during deactivation (Eq. 2). It is clear that kinetic mechanisms that ignore desensitization as a pathway for closed $\Rightarrow$ open burst termination cannot explain these findings. Moreover, the results in Figs. 5 and 6 are consistent with the assumption (Eqs. 1 and 2) that desensitization of the diliganded wild-type AChR can only proceed from the open state. Evidently, if desensitization proceeded only from the closed state (as suggested for other neurotransmitter-gated ion channels), the extent of desensitization upon neurotransmitter removal would not increase as the $\beta_2/\alpha_2$ ratio (see Fig. 1) does (i.e., in going from $\delta$S268A to $\delta$S268Q).

It could be argued that the behavior illustrated in Figs. 5 and 6 can also be due to other factors, in addition to the increase in the $\beta_2/\alpha_2$ ratio upon mutation (Eq. 2). Certainly, a faster rate constant of entry into desensitization and/or slower kinetics of recovery from desensitization upon ACh removal ($DA_2 \rightarrow DA \rightarrow D \rightarrow C$ or $DA_2 \rightarrow DA \rightarrow CA \rightarrow C$; Fig. 1) would contribute to the progressive depression of the macroscopic peak currents observed. And, although large changes in the kinetics of entry into desensitization were not evident in previous cell-attached single-channel recordings from these mutants (Grosman and Auerbach, 2001), the kinetics of recovery upon neurotransmitter washout cannot be studied during steady applications of neurotransmitter.

To study the effect of the tested mutations on the kinetics of entry into desensitization, we exposed outside-out patches to step changes in the concentration of ACh from 0 to 100 $\mu$M (Fig. 7), and analyzed the decaying phase of the resulting current transients (Table 1). The time constant(s) governing this current decay reflects the rate constant(s) of entry into desensitization without much interference from the kinetics of gating, reopening of desensitized receptors, or ACh binding and unbinding. This is because (a) the diliganded gating equilibrium constant values of the wild-type and mutant AChRs studied here are large ($>15$), (b) reopening of diliganded desensitized receptors is comparatively slow, (c) desensitization of diliganded AChRs proceeds mainly from the open state, and (d) a value of 100 $\mu$M for the concentration of ACh is high enough to temporarily segregate the time course of ACh binding (which occurs, largely, during the rising phase of the transient) from that of desensitization (which occurs, largely, during the decaying phase). We can illustrate this point with a simple calculation. For example, using the full model of Fig. 1 and adult wild-type values (which include a value of $\sim 25$ s$^{-1}$ for the OA$_2 \rightarrow$ DA$_2$ rate constant and zero values for the other five rate constants of entry into desensitization), it can be calculated (using Q-matrix methods; Colquhoun and Hawkes, 1995) that upon stepping the concentration of ACh from 0 to 100 $\mu$M, the decaying phase of the current transient would be dominated by an $\sim 46$-ms time constant. Indeed, the value of this time constant is quite close to 40 ms, that is, the reciprocal of the OA$_2 \rightarrow$ DA$_2$ entry-into-desensitization rate constant. Of course, the use of ACh concentrations
$100 \mu M$ would be more effective at temporally separating ACh binding from desensitization (and would, thus, allow more accurate estimates of the entry-into-desensitization rate constant) but pore blockade ($K_{B, ACh} \approx 2.0 \text{ mM at } -80 \text{ mV}$) would, then, become a new problem. Therefore, sustained applications of $100 \mu M$ ACh seem appropriate to probe the effect of mutations, specifically, on the entry-into-desensitization rate constant (note that desensitization of activated receptors occurs as a single-step isomerization in this scheme) was calculated from the half-time values in Table I, as $\ln 2/t_{1/2}$. In turn, these half-times were calculated as explained in Materials and methods and in the legend to Table I. For those mutants displaying double-exponential desensitization time courses, the use of a single desensitization rate constant is, of course, an oversimplification. However, it can be shown that the monoexponential decays calculated using these half-times approximate very well the experimentally obtained double-exponential desensitization time courses during the first tens of milliseconds (see Materials and methods).

>100 $\mu M$ would be more effective at temporally separating ACh binding from desensitization (and would, thus, allow more accurate estimates of the entry-into-desensitization rate constant) but pore blockade ($K_{B, ACh} \approx 2.0 \text{ mM at } -80 \text{ mV}$) would, then, become a new problem. Therefore, sustained applications of $100 \mu M$ ACh seem appropriate to probe the effect of mutations, specifically, on the entry-into-desensitization rate constant of open, diliganded AChRs. The experimentally obtained values in Table I (expressed as desensitization half-times, as explained in Materials and methods) clearly show that, if anything, entry into desensitization is slower in these gain-of-function mutants, not faster.

The effect of the mutations on the kinetics of recovery, on the other hand, was assessed using pairs of conditioning (1 s) and test (100 ms) $100 \mu M$ ACh pulses separated by intervals of variable duration (Fig. 8 and Table I). The long duration and high ACh concentration during the conditioning pulse ensured that the macroscopic currents decayed almost completely before the ACh washout interval started. In this manner, entry into and recovery from desensitization are dissected, the former occurring during the conditioning pulse, and the latter occurring during the interval between the conditioning and test pulses. The important caveat here, though, is that the kinetics of recovery are known to depend on the duration of the conditioning pulse (Reitstetter et al., 1999; Elenes and Auerbach, 2002) and, thus, our particular paired-pulse protocol, using 1-s conditioning pulses, may not really have probed the kinetics of recovery that are relevant during a high-frequency train of brief ACh pulses. During such trains,
the neat separation between entry into desensitization and recovery is lost because both phenomena occur during the interpulse intervals, when the concentration of ACh is close to zero. The problem of estimating the kinetics of recovery within a train of neurotransmitter pulses is addressed in the sections below.

A Quantitative Description of the Response of the Muscle AChR to Trains of ACh Pulses

To gain a clearer insight into what happens during the deactivation phase of macroscopic currents upon repetitive exposure to ACh, we analyzed the kinetic scheme in Fig. 9 A, a simplified version of that in Fig. 1. In this reduced scheme, the various states in Fig. 1 are grouped into activated (CA₂ and O₂A), activatable (C and CA), and desensitized (D, DA, and DA₂) sets of states. Furthermore, since unliganded and monoliganded open channels (O and OA) close very fast, they are considered to be part of the activatable pool of receptors, as well. One of the main advantages of using this simplified model is that the time courses of the three relevant sets of states upon stepping the ACh concentration to zero (i.e., activated, activatable, and desensitized) can be expressed as rather simple analytical expressions.

We can assume that, in the absence of ACh, all AChRs are closed and, therefore, activatable. Thus, upon stepping the ACh concentration from zero to a high-concentration value, most receptors become rapidly diliganded (i.e., activated), as they quickly bind the applied ACh. On termination of the pulse, activated channels keep oscillating between the diliganded closed and open conformations until they either lose the bound ACh (Activated → Activatable) or adopt a desensitized conformation (Activated → Desensitized). Desensitized receptors, in turn, can recover while the concentration of ACh remains at near-zero levels, thus joining the pool of activatable receptors (Desensitized → Activatable). Fig. 9 B shows the calculated time course of interconversion among the activated, activatable, and desensitized sets of states during one example interpulse interval for a hypothetical gain-of-function AChR. On arrival of the following pulse of ACh, activated receptors remain activated, desensitized receptors stay desensitized and become fully liganded, and most activatable receptors become activated, as they bind the newly applied ACh.

Fig. 10 (A–D) shows the predicted effect of the different variables (i.e., deactivation time constant, rate...
constants of entry into and recovery from desensitization, and stimulation frequency) on the response of a hypothetical ensemble of channels to a train of neurotransmitter pulses. It can be seen that, at a given train frequency, a slower deactivation (Fig. 10 A), a faster entry into desensitization (Fig. 10 B), and a slower recovery (Fig. 10 C) all lead to a more profound depression of the macroscopic current response.

A salient feature of the response of multichannel outside-out patches to high-frequency repetitive stimulation is that the decline in the peak current values continues until a nonzero plateau level is attained (Figs. 5 and 6). This is compelling evidence that desensitized receptors recover appreciably during the 20-ms interpulse intervals. Indeed, if this recovery were negligible, then this decline would continue until no more currents can be elicited by subsequent pulses. Early in each train, the peak current values decrease because the number of receptors that desensitize exceeds the number of receptors that recover. However, the number of receptors that desensitize during interpulse intervals decreases, whereas the number of receptors that recover increases, along a train. Eventually, these two numbers become equal, and the peak current reaches a steady-state value. Fig. 10 (E and F) shows experimental data (of the kind shown in Fig. 6) recorded at different train frequencies from two of the gain-of-function mutants. Reassuringly, the observed depression of peak currents displays the frequency dependence predicted by Fig. 10 D. That is, as the frequency of the stimulation train increases, the steady-state peak current level decreases, eventually becoming equal to the steady-state current in response to a sustained application of neurotransmitter.

As mentioned above, when the response within a stimulation train is under study, the recovery that matters is the one occurring during the interpulse intervals. To estimate its kinetics in the different constructs, we “fitted” the experimental observations in Fig. 6 with the simplified mechanism in Fig. 9 A. We did this, essentially, as for Fig. 10, calculating the fraction of channels that become activated upon each consecutive pulse, using the analytical expressions for the time courses of the different sets of states. In this particular case, though, the rate/time constants of deactivation and entry into desensitization of each construct were taken from their experimentally estimated values (Table I), the interpulse interval was fixed at 20 ms, and the (unknown) value of the recovery rate constant (i.e., the rate constant governing the Desensitized→Activatable step in Fig. 9 A) was varied manually until a reasonably good fit (judged by eye) to the experimental data in Fig. 6 (black circles) was reached. The fits are shown with red circles in Fig. 6, and the estimated recovery rate constants are listed in Table I, expressed as their reciprocals (for easier comparison with the time constant estimates obtained from paired-pulse protocols). Somewhat unexpectedly, we found that the kinetics of recovery during interpulse intervals are not very different from those after 1-s exposures to 100 μM ACh. Perhaps, even longer applications of ACh (>1 s) are needed for the AChR to adopt the more reluctant, slower-to-recover conformations reported in the literature (Reitstetter et al., 1999).

A cursory inspection of Table I indicates that the tested mutations affect mostly the kinetics of deactivation; the kinetics of entry into and recovery from desensitization are affected to a much smaller degree. We conclude, then, that it is mainly the higher $\beta_2/\alpha_2$ ratio of these mutants that underlies the larger extent of desensitization (Eq. 2). In other words, the comparatively small depression of the macroscopic currents observed upon repetitive stimulation of the wild-type AChR is due, not to desensitization being exceedingly slow or to...
recovery being extremely fast but, rather, to its gating equilibrium constant not being large enough.

**DISCUSSION**

The main finding of this paper is that gain-of-function mutations increase the extent to which AChRs desensitize during deactivation. This is important because most of the mutations that have been engineered, thus far, to probe the relationship between structure and function of the muscle AChR lead to gain-of-function phenotypes, and the functional impact of these mutations is (in the best cases) inferred from steady-state kinetic modeling studies in which desensitization is usually ignored. One clear concept that emerges from this study is that, although this useful simplification is certainly valid in the wild type, the extent to which a diliganded closed ≡ open burst is curtailed by entry into desensitization is considerable in mutants of the gain-of-function type (Figs. 5 and 6). In other words, if it were not for desensitization, endplate currents through gain-of-function mutants would decay even more slowly. For example, in the case of the Gln mutant (δS268Q), it can be calculated (using Eq. 1 and the values in Table I) that, if desensitization did not contribute to channel deactivation, the deactivation time constant would be ≈52 ms,
almost twice as long as the measured value. Thus, the effect of desensitization on the observed channel kinetics should not be disregarded. Evidently, physically realistic conclusions (i.e., the only kind of conclusion that can help us understand how structure gives rise to function) can only be drawn in the framework of correct kinetic mechanisms. More generally, our results underscore the critical importance of complementing the insight provided by steady-state applications of agonist with that stemming from concentration-jump experiments.

Another reason why the finding we report here is important is that desensitization can limit the availability of activatable receptors during repetitive stimulation. Indeed, it is quite tempting to extrapolate our results to a more physiological situation and entertain the possibility that gain-of-function AChRs accumulate in desensitized conformations also in the context of an intact neuromuscular junction, in response to high-frequency action potential firing. Further, it is intriguing to ponder that this depression might contribute to the impaired neuromuscular transmission that characterizes the slow-channel congenital myasthenic syndrome.

However, it is important to realize here that, unlike the situation in the outside-out patch-clamp configuration, the quanta of neurotransmitter released by a presynaptic neuron do not impinge on exactly the same subset of postsynaptic receptors every time an action potential arrives. Therefore, any given receptor interacts with the neurotransmitter at only a fraction of the firing frequency and, eventually, only once per burst of action potentials. This is relevant because, as shown by the outside-out recordings in Fig. 10 (E and F), the extent of depression decreases as the frequency of stimulation is reduced. The question, then, arises as to how much lower this “effective frequency” gets to be.

The value of this effective frequency depends directly on the degree to which the areas of postsynaptic membrane covered by the neurotransmitter released in response to successive action potentials overlap. This, in turn, depends on the area of postsynaptic membrane covered by single synaptic vesicles (sometimes referred to, simply, as the “postsynaptic area”), the detailed morphology of the endplate, the quantal parameters, and the kinetics of vesicle-pool cycling. Thus, this frequency might be different for different species, and even for different muscle fibers of the same species.

The issue of the size of the postsynaptic area has been addressed in the past in the context of both the neuromuscular junction (Hartzell et al., 1975) and neuron–neuron synapses (Trussell et al., 1993; Otis et al., 1996; Barbour and Häusser, 1997; Tureček and Trussell, 2000; Chen et al., 2002; Pugh and Raman, 2005), and is key to any treatment of short-term depression of postsynaptic origin. The extent to which these areas overlap, however,
has remained elusive in the case of the vertebrate endplate. In the snake neuromuscular junction, Hartzell et al. (1975) have clearly shown that the postsynaptic areas corresponding to vesicles “recruited” by the same action potential overlap partially if the acetylcholinesterase (AChE) activity is inhibited, but a firm conclusion could not be drawn from their experiments for the situation in which AChE is fully active. Simulation studies addressing this particular issue have subsequently suggested that, under normal conditions (i.e., intact AChE activity), these areas do not overlap at all (Salpeter, 1987), as if the rapid hydrolyzing effect of AChE limited the lateral spread of the released ACh and completely isolated postsynaptic areas from one another. This would maximally reduce the frequency at which any given receptor interacts with ACh during repetitive stimulation. Although this is clearly a possibility, it would be desirable to address this crucial aspect of synaptic transmission directly, with experiments.

In summary, the data presented here support the well-established notion that, in a physiological context, wild-type muscle AChRs are largely unaffected by desensitization (Edmonds et al., 1995). Indeed, it seems that the behavior of this receptor would not be much different if desensitization did not occur at all. This apparent lack of physiological relevance for such a highly conserved functional property of a protein remains most puzzling. More importantly, our results provide compelling evidence that entry into desensitization contributes to the time course of deactivation in gain-of-function mutants of the muscle AChR. This raises the interesting possibility that, at mutant endplates, the availability of activatable receptors decreases along a train of repetitive stimulation. Experiments using approaches that mimic synaptic transmission more closely than the fast perfusion of outside-out patches used here are, undoubtedly, needed.

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