Dependence of Nicotinic Acetylcholine Receptor Recovery from Desensitization on the Duration of Agonist Exposure

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ABSTRACT

When subjected to prolonged exposure to nicotinic agonists, nicotinic acetylcholine receptors undergo desensitization, resulting in an inactive receptor that does not allow for the passage of ions. The induction of desensitization of diverse nicotinic acetylcholine receptor subtypes in muscle, ganglia, or brain is likely to play important modulatory roles in synaptic transmission. Furthermore, nicotinic receptor desensitization may contribute to behavioral changes in humans or animals subjected to prolonged nicotine exposure pharmacologically or through the use of tobacco products. We investigated the recovery from desensitization of muscle-type nicotinic acetylcholine receptors in TE671/RD cells induced by exposure to acetylcholine or nicotine. Rates of recovery from desensitization are dependent on the length of agonist exposure and on the agonist used to induce desensitization. Increasing the time of exposure results in an increase in the time constant of recovery for both agonists. The recovery from nicotine-induced desensitization is consistently faster than the recovery from acetylcholine-induced desensitization regardless of whether nicotine or acetylcholine is used to assess levels of desensitization. These findings suggest the existence of more than one state of receptor desensitization and that nicotinic agonists vary in their efficiency of inducing receptors to states of differing depths of desensitization.

Nicotinic acetylcholine receptors (nAChRs) represent a diverse family of ligand-gated ion channels (Lindstrom, 1996; Gotti et al., 1997; Lukas, 1998). They are involved in cholinergic transmission in the central nervous system, the peripheral nervous system, and at the neuromuscular junction. nAChRs are composed of five subunits that are arranged like the staves of a barrel surrounding a central pore through which cations can pass. So far, 16 different subunits have been identified, with a2 through a9 and b2 through b4 subunits being expressed in autonomic and/or central nervous systems, and a1, b1, g, e, and d subunits being expressed in muscle in a developmentally regulated fashion.

Extensive studies on the function and structure of nAChR channels have been done using muscle-type receptors from electric fish and preparations from the vertebrate neuromuscular junction. The phenomenon of nAChR desensitization was first described in muscle by Katz and Thesleff (1957). Resting nAChR channels open in response to agonist binding to allow the passage of ions. Prolonged presence of an agonist produces a desensitized state that no longer permits ion passage across the receptor channel. Recovery from this state occurs after the agonist has been removed. Desensitization can also occur without channel activation, thus bypassing the open state (Magleby and Pallotta, 1981; Changeux, 1990).

nAChR desensitization has been found to also occur for nAChRs located in neuronal tissues. Desensitization has been suggested to play a role in synaptic plasticity (Changeux and Heidmann, 1987; Galzi and Changeux, 1994) and in nicotine dependence in smokers (Lukas et al., 1996). Desensitization on prolonged exposure to nicotine, as a consequence of use of tobacco products, has been implicated in cognitive enhancement, relief of depression, anxiolysis, and other responses but also withdrawal symptoms and substance dependence in smokers (Lindstrom, 1996, Lukas et al., 1996). Furthermore, ion flux experiments suggest that nAChRs undergo a longer-lasting and slower-onset loss of function, termed “persistent inactivation”, and suggest that this is distinct from desensitization, when exposed to agonist for 1 to 24 h or longer (Lukas, 1991; Ke et al., 1998).

In the present study, we used whole-cell current recordings of muscle-type nAChRs (a1b2d2g2) in TE671/RD cells to investigate receptor desensitization as a function of agonist exposure time. Receptor currents were elicited with acetylcholine (ACh) to test for the action of a physiological agonist and with nicotine to test for the influence of a drug self-administered by smokers. In agreement with previous studies, our results indicate that the time needed for recovery from desensitization induced by exposure to either agonist increases with increased time of receptor exposure to agonist. Furthermore, rates of recovery from desensitization differ as a function of ligand used to induce desensitization.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine.
Materials and Methods

TE671/RD cells were maintained in high glucose Dulbecco’s modified Eagle’s medium containing 10% horse serum, 5% fetal calf serum, 0.05 mg/ml streptomycin, and 2.5 U/ml penicillin in 7% CO₂. Cells were passaged every 3 to 5 days at 60 to 70% confluence. For recordings, TE671/RD cells were plated on poly-L-lysine-coated coverslips, which formed the floor of a recording chamber (Warner Instruments, CT). The chamber was mounted on the stage of an inverted microscope and continuously superfused with bath solution (120 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 25 mM d-glucose, 10 mM HEPES, 0.001 mM atropine sulfate, pH 7.4).

Experiments were carried out at room temperature (20–22°C). Recording pipettes for whole-cell recordings were made from soft glass (75-μl micropipettes; VWR, OH; PG150T-10; Warner Instruments) in a two-stage puller (L/M-3P-A; List Medical). The tips were heat-polished and had a resistance of 1–4 MΩ (ments) in a two-stage puller (L/M-3P-A; List Medical). The tips were heat-polished and had a resistance of greater than 2 GΩ.

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Agonist-induced currents were elicited by the application of 1 mM ACh or nicotine in the bath saline. Agonists were applied by means of a U-tube application system (modified after Alkondon and Albuquerque, 1993) or a stepper motor-operated streaming filament application system (SF-77B Perfusion Fast Step, Warner Instruments). The streaming filament application system was used for the short application protocols (1 and 30 s) because agonist removal after the end of the desensitization pulse was not sufficiently rapid with the U-tube system. The latter was used in the long-lasting desensitization protocols (3 and 10 min). Agonist test pulses were 1 s in duration; agonist desensitization pulses for short-term exposure were either 1 or 30 s long. For longer term drug exposure (3 and 10 min), the agonist was applied in the superfusion bath solution. Agonist-evoked currents were recorded with an EPC-7 patch-clamp amplifier (List Medical), digitized online with a Digidata 1200 series A/D-board (Axon Instruments, Burlingame, CA), and stored on computer hard disk. The experiment protocol, data acquisition, and analysis were carried out using pClamp (version 6.0; Axon Instruments).

After the whole-cell clamp configuration was achieved, an initial desensitizing pulse (d) was delivered. This pulse was paired with a test pulse that followed the d pulse at various intervals. Subsequent desensitizing and test pulse pairs were given after complete desensitization was achieved. Each pair of current recordings, consisting of the desensitization pulse (which also elicited the initial maximum current response of the cell under investigation) and the test pulse, was analyzed for the peak current response. The extent of recovery from desensitization was expressed as the fraction of the test pulse response relative to the desensitization pulse (maximum) response. Mean values of fractional responses were plotted versus the interval time between desensitization and test pulses to show the time course of recovery. The data were best fitted with a single exponential. Data points were plotted and fitted using Prism (GraphPAD Software Inc., San Diego, CA). Differences in time constants for recovery from agonist exposure of a given duration across agonists were tested for statistical significance (p < .05) using unpaired t tests (Instat version 3.0; GraphPAD). Differences in time constants for recovery from agonist exposures of different durations were tested for significance using ANOVA (GraphPAD).

Results

A family of whole-cell current responses of TE671/RD cells to 1-s pulses of ACh is given in Fig. 1. The peak current from each trace was used to quantify the efficacy of agonist exposure, and the response of cells to an initial exposure to ACh (a desensitizing pulse; trace d) was used as a normalization control. If test pulses were given shortly after the desensitizing pulse, the peak current measured was markedly reduced (trace 1, 10-s interval between desensitizing and test pulses), demonstrating induction of nAChR desensitization. However, as the interval between desensitizing and test pulses increased, the peak current response to a test pulse increased (traces 2–5 for interpulse intervals of 40–200 s). Rates of recovery from desensitization were monitored by plotting peak current responses to test pulses normalized to the peak current response to the desensitizing pulse as a function of the interval between desensitizing and test pulses and for different durations of desensitizing pulses.

First, we followed the time-dependent recovery from desensitization after exposure of cells for 1 s to either 1 mM ACh or 1 mM nicotine (Fig. 2A). Receptors recovered completely from this short exposure with mean ± S.E.M. time constants of 31.5 ± 4.0 s for ACh-induced desensitization and 18.8 ± 2.6 s for nicotine-induced desensitization (Table 1). Next, we assessed whether recovery from desensitization depends on the duration of exposure to the agonist. Increasing the exposure time to 30 s resulted in a prolonged recovery phase from desensitization induced either by ACh or nicotine (Fig. 2B). Time constants for recovery were 112.8 ± 20.9 s for ACh-induced desensitization and 50.0 ± 5.4 s for nicotine-induced desensitization (Table 1). Recovery from desensitization, under these conditions, was faster for cells exposed to nicotine than for cells exposed to ACh.

Ion flux assays showed (Lukas, 1991; Lukas et al., 1996; Ke et al., 1998) that prolonged exposure (minutes to hours) to agonist induced a prolonged loss of nAChR function termed “persistent inactivation”. We investigated the recovery from desensitization of receptors after exposure to either ACh or nicotine for 3 or 10 min as a point of comparison. The time needed to carry out such experiments was well within the survival time of patched cells under investigation. After exposure to ACh or nicotine for 3 min, the recovery from desensitization had time constants of 8.9 ± 1.4 and 3.9 ± 0.7 min, respectively (Fig. 3A), considerably longer than the recovery times after shorter (seconds) agonist exposures. The previously established trend of ACh-induced desensitization requiring longer recovery periods compared

Fig. 1. Examples of whole-cell current recordings used to monitor responses of TE671/RD cells to 1-s pulses of 1 mM ACh. Current traces illustrate the recovery from ACh-induced desensitization. A response to a 1-s desensitizing pulse of ACh (trace d) is shown on the left followed by responses to test pulses of ACh applied 10 s (trace 1), 40 s (trace 2), 80 s (trace 3), 120 s (trace 4), or 200 s (trace 5) after a desensitizing pulse.
with nicotine-induced desensitization also occurred for these longer exposures (Table 1). Increasing the agonist exposure time to 10 min resulted in an even more prolonged recovery with a time constant of 16.9 ± 4.8 min for ACh-induced desensitization and a time constant of 4.7 ± 0.9 min for nicotine-induced desensitization (Fig. 3B, Table 1). Differences in recovery time constants with increasing duration of agonist exposure (Table 1) were statistically significant at the .002 level for ACh and at the .0003 level for nicotine (ANOVA). Differences in recovery time constants between ACh and nicotine for a given duration of treatment were also statistically significant (Table 1, column 4; t test).

These results show that recovery from agonist-induced desensitization depends on the duration of agonist exposure, as well as on the type of agonist that was used to induce desensitization. To test whether the agonist that induces desensitization determines recovery rates or whether recovery depends on the identity of the agonist used to elicit current responses during recovery, we used a double-pulse protocol. Receptors were desensitized by a 1-s application of one agonist, and recovery was monitored by the application of pulses using a second agonist. When receptors were desensitized by the application of 1 mM ACh for 1 s, followed by screening for recovery with pulses of 1 mM nicotine, the time constant for recovery (38.4 ± 4.2 s) was not statistically different from that derived using ACh in both desensitizing and test pulses (31.5 ± 4.0 s; Fig. 4, Table 1; unpaired t test, \( p = .26 \)). Similarly, desensitization of the receptors with 1 mM nicotine for 1 s, followed by screening for recovery with 1 mM ACh pulses, resulted in a time constant for recovery of 22.1 ± 2.4 s, which is not significantly different from that derived using nicotine for desensitizing and test pulses (18.8 ± 2.6 s; Fig. 4 and Table 1; unpaired t test, \( p = .37 \)). However, time constants are significantly or nearly significantly different, respectively, for the ACh—nicotine versus the nicotine—nicotine paradigm (\( p = .003 \)) or for the nicotine—ACh versus the ACh—ACh paradigm (\( p = .06 \)). Therefore, we conclude that recovery of nAChR channels depends both on the duration of agonist treatment and on identity of the agonist inducing desensitization but not on the identity of the agonist used in test pulses.

**Discussion**

When nAChRs are exposed to agonists for prolonged times, they enter an inactive, desensitized state from which they can recover on agonist removal. We show here that the time needed to recover from desensitization depends on the duration of agonist exposure. In addition, recovery from nicotine-induced desensitization occurs faster (about 2-fold) than recovery from ACh-induced desensitization when the desensitizing pulse duration is the same for both agonists. When receptor channels were desensitized by the application of one agonist followed by monitoring for recovery using a different agonist, the recovery rate depended on the agonist used for desensitization and not on the agonist used for monitoring recovery.

Our data obtained with whole-cell current recordings are in good agreement with ion flux measurements obtained from large populations of cells (Lukas et al., 1996; Ke et al., 1998). When the corresponding exposure and recovery times are compared, the fractional responses are very similar, indicating that both techniques yield similar results. Practical limitations of the whole-cell clamp recordings preclude a systematic study of the effects of agonist exposure exceeding about 10 min in duration. Ion flux studies show that after agonist exposure times of 24 h or longer, the time constant for recovery becomes very long and there is less-than-full recovery from desensitization (Boyd, 1987; Lukas, 1991). This virtually irreversible desensitization state has been suggested to result from receptor phosphorylation (Boyd, 1987, Hsu et al., 1997).

Single-channel recordings (Sakmann et al., 1980) and ion flux experiments (Boyd, 1987; Lukas, 1991; Lukas et al., 1996; Ke et al., 1998) indicate that receptors enter more than one desensitized state. Our results indicate that time constants for recovery from desensitization increase as a function of agonist exposure time. At agonist concentrations of 1 mM, the entire receptor channel population on the cell surface is expected to be activated (Dudel et al., 1992). Consistent with this expectation, maximal efficacy in acute assays of human muscle-type nAChR function in TE671/RD cells is observed at 1 mM ACh or nicotine (Lukas, 1989; Ke and Lukas, 1996); therefore, time-dependent fluctuations in the number of available receptor channels would.
Time constants for recovery from agonist-induced desensitization

Time constants for recovery from agonist exposure to 1 mM ACh or 1 mM nicotine are based on test pulses using the same agonist that was used for desensitization. For mixed agonist experiments, a different agonist was used for the test pulse than for the desensitization pulse. In this case, the time constants are listed under the agonist used for the desensitization pulse. Time constants were derived by fitting the data with a single exponential. Values are expressed as mean ± S.E. The number of cells per trial is given in parentheses. The \( p \) values are for statistical comparisons at the indicated exposure time between ACh and nicotine applied during desensitization pulses or for the 1-s nicotine—ACh and 1-s ACh—nicotine comparison \( (t \) test). ANOVA analyses for a given agonist across exposure durations indicate that the time constant for any duration of agonist exposure is significantly different from that derived for any other duration of agonist exposure \( (p < .002 \text{ for ACh}, p < .0003 \text{ for nicotine}) \).

<table>
<thead>
<tr>
<th>Duration of Agonist Exposure</th>
<th>ACh</th>
<th>Nicotine</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 s</td>
<td>31.5 ± 4.0 s (6)</td>
<td>18.8 ± 2.6 s (6)</td>
<td>.024</td>
</tr>
<tr>
<td>30 s</td>
<td>112.8 ± 20.9 s (4)</td>
<td>50.0 ± 5.4 s (4)</td>
<td>.027</td>
</tr>
<tr>
<td>3 min</td>
<td>8.9 ± 1.4 min (8)</td>
<td>3.9 ± 0.7 min (8)</td>
<td>.007</td>
</tr>
<tr>
<td>10 min</td>
<td>16.9 ± 4.8 min (7)</td>
<td>4.7 ± 0.9 min (7)</td>
<td>.028</td>
</tr>
<tr>
<td>Nicotine—ACh, 1 s</td>
<td>22.1 ± 2.4 s (7)</td>
<td>.005</td>
<td></td>
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<tr>
<td>ACh—nicotine, 1 s</td>
<td>38.4 ± 4.2 s (6)</td>
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**Fig. 3.** Recovery from 3- and 10-min agonist exposure-induced desensitization of nAChR channels in TE671/RD cells. Results were obtained and analyzed as described in Fig. 1. Time constants for single-exponential fits to the data shown as solid lines are reported in Table 1. A, recovery of nAChR function after exposure to 1 mM ACh (●) or nicotine (○) for 3 min \( (r^2 = 0.92 \text{ for ACh and 0.99 for nicotine; eight cells}) \). B, recovery after 10-min exposure to 1 mM ACh (●) or nicotine (○) \( (r^2 = 0.99 \text{ for ACh and 0.98 for nicotine; seven cells}) \).

**Fig. 4.** Recovery from agonist-induced desensitization of nAChR channels in TE671/RD cells measured using a second agonist. Data were obtained and analyzed as described in Fig. 1 except that the agonist used for the desensitizing pulse was different from the agonist used in the test pulses during the recovery phase. Recovery of the normalized current responses elicited by 1 mM nicotine after desensitization with 1 mM ACh (●; \( r^2 = 0.98 \); six cells) or tested with 1 mM ACh pulses after desensitization with 1 mM nicotine (○; \( r^2 = 0.99 \); seven cells) is shown and yields time constants presented in Table 1. That our data were best fit to a single-exponential model for all agonist exposure times (based on \( F \) test analyses). Attempts to fit with higher order models (two and three exponentials) resulted in no convergence to the data, poorer fits to the data, or derivation of two rate constants that were inconsequentially different. Consequently, time constants derived from these studies probably are dominated by recovery from the most deeply desensitized state induced during a given period of agonist exposure. Therefore, time constants for recovery are influenced minimally by rates of recovery from less deeply desensitized states and/or the number of receptors in such states is relatively small. On the other hand, it would be difficult to explain the phenomena observed in this study if the rate-limiting event for recovery from desensitization was from the initially induced and least-desensitized state. We considered the possibility that even with the continuous perfusion (washout) method used, time-dependent accumulation of ligand in intracellular pools may dictate rates of recovery from desensitization. However, this possibility may be discounted because nicotine is considerably more membrane permeable than ACh and nevertheless produced a shorter lasting desensitization. Another feature of the results, which we report here, is the dependence of the rate of recovery from desensitization on the agonist used to induce this desensitization (slower recovery from desensitization induced by the same duration of exposure to ACh than to nicotine). This phenomenon could be
The possibility of the presence of even more states is indicated by $D_n$ explained by the sequential model (Fig. 5) if ACh drives nAChR into more deeply desensitized states more efficiently than does nicotine. It also could be explained if each agonist is capable of inducing different receptor conformations differing in their depths of desensitization and if ACh induces more of the more deeply desensitized states than does nicotine. Even though each of these agonists is maximally efficacious at 1 mM, the acute functional potency of ACh at TE671/RD cell muscle-type nAChR is higher than that for nicotine ($EC_{50} = \sim 5$ and $\sim 100$ μM, respectively), and nicotine has a lower efficacy than ACh (Lukas, 1989). Perhaps higher efficacy coupled with higher potency allows ACh to more strongly or rapidly induce deeper states of desensitization. On the other hand, nicotine is more potent than ACh in terms of inhibition of nAChR function at high doses of agonist ($IC_{50} = \sim 5$ and $>10$ mM, respectively; Lukas, 1989; Ke and Lukas, 1996). Studies with a wider range of ligands might illuminate features of the compounds that dictate their abilities to induce different states of nAChR desensitization.

Desensitization has been proposed to be due to conformational changes of the receptor that result in closure of the permeation path (Sakmann et al., 1980; Unwin, 1995). The deeper desensitization states observed in our studies could reflect induction of additional conformational changes within the pore region of the receptor channel that act to stabilize the initial desensitized state or by conformational changes that stabilize binding of the agonist molecule in the receptor active (or regulatory) site or sites. If the deeper desensitized states reflect post-translational modifications, such as phosphorylation, then recovery from desensitization, presumably via dephosphorylation, must occur quickly, and there must be agonist dependence in either phosphorylation or dephosphorylation steps. Moreover, conformational changes might occur in, or be propagated to, the cytoplasmic domain to account for changes in phosphorylation state.

Our experiments show that several desensitization states exist in the muscle-type nAChR in TE671/RD cells. The presence of the receptor in these states depends on the agonist exposure time, with increasing exposure time resulting in deeper desensitization states, and on the identity of the agonist used to induce desensitization, with ACh inducing desensitization more effectively than does nicotine. These findings are of interest in terms of synaptic plasticity and neural changes that are the consequence of nicotine use (Lukas et al., 1996; Wonnacott et al., 1996).

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References


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