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 α2 through α9 and β2 through β4 subunits being expressed in autonomic and/or central nervous systems, and α1, β1, γ, ɛ, and δ 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 (α1β1γδ) 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 when filled with recording saline (140 mM KCl, 5 mM NaCl, 2 mM MgCl2, 10 mM HEPES, 100 μg/ml nystatin, pH 7.4). Recording pipette-cell membrane seals were formed under microscopic control, producing seal resistances of greater than 2 GΩ. The transition to whole-cell mode, due to partitioning of nystatin into the cell membrane underlying the pipette tip area, was monitored by application of voltage steps from −60 to 60 mV, which triggered voltage-gated Na+ and K+ currents. Whole-cell mode was usually attained 15 to 20 min after seal formation. Cells were voltage-clamped at −80 mV.

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 Alkkon 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 6.09; 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 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 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
ACh and for nicotine; four cells). For nicotine, four cells.

Recovery of nAChR function after exposure to 1 mM ACh (r = 0.97 for ACh and 0.96 for nicotine; six cells).

B, recovery of nAChR function after exposure to 1 mM nicotine (○) for 30 s. Solid lines indicate best fit of the data to a single exponential (mean ± S.E.M.), yielding time constants shown in Table 1 (r² = 0.98 for ACh and for nicotine; four cells).

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 identity of the agonist used in test pulses.
TABLE 1

Time constants for recovery from agonist-induced desensitization

<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 ± 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>38.4 ± 4.2 s (6)</td>
<td>22.1 ± 2.4 s (7)</td>
<td>.005</td>
</tr>
</tbody>
</table>

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 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 ($r^2 = 0.92$ for ACh and 0.99 for nicotine; eight cells). B, recovery after 10-min exposure to 1 mM ACh (●) or nicotine (○) ($r^2 = 0.99$ 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.

not be expected to influence our findings. Thus, the fact that we see different recovery time constants at each agonist exposure time (Figs. 2 and 3) suggests that the muscle-type nAChR can attain several different states of desensitization. One interpretation of these findings is that different states of desensitization are achieved sequentially (Fig. 5) as longer ligand occupancy drives the receptor molecule into deeper states of desensitization, each requiring progressively longer times for recovery to the ground state after the removal of agonist. The use of a sequential model for desensitization may also explain the fact that our data were best fit to a single-exponential model for all agonist recovery 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
Fig. 5. A simplified model for different states of nAChR desensitization. The closed state (C) can undergo a conformational transition to form an open, ion-permeating state (O), which in turn will undergo a transition to a desensitized, inactive state (D1) when the agonist is present for prolonged periods of time. Our experimental results indicate that increased agonist exposure results in increasingly longer recovery times suggest the existence of several, additional desensitized states (D2, D3, D4). The possibility of the presence of even more states is indicated by Dn.

...explained by the sequential model (Fig. 5) if ACh drives nAChR into more deeply desensitized states more effectively 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 (EC50 = ~5 and ~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 (IC50 = ~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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