Regulation of Nicotinic Acetylcholine Receptor Desensitization by \( \text{Ca}^{2+} \)

Xiaochuan Guo\(^1,2\) and Robin A. J. Lester\(^1,2\)

\(^1\)Department of Neurobiology, McKnight Brain Institute; \(^2\)Civitan International Research Center, and the \(^3\)Vision Science Graduate Program, University of Alabama at Birmingham, Birmingham, Alabama

Submitted 4 October 2005; accepted in final form 11 October 2006

Guo X, Lester RA. Regulation of nicotinic acetylcholine receptor desensitization by \( \text{Ca}^{2+} \). J Neurophysiol 97: 93–101, 2007. First published October 18, 2006; doi:10.1152/jn.01047.2005. The relationship between the concentration of intracellular \( \text{Ca}^{2+} \) ([\( \text{Ca}^{2+} \)]\(_i\)) and recovery from desensitization of nicotinic acetylcholine receptors (nAChRs) in rat medial habenula (MHb) neurons was investigated using the whole cell patch-clamp techniques in combination with microfluorescent [\( \text{Ca}^{2+} \)]\(_i\) measurements. Recovery from desensitization was assessed with a paired-pulse agonist application protocol. Application of 100 \( \mu \text{M} \) nicotine (5 s) caused pronounced desensitization of nAChRs, after which recovery proceeded with two components. The relative weight of the two phases of recovery was sensitive to the nature of the intracellular \( \text{Ca}^{2+} \) chelator, with a greater fraction of channels recovering during the fast phase in the presence of BAPTA than EGTA. Recovery was affected by differential \( \text{Ca}^{2+} \) buffering only when \( \text{Ca}^{2+} \) was present in the extracellular solution, implying that \( \text{Ca}^{2+} \) influx through nAChRs was responsible for slowing the recovery. Simultaneous [\( \text{Ca}^{2+} \)]\(_i\), measurements showed that recovery from desensitization was inversely correlated with the instantaneous [\( \text{Ca}^{2+} \)]\(_i\), further supporting the suggestion that elevation of [\( \text{Ca}^{2+} \)]\(_i\), limits the return of nAChRs to the resting state. In a separate set of experiments, activation of voltage-gated \( \text{Ca}^{2+} \) channels during the recovery phase produced a sufficiently large increase in [\( \text{Ca}^{2+} \)]\(_i\) to reduce recovery from desensitization even in the absence of \( \text{Ca}^{2+} \) influx through nAChRs. Overall, it is suggested that \( \text{Ca}^{2+} \) entry through both nAChRs and voltage-gated \( \text{Ca}^{2+} \) channels exerts a negative feedback on nAChR activity through stabilization of desensitized states. The interaction of these two \( \text{Ca}^{2+} \) sources could form the basis of a coincidence detector under specific circumstances.

INTRODUCTION

Desensitization represents a decrease or loss of biological response after prolonged or repetitive stimulation by agonist or neurotransmitter and is observed in a wide variety of ligand-gated channels (Quick and Lester 2002). Even though it is generally accepted that receptor desensitization can influence synaptic amplitude and time course (Jones and Westbrook 1996), the physiological significance of desensitization of central nicotinic acetylcholine receptors (nAChRs) remains unresolved largely because of a lack of knowledge concerning their activation by endogenously released transmitter (Brown 2000; Giniatullin et al. 2005). Conversely, the pathological relevance of desensitization of nAChRs has been recognized for many years, particularly with respect to chronic nicotine (Collins et al. 1990; Dani and Heinemann 1996) and likely becomes an important factor during treatment of Alzheimer’s disease with cholinesterase inhibitors (Paterson and Nordberg 2000).

In the absence of a comprehensive understanding of the function of nAChRs, however, arguments in favor of examining desensitization can be made. In multistate systems of which ligand-gated ion channels are members (Katz and Thesleff 1957), the fractional distribution of receptors between those states becomes relevant for predicting how the system will behave under a given set of circumstances. Specifically, the balance between activatable and desensitized states will set the maximum response amplitude and thus any alteration of this balance will confer plasticity to the system (Changeux et al. 1998). Interestingly many receptors have a built-in mechanism for performing this task—by allosteric modulation of the kinetics of receptor desensitization using \( \text{Ca}^{2+} \) and/or protein phosphorylation (Huganir and Greengard 1990). Taken to one extreme, such modulation acts as a time-dependent shuffle between functional and nonfunctional receptor pools.

The impact of this form of modulation becomes clearer if information about cholinergic–nicotinic signaling in the CNS is formalized. Cholinergic synaptic contacts in many parts of the brain are randomly distributed and, in the hippocampus, \(<10%\) are in apposition to postsynaptic sites (Descaries et al. 1997). Even assuming the transmitter is released at a high concentration, it will be in the low micromolar range after only a few microns of diffusion (Clements 1996), providing an explanation for lack of fast synaptic responses in many areas of the brain, including the medial habenula (Edwards et al. 1992), despite the strong expression of nAChRs in this region (Lester and Dani 1994; McCormick and Prince 1987; Mulle and Changeux 1990). Moreover, desensitization becomes important if postsynaptic receptors are required to detect low levels of ambient transmitter because the concentration “window” over which they remain sensitive is determined by the concentration dependency of both activation and desensitization (Steinbach 1990). Desensitization dominates at lower doses of agonist, but there will be a narrow concentration range over which receptors will start to activate before becoming fully desensitized by higher steady-state levels of transmitter (Lester 2004; Lester and Dani 1995). Under these conditions, any change in the sensitivity of conformational states to agonist will alter the responsiveness of the cell to available transmitter.

There is little information describing the modulation of desensitization of nAChRs in the CNS (Quick and Lester 2002). Results from peripheral tissue and heterologous expression systems indicate that desensitization is regulated by \( \text{Ca}^{2+} \), although the effect of \( \text{Ca}^{2+} \) may depend on the subtype of nAChR (Quick and Lester 2002). For example, in putative \( \alpha_3\beta_4^+\)-subunit–containing receptors on chromaffin cells, an

Address for reprint requests and other correspondence: R.A. J. Lester, Department of Neurobiology, SHEL 1006, University of Alabama at Birmingham, 1825 University Boulevard, Birmingham, AL 35294-0021 (E-mail: rlester@nrc.uab.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
elevation in intracellular Ca\(^{2+}\) suppressed recovery from desensitization (Khiroug et al. 1997, 1998), whereas the opposite is true for α4β2 nAChRs (Fenster et al. 1999). Receptors in the peripheral nervous system (PNS) and MHb cells are considered to be primarily of the α3β4* subtype (McGehee and Role 1995), which gives rise to the prediction that their Ca\(^{2+}\) modulation should be similar. However, the precise physiological and pharmacological characteristics are different for the two groups of receptors. MHb nAChRs have a different rank order of agonist efficacy (Coverton et al. 1994; Mulle and Changeux 1990) as well as single-channel conductance (Mulle and Changeux 1990; Sivilotti et al. 1997) than nAChRs in the PNS, implying that their underlying subunit composition is not exactly the same and, as such, they may exhibit differential regulation of desensitization. Thus here we have addressed 1) whether Ca\(^{2+}\) can modulate recovery from desensitization of nAChRs in the MHb and 2) whether this resembles regulation of a similar nAChR subtype in the PNS. Results from these studies may reveal whether there is a universal mechanism for the regulation of desensitization of nAChRs by Ca\(^{2+}\). In addition, because many nAChRs are present on nerve terminals, including MHb axons (Marks et al. 1998), we consider whether Ca\(^{2+}\) entry by voltage-gated Ca\(^{2+}\) channels can influence nAChR function.

METHODOLOGICAL

Methods

Acute isolation of habenula neurons, electrophysiology, and calcium measurements

All experiments were performed on neurons isolated from medial habenula nuclei of 10- to 20-day-old rats using methods described previously (Quick et al. 1999). Methods for electrophysiology, drug application, and intracellular Ca\(^{2+}\) measurements were as described in the companion paper (Guo and Lester 2007). Deviations from standard solutions are described in the text.

Experimental protocols and data fitting

All experiments were designed to assess recovery from agonist-induced desensitization. Thus a standard paired-pulse protocol was used throughout and the interval between different trials was 3 min. The protocol consisted of an initial brief (2- to 10-s) desensitizing application of a high (100 μM) concentration of nicotine followed by a second pulse of nicotine (100 μM) at various interpulse intervals (250 ms to 10 s). Recovery from desensitization was assessed as the ratio of the peak amplitude of the second response \(I_2\) with respect to the peak amplitude of the first response \(I_1\). The time course of recovery was quantified from the time constants \(\tau\) and relative amplitudes \(A\) of single- and double-exponential components fitted to the data. In all cases recovery was constrained to be complete (i.e., \(I_2/I_1 = 1\)) using the equation:

\[
I_2/I_1 = \sum A e^{-r\tau}
\]

Statistical analysis

The significance of the linear regression and comparisons among multiple groups were tested with one-way ANOVA using the program SPSS 1.0 (SPSS, Chicago, IL). In addition, paired and unpaired Student’s t-tests were used. Significance was taken as \(P < 0.05\) (*) and \(P < 0.01\) (**). All data are presented as means ± SE.

Receptor nomenclature

Subtypes of nAChRs are referred to by their putative subunit composition with an asterisk to represent likely inclusion of additional subunits (Lukas et al. 1999).

RESULTS

Effects of intracellular Ca\(^{2+}\) buffering on recovery from desensitization

The goals of the study were to assess the influence of intracellular Ca\(^{2+}\) on recovery from desensitization of nAChRs in MHb neurons. Even though many different types of nAChR subunit mRNAs are expressed in the MHb region (Sheffield et al. 2000), previous studies showed that MHb neurons possess a reasonably homogeneous population of functional receptors with a minimal subunit composition of α3 and β4 subunits (Mulle et al. 1991; Quick et al. 1999; Sheffield et al. 2000). Extensive desensitization of nAChRs in MHb, similar to that of all types of nAChRs (Quick and Lester 2002), can be produced by quite brief applications of high concentrations of agonist (Lester and Dani 1995). In the current experiments, 5-s pulses of nicotine (100 μM) were used to induce desensitization and recovery from desensitization was measured with a second application at a number of different interpulse intervals. Recovery was quantified as the ratio of the peak of the second current to the peak of the first desensitizing current. Under standard intracellular Ca\(^{2+}\) buffering conditions (10 mM EGTA), recovery from this type of desensitization proceeded with two components, quantified by fast \(\tau_f\) and slow \(\tau_s\) exponential time constants having relative amplitudes of 39 ± 5 and 61 ± 5%, respectively (Fig. 1, A and B; Table 1). It was previously argued that the biphasic nature of desensitization in MHb nAChRs arises from a single receptor rather than from two separate receptor populations with fast and slow kinetics (Lester and Dani 1995).

To obtain an initial assessment of the effects of intracellular Ca\(^{2+}\) buffering on recovery from desensitization, recovery was compared in the presence of EGTA and BAPTA, two Ca\(^{2+}\) chelators with relatively slow and fast binding kinetics (Tsien 1980). Irrespective of the nature of the chelator, recovery from desensitization proceeded biexponentially (Fig. 1C). However, in the presence of BAPTA, recovery was more complete at longer interpulse intervals. Quantitatively this was accounted for by a greater contribution of the fast phase of recovery (71 ± 7% compared with 39 ± 5%) when BAPTA was used (Table 1). The process of desensitization in nicotinic receptors is usually described with a two-desensitized-states model, which posits that nAChRs may exist in both fast (shallow) and slowly accessible (deep) desensitized conformations (Boyd 1987; Feltz and Trautmann 1982). In terms of this model, our results with the Ca\(^{2+}\) chelators imply that intracellular Ca\(^{2+}\) acts to stabilize nAChRs in the deep desensitized conformation because there is less accumulation of receptors in the deep state (i.e., more receptors recover with fast kinetics) when Ca\(^{2+}\) is rapidly buffered. In further support of this idea, the fraction of nondenitized receptors, estimated from the steady-state/peak ratios at the end of the first 5-s pulse of 100 μM nicotine was unaffected by the nature of the chelator. These ratios were 0.13 ± 0.02 (n = 8) and 0.13 ± 0.02 (n = 8) for EGTA and BAPTA, respectively. Although this pseudosteady-state pa...
most likely source of the $\text{Ca}^{2+}$ increase is direct influx through the nAChR (Mülle et al. 1992; Vernino et al. 1992). If so, then any differences in the recovery profiles recorded using either EGTA or BAPTA should be eliminated in the nominal absence of extracellular $\text{Ca}^{2+}$. This result was confirmed (Fig. 1D) and, in addition, the percentage recovery of the fast phase in EGTA/$\text{Ca}^{2+}$-free conditions was increased and resembled that observed with BAPTA/extracellular $\text{Ca}^{2+}$ (Table 1). These data imply that BAPTA effectively buffers $\text{Ca}^{2+}$ influx and the differential effects of EGTA and BAPTA on the slow phase of recovery in the presence of extracellular $\text{Ca}^{2+}$ are the direct result of their differential $\text{Ca}^{2+}$-chelating properties.

To confirm that the fast phase of desensitization is relatively insensitive to $\text{Ca}^{2+}$, recovery from desensitization was assessed after a shorter (2-s) desensitizing application of nicotine (100 $\mu$M). During very brief exposure to agonist, there is less time for equilibration with slowly accessible deep desensitized states and recovery will largely occur from the fast shallow desensitized state (Fenster et al. 1999; Lester and Dani 1995). With 10 mM EGTA in the recording pipette, recovery was similar in both the absence and presence of extracellular $\text{Ca}^{2+}$ and could be reasonably well described by a single-exponential function with time constants of a magnitude similar to those for the fast component ($\tau_f$) of recovery during longer agonist exposure (Fig. 1E; Table 1). Double-exponential functions revealed a minor slow component (roughly 17%) that was not well defined (Table 1). Provided that nAChRs are the major contributor to intracellular $\text{Ca}^{2+}$ transients under these conditions (see following text), these findings suggest that $\text{Ca}^{2+}$ has little or no effect on the fast phase of recovery from desensitization.

Relationship between intracellular $\text{Ca}^{2+}$ and recovery from desensitization

We have measured the fractional current carried by $\text{Ca}^{2+}$ through nAChRs in MHb neurons to be around 3–4% in physiological conditions (Guo and Lester 2007), a percentage sufficient to cause a measurable rise in intracellular $[\text{Ca}^{2+}]$. Thus MHb neurons are a suitable model to study the relationship between $\text{Ca}^{2+}$ influx through nAChRs and the recovery from desensitization in detail. It is difficult, however, to maintain normal $\text{Ca}^{2+}$ homeostasis in intact cells once a whole cell configuration is formed because cytoplasmic components are inevitably washed out. Fortunately, with few exceptions (Roberts 1993), $\text{Ca}^{2+}$ buffers are resistant to diffusion in most cells studied (Neher 1995). Thus as so not to confound physiological buffering capacity and to allow $\text{Ca}^{2+}$ detection by indo-1 (40 $\mu$M), a low concentration of EGTA (0.6 mM) was used in these experiments.

Simultaneous measurements of intracellular $[\text{Ca}^{2+}]$ and membrane currents were used to assess the relationship between recovery from desensitization and intracellular $[\text{Ca}^{2+}]$. Recovery was assessed from the relative amplitude of the second response in a paired-pulse protocol (5 s; 100 $\mu$M nicotine) and compared with the instantaneous intracellular $[\text{Ca}^{2+}]$ immediately before the second pulse (Fig. 2A). Interpulse intervals were set between 2 and 10 s because our previous experiments (see Fig. 1, C and E) indicated that the major effect of $\text{Ca}^{2+}$ was on the slow component of recovery. Not surprisingly, as the interpulse interval was increased,
recovery from desensitization was facilitated and intracellular [Ca$^{2+}$] moved closer to resting levels (Fig. 2B). However, although these results per se do not imply modulation of desensitization by Ca$^{2+}$, an inverse relationship between recovery and [Ca$^{2+}$] could be extracted from the variability in individual cells. Example traces from cells with high and low intracellular [Ca$^{2+}$] are illustrated in Fig. 2C. A plot of the instantaneous [Ca$^{2+}$]$_i$ versus recovery at the 2-s interval revealed a significantly greater recovery at lower [Ca$^{2+}$]$_i$ (Fig. 2D; the linear regression is significant with a correlation coefficient, R = 0.59; n = 37; P < 0.001, ANOVA).

The increase in intracellular [Ca$^{2+}$] after activation of nACHRs could have been partially the result of a secondary release from endoplasmic reticulum Ca$^{2+}$ stores (CICR) induced by Ca$^{2+}$ influx through nACHRs ( Rathouz et al. 1996). It was therefore necessary to test whether CICR affected recovery in our experiments. This question was addressed by depleting intracellular Ca$^{2+}$ stores using bath application of 1 μM thapsigargin. Recovery from desensitization was similar before (0.6 ± 0.05) and after (0.61 ± 0.07; n = 7; P > 0.05) thapsigargin treatment, suggesting that CICR does not contribute to the effect of intracellular Ca$^{2+}$ transients on recovery. Mitochondria constitute another important intracellular Ca$^{2+}$ store, which can effectively sequester Ca$^{2+}$ during a rapid rise of intracellular Ca$^{2+}$ (Pivovarova et al. 2002). However, because mitochondria buffering does not affect the nicotinic Ca$^{2+}$ response in a physiological (2 mM external Ca$^{2+}$) solution (Guo and Lester 2007), it is unlikely that these organelles would contribute to the modulation of nACHR desensitization by Ca$^{2+}$.

Voltage-dependent Ca$^{2+}$ influx and recovery from desensitization

Many nACHRs exist presynaptically (Wonnacott 1997) where they may readily interact with voltage-gated Ca$^{2+}$ channels to synergistically facilitate the release of neurotransmitter (Kulak et al. 2001; Tredway et al. 1999). Thus Ca$^{2+}$ entering through voltage-gated Ca$^{2+}$ channels during depolarization induced by nACHR activation or otherwise could act to modulate nACHR function. Although spontaneous Ca$^{2+}$ transients or those induced by membrane depolarization have no direct role in promoting nACHR desensitization (Khiroug et al. 1998), it is suggested here that voltage-dependent Ca$^{2+}$ influx may act as an additional means of attenuating recovery from desensitization.

Simultaneous monitoring of membrane current and [Ca$^{2+}$]$_i$ during a 1-s depolarizing step from −50 to 0 mV demonstrates the existence of voltage-gated Ca$^{2+}$ channels in MHb neurons (Fig. 3A, top traces). The inward current (possibly a mixture of voltage-gated Na$^+$ and Ca$^{2+}$ currents) and its associated [Ca$^{2+}$]$_i$ transient could both be blocked with 200 μM Cd$^{2+}$ (Fig. 3A, bottom traces). In addition, 200 μM Cd$^{2+}$ by itself

---

**TABLE 1. Effects of differential intracellular Ca$^{2+}$ buffering on recovery from desensitization**

<table>
<thead>
<tr>
<th>Chelator (n)</th>
<th>[Ca$^{2+}$]$_{rest}$, mM</th>
<th>Agonist Duration, s</th>
<th>$\tau_f$, s</th>
<th>$\tau_s$, s</th>
<th>$A_f$, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1EGTA (5)</td>
<td>2</td>
<td>5</td>
<td>0.30 ± 0.05</td>
<td>6.3 ± 1.6</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>2BAPTA (6)</td>
<td>2</td>
<td>5</td>
<td>1.12 ± 0.18</td>
<td>8.6 ± 2.6</td>
<td>71 ± 7</td>
</tr>
<tr>
<td>3EGTA (6)</td>
<td>0</td>
<td>5</td>
<td>0.89 ± 0.21</td>
<td>21.0 ± 9.0</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>4BAPTA (5)</td>
<td>0</td>
<td>5</td>
<td>1.37 ± 0.29</td>
<td>18.3 ± 9.0</td>
<td>76 ± 10</td>
</tr>
<tr>
<td>EGTA (4)</td>
<td>2</td>
<td>2</td>
<td>2.40 ± 0.25</td>
<td>—</td>
<td>83 ± 6b</td>
</tr>
<tr>
<td>EGTA (3)</td>
<td>0</td>
<td>2</td>
<td>2.23 ± 0.14</td>
<td>—</td>
<td>83 ± 4b</td>
</tr>
</tbody>
</table>

Values are means ± SE; n values are in parentheses. The time courses of recovery in individual cells were fitted with single- or double-exponential components using Eq. 1. $\tau_f$ and $\tau_s$ are the time constants for the fast and slow components. $A_f$ is the relative amplitude of the fast component. With respect to the superscripts 1–4 in the first column, when $\tau_f$, $\tau_s$, and $A_f$ values for these four groups were compared, significant differences were found for $\tau_f$ and $A_f$, but not for $\tau_s$ (P < 0.05, one-way ANOVA). The difference between pairs of individual groups was tested by the LSD (least significant difference) multiple comparison test and values of P < 0.05 were considered to be significant. The $\tau_f$ value of group 1 was different from that of groups 2 and 3 and the $\tau_s$ value of group 1 was different from that of groups 2, 3 and 4. No significant difference was found for any other group pairs. *These data were better fit with a single-exponential function and there was no difference in time constants of recovery from 2-s desensitization in the presence or absence of extracellular Ca$^{2+}$ (P > 0.05, t-test). **The relative percentage of fast phase when recovery was fitted to double-exponential components.

---

**Fig. 2. Recovery from desensitization is inversely proportional to the intracellular Ca$^{2+}$, A: simultaneous measurements of agonist-induced currents (bottom traces) and intracellular [Ca$^{2+}$]$_i$ (top traces) resulting from a paired-pulse application of nicotine (100 μM; 5 s; interpulse interval = 2 s). Pipette solution contained 50 μM indo-1 and 0.6 mM EGTA. Arrowhead in A indicates that the instantaneous [Ca$^{2+}$]$_i$ was estimated immediately before the second pulse of agonist. B: recovery from desensitization (open symbols) and instantaneous [Ca$^{2+}$]$_i$ (filled symbols) are plotted against the interpulse interval (n = 7). C: example traces from two cells showing different paired-pulse recovery at 2 s (bottom traces) with respects to different intracellular Ca$^{2+}$ (top traces). Arrowheads indicate the points at which instantaneous [Ca$^{2+}$]$_i$ was measured. D: plotting the relationship between recovery from desensitization and the instantaneous [Ca$^{2+}$]$_i$. Linear regression was significant (n = 37; P < 0.001, ANOVA) with a coefficient R = 0.59.**
confirming that Ca\(^{2+}\) entry through voltage-gated calcium channels was responsible for the change in nAChR function.

MHB neurons (both in slice and acutely isolated) fire spontaneous action potentials at a frequency from 1 to 20 Hz, with a mean frequency of about 5 Hz (McCormick and Prince 1987). As a result, the intracellular Ca\(^{2+}\) would likely be constantly oscillating. To mimic this type of activity, cells were challenged with a series of brief membrane depolarizations during the recovery phase (Fig. 4). Here, the paired-pulse protocol constituted a long desensitizing pulse of 10 s and a test pulse of 1 s separated by an interpulse interval of 8 s. A 7-s train of depolarizing pulses (from −50 to 0 mV; 100 ms) at 2.5 or 5 Hz was applied 500 ms after the end of the first pulse (Fig. 4, A and B). The relative changes in [Ca\(^{2+}\)]\(_{i}\), (Δ[Ca\(^{2+}\)]\(_{i}\)), calculated by subtracting the resting level from instantaneous [Ca\(^{2+}\)]\(_{i}\), at the start of the second pulse of nicotine) under control, and at 2.5- and 5-Hz depolarization were 0.04 ± 0.02, 0.09 ± 0.03, and 0.11 ± 0.02 (n = 7), respectively (Fig. 4C). The associated fractional recoveries from desensitization were 0.86 ± 0.03, 0.71 ± 0.04, and 0.58 ± 0.04, respectively (Fig. 4D), further indicating that recovery was inversely correlated with intracellular [Ca\(^{2+}\)]\(_{i}\). To test the possible involvement of Ca\(^{2+}\)-induced Ca\(^{2+}\) release during the prolonged Ca\(^{2+}\) influx, intracellular Ca\(^{2+}\) stores were again depleted by bath application of 1 μM thapsigargin. In this case, the 10-s desensitizing...

**FIG. 3.** Interaction between voltage-dependent Ca\(^{2+}\) influx and recovery from desensitization of nicotinic acetylcholine receptors (nAChRs). A: example of the inward current and the increase in [Ca\(^{2+}\)]\(_{i}\) (top 2 traces) caused by a 1-s membrane depolarization step (from −50 to 0 mV). Both the current and the increase in [Ca\(^{2+}\)]\(_{i}\) were blocked by 200 μM Cd\(^{2+}\) (bottom 2 traces). B: intracellular Ca\(^{2+}\) transients (top traces) and inward currents (bottom traces) in medial habenula (MHb) cells. Paired-pulse agonist protocol (100 μM nicotine; 5 s; interpulse interval = 4 s) was given in the absence (left traces) and presence (middle and right traces) of a 1-s voltage step (from −50 to 0 mV) positioned 1 s after the first agonist pulse. Note that experiments with Cd\(^{2+}\) were done in a separate set of cells and there is no additional Ca\(^{2+}\) increase during the interpulse interval in the presence of Cd\(^{2+}\). Bar graph in C shows the relative change (Δ) in instantaneous [Ca\(^{2+}\)]\(_{i}\) before the second agonist pulse with and without depolarization. Δ[Ca\(^{2+}\)]\(_{i}\), was calculated by subtracting the resting [Ca\(^{2+}\)]\(_{i}\), from the instantaneous [Ca\(^{2+}\)]\(_{i}\). In these experiments, [Ca\(^{2+}\)]\(_{i}\) is expressed as the raw fluorescence ratio (405 nm/490 nm). D: bar graph showing the fractional recovery from desensitization after 4 s under the same conditions shown in C.

had no effect on either the nicotinic current or recovery from desensitization. The peak current amplitude induced by 100 μM nicotine was 4,376 ± 910 pA in control and 4,161 ± 783 pA in the presence of extracellular Cd\(^{2+}\) (n = 7; P > 0.05), and the fractional recoveries (at the 6-s interpulse interval) were 0.68 ± 0.03 and 0.66 ± 0.02 in the absence and presence of Cd\(^{2+}\), respectively (n = 7; P > 0.05). Although the anatomical relationship between Ca\(^{2+}\) channels and nAChRs is unknown, it was reasoned that appropriately timed voltage-dependent Ca\(^{2+}\) entry should retard nAChR recovery from desensitization. To study this interaction, a paired-pulse protocol (5 s; 100 μM nicotine) at a fixed interpulse interval of 4 s was applied with or without a voltage step (1 s; −50 to 0 mV) 1 s after the first pulse. Examples of currents and intracellular [Ca\(^{2+}\)]\(_{i}\) transients are shown in Fig. 3B. Intracellular [Ca\(^{2+}\)]\(_{i}\) was significantly higher at the start of the second pulse when it was preceded by a voltage-step that produced a clear additional Ca\(^{2+}\) transient (Fig. 3C). Furthermore, the additional rise in intracellular [Ca\(^{2+}\)]\(_{i}\) was associated with less recovery from desensitization (Fig. 3D). In a separate series of experiments, Cd\(^{2+}\) was shown to selectively block Ca\(^{2+}\) entry through voltage-gated channels (Fig. 3B, right traces) and restore recovery from desensitization to control levels (Fig. 3D), thus...

**FIG. 4.** Regulation of recovery from desensitization is proportional to voltage-dependent Ca\(^{2+}\) influx. Intracellular Ca\(^{2+}\) transient (top trace) and inward current (bottom trace) induced by a paired-pulse application of nicotine (100 μM nicotine; interpulse interval was 8 s) in the absence of membrane depolarization (A) and when a train of depolarizing pulses (−50 to 0 mV; 100 ms; at a frequency of 5 Hz for 7 s) was introduced 0.5 s after the first desensitizing application of nicotine (B). A slowly developing inward current occurred as a result of the depolarization train and the arrow indicates the starting point of the second pulse. Δ[Ca\(^{2+}\)]\(_{i}\) (calculated by subtracting the resting [Ca\(^{2+}\)]\(_{i}\), from the instantaneous [Ca\(^{2+}\)]\(_{i}\), before the second agonist application) and recovery under different conditions are shown in C and D. [Ca\(^{2+}\)]\(_{i}\) is shown as the raw fluorescence ratio (405 nm/490 nm).
agonist application was followed by 2-s depolarization at 5 Hz. Fractional recoveries assessed at an interpulse interval of 3 s before and after thapsigargin treatment were 0.51 ± 0.05 and 0.55 ± 0.04 (n = 7; P > 0.05; data not shown), suggesting the effects of depolarization on recovery were solely attributable to Ca$^{2+}$ influx through voltage-gated calcium channels.

A further set of experiments was performed to address whether Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels alone would be sufficient to modulate the recovery from desensitization of nAChRs. To test this hypothesis, both the desensitizing (100 μM; 10 s) and test agonist applications (100 μM; 1 s) of nicotine were performed in a nominally Ca$^{2+}$-free external solution, so that any Ca$^{2+}$ influx would occur solely as a result of membrane depolarization during the interpulse interval in which extracellular [Ca$^{2+}$] was changed to 2 mM. A shorter interpulse interval of 4 s was chosen because recovery appeared to be faster when nAChRs were desensitized in Ca$^{2+}$-free solution (Fig. 5A). A 2-s train of depolarizing pulses (5 Hz) during the interpulse interval appreciably elevated [Ca$^{2+}$], (n = 6; P < 0.05; Fig. 5B) and was associated with a decreased recovery from desensitization (n = 6; P < 0.01; Fig. 5C). These results demonstrate that Ca$^{2+}$ influx through nAChRs is not an absolute requirement for either inducing desensitization or for regulation of its recovery and, provided that nAChRs are desensitized, Ca$^{2+}$ from other sources can activate the mechanisms that alter nAChR function.

An important point with respect to the role of Ca$^{2+}$ is whether its actions are limited to the regulation of receptor desensitization. It is possible that intracellular Ca$^{2+}$ has a more generalized role in attenuating the responsiveness of nAChRs. Two experiments were designed to test whether either Ca$^{2+}$ entry and/or intracellular Ca$^{2+}$ altered nAChR sensitivity to agonist. First, the paired-pulse experiments were repeated with the voltage-step timed to occur 1 s before the first agonist exposure. Thus intracellular [Ca$^{2+}$] would be elevated before receptor activation (Fig. 6A). Under these conditions, currents...
mediated by nAChRs were unaffected (Fig. 6B) and neither was recovery from desensitization (Fig. 6D), presumably because the influence of the voltage step on $[Ca^{2+}]$ was negligible by the time of the second agonist exposure (Fig. 6C). Second, to confirm that intracellular $Ca^{2+}$ does not regulate the activation of nAChRs, the relative open probability of these channels was estimated during intracellular perfusion with differential $[Ca^{2+}]/[Ca^{2+}]$-buffering capacity: 10 mM BAPTA versus 200 nM free $[Ca^{2+}]$, (6.67 mM Ca$^{2+}$ and 10 mM EGTA). An open channel blocker, chlorisondamine, was used to assess open probability as it becomes trapped inside when receptors close (Amador and Dani 1995; Hicks et al. 2000; Neely and Lingle 1986). As predicted during coapplication of nicotine (30 $\mu$M) and chlorisondamine (1 $\mu$M) the measured currents are progressively attenuated (Fig. 6E). Normalization of the responses to a control application of nicotine revealed no differences in either the rate or extent of block by chlorisondamine (Fig. 6F).

DISCUSSION

In the present study we demonstrate that $Ca^{2+}$ flux through nAChRs can influence the rate of recovery from desensitization. Specifically it is shown that the extent of recovery is inversely correlated with intracellular $[Ca^{2+}]$ and that $Ca^{2+}$ entry through voltage-gated calcium channels acts in a similar manner. We discuss these data in terms of mechanism, activation of nAChRs by diffusely released transmitter, and a form of coincidence detection.

Mechanism of $Ca^{2+}$ action

Autoregulation of recovery from desensitization arising from $Ca^{2+}$ influx through nAChR channels was previously reported in brain. The mechanism appears to be universal because it is common to all major types of nAChRs, putative homomeric $\alpha 7$ receptors (Khirou et al. 2003), and heteromeric $\alpha 3\beta 4\gamma$ receptors in both the peripheral (Khirou et al. 1997, 1998) and central (this study) nervous system. For $\alpha 4\beta 2$ receptors, expressed in Xenopus oocytes, $Ca^{2+}$ also affects recovery from desensitization, but in the opposite direction (Fenster et al. 1999). Although $Ca^{2+}$ may have a direct influence on nAChR desensitization (Cachelin and Colquhoun 1989; Miledi 1980), it is more likely that its effects are mediated through protein kinases and phosphatases, as reported for peripheral nAChRs (Khirou et al. 1998), and the disparate effects of $Ca^{2+}$ may have resulted from a differential interaction with downstream second messengers in different systems.

If $Ca^{2+}$ influx through nAChRs, which accounts for 3–4% of total charge influx (Guo and Lester 2007), is sufficient to affect recovery from desensitization, other means of increasing intracellular $[Ca^{2+}]$ in the vicinity of nAChRs should also influence the process. Indeed, $Ca^{2+}$ entry through voltage-gated calcium channels is sufficient to attenuate recovery. It should be noted that, although our data suggest $Ca^{2+}$-induced $Ca^{2+}$ release does not affect the recovery, other means of releasing $Ca^{2+}$ from stores, such as activation of the IP3 pathway, may be more effective.

It is now generally accepted that there are two major desensitized conformations of the nAChR (Quick and Lester 2002). Moreover, when regulation of these channels exists, it appears to largely but not exclusively affect the transitions between the short-lived shallow and long-lived deep desensitized states. This is the case for protein kinase A (PKA) modulation of muscle type nAChRs (Paradiso and Brehm 1998), the marked temperature dependency of receptor desensitization in PC12 cells (Boyd 1987), and the protein kinase C (PKC)–dependent regulation of both expressed (Fenster et al. 1999) and native $\alpha 4\beta 2$ nAChRs (Marszalec et al. 2005). In Markov models of desensitization (e.g., see Fenster et al. 1999), accumulation of receptors in any conformation will depend on the rate constants governing the movement into and out of that particular state. Thus in simple terms, $Ca^{2+}$ could act either by promoting entry into or by restricting exit from the “deep” desensitized state. The data in the current investigation do not permit us to fully distinguish between these two options. On the one hand, our findings seem to imply that the rate into the deep desensitization is most likely the target of $Ca^{2+}$ because the return transition from deep to shallow states, as indicated by the time constant determining recovery from the slow phase of desensitization ($\tau_r$), was not affected by $Ca^{2+}$ (see Table 1). Conversely, recovery from desensitization is slowed when $Ca^{2+}$ is available only during the recovery phase (see Fig. 5), which is more consistent with regulation of the rate out of the deep desensitized conformation. Irrespective of the precise mechanism, our findings argue that high intracellular $[Ca^{2+}]$ promotes stabilization of the deep desensitized conformation. Such a negative-feedback mechanism could serve to further dampen nAChR activation during a prolonged period of stimulation.

Activation of central nAChRs by ambient levels of transmitter

The exact relevance of nAChR desensitization is unknown, particularly with respect to how it relates to synaptic function (Giniatullin et al. 2005). Typically fast ligand-gated channels are transiently stimulated only by transmitter and agonist is not present sufficiently long for desensitization to become physiologically relevant (Colquhoun and Ogden 1988; Colquhoun and Sakmann 1998). However, despite the widespread presence of nAChRs, there are relatively few examples of fast nicotinic-cholinergic transmission in the brain (Alkondon et al. 1998; Frazier et al. 1998; Futami et al. 1995; Matsubayashi et al. 2004). Specifically, with respect to the current study, there is no evidence for conventional synaptic activation of nAChRs in the MHB even given strong neurochemical and anatomical support (Brown 2000; Edwards et al. 1992). To produce a fast synaptic response, pre- and postsynaptic structures have to be in close proximity so that a high concentration of neurotransmitter can activate postsynaptic receptors. However, in other parts of the brain, extensive studies have revealed that only about 10% of the cholinergic terminals form morphologically defined synapses, whereas the majority are distributed randomly in the neuropil (Descarries et al. 1997). In addition, contrary to the well-studied neuromuscular junction, nAChRs in the CNS may localize outside synaptic structures (Hill et al. 1993). Therefore the transmitter acetylcholine (ACh) may have to diffuse some distance and will likely be in the nanomolar to micromolar range in the vicinity of nAChRs (Descarries et al. 1997; Zoli et al. 1999). Prolonged activation of receptors in this
low-concentration range will favor slowly accessible high-affinity desensitized states (Changeux et al. 1984). Thus if nAChRs are capable of detecting low levels of ambient transmitter, agonists must be within the concentration “window” over which receptors start to activate even though desensitization is not complete (Steinbach 1990). Within such a “window,” activatable and desensitized nAChRs are in a dynamic equilibrium, such that agonist could persistently open a fraction of nAChRs (Lester 2004). In the preceding scenario, modulation of desensitization becomes an important mechanism for adjusting the “window” and determining the level of activation at a given concentration of ACh. Higher intracellular $[\text{Ca}^{2+}]$ would “trap” more nAChRs in deep desensitized conformations and decrease the total number of activatable receptors. In terms of a concentration–response interaction, the desensitization curve would shift to the left and the “window” concentration range would become narrower (Lester 2004), thereby dampening the response to ambient/diffuse levels of agonist.

State-dependent modulation and coincidence detection

Our data show that $\text{Ca}^{2+}$ entry through voltage-gated calcium channels slowed the process of recovery, but only when the nAChRs were already desensitized. Similar experiments in chromaffin cells showed that spontaneous intracellular $\text{Ca}^{2+}$ transients do not affect nAChR current amplitude or desensitization onset (Khiroug et al. 1998). Likewise, intracellular perfusion of MHB cells with elevated $[\text{Ca}^{2+}]$ did not change the channel open probability—a measure of the fractional activation of nAChRs. Together these results imply that the modulation of nAChRs by intracellular $\text{Ca}^{2+}$ is state dependent: The effects of $\text{Ca}^{2+}$ alter transitions only between shallow and deep desensitized states. One possibility is that conformational changes on desensitization (Unwin et al. 1988) reveal sites for either $\text{Ca}^{2+}$ binding or phosphorylation. Similar conformation-dependent effects are important in voltage-gated sodium channels, where large structural rearrangements are responsible for local anesthetic inhibition after depolarization-induced inactivation (Catterall 1999). Alternatively, the binding of $\text{Ca}^{2+}/$phosphorylation may not occur in a state-dependent manner, but merely allow alteration of transition probabilities between certain states.

One consequence of this type of regulation is that transient activation of nAChRs, such as fast synaptic transmission, would proceed independently of the level of intracellular $\text{Ca}^{2+}$. Only when receptors become saturated with agonist for prolonged periods of time would the effects of $\text{Ca}^{2+}$ become realized. Moreover, true state-dependent tagging could serve as a signal for receptor desensitization/internalization, as occurs with G-protein–coupled receptors (Pierce and Lofkowitz 2001). Indeed, after prolonged desensitization of muscle nAChRs recovery is often incomplete (Katz and Thesleff 1957). A similar mechanism could occur under conditions of prolonged exposure to nicotine, when many nAChRs are presumed to be desensitized (Quick and Lester 2002). If this were to operate at presynaptic locations, the state-dependent modulation may serve as a coincidence-detection mechanism: $\text{Ca}^{2+}$ influx through voltage-gated calcium channels during sustained action potential firing could trap nAChRs in deep desensitized states, even in the absence of $\text{Ca}^{2+}$ influx through the nAChR channel.

GRANTS

This work was supported by National Institute of Neurological Disorders and Stroke/Public Health Service Grant NS-31669.

REFERENCES


