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Protons Enhance the Gating Kinetics of the $\alpha3/\beta4$ Neuronal Nicotinic Acetylcholine Receptor by Increasing Its Apparent Affinity to Agonists

GALYA ABDRAKHMANOVA, JULIA DORFMAN, YINGXIAN XIAO, and MARTIN MORAD

Department of Pharmacology, Georgetown University School of Medicine, Washington DC

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are widely distributed in the nervous system. Although there is a vast literature on the molecular, structural and pharmacological properties of neuronal nAChR, little is known of their pH regulation. Here we report that rapid acidification (pH 6.0) enhances the current through the $\alpha3/\beta4$ recombinant nAChRs expressed stably in human embryonic kidney 293 cells and accelerates its activation kinetics without altering selectivity. Acidification also strongly accelerates the decay kinetics ("desensitization") of cytisine- and nicotine-evoked currents ($pK_a \sim 6.1$), but the effect is somewhat smaller with acetylcholine and carbachol (undetermined pK_a values), suggesting that protonation of the agonist contributes to the relaxation of the current. Transient increases of $[H^+]_o$ from pH 7.4 to 6.0, during the time course of decay of the current, enhances the current and accelerates its decay kinetics in a manner similar to reactivation of current by higher concentrations of agonists. We suggest that protons interact with multiple extracellular sites on $\alpha3/\beta4$ nAChRs, decreasing the effective EC_{50} values of the agonist and accelerating gating kinetics, in part by promoting agonist-induced block. We speculate that corelease of protons with ACh from the secretory vesicles may induce rapid and reversible conformational changes in the slowly "desensitizing" $\alpha3/\beta4$ nAChRs, leading to accelerated signaling.

Neuronal nAChRs are widely distributed in the nervous system, mediating both pre- and postsynaptic signaling. Thus far, nine α ($\alpha2$–$\alpha10$) and three β ($\beta2$–$\beta4$) neuronal nAChR subunits have been identified, cloned, and functionally expressed [for recent reviews, see Lindstrom (1997); Lindstrom et al. (1998); McGehee (1999); Corringer et al. (2000); Dani (2001); Elgoyhen et al. (2001); Hsiao et al. (2001)]. Recombinant neuronal nAChRs composed of different subunit combinations expressed in *Xenopus laevis* oocytes or eukaryotic cell lines provide direct evidence for the variability of their affinity to agonists, antagonists, Ca^{2+} permeability, and rate of desensitization. However, little is known about the pH sensitivity of the neuronal nAChRs, even though there are many reports on the pH sensitivity of the muscle and electric organ nicotinic receptors (Trautmann and Zilber-Gachelin, 1976; Landau et al., 1981; Palma et al., 1991; Li and McNamee, 1992). These reports show that acidic pH generally inhibits the muscle nAChRs by reducing their single-channel conductance. In addition, kinetic analysis suggests a biphasic pH-dependence of the mean open time of the channel, with a maximum near the physiological pH (Landau et al., 1981; Palma et al., 1991). The rate of desensitization of the receptor seems also to be accelerated at both alkaline and acidic pH values (Li and McNamee, 1992). The proton-induced effects were found to be neither voltage-dependent nor mediated by changes in the ionic selectivity of the receptor.

Here we have examined the effect of rapid (<20 ms) and transient coapplication of various proton and agonist concentrations on the recombinant rat $\alpha3/\beta4$ nAChRs stably transfected in HEK cells. The rapid coapplication of protons and agonist was undertaken to approximate, in part, the transient local changes in pH that might occur as protons and the transmitter are coreleased from the secretory vesicles (Johnson, 1987; Miesenbock et al., 1998) during synaptic signaling. Our data suggest that protons interact with multiple extracellular sites on $\alpha3/\beta4$ nAChRs. The coapplication of protons enhances the agonist-induced current and accelerates its kinetics in a manner resembling the effects of a higher agonist concentration. We describe this as a decrease in the EC_{50} of the receptor, or an increase in its "apparent affinity", not to the exclusion of other mechanism. We speculate that transient changes of pH in the synaptic cleft might play a critical role in the regulation of synaptic signaling. A preliminary report of this work has already appeared (Abdrakhmanova et al., 2001).

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; HEK, human embryonic kidney; ACh, acetylcholine; GABA, γ-aminobutyric acid.

Materials and Methods

Cell Transfection and Culture. Stably transfected HEK 293 cells (American Type Culture Collection, Manassas, VA) expressing rat $\alpha3/\beta4$ neuronal nAChRs (cell line designation, KX$\alpha3\beta4R_2$) were prepared as described previously (Xiao et al., 1998). HEK 293 cells were maintained at 37°C with 5% CO_2 in the incubator. Growth medium for HEK 293 cells was minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfection was conducted by calcium phosphate method (Chen and Okayama, 1987) using a method identical to that described earlier (Zhang et al., 1999). Stably transfected cell lines were raised in selective growth medium containing 0.7 mg/ml of Geneticin (G418). Cells were plated in tissue culture medium (Invitrogen, Carlsbad, CA) containing bovine serum and antibiotics. ACh, nicotine, cytisine, and carbamylcholine chloride (carbachol) were purchased from Sigma Chemical Co. (St. Louis, MO) and were used at the indicated concentrations.

Electrophysiological Measurements. Functional expression of nicotinic receptors was evaluated in the whole-cell configuration of the patch-clamp technique using a Dagan 8900 amplifier (Dagan Corp., Minneapolis, MN). The patch electrodes, pulled from borosilicate glass capillaries, had a resistance of 3 to 4 MΩ when filled with internal solutions containing either 80 mM tetraethylammonium chloride, 60 mM NaCl, 5 mM MgATP, 10 mM glucose, 10 mM EGTA, 10 mM HEPES, and 0.1 mM cAMP (titrated to pH 7.4 with NaOH) or 110 mM CsCl, 20 mM tetraethylammonium chloride, 5 mM MgATP, 14 mM EGTA, and 20 mM HEPES (pH adjusted to 7.4 with CsOH). Higher $[Na^+]_i$ were used to provide for accurate measurements of reversal potential at moderate positive potentials in this highly rectifying nicotinic receptor (Zhang et al., 1999). In some experiments, the pH of the internal solution was adjusted to 6.0. About 90% of electrode resistance in the cell was compensated electronically, so that the effective series resistance in the whole-cell configuration was always less than 1 MΩ. Stably transfected HEK cells were studied for 2 to 4 days after plating the cells on the cover slips. Generation of voltage-clamp protocols and acquisition of data were carried out using pCLAMP software (Axon Instruments, Inc., Union City, CA). Sampling frequency was 0.5 to 2.0 kHz and current signals were filtered at 10 kHz before digitization and storage. All experiments were performed at room temperature (23–25°C). The measured currents were normalized relative to the membrane capacitance ranging between 18 and 40 pF and were quantified as the mean ± S.E.M. for the number of cells (n). To estimate the time constants of the decay of agonist-induced currents, the time course of the relaxation of current in the presence of agonists was determined by dividing the maximal slope (linear regression) by the peak amplitude of the current. This ad hoc method was preferred to exponential analysis because a clear monoexponential decay often was not observed (e.g., due to our relatively brief drug exposure times).

Application of Agonists and the Perfusion System. Cells plated on 15-mm round plastic cover slips (Thermanox; Nunc, Inc., Napierville, IL) were transferred to an experimental chamber mounted on the stage of an inverted microscope (Diaphot; Nikon, Nagano, Japan) and were bathed in a solution containing 137 mM NaCl, 10 mM HEPES, 1 mM $MgCl_2$, 10 mM glucose, 5.4 mM KCl, and 2 mM $CaCl_2$ (pH adjusted to 7.4 with NaOH). The experimental chamber was constantly perfused with the control bathing solution (1 ml/min). KCl was omitted from the control and agonist-containing puffing solutions to suppress possible K^+ currents in the voltage-clamped cell.

The amplitude and time course of the nicotine-activated current was highly dependent on the speed of application of nicotine. A reduction in flow rate significantly slowed the activation, decreased the amplitude, and slowed the desensitization of the nicotinic current (Callewaert et al., 1991). Therefore, we used servo-controlled miniature solenoid valves (Lee Company, Westbrook, CT) for rapid switching between control and test solutions (Cleemann and Morad, 1991; Zhang et al., 1999). The effective switching time was determined in rat ventricular cells by measuring the peak Na^+ currents at various times after triggering a change in $[Na^+]_o$ or by measuring the holding current at the tip of an open patch pipette subjected to a solution of low Cl^- (Davies et al., 1988). Under optimal conditions, such changes in solution had a delay of 6 to 8 ms (corresponding mainly to the pull and release of the solenoid valves and replacement of fluid in the common outlet of the perfusion manifold) followed by a transition period in which the measured current changed with a time constant of 5 to 10 ms. In repeated applications, the delay was fairly reproducible, but it did show variation in different experiments with different hydrostatic pressures and perfusion manifolds. In experiments aiming to measure the rate of activation of the nicotinic current (Fig. 8), fluid was applied under high hydrostatic pressure and the transition time was typically about 20 ms. Because the rapid flow of solution tended to dislodge the voltage-clamped cell, in less critical experiments, we lowered the hydrostatic pressure, resulting in slower fluid exchange periods (~50 ms; see Figs. 1, 5, and 10).

Results

Stably transfected HEK 293 cells expressing $\alpha3/\beta4$ nAChRs generated a rapidly activating but slowly desensitizing cationic current in response to step-changes in extracellular nicotine. Figure 1A compares the current recorded from a cell clamped at −80 mV in response to step application of 40 μM nicotine buffered at a pH value of either 7.4 or 6.0. The superimposed original traces show that nicotine solution buffered at pH 6.0 not only enhanced the current but also accelerated its decay kinetics, such that at the end of 1-s drug application at pH 6.0 the magnitude of current was significantly smaller. Similar enhancement of decay kinetics of the current induced by higher concentrations of agonist have been observed before and referred to as "rapid desensitization" (Lester and Dani, 1995) or "agonist-induced channel block" (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985; Luetje and Patrick, 1991; Maconochie and Steinbach, 1995; Philipson et al., 2001).

Figure 1B explores the time course and the magnitude of nicotine-activated current, when the proton concentrations were elevated before the application of nicotine. There was no significant difference in the magnitude or the kinetics of the nicotine-activated current whether the proton concentrations were elevated before or simultaneously with the application of nicotine. Furthermore, this experiment suggests that elevation of $[H^+]_o$ does not activate a significant current by itself.

In another series of experiments, we also tested the possible effects of step elevation of proton concentrations after the current was first activated by application of 40 μM nicotine at pH 7.4. Figure 1C shows superimposed traces of repeated application of nicotine at pH 6.0, at various intervals after the activation of current by 40 μM nicotine buffered at pH 7.4. Note that significant current is induced during the course of "rapid desensitization" by step elevation of proton concentration (pH 6.0). The effect of elevation of $[H^+]_o$ was accompanied not only by transient enhancement of the current but also by acceleration of its decay kinetics resulting in smaller steady-state current (see also Fig. 1A). Thus, irrespective of the timing of step-increase of $[H^+]_o$, the nicotine-activated current was enhanced and its gating kinetics were accelerated.

pH Effect on the Amplitude of Agonist-Induced Current. Quantification of the whole-cell currents induced by

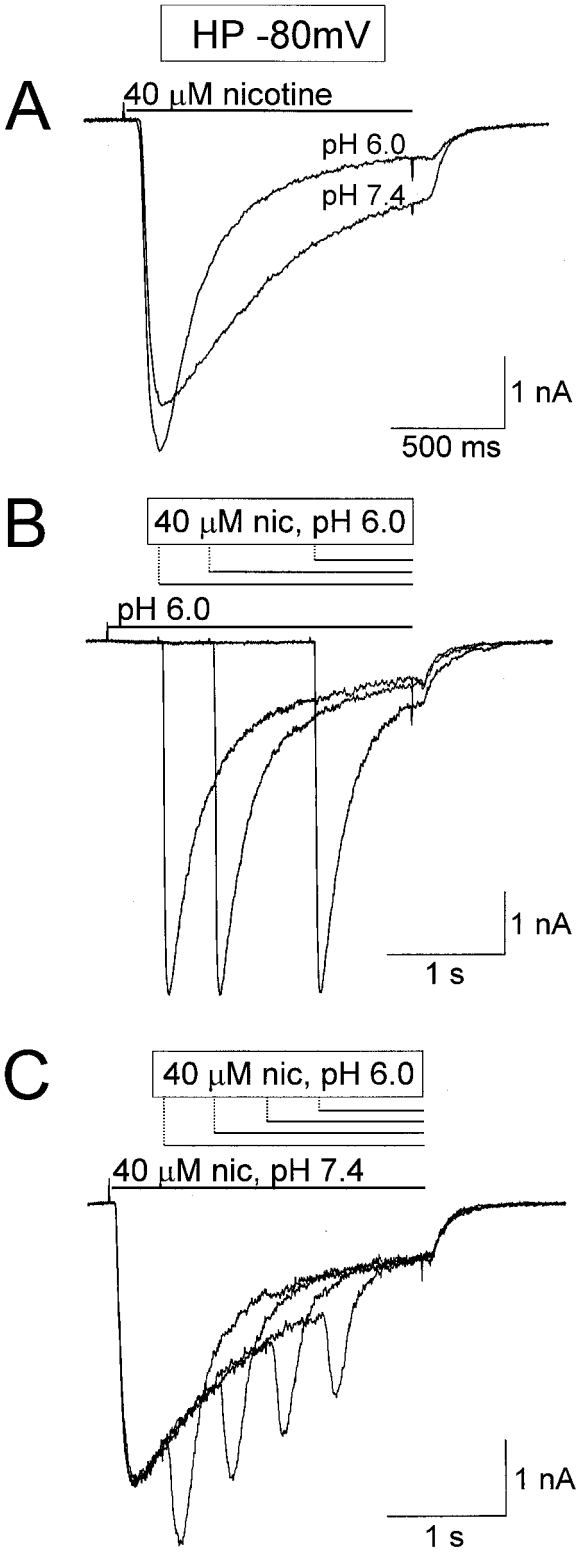


Fig. 1. Acidification enhances and accelerates the gating kinetics of nicotine-induced current. A, superimposed traces of currents activated by 40 μM nicotine at pH 7.4 and 6.0, recorded from a cell voltage-clamped at −80 mV. B, exposure of the cell to pH 6.0 for 415, 830 and 1660 ms, before application of 40 μM nicotine did not alter the magnitude or the kinetics of the current induced by nicotine. C, step-changes of pH from 7.4 to 6.0 applied at different times (415, 830, 1245, and 1660 ms) during the course of "desensitization" reactivate the nicotinic current. Recordings of A and B were obtained from the same cell (capacitance 27 pF), and those of C from another cell (capacitance 44 pF).

different nicotine concentrations (10, 40, 200, and 1000 μM) showed that increasing the $[H^+]_o$ generally enhanced the current and accelerated its decay kinetics. The pH effect on the magnitude of nicotine-evoked current was more pronounced at lower nicotine concentrations, becoming smaller at 200 μM and negligible at 1 mM (see legend of Fig. 2). Figure 2A compares the effect of elevation of $[H^+]_o$ at different agonist concentrations. Quantification of the data suggests a shift of concentration response curve, such that the proton effects are minimized at higher nicotine concentrations. The data points for activation of nicotine-induced current were fit with the empirical Hill equation $y = 1 / [1 +$

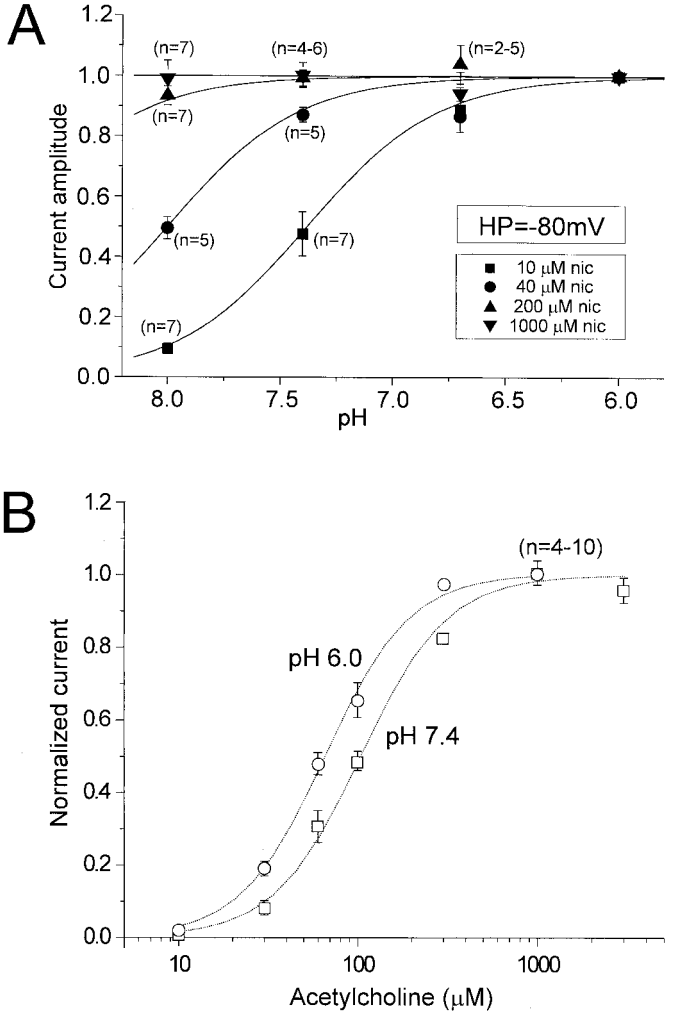


Fig. 2. Increasing $[H^+]_o$ enhances the apparent affinity of α3/β4 receptor to nicotine and ACh. A, dose-response curves for the peak currents induced by nicotine (■, 10 μM; ●, 40 μM; ▲, 200 μM; and ▼, 1000 μM) at different pH values (8.0, 7.4, 6.7, and 6.0) in cells voltage-clamped at −80 mV. The amplitude of the currents was normalized relative to the peak current at pH 6.0. Each symbol is labeled with the number of cells tested and a vertical error bar indicating the S.E.M. The maximal currents induced by 1 mM nicotine were 247 ± 8 pA/pF at pH 7.4 and 258 ± 11 pA/pF at pH 6.0 in paired measurements on four cells. B, dose-response curves for the peak currents induced by ACh at two different pH values (□, 7.4; ○, 6.0) in cells voltage-clamped at −80 mV. The currents recorded from each cell were normalized relative to the current induced by 300 μM ACh. The data points were fit with least-squares determination of EC_{50} and normalized to give a maximum response at one. The maximal currents induced by 1 mM ACh measured in two sets of cells were 259 ± 18 pA/pF at pH 7.4 (n = 5) and 304 ± 36 pA/pF at pH 6.0 (n = 4). Each symbol is labeled with a vertical error bar indicating the SEM (n = 4–10). The continuous curves represent a fit to Hill equation ($^{n_H} \sim 1.85$).

(EC_{50} / [protons]^{n_H})] yielding an apparent Hill coefficient (n_H) of ~1.5. This finding suggests that acidification may increase the apparent affinity of the $\alpha3/\beta4$ nAChRs to nicotine.

To examine more specifically whether protonation enhances the apparent affinity of $\alpha3/\beta4$ receptor to ACh, we measured the dose dependence of ACh-induced current at two different pH values. In this set of experiments, individual cells were exposed either to pH 7.4 or 6.0, while varying the ACh concentrations. Figure 2B shows that reduction of pH from 7.4 to 6.0, decreased the EC_{50} of ACh-evoked current from 105 to 67 μM, but had little effect on the cooperativity factor ($n_H \sim 1.85$). As for nicotine, acidification had no significant effect on the amplitude of the currents measured at saturating ACh concentrations (see legend of Fig. 2). Because ACh is unlikely to be protonated at pH 6.0, the data suggest a direct effect of protons on the $\alpha3/\beta4$ receptor.

It is well known that agonists of nAChRs differ in their pK_a values. Some, like nicotine or cytisine, are protonated [pK_a = 6.16 and 6.11, respectively (Windholz, 1983)], whereas others (e.g., ACh or carbachol) have no pK_a in the physiological range and are permanently ionized. To distinguish whether changes in pH modulate the channel function by acting at a proton sensing site on the receptor or by altering the ionization state of the ligand, we tested the pH effects of these four well-known agonists with different affinities to the receptor. The agonists were applied at concentrations approximating their EC_{50} values [for instance, 40, 60, 100, and 450 μM for nicotine, cytisine, ACh, and carbachol, respectively (Zhang et al., 1999; Meyer et al., 2001)]. Figure 3 shows that acidification causes an enhancement of the agonist-induced current for all four agonists in a similar range of pH values. The effect on the decay kinetics of the agonist-induced current, however, varied greatly depending on the type of the agonist. That is, in those with pK_a values around 6.1 (nicotine and cytisine), elevation of $[H^+]_o$ strongly accelerated the decay kinetics of their current, whereas those permanently ionized (ACh and carbachol) were less affected by acidic pH values (see also Fig. 6). These findings suggest that although the enhancement of the current by nicotinic agonists was not significantly dependent on the ionization state of the agonist, the decay kinetics of the current seemed to be modulated in part by the protonation of the agonists.

Comparison of Reactivation of the Current by Higher Agonist Concentration or Lower pH. Consecutive step-changes of pH from 7.4 to 6.0 during the course of decay of the current induced by 20 μM nicotine resulted in transient enhancement of the current and acceleration of its relaxation kinetics (Fig. 4A). To test whether this effect also resulted from increased apparent affinity of the receptor to the agonist (see Fig. 2), we compared the effects of step-changes of elevation of $[H^+]_o$ with step-increases of the agonist concentration (20 to 60 μM) in the same cell. Step-increase of nicotine from 20 to 60 μM augmented both the current and its relaxation (Fig. 4B) in a manner similar to step elevation of $[H^+]_o$ (Cf. Figure 4A). As the step-changes of either pH 6.0 or 60 μM nicotine were done repeatedly at different times during the course of decay of the current activated by 20 μM nicotine, we compared the magnitude of reactivated current measured in five cells in which both proton and nicotine concentrations were increased (Fig. 4C). The magnitude of reactivated currents at later times (Fig. 4C, traces 2, 3, and 4) were measured relative to the first reactivated current (Fig. 4C, trace 1). Figure 4C shows that current reactivated by step-change to pH 6.0 remained almost constant (within 6%) during the course of "desensitization", whereas the current reactivated by step-increase in nicotine concentration decreased significantly (38%) in 1245 ms. These data demonstrate that the effects produced by acidification and increased agonist concentration may look similar but, in fact, are qualitatively different. Thus, it seems that step elevation of proton concentrations, in addition to increasing the apparent receptor affinity to agonist, may partially protect the receptor against events that lead to suppression of the current in the presence of agonist ("rapid desensitization").

pH Modulation of Decay of Agonist-Induced Current, "Rapid Desensitization". One of the consistent observations of this study was that the decay kinetics of nAChR

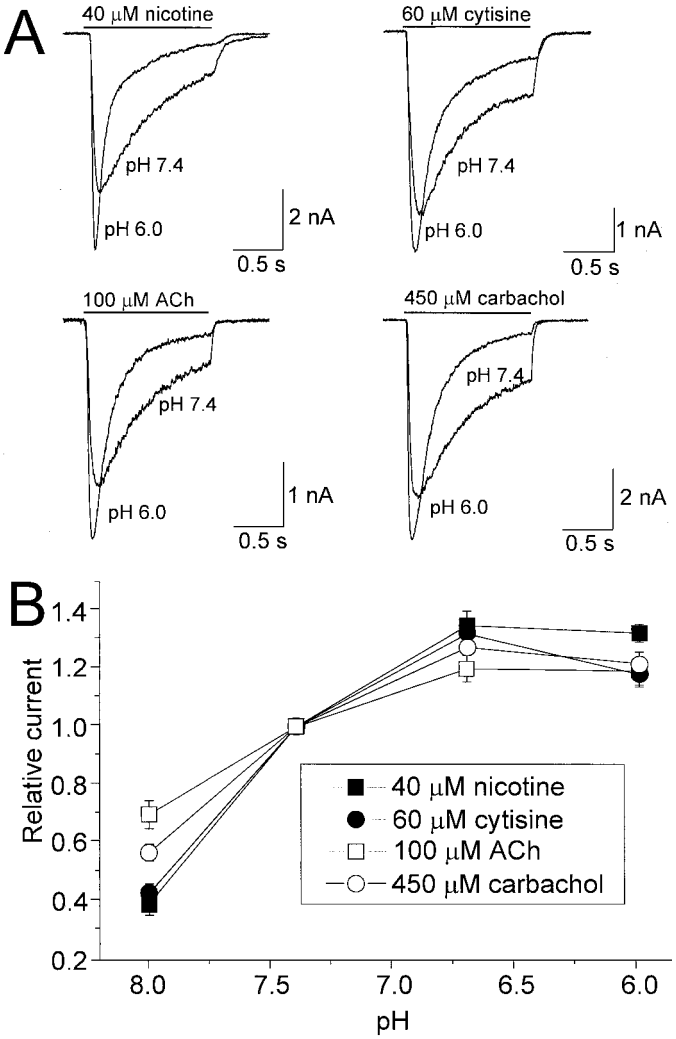
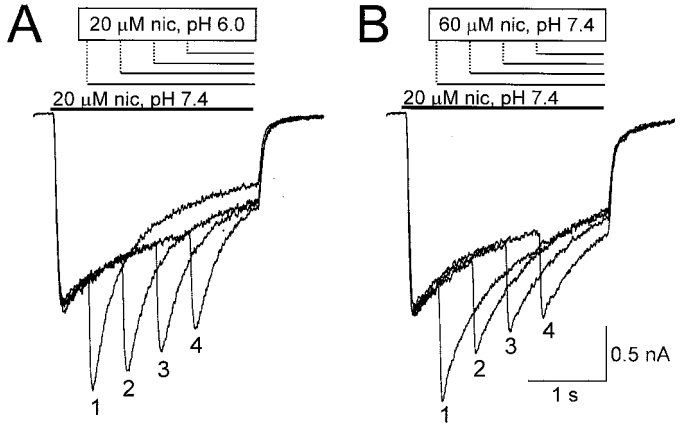


Fig. 3. Comparison of the pH effect on the amplitude of the current activated by different agonists. A, superimposed traces of currents evoked at pH values 7.4 and 6.0 by nicotine (■, 40 μM), cytisine (●, 60 μM), ACh (□, 100 μM), and carbachol (○, 450 μM). The concentrations chosen approximate the EC_{50} value for each agonist, and the duration of agonist application is indicated by the horizontal bar. B, pH-dependence of the current amplitude. Agonist-activated current was recorded in each cell at pH 8.0, 7.4, 6.7, and 6.0 (n = 4–5). The amplitude of the currents activated at different pH values, when normalized relative to the peak current measured at pH 7.4, produced a similar relationship for different agonists.

current were significantly accelerated by rapid elevation of $[H^+]_o$, irrespective of the nature of the agonist. Figure 5 compares the effect of application of 40 μM ACh, carbachol, nicotine, and cytisine at four different pH values (8.0, 7.4, 6.7, and 6.0). Simultaneous application of any of the four agonists at higher proton concentrations not only enhanced the current through the receptor, but also accelerated its relaxation.

Figure 6 quantifies the effects of changes in pH on the kinetics of relaxation of the currents induced by four different agonists: nicotine, cytisine, ACh, and carbachol. In this set of experiments, the concentration of agonist chosen was close to the EC_{50} of each drug. The analysis is based on records similar to those shown in Fig. 3A. Such analysis shows that the rates of decay of cytisine- and nicotine-activated currents (A) were strongly enhanced at acidic pH values (~10-fold from pH 6.0 to 8.0), whereas those of carbachol- and ACh-evoked currents (B) were less strongly modified (~4 fold from pH 6.0 to 8.0). This finding is consistent with the idea that increased ionization of cytisine and nicotine ($pK_a \sim 6.1$) at lower pH values provides the agonist with better access to the mouth of the channel, or to sites that regulate "desensitization". On the other hand, the significant effect of protons on carbachol and ACh-induced currents (Fig. 6B) supports the notion that pH-dependence of the decay kinetics is not determined exclusively by the ionization state of the agonist but may be directly mediated by the receptor.

Figure 7 quantifies the effect of elevation of $[H^+]_o$ on the kinetics of decay of the currents measured in the experiments illustrated in Fig. 2. Figure 7A quantifies the pH effect at two different nicotine concentrations, 40 μM (~EC_{50}) and 1 mM



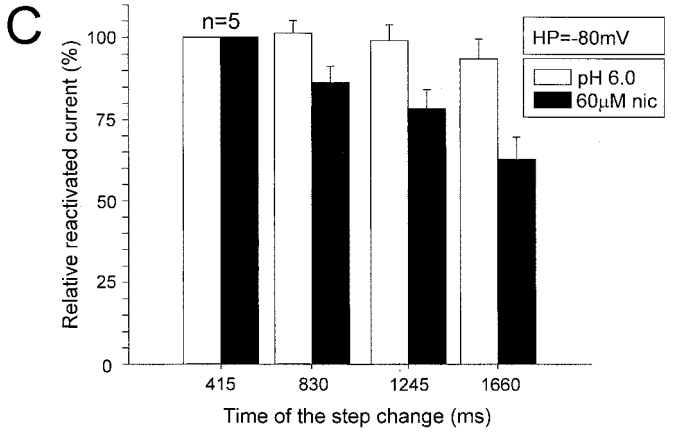


Fig. 4. Reactivation of nicotinic current by acidification and higher nicotine concentration during the time course of "desensitization". Consecutive step-changes in $[H^+]_o$ to pH 6.0 (A) or to 60 μM nicotine (B) were applied to the same cell (holding potential −80 mV). The reactivated current was evoked induced at time intervals of 415 (1), 830 (2), 1245 (3), and 1660 (4) ms, after the initial application of 20 μM nicotine. Bar graph in C represents the mean ± S.E.M. ($n = 5$ cells) of normalized reactivated currents at the indicated times evoked either by step-changes to pH 6.0 (□) or by step-increases of nicotine concentration at the indicated times (■).

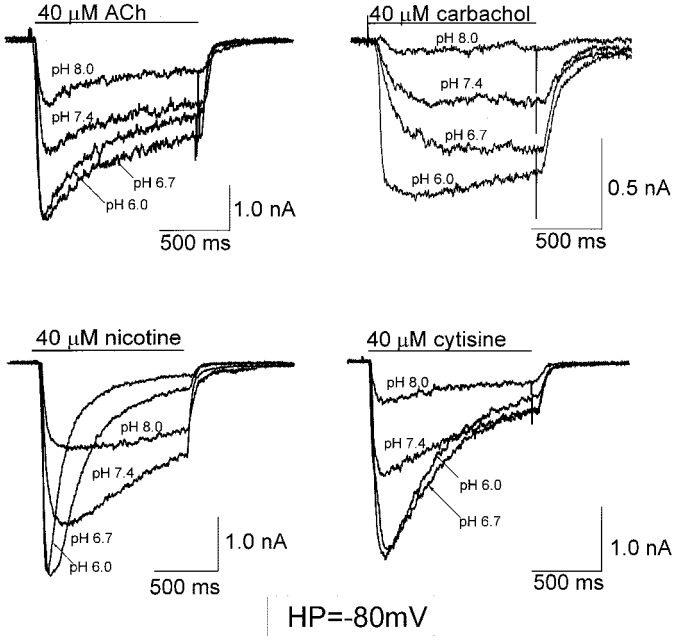


Fig. 5. Agonist-dependent pH response. Each shows superimposed current traces of the indicated agonist coapplied with proton concentrations of pH 8.0, 7.4, 6.7, and 6.0 recorded on different representative cells (holding potential, −80 mV). Each agonist was applied at 40 μM concentrations. Acidification uniformly enhanced the current activated by every agonist.

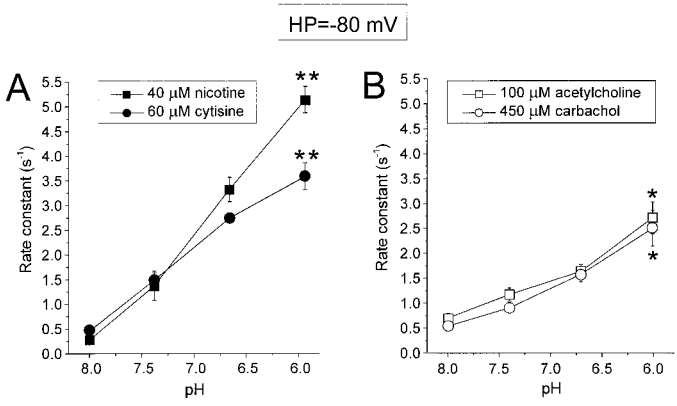


Fig. 6. Acceleration of decay kinetics of the currents by acidic pH is influenced by the pK_a of the agonists. Rate constants of decay of the whole-cell currents induced by: 40 μM nicotine (■) and 60 μM cytisine (●) (A) or 100 μM ACh (□) and 450 μM carbachol (○) (B) are compared at pH values of 8.0, 7.4, 6.7, and 6.0. The rates of decay, in pH range from 8.0 to 6.0, accelerated markedly for the nicotine (pK_a 6.16)- and cytisine (pK_a 6.11)-induced currents (A). The acceleration of the rates of decay was smaller for ACh- and carbachol-activated currents (with no known pK_a; $n = 4$–5 for each agonist). For each agonist, paired t test showed that the rate constants of decay of the currents at pH 6.0 were significantly faster than that measured at pH 8.0 (**, $P < 0.002$; *, $P < 0.02$).

(saturating concentration). Analysis of the data in 5 to 6 cells per concentration of the agonist at pH values from 8.0 to 6.0 shows that the rate constants of decay of the current were dependent on the agonist concentrations, such that at 1 mM nicotine increases in $[H^+]_o$ had little or no effect on the time course of relaxation, consistent with the finding of Fig. 2 and 4 that elevation of proton concentration mimics the effect of increased agonist concentrations. Figure 7B compares the dose dependence of the rate constants of decay of current when pH is changed from 7.4 to 6.0. Similar to the effect of pH 6.0 on ACh-induced peak current in the same set of cells (Fig. 2B), acidification decreased the mean EC_{50} value for the rate of decay of the ACh-induced currents (from 371 to 139 μM). Furthermore, in a manner similar to the nicotine-induced responses (Fig. 7A), the rates of decay of the ACh-activated currents were not significantly altered by acidification at saturating ACh concentrations.

pH Effect on Rate of Activation of Agonist-Induced Current. Elevation of $[H^+]_o$ also seemed to enhance the rate of activation of the receptor. This effect was most pronounced when using carbachol as an agonist (Fig. 8). Similar, but less pronounced effects were also observed with nicotine when comparing the rate of activation of the current at pH 8.0 and 6.0. Consistent with this idea, comparison of rise time of responses induced by nicotine (high-affinity agonist) and carbachol (low-affinity agonist) induced responses at pH 7.4 and 6.0 (using different agonist concentrations), indicated that for both agonists, currents activated markedly faster in acidic pH values, and the effect was more pronounced at lower agonist concentrations. The original traces in Fig. 8, A and B, illustrate representative examples of the pH effect on the rate of activation of nicotine- and carbachol-evoked currents when the agonists were applied at comparable effective concentrations of 20 and 200 μM, respectively. The data from a number of cells, quantified in lower graphs of Fig. 8, show that even though the rise times (10–90%) of nicotine-induced currents at pH 7.4 and 6.0 were similar for 100 or 200 μM nicotine concentrations, the current accelerated on average by 26% (from 38 to 28 ms) for 40 μM, and 39% (from 80 to 49 ms) for 20 μM nicotine (Fig. 8, lower left). For carbachol-induced currents, on the other hand, the pH effect on the rise time was minimal only at 1000 μM concentration but became larger at smaller carbachol concentrations at acidic pH values. For instance, current accelerated ~4 fold for responses induced by 200 and 40 μM carbachol (from 120 to 28 ms and from 261 to 99 ms, respectively). The pronounced effect of changes in pH on activation kinetics of carbachol-induced

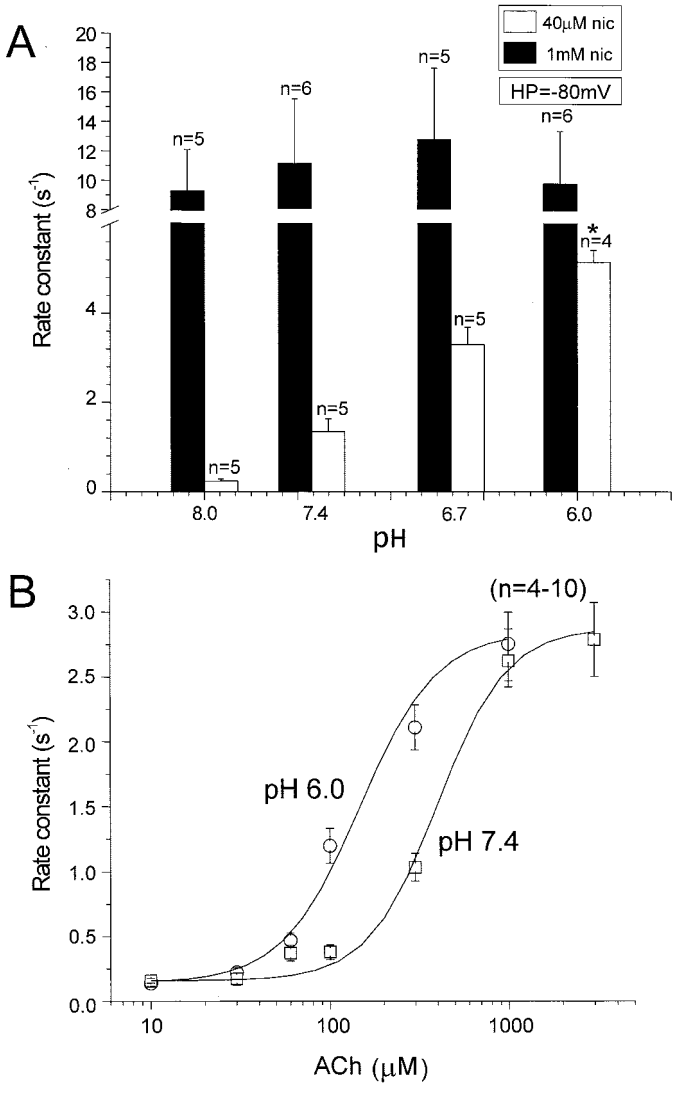


Fig. 7. Rate of decay of current at different pH values and agonist concentrations. A, the pH effect on the rate of decay of nicotine-induced current is dependent on concentration of the agonist. Bar graph represents the rate constants of decay of the currents induced by 40 μM nicotine (□) and 1 mM nicotine (■) (n = 5–6) at pH values 8.0, 7.4, 6.7, and 6.0. At 1 mM nicotine concentration, the rate of decay is not significantly altered by the acidic pH values. * indicates statistically significant difference between rate constants of decay compared at pH values 8.0 to 6.0 for currents induced by 40 μM nicotine ($P < 0.002$). B, acidification accelerates and shifts the rate of decay of the ACh-induced current toward smaller ACh concentration (holding potential, −80 mV). The data points (n = 4–10) were fit by the Hill equation (n_H = 2.0) with EC_{50} values of 371 μM at pH 7.4 and 139 μM at pH 6.0.

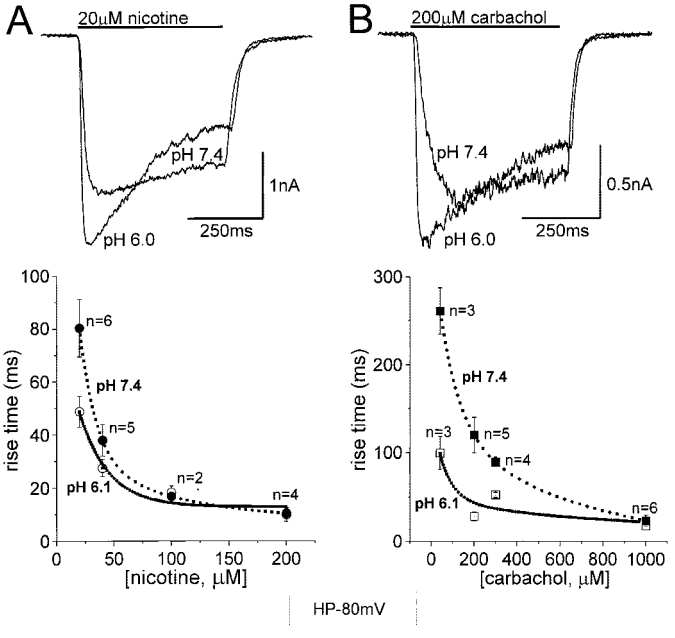


Fig. 8. The effect of acidification on the rate of activation of currents evoked by high- and low-affinity agonists. Effect of pH 6.0 on rise time of currents activated by nicotine concentrations of 20 to 200 μM (A), and by carbachol (40–1000 μM) (B). Top, representative superimposed traces of rise time of currents induced at pH 7.4 and 6.0 by 20 μM nicotine (A) and 200 μM carbachol (B) in the same cell (holding potential, −80 mV).

current may result, in part, from the lower affinity of this drug for $\alpha 3/\beta 4$ nAChRs.

The Site and Mechanism of Proton-Induced Modulation. To determine the site of the proton effect on the nicotinic receptors, a number of cells ($n = 9$) were dialyzed with pipette solutions buffered at pH 6.0. Figure 9A compares nicotine-induced currents in two representative cells dialyzed, respectively at pH_i values of 7.4 or 6.0. There was no significant difference in the kinetics of nicotine-activated current between cells dialyzed with pH_i values of 7.4 and 6.0 (Fig. 9B). Furthermore, elevation of extracellular proton concentrations continued to enhance the current and accelerate its decay kinetics, irrespective of $[H^+]_i$ (Fig. 9B). These data are consistent with those of Fig. 1 and suggest that protons interact primarily with the extracellular domains of the receptor.

Whether pH-induced altered cationic selectivity of nAChRs could be responsible for changes in the magnitude and kinetics of the agonist-induced current was also tested. Figure 10 shows data from two cells in which the effects of nicotine and ACh at EC_{50} values on the voltage-dependence of the activated current were quantified at pH 6.0, 7.4, and 8.0. Currents induced by ACh or nicotine showed strong rectification between 0 and 100 mV, a characteristic of $\alpha 3/\beta 4$ receptor (Zhang et al., 1999; Haghighi and Cooper, 2000), but there was no measurable change in the reversal potential of the current activated at different $[H^+]_o$ ($n > 20$). Note that both ACh and nicotine significantly enhanced the current and its kinetics as the pH was changed from 8.0 to 7.4 and 6.0. The data are consistent with the idea that enhancement of the current and acceleration of its kinetics is brought about by a mechanism independent of the change in selectivity of the channel.

Experiments of the type shown in Fig. 10 were analyzed in greater detail to test whether the pH effect was modulated by the holding potential. The top of Fig. 11 shows the manner in which the peak currents at different potentials evoked by 40 μM nicotine (A) or 100 μM ACh (B) were altered by pH 6.0 and pH 8.0 relative to the currents measured at pH 7.4. The effects of pH on the current amplitude was not significantly voltage-dependent (Fig. 11). A similar analysis was performed for the decay kinetics of the current. The rates of decay of the current induced by nicotine (C) and ACh (D) were independent of voltage at pH 6.0. Consistent with our finding that protons increase the affinity of $\alpha 3/\beta 4$ receptors to the agonists (see Fig. 2), it is possible that at pH 6.0, the current activated by 40 μM nicotine mimics that induced by saturating nicotine concentrations at which the effects of protons on the decay kinetics of the current are markedly reduced or negligible (see Fig. 7). At pH 7.4, the rates of decay of the nicotine- and ACh-induced currents (C and D) were accelerated at very negative holding potentials, consistent with the idea that protons or positively charged agonist may modulate the rate of "desensitization" slightly by moving through a small fraction of the membrane field. This effect was quantified in terms of a Boltzmann factor, $\exp(\alpha FV_m/RT)$ [where V_m is the membrane potential, R is the gas constant,

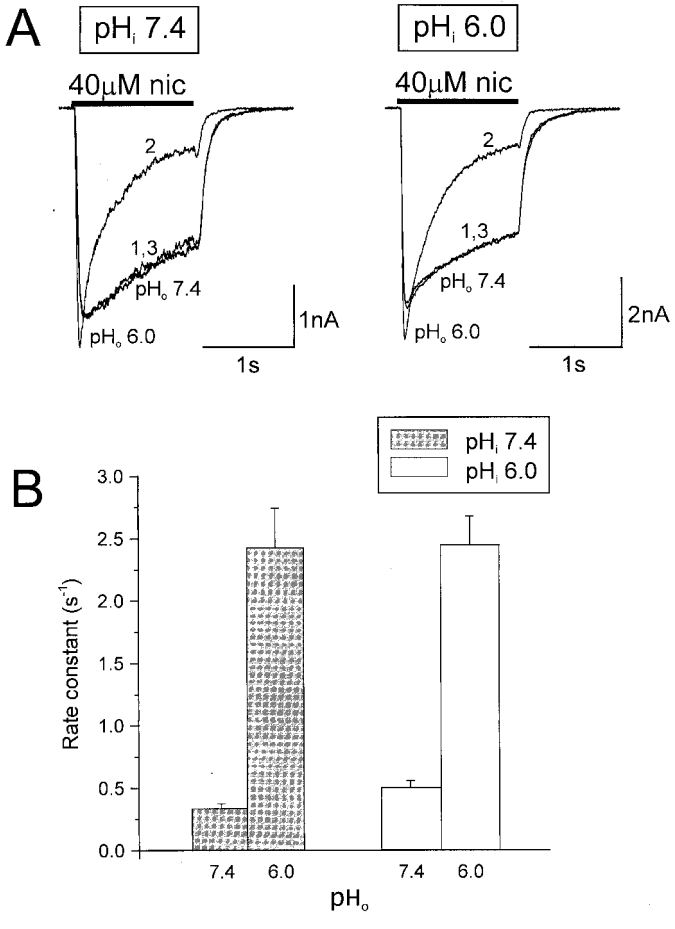


Fig. 9. Comparison of the effect of external acidification on the nicotine-activated current in cells buffered with two different intracellular pH values. A, representative superimposed traces of currents evoked by 40 μM nicotine coapplied with either pH 7.4 (trace 1) or 6.0 (trace 2) and return to pH 7.4 (trace 3) in two different cells dialyzed by pipette solutions of either pH 7.4 (left) or pH 6.0 (right). B, average effects of external acidification on nicotine-induced currents recorded from cells dialyzed by pipette solution of pH 7.4 ($n = 3$) or pH 6.0 ($n = 9$). Holding potential, −80 mV.

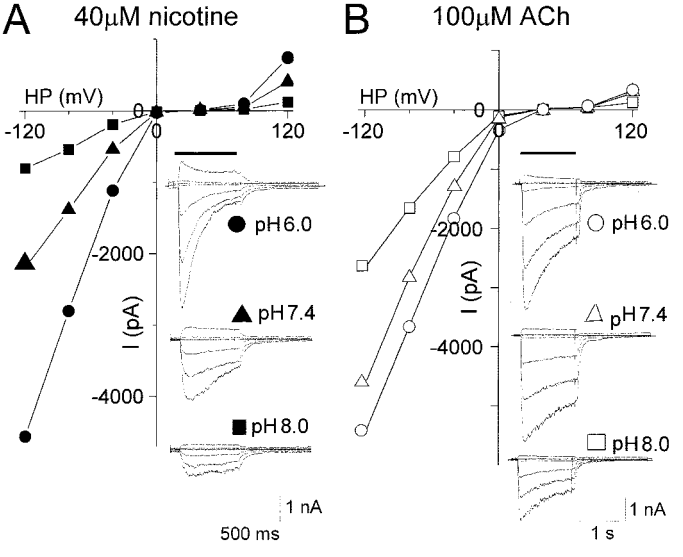


Fig. 10. Voltage-dependence of nicotine- and ACh- induced currents at different pH values. Each shows data obtained from a single representative cell. Current was quantified at its maximal value by activating it with either 40 μM nicotine (A) or 100 μM ACh (B). Reversal potentials for nicotine- (26.6, 26.7, and 28.5 mV) or ACh-evoked currents (33.3, 34.6, and 37.6 mV) were not significantly different at pH 8.0, 7.4, and 6.0. The three insets in A and B represent superimposed traces of the evoked currents at the indicated pH values.

T is the absolute temperature (RT/F = 25 mV), and α is the fraction of membrane field seen by monovalention]. The values of α, calculated between −120 mV and 0 mV (0.18 for nicotine, 0.11 for ACh), in combination with the apparent Hill-coefficient (~2; Fig. 7B), suggest that H^+ moves through only a small fraction (<9%) of the electric field of the membrane. This fraction is even smaller if the rate constants measured at +120 mV are included in the analysis (<6% for nicotine, < 2% for ACh). These approximate calculations support the notion that protons do not move appreciably into the channel pore but modulate the nAChR from extracellular sites.

Discussion

The major finding of this report is that rapid coapplication of agonists and protons first enhances and then suppresses the evoked current through the $\alpha3/\beta4$ nAChR. The eventual suppression of the current is most likely caused by acceleration of the decay kinetics of the agonist-induced current in acidic pH values, depending, in part, on the pK_a value of the agonist. The pH effect was specific to the extracellular site of the receptor, was mostly independent of potential, and was not accompanied by significant changes in the cationic selectivity of the $\alpha3/\beta4$ receptor. In this respect, the response of $\alpha3/\beta4$ nAChR to acidification was similar to that described for non-neuronal nAChR in frog neuromuscular junction (Trautmann and Zilber-Gachelin, 1976; Landau et al., 1981), the *N*-methyl-D-aspartate (Tang et al., 1990), and $GABA_A$ receptor (Krishek et al., 1996). However, in sharp contrast to the effect of acidification on other ligand- (Traynelis, 1998) or voltage-gated channels (Tombaugh and Somjen, 1996), the cationic current through the $\alpha3/\beta4$ receptor was transiently enhanced by acidic pH values, suggesting that protons may rapidly and reversibly alter nAChR gating without interfering with the permeation path of the receptor. The acceleration of decay kinetics in acidic pH values led eventually to suppression of steady-state evoked current consistent with the idea that protons may increase the rate and extent of desensitization or block of the channel either by interfering with its permeation site (Imoto et al., 1988; Prod'hom et al., 1989) and/or by allowing the agonist to serve as an open channel blocker (Sine and Steinbach, 1984; Ogden and Colquhoun, 1985; Luetje and Patrick, 1991; Maconochie and Steinbach, 1995; Philipson et al., 2001).

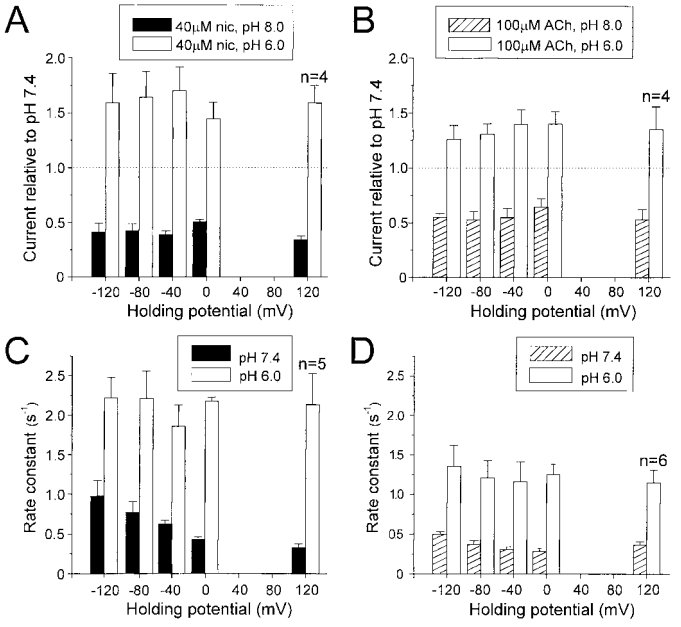


Fig. 11. Analysis of voltage-dependence of agonist-induced current and its "desensitization". A, mean (± S.E.M.) amplitude of the currents activated by 40 μM nicotine (n = 4) at pH 8.0 (■) and at pH 6.0 (▨), and that activated by 100 μM ACh (B; n = 4) at pH 8.0 (▨) and at pH 6.0 (squlo]) measured at different holding potentials and normalized to pH 7.4. Similarly, in the same set of cells, the voltage-dependence of mean (±S.E.M.) rate constants of decay ("desensitization") of the currents activated by 40 μM nicotine (C; n = 5) at pH 7.4 (■) and at pH 6.0 (□) are compared with those activated by 100 μM ACh (D; n = 6) at pH 7.4 (▨) and pH 6.0 (□). Because few if any currents were activated at 40 and 80 mV, these data points were excluded from the analysis.

One critical procedure used in the present study that may bear on comparing the present results with those of other investigators was that the agonist and the higher proton concentrations were coapplied rapidly (~ 20 ms) for periods less than 2.0 s, making it possible to quantify both the transient and quasi–steady-state responses to extracellular acidification. In this respect, it was noted that the potentiating effects of acidification on the $\alpha3/\beta4$ occurred within 50 ms, allowing the nicotinic agonists to both transiently enhance and then suppress the current (Fig. 3). In part, some of the differences between pH modulation of $\alpha3/\beta4$ neuronal nAChR reported here and those reported for the muscle and *Torpedo californica* nicotinic receptors may be related to much slower application of agonists used in the data published previously.

Does $[H^+]_o$ Change the Affinity of $\alpha3/\beta4$ nAChR? Figs. 1, 2, 3, 5, and 8 clearly show that coapplication of agonists with higher proton concentrations enhanced the magnitude and accelerated the rate of activation of the current. These effects were more pronounced at lower agonist concentrations (Fig. 2 and 8) and/or when using lower affinity agonists (Fig. 5). In fact, at nicotine concentrations between 0.2 to 1 mM, the amplitude and the rate of rise of the current were only minimally modified by elevation of $[H^+]_o$ (Figs. 2A and 8A). This finding suggests that as the receptors become fully saturated, their gating can no longer be modified by elevation of $[H^+]_o$, as if increasing the $[H^+]_o$ emulated the increase in agonist concentration. For carbachol and ACh immunity to pH modulation occurred at saturating concentrations of ~1 mM (Figs. 2B and 8B). In the case of acidic agonists, such as nicotine and cytisine (pK_a ~ 6.1), the effects of pH may, in part, reflect preferential binding of the protonated agonist to receptor sites. In this respect, it is interesting that the natural neurotransmitter ACh, which is ionized and not further protonated at pH 6.0 to 8.0, has a highly pH-sensitive dose-response curve (shifting its EC_{50} from 105 μM to 67 μM; Fig. 2B) exhibiting similar pH-induced changes in the amplitude and kinetics of the current.

The fast activation kinetics of AChRs have been analyzed previously in considerable detail, making it possible to distinguish, for instance, between ligand-binding and channel opening (Maconochie et al., 1994; Maconochie and Steinbach, 1995). The role of protons in such multistate schemes could be important in evaluation of their potential role as modulators of fast neuronal signaling. In this context, it should be noted that the resolution of faster (<20 ms) activation times often observed with high affinity agonists or at higher drug concentrations (Fig. 8), maybe limited in our study by the

inherent delays (~10 ms) in the application speed of the solutions.

The possibility that elevation of $[H^+]_o$ may increase the apparent affinity of the receptor for the agonists is not supported by studies on frog, chick, and *T. californica* non-neuronal nAChRs, where acidification has been shown to decrease both the conductance and kinetics of the channels without affecting the agonist binding affinity (Huang et al., 1978). In muscle nAChR, elevation of $[H^+]_o$ decreases both the single-channel conductance and its mean open time, consistent with the idea that carboxylic side chains near the vestibule of the channel were protonated (Imoto et al., 1988). Similarly, elevation of $[H^+]_o$, suppresses the voltage-gated Ca^{2+} channels by reducing their single-channel conductance (Prod'hom et al., 1989; Tombaugh and Somjen, 1996). The mean open time of non-neuronal nAChR channel, however, shows bell-shaped dependence on pH_o, with a maximum around pH 7.4, consistent with the idea that protonation may also affect the alkaline pK_a sites (e.g., histidine moieties) (Landau et al., 1981). On the other hand, somewhat similar to our finding, in $GABA_A$ receptor of cerebellar granule cells or in the recombinant GABA receptor $\alpha1\beta1$, enhancement of the anionic current at low pH values seems to be mediated by the higher affinity of the receptors to the agonist (Robello et al., 1994). Thus, the proton-induced modulation of $\alpha3/\beta4$ nAChR seems to be qualitatively different from that reported for the muscle or *T. californica* nAChRs (Trautmann and Zilber-Gachelin, 1976; Landau et al., 1981; Palma et al., 1991; Li and McNamee, 1992) and more consistent with the possibility that step-increases in $[H^+]_o$ induce a transient conformational change of the receptor, resulting in higher apparent receptor affinity.

Does pH Alter the Decay Kinetics of the Agonist-Induced Current? The other major observation of this study is that elevation of proton concentrations accelerates the decay kinetics of the $\alpha3/\beta4$ nAChRs (Fig. 1, 4, 5, 6, and 7). It is likely that ionization state of the ligand contributes, in part, to acceleration of relaxation of the current on increasing the $[H^+]_o$, because the decay kinetics of nicotine- and cytisine-activated current (pK_a ~6.1) were more strongly accelerated at pH 6.0 than those of ACh and carbachol (with no known pK_a) (Fig. 6). This finding suggests that the protonated forms of nicotine and cytisine may have better access to the channel pore, consistent with the idea that the decay of the nicotinic current reflects not only receptor desensitization but also the agonist-induced channel block (Sine and Steinbach, 1984; Webster et al., 1999). Models for the agonist-induced channel block of AChRs suggest that the blocked channels may isomerize into states comparable with a drug-bound closed state or the "desensitized" state (Maconochie and Steinbach, 1995). Alternatively, nicotine may bind to some inhibitory site with lower off-rate (dissociation rate) that becomes available when the receptor is subjected to superpharmacological concentrations of the agonist (Webster et al., 1999).

In some sets of experiments, the rate constants of decay of the agonist-induced currents were found to be different even under identical conditions; for instance, the values presented in Figs. 9 and 11 are somewhat smaller than those in Figs. 6 and 7. Such variations in the rate of "desensitization" were reported earlier (Zhang et al., 1999) in this cell line, and may be related to the stoichiometry of subunit expression.

In contrast to our observations, the bell-shaped dependence of pH modulation in frog neuromuscular junction (Landau et al., 1981) was interpreted to be caused by the electrostatic interaction between two fixed and titratable ionic groups and a mobile charge in the receptor molecule. It is likely, therefore, that different nicotinic receptor subtypes are differentially modulated by $[H^+]_o$, depending, in part, on their molecular structure.

The action of protons seems to occur at extracellular sites of the $\alpha3/\beta4$ receptor, because intracellular acidification produced no effects (Fig. 9). Similarly, the protons do not seem to penetrate sufficiently into the channel pore or membrane field to alter ionic selectivity (Fig. 10) or voltage dependence (Figs. 10 and 11), respectively. This finding is consistent with those reported for the nAChR in frog neuromuscular junction (Trautmann and Zilber-Gachelin, 1976; Landau et al., 1981).

Physiological Implications. Rapid coapplication of the nicotinic agonists and protons provides an approximation to physiological release of the contents of the vesicle into the synaptic cleft. Considering the high vesicular proton concentration (pH ~ 5.5; Miesenbock et al., 1998), it is likely that significant transient acidification of the postsynaptic receptor takes place before activation of the current. If indeed the receptors in vivo were to behave like the recombinant $\alpha3/\beta4$ receptors, then ACh, a low-affinity agonist, would transiently develop the properties of a high-affinity ligand, thus evoking a larger and faster current, especially at lower ACh concentrations (Fig. 5). The advantage of protons as cotransmitters includes rapid diffusion speeds, ability to be rapidly buffered by the extracellular buffers, and allowing the low-affinity ligand to have both fast on- and off-rates. The proposed pH-modulation of neuronal signaling is presently rather speculative because it will depend on the yet-to-be-determined ability of nAChR to respond to a possible pH change in the synaptic clefts. Irrespectively, such a mechanism would be quite different from the well-studied suppression of nicotinic current by longer lasting acidification.

We also considered how the enhanced relaxation of current might accelerate synaptic signaling. The finding that progressively longer exposures to acidic pH values during the onset of "desensitization" of $\alpha3/\beta4$ nAChRs results in the enhancement of the reactivated nicotinic current (Fig. 4) might suggest that as protons accelerate the relaxation of the current, they may also protect the $\alpha3/\beta4$ receptor against "rapid desensitization". This is consistent with the finding that the pH-induced reactivated current remained fairly constant during the time course of "rapid desensitization" compared with that induced by step-increases in nicotine concentrations (Fig. 4, see also Fig. 1C). It is possible that as protons increase the apparent affinity of $\alpha3/\beta4$ receptors to the agonist, they may also slow down the processes that leads to "rapid desensitization" of the receptor (Fig. 4). We speculate that during repetitive firing of a cholinergic synaptic pathway, as protons and ACh are coreleased from the secretory vesicles for a few milliseconds into the synaptic cleft, they provide the conditions that are essential for generating rapidly relaxing currents mediated by the slow $\alpha3/\beta4$ receptor (Fenster et al., 1997) without causing significant "desensitization" that would preclude its reactivation.

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Address correspondence to: Dr. Martin Morad, Department of Pharmacology, Georgetown University School of Medicine, 4000 Reservoir Road Building D, Washington DC 20007. E-mail: moradm@georgetown.edu