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Differential Modulation of β2 and β4 Subunits of Human Neuronal Nicotinic Acetylcholine Receptors by Acidification

Galya Abdrakhmanova, Lars Cleemann, Jon Lindstrom, and Martin Morad

Department of Pharmacology, Georgetown University School of Medicine, Washington DC (G.A., L.C., M.M.); and Department of Neuroscience, Medical School of the University of Pennsylvania, Philadelphia, Pennsylvania (J.L.)

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ABSTRACT

We have shown previously that acidification increases the affinity of agonists to rat $\alpha3\beta4$ nicotinic acetylcholine receptors (nAChR) and accelerates both the activation and decay kinetics of agonist-induced currents recorded from human embryonic kidney 293 cells stably expressing the receptor (Abdrakhmanova et al., 2002b). Here, we report on experiments examining the effect of rapid acidification on four different subtypes ($\alpha3\beta4\alpha5$, $\alpha4\beta2$, $\alpha3\beta2$, and $\alpha3\beta2\alpha5$) of human neuronal nAChRs stably expressed in tsA201 cells using a piezoelectric device for rapid ($<$5 ms) solution application. Application of ACh, at its EC_{50} concentration for each nAChR subtype, at pH values 7.4 and 6.0, showed that acidification, similarly to that reported for rat $\alpha3\beta4$ acetylcholine receptors (AChRs), increased the amplitude and accelerated the activation and decay kinetics of the currents in human $\alpha3\beta4\alpha5$ AChRs by increasing their affinity to the agonist. In sharp contrast, acidification reduced the amplitude but accelerated the decay kinetics of the current in all human $\beta2$-containing nAChR subtypes ($\alpha3\beta2$, $\alpha3\alpha5\beta2$, $\alpha4\beta2$) examined in this study. Brief application of ACh at saturating concentration (1 mM) on $\alpha3\beta4\alpha5$ AChRs induced a "rebound current" upon rapid washout of the agonist at pH 7.4, but no "rebound current" was observed in $\alpha3\beta2$ AChRs. Surprisingly, acidification, pH 6.0, applied only during the agonist pulse also accelerated the decay kinetics of the "rebound current". Our data provide evidence for the specificity of proton-induced modulation of neuronal nAChRs based on their β subunit composition. Furthermore, in $\alpha3\beta4\alpha5$ AChR, we find that protonation effects may persist, after washout of acidic solutions, consistent with proton-induced conformational changes of the receptor.

Cellular functions of mammalian nervous systems depend on tight regulation of both extracellular and intracellular pH. It is becoming clear that extracellular pH is far from static and at times may be subjected to both long and transient acidification in response to normal neuronal activity, ischemia, hypoxia, epilepsy, etc. (Chesler, 1990; Chesler and Kaila, 1992). Irrespective of cause, acidification typically suppresses cationic channels. Although local fluctuations in pH at synapses have not been directly quantified, the processes that may induce such changes have been described. For instance, because synaptic vesicles have a low pH (Miesenbock et al., 1998) and the buffering capacity of the synaptic cleft is limited during brief synaptic events (Tong et al., 2000; Chesler, 2003), protons coreleased with the neurotransmitter may transiently acidify the synaptic cleft. This idea is supported by the finding that protons, stored in the vesicles and coreleased with transmitters, modulate presynaptic Ca^{2+} channels (Callewaert et al., 1991; DeVries, 2001). It was shown recently that pH may also regulate miniature inhibitory postsynaptic currents mediated by $GABA_A$ receptors (Mozrzymas et al., 2003). These findings support, but do not prove, that rapid and brief pH changes in the synaptic cleft could modulate synaptic transmission by direct interaction of protons with postsynaptic receptors.

The wide distribution of nAChRs in the central and peripheral nervous systems includes some receptors that are expressed postsynaptically and mediate fast excitatory synaptic signaling (for review, see Jones et al., 1999; Hogg et al., 2003). Different mammalian cholinoceptive neuronal populations express distinct but usually heterogeneous combinations of neuronal nAChR subunit genes. In general, it is believed that $\alpha4\beta2$ is the predominant nAChR subtype in the brain, whereas $\alpha3\beta4$ AChRs are more specific for the peripheral nervous system. However, comparison of the pattern of subunit mRNA distribution suggests more extensive involvement of $\beta4$-containing AChRs in the brain cholinergic signaling of higher mammals than in rodents (Boulter et al., 1990; Han et al., 2000; Quik et al., 2000).

Because our previous study of pH effects on rat $\alpha3\beta4$ neuronal nAChR indicated that rapid acidification potentiates the agonist-induced current and markedly accelerates its decay kinetics (Abdrakhmanova et al., 2002b), we examined

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; AChR, acetylcholine receptor.

whether acidification would be equally effective in altering the amplitude and gating kinetics of human "slow" β4- and "fast" β2-containing neuronal nAChRs (Cachelin and Jaggi, 1991; Fenster et al., 1997; Bohler et al., 2001; Quick and Lester, 2002). Using rapid coapplication of protons (pH 6.0) and ACh, we found a significant element of subunit dependence to the proton effects. Acidic pH enhanced the activation of the ACh-induced current of human nAChRs that contain β4 but not β2 subunits. Nevertheless, acidification accelerated the decay kinetics of the current in both β4- and β2-containing AChRs. On the other hand, alkalization (pH 8.0) suppressed the ACh-induced current in all of these receptors. Thus, the acidification effects seem to be subunit-dependent. Preliminary reports of this work have already appeared (Abdrakhmanova et al., 2002a, 2003).

Materials and Methods

Cell Transfection and Culture. Transfected tsA201 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Inc.), and 2 mM L-glutamine (Invitrogen) in an incubator maintained at 5% CO_2, 37°C, and saturating humidity (Wang et al., 1998). Selective growth medium contained Geneticin (600 μg/ml; Invitrogen) and Zeocin (500 μg/ml; Invitrogen) for the α3β2 and α4β2 cell lines. For the α3β2α5 and α3β4α5 cell lines, the third antibiotic drug, Hygromycin (200 μg/ml; Roche Diagnostics, Indianapolis, IN), was added to the above selection medium. At least one day before recordings, cells were plated onto plastic cover slips (15-mm circular Thermanox; Nalge Nunc, Inc., Naperville, IL) coated with calf skin collagen (type 1; Sigma, St. Louis, MO).

In accordance with procedures that have been used to increase the level of functional nAChRs in these cell lines (Wang et al., 1998; Nelson et al., 2003), coverslips with β2-subunit-containing AChRs were exposed to 100 μM nicotine for at least 8 h before electrophysiological recordings. Figure 1 shows a typical example of ACh application on control (A) and nicotine incubated (B) tsA201 cells stably expressing α3β2 nAChRs.

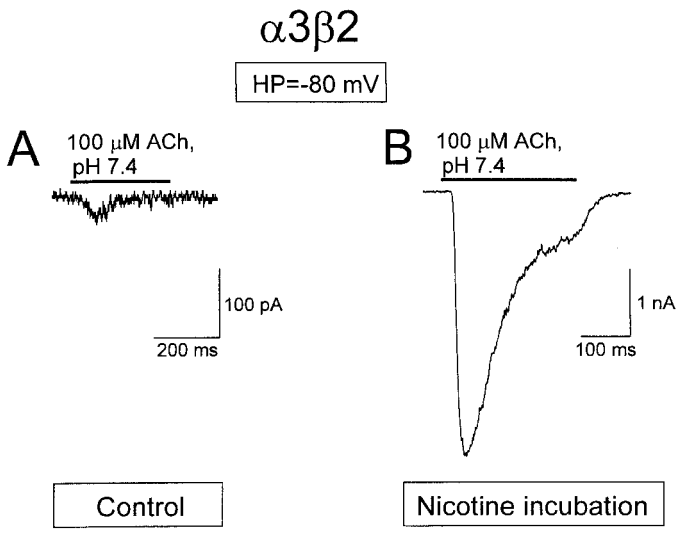

Fig. 1. Effect of pretreatment with nicotine in human neuronal α3β2 nAChRs stably expressed in tsA 201 cells. Application of 100 μM ACh did not induce any measurable whole-cell current through the human α3β2 nAChRs ($n > 30$) in control cells (not treated with nicotine) (A). Incubation for >8 h in 50 μM nicotine of the same cell line significantly increased the level of functional α3β2 AChRs (B). The cells incubated in nicotine were washed out with control extracellular solution for 1 h before recording to remove desensitization of the receptors (holding potential, −80 mV; servo-controlled miniature solenoid valve multibarreled application system was used in these experiments).

We were aware that acidification also activates a proton-gated channel in tsA201 cells. It is noteworthy that the expression of the proton-gated channel was somewhat variable in the tsA201 cells stably expressing human neuronal nAChRs. The proton-gated currents were characterized by slow onset and decay kinetics, and were sensitive to amiloride in μM concentration. We did not use amiloride to block this current, however, because the drug also affected the nicotinic current (G. Abdrakhmanova, L. Cleeman, and M. Morad, unpublished data). Control experiments were carried out to determine the extent of functional expression of proton-gated current. The cells that exhibited more than 50 to 100 pA of current induced by acidification alone (corresponding to more than 5% of ACh-induced current) were excluded from the analysis of the data. ACh was purchased from Sigma and was used at the concentrations indicated in the text and figures.

Electrophysiological Measurements. Functional expression of nAChRs was evaluated in the whole-cell configuration of the patch-clamp technique using a Dagan 8900 amplifier (Dagan, Minneapolis, MN). The patch electrodes, pulled from borosilicate glass capillaries, had a resistance of 3 to 4 MΩ when filled with internal solution containing 110 mM CsCl, 20 mM tetraethylammonium chloride, 5 mM MgATP, 14 mM EGTA, and 20 mM HEPES, pH adjusted to 7.3 with CsOH. About 90% of electrode resistance was compensated for electronically, so that the effective series resistance in the whole-cell configuration was always less that 1 MΩ. Stably transfected tsA201

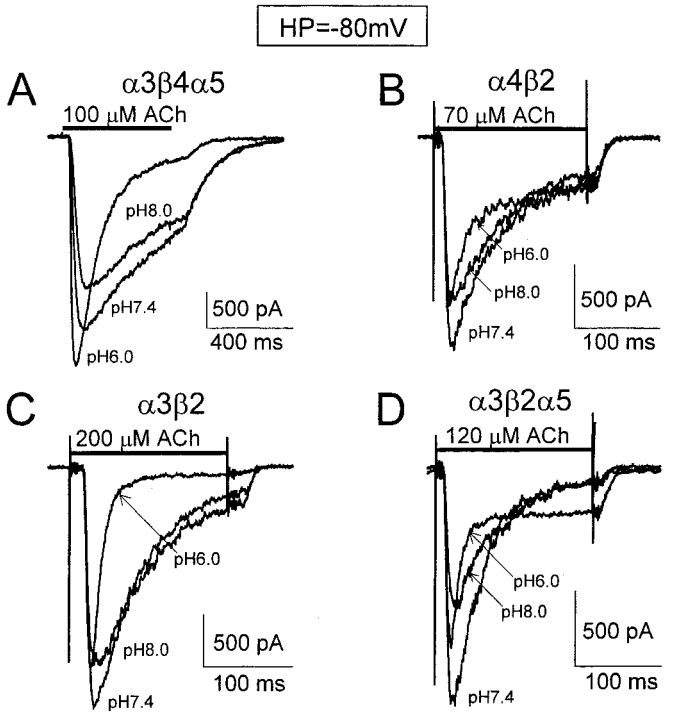

Fig. 2. Comparison of the pH effect on the amplitude and decay kinetics of ACh-induced currents in four different recombinant human neuronal nAChR subtypes. Shown are superimposed current traces evoked at pH values 7.4, 6.0, and 8.0 by ACh in one of the subtypes. The nAChR subtypes were α3β4α5 (A), α4β2 (B), α3β2 (C), and α3β2α5 (D) nAChRs. ACh was applied at its EC_{50} concentration for each AChR subtype (100, 70, 200, and 120 μM for α3β4α5, α4β2, α3β2, or α3β2α5, respectively). Rapid coapplication of protons, pH 6.0, and ACh enhanced the peak current only in α3β4α5 AChRs. In contrast, acidification suppressed the current in β2-containing AChRs (α4β2, α3β2, and α3β2α5). pH 6.0 induced an acceleration of decay of the current in both β4- and β2-containing AChRs. pH 8.0 suppressed and slowed the decay of the ACh-induced current independently of β-subunit present (holding potential, −80 mV; servo-controlled, miniature solenoid valve, multibarreled system was used in cell expressing α3β4α5 AChRs).

TABLE 1

Comparison of the effect pH or acidification on the amplitude and decay kinetics of the ACh (EC_{50})-induced current in different subtypes of recombinant neuronal nAChRs (holding potential, −80 mV)

The peak currents activated at pH values 6.0 and 8.0 were normalized relative to the peak current at pH 7.4 (100%). Values are presented as mean ± S.E.M.

	Δ of Peak I_{ACh}		Rate Constant of Decay		
Human Neuronal nAChR Subtypes	pH 6.0	pH 8.0	pH 6.0	pH 7.4	pH 8.0
	%			s^{-1}	
$\alpha3\beta4\alpha5$	↑ 66.0 ± 15.0	↓ 34 ± 5.7	4.8 ± 1.04	1.27 ± 0.09	1.07 ± 0.18
$\alpha4\beta2$	↓ 13.0 ± 3.2	↓ 27.8 ± 2.2	28.32 ± 3.95	11.98 ± 0.32	9.6 ± 0.42
$\alpha3\beta2$	↓ 21.1 ± 2.78	↓ 23.5 ± 4.0	53.5 ± 5.1	18.8 ± 0.87	13.14 ± 1.01
$\alpha3\beta2\alpha5$	↓ 19.4 ± 11.7	↓ 24.8 ± 1.7	40.42 ± 2.3	16.34 ± 0.61	10.70 ± 0.49

cells were studied 2 to 4 days after plating onto the cover slips. Generation of voltage-clamp protocols and acquisition of data were carried out using pCLAMP software (Axon Instruments, Inc., Burlingame, 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 amplitudes of the currents, recorded from the same cell at pH values 6.0 or 8.0, were normalized to that at pH 7.4 and were then quantified as the mean ± S.E.M. for the number of cells (n). The time course of decay of the ACh-induced current, in the presence of the agonist, was determined by dividing the maximal slope (linear regression) by the peak amplitude of the current. This ad hoc method, used also in our previous study on rat $\alpha3\beta4$ nAChRs (Zhang et al., 1999; Abdrakhmanova et al., 2002), was preferred to exponential analysis, because clear exponential decay of the currents was not often observed (e.g., because of our relatively brief drug exposure times) and was applied to compare the effect of acidification on decay kinetics of $\beta2$- and $\beta4$-containing subtypes of nAChRs.

Application of Agonists and the Perfusion System. Cells plated onto plastic cover slips (15-mm round Thermanox; Nalge Nunc, Inc.) coated with calf skin collagen (type 1; Sigma) 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 (2 ml/min). KCl was omitted from the control and agonist-containing puffing solutions to suppress possible K^+ currents in the voltage-clamped cell. Cells preincubated in to nicotine (see *Materials and Methods*) were washed out with a bathing solution 1 h before recording.

The amplitude and time course of the nicotinic current were highly dependent on the speed of the agonist application. Therefore, in addition to a servo-controlled, miniature solenoid valve (Lee Company, Westbrook CT), multibarreled system (Cleemann and Morad, 1991) (see Methods section in Abdrakhmanova et al., 2002b), we used a piezoelectric puffing device developed in our laboratory to change the superfusing solution in 1 to 5 ms. After establishment of the whole-cell configuration, the cell was lifted off the bottom of the perfusion chamber and placed in the middle of one of the two adjacent streams flowing from a short piece of θ-tubing. One of the solutions was the control, the other contained ACh at the desired pH and had been flushed for a few seconds before its use for another test solution. The solution around the cell was changed by moving the θ-tubing (~200 μm) such that the other stream enveloped the cell. The θ-tubing cantilevered upon a two-layered piezoelectric motor beam (6 × 25 mm; Piezo Systems, Inc., Cambridge, MA), was driven by a high-voltage DC-coupled amplifier (~100 V). The voltage was applied to the central conductor to bend the beam by slightly stretching one layer while compressing the other. This drive mode, in conjunction with an external casing/shielding, was chosen to reduce electrical artifacts on the recorded current at the time of switching. A high-speed camera (734 f/s) was used to check the motion of the device and reduce vibrations by adjusting the parameters of a filter shaping the time course of the drive voltage. The overall performance of the device was checked by measuring the resistance of a bare electrode switching between solutions of high and low conductance. The system had a delay of about 5 ms that was followed by a much shorter transition (10–90%) of about 0.4 ms. The delay depended on the flow rate and the distance of the cell from the tip of the θ-tubing and was highly reproducible (±0.15 ms).

Results

As reported by others, ACh at pH 7.4 activated currents of different kinetics in $\alpha3\beta4\alpha5$, $\alpha4\beta2$, $\alpha3\beta2$, and $\alpha3\beta2\alpha5$ neuronal nAChR subtypes (for review, see Quick and Lester, 2002). In general, $\beta4$-containing AChRs activate and decay significantly more slowly than $\beta2$-containing AChRs, and $\alpha3\beta2$ AChRs possess significantly faster kinetics than $\alpha4\beta2$ AChRs.

pH Effect on the Amplitude and Rate of Decay of ACh-Induced Current. In this set of experiments, we compared the effect of rapid and transient changes of pH on ACh-activated currents in human $\beta4$- ($\alpha3\beta4\alpha5$) and $\beta2$- ($\alpha4\beta2$, $\alpha3\beta2$, and $\alpha3\beta2\alpha5$) containing nAChRs. It should be noted that the $\alpha5$ subunit is not always certain to be present in all the nicotinic receptor complexes of the trimers used in

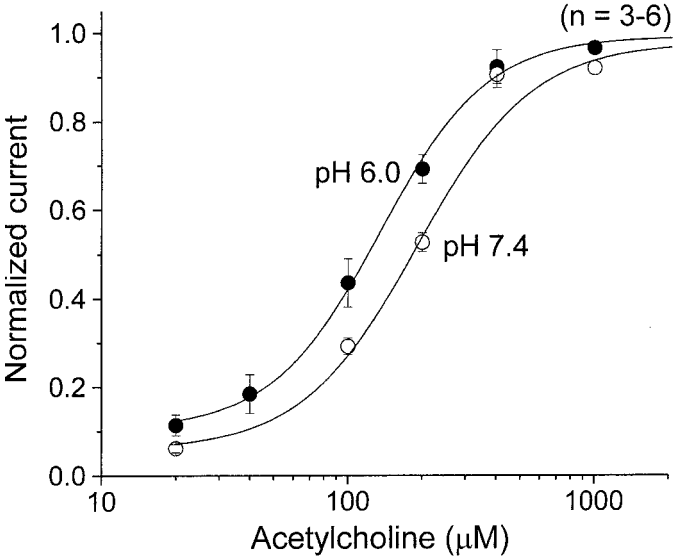

Fig. 3. Increasing $[H^+]o$ enhances the apparent affinity of $\alpha3\beta4\alpha5$ nAChR to ACh. Dose-response curves for the peak currents induced by ACh at two different pH values (○, 7.4; ●, 6.0) in cells voltage-clamped at −80 mV. The currents recorded from each cell were normalized relative to the current induced by 1000 μM ACh and renormalized after curve-fitting. Each symbol is labeled with a vertical error bar indicating the S.E.M. (n = 3–6). The continuous curves represent a fit to the Hill equation (n_H ~ 1.9) (servo-controlled, miniature solenoid valve, multibarreled system was used in these experiments).

this study (Wang et al., 1998). Figure 2 shows the effect of coapplication of H^+ (pH 7.4, 6.0, and 8.0) and ACh on four distinct subtypes of human nAChRs stably expressed in tsA201 cells. ACh was applied at its EC_{50} concentrations for each AChR subtype (Wang et al., 1998; Nelson et al., 2003). Acidification from pH 7.4 to 6.0 enhanced the current only through the $\alpha3\beta4\alpha5$ AChR (Fig. 2A) but not through the other three subtypes containing $\beta2$ subunit ($\alpha4\beta2$, $\alpha3\beta2$, $\alpha3\beta2\alpha5$) (Fig. 2, B–D). The enhancement of the current at pH 6.0 in $\alpha3\beta4\alpha5$ AChRs was transient; thus, the maintained phase of the nicotinic current was consistently suppressed by acidification (e.g., pH 6.0 trace in Fig. 2A), as has been described previously in rat $\alpha3\beta4$ AChR (Abdrakhmanova et al., 2002b). In all $\beta2$-containing AChRs, acidification suppressed the peak and the maintained components of ACh-activated current independent of their α subunit composition ($\alpha4$, Fig. 2B; $\alpha3$, Fig. 2C; and $\alpha3\alpha5$, Fig. 2D). It is interesting that elevation of pH from 7.4 to 8.0 suppressed the currents independent of the subtype of β present. Likewise, acidification accelerated the rate of decay of both $\beta4$- and $\beta2$-containing receptors, and the elevation of pH (7.4 to 8.0) slowed the decay kinetics of the current (Fig. 2).

Table 1 summarizes the pH effects on the amplitude and the decay kinetics of the ACh-induced current in four different subtypes of human neuronal nAChRs (n = 4–5 for each AChR subtype). In human $\alpha3\beta4\alpha5$ AChRs, pH 6.0 enhanced the current on average by 66% but suppressed the current in all $\beta2$-containing AChRs equally (13% for $\alpha4\beta2$, for 21% $\alpha3\beta2$, and 19% for $\alpha3\beta2\alpha5$). On the other hand, acidification, pH 6.0, accelerated the rate constant of the decay of the current in all $\beta2$-containing AChRs to the same extent. The effect was somewhat larger in $\beta4$- (3.8-fold for $\alpha3\beta4\alpha5$), compared with $\beta2$-containing AChRs (2.4 for $\alpha4\beta2$, 2.8 for $\alpha3\beta2$, and 2.5 for $\alpha3\beta2\alpha5$). These findings and the similarity of pH effects on the amplitude and decay kinetics of the ACh-induced currents in human $\alpha3\beta4\alpha5$ to those described in rat

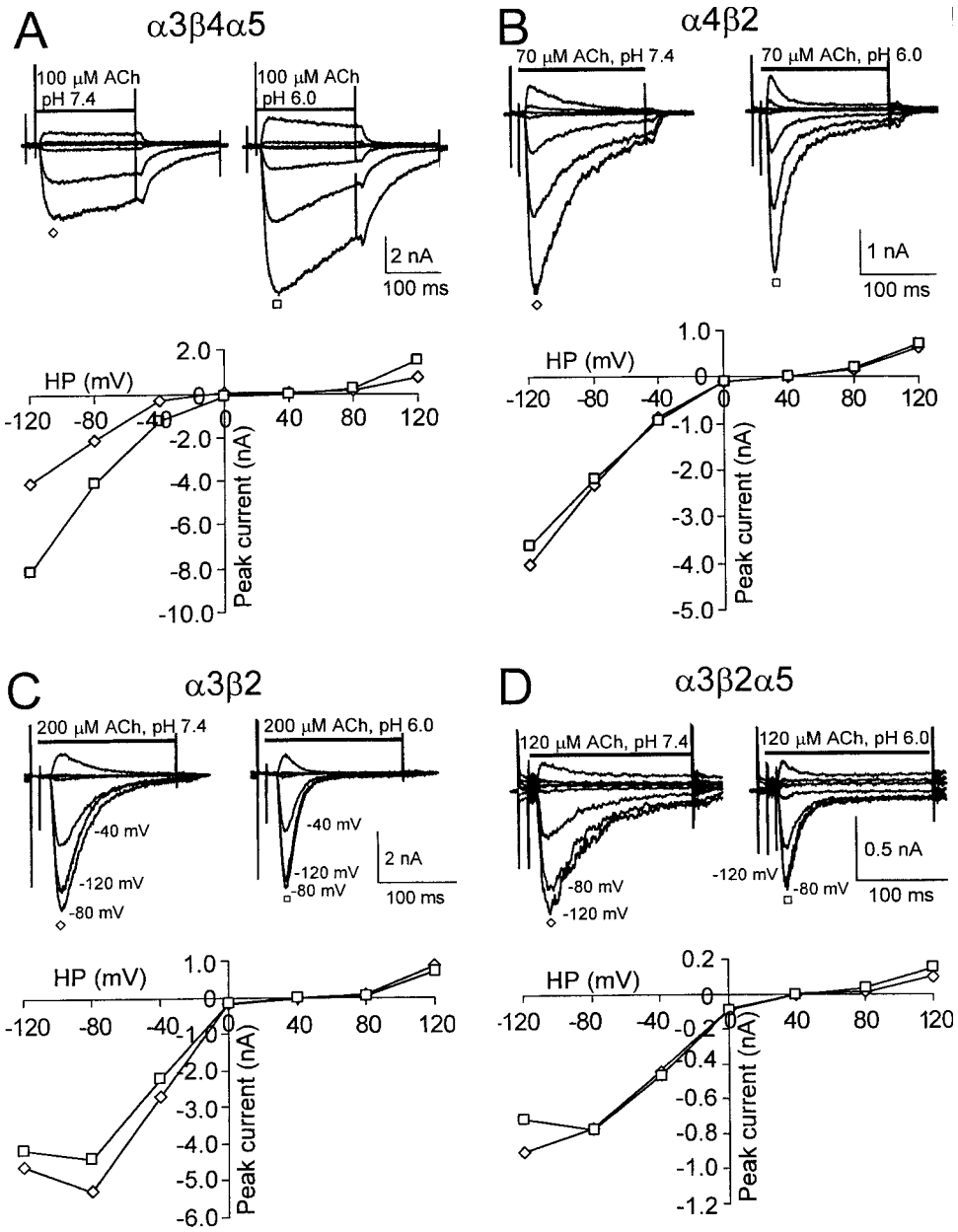

Fig. 4. Current-voltage relationship of ACh-induced currents recorded at pH values 7.4 and 6.0 from single representative cells expressing four different subtypes of human neuronal nAChRs. Shown is the voltage-dependence of the currents activated by ACh at its EC_{50} concentration for the indicated AChR subtype quantified at the maximal value (peak current) at both pH values 7.4 (◇) and 6.0 (□). The insets represent superimposed traces of the evoked currents at different holding potentials from −120 to +120 mV by 40 mV step at the indicated pH values of 7.4 (left) and 6.0 (right). Note a substantial enhancement of the peak [by (Δ) 100, 98, and 120% at −120, −80, and +120 mV, respectively] current at pH 6.0 in $\alpha3\beta4\alpha5$ AChRs (A). The current was not affected or rather slightly suppressed by pH 6.0 at its peak in $\alpha4\beta2$ (B), $\alpha3\beta2$ (C), and $\alpha3\beta2\alpha5$ (D) nAChRs.

$\alpha3\beta4$ nAChRs (Abdrakhmanova et al., 2002b) suggest that acidification differentially modulates only the magnitude of the current generated by neuronal nAChRs, depending on their β-subunit subtype. Alkalization shows no such subunit specificity on either the magnitude or kinetics of the current (Table 1).

To examine whether the effect of acidification in $\alpha3\beta4\alpha5$ nAChR is caused by an enhancement of the apparent affinity of the receptor to ACh, we measured the dose dependence of ACh-induced current at two different pH values, 7.4 and 6.0. In this set of experiments, individual cells were exposed to either pH 7.4 or 6.0 and varying ACh concentrations (20, 40, 100, 200, 400, and 1000 μM). The data points were fit with the empirical Hill equation, $y = 1/[1 + (EC_{50}/[\text{protons}]^{n_H})$. Figure 3 shows that reduction of pH from 7.4 to 6.0 decreased the EC_{50} of ACh-evoked currents from 191 to 134 μM but had little effect on the cooperativity factor ($n_H \sim 1.9$). The effect of acidification on the magnitude of ACh-induced current was more pronounced at lower ACh concentrations and became not significant at the saturating concentrations. Because ACh is unlikely to be protonated at pH 6.0, the data suggest a direct effect of protons on the $\alpha3\beta4\alpha5$ nAChR.

Voltage-Dependence of ACh-Induced Currents at pH Values 7.4 and 6.0. In this set of experiments, the effect of rapid acidification on the peak of ACh-induced currents was quantified at different potentials from −120 mV to +120 mV. Whole-cell currents in all four cell lines showed strong inward rectification such that ACh-activated currents were small or not clearly resolvable between 0 and 80 mV at pH 7.4 or 6.0, making accurate measurements of the reversal potential somewhat difficult.

Figure 4A shows the data from a representative cell stably expressing the human $\alpha3\beta4\alpha5$ nAChRs. Acidification enhanced the peak ACh-induced current on average by (Δ) 82 ± 18%, 81 ± 16%, and 83 ± 37% ($n = 3$) at −120, −80, and +120 mV, respectively. Thus, there seems to be no significant voltage-dependence to the acidification effect, quite similar to the data reported for rat $\alpha3\beta4$ AChRs (Abdrakhmanova et al., 2002b).

Figure 4, B and C, compares the effect of rapid coapplication of ACh and protons, pH 6.0, at different holding potentials in human $\alpha4\beta2$ and $\alpha3\beta2$ AChRs. Acidification did not significantly affect the peak current amplitude in $\alpha4\beta2$ nAChR subtype (Fig. 4B). There was approximately only 10 ± 2% and 8 ± 4% suppression of the peak current at −120 mV and −80 mV, respectively ($n = 2$–3). The suppression in $\alpha3\beta2$ nAChR was somewhat larger: (Δ) 17 ± 8%, 24 ± 7%, and 27 ± 12% at −120, −80, and +120 mV, respectively ($n = 3$, Fig. 4C). Figure 4D shows that acidification did not alter substantially the peak current amplitude in cells expressing $\alpha5$, in addition to $\alpha3\beta2$ subunits. These data suggest that rapid acidification of $\beta2$-, in contrast to $\beta4$-containing AChRs, may suppress the current through the AChR. This effect seems to be independent of incorporation of $\alpha5$ subunit in the AChR.

Effect of Acidification on the Rate of Activation. Elevation of $[H^+]_o$ seemed to enhance the rate of activation of $\alpha3\beta4\alpha5$ AChRs as measured from the rising phase of the current from 10 to 90% of the peak current. To estimate whether there was a significant difference in the rise time (10–90%) of the currents between that activated at pH values 7.4 and 6.0, the duration of ACh application was shortened to ~100 ms. The original traces of Fig. 5A illustrate representative examples of the effect of rapid acidification on the rate of activation of ACh-induced current in $\alpha3\beta4\alpha5$ expressing cells using the rapid <5-ms piezoelectric puffing device (note that on this time scale, the nicotinic currents barely desensitize at pH 7.4). The rise time of human $\alpha3\beta4\alpha5$ AChRs was

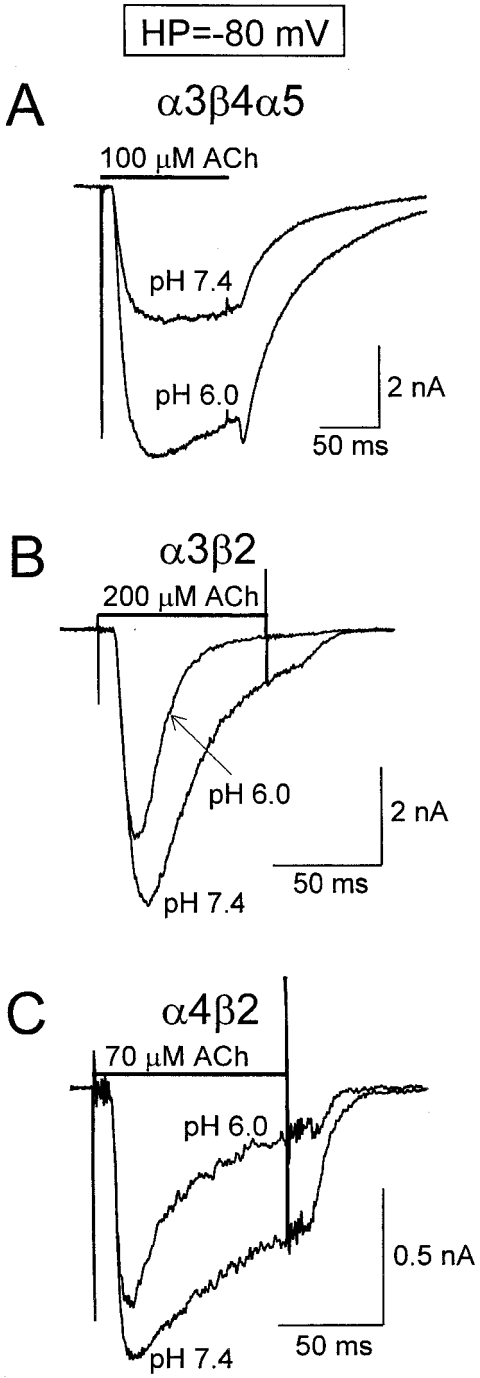

Fig. 5. Comparison of the effect of pH 6.0 on the rate of activation of ACh-induced current in human $\beta4$- and $\beta2$-containing nAChRs. Shown are representative superimposed traces of the currents induced at pH values 7.4 and 6.0 on the indicated nAChR subtype. Pulses of a shorter duration (105 ms in A, 80 ms in B and C) than in experiments presented above (105 ms) were used (holding potential, −80 mV). Rise time (10–90%) of the current in $\alpha3\beta4\alpha5$ AChRs decreased by 30% (from 18.57 to 12.83 ms) (A) but did not change significantly in $\alpha3\beta2$ (B, 7.59 versus 6.1 ms) or $\alpha4\beta2$ (C, 4.7 versus 3.9 ms) AChRs.

shortened on average by 28% (from 20.2 ± 2.6 to 14.5 ± 2.2 ms, $n = 5$, $p < 0.001$ by paired t test). This finding is similar to that found in rat $\alpha3\beta4$ AChRs (Abdrakhmanova et al., 2002b), where the rate of activation was enhanced by 26% at EC_{50} concentration of nicotine (40 μM). In $\alpha3\beta4\alpha5$ AChRs, the deactivation of the current after removal of ACh was faster at pH 7.4 ($\tau_1 = 54.7$ ms, $\tau_2 = 10.3$ ms) than at pH 6.0. ($\tau_1 = 80.16$ ms, $\tau_2 = 16.9$ ms), consistent with the increased affinity of $\beta4$-containing AChRs to ACh at acidic pH (see Fig. 3 and Abdrakhmanova et al., 2002b) and possibly related to slower unbinding of the agonist.

In $\beta2$-containing AChRs, with much faster activation and decay kinetics, acidification did not seem to change the rate of activation of ACh-induced currents in either $\alpha3\beta2$ or $\alpha4\beta2$ AChRs (Fig. 5, B and C). The rise times (10–90%) at pH values 7.4 and 6.0 were 5.2 ± 0.3ms and 4.7 ± 0.1ms ($n = 3$; $p = 0.528$ paired t test) in $\alpha4\beta2$, and 4.9 ± 0.5ms and 4.1 ± 0.2ms in $\alpha3\beta2$ AChR ($n = 3$; $p = 0.454$ paired t test). The data showed that the activation rate of the current was markedly faster in $\beta2$- than in $\beta4$-containing receptors. Although there seemed to be no significant difference in the activation rates of $\beta2$-containing receptors at pH values of 7.4 and 6.0, this may reflect limitations in speed of drug application (see *Discussion*).

Persistence of Acidification Effect after Removal of Acid Solution. We tested whether rapid acidification of the neuronal nAChR is accompanied by its conformational changes that persist after removal of protons. In particular, we studied a "rebound current" that is activated upon rapid withdrawal of high concentrations of the agonist and tested whether it would reflect the properties of the receptors exposed to acid. The "rebound current" is thought to be activated as a result of rapid relief of agonist-induced channel block at high concentration of the agonist and has been reported previously for muscle nAChRs (Drapeau and Legendre, 2001; Philipson et al., 2001) but not for neuronal nAChRs. Figure 6 shows that in $\beta4$-containing neuronal nAChRs, rapid removal of 1 mM ACh, applied at pH 7.4, induced a "rebound current" that was pronounced at negative potentials (Fig. 6A, left). Coapplication of ACh and pH 6.0 did not significantly alter the kinetics or the amplitude of the current induced by 1 mM ACh, but the rapid removal of acid solution accelerated the decay of the "rebound current" (from 55 to 74 s^{-1} at −80 mV) long after the washout (Fig. 6A, right). Likewise, removal of 1 mM nicotine applied on rat $\alpha3\beta4$ nAChRs induced a "rebound current" at pH 7.4, the rate of decay of which was also accelerated on preacidification (from 3.1 to 5.9 s^{-1} at −60 mV) (Fig. 6B). One possible explanation for the persistence of proton effect on the kinetics of "rebound current" is acidification-induced conformational changes of $\beta4$-containing neuronal nAChR that outlast the presence of protons.

We also examined possible activation of the "rebound current" in $\alpha3\beta2$ AChRs, that seemed to have the fastest kinetic properties compared with the other $\beta2$-containing nAChR subtypes used in this study. In contrast to $\beta4$-containing AChRs, no "rebound current" was detected upon rapid withdrawal of 1 mM ACh in $\alpha3\beta2$ AChRs (Fig. 7A). Considering the possibility that this might reflect desensitization of the $\alpha3\beta2$ AChR, we also used much briefer exposures to ACh (Fig. 7, A and B, 180 versus 40 ms, respectively) to confirm the absence of a "rebound current". Application of 1 mM ACh at pH 7.4 induced a response characterized by significantly faster decay kinetics (27.8 ± 1.49 s^{-1}) than at the EC_{50}

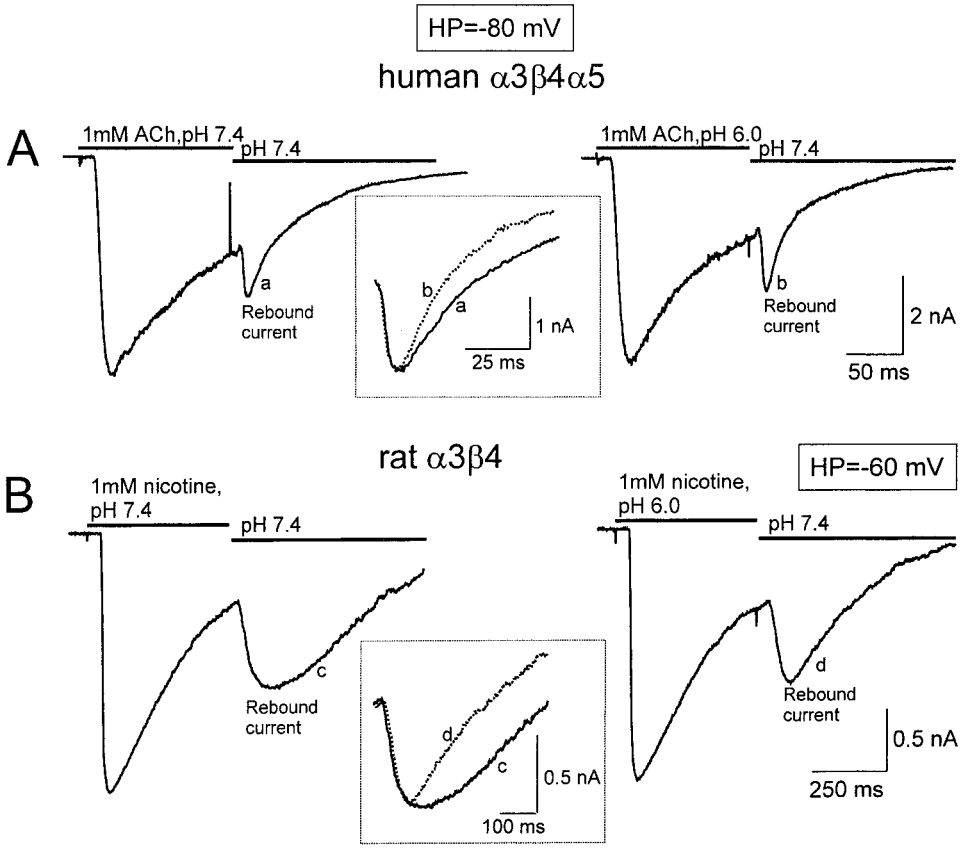

Fig. 6. Effect of prior acidification on a "rebound current" in $\beta4$-containing nAChRs. Original traces represent whole-cell responses to exposure of two cells expressing human $\alpha3\beta4\alpha5$ receptors to 1 mM ACh (A) or rat $\alpha3\beta4$ AChRs to 1 mM nicotine (B) at pH values 7.4 (left) and 6.0 (right). The marked "rebound current" appeared after rapid withdrawal of the agonist. Insets show superimposed rebound currents after agonist application at pH values 7.4 and 6.0 (a versus b in A; c versus d in B). The rate of decay of the "rebound current" was accelerated by AChR preacidification (pH 6.0) during the agonist application, even though the cells were exposed to pH 7.4 buffered control solution immediately after the agonist removal. (Servo-controlled, miniature solenoid valve, multibarreled system was used in human embryonic kidney 293 cells expressing rat $\alpha3\beta4$ AChRs.)

concentration (see Table 1) (Fig. 7A, left). Coapplication of pH 6.0 and ACh, did not significantly affect the amplitude of the current but slightly accelerated its decay kinetics ($56.69 \pm 5.37\ s^{-1}$) (Fig. 7A, right).

Discussion

The major finding of this report is that rapid coapplication of ACh and protons differentially regulates human neuronal nAChRs and that the β subunit composition of the AChR is critical for this effect. Rapid acidification enhances the peak current through the β4-containing human AChR ($\alpha3\beta4\alpha5$) but, in faster β2-containing AChRs ($\alpha4\beta2$, $\alpha3\beta2$ and $\alpha3\beta2\alpha5$), acidification causes suppression of the activated current even in the first 50 ms of drug exposure. This finding implies that the β subunit not only determines the decay kinetics of the ACh-induced current at physiological pH values but probably also contains the proton modulatory site. Somewhat unexpected was the finding that the effect of protons, especially in β4-containing AChRs, persists long after removal of extracellular acidification, suggesting possible longer-lasting proton-induced conformational changes of nAChR.

Effect of pH on the Ach-Activated Current Amplitude. The transient enhancement of the cationic current through the human and rat β4-containing neuronal nAChRs seems to differ from the findings that acidification generally suppresses cationic voltage- (Tombaugh and Somjen, 1998) or ligand-gated (Traynelis, 1998) channels, except in GluR6(R)/KA1 receptor (Mott et al., 2003), where low pH also enhances the agonist-induced current. The acidification-induced enhancement of the current occurs mostly at low concentrations of ACh and in the midrange near EC_{50} (Figs. 2A, 3, and 4A). This effect, however, is relatively less important above EC_{50} and is not present at saturating concentration of ACh (1 mM; Figs. 3 and 6, B-D). In contrast to β4-containing AChRs, in β2-containing AChRs, acidification consistently suppressed the ACh-induced current, independent of their α subunit composition.

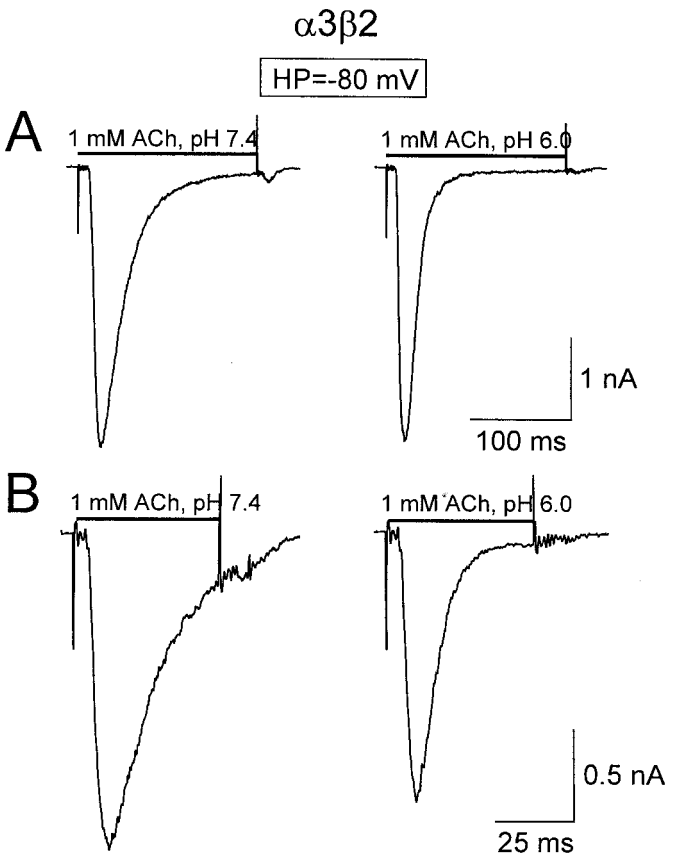

Fig. 7. The rebound current is absent in β2-containing AChRs independent of pulse duration. Representative current traces induced by 1 mM ACh at pH values 7.4 (left) and 6.0 (right) in the same cell, expressing $\alpha3\beta2$ AChRs, at two different pulse durations of 180 (A) and 45 (B) ms (holding potential, −80mV). Note that no "rebound current" appeared upon rapid withdrawal of 1 mM ACh.

Figures 5A clearly shows that coapplication of agonists with the higher proton concentration not only enhanced the magnitude but also accelerated the rate of activation of the current in human $\alpha3\beta4\alpha5$ AChR. Consistent with acidification-induced increase of the agonist affinity to the receptor (Fig. 3), this effect was identical to that reported for rat $\alpha3\beta4$ AChR (Abdrakhmanova et al., 2002b), and contrasted sharply with the data for human β2-containing AChRs (Fig. 5B), where acidification failed to alter significantly the activation rate of the current. It is likely that different mechanisms of the pH modulation are at play for β4- and β2-containing neuronal nAChRs.

***α5* Subunit Incorporation into the nAChR and Acidification.** Immunoprecipitation data show that when expressed in tsA201cells, only 49% of $\alpha3\beta2\alpha5$ or 14% of $\alpha3\beta4\alpha5$ AChRs actually incorporate the α5 subunit (Wang et al., 1998). It is unfortunate that we could not identify which whole-cell clamped cells of our study expressed the α5 subunit because of the unavailability of the α5 subunit-specific pharmacological agents. Nevertheless, there was no great variation within a specific cell line or significant differences in proton modulation of either human $\alpha3\beta2$ and $\alpha3\beta2\alpha5$ (Fig. 2, C-D and 4C-D), or human $\alpha3\beta4\alpha5$ (Fig. 2A, 4A, 5A) and rat $\alpha3\beta4$ (Abdrakhmanova et al., 2002b) AChRs.

Possible Mechanisms for Differential Proton Modulation of *β*2- and *β*4-Containing AChRs. Two possibilities were considered in evaluating the opposite effects of protons on ACh-induced current in β4- and β2-containing AChRs: 1) a difference in the molecular structure of the two receptors and/or 2) a faster rate of activation and desensitization kinetics of β2-containing AChRs that masks the effect of solution changes occurring in millisecond domains.

1. Comparison of the sequences of human nAChR β2 and β4 subunits in their first three transmembrane segments from residues 210 to 297 (Rush et al., 2002) indicates the following four amino acid differences: isoleucine versus threonine (position 224), serine versus leucine (position 226), valine versus phenylalanine (position 254), and tyrosine versus isoleucine (position 273). There are no structural grounds to think that these differences in β2 and β4 AChRs may underlie the subunit-dependent differential proton modulation of the receptors, because none of these amino acids is charged or located extracellularly. On the other hand, it has been shown that much slower desensitization rates of β4-containing AChRs rely on the extracellular N-terminal (Bohler et al., 2001). Chimeras constructed with the β2 and β4 subunits, where the whole N-terminal or some of its segments in β2 sequence (1–45, 1–95, 81–77, 72–95) were transferred to that of the β4 subunit, exhibited significantly faster kinetics similar to

those of β2-containing AChRs. However, none of these chimeras were characterized for their possible altered sensitivity to ACh, because the studies were carried out in saturating ACh concentration (1 mM). It should be noted that comparison of N-terminal sequence shows only one negatively charged glutamic acid (E) site in the rat β4 subunit (position 38), whereas in rat β2 subunit, the corresponding position is occupied by an uncharged methionine (M) (sequence from Bohler et al., 2001). Likewise, in human β4, position 34 is occupied by a negatively charged aspartic acid, but in human β2 by a positively charged histidine (sequence from Elliott et al., 1996; Groot Kormelink and Luyten, 1997). Whether such site(s) could be responsible for the differential modulation of β2 and β4-containing AChRs remains to be determined.

2. The piezoelectric device for application of solutions in the present study allowed us to coapply ACh and protons in ~5 ms, making it possible to quantify critically transient responses of neuronal AChRs to more rapid acidification. This was particularly important for "fast" β2-containing AChRs. Using this approach, we found that elevation of protons also accelerates the decay kinetics of human β2-containing AChRs (see Table 1), even at the EC_{50} concentration of ACh. This finding is consistent with, but not a proof of, the idea that the more rapid acidifications may induce conformational changes of β2-containing AChRs that promote agonist-induced channel block in a manner similar to β4-containing AChRs (Fig. 6; Sine and Steinbach, 1984). However, because our approach is limited by solution application time of ~5 ms, we cannot rule out that faster application times may not in fact reveal faster rise times and evoke "rebound current" on withdrawal of agonist. Given these reservations, our data show that the faster decay kinetics observed on acidification of β2-containing AChRs arises from a direct effect of protons on desensitization kinetics of the receptors.

Physiological Implication of pH Modulation in Microdomain of Synaptic Cleft. Considering several lines of experimental evidence obtained in different laboratories that: 1) postsynaptic potentials may be accompanied by brief (<10-ms) acidic shifts (Krishtal et al., 1987); 2) vesicular proton concentrations are high (pH ~5.5; Miesenbock et al., 1998); 3) the transient acidification induced rapid inhibition of presynaptic Ca^{2+} channels (Callewaert et al., 1991; DeVries, 2001); and 4) the extracellular buffering capacity of the cleft for H^+ is limited (Tong et al., 2000) within the time frame of synaptic events, it is likely that significant transient acidification of the postsynaptic AChRs takes place before and during activation of ACh-induced current.

The concentrations of ACh that activate postsynaptic AChRs during synaptic events remain unclear. The classic model of exocytosis, known as a "total fusion", predicts that approximately 1 mM ACh is rapidly released into the cleft (Clements, 1996), causing nearly synchronous activation of all of the postsynaptic nAChRs, in approximately 1 ms. An alternative model of exocytosis, known as "kiss and run", suggests that in some neurons and neuroendocrine cells, the release of intravesicular contents into the extracellular space is not complete (Klingauf et al., 1998), so that that only few neurotransmitter molecules (e.g., ACh) are released from a single vesicle. If such a "kiss and run" event is accompanied by a significant release of protons stored in the vesicle, and if protons do in fact sensitize the AChR to ACh, then the activation of the postsynaptic AChRs may occur, not at the saturating concentration of the agonist, as the "total model" predicts, but at much lower concentrations of the neurotransmitter. The "kiss and run" model seems to be in agreement with recent findings suggesting that subsaturating concentrations of glutamate and GABA are present during the signaling of single synaptic events in hippocampal neurons (Liu et al., 1999; Mozrzymas et al., 2003) and the brainstem giant nerve terminal (the calyx of Held) (Ishikawa et al., 2002).

Our data on rapid coapplication of protons and ACh not only provide an approximation for the transient local changes in pH that might occur in the synaptic cleft but also suggest possible plasticity of proton modulation of nAChRs based on their subunit composition. We speculate that rapid acidification of the postsynaptic neuronal AChRs (Jones et al., 1999) may play a critical role in signaling of specific groups of cholinergic neurons expressing different subtypes of neuronal nAChRs.

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