0022-3565/03/3043-1143–1152$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 304, No. 3
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44537/1044503
JPET 304:1143–1152, 2003
Printed in U.S.A.

The Journal of PHARMACOLOGY And Experimental Therapeutics

# Dual Action of *n*-Butanol on Neuronal Nicotinic $\alpha4\beta2$ Acetylcholine Receptors

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Received September 18, 2002; accepted November 26, 2002

## ABSTRACT

*n*-Alcohols exert a dual action on neuronal nicotinic acetylcholine (ACh) receptors with short-chain alcohols exhibiting potentiating action and long-chain alcohols exhibiting inhibitory action. *n*-Butanol lies at the transition point from potentiation to inhibition. To elucidate the mechanism of dual action of alcohols, the effects of *n*-butanol on the human $\alpha4\beta2$ ACh receptors expressed in the HEK293 cell line were analyzed in detail by the whole-cell patch-clamp technique. Prolonged applications of *n*-butanol evoked small currents with an $EC_{50}$ value of 230 ± 90 mM and a Hill coefficient of 1.8 ± 0.4. This current was blocked by either the ACh channel blocker mecamylamine or the receptor blocker dihydro-$\beta$-erythroidine, indicating that butanol activated receptors as a partial agonist. As expected from its partial agonist action, *n*-butanol also modulated ACh-induced currents in a concentration-dependent manner. Butanol at 300 mM potentiated currents induced by low concentrations of ACh (≤30 $\mu$M), while inhibiting the currents induced by high concentrations of ACh (100–3000 $\mu$M). In addition, butanol at a low concentration (10 mM) suppressed the currents evoked by 10 to 3000 $\mu$M ACh, a result consistent with a channel-blocking action. Most features of *n*-butanol effects were satisfactorily simulated by a model in which butanol acts as a partial agonist and as a channel blocker.

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The importance of neuronal nicotinic acetylcholine receptors (nnAChRs) in brain function and drug action is increasingly recognized. Because nnAChRs are located on soma, preterminal, and presynaptic regions of GABAergic and other interneurons in the cortex and hippocampus, their modulation caused by various drugs could lead to a cascade of synaptic events involving multiple neurotransmitters, resulting in complex behavioral changes.

*n*-Alcohols have been shown to exert a dual action on nAChRs, depending on the carbon chain length. In muscle nAChRs, ethanol and other short-chain alcohols prolong the decay phase of the miniature end-plated currents (EPCs) (Gage et al., 1975), increase the peak EPC amplitude, and prolong the channel lifetime (Bradley et al., 1980; Linder et al., 1984). In contrast, longer chain alcohols (*n*-butanol, *n*-hexanol, and *n*-octanol) accelerate the EPC decay and reduce the peak EPC amplitude (Bradley et al., 1984). At the single-channel level, butanol and pentanol increase the burst frequency, resulting from an increase in the opening rate of the ACh receptor channel (Dilger et al., 1994; Liu et al., 1994). This effect would account for the observation that ethanol increased the saturating response induced by high concentrations of ACh (Aistrup et al., 1999; Zuo et al., 2001). In addition, *n*-alcohols reduce single-channel conductance and shorten the mean channel open time in muscle nAChRs, both of which are thought to be the basis for the inhibitory action of alcohols (Murrell et al., 1991; Forman and Zhou, 1999).

Experiments performed with nnAChRs of native neurons have shown that ethanol potentiates ACh-induced currents in $\alpha4\beta2$-type nnAChRs in rat cortical neurons in primary culture (Aistrup et al., 1999). Using the $\alpha4\beta2$ nnAChRs stably expressed in human embryonic kidney (HEK) cell line, we have previously found that shorter chain alcohols from methanol to *n*-propanol potentiate the currents induced by ACh, whereas longer chain alcohols from *n*-pentanol to *n*-dodecanol (C5–C12) inhibit the currents (Zuo et al., 2001). *n*-Butanol (C4) is at the transition position from potentiation to inhibition and exerts a biphasic effect, either potentiating or inhibiting the currents depending on the concentrations of ACh and butanol. At low ACh concentrations, butanol exhibits a biphasic inhibition being accentuated by increasing butanol concentration from 1 to 100 mM, but becoming less pronounced or even being converted to potentiation at concentrations higher than 100 mM (Zuo et al., 2001).

Because *n*-butanol is at the transition point from potentiation to inhibition in the action of a series of aliphatic chain *n*-alcohols, and also because *n*-butanol itself exhibits a dual

This work was supported by National Institutes of Health Grant AA07836.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.102.044537.

**ABBREVIATIONS:** nAChR, nicotinic acetylcholine receptor; EPC, end-plated current; ACh, acetylcholine; nnAChR, neuronal nAChR; HEK, human embryonic kidney; DH$\beta$E, dihydro-$\beta$-erythroidine.

action, it might hold a clue to the mechanism of the dual action of alcohols. Several questions are asked. Is the relief of inhibition observed at high butanol concentrations due to butanol potentiation overcoming the inhibition? Alternatively, is it due to butanol's direct activation of receptor as a partial agonist? Is butanol less effective in blocking the butanol-activated receptor than the ACh-activated receptor?

It was found that *n*-butanol at high concentrations evoked small currents that were blocked by both mecamylamine and dihydro-$\beta$-erythroidine (DH$\beta$E), suggesting that butanol acted as a weak partial agonist on nnAChRs. *n*-Butanol also modulated ACh-induced currents in a concentration-dependent manner. At a low concentration (10 mM), butanol shifted the ACh dose-response curve toward higher concentrations and suppressed the maximum response, whereas at a high concentration (300 mM) butanol potentiated the currents induced by low ACh concentrations and inhibited the currents induced by high ACh concentrations.

These results could be simulated using a model in which butanol acted both as a partial agonist and as an open channel blocker. In this model, we assumed that receptors bound by two ACh molecules or two butanol molecules were able to open the channel and that butanol was able to block the two ACh-opened channel but not the butanol-activated channel.

## Materials and Methods

**Cell Preparations.** Human $\alpha4\beta2$ AChR subunit combination was stably expressed in the HEK293 cell line. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 $\mu$g of streptomycin (Invitrogen, Carlsbad, CA), 6% iron-supplemented calf serum (Sigma-Aldrich, St. Louis, MO), and 100 $\mu$g/ml G418 (Mediatech, Herndon, VA). Cells were kept at 34.7°C in air + $CO_2$ (93 + 7%, by volume). For patch-clamp experiments, cells were plated on glass coverslips coated with poly-L-lysine and cultured for 1 to 5 days.

**Electrophysiological Recording.** Whole-cell currents were recorded with an Axopatch 200 patch-clamp amplifier (Axon Instruments, Inc., Foster City, CA) at room temperature (20–25°C). Recorded currents were directly digitized at 1 to 10 kHz via a Digidata 1200 ADC/DAC interfaced with a microcomputer under the control of the ClampEx module of the pClamp6 software package (Axon Instruments, Inc.). The holding potential was −50 mV. The external solution contained 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES acid, and 4.5 mM Na-HEPES, pH adjusted to 7.3 with HCl, and osmolarity 320 mOs. The internal solution contained 140 mM K-gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES acid, 2 mM $Mg^{2+}$ ATP, and 0.2 mM $Na^+$ GTP, pH adjusted to 7.3 with KOH, and osmolarity 300 mOs. The patch-clamp pipettes were pulled from Clark patch glass capillaries (PG120T-10, 1.2-mm o.d., 0.93-mm i.d., 10-cm length; Warner Instrument, Hamden, CT) in two stages on a vertical pipette puller (PP-830; Narishige, Tokyo, Japan) and lightly fire-polished to a final resistance of 1.5 to 2 M$\Omega$ when filled with internal solution. Recording was started about 5 to 10 min after the rupture of the cell membrane to allow for the internal solution to reach the equilibration with intracellular milieu.

**Drug Application.** All drugs were applied to the cell by a modified computer-operated U-tube perfusion system (Marszalec and Narahashi, 1993). The speed of solution exchange was measured by the junction potential change using the patch pipette placed at a distance from the opening of the U-tube or near the U-tube opening. The rise time (10–90%) of junction potential change was found to be 50 ms when the patch pipette was placed 200 $\mu$m from the opening of the U-tube and was reduced to less than 10 ms when the patch pipette was placed near the opening of the U-tube. When the recording cell was placed at 200 $\mu$m from the opening of the U-tube, the solution exchange on the cell surface was, however, found to complete within around 200 ms by measuring the rate of changes in ACh-induced current in response to changes in sodium ion concentration (Liu and Dilger, 1991; Mori et al., 2001). In a few experiments, the recording cell was brought near the opening of the U-tube. The rise time of the ACh response was less than 10 ms. In the present experiments, short-pulse drug exposure time was 250 ms unless otherwise stated, and long-pulse experiments were performed with 1 to 10 s pulses. In all these cases, the intervals between pulses were 2 min to avoid current reduction due to agonist-induced desensitization. Control currents (30 $\mu$M ACh alone) were usually checked before and after each application of a test drug to ensure the stability of control responses. *n*-Butanol was purchased from Aldrich Chemical Co. (Milwaukee, WI). DH$\beta$E and mecamylamine were purchased from Sigma/RBI (Natick, MA).

**Data Analysis.** Recorded currents were initially analyzed by the Clamp-Fit module of the pClamp6 to assess whole-cell current amplitudes and decay kinetics. Data were then exported from pClamp6 to a Microsoft Excel program (Microsoft Office 2000) for statistical analysis. The concentration-response data were subsequently fitted to a single or double Hill logistic equations and compiled for graphical analysis in SigmaPlot 5.0 (SPSS Science, Inc., Chicago, IL). Data were expressed as mean ± S.E.M. unless otherwise mentioned.

The ACh dose-response curve was fitted by a single Hill equation or by the sum of two Hill equations:

$$y = [p_1/(1 + (EC_{50H}/x)^{n_{H1}})] + [(1 - p_1)/(1 + (EC_{50L}/x)^{n_{H2}}]$$

where $y$ is the normalized peak currents, and $x$ is the agonist concentration. $EC_{50H}$ and $EC_{50L}$ are the half-effective concentrations for the high- and low-affinity receptors, respectively. $n_{H1}$ and $n_{H2}$ are the Hill coefficients of the high- and low-affinity receptors, respectively, and $p_1$ is the percentage of receptors in the high-affinity state.

**Simulation.** The kinetic simulation was carried out with $C^{++}$ programs for numerical solution of differential equations based on a kinetic scheme (Scheme I in Fig. 9). Butanol is assumed to have two kinds of action on nnAChRs, as a partial agonist and as a channel blocker.

## Results

**Both ACh and *n*-Butanol Activate nnAChR Currents.** The currents of the $\alpha4\beta2$ AChRs expressed in HEK cells rose rapidly, reaching a peak in less than 100 ms at low ACh concentrations, and its rising phase became faster with increasing ACh concentrations. At 3 mM, the time to peak is less than 10 ms (Figs. 1A and 2A). During application of 10-s pulse of 3 mM ACh, the ACh-induced currents decayed with two exponential phases (Fig. 1A), reflecting receptor desensitization. The time constants ($\tau$) of fast and slow desensitization were 350 ± 30 ms ($\tau_{fast}$) and 2270 ± 70 ms ($\tau_{slow}$), respectively ($n$ = 7). About 22% of the current was associated with the fast component, whereas the rest was associated with the slow one.

When the dose-response relationship was fitted to a single Hill equation (Fig. 1C, dotted line), an $EC_{50}$ value of 68 $\mu$M and an $n_H$ of 0.7 ± 0.1 were obtained ($n$ = 7–28). This $EC_{50}$ value is much higher than that measured on the $\alpha$-bungarotoxin-insensitive nnAChRs in rat cortical neurons ($EC_{50}$ of 2.7 $\mu$M; Aistrup et al., 1999). The high $EC_{50}$ value and a small $n_H$ of the ACh dose-response relationship for the expressed $\alpha4\beta2$ nnAChRs has also been reported previously (Chavez-Noriega et al., 1997; Zuo et al., 2001; Buisson and Bertrand, 2001).

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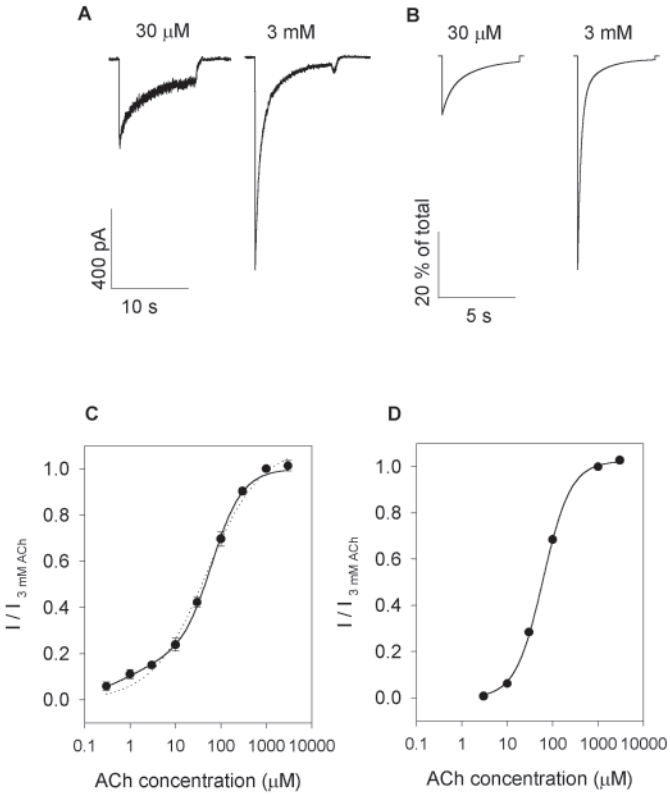


**Fig. 1.** Dose-response relationship for ACh-induced currents in the $\alpha4\beta2$ receptors expressed in HEK cells. Currents were recorded at a holding potential of −50 mV. A, currents recorded from one cell in response to 10 s perfusion of 30 and 3000 $\mu$M ACh. B, simulated currents induced by 30 and 3000 $\mu$M ACh. C, dose-response relationship of peak currents. The dotted line represents the best fit using a single Hill equation ($EC_{50}$ of 48 ± 9 $\mu$M and $n_H$ of 0.7 ± 0.1). A better fit is obtained with the sum of two Hill equations (solid line; see equation, *Materials and Methods*). In this case, 20% ($p_1$) of the receptors have a high affinity for ACh, with an $EC_{50H}$ of 1.0 ± 2.5 $\mu$M and an $n_{H1}$ of 0.8 ± 0.7, whereas the remaining 80% receptors have a low affinity, with an $EC_{50L}$ of 63 ± 14 $\mu$M and an $n_{H2}$ of 1.3 ± 0.2 ($n$ = 7–28) (mean ± S.E.M., $n$ = 7–28). D, simulated dose-response relationship of peak currents of the low-ACh-affinity receptors. Kinetic parameters are given in Scheme I (Fig. 9) and Fig. 9 legend and text. The simulated current amplitudes at various ACh concentrations were normalized to the peak current obtained at 3000 $\mu$M ACh. The fit to the simulated data gives an $EC_{50}$ of 61 $\mu$M and an $n_H$ of 1.4.

One possibility of the low $n_H$ for the ACh dose-response curve is due to the existence of two receptor pools with different affinities for ACh, as suggested by Buisson and Bertrand (2001). To examine this possibility, additional experiments covering a wider range of ACh concentrations were performed and the dose-response relationship was fitted with the sum of two Hill equations. In this case, 20% ($p_1$) of the receptors have a high affinity for ACh, with an $EC_{50H}$ value of 1.0 ± 2.5 $\mu$M and an $n_{H1}$ of 0.8 ± 0.7, whereas the remaining 80% receptors have a low affinity, with an $EC_{50L}$ value of 63 ± 14 $\mu$M and an $n_{H2}$ value of 1.3 ± 0.2 ($n$ = 7–28) (Fig. 1C, solid line). The large standard deviations for the $EC_{50L}$ and $n_{H2}$ for the high-affinity receptor are probably due to the fact that it constitutes a minor component of the total responses. These results resemble those of Buisson and Bertrand (2001) in that high-affinity receptors have an $EC_{50H}$ value of 1.60 $\mu$M and an $n_{H1}$ of 0.92, whereas the low-affinity receptors have an $EC_{50L}$ value of 68 $\mu$M and an $n_{H2}$ of 1.60.

To test an alternative hypothesis that the small Hill coefficient is due to rapid receptor desensitization, an improvement in our perfusion system was made by bringing a smaller cell to the site close to the opening of the U-tube so that the solution exchange was greatly speeded up to less than 10 ms. Figure 2 depicts examples of current traces and the dose-response relationship. Even at low ACh concentrations, the ACh-induced currents reached the peak in less than 20 ms. The decay time constant estimated from the current induced by 3 mM ACh was 310 ± 40 ms ($n$ = 5). No decay time constant faster than 100 ms was detected. When the data were fitted to the single Hill equation, the $EC_{50}$ and $n_H$ were estimated to be 38 ± 9 $\mu$M and 0.8 ± 0.2, respectively (Fig. 2B, dotted line). Thus, it is rather unlikely that the small $n_H$ is due to the complication from the receptor desensitization because the peak of ACh current was measured at less than 20 ms and because the receptor underwent desensitization with a time constant of more than 300 ms.

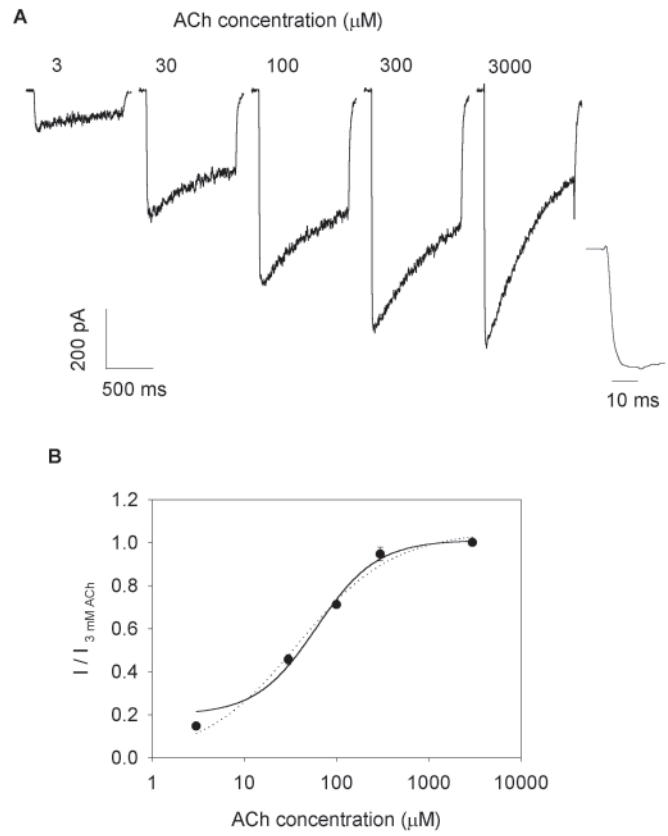


**Fig. 2.** Dose-response relationship for ACh-induced currents in the $\alpha4\beta2$ receptors expressed in HEK cells using an improved perfusion system. Cells were lifted to near the opening of the U-tube so that the solution exchange was greatly speeded up. A, currents recorded from one cell in response to 500 ms of perfusion of 3 to 3000 $\mu$M ACh. The current induced by 3000 $\mu$M ACh reached the peak in less than 10 ms with a rise time of 3 ms (see inset). Currents were recorded at a holding potential of −50 mV. The decay phase of the current induced by 3000 $\mu$M ACh was fitted by a single exponential function to obtain the fast time constant of desensitization of 340 ± 40 ms ($n$ = 5). B, dose-response relationship of peak currents. Current amplitudes were normalized to the current obtained at 3000 $\mu$M ACh (mean ± S.E.M., $n$ = 6). The dotted line represents the best fit using a single Hill equation ($EC_{50}$ of 38 ± 9 $\mu$M; $n_H$ = 0.8 ± 0.2). The solid line represents the best fit of the low-affinity receptors only, assuming 80% of the data coming from the low-affinity receptor with 20% from the high-affinity receptor ($EC_{50}$ of 60 ± 13 $\mu$M and $n_H$ = 1.3 ± 0.3). The 20% high-affinity receptor was based on the analysis of Fig. 1C.

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However, when one assumed 80% of the data coming from the low-affinity receptor with 20% from the high-affinity receptor, an $EC_{50}$ and an $n_H$ of 60 ± 13 μM and 1.3 ± 0.3, respectively, were obtained for the low-affinity receptor (Fig. 2B, solid line). The 20% high-affinity receptor was based on the analysis of Fig. 1C. To simplify the simulation, we simulated the effect of butanol only on the low-affinity receptor, because the low-affinity receptor represents the majority of the receptors in our cell lines, and also because most of the experiments were performed at high concentrations of ACh (>10 μM).

In the absence of ACh, application of long pulses (2–5 s) of *n*-butanol generated inward currents in a dose-dependent manner at a holding potential of −50 mV (Fig. 3A). The currents induced by *n*-butanol were small compared with ACh-induced currents. For example, the currents induced by 300 mM butanol ranged from 40 to 80% of the currents induced by 30 μM ACh. Unlike ACh-induced currents, the butanol-induced currents decayed with a single exponential time course (Fig. 3A). For currents induced by 300 mM butanol, the time constant of decay was around 2 s. A logistic equation with three parameters was used to fit the butanol data, giving an $EC_{50}$ value of 230 ± 90 mM, an $n_H$ of 1.8 ± 0.4, and the saturation currents estimated to be 1.6 ± 0.5 times that of the currents induced by 300 mM butanol ($n$ = 6) (Fig. 3B). The butanol-induced current had a much slower onset (200–300 ms) compared with the ACh-induced current, suggesting either a lower binding rate of butanol to nnAChRs or a slower open rate of butanol-bound receptors. Furthermore, no current was evoked by butanol in the cells that failed to respond to ACh. These results suggest that *n*-butanol is capable of activating nnAChRs albeit with less potency and less efficacy than ACh.

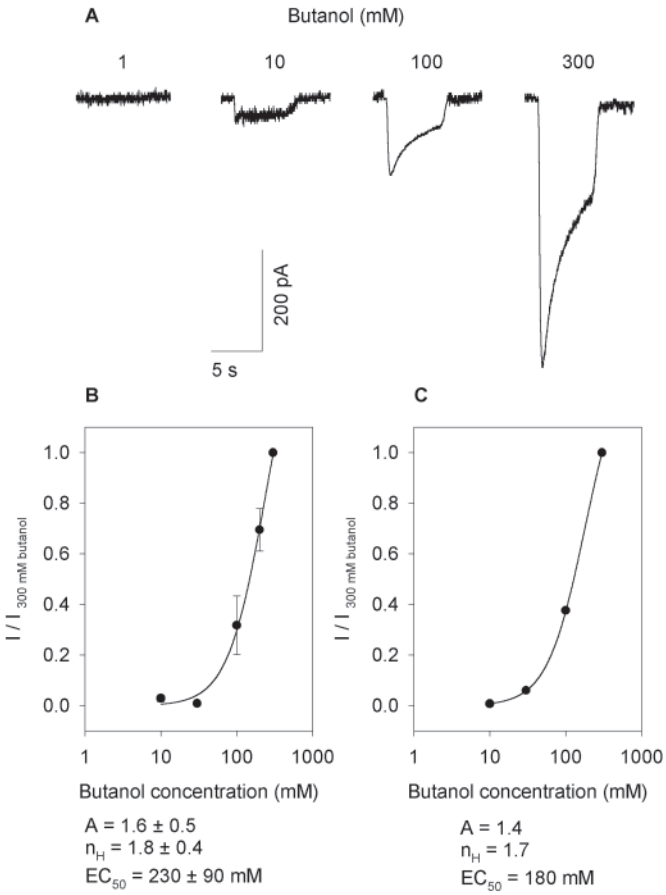


**Fig. 3.** Dose-response relationship for *n*-butanol-induced currents in the α4β2 receptors expressed in HEK cells. A, currents recorded from one cell in response to 1 to 300 mM butanol, which was applied for 5 s at intervals of 2 min using the U-tube system. B, dose-response relationship of the peak currents. Current amplitudes were normalized to the current obtained in 300 mM butanol. A maximum response could not be evoked because of the limited solubility of butanol in water, but fitting the data to a logistic equation gave the estimated maximum response of 1.5 times of the response induced by 300 mM butanol. Three parameters were used to fit the data: $EC_{50}$ of 230 ± 70 mM; $n_H$ of 1.8 ± 0.4; and the saturation current estimated to be 1.6 ± 0.5 times that of the currents induced by 300 mM butanol (mean ± S.E.M., $n$ = 4). C, simulated dose-response relationship of peak currents. Kinetic parameters are given in Scheme I (Fig. 9), and Fig. 9 legend and text. The simulated current amplitudes were normalized to the current obtained at 300 mM butanol. The same fit used for experimental results to the simulated data gave an $EC_{50}$ value of 180 mM and an $n_H$ of 1.7, and saturation currents was estimated to be 1.4 times that of the currents induced by 300 mM butanol.

To further test the hypothesis that butanol-induced current was due to the activation of nnAChRs, we tested whether specific AChR inhibitors could block the currents. DHβE has been widely used as a nonselective and competitive nAChR antagonist. It has a nanomolar affinity for α4β2 nnAChRs (Dwoskin and Crooks, 2001). The previous study of human α4β2 nnAChRs showed that the inhibitory action of mecamylamine is voltage-dependent and noncompetitive, suggesting that it acts as an open channel blocker (Papke et al., 2001). Both 1 μM DHβE (ACh receptor blocker) and 50 μM mecamylamine (ACh channel blocker) blocked the currents induced by either 300 mM butanol or 30 μM ACh when preperfused in the bath for 2 min and coapplied with the agonist for 2 or 5 s (Fig. 4). Both DHβE and mecamylamine blocked the peak ACh- and butanol-induced currents almost to the same extent (Table 1).

The blocking kinetics in the presence of DHβE and mecamylamine were significantly different between the ACh-opened (Fig. 4, A and B) and the butanol-opened channels (Fig. 4, C and D). In the presence of 1 μM DHβE, the butanol-induced current rose more slowly than the butanol control current (Fig. 4C), whereas the ACh-induced current was simply scaled down without changing kinetics (Fig. 4A). When the block was measured at the end of agonist pulse, ACh-induced currents were more sensitive than butanol-induced currents to both blockers (Table 1). DHβE at 1 μM reduced butanol-induced currents at the end of agonist pulse to 33 ± 1% ($n$ = 3) of control, while reducing ACh-induced currents to 12 ± 3% ($n$ = 3) of control. Similarly, 50 μM mecamylamine reduced butanol-induced currents to 29 ± 2% ($n$ = 3) of control compared with 1.0 ± 0.4% ($n$ = 5) for ACh-induced currents (Table 1). The slow rise in the butanol-induced current in the presence of DHβE is consistent with the notion that butanol at high concentrations might compete against DHβE for binding to the receptors. Thus, the DHβE-bound receptors become unblocked as more butanol molecules competitively bind to the receptors to open the channel. Without doing more detailed Schild analysis of competition between butanol and DHβE, one could not rule out an alternative explanation, namely, a different conformational change of the receptor occurs in the presence of butanol and DHβE. Mecamylamine blocked the ACh-induced current in a time-dependent manner (Fig. 4B), leading to a near complete block at the end of 2 s pulse (Table 1). The time-dependent

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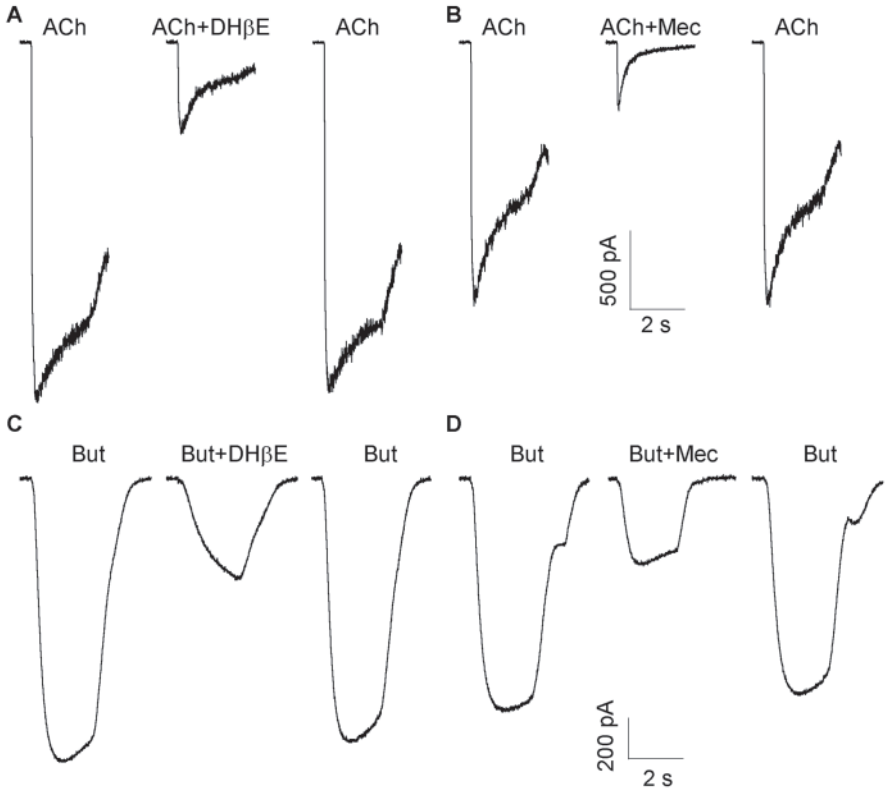


**Fig. 4.** Effects of DHβE and mecamylamine on ACh-induced currents (A and B) and butanol-induced currents (C and D). The blockers were preperfused for 2 min and then coapplied with the agonist. DHβE (1 μM) suppressed the 30 μM ACh-induced currents to 28 ± 1% of control at the peak and to 12 ± 3% at the end of drug application pulse ($n$ = 3) (A) and the 300 mM butanol-induced currents to 26 ± 3% of control at the peak and 33 ± 1% at the end of drug application pulse ($n$ = 3) (C). Mecamylamine (50 μM) suppressed the 30 μM ACh-induced currents to 21 ± 3% of control at the peak and 1.0 ± 0.4% at the end of drug application pulse ($n$ = 5) (B) and the 300 mM butanol-induced currents to 17 ± 4% of control at the peak and 29 ± 2% at the end of drug application pulse ($n$ = 3) (D). All these inhibitions were reversible after washing with inhibitor-free solution for 2 to 10 min. The U-tube application time was 2 or 5 s with 2-min intervals between each application.

TABLE 1
Block of ACh and/or butanol-induced currents by DHβE and mecamylamine
Data are given as percentages of current amplitude relative to control with the number of experiments in parentheses.

| $I/I_{control}$ | ACh (30 μM) | | Butanol (300 mM) | |
|---|---|---|---|---|
| | At Control Peak | At Pulse End | At Control Peak | At Pulse End |
| DHβE (1 μM) | 27.7 ± 1.4% (3) | 12.4 ± 3.4% (3) | 25.7 ± 2.5% (3) | 33.4 ± 1.2% (3) |
| Mecamylamine (50 μM) | 20.7 ± 2.6% (5) | 1.0 ± 0.4% (5) | 17.4 ± 3.9% (3) | 29.1 ± 2.4% (3) |

enhancement of block of mecamylamine was not seen with butanol-induced current (Fig. 4D).

Thus, the butanol-induced current seems to be generated by the activation of AChRs, and butanol acts as a partial agonist for nnAChRs. However, the butanol-opened channels seem to be less sensitive to DHβE and mecamylamine than the ACh-opened channels. The reduced sensitivity to DHβE is probably due to the displacement of DHβE from the binding site by high concentrations of butanol, whereas the reduced sensitivity to mecamylamine block may represent a less sensitivity of the butanol-activated channel to open channel blockers.

**Modulation of ACh Dose-Response Curve by Butanol.** In our previous study (Zuo et al., 2001), butanol was thought to have a direct inhibitory action and its $IC_{50}$ value was estimated to be 6.8 mM by extrapolation of the relationship between the $IC_{50}$ and carbon number of longer chain $n$-alcohols. Because the estimated $IC_{50}$ value of butanol is far lower than its $EC_{50}$ value of 230 mM, one could evaluate its direct inhibitory action on ACh dose-response relationship. Figure 5A shows the effects of a low concentration (10 mM) of butanol on the currents induced by various concentrations of ACh. Butanol inhibited currents induced by 10 to 3000 μM ACh about 30%. The $EC_{50}$ value of ACh dose-response curve was slightly and insignificantly ($P > 0.5$) shifted in the direction of higher ACh concentrations by 10 mM butanol coapplication, without change in the Hill coefficient ($EC_{50}$ of 66 ± 25 μM; $n_H$ of 0.6 ± 0.1 without butanol versus $EC_{50}$ of 96 ± 45 μM; $n_H$, 0.6 ± 0.1 with 10 mM butanol; Fig. 6A). At lower concentrations, butanol clearly suppressed the ACh-induced current, but the suppression was less than that predicted by $IC_{50}$ value of 6.8 mM. These results suggest that butanol is not a simple channel blocker.

The effect of $n$-butanol on the ACh dose-response relationship was also evaluated in the presence of high butanol concentration at which it could act as a partial agonist. Butanol at 300 mM modulated ACh-induced currents in a complex manner (Fig. 5B). Currents induced by low concentrations of ACh (3 and 10 μM) were potentiated by butanol, whereas those induced by higher concentrations of ACh (30–3000 μM) were inhibited, with the maximum inhibition occurring at 100 μM ACh (Fig. 6A). Again, this V-shape (Fig. 6A) ACh-dose-response relationship in the presence of 300 mM butanol is not consistent with a simple partial agonist model, which predicts a monophasic ACh dose-response curve with butanol raising the foot at low ACh concentrations without altering the maximum response.

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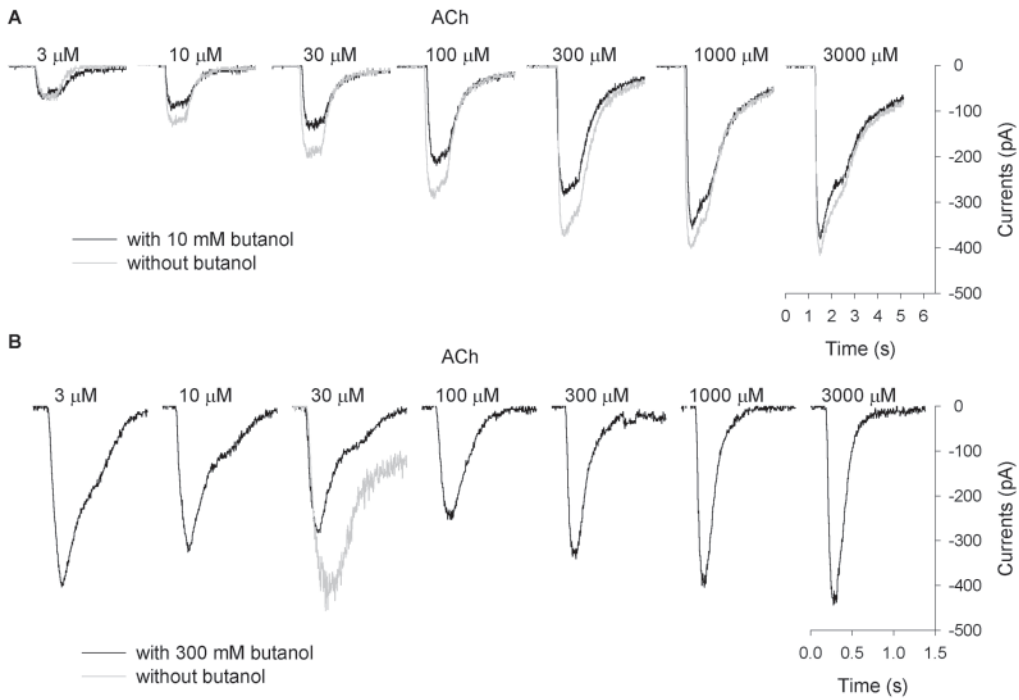


**Fig. 5.** Effects of *n*-butanol at concentrations of 10 mM (A) and 300 mM (B) on ACh-induced currents. A and B were obtained from two different HEK cells expressing $\alpha4\beta2$ nnAChRs. ACh (3–3000 $\mu$M) was coapplied with butanol by U-tube system for 1 s at an interval of 2 min. Butanol at 10 mM exerts an inhibitory action at all ACh concentrations tested (A). Butanol at 300 mM potentiates the currents induced by low ACh concentrations, but inhibits the currents induced by high ACh concentrations (B). The shaded currents in A and B are controls without butanol.

**Interaction between Butanol and ACh.** Because butanol acts as a partial agonist only at higher concentrations and as a channel blocker at lower concentrations, one would expect that the interaction between butanol and ACh on the $\alpha4\beta2$ receptor depends on the concentrations of both butanol and ACh. Thus, the dose-response relationship for butanol action was examined in the presence of 30 $\mu$M and 1 mM ACh.

The inhibition of ACh-induced currents by low-to-medium butanol concentrations up to 100 mM was slightly dependent on the ACh concentration (Figs. 7 and 8A). However, the effect of butanol at 300 mM was greatly dependent on ACh concentration: either no change or further inhibition was observed. Butanol exhibited a biphasic inhibitory dose-response curve at low ACh concentrations (30 and 100 $\mu$M ACh, around ACh $EC_{50}$) (Figs. 7A and 8A). At 1 mM ACh, which activates most receptors, butanol exhibited a monophasic dose-response relationship for inhibiting the ACh-induced current (Figs. 7B and 8A), because butanol not only competed with ACh for the binding site but also acted as a blocker. Thus, the inhibition by low-to-medium butanol concentrations up to 100 mM was slightly dependent on the ACh concentration (Figs. 7 and 8A). At 300 mM butanol one would not expect to see much current because butanol could effectively abolish the current by competing with ACh for binding to the receptor and by blocking the open channel. Because a substantial current actually remains, butanol must be less effective in blocking the butanol-opened channel than the ACh-opened channel, a result consistent with the weak blocking action of mecamylamine (Fig. 4D). At higher concentrations of ACh, a monophasic inhibition is expected to be observed because both the partial agonist action and the channel blocking action would contribute to the butanol inhibitory action.

Experimentally, the coapplication of 30 $\mu$M ACh and 300 mM butanol produces highly variable results compared with the control current produced by 30 $\mu$M ACh. The examples shown in Figs. 7 and 10 represent extremes of the inhibitory and potentiating responses. This is due to the fact that butanol is a weak agonist with an $EC_{50}$ value around 230 mM and with a large S.E.M. of 90 mM.

**Simulation of Butanol Action.** We previously performed a kinetic simulation for the dual action of *n*-alcohols on nnAChRs (Zuo et al., 2001). The original scheme based on that study was expanded to Scheme I (Fig. 9) to include the assumption that butanol acts as a partial agonist on nnAChRs. In addition, we assumed that the ACh receptor-channel can be opened by two ACh molecules or two butanol

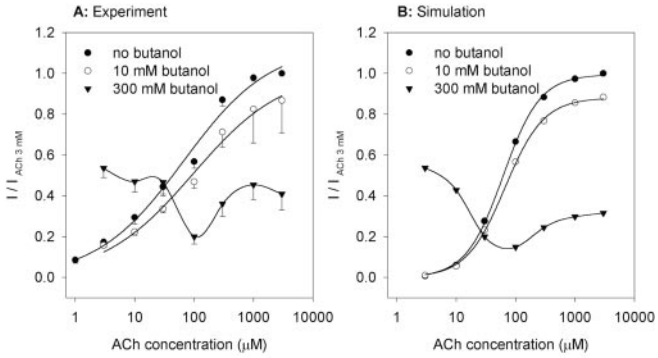


**Fig. 6.** The ACh dose-response curve is differentially modified by 10 and 300 mM butanol in the $\alpha4\beta2$ HEK cells. A, experiment data. ACh (3–3000 $\mu$M) was coapplied with butanol for 250 ms at intervals of 2 min. The peak current amplitudes were normalized to the current obtained at 3 mM ACh alone. Data are presented as mean ± S.E.M. ($n$ = 5–6). The $EC_{50}$ value for ACh dose-response curve without butanol is 66 ± 25 $\mu$M with an $n_H$ of 0.6 ± 0.1 and the $EC_{50}$ value for that with 10 mM butanol is 96 ± 45 $\mu$M with an $n_H$ of 0.6 ± 0.1. Butanol at 300 mM produces a dual action: potentiating action at low ACh concentrations and a biphasic inhibition at moderate-to-high ACh concentrations. B, simulation. The symbols represent the simulated values, which were fitted by a single Hill equation. The $EC_{50}$ value for ACh dose-response curve without butanol is 61 $\mu$M with an $n_H$ of 1.4, and the $EC_{50}$ value with 10 mM butanol is 66 $\mu$M with an $n_H$ of 1.3. Both experimental data and the simulated value in the presence of 300 mM butanol were connected by a line. See the legend of Fig. 9 and the text for kinetic parameters and further explanation.

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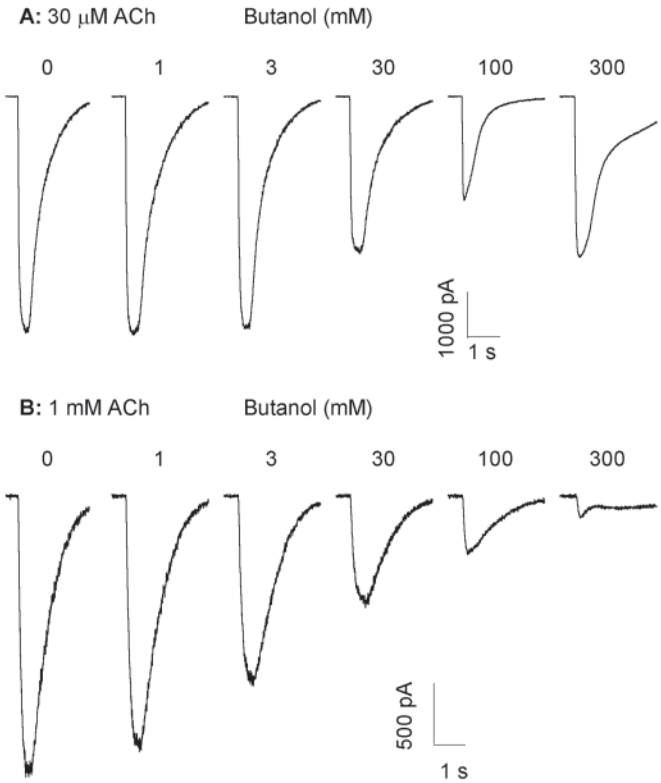


**Fig. 7.** Butanol inhibition of currents evoked by 30 $\mu$M (A) and 1 mM (B) ACh in the $\alpha4\beta2$ HEK cells. Butanol caused a biphasic inhibition on the current induced by 30 $\mu$M ACh (A) and a monophasic inhibition on the currents induced by 1 mM ACh (B). ACh and butanol were coapplied by U-tube system for 250 ms at intervals of 2 min.

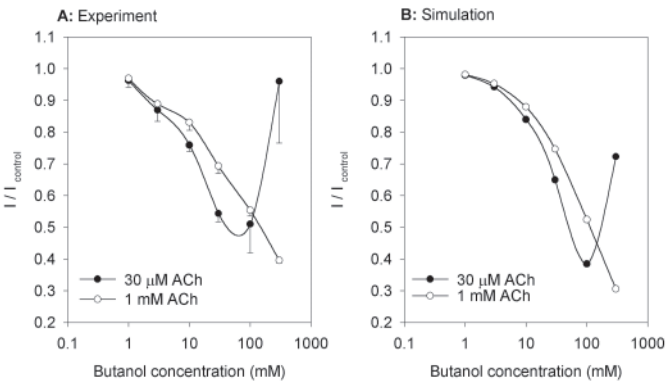


**Fig. 8.** Butanol inhibitory dose-response curves at 30 $\mu$M and 1 mM ACh in the $\alpha4\beta2$ HEK cells. A, ACh and butanol were applied by U-tube system for 250 ms at intervals of 2 min. Current amplitudes were normalized to the current induced by ACh alone. Data are presented as mean ± S.E.M. (ACh 30 $\mu$M, $n$ = 6; and 1 mM, $n$ = 5). B, similar dose-response relationships obtained by simulation. See text for kinetic parameters and further explanation.

molecules, but not by one ACh molecule and one butanol molecule. In addition, butanol is able to block the two ACh-opened channel but not the butanol-activated channel. Despite some indication of ACh "self-block" at higher concentrations (3 mM; Figs. 1A and 10, F and G), it was not included in the kinetic simulation. This is because most of butanol and ACh interaction studies were performed at ACh concentrations less than 3 mM. To simplify the simulation, we focused on the effect of butanol on the low-affinity receptor, which constitutes at least 80% of the total current we measured.

Because data on single-channel recording and kinetic analysis of $\alpha4\beta2$ nnAChRs are limited, most of the parameters used in our simulation (Fig. 9) were chosen based on previous publications on muscle nAChRs (Colquhoun and Sakmann, 1985; Franke et al., 1991, 1993). We started with the parameters from Colquhoun and Sakmann (1985): $k_{on1} = 10^8\ M^{-1}\ s^{-1}$, $k_{off1} = 8{,}000\ s^{-1}$, $\alpha = 700\ s^{-1}$, and $\beta = 30{,}000\ s^{-1}$. Their rate constants were modified based on our experimental results and for best fitting to our data. The modification of parameters was performed in several steps. First, the rate constants for ACh binding and unbinding rates used in our simulation were as follows: a $k_{on1}$ of $10^8\ M^{-1}\ s^{-1}$ and a $k_{off1}$ of 6,000 $s^{-1}$. These values gave a $K_d$ of 60 $\mu$M for ACh binding, very close to the observed $EC_{50L}$. Second, for butanol binding, the slower onset of butanol-induced currents compared with ACh-induced currents (200–300 ms versus less than 20 ms) can be explained by either the slow binding of butanol ($k_{on}$), or the slow opening ($\beta$) of the butanol-bound channel. In our model, butanol binds to the receptor much more slowly than ACh, whereas the opening rates are assumed to be the same in both cases. The rate constants $k_{on2}$ (400 $M^{-1}\ s^{-1}$) and $k_{off2}$ (100 $s^{-1}$) were chosen to generate a butanol activation dose-response relationship similar to experimental observation ($K_d$ = 250 mM for each binding step). Both rate constants for butanol were smaller than those of ACh, because butanol induced currents with a much slower onset. Third, Two-step desensitization process, $RA_2^{D1}$ and $RA_2^{D2}$, was used because the ACh-induced current decayed with a biexponential time course (Fig. 1A). The desensitization rates $\delta_1$ and $\delta_3$ were chosen as 3 and 0.4 $s^{-1}$, respectively, with the resensitization rates ($\gamma$) 1/10 of desensitization rates. Fourth, desensitization occurred much more slowly for butanol-activated currents than for ACh-activated currents. The decay for the current induced by 300 mM butanol is well fitted with one exponential time course, with $\tau$ around 2 s. Thus, $\delta_2$ is estimated to be around 0.5 $s^{-1}$, with $\gamma_2$ 1/10 of $\delta_2$. Fifth, single-channel study of the muscle receptor showed a very high open probability [$P_{open} = \beta/(\alpha+\beta)$] of about 0.98 (Colquhoun and Sakmann, 1985). Previous data suggest that the nnAChRs have a much lower open probability, although no maximum $P_{open}$ value is available (Weaver and Chiappinelli, 1996). Because no such data are available, we chose 0.67 for $P_{open}$ of ACh-opened channels, as in our previous simulations (Zuo et al., 2001). The same opening and closing rate constants are assigned to butanol-opened channels to simplify the simulation.

The currents were simulated from Scheme I (Fig. 9) at various concentrations of ACh. The simulated current traces are illustrated in Fig. 1B and the peak amplitude normalized to that evoked by 3 mM ACh is plotted against ACh concentration (Fig. 1C). The $EC_{50}$ and $n_H$ values were estimated to be 61 $\mu$M and 1.4, respectively. The simulated butanol dose-response relationship exhibited an $EC_{50}$ value of 180 mM and an $n_H$ of 1.7 (Fig. 3C). The $EC_{50}$ values for both ACh and butanol are close to those of the experimental results.

Butanol effects on ACh dose-response curve were simulated with the incorporation of the blocking action of butanol on the ACh-activated currents. The following blocking parameters were used: the rate constant for butanol block of the two ACh-opened channel ($b_1$), $1.5 \times 10^4\ M^{-1} \cdot s^{-1}$; and the butanol unbinding rate constant ($ub_1$), 100 $s^{-1}$. These values give us the $K_d$ around 6.7 mM, similar to the extrapolated

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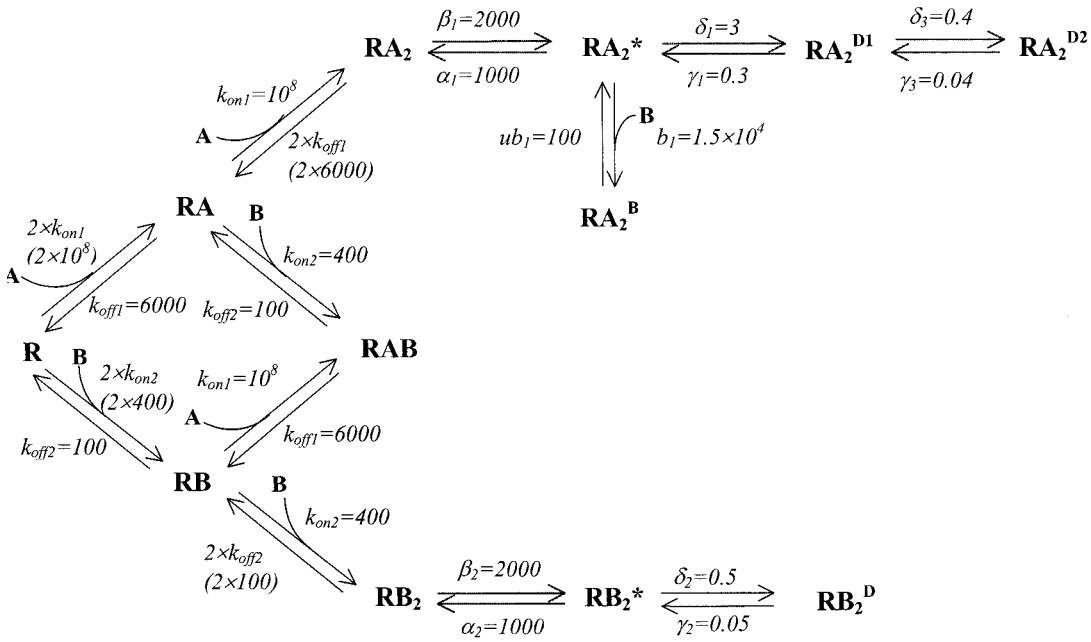


**Fig. 9.** A kinetic scheme based on the assumption that *n*-butanol acts as a partial agonist and as an open channel blocker. R, nnAChR; A, ACh; B, *n*-butanol; RA and RB, receptors bound by one agonist molecule; $RA_2$, RAB and $RB_2$, receptors bound by two agonist molecules; $RA_2$* and $RB_2$*, agonist-induced activated receptors; $RA_2^{D1}$, $RB_2^{D}$, and $RA_2^{D2}$, desensitized receptors; $RA_2^{B}$, butanol-blocked receptors; $k_{on1}$ and $k_{on2}$, ACh and butanol binding rates (molar per second); $k_{off1}$ and $k_{off2}$, ACh and butanol unbinding rates (per second); $\alpha_1$ and $\alpha_2$, channel closing rates (per second); $\beta_1$ and $\beta_2$, channel opening rates (per scond); $\delta_1$, $\delta_2$, and $\delta_3$, the rates of entry into desensitization (per second); $\gamma_1$, $\gamma_2$, and $\gamma_3$, the exit rates from desensitization (per second); $b_1$, butanol blocking rate for ACh-opened channels (molar per second); and $ub_1$, unblocking rates of butanol (per second). These rate constants were chosen as described in the text.

$IC_{50}$ value for butanol block (Zuo et al., 2001). Based on weak blocking action of the open channel blocker mecamylamine on the butanol-activated currents, we assumed that the butanol-opened channels are not sensitive to butanol block at all. The simulated results are plotted in Fig. 6B, which captures many features of the observed ACh dose-response curves with both 10 and 300 mM butanol.

A similar simulation was also performed for the butanol inhibitory dose-response relationship (Fig. 8B). A biphasic inhibitory dose-response curve was obtained using 30 $\mu$M ACh with a maximal inhibition at 100 mM butanol, whereas a monophasic inhibitory relationship was observed at 1 mM ACh. Thus, simulation yields the butanol dose-response relationships similar to those of experimental results.

**Butanol as Channel Blocker.** *n*-Butanol also acted as a channel blocker for nnAChRs, in a way similar to other long-chain alcohols (Murrell and Haydon, 1991). Although butanol was a weak agonist with an $EC_{50}$ value around 200 mM, it was much more potent as a channel blocker of the ACh-opened channels. The blocking rate constant of $1.5 \times 10^4\ M^{-1} \cdot s^{-1}$ for the two ACh-opened channel, combined with the unbinding rate constant of 100 $s^{-1}$ gave rise to a $K_d$ of 6.7 mM. This difference between the agonist $EC_{50}$ value and the blocking $K_d$ was reflected in currents induced by coapplication of ACh and butanol (Fig. 10). Butanol at 10 mM showed hardly any agonist action (Fig. 10A), but caused a significant inhibition of currents induced by 30 and 3000 $\mu$M ACh (Fig. 10, D and G). Butanol at 300 mM had a significant agonist action (Fig. 10B), potentiated the current evoked by 30 $\mu$M ACh (Fig. 10E), and inhibited the current evoked by 3000 $\mu$M ACh (Fig. 10H).

## Discussion

**Multiple Action of *n*-Butanol.** The present study showed that *n*-butanol exerted multiple actions on the $\alpha4\beta2$ nnAChRs stably expressed in HEK293 cells. In the absence of ACh, *n*-butanol generated a small current, and in the presence of ACh, it either potentiated or inhibited ACh-induced currents, depending on the concentrations of ACh and butanol. Most of the features of these multiple actions could be simulated by a model based on the hypothesis that *n*-butanol acts both as a partial agonist to induce currents and as an open-channel blocker (Fig. 9).

**Contributions of Different Actions of Butanol to the Biphasic Dose-Response Relationship.** To understand the contributions of two major actions of butanol as a partial agonist and as a channel blocker to the overall action of butanol on nnAChRs, we analyzed the biphasic nature of the ACh dose-response curve at 300 mM butanol in detail. At low concentrations of ACh, 3 $\mu$M for example, we observed over 3-fold potentiation by coapplication of 300 mM butanol (Fig. 6A). This is mainly due to the agonist action of butanol at such a high concentration to open the ACh channel. Based on our simulation (Fig. 11), 300 mM butanol itself can open about 40% of the total AChR channels, similar to what 60 $\mu$M ACh does. Thus, with 3 $\mu$M ACh and 300 mM butanol, over 99.8% of the open channels are occupied by two butanol molecules. However, because the affinity of ACh for the receptor is more than 4000 times higher than that of butanol (ACh $K_d$ value of 60 $\mu$M versus butanol $EC_{50}$ value of 250 mM), butanol's contribution as a partial agonist decreases dramatically as the ACh concentration increases, because more and more receptors are bound by ACh instead of buta-

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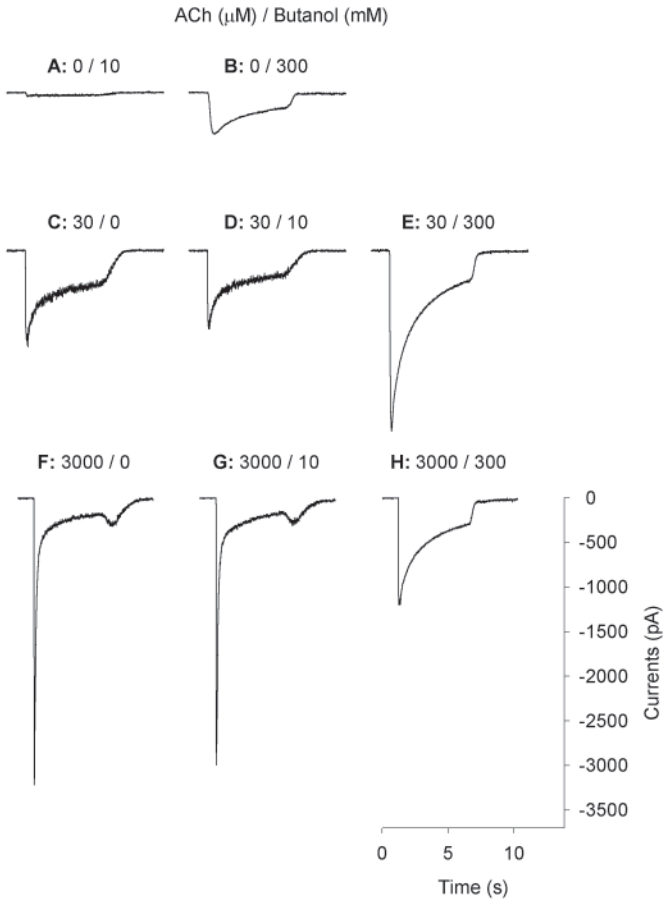


**Fig. 10.** Long-pulse (5-s) application of ACh, *n*-butanol, or both to the nnAChRs stably transfected in the HEK293 cells. Drugs were applied through U-tube and the recordings were performed at a holding potential of −50 mV at intervals of 2 min. A, 10 mM butanol alone. B, 300 mM butanol alone. C, 30 μM ACh alone. D, 30 μM ACh plus 10 mM butanol. E, 30 μM ACh plus 300 mM butanol. F, 3000 μM ACh alone. G, 3000 μM ACh plus 10 mM butanol. H, 3000 μM ACh plus 300 mM butanol.

nol. The most significant change in the composition of the overall open channels occurs between the ACh concentrations of 30 and 100 μM: the fraction of the two ACh-open channel increases from 10% of total opened channels at 30 μM ACh to more than 99% at 100 μM ACh, and at the same time, the two butanol-opened channel drops from 90% of total open channels to less than 1%. This trend continues until the concentrations are increased to 3000 μM ACh, when almost all the open channels are occupied by two ACh molecules, whereas butanol hardly opens any channel by itself (<0.01%).

Figure 11 depicts the results of simulation of the fraction of various receptor states at the peak current in the presence of various concentrations of ACh (Fig. 11A) and butanol (Fig. 11B). Complex dose-response curves are obtained showing an apparent potentiating action of butanol at low ACh concentrations and a biphasic inhibitory action at moderate-to-high ACh concentrations (Fig. 11A, open squares) as seen experimentally (Fig. 6A). Figure 11A illustrates that the initial level (~35%) occurring at 1 μM ACh is mainly due to the activation of the receptors by butanol. Because butanol blocks the ACh-opened channels only, the blocking action takes place only when there are many ACh-opened channels.

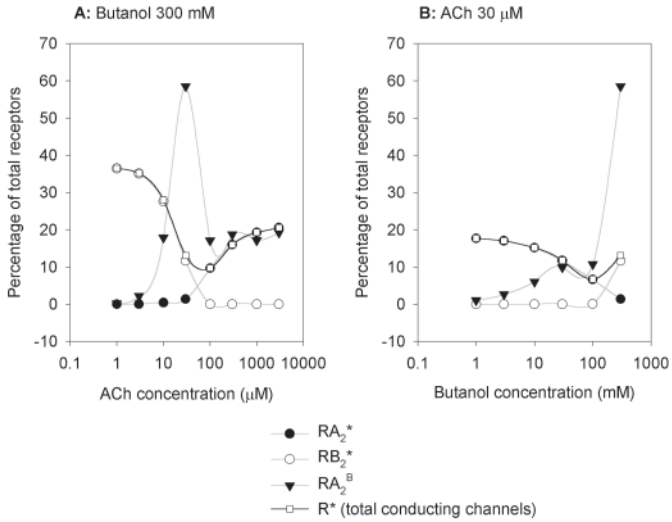


**Fig. 11.** Compositions of different receptor states at the peak of the simulated currents induced by ACh and butanol. The parameters used for simulation are given in Fig. 9. A, effects of 300 mM butanol on ACh dose-response relationship (1–3000 μM). B, butanol inhibition (1–300 mM) at 30 μM ACh. Filled circles, $RA_2$*, channel opened by two ACh molecules; open circles, $RB_2$*, channel opened by two butanol molecules; triangles, $RA_2^B$, the two ACh-opened channel blocked by butanol; and squares, R* (total conducting channels), the total open channels considering both the partial agonist action and the channel blocking action of butanol.

This is shown by the fact that the percentage of butanol block of the total active receptors is 1% at 1 μM ACh compared with 82% at 30 μM ACh. Because the blocking action of butanol takes place at a rate much lower than that of ACh to open the channel, as the concentration of ACh increases, the current peaks sooner and butanol blocks less. Thus, only 48% block occurs at 3000 μM ACh compared with 82% block at 30 μM ACh. Thus, the combination of both the partial agonist action and the kinetics of channel blocking action generates a V-shaped ACh dose-response curve at 300 mM butanol (Fig. 11A, squares).

The simulation of the V-shape dose-response curve for butanol inhibition at 30 μM ACh (Fig. 8B) could be similarly accounted for as illustrated in Fig. 11B (squares). At low concentrations of butanol (1–30 mM), more than 99% of the total open channels bind two ACh molecules and are very sensitive to butanol blocking action. However, as the butanol concentration increases, butanol starts competing with 30 μM ACh to open the channel. For example, 100 and 300 mM butanol contribute 11 and 90%, respectively, to the total channel conductance in the presence of 30 μM ACh and butanol. Thus, a biphasic inhibitory dose-response relationship was obtained at 30 μM (Fig. 8).

When the $\alpha4\beta2$ receptors were activated by 1 mM ACh, butanol exerted a monophasic inhibition, yielding an apparent $IC_{50}$ value around 100 mM. A similar dose-response relationship was simulated by the kinetic model in which butanol acted as a partial agonist and as an open channel blocker. In the simulation, however, the $IC_{50}$ value for open channel block was assumed to be 6.7 mM, a value similar as the extrapolated $IC_{50}$ value from the previous study of the chain-length dependence of long-chain alcohols to inhibit the whole-cell current of $\alpha4\beta2$ receptors (Zuo et al., 2001).

The obvious discrepancy between the measured $IC_{50}$ value of 100 mM and the extrapolated one of 6.7 mM is due to two

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factors, both of which would reduce $IC_{50}$ value measured at the whole-cell level. One factor is the butanol's dual action because the channel blocking action could not be cleanly separated from its partial agonist action at the whole-cell level. Another factor contributing to the lesser potency of block is the kinetic effect of channel gating on the equilibrium block. Given the blocking and unblocking rate constants used in the simulation, butanol should reach an equilibrium block at the peak of ACh current. However, in the constant presence of ACh during coapplication of ACh and butanol, the rate constants governing channel opening and closing would reduce the butanol block. This gating effect on the open channel block was recognized in open channel block of other channels as well (Coronado and Miller, 1979; French and Shoukimas, 1981).

**Comparison of multiple actions with previous studies.** Multiple actions have been reported previously for the *n*-octanol action on the $GABA_A$ receptor (Kurata et al., 1999); the actions of *d*-tubocurarine (Steinbach and Chen, 1995), metocurine, and atracurium (Fletcher and Steinbach, 1996) on the fetal nAChRs; and the actions of *d*-tubocurarine on nnAChRs containing $\beta4$ subunit (Cachelin and Rust, 1994). However, in most cases, all the observations were satisfactorily explained by a model in which the drug acted as a weak agonist to open the channel either by itself or by cobinding with another agonist. The latter example was used in the model to explain the dual action of atropine (Zwart and Vijverberg, 1997) and *d*-tubocurarine (Cachelin and Rust, 1994) on nnAChRs because the receptors occupied by two atropine or tubocurarine molecules do not conduct current. In our case, butanol acts as a partial agonist at high concentrations and as channel blocker at low concentrations on the $\alpha4\beta2$ receptor.

Inasmuch as channel opening and block are such distinct actions, the multiple effects of the agents suggest multiple sites of action on nnAChRs. Our previous simulation based on a model in which long-chain alcohols both block the open ACh receptor-channel and interfere with ACh binding explains satisfactorily the alcohol's inhibitory action (Zuo et al., 2001). It was also suggested that the inhibitory action of long-chain alcohols is due to both alcohol block of open channels and alcohol interference with ACh binding (decrease in $k_{on}$). One possible mechanism of $k_{on}$ reduction is that long-chain alcohols can compete with ACh for the ACh binding site. Because butanol has a similar size to ACh, it is capable of opening the channel after binding to ACh binding site. This competition between long-chain alcohols and ACh for binding to the agonist sites would manifest as a slowing in $k_{on}$, the rate constant for ACh to bind the agonist site in the previous simulation (Zuo et al., 2001).

**Acknowledgments**

We thank Nayla Hasan for technical assistance and Julia Irizarry for secretarial assistance. HEK cell lines expressing the $\alpha4\beta2$ nnAChR subunits were provided by SIBIA Neurosciences, Inc. (La Jolla, CA) (now Merck Laboratories, San Diego, CA).

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