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Block by 5-hydroxytryptamine and apomorphine of recombinant human neuronal nicotinic receptors

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Abstract

The effects of 5-hydroxytryptamine and apomorphine on human neuronal nicotinic acetylcholine receptor/channels were examined by expressing these channels in *Xenopus* oocytes. Functional channels were expressed by combining one type of α subunits (α3 or α4) and one type of β subunits (β2 or β4). 5-Hydroxytryptamine (100 μM to 1 mM) and apomorphine (10 to 100 μM) inhibited an inward current activated by acetylcholine in the oocytes expressing the channels. The sensitivity to 5-hydroxytryptamine or apomorphine depended on subunit combinations. When concentration–response relationship was obtained for the acetylcholine-activated current, the maximal response was reduced by these compounds. The inhibition by these compounds exhibited voltage-dependence: the inhibition was augmented at negative potentials. The results suggest that 5-hydroxytryptamine and apomorphine noncompetitively inhibits human recombinant nicotinic acetylcholine receptor/channels, presumably by acting on channel pores.

Keywords: Human nicotinic receptor; 5-Hydroxytryptamine; Apomorphine; Allosteric modulation

1. Introduction

Nicotinic acetylcholine receptors are channel forming receptors which promote excitatory cellular functions in postsynaptic cells, including spike generation in neurons, the contraction of skeletal muscle and catecholamine secretion from adrenal medulla, by allowing extracellular cations to enter cells. Allosteric modulations of nicotinic responses have been reported (Arias, 1998). Among endogenous substances, 5-hydroxytryptamine, a neuroamine, is known to inhibit cellular responses mediated through nicotinic acetylcholine receptors. 5-Hydroxytryptamine inhibits fast excitatory postsynaptic potentials in bull–frog sympathetic neurons (Akasu and Kotetsu, 1986) and nicotine-induced catecholamine release from cultured bovine adrenal chromaffin cells (Vijayaraghavan et al., 1993). More recently, 5-hydroxytryptamine has been shown to inhibit an ionic current mediated through nicotinic acetylcholine receptor/channels expressed from cDNA's encoding receptor subunits in *Xenopus* oocytes. 5-Hydroxytryptamine inhibits ionic current mediated through recombinant mouse neuronal and muscular nicotinic receptors (Cross et al., 1995), rat neuronal nicotinic receptors (García-Colunga and Miledi, 1995; Nakazawa et al., 1995) and chicken neuronal nicotinic receptors (Palma et al., 1996). In addition to 5-hydroxytryptamine, it has been reported that compounds related to dopamine, another neuroamine, inhibit cellular responses mediated through nicotinic receptors. Dopamine receptor agonists, including apomorphine, decrease catecholamine secretion induced by nicotinic stimulation in bovine adrenal chromaffin cells (Sontag et al., 1990), perfused cat adrenal glands (Artalejo et al., 1985) and rat pheochromocytoma cells (Althaus et al., 1991). We previously reported that apomorphine and other compounds related to dopamine receptors (but not dopamine itself) reduces ionic current mediated through recombinant rat neuronal nicotinic receptors expressed *Xenopus* oocytes (Nakazawa et al., 1994). However, the effects of these allosteric modulators on human nicotinic acetylcholine receptor/channels have not been reported.

In the present study, we investigated the effects of 5-hydroxytryptamine and apomorphine on human nicotinic receptors expressed in *Xenopus* oocytes. We found that

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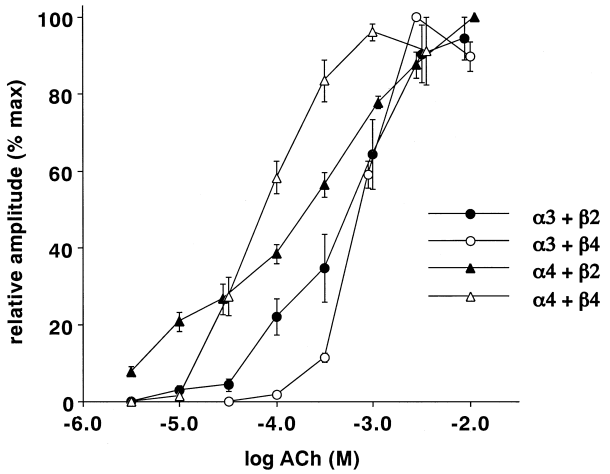

Fig. 1. Concentration–response relationship for acetylcholine-evoked current in *Xenopus* oocytes expressing α3β2, α3β4, α4β2 and α4β4 receptor/channels. The oocytes were held at −50 mV. Current responses were normalized to maximal responses in individual oocytes. Each symbol and bar represent the mean and S.E. obtained from four to six oocytes.

the compounds inhibited these recombinant channels in a noncompetitive and a voltage-dependent manner.

2. Methods

Human neuronal nicotinic receptor clones were obtained from Dr. Jon Lindstorm of University of Pennsylvania. All the clones were isolated and subcloned into plasmids in his laboratory (β4: Kuryatov et al., 1997; Olale et al., 1997; the data for the remaining clones were personally provided). The α3 and β2 clones provided had been subcloned into pcDNAI plasmid (Invitrogen, The Netherlands), and the α4 and β4 clones provided had been subcloned into pSP64 poly(A +) plasmid (Promega, Madison, WI, USA). The α3 clone was resubcloned into pSP64 poly(A +) plasmid at *Hin*dIII and *Bam*HI sites, because current responses to acetylcholine were too small to analyze (less than 10 nA at −80 mV after a 5-day incubation) with the original clone. The plasmids containing cDNA's were linearized at *Bam*HI (α3 and α4), *Sac*I (β2) or *Xho*I site (β4), and cRNA's were transcribed with SP6 (α3, α4 and β2) or T7 (β4) RNA polymerase. Expression of channels and membrane current measurements were conducted as in our previous report (Nakazawa et al., 1994). Defolliculated *Xenopus* oocytes were injected with the transcribed cRNA's, and incubated for 2 to 6 days at 18°C. Membrane current was measured by the

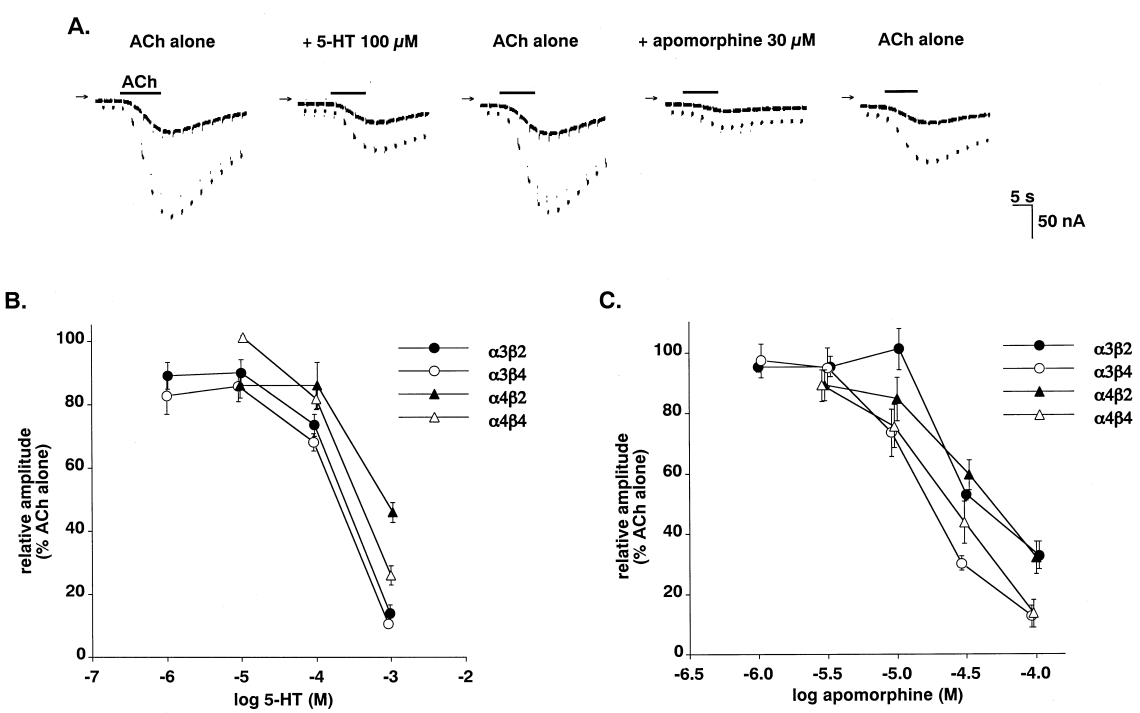

Fig. 2. Inhibition by 5-hydroxytryptamine and apomorphine of acetylcholine-evoked current. (A) Current responses to five consecutive applications of 1 mM acetylcholine in an oocyte expressing the α3β2 receptor/channel in the absence (ACh alone) or the presence of 100 μM 5-hydroxytryptamine (5-HT) or 30 μM apomorphine. The oocyte was held at −50 mV, and a 200-ms hyperpolarizing step to −80 mV was applied every 2 s. Arrows indicate zero current levels. (B, C) Inhibition by 5-hydroxytryptamine (A) or apomorphine (B) of acetylcholine-evoked current with various subunit combinations. The current was evoked by 100 μM (α4β2), 300 μM (α4β4) or 1 mM (α3β2 and α3β4) acetylcholine. Current responses at −50 mV were measured and normalized to those to acetylcholine alone in individual oocytes. Each symbol and bar represent the mean and S.E. obtained from five to six oocytes tested.

conventional two-microelectrode voltage-clamp technique from oocytes bathed in an experimental chamber of about 0.1 ml in volume filled with an extracellular solution [composition (mM): NaC1 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 5; pH 7.5 with NaOH]. Acetylcholine and other substances were applied to oocytes by superfusion at a constant flow rate of about 0.5 ml/s. The application periods of acetylcholine were brief (4 to 10 s, depending on the concentrations of acetylcholine), and each application of acetylcholine was separated by 2 min. With this application protocol, stable inward current was evoked by acetylcholine without obvious desensitization for 30 min or longer.

EC_{50} for acetylcholine and IC_{50} for 5-hydroxytryptamine or apomorphine were calculated from data from individual oocytes, to which various concentrations of the drugs had been applied successively, according to the following equation (Tallarida and Jacob, 1977):

$$\log\left[E/(E_{\max}-E)\right] = k\log A - \log K, \quad (1)$$

where E, $E_{\max}$, A, K and k are effect, maximal effect, drug concentration and Hill coefficient, respectively. Data were fit to Eq. (1) by the least square method, and K and k were obtained. EC_{50} or IC_{50} was then calculated from these values (EC_{50} or $IC_{50} = K^{1/k}$).

Acetylcholine chloride, 5-hydroxytryptamine creatinine sulfate complex and apomorphine hydrochloride was purchased from Sigma (St. Louis, MO, USA).

All the data were given as mean ± S.E. Statistical analysis was done with the paired *t*-test when comparing the data obtained from individual oocytes, and done with Duncan's multiple comparison when comparing the data obtained from different oocytes. Significant difference was judged when $P < 0.05$.

3. Results

3.1. Acetylcholine-evoked current through recombinant human nicotinic acetylcholine receptor / channels

When one type of α subunits was combined with one type of β subunits to express functional channels, acetylcholine evoked inward currents of about 30 nA to 1 μA at −50 mV in cRNA-injected *Xenopus* oocytes after 2- to

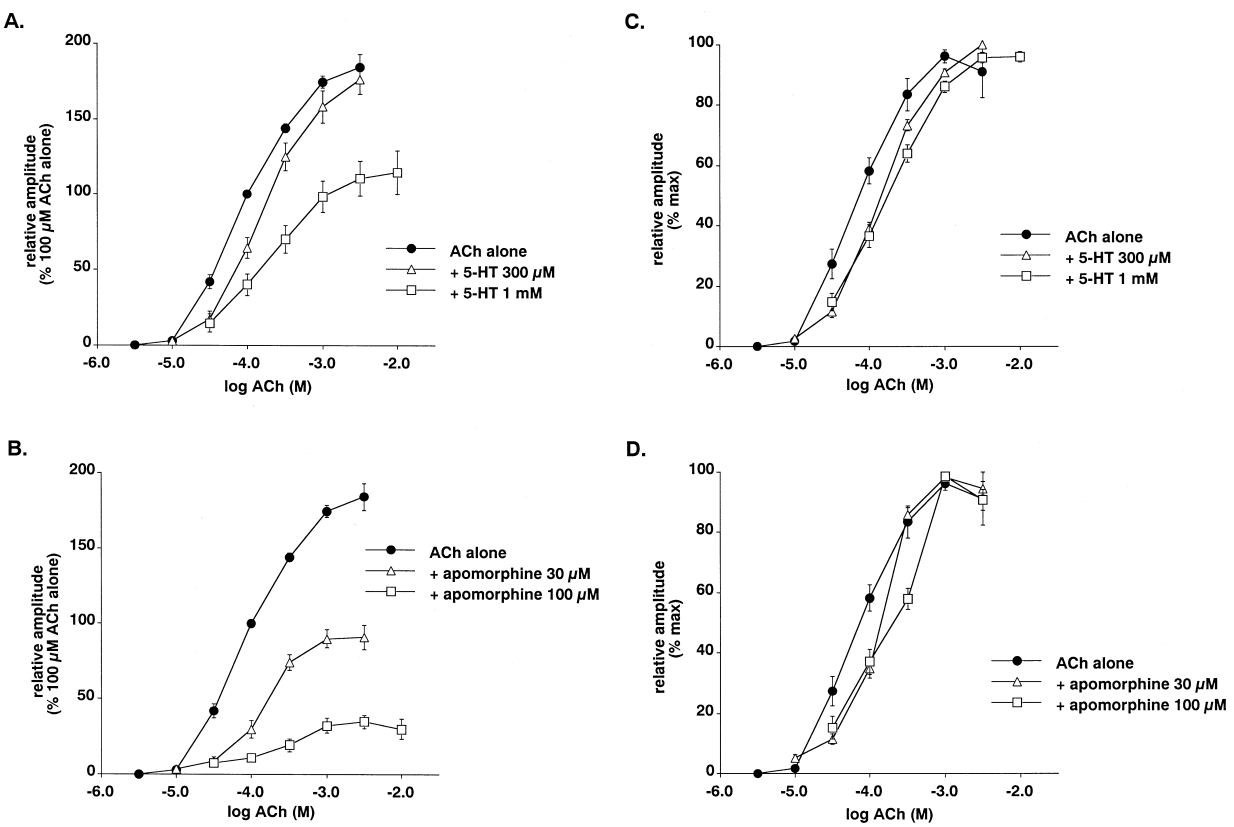

Fig. 3. (A, B) Concentration–response relationship for acetylcholine-evoked current in the absence or the presence of 5-hydroxytryptamine (300 μM and 1 mM; A) or apomorphine (30 and 100 μM; B) in the α4β4 receptor/channel. Current responses were normalized to that to 100 μM acetylcholine alone in each oocyte. Holding potential was −50 mV. Each symbol and bar represent the mean and S.E. obtained from four to six oocytes tested. (C, D) Concentration–response curves for the comparison of the sensitivity to acetylcholine. The same data in A or B were normalized to the maximal response among responses to a series of acetylcholine concentrations in each oocyte.

6-day incubation periods. Fig. 1 shows dose–response relationship for the acetylcholine-evoked current obtained for four subunit combinations. The subunit combinations involving α4 subunits were more sensitive to acetylcholine than those involving α3 subunits. EC_{50} values for acetylcholine were 782 ± 216 μM (α3β2, $n = 4$), 844 ± 41 μM (α3β4, $n = 4$), 145 ± 18 μM (α4β2, $n = 6$) and 98 ± 19 μM (α4β4, $n = 4$). Among these values, significant difference was found between α3β4 and α4β4. The slope of the dose–response relationship was steeper with the subunit combinations involving β4 subunit than with those involving β2 subunit. The slope calculated was 0.94 ± 0.06 (α3β2, $n = 4$), 1.94 ± 0.03 (α3β4, $n = 4$), 0.59 ± 0.04 (α4β2, $n = 7$) or 1.66 ± 0.20 (α4β4, $n = 4$), and significant difference was found between α3β2 and α3β4 and between α4β2 and α4β4. These properties are similar to the results obtained with recombinant human nicotinic receptors expressed in *Xenopus* oocytes (Chavez-Noriega et al., 1997) or human embryonic kidney (HEK) 293 cells (Stauderman et al., 1998).

3.2. Block by 5-hydroxytryptamine and apomorphine of acetylcholine-evoked current

To investigate the effects of 5-hydroxytryptamine and apomorphine, equipotent concentrations of acetylcholine were selected for the subunit combinations based on the data shown in Fig. 1, namely, 100 μM for α4β4, 300 μM for α4β2, and 1 mM for α3β2 and α3β4. Fig. 2A illustrate five consecutive trials of 1 mM acetylcholine on an oocyte expressing the α4β2 receptor/channel in the absence or presence of 100 μM 5-hydroxytryptamine or 30 μM apomorphine. 5-Hydroxytryptamine reduced the acetylcholine-evoked current (the second left panel), and the reduced current was readily recovered after washout of 5-hydroxytryptamine (middle panel). Apomorphine also reduced the acetylcholine-evoked current (the second right panel), and the reduced current was not readily recovered (right panel). The current reduced by apomorphine was gradually recovered during several additional trials of acetylcholine alone, but full recovery was not achieved: the current was recovered to 70 to 80% of the level before the application of apomorphine. Reversible inhibition by 5-hydroxytryptamine and partially reversible inhibition by apomorphine were observed irrespective of the subunit combinations tested.

3.3. Dependence on subunit combinations

Fig. 2B and C compare the inhibition by 5-hydroxytryptamine or apomorphine of the acetylcholine-evoked current among the subunit combinations. The subunit combinations involving α3 subunit were more sensitive to 5-hydroxytryptamine than those involving α4 subunit (i.e., α3β2 > α4β2 and α3β4 > α3β4; Fig. 2B). IC_{50} values calculated were significantly different between α3β2 (159 ± 35 μM, $n = 4$) and α4β2 (867 ± 114 μM, $n = 4$), and between α3β4 (113 ± 42 μM, $n = 4$) and α4β4 (418 ± 71 μM, $n = 4$). On the other hand, the subunit combinations involving β2 subunit was more sensitive to apomorphine than those involving β4 subunit (i.e., α3β2 > α3β4 and α4β2 > α4β4; Fig. 2C), though IC_{50} values calculated were significantly different only between α3β2 (54.0 ± 10.1 μM, $n = 4$) and α3β4 (20.8 ± 3.5 μM, $n = 4$), but not between α4β2 (48.5 ± 12.5 μM, $n = 4$) and α4β4 (26.2 ± 7.0 μM, $n = 4$).

3.4. Influence on dose–response relationship for acetylcholine-evoked current

The concentration–response relationship was obtained for the α4β4 receptor/channel, which exhibited the high-

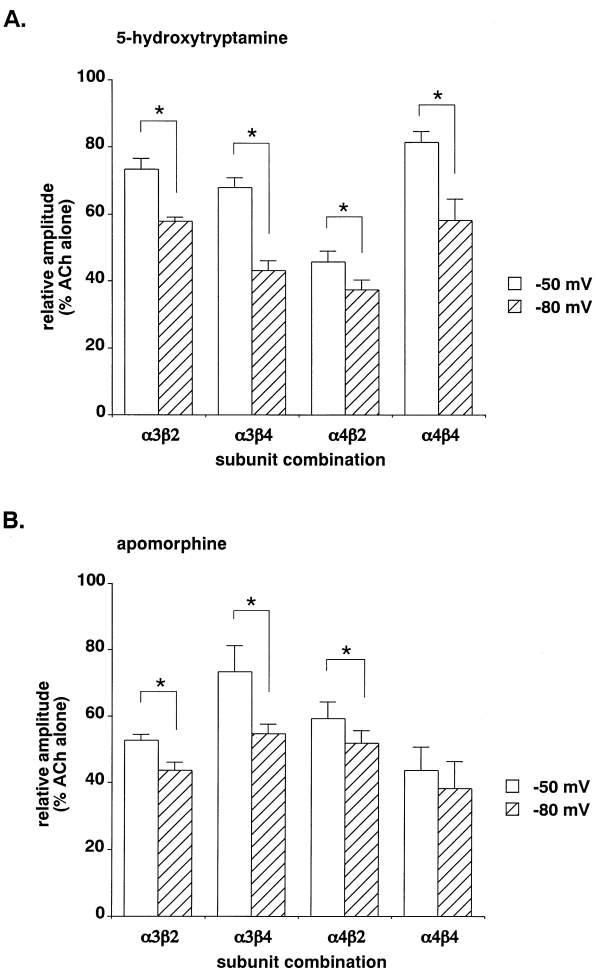

Fig. 4. (A, B) Comparison of acetylcholine-evoked current at −50 and −80 mV in the presence of 5-hydroxytryptamine (A) or apomorphine (B). Oocytes were held at −50 mV and a hyperpolarizing step to −80 mV was periodically applied, as shown in Fig. 2A. The concentration of 5-hydroxytryptamine in A was 100 μM (α3β2, α3β4 and α4β4) or 1 mM (α4β2), and the concentration of apomorphine in B was 10 μM (α3β4) or 30 μM (α3β2, α4β2 and α4β4). Other conditions were the same as in Fig. 3. Asterisks indicate significant difference determined by the paired *t*-test.

est sensitivity to acetylcholine among the subunit combinations tested (Fig. 1), in the presence of 5-hydroxytryptamine or apomorphine (Fig. 3). To compare the first responses to a series of stepwisely increased concentrations of acetylcholine, a standard response to 100 μM acetylcholine alone was always obtained from oocytes, and responses to various concentrations of acetylcholine in the absence and the presence of 5-hydroxytryptamine or apomorphine were normalized to this standard response (Fig. 3A and B). 5-Hydroxytryptamine shifted the dose–response toward right without largely affecting the maximal response at 300 μM, and it reduced the maximal value by 40% at 1 mM (Fig. 3A). Apomorphine (30 and 100 μM) concentration-dependently decreased the maximal response (Fig. 3B). The sensitivity to acetylcholine was compared in Fig. 3C and D. In this case, current responses to a series of the stepwisely increased concentrations of acetylcholine were normalized to the maximal response among the responses in each oocyte. EC_{50} value was significantly increased from 98 ± 19 μM ($n = 4$) to 151 ± 15 μM or 176 ± 19 μM with 300 μM ($n = 6$) or 1 mM ($n = 6$) 5-hydroxytryptamine, respectively (Fig. 3C). The sensitivity to acetylcholine also appeared to be reduced by apomorphine (Fig. 3D). However, EC_{50} values calculated [112 ± 13 μM with 30 μM apomorphine ($n = 6$) and 129 ± 14 μM with 100 μM apomorphine ($n = 6$)] were not significantly different from the control value (98 ± 19 μM, $n = 4$).

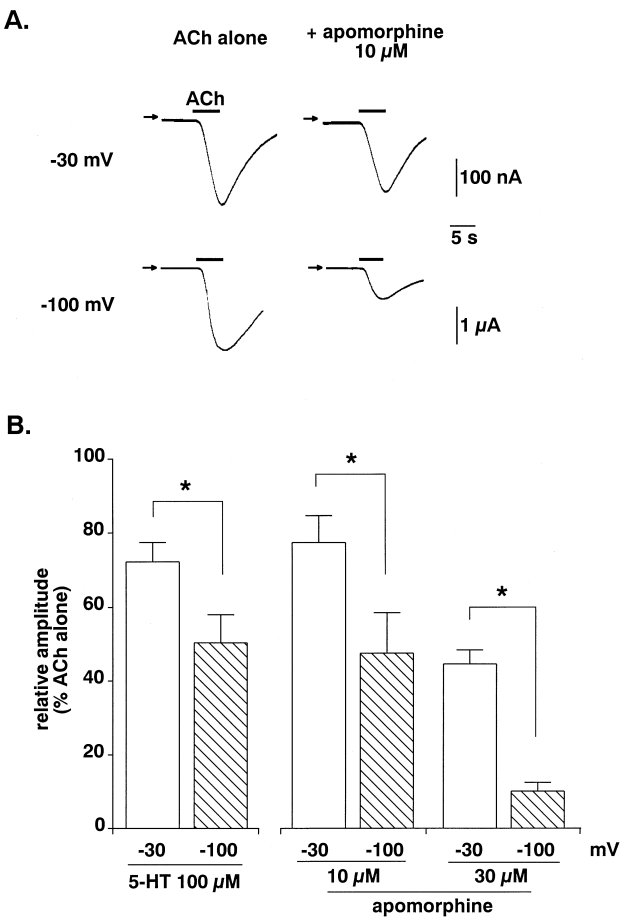

Fig. 5. Voltage-dependent inhibition by 5-hydroxytryptamine and apomorphine of acetylcholine-evoked current demonstrated by holding oocytes at different potentials. (A) Current responses to 1 mM acetylcholine at −30 (upper traces) and −100 mV (lower traces) in the absence (left) and presence of 10 μM apomorphine (right) in oocytes expressing the α3β4 receptor/channel. The upper and the lower traces were obtained from different oocytes. Arrows indicate zero current levels. (B) Summarized data for inhibition by 5-hydroxytryptamine (100 μM) or apomorphine (10 and 30 μM) of the acetylcholine-evoked current. Current measurement was made as in A. Current responses in the presence of 5-hydroxytryptamine or apomorphine were normalized to those before application of these compounds. Each column and bar represent the mean and S.E. from four to five oocytes tested. Asterisks indicate significant difference determined by the paired *t*-test.

3.5. Voltage-dependent block of acetylcholine-evoked current

Fig. 4 compares the current inhibition by 5-hydroxytryptamine and apomorphine at −50 and −80 mV. The data were obtained by holding oocytes at −50 mV and applying a 200-ms hyperpolarizing step to −80 mV every 2 s, as shown in Fig. 2A. Fractional current in the presence of 5-hydroxytryptamine or apomorphine was always smaller, or, in other words, the current inhibition by these compound was always more remarkable at −80 mV than at −50 mV irrespective of the subunit combinations tested, suggesting that these compounds exhibit voltage-dependent block. The voltage-dependent block was also confirmed by the experiment using different holding potentials (Fig. 5). The current response at a holding potential of −100 mV was more markedly inhibited by 100 μM 5-hydroxytryptamine or 10 and 30 μM apomorphine than that at a holding potential of −30 mV.

4. Discussion

We have demonstrated that 5-hydroxytryptamine and apomorphine inhibit human recombinant nicotinic acetylcholine receptor/channels, as has been reported for rodent (Cross et al., 1995; García-Colunga and Miledi, 1995; Nakazawa et al., 1995) or avian (Palma et al., 1996) nicotinic receptors. Although 5-hydroxytryptamine or apomorphine reduced the sensitivity of the recombinant channels to acetylcholine, the inhibition may not be attributed solely to competition with acetylcholine for the binding-site of nicotinic receptors, judging from the concentration–response curves for the acetylcholine-evoked current, where a dose-dependent, parallel shift of the curve was not observed (Fig. 3). Instead, the voltage-dependence of the inhibition (Figs. 4 and 5) suggests that these compounds act on the channel pore of nicotinic receptor/channels. This view may be supported by the fact that both 5-hy-

droxytryptamine and apomorphine possess positive charges in their structures as pore blockers of nicotinic acetylcholine receptor/channels do (Ascher et al., 1979; Buisson and Bertrand, 1998). Palma et al. (1996) have also concluded that 5-hydroxytryptamine acts on the pore region of avian homomeric α7 nicotinic receptor/channels, from their finding of the conversion of this compound from an antagonist to an agonist upon the replacement of an amino acid residue at the pore region. The pore block can also account for the observed reduction of the sensitivity to acetylcholine assuming if the pore block secondarily affects the coupling between agonist binding and a conformational change to an open-state. However, in addition to the pore block, a possibility of direct block of the binding of acetylcholine cannot be excluded, especially for 5-hydroxytryptamine, because EC_{50} value of acetylcholine was significantly increased by this compound (Fig. 3).

The location of the binding-site for channel blocking particles can be estimated by an equation based on the constant-field theory (Woodhull, 1973). If calculation was made from this equation and the values shown in Fig. 5, the location of binding-site for 5-hydroxytryptamine is estimated to be 34% of total electrical distance from the outer mouth of the channel pore. Similarly, the location of the binding-site for apomorphine, when calculated from the data with 10 μM of this compound, is estimated to be 49% of total electrical distance. These values are comparable to those reported for various pore-blocking agents in human nicotinic receptor/channels expressed with a combination of α4 and β2 (Buisson and Bertrand, 1998). Thus, these positively charged compounds may interact with some negatively charged or polarized amino acid residue existing halfway along the channel pore.

The current inhibition by 5-hydroxytryptamine or apomorphine exhibited dependence on channel subunit combinations (Fig. 2). Such dependence on subunit combinations has not been definitely demonstrated for the clones of other animals, though our previous study (Nakazawa et al., 1994) showed that the sensitivity to apomorphine was lower with β2 subunit than β4 subunit when the same (not equipotent) concentration of acetylcholine was used to activate inward currents. The subunit dependence suggests that the structure of the binding-site for 5-hydroxytryptamine or apomorphine is different among the subunit combinations. The channel pore of nicotinic acetylcholine receptor/channels are formed by the association of transmembrane (M2) regions of five subunits (Hille, 1992a,b). If the binding-site for 5-hydroxytryptamine or apomorphine is formed by this association, different sensitivity may be attributable to some amino acid residues that are different between subunits. The amino acid residues in M2 regions are highly conserved, and, for human clones, only one or three amino acid is different between α3 and α4 or between β2 or β4, respectively. We have tried to substitute these 'nonconserved' amino acids with site-directed mutagenesis, but have not succeeded presumably because DNA sequences coding the pore regions are rather unsuitable for the design of mutation primers (unpublished results).

The results obtained in the present study have raised a possibility that 5-hydroxytryptamine and apomorphine inhibit nicotinic acetylcholine receptors in human. The interaction between nicotinic acetylcholine receptors and 5-hydroxytryptamine, apomorphine or other compounds related to dopamine or 5-hydroxytryptamine receptors (Nakazawa et al., 1994, 1995) may be taken into account when investigations are made on physiological and pathological conditions of the human nervous system including the brain. The block by 5-hydroxytryptamine of nicotinic receptors may have implications in regulation of normal brain functions. It has been shown that nicotine stimulates serotoninergic dorsal raphe neurons and increases the release of 5-hydroxytryptamine by acting on presynaptic and/or postsynaptic receptors (Li et al., 1998; Mihailescu et al., 1998). The results presented here suggest that 5-hydroxytryptamine may limit its own liberation by directly blocking nicotinic receptors. This negative feedback mechanism is additional to negative feedback mediated through $5\text{-}HT_{1A}$ receptors in serotoninergic dorsal raphe neurons (Sprouse and Aghajanian, 1987). It is also possible that the increase of 5-hydroxytryptamine levels induced by reuptake inhibitors, which are currently used as antidepressant drugs, affect globally the release of various neurotransmitters in the brain by blocking presynaptic nicotinic receptors (Wonnacott, 1997).

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