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Modulation by estrogens and xenoestrogens of recombinant human neuronal nicotinic receptors

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Abstract

The effects of estrogens and xenoestrogens on human neuronal nicotinic acetylcholine receptor/channels were examined by expressing recombinant channels in *Xenopus* oocytes. When functional channels were expressed with α3 and β4 subunits, estrogens (17β-estradiol, 17α-estradiol, 17α-ethynylestradiol and diethylstilbestrol) and xenoestrogens (bisphenol A, *p*-nonylphenol and *p*-octylphenol) inhibited an ionic current activated by acetylcholine at concentrations up to 100 μM. When the subunit combination was changed to α4β2, diethystilbestrol and the xenoestrogens inhibited the acetylcholine-activated current, but 17β-estradiol or 17α-estradiol did not. For 17α-ethynylestradiol, the current through the α4β2 receptor/channel was inhibited at 1 μM, but it was markedly enhanced at 10 and 100 μM. Tamoxifen (10 μM), an antiestrogen, itself inhibited the acetylcholine-activated current but did not antagonize the current modulations induced by the estrogens and the xenoestrogens. These and additional results suggest that human neuronal nicotinic acetylcholine receptors are the targets of non-genomic actions of estrogens and xenoestrogens.

Keywords: Nicotinic receptor, (Human); Estrogen; Xenoestrogen; Non-genomic action

1. Introduction

The hormone 17β-estradiol regulates gene transcription through intracellular estrogen receptors. A number of environmental agents are known or suspected to act on estrogen receptors. These compounds (except for those structurally related to 17β-estradiol) are called xenoestrogens, and scientists have warned of their influence on humans and animals (Corbone et al., 1993; Korach, 1993; Stone, 1994). In addition to a genomic action through intracellular estrogen receptors, 17β-estradiol has been shown to exhibit relatively rapid non-genomic actions which may be mediated through receptors or other functional proteins in the cell membrane (see reviews, Moss et al., 1997; Kelly and Lagrange, 1998; Kelly and Wagner, 1999). It has scarcely been clarified whether or not xenoestrogens produce similar non-genomic actions or not, though Nadal et al. (2000) recently suggested that catecholamine receptors may serve as non-genomic receptors for both estrogens and xenoestrogens.

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 the adrenal medulla, by allowing extracellular cations to enter cells. It has been reported that nicotinic acetylcholine receptors are the targets of the non-genomic actions of 17β-estradiol. Ke and Lukas (1996) showed that 17β-estradiol as well as other steroids such as progesterone inhibited $^{86}Rb^{+}$ efflux induced by carbamylcholine in both human neuronal and muscle cell lines expressing nicotinic acetylcholine receptors. Uki et al. (1999) found that steroids including 17β-estradiol and 17α-estradiol suppressed an ionic current activated by acetylcholine in rat superior cervical ganglionic neurons.

We previously showed that *Xenopus* oocytes are suitable for pharmacological studies of cloned human neuronal nicotinic acetylcholine receptors (Nakazawa and Ohno, 1999). In the present study, we used this *Xenopus* oocyte expression system to examine the effects of estrogens and xenoestrogens on the cloned human neuronal nicotinic acetylcholine receptor.

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2. Methods

Cloned human neuronal nicotinic receptors (Kuryatov et al., 1997; Olale et al., 1997) were expressed in *Xenopus* oocytes as described previously (Nakazawa and Ohno, 1999). Defolliculated *Xenopus* oocytes were injected with in vitro transcribed cRNAs encoding α and β subunits, and incubated for 2 to 6 days at 18 °C. Membrane current was measured by the conventional two-microelectrode voltage-clamp technique under the conditions described before (Nakazawa et al., 1994a). Oocytes were bathed in an experimental chamber of about 0.1 ml 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] and held at −50 mV. A 400-ms hyperpolarizing voltage step to −80 mV was applied every 2 s to confirm clamp conditions and to observe the voltage dependence of current responses. Acetylcholine and other substances were applied to oocytes by superfusion at a constant flow rate of about 0.5 ml/s. Because the sensitivity to acetylcholine was different among subunit combinations (Nakazawa and Ohno, 1999), the current was activated by acetylcholine at a concentration sufficient to obtain about 60% of the maximal response for each subunit combination (α3β4, 300 μM; α4β2, 1 mM; α3β2, 1 mM; α4β4, 100 μM), except for the measurement of the concentration–response relationship for acetylcholine-activated currents. The period of acetylcholine application was brief (6 to 10 s, depending on the concentration of acetylcholine), and each application of acetylcholine was separated by 2 min. With this application protocol, a stable inward current was activated by acetylcholine without obvious desensitization for 30 min or longer.

Acetylcholine chloride, 17β-estradiol, 17α-estradiol, 17α-ethynylestradiol, diethylstilbestrol and tamoxifen were purchased from Sigma (St. Louis, MO, USA). Bisphenol A and *p*-nonylphenol (*p*-*n*-nonylphenol) were purchased from Nacalai Tesque (Kyoto, Japan), and *p*-octylphenol (*p*-*n*-octylphenol) and D-tubocurarine hydrochloride were purchased from Wako (Osaka, Japan). The chemical structures of the estrogens and the xenoestrogens used are shown in Fig. 1. 17β-Estradiol and bisphenol A were first dissolved in ethanol, and 17α-estradiol, 17α-ethynylestradiol, *p*-nonylphenol, *p*-octylphenol and tamoxifen

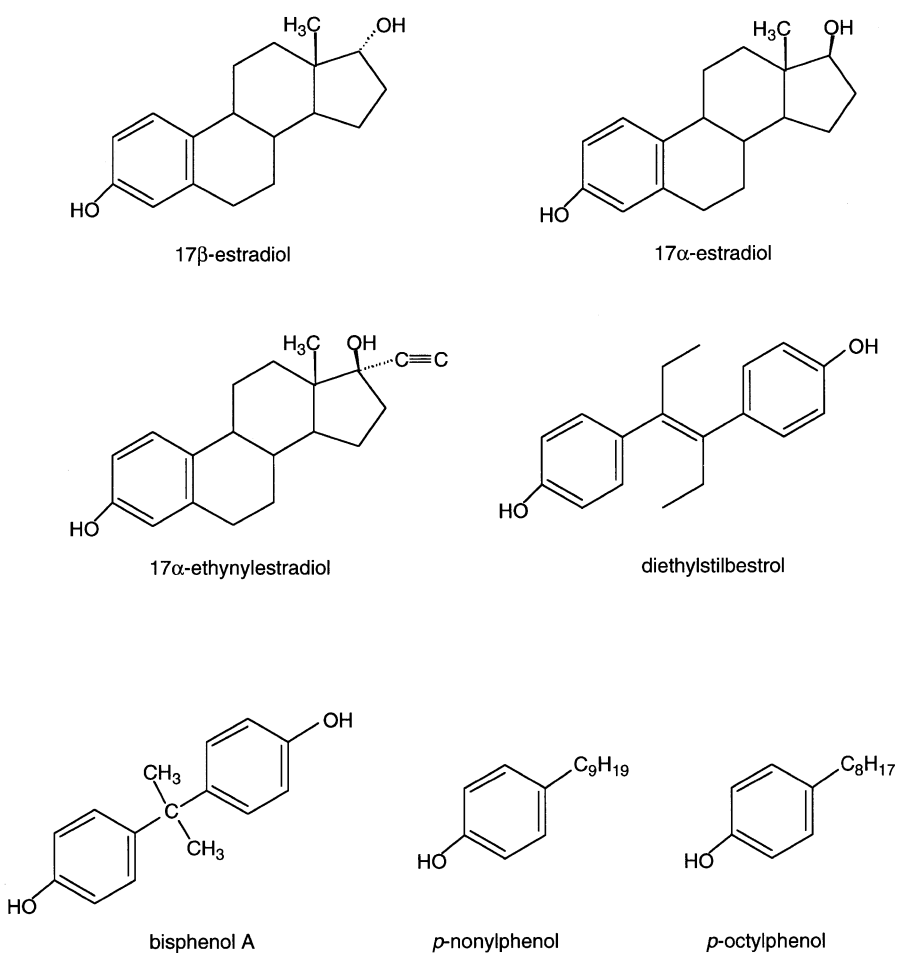

Fig. 1. Chemical structures of the estrogens and xenoestrogens used in the present study.

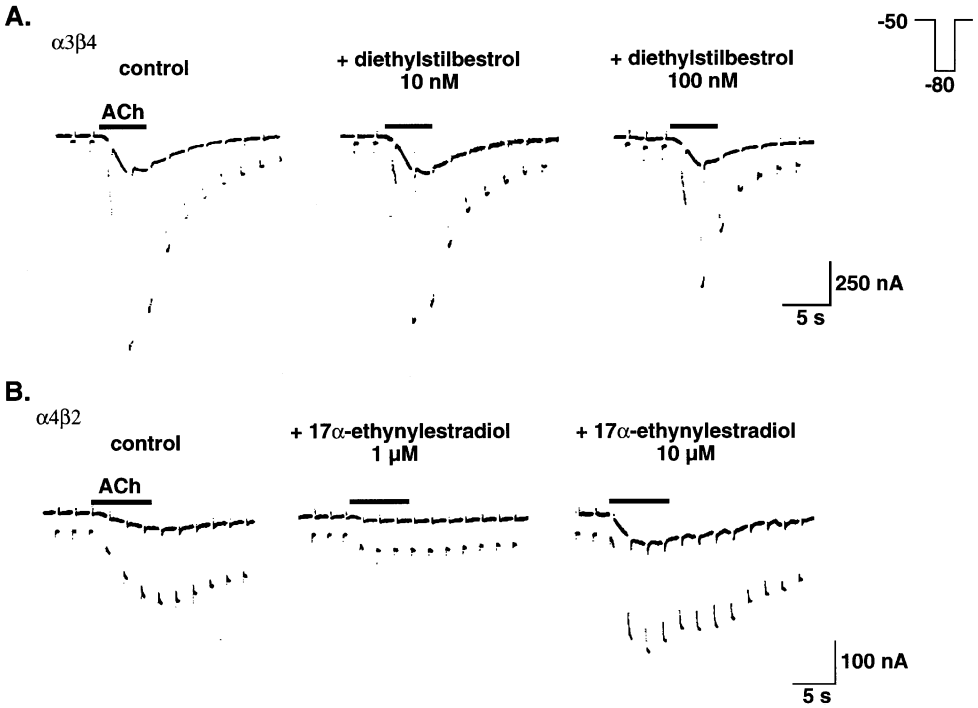

Fig. 2. Modulation by diethylstilbestrol (A) and 17α-ethynylestradiol (B) of acetylcholine-activated currents in *Xenopus* oocytes expressing human nicotinic receptors. Oocytes were held at −50 mV, and a 400-ms hyperpolarizing step to −80 mV was applied every 2 s. (A) Inhibition by diethylstilbestrol of the acetylcholine-activated current through α3β4 receptor/channels. The current activated by 300 μM acetylcholine (ACh; left) was reduced by 10 (middle) or 100 nM diethylstilbestrol (right). (B) Dual modulation by 17α-ethynylestradiol of acetylcholine-activated current through α4β2 receptor/channels. The current activated by 1 mM acetylcholine (left) was inhibited by 1 μM 17α-ethynylestradiol (middle), but the current was enhanced by 10 μM 17α-ethynylestradiol (right).

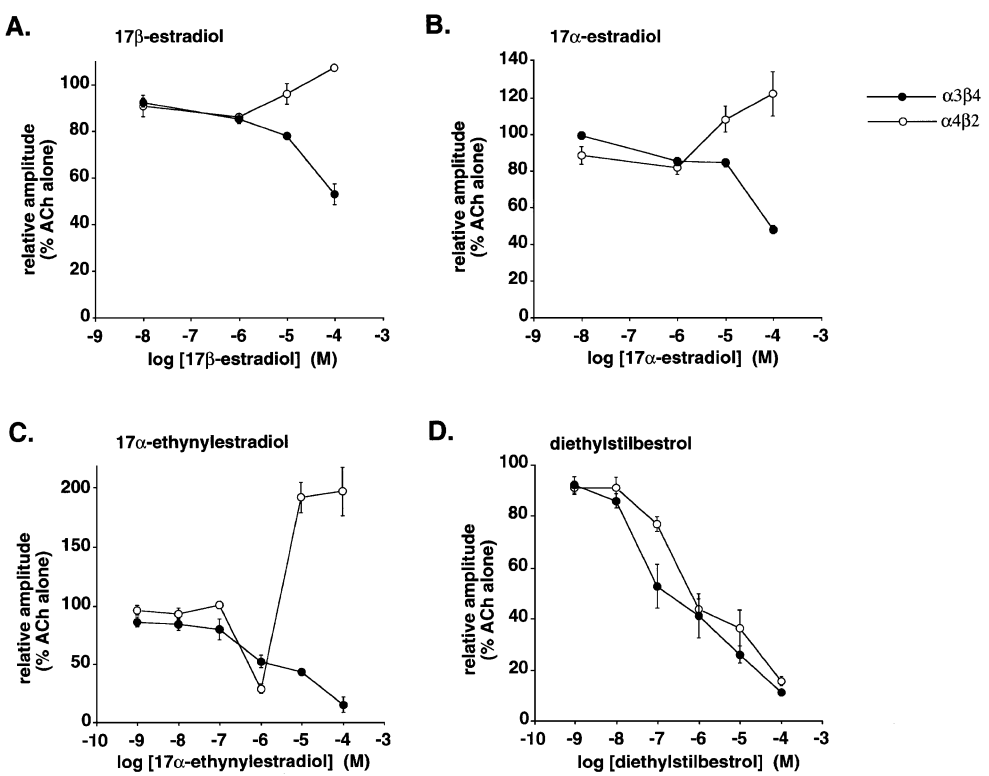

Fig. 3. Effects of 17β-estradiol (A), 17α-estradiol (B), 17α-ethynylestradiol (C) and diethylstilbestrol (D) on acetylcholine-activated currents through α3β4 and α4β2 receptors. The current was recorded as shown in Fig. 2, and current responses to 300 μM (α3β4) or 1 mM (α4β2) acetylcholine at −80 mV in the presence of the compounds were normalized to those in the absence of the compounds. Each symbol and bar represent the mean and S.E. obtained from four to six oocytes.

were first dissolved in dimethylsulfoxide. The final concentration of ethanol or demethylsulfoxide was 0.1% or lower, and these vehicles at 0.1% did not affect the acetylcholine-activated current. Oocytes were continuously exposed to drugs 20 s before and during the application of acetylcholine.

All the data are given as means ± S.E. Statistical analysis was done with the paired *t*-test by comparing the data obtained from individual oocytes. A difference was judged significant when $P < 0.05$. In the graphs for concentration–response data, the data points are connected by straight lines, and IC_{50} values were estimated by eye.

3. Results

3.1. Modulation by estrogens and xenoestrogens of acetylcholine-activated current through α3β4 and α4β2 receptor / channels

Fig. 2 illustrates examples of recordings of acetylcholine-activated currents in the absence and the presence of estrogens. Among the estrogens tested, diethylstilbestrol was the most potent in inhibiting the acetylcholine-activated current through both α3β4 and α4β2 receptor/channels (Figs. 2A and 3D). The current was almost abolished at 100 μM, and IC_{50} values were about 100 nM for α3β4 receptor/channels and about 300 nM for α4β2 receptor/channels. 17β-Estradiol and 17α-estradiol partially inhibited the acetylcholine-activated current through α3β4 receptor/channels (about 50% inhibition at 100 μM; Fig. 3A,B). Remarkable current inhibition was not observed with 17β-estradiol for α4β2 receptor/channels (Fig. 3A), and weak enhancement, rather than inhibition, was observed with 17α-estradiol for α4β2 receptor/channel (Fig. 3B). Different responses between these subunit combinations were most remarkable for 17α-ethynylestradiol (Figs. 2B and 3C). 17α-Ethynylestradiol inhibited the acetylcholine-activated current through α3β4 receptor/channels at concentrations of 1 μM and higher. It markedly enhanced the current through α4β2 receptor/channels at concentrations of 10 and 100 μM, though it inhibited the current at 1 μM.

The acetylcholine-activated current was also inhibited by xenoestrogens (Fig. 4). Bisphenol A inhibited the current through both α3β4 and α4β2 receptor/channels with an IC_{50} of about 10 μM. *p*-Nonylphenol also inhibited the current through both α3β4 and α4β2 channels at 10 and 100 μM, but the magnitude of the current inhibition was larger for α4β2 receptor/channels than for α3β4 receptor/channels. *p*-Octylphenol preferentially inhibited α4β2 receptor/channels: it reduced the current through α4β2 receptor/channels at concentrations of 1 μM and higher, and reduced the current through α3β4 receptor/channels at 100 μM.

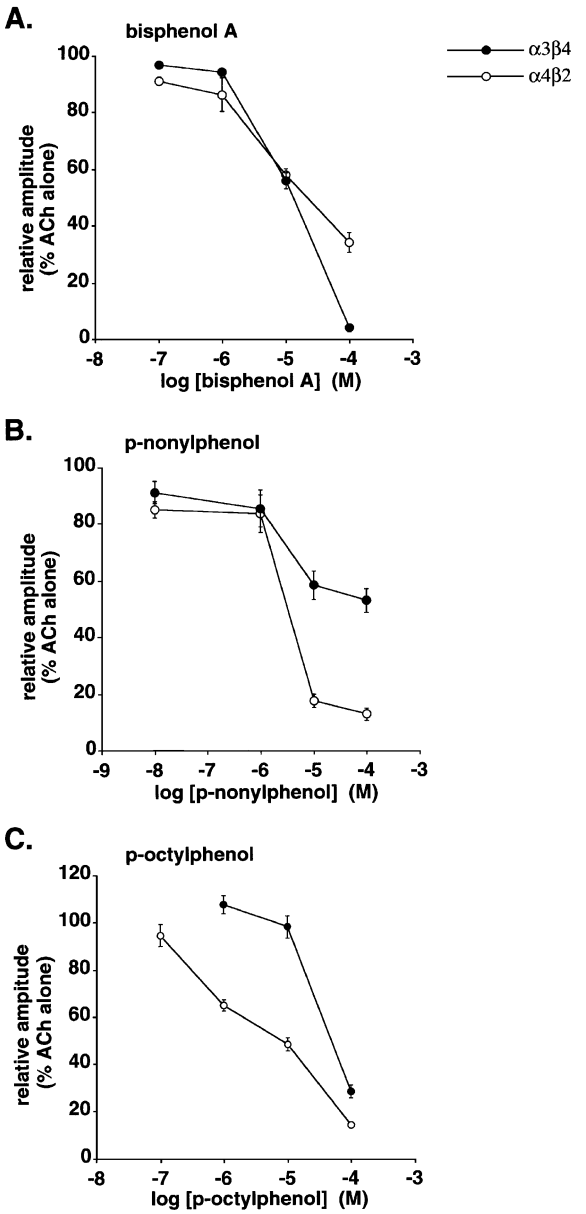

Fig. 4. Effects of bisphenol A (A), *p*-nonylphenol (B) and *p*-octylphenol (C) on acetylcholine-activated currents. The data were obtained and shown as in Fig. 3. Each symbol and bar represent the mean and S.E. obtained from four to six oocytes.

The current inhibition elicited by these compounds described above was reversible, and the current response to acetylcholine was restored within acetylcholine applications without drugs (equivalent to 6 min). None of the compounds significantly changed the basal current before acetylcholine application, except that 17α-ethynylestradiol produced small fluctuations (less than 30 nA) in the basal current at 100 μM in some cases. No significant voltage-dependent inhibition was observed when the inhibition at −50 mV and that at −80 mV were compared for each compound tested.

The effect of D-tubocurarine on the current enhancement by 17α-ethynylestradiol was tested to determine

whether or not the enhanced current is mediated through the expressed α4β2 receptor/channel. D-Tubocurarine (10 μM) reduced the acetylcholine (300 μM)-activated current through α4β2 receptor/channels to 46.7 ± 5.9% of the control current ($n = 4$). The acetylcholine-activated current in the presence of 100 μM 17α-ethynylestradiol was also reduced by 10 μM D-tubocurarine to 47.2 ± 3.6% ($n = 4$), suggesting that the enhanced current is also mediated through the expressed α4β2 receptor/channel.

3.2. Effects of tamoxifen on the current modulation by estrogens and xenoestrogens

The effects of tamoxifen, an antiestrogen, on the current modulation induced by estrogens and xenoestrogens were examined. Tamoxifen (10 μM) itself inhibited the acetylcholine-activated current through both α3β4 (Fig. 5A–D) and α4β2 (Fig. 5E) receptor/channels by about 40%. The current partially reduced by tamoxifen was further inhib-

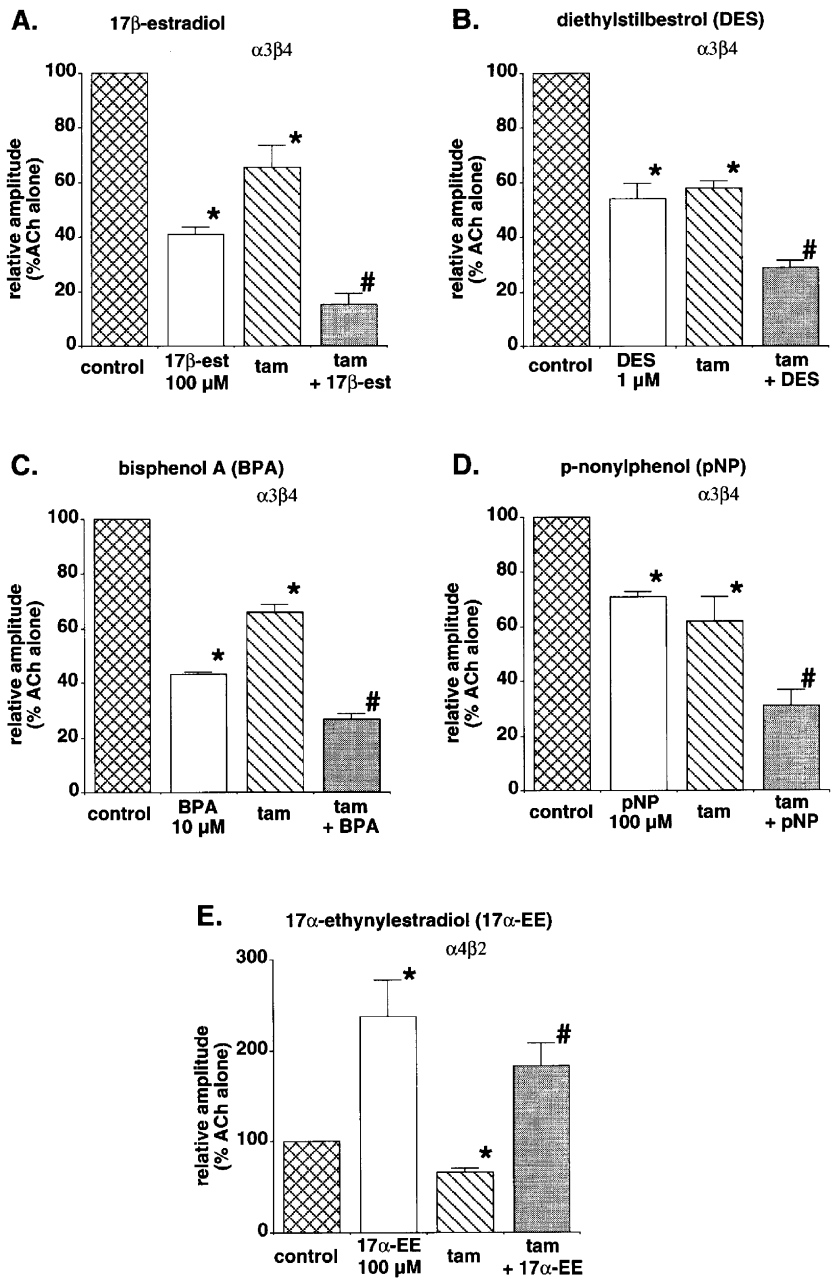

Fig. 5. Effects of tamoxifen (tam; 10 μM) on acetylcholine-activated currents in the absence or presence of 100 μM 17β-estradiol (17β-est; A), 1 μM diethylstilbestrol (DES; B), 10 μM bisphenol A (BPA; C), 100 μM *p*-nonylphenol (pNP; D) and 100 μM 17α-ethynylestradiol (17α-EE; E). Current responses to 300 μM (α3β4; A to D) or 1 mM (α4β2; E) acetylcholine at −80 mV in the presence of the compounds were normalized to those in the absence of the compounds. Each column and bar represent the mean and S.E. obtained from four to five oocytes. Statistically significant differences from control (*) or tamoxifen (#) determined by the paired *t*-test using non-normalized values are indicated.

ited by 17β-estradiol (Fig. 5A), diethylstilbestrol (Fig. 5B), bisphenol A (Fig. 5C) or *p*-nonylphenol (Fig. 5D) for α3β4 receptor/channels. Like the acetylcholine-activated current in the absence of tamoxifen, the current reduced by tamoxifen was also subsequently enhanced by 17α-ethynylestradiol for α4β2 receptor/channels (Fig. 5E).

3.3. Effects of estrogens and xenoestrogens on the concentration–response relationship for the acetylcholine-activated current

Fig. 6 compares the concentration–response relationship for the acetylcholine-activated current in the absence and the presence of estrogens or xenoestrogens. In the absence of drugs, the acetylcholine-activated current through α3β4 receptor/channels was saturated at 3 mM (Fig. 6A and C; see also Nakazawa and Ohno, 1999), suggesting that the current reaches its maximal at this concentration. 17β-Estradiol (Fig. 6A), diethylstilbestrol (Fig. 6B), bisphenol A (Fig. 6C) and *p*-nonylphenol (Fig. 6D) inhibited the current activated by various concentrations of acetylcholine up to 3 or 10 mM. Conversely, 17α-ethynylestradiol enhanced the current though α4β2 receptor/channels activated by various concentrations of acetylcholine up to 3 mM (Fig. 6E). We did not test acetylcholine at concentrations higher than 3 mM with the α4β2 receptor/channel because (1) this receptor/channel exhibits a concentration–response relationship with a very

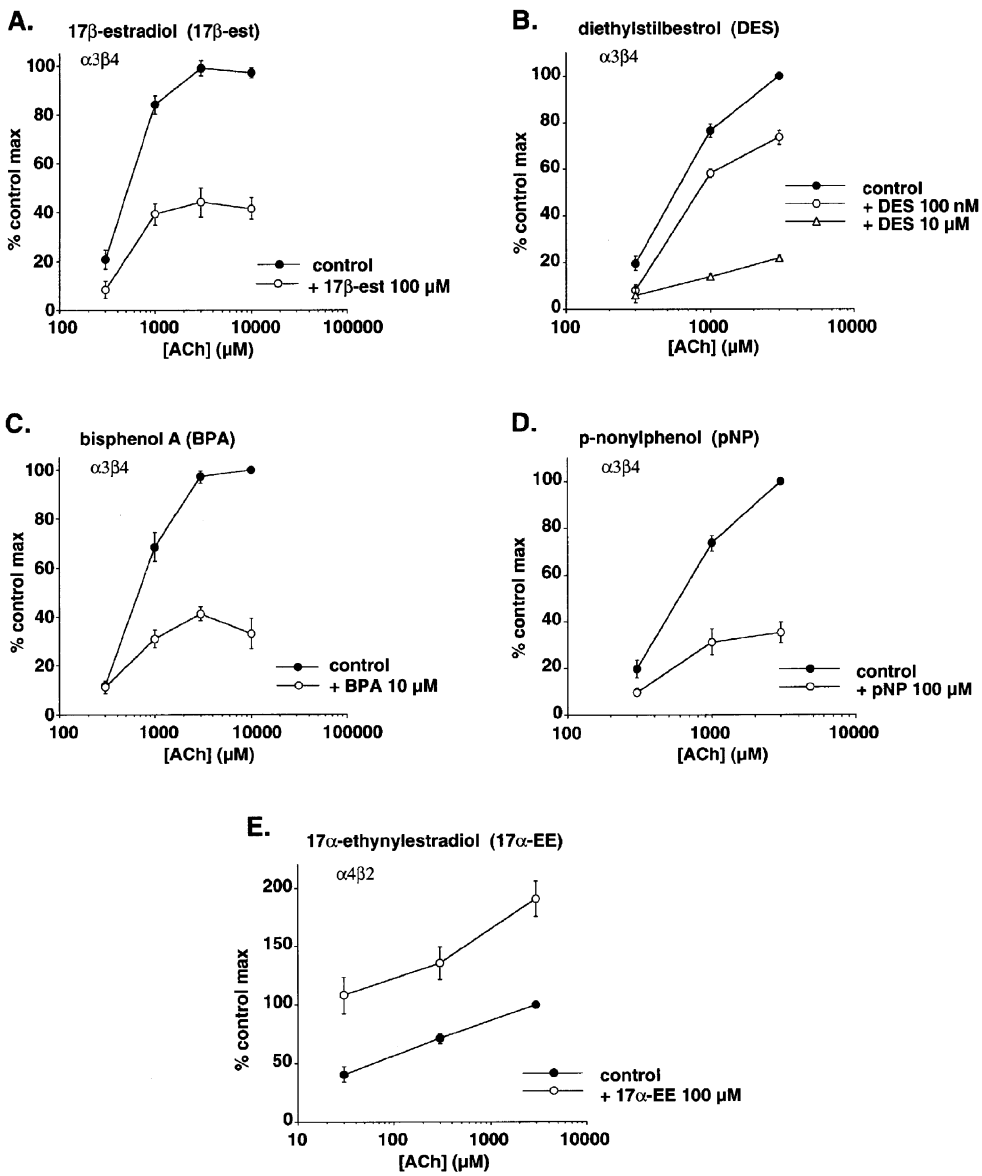

Fig. 6. Concentration–response curves for acetylcholine-activated currents through α3β4 (A to D) or α4β2 (E) receptor/channels in the absence and the presence of 17β-estradiol (17β-est, 100 μM; A), diethylstilbestrol (DES, 100 nM and 10 μM; B), bisphenol A (BPA, 10 μM; C), *p*-nonylphenol (pNP, 100 μM; D) or 17α-ethynylestradiol (17α-EE). Current responses to acetylcholine at −80 mV were normalized to maximal responses in the absence of the compounds. Each symbol and bar represent the mean and S.E. obtained from four to five oocytes.

gentle slope and it does not saturate even at 10 mM (Nakazawa and Ohno, 1999), and (2) acetylcholine at concentrations higher than 10 mM itself appears to block the channel as a large cation and cannot be used as a pure agonist (K. Nakazawa, unpublished observation).

3.4. Effects of subunit exchange on the current modulations

The effects of the different subunits of acetylcholine receptor/channels were examined for the current inhibition exerted by *p*-octylphenol and the current enhancement elicited by 17α-ethynylestradiol because these current modulations were selective for α4β2 receptor/channels (Figs. 3 and 4). The preferential inhibition by *p*-octylphenol of α4β2 receptor/channels was attenuated by replacement of the α4 subunit by the α3 subunit or by replacement of the β2 subunit by the β4 subunit: the current through α3β2 or α4β4 receptor/channels exhibited a sensitivity to *p*-octylphenol similar to that of the current through α3β4 receptor/channels (Fig. 7A). As for the enhancement by 17α-ethynylestradiol of the current through α4β2 receptor/channels, the subunit exchange resulted in the disappearance of the enhancement (Fig. 7B). The current through α3β2 or α4β4 receptor/channels was only weakly inhibited by 17α-ethynylestradiol, and the inhibition was weaker than that of the current through α3β4 receptor/channels.

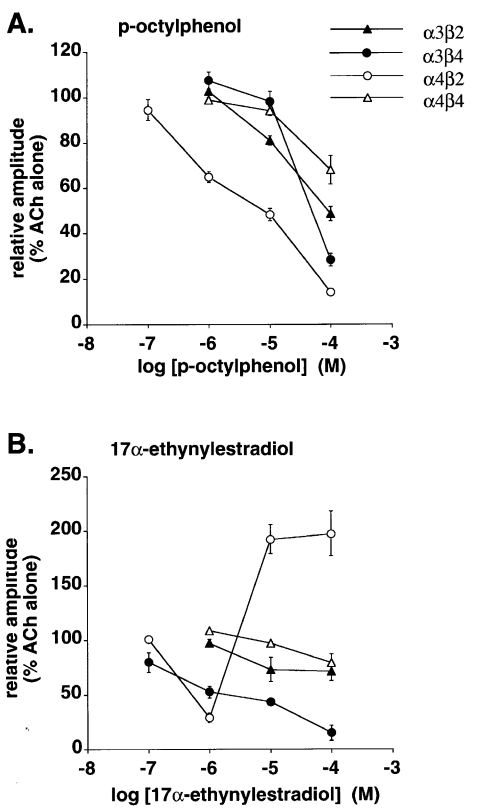

Fig. 7. Comparison of the effects of *p*-octylphenol (A) and 17α-ethynylestradiol (B) on four different subunit combinations (α3β2, α3β4, α4β2 and α4β4). The data were collected and shown as in Fig. 3. Each symbol and bar represent the mean and S.E. obtained from four to six oocytes.

4. Discussion

We have demonstrated that estrogens and xenoestrogens affect human recombinant neuronal nicotinic acetylcholine receptor/channels. Most of the modulation by these compounds was inhibitory, and diethylstilbestrol exhibited the highest potency in inhibiting both α3β4 and α4β2 receptor/channels. The modulation by estrogens or related compounds of nicotinic acetylcholine receptors has scarcely been reported, except for that elicited by 17β-estradiol. Ke and Lukas (1996) reported that 17β-estradiol inhibits $^{86}Rb^{+}$ efflux mediated through native human muscular and neuronal nicotinic acetylcholine receptors with IC_{50} values of 56 and 43 μM, respectively. The neuronal acetylcholine receptors in their report were regarded as being of the α3β4 type, and thus, the susceptibility of the recombinant α3β4 receptor to 17β-estradiol in the present study (Fig. 3A) accords with their results obtained with the corresponding native receptor. In rat superior cervical ganglionic neurons, the ionic current activated by acetylcholine was inhibited by both 17β-estradiol and 17α-estradiol at 100 μM (Uki et al., 1999), which also corresponds with the inhibition exerted by these compounds of the current through α3β4 receptor/channels in the present study (Fig. 3A,B).

The acute inhibition by estrogens and xenoestrogens of the cloned human nicotinic acetylcholine receptors in the present study may be attributable to non-genomic actions on the expressed receptors because at least 30 min is required for the genomic response to estrogens to occur (e.g., Orimo et al., 1993). The binding site responsible for the current inhibition, if estrogens and xenoestrogens share the same binding site, may be quite different from the classical estrogen receptor (estrogen receptor α). 17β-Estradiol binds to estrogen receptor α at least 100-fold more strongly than bisphenol A or *p*-nonylphenol, when determined by competitive binding study (Bergeron et al., 1999) or surface plasmon resonance analysis (Nishikawa et al., 1999). The agonist potency of 17β-estradiol is also at least 100-fold greater than that of bisphenol A or *p*-nonylphenol when determined from their activity to induce transcription through estrogen receptor α (Gould et al., 1998; Nishikawa et al., 1999). In contrast to these findings, bisphenol A or *p*-nonylphenol inhibited the cloned acetylcholine receptors more strongly than 17β-estradiol did (Figs. 3 and 4). The inhibition by tamoxifen, an antiestrogen, of the cloned acetylcholine receptors without antagonism of the modulation elicited by estrogens and xenoestrogens (Fig. 5) also supports the structural difference between the hypothetical binding site and the estrogen receptor α. Tamoxifen has been shown to inhibit recombi-

nant human muscle nicotinic acetylcholine receptors (Allen et al., 1998). Tamoxifen may act on some motif in common between neuronal and muscle nicotinic receptors. It is not clear at present whether or not tamoxifen shares the binding sites associated with current inhibition with estrogens or xenoestrogens.

With regard to the concentration–response relationship for the acetylcholine-activated current, 17β-estradiol, diethylstilbestrol, bisphenol A and *p*-nonylphenol reduced the maximal current induced by acetylcholine without remarkably affecting the sensitivity to acetylcholine (Fig. 6A–D). The result suggests that the inhibition exerted by these compounds is noncompetitive, and that the inhibition may be mediated through some binding site distinct from the acetylcholine binding site.

17α-Ethynylestradiol inhibited the acetylcholine-activated current through α3β4 receptor/channels at 1 μM and higher concentrations. This compound inhibited the current through α4β2 receptor/channels only at 1 μM, but it enhanced the current at 10 and 100 μM. 17α-Ethynylestradiol may exhibit dual effects on α4β2 receptor/channels, and the inhibition observed at 1 μM may be replaced by enhancement at higher concentrations. The enhancement of the current through α4β2 receptor/channels was observed at all concentrations of acetylcholine tested (Fig. 6E). Because the maximal current was not obvious with the channel of this subunit combination, it could not be determined whether 17α-ethynylestradiol increases the number of available channels or individual channel activity. It is also unclear whether this compounds acts as an allosteric modulator or as a partial agonist.

The preferential inhibition of the current through the α4β2 receptor/channel exerted by *p*-octylphenol and the selective current enhancement elicited by 17α-ethynylestradiol disappeared when one of the constituent subunits was replaced (Fig. 7). The results suggest that none of the single subunits determine the magnitude of the inhibition or the enhancement. Because estrogens and xenoestrogens are hydrophobic, they may influence the activity of nicotinic receptors via some indirect action through the cell membrane, rather than via a direct action on the receptor subunits. Contrary to this speculation, Garbus et al. (2001) have shown that the potency of steroids in inhibiting nicotinic receptor/channels correlates well with their hydrophilicity.

The compounds which inhibited both α3β4 and α4β2 receptor/channels, namely diethylstilbestrol, bisphenol A, *p*-nonylphenol and *p*-octylphenol, have a common molecular structure: they possess *p*-hydroxyalkylphenyl moieties in their structures (see Fig. 1). Similar carbon-substituted *p*-hydroxyphenyl moieties are also found in several compounds that inhibit nicotinic receptors: capsaicin (Nakazawa et al., 1994b) and alkyl *p*-hydoxybenzoates (Inoue et al., 1994) inhibit ionic current and other cellular responses mediated through nicotinic receptors in rat pheochromocytoma cells. This carbon-substituted *p*-hydroxyphenyl structure may be regarded as an indicator for possible inhibition of nicotinic receptors.

Although we assume that the inhibition by estrogens and xenoestrogens of the cloned human nicotinic receptors is attributable to actions on the receptor subunits themselves or on membrane materials surrounding receptor subunits, the contribution of second messenger systems to the inhibition cannot be excluded at present. The contribution of protein kinase A, protein kinase C or phospholipase C, however, has been ruled out as being involved in the mechanisms underlying the acute effects of 17β-estradiol on nicotinic receptor/channels (Uki et al., 1999) or other ion channels (Kim et al., 2000).

The results obtained in the present study have raised a possibility that xenoestrogens as well as estrogens inhibit nicotinic acetylcholine receptors in humans. The possible involvement of nicotinic acetylcholine receptors in pathological conditions, including epilepsy or schizophrenia, has been reported (Gotti et al., 1997). In addition to the potential endocrine-disrupting actions of exogenous estrogens and xenoestrogens, these compounds may affect the human nervous system when they accumulate in the human body.

Acknowledgements

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