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Potentiation of Human $\alpha4\beta2$ Neuronal Nicotinic Acetylcholine Receptor by Estradiol

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ABSTRACT

The modulation of neurotransmitter receptors by various substances can reflect important physiological mechanisms involved in the regulation of neural function. Furthermore, such substances, in particular specific allosteric modulators, can reveal promising therapeutic targets for diseases of the nervous system. From this perspective, we investigated the effects of the steroid hormone estradiol on human neuronal nicotinic acetylcholine receptors expressed either in *Xenopus laevis* oocytes or human embryonic kidney cells. Acetylcholine-evoked currents were potentiated both by pre- and coapplications of estradiol in $\alpha4\beta2$ and $\alpha4\beta4$ receptors, but not in $\alpha3\beta2$ or $\alpha3\beta4$ receptors. The reversible potentiation of $\alpha4$-containing receptors could be induced within seconds in *X. laevis* oocytes and at micromolar concentrations of estradiol. The potentiation was greatest for responses evoked by low concentrations of acetylcholine, resulting in an apparent increase of receptor affinity. At the single channel level, estradiol potentiation resulted from an increase in opening probability. Finally, the use of functional chimeric or truncated $\alpha4$ subunits demonstrated that a site at the C-terminal tail of the $\alpha4$ subunit is required for estradiol potentiation. These results suggest the presence of a specific site at the human nicotinic acetylcholine receptor $\alpha4$ subunit through which estradiol can cause an allosteric potentiation of acetylcholine-evoked responses.

Nicotinic acetylcholine receptors (nAChRs) are members of a large, structurally related ligand-gated ion channel (LGIC) superfamily including γ-aminobutyric acid$_A$, glycine, and serotonin receptors (Ortells and Lunt, 1995). Like all members of the LGIC superfamily, nAChRs are transmembrane proteins formed by the assembly of five homologous subunits arranged quasi-symmetrically around a central axis corresponding to the receptor's ion channel. Each subunit has a large extracellular N-terminal segment, four transmembrane domains (1–4), and a small extracellular C-terminal tail. The ACh binding site is at the interface between two subunits in the N-terminal extracellular region (Changeux et al., 1998). The physiological and pharmacological properties of different nAChRs are determined principally by their specific subunit combination (Colquhoun and Patrick, 1997). To date, 17 nAChR subunits have been identified ($\alpha1$–10, $\beta1$–4, δ, ϵ, and γ). Neuronal nAChRs are widely expressed throughout different structures of the nervous system and can be formed from a wide repertoire of subunits ($\alpha2$–10 and $\beta2$–4). More than 10 neuronal subunit combinations have been identified in vivo, although about twice that number can form functional receptors in vitro. The most abundant and well documented nAChR types of the central nervous system (CNS) include the homomeric $\alpha7$ nAChR (five $\alpha7$ subunits) and the heteromeric $\alpha4\beta2$ (two $\alpha4$ and three $\beta2$ subunits) (Changeux et al., 1998). Several other subunit combinations are expressed to a lesser degree in the CNS, although the $\alpha3\beta4$ nAChR subtype is also expressed at a high level in the peripheral nervous system, where it is thought to mediate synaptic transmission.

NAChRs of the CNS mediate the cognitive effects of nicotinic agonists such as memory and attention enhancement and are implicated in the addictive properties of tobacco. They have also been critically implicated in several brain pathologies (Léna and Changeux, 1998). $\alpha7$ nAChRs have been proposed as candidates in the pathogenesis of schizophrenia and mutations of either the $\alpha4$ or $\beta2$ nAChR subunits can cause a particular form of genetic epilepsy (Weiland et al., 2000; Phillips et al., 2001). Furthermore, certain neurodegenerative disease such as Alzheimer's are characterized by a significant depletion of $\alpha4\beta2$ nAChRs (Court et al., 2001).

The implication of these receptors in such pathologies gives some insight into the complexities of neuronal nAChRs physiological roles. Neuronal nAChRs are not only targets of the

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; LGIC, ligand-gated ion channel; ACh, acetylcholine; CNS, central nervous system; E, 17β-estradiol, estradiol; PCR, polymerase chain reaction; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEK, human embryonic kidney; H, high-affinity component; L, low-affinity component.

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natural agonist ACh but also a number of endogenous modulators that can increase or decrease the activating effect of ACh. Of particular interest are the allosteric modulators, which can alter nAChR function by direct receptor interaction (Buisson and Bertrand, 1998). For example, Lynx1, a protein expressed in murine brains, was shown to bind to nAChRs and potentiate their function (Miwa et al., 1999). Several divalent ions have been shown to potentiate nAChR function. Initial experiments revealed nAChR potentiation by Ca^{2+} and identified an extracellular binding site for Ca^{2+} on the $\alpha7$ subunit (Eiselé et al., 1993; Galzi et al., 1996). More recently, potentiation of ACh-induced currents by Mg^{2+}, Pb^{2+}, Zn^{2+}, and Cd^{2+} has been described for certain types of nAChRs (Zwart et al., 1995; Palma et al., 1998; Hsaio et al., 2001).

Allosteric potentiation of nAChRs has also been suggested as a promising therapeutic strategy for diseases characterized by decreased nicotinic function (Léna and Changeux, 1998). Of potential relevance has been the discovery that the anticholinesterase drug galantamine, used in the treatment of Alzheimer's, may also be an allosteric potentiator of $\alpha4\beta2$ nAChRs, although the precise mechanism of action remains to be elucidated (Coyle and Kershaw, 2001).

Steroid hormones and their metabolites represent a large family of molecules with varying allosteric modulatory effects on nAChRs as well as other members of the LGIC superfamily (Buisson and Bertrand, 1999; Rupprecht and Holsboer, 1999). In certain cases, these modulations undoubtedly represent physiological regulatory mechanisms because many steroids are locally synthesized in the brain (Compagnone and Mellon, 2000). They also are of great pharmaceutical interest because the highly liposoluble steroid molecules easily penetrate into the CNS (Pardridge et al., 1980). Almost all steroids tested to date on nAChRs have mainly inhibitory effects (Valera et al., 1992; Ke and Lukas, 1996; Paradiso et al., 2000). However, recent reports have described potentiating effects of 17β-estradiol (E) on human nAChR function (Buisson et al., 1998; Paradiso et al., 2001), which present an intriguing and so far unique example of positive modulation of the neuronal nAChR by an endogenous steroid that this study has further investigated.

Our results demonstrate a positive modulation of certain human neuronal nicotinic receptor subtypes expressed either in *Xenopus laevis* oocytes or in human embryonic kidney cells by E. We further analyzed the kinetics, concentration dependence, and structural requirements involved. Our results suggest that E is an allosteric modulator that can potentiate human $\alpha4\beta2$ nAChR function by interacting with a specific site on the $\alpha4$ subunit.

Materials and Methods

Oocyte Preparation and cDNA Injection. *X. laevis* oocytes were isolated and prepared as described previously (Bertrand et al., 1991). The oocytes were injected intranuclearly with 2 ng of expression vector cDNA and maintained at 18°C in Barth's medium [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, pH 7.4 adjusted with NaOH] supplemented with antibiotics (20 μg/ml kanamycin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B). To improve cell survival and minimize possible contamination, each oocyte was placed in one well of a 96-well microtiter plate (Nunc, Naperville, CT).

Drugs and Solutions. Drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO) or Fluka Chemical (Ronkonkoma, NY). E used was a water-soluble form of 17β-estradiol encapsulated in 2-hydroxypropyl-β-cyclodextrin (45 mg of E/g). Application of β-cyclodextrin alone up to 2.5 mg/ml (equivalent to 4 times the highest concentration of E used) had no detectable effect on ACh currents in oocytes expressing $\alpha4\beta2$ nAChRs, either in coapplication or 20-s preapplication. All recordings were performed in OR2 bath solution (82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 adjusted with NaOH) supplemented with 0.5 μM atropine to block possible endogenous muscarinic responses.

cDNA Construction. Chimeric and mutant $\alpha3/\alpha4$ subunits were generated using a PCR-based method described previously (Curtis et al., 2000). A3(202)$\alpha4$ and $\alpha4(202)\alpha3$ cDNAs were constructed through two successive PCRs (conditions identical to Curtis et al., 2000) by using the following primers: $\alpha3$-F (5′-AGCTTATGGCTCTGGCCGTCTC-3′); $\alpha3(202)\alpha4$-R (5′-CATAGGTGATGTCGGGGTAGATCTCC-3′); $\alpha3(202)\alpha4$-F (complementary to $\alpha3(202)\alpha4$-R); $\alpha4$-R (5′-CGCACTTCCTAGATCATGCCAGCC-3′); $\alpha4$-F (5′-TCGATCTAGAGCCCGCGAGGTG-3′); $\alpha4(202)\alpha3$-R (5′-GTGATGTCGGGGTAGATCTCG-3′); $\alpha4(202)\alpha3$-F (complementary to $\alpha4(202)\alpha3$-R); and $\alpha3$-R (5′-GCAAGGCAGGCACACAGCTTAG). The $\alpha4(588)$stop cDNA was constructed using one PCR (conditions identical to first PCR described in Curtis et al., 2000) and the following primers: $\alpha4$-F and $\alpha4(588)$stop-R (5′-CTACTAGGGCGGTAGGAAGAGGC-3′). $\alpha3(202)\alpha4$, $\alpha4(202)\alpha3$, and $\alpha4(588)$stop cDNAs were cloned into pCR 3.1 or pRc/CMV expression vectors (Invitrogen, Carlsbad, CA).

Electrophysiological Recordings. Perfusion solution was fed by gravity at a rate of ~6 ml/min. The oocytes were superfused continuously with OR2 and solution exchange was controlled by computer-driven electromagnetic valves. Electrophysiological recordings were made 2 to 4 days after injection by using a two-electrode voltage clamp (GeneClamp amplifier; Axon Instruments, Foster City, CA). Electrodes made from borosilicate glass were filled with a filtered solution of 3 M KCl. Holding potential was −100 mV. All experiments were performed at 18°C.

Cell line, Culture, and Recordings. Human embryonic kidney cells (293 cells) transfected with plasmids containing the human $\alpha4$ and $\beta2$ cDNAs (K177 cell line) were maintained in culture according to the method described previously (Buisson et al., 1996). Cells were plated onto 35-mm Petri dishes 2 to 5 days before recording. During electrophysiological experiments, cells were placed in a medium containing 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM glucose, 10 mM HEPES, and 0.5 μM atropine, adjusted to pH 7.4 with NaOH. Pipettes were pulled from borosilicate glass, filled with 120 mM KF, 10 mM KCl, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, and 10 mM BAPTA, adjusted to pH 7.4 with KOH, and mounted on the head-stage of an Axopatch 200B amplifier (Axon Instruments). Drugs were applied using a liquid filament system based on a theta tube mounted on a piezoquartz actuator (Physics Instruments, Waldbrunn, Germany).

Data Analysis and Computation. Data were captured on-line by an analog-to-digital converter (National Instruments, Austin, TX) and stored on the hard disk of Macintosh for later analysis. Values for currents correspond to peak response currents. Curve fitting was done using a least-squares minimization algorithm (SIMPLEX). Time courses for potentiation in oocytes were fitted with the function: $I = a \times (0.65 \times e^{(-t/\tau1)} + 0.35 \times e^{(-t/\tau2)}) + b$, where I is current, a and b are scaling factors, and $\tau1$ and $\tau2$ are time constants. Dose-response curves were calculated using the empirical two-component Hill equation: $I = \{a / [1 + (EC_{50}H / x)^{nH}]\} + \{1 - a / [1 + (EC_{50}L / x)^{nL}]\}$, where I is the fraction of activated current, a is the proportion of high-affinity component (H), $EC_{50}H$ and $EC_{50}L$ are the concentration of half-activation for high- and low-affinity (L) components, n_H and n_L are the respective Hill constants of each component, and x is the agonist concentration. Unless otherwise indicated, values are stated as mean ± S.E.M.

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Results

Estradiol Potentiates Human nAChRs Containing Human α4 Subunit. Progesterone modulation of nAChRs has been shown to be dependent on ACh concentration (Valera et al., 1992). To compare E effect on different nAChR subtypes, the I_{max} was determined for voltage-clamped oocytes, expressing α4β2, α4β4, α3β2, or α3β4 human nAChRs. Oocytes were superfused with 1 mM ACh and evoked currents were in the 10-μA range (I_{max} = 15.8 ± 2.3 μA for α4β2, 27.4 ± 7.1 μA for α4β4, 17.1 ± 1.2 μA for α3β2, and 29.3 ± 9 μA for α3β4; n = 11, 5, 4, and 4, respectively). To best assess E effects, low ACh concentrations evoking less than 10% of the saturating current were used. ACh test pulses were applied for 5 s before, and immediately after a 20-s pulse of 30 μM E for each receptor type (Fig. 1A). As shown in this figure, E potentiated ACh responses in oocytes expressing α4-containing receptors (Fig. 1B). ACh responses from oocytes expressing α4β2 receptors were significantly potentiated (after E, ACh currents 342 ± 50% of control for n = 11; Fig. 1B). α4β4 receptors were also potentiated, but to a lesser degree (199 ± 12%; n = 5). Conversely, E failed to potentiate ACh responses in oocytes expressing α3-containing receptors. The α3β2 receptors were inhibited (88 ± 2%; n = 4) and α3β4 receptors were not significantly affected (104 ± 2%; n = 4). For all nAChR types tested, the effects of E were reversible; ACh responses evoked 2 min after E prepulses were equivalent to the initial ACh control currents (99 ± 3%; n = 12; data not shown). Because E potentiates both α4β2 and α4β4, but not α3β2 or α3β4 nAChRs, the positive modulation can be attributed to the α4 subunit.

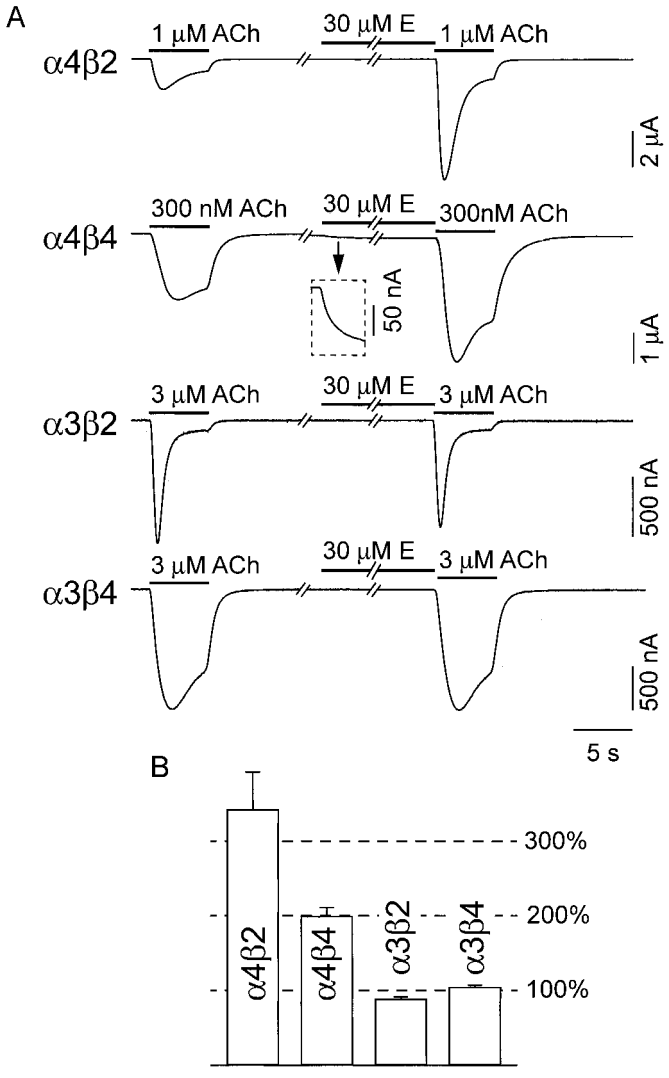

Fig. 1. E potentiates human nAChRs containing the α4 subunit expressed in *X. laevis* oocytes. A, typical current traces for human α4β2, α4β4, α3β2, and α3β4 nAChRs expressed in *X. laevis* oocytes. ACh-evoked currents were recorded before and immediately after a 20-s preapplication of 30 μM E. E alone acts as a very weak partial agonist at the α4β4 nAChR (inset, arrow) and has no detectable effect on the other nAChR types tested. B, average modulation of ACh-evoked currents by a 20-s preapplication of 30 μM E for human nAChR subtypes and corresponding ACh concentrations shown in A. Values are from 11, 5, 4, and 4 oocytes, respectively.

To investigate the possible involvement of endogenous oocyte E receptors in the potentiation process, we also applied the biologically inactive 17α stereoisomer of E by using the same protocol. In four of four oocytes expressing the α4β2 nAChR, potentiation of 1 μM ACh evoked responses by a 30 μM, 20-s 17α-estradiol prepulse was equivalent to the potentiation seen with 17β-estradiol (in these four cells, 17α-estradiol-potentiated responses were 251 ± 65% of control compared with 226 ± 53% for 17β-estradiol; data not shown). Furthermore the effects E alone were evaluated on oocytes expressing all receptor types tested. Twenty-second applications of up to 300 μM E did not generate detectable currents in any α4β2-, α3β2-, or α3β4-expressing oocytes. However, in all α4β4-expressing oocytes tested, 20 s 30 μM E superfusion resulted in small, but significant currents (68 ± 31.7 nA; n = 5), representing 2.5 ± 1.2% of maximal ACh current.

Estradiol Potentiates α4β2 nAChRs Rapidly. To determine the rapidity with which E modulated the α4β2 receptor, 1 μM ACh-evoked responses were preceded by 30 μM E pulses of 1, 3, 10, or 20 s, and compared with the control responses (i.e., preceded by 0-s E) in three oocytes (Fig. 2A). A plot of the relative response as a function of the duration of E preapplication could be fit by a biexponential function with time constants τ1 and τ2 of 1 and 10 s, respectively. E applied during an ACh pulse also potentiated evoked responses within seconds; oocytes expressing α4β2 were superfused with 50 nM ACh for 50 s. This gave rise to small currents, almost deprived of desensitization, which could be potentiated by a coapplication of 30 μM E (Fig. 2B). The time course of coapplication potentiation onset was comparable with that of prepulse potentiation (The dashed curve in Fig. 2B is equivalent to the function curve in Fig. 2A, although with different scaling factors). The t_{50} of maximal potentiation as predicted by the time course function is 1.23 s. This is quite rapid and comparable with the time course of low-concentration ACh activation. However, the time course of drug-receptor interaction can be considerably increased when studied in oocyte systems, due to large cell size and access restriction in experimental conditions (Madeja et al., 1997). Accordingly, E potentiation of α4β2 nAChRs expressed in HEK 293 was considerably faster. The rise time of potentiation for a 10 μM E concentration jump during a long 10 μM ACh pulse was 20.8 ± 3.9 ms (n = 5; Fig. 2C).

Estradiol Potentiates α4β2 by Increasing Apparent ACh Affinity. To best evaluate E potency in inducing α4β2 potentiation, E dose-response profiles were determined for different ACh concentrations (Fig. 3A). The results demon-

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strated that the degree of relative potentiation of an ACh current by preapplication of E was dependent on both the ACh concentration and the E concentration. Responses evoked by low concentrations of ACh were highly potentiated by E preapplications and increasing E concentrations had considerably greater effect (Fig. 3A, left). Conversely, responses evoked by high concentrations of ACh were only marginally potentiated by E and increasing E concentrations have relatively little effect (Fig. 3A, right). Illustrating this, E potentiation of 100 nM ACh responses ranged from 198 ± 47% for 3 μM E to 646 ± 178% for 100 μM E (n = 5). E potentiation of 100 μM ACh responses ranged from 105 ± 4% for 3 μM E to 108 ± 7% for 100 μM E (n = 5). These dose-response profiles could be fitted with one-component empirical Hill curves with calculated EC_{50} values (that is, E concentrations necessary for half-maximal potentiation) of 33.2, 13.9, 6.8, and 2.9 μM for ACh 0.1, 1, 10, and 100 μM, respectively (data not shown). Shown in Fig. 3B is the dose-response relationship of E potentiation at 1 μM ACh. Accordingly, a fixed concentration of 30 μM E applied before varying concentrations of ACh modified the ACh dose-response relationship of the $\alpha4\beta2$ receptor (Fig. 3C). Overall, the mean ACh concentration evoking half-maximal current in $\alpha4\beta2$

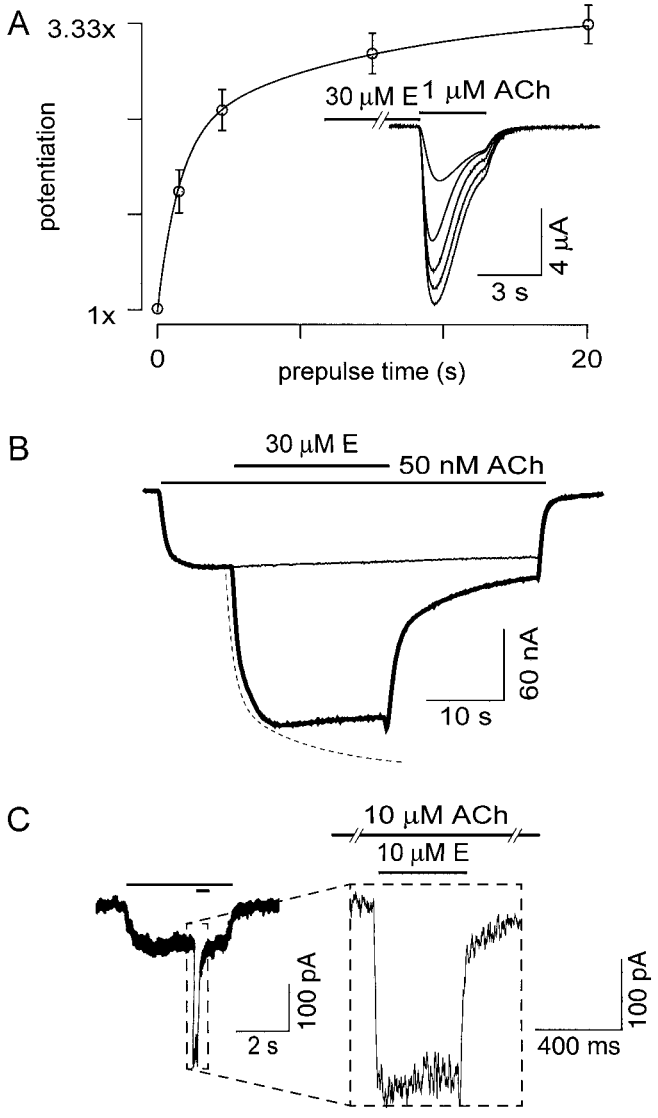

Fig. 2. E potentiates $\alpha4\beta2$ expressed in *X. laevis* oocytes within seconds. A, E potentiation of ACh current plotted against duration of steroid preapplication. Currents evoked by 1 μM ACh were recorded after 1-, 3-, 10-, and 20-s preapplications of E in $\alpha4\beta2$ expressing *X. laevis* oocytes (inset; also shown is initial evoked current before preapplications). E potentiation is time-dependent and can be fitted by the exponential function under *Materials and Methods*, where $\tau1$ = 1 s and $\tau2$ = 10 s. Values from three oocytes. B, in a single *X. laevis* oocyte expressing $\alpha4\beta2$ nAChRs, application of 50 nM ACh for 50 s elicited a stable, slowly desensitizing current (thin trace). Coapplication of 30 μM E for 20 s caused rapid potentiation (thick trace superimposed on control trace). Dashed line shows the exponential function from A, with modified scaling constants, for comparison. C, concentration jump of E during a long ACh pulse (10 μM, top line) in human embryonic kidney cells expressing $\alpha4\beta2$. Chart paper recording (left) illustrates experimental protocol. Enlargement (right) of the E concentration jump (10 μM; 400 ms) illustrates the fast rising time for E potentiation (≤25 ms).

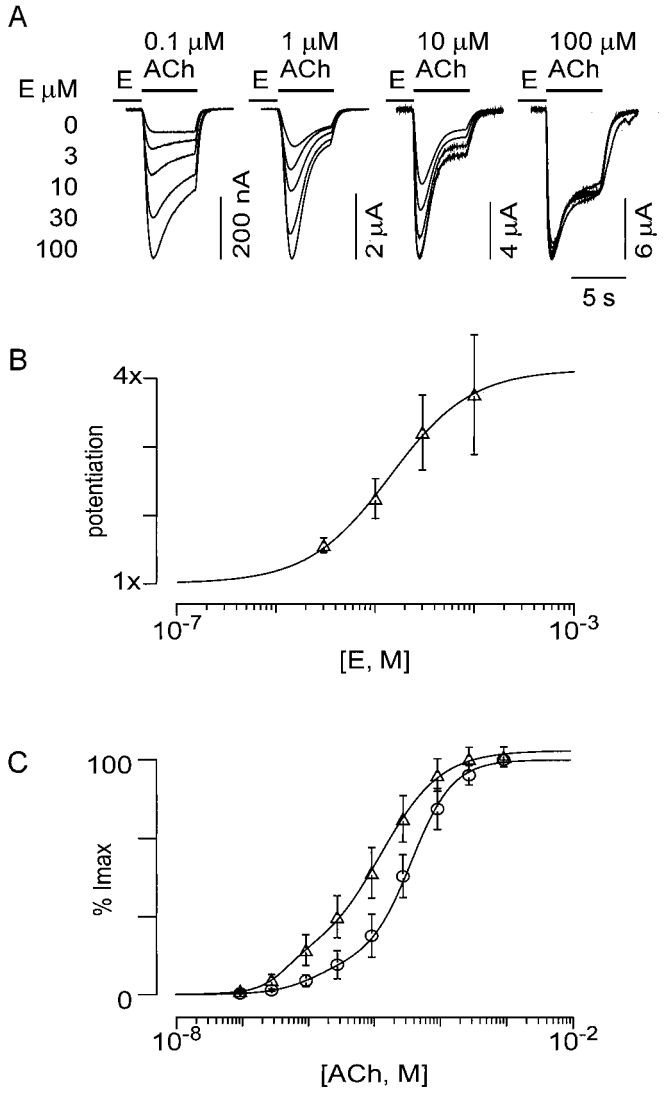

Fig. 3. E increases apparent affinity of $\alpha4\beta2$ receptors in *X. laevis* oocytes. A, typical current traces for $\alpha4\beta2$ expressed in *X. laevis* oocytes. Currents evoked by 0.1, 1, 10, and 100 μM ACh were recorded before and after 20-s preapplication of 3, 10, 30, and 100 μM E. B, E dose-dependent potentiation of ACh responses. Relative potentiation of currents evoked by 1 μM ACh was plotted as a function of E concentration. This dose-response profile could be fitted with an empirical Hill equation with an EC_{50} of 13.9 μM E. Values are from five oocytes. C, ACh dose-response curves: control (○) and after 20-s preapplication of 30 μM E (△). Curves are best fits obtained with the two-component empirical Hill equations by using values given in Table 1. Values are from 11 oocytes.

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expressing oocytes was nearly 3 times smaller subsequent to E application (11.3 ± 5.5 versus 33.6 ± 14.8 μM). However, in agreement with the work of Covernton and Connolly (2000) and our lab (Buisson and Bertrand, 2001; Phillips et al., 2001), α4β2 dose-response relationships, both before after E potentiation, were best fit by two-component Hill equations. Values for the two components [high- (H) and low (L)-affinity components] are given in Table 1. The modification of the α4β2 dose-response relationship by E was best described by both a ~2-fold decrease in the EC_{50} values of both components and a ~2-fold increase in the relative contribution of the high-affinity component. Furthermore, calculated I_{max} was not significantly different before and after E application (E conditioned I_{max}, 104 ± 4% of control).

Estradiol Increases Probability of Opening of α4β2 nAChRs Expressed in HEK Cells. The observed macroscopic current increases could most readily be explained either by an increase in unitary channel conductance, an increase in mean open time, and/or an increase of opening probability. To investigate these possibilities, single channel events of α4β2 nAChRs were studied in transfected HEK 293 cells. Single channel openings were recorded during a 500-ms application of 100 nM ACh before and after a 10-s 30 μM exposure to E (Fig. 4). Single channel conductance was not significantly different before (40.8 ± 1.5 pS) or after E application (40.3 ± 1.3 pS; n = 5). However, a significant increase in channel openings was observed after E preapplication (Fig. 4A). Quantification, from the all points amplitude histogram (Fig. 4B), of the probability of opening in control and after E exposure yielded values of 0.33 ± 0.19 and 0.61 ± 0.3, respectively (n = 5). In these experiments, α4β2 activity diminishes with ACh exposure due to fast run down (Buisson et al., 1996). This would explain the diminished activity during washout after E exposure (Fig. 4A) and suggest that the increase of the probability of opening by E is underestimated.

C-Terminal Fragment of α4 Subunit Is Sufficient and Necessary for E Potentiation. Given that α4 subunits confer potentiation and α3 subunits do not, chimeric subunits were created by fusing the extracellular N-terminal moiety of the α3 subunit with the C-terminal moiety of the α4 subunit [α3(202)α4; Fig. 5]. The reverse chimera was also constructed [α4(202)α3] and oocytes coinjected with either chimeric subunit cDNA combined with the β2 subunit cDNA-expressed receptors responsive to ACh [I_{max} = 8.8 ± 2.6 μA for α3(202)α4β2 and 10 ± 2.2 μA for α4(202)α3β2; n = 5 and 3, respectively]. Furthermore, an α4 subunit mutant [α4(588)stop] was created lacking the five C-terminal amino acids (WLAGMI) directly C terminal to the fourth transmembrane domain. Oocytes coinjected with α4(588)stop and β2 cDNA also expressed ACh-responsive receptors (I_{max} = 9.7 ± 2.9 μA; n = 7). Responses of α3(202)α4β2 receptors to 3 μM ACh (evoking less than 10% of I_{max}) were potentiated after a 20-s pulse of 30 μM E (after E, ACh currents 159 ± 5% of control for n = 5) (Fig. 5). Conversely, α4(202)α3β2 receptor responses to 3 μM ACh (also <10% of I_{max}) were inhibited by E (88 ± 3% of control for n = 3). Similarly, α4(588)stopβ2 receptor responses to 300 nM ACh (<10% of I_{max}) were inhibited after a 20-s pulse of 30 μM E (90 ± 8% of control for n = 7). These observations demonstrate that the extracellular C-terminal tail of the α4 subunit is necessary for E potentiation. Nonetheless, although the α3β2 and α4(202)α3β2 nAChRs are inhibited to a similar degree (88 ± 2 and 88 ± 3%, respectively), the potentiation observed in α3(202)α4β2 nAChRs (159 ± 5%) is considerably smaller than that observed in α4β2 nAChRs (342 ± 50%). As for α4β2, EC_{50} values for both the high- and low-affinity components of a two-component α3(202)α4β2 ACh dose-response curve were diminished by E preapplication (Table 1; Fig. 6). Furthermore, the decrease of mean ACh concentration evoking half-maximal current induced by a 20-s 30 μM E preapplication was of similar magnitude (from 53.6 ± 6.9 to 20.6 ± 4.7 μM ACh).

However, as evidenced by significant reduction of high ACh concentration responses by E, the E-modulated dose-response curve (triangles) had an I_{max} 66 ± 10% that of the control ACh curve. This would suggest that E simultaneously increases the apparent affinity of the α3(202)α4β2 receptor while acting as a noncompetitive inhibitor. In comparison E preapplication did not significantly alter mean ACh concentrations evoking half-maximal current for the α3β2, α4(202)α3β2, or α4(588)stopβ2 receptors [from 41.8 ± 4.9 to 41.3 ± 4.6 μM ACh for α3β2; 32.7 ± 15.8 to 31 ± 13.3 μM ACh for α3(202)α4β2; and 2.1 ± 0.5 μM to 1.8 ± 0.3 μM for α4(588)stopβ2]. I_{max} was comparably reduced for both α3β2 and α4(202)α3β2receptors [E reduced I_{max} by 12 ± 1% for α3β2 and by 11 ± 5% for α3(202)α4β2] and slightly more inhibited in α4(588)stopβ2 receptors (E reduced I_{max} by 17 ± 12%). The ACh concentration dependence of E modulation is more apparent when plotting the ratio of 30 μM E-modulated ACh current to control ACh current as a function of ACh concentration (Fig. 7). A sigmoid relationship between the degree E potentiation for α4β2 and α3(202)α4β2 receptors and corresponding ACh concentration is observed. For both

TABLE 1
Properties of different receptor types before and after E modulation

Subunit Combination after 30 μM E	$EC_{50}H^a$	$EC_{50}L^a$	n_H	n_L	% (H)	% (L)	~10% I_{max}	I_{max}
α4β2	1.43 ± 0.82	40.3 ± 10.2	1.3 ± .2	1.4 ± .3	14 ± 11	86 ± 11	100%	100%
	0.76 ± 0.24	18.9 ± 2.9	1.5 ± .2	1.1 ± .1	26 ± 14	74 ± 14	342 ± 50%	104 ± 4%
α3β2	13.9 ± 1.9	136 ± 35	1.6 ± .1	1.5 ± .2	56 ± 8	44 ± 8	100%	100%
	13.9 ± 1.9	136 ± 35	1.6 ± .1	1.5 ± .2	56 ± 8	44 ± 8	88 ± 2%	82 ± 5%
α4(202)α3β2	3.45 ± 1.26	67.7 ± 7.9	1.3 ± .2	1.3 ± .1	37 ± 11	63 ± 11	100%	100%
	3.45 ± 1.26	67.7 ± 7.9	1.3 ± .2	1.3 ± .1	37 ± 11	63 ± 11	88 ± 3%	92 ± 3%
α3(202)α4β2	7.35 ± 0.60	74.2 ± 1.1	0.9 ± .1	1.1 ± .1	19 ± 4	81 ± 4	100%	100%
	1.27 ± 0.21	41.6 ± 8.9	1 ± .1	1.3 ± .2	27 ± 6	73 ± 6	159 ± 5%	66 ± 10%
α4(588)stopβ2	1.65 ± 0.23	47.3 ± 3.6	1.2 ± .2	1.6 ± .2	90 ± 5	10 ± 5	100%	100%
	1.65 ± 0.23	47.3 ± 3.6	1.2 ± .2	1.6 ± .2	90 ± 5	10 ± 5	90 ± 8%	89 ± 5%

[a] Values for EC_{50} values are in micromolar ACh.

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these receptors, this relationship follows a sigmoid curve with the greatest potentiation seen at lower concentrations of ACh (Fig. 7, top). In contrast, the slight inhibition of responses to ACh by 30 μM E seen in $\alpha3\beta2$, $\alpha4(202)\alpha3\beta2$, and $\alpha4(588)$stop$\beta2$ receptors is proportionally similar at all ACh concentrations tested.

Discussion

Our results describe a potentiating effect of E on human $\alpha4\beta2$ function that is reversible, rapid in onset, dependent on ACh concentration, and occurs in the micromolar range. Combinations of different subunits reveal that the presence of human $\alpha4$ subunit is necessary for E potentiation, because only $\alpha4$ subunit containing combinations are potentiated. Our results confirm recent reports of E potentiation of the human $\alpha4\beta2$ nAChR (Buisson et al., 1998; Paradiso et al., 2001), as well as previous findings showing inhibition of $\alpha3\beta4$ nAChRs by micromolar concentrations of E (Ke and Lukas, 1996). Previous reports have also demonstrated inhibition of the homomeric $\alpha7$ nAChRs by E (Buisson et al., 1998). Consequently, of the predominant species of nAChR expressed in human brain, only $\alpha4\beta2$ would seem to be potentiated by E. Although the β-subunit does not seem to determine modulation type, it is interesting to note that $\beta4$-containing receptors seem less susceptible to the effects of E, whether potentiating ($\alpha4$) or inhibitory ($\alpha3$), compared with $\beta2$-containing receptors.

The potentiation of $\alpha4$-containing nAChRs by E is relatively specific, because many other endogenous steroids, including both progesterone and testosterone, have previously been shown to inhibit the $\alpha4\beta2$ nAChR, also in the micromolar range, and cholesterol and pregnenolone had no effect (Valera et al., 1992; Buisson et al., 1998; Paradiso et al., 2001). Our data are highly suggestive of direct allosteric modulation by E of the nAChR. The speed and reversibility of the effect are consistent with allosteric modulation, because second-messenger- and gene expression-mediated phenomena have generally been described in a time frame of minutes to hours (Rupprecht and Holsboer, 1999). Although several seconds are required to achieve maximal E potentiation in oocytes, our results with HEK 293 cells suggest this may be due in part to oocyte properties and that potentiating interaction of E with the $\alpha4\beta2$ nAChR actually occurs on a millisecond time scale. Observed in HEK 293 cells, this is comparable with the time scale of ion exchange (Buisson et al., 1996). In comparison, G protein-dependent E potentiation of kainate currents in acutely dissociated neurons had a t_{50} of several minutes (Gu and Moss, 1996). Interestingly, the speed of potentiation onset in $\alpha4\beta2$-expressing oocytes was

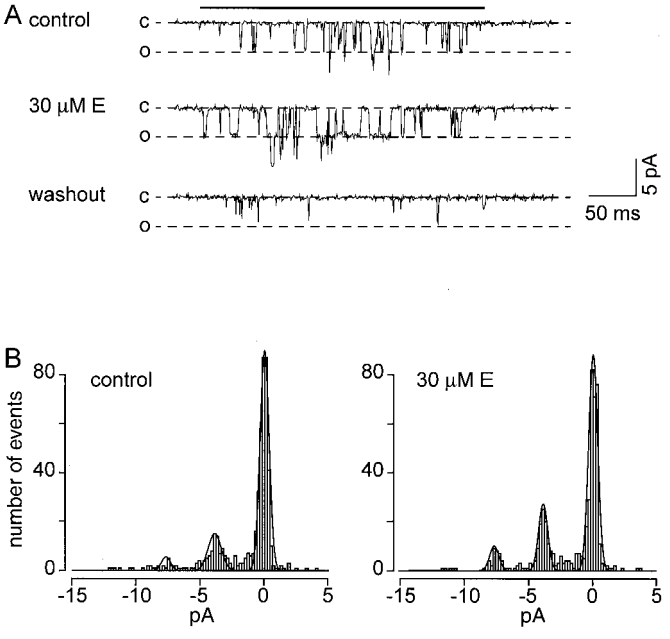

Fig. 4. Effects of E on $\alpha4\beta2$ elementary events. A, single channel currents recorded in an outside out patch obtained from a K177 cell. ACh (1 μM; 300 ms; horizontal bar) was delivered once every 20 s; 30 μM E was preapplied during 20 s. Because of a strong run down (Buisson et al., 1996), the channel activity almost completely disappeared during washout. B, point amplitude histograms computed at a 0.2-pA resolution from ACh-evoked currents (100 nM ACh; 500 ms) recorded in another outside out patch in control (left) and after a 20-s preapplication of 30 μM E (right). Data were fitted by the sum of three Gaussian functions with mean values of 0, −3.9, and −7.8 pA in control and after E. The variance was adjusted at 0.5 for the three Gaussians.

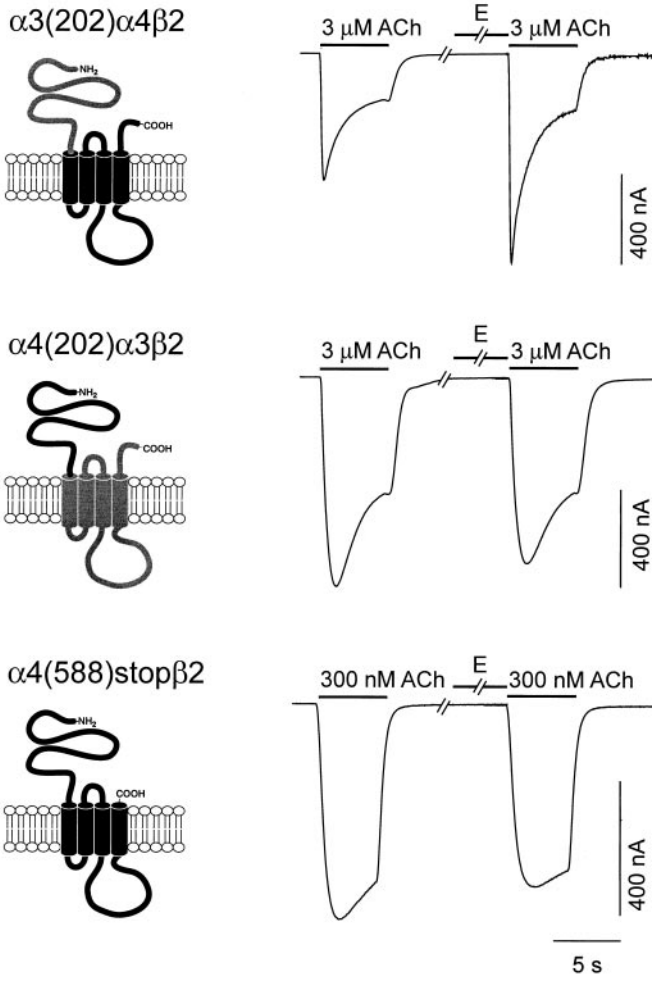

Fig. 5. E potentiates ACh-evoked responses in $\alpha4(202)\alpha3\beta2$ receptors and inhibits ACh-evoked responses in $\alpha3(202)\alpha4\beta2$ and $\alpha4(588)$stop$\beta2$ receptors expressed in *X. laevis* oocytes. Left, schematic illustration of transmembrane topology of the $\alpha3(202)\alpha4$ (top), $\alpha4(202)\alpha3$ (middle), and $\alpha4(588)$stop (bottom) chimeric subunits. Light shading corresponds to an $\alpha3$ subunit segment and dark shading to $\alpha4$. Right, typical current traces for the corresponding chimeric nAChR by ACh concentrations evoking less than 10% of I_{max} before and after 20-s preapplication of 30 μM E.

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similar to the speed of E activation in α4β4 expressing oocytes (compare Fig. 2 with Fig. 1), suggesting that analogous or related mechanisms may be involved. Furthermore, the similar potentiation by both 17β-estradiol and the biologically inactive 17α-estradiol makes it unlikely that specific E receptors located either at the cell membrane or cytoplasm are involved. Finally, in six outside out patches from HEK 293 cells, repetitive potentiation of single channel activity by E (up to 3 times) could be observed in the absence of either GTP or ATP (data not shown), which would argue against the implication of a second messenger pathway.

In support of the relevance of an allosteric model are the modifications observed in the dose-response profiles and the small yet detectable activation of α4β4 receptors by E. The action of a positive allosteric modulator, according to the allosteric model (Edelstein et al., 1996), would reduce the equilibrium constant (L value, which describes the equilibrium between a basal and active state). A reduction of L value would lead to an increase of leak currents in the absence of ACh and a higher apparent affinity and response cooperativity in the presence of ACh. In oocytes expressing α4β4 nAChRs, we do indeed observe a very small increase of the leak currents. Presumably this is best observed in the α4β4 receptor because these nAChR types have a lower initial L value. The modifications of the two-component dose-response Hill curves for the various nAChRs are further compatible with predictions from an allosteric model that would include a high- and low-affinity state. Although a physical basis of the recently described two components has not been definitively demonstrated, it has been suggested that these components may correspond to distinct functional receptor types (Covernton and Connolly, 2000; Buisson and Bertrand, 2001). Under this assumption, E seems to predominantly potentiate the high-affinity component of both the α4β2 and α3(202)α4β2 nAChRs in a manner consistent with a positive allosteric modulator (decrease of EC_{50}, increase of both n_H and relative contribution of component). In contrast, the observed effect of E on the α4(202)α3β2 and α4(588)stopβ2 nAChRs was purely inhibitory, comparable with that observed for α3β2 nAChRs. A striking feature of the α4(588)stopβ2 nAChR ACh dose-response relationship was that although the EC_{50} values and Hill constants of both the high- and low-affinity components were roughly equivalent to those observed for α4β2, the relative contribution of the high-affinity component was greater for the α4(588)stopβ2 nAChRs, suggesting that removal of the α4 WLAGMI C-terminal tail favored a high-affinity configuration. Despite presenting a majority of high-affinity receptors, α4(588)stopβ2 nAChRs were inhibited by E at all ACh concentrations tested. These results, in conjunction with the observed potentiation of the α3(202)α4β2 nAChRs and inhibition of the α4(202)α3β2 nAChRs, are in accordance with those of Paradiso et al. (2001), which implicate the WLAGMI C-terminal tail of the α4 subunit in E potentiation.

Paradiso et al. (2001) further presented evidence suggesting that this region constitutes a binding site for E. Our

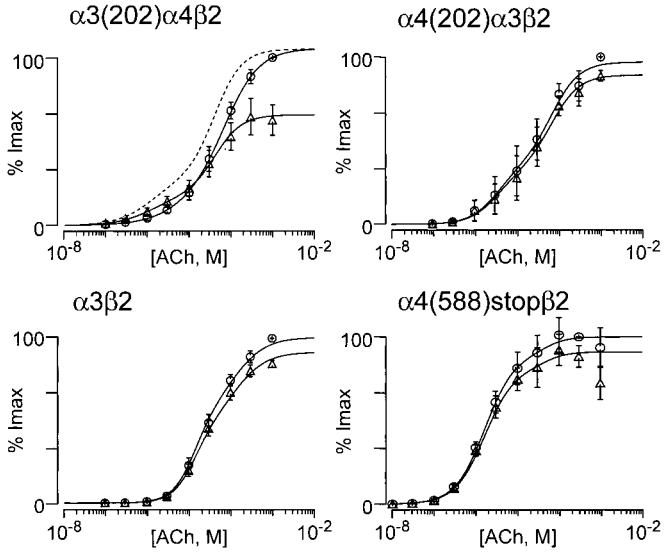

Fig. 6. E modification of ACh dose-response relationships for α3β2 or receptors constituted of α3α4 chimeric and β2 subunits expressed in *X. laevis* oocytes. ACh dose-response curves [control (○) and after 20-s preapplication of 30 μM E(Δ)] for α3(202)α4β2, α4(202)α3β2 receptor, α3β2, and α4(588)stopβ2. Dashed curve at top [α3(202)α4β2] represents normalized dose-response curve after modulation. Curves are best fits obtained with two-component empirical Hill equations. Values are given in Table 1. Values from five, three, four, and eight oocytes for α3(202)α4β2, α4(202)α3β2, α3β2, and α4(588)stopβ2, respectively.

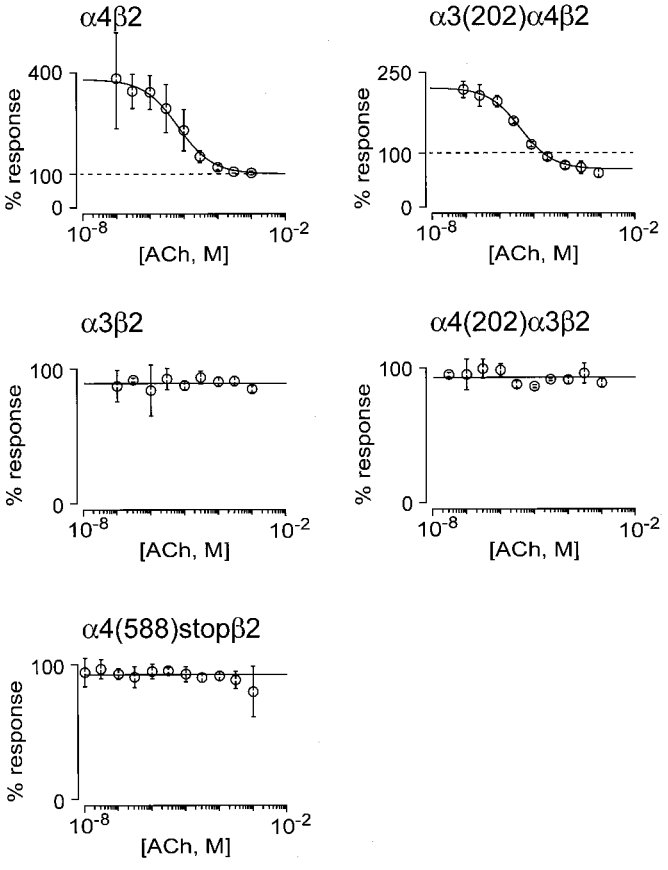

Fig. 7. The C-terminal moiety of the human α4 subunit is sufficient and necessary for an ACh concentration-dependent E potentiation of ACh-evoked responses. E potentiation/inhibition is plotted as a function of ACh concentration (dashed lines indicate 100% response). α-3β2, α4(202)α3β2, and α4(588)stopβ2 values could be fitted with horizontal lines at 88, 93, and 90%, respectively. α-4β2 and α3(202)α4β2 values were fitted with the Hill equation ($I = P - p / (1 + (EC_{50} / [ACh])^{n_H})$). For α4β2, P = 3.75, p = 1.75, EC_{50} = 8 μM, and n_H = 0.95. For α3(202)α4β2, P = 2.2, p = 1.5, EC_{50} = 5 μM, and n_H = 0.95. Values from 11, 5, 4, 3, and 8 oocytes for α4β2, α3(202)α4β2, α3β2, α4(202)α3β2, and α4(588)stopβ2, respectively.

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results cannot distinguish between this possibility and the alternative that E is indirectly involved in the allosteric coupling. However, in further support of the possibility of direct interaction, it should be noted that this region is immediately adjacent to the highly hydrophobic fourth transmembrane domain that, lying at the lipid-protein interface (Blanton et al., 1999), is compatible with direct interaction with the hydrophobic E. Moreover, related steroid molecules such as cholesterol and promegestone have been shown to bind to residues of the fourth transmembrane domain of the *Torpedo californica* nAChR α subunit (Blanton et al., 1998; Corbin et al., 1998). Finally, a functional correlate can be suggested by previous studies demonstrating that point mutations within the fourth transmembrane domain can alter probability of opening of mouse muscle nAChRs (Bouzat et al., 2000).

Despite the potentiating effects of E on the $\alpha4\beta2$ and $\alpha3(202)\alpha4\beta2$ nAChRs, the similar maximum currents observed before and after E modulation for $\alpha4\beta2$ nAChRs and in particular the surprising E inhibition of I_{max} in $\alpha3(202)\alpha4\beta2$ nAChRs suggest that the positive allosteric modulation observed in these receptors is concomitant to a noncompetitive inhibition by E. Interestingly, analysis of E modulation of the $\alpha3\alpha4$ chimeras indicates that although the site responsible for potentiation can be isolated to the C-terminal moiety [the affinity shift for $\alpha4\beta2$ and $\alpha3(202)\alpha4\beta2$ being equivalent], E inhibition, which we observe to a varying degree for all nAChR types tested, does not seem dependent on a discrete portion of the receptor. Of particular interest is the small but significant inhibition observed with the $\alpha4(588)$stop$\beta2$ nAChR, demonstrating that despite the absence of the critical WLAGMI tail, E still interacts with this receptor. Because noncompetitive inhibition of several different nAChR subtypes and even other LGICs has been reported for several steroids related to E such as progesterone or testosterone (Valera et al., 1992; Ke and Lukas, 1996; Paradiso et al., 2000), the mechanisms of inhibition may be similar and could involve several sites in a relatively nonspecific manner. In contrast, the nAChR potentiating effect of E seems to be very specific, requiring E and a discrete portion of the human $\alpha4$ subunit and can apparently occur concomitantly to E inhibition. Thus, alternative sites to the WLAGMI C-terminal sequence may be present on nAChRs where E interaction would cause a receptor inhibition.

Considering the C-terminal WLAGMI fragment necessary for E potentiation, an intriguing observation concerns rat nAChRs. Alternative splicing of the rat $\alpha4$ subunit ($\alpha4$-1 and $\alpha4$-2) results in two variants of the rat $\alpha4\beta2$ nAChR (Connolly et al., 1992). The $\alpha4$-1 and $\alpha4$-2 splice variant sequences are identical except for the last three amino acids changing a WLAAC C-terminal tail to WLAGMI. Rat $\alpha4-1\beta2$ nAChRs have previously been shown to be inhibited by E (Paradiso et al., 2000), yet our observations would suggest that the relatively minor splice variation found in $\alpha4$-2$\beta2$ nAChRs (widely expressed in rat brain and described previously as pharmacologically indistinguishable from $\alpha4$-1$\beta2$; Connolly et al., 1992) could confer the capability of E potentiation to these receptors.

The potentiating effects of E on the human $\alpha4\beta2$ nAChR occur in the micromolar range. Although, these concentrations are comparable with those of other steroid modulators of the nAChR, they are higher than what is commonly accepted to be physiological serum concentrations of E (i.e., the nanomolar range). Furthermore, studies on both rodent and human brains suggest that average E concentrations in brain tissues approximate the serum values (Backstrom et al., 1976; Bixo et al., 1986). However, these studies do not exclude the possibility of localized areas of high E concentrations in restricted brain areas. Because all enzymes required for E synthesis have been isolated from brain tissue (Mellon and Compagnone, 1999) and high activities of P450 aromatase have been demonstrated in prenatal brainstem structures, which are also areas of high nAChR densities (Lephart et al., 1996), it can even be postulated that localized synthesis of this steroid could produce micromolar concentrations, although no explicit evidence yet supports this hypothesis.

In conclusion, our results confirm a steroid potentiation of the human $\alpha4\beta2$ nAChR, which, to our knowledge, is the only potentiation by a steroid molecule described for a member of the LGIC superfamily other than the γ-aminobutyric acid$_A$. Furthermore, observations in both oocytes and HEK cells support an allosteric mechanism of potentiation. In accordance with recent results (Paradiso et al., 2001), our results also demonstrate that the structural requirements conferring potentiation capacity are relatively minor, suggesting a very specific interaction between steroid and a discrete receptor area. In contrast, steroid inhibition of nAChRs would seem not only to involve other receptor areas but also to be less specific, perhaps implicating multiple sites. The physiological relevance of the effects we describe remains to be elucidated, given the seemingly high E concentrations required. However, the specific presence of the amino acid fragment necessary for potentiation in the human $\alpha4$ subunit as well the splice variation of precisely this fragment described for rat strongly support a functional basis of our observations. Our hypotheses include the possibility of high, localized synaptic E expression or perhaps the existence of a structurally similar neurosteroid with equivalent potentiating effects. Finally, our results present the major human nAChR of the CNS as a potential therapeutic target for highly specific positive nicotinic allosteric modulators.

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References

Backstrom T, Carstensen H, and Sodergard R (1976) Concentration of estradiol, testosterone and progesterone in cerebrospinal fluid compared to plasma unbound and total concentrations. *J Steroid Biochem* **7:**469–472.

Bertrand D, Cooper E, Valera S, Rungger D, and Ballivet M (1991) Electrophysiology of neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes following nuclear injection of genes or cDNAs. *Methods Neurosci* **4:**174–193.

Bixo M, Backstrom T, Winblad B, Selstam G, and Andersson A (1986) Comparison between pre- and postovulatory distributions of oestradiol and progesterone in the brain of the PMSG-treated rat. *Acta Physiol Scand* **128:**241–246.

Blanton MP, Xie Y, Dangott LJ, and Cohen JB (1999) The steroid promegestone is a noncompetitive antagonist of the *Torpedo* nicotinic acetylcholine receptor that interacts with the lipid-protein interface. *Mol Pharmacol* **55:**269–279.

Bouzat C, Barrantes F, and Sine S (2000) Nicotinic receptor fourth transmembrane domain hydrogen bonding by conserved threonine contributes to channel gating kinetics. *J Gen Physiol* **115:**663–671.

Buisson B and Bertrand D (1998) Allosteric modulation of neuronal nicotinic acetylcholine receptors. *J Physiol (Lond)* **92:**89–100.

Buisson B and Bertrand D (1999) Steroid modulation of nicotinic receptors, in *Neurosteroids—A New Regulatory Function in the Nervous System* (Baulieu EE, Robel P and Schumacher M eds) pp 207–223, Humana Press, Totowa, NJ.

Buisson B and Bertrand D (2001) Chronic exposure to nicotine upregulates the human $\alpha4\beta2$ nicotinic acetylcholine receptor function. *J Neurosci* **21:**1819–1829.

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Buisson B, Bertrand S, and Bertrand D (1998) Inhibition and potentiation of human nicotinic receptors by sex hormones. *Soc Neurosci Abstr* **24:**530.19.

Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP, and Bertrand D (1996) Human $\alpha4\beta2$ neuronal nicotinic acetylcholine receptor in HEK 293 cells: a patch-clamp study. *J Neurosci* **16:**7880–7891.

Changeux JP, Bertrand D, Corringer PJ, Dehaene S, Edelstein S, Léna C, Le Novère N, Marubio L, Picciotto M, and Zoli M (1998) Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. *Brain Res Brain Res Rev* **26:**198–216.

Colquhoun LM and Patrick JW (1997) Pharmacology of neuronal nicotinic acetylcholine receptor subtypes. *Adv Pharmacol* **39:**191–220.

Compagnone NA and Mellon SA (2000) Neurosteroids biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol* **21:**1–56.

Connolly J, Boulter J, and Heinemann SF (1992) Alpha 4–2 beta 2 and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs. *Br J Pharmacol* **105:**657–666.

Corbin J, Wang HH, and Blanton MP (1998) Identifying the cholesterol binding domain in the nicotinic acetylcholine receptor with [^{125}I]azido-cholesterol. *Biochim Biophys Acta* **1414:**65–74.

Court J, Martin-Ruiz C, Piggott M, Spurden D, Griffiths M, and Perry E (2001) Nicotinic receptor abnormalities in Alzheimer's disease. *Biol Psychiatry* **49:**175–184.

Covernton PJ and Connolly JG (2000) Multiple components in the agonist concentration-response relationships of neuronal nicotinic acetylcholine receptors. *J Neurosci Methods* **96:**63–70.

Coyle J and Kershaw P (2001) Galantamine, a cholinesterase inhibitor that allosterically modulates nicotinic receptors: effects on the course of Alzheimer's disease. *Biol Psychiatry* **49:**289–299.

Curtis L, Chiodini F, Spang JE, Bertrand S, Patt JT, Westera G, and Bertrand D (2000) A new look at the neuronal nicotinic acetylcholine receptor pharmacophore. *Eur J Pharmacol* **393:**155–163.

Edelstein SJ, Schaad O, Henry E, Bertrand D, and Changeux JP (1996) A kinetic mechanism for nicotinic acetylcholine receptors based on multiple allosteric transitions. *Biol Cybern* **75:**361–379.

Eiselé JL, Bertrand S, Galzi JL, Devillers-Thiery A, Changeux JP, and Bertrand D (1993) Chimaeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. *Nature (Lond)* **366:**479–483.

Hsaio B, Dweck D, and Luetje CW (2001) Subunit-dependent modulation of neuronal nicotinic receptors by zinc. *J Neurosci* **21:**1848–1856

Galzi JL, Bertrand S, Corringer PJ, Changeux JP, and Bertrand D (1996) Identification of calcium binding sites that regulate potentiation of a neuronal nicotinic receptor. *EMBO (Eur Mol Biol Organ) J* **15:**5824–5832.

Gu Q and Moss RL (1996) 17 β-Estradiol potentiates kainate-induced currents via activation of the cAMP cascade. *J Neurosci* **16:**3620–3629.

Ke L and Lukas RJ (1996) Effects of steroid exposure on ligand binding and functional activities of diverse nicotinic acetylcholine receptor subtypes. *J Neurochem* **67:**1100–1112.

Léna C and Changeux JP (1998) Allosteric nicotinic receptors, human pathologies. *J Physiol (Lond)* **92:**63–74.

Lephart ED, Ladle DR, Jacobson NA, and Rhees RW (1996) Inhibition of brain 5 α-reductase in pregnant rats: effects on enzymatic and behavioral activity. *Brain Res* **739:**356–360.

Madeja M, Musshoff U, and Speckmann EJ (1997) Follicular tissues reduce drug effects on ion channels in oocytes of *Xenopus laevis*. *Eur J Neurosci* **9:**599–604.

Mellon SH and Compagnone NA (1999) Molecular biology and developmental regulation of the enzymes involved in the biosynthesis and metabolism of neurosteroids, in *Neurosteroids—A New Regulatory Function in the Nervous System* (Baulieu EE, Robel P and Schumacher M eds) pp 27–49, Humana Press, Totowa, NJ.

Miwa JM, Ibanez-Tallon I, Crabtree JW, Sanchez R, Sali A, Role LW, and Heintz N (1999) lynx1, an endogenous toxin-like modulator of nicotinic acetylcholine receptors in the mammalian CNS. *Neuron* **23:**105–114.

Ortells MO and Lunt GG (1995) Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *Trends Neurosci* **18:**121–127.

Palma E, Maggi L, Miledi R, and Eusebi F (1998) Effects of Zn^{2+} on wild and mutant neuronal $\alpha7$ nicotinic receptors. *Proc Natl Acad Sci USA* **95:**10246–10250.

Paradiso K, Sabey K, Evers AS, Zorumski CF, Covey DF, and Steinbach JH (2000) Steroid inhibition of rat neuronal nicotinic $\alpha4\beta2$ receptors expressed in HEK 293 cells. *Mol Pharmacol* **58:**341–351.

Paradiso K, Zhang J, and Steinbach JH (2001) The C terminus of the human nicotinic $\alpha4\beta2$ receptor forms a binding site required for potentiation by an estrogenic steroid. *J Neurosci* **21:**6561–6568.

Pardridge WM, Moeller TL, Mietus LJ, and Oldendorf WH (1980) Blood-brain barrier transport and brain sequestration of steroid hormones. *Am J Physiol* **239:**E96–E102.

Phillips HA, Favre I, Kirkpatrick M, Zuberi SM, Goudie D, Heron SE, Scheffer IE, Sutherland GR, Berkovic SF, Bertrand D, et al. (2001) CHRNB2 is the second acetylcholine receptor subunit associated with autosomal dominant nocturnal frontal lobe epilepsy. *Am J Hum Genet* **68:**225–231.

Rupprecht R and Holsboer F (1999) Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci* **22:**410–416.

Valera S, Ballivet M, and Bertrand D (1992) Progesterone modulates a neuronal nicotinic acetylcholine receptor. *Proc Natl Acad Sci USA* **89:**9949–9953.

Weiland S, Bertrand D, and Leonard S (2000) Neuronal nicotinic acetylcholine receptors: from the gene to the disease. *Behav Brain Res* **113:**43–56.

Zwart R, Van Kleef RG, Milikan JM, Oortgiesen M, and Vijverberg HP (1995) Potentiation and inhibition of subtypes of neuronal nicotinic acetylcholine receptors by Pb^{2+}. *Eur J Pharmacol* **291:**399–406.

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