

$\alpha 5$ Subunit Alters Desensitization, Pharmacology, Ca^{++} Permeability and Ca^{++} Modulation of Human Neuronal $\alpha 3$ Nicotinic Receptors¹

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ABSTRACT

Functional effects of human $\alpha 5$ nicotinic ACh receptor (AChR) subunits coassembled with $\alpha 3$ and $\beta 2$ or with $\alpha 3$ and $\beta 4$ subunits, were investigated in *Xenopus* oocytes. The presence of $\alpha 5$ subunits altered some properties of both $\alpha 3$ AChRs and differentially altered other properties of $\alpha 3\beta 2$ AChRs vs. $\alpha 3\beta 4$ AChRs. $\alpha 5$ subunits increased desensitization and Ca^{++} permeability of all $\alpha 3$ AChRs. The Ca^{++} permeabilities of both $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs were comparable to that of $\alpha 7$ AChRs. As we have shown previously, $\alpha 5$ subunits increased the ACh sensitivity of $\alpha 3\beta 2$ AChRs 50-fold but had little effect on $\alpha 3\beta 4$ AChRs. $\alpha 5$ caused only subtle changes in the activation potencies of $\alpha 3$ AChRs for nicotine, cytosine and 1,1-

dimethyl-4-pyrenylpiperazinium (DMPP). However, $\alpha 5$ increased the efficacies of nicotine and DMPP on $\alpha 3\beta 2$ AChRs but decreased them on $\alpha 3\beta 4$ AChRs. Immunolocalization of cloned human AChRs expressed in oocytes showed that $\alpha 5$ efficiently coassembled with $\alpha 3$ plus $\beta 2$ and/or $\beta 4$ subunits. As expected, human AChRs immunolocalized from SH-SY5Y neuroblastoma cells showed that AChRs containing $\alpha 3$ and probably $\alpha 5$ subunits were present, but $\alpha 4$ AChRs were not. In brain, by contrast, $\alpha 4\beta 2$ AChRs were shown to predominate over $\alpha 3$ AChRs. Some of the brain $\alpha 4\beta 2$ AChRs were found to contain $\alpha 5$ subunits.

Neuronal nicotinic AChRs are thought to be formed by pentameric assemblies of certain combinations of $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\alpha 9$, $\beta 2$, $\beta 3$ and $\beta 4$ subunits (Deneris *et al.*, 1991; Role, 1992; Sargent, 1993; Le Novere and Changeux, 1995; Lindstrom *et al.*, 1995; McGehee and Role, 1995; Lindstrom, 1996). The homologous subunits of an AChR are thought to be organized around a central cation channel like barrel staves so that parts of the M1 and M2 transmembrane domains of all subunits contribute to the lining of the channel. In the case of muscle-type AChRs, which are known to have their subunits organized around the channel in the order $\alpha 1\gamma\alpha 1\delta\beta 1$, there are two ACh binding sites at interfaces between $\alpha 1$ and γ or between $\alpha 1$ and δ subunits, but the $\beta 1$ subunit is not thought to contribute contact amino acids to these binding sites (Karlin and Akabas, 1995). The stoichiometry of $\alpha 4\beta 2$ AChRs expressed in oocytes is known to be $(\alpha 4)_2(\beta 2)_3$ (Anand *et al.*, 1991; Cooper *et al.*, 1991), and it is thought that these subunits are similarly organized around the channel in the order $\alpha 4\beta 2\alpha 4\beta 2\beta 2$, which results in two ACh binding sites at interfaces between $\alpha 4$ and $\beta 2$ subunits. $\alpha 3$ subunits can form functional AChRs in combination with

$\beta 2$ or $\beta 4$ subunits, and it is presumed that these also probably have two ACh binding sites. $\alpha 5$ is known to be a subunit of AChRs containing $\alpha 3$, $\beta 4$ and/or $\beta 2$ subunits in chick ganglia (Conroy *et al.*, 1992; Vernallis *et al.*, 1993), in a human neuroblastoma (Wang *et al.*, 1996), and associated with a small fraction of the $\alpha 4\beta 2$ AChRs in chick brain (Conroy and Berg, 1995). The stoichiometry of $\alpha 5$ containing AChRs has not been directly determined. However, the observation that $\alpha 5$ does not form functional AChRs when expressed in *Xenopus* oocytes alone or in paired combination with $\alpha 3$, $\beta 2$ or $\beta 4$ (Wang *et al.*, 1996) suggests that $\alpha 5$ subunits, like $\beta 1$ subunits, cannot interface with the sides of these subunits that are involved in forming ACh binding sites (Karlin and Akabas, 1995). Thus it has been suggested that $\alpha 5$ may occupy a position homologous to that of $\beta 1$ in muscle-type AChRs (Wang *et al.*, 1996). For example, the order of subunits around the channel might be $\alpha 3\beta 2\alpha 3\beta 2\alpha 5$.

Our initial studies of human $\alpha 5$ subunits expressed in *Xenopus* oocytes showed that they assembled efficiently with human $\alpha 3$ and $\beta 2$ or human $\alpha 3$ and $\beta 4$ subunits to form AChRs that desensitized more rapidly and that, especially in the case of $\alpha 3\beta 2\alpha 5$ AChRs, exhibited altered pharmacological properties (Wang *et al.*, 1996). Here we extend these electrophysiological studies in *Xenopus* oocytes and conduct immunoprecipitation studies to investigate the fraction of various

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ABBREVIATIONS: AChR, acetylcholine receptor; mAb, monoclonal antibody; DMPP, 1,1-dimethyl-4-phenylpiperazinium.

AChR subunits in extracts of rat and human brain that have $\alpha 5$ associated with them.

Materials and Methods

cDNAs. The cDNA sequences for human $\alpha 3$ (unpublished EMBL accession no. X53559) and $\beta 2$ (Anand and Lindstrom, 1990) were subcloned in expression vectors pcDNA1 (Invitrogen, San Diego, CA) and pSP64poly(A) (Promega, Madison, WI), respectively. The cDNA for human $\alpha 5$ was first described by Chini *et al.* (1992) and was kindly provided by Dr. Francesco Clementi (University of Milan). It was subcloned in the pSP64poly(A) vector. The cDNA for human $\beta 4$ was cloned in this lab from a cDNA library from the neuroblastoma cell line SH-SY5Y (Gerzanich *et al.*, 1997). It was then subcloned into the pcDNA1 vector. $\alpha 1$ and δ cDNAs were described previously (Luther *et al.*, 1989). Epitope tagged $\alpha 5^c$ cDNA was described previously (Wang *et al.*, 1996). Human β , ϵ and γ cDNAs were kindly provided by Dr. Andrew Engel (Mayo Clinic).

Expression of human $\alpha 3$ AChRs in *Xenopus* oocytes. cRNAs for human AChR subunits $\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$ were synthesized *in vitro* using T7 (if the cDNA was in the pcDNA1 vector) or SP6 (if the cDNA was in the pSP64poly(A) vector) RNA polymerase (mMESSAGEmMACHINE, Ambion, Austin, TX). Oocytes were prepared for microinjection as described previously (Gerzanich *et al.*, 1995) and injected with equal amounts (5–15 ng) of cRNA for each of the subunits. They were incubated for 3 to 4 days after injection in media containing 50% L15 (GIBCO BRL), 10 mM HEPES buffer, pH 7.5, 10 U/ml penicillin and 10 mg/ml streptomycin at 18°C.

Electrophysiological procedures and drug application. Currents in oocytes were measured using a standard two-microelectrode voltage-clamp amplifier (Oocyte Clamp OC-725, Warner Instrument Corp., Hamden, CT). Electrodes were filled with 3 M KCl and had resistances of 0.5 to 1.0 M Ω for the voltage electrode and 0.4 to 0.6 M Ω for the current electrode. All records were digitized (MacLab/2e interface and Scope software (AD Instruments, Castle Hill, Australia), stored on a Macintosh IIcx computer and analyzed using AXOGRAPH software (Axon Instruments, Foster City, CA). The recording chamber was continually perfused at a flow rate of 10 ml/min with saline solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.6. Atropine (0.5–1 μ M) was included in all solutions to block responses of endogenous muscarinic AChRs. Application of agonists was performed as described in detail previously (Gerzanich *et al.*, 1995). In summary, all agonists were applied by means of a set of 2-mm glass tubes directed on the animal pole of the oocyte. Application was achieved by manual unclamping and clamping of a flexible tube connected to the syringe with the test solution. Typically delay between beginning of the application and first deflection of the induced current was about 0.25 sec. The Hill equation was fitted to the concentration-response dependencies using a nonlinear least-squares error curve fit method (KaleidaGraph, Abelbeck Software): $I(x) = I_{\max} [x^n / (x^n + EC_{50}^n)]$, where $I(x)$ is current measured at the agonist concentration x , I_{\max} is the maximal current response at the saturating agonist concentration, EC_{50} is the agonist concentration required for the half-maximal response and n is the Hill coefficient.

For experiments measuring the effect of extracellular Ca⁺⁺ on the current amplitude and reversal potentials, intracellular electrodes were filled with 2.5 M potassium aspartate. In order to prevent activation of the endogenous Ca⁺⁺-dependent Cl⁻ channels, Cl⁻-free solutions were used for oocyte preincubation (6–12 hr) and for the perfusion during recordings (Francis and Papke, 1996). The “normal”-Ca⁺⁺ solution included 90 mM NaMeSO₃, 2.5 mM KOH, 10 mM HEPES and 1.8 mM Ca(OH)₂. Additionally, 48 mM dextrose was supplemented in the normal solution in order to yield osmolarity equal to the “high”-Ca⁺⁺ solution, which contained 18 mM Ca(OH)₂ and the same concentration of the other ions as the “normal”-Ca⁺⁺ solution. Both solutions were buffered with methanesulfonic acid to pH 7.3. Reversal potentials of the currents were determined either

by 6-sec agonist applications at different holding potentials or by 2-sec ramps of the holding potential from -50 to +50 mV during agonist application after the current reached a steady state. Both protocols gave similar estimates for the reversal potential. Control ramp currents obtained before agonist applications were subtracted from the ramp currents during AChR activation.

Purification and radioimmunoassay of AChRs from oocytes, SH-SY5Y cells and human brain. Purification, immunodepletion and solid phase radioimmunoassay of AChRs from oocytes were performed as described previously (Wang *et al.*, 1996). AChRs from the human neuroblastoma cell line SH-SY5Y, neocortex from post-mortem human brain and whole rat brain tissue were isolated in accordance with the method of Whiting and Lindstrom (1986) and Wang *et al.* (1996). For radioimmunoassay, 250- μ l aliquots of tissue extract either were mixed directly with 50 μ l of the mAb-Actigel and [³H]-epibatidine (5.3 nM) or were preabsorbed with 50 μ l of mAb-Actigel before mixing with [³H]-epibatidine and a fresh aliquot of the mAb-Actigel. mAb-Actigel contained 5 mg/ml of mAb. After 8 to 12 hr of incubation at 4°C, the Actigel was rinsed three times with ice-cold PBS, 0.05% Tween buffer. The amount of bound AChRs was determined by labeling with 5 nM [³H]-epibatidine, followed by liquid scintillation counting (Wang *et al.*, 1996). Nonspecific binding of the AChRs to mAb-Actigel was determined by incubation of aliquots of tissue extracts with an irrelevant mAb or normal rat IgG-Actigel under the same conditions.

Results

$\alpha 5$ subunit enhances desensitization in recombinant human neuronal $\alpha 3$ AChRs. The time course of the currents induced by saturating concentrations of ACh in oocytes expressing AChRs after coinjection of $\alpha 3\beta 2\alpha 5$ or $\alpha 3\beta 4\alpha 5$ cRNA combinations are compared with those after $\alpha 3\beta 2$ and $\alpha 3\beta 4$ cRNA coinjections in figure 1. ACh-evoked currents reached a maximum and then decayed biphasically, showing both a transient and a plateau phase. Small “rebound” currents, commonly explained as channel block by agonist, were observed only for AChRs containing $\beta 4$ subunits (fig. 1, bottom two traces). The onset of the current in the AChRs containing $\beta 2$ subunits (fig. 1, top two traces) was significantly steeper (0.23 ± 0.1 and 0.17 ± 0.06 sec to peak for $\alpha 3\beta 2$ and $\alpha 3\beta 2\alpha 5$ combinations, respectively) compared to $\beta 4$ subunit-containing AChRs (0.77 ± 0.34 and 0.43 ± 0.23 sec to peak for $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$, respectively) (bottom two traces). Listed data represent the mean of 7 to 9 oocytes for each subunit combination \pm S.D. Resolution of the current onset for $\beta 2$ -containing AChRs was limited by the perfusion time (see “Materials and Methods”).

Addition of $\alpha 5$ subunits to the $\alpha 3\beta 2$ combination resulted in AChRs with notably faster desensitization. $T_{1/2}$ of the current decay upon exposure to a saturating concentration of ACh decreased from 1.1 to 0.64 sec (fig. 1, left plot on the top panel). In addition, the amount of desensitization (percent of current from the peak to plateau) increased from 46% to 68% (fig. 1, right plot on the top panel). A similar phenomenon was observed when $\alpha 5$ subunits were coexpressed together with $\alpha 3$ and $\beta 4$ subunits. Both the rate of desensitization ($T_{1/2}$ of decay decreased from 1.8 to 0.7 sec) and amount of desensitization (increased from 21% to 41%) were enhanced in $\alpha 3\beta 4\alpha 5$ compared with $\alpha 3\beta 4$ AChRs (fig. 1, bottom panel).

$\alpha 5$ subunit alters pharmacology of recombinant human neuronal $\alpha 3$ AChRs. Pharmacological profiles of $\alpha 3$ AChRs were investigated using four nicotinic agonists: ACh, nicotine, cytisine and DMPP. Concentration-response curves

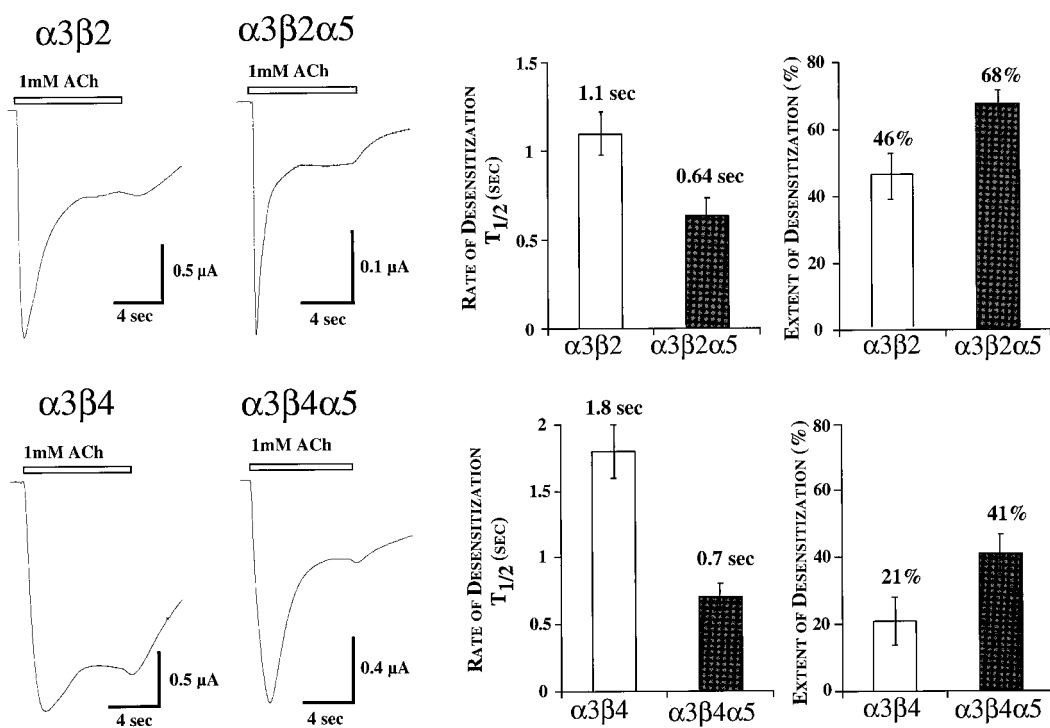


Fig. 1. $\alpha 5$ subunits increase both rate and amount of desensitization of $\alpha 3$ AChRs. Typical responses to saturating concentrations of the ACh are shown on the left for AChRs formed by four different subunit combinations. Graphs on the right depict averaged data on $T_{1/2}$ of the current decay and percent of the transient peak component. Open bars represent values obtained from $\alpha 5$ -less AChRs; filled bar, from AChRs with $\alpha 5$. Values represent the mean \pm S.E. from at least seven separate experiments. Current plots and bar graphs were obtained from oocytes clamped at -30 mV.

for these agonists were built from data collected from oocytes expressing four different $\alpha 3$ neuronal AChR subtypes (fig. 2). Concentration-response curves for ACh and nicotine, which are shown for comparison with the effects of DMPP and cytisine, are from our previous study (Wang *et al.*, 1996). All currents were normalized to the maximal currents induced by ACh for each AChR subtype. ACh was used for normalization of efficacy of the nicotinic agonists because it is the endogenous agonist. Values for the EC_{50} , Hill coefficients and the relative maximal responses are listed in table 1. Comparison of the families of the concentration/response curves built for $\alpha 3\beta 2$, $\alpha 3\beta 2\alpha 5$, $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ AChRs revealed striking differences in pharmacological properties among these AChRs.

Substitution of $\beta 2$ subunits for $\beta 4$ subunits in $\alpha 3$ AChRs resulted in decreases of potency for ACh, nicotine and DMPP (table 1). Furthermore, this resulted in increased efficacy of nicotine, changing it from a partial to a full agonist. Efficacy for cytisine also increased from 23% to 56% with no significant changes in apparent affinity. In addition, concentration-response curves for the agonists tested had higher Hill coefficients for $\alpha 3\beta 4$ AChRs than for $\alpha 3\beta 2$ AChRs.

Notable changes in pharmacological properties were observed when $\alpha 5$ subunits were added to $\alpha 3\beta 2$ AChRs (fig. 3; table 1). Thus, as we have shown previously (Wang *et al.*, 1996), $\alpha 3\beta 2\alpha 5$ AChRs had almost 50 times higher sensitivity to ACh compared with $\alpha 3\beta 2$ AChRs. Less significant increases of apparent affinity were observed for nicotine and DMPP. In contrast, efficacies of these agonists changed dramatically, nicotine switching from a partial (55%) to a full agonist (Wang *et al.*, 1996), and DMPP increasing in efficacy from 107% to 187% compared with ACh. This, in essence, converted ACh and nicotine into partial agonists.

Addition of $\alpha 5$ subunits to $\alpha 3\beta 4$ AChRs caused less significant changes in apparent affinities for the agonists tested (fig. 2; table 1). Only cytisine exhibited a moderate increase of

apparent affinity for $\alpha 3\beta 4\alpha 5$ AChRs compared with $\alpha 3\beta 4$ AChRs, and there was basically no change in the rank order of potencies of agonists. In contrast to $\alpha 3\beta 2$ AChRs, where addition of $\alpha 5$ subunits increased the efficacy of DMPP to greater than that of ACh, addition of $\alpha 5$ to $\alpha 3\beta 4$ AChRs decreased the efficacy of DMPP from 100% to 13%. Overall, concentration-response curves for $\alpha 3\beta 4\alpha 5$ AChRs had higher Hill coefficients than curves built for $\alpha 3\beta 2\alpha 5$ AChRs (table 1).

$\alpha 5$ subunits enhance Ca^{++} permeability and Ca^{++} modulation of recombinant human neuronal $\alpha 3$ AChRs. Relative permeability of Ca^{++} through AChRs was evaluated by the shifts of reversal potential caused by changes in extracellular Ca^{++} concentration. More precise estimates of the permeability ratios were constrained by our inability to monitor intracellular cation concentrations while using the two-electrode voltage-clamp method. $\alpha 7$ AChRs were shown previously to have exceptionally high permeability for Ca^{++} ions, comparable to that of NMDA receptors (Bertrand *et al.*, 1993; Seguela *et al.*, 1993; Castro and Albuquerque 1995; Delbono *et al.*, 1997). In contrast, muscle AChRs have rather low Ca^{++} permeability (Vernino *et al.*, 1992; Dani and Mayer 1995; Francis and Papke 1996). These two AChRs were used to "calibrate" the range of the extracellular Ca^{++} -dependent shift of reversal potential (fig. 4) and, subsequently, to compare the relative Ca^{++} permeabilities of $\alpha 3$ AChR subtypes. Human $\alpha 7$ AChRs exhibited a 17.8 ± 0.9 mV ($n = 12$) positive shift of reversal potential as a result of a 10-fold increase of Ca^{++} concentration from 1.8 to 18 mV. Human muscle AChRs formed from $\alpha 1$, $\beta 1$, δ and ϵ subunits exhibited a shift of only 0.8 ± 0.9 mV ($n = 4$). $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs had similar shifts of reversal potential upon increase of Ca^{++} concentration (5.8 ± 0.8 mV ($n = 7$) and 6.1 ± 1.2 mV ($n = 6$), respectively). This suggests similar contributions by both $\beta 2$ and $\beta 4$ subunits to the AChR channel lining. Incorporation of $\alpha 5$ subunits in both $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs dramatically increased the Ca^{++} -dependent

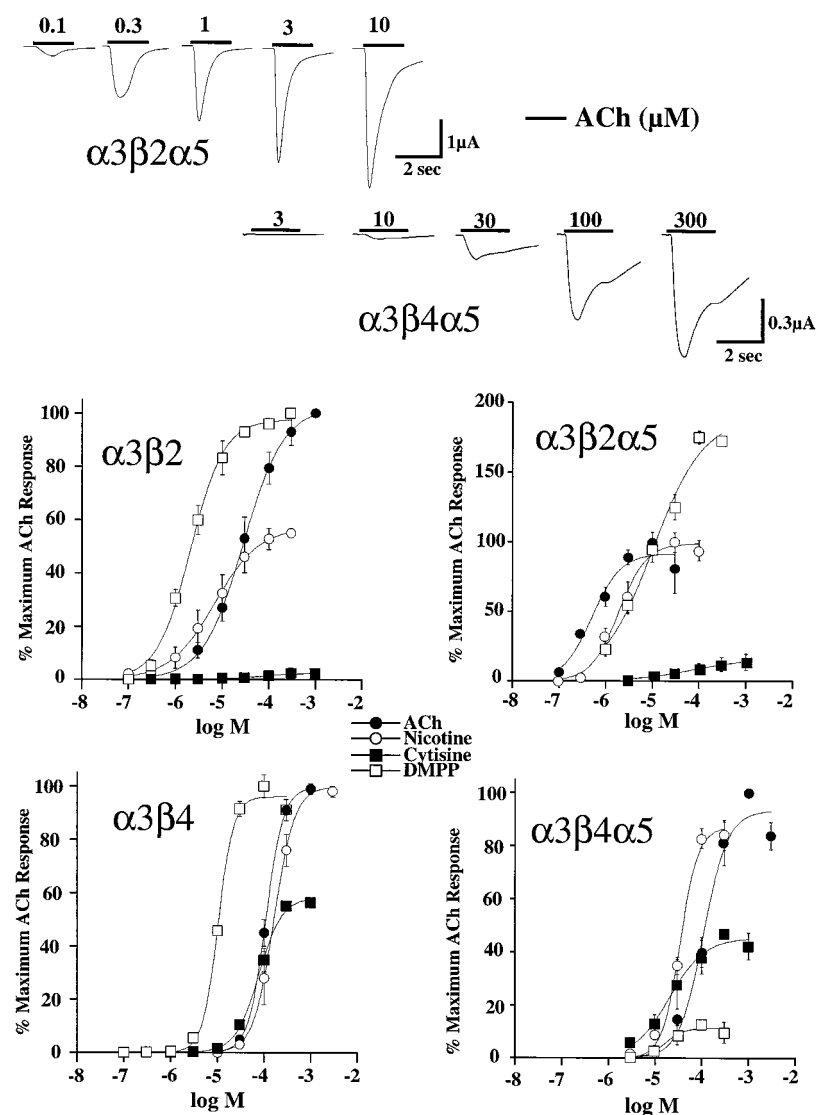


Fig. 2. $\alpha 5$ subunits, like $\beta 2$ and $\beta 4$ subunits, extensively contribute to pharmacological profiles of $\alpha 3$ AChRs. *Top*) Representative currents induced in *Xenopus* oocytes expressing human $\alpha 3$, $\beta 2$ and $\alpha 5$ or human $\alpha 3$, $\beta 4$ and $\alpha 5$ AChR subunits. Responses to consecutive applications of increasing concentrations of ACh to oocytes voltage-clamped at -30 mV are displayed for both AChR subunit combinations. Traces shown were obtained from oocytes 3 days after cRNA injections in a 1:1:1 ratio. Bars and numbers above each trace mark the duration of the application and the concentration of ACh. *Middle and bottom*) Families of concentration-response curves for ACh (\bullet), nicotine (\circ), cytosine (\blacksquare) and DMPP (\square) were built for the four combinations of the subunits tested. Curves for ACh and nicotine are from Wang *et al.* (1996). Data were obtained from 3 to 5 oocytes clamped at -50 mV. Current amplitudes were normalized to maximal responses induced by ACh and averaged. Values of EC_{50} and Hill coefficients are listed in table 1.

TABLE 1

Comparison of the potency and efficacy of nicotinic agonists for recombinant human $\alpha 3$ AChRs

	ACh***		Nicotine***			Cytosine			DMPP		
	EC_{50} *	nH	EC_{50} *	nH	Efficacy**	EC_{50} *	nH	Efficacy**	EC_{50} *	nH	Efficacy**
$\alpha 3\beta 2$	26 ± 0.3	0.9	6.8 ± 0.5	1.0	55%	71 ± 20	1.1	23%	2.1 ± 0.2	1.2	107%
$\alpha 3\beta 2\alpha 5$	0.5 ± 0.6	1.4	1.9 ± 0.3	1.5	100%	70 ± 23	1.0	13%	10 ± 3	1.0	183%
$\alpha 3\beta 4$	163 ± 6	1.9	106 ± 4	2.0	100%	76 ± 4	1.7	56%	10 ± 2.2	2.2	100%
$\alpha 3\beta 4\alpha 5$	122 ± 20	1.7	105 ± 4.0	2.0	100%	20 ± 4	1.2	47%	20 ± 5	2.0	13%

* μM

** Maximum currents normalized to the maximum current induced by a saturating concentration of ACh.

*** Values from Wang *et al.* (1996) are shown for comparison.

shift of the reversal potential to 13.7 ± 1.4 mV ($n = 11$) and 11.7 ± 1.1 mV ($n = 10$), respectively. This indicates that the Ca^{++} permeabilities of human $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs approach that of homomeric $\alpha 7$ AChRs.

Increase of the extracellular Ca^{++} concentration also augmented the amplitude of currents mediated by $\alpha 3$ AChRs (fig. 4). Although for $\alpha 3\beta 4$ AChRs this increase of amplitude could be attributed solely to the increase in the driving force due to the change of the reversal potential upon increase of the Ca^{++} concentration, for $\alpha 3\beta 2$ AChRs, the increase of amplitude in 18 mM Ca^{++} was 3-fold larger. Addition of $\alpha 5$ sub-

units increased the $\alpha 3\beta 2$ AChR-mediated current, whereas no increase was observed for $\alpha 3\beta 4$ AChRs. Thus $\beta 2$ and $\beta 4$ subunits clearly contributed differently to extracellular Ca^{++} modulation of $\alpha 3$ AChRs, and $\alpha 5$ further enhanced this modulation for $\alpha 3\beta 2\alpha 5$ AChRs.

Evidence that $\alpha 5$ subunits can assemble in AChRs with four different subunits. Neurons frequently express $\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$ subunits (*e.g.*, Conroy and Berg, 1995; Wang *et al.*, 1996). Coinjection of equal amounts of all four subunit cRNAs— $\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$ —resulted in AChRs that responded to ACh application in a distinct manner. The time

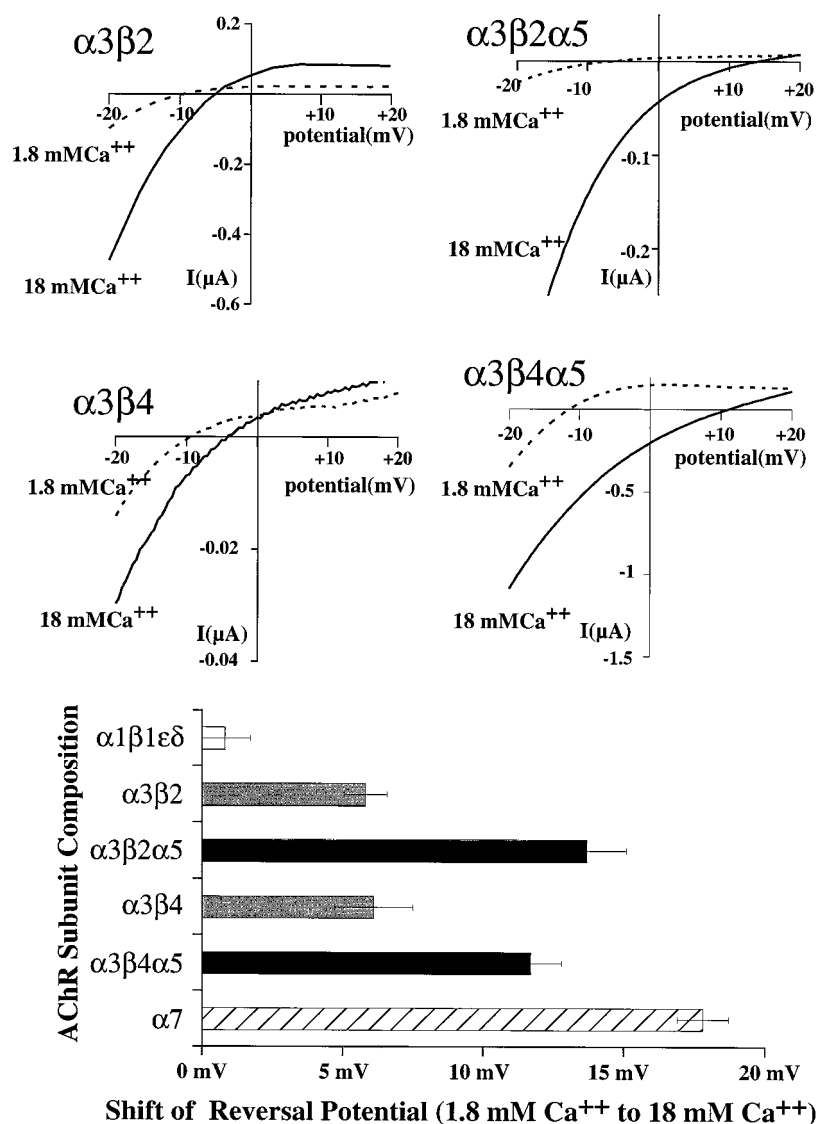


Fig. 3. $\alpha 5$ subunits significantly increase Ca^{++} permeability of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs. *Top and middle*) Shift of the reversal potential of $\alpha 5$ -containing and $\alpha 5$ -less AChRs induced by a 10-fold increase of Ca^{++} concentration from 1.8 to 18 mM. Representative currents induced by the application of voltage ramps to oocytes perfused by 100 μM ACh with 1.8 mM (dashed trace) or 18 mM Ca^{++} (solid trace) in the extracellular solution are plotted against membrane potential. Currents induced by the ramps in agonist-free solutions are subtracted. Recordings were performed in Cl^- -free solutions on oocytes preincubated in Cl^- -free media (see "Materials and Methods"). *Bottom*) Plot of the reversal potential shifts induced by a 10-fold increase of extracellular Ca^{++} concentration (from 1.8 to 18 mM) for muscle-type $\alpha 1\beta 1\epsilon\delta$ and neuronal $\alpha 7$ AChRs (open bars), $\alpha 3\beta 2$ and $\alpha 3\beta 4$ (gray bars) and $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs (black bars). Averaged data were obtained from 7 to 14 oocytes as described on the top panel and represent mean \pm S.E.

course of activation and desensitization of currents from $\alpha 3\beta 2\beta 4\alpha 5$ AChRs (fig. 5) resembled most closely the time course of $\alpha 3\beta 4\alpha 5$ AChRs (fig. 1), though current rise and decay were both slower. Higher concentrations of ACh were required in order to saturate the response, and a small rebound current was observed upon removal of 3 mM ACh (fig. 5, left). The concentration-response curve yielded a satisfactory fit with a two-site Hill equation. The higher-affinity site (S_1), with an EC_{50} of 24 μM , constituted $\sim 35\%$ of the maximal response. The lower-affinity site (S_2), with an EC_{50} of 345 μM , constituted $\sim 65\%$ of the maximal response. DMPP behaved as a partial agonist with a maximal response equal to 65% of the response induced by the maximal concentration of ACh. Efficacy of DMPP for the $\alpha 3\beta 2\beta 4\alpha 5$ subunit combination did not match efficacies for other double and triple subunit combinations tested (table 1). S_1 for DMPP ($\sim 45\%$ of all sites) had an EC_{50} of 3.3 μM . S_2 ($\sim 55\%$ of all sites) had an EC_{50} of 110 μM . The S_2 site detected by both ACh and DMPP differed in EC_{50} from those observed for these agonists on $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs.

In order to evaluate the yield of assembly of $\alpha 5$ into $\alpha 3$ AChRs expressed in oocytes, we immunisolated [^3H]-epiba-

tidine labeled AChRs with subunit-specific mAbs (fig. 5). Precise evaluation of the composition of the AChRs formed in these conditions was constrained by the availability of mAbs. mAb210 crossreacts with both human $\alpha 3$ and human $\alpha 5$ AChR subunits (Wang *et al.*, 1996). The efficiency of $\alpha 5$ subunit incorporation into $\alpha 3$ AChRs was estimated by an mAb 142 epitope-tagged $\alpha 5$ subunit (Wang *et al.*, 1996) termed $\alpha 5^t$. A specific mAb is not available for human $\beta 4$ subunits.

Virtually all [^3H]epibatidine binding sites were absorbed by the $\beta 2$ -specific mAb290 (Peng *et al.*, 1994) from oocytes expressing $\alpha 3$, $\beta 2$ and $\alpha 5$ subunits (fig. 5), and virtually none from oocytes expressing $\alpha 3$, $\beta 4$ and $\alpha 5$ subunits (fig. 5). When all four subunits were expressed, more than 85% of the AChRs were found to contain $\beta 2$ subunits. Efficiency of $\alpha 5$ coassembly with $\alpha 3$ and $\beta 2$ subunits was 65%, and with $\alpha 3$ and $\beta 4$ subunits was about 50% (fig. 5). When all four subunits were expressed, more than 70% of the AChRs contained $\alpha 5$ subunits (fig. 5). Hence, when all four ($\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$) AChR subunits are expressed in oocytes, the majority of AChRs contain $\alpha 3$, $\alpha 5$ and $\beta 2$ subunits. The differences in expression levels of $\alpha 3\beta 2\alpha 5$ (~ 10 fM/oocyte) and $\alpha 3\beta 4\alpha 5$ (~ 2

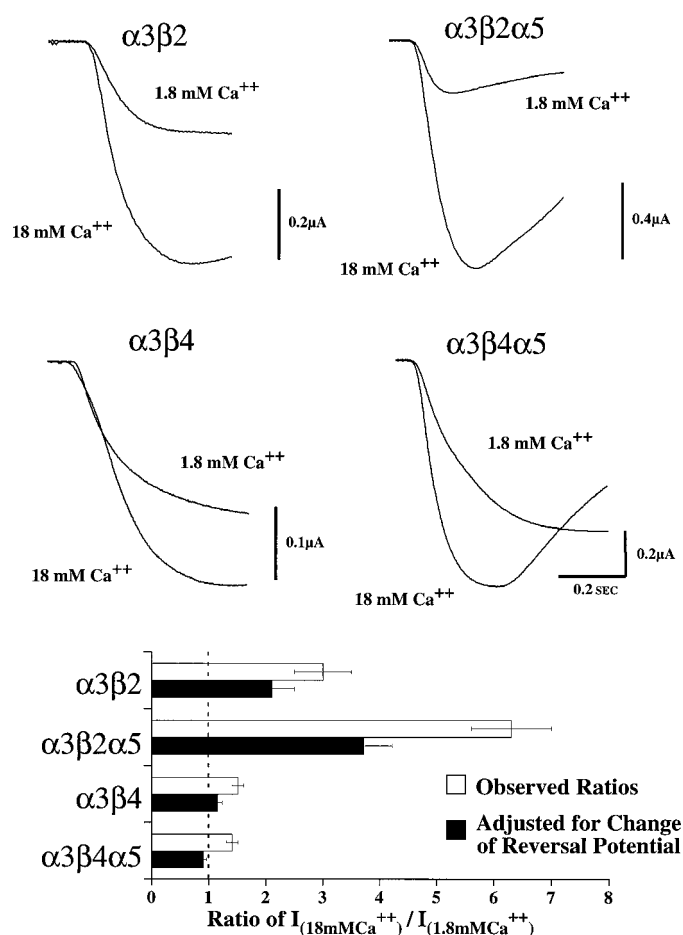


Fig. 4. Both $\beta 2$ and $\alpha 5$ subunits contribute to the modulation of $\alpha 3$ AChRs by extracellular Ca^{++} ions. *Top and middle*) ACh-induced currents are shown from oocytes expressing $\alpha 3\beta 2$, $\alpha 3\beta 2\alpha 5$, $\alpha 3\beta 4$ and $\alpha 3\beta 4\alpha 5$ AChRs. The extracellular Ca^{++} concentration is shown beside each trace. The ACh concentration was 100 μM , and the holding potential was -30 mV. Time scale bar is the same for all traces. Recordings were performed in Cl^- -free solutions on oocytes preincubated in Cl^- -free media (see "Materials and Methods"). *Bottom*) Plot of the ratio of the current amplitude in 18 mM Ca^{++} to the amplitude in 1.8 mM Ca^{++} for $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs and for $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs (open bars). Filled bars indicate ratios adjusted for the changes of the reversal potentials. Averaged data were obtained from 5 to 11 oocytes as shown on the top panel and represent mean \pm S.E.

fM/oocyte) AChRs (fig. 5) did not allow for evaluation of efficiency of the incorporation of $\beta 4$ subunits when all four subunits were expressed in oocytes.

Analysis of subunit composition of native human AChRs using mAbs. We used the available mAbs to assay incorporation of $\alpha 5$ subunits in AChRs from neuronal tissues of central and peripheral origin. The human neuroblastoma cell line SH-SY5Y expressing postsynaptic type $\alpha 3$ AChRs (Wang *et al.*, 1996) was used as a model of ganglionic type AChRs. Post-mortem human brain tissue from neocortex was used to characterize central $\alpha 3$ AChRs. For comparison, the expression level of $\alpha 4$ AChRs was evaluated using mAb299 (Peng *et al.*, 1994). Quantities of the different AChRs immunisolated in the same experiment are compared for both tissues in figure 6.

$\alpha 3$ AChRs predominate in SH-SY5Y cells, and about half of these contain $\beta 2$ subunits. mAbs were not available with which to determine independently the fraction of these

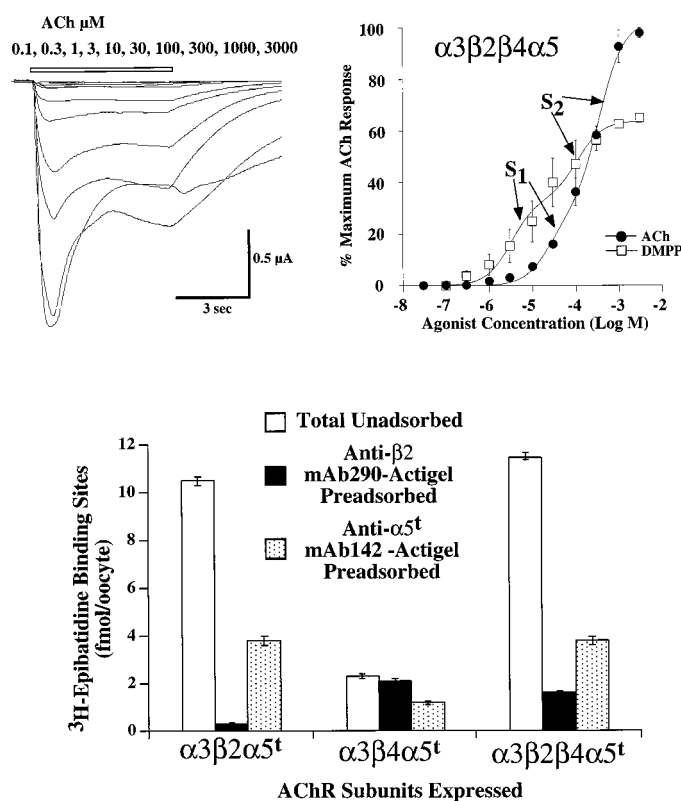


Fig. 5. $\alpha 5$ and $\beta 2$ subunits efficiently assemble into four subunit $\alpha 3\beta 2\beta 4\alpha 5$ AChRs. *Top left*) ACh-induced currents in oocytes expressing $\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$ AChR subunits. Responses to consecutive applications of increasing concentrations of ACh to the oocytes voltage-clamped at -50 mV are displayed on the left. Traces shown were obtained from oocytes 3 days after injections of 10 ng of each cRNA. Bar and numbers above the traces mark duration of the application and concentration of the ACh. *Top right*) Concentration-response curves for ACh and DMPP. Experimental data were fitted using the sum of two Hill equations yielding both high-affinity (S_1) and low-affinity (S_2) sites for both agonists. Hill coefficients were fixed to 1.5. Fit with one Hill equation resulted in Hill coefficient values below 0.7 for both curves. The ACh concentration-response curve had EC_{50} values of 24 ± 7 μM ($S_1 \sim 35\%$ of all sites) and 344 ± 43 μM ($S_2 \sim 65\%$ of all sites). The DMPP concentration-response curve had EC_{50} values of 24 ± 7 μM ($S_1 \sim 45\%$ of all sites) and 344 ± 43 μM ($S_2 \sim 55\%$ of all sites) with maximal currents reaching 65% of the current induced by 3 mM ACh. Data for ACh and DMPP were obtained from two different sets of oocytes and normalized as described for figure 4. *Bottom*) Assembly of $\alpha 3$ AChRs evaluated by specific mAbs. Equal (10-ng) amounts of cRNAs for $\alpha 3$, $\beta 2$, $\beta 4$ and reporter epitope tagged $\alpha 5^t$ subunits were injected into oocytes in the combinations listed below the groups of bars on the graph. Aliquots of the oocyte extracts were immunodepleted extensively with mAb142-Actigel, which removed all the $\alpha 5^t$ -containing AChRs, or with mAb290-Actigel, which removed all the $\beta 2$ -containing AChRs. By comparing the ^3H -epibatidine binding sites in the extracts before and after adsorption with mAb142 or mAb210, we determined the efficiency of incorporation of $\alpha 5$ and $\beta 2$ subunits into three- and four-subunit AChRs. Values represent the mean \pm S.E. from at least three separate experiments.

AChRs that contain $\alpha 5$ or $\beta 4$ subunits. As expected, no $\alpha 4$ AChRs were found.

Most (63%) of the human neocortex extract AChRs that contained $\beta 2$ subunits also contained $\alpha 4$ subunits. Of these $\alpha 4\beta 2$ AChRs, 36% may also contain $\alpha 5$ subunits because they could be adsorbed by mAb210.

In order to evaluate the relative amounts of various AChR subtypes in whole brain, we performed a similar immunoisolation of ^3H -epibatidine binding sites from extracts of complete rat brains. As in human neocortex, the major ^3H -epibatidine binding component was adsorbed by both mAb299 to $\alpha 4$ and

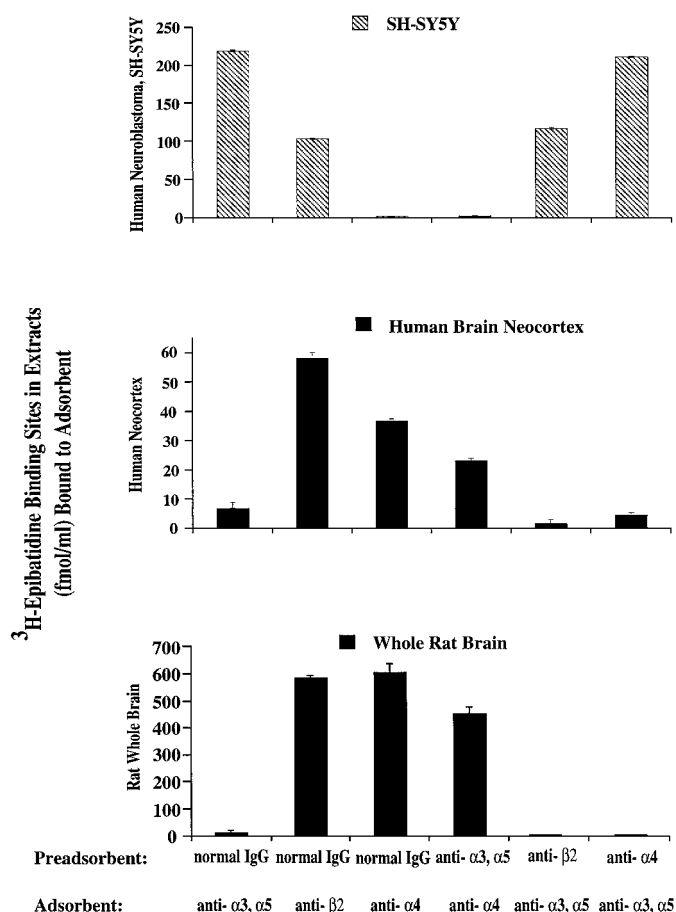


Fig. 6. Differences in expression of $\alpha 3$, $\alpha 4$ and $\alpha 5$ AChR subunits immunoprecipitated from ganglionic and brain tissue. Aliquots of tissue extracts were immunodepleted extensively with mAb210-Actigel (which removed all the $\alpha 5$ - and $\alpha 3$ -containing AChRs) or with mAb290-Actigel (which removed all the $\beta 2$ -containing AChRs) or with mAb299-Actigel (which removed all the $\alpha 4$ -containing AChRs). By comparing [3 H]epibatidine binding sites adsorbed by the mAb-Actigels in the extracts before and after immunodepletion, we determined the levels of expression and coassembly among $\alpha 3$, $\alpha 5$, $\alpha 4$ and $\beta 2$ subunits. The upper histogram represents [3 H]epibatidine binding data obtained from extracts of the human ganglionic neuron-like SH-SY5Y cell line from the human neocortex extract (middle) and from the total rat brain extract (bottom). Values represent the mean \pm S.E. from at least three separate experiments.

mAb290 to $\beta 2$ (fig. 6), which confirms that $\alpha 4\beta 2$ is the dominant central neuronal AChR with high affinity for epibatidine. About 20% of these $\alpha 4\beta 2$ AChRs appeared to have $\alpha 5$ associated with them, because they could be preadsorbed with mAb210. The amount of $\alpha 3$ or $\alpha 5$ AChRs in this tissue was about 4% of the $\alpha 4\beta 2$ AChRs. Most or all of these appeared to contain $\beta 2$ subunits, but this measurement was difficult because so few $\alpha 3$ AChRs were present.

Discussion

Our results prove that, when expressed in *Xenopus* oocytes, human $\alpha 5$ subunits are efficiently incorporated with $\alpha 3$ and $\beta 2$ or with $\alpha 3$ and $\beta 4$ subunits to form AChRs that differ in both dose dependence of activation and cation channel properties from AChRs containing only $\alpha 3$ and $\beta 2$ subunits or $\alpha 3$ and $\beta 4$ subunits. These results suggest that $\alpha 5$ subunits alter channel properties because they contribute directly to structure and can alter the EC_{50} or efficacy of

some agonists. Although they may not be part of the structure of the agonist binding sites, the $\alpha 5$ subunit contribution to the overall structure of the AChR influences the ability of the AChR to make the concerted changes in subunit orientation or conformation that are required for channel opening or desensitization.

It was shown recently that chick $\alpha 5$ subunits can efficiently assemble together with $\alpha 4$ and $\beta 2$ subunits to form AChRs with distinct properties (Ramirez-Latorre *et al.*, 1996). Immunoprecipitation studies have shown that only a minor fraction of native chick brain $\alpha 4\beta 2$ AChRs contain $\alpha 5$ subunits (Conroy and Berg 1995). In contrast, a majority of native $\alpha 3$ -containing AChRs, at least in autonomic ganglia, are thought to have $\alpha 5$ subunits incorporated (Conroy *et al.*, 1992; Vernallis *et al.*, 1993; Conroy and Berg 1995). Thus determination of the functional impact of $\alpha 5$ subunit on $\alpha 3$ AChRs is crucial to understanding the physiological contributions of individual subunits to native "ganglionic-type" neuronal nicotinic AChRs.

Pharmacology. When $\alpha 5$ is coexpressed with $\alpha 3$ and $\beta 2$ subunits, two types of AChRs may be formed: $\alpha 3\beta 2$ and $\alpha 3\beta 2\alpha 5$. As we have shown previously by immune precipitation and have confirmed here, in these conditions more than 70% of the $\alpha 3$ AChRs contain $\alpha 5$ subunits (Wang *et al.*, 1996). The presence of $\alpha 5$ subunits produces a uniform change in functional properties. Concentration-response curves for the $\alpha 3\beta 2\alpha 5$ subunit combination do not resolve two subpopulations of AChRs. EC_{50} for ACh differs 50-fold between $\alpha 3\beta 2$ and $\alpha 3\beta 2\alpha 5$ AChRs. Additionally, the efficacy of DMPP changed dramatically between these two subunit combinations. DMPP had significantly higher efficacy (183%) than ACh for $\alpha 3\beta 2\alpha 5$ AChRs. Higher efficacy of DMPP compared with ACh was reported previously for rat $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs expressed in the *Xenopus* oocytes (Cachelin and Jaggi, 1991). Oddly, however, when rat $\alpha 3\beta 4$ AChRs were transiently expressed in HEK-293 cells, DMPP was reported to behave as a partial agonist with less than 30% efficacy compared with ACh (Wong *et al.*, 1995). Overall, DMPP exhibited remarkable sensitivity to the human AChR subunit combination expressed. Despite only moderate changes in EC_{50} for the four $\alpha 3$ AChRs tested, DMPP exhibited large differences in efficacy. DMPP had only 13% efficacy for $\alpha 3\beta 4\alpha 5$ AChRs, was as efficacious as ACh on $\alpha 3\beta 4$ AChRs, was slightly more efficacious than ACh on $\alpha 3\beta 2$ AChRs and was almost twice as efficacious as ACh on $\alpha 3\beta 4$ AChRs. This characteristic of DMPP could prove useful in identification of the subunit composition of native human $\alpha 3$ AChRs.

Cytisine exhibited poor efficacy for all the human $\alpha 3$ AChRs tested. It had higher efficacy (50% for $\beta 4$ -containing AChRs than for $\beta 2$ -containing AChRs (20%). This difference in efficacy for cytisine between $\beta 2$ - and $\beta 4$ -containing AChRs was also observed for rat $\alpha 3$ AChRs (Papke and Heinemann, 1993). However, for rat $\alpha 3\beta 4$ AChRs transiently expressed in the HEK-293 cells, cytisine behaved as a full agonist compared with ACh (Wong *et al.*, 1995).

Of the four subunit combinations tested, concentration-response curves built for AChRs containing $\beta 4$ subunits compared with AChRs containing $\beta 2$ subunits were significantly steeper, with Hill coefficients closer to 2 for all agonists but cytisine. This could reflect the slower desensitization rates observed for $\beta 4$ -containing AChRs, which could permit better resolution of responses at high agonist concentrations. Alter-

natively, the presence of a subpopulation of AChRs with different agonist affinity could modify the slopes of concentration-response curves. Covernton *et al.* (1994) reported significantly higher Hill slopes in *Xenopus* oocytes for rat $\alpha 3\beta 4$ AChRs than for $\alpha 3\beta 2$ AChRs.

Desensitization. For both $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs, rates and magnitude of desensitization were higher than for $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs. Addition of the rat $\alpha 5$ subunit to $\alpha 4\beta 2$ has also been reported to cause acceleration of desensitization (Ramirez-Latorre *et al.*, 1996). Enhancement of desensitization in $\alpha 5$ -containing AChRs might be expected to shift EC_{50} values for activation to higher concentrations. However, increases of apparent affinity for ACh and nicotine were observed when $\alpha 5$ subunits were added to $\alpha 3\beta 2$ AChRs. Thus the pharmacological effects of $\alpha 5$ subunits probably do not reflect changes only in rates of desensitization.

Comparison of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs indicates that switching of $\beta 2$ for $\beta 4$ structural subunits significantly influences both the kinetics and the pharmacological properties of the AChRs. Similar phenomena were described previously for heterologously expressed chick and rat $\alpha 3$ -containing AChRs (Luetje and Patrick, 1991; Papke, 1993; Hussy *et al.*, 1994; Gerzanich *et al.*, 1995; Fenster *et al.*, 1997). It was suggested that $\beta 2$ and $\beta 4$ subunits contribute directly to the ligand binding pocket on the interface with α subunits. This raises a question of the possible position of the $\alpha 5$ subunit in the $\alpha 3$ AChR pentamer and the mechanisms by which $\alpha 5$ might influence functional properties. Pentameric structure of $\alpha 3$ AChRs is assumed on the basis of homology within the gene family and from comparison of the sizes of AChRs obtained in sucrose-gradient experiments (Wang *et al.*, 1996). The inability of $\alpha 5$ subunits to assemble directly with $\alpha 3$ or β subunits to form functional AChRs, together with lack of $\alpha 5$ influence on the ligand affinities in the equilibrium binding experiments (Wang *et al.*, 1996) suggests that $\alpha 5$ subunits do not contribute to the ligand binding pocket at the interface between $\alpha 3$ and β subunits. This indicates that changes in the macroscopic kinetic properties and pharmacological profiles of $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4\alpha 5$ AChRs observed electrophysiologically are determined not by the $\alpha 5$ subunit's direct interaction with agonists but by the overall conformational changes that it induces in AChRs. In addition, an $\alpha 5$ subunit present in an AChR would be expected to contribute one-fifth of the amino acids lining the cation channel and thereby potentially affect ion flow directly.

Ca⁺⁺ permeability and modulation. Native and recombinant $\alpha 7$ AChRs were shown to have Ca⁺⁺ permeabilities comparable to that of NMDA receptors (Bertrand *et al.*, 1993; Seguela *et al.*, 1993; Castro and Albuquerque, 1995). Previously it was shown that native and recombinant rat $\alpha 3$ AChRs have significant Ca⁺⁺ permeability (Fieber and Adams, 1991; Adams and Nutter, 1992; Vernino *et al.*, 1992; Rogers and Dani, 1995). Dependence of the reversal potential on extracellular Ca⁺⁺ indicates that human $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs could conduct a significant amount of Ca⁺⁺ ions. Because of the much slower desensitization rates of $\alpha 3$ AChRs compared with $\alpha 7$ AChRs, $\alpha 3$ AChRs could potentially, over prolonged periods, conduct more Ca⁺⁺ than could $\alpha 7$ AChRs. Moreover, introduction of $\alpha 5$ subunits further increases the Ca⁺⁺ permeability of $\alpha 3$ AChRs, producing, after a 10-fold increase of extracellular Ca⁺⁺, a shift of the reversal potential comparable to that of $\alpha 7$ AChRs. This

suggests that $\alpha 3\alpha 5\beta 2$ and $\alpha 3\alpha 5\beta 4$ AChRs may play more important roles than previously suspected in ACh-induced Ca⁺⁺-mediated effects in both the peripheral nervous system and the CNS.

Ca⁺⁺ permeability of neuronal AChRs is important because of the well-established role of Ca⁺⁺ influx in many physiological and pathophysiological processes. In autonomic ganglia, $\alpha 3$ AChRs are directly involved in synaptic transmission from preganglionic neurons. Ca⁺⁺ ions entering neurons through postsynaptic AChRs during EPSCs were shown to trigger a Ca⁺⁺-dependent K⁺ current (Tokimasa and North, 1984). In the CNS, presynaptic nicotinic AChRs were shown to exert facilitatory effects by increasing presynaptic Ca⁺⁺ concentration (Mulle *et al.*, 1992).

Potentialiation by extracellular Ca⁺⁺ of recombinant and native AChRs is viewed as an important mechanism of modulation (Mulle *et al.*, 1992; Vernino *et al.*, 1992; Amador and Dani, 1995; Galzi *et al.*, 1996). For chicken homomeric $\alpha 7$ AChRs, it was shown that divalent cation binding sites in extracellular domains are likely to mediate potentiation of the response by extracellular Ca⁺⁺. It was proposed that Ca⁺⁺ potentiates responses by direct interaction with the nicotinic ligand binding site of the AChRs. Substitution of $\beta 2$ for $\beta 4$ subunits virtually eliminates Ca⁺⁺ potentiation of the human $\alpha 3$ AChR responses. This suggests that extracellular Ca⁺⁺ can modulate AChR function *via* "structural subunits" as well. Considering that the ligand binding pocket is formed by the interface of the α and β AChR subunits, a $\beta 2$ -located site of the domain responsible for the Ca⁺⁺ potentiation is not unexpected. Differential Ca⁺⁺ potentiation of the $\alpha 3\beta 2$ and $\alpha 3\beta 4$ AChRs could account for differences of Ca⁺⁺ flux observed for these AChRs recombinantly expressed in HEK-293 cells (Mahaffy *et al.*, 1996).

Recombinant and native $\alpha 3$ AChRs. As shown by Conroy and Berg (1995) on neurons of chick ciliary ganglia, immunoprecipitation and immunoblot analysis strongly suggests that at least a portion of $\alpha 3$ AChRs contain four kinds of subunits: $\alpha 3$, $\beta 2$, $\beta 4$ and $\alpha 5$. Coexpression of the corresponding human subunits in *Xenopus* oocytes resulted in functional AChRs with a distinct concentration-response curve for ACh. Hill equation fit indicated at least two populations of AChRs. One population (55%–65% of the total) had significantly lower affinity for ACh ($EC_{50} = 345 \mu M$) and DMPP ($EC_{50} = 110 \mu M$) compared with the other subunit combinations tested (table 1), which suggests that it might result from the combination of four kinds of subunits. The higher-affinity site had affinities for both ACh and DMPP close to the values for $\alpha 3\beta 2$ AChRs. The distribution of affinities for ACh estimated for oocytes expressing all four subunits indicates that the contribution of $\alpha 3\beta 2\alpha 5$ AChRs to the mixture of AChRs expressed was negligible. Immune precipitation analysis showed that greater than 70% of the $\alpha 3$ AChRs contained both $\alpha 5$ and $\beta 2$ subunits. This strongly suggests that the population of $\alpha 3$ AChRs with unusually low affinity for ACh contains all four subunits. Overall, data on immunoidentification confirm not only the high efficiency of coassembly of $\alpha 5$ subunits with $\alpha 3$ and $\beta 2$ or with $\alpha 3$ and $\beta 4$ AChR subunits as previously determined (Wang *et al.*, 1996) but also indicate the incorporation of $\alpha 5$ subunits into $\alpha 3$ AChRs containing both $\beta 2$ and $\beta 4$ subunits.

Examination of AChR subunit expression in human neocortex confirmed that $\alpha 4\beta 2$ AChRs are the dominant non-

α bungarotoxin binding neuronal AChR in the brain (Whiting and Lindstrom, 1986, Flores *et al.*, 1992). A significant part (up to 25%) of human neocortex $\alpha 4$ -containing AChRs could be immunodepleted by preadsorption with mAb 210, which binds to both $\alpha 3$ and $\alpha 5$ subunits. The amount of mAb210-immunodepleted $\alpha 4$ containing AChRs appears to be larger than the amount of AChRs that could be immunodepleted from neocortex by mAb210 alone. This discrepancy might be in part due to degradation of the AChRs during the day required for the additional step of immunodepletion. A majority of the AChRs that bind to mAb210 could be depleted by the $\alpha 4$ -specific mAb299. These data strongly suggest that the $\alpha 5$ subunit is incorporated in some $\alpha 4\beta 2$ AChRs, although incorporation of $\alpha 3$ or of some other unknown AChR subunit that has affinity for mAb210 could not be excluded. According to the *in situ* hybridization studies, expression of $\alpha 3$, that of $\alpha 4$ and that of $\alpha 5$ have different but overlapping patterns in mammalian brain. Cerebral cortex contains messages for all of these AChR subunits as well as for $\beta 2$ subunits (Deneris *et al.*, 1991).

As expected, ganglionic-type neurons from the human neuroblastoma cell line SH-SY5Y were found to have a significantly different pattern of AChR subunit expression. $\alpha 3\alpha 5$ subunit-containing AChRs account for all of the high-affinity [3 H]epibatidine binding sites in these cells, with no detectable expression of $\alpha 4$ subunits. Half of these $\alpha 3\alpha 5$ AChRs contain $\beta 2$ subunits. $\beta 4$ subunits probably substitute for $\beta 2$ in the rest of the AChRs.

Comparison of the data on AChR subunit expression in the human neocortex with the data obtained from the rat total brain extract reveals significant differences in levels of expression of mAb210 binding AChRs. A small but significant part of the $\alpha 4$ -containing AChRs from the rat brain could be immunodepleted by binding to mAb210. The overall level of $\alpha 3$ and $\alpha 5$ AChR subunits is very small ($\sim 4\%$) relative to $\beta 2$ and $\alpha 4$ subunits, a level much lower than in the human neocortex. These differences could result from differences in the origin of the brain tissue, with human cortex representing only its local distribution of the AChRs. Alternatively, differences in expression could be interpreted as due to differences between rats and humans.

Unlike message for the $\alpha 4$ AChR subunits, which has a rather diffuse and diverse pattern of expression in the vertebrate brain, the patterns of $\alpha 3$ and $\alpha 5$ subunit expression are much more localized. $\alpha 5$ subunit mRNAs are present at modest levels in the cortex, at higher levels in the interpeduncular nucleus and at the highest levels in the ventral tegmental area and substantia nigra pars compacta (Wada *et al.*, 1989; Boulter *et al.*, 1990). These areas include regions in which there is nicotinic facilitation of dopamine release. Recently it has been suggested (Le Novere and Changeux, 1995) that some of the regions thought to contain $\alpha 3$ on the basis of the *in situ* hybridization studies actually contain the closely related but pharmacologically distinct $\alpha 6$ subunit (Gerzanich *et al.*, 1997). This prediction has been confirmed immunohistochemically (Goldner *et al.*, 1997).

Pharmacological, kinetic and Ca^{++} -dependent effects of $\alpha 5$ subunits on $\alpha 3$ AChRs imply that $\alpha 5$ subunits could be utilized effectively for fine-tuning neuronal nicotinic AChR function *in vivo*. In the periphery, synaptic $\alpha 3$ AChRs from human autonomic neurons are the most likely to be functionally affected by the presence of $\alpha 5$ AChR subunits. In the

human brain, $\alpha 5$ subunits may be associated with a small fraction of $\alpha 4\beta 2$ AChRs as well as with $\alpha 3$ AChRs.

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