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Expression and functional characterisation of a human chimeric nicotinic receptor with α6β4 properties

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Abstract

Despite being cloned several years ago, the expression of functional nicotinic acetylcholine receptors containing the human α6 subunit in recombinant mammalian cell lines has yet to be demonstrated. The resulting lack of selective ligands has hindered the evaluation of the role of α6-containing nicotinic receptors. We report that functional channels were recorded following co-transfection of human embryonic kidney (HEK-293) cells with a chimeric α6/α4 subunit and the β4 nicotinic receptor subunit. They displayed an agonist rank order potency of epibatidine ≫ 1,1-dimethyl-4-phenylpiperazinium (DMPP) ≥ cytisine>acetylcholine>nicotine measured in a fluorescent imaging plate reader assay. Nicotine, cytisine, DMPP and epibatidine displayed partial agonist properties whilst α-conotoxin MII and methyllycaconitine blocked the functional responses elicited by acetylcholine stimulation. Co-transfection of the α6/α4 chimera with the β2 nicotinic receptor subunit did not result in functional receptors. The human α6/α4β4 chimeric nicotinic receptor expressed in HEK-293 cells may provide a valuable tool for the generation of subtype specific ligands.

Keywords: Nicotinic receptor; α6 Subunit; Chimera; HEK-293 cell; (Human)

1. Introduction

Nicotinic acetylcholine receptors represent a family of ligand-gated ion channels distributed throughout the peripheral and central nervous systems. They include a variety of subtypes, with distinct pharmacological and functional profiles, reflecting the diversity of the genes encoding the five constituent subunits of each receptor. To date, 12 vertebrate genes coding for neuronal nicotinic receptor subunits, α2 to α10 and β2 to β4, have been cloned. In heterologous expression systems, homomeric channels can be formed only by α7, α8 or α9 nicotinic receptor subunits, whereas heteromeric channels can be formed by the co-expression of different combinations of α2, α3, α4, α6 and β2 or β4, with or without α5 or β3 nicotinic receptor subunits (reviewed by Mc Gehee and Role, 1995; Lindstrom, 2000; Clementi et al., 2000).

Nicotinic acetylcholine receptors are involved in numerous brain functions such as locomotor control (Clarke et al., 1988), learning and memory (Picciotto et al., 1995; Levin and Simon, 1998) and reward mechanisms (Picciotto et al., 1998), and they have been implicated in a number of central nervous system disorders (reviewed by Jones et al., 1999; Dani, 2001; Quik and Kulak, 2002). The most abundant and widely expressed nicotinic acetylcholine receptors in the mammalian brain are the α4β2 and α7 subtypes. In contrast, the distribution of α2, α5, α6, β3 and β4 nicotinic receptor subunits appears to be limited to specific areas (McGehee and Role, 1995; Lindstrom, 2000). In particular, the expression of α6 nicotinic receptor subunit mRNA and protein in rat brain is highly restricted and is most abundant in the locus coeruleus and dopaminergic nuclei of the midbrain, especially the substantia nigra and ventral tegmental area (Le Novere et al., 1996; Goldner et al., 1997; Quik et al., 2000a; Azam et al., 2002). This distribution suggests a role for the α6 receptor subunit in the nicotinic modulation of

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dopaminergic transmission (Kulak et al., 1997; Champtiaux et al., 2002) which may be important in locomotion, mediating the addictive properties of nicotine and other drugs of abuse, in schizophrenia and in Parkinson's disease (Pontieri et al., 1996; Le Novere et al., 1999; Quik and Kulak, 2002; Quik et al., 2002).

Initial reports of rat and avian α6 nicotinic receptor subunits appeared in the literature over 10 years ago (Lamar et al., 1990), whilst 6 years later, Elliot et al. (1996) first described the cDNA sequence of the human α6 nicotinic receptor subunit. It is most closely related to the α3 nicotinic receptor subunit, with which it shares a high level of amino acid sequence homology across species (~ 67% for chick, rat and human). This would suggest that, as for the α3 nicotinic receptor subunit, α6 should act as a ligand-binding subunit partner to form functional acetylcholine receptors in conjunction with β2 or β4 nicotinic receptor subunits. However, heterologous expression of functional α6-containing nicotinic receptor subtypes has proved to be difficult. Gerzanich et al. (1997) were the first to report that, when co-expressed with the human β4 nicotinic receptor subunit in *Xenopus* oocytes, chick or rat α6 receptor subunits formed functional channels with novel pharmacological properties. In the same study, α6β2 nicotinic receptor subunit combinations failed to form functional channels. It has since been demonstrated that chick α6 nicotinic receptor subunits form functional receptors when co-expressed with chick β2 or β4 nicotinic receptor subunits in BOSC 23 cells (Fucile et al., 1998). More recently, Kuryatov et al. (2000) reported on the functional expression of human α6-containing nicotinic receptors in *Xenopus* oocytes. To date, there have been no reports of functional expression of nicotinic acetylcholine receptor subtypes containing the human α6 subunit in a mammalian expression system.

It has been shown that, despite a generally high degree of sequence homology across species for individual subunits (Elliot et al., 1996), pharmacological differences are apparent between species homologues (Chavez-Noriega et al., 1997; Hussy et al., 1994; Kuryatov et al., 2000; Meyer et al., 1998) and also between host expression systems (Lewis et al., 1997). These differences highlight the need to generate functional human nicotinic acetylcholine receptor constructs, expressed in mammalian cells, for the evaluation of subtype specific ligands as potential therapeutic agents.

To this end, we have generated a chimeric subunit composed of the extracellular N-terminus of the human α6 nicotinic receptor subunit linked to the transmembrane regions and C-terminus of the human α4 nicotinic receptor subunit. In accordance with previously published results (Kuryatov et al., 2000), this construct produced functional receptors in *Xenopus* oocytes when co-expressed with human β4 nicotinic receptor subunits. We now report that co-expression of the human nicotinic α6/α4 chimeric receptor subunit with the human nicotinic β4 receptor subunit in mammalian human embryonic kidney (HEK-293) cells results in functional nicotinic acetylcholine receptors with a pharmacological profile consistent with that of receptors containing the nicotinic α6 subunit.

2. Materials and methods

2.1. cDNA and construction of human nicotinic receptor α6/α4 subunit chimera

The cDNAs for the human acetylcholine nicotinic receptor α4, α6, β2 and β4 subunits cloned into pcDNA3 were obtained from Merck Research Laboratories, La Jolla, CA, USA. They had been isolated from cDNA libraries prepared from human hippocampus, substantia nigra, prefrontal cortex and the human IMR32 neuroblastoma cell line, respectively (Elliot et al., 1996).

The nicotinic α6/α4 chimeric receptor subunit, composed of the extracellular coding domain of the α6 subunit cDNA and the remaining part of α4 subunit cDNA, was generated using polymerase chain reaction (PCR) amplification to introduce a *Kpn 2I* site, without changing the amino acid sequence, at the position corresponding to Arg ^{207}. The nicotinic α6 receptor subunit component was generated using a forward primer within pcDNA3 (GCTTACTGGCTTATCGAAATT) and a specific reverse primer to introduce the 3′ *Kpn 2I* site (CGGCAATCTCCGGATGTAGAAAGA). The nicotinic α4 receptor subunit component was generated by first amplifying the cDNA with specific forward (GACATCACCTATGCCTTCGTCATC) and reverse primers (CCCGGTCCCTTCCTAGATCAT) to produce a template which was then used with nested primers to introduce the 5′ *Kpn 2I* site (TTCGTCATCCGGAGGCTGCCGCTC) and a 3′ *XhoI* site (ACGTTACTCGAGCTAGATCATGCC) for cloning.

The nicotinic α6 receptor subunit component was first cloned with blunt ends into an intermediate vector, pCR®4Blunt-TOPO® vector, excised by digestion with *Not I* and *Hind III* and cloned into pcDNA 3.1 (+) (both vectors from Invitrogen life technologies, Paisley, UK) at these restriction sites. Both the α6 receptor subunit/pcDNA 3.1 (+) construct and the α4 receptor subunit PCR generated fragment were digested with *Kpn 2I* and *Xho I* and ligated together at these sites. This resulted in the nicotinic receptor subunit chimeric construct α6 (amino acid 1 to Arg ^{207})/α4 (α6/α4) in the expression vector pcDNA3.1 (+) which was then verified by automated sequencing (Cytomyx, Cambridge, UK).

2.2. Expression in Xenopus laevis oocytes and electrophysiological characterisation

Adult female *Xenopus laevis* frogs were obtained from Blades Biological (Edenbridge, UK). Oocytes (stages V–VI) were removed from schedule I, sacrificed frogs and defolliculated manually after treatment with collagenase

type I (4 mg/ml calcium-free Barth's solution) for 1.5 h at room temperature. cDNAs were injected into the nuclei of stages V and VI oocytes within 4 h after harvesting, using a Drummond variable volume microinjector (Broomall, PA, USA). One nanogram of each subunit cDNA was injected for the combinations of nicotinic α6/α4β4, α6β4, α4β4, α6/α4β2 α6β2 and α4β2 receptor subunits in a total volume of 20 nl. After injection, oocytes were incubated at 18 °C in a modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 15 mM HEPES and 50 mg/l neomycin (pH 7.6 with NaOH; osmolarity 235 mOsm). Experiments were performed on oocytes after 3–5 days of incubation.

Oocytes were placed in a recording chamber (∅ 3 mm), which was continuously perfused with a saline solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.3 with NaOH, 235 mOsm) at a rate of ~ 10 ml/min. Drugs were prepared in external saline immediately before the experiments. A BPS-8 solution exchange system (ALA Scientific, Westbury, NY, USA) was used in order to switch between control and drug-containing saline. Alternation between superfusion with agonist and agonist-free saline applications (5-min cycles for the chimeric receptor subunit) were made in order to allow the receptors to recover from desensitisation.

Oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–2.5 MΩ) and voltage clamped using a Geneclamp 500B amplifier (Axon Instruments, Foster City, CA, USA). The external saline was clamped at ground potential by means of a virtual ground circuit using an Ag/AgCl reference electrode and a Pt/Ir current passing electrode. The membrane potential was held at −60 mV and the current required to keep the oocyte membrane at the holding potential was measured. Membrane currents were low-pass filtered (4-pole low pass Bessel filter, -3dB at 1 kHz), digitised (1000 samples/record) and stored on disk for off-line computer analysis. All experiments were performed at room temperature.

2.3. Cell culture

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine, (95% air, 5% CO_2). For transient transfections, cells were sub-cultured and plated to achieve ~ 60–70% confluency within 24 h. The cells were then transfected with various nicotinic receptor subunit combinations (α6β4, α6/α4β4, α6β2, α6/α4β2, α4β4 and α4β2) using FuGENE 6 transfection reagent (Roche, Lewes, East Sussex), according to the manufacturer's instructions. Optimal transfection conditions were obtained using a 1:1 ratio of each subunit cDNA and a 1:3 ratio of DNA/FuGENE reagent. Unless otherwise stated, the cells were incubated for 48 h at 29 °C before being assayed.

HEK-293 cells stably expressing human α4β4 nicotinic receptors, obtained from Merck Research Laboratories (Stauderman et al., 1998), were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 50 μg/ml geneticin.

All cells were grown in a humidified incubator maintained at 37 °C. Cell culture reagents were from Sigma (Poole, Dorset, UK) or Invitrogen life technologies.

2.4. Intact cell binding assay

Estimation of surface [^{3}H]epibatidine binding was performed according to Whiteaker et al. (1998), with slight modification. Briefly, transiently transfected cells plated on poly-D-lysine-coated 24-well plates were washed extensively in ice-cold buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride) before assay. The cells were incubated with 5 nM [^{3}H]epibatidine (specific activity 55 Ci/mmol, NEN, Perkin-Elmer, Cambridge, UK) and displacing ligands for 2 h at room temperature in a final volume of 1 ml. Total binding was determined with buffer alone, and non-specific binding was determined in the presence of 10 μM unlabelled epibatidine. Cell surface binding was determined as the component of specific binding inhibited by the membrane impermeant ligand, methylcarbamylcholine (300 μM). Cells were then washed extensively in phosphate-buffered saline (PBS) and lysed with 0.2 M NaOH before being transferred to 24-well plates (Wallac, Perkin-Elmer Liquid scintillantion cocktail (Optiphase supermix, Perkin-Elmer) was added and the radioactivity in each well was counted on a 1450 Microbeta Trilux Wallac Counter.

2.5. Single-cell calcium measurement

Transiently transfected cells plated on poly-D-lysine-coated glass bottomed 35 mm micro-well dishes (MatTEK, MA, USA) were loaded with 2 μM Fura-2- acetoxymethylester (AM) diluted in growth medium, at 37 °C for 30 min. Cells were then washed and incubated for at least a further 30 min at room temperature in a HEPES-buffered saline solution (HBSS), containing 135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.3. Cells in dishes were mounted in a chamber onto the stage of an inverted epifluorescence microscope (Axiovert 100TV, Zeiss, Germany) and viewed using a × 10 (air) or × 40 (oil immersion) fluorescence objective. Cells were alternatively excited by light of 340 and 380 nm wavelength, provided by a polychrome II from T.I.L.L. photonics (Planegg, Germany) housing a Xe lamp and monochromator. Emitted light was captured by a SensiCam cooled CCD camera (PCO CCD Imaging, Kelheim, Germany) after passage through a dichroic mirror (400 nm) and high pass barrier filter (480 nm). Digitised images were stored and processed using Axon Imaging Workbench software (Version 2.2 and 4.0, Axon Instruments) and

Origin 6.1 software (OriginLab, MA, USA). The cells were continuously perfused with HBSS and stimulated by a rapid local application of drugs. Acetylcholine was applied in the presence of 1 μM atropine to block endogenous muscarinic receptors.

2.6. Cell population calcium measurements using FLIPR

Both stable and transiently transfected cells were plated overnight in black-walled, transparent bottomed, poly-D-lysine coated 96-well plates (Becton Dickinson, Marathon labs, London) at a density of 0.5×10^6 cells/ml. Growth medium was removed from the cells before the addition of HBSS containing 10 μM Fluo-4/0.05% pluronic F-127 using an automated multidrop dispenser (Lab Systems, Helsinki, Finland). The cells were incubated with the dye for 1 h at room temperature before replacing the medium with HBSS in the absence of Fluo-4-AM. The plates were then transferred to a fluorescent imaging plate reader (FLIPR) system (Molecular Devices, Winnersh, UK) for fluorescence recordings. The cells were excited with a 488-nm wavelength light from a 4 W Argon-ion laser, and the emitted fluorescence was passed through a 510–570 nm bandpass interference filter before being detected with a cooled CCD camera (Princeton Instruments, NJ, USA). Image data were transferred to a Dell Optiplex GX110 computer and stored for offline analysis using FLIPR system software and Origin 6.1 software (OriginLab). Drug dilutions were prepared in a separate 96-well plates using a Biomek 2000 (Beckman Instruments, Fullerton, CA, USA). Parameters for drug addition to the cell plate were programmed into the computer and delivery was automated through a 96-tip head pipettor. The results obtained were normalised to the maximal responses obtained with the non-selective nicotinic receptor agonist acetylcholine (300 μM) or an EC_{90} value of epibatidine (100 nM), as indicated.

Acetylcholine, 1,1-dimethyl-4-phenylpiperazinium (DMPP), nicotine and cytisine were purchased from Sigma-RBI. Methyllycaconitine citrate, α-conotoxin MII, and epibatidine were from Tocris Cookson (Bristol, UK). Fura-2-AM, Fluo-4-AM and pluronic F-127 were from Molecular Probes (Leiden, The Netherlands).

3. Results

3.1. The nicotinic α6/α4 chimeric receptor subunit forms functional receptors when co-expressed with β4 but not β2 nicotinic receptor subunits in Xenopus oocytes

The functional properties of the nicotinic α6/α4 chimeric receptor subunit were first assessed and compared with wild type nicotinic α6 and α4 receptor subunits co-injected with the β2 or β4 nicotinic receptor subunits in *Xenopus* oocytes. As expected, both nicotinic α4β2 and α4β4 receptors were functional whereas injection of the wild type nicotinic α6 receptor subunit with either the nicotinic β2 or β4 receptor subunits failed to produce functional receptors, as did the nicotinic α6/α4 subunit chimera with the nicotinic β2 receptor subunit. Co-expression of the nicotinic α6/α4 subunit chimera with the nicotinic β4 receptor subunit, however, generated functional receptors that gave large, robust responses to acetylcholine (Fig. 1A).

The effects of acetylcholine and α-conotoxin MII on nicotinic α6/α4β4 receptors were investigated and compared with their effects on human nicotinic α4β4 receptors. Superfusion of oocytes expressing α6/α4β4 nicotinic receptor subunits with 1 mM acetylcholine evoked inward currents in 67% of injected oocytes ($n = 18$) with amplitudes

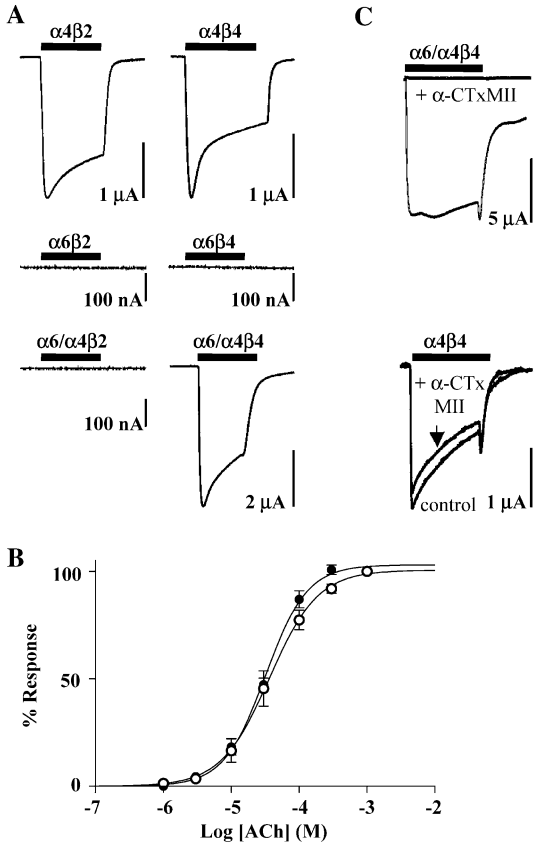

Fig. 1. Expression of the α6/α4 nicotinic chimera in *Xenopus* oocytes forms functional, α-conotoxin MII-sensitive nicotinic receptors when co-expressed with the wild type β4 nicotinic subunit. (A) Application of 1 mM acetylcholine to oocytes resulted in functional responses in oocytes injected with α4β2, α4β4 and α6/α4β4 nicotinic receptor subunit combinations of cDNAs. Oocytes injected with α6β2 ($n = 70$), α6β4 ($n = 57$) and α6/α4β2 ($n = 68$) nicotinic receptor subunit combinations did not respond to 1 mM acetylcholine, demonstrating that these subunit combinations do not form functional receptors. Examples of recordings are shown with 10-s acetylcholine applications (black bars) for the subunit combinations indicated. (B) Concentration–response curves of acetylcholine obtained from oocytes expressing α4β4 (filled circles) and α6/α4β4 nicotinic receptors (open circles). (C) Acetylcholine-induced ion currents (1 mM) mediated by α6/α4β4 nicotinic receptors are almost completely (96 ± 5%; $n = 3$) blocked by 10–15 min pre-exposure to 100 nM α-conotoxin MII. The same toxin treatment resulted in only a slight inhibition (14 ± 3%; $n = 4$) of α4β4 nicotinic receptor-mediated ion currents.

ranging between 0.7 and 18.1 μA. Acetylcholine concentration–response curves were obtained from oocytes expressing α6/α4β4 and α4β4 nicotinic receptors. Peak amplitudes of these currents, normalised to the peak amplitude of the 1 mM acetylcholine-induced currents in the same oocytes, were plotted against agonist concentration. Mean values for EC_{50} and nH estimated from these curves were 37 ± 12 μM and 1.2 ± 0.2 for α6/α4β4 and 32 ± 5 μM and 1.4 ± 0.1 for α4β4, respectively (Fig. 1B). Application of 100 nM of α-conotoxin MII blocked 1 mM acetylcholine-induced α6/α4β4 nicotinic receptor currents by 96 ± 5% whereas it only blocked α4β4 nicotinic receptor currents by 14 ± 3% (Fig. 1C), suggesting a specific interaction of α-conotoxin MII with the binding site formed by the α6 and β4 nicotinic receptor subunit interfaces.

3.2. The nicotinic α6/α4 chimeric receptor subunit is expressed at the cell surface with both β2 and β4 nicotinic receptor subunits in transiently transfected HEK-293 cells

Binding studies were initially performed as a first step in the characterisation of mammalian cell lines expressing nicotinic α6 receptor subunits. The cell surface expression of both the wild type nicotinic α6 subunit and the α6/α4 chimera after transient transfection with either the β2 or β4 nicotinic receptor subunits was investigated in HEK-293.

We found that the wild type nicotinic α6 receptor subunit can assemble and generate surface [^{3}H]epibatidine binding sites when co-transfected with β2 but not β4 nicotinic receptor subunits (58% and 7% cell-surface binding, respectively, Fig. 2). In contrast, the nicotinic α6/α4 chimera was able to assemble and induce surface [^{3}H]epibatidine binding

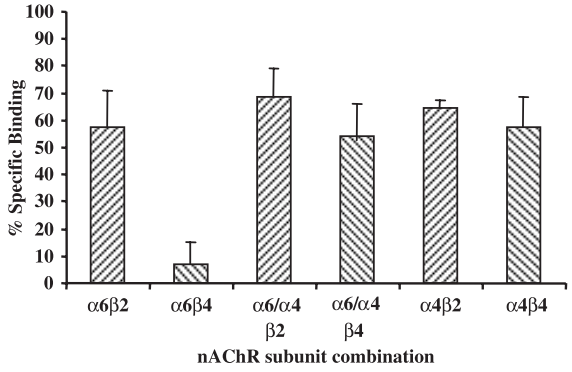

Fig. 2. The α6/α4 chimeric nicotinic receptor subunit co-expressed with β2 or β4 nicotinic receptor subunits forms cell surface epibatidine binding sites. [^{3}H]Epibatidine (5 nM) binding to HEK-293 cells transiently transfected with the subunit combinations indicated were measured in the presence or absence of displacing ligands 48 h post transfection. Total binding was determined in the presence of buffer alone, non-specific binding in the presence of unlabelled epibatidine (10 μM) and cell surface binding was determined as the component of specific binding inhibited by the membrane impermeant ligand, methylcarbamylcholine (300 μM). The cell surface binding is shown for each of the nicotinic receptor subunit combinations expressed as a percentage of specific epibatidine binding. Data are mean values ± S.D. from three experiments performed in quadruplicate.

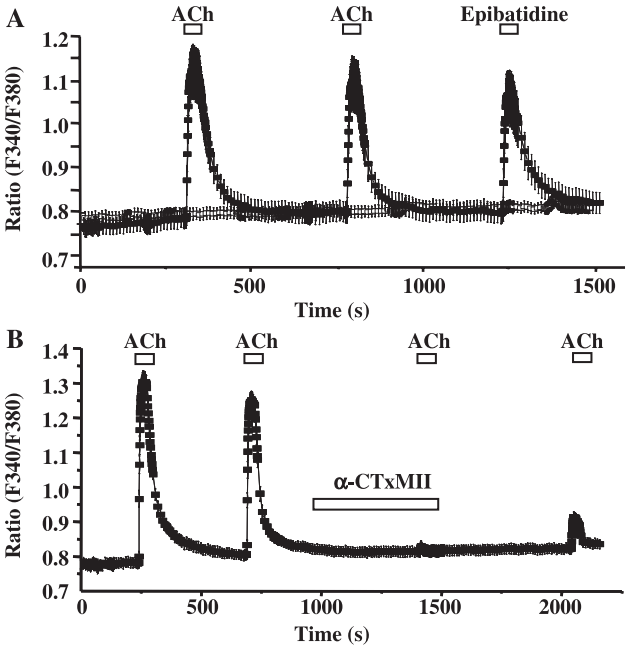

Fig. 3. α6/α4β4 chimeric nicotinic receptor subunits transiently transfected into mammalian cells, form functional receptors sensitive to nicotinic agonists and antagonists. Fura-2 loaded HEK-293 cells transiently transfected with α6/α4β4 nicotinic subunits (■) or untransfected control cells (□) were superfused (30 s applications) with HBSS containing nicotinic agonists, acetylcholine (300 μM, in the presence of 1 μM atropine to block endogenous muscarinic receptors) or epibatidine (1 μM), as indicated (A and B). Control cells failed to respond to nicotinic agonists (A). Responses to acetylcholine (300 μM) in transfected cells were fully blocked by a 10-min pre-incubation with 100 nM α-conotoxin MII, which was partially reversible on washout (B). Results are mean values ± S.E.M., n>10 cells, from single experiments, representative of three similar experiments.

sites in conjunction with both β2 and β4 nicotinic receptor subunits (69% and 54% cell-surface binding, respectively, Fig. 2). No specific epibatidine binding was observed in untransfected HEK-293 cells.

3.3. Human α6/α4β4 chimeric nicotinic receptors form functional channels after transient transfection in mammalian cells

The functional properties of transiently transfected HEK-293 cells were assessed using single-cell Ca^{2+} imaging of Fura-2 loaded cells. Co-expression of the nicotinic α6/α4 chimera with the β4 receptor subunit generated functional receptors that responded to various nicotinic receptor ligands. Rapid, local application of either acetylcholine (300 μM) or epibatidine (1 μM) caused increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in 30–40% of transfected cells (Fig. 3A). Consistent with the results in oocytes, these responses were fully blocked by α-conotoxin MII (100 nM, Fig. 3B). Expression of the nicotinic α6/α4 chimera with the β2 receptor subunit failed to produce functional receptors, as did the wild type human nicotinic α6 receptor subunit co-expressed with either the β2 or the β4 nicotinic receptor subunits (results not shown).

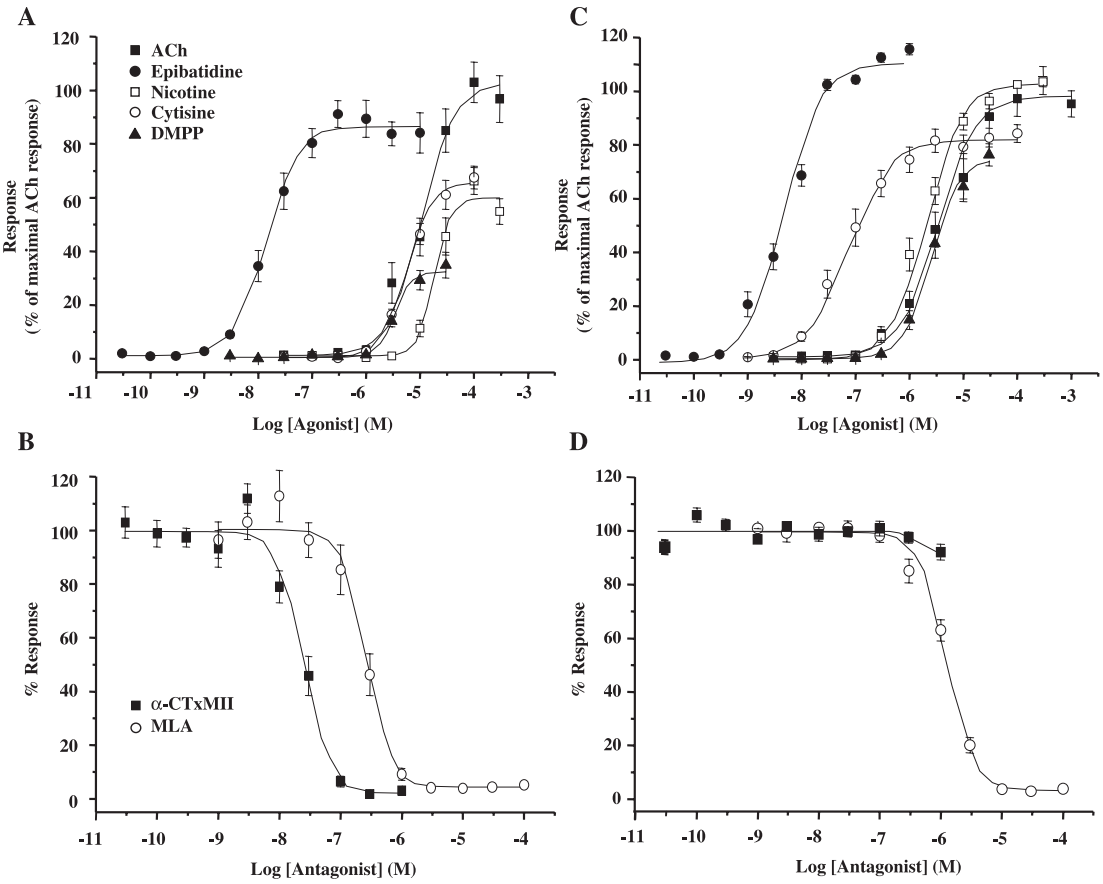

Fig. 4. Nicotinic agonist and antagonist profiles of α6/α4β4 chimeric and α4β4 wild-type nicotinic receptors expressed in mammalian cells. Fluo-4 loaded HEK-293 cells either transiently transfected with α6/α4β4 (A and B) or stably expressing α4β4 (C and D) nicotinic receptors were challenged with nicotinic agonists (acetylcholine, epibatidine, nicotine, cytisine and DMPP) (A and C) or antagonists (methyllycaconitine, α-conotoxin MII) (B and D). Antagonists were pre-applied for 20 min before addition of an EC_{90} value of epibatidine (100 nM) to assess receptor blockade. Concentration–response curves are mean values ± S.E.M. from $n = 8–12$ independent experiments.

3.4. Comparison of the pharmacological profile of the α6/α4β4 chimeric nicotinic receptors with nicotinic α4β4 receptors expressed in HEK-293 cells

To assess the pharmacology of α6/α4β4 chimeric nicotinic receptors, the population Ca^{2+} responses of Fluo-4-AM loaded, transiently transfected, HEK-293 cells were monitored using a FLIPR and compared with HEK-293 cells stably expressing α4β4 nicotinic receptors. The profiles of a number of nicotinic receptor agonists (acetylcholine, cytisine, 1,1-dimethyl-4-phenylpiperazinium (DMPP), epibatidine and nicotine) and antagonists (methyllycaconitine and α-conotoxin MII) were assessed. Agonist activity was measured in naive cells, whilst antagonist activity was assessed after a 20-min pre-treatment of cells with the respective antagonists, followed by the addition of an EC_{90} value of epibatidine (100 nM).

The order of agonist potency at the α6/α4β4 chimeric nicotinic receptor was epibatidine ≫ DMPP ≥ cytisine > acetylcholine > nicotine. Epibatidine, cytisine, nicotine and

Table 1
Pharmacological profiles of the α6/α4β4 chimeric nicotinic receptors and α4β4 nicotinic receptors expressed in HEK-293 cells

Ligand	α6/α4β4		α4β4	
	EC_{50}μM	E_{max}	EC_{50}μM	E_{max}
Epibatidine	0.015 ± 0.003	83 ± 3% ($p < 0.001$)	0.006 ± 0.001	114 ± 3% ($p < 0.05$)
Nicotine	18 ± 3.8	58 ± 4% ($p < 0.001$)	2.1 ± 0.3	105 ± 1% (not significant)
ACh	10.8 ± 2.4	100 ± 7% –	3.3 ± 0.60	100 ± 3% –
Cytisine	5.8 ± 0.9	64 ± 5% ($p < 0.001$)	0.70 ± 0.10	85 ± 2% ($p < 0.05$)
DMPP	3.4 ± 0.4	31 ± 3% ($p < 0.001$)	2.6 ± 0.30	77 ± 3% ($p < 0.001$)

Fluo-4-AM loaded HEK-293 cells either transiently transfected with α6/α4β4 nicotinic subunits or stably expressing α4β4 nicotinic receptors were challenged with nicotinic agonists. Cell population Ca^{2+} measurements were recorded using FLIPR. Each value represents the mean ± S.E.M. from $n = 8$ independent experiments. *P* values are based on differences between means using Dunnett's test with ACh as control.

DMPP displayed a range of efficacies, with DMPP being the weakest partial agonist (Fig. 4A, Table 1). The α6/α4β4 chimeric nicotinic receptors were sensitive to blockade by α-conotoxin MII and methyllycaconitine with IC_{50} values of 24.2 ± 0.5 nM and 260 ± 4 nM, respectively ($n = 12$) (Fig. 4B). This pharmacological profile was quite distinct from that of α4β4 nicotinic receptors whose agonist order of potency was epibatidine ≫ cytisine > nicotine ≥ DMPP > acetylcholine, with all agonists displaying high efficacy values (Fig. 4C, Table 1). Furthermore, the α4β4 nicotinic receptors were insensitive to α-conotoxin MII and an order of magnitude less sensitive to blockade by methyllycaconitine (IC_{50} value of 1.25 ± 0.12 μM, $n = 12$) than the α6/α4β4 chimeric nicotinic receptor (Fig. 4D).

4. Discussion

The present study reports our attempts to express and characterise human α6 nicotinic acetylcholine receptor subunits in a mammalian heterologous system. As functional expression of wild type nicotinic α6 receptors with either β2 or β4 nicotinic receptor subunits was not demonstrated, we focused on a nicotinic α6/α4 chimeric receptor subunit in which the pharmacologically relevant N-terminus is from the nicotinic α6 receptor subunit. Whilst no functional expression was obtained with this chimera when co-expressed with nicotinic β2 receptor subunit, co-expression of the human α6/α4 chimeric nicotinic subunit with the human nicotinic β4 receptor subunit in mammalian HEK-293 resulted in functional receptors.

The functional properties of the α6/α4 chimeric nicotinic receptor subunit were first assessed in *Xenopus* oocytes and compared with literature reports of expression of both wild type and chimeric α6 nicotinic receptor subunits in this system. In accordance with a previous report by Kuryatov et al. (2000), co-expression of the human α6/α4 chimera with the human β4 nicotinic receptor subunit produced functional receptors that gave large responses to acetylcholine. The currents recorded displayed relatively slow activation and desensitisation kinetics consistent with those reported for wild type nicotinic α6β4 receptors expressed in oocytes (Gerzanich et al., 1997; Kuryatov et al., 2000). These were blocked by the antagonist α-conotoxin MII, which is selective for α3β2 and α6-containing nicotinic receptor subtypes (Cartier et al., 1996; Vailati et al., 1999; Astles et al., 2002). Co-injection of the α6/α4 chimeric nicotinic receptor subunit with the nicotinic β2 receptor subunit failed to produce functional receptors. This is in contrast to previously reported results with the same chimera (Kuryatov et al., 2000) but is consistent with reports of attempts to express functional wild type nicotinic α6β2 receptors in oocytes (Kuryatov et al., 2000; Gerzanich et al., 1997).

In order to assess the function and pharmacology of the human α6/α4 chimeric nicotinic receptor subunit in a mammalian system, HEK-293 cells were transiently co-transfected with the chimera and either human β2 or β4 nicotinic receptor subunits. As seen in oocytes, expression of α6/α4 with β4 nicotinic receptor subunits produced large, reproducible Ca^{2+} responses following acetylcholine stimulation whereas co-expression with the β2 nicotinic receptor subunit failed to produce functional receptors. The cell-surface expression of these subunit combinations was assessed to determine if the lack of function was due to inefficient expression and/or assembly. Co-expression of the β2 nicotinic receptor subunit with the α6/α4 chimera, wild type α6 or wild type α4 nicotinic receptor subunits resulted in similar levels of total and cell surface binding sites but only nicotinic α4β2 receptors formed functional channels. However, similar levels of cell surface expression were observed following co-transfection of the nicotinic β4 receptor subunit with the α6/α4 chimera or the α4 nicotinic receptor subunit and both combinations resulted in functional receptors. In contrast, with nicotinic α6β4 receptor subunits, both expression and function were low or not detectable. Thus, in co-expression studies with the nicotinic β4 receptor subunit in HEK-293 cells, surface binding correlated with functional expression of nicotinic receptors but this was not the case for the β2 nicotinic receptor subunit. Co-expression of α6/α4 or α6 with β2 nicotinic receptor subunits was sufficient for these subunits to reach the cell surface and to form pharmacologically "active" interfaces but not functional channels. The expression of another subunit with α6β2 and α6/α4β2 nicotinic receptors may be necessary to produce functional receptors in this system. Kuryatov et al. (2000) found that less than 2% of the high affinity [^{3}H]epibatidine binding sites formed by the expression of human α6β2 nicotinic receptors in oocytes were cell surface and no functional channels were formed, whereas the addition of nicotinic α5 receptor subunits resulted in the formation of functional receptors. However, these expression properties appear to be species and/or host cell specific as demonstrated by the formation of functional chick α6β2 nicotinic receptors in BOSC 23 cells (Fucile et al., 1998). Previous studies have shown that the ion channel and pharmacological properties of nicotinic receptor subtypes can be influenced by the kind of heterologous system in which they are expressed. Lewis et al. (1997) reported that the properties of recombinant rat α3β4 nicotinic receptors expressed in oocytes differed considerably from the same subunit combination expressed in a mammalian expression system, mouse fibroblast L929 cells. Furthermore, the latter more closely resembled endogenous nicotinic receptors expressed in rat superior cervical ganglion cells.

The pharmacological profile we obtained for nicotinic α6/α4β4 chimeric receptors with the FLIPR assay is similar to that reported by Kuryatov et al. (2000) for wild type nicotinic α6β4 receptors expressed in oocytes. Both assays revealed high sensitivity to epibatidine but not cytisine, which is instead a potent agonist on α4β4 nicotinic receptors (Chavez-Noriega et al., 1997; Stauderman et al., 1998).

In agreement with their effects on human β4 nicotinic receptor subunits expressed in oocytes with human, rat or chick α6 nicotinic receptor subunits (Kuryatov et al., 2000; Gerzanich et al., 1997), DMPP, cytisine and nicotine all displayed partial agonist activity at the chimeric α6/α4β4 nicotinic receptor in HEK-293 cells. In contrast, nicotine is a full agonist on α4β4 nicotinic receptors (present study; Stauderman et al., 1998). In accordance with reports on human α6β4 nicotinic receptors expressed in oocytes (Kuryatov et al., 2000), epibatidine was also found to behave as a partial agonist on the human α6/α4β4 chimeric nicotinic receptor in this system, whereas it has been reported to be a full agonist on chick α6 expressed with human β4 nicotinic receptor subunits in oocytes (Gerzanich et al., 1997). This observation again suggests that the pharmacology of nicotinic receptor subtypes may differ between species, a finding already reported for other nicotinic receptors, including α7 (Meyer et al., 1998) and α3β2 receptors (Hussy et al., 1994; Chavez-Noriega et al., 1997). The antagonist profile of the α6/α4 chimera expressed with the β4 nicotinic receptor subunit clearly distinguished it from nicotinic α4β4 receptors as α-conotoxin MII fully blocked the agonist-induced responses. Furthermore, methyllycaconitine was an order of magnitude more potent on the α6/α4β4 chimeric receptor than on α4β4 nicotinic receptors. These high antagonist potencies are consistent with values from competitive binding studies on reconstituted native chick α6β4 (Vailati et al., 1999) and recombinant chick α6β4 nicotinic acetylcholine receptors expressed in BOSC 23 cells (Barabino et al., 2001).

The limited distribution of the α6-containing nicotinic receptors in the central nervous system makes them an attractive therapeutic target. They are most abundant in the locus coeruleus and dopaminergic nuclei of the midbrain (Goldner et al., 1997; Le Novere et al., 1996; Azam et al., 2002; Quik et al., 2000a) where the β4 nicotinic receptor subunit mRNA and protein have also been shown to be localised (Dineley-Miller and Patrick, 1992; Quik et al., 2000a; Han et al., 2000; Johnson et al., 2002). This suggests that native α6β4-containing nicotinic receptor subtypes may be present in these areas. Infusion of rats with α6 nicotinic receptor subunit antisense oligonucleotides has been shown to suppress the enhanced locomotor effect of nicotine (Le Novere et al., 1999), whilst increased levels of α6 nicotinic receptor subunit mRNA together with decreased levels of [^{125}I]α-conotoxin MII and [^{125}I]epibatidine binding have been reported in monkey basal ganglia following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced nigrostriatal degeneration (Quik et al., 2000b, 2001). Furthermore, the majority of the [^{125}I]α-conotoxin MII sensitive sites in the striatum were selectively lost (Quik et al., 2002) and studies in knockout mice have demonstrated that these are all α6-containing nicotinic receptors (Champtiaux et al., 2002). It has also been demonstrated that nicotine is protective in primary mesencephalic cultures exposed to MPTP (Jeyarasasingam et al., 2002). Taken together, these results suggest that α6-containing nicotinic receptors may be good targets for symptomatic or indeed neuroprotective treatment of Parkinson's disease. Results of polymorphic analysis of recombinant inbred mouse strains have suggested that α6-containing nicotinic receptors may also play a role in regulating nicotine-induced seizures (Stitzel et al., 2000). The catecholaminergic nuclei play key roles in several other central nervous system disorders such as depression, cognition and addiction. Therefore, modulation of these nuclei by selective α6 nicotinic receptor subunit agonists or antagonists may offer new approaches to the treatment of a number of central nervous system disorders.

In conclusion, we have shown that co-expression of a human α6/α4 chimeric nicotinic receptor subunit with the human β4 nicotinic receptor subunit in HEK-293 cells results in the formation of a functional receptor with a pharmacological profile consistent with that of wild type α6-containing nicotinic receptors. The variations in function and pharmacology observed across different species and between different heterologous expression systems highlight the fact that the choice of both is critical when evaluating subunit specific ligands. Thus, in the absence of a mammalian system expressing functional wild type or native human α6-containing nicotinic receptors, this chimeric α6 nicotinic acetylcholine receptor expressed in HEK-293 may be a valuable tool for the identification of potential therapeutic agents acting at this subtype.

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