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Up-regulation of human $\alpha 7$ nicotinic receptors by chronic treatment with activator and antagonist ligands

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Abstract

This study examined the binding and functional properties of human $\alpha 7$ neuronal nicotinic acetylcholine receptors stably expressed in human embryonic kidney (HEK) 293 cells following chronic treatment with nicotinic receptor ligands. Treatment of cells with (−)-nicotine (100 μM) for 120 h increased the B_{max} values of [^{125}I]α-bungarotoxin binding 2.5-fold over untreated cells. This effect was concentration-dependent ($EC_{50} = 970$ μM) and a 6-fold upregulation was observed with the maximal concentration of (−)-nicotine tested. Also, treatment of cells with ligands of varying intrinsic activities including (±)-epibatidine, (2,4)-dimethoxybenzylidene anabaseine (GTS-21) and 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP) also upregulated [^{125}I]α-bungarotoxin binding. A concentration-dependent upregulation of binding sites was also observed following treatment with the $\alpha 7$ nicotinic receptor antagonist, methyllycaconitine ($EC_{50} = 92$ μM) with a maximal upregulation of about 7-fold. Functionally, the peak amplitude of the whole-cell currents recorded by fast application of (−)-nicotine after chronic treatment of cells with concentrations of (−)-nicotine (1000 μM) or methyllycaconitine (10 μM) that elicited similar increases in binding levels (3.5-fold) resulted in increases of 2-fold (505 ± 21 pA) and 6-fold (1820 ± 137 pA) respectively in whole cell current amplitude compared to untreated cells (267 ± 24 pA). These studies clearly demonstrate that long-term exposure to both activator and antagonist ligands can increase the density of $\alpha 7$ nicotinic receptors and can differentially enhance nicotinic receptor function. © 1998 Elsevier Science B.V.

Keywords: Nicotinic receptor, human; Regulation; Nicotine; Methyllycaconitine; α-bungarotoxin

1. Introduction

Neuronal nicotinic acetylcholine receptors belong to a family of ligand-gated cation-selective ion channels. Functionally, distinct nicotinic receptors are formed by assembly of subunits derived from at least 11 gene products, $\alpha 2$–$\alpha 9$ and $\beta 2$–$\beta 4$ (reviewed in: McGehee and Role, 1995; Lindstrom, 1997). Heterologous expression studies of recombinant subunits indicate that the $\alpha 2$–$\alpha 4$ subunits in combination with $\beta 2$ and $\beta 4$ subunits may form pentameric channels in a $(\alpha_x)_2(\beta_y)_3$ stoichiometry while the α_5 subunit participates in the formation of functional ion channels if coexpressed with both another α (non $\alpha 5$) and β subunit (Boulter et al., 1987; Ramirez-Latorre et al., 1996). In contrast, the $\alpha 7$–$\alpha 9$ subunits are capable of forming functional ion channels when expressed as homo-oligomers in *Xenopus* oocytes or in mammalian cell lines (Couturier et al., 1990; Elgoyhen et al., 1994; Gopalakrishnan et al., 1995).

The two major classes of nicotinic receptors identified in the brain using radioligand binding techniques include those with high affinity for [^{3}H]nicotine which corresponds to the $\alpha 4\beta 2$ nicotinic receptor subtype and those that recognize [^{125}I]α-bungarotoxin with high affinity that correspond to nicotinic receptors containing the $\alpha 7$ subunit (Lindstrom, 1997). Previous studies have indicated that chronic nicotine treatment increases the levels of high affinity [^{3}H]nicotine binding sites in the brain (Wonnacott, 1990). More recently, a number of mechanisms have been postulated to explain this phenomenon including decreased

Abbreviations: HEK-293, Human embryonic kidney 293

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receptor turnover, increased recruitment from a reserve pool and possibly involving protein kinases (Peng et al., 1994; Bencherif et al., 1995; Gopalakrishnan et al., 1997). Although nicotine has been shown to evoke upregulation of [^{125}I]α-bungarotoxin binding sites in the brain (Pauly et al., 1991), the nature and mechanisms underlying this phenomenon have been less well studied. It is now becoming increasingly clear that activation of $\alpha 7$ nicotinic receptors could have profound physiological consequences as revealed by the observations that this subtype desensitizes rapidly and displays a permeability to Ca^{2+} ions that is significantly higher than those observed for other ligand-gated ion channels (Couturier et al., 1990; Bertrand et al., 1992). Activation of $\alpha 7$ nicotinic receptors in mammalian cells can elevate intracellular Ca^{2+} concentrations to levels that has been shown to support cell survival in the absence of trophic factors (Franklin and Johnson, 1992; Delbono et al., 1997) and numerous studies have pointed out that influx of Ca^{2+} is of importance in processes such as neurite outgrowth and retraction, modulation of neurotrophic factors, cell survival and cytoprotection (Rathouz et al., 1996). A possible role for $\alpha 7$ nicotinic acetylcholine receptors in neuroprotection has been suggested by studies showing that $\alpha 7$ nicotinic receptor activation promotes a Ca^{2+}-mediated increase in spinal cord motor neuron survival (Messi et al., 1997), that the levels of neurotrophic factors can be modulated by α-bungarotoxin (Freedman et al., 1993) and that the neuroprotective properties of certain ligands observed in vitro and in vivo may be mediated through $\alpha 7$ receptors. (−)-Nicotine and other novel ligands with potential for the treatment of neurodegenerative diseases, such as (*S*)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole (ABT-418) and (2,4)-dimethoxybenzylidene anabaseine (GTS-21) have been shown to have neuroprotective properties in in vitro models of cytotoxicity (Donnelly-Roberts et al., 1996). However, little is known about the modulation of expression and function of the $\alpha 7$ nicotinic receptor subtype following long-term exposure to these ligands, especially in humans.

Thus, the objective of this study was to characterize human $\alpha 7$ nicotinic receptor dynamics following chronic treatment with nicotinic receptor ligands that differentially modulate receptor function using stably transfected HEK-293 cells as a well defined model system. Some of these results have appeared previously in the form of an abstract (Gopalakrishnan et al., 1996).

2. Materials and methods

2.1. Materials

[^{125}I]α-Bungarotoxin (110–120 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Methyllycaconitine citrate, (±)-epibatidine dihydrochloride and 1,1-dimethyl-4-phenyl piperazinium iodide were purchased from Research Biochemicals International (Natick, MA). (2,4)-dimethoxybenzylidene anabaseine (GTS-21) was synthesized at Abbott Laboratories (Abbott Park, IL). α-bungarotoxin was purchased from Molecular Probes (Eugene, OR) and (−)-nicotine was purchased from Sigma (St. Louis, MO). Cell culture media, fetal bovine serum, geneticin (G 418) and other antibiotics were obtained from Life Technologies (Grand Island, NY).

2.2. Cell culture and drug treatments

HEK-293 cells stably expressing human $\alpha 7$ nicotinic receptors (Doucette-Stamm et al., 1993) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 250 μg/ml geneticin in a humidified atmosphere (5% CO_2: 95% air) at 37°C. Briefly, this cell line was obtained as previously described (Gopalakrishnan et al., 1995; Delbono et al., 1997) following transfection of HEK-293 cells with the human $\alpha 7$ nicotinic receptor subunit, subcloned in the expression vector pRcCMV, followed by appropriate antibiotic selection and propagation. Previous studies from our laboratories have demonstrated stable expression of the $\alpha 7$ nicotinic receptors in these cells by [^{125}I]α-bungarotoxin binding, whole cell patch clamp and by intracellular Ca^{2+} imaging techniques. For radioligand binding experiments, cells were plated in 6-well culture dishes at a density of 2.5×10^5 cells/well and compounds were added 24 h after plating. For electrophysiologic studies, cells were plated on poly-L-lysine coated coverslips and compounds were added 24 h after plating. Cells were treated with varying concentrations of compounds for 120 h during which fresh media containing ligands was replaced every 48 h.

2.3. Radioligand binding

After chronic treatment with compounds, cells were rinsed twice with ice-cold binding buffer (composition, mM: KH_2PO_4, 50; EDTA, 1 and phenylmethylsulfonyl fluoride, 0.1; pH 7.4 at 25°C), mechanically disaggregated and homogenized using a polytron for 10 s. The homogenate was centrifuged at $45\,000 \times g$ for 20 min at 4°C and the pellet washed two times by repeated centrifugation to remove any residual drug as described previously (Gopalakrishnan et al., 1997) and finally resuspended in ice-cold buffer for use in [^{125}I]α-bungarotoxin binding studies. Cell membranes (50–60 μg protein/assay tube) were incubated in a final volume of 250 μl with 5 nM radioligand for 120 min at 37°C. Specific binding was defined by the addition of 1 μM unlabeled α-bungarotoxin to a duplicate set of tubes. For saturation studies, increasing concentrations of [^{125}I]α-bungarotoxin (100 pM–2.4 nM) were employed. Incubations were terminated by rapid vacuum filtration through GF/B glass fiber filters presoaked in 0.5% polyethyleneimine and filters washed four times with 2.5 ml of ice-cold buffer. Bound radioac-

tivity was quantitated by gamma counting (80% efficiency) and protein determined using bovine serum albumin as the standard (Lowry et al., 1951).

2.4. Electrophysiology

Cells were voltage-clamped using the whole-cell configuration of the patch-clamp with an Axopatch-200A amplifier (Axon Instruments, Foster City, CA) using standard techniques (Hamill et al., 1981) as previously described (Delbono et al., 1997). Coverslips were mounted in a small flow-through Lucite chamber positioned on the stage of an Axiovert 135 microscope (Zeiss, Germany). The recording chamber was provided with a central hole to allow direct access to the plated cells. A coverslip was fixed to the recording chamber by means of a Vaseline ring in such a way that only one coverslip was interposed between the cells and the microscope objective (100×, NA 1.3, Fluar, Zeiss). Continuous cell perfusion with a bathing external solution was done with a push–pull syringe pump (WPI, Sartoga, FL). Micropipettes were pulled from borosilicate glasses (Boralex) using a Flaming–Brown micropipette puller (P97, Sutter Instrument, Novato, CA) to obtain electrode resistance ranging from 2–4 MΩ. The composition of the internal (pipette) solution was (mM): 120 KF, 20 KCl, 2 $MgCl_2$, 0.1 EGTA, 10 HEPES, pH 7.4 adjusted with KOH. The composition of the external solution was (mM): 120 NaCl, 5 KCl, 2 $MgCl_2$, 10 HEPES, 25 D-glucose, pH 7.4 adjusted with NaOH. Whole cell currents were acquired at 5 Hz and filtered at 2 Hz using the pClamp software 6.0 (Axon Instruments) run on a personal computer. (−)-Nicotine (100 μM) was delivered by puffing the contents of a reservoir with a Harvard syringe pump through one barrel of a theta-tube micropipette as previously described (Delbono et al., 1997). Digidata 1200 interface (Axon Instruments) and a Hewlett-Packard 1300T Optical disk drive were used for A–D conversion and data storing respectively. Before recording, cells were washed out to remove any residual ligand by rinsing the coverslips five times with fresh bathing solution.

2.5. Data analysis and statistics

The binding parameters of [^{125}I]α-bungarotoxin were determined from analysis of saturation binding isotherm using a nonlinear curve fitting program (LIGAND; Munson and Rodbard, 1980). Significant differences between groups of means were assessed by the unpaired Student's *t*-test using Instat (Graphpad Software, San Diego, CA). When comparing multiple group means, one-way analysis of variance (ANOVA) was employed. The EC_{50} values, wherever reported, were determined by nonlinear least-squares regression analysis using Inplot (GraphPad Software, San Diego, CA). In cases where plateau responses were not attained, the EC_{50} values were estimated. Values are expressed as mean ± S.E.M. unless otherwise indicated.

3. Results

3.1. Upregulation of human α7 nicotinic receptors by activator ligands

Treatment of HEK-293 cells stably expressing the human α7 nicotinic receptors with (−)-nicotine for 120 h resulted in significant increases in the levels of [^{125}I]α-

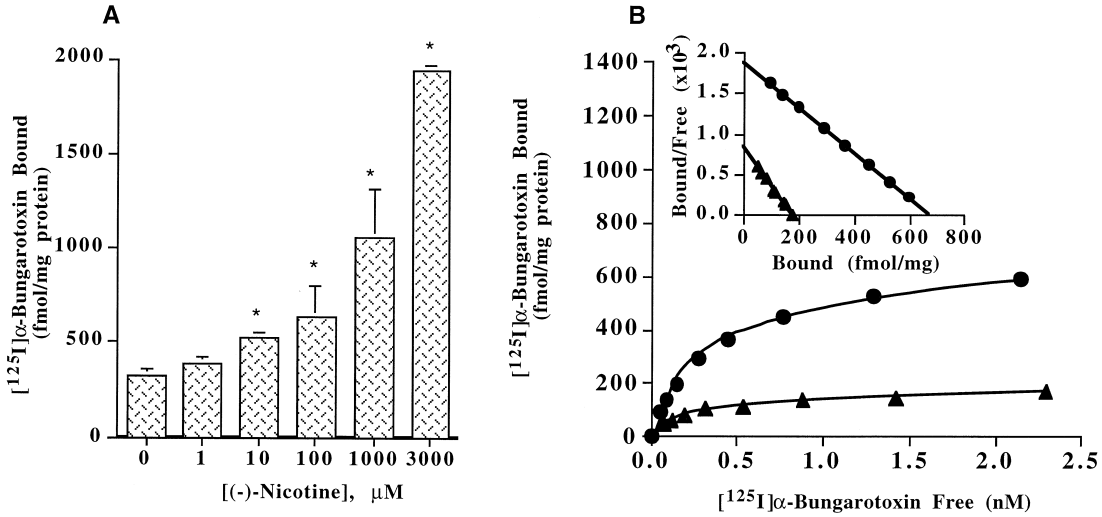

Fig. 1. Effect of chronic (−)-nicotine treatment on [^{125}]α-bungarotoxin binding in HEK-293 cells expressing human α7 nicotinic receptors. (A) Concentration-dependence of upregulation of [^{125}I]α-bungarotoxin binding. Cells were treated with varying concentrations of (−)-nicotine for 120 h following which radioligand binding was performed as discussed in Section 2. * represent values significantly ($P < 0.05$) different from untreated cells. (B) Saturation analysis of [^{125}I]α-bungarotoxin binding to cells treated with vehicle (▲) or 1 mM (−)-nicotine (●). Inset: Scatchard analysis of specific binding data.

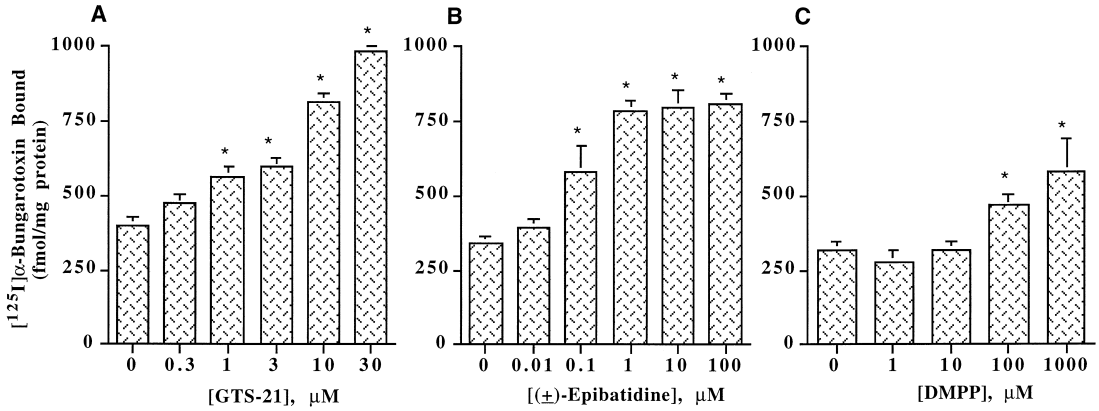

Fig. 2. Concentration-dependence of upregulation of [^{125}I]α-bungarotoxin binding in HEK-293 cells transfected with human $\alpha 7$ nicotinic receptor. Cells were treated with varying concentrations of (2,4)-dimethoxybenzylidene anabaseine (GTS-21) (A), (±)-epibatidine (B) or 1,1-dimethyl-4-phenyl piperazinium iodide (C) as indicated for 120 h. [^{125}I]α-bungarotoxin (5 nM) binding to membranes were performed as described under Section 2. ∗represent values significantly ($P < 0.05$) different from untreated cells.

bungarotoxin binding. Fig. 1A shows the concentration dependency of this process. Significant increase in binding sites were observed following treatment with 10 μM (−)-nicotine and an increase of about 6-fold was observed at the maximal concentration of (−)-nicotine (3000 μM) tested. Since the binding levels observed following treatment of (−)-nicotine continues to increase even at the highest concentrations, the EC_{50} values had to be estimated. The estimated EC_{50} value for upregulation by (−)-nicotine was 970 ± 290 μM ($n = 3$). Under the present treatment conditions, (−)-nicotine (1 and 3 mM) did not alter the pH of the media in which the cells were incubated in and did not alter the proliferation of cells. A similar range of (−)-nicotine concentrations have been used previously in studies with human neuroblastoma SH-SY5Y cells (Peng et al., 1997). Saturation experiments performed using a wide range of radioligand concentrations showed that (−)-nicotine treatment resulted in a significant increase in the B_{max} value of [^{125}I]α-bungarotoxin binding sites in transfected cells (nicotine 1000 μM, 756 ± 40 fmol/mg; control, 298 ± 50 fmol/mg; $n = 3$; Fig. 1B) with no significant change in the K_d value of the ligand (nicotine, 0.34 ± 0.05 nM; control, 0.23 ± 0.04 nM; $n = 3$).

To determine whether the increase in receptor levels were unique to (−)-nicotine, studies were carried out

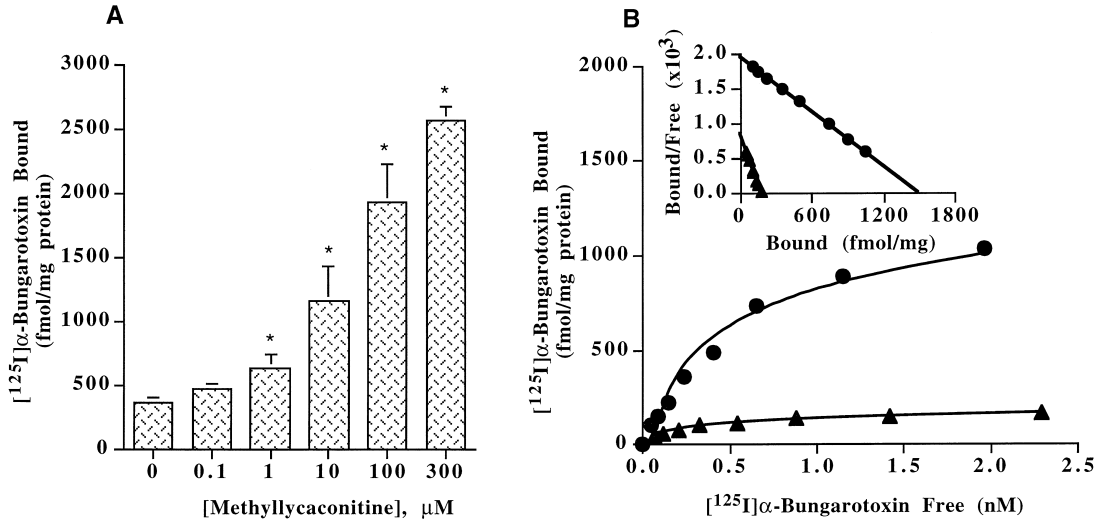

Fig. 3. Effect of chronic methyllycaconitine treatment on [^{125}I]α-bungarotoxin binding in HEK-293 cells expressing human $\alpha 7$ nicotinic receptors. (A) Concentration-dependence of upregulation of [^{125}I]α-bungarotoxin binding. Cells were treated with varying concentrations of methyllycaconitine for 120 h following which radioligand binding was performed as discussed in Section 2. ∗represent values significantly ($P < 0.05$) different from untreated cells. (B) Saturation analysis of [^{125}I]α-bungarotoxin binding to cells treated with vehicle (▲) or 30 μM MLA (●). Inset: Scatchard analysis of specific binding data.

using other activator ligands including (±)-epibatidine, (2,4)-dimethoxybenzylidene anabaseine (GTS-21) and 1,1-dimethyl phenyl piperazinium. As shown in Fig. 2, concentration-dependent upregulation of [^{125}I]α-bungarotoxin binding was also observed following treatment with these ligands. The EC_{50} values for (±)-epibatidine, (2,4)-dimethoxybenzylidene anabaseine and 1,1-dimethyl phenyl piperazinium were 89 ± 7 nM, 4 ± 1 μM and > 1000 μM respectively and the maximal increase in [^{125}I]α-bungarotoxin binding was 139 ± 3%, 146 ± 8% and 83 ± 35%, respectively over control values. At the maximal concentrations tested, the increases in binding levels observed with these ligands were considerably lower than those attained by (−)-nicotine.

3.2. Upregulation of human α7 nicotinic receptors by methyllycaconitine.

Treatment of cells with the α7 nicotinic receptor selective antagonist, methyllycaconitine, also elicited a concentration-dependent upregulation of [^{125}I]α-bungarotoxin binding levels. As shown in Fig. 3A, significant increases in [^{125}I]α-bungarotoxin binding levels were evoked by 1 μM methyllycaconitine and at the maximal concentration tested (300 μM), an increase of about 7-fold was attained. The estimated EC_{50} value of upregulation by methyllycaconitine was 92 ± 44 μM ($n = 3$). Saturation experiments revealed that treatment of cells with methyllycaconitine (30 μM) evoked a significant increase of about 4.7 fold in the B_{max} value of [^{125}I]α-bungarotoxin binding (1692 ± 145 fmol/mg vs. control, 298 ± 50 fmol/mg; Fig. 3B). The ligand binding affinity in cells treated with methyllycaconitine was 0.86 ± 0.14 nM ($n = 3$) which is about 2.5-fold higher than those observed in untreated cells. Although the membrane preparation was extensively washed prior to performing the assay, it is possible that residual MLA present, if any, could contribute to a shift in the affinity of the radioligand.

Table 1
Whole cell current responses in α7-nicotinic receptor expressing HEK-293 cells following chronic treatment of cells with (−)-nicotine or methyllycaconitine

Compound	Current amplitude (pA)	*n*
(−)-Nicotine (μM)		
Control	275 ± 21	50
1.0	320 ± 25	15
10	405 ± 17[a]	15
100	495 ± 18[a]	15
1000	505 ± 21[a]	15
Methyllycaconitine (μM)		
Control	259 ± 27	50
0.001	421 ± 36[a]	12
0.01	526 ± 41[a]	12
0.1	539 ± 45[a]	12
10	1820 ± 137[a]	12

Values of current amplitude are expressed as mean ± S.D.; [a]Represent values significantly ($P < 0.01$) different from the corresponding control amplitude values. Cells were incubated with varying concentrations of (−)-nicotine or methyllycaconitine as indicated for 120 h following which compounds were washed out and current responses were evoked by application of 100 μM (−)-nicotine as described in Section 2.

3.3. Modulation of functional activity of α7 nicotinic receptors

Whole cell patch clamp studies were performed to evaluate the functional properties of α7 nicotinic receptors following chronic treatment with (−)-nicotine or methyllycaconitine. Functional responses were evaluated by fast application of 100 μM (−)-nicotine, a concentration that evokes near maximal current response in these cells (Gopalakrishnan et al., 1995; Delbono et al., 1997). As shown in Fig. 4A, agonist-evoked whole cell current responses were potentiated following chronic treatment of cells with (−)-nicotine. The effects on current amplitude were concentration-dependent (Table 1). When treated with 1 mM (−)-nicotine, the whole cell current responses were

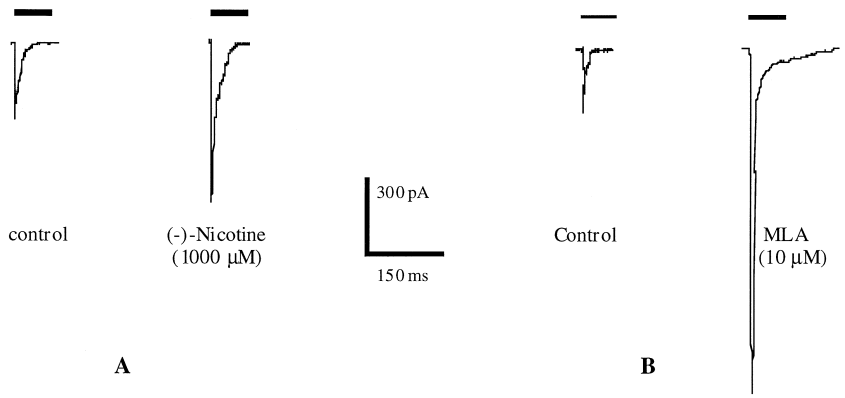

Fig. 4. Whole cell current responses to (−)-nicotine in HEK 293 cells stably expressing the human α7 nicotinic receptors following chronic treatment of cells with (−)-nicotine (1000 μM) (A) or methyllycaconitine (10 μM) (B) for 120 h. Cells were voltage-clamped at a holding potential of −100 mV and (−)-nicotine (100 μM) was perfused for 75 ms (indicated by horizontal bars) through a barrel theta tube mounted in a piezoelectric device as indicated in Section 2.

enhanced about 2-fold compared to untreated cells. Under similar conditions, cells treated chronically with methyllycaconitine (10 μM) showed an almost 6-fold increase in whole cell current amplitude responses. The concentrations of (−)-nicotine (1000 μM) and methyllycaconitine (10 μM) employed in this case elicited comparable levels of $\alpha 7$ nicotinic receptor (3.5-fold), as revealed by [^{125}I]α-bungarotoxin binding, (Fig. 1A and Fig. 3A).

4. Discussion

Using recombinant human $\alpha 7$ nicotinic receptors expressed stably in HEK-293 cells, our studies present evidence that the expression and function of this nicotinic receptor subtype can be upregulated both by (−)-nicotine and other cholinergic channel activators including 1,1-dimethyl phenyl piperazinium, (±)-epibatidine and (2,4)-dimethoxybenzylidene anabaseine (GTS-21) and, by methyllycaconitine, a selective $\alpha 7$ nicotinic receptor antagonist. (−)-Nicotine or methyllycaconitine-evoked increase in [^{125}I]α-bungarotoxin binding densities were accompanied by differential enhancements in nicotinic receptor function as assessed by agonist-evoked peak whole cell current amplitudes.

Our present studies confirm previous findings, both in vivo and in vitro, that chronic (−)-nicotine treatment results in increased levels of [^{125}I]α-bungarotoxin binding sites (Pauly et al., 1991; Barrantes et al., 1995; Peng et al., 1997). In addition, we also show upregulation by other cholinergic channel activators and by the selective $\alpha 7$ antagonist, methyllycacontine, suggesting that effects are not unique to (−)-nicotine and could be evoked by both activator and antagonist ligands. Previous studies in hippocampal neurons have provided preliminary evidence for the modulation of [^{125}I]α-bungarotoxin binding sites following chronic exposure to nicotine (Barrantes et al., 1995). The increase in binding sites observed in our study with 10 μM (−)-nicotine (58%) is comparable to those reported in rat hippocampal cultures treated with a similar concentration of (−)-nicotine for 14 days (40%). The observation that (−)-nicotine-evoked up-regulation of $\alpha 7$ nicotinic receptors occurs in a kidney cell line indicates the lack of participation of any transcriptional factors or receptor gene promoter elements that are likely associated with the expression of these subunits in intact neurons that mediate the upregulation process. The EC_{50} value for upregulation of the human $\alpha 7$ receptors by (−)-nicotine is some 2000-fold higher than those observed with the $\alpha 4\beta 2$ receptors expressed in HEK-293 cells (0.5 μM, Gopalakrishnan et al., 1997) or in M10 cells (0.2 μM; Peng et al., 1994). This is consistent with previous in vivo studies where the doses of (−)-nicotine required to upregulate [^{125}I]α-bungarotoxin sites were higher than those required to upregulate [^{3}H]nicotine binding sites (Pauly et al., 1991). More recently, studies in human neuroblastoma SH-SY5Y cells have also shown that high concentrations of (−)-nicotine (1 mM) are required to upregulate $\alpha 7$ nicotinic receptors (Peng et al., 1997). This difference in sensitivity of nicotine in upregulating the $\alpha 7$ and $\alpha 4\beta 2$ nicotinic receptor subtypes may be related to the differences in binding affinities of (−)-nicotine towards these subtypes ($\alpha 7$, 1.6 μM vs. $\alpha 4\beta 2$, 1 nM). It should also be pointed out that the magnitude of increase in [^{125}I]α-bungarotoxin binding was about 6-fold after treatment of cells with (−)-nicotine 3000 μM. Whereas, at the human $\alpha 4\beta 2$ nicotinic receptors, an increase of about 15-fold in the levels of $\alpha 4\beta 2$ nicotinic receptor binding sites was observed with (−)-nicotine 10 μM (Gopalakrishnan et al., 1997). The significant increase in B_{max} values following nicotine treatment is indicative of an increase in receptor levels in membrane preparations. However, since the [^{125}I]α-bungarotoxin binding protocol employed does not distinguish between nicotinic receptors present on cell surface or in intracellular membrane components, further studies will be necessary to address the issue of intracellular vs. cell surface localization of the upregulated nicotinic receptors.

(±)-Epibatidine binds to the $\alpha 7$ receptor with a K_i of 21 nM and is a potent agonist ($EC_{50} = 3$ μM) at the $\alpha 7$ homo-oligomeric receptors whereas GTS-21 is at least 10-fold weaker in displacing binding ($K_i = 652$ nM) than (±)-epibatidine and is a very weak partial agonist at the human $\alpha 7$ receptor (Briggs et al., 1995). Treatment of transfected cells with GTS-21 or with (±)-epibatidine elicited about 1.5 to 2-fold increase in binding levels. Although the upregulation of [^{125}I]α-bungarotoxin binding sites evoked by (±)-epibatidine or GTS-21 at the highest concentrations tested were lower than those evoked by (−)-nicotine, the effects were observed at 1 μM, a concentration that did not evoke any significant effect in the case of (−)-nicotine. It thus appears that the potencies, but not the efficacies, for evoking receptor upregulation may be related to the ligand binding affinities, a paradigm previously observed at the $\alpha 4\beta 2$ nicotinic receptor subtype (Gopalakrishnan et al., 1997). However, as observed previously with the $\alpha 4\beta 2$ subtype, the concentrations required to evoke upregulation are higher than those for receptor binding or function. For example, (−)-nicotine has a binding affinity of 1.6 μM and activates the $\alpha 7$ receptor with an EC_{50} of 49 μM (Gopalakrishnan et al., 1995), but the EC_{50} value for receptor upregulation is about 970 μM. The lack of 1:1 correlation between the concentrations for ligand binding or receptor activation to those that result in upregulation indicates involvement of other mechanisms in the upregulation process.

Methyllycaconitine displaces [^{125}I]α-bungarotoxin binding with high affinity ($K_i \cong 10$ nM) and blocks homomeric $\alpha 7$ nicotinic receptors at nanomolar concentrations (Gopalakrishnan et al., 1995). Chronic treatment of cells with methyllycaconitine resulted in a concentration-dependent upregulation of [^{125}I]α-bungarotoxin binding sites

(EC_{50} = 92 μM) with a significant increase at 1 μM and a maximal 6-fold increase at 300 μM. Although at micromolar concentrations methyllycaconitine could interact with other nicotinic receptor subtypes, the effects reported here are mediated via interactions at the $\alpha 7$ nicotinic receptors since the recombinant receptors have been stably expressed in a well defined heterologous system. The observation that methyllycaconitine, like (−)-nicotine, also elicited upregulation indicates that nicotinic receptor activation and/or ion flow through the channel are not prerequisites for ligand-evoked upregulation. This situation is analogous to the findings at the $\alpha 4\beta 2$ nicotinic receptor subtype where antagonists including dihydro-β-erythroidine and D-tubocurarine, like (−)-nicotine, were capable of evoking receptor upregulation (Gopalakrishnan et al., 1997). Further, the observation that upregulation is observed regardless of whether (−)-nicotine or methyllycaconitine were used for chronic treatment indicates that receptor occupancy, either by an activator or an antagonist ligand, may be sufficient to trigger this increase in receptor levels. This process may involve post-translational mechanisms, as for example, persistent receptor occupation and stabilization due to ligand binding. Previous studies in SH-SY5Y cells have shown that the levels of mRNA are not altered after nicotine treatment (Peng et al., 1997).

The increase in binding sites in transfected cells following chronic ligand treatments was accompanied by an increase in agonist-evoked functional responses (Fig. 4). The potentiation of the macroscopic current could be associated with an increase in channel density as indicated by the observed increase in the B_{max} value without changes in ligand affinities (Fig. 1BFig. 3B). It is interesting to note that treatment of cells with concentrations of (−)-nicotine (1000 μM) and methyllycaconitine (10 μM) that elicited equivalent degree of upregulation of binding sites (about 3.5-fold) resulted in almost 2-fold and 6-fold enhancement respectively in peak current amplitudes compared to untreated cells. This differential modulation of nicotinic receptors by activator and antagonist ligands may be related to the fact that although the magnitude of upregulation by (−)-nicotine or methyllycaconitine are similar, those nicotinic receptors upregulated by (−)-nicotine could predominantly be in a desensitized state and thus may not effectively coordinate agonist-evoked response, in contrast to those nicotinic receptors upregulated by methyllycaconitine. The increase in $\alpha 7$ receptor function following (−)-nicotine treatment differs from observations at the $\alpha 4\beta 2$ receptors where there is an increase in function at lower concentrations of (−)-nicotine (0.1 and 1 μM) and a persistent decrease in function predominantly at higher concentrations (100 and 1000 μM; Gopalakrishnan et al., 1997). It is possible that much higher concentrations of (−)-nicotine may be required for functional downregulation especially in view of the fact that the EC_{50} for upregulation of $\alpha 7$ receptors is some 2000-fold higher than those at the $\alpha 4\beta 2$ subtype.

$\alpha 7$ Nicotinic receptors has been shown to be regulated by a number of factors including persistent neuronal activity and membrane depolarization (Geertsen et al., 1988; De Koninck and Cooper, 1995; Broide et al., 1996), by neuron-glia interactions (Jensen et al., 1997) and by chronic nicotine treatment. The present study provides direct evidence that the recombinant $\alpha 7$ nicotinic receptors in a heterologous expression system could be upregulated in number and function following chronic treatment with both activator and antagonist nicotinic receptor ligands. This upregulation of $\alpha 7$ receptors by both activator and antagonist ligands is similar to those previously observed at the $\alpha 4\beta 2$ nicotinic receptor subtype and may share common mechanisms, as for example, involving second messenger pathways. Previous studies have shown that changes in membrane excitability such as persistent depolarization could upregulate α-bungarotoxin binding sites (De Koninck and Cooper, 1995; Geertsen et al., 1988; Quik et al., 1997). In rat sympathetic neurons, chronic membrane depolarization increased $\alpha 7$ RNA levels and cell surface $\alpha 7$ receptor expression, but interestingly had no effect on the magnitude or kinetics of acetylcholine-evoked currents (De Koninck and Cooper, 1995). Studies with the $\alpha 4\beta 2$ nicotinic receptor subtype have suggested mechanisms including decreased receptor turnover, increased recruitment from reserve pool and perhaps involving protein kinase A to explain nicotinic receptor upregulation (Geertsen et al., 1988; Peng et al., 1994; Bencherif et al., 1995; Gopalakrishnan et al., 1997). The intracellular domain of the $\alpha 7$ subunit containing a conserved consensus sequence for protein kinase A (Ser-342) has been shown to be specifically phosphorylated by cAMP-dependent protein kinase and this event has been suggested to play a role in mediating cellular regulation of neuronal nicotinic receptors containing the $\alpha 7$ subunit (Moss et al., 1996).

The physiological significance of $\alpha 7$ nicotinic receptor upregulation following prolonged exposure to levels of nicotine or nicotinic ligands as in heavy smoking or following potential long-term treatment with selective cholinergic channel modulators remains to be assessed; however, it is conceivable that any such changes by continued ligand exposure, especially at lower concentrations, could modulate fast synaptic transmission and neuronal plasticity involving nicotinic receptors containing the $\alpha 7$ nicotinic receptor subunit. Although the levels of nicotine present in the serum of smokers (0.15 μM; Benowitz, 1996) cannot be strictly correlated with the concentration required for upregulation (significant effects observed only at 10 μM in our study), it is possible that a much longer duration of sustained exposure, typical in smokers, could eventually influence $\alpha 7$ receptor dynamics. Studies performed in vivo in animals have not consistently shown an upregulation of α-bungarotoxin binding sites (Pauly et al., 1991; Collins et al., 1994); such discrepancies could be related to varying doses of ligands administered and/or time of

chronic treatment. More recent studies have shown that neuroprotective properties observed in vitro following chronic treatment with subtype-selective nicotinic receptor ligands such as 2-methyl-3-(2-(*S*)-pyrrolidinylmethoxy)pyridine dihydrochloride (ABT-089) appear to be mediated via an interaction with nicotinic receptors containing the $\alpha 7$ subunit and it has been suggested that possible upregulation of $\alpha 7$ nicotinic receptors and consequent changes in intracellular calcium levels may trigger changes in the levels of neurotrophic factors, transcriptional factors or genes that regulate apoptotic processes (Sullivan et al., 1997). Indeed, chronic exposure of rat spinal cord motor neuron cultures to (−)-nicotine has been shown to promote cell survival in a manner related to the levels of intracellular Ca^{2+} triggered through activation of the $\alpha 7$ nicotinic receptors (Messi et al., 1997).

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