Functional expression of human α_7 nicotinic acetylcholine receptor in human embryonic kidney 293 cells

YUAN GONG, JI-HONG JIANG and SHI-TONG LI

Department of Anesthesiology, First People's Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200030, P.R. China

Received July 5, 2015; Accepted June 27, 2016

DOI: 10.3892/mmr.2016.5493

Abstract. The functional expression of recombinant α_7 nicotinic acetylcholine receptors in human embryonic kidney (HEK) 293 cells has presented a challenge. Resistance to inhibitors of cholinesterase 3 (RIC-3) has been confirmed to act as a molecular chaperone of nicotinic acetylcholine receptors. The primary objectives of the present study were to investigate whether the co-expression of human (h)RIC-3 with human α_7 nicotinic acetylcholine receptor in HEK 293 cells facilitates functional expression of the α_7 nicotinic acetylcholine receptor. Subsequent to transfection, western blotting and polymerase chain reaction were used to test the expression of α7 nicotinic acetylcholine receptor and RIC-3. The α_7 nicotinic acetylcholine receptor was expressed alone or co-expressed with hRIC-3 in the HEK 293 cells. Drug-containing solution was then applied to the cells via a gravity-driven perfusion system. Calcium influx in the cells was analyzed using calcium imaging. Nicotine did not induce calcium influx in the HEK 293 cells expressing human α_7 nicotinic acetylcholine receptor only. However, in the cells co-expressing human RIC-3 and α_7 nicotinic acetylcholine receptor, nicotine induced calcium influx via the α_7 nicotinic acetylcholine receptor in a concentration-dependent manner (concentration required to elicit 50% of the maximal effect=29.21 μM). Taken together, the results of the present study suggested that the co-expression of RIC-3 in HEK 293 cells facilitated the functional expression of the α_7 nicotinic acetylcholine receptor.

Introduction

Nicotinic acetylcholine (ACh) receptors are members of the pentameric ligand-gated ion channel superfamily, and are expressed at neuromuscular junctions and within the central and peripheral nervous system, where they are activated by nicotine and the endogenous neurotransmitter, ACh. A total of 17 nicotinic ACh receptor subunits have been identified in vertebrates (α_1-α_{10}, β_1-β_4, γ, δ and ε), and these subunits can assemble into a variety of heteropentameric and homopentameric receptors (1,2).

Of the nicotinic ACh receptor subtypes, the homopentameric α_7 nicotinic ACh receptor is known to be the most permeable to calcium ions (Ca^{2+}). Calcium influx through the α_7 nicotinic ACh receptor is involved in increasing cytoplasmic calcium levels, which in turn triggers a series of calcium-dependent intracellular processes. Following the suggestion that the α_7 nicotinic ACh receptor regulates inflammation, it has been the focus of intense investigation since the early 21st century (3). Consequently, there has been substantial interest in the identification and characterization of the α_7 nicotinic ACh receptor.

However, the expression of functional recombinant α_7 nicotinic ACh receptors in mammalian cell types, including human embryonic kidney (HEK) 293 cells, has been problematic, as the assembly of the α_7 nicotinic ACh receptor is a slow and inefficient process. Individual subunits require appropriate transmembrane topology and undergo a series of critical post-translational modifications (4). In addition, to enable folding into the correct conformation, the receptors require appropriate inter-subunit interactions. The early steps of receptor folding and assembly occur within the endoplasmic reticulum, an intracellular compartment containing several proteins required for efficient protein folding and post-translational modification (4). Although there have been reports of the successful functional expression of the recombinant α_7 nicotinic ACh receptor in certain mammalian cell lines (5-8), measurable levels of functional receptors have been difficult to achieve in several cell types. This effect appears to be host-cell dependent (9,10), as functional α_7 nicotinic ACh receptors can be generated in mammalian cell lines when co-expressed with either *Caenorhabditis elegans* resistance to inhibitors of cholinesterase 3 (CeRIC-3) or its human homolog (hRIC-3).

To the best of our knowledge, the functional expression of recombinant human α_7 nicotinic ACh receptors in HEK 293 cells co-expressing hRIC-3 has not been reported. In the present study, heterologously expressed nicotinic ACh receptors in HEK 293 cells were investigated and the functional expression levels of recombinant α_7 nicotinic ACh receptors in the HEK 293 cells were examined, in order to aid in the development of novel pharmaceutical agents.

Correspondence to: Professor Shi-Tong Li, Department of Anesthesiology, First People's Hospital, School of Medicine, Shanghai Jiaotong University, 100 Hai-Ning Road, Shanghai 200030, P.R. China
E-mail: woodyyc@hotmail.com

Key words: resistance to inhibitors of cholinesterase 3, human α_7 nicotinic acetylcholine receptors, calcium imaging

Materials and methods

Drugs. The drugs used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. Fluo-4 AM (1 mM) and Pluronic® F-127 (5%) were prepared in dimethyl sulfoxide and stored at -20˚C. All solutions were prepared and diluted appropriately prior to experimentation.

Cell culture and transfection. Transfection was performed, as described previously (10). Expression plasmids (hRIC-3 and hα_7) containing complementary DNA sequences for hRIC-3 and α_7 nicotinic ACh receptor subunits, respectively, were used. The subunits were subcloned into pcDNA3.1$^+$ (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). HEK 293 cells were cultured at 2x10^4 cells/ml in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator. The medium was renewed every 3 days. The HEK 293 cells were then transfected with the expression plasmids using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The transfected cells were incubated for 24 h prior to obtaining recordings.

Immunohistochemistry. The transfected HEK 293 cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai) were grown on glass chamber slides. The cells were washed twice, (5 min for each wash) in 0.05 M phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. Following washing five times with 0.05 M PBS, the cells were incubated in a blocking solution [10% normal goat serum (Sigma-Aldrich) and 1% Triton™ X-100 in PBS] for 1 h at room temperature. The cells were then incubated for 4 h at 4˚C with rabbit anti-human α_7 nicotinic ACh receptor polyclonal antibody (cat. no. sc-5544; Santa-Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a dilution of 1:500 in 0.05 M PBS containing 1% Triton™ X-100 and 2% normal goat serum. The slides were then washed with 0.05 M PBS four times, (5 min for each wash). The washed sections were then incubated for 2 h at 37˚C with 5% CO_2 with a secondary antibody (goat anti-rabbit Alexa Fluor 488; Molecular Probes, Invitrogen; Thermo Fisher Scientific, Inc.) at a dilution of 1:1,000 in 10% normal goat serum/PBS/Triton™ X-100 solution. The cells were then washed with 0.05 M PBS four times (5 min for each wash) prior to incubation with 4',6-diamidino-2-phenylindole (1:15,000) for 5 min. The slides were stored at 4˚C until further use. The cells were visualized with the fluorescence microscope (Leica DMI4000 B; Leica Microsystems GmbH, Wetzlar, Germany) and an optical microscope (BX51, U-TV0.5XC-3; Olympus Corporation, Tokyo, Japan) and camera (DFC320; Leica Microsystems GmbH).

Western blot analysis. Cell lysates were prepared by incubating the HEK 293 cells with lysis buffer, containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.4), 0.02% NaN_3, 1% Triton™ X-100 and protease inhibitor cocktail, on ice for 90 min. Protein concentrations were determined using the Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Equal quantities (5 μg) of total protein were subjected to 12% SDS-polyacrylamide electrophoresis and were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline prior to western blot analysis. The membranes wer incubated with the rabbit anti-human α_7 nicotinic ACh receptor polyclonal primary antibody (1:2,000; cat. no. sc-5544; Santa-Cruz Biotechnology, Inc.) at 4˚C overnight, followed by incubation at 37˚C for 2 h with the horseradish peroxidase-conjugated secondary antibodies (1:5,000; ab6721; Abcam, Cambridge, MA, USA). The signals were detected using enhanced chemiluminescence reagent (EMD Millipore). The expression of each target protein was relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and was calculated based on the grey level.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was prepared from the *in vitro* HEK 293 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-PCR analysis was performed using a PrimeScript RT reagent kit and SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. The reaction mixture (25 μl) was comprised of 2X Subgreen mix (12.5 μl), forward and reverse primers (each 10 μM; 1 μl), cDNA (1 μg; 0.5 μl) and diethylpyrocarbonate-treated ddH_2O (10 μl). The primer sets for reverse transcription were as follows: RIC-3, forward 5'-TTC AGACTGTATCAAGCGTAGGC-3' and reverse 5'-TGGATC ACACGAGGTAACAGAA-3'; GAPDH, Forward 5'-ACAACT TTGGTATCGTGGAAGG-3' and reverse 5'-GCCATCACG CCACAGTTTC-'3. Cycling was conducted using and ABI 7900 cycling machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the conditions were as follows: 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec.

Calcium imaging. Changes in cytosolic free calcium concentration were measured using fluorescence imaging with the Ca^{2+}-sensitive dye, Fluo-4. The transfected HEK 293 cells were treated with 2 μM Fluo-4 AM for 30 min at 37˚C, in a medium containing 120 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM glucose and 10 mM HEPES (pH 7.3, adjusted with Tris) prior to imaging. Following treatment with the dye, the cells were observed under an inverted microscope (Leica DMI4000 B) and images were captured using a charge-coupled device camera (Leica DF350, Leica Microsystems GmbH), as shown in Fig. 1. The fluorescence intensities of individual cells in regions of interest were recorded and analyzed using Leica Advanced Florescence Application software (AF6000; Leica Microsystems GmbH). Nicotine (10, 30, 100, 300 and 1,000 nM) and adenosine diphosphate (ADP; 10 μM) were applied to the cells by gravity using a microperfusion apparatus. Between each drug application for 5 sec, a 15-min washout period with fresh medium was included to allow clearance of the drug.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Two-tailed unpaired Student's *t*-tests were used for all comparisons, unless otherwise indicated.

Results

Surface α_7 receptors are detected on the surface of HEK 293 cells expressing the human α_7 receptor and/or co-expressing

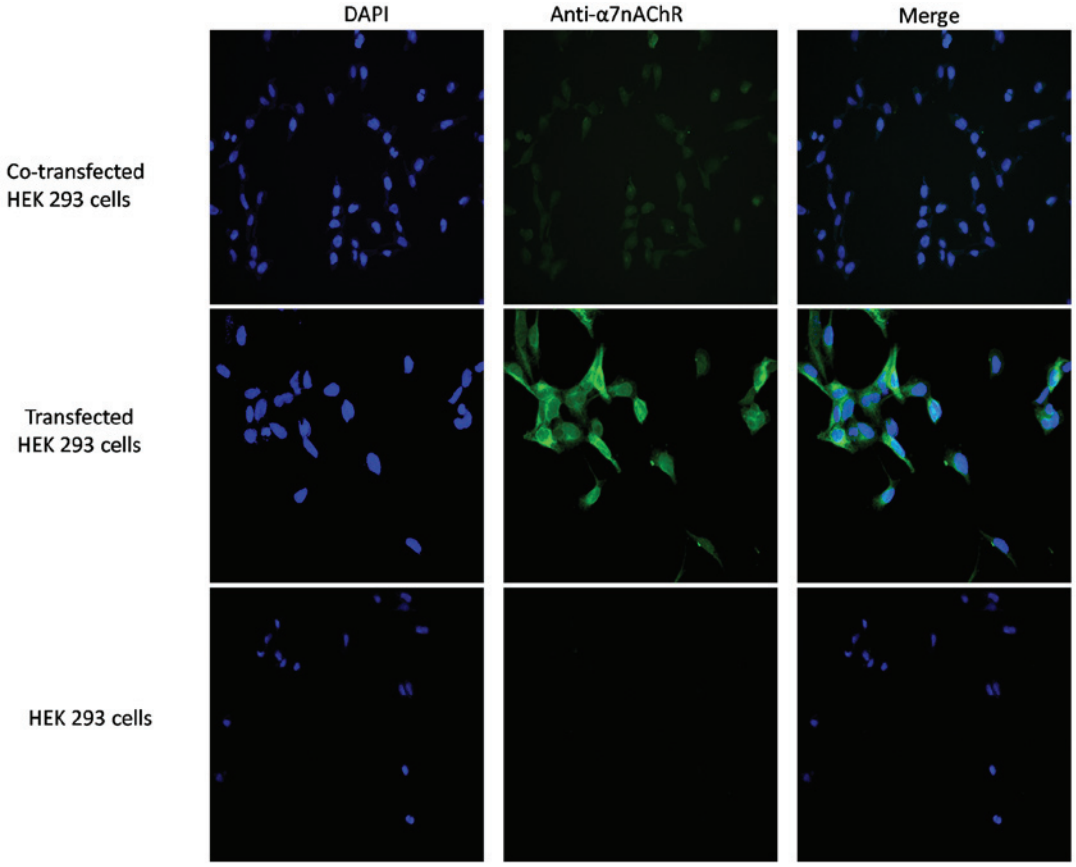

Figure 1. Immunostaining of α_7 protein on the surface of HEK 293 cells co-expressing the human α_7 receptor and hRIC-3. HEK 293 cells, transfected HEK 293 cells and co-transfected HEK 293 cells were fixed on slides and immunostained with rabbit anti-human α_7 nicotinic acetylcholine receptor polyclonal antibody. Images of the cells were then captured using a charge-coupled device and a camera fitted with a 40x objective lens. Human α7 nicotinic acetylcholine receptors were detected on transfected HEK 293 cells and co-transfected HEK 293 cells, however were not detected on HEK 293 cells. HEK, human embryonic kidney; hRIC-3, human resistance to inhibitors of cholinesterase 3; α_7nAChR, α_7 nicotinic acetylcholine receptor.

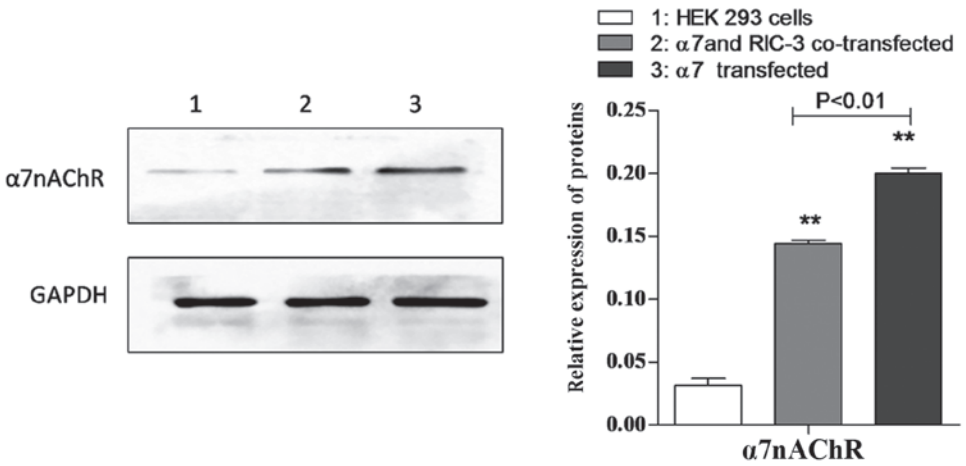

Figure 2. Protein expression levels of human α_7 in transfected and co-transfected HEK 293 cells. The protein expression of the α_7 subunit was examined using western blot analysis. Incubation with rabbit anti-human α_7nAChR polyclonal antibody revealed a clear band in the transfected HEK 293 cells and co-transfected HEK 293 cells. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. HEK 293 cells. HEK, human embryonic kidney; RIC-3, resistance to inhibitors of cholinesterase 3; α_7nAChR, α_7 nicotinic acetylcholine receptor.

hRIC-3. To detect the protein expression of α_7, HEK 293 cells were incubated and transfected, and the HEK 293 cells were treated with antibody directed against the α_7 protein. This was followed by incubation with a fluorescent-labeled secondary antibody. No discernible binding of the anti-α_7 antibody to the HEK 293 control cells was observed (Fig. 1). Immunostaining of the HEK 293 cells co-transfected with hRIC-3 revealed binding of anti-α_7 antibodies to the surface of the co-transfected cells, suggesting that these cells expressed α_7 nicotinic ACh receptors on their membrane surface.

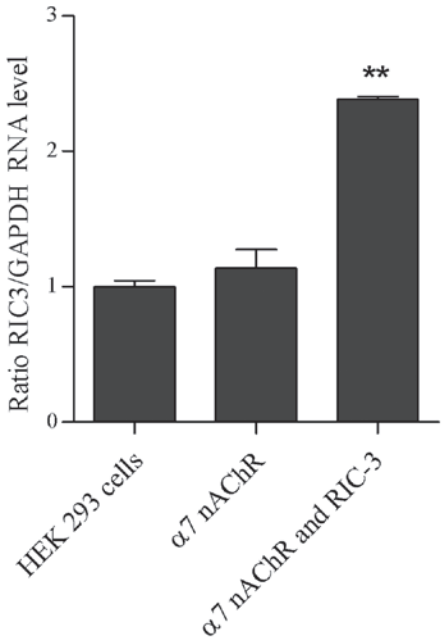

Figure 3. hric-3 transcripts are present in co-transfected HEK 293 cells, and absent in HEK 293 cells and transfected HEK 293 cells. Reverse transcription-polymerase chain reaction analysis was used to examine the levels of the hric3 transcript in HEK 293 cells, transfected HEK 293 cells and co-transfected HEK 293 cells. hric3 was detected in the co-transfected HEK 293 cells, whereas no hric-3 transcripts were detected in the HEK 293 cells and transfected HEK 293 cells. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. HEK 293 cells. HEK, human embryonic kidney; RIC3, resistance to inhibitors of cholinesterase 3; α_7nAChR, α_7 nicotinic acetylcholine receptor.

α_7 protein is detected in HEK 293 cells expressing human α_7 receptors and/or co-expressing hRIC-3. To detect the protein expression of α_7, the present study examined HEK 293 cells, transfected HEK 293 cells and co-transfected HEK 293 cells using western blot analysis with antibody against α_7 protein. As shown in Fig. 2, α_7 protein was detected in the transfected and co-transfected HEK 293 cells.

hric3 mRNA is expressed in co-transfected HEK 293 cells, and is absent in HEK 293 cells and transfected HEK 293 cells. RT-PCR analysis was used to examine the expression of hric3 in HEK 293 cells, transfected HEK 293 cells and co-transfected HEK 293 cells. As shown in Fig. 3, hric3 transcripts were detected in the co-transfected HEK 293 cells only; hric3 transcripts were not detected in the HEK 293 cells or HEK 293 cells transfected with α_7 nicotinic ACh receptor alone.

Nicotine does not induce calcium transients in HEK 293 cells expressing human α_7 receptors. Subsequently, to analyze the changes in the concentration of cytosolic free calcium induced by the opening of the α_7 nicotinic ACh receptors, HEK 293 cells expressing human α_7 receptors were treated with various concentrations of nicotine for 30 sec. Following 15 min of washout with fresh medium, the cells were treated with 10 μM ADP. Nicotine did not induce calcium influx in the HEK 293 cells expressing human α_7 receptors at any concentration (Fig. 4).

Nicotine induces calcium transients in HEK 293 cells co-expressing hRIC-3 and human α_7 receptors. The present study then analyzed the changes in the concentration of cytosolic free calcium induced by the opening of α_7 nicotinic ACh receptors in HEK 293 cells transiently co-expressing hRIC-3 and the human α_7 nicotinic ACh receptor. In these cells, high levels of functional α_7 nicotinic ACh receptors were expressed; the activity of these receptors has been confirmed in a previous study using whole-cell patch-clamp recording (7).

To assess the effect of nicotine on functional α_7 nicotinic ACh receptors, the co-transfected HEK 293 cells were treated with different concentrations of nicotine for 30 sec. Nicotine induced calcium influx in a concentration-dependent manner (Fig. 5). The data obtained were fitted to a logistic equation, and the nicotine concentration required to elicit 50% of the maximal response was calculated to be 29.42 μM, with a 95% confidence interval of 13.32-65.42 μM).

Discussion

Although the α_7 subunit is able to generate functional nicotinic ACh receptors when expressed in *Xenopus* oocytes, considerable difficulty has been encountered in the efficient expression of functional α_7 nicotinic ACh receptors in cultured mammalian cell lines (9,11-13). Previous studies (10,14) have demonstrated that α_7 can efficiently generate functional nicotinic ACh receptors in mammalian cell lines when co-expressed with either CeRIC-3 or its human homolog, hric-3. RIC-3 is required for efficient receptor folding, assembly and functional expression of homomeric α_7 nicotinic ACh receptors (15).

In the present study, the expression of functional recombinant nicotinic ACh receptors in HEK 293 cells was induced

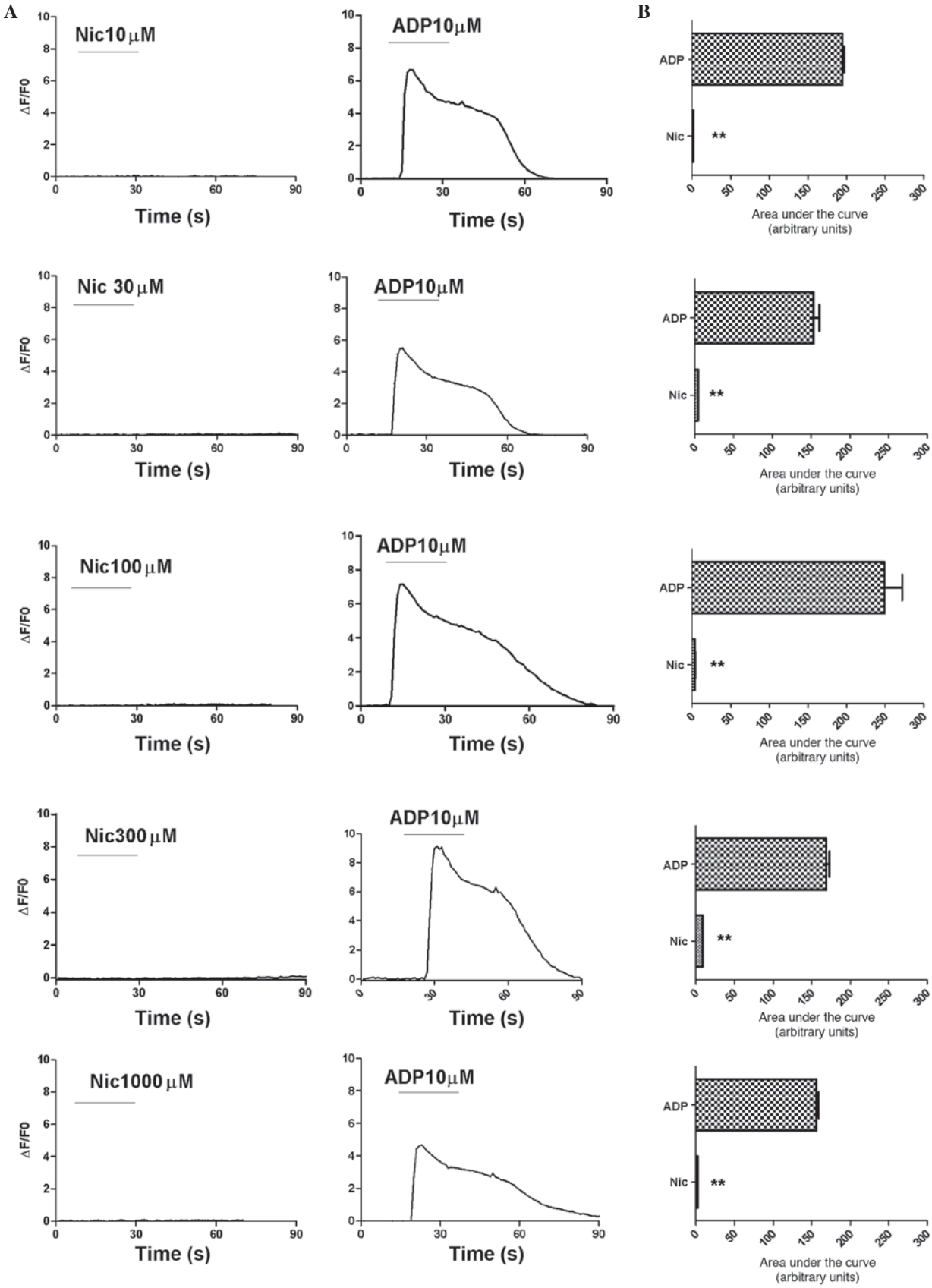

Figure 4. Nicotine does not induce calcium transients in transfected HEK 293 cells. Fluo-4-loaded transfected HEK 293 cells were observed under an inverted microscope (Leica DMI4000 B) and images were captured using a charge-coupled device camera (Leica DF350). Changes in the fluorescence intensity of the fluo-4 images were normalized to the intensity of the first image (ΔF/F0). (A) Nicotine did not induce calcium transients at any concentration, however, 100 μM ADP induced calcium transients in the HEK 293 cells. (B) Statistical evaluation of the data in the graphs in (A). Data are presented as the mean ± standard error of the mean of responses integrated for 60 sec following nicotine and ADP treatment in 51, 29, 51, 49 and 44 cells treated with 10, 30, 100, 300 and 1,000 μM nicotine, respectively. **P<0.01. Certain graphs show no error bars, as they are smaller than the symbols. HEK, human embryonic kidney; Nic, nicotine; ADP, adenosine diphosphate.

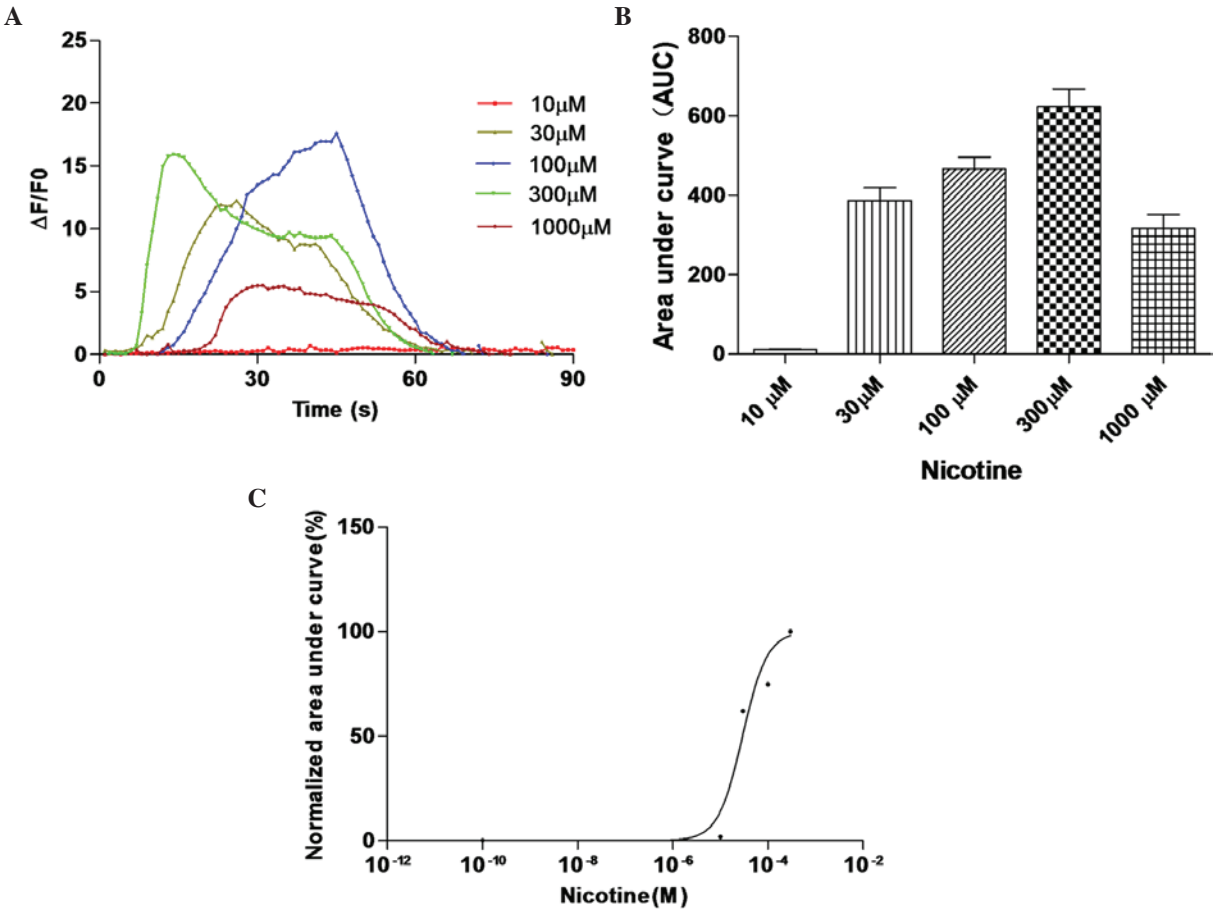

Figure 5. Nicotine induces calcium transients in co-transfected HEK 293 cells. (A) Co-transfected HEK 293 cells were treated with different concentrations of nicotine to induce calcium influx. Fluorescence intensity changes in each transfected HEK 293 cell were recorded to generate concentration-response curves. (B) Area under the curve for each transfected HEK 293 cell was calculated, and the (C) response in each transfected HEK 293 cell was normalized to the maximum area under the curve in each cell. Data are presented as the mean ± standard error of the mean of ΔF/F0 responses integrated for 60 sec following application of 10, 30, 100, 300 and 1,000 μM nicotine in 18, 26, 19, 29 and 15 co-transfected HEK 293 cells, respectively. **P<0.01. HEK, human embryonic kidney.

by co-expression with hRIC-3. In addition, immunohistochemistry and western blot analysis were performed to confirm the protein expression of the human α_7 nicotinic ACh receptor, and RT-PCR analysis was used to detect hric3 transcripts. Native HEK 293 cells did not express the human α_7 nicotinic ACh receptor pr hric-3 transcripts, whereas the human α_7 nicotinic ACh receptor was detected following transfection. Even in the absence of hric-3, α_7 protein was detected in the HEK 293 cells transiently expressing α_7.

Using the calcium dye, Fluo-4, to record intracellular calcium signals generated by the opening of α_7 nicotinic ACh receptors, images of calcium transients were captured in the transfected HEK 293 cells expressing a high level of α_7 nicotinic ACh receptors. It was demonstrated that these HEK 293 cells did not express detectable levels of hric-3 transcripts, which was associated with a lack of functional human α_7 nicotinic ACh receptors. Subsequently, images of calcium transients were captured in co-transfected HEK 293 cells with high expression levels of α_7 nicotinic ACh receptors and hric-3 transcripts. The observed calcium transients were predominantly derived from the opening of membrane α_7 nicotinic ACh receptors, as evidenced by the following observations: (i) the signals were induced by treatment with nicotine and (ii) human α_7 nicotinic ACh receptors were detectable in the co-transfected HEK 293 cells using immunohistochemical and western blot analyses.

In conclusion, the findings of the present study suggested that hRIC-3, when co-expressed with human α_7 nicotinic ACh receptors in HEK 293 cells, supported the functional expression of α_7 nicotinic ACh receptors. These observations may aid in the development of treatment strategies for inflammation.

Acknowledgements

The authors would like to thank Editage for English language editing. This study was supported by the National Natural Science Foundation of China (grant no. 81171845) and the Songjiang Foundation (grant no. 2011PD13).

References

1. Le Novere N, Corringer PJ and Changeux JP: The diversity of subunit composition in nAChRs: Evolutionary origins, physiologic and pharmacologic consequences. J Neurobiol 53: 447-456, 2002.

2. Millar NS and Gotti C: Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology 56: 237-246, 2009.
3. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susaria S, Li JH, Wang H, Yang H, Ulloa L, *et al*: Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 421(6921): 384-388, 2003.
4. Green WN and Millar NS: Ion-channel assembly. Trends Neurosci 18: 280-287, 1995.
5. Quik M, Choremis J, Komourian J, Lukas RJ and Puchacz E: Similarity between rat brain nicotinic alpha-bungarotoxin receptors and stably expressed alpha-bungarotoxin binding sites. J Neurochem 67: 145-154, 1996.
6. Peng JH, Lucero L, Fryer J, Herl J, Leonard SS and Lukas RJ: Inducible, heterologous expression of human alpha7-nicotinic acetylcholine receptors in a native nicotinic receptor-null human clonal line. Brain Res 825: 172-179, 1999.
7. Puchacz E, Buisson B, Bertrand D and Lukas RJ: Functional expression of nicotinic acetylcholine receptors containing rat alpha 7 subunits in human SH-SY5Y neuroblastoma cells. FEBS Lett 354: 155-159, 1994.
8. Zhao L, Kuo YP, George AA, Peng JH, Purandare MS, Schroeder KM, Lukas RJ and Wu J: Functional properties of homomeric, human alpha 7-nicotinic acetylcholine receptors heterologously expressed in the SH-EP1 human epithelial cell line. J Pharmacol Exp Ther 305: 1132-1141, 2003.
9. Cooper ST and Millar NS: Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor alpha7 subunit. J Neurochem 68: 2140-2151, 1997.
10. Williams ME, Burton B, Urrutia A, Shcherbatko A, Chavez-Noriega LE, Cohen CJ and Aiyar J: Ric-3 promotes functional expression of the nicotinic acetylcholine receptor alpha7 subunit in mammalian cells. J Biol Chem 280: 1257-1263, 2005.
11. Chen D, Dang H and Patrick JW: Contributions of N-linked glycosylation to the expression of a functional alpha7-nicotinic receptor in *Xenopus* oocytes. J Neurochem 70: 349-357, 1998.
12. Kassner PD and Berg DK: Differences in the fate of neuronal acetylcholine receptor protein expressed in neurons and stably transfected cells. J Neurobiol 33: 968-982, 1997.
13. Rangwala F, Drisdel RC, Rakhilin S, Ko E, Atluri P, Harkins AB, Fox AP, Salman SS and Green WN: Neuronal alpha-bungarotoxin receptors differ structurally from other nicotinic acetylcholine receptors. J Neurosci 17: 8201-8212, 1997.
14. Lansdell SJ, Gee VJ, Harkness PC, Doward AI, Baker ER, Gibb AJ and Millar NS: RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells. Mol Pharmacol 68: 1431-1438, 2005.
15. Millar NS: RIC-3: A nicotinic acetylcholine receptor chaperone. Br J Pharmacol 153 (Suppl 1): S177-S183, 2008.