MOL 17459

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RIC-3 enhances functional expression of multiple nicotinic acetylcholine receptor subtypes in mammalian cells

Stuart J. Lansdell, Veronica J. Gee, Patricia C. Harkness, Anne I. Doward,

Elizabeth R. Baker, Alasdair J. Gibb and Neil S. Millar

Department of Pharmacology, University College London,

Gower Street, London, WC1E 6BT, UK

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Running title: RIC-3 enhances functional expression of nAChRs

Corresponding author: Dr Neil Millar, Department of Pharmacology, University College London, Gower Street, London, WC1E 6BT. Tel.: +44-(0)20-7679-7241; email: n.millar@ucl.ac.uk

Number of pages of text: **34**

Number of tables: **0**

Number of figures: **6**

Number of references: **40**

Number of words in the Abstract: **249**

Number of words in the Introduction: **675**

Number of words in the Discussion: **1480**

Abbreviations: 5HT, 5-hydroxytryptamine; αBTX, α-bungarotoxin; GABA, γ-aminobutyric acid; mAb, monoclonal antibody; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; RT-PCR, reverse transcriptase-polymerase chain reaction.

Abstract

Recent studies have shown that RIC-3, originally identified in *C. elegans* as the protein encoded by the gene *ric-3* (resistance to inhibitors of cholinesterase), can enhance functional expression of $\alpha7$ nicotinic acetylcholine receptors (nAChRs). In the present study, the influence of RIC-3 upon multiple homomeric ($\alpha7$, $\alpha8$ and $\alpha9$) and heteromeric ($\alpha3\beta2$, $\alpha3\beta4$, $\alpha4\beta2$, $\alpha4\beta4$ and $\alpha9\alpha10$) nAChR subtypes has been examined in transfected mammalian cells by radioligand binding and functional characterisation. Coexpression of RIC-3 facilitates a dramatic enhancement of the ability of $\alpha7$ (and the closely related $\alpha8$ subunit) to generate functional nAChRs in otherwise non-permissive mammalian cells. In contrast, coexpression of RIC-3 did not facilitate functional expression of either homomeric $\alpha9$ or heteromeric $\alpha9\alpha10$ nAChRs in mammalian cell lines. Interestingly, whereas RIC-3 has been reported to cause a marked functional inhibition of heteromeric nAChRs such as $\alpha3\beta4$ and $\alpha4\beta2$ expressed in *Xenopus* oocytes, RIC-3 significantly enhances levels of functional expression of these and other ($\alpha3\beta2$ and $\alpha4\beta4$) heteromeric nAChRs when expressed in mammalian cell lines. In addition, the interaction of multiple nAChR subunits ($\alpha3$, $\alpha4$, $\alpha7$, $\beta2$ and $\beta4$) with RIC-3 has been demonstrated by coimmunoprecipitation from metabolically labelled transfected cells. Significantly, coimmunoprecipitation experiments have provided evidence that RIC-3 associates with unassembled nAChR subunits, a finding which is consistent with previous suggestions that RIC-3 may act by enhancing the maturation (subunit folding and assembly) of nAChRs. We conclude that RIC-3 is a nAChR-associated protein which can enhance functional expression of multiple nAChR subtypes in transfected mammalian cells.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are neurotransmitter-gated ion channels expressed at the neuromuscular junction and within the central and peripheral nervous system. Seventeen vertebrate nAChR subunits have been identified and cloned (α1-α10, β1-β4, γ, δ and ε) which coassemble to generate a diverse family of pentameric receptors (Le Novère et al., 2002; Millar, 2003). In parallel with studies of native nAChRs, molecular cloning has enabled the characterisation of recombinant nAChRs in a variety of artificial expression systems, such as *Xenopus* oocytes and cultured mammalian cell lines. A great advantage of these approaches is that nAChRs generated from defined subunit combinations can be studied. This has provided important insights into the relationship between subunit composition and the pharmacological and physiological properties of nAChRs.

In addition to the well characterised nAChRs expressed at the skeletal neuromuscular junction (which are assembled from α1, β1, γ, δ and ε subunits), a family of nAChRs is expressed in the central and peripheral nervous system, the "neuronal nAChRs". Nine neuronal nAChR subunits (α2-α7 and β2-β4) are expressed in the mammalian nervous system and coassemble to generate neuronal nAChRs with a variety of distinct subunit combinations (Le Novère et al., 2002; Millar, 2003). An α8 subunit, which is closely related in primary amino acid sequence to the α7 subunit, has been identified in avian, but not mammalian, species. The two most recently identified nAChR subunits (α9 and α10) appear to be expressed primarily in hair cells of the inner ear (Elgoyhen et al., 1994; Elgoyhen et al., 2001) and have been implicated in auditory processing. Most neuronal nAChRs, like the muscle-type nAChR, are heteromeric complexes of more than one subunit type. The main exception is the α7 subunit (and the closely related α8 subunit). There is evidence to suggest that native α7 nAChRs are

homomeric (Chen and Patrick, 1997; Drisdel and Green, 2000). The α7 subunit has also been

shown to generate functional homomeric nAChRs when expressed in *Xenopus* oocytes

(Couturier et al., 1990), as has α8 (Gerzanich et al., 1994; Gotti et al., 1994). Whilst the α9

subunit has been reported to form homomeric channels in oocytes (Elgoyhen et al., 1994), it

appears to form heteromeric nAChRs (with α10) more efficiently (Elgoyhen et al., 2001).

Although the α7 subunit is able to generate functional nAChRs when expressed in *Xenopus*

oocytes, considerable difficulties have been encountered in the efficient expression of

functional α7 nAChRs in many cultured mammalian cell lines (Cooper and Millar, 1997;

Kassner and Berg, 1997; Rangwala et al., 1997; Chen et al., 1998). Similar problems have

been encountered with expression of homomeric nAChRs from α8 and α9 subunits in

mammalian cells (Cooper and Millar, 1998; Baker et al., 2004). In contrast, functional

neuronal nAChRs have been generated successfully from numerous heteromeric subunit

combinations (such as α3β2, α3β4, α4β2 and α4β4) in cultured mammalian cells (see, for

example, Whiting et al., 1991; Lewis et al., 1997; Ragozzino et al., 1997). Despite success in

expression of such heteromeric nAChRs, a common finding has been that only relatively low

levels of correctly folded cell-surface nAChRs are expressed, a conclusion which is supported

by evidence of substantially enhanced levels of cell-surface expression obtained with artificial

subunit chimeras (Cooper et al., 1999; Harkness and Millar, 2002).

Recent studies with the nematode *Caenorhabditis elegans* have identified a protein (RIC-3),

encoded by the gene *ric-3*, <u>r</u>esistant to <u>i</u>nhibitors of <u>c</u>holinesterase, which contains two

putative transmembrane domains (Halevi et al., 2002). Studies performed in *Xenopus* oocytes

have revealed that coexpression of the *C. elegans* RIC-3 protein (CeRIC-3) or its human

homologue (hRIC-3) can enhance levels of functional α7 nAChRs (Halevi et al., 2002; Halevi

et al., 2003) but causes a marked inhibition of functional responses with heteromeric nAChRs

such as α3β4 and α4β2. Recent studies have also demonstrated that coexpression of RIC-3

can facilitate functional expression of α7 in mammalian cells (Williams et al., 2005). In the

present study we have examined the influence of RIC-3 upon multiple homomeric (α7, α8

and α9) and heteromeric (α3β2, α3β4 α4β2, α4β4 and α9α10) nAChR subtypes expressed in

transfected mammalian cells.

Material and Methods

Plasmids, antibodies and cell lines. Rat $\alpha3$, $\alpha4$, $\alpha7$, $\beta2$ and $\beta4$ nAChR subunit cDNAs were provided by Jim Patrick (Baylor College of Medicine, TX. USA). Human $\alpha7$ and chick $\alpha8$ nAChR subunit cDNAs were provided by Jon Lindstrom (University of Pennsylvania, PA. USA). Rat $\alpha9$ nAChR subunit cDNA was provided by Belén Elgoyhen (Universidad de Buenos Aires, Argentina). Rat $\alpha10$ nAChR subunit cDNA was provided by Jim Boulter (University of California, Los Angeles, CA. USA). Mouse GABA$_A$ receptor $\alpha1$ subunit tagged at its *N*-terminus with a myc epitope (GABA$_A$R $\alpha1^{MYC}$) (Connolly et al., 1996) was provided by Steve Moss (University of Pennsylvania, PA. USA). *C. elegans* RIC-3 cDNA was provided by Millet Treinin (Hebrew University, Jerusalem, Israel). Monoclonal antibody (mAb) mAb319, raised against the nAChR $\alpha7$ subunit, and mAbFLAG-M2, raised against the FLAG epitope, were obtained from Sigma. A mAb specific for the myc epitope tag (mAb9E10) was purified from the Myc1-9E10 hybridoma cell line (European Collection of Cell Cultures, No. 85102202)

Detection of RIC-3 transcripts. Total RNA was prepared from $\sim5\times10^6$ cells, by methods described previously (Gough, 1988). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed using the OneStep PCR Kit (Qiagen) with 0.5 μg total RNA template. Oligonucleotide primers spanning intron-exon boundaries were designed to the human RIC-3 gene sequence (5' GGGACCTAATGGTGAGAGAGCAC 3' and 5' CCTACGCTTGATACAGTCTGAAAGG 3') and to $\alpha1$ tubulin gene sequence (5'ACACCTTCTTCAGTGAGACAGG 3' and 5'CTCATTGTCTACCATGAAGGCAC 3').

Molecular cloning and epitope tagging of hRIC-3. Oligonucleotide primers were synthesised which correspond to the predicted 5' and 3' untranslated regions of hRIC-3 and were used to amplify hRIC-3 cDNA from a human cDNA library. The resulting fragment was subcloned into pCRII (Invitrogen) and then excised with *Eco*RI and subcloned into pRK5 to create pRK5-hRIC-3. The eight amino acid FLAG epitope tag (DYKDDDDK) was introduced into hRIC-3 by ligation of synthetic oligonucleotides at an endogenous *Bst*EII site located near the *C*-terminus, after a glycine residue at position 224, to create pRK5-hRIC-3[FLAG].

Heterologous expression. Human kidney tsA201 cells were cultured in Dulbecco's Modified Eagles Medium (Invitrogen) containing 10% foetal calf serum (Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Life Technologies). Cells were maintained in a humidified incubator containing 5% CO_2 at 37°C. Cells were transfected using the Effectene reagent (Qiagen) according to the manufacturer's instructions. After overnight incubation in Effectene, cells were incubated at 37°C for 24 h before being assayed for radioligand binding.

Radioligand binding. [3H]epibatidine (55.5 Ci/mmol) was purchased from Perkin Elmer Life Sciences and [125I]α-bungarotoxin ([125I]αBTX; 150-200 Ci/mmol) from GE Healthcare. Radioligand binding to transiently transfected tsA201 cells was performed as described previously (Cooper and Millar, 1997; Baker et al., 2004). Samples were assayed by filtration onto Whatman GF/B filters (for [3H]epibatidine) or GF/A filters (for [125I]αBTX), followed by rapid washing using a Brandel cell harvester. Prior to use, filters were pre-soaked in 0.5% polyethyleneimine (PEI). All binding experiments with [125I]αBTX were performed in buffer containing 0.5% BSA to reduce non-specific binding.

Metabolic labelling and immunoprecipitation. Transfected tsA201 cells were metabolically labelled as described previously (Cooper and Millar, 1997). After growth in methionine-free medium for 15 min, cells were labelled with 250 µCi Pro-mix, a mixture of $[^{35}S]$methionine and $[^{35}S]$cysteine, (GE Healthcare) in 3.5 ml methionine-free medium for 3 hours. Medium containing 30 mg/L methionine and 10% heat-inactivated FCS was then added and the cells incubated for a further 90 min. Cells were washed twice with 10 ml phosphate buffered saline (PBS) and harvested into 300 µl ice-cold lysis buffer (150 mM NaCl, 50 mM Tris/Cl pH 8.0, 5 mM EDTA and 1% Triton-X100) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 mM *N*-ethylmaleimide and 10 µg/ml, each, of leupeptin, aprotinin and pepstatin). Solubilisation and all subsequent steps were performed at 4°C. After 1 h solubilisation, the cell lysate was pre-cleared by incubation overnight with 30 µl protein G-sepharose (GE Healthcare) in a 1:1 mixture with lysis buffer. Non-solubilised material was pelleted by centrifugation at 14,000 x g for 15 min. Cell lysates were incubated with primary antibody for 3.5 hours. The antibody-receptor complex was immunoprecipitated by the addition of 35 µl protein G-sepharose, incubated for a further 3.5 h and isolated by centrifugation. Samples were washed with 4 x 1 ml lysis buffer. Samples were examined by SDS polyacrylamide gel electrophoresis followed by autoradiography as described previously (Lansdell et al., 1997).

Electrophysiology. Cells, grown on glass coverslips coated in collagen and polylysine (both 10 µg/ml), were cotransfected with pEGFP-C2 (Clontech) encoding green fluorescent protein (GFP), and with plasmids containing RIC-3 and nAChR subunit constructs. Whole-cell recordings were performed at room temperature, 36-48 hours after transfection, from cells identified under fluorescence microscopy as GFP-positive. Recording solution contained (in mM): 110 NaCl, 5.4 KCl, 0.8 MgCl$_2$, 1.8 CaCl$_2$, 25 glucose, 0.9 NaH$_2$PO$_4$, 44 NaHCO$_3$.

Borosilicate electrodes (Harvard GC150F-7.5) of resistance 6-12 MΩ contained (in mM) 140 CsCl, 10 Hepes, 10 EGTA, 0.5 CaCl$_2$, 29.53 CsOH, pH adjusted to 7.26, osmolarity 283 mOsm/Kg H$_2$O. Unless otherwise specified, the holding potential was –60 mV. Fast cell superfusion of agonists was achieved with a theta-barrelled application pipette made from 1.5 mm diameter theta tubing (Harvard AH-30-0114), which was moved laterally using a stepper motor. Agonist-evoked currents were recorded using an Axopatch 200B amplifier, filtered at 2 kHz and digitised at 10 kHz.

Intracellular calcium recording. Transfected tsA201 cells were replated onto poly-L-lysine coated black-walled 96-well plates (Marathon Laboratories, London, UK) approximately 18-20 hours post transfection. Approximately 24 hours later cell medium was removed and the cells incubated in 50-100 μl of 1 μM Fluo-4 acetoxymethyl ester (Molecular Probes) in Hanks' Balanced Salt Solution (HBSS) with 0.02 % Pluronic$^{\circledR}$ F-127 (Molecular Probes) for 30-60 minutes at room temperature. Cells were rinsed once with 160 μl assay buffer (HBSS supplemented with 18.8 mM CaCl2, 8.8 mM sucrose and 6.3 mM HEPES) and the cells assayed using the Fluorometric Imaging Plate Reader system (FLIPR) (Molecular Devices, Winnersh, UK). Cells were excited by light of 488 nm wavelength from a 4 W argon-ion laser and the emitted fluorescence passed through a 510 to 570 nm bandpass interference filter before detection with a cooled 'charge coupled device' (CCD) camera (Princeton Instruments). Drug dilutions in assay buffer were prepared in a separate 96-well plate. Parameters for drug addition to the cell plate were pre-programmed and delivery was automated through a 96-tip head pipettor.

Results

Endogenous expression of RIC-3 in mammalian cells

Oligonucleotide primers designed to the human RIC-3 (hRIC-3) cDNA sequence (Halevi et al., 2003) were used to detect RIC-3 transcripts in a variety of cultured mammalian cell lines by reverse transcriptase-polymerase chain reaction (RT-PCR). A single band of the size expected for PCR-amplified hRIC-3 transcripts (220 bp) was detected in SH-SY5Y cells, a human neuroblastoma cell line which expresses endogenous $\alpha7$ nAChRs (Lukas et al., 1993; Cooper and Millar, 1997). In contrast, no evidence of hRIC-3 mRNA could be detected in the human kidney cell line tsA201 which has been shown previously to be a host cell type in which $\alpha7$ fails to form functional nAChRs (Cooper and Millar, 1997). These findings are in agreement with those which have been reported previously (Williams et al., 2005).

RT-PCR amplification was also performed on mRNA samples isolated from several rat cultured cell lines. Previous studies have reported that different isolates of the rat phaeochromocytoma PC12 cell line differ in their expression of endogenous $\alpha7$ nAChRs and in their ability to express functional recombinant $\alpha7$ nAChRs (Blumenthal et al., 1997). In the present study, two isolates of PC12 cells (PC12-a and PC12-b) were examined which exhibited different levels of specific binding of [^{3}H]MLA, an $\alpha7$-selective nicotinic antagonist. PC12-a expressed specific [^{3}H]MLA binding (230 ± 83 fmol/mg protein; $n=4$) whilst little or no specific binding of [^{3}H]MLA could be detected in PC12-b cells. In addition, the rat pituitary GH_4C_1 cell line was examined. The GH_4C_1 cell line does not express endogenous $\alpha7$ nAChRs but is a suitable host cell type in which to express functional recombinant $\alpha7$ nAChRs (Quik et al., 1996; Cooper and Millar, 1998). A single PCR fragment of a size similar to that amplified from human SH-SY5Y cells was detected by RT-

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PCR amplification of mRNA isolated from GH_4C_1 and PC12-a cells, demonstrating expression of RIC-3 transcripts and indicating that the primers designed to the human RIC-3 nucleotide sequence are able to detect rat RIC-3 transcripts. In three separate preparations of mRNA isolated from PC12-b cells (in which endogenous [3H]MLA binding was not detected), no evidence of RIC-3 mRNA was detected by RT-PCR amplification. In all cases, a band of the size expected for PCR-amplified transcripts of the ubiquitously expressed α1 tubulin (473 bp) was detected (data not shown). These and previous (Williams et al., 2005) RT-PCR studies provide evidence of a correlation between the endogenous expression of RIC-3 transcripts and the ability of a cell to express functional α7 nAChRs (either endogenously or from heterologous expression of α7 cDNA).

Heterologous coexpression of α7 and RIC-3

In agreement with previous studies (Eiselé et al., 1993; Cooper and Millar, 1997; Cooper and Millar, 1998), no specific binding of the α7-selective radioligand [125I]αBTX could be detected when either the human α7 subunit (hα7) or rat α7 subunit (rα7) was expressed by transient transfection in human kidney tsA201 cells (Fig. 1). In marked contrast, however, when either the hα7 or rα7 subunit was coexpressed with CeRIC-3 or hRIC-3, high levels of specific radioligand binding were detected (Fig. 1), findings which are consistent with previous studies (Williams et al., 2005).

Functional expression of α7 nAChRs

To determine whether functional α7 nAChRs are formed in tsA201 cells after cotransfection with RIC-3, cells were examined by whole-cell patch-clamp recording. Rapidly desensitising responses to brief applications of 200 μM acetylcholine (Fig. 2), characteristic of α7 nAChRs, were detected in cells transfected with all combinations of α7 and RIC-3 examined:

rα7+CeRIC-3 (6/7 cells), hα7+CeRIC-3 (9/11 cells), rα7+hRIC-3 (8/8 cells) and hα7+hRIC-3 (13/17 cells). No significant differences were observed in the magnitude of whole-cell responses from cells transfected with α7 and RIC-3 cDNA combinations from different species (mean 119±28 pA). We have been unable to detect functional responses from α7 expressed alone in transfected tsA201 cells by whole-cell electrophysiological recording (0/18 cells examined).

Coassembly of α7 with RIC-3

The ability of RIC-3 to coassemble with α7 was investigated by coimmunoprecipitation. To enable detection of the hRIC-3 protein, an eight amino acid FLAG epitope was introduced into hRIC-3 to generate hRIC-3^{FLAG}. Cells were transfected with combinations of hα7 and hRIC-3^{FLAG} cDNAs and examined by immunoprecipitation with either the α7-specific antibody mAb319 or with mAbFLAG-M2, an antibody which recognises the FLAG epitope. Specific bands corresponding to hα7 and hRIC-3^{FLAG} were detected in cells transfected with these cDNAs alone and were absent from untransfected cells (Fig. 3A). Coprecipitation of hα7 with hRIC-3^{FLAG} was observed in cells cotransfected with hα7 and hRIC-3^{FLAG} using mAbFLAG-M2 (Fig. 3A, lane 3). Similarly, coprecipitation of hRIC-3^{FLAG} with hα7 was observed in cells cotransfected with hα7 and hRIC-3^{FLAG} using mAb319 (Fig. 3A, lane 7). The absence of cross-reactivity of mAbFLAG-M2 with hα7 and of mAb319 with hRIC-3^{FLAG} was also confirmed (Fig. 3A, lanes 4 and 10). This provides strong evidence of coassembly of the hα7 and hRIC-3 proteins. To eliminate the possibility that the coimmunoprecipitation of α7 and RIC-3 was a consequence of non-specific protein aggregation, a control experiment was performed in which hα7 and hRIC-3 were expressed separately by transfection of tsA201 cells. Transfected cells were metabolically labelled, disrupted by detergent solubilisation, and

the two cell extracts mixed prior to immunoprecipitation. In contrast to the clear evidence for coprecipitation when $h\alpha 7$ and hRIC-3 were coexpressed, no coimmunoprecipitation was observed when proteins were mixed after having been expressed separately (data not shown).

To examine the specificity of this interaction, coimmunoprecipitation studies were performed with hRIC-3^{FLAG} and the GABA$_A$R $\alpha 1$ subunit, using a previously characterised GABA$_A$R $\alpha 1$ subunit tagged with a myc epitope ($\alpha 1^{MYC}$) which has been shown to be functionally silent (Connolly et al., 1996). Although both $\alpha 1^{MYC}$ and hRIC-3^{FLAG} could be detected by immunoprecipitation (Fig. 3B, lanes 2 and 6), no evidence of coassembly between the GABA$_A$R subunit and hRIC-3^{FLAG} could be detected (Fig. 3B, lanes 3 and 7).

Influence of RIC-3 upon $\alpha 8$, $\alpha 9$ and $\alpha 10$-containing nAChRs

Whilst the successful functional heterologous expression of many heteromeric nAChR subunit combinations has been reported in several mammalian cell expression systems, considerable problems have been encountered with the expression of homomeric nAChRs (such as $\alpha 7$) in cultured cell lines (Cooper and Millar, 1997; Kassner and Berg, 1997; Rangwala et al., 1997; Chen et al., 1998). Functional homomeric nAChRs have been reported for both $\alpha 8$ and $\alpha 9$ subunits expressed in *Xenopus* oocytes (Elgoyhen et al., 1994; Gerzanich et al., 1994; Gotti et al., 1994) but, as for $\alpha 7$, difficulties have been encountered in heterologous expression of homomeric $\alpha 8$ and $\alpha 9$ in cultured cell lines (Cooper and Millar, 1998; Baker et al., 2004). As might have been expected from the very close sequence similarity between $\alpha 7$ and $\alpha 8$ subunits, clear evidence of specific cell-surface [^{125}I]αBTX binding was also detected when the chick $\alpha 8$ nAChR subunit was coexpressed with either CeRIC-3 (53.0 ± 13.8 fmol/10^6 cells; $n=3$) or hRIC-3 (24.0 ± 6.2 fmol/10^6 cells; $n=3$). In contrast, no significant binding of [^{125}I]αBTX was detected when the $\alpha 8$ subunit was

expressed in the absence of RIC-3 (1.1 ± 1.7 fmol/10^6 cells; $n=3$). Despite the ability of RIC-3 to facilitate binding of [^{125}I]αBTX to homomeric α7 and α8 nAChRs, no evidence of specific nicotinic radioligand binding was detected when the rat α9 nAChR subunit was cotransfected with either CeRIC-3 or hRIC-3. Since there is strong evidence for coassembly of α9 and α10 subunits into heteromeric nAChRs (Elgoyhen et al., 2001; Baker et al., 2004), the influence of RIC-3 constructs upon coexpressed α9 and α10 subunits was examined, but no evidence of specific binding of nicotinic radioligands was detected when these subunits were coexpressed with either CeRIC-3 or hRIC-3.

Despite the well documented problems associated with efficient functional expression of α8 in transfected mammalian cells (Cooper and Millar, 1998), in three of eleven cells examined, whole-cell responses to acetylcholine (72 ± 26 pA) were observed. However, coexpression of α8 with hRIC-3 generated whole-cell responses to acetylcholine (124 ± 24 pA) in all cells examined (14/14 cells). The rapidly desensitising whole-cell responses to acetylcholine were completely and reversibly blocked by d-tubocurarine (data not shown).

Influence of RIC-3 upon α3, α4, β2 and β4-containing nAChRs

Previous studies conducted in oocytes have reported that coexpression of RIC-3 causes a marked inhibition of functional responses with heteromeric α3β4 and α4β2 nAChRs (Halevi et al., 2003). We have examined the influence of hRIC-3 upon levels of radioligand binding to four nAChRs (α3β2, α3β4 α4β2 and α4β4) expressed in mammalian cells. Coexpression of hRIC-3 resulted in significantly enhanced levels of specific [^{3}H]epibatidine binding to all subunit combinations (Fig. 5). To examine the influence of RIC-3 upon levels of functional nAChRs, agonist-induced changes in intracellular calcium were examined by use of an automated 96-well format fluorometric imaging plate reader (FLIPR). The advantage of this

approach is that responses in a population of cells can be examined and the magnitude of responses in the presence and absence of RIC-3 can be determined simultaneously in a single experiment. Figure 6A illustrates responses obtained from cells in which heteromeric nAChR subunit combinations were expressed in the presence and absence of hRIC-3 (paired data from a single 96-well plate in which responses from four different wells have been averaged). In all cases a significant increase in agonist-induced responses was observed. Data pooled from several such experiments reveals a consistent and significant increase in functional responses for α3β2, α3β4, α4β2 and α4β4 nAChRs when cotransfected with hRIC-3 (Fig. 6B).

Although fewer problems have been encountered in the functional expression of heteromeric nAChRs such as α3β4 and α4β2 in transfected mammalian cells, we and others have encountered particular difficulties in detecting functional expression of α3β2 nAChRs by patch-clamp recording. In tsA201 cells transfected with α3 and β2 subunits in the absence of coexpressed RIC-3, small whole-cell responses (~18 pA) were detected in only 2/10 cells examined. In contrast, when α3 and β2 were cotransfected with hRIC-3, significantly larger whole-cell responses (55-170 pA) were detected in all nine cells examined).

Experiments were also performed to examine whether heteromeric nAChR subunits such as α4 and β2 were coimmunoprecipitated with hRIC-3 (Fig. 4). As shown previously for α7 (Fig. 3A), clear evidence for the coimmunoprecipitation of other nAChR subunits (α3, α4, β2 and β4) with hRIC-3 was obtained (coprecipitation of α4 and β2 is illustrated in Fig. 4). Since α3, α4, β2 and β4 form functional nAChRs only when assembled into heteromeric complexes (e.g. α4β2 and α3β4), we were interested to examine whether coassembly of RIC-3 could be detected when these subunits were expressed individually. Clear evidence of coassembly with

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RIC-3 was detected for all four subunits examined (see Fig. 4 for data obtained with α4 and

β2), indicating that RIC-3 is able to associate with unassembled nAChR subunits.

Discussion

The RIC-3 protein from *C. elegans* (CeRIC-3) and its human homologue (hRIC-3) have been shown to enhance levels of functional nAChRs when coexpressed with α7 in *Xenopus* oocytes (Halevi et al., 2002; Halevi et al., 2003). A similar enhancement is seen with a *C. elegans* nAChR containing the DEG-3 and DES-2 subunits (Halevi et al., 2002; Halevi et al., 2003). An enhancement of cell-surface expression of the $5HT_3$ receptor subunit $5HT_{3A}$ (a subunit which shows close sequence similarity to nAChR subunits) has also been reported in mammalian cells (Cheng et al., 2005). In contrast, RIC-3 does not appear to influence functional expression levels of GABA, glutamate and glycine receptors (Halevi et al., 2002; Halevi et al., 2003). Despite the enhancement of α7 nAChR responses caused by coexpression of RIC-3, a marked inhibition of functional responses to heteromeric nAChRs such as α3β4 and α4β2 has been reported on coexpression of hRIC-3 in oocytes (Halevi et al., 2003).

Despite the relative ease with which functional nAChRs can be generated by expression in *Xenopus* oocytes, considerable problems have been encountered in functional expression in transfected mammalian cells and are particularly pronounced for homomeric nAChRs such as α7 (Millar, 1999). When expressed in *Xenopus* oocytes, the nAChR α7 subunit forms functional ion channels which are blocked by the snake neurotoxin αBTX (Couturier et al., 1990). Whilst there have been reports of the successful heterologous expression of functional α7 nAChRs in some cultured mammalian cell lines (Puchacz et al., 1994; Gopalakrishnan et al., 1995; Quik et al., 1996), considerable difficulties have been reported in the functional expression of homomeric α7 nAChRs in many other mammalian cell types (Cooper and Millar, 1997; Kassner and Berg, 1997; Rangwala et al., 1997; Chen et al., 1998). Problems

associated with heterologous expression of $\alpha 7$ in non-permissive host cell types can be circumvented by the expression of subunit chimeras containing the extracellular domain of $\alpha 7$ fused to the transmembrane and *C*-terminal domain of the 5-hydroxytryptamine type 3 receptor (5HT$_3$R) subunit 5HT$_{3A}$ (Eiselé et al., 1993; Blumenthal et al., 1997; Rangwala et al., 1997; Cooper and Millar, 1998). This provides evidence that problems associated with the efficient formation of correctly assembled $\alpha 7$ nAChRs are due to sequences present in its *C*-terminal region, but does not explain the reason for differences between host cell types.

Until recently (Williams et al., 2005), there have been no reports of the influence of RIC-3 upon nAChRs expressed in transfected mammalian cell lines. The recent study of Williams et al. (2005), and the work reported here, demonstrate that $\alpha 7$ is able to efficiently generate functional nAChRs in mammalian cell lines when coexpressed with either CeRIC-3 or hRIC-3. We have also demonstrated that coexpression of RIC-3 promotes functional expression of homomeric $\alpha 8$ (but not $\alpha 9$) nAChRs. An interesting aspect of the present study is the evidence that coexpression of RIC-3 enhances functional expression of heteromeric nAChRs such as $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$ and $\alpha 4\beta 4$ in transfected mammalian cells. This is in marked contrast to the report that coexpression of RIC-3 causes a marked inhibition of functional responses to $\alpha 3\beta 4$ and $\alpha 4\beta 2$ in oocytes (Halevi et al., 2003). It appears, therefore, that the influence of RIC-3 upon functional expression of nAChRs can be influenced by other host cell factors.

We have obtained clear evidence for the coprecipitation of RIC-3 with multiple nAChR subtypes ($\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 2$ and $\beta 4$). Our inability to detect coprecipitation of the GABA$_A$R $\alpha 1$ subunit with RIC-3 indicates that the association with multiple nAChR subunits does not extend to all ligand-gated ion channel subunits and is consistent with evidence that functional

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expression levels of GABA, glutamate and glycine receptors are unaffected by coexpression of RIC-3 (Halevi et al., 2002; Halevi et al., 2003). Interestingly, a recent study has demonstrated the coprecipitation of RIC-3 with the $5HT_3$ receptor subunit $5HT_{3A}$ (Cheng et al., 2005), a subunit with close sequence similarity to nAChR subunits. An interesting aspect of the present study was evidence for the coassembly of RIC-3 with unassembled nAChR subunits (e.g. $\alpha4$ and $\beta2$; see Fig. 4). It is plausible that RIC-3 promotes the formation of functional assembled nAChRs by interacting with unassembled subunits. A consequence of such interactions may be to promote appropriate subunit folding, thereby enhancing productive subunit-subunit interactions. This conclusion is consistent with other recent studies which provide evidence that RIC-3 can enhance maturation of nAChRs (Halevi et al., 2002; Cheng et al., 2005). It is also consistent with evidence that RIC-3 interacts with $5HT_{3A}$ subunits within the endoplasmic reticulum (Cheng et al., 2005).

The apparent molecular weight of the hRIC-3^{FLAG} protein (~58 kDa), determined by immunoprecipitation and SDS-PAGE, is considerably larger than would have been predicted from the primary amino acid sequence (41 kDa). When precipitated with mAbFLAG-M2 (Fig. 3A, lane 2 and Fig. 3B, lane 2), a single band was detected which was absent from untransfected cells. This was a consistent observation in several independent immunoprecipitation experiments and is strong evidence that this band corresponds to hRIC-3^{FLAG}. In addition, a band of the same apparent molecular weight was found to coprecipitate with $\alpha7$ (Fig. 3A, lane 7). This band was observed only in cells transfected with hRIC-3^{FLAG} (Fig. 3A). We have found previously that inclusion of a FLAG epitope tag can cause a significant, and unexpectedly large, increase in the apparent molecular weight of a protein determined by SDS-PAGE (Lansdell and Millar, 2002). In agreement with these previous observations, a band of lower apparent molecular weight (~55 kDa) was found to

coprecipitate with α7 in cells transfected with hRIC-3 lacking the FLAG epitope tag (Fig. 3A, lane 8). This provides further evidence for coprecipitation of hRIC-3 with α7. Although the FLAG epitope is responsible, at least in part, for the high apparent molecular weight of hRIC-3^{FLAG}, the untagged hRIC-3 also migrated at a higher apparent molecular weight than would have been predicted from its primary amino acid sequence. There are no potential *N*-linked glycosylation sites (N-X-S/T) within the hRIC-3 coding sequence, so the high apparent molecular weight can not be attributed to *N*-linked glycosylation, as is common in nAChR subunits. It is not clear what is responsible for the high apparent molecular weight of hRIC-3 determined by SDS-PAGE but, as has been discussed previously (Dunker and Rueckert, 1969), it is not uncommon for proteins to migrate on SDS-PAGE gels with apparent molecular weights different from that predicted from their amino acid sequence.

The phenotypic consequence of mutations in the gene encoding RIC-3 in *C. elegans* has been reported to be the intracellular accumulation of nAChR proteins (Halevi et al., 2002). This has led to the suggestion that CeRIC-3 is involved in the maturation pathway of nAChRs (Halevi et al., 2002). The identification of a related protein in humans (Halevi et al., 2003), together with the results presented here, suggest that RIC-3 is a nAChR-associated protein which is required for efficient and accurate folding, assembly and functional expression of nAChRs in diverse species.

Many receptor-associated proteins have been identified in recent years, several of which appear to be important in assembly or intracellular trafficking. In comparison to several other neurotransmitter receptors, relatively few nAChR-associated proteins have been identified. Rapsyn (or 43K protein) is associated with nAChRs expressed at the neuromuscular junction and in the electric organ of fish such as *Torpedo*. Whilst rapsyn plays an important role in

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organisation of nAChRs at the neuromuscular junction, expression studies reveal that it is not required for functional heterologous expression of muscle-type nAChRs (Phillips et al., 1991). RIC-3 is likely to be one of several proteins involved in the folding and assembly of nAChRs. Chaperone proteins such as BiP, calnexin and 14-3-3η have been shown to have an effect on the maturation of nAChRs (Blount and Merlie, 1991; Gelman et al., 1995; Jeanclos et al., 2001), but are ubiquitously expressed and interact with a diverse group of proteins. The work reported here and previously (Halevi et al., 2002; Halevi et al., 2003; Cheng et al., 2005; Williams et al., 2005) suggests that RIC-3 may play a role which is restricted to a narrower group of proteins, such as nAChRs and the closely related $5HT_3$ receptor.

In summary, we have obtained evidence for a correlation between the expression of endogenous RIC-3 transcripts and the ability of mammalian cell types to permit accurate folding and assembly of nAChR subtypes such as α7. In mammalian cell lines which do not permit efficient folding and functional expression of α7 nAChRs, coexpression of RIC-3 facilitates both subunit assembly (assayed by nicotinic radioligand binding) and formation of functional nAChRs (assayed by patch-clamp recording). In contrast to studies conducted in *Xenopus* oocytes, enhanced functional expression of several heteromeric nAChR subtypes (α3β2, α3β4, α4β2 and α4β4), when coexpressed with RIC-3, has also been demonstrated in transfected mammalian cells. We conclude that RIC-3 is a nAChR-associated protein which acts to enhance the efficient folding and assembly of multiple nAChR subunits into functional receptors.

Acknowledgements

References

Baker ER, Zwart R, Sher E and Millar NS (2004) Pharmacological properties of α9α10 nicotinic acetylcholine receptors revealed by heterologous expression of subunit chimeras. *Mol. Pharmacol.* 65:453-460.

Blount P and Merlie JP (1991) BIP associates with newly synthesized subunits of the mouse muscle nicotinic receptor. *J. Cell Biol.* 113:1125-32.

Blumenthal EM, Conroy WG, Romano SJ, Kassner PD and Berg DK (1997) Detection of functional nicotinic receptors blocked by α-bungarotoxin on PC12 cells and dependence of their expression on post-translational events. *J. Neurosci.* 17:6094-6104.

Chen D, Dang H and Patrick JW (1998) Contributions of N-linked glycosylation to the expression of a functional α7-nicotinic receptor in *Xenopus* oocytes. *J. Neurochem.* 70:349-357.

Chen D and Patrick JW (1997) The α-bungarotoxin-binding nicotinic acetylcholine receptor from rat brain contains only the α7 subunit. *J. Biol. Chem.* 272:24024-24029.

Cheng A, McDonald NA and Connolly CN (2005) Cell surface expression of 5-hydroxytryptamine type 3 receptors is promoted by RIC-3. *J. Biol. Chem.* 280:22502-22507.

Connolly CN, Krishek BJ, McDonald BJ, Smart TG and Moss SJ (1996) Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. *J. Biol. Chem.* 271:89-96.

Cooper ST, Harkness PC, Baker ER and Millar NS (1999) Upregulation of cell-surface α4β2 neuronal nicotinic receptors by lower temperature and expression of chimeric subunits. *J. Biol. Chem.* 274:27145-27152.

Cooper ST and Millar NS (1997) Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor α7 subunit. *J. Neurochem.* 68:2140-2151.

Cooper ST and Millar NS (1998) Host cell-specific folding of the neuronal nicotinic receptor α8 subunit. *J. Neurochem.* 70:2585-2593.

Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T and Ballivet M (1990) A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron* 5:847-856.

Drisdel RC and Green WN (2000) Neuronal α-bungarotoxin receptors are α7 subunit homomers. *J. Neurosci.* 20:133-139.

Dunker AK and Rueckert RR (1969) Observations on molecular weight determinations on polyacrylamide gel. *J. Biol. Chem.* 244:5074-5080.

Eiselé J-L, Bertrand S, Galzi J-L, Devillers-Thiéry A, Changeux J-P and Bertrand D (1993) Chimaeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. *Nature* 366:479-483.

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE and Heinemann S (1994) α9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* 18:705-715.

Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heineman SF and Boulter J (2001) α10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc. Natl. Acad. Sci. USA* 98:3501-3506.

Gelman MS, Chang W, Thomas DY, Bergeron JJM and Prives JM (1995) Role of the endoplasmic reticulum chaperone calnexin in subunit folding and assembly of nicotinic acetylcholine receptors. *J. Biol. Chem.* 270:15085-15092.

Gerzanich V, Anand R and Lindstrom J (1994) Homomers of α8 and α7 subunits of nicotinic receptors exhibit similar channels but contrasting binding site properties. *Mol. Pharmacol.* 45:212-220.

Gopalakrishnan M, Buisson B, Touma E, Giordano T, Campbell JE, Hu IC, Donnelly-Roberts D, Arneric SP, Bertrand D and Sullivan JP (1995) Stable expression and pharmacological properties of the human α7 nicotinic acetylcholine receptor. *Eur. J. Pharmacol.* 290:237-246.

Gotti C, Hanke W, Maury K, Moretti M, Ballivet M, Clementi F and Bertrand D (1994) Pharmacology and biophysical properties of α7 and α7-α8 α-bungarotoxin receptor subtypes immunopurified from the chick optic lobe. *Eur. J. Neurosci.* 6:1281-1291.

Gough NM (1988) Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Anal. Biochem.* 173:93-95.

Halevi S, McKay J, Palfreyman M, Yassin L, Eshel M, Jorgensen EM and Treinin M (2002) The *C.elegans ric-3* gene is required for maturation of nicotinic acetylcholine receptors. *EMBO Journal* 21:1012-1020.

Halevi S, Yassin L, Eshel M, Sala F, Sala S, Criado M and Treinin M (2003) Conservation within the RIC-3 gene family: effectors of mammalian nicotinic acetylcholine receptor expression. *J. Biol. Chem.* 278:34411-34417.

Harkness PC and Millar NS (2002) Changes in conformation and sub-cellular distribution of α4β2 nicotinic acetylcholine receptors revealed by chronic nicotine treatment and expression of subunit chimeras. *J. Neurosci.* 22:10172-10181.

Jeanclos EM, Lin L, Treuil MW, Rao J, DeCoster MA and Anand R (2001) The chaperone protein 14-3-3η interacts with the nicotinic acetylcholine receptor α4 subunit. *J. Biol. Chem.* 276:28281-28290.

Kassner PD and Berg DK (1997) Differences in the fate of neuronal acetylcholine receptor protein expressed in neurons and stably transfected cells. *J. Neurobiol.* 33:968-982.

Lansdell SJ and Millar NS (2002) Dβ3, an atypical nicotinic acetylcholine receptor subunit from *Drosophila*: molecular cloning, heterologous expression and coassembly. *J. Neurochem.* 80:1009-1018.

Lansdell SJ, Schmitt B, Betz H, Sattelle DB and Millar NS (1997) Temperature-sensitive expression of *Drosophila* neuronal nicotinic acetylcholine receptors. *J. Neurochem.* 68:1812-1819.

Le Novère N, Corringer P-J and Changeux J-P (2002) The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences. *J. Neurobiol.* 53:447-456.

Lewis TM, Harkness PC, Sivilotti LG, Colquhoun D and Millar NS (1997) The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. *J. Physiol.* 505:299-306.

Lukas RJ, Norman SA and Lucero L (1993) Characterization of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y neuroblastoma clonal line. *Mol. Cell. Neurosci.* 4:1-12.

Millar NS (1999) Heterologous expression of mammalian and insect neuronal nicotinic acetylcholine receptors in cultured cell lines. *Biochem. Soc. Trans.* 27:944-950.

Millar NS (2003) Assembly and subunit diversity of nicotinic acetylcholine receptors. *Biochem. Soc. Trans.* 31:869-874.

Phillips WD, Kopta C, Blount P, Gardner PD, Steinbach JH and Merlie JP (1991) ACh receptor-rich membrane domains organized in fibroblasts by recombinant 43-kilodalton protein. *Science* 251:568-70.

Puchacz E, Buisson B, Bertrand D and Lukas RL (1994) Functional expression of nicotinic acetylcholine receptors containing rat α7 subunits in human SH-SY5Y neuroblastoma cells. *FEBS Letters* 354:155-159.

Quik M, Choremis J, Komourian J, Lukas RJ and Puchacz E (1996) Similarity between rat brain nicotinic α-bungarotoxin receptors and stably expressed α-bungarotoxin binding sites. *J. Neurochem.* 67:145-154.

Ragozzino D, Fucile S, Giovannelli A, Grassi F, Mileo AM, Ballivet M, Alema S and Eusebi F (1997) Functional properties of neuronal nicotinic acetylcholine receptor channels expressed in transfected human cells. *Eur. J. Neurosci.* 9:480-488.

Rangwala F, Drisdel RC, Rakhilin S, Ko E, Atluri P, Harkins AB, Fox AP, Salman SB and Green WN (1997) Neuronal α-bungarotoxin receptors differ structurally from other nicotinic acetylcholine receptors. *J. Neurosci.* 17:8201-8212.

Whiting P, Schoepfer R, Lindstrom J and Priestley T (1991) Structural and pharmacological characterization of the major brain nicotinic acetylcholine receptor subtype stably expressed in mouse fibroblasts. *Mol. Pharmacol.* 40:463-472.

Williams ME, Burton B, Urrutia A, Shcherbatko A, Chavez-Noriega LE, Cohen CJ and Aiyar J (2005) Ric-3 promotes functional expression of the nicotinic acetylcholine receptor α7 subunit in mammalian cells. *J. Biol. Chem.* 280:1257-1263.

Footnotes

Address correspondence to: Dr Neil Millar, Department of Pharmacology, University College
London, Gower Street, London, WC1E 6BT. Tel.: +44-(0)20-7679-7241; email:
n.millar@ucl.ac.uk

Legends for Figures

Figure 1. Influence of RIC-3 upon radioligand binding to α7 nAChR. Specific binding of [^{125}I]αBTX to the human and rat α7 nAChR subunit (hα7 and rα7) was detected in tsA201 cells cotransfected with CeRIC-3 or hRIC-3. Binding with [^{125}I]αBTX was performed on intact cells to examine levels of binding to cell surface receptors. Data are means of 7-8 independent experiments, each performed in triplicate Binding has been presented as fmol/10^6 cells to provide an indication of the density of cell-surface binding sites, but 10^6 cells corresponds to ~1-2 mg membrane protein.

Figure 2. Functional expression of α7 coexpressed with RIC-3 in tsA201 cells. Functional expression of α7 nAChRs was detected by whole-cell patch-clamp recording when coexpressed in human tsA201 cells with either CeRIC-3 or hRIC-3. Representative whole-cell recordings are shown of hα7 coexpressed with CeRIC-3 (*A* and *B*). Rapid application of 200 μM acetylcholine is indicated by a solid black line. Block of whole-cell responses by bath application of 100 nM MLA is indicated by a horizontal double line.

Figure 3. Coprecipitation of the α7 subunit with RIC-3. Human kidney tsA201 cells were transfected with combinations of hα7 nAChR subunit and hRIC-3 cDNAs (*A*) or with the α1 GABA$_A$R subunit and hRIC-3 (*B*). Proteins were immunoprecipitated from metabolically labelled cells and analysed by SDS-PAGE, followed by autoradiography. The nAChR hα7 subunit was detected by mAb319, a mAb raised against an endogenous α7 epitope, hRIC-3 (tagged with a FLAG epitope; hRIC-3^{FLAG}) was detected with mAbFLAG-M2 and the GABA$_A$R α1 subunit (tagged with a myc epitope; α1^{MYC}) was detected with mAb9E10. *A*) A band (apparent MW ~58 kDa) corresponding to hRIC-3^{FLAG} was detected in cells transfected

with hRIC-3^{FLAG} (lane 2) which was absent from untransfected cells (lane 1). A band of the

size expected of hα7 (~50 kDa) was coprecipitated with hRIC-3^{FLAG} from cells cotransfected

with hRIC-3^{FLAG} and hα7 (lane 3). The absence of cross-reactivity of mAbFLAG-M2 with

hα7 was confirmed (lane 4). A band corresponding to hα7 (~50 kDa), which was absent from

untransfected cells (lane 5), was detected in cells transfected with hα7 (lane 6). A band of ~58

kDa, corresponding to hRIC-3^{FLAG} (lane 7), and of ~55 kDa, corresponding to hRIC-3 (lane

8), was coprecipitated with hα7. The absence of cross-reactivity of mAb319 with hRIC-3^{FLAG}

and hRIC-3 was confirmed (lanes 9 and 10). *B*) A band (apparent MW ~58 kDa)

corresponding to hRIC-3^{FLAG}, which was absent from untransfected cells (lane 1), was

detected in cells transfected with hRIC-3^{FLAG} (lane 2). No evidence of a coprecipitated protein

was observed in cells cotransfected with hRIC-3^{FLAG} and α1^{MYC} (lane 3). A band

corresponding to α1^{MYC} (~50 kDa) was detected in cells transfected with α1^{MYC} (lane 6) and

was absent from untransfected cells (lane 5). No evidence of a coprecipitated protein was

observed in cells cotransfected with hRIC-3^{FLAG} and α1^{MYC} (lane 7). The position of

molecular weight markers is shown.

Figure 4. Coprecipitation of α4 and β2 subunits with RIC-3. Coprecipitation of hRIC-3

with the α4 and β2 nAChR subunits was examined by cotransfection in tsA201 cells. As

previously (Fig. 3), proteins were immunoprecipitated from metabolically labelled cells and

analysed by SDS-PAGE, followed by autoradiography. To examine whether coprecipitation

might be a consequence of non-specific interaction or aggregation, experiments were

performed in parallel in which hRIC-3 and nAChR subunits were transfected into separate

populations of cells (followed by solubilisation and mixing prior to immunoprecipitation). A

band (apparent MW ~58 kDa) corresponding to hRIC-3^{FLAG}, which was absent from

untransfected cells (lane 1), was detected in all cells transfected with hRIC-3^{FLAG} (lanes 2-7).

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Bands of the size expected for α4 and β2 were detected in cells in which these subunits were coexpressed alone (lanes 5 and 6) or together (lane 7). No evidence of coprecipitated α4 or β2 was observed when proteins were expressed alone and then mixed (lanes 2-4). Lane 1 shows the absence of cross-reactivity of α4 and β2 with mAbFLAG-M2. Experiments involving the mixing of proteins which were expressed separately is indicated by an asterisk. The position of molecular weight markers is shown.

Figure 5. Influence of hRIC-3 on specific [^{3}H]epibatidine binding to heteromeric nAChRs. Specific binding of [^{3}H]epibatidine was determined with membrane preparations of tsA201 cells transfected with α3β2, α3β4, α4β2 and α4β4 subunit combinations in the presence and absence of cotransfected hRIC-3. Data are means of 3-7 independent experiments, each performed in triplicate. Significant differences, determined by two-tailed Student's *t*-test, are indicated (* $p < 0.05$; ** $p < 0.02$; *** $p < 0.002$).

Figure 6. Enhancement of functional expression of heteromeric nAChRs by RIC-3. *A*) Functional expression of α3β2, α3β4, α4β2 and α4β4 nAChRs examined by agonist-induced changes in intracellular calcium in populations of transfected tsA201 cells. Cells loaded with the calcium-sensitive dye fluo-4 were plated in 96-well plates. Agonist-induced changes in fluorescence were recorded simultaneously in all wells of the 96 well plate using a fluorometric imaging plate reader (FLIPR). Traces show representative paired experiments in which responses to different nAChR subunit combinations are compared in the absence (lower trace) and presence of hRIC-3 (upper trace). Data points, recorded at 1 sec intervals, are means of four separate wells from a single 96 well plate. Error bars are shown for every 10th data point. *B*) The influence of coexpression of hRIC-3 on functional responses measured as agonist-induced elevations in intracellular calcium is presented as a fold effect, normalised

to paired responses for each subunit combination in the absence of hRIC-3. Data are means of

3-5 independent experiments, each performed in quadruplicate. Significant differences,

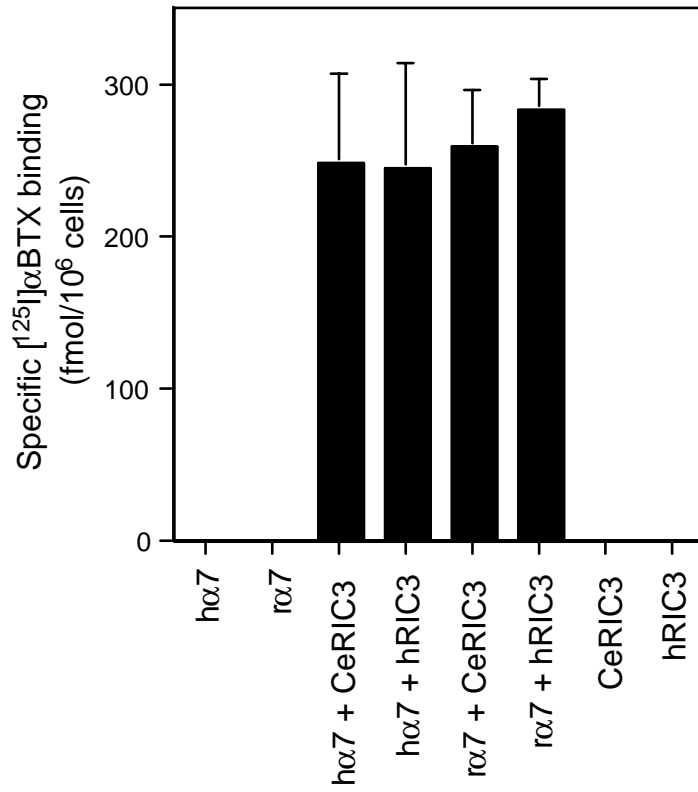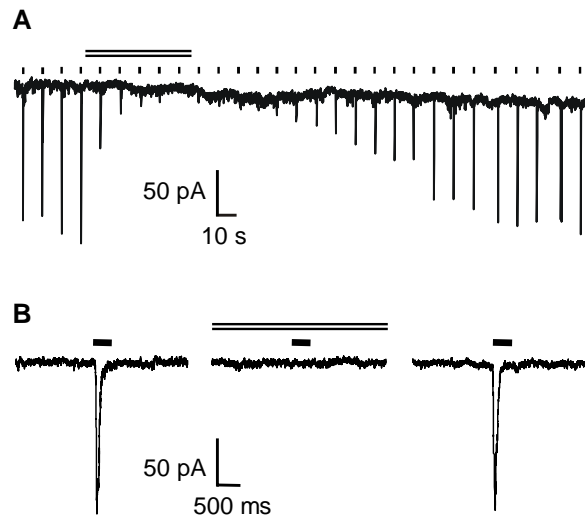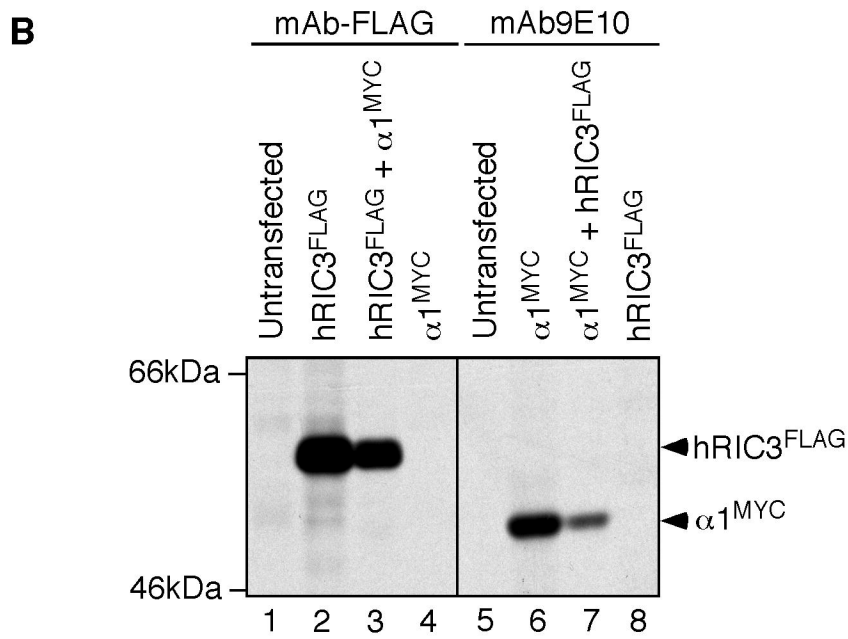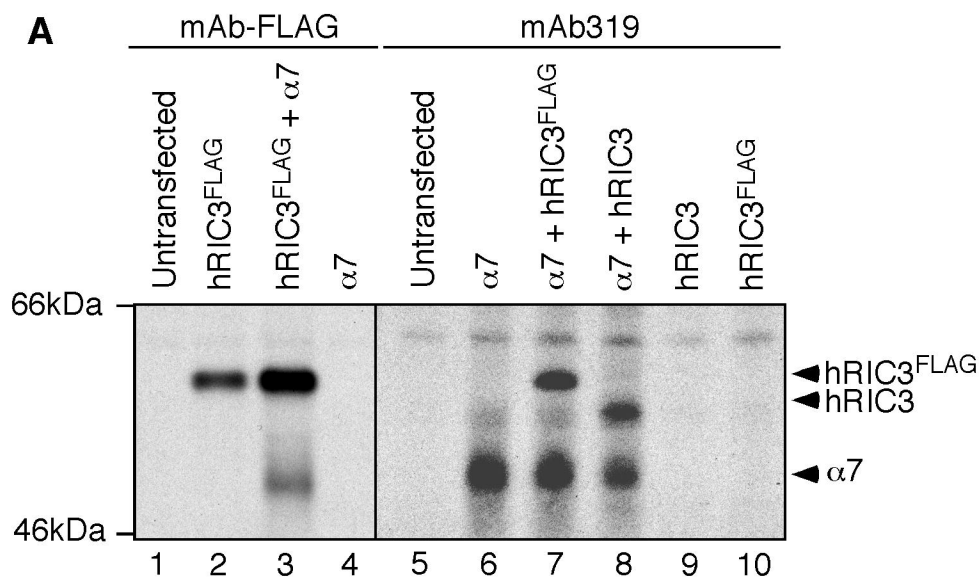determined by two-tailed Student's *t*-test, are indicated (* $p < 0.05$)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6