

# RIC-3 Enhances Functional Expression of Multiple Nicotinic Acetylcholine Receptor Subtypes in Mammalian Cells

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Received July 29, 2005; accepted August 24, 2005

## ABSTRACT

Recent studies have shown that RIC-3, originally identified in *Caenorhabditis elegans* as the protein encoded by the gene resistance to inhibitors of cholinesterase (*ric-3*), can enhance functional expression of  $\alpha 7$  nicotinic acetylcholine receptors (nAChRs). In the present study, the influence of *C. elegans* and human RIC-3 upon multiple homomeric ( $\alpha 7$ ,  $\alpha 8$ , and  $\alpha 9$ ) and heteromeric ( $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ ,  $\alpha 4\beta 4$ , and  $\alpha 9\alpha 10$ ) nAChR subtypes has been examined in transfected mammalian cells by radioligand binding and functional characterization. Coexpression of RIC-3 facilitates a dramatic enhancement of the ability of  $\alpha 7$  (and the closely related  $\alpha 8$  subunit) to generate functional nAChRs in otherwise nonpermissive mammalian cells. In contrast, coexpression of RIC-3 did not facilitate functional expression of either homomeric  $\alpha 9$  or heteromeric  $\alpha 9\alpha 10$  nAChRs in mammalian cell lines. It is noteworthy that whereas RIC-3 has been reported to cause a marked functional inhibition of het-

eromeric nAChRs such as  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$  expressed in *Xenopus laevis* oocytes, RIC-3 significantly enhances levels of functional expression of these and other ( $\alpha 3\beta 2$  and  $\alpha 4\beta 4$ ) heteromeric nAChRs when expressed in mammalian cell lines. In addition, the interaction of multiple nAChR subunits ( $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$ ) with RIC-3 has been demonstrated by coimmunoprecipitation from metabolically labeled transfected cells. It is significant that coimmunoprecipitation experiments have provided evidence that RIC-3 associates with unassembled nAChR subunits, a finding that 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 an nAChR-associated protein that can enhance functional expression of multiple nAChR subtypes in transfected mammalian cells.

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 ( $\alpha 1$ – $\alpha 10$ ,  $\beta 1$ – $\beta 4$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ) that 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 characterization of recombinant nAChRs in a variety of artificial expression systems, such as *Xenopus laevis* 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 characterized nAChRs expressed at the skeletal neuromuscular junction (which are assembled from  $\alpha 1$ ,  $\beta 1$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  subunits), a family of nAChRs is expressed in the central and peripheral nervous system, the “neuronal nAChRs”. Nine neuronal nAChR subunits ( $\alpha 2$ – $\alpha 7$  and  $\beta 2$ – $\beta 4$ ) 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  $\alpha 8$  subunit, which is closely related in primary amino acid sequence to the  $\alpha 7$  subunit, has been identified in avian, but not mammalian, species. The two most recently identified nAChR subunits ( $\alpha 9$  and  $\alpha 10$ ) seem to be expressed primarily in hair cells of the inner ear (Elgoyhen et al., 1994, 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  $\alpha 7$  subunit (and the

This work was supported by grants from the Biological and Biotechnology Research Council, the Medical Research Council, and The Wellcome Trust.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.105.017459.

**ABBREVIATIONS:** nAChR, nicotinic acetylcholine receptor; mAb, monoclonal antibody; RT-PCR, reverse transcriptase-polymerase chain reaction;  $\alpha$ BTX,  $\alpha$ -bungarotoxin; FLIPR, fluorometric imaging plate reader; PCR, polymerase chain reaction; MLA, methyllycaconitine; 5HT, 5-hydroxytryptamine; PAGE, polyacrylamide gel electrophoresis.

closely related  $\alpha 8$  subunit). There is evidence to suggest that native  $\alpha 7$  nAChRs are homomeric (Chen and Patrick, 1997; Drisdell and Green, 2000). The  $\alpha 7$  subunit has also been shown to generate functional homomeric nAChRs when expressed in *X. laevis* oocytes (Couturier et al., 1990), as has  $\alpha 8$  (Gerzanich et al., 1994; Gotti et al., 1994). Although the  $\alpha 9$  subunit has been reported to form homomeric channels in oocytes (Elgoyhen et al., 1994), it seems to form heteromeric nAChRs (with  $\alpha 10$ ) more efficiently (Elgoyhen et al., 2001).

Although the  $\alpha 7$  subunit is able to generate functional nAChRs when expressed in *X. laevis* oocytes, considerable difficulties have been encountered in the efficient expression of functional  $\alpha 7$  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  $\alpha 8$  and  $\alpha 9$  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  $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 4\beta 4$ ) in cultured mammalian cells (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 that 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 resistant to inhibitors of cholinesterase (*ric-3*), which contains two putative transmembrane domains (Halevi et al., 2002). Studies performed in *X. laevis* oocytes have revealed that coexpression of the *C. elegans* RIC-3 protein (CeRIC-3) or its human homolog (hRIC-3) can enhance levels of functional  $\alpha 7$  nAChRs (Halevi et al., 2002, 2003) but causes a marked inhibition of functional responses with heteromeric nAChRs such as  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$ . Recent studies have also demonstrated that coexpression of RIC-3 can facilitate functional expression of  $\alpha 7$  in mammalian cells (Williams et al., 2005). In the present study, we have examined the influence of RIC-3 upon multiple homomeric ( $\alpha 7$ ,  $\alpha 8$ , and  $\alpha 9$ ) and heteromeric ( $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ ,  $\alpha 4\beta 4$ , and  $\alpha 9\alpha 10$ ) nAChR subtypes expressed in transfected mammalian cells.

## Materials and Methods

**Plasmids, Antibodies, and Cell Lines.** Rat  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 7$ ,  $\beta 2$ , and  $\beta 4$  nAChR subunit cDNAs were provided by Jim Patrick (Baylor College of Medicine, TX). Human  $\alpha 7$  and chick  $\alpha 8$  nAChR subunit cDNAs were provided by Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). Rat  $\alpha 9$  nAChR subunit cDNA was provided by Belén Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). Rat  $\alpha 10$  nAChR subunit cDNA was provided by Jim Boulter (University of California, Los Angeles, CA). Mouse GABA<sub>A</sub> receptor  $\alpha 1$  subunit tagged at its N terminus with a myc epitope (GABA<sub>A</sub>R  $\alpha 1^{MYC}$ ) (Connolly et al., 1996) was provided by Steve Moss (University of Pennsylvania). *C. elegans* RIC-3 cDNA was provided by Millet Treinin (Hebrew University, Jerusalem, Israel). Monoclonal antibody (mAb) mAb319, raised against the nAChR  $\alpha 7$  subunit, and mAbFLAG-M2, raised against the FLAG epitope, were obtained from Sigma-Aldrich (St. Louis, MO). A mAb specific for the myc epitope

tag (mAb9E10) was purified from the Myc1-9E10 hybridoma cell line (European Collection of Cell Cultures 85102202).

**Detection of RIC-3 Transcripts.** Total RNA was prepared from  $\sim 5 \times 10^6$  cells, by methods described previously (Gough, 1988). Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was performed using the OneStep PCR kit (QIAGEN, Valencia, CA) with 0.5  $\mu$ g of 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  $\alpha 1$  tubulin gene sequence (5' ACACCTTCTTCAGTGAGACAGG 3' and 5' CTCATTGTCTACCATGAAGGCAC 3').

**Molecular Cloning and Epitope Tagging of hRIC-3.** Oligonucleotide primers were synthesized 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, Carlsbad, CA) and then excised with EcoRI 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 BstEII site located near the C terminus, after a glycine residue at position 224, to create pRK5-hRIC-3<sup>FLAG</sup>.

**Heterologous Expression.** Human kidney tsA201 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (Sigma-Aldrich), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml) (Invitrogen). Cells were maintained in a humidified incubator containing 5% CO<sub>2</sub> 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.** [<sup>3</sup>H]Epibatidine (55.5 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences, and [<sup>125</sup>I]- $\alpha$ -bungarotoxin (<sup>125</sup>I- $\alpha$ BTX; 150–200 Ci/mmol) from GE Healthcare (Little Chalfont, Buckinghamshire, UK). 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 [<sup>3</sup>H]epibatidine) or GF/A filters (for [<sup>125</sup>I]- $\alpha$ BTX), followed by rapid washing using a Brandel cell harvester. Before use, filters were presoaked in 0.5% polyethylenimine. All binding experiments with [<sup>125</sup>I]- $\alpha$ BTX were performed in buffer containing 0.5% bovine serum albumin to reduce nonspecific binding.

**Metabolic Labeling and Immunoprecipitation.** Transfected tsA201 cells were metabolically labeled as described previously (Cooper and Millar, 1997). After growth in methionine-free medium for 15 min, cells were labeled with 250  $\mu$ Ci of Pro-mix, a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (GE Healthcare) in 3.5 ml of methionine-free medium for 3 h. Medium containing 30 mg/l methionine and 10% heat-inactivated fetal calf serum was then added, and the cells incubated for a further 90 min. Cells were washed twice with 10 ml of phosphate-buffered saline (PBS) and harvested into 300  $\mu$ l of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 5 mM EDTA, and 1% Triton X-100) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, and 10  $\mu$ g/ml each of leupeptin, aprotinin, and pepstatin). Solubilization and all subsequent steps were performed at 4°C. After 1-h solubilization, the cell lysate was precleared by incubation overnight with 30  $\mu$ l of protein G-Sepharose (GE Healthcare) in a 1:1 mixture with lysis buffer. Nonsolubilized material was pelleted by centrifugation at 14,000g for 15 min. Cell lysates were incubated with primary antibody for 3.5 h. The antibody-receptor complex was immunoprecipitated by the addition of 35  $\mu$ l of protein G-Sepharose, incubated for a further 3.5 h, and isolated by centrifugation. Samples were washed with 4  $\times$  1 ml of 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  $\mu\text{g}/\text{ml}$ ), were cotransfected with pEGFP-C2 (BD Biosciences Clontech, Palo Alto, CA) encoding green fluorescent protein and with plasmids containing RIC-3 and nAChR subunit constructs. Whole-cell recordings were performed at room temperature, 36 to 48 h after transfection, from cells identified under fluorescence microscopy as green fluorescent protein-positive. Recording solution contained 110 mM NaCl, 5.4 mM KCl, 0.8 mM  $\text{MgCl}_2$ , 1.8 mM  $\text{CaCl}_2$ , 25 mM glucose, 0.9 mM  $\text{NaH}_2\text{PO}_4$ , and 44 mM  $\text{NaHCO}_3$ . Borosilicate electrodes (GC150F-7.5; Harvard Apparatus Inc., Holliston, MA) of resistance 6 to 12 M $\Omega$  contained 140 mM CsCl, 10 mM HEPES, 10 mM EGTA, 0.5 mM  $\text{CaCl}_2$ , and 29.53 mM CsOH, pH adjusted to 7.26, osmolality 283 mOsmol/kg  $\text{H}_2\text{O}$ . Unless otherwise specified, the holding potential was  $-60$  mV. Fast cell superfusion of agonists was achieved with a theta-barreled application pipette made from 1.5-mm-diameter theta tubing (AH-30-0114; Harvard Apparatus Inc.), which was moved laterally using a stepper motor. Agonist-evoked currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized 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 to 20 h post-transfection. Approximately 24 h later, cell medium was removed, and the cells incubated in 50 to 100  $\mu\text{l}$  of 1  $\mu\text{M}$  Fluo-4 acetoxymethyl ester (Invitrogen) in Hanks' balanced salt solution with 0.02% Pluronic F-127 (Invitrogen) for 30 to 60 min at room temperature. Cells were rinsed once with 160  $\mu\text{l}$  of assay buffer (Hanks' balanced salt solution supplemented with 18.8 mM  $\text{CaCl}_2$ , 8.8 mM sucrose, and 6.3 mM HEPES), and the cells were assayed using the fluorometric imaging plate reader (FLIPR) system (Molecular Devices, Warriner, 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 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 preprogrammed, and delivery was automated through a 96-tip head pipetter.

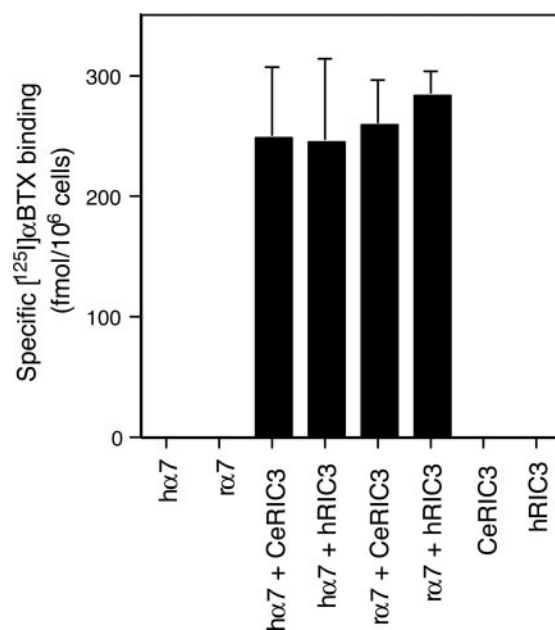
## Results

**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 RT-PCR. A single band of the size expected for PCR-amplified hRIC-3 transcripts (220 base pairs) was detected in SH-SY5Y cells, a human neuroblastoma cell line that expresses endogenous  $\alpha 7$  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 that has been shown previously to be a host cell type in which  $\alpha 7$  fails to form functional nAChRs (Cooper and Millar, 1997). These findings are in agreement with those that 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 pheochromocytoma PC12 cell line differ in their expression of endogenous  $\alpha 7$  nAChRs and in their ability to express functional recombinant  $\alpha 7$  nAChRs (Blumenthal et al., 1997). In the present study, two isolates of PC12 cells (PC12-a and PC12-b) were examined that exhibited different levels of specific binding of [ $^3\text{H}$ ]MLA, an  $\alpha 7$ -selective nicotinic antagonist.

PC12-a expressed specific [ $^3\text{H}$ ]MLA binding ( $230 \pm 83$  fmol/mg of protein;  $n = 4$ ), whereas little or no specific binding of [ $^3\text{H}$ ]MLA could be detected in PC12-b cells. In addition, the rat pituitary  $\text{GH}_4\text{C}_1$  cell line was examined. The  $\text{GH}_4\text{C}_1$  cell line does not express endogenous  $\alpha 7$  nAChRs but is a suitable host cell type in which to express functional recombinant  $\alpha 7$  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-PCR amplification of mRNA isolated from  $\text{GH}_4\text{C}_1$  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 [ $^3\text{H}$ ]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  $\alpha 1$  tubulin (473 base pairs) 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  $\alpha 7$  nAChRs (either endogenously or from heterologous expression of  $\alpha 7$  cDNA).

**Heterologous Coexpression of  $\alpha 7$  and RIC-3.** In agreement with previous studies (Eiselé et al., 1993; Cooper and Millar, 1997, 1998), no specific binding of the  $\alpha 7$ -selective radioligand [ $^{125}\text{I}$ ]- $\alpha\text{BTX}$  could be detected when either the human  $\alpha 7$  subunit (h $\alpha 7$ ) or rat  $\alpha 7$  subunit (r $\alpha 7$ ) was expressed by transient transfection in human kidney tsA201 cells (Fig. 1). In marked contrast, however, when either the h $\alpha 7$  or r $\alpha 7$  subunit was coexpressed with CeRIC-3 or hRIC-3,



**Fig. 1.** Influence of RIC-3 upon radioligand binding to  $\alpha 7$  nAChR. Specific binding of [ $^{125}\text{I}$ ]- $\alpha\text{BTX}$  to the human and rat  $\alpha 7$  nAChR subunit (h $\alpha 7$  and r $\alpha 7$ ) was detected in tsA201 cells cotransfected with CeRIC-3 or hRIC-3. Binding with [ $^{125}\text{I}$ ]- $\alpha\text{BTX}$  was performed on intact cells to examine levels of binding to cell surface receptors. Data are means of seven to eight independent experiments, each performed in triplicate. Binding has been presented as femtomoles per  $10^6$  cells to provide an indication of the density of cell surface binding sites, but  $10^6$  cells corresponds to  $\sim 1$  to 2 mg of membrane protein.

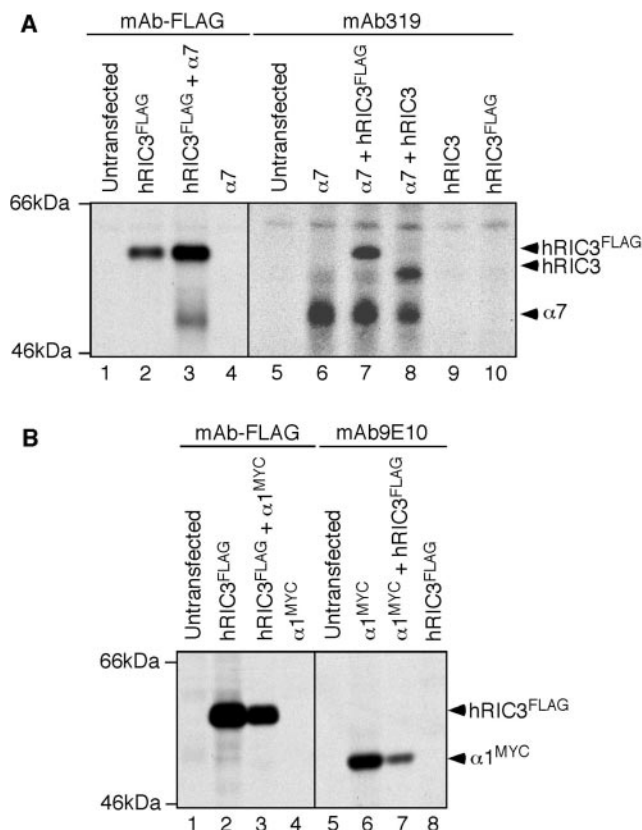


high levels of specific radioligand binding were detected (Fig. 1), findings that are consistent with previous studies (Williams et al., 2005).

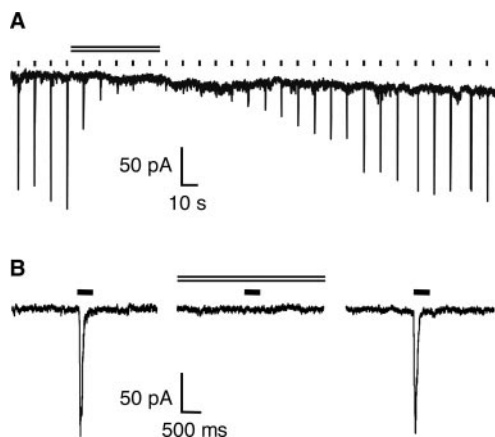
**Functional Expression of  $\alpha 7$  nAChRs.** To determine whether functional  $\alpha 7$  nAChRs are formed in tsA201 cells after cotransfection with RIC-3, cells were examined by whole-cell patch-clamp recording. Rapidly desensitizing responses to brief applications of 200  $\mu$ M acetylcholine (Fig. 2), characteristic of  $\alpha 7$  nAChRs, were detected in cells transfected with all combinations of  $\alpha 7$  and RIC-3 examined:  $\alpha 7$ +CeRIC-3 (six of seven cells),  $\alpha 7$ +hRIC-3 (nine of 11 cells),  $\alpha 7$ +hRIC-3 (eight of eight cells), and  $\alpha 7$ +hRIC-3 (13 of 17 cells). No significant differences were observed in the magnitude of whole-cell responses from cells transfected with  $\alpha 7$  and RIC-3 cDNA combinations from different species (mean  $119 \pm 28$  pA). We have been unable to detect functional responses from  $\alpha 7$  expressed alone in transfected tsA201 cells by whole-cell electrophysiological recording (zero of 18 cells examined).

**Coassembly of  $\alpha 7$  with RIC-3.** The ability of RIC-3 to coassemble with  $\alpha 7$  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<sup>FLAG</sup>. Cells were transfected with combinations of  $\alpha 7$  and hRIC-3<sup>FLAG</sup> cDNAs and examined by immunoprecipitation with either the  $\alpha 7$ -specific antibody mAb319 or with mAbFLAG-M2, an antibody that recognizes the FLAG epitope. Specific bands corresponding to  $\alpha 7$  and hRIC-3<sup>FLAG</sup> were detected in cells transfected with these cDNAs alone and were absent from untransfected cells (Fig. 3A). Coprecipitation of  $\alpha 7$  with hRIC-3<sup>FLAG</sup> was observed in cells cotransfected with  $\alpha 7$  and hRIC-3<sup>FLAG</sup> using mAbFLAG-M2 (Fig. 3A, lane 3). Likewise, coprecipitation of hRIC-3<sup>FLAG</sup> with  $\alpha 7$  was observed in cells cotransfected with  $\alpha 7$  and hRIC-3<sup>FLAG</sup> using mAb319 (Fig. 3A, lane 7). The absence of cross-reactivity of mAbFLAG-M2 with  $\alpha 7$  and of mAb319 with hRIC-3<sup>FLAG</sup> was also confirmed (Fig. 3A, lanes 4 and 10). This provides strong evidence of coassembly of the  $\alpha 7$  and hRIC-3 proteins. To eliminate the possibility that the coimmunoprecipitation of  $\alpha 7$  and RIC-3 was a con-

sequence of nonspecific protein aggregation, a control experiment was performed in which  $\alpha 7$  and hRIC-3 were expressed separately by transfection of tsA201 cells. Transfected cells were metabolically labeled, disrupted by detergent solubilization, and the two cell extracts were mixed before immunoprecipitation. In contrast to the clear evidence for coprecipitation when  $\alpha 7$  and hRIC-3 were coexpressed, no coimmunoprecipitation was observed when proteins were mixed after having been expressed separately (data not shown).



**Fig. 3.** Coprecipitation of the  $\alpha 7$  subunit with RIC-3. Human kidney tsA201 cells were transfected with combinations of  $\alpha 7$  nAChR subunit and hRIC-3 cDNAs (A) or with the  $\alpha 1$  GABA<sub>A</sub> subunit and hRIC-3 (B). Proteins were immunoprecipitated from metabolically labeled cells and analyzed by SDS-PAGE, followed by autoradiography. The nAChR  $\alpha 7$  subunit was detected by mAb319, a mAb raised against an endogenous  $\alpha 7$  epitope, hRIC-3 (tagged with a FLAG epitope; hRIC-3<sup>FLAG</sup>) was detected with mAbFLAG-M2 and the GABA<sub>A</sub>  $\alpha 1$  subunit (tagged with a myc epitope;  $\alpha 1^{MYC}$ ) was detected with mAb9E10. A, a band (apparent molecular mass ~58 kDa) corresponding to hRIC-3<sup>FLAG</sup> was detected in cells transfected with hRIC-3<sup>FLAG</sup> (lane 2) that was absent from untransfected cells (lane 1). A band of the size expected of  $\alpha 7$  (~50 kDa) was coprecipitated with hRIC-3<sup>FLAG</sup> from cells cotransfected with hRIC-3<sup>FLAG</sup> and  $\alpha 7$  (lane 3). The absence of cross-reactivity of mAbFLAG-M2 with  $\alpha 7$  was confirmed (lane 4). A band corresponding to  $\alpha 7$  (~50 kDa), which was absent from untransfected cells (lane 5), was detected in cells transfected with  $\alpha 7$  (lane 6). A band of ~58 kDa, corresponding to hRIC-3<sup>FLAG</sup> (lane 7), and of ~55 kDa, corresponding to hRIC-3 (lane 8), was coprecipitated with  $\alpha 7$ . The absence of cross-reactivity of mAb319 with hRIC-3<sup>FLAG</sup> and hRIC-3 was confirmed (lanes 9 and 10). B, a band (apparent molecular mass ~58 kDa) corresponding to hRIC-3<sup>FLAG</sup>, which was absent from untransfected cells (lane 1), was detected in cells transfected with hRIC-3<sup>FLAG</sup> (lane 2). No evidence of a coprecipitated protein was observed in cells cotransfected with hRIC-3<sup>FLAG</sup> and  $\alpha 1^{MYC}$  (lane 3). A band corresponding to  $\alpha 1^{MYC}$  (~50 kDa) was detected in cells transfected with  $\alpha 1^{MYC}$  (lane 6) and was absent from untransfected cells (lane 5). No evidence of a coprecipitated protein was observed in cells cotransfected with hRIC-3<sup>FLAG</sup> and  $\alpha 1^{MYC}$  (lane 7). The position of molecular mass markers is shown.



**Fig. 2.** Functional expression of  $\alpha 7$  coexpressed with RIC-3 in tsA201 cells. Functional expression of  $\alpha 7$  nAChRs was detected by whole-cell patch-clamp recording when coexpressed in human tsA201 cells with CeRIC-3. Representative whole-cell recordings are shown of  $\alpha 7$  coexpressed with CeRIC-3 (A and B). Rapid application of 200  $\mu$ M 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.

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

**Influence of RIC-3 upon  $\alpha 8$ -,  $\alpha 9$ -, and  $\alpha 10$ -Containing nAChRs.** Although 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 *X. laevis* 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 <sup>125</sup>I- $\alpha$ BTX binding was also detected when the chick  $\alpha 8$  nAChR subunit was coexpressed with either CeRIC-3 ( $53.0 \pm 13.8$  fmol/ $10^6$  cells;  $n = 3$ ) or hRIC-3 ( $24.0 \pm 6.2$  fmol/ $10^6$  cells;  $n = 3$ ). In contrast, no significant binding of <sup>125</sup>I- $\alpha$ BTX was detected when the  $\alpha 8$  subunit was expressed in the absence of RIC-3 ( $1.1 \pm 1.7$  fmol/ $10^6$  cells;  $n = 3$ ). Despite the ability of RIC-3 to facilitate binding of <sup>125</sup>I- $\alpha$ BTX to homomeric  $\alpha 7$  and  $\alpha 8$  nAChRs, no evidence of specific nicotinic radioligand binding was detected when the rat  $\alpha 9$  nAChR subunit was cotransfected with either CeRIC-3 or hRIC-3. Because there is strong evidence for coassembly of  $\alpha 9$  and  $\alpha 10$  subunits into heteromeric nAChRs (Elgoyhen et al., 2001; Baker et al., 2004), the influence of RIC-3 constructs upon coexpressed  $\alpha 9$  and  $\alpha 10$  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.

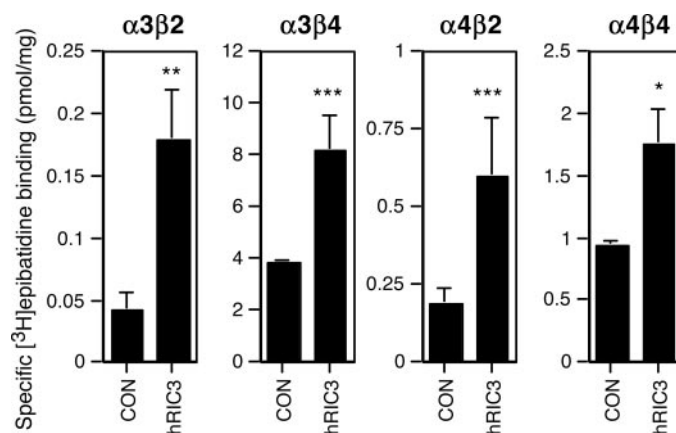
Despite the well documented problems associated with efficient functional expression of  $\alpha 8$  in transfected mammalian cells (Cooper and Millar, 1998), in three of 11 cells examined, whole-cell responses to acetylcholine ( $72 \pm 26$  pA) were observed. However, coexpression of  $\alpha 8$  with hRIC-3 generated whole-cell responses to acetylcholine ( $124 \pm 24$  pA) in all cells examined (14 of 14 cells). The rapidly desensitizing whole-cell responses to acetylcholine were completely and reversibly blocked by *d*-tubocurarine (data not shown).

**Influence of RIC-3 upon  $\alpha 3$ -,  $\alpha 4$ -,  $\beta 2$ -, and  $\beta 4$ -Containing nAChRs.** Previous studies conducted in oocytes have reported that coexpression of RIC-3 causes a marked inhibition of functional responses with heteromeric  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$  nAChRs (Halevi et al., 2003). We have examined the influence of hRIC-3 upon levels of radioligand binding to four nAChRs ( $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 4\beta 4$ ) expressed in mammalian cells. Coexpression of hRIC-3 resulted in significantly enhanced levels of specific [<sup>3</sup>H]epibatidine binding to all subunit combinations (Fig. 4). To examine the influence of RIC-3 upon levels of functional nAChRs, agonist-induced changes in intracellular calcium were examined by use of an auto-

mated 96-well format 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 5A 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  $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 4\beta 4$  nAChRs when cotransfected with hRIC-3 (Fig. 5B).

Although fewer problems have been encountered in the functional expression of heteromeric nAChRs such as  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$  in transfected mammalian cells, we and others have encountered particular difficulties in detecting functional expression of  $\alpha 3\beta 2$  nAChRs by patch-clamp recording. In tsA201 cells transfected with  $\alpha 3$  and  $\beta 2$  subunits in the absence of coexpressed RIC-3, small whole-cell responses ( $\sim 18$  pA) were detected in only two of 10 cells examined. In contrast, when  $\alpha 3$  and  $\beta 2$  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  $\alpha 4$  and  $\beta 2$  were coimmunoprecipitated with hRIC-3 (Fig. 6). As shown previously for  $\alpha 7$  (Fig. 3A), clear evidence for the coimmunoprecipitation of other nAChR subunits ( $\alpha 3$ ,  $\alpha 4$ ,  $\beta 2$ , and  $\beta 4$ ) with hRIC-3 was obtained (coprecipitation of  $\alpha 4$  and  $\beta 2$  is illustrated in Fig. 6). Because  $\alpha 3$ ,  $\alpha 4$ ,  $\beta 2$ , and  $\beta 4$  form functional nAChRs only when assembled into heteromeric complexes (e.g.,  $\alpha 4\beta 2$  and  $\alpha 3\beta 4$ ), we were interested to examine whether coassembly of RIC-3 could be detected when these subunits were expressed individually. Clear evidence of coassembly with RIC-3 was detected for all four subunits examined (see Fig. 6 for data obtained with  $\alpha 4$  and  $\beta 2$ ), indicating that RIC-3 is able to associate with unassembled nAChR subunits.



**Fig. 4.** Influence of hRIC-3 on specific [<sup>3</sup>H]epibatidine binding to heteromeric nAChRs. Specific binding of [<sup>3</sup>H]epibatidine was determined with membrane preparations of tsA201 cells transfected with  $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 4\beta 4$  subunit combinations in the presence and absence of cotransfected hRIC-3. Data are means of three to seven independent experiments, each performed in triplicate. Significant differences, determined by two-tailed Student's *t* test, are indicated (\*,  $p < 0.05$ ; \*\*,  $p < 0.02$ ; \*\*\*,  $p < 0.002$ ).

## Discussion

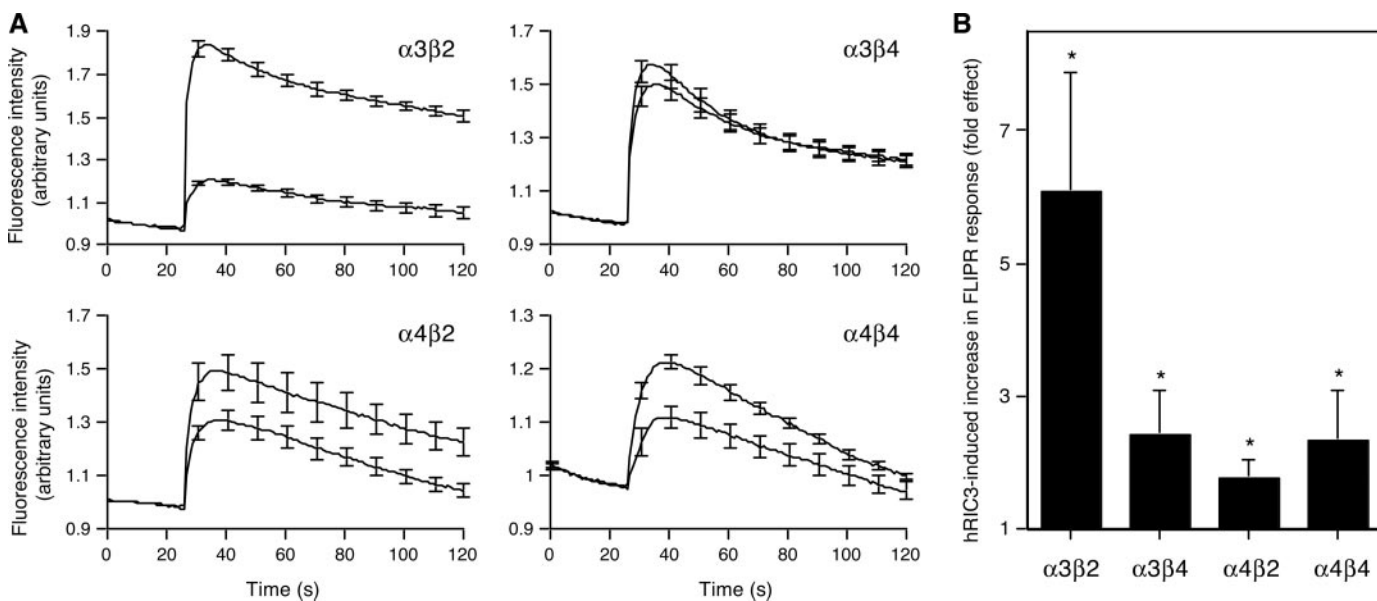
The RIC-3 protein from *C. elegans* (CeRIC-3) and its human homolog (hRIC-3) have been shown to enhance levels of functional nAChRs when coexpressed with  $\alpha 7$  in *X. laevis* oocytes (Halevi et al., 2002, 2003). A similar enhancement is seen with a *C. elegans* nAChR containing the DEG-3 and DES-2 subunits (Halevi et al., 2002, 2003). An enhancement of cell surface expression of the 5HT<sub>3</sub> receptor subunit 5HT<sub>3A</sub> (a subunit that shows close sequence similarity to nAChR subunits) has also been reported in mammalian cells (Cheng et al., 2005). In contrast, RIC-3 does not seem to influence functional expression levels of GABA, glutamate, and glycine receptors (Halevi et al., 2002, 2003). Despite the enhancement of  $\alpha 7$  nAChR responses caused by coexpression of RIC-3, a marked inhibition of functional responses to heteromeric nAChRs such as  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$  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 *X. laevis* oocytes, considerable problems have been encountered in functional expression in transfected mammalian cells and are particularly pronounced for homomeric nAChRs such as  $\alpha 7$  (Millar, 1999). When expressed in *X. laevis* oocytes, the nAChR  $\alpha 7$  subunit forms functional ion channels that are blocked by the snake neurotoxin  $\alpha$ BTX (Couturier et al., 1990). Although there have been reports of the successful heterologous expression of functional  $\alpha 7$  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  $\alpha 7$  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

nonpermissive 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 subunit 5HT<sub>3A</sub> (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 it 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 seems, 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<sub>A</sub>  $\alpha 1$  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 func-



**Fig. 5.** Enhancement of functional expression of heteromeric nAChRs by RIC-3. **A**, functional expression of  $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 4\beta 4$  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 FLIPR. Traces show representative paired experiments in which responses to different nAChR subunit combinations are compared in the absence (bottom trace) and presence of hRIC-3 (top trace). Data points, recorded at 1-s intervals, are means of four separate wells from a single 96-well plate. Error bars are shown for every 10th data point. **B**, influence of coexpression of hRIC-3 on functional responses measured as agonist-induced elevations in intracellular calcium is presented as a fold effect, normalized to paired responses for each subunit combination in the absence of hRIC-3. Data are means of three to five independent experiments, each performed in quadruplicate. Significant differences, determined by two-tailed Student's *t* test, are indicated (\*,  $p < 0.05$ ).



tional expression levels of GABA, glutamate, and glycine receptors are unaffected by coexpression of RIC-3 (Halevi et al., 2002, 2003). A recent study demonstrated the coprecipitation of RIC-3 with the 5HT<sub>3</sub> receptor subunit 5HT<sub>3A</sub> (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.,  $\alpha 4$  and  $\beta 2$ ; Fig. 6). 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 that 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<sub>3A</sub> subunits within the endoplasmic reticulum (Cheng et al., 2005).

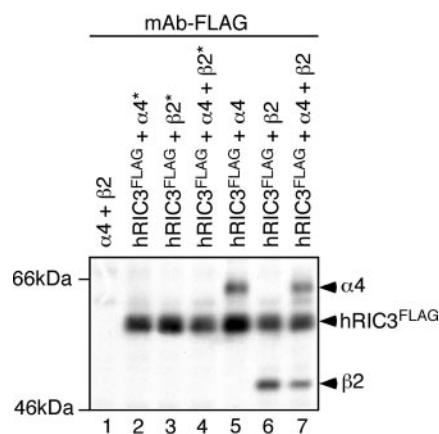
The apparent molecular mass of the hRIC-3<sup>FLAG</sup> protein (~58 kDa), determined by immunoprecipitation and SDS-PAGE, is considerably larger than would have been predicted from the primary amino acid sequence (41 kDa). When precipitated with mAb FLAG-M2 (Fig. 3A, lane 2 and 3B, lane 2), a single band was detected that 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<sup>FLAG</sup>. In addition, a band of the same apparent molecular mass was found to coprecipitate with  $\alpha 7$  (Fig. 3A, lane 7). This band was observed only in cells transfected with hRIC-3<sup>FLAG</sup> (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 mass of a protein determined by SDS-PAGE (Lansdell and Millar, 2002). In agreement with these previous observations, a band of lower apparent molecular mass (~55 kDa) was found to coprecipitate with  $\alpha 7$  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  $\alpha 7$ . Although the FLAG epitope is responsible, at least in part, for the high apparent molecular mass of hRIC-3<sup>FLAG</sup>, the untagged hRIC-3 also migrated at a higher apparent molecular mass 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 mass cannot be attributed to N-linked glycosylation, as is common in nAChR subunits. It is not clear what is responsible for the high apparent molecular mass 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 masses 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 an nAChR-associated protein that 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 seem to be important in assembly or intracellular trafficking. In comparison with 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 such fish as *Torpedo californica*. Although rapsyn plays an important role in organization 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 14-3-3 $\eta$  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 they are ubiquitously expressed and interact with a diverse group of proteins. The work reported here and previously (Halevi et al., 2002, 2003; Cheng et al., 2005; Williams et al., 2005) suggests that RIC-3 may play a role that is restricted to a narrower group of proteins, such as nAChRs and the closely related 5HT<sub>3</sub> 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  $\alpha 7$ . In mammalian cell lines that do not permit efficient folding and functional expression of  $\alpha 7$  nAChRs, coexpression of RIC-3 facilitates both subunit assembly (assayed by nicotinic radioligand binding) and formation of functional nAChRs (assayed



**Fig. 6.** Coprecipitation of  $\alpha 4$  and  $\beta 2$  subunits with RIC-3. Coprecipitation of hRIC-3 with the  $\alpha 4$  and  $\beta 2$  nAChR subunits was examined by cotransfection in tsA201 cells. As shown previously (Fig. 3), proteins were immunoprecipitated from metabolically labeled cells and analyzed by SDS-PAGE, followed by autoradiography. To examine whether coprecipitation might be a consequence of nonspecific interaction or aggregation, experiments were performed in parallel in which hRIC-3 and nAChR subunits were transfected into separate populations of cells (followed by solubilization and mixing before immunoprecipitation). A band (apparent molecular mass ~58 kDa) corresponding to hRIC-3<sup>FLAG</sup>, which was absent from untransfected cells (lane 1), was detected in all cells transfected with hRIC-3<sup>FLAG</sup> (lanes 2–7). Bands of the size expected for  $\alpha 4$  and  $\beta 2$  were detected in cells in which these subunits were coexpressed alone (lanes 5 and 6) or together (lane 7). No evidence of coprecipitated  $\alpha 4$  or  $\beta 2$  was observed when proteins were expressed alone and then mixed (lanes 2–4). Lane 1 shows the absence of cross-reactivity of  $\alpha 4$  and  $\beta 2$  with mAbFLAG-M2. Experiments involving the mixing of proteins that were expressed separately is indicated by an asterisk. The position of molecular mass markers is shown.

by patch-clamp recording). In contrast to studies conducted in *X. laevis* oocytes, enhanced functional expression of several heteromeric nAChR subtypes ( $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 4\beta 4$ ), when coexpressed with RIC-3, has also been demonstrated in transfected mammalian cells. We conclude that RIC-3 is a nAChR-associated protein that acts to enhance the efficient folding and assembly of multiple nAChR subunits into functional receptors.

#### Acknowledgments

We thank Jim Boulter, Belén Elgoyhen, Jon Lindstrom, Steve Moss, Jim Patrick, and Millet Treinin for generously providing cDNAs used in this study. We thank Sam Ranasinghe for assistance with FLIPR experiments.

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