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REVIEW

RIC-3: a nicotinic acetylcholine receptor chaperone

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RIC-3 is a transmembrane protein which acts as a molecular chaperone of nicotinic acetylcholine receptors (nAChRs). For some nAChR subtypes (such as homomeric α7 neuronal nAChRs), RIC-3 is required for efficient receptor folding, assembly and functional expression. In contrast, for other nAChR subtypes (such as heteromeric α4β2 neuronal nAChRs) there have been reports that RIC-3 can both enhance and reduce levels of functional expression. There is also evidence that RIC-3 can modulate maturation of the closely related 5-hydroxytryptamine (5-HT) receptor (5-HT_3R). As with heteromeric nAChRs, apparently contradictory results have been reported for the influence of RIC-3 on 5-HT_3R maturation in different expression systems. Recent evidence indicates that these differences in RIC-3 chaperone activity may be influenced by the host cell, suggesting that other proteins may play an important role in modulating the effects of RIC-3 as a chaperone. RIC-3 was originally identified in the nematode *Caenorhabditis elegans* as the protein encoded by the gene *ric-3* (*r*esistance to *i*nhibitors of *c*holinesterase) and has subsequently been cloned and characterized from mammalian and insect species. This review provides a brief history of RIC-3; from the identification of the *ric-3* gene in *C. elegans* in 1995 to the more recent demonstration of its activity as a nAChR chaperone.
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Abbreviations: 5-HT, 5-hydroxytryptamine; ER, endoplasmic reticulum; nAChR, nicotinic acetylcholine receptor; RIC, resistant to inhibitors of cholinesterase

Introduction

Resistant to inhibitors of cholinesterase (RIC)-3 is a transmembrane protein, which exerts a dramatic influence upon the maturation (folding and assembly) of neuronal nicotinic acetylcholine receptors (nAChRs). Recent evidence suggests that RIC-3 acts by interaction with unassembled receptor subunits within the endoplasmic reticulum (ER), thereby facilitating subunit folding and receptor assembly (Lansdell *et al*., 2005). Unlike many chaperone proteins, RIC-3 appears to be highly specific in its chaperone activity. In addition to its effect on nAChRs, RIC-3 interacts with and modulates maturation of the closely related 5-hydroxytryptamine (5-HT) type 3 receptor (5-HT_3R) (Halevi *et al*., 2003; Cheng *et al*., 2005). In contrast, RIC-3 appears to have a little or no effect upon other neurotransmitter-gated ion channels, including those activated by GABA and glutamate (Miller *et al*., 1996; Halevi *et al*., 2002; Halevi *et al*., 2003; Lansdell *et al*., 2005).

Nicotinic acetylcholine receptors are members of the Cys-loop family of neurotransmitter-gated ion channels, all of which are pentameric transmembrane receptors (Lester *et al*., 2004; Millar, 2006). Nicotinic receptors are assembled from a diverse collection of subunits (Le Novère *et al*., 2002; Millar, 2003). In vertebrate species, 17 subunits (α1–α10, β1–β4, γ, δ and ε) have been identified, five of which (α1, β1, γ, δ and ε) are expressed at the neuromuscular junction. The remaining subunits (α2–α10 and β1–β4) are commonly referred to as neuronal nAChR subunits and, with some exceptions (Sharma and Vijayaraghavan, 2002), are located within the central and peripheral nervous system (McGehee and Role, 1995; Dani and Bertrand, 2007). Each subunit has a complex membrane topology, comprising a large N-terminal extracellular domain and four α-helical transmembrane domains. Compared with several other transmembrane proteins, the assembly of ion channels such as nAChRs appears to be both a slow and inefficient process (Green and Millar, 1995). Individual subunits must adopt an appropriate transmembrane topology and undergo a series of critical post-translational modifications (Green and Millar, 1995). In addition to the requirement for subunits to fold into an appropriate conformation, they must also make appropriate subunit–subunit interactions to form fully assembled pentameric receptors. The early steps of receptor folding and assembly occur within the ER, an intracellular compartment containing several proteins required for efficient protein folding and post-translational modification (Green and Millar, 1995).

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There is evidence that nAChR folding, assembly and trafficking are influenced by several chaperone-type proteins. In particular, the 14-3-3η protein (Jeanclos *et al*., 2001) and the ER-resident chaperone proteins, BiP and calnexin (Blount and Merlie, 1991; Paulson *et al*., 1991; Forsayeth *et al*., 1992; Gelman *et al*., 1995; Chang *et al*., 1997), have been shown to associate with and/or influence trafficking of nAChR subunits. Whereas 14-3-3η, BiP and calnexin interact with a diverse range of target proteins (Shaw, 2000; Kleizen and Braakman, 2004), it appears, as will be discussed below, that RIC-3 is a chaperone protein, which acts much more selectively (on nAChRs and the closely related 5-HT_3R).

Identification of a role for RIC-3 in cholinergic signalling

The gene encoding RIC-3 (*ric-3*) was identified in 1995 as one of several genes in *Caenorhabditis elegans*, which, when mutated, confers resistance to inhibitors of acetylcholinesterase (Nguyen *et al*., 1995). The nematode *C. elegans* has been used extensively in the field of neurobiology, due, in large part, to its relatively simple but well-characterized nervous system (White *et al*., 1986; Chalfie and Jorgensen, 1998). Several recessive mutations were identified in *C. elegans*, which confer resistance to aldicarb and trichlorfon (Nguyen *et al*., 1995; Miller *et al*., 1996), both of which are pesticides which act by inhibiting the enzyme acetylcholinesterase. Mutations conferring this phenotype were identified in about 20 genes. Those genes that had not previously been assigned a name in *C. elegans* were designated '*ric*', a term derived from the phenotype '*r*esistance to *i*nhibitors of *c*holinesterase'. Several of the genes identified in these studies encode synaptic proteins, such as SNAP-25 (*ric-4*), synaptotagmin (*snt-1*), synaptojanin (*unc-26*) and syntaxin (*unc-64*), or proteins involved in cholinergic signalling, such as choline acetyltransferase (*cha-1*), vesicular acetylcholine transporter (*unc-13*) and the *C. elegans* nAChR subunit UNC-63 (*unc-63*). Most of the mutations conferring resistance to inhibitors of cholinesterase have been shown to also cause deficits in GABA-dependent processes (Miller *et al*., 1996), suggesting that they encode proteins involved in general, rather than cholinergic-specific, synaptic transmission. In contrast, mutations in *ric-3* appear to cause a more specific cholinergic deficit (Miller *et al*., 1996).

Further genetic screening in *C. elegans* revealed that mutations within the *ric-3* gene also confer resistance to levamisole (Miller *et al*., 1996), an antiparasitic (anthelmintic) drug, which acts on nAChRs (Lewis *et al*., 1987). Resistance to levamisole is a phenotype that is also displayed by *C. elegans* strains containing mutations in genes encoding the nAChR subunits LEV-1, UNC-29 and UNC-38 (Miller *et al*., 1996; Fleming *et al*., 1997), a finding that led to the suggestion that the protein encoded by *ric-3* gene might act at a postsynaptic site (Halevi *et al*., 2002). Interestingly, one of the mutations that was identified as conferring resistance to inhibitors of cholinesterase is located within the *C. elegans* gene *unc-63* (Nguyen *et al*., 1995), which has subsequently been shown to encode a levamisole-sensitive nAChR subunit (Culetto *et al*., 2004). Thus, these early genetic studies provide strong evidence implicating *ric-3* as a gene involved in cholinergic signalling.

Identification of a role for RIC-3 in nAChR maturation

Evidence indicating that the protein encoded by *ric-3* is required for the maturation of nAChRs was obtained by screening for suppressor mutations in *C. elegans* (Halevi *et al*., 2002). Previous work had identified a missense mutation, *deg-3(u662)*, within the *C. elegans* nAChR subunit DEG-3, which caused neuronal degeneration and uncoordinated movements (Treinin and Chalfie, 1995). The *deg-3(u662)* mutation alters an amino-acid residue, which is located within the pore-forming transmembrane domain of DEG-3 and which appears to cause neuronal degeneration due to altered nAChR function (Halevi *et al*., 2002). It was assumed that screening for mutations that cause suppression of the *deg-3(u662)* phenotype might identify genes, which are required for nAChR function. Several suppressor mutations were identified within the *deg-3* gene itself (Halevi *et al*., 2002; Yassin *et al*., 2002). Other suppressor mutations were identified within *des-2* (Halevi *et al*., 2002), which encodes a nAChR subunit (DES-2) that co-assembles with DEG-3 (Treinin *et al*., 1998; Yassin *et al*., 2001). In addition to suppressor mutations located within *deg-3* and *des-2*, several *deg-3(u662)*-suppressor mutations were identified within the *ric-3* gene (Halevi *et al*., 2002).

Electrophysiological recordings from pharyngeal and body muscles of *C. elegans* revealed that mutations in *ric-3* are associated with deficits in cholinergic transmission, whereas glutamate and GABA currents are unaffected (Miller *et al*., 1996; Halevi *et al*., 2002). More direct evidence for an effect on nAChRs came from immunohistochemical staining, which demonstrated that *C. elegans* strains containing mutations within *ric-3* have an altered subcellular distribution of the nAChR subunit DEG-3. In particular, reduced DEG-3 staining is observed in cell processes, a finding that led to the conclusion that RIC-3 is required for correct nAChR maturation in *C. elegans* (Halevi *et al*., 2002). More recent immunohistochemical studies in *C. elegans* have demonstrated that mutations in *ric-3* also cause a reduction in cell-surface expression of the nAChR LEV-1 subunit (Gottschalk and Schafer, 2006). Interestingly, a similar reduction in cell-surface LEV-1 subunit expression is observed in *C. elegans* strains containing a mutation within *unc-38* (Gottschalk and Schafer, 2006). As *unc-38* encodes a nAChR subunit (UNC-38) that co-assembles with LEV-1 (Fleming *et al*., 1997), it appears that mutations in either RIC-3 or in nAChR subunits that co-assemble with LEV-1 can result in a similar disruption of LEV-1 maturation and cell-surface expression.

Heterologous expression of RIC-3

A common problem that has been encountered with several nAChR subtypes is that of inefficient functional expression of recombinant receptors in artificial expression systems

(Millar, 1999). For some nAChR subunits, such as α7, much greater success has been achieved by expression in *Xenopus* oocytes, rather than in cultured mammalian cell lines (Couturier *et al*., 1990; Cooper and Millar, 1997). Heterologous expression studies performed in *Xenopus* oocytes have demonstrated that co-expression of *C. elegans* RIC-3 causes enhanced levels of functional expression of nAChR subtypes, such as α7 (Halevi *et al*., 2002), and similar results have been reported with a human homologue of RIC-3 (Halevi *et al*., 2003). These findings strongly support the idea that RIC-3 is able to enhance nAChR maturation.

Perhaps, the most dramatic effect of RIC-3 is its ability to facilitate the folding and functional expression of nAChRs such as α7 in host cell types, which would otherwise fail to generate functional nAChRs (Castillo *et al*., 2005; Lansdell *et al*., 2005; Williams *et al*., 2005). It has been known for some time that the extent to which some nAChR subunits generate functional receptors in artificial expression systems is influenced dramatically by the host cell type (Cooper and Millar, 1997; Kassner and Berg, 1997; Rangwala *et al*., 1997). The α7 subunit, which efficiently forms functional homomeric nAChRs when expressed in *Xenopus* oocytes (Couturier *et al*., 1990) and in some cultured cell lines (Puchacz *et al*., 1994; Gopalakrishnan *et al*., 1995; Quik *et al*., 1996), fails to do so when expressed in many other cell lines (Cooper and Millar, 1997; Kassner and Berg, 1997; Rangwala *et al*., 1997). Reverse transcription-PCR studies have demonstrated a correlation between the expression of RIC-3 mRNA in cultured cell lines and the ability of cell lines either to express endogenous α7 nAChRs or to permit the functional expression of recombinant α7 nAChRs (Lansdell *et al*., 2005; Williams *et al*., 2005).

In the absence of RIC-3, little or no specific binding of nicotinic radioligands is detected when the α7 subunit is expressed in mammalian cell lines, which lack endogenous RIC-3 (such as simian COS cells and some human HEK293-derived cell lines). In contrast, co-expression of RIC-3 with α7 facilitates both high levels of specific [^{125}I]α-bungarotoxin binding and the expression of functional α7 nAChRs (Castillo *et al*., 2005; Lansdell *et al*., 2005; Williams *et al*., 2005).

In contrast to homomeric α7 nAChRs, many other nAChR subtypes (such as α3β4 and α4β2) are able to generate functional nAChRs in a wide range of cultured cell lines (Whiting *et al*., 1991; Wong *et al*., 1995; Gopalakrishnan *et al*., 1996; Lewis *et al*., 1997), including those known to lack endogenous RIC-3 (Lansdell *et al*., 2005). There is, however, evidence that co-expression of RIC-3 can modulate levels of functional expression of heteromeric nAChR subtypes, such as α3β4 and α4β2 (Halevi *et al*., 2003; Castillo *et al*., 2005; Lansdell *et al*., 2005). Whereas the influence of RIC-3 upon α7 nAChRs is consistent in all published studies (causing either enhancement or facilitation of functional expression), the influence of RIC-3 upon heteromeric nAChRs is much less consistent between studies. Co-expression of RIC-3 with heteromeric nAChRs such as α3β4 and α4β2 in a human kidney cell line has been shown to enhance levels of specific radioligand binding and functional expression (Lansdell *et al*., 2005). In contrast, there are reports that co-expression of RIC-3 suppresses levels of functional expression of α3β4 and α4β2 nAChRs in *Xenopus* oocytes (Halevi *et al*., 2003). Similarly, there are apparent inconsistencies between the reported effects of co-expressed RIC-3 upon 5-HT$_3$Rs (Halevi *et al*., 2003; Castillo *et al*., 2005; Cheng *et al*., 2005; Cheng *et al*., 2007). Although co-expression of RIC-3 with the 5-HT$_{3A}$ subunit in *Xenopus* oocytes causes an almost complete suppression of functional expression of 5-HT$_3$Rs (Halevi *et al*., 2003; Castillo *et al*., 2005), RIC-3 is reported to cause enhanced functional expression of these receptors in a human kidney cell line (Cheng *et al*., 2005; Cheng *et al*., 2007).

It is possible that the apparent differences, which have been reported concerning the influence of RIC-3 upon 5-HT$_3$Rs and heteromeric nAChRs, may be a consequence of the different expression systems employed. Recent data to support this conclusion have come from studies with a *Drosophila* RIC-3 homologue. Studies in which *Drosophila* and human RIC-3 constructs were expressed in both insect and mammalian cultured cell lines have provided evidence that the ability of RIC-3 to act as a chaperone of nAChRs is influenced by the nature of the host cell (Lansdell *et al*., 2008). Specifically, it was found that *Drosophila* RIC-3 enhances maturation of nAChRs (containing either mammalian or *Drosophila* nAChR subunits) more efficiently in a *Drosophila* cell line, whereas human RIC-3 does so more efficiently in a human cell line (Lansdell *et al*., 2008). A plausible explanation for these findings might be that the extent to which RIC-3 is able to modulate the folding and maturation of nAChRs is influenced by other host-cell-specific proteins. Such interactions with host cell factors might also cause RIC-3 to suppress, rather than enhance, maturation of some receptor subtypes, thereby explaining some of the apparently contradictory results with 5-HT$_3$Rs and heteromeric nAChRs.

An interesting finding has been the observation that, in contrast to its effect on α7, co-expression of RIC-3 with several α7/5-HT$_{3A}$ subunit chimeras causes a dramatic reduction in the level of receptor cell-surface expression (Castillo *et al*., 2006; Gee *et al*., 2007). Such effects have been observed consistently in both oocytes and mammalian cell expression systems. Further studies of these chimeras may help to establish the mechanism by which RIC-3 modulates receptor maturation. All published studies with recombinant nAChRs have focused on RIC-3's effects on neuronal nAChRs, which raises the question of whether RIC-3 can also modulate the maturation of muscle-type nAChRs. Recent preliminary studies indicate that this is the case. Co-expression of human RIC-3 has been found to result in a 60% enhancement in levels of α-bungarotoxin to the human muscle-type nAChR in transfected HEK-293 cells (personal communication; Andrew G. Engel, Mayo Clinic, Rochester, USA).

Identification of protein domains in RIC-3

Sequence analysis of the predicted open-reading frame of the *C. elegans ric-3* gene led to the proposal that the *C. elegans* RIC-3 protein is a membrane protein containing two transmembrane domains in which both the N- and

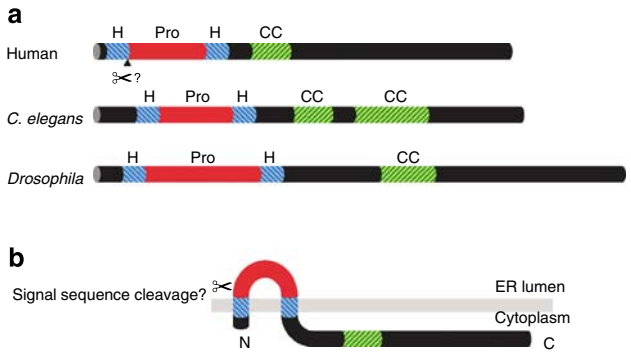

Figure 1 Protein domains and predicted membrane topology of RIC-3. (**a**) The location of hydrophopic domains (blue diagonal stripes), predicted coiled-coil domains (green diagonal stripes) and proline-rich domains (red) are shown for the RIC-3 proteins of *C. elegans*, *D. melanogaster* and human (approximately to scale). Although several alternatively spliced isoforms of RIC-3 have been reported in the literature, those illustrated here correspond to the 378 amino acid *C. elegans* RIC-3 isoform described by Halevi *et al.* (2002), accession number NM_068898; the 369 amino acid human RIC-3 isoform A (Halevi *et al.*, 2003), accession number AY326435 and the 472 amino acid *Drosophila* RIC-3 isoform DmRIC-3[6,7,9] (Lansdell *et al.*, 2008), accession number AM902271. The position of the predicted signal-sequence cleavage site in human RIC-3 is indicated by a scissors symbol and arrowhead. (**b**) The predicted transmembrane topology of RIC-3 is illustrated. Two hydrophobic regions are present in RIC-3 proteins from all species that have been examined. In all cases, it appears that the second of these hydrophobic regions is a transmembrane domain. The first of these hydrophobic regions is also predicted to be a transmembrane domain in RIC-3 proteins from invertebrate species, such as *C. elegans* and *Drosophila*. In contrast, it appears that the first hydrophobic region may be a cleaved N-terminal signal sequence in mammalian species, such as human, rat and mouse. Therefore, in invertebrate species RIC-3 is predicted to have both its N- and C-terminus located on the cytoplasmic side of the membrane. In contrast, if the postulated N-terminal signal sequence is cleaved (as is predicted in mammalian RIC-3 proteins), this would be expected to result in a single-transmembrane protein with only the C-terminus of the mature protein on the cytoplasmic side of the membrane. The position of the predicted signal-sequence cleavage site (in human RIC-3) is indicated by a scissors symbol. Predictions of protein secondary structure (such as signal sequence cleavage sites and coiled-coil domains) and of membrane topology were based on computer programs, such as COILS (Lupas *et al.*, 1991; Lupas, 1996), Phobius (Käll *et al.*, 2004) and PONGO (Amico *et al.*, 2006). RIC, resistant to inhibitors of cholinesterase.

C-terminus are located in the cytoplasm (Halevi *et al.*, 2002) (Figure 1). The cloning of a human homologue of RIC-3 also revealed two hydrophobic domains (Halevi *et al.*, 2003); however, analysis of the amino-acid sequence of human RIC-3 indicates that its N-terminal hydrophobic domain resembles a cleaveable signal sequence (Castillo *et al.*, 2005; Cheng *et al.*, 2007). Analysis of predicted RIC-3 proteins from other species suggests that RIC-3 proteins from other mammalian species contain potential cleavable signal sequences, whereas RIC-3 proteins from invertebrate species do not. For example, a RIC-3 homologue, which has been cloned from the model insect species *Drosophila melanogaster*, and which has been shown to have nAChR chaperone activity (Lansdell *et al.*, 2008), resembles *C. elegans* RIC-3, contains two hydrophobic domains but lacks a predicted cleaved signal sequence. Further work will be required to establish whether these differences in predicted signal sequence cleavage sites reflect real differences in protein processing and transmembrane topology. Experimental evidence has been obtained which supports the presence of a cleavable signal sequence in the human RIC-3 protein (Cheng *et al.*, 2007), but another recent study has obtained evidence which argues against proteolytic cleavage of human RIC-3 (Castelán *et al.*, 2008).

A consistent feature of RIC-3 from all species is the presence of one or more predicted coiled-coil domains located within the C-terminal region (Halevi *et al.*, 2002; Halevi *et al.*, 2003) (Figure 1), a motif that has been implicated in protein–protein interactions (Burkhard *et al.*, 2001). The possible role of the coiled-coil domains in RIC-3 is unclear, although there is evidence to suggest that they are involved in aggregation of RIC-3 (Cheng *et al.*, 2007). In contrast, there is evidence that they are not required for RIC-3's chaperone activity. Eleven alternatively spliced isoforms of *Drosophila* RIC-3 have been identified and characterized (Lansdell *et al.*, 2008). Of these, those isoforms containing exon 7 contain a predicted coiled-coil domain, but those containing the alternative exon 7A lack a predicted coiled-coil motif. Despite this, no significant difference in chaperone activity has been detected between *Drosophila* RIC-3 isoforms containing either exon 7 or 7A (Lansdell *et al.*, 2008). Studies with truncated RIC-3 proteins also indicates that these predicted coiled-coil domains are not essential for RIC-3 to function as a molecular chaperone (Ben-Ami *et al.*, 2005; Cheng *et al.*, 2007; Lansdell *et al.*, 2008).

Another common feature of RIC-3 proteins is the presence of a proline-rich domain located between the two hydrophobic domains (Halevi *et al.*, 2002; Halevi *et al.*, 2003) (Figure 1). The importance of this region is unclear, but studies with alternatively spliced isoforms of *Drosophila* RIC-3 suggest that this region is critical to RIC-3's chaperone activity. *Drosophila* RIC-3 isoforms that either contain or lack a 40 amino-acid exon (exon 2), located within the proline rich domain, have been identified. Despite maintaining the open-reading frame, the presence of exon 2 (which is not rich in proline residues) dramatically reduces the nAChR-chaperone activity of *Drosophila* RIC-3 (Lansdell *et al.*, 2008).

RIC-3 is a nAChR-associated protein

Evidence for an interaction between RIC-3 and the nAChR α7 subunit has been obtained by co-immunoprecipitation studies (Lansdell *et al.*, 2005; Williams *et al.*, 2005). Similarly, co-immunoprecipitation of RIC-3 has been demonstrated with other nAChR subunits, including α3, α4, β2 and β4 (Lansdell *et al.*, 2005). In addition, RIC-3 has been shown to co-precipitate with 5-HT_{3A} (Cheng *et al.*, 2005), a subunit with close sequence similarity to nAChR subunits. In contrast, co-immunoprecipitation of RIC-3 has not been observed with subunits from other members of the Cys-loop ligand-gated ion channel family, such as the $GABA_A$ receptor α1 subunit (Lansdell *et al.*, 2005).

Subcellular localization of RIC-3

In both vertebrate and invertebrate species, RIC-3 is expressed in muscle and nerve cells (Halevi *et al.*, 2002;

Halevi *et al*., 2003). RIC-3 protein is concentrated in cell bodies and appears to be located predominantly within the ER (Halevi *et al*., 2002; Castillo *et al*., 2005; Cheng *et al*., 2007). Immunoprecipitation studies have revealed that RIC-3 is able to associate with unassembled nAChR subunits (Lansdell *et al*., 2005), which are not thought to be exported from the ER to the cell surface (Green and Millar, 1995). Although there is strong evidence that RIC-3 is expressed within the ER, there is some debate about whether it is also transported to the cell surface. Studies conducted in a human kidney cell line have led to the conclusion that human RIC-3 is expressed on the cell surface (Williams *et al*., 2005), but other studies do not appear to support this conclusion. Experiments performed with a pH-sensitive variant of green fluorescent protein (PHluorin) have provided evidence that human RIC-3 is located predominantly within the ER, with no evidence of endosomal or cell-surface expression (Cheng *et al*., 2007). Similar conclusions have been made from studies using rat RIC-3 with a fluorescent protein tag, in which no evidence was obtained for expression of RIC-3 on the cell surface (Roncarati *et al*., 2006).

Mechanism of action

There are apparent similarities between the ability of RIC-3 to upregulate levels of cell-surface nAChRs and receptor upregulation caused by chronic exposure to nicotine. This effect of nicotine has been described as that of a 'pharmacological chaperone' (Kuryatov *et al*., 2005; Sallette *et al*., 2005). It seems likely, however, that receptor upregulation caused by RIC-3 and by chronic exposure to nicotine may occur by different mechanisms. There is extensive evidence to indicate that chronic exposure to nicotine, as occurs during tobacco smoking, can result in an upregulation of brain nAChRs. Evidence for nicotine-induced upregulation of nAChRs in the brain has come from studies with post-mortem brain tissue from human tobacco smokers (Benwell *et al*., 1988) and from animal studies (Marks *et al*., 1985; Schwartz and Kellar, 1985). Nicotine-induced receptor upregulation occurs by a post-translational mechanism (Marks *et al*., 1992) and can be mimicked in cultured cell lines by exposure to low concentrations of nicotine for 24–48 h. Receptor upregulation has been observed in cultured cell lines both for $\alpha4\beta2$ nAChRs (Peng *et al*., 1994; Zhang *et al*., 1994; Bencherif *et al*., 1995; Gopalakrishnan *et al*., 1996) and $\alpha7$ nAChRs (Quik *et al*., 1996; Peng *et al*., 1997). With nAChR subtypes such as $\alpha4\beta2$, there is evidence that chronic nicotine treatment can enhance subunit folding and induce conformational changes (Harkness and Millar, 2002). As the binding site for agonists, such as nicotine, is located at subunit interfaces (Arias, 2000; Celie *et al*., 2004) and is generated only after subunit co-assembly, it seems reasonable to assume that nicotine causes upregulation by an interaction with assembled or partially assembled nAChRs, rather than with unassembled subunits, a conclusion that is supported by experimental evidence (Harkness and Millar, 2002). Indeed, it has been proposed that nicotine causes receptor upregulation by acting as a pharmacological chaperone (Kuryatov *et al*., 2005; Sallette *et al*., 2005) and via a direct action at the agonist binding site (Kishi and Steinbach, 2006). In contrast, as discussed earlier, there is evidence that RIC-3 associates with unassembled nAChR subunits within the ER (Lansdell *et al*., 2005). As unassembled subunit do not form a nicotine-binding site, it is possible that the chaperone activity of RIC-3 is distinct from that of pharmacological chaperones, such as nicotine.

Conclusion

It is over a decade since the *ric-3* mutant phenotype was first identified in *C. elegans* (Nguyen *et al*., 1995), but it is only relatively recently that the role of RIC-3 as a nAChR molecular chaperone been established (Halevi *et al*., 2002). In addition to studies in *C. elegans*, RIC-3 has now been cloned and characterized from mammalian and insect species (Halevi *et al*., 2003; Lansdell *et al*., 2008). The ability of RIC-3 to enhance the maturation of homomeric nAChRs, such as $\alpha7$, is now well established. Of particular note is the ability of RIC-3 to facilitate the functional expression of $\alpha7$ nAChRs in otherwise non-permissive cultured cell lines (Castillo *et al*., 2005; Lansdell *et al*., 2005; Williams *et al*., 2005). A considerable amount has been discovered about the role of RIC-3 in modulating the maturation of nAChRs, but several important questions are unresolved. Particularly intriguing are the apparently contradictory reports concerning the influence of RIC-3 upon heteromeric nAChR subtypes, such as $\alpha4\beta2$ (Halevi *et al*., 2003; Lansdell *et al*., 2005). It is possible that these opposing effects (that is either enhancement or suppression of functional expression) are determined by the choice of expression system, but this remains to be confirmed and explained. It seems likely that there may be additional cell-specific proteins, which modulate the effect of RIC-3 (Lansdell *et al*., 2008), but these remain to be identified. Another possibility is that there are additional but currently unidentified proteins, which, themselves, have nAChR-chaperone activity. This possibility is suggested by the apparent inability of RIC-3 to resolve problems, which have been encountered in the heterologous expression of some nAChR subtypes. Of particular note are problems associated with functional expression in cultured cell lines of mammalian nAChRs subtypes such as $\alpha9\alpha10$ (Lansdell *et al*., 2005) and of some invertebrate nAChRs (Lansdell *et al*., 2008). Further work will also be required to establish more precisely the membrane topology and structure of RIC-3 and to obtain a better understanding of its mechanism of action as a molecular chaperone.

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Conflict of interest

The author states no conflict of interest.

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