RIC-3 Affects Properties and Quantity of Nicotinic Acetylcholine Receptors via a Mechanism That Does Not Require the Coiled-coil Domains*

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Members of the RIC-3 gene family are effectors of nicotinic acetylcholine receptor (nAChR) expression in vertebrates and invertebrates. In Caenorhabditis elegans RIC-3 is needed for functional expression of multiple nAChRs, including the DEG-3/DES-2 nAChR. Effects of RIC-3 on DEG-3/DES-2 functional expression are found in vivo and following heterologous expression in Xenopus leavis oocytes. We now show that in X. leavis oocytes RIC-3 also affects the kinetics and agonist affinity properties of the DEG-3/DES-2 receptor. Because these effects are mimicked by increasing the ratio of DEG-3 subunits within DEG-3/DES-2 receptors, this suggests that RIC-3 may preferentially promote maturation of DEG-3-rich receptors. Indeed, effects of RIC-3 on functional expression of DEG-3/DES-2 positively correlate with the DEG-3 to DES-2 ratio. All RIC-3 family members have two transmembrane domains followed by one or two coiled-coil domains. Here we show that the effects of RIC-3 on functional expression and on receptor properties are mediated by the transmembrane domains and do not require the coiled-coil domains. In agreement with this, mammals express a RIC-3 transcript lacking the coiled-coil domain that is capable of promoting DEG-3/DES-2 functional expression. Last, we show that RIC-3 affects DEG-3 quantity, suggesting stabilization of receptors or receptor intermediates by RIC-3. Together our results suggest that subunit-specific interactions of RIC-3 with nAChR subunits, mediated by the transmembrane domains, are sufficient for the effects of RIC-3 on nAChR quantity and quality.

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Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels expressed in many tissues and organisms. These channels are pentamers, usually heteromers. Subunits of the nAChRs belong to one gene family, having four transmembrane domains, a large N-terminal extracellular loop, and an intracellular loop in between transmembrane domains III and IV (1). Biogenesis of functional nAChRs requires folding of each subunit and assembly into pentamers having the appropriate stoichiometry and arrangement. Subunits are also modified by glycosylation and disulfide bond formation. Folding, assembly, and most post-translational modifications occur in the endoplasmic reticulum and are a prerequisite for endoplasmic reticulum exit, which is followed by trafficking through the Golgi apparatus to the surface membrane (2–4). The complex and time-consuming process leading to functional expression of nAChRs is likely to require activity of multiple proteins. One such protein is RIC-3. Work on the Caenorhabditis elegans ric-3 suggests that it affects nAChR maturation within the endoplasmic reticulum (5). However, the mechanism enabling the effects of RIC-3 on nAChR maturation is still unknown.

Mutations in the C. elegans ric-3 lead to reduced activity of multiple nAChRs, resulting in defective cholinergic signaling (5). The RIC-3 gene belongs to a conserved family (6). Members of this family, when co-expressed with nAChRs, have significant effects on functional surface expression of these receptors. RIC-3 family members are predicted to encode two membrane-spanning domains followed by one or two coiled-coil domains, but the contributions of these domains to RIC-3 function are still unknown. The ability of RIC-3 to interact with multiple divergent nAChRs is demonstrated by its effects on their expression. However, effects of these interactions are divergent, as RIC-3 enhances functional expression of some receptors (C. elegans DEG-3/DES-2 and mammalian α7 nAChRs) while reducing or eliminating the expression of others (mammalian α4β2 and α3β4 nAChRs) (6).

Here we used heterologous expression of RIC-3 in Xenopus leavis oocytes to analyze its effects on the C. elegans DEG-3/DES-2 nAChR. Previously we have shown that RIC-3 is required for normal activity and localization of this receptor in vivo (5). We have also shown that expression of RIC-3 in X. leavis oocytes with the DEG-3 and the DES-2 subunits, both necessary subunits for DEG-3/DES-2 receptor function (7), enhances functional expression of the DEG-3/DES-2 receptor (6). We now show that in addition to these quantitative effects on functional surface expression, RIC-3 also affects receptor properties. We show that these effects are similar to the effects of having a high DEG-3 to DES-2 ratio, and we provide evidence for a link between subunit stoichiometry and the effects of RIC-3. Both quantitative and qualitative changes in DEG-3/DES-2 activity are obtained when a truncated (118 amino acid) “transmembrane domain” RIC-3 protein is expressed, demonstrating that the coiled-coil domains are not needed for RIC-3 function in Xenopus oocytes. A naturally occurring transcript encoding for a similar transmembrane domain isoform is found in mammals (6) and has similar effects on DEG-3/DES-2 functional expression. Last, we show that RIC-3 expression leads to an increase in the quantity of DEG-3, suggesting that interaction with RIC-3 stabilizes nAChRs or nAChR maturation in-
termediates. Overall, this analysis suggests that interactions between the RIC-3 transmembrane domains and nAChRs (or nAChR intermediates) stabilize these proteins, thereby altering the maturation process in a subunit-specific manner.

MATERIALS AND METHODS

Heterologous Expression and Electrophysiology—DEG-3-, DES-2-, RIC-3-, and hRIC3-expressing clones were described previously (5–7). In vitro transcribed and capped cRNAs were injected at final concentrations of 0.1–0.2 μg/μl for DEG-3 and DES-2 (unless otherwise stated), 0.04–0.1 μg/μl for RIC-3 and RIC-3 derivatives, and 0.01 μg/μl for hric3 and its transmembrane domain isoform. For the stoichiometry experiments, the total RNA was kept constant (0.4 μg/μl). Injections and recordings were done as described in Ref. 7. The effects of co-expressing RIC-3, hRIC3, or their derivatives on DEG-3/DES-2 receptor-mediated whole cell current amplitudes were assayed between the first and third days following injections.

Data Analysis—Data analysis was done using Origin versions 5.0 and 7.1. Results are presented as the mean ± S.E. (N = number of frogs, n = number of oocytes). Significance was examined using the paired t test, unless stated otherwise. In Figs. 2, 5, and 7, p values were adjusted using the Bonferroni correction for multivariable analysis; results are considered significant when p < 0.01. To reduce noise due to variability in expression levels between different experiments, in Figs. 5 and 7 we normalized the current amplitudes in each experiment to the average response for oocytes expressing only DEG-3 and DES-2. For dose-response analysis, the current amplitudes were normalized to the amplitudes elicited by 3 mM choline for each oocyte (N = 2, n = 4).

RIC-3 Function Does Not Require the Coiled-coil Domains
Dose-response relationship was fitted using the Sigma Plot 2000 program using the double component Hill equation as shown in Equation 1.

\[
y = \frac{a_1}{1 + (EC_{50,1}x)^n_1} + \frac{a_2}{1 + (EC_{50,2}x)^n_2} \quad \text{(Eq. 1)}
\]

\(y\) = amplitude of the evoked current normalized to unity, \(a_1\) = fraction of receptor population type 1, \(EC_{50,1}\) = half-maximal activation for receptor type 1, \(x\) = agonist concentration, \(n_1\) = Hill coefficient for receptor type 1, \(a_2\) = fraction of receptor population type 2, \(EC_{50,2}\) = half-maximal activation for receptor type 2, \(n_2\) = Hill coefficient for receptor type 2.

Time decay was fitted by a double exponential using the Clampfit program (Axon Instruments). Exponential equation: \(y = A_1 e^{-\tau_1 x} + A_2 e^{-\tau_2 x} + y_o\). \(A_1\) is current amplitude corresponding to \(\tau_1\), \(A_2\) is current amplitude corresponding to \(\tau_2\), \(\tau_1\) and \(\tau_2\) are the time decay constants (\(\tau_{1,2}\) respectively), and \(y_o\) is the offset (residual current). Desensitization efficacy was calculated as follows: \(D = \frac{\text{peak amplitude} - \text{steady state amplitude}}{\text{peak amplitude}}\).

**Fig. 2. DEG-3 to DES-2 ratio affects properties of the DEG-3/DES-2 channel.**

**A**, representative traces of currents elicited upon application of 3 mM choline from oocytes expressing different ratios of DEG-3 to DES-2, (1:5, 1:1, and 5:1). The total amount of RNA was kept constant in all experiments. Bars indicate the time of agonist application. For comparison only responses with similar amplitudes are shown. **B, D** values from oocytes expressing different DEG-3 to DES-2 ratios: 1:5 (black), 1:1 (white), and 5:1 (hatched) (\(N = 4, n = 28\)). Effects of ratio manipulations are significant for the 1:5 and 5:1 ratios relative to the 1:1 ratio. **C**, time constant decay fitted by double exponential (\(N = 4, n = 28\)). Differences in the decay constants \(\tau_{1,2}\) are significant for the 5:1 ratio relative to the 1:1 ratio. **D**, dose-response curves from oocytes expressing the DEG-3/DES-2 receptor in DEG-3 to DES-2 ratio of 1:1 (circles), 1:5 (triangles), and 5:1 (squares). Note that the curves for the 1:1 and 1:5 ratios overlap, making them indistinguishable. The results are fitted by a double Hill equation (see "Materials and Methods"). Values are mean of \(N = 2\), \(n = 4\); \(N = 3\), \(n = 5\); \(N = 3\), \(n = 5\) for 1:1, 1:5, and 5:1 DEG-3 to DES-2 ratios, respectively. Responses are normalized to current amplitude elicited by 3 mM choline/each oocyte.
RIC-3 Function Does Not Require the Coiled-coil Domains

Construction of RIC-3 Derivatives—To tag RIC-3 with GFP we amplified the GFP fragment from plasmid pGFP-C1 (BD Biosciences, Clontech) using primers GCTAGGCCTACCGTTGGACCATGGTG-GAGGATCAGCGGAAAGGAC and a primer for the GFP sequence. This fragment was digested by BamHI and SaII and inserted into a RIC-3-GFP construct in which the non-conserved C-terminal domain was deleted using EcoRI digestion followed by self ligation. The transmembrane domain derivative (RIC-3 TM) was generated by amplification of the transmembrane domain region using primers GCCGAATACGACT-CCTATAGGGGCAATGATGTGTAGATGTTATGTTA-AACAGCG followed by digestion with BamHI and SaII and ligation into the minimal derivative construct in place of the longer BamHI-SaII fragment that contains the entire conserved regions. A similar manipulation was done to clone the first coiled-coil domain (RIC-3 CC) following amplification using GTATACCGTTGGATACATCGTGGTGGAAGAAAG and TGTACGAGTCATACGGTACATCGTGGTGCACTG.
of low affinity receptors is 1.78 ± 0.12 mM). Thus it is possible that RIC-3 expression preferentially promotes functional expression of DEG-3-rich receptors having higher desensitization efficiency and higher affinity for choline.

To further examine the relationship between subunit stoichiometry and the effects of RIC-3 we combined the two manipulations. This analysis shows that effects of RIC-3 on whole cell current amplitude are affected by subunit ratio and positively correlate with DEG-3 ratio (Fig. 3). Such results support the suggestion that RIC-3 preferentially promotes maturation of receptors having a high DEG-3 to DES-2 ratio.

The Coiled-coil Domains Are Not Required for RIC-3 Activity

To better understand how RIC-3 affects nAChR expression we sought to dissect the roles of the different RIC-3 domains. Structural predictions based on the sequence of the C. elegans ric-3 suggest that RIC-3 is composed of an N-terminal cytosolic domain, two transmembrane domains separated by a proline-rich spacer, and a cytosolic C-terminal domain having two coiled-coil domains (5). Similar analysis of other members of the ric-3 gene family shows conservation of the transmembrane domains, proline-rich spacer, and the first coiled-coil domain (6). To examine the role of each of these domains in RIC-3 function we started by deleting the non-conserved N- and C-terminal domains, thus producing a 184-amino acid "Minimal RIC-3" (Fig. 4). This protein reproduced both the quantitative and qualitative effects of the full-length RIC-3, demonstrating that the non-conserved domains are not required for RIC-3 function in oocytes (Fig. 5). To further dissect the structure of RIC-3 we created two smaller fragments of RIC-3, a transmembrane domain fragment (TM) and a coiled-coil domain fragment (CC) (Fig. 4). Expression of each fragment with the DEG-3/DES-2 receptor shows that the trans-
membrane domain fragment, but not the coiled-coil domain fragment, is sufficient for eliciting the effects of RIC-3 on the DEG-3/DES-2 receptor (Fig. 5; both deletion mutants express a stable protein as seen by Western analysis; results not shown). Effects of this truncated RIC-3 TM protein on whole cell current amplitude are comparable with the effects of the full-length RIC-3. This truncated protein also produces similar effects on DEG-3/DES-2 current kinetics. Last, the truncated RIC-3 interacts with the DEG-3/DES-2 nAChR, as shown by co-immunoprecipitation analysis (Fig. 6). Thus the conserved first coiled-coil domain is not required for interaction between RIC-3 and nAChRs or for the resulting effects on nAChR activity in oocytes.

Analysis of available human ric-3 (hric3) cDNAs has identified a number of putative hric3 isoforms. Among these, one isoform, AY326436, is similar to our RIC-3 TM deletion mutant in encoding for the transmembrane domain region alone (6). Although the function of this alternative hric3 transcript is unclear, it may be a product of aberrant RNA processing, it is interesting to note that mice also express a similar transcript, AK038724. Mice appear to express a more limited repertoire of ric-3 transcripts, and thus conservation of this transmembrane domain transcript is indicative of a functional role for the resulting protein. To examine this function we expressed the human transmembrane domain isoform in oocytes. As shown for the C. elegans RIC-3 deletion mutant, expression of this human transmembrane domain RIC-3 protein also increases whole cell current amplitude in oocytes expressing the DEG-3/DES-2 receptor (Fig. 7). However, unlike the C. elegans transmembrane domain protein that we engineered and whose effects are indistinguishable from the effects of the full-length RIC-3, effects of the human RIC-3 isoform are smaller relative to those of the full-length human RIC-3.

Expression of RIC-3 Affects DEG-3 Protein Quantity—Our analysis of the effects of RIC-3 on DEG-3/DES-2 properties suggests that it alters the outcome of the assembly process, leading to preferential expression of DEG-3-rich receptors. Such effects could be a result of RIC-3 directly affecting the assembly process. Another possibility is that RIC-3 functions through stabilization of receptors or receptor assembly intermediates in a subunit-specific way. To examine whether RIC-3 is capable of stabilizing nAChR subunits we examined its effects on the DEG-3 subunit. Western analysis of oocytes expressing the DEG-3/DES-2 receptor with and without RIC-3 co-expression reproducibly showed increased DEG-3 signal in the presence of RIC-3. Quantification of this effect shows a 2-fold increase in total DEG-3 quantity in the presence of RIC-3 (Fig. 8). As effects of RIC-3 are probably post-translational (5), this suggests an effect on stability of the DEG-3/DES-2 receptor or of DEG-3/DES-2 receptor maturation intermediates. To examine whether DES-2 co-expression is required for the effects of RIC-3 on DEG-3 quantity, we examined the effects of RIC-3
on DEG-3 quantity in oocytes not expressing DES-2. This analysis shows a significant 1.49 ± 0.49-fold difference in the quantity of DEG-3 (N = 8, n = 160). This result demonstrates that the effects of RIC-3 on DEG-3 quantity do not require DES-2. Thus, the stabilizing effects of RIC-3 do not require formation of a functional receptor. However, we cannot rule out an interaction of RIC-3 with non-functional DEG-3 pentamers.

Effects of RIC-3 on untagged (wild-type) DEG-3 quantity are reproducible, and similar effects on receptor quantity were seen when human RIC-3 was expressed with serotonin (5-hydroxytryptamine) type 3 (5HT3) receptors (11). These effects, however, are not a prerequisite for the effects of RIC-3 on functional expression, as effects of RIC-3 on functional expression are seen in the absence of effects on subunit quantity in oocytes expressing DEG-3 and Myc-tagged DES-2 (results not shown) and in human embryonic kidney 293 cells expressing α7 receptors (12). The absence of the stabilizing effects of RIC-3 in these experiments is compatible with the idea that RIC-3 interacts with and stabilizes only a small subset of receptors/receptor intermediates. The fraction of this subset relative to the total receptor subunits may differ between experiments and may depend on expression levels and stability of the nAChR subunits used. When this fraction is smaller, the stabilizing effects of RIC-3 may be masked. Unfortunately, the fact that Myc-tagging DES-2 interferes with the effects of RIC-3 on DEG-3 quantity and the absence of antibodies directed to DES-2 made it impossible to examine the effects of RIC-3 on the stability of the DES-2 subunit.

DISCUSSION

The *C. elegans* RIC-3 is required for normal functional expression of multiple nAChRs in vivo (5), and the human RIC-3 homolog is required for functional expression of α7 receptors in human embryonic kidney 293 cells (12) and promotes surface expression of serotonin (5-hydroxytryptamine) type 3 (5HT3) receptors in COS-7 cells (11). In Xenopus oocytes both *C. elegans* and human RIC-3 promote or inhibit functional expression of co-expressed receptors in a subtype-specific manner (6). We now show that RIC-3 expression also affects the properties, kinetics, and affinity of the *C. elegans* DEG-3/DES-2 receptor. Effects on receptor properties are usually attributed to auxiliary subunits interacting with the mature receptor on the membrane, but RIC-3 is unlikely to function on the surface membrane (5, 11). Instead, we show that effects of RIC-3 on receptor properties can be mimicked by increasing the ratio of DEG-3 to DES-2, suggesting that RIC-3 may preferentially promote expression of receptors having high DEG-3 to DES-2 stoichiometry. Support for this suggestion comes from the preferential effects of RIC-3 on whole cell current amplitudes in oocytes expressing high DEG-3 to DES-2 ratios. Together with previously reported results on receptor subtype-specific effects of RIC-3 (6), this suggests that although RIC-3 interacts with multiple divergent nAChRs, the implications of these interactions differ in a subtype-specific manner.

Members of the RIC-3 family have two transmembrane domains followed by a coiled-coil domain (6). We now show that this core structure, as seen in the RIC-3 minimal protein, is sufficient for RIC-3 function, as deletion of the non-conserved, *C. elegans*-specific N- and C-terminal domains has no effects on the function of RIC-3 in *Xenopus* oocytes. Coiled-coil domains have been implicated in mediating protein-protein interactions (13); thus, conservation of the first coiled-coil domain in all RIC-3 homologs suggested that they may function as part of a protein complex needed for efficient maturation of nAChRs (6). However, we now show that the coiled-coil domains are not necessary for the effects of RIC-3 in *Xenopus* oocytes, demonstrating that RIC-3 does not require assistance of "maturation-promoting factors" brought in through interactions with the coiled-coil domain for its activity. The finding that only a short sequence within RIC-3 is required for its function raises the question of what is the role of the "non-essential" domains. Specifically, we were surprised by the finding that the conserved coiled-coil, a domain conserved in all RIC-3 homologs (6), is not necessary for function. One explanation for this finding is that in *vivo* RIC-3 has additional effects that cannot be detected in oocytes expressing the DEG-3/DES-2 receptor. A second explanation and our preferred hypothesis is that these domains are needed for regulating RIC-3 function.

Our deletion analysis shows that a short 118-amino acid region encoding for the two transmembrane domains and the proline-rich spacer is sufficient for the effects of RIC-3 on the DEG-3/DES-2 receptor. Within this short region, the second transmembrane domain is the most conserved, whereas conservation within the proline-rich spacer is limited to having high frequency of prolines (6). In addition, signal peptide predictions (www.cbs.dtu.dk/services/SignalP/) show that in all vertebrate RIC-3 homologs, but not in the invertebrate homologs, the first transmembrane domain is likely to function as a signal peptide (12).2 Thus, its sole role is likely to be proper

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2 M. Treinin, unpublished results.
orientation of RIC-3 relative to the membrane, further reducing the size of the protein needed for interaction with and regulation of nAChR expression. These sequence analysis and sequence conservation data suggest that conserved functions of RIC-3 may be mediated solely by the second transmembrane domain. Such a short sequence is less likely to recruit additional maturation-promoting factors for its activity; rather, it may act similarly to pharmacological chaperones, small molecules that specifically bind and stabilize proteins within the endoplasmic reticulum (14). This binding may stabilize receptor intermediates in a conformation that either promotes or interferes with further maturation, thus providing an explanation for the diverse effects of RIC-3 on co-expressed nAChRs.

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