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Evolution of Developmental Control Mechanisms

Developmental expression of a molluscan RXR and evidence for its novel, nongenomic role in growth cone guidance

Christopher J. Carter, Nathan Farrar, Robert L. Carlone, Gaynor E. Spencer *

Dept. Biological Sciences, Brock University, 500 Glenridge Ave. St. Catharines, Ontario, Canada L2S 3A1

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ABSTRACT

It is well known that the vitamin A metabolite, retinoic acid, plays an important role in vertebrate development and regeneration. We have previously shown that the effects of RA in mediating neurite outgrowth, are conserved between vertebrates and invertebrates (Dmetrichuk et al., 2005, 2006) and that RA can induce growth cone turning in regenerating molluscan neurons (Farrar et al., 2009). In this study, we have cloned a retinoid receptor from the mollusc *Lymnaea stagnalis* (*Lym*RXR) that shares about 80% amino acid identity with the vertebrate RXRα. We demonstrate using Western blot analysis that the *Lym*RXR is present in the developing *Lymnaea* embryo and that treatment of embryos with the putative RXR ligand, *9-cis* RA, or a RXR pan-agonist, PA024, significantly disrupts embryogenesis. We also demonstrate cytoplasmic localization of *Lym*RXR in adult central neurons, with a strong localization in the neuritic (or axonal) domains. Using regenerating cultured motor neurons, we show that *Lym*RXR is also present in the growth cones and that application of a RXR pan-agonist produces growth cone turning in isolated neurites (in the absence of the cell body and nucleus). These data support a role for RXR in growth cone guidance and are the first studies to suggest a nongenomic action for RXR in the nervous system.

Introduction

Retinoic acid (RA) is the active metabolite of vitamin A and is well known to influence morphogenesis during vertebrate development (Maden and Hind, 2003; Maden, 2007). It can also act as a trophic factor and has been implicated in neurite outgrowth (Corcoran et al., 2000; Maden et al., 1998; Wuarin et al., 1990) and regeneration (Dmetrichuk et al., 2005) of the nervous system. Retinoic acid classically acts through nuclear receptors that act as transcription factors to affect downstream activation of various genes, including neurotrophins, cytokines, cell surface molecules (reviewed in Gudas, 1994; Mey and McCaffery, 2004), as well as specific genes involved in neurite outgrowth, such as *NEDD9* (Knutson and Clagett-Dame, 2008) and *neuron navigator 2* (Muley et al., 2008). The nuclear receptors responsive to RA include the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), and at least three classes of each have been identified (α, β, and γ). RARs bind both *all-trans* and *9-cis* RA isomers, whereas the RXRs (at least in vertebrates) bind only *9-cis* RA (Heyman et al., 1992). There is evidence that both RARs and RXRs play a role in neurite outgrowth and/or neurite regeneration; RARβ plays a major role in the induction of neurite outgrowth from both embryonic (Corcoran et al., 2000) and adult (Dmetrichuk et al., 2005) spinal cord neurons, while RXR has been suggested to play a role in motor neuron innervation of limbs in mice (Solomin et al., 1998). Both RARs and RXRs are found in vertebrate nervous systems, but until recently, it was generally believed that nonchordates possessed only RXRs. However, evidence for putative RARs has now emerged from EST/genomic databases in annelids and molluscs (Albalat and Canestro, 2009), and a molluscan RAR has now been cloned (Carter and Spencer, 2009; accession no. GU932671).

It has become increasingly evident that many effects of retinoic acid are conserved between vertebrate and invertebrate species. The presence of RA in invertebrates has been implicated by the presence of retinoic acid binding proteins in the insect (Mansfield et al., 1998), shrimp (Gu et al., 2002), and marine sponge (Biesalski et al., 1992). RA has also been detected in fiddler crab limb blastemas (Chung et al., 1998) and in the locust embryo (Nowickyj et al., 2008), suggesting a role in both limb regeneration and embryonic development. More recently, we have shown for the first time that RA is present in the invertebrate CNS (Dmetrichuk et al., 2008) and demonstrated that (in the absence of other neurotrophic factors) it induces neurite outgrowth as well as growth cone turning in cultured neurons of the mollusc, *Lymnaea stagnalis* (Dmetrichuk et al., 2006, 2008; Farrar et al., 2009). The mechanisms underlying the neurotrophic and chemotropic effects of retinoic acid in *Lymnaea* are, at present, largely unknown, although we have recently shown that the RA-induced growth cone turning involves a nongenomic mechanism that requires protein synthesis and calcium influx (Farrar et al., 2009).

In this study, we have cloned a RXR from the CNS of *Lymnaea* that demonstrates a high sequence homology with the vertebrate RXRα.

* Corresponding author. Fax: +1 905 688 1855.
E-mail address: gspencer@brocku.ca (G.E. Spencer).

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Our aim was then to determine whether this *Lymnaea* RXR plays a role in either embryonic development and/or neuronal regeneration of central neurons in *L. stagnalis*.

Materials and methods

Cloning of L. stagnalis RXR

L. stagnalis used in this study were laboratory-bred and kept in aerated, artificial pond water and fed lettuce and NutraFin Max Spirulina fish food (Hagen). RT–PCR was performed on cDNA generated from total RNA extracted from the *Lymnaea* CNS. Briefly, reverse transcription was carried out with a mixture of poly A and random hexamer primers according to the iScript cDNA synthesis kit (BioRad). cDNA was then amplified with 40 pmol of forward (5′-CGA CAA AAG ACA GAG AAA CAG ATG YCA RTA YTG-3′) and reverse (5′-GTC TCT GAA GTG TGG GAT TCT TTT NGC CCA YTC-3′) degenerate primers. After 3 min of denaturation at 95 °C, 35 cycles at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 1 min were performed with an Eppendorf Mastercycler Personal Thermocycler. Analysis of the product was carried out on 1% agarose gels in TAE buffer stained with ethidium bromide. Fragments of interest were excised from the gel and cloned into the pGemTEasy vector system (Promega). Sequencing was performed by GénomeQuébec (Montréal, Canada) using 3730xl DNA Analyzer systems from Applied Biosystems. Full-length cDNA was obtained by performing multiple rounds of 5′ and 3′ RACE with cDNA prepared from the same *Lymnaea* CNS preparations with the SMART RACE cDNA amplification kit (Clontech).

Antibodies

We designed antibodies against a synthetic peptide from the predicted ‘hinge’ region of the *Lymnaea* RXR covering the amino acid residues 183–198 between the DNA binding domain (DBD) and ligand binding domain (LBD). This custom-made *Lym*RXR antibody was produced in New Zealand white rabbits and affinity-purified from the antisera by Pacific Immunology Corp. (Ramone, CA, USA). In some Western blotting procedures, a commercial antibody against human GAPDH (Abcam Inc.) was used as a cytosolic fraction marker, and a commercial antibody against human actin (Sigma-Aldrich) was used as a control.

Lymnaea embryos

To investigate *Lym*RXR protein levels during *Lymnaea* development, egg masses were first incubated in pond water at room temperature and allowed to reach various stages of development, as described in Nagy and Elekes (2000). These stages included day 0 of embryogenesis (when egg mass is first laid and before first cleavage), the trochophore stage (approx. 36–60 h of embryogenesis), the veliger stage (approx. 60–96 h of embryogenesis), the adult-like stage just before hatching (96–192 h of embryogenesis), and hatchlings. When the embryos reached the desired stage, the capsules were removed from their gelatinous surroundings, total protein was extracted, and Western blotting was performed according to the protocol listed below for adult CNSs. These experiments were performed twice.

To determine if *Lym*RXR plays a role in *Lymnaea* development, embryos were incubated in a synthetic RXR agonist (PA024, a kind gift from Dr. H. Kagechika, Tokyo) and *9-cis* RA (Sigma-Aldrich). At day 0 of embryogenesis (when egg mass is first laid), capsules were teased out of the gelatinous surroundings in which they were embedded and maintained in pond water at room temperature for 30 h (until the end of the gastrulation stage). This separation of the capsules increased probability of penetration of the agonist PA024 and *9-cis* RA while still allowing normal development of the egg inside the capsule (Creton et al., 1993). The RXR agonist PA024 (10^{-7} M), *9-cis* RA (10^{-7} M), or DMSO (0.001%, vehicle control) was added to separate dishes of embryos at 30 h of embryogenesis (this was previously shown to be the most sensitive stage to disruption by RA; Creton et al., 1993). At days 6–7 of embryogenesis, embryos showing eye and/or shell malformations, or arrested development at the trochophore stage, were scored and compared with the DMSO control embryos. These experiments were also performed twice with incubation of embryos in RXR antagonists alone (PA452 = 10^{-6} M and HX531 = 10^{-6} M both a kind gift from Dr. H. Kagechika, Tokyo) in the exact same manner as described above. Statistical analysis was composed of multiple Fisher exact tests that were then Bonferroni–Holm-corrected.

Western blotting

To investigate the expression of *Lym*RXR protein, the adult *Lymnaea* CNS (or whole embryos) were homogenized in lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 10 mM EDTA, 1% Trition X-100, 1 mM PMSF, and 0.01% Protease Inhibitor Cocktail (Sigma-Aldrich) with a PowerGen handheld homogenizer (Fisher Scientific). The homogenates were centrifuged 20,000 × *g* at 4 °C for 30 min. Fifteen micrograms of protein from each extract was separated on a discontinuous SDS–polyacrylamide gel (12% resolving and 4% stacking) and electroblotted onto a nitrocellulose membrane (BioRad). For embryo experiments, gels were electrophoresed in duplicate under identical conditions; one was stained with Coomassie blue to ensure equal loading of protein and the other was subjected to Western blot analysis. The membranes were washed for 5 min in 1× PBS and then blocked in 1× PBS/0.1% Tween-20 (PBT) with 3% skim milk powder (wt./vol.) for 1 h. The membranes were then incubated with affinity purified *Lymnaea* RXR antibody at a dilution of 1:2500 in PBT/3% skim milk overnight at 4 °C with gentle horizontal shaking. This was followed by 4 × 5 min washes at room temperature in PBT and incubation with a 1:15,000 dilution of Alexa Fluor 680 goat antirabbit secondary antibody (Invitrogen). After 4 × 5 min washes in PBT and one wash in 1× PBS, the membranes were imaged with the LI-COR Odyssey Infrared Imaging System at a wavelength of 700 nm.

To investigate the subcellular expression of *Lym*RXR in the *Lymnaea* CNS, as well as in embryos just before hatching, total proteins from the cytoplasmic, membrane, and nuclear compartments were isolated according to directions from the Qproteome Cell Compartment kit (Qiagen). These protein fractions were loaded onto a discontinuous SDS–polyacrylamide gel, and *Lym*RXR was detected by Western blotting technique as previously described above. Anti-GAPDH (Abcam Inc.) was used as a cytosolic fraction marker, and successful isolation of protein from all three compartments of the CNS was confirmed by staining for actin. These experiments were performed twice for embryos and four times for the adult CNS.

Immunostaining

For immunostaining, the CNSs isolated from the snails were fixed in 4% paraformaldehyde in PBS at 4 °C overnight and washed in 10% sucrose/PBS for 2 h, 20% sucrose/PBS for 2 h, and then 30% sucrose/PBS overnight at 4 °C. After embedding the fixed CNSs in Optimal Cutting Temperature (OCT) Compound (Tissue-Tek), serial 20 μm sections were cut using a cryostat (Leica Microsystems) and placed on SuperFrost Plus slides (Fisher Scientific). For immunostaining of cultured neurons following outgrowth (24–36 h), cells were fixed in 4% paraformaldehyde in PBS at 4 °C overnight. From this point on, all immunostaining procedures were the same for CNSs and cultured neurons. The samples were washed in PBS and then permeabilized in 0.3% Triton X-100 in PBS (PBT) for 20 min and blocked in 5% normal goat serum (NGS) in PBT for 1 h at room temperature. The samples were then incubated with the primary *Lym*RXR antibody diluted 1:100 in blocking solution at 4 °C overnight. As a control, preparations