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THE ROYAL SOCIETY

Processing of ARIA and release from isolated nerve terminals

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The neuromuscular junction is a specialized synapse in that every action potential in the presynaptic nerve terminal results in an action potential in the postsynaptic membrane, unlike most interneuronal synapses where a single presynaptic input makes only a small contribution to the population postsynaptic response. The postsynaptic membrane at the neuromuscular junction contains a high density of neurotransmitter (acetylcholine) receptors and a high density of voltage-gated Na^+ channels. Thus, the large acetylcholine activated current occurs at the same site where the threshold for action potential generation is low. Acetylcholine receptor inducing activity (ARIA), a 42 kD protein, that stimulates synthesis of acetylcholine receptors and voltage-gated Na^+ channels in cultured myotubes, probably plays the same roles at developing and mature motor endplates *in vivo*. ARIA is synthesized as part of a larger, transmembrane, precursor protein called proARIA. Delivery of ARIA from motor neuron cell bodies in the spinal cord to the target endplates involves several steps, including proteolytic cleavage of proARIA. ARIA is also expressed in the central nervous system and it is abundant in the molecular layer of the cerebellum. In this paper we describe our first experiments on the processing and release of ARIA from subcellular fractions containing synaptosomes from the chick cerebellum as a model system.

Keywords: neuregulin; secretion; synaptosomes; depolarization

1. INTRODUCTION

Neuregulins (NRGs) are a family of proteins that bind to and activate EGF receptor-like tyrosine kinases, erbB2, erbB3 and erbB4. The NRG family includes neu differentiation factor (NDF), heregulin (the human homologue of NDF), acetylcholine receptor inducing activity (ARIA) and glial growth factor (GGF). These proteins were initially characterized by their ability to phosphorylate tyrosine residues in a 185 kD transmembrane protein, presumed to be the product of the *neu* or *her2* oncogene (NDF or heregulin) (Holmes *et al*. 1992; Peles *et al*. 1992; Wen *et al*. 1992), to stimulate acetylcholine (ACh) receptor synthesis in cultured chick and rat myotubes (ARIA) (Falls *et al*. 1993; Usdin & Fischbach 1986), or to stimulate proliferation of Schwann cells (GGF) (Marchionni *et al*. 1993). Molecular cloning of full-length cDNAs revealed that they are all derived from a single gene (NRG1) by alternative RNA splicing. Two other NRG-related genes (NRG2 and NRG3) have been identified recently (Busfield *et al*. 1997; Carraway III *et al*. 1997; Chang *et al*. 1997; Zhang *et al*. 1997), but they will not be discussed further here.

Many NRG1 isoforms are synthesized as transmembrane precursors (proNRG). Presumably, proteolytic processing of the extracellular domain liberates soluble polypeptide (NRG) that interacts with target tissues (figure 1). All NRGs contain an EGF-like domain with six cysteins arranged in three disulfide bonds that is essential for high-affinity binding, and this 50–55 amino acid sequence can reproduce all known NRG actions. The spacing between the cysteins is different from that of EGF, and members of NRG family do not directly activate EGF receptor at the same low concentration (10^{-11} M) as they activate other erbB tyrosine kinases. ARIA and several other NRG1 isoforms contain an Ig-like domain N-terminal to the EGF-like domain. This part of the protein is apparently essential for the binding of NRGs to heparan sulphate proteoglycans in the extracellular matrix (Loeb & Fischbach 1995). The role of this extracellular reservoir of neuregulin binding sites remains to be determined.

Our laboratory has attempted to characterize the actions of ARIA at developing and mature neuromuscular junctions (NMJs) and also at interneuronal synapses. After a brief review on this work, we describe our first experiments on the processing and release of ARIA from subcellular fractions containing synaptosomes and synaptic vesicles. Understanding the biological role of ARIA at synapses would not be complete without knowing how this molecule is delivered to nerve terminals, and how it is released into the extracellular space.

2. ARIA AT THE NEUROMUSCULAR JUNCTION

Postsynaptic membranes at the NMJ contain an extremely high density of ACh receptors (AChR) ($>10\,000$ molecules μm^{-2}). This ensures that a sufficient number of channels are opened following each presynaptic nerve impulse to trigger an all-or-none action potential in the muscle membrane. The safety factor for neuromuscular transmission is about four to five. That is, even when the amount of ACh released from nerve terminus is reduced by four- to fivefold, enough numbers

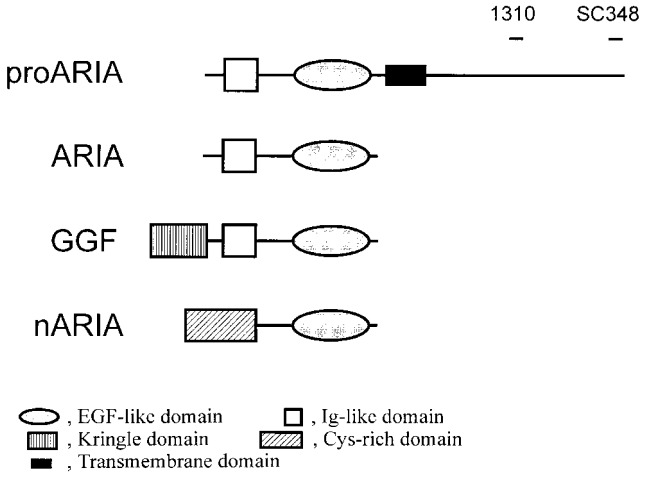

Figure 1. Structure of NRG isoforms and epitopes for antibodies. NRGs contain EGF-like domain which can reproduce all known biological functions of NRG. Many isoforms including ARIA and GGF contain additional Ig-like domain while nARIA (for neuronal ARIA) contains Cys-rich domain in place of Ig-like domain. Many NRG isoforms including ARIA are made as a transmembrane form (proARIA). Orientation of proARIA is such that EGF-like domain is in the extracellular space when it is expressed on the cell surface. Two antibodies, 1310 and SC348, raised against two different regions in the cytoplasmic domain of proARIA recognized common bands from Western blots of synaptosomal membranes (see text).

of AChRs are activated to trigger action potential in the muscle membrane. The high AChR density is clearly dependent on the presence of motor nerve terminals. AChR clusters do not appear on never-innervated myotubes following ablation of the neural tube. The receptor density remains high at mature endplates following denervation, but it declines over the course of several weeks. It may be that a nerve-derived factor remains lodged in the basal lamina at the vacated synapse.

The receptor density in the extrasynaptic membrane is low (<10 molecules μm^{-2}) in mature, innervated muscle fibres, but it is one to two orders of magnitude higher in embryonic myotubes and in mature fibres following denervation. Muscle activity, probably via a protein kinase C-dependent mechanism, turns off AChR synthesis in extrasynaptic nuclei. The influence of the nerve is remarkable in that it stimulates AChR synthesis in the subsynaptic nuclei and keeps the local AChR density high despite this powerful downregulating influence.

Motor neurons keep endplate AChR density high by stimulating AChR gene expression in the endplate nuclei and by immobilizing AChRs once they appear in the membrane (Miledi & Zelena 1966; Role *et al*. 1985; see Hall & Sanes (1993) for a review). Two motor neuron-derived proteins are thought to account for these effects of motor nerve terminals. ARIA, a 42 kD glycoprotein, was purified from chick brain based on its ability to stimulate incorporation of new AChRs into cell surface membranes in cultured embryonic myotubes (reviewed in Fischbach & Rosen 1997). Agrin, a large proteoglycan, was purified from torpedo electric organ (Nitkin *et al*. 1987) based on its ability to promote aggregation of AChRs into macroscopic clusters. Agrin does not increase the total number of AChRs, but it does immobilize AChRs as measured by fluorescence photobleaching recovery (Meier *et al*. 1995).

Several experiments indicate that ARIA and closely related molecules do indeed play a role at developing neuromuscular junctions. ARIA mRNA is detectable in the ventral half of the neural tube soon after motor neurons are born (Goodearl *et al*. 1995), and ARIA immunoreactivity can be detected in spinal cord motor neurons as soon as they migrate out of the germinal epithelium and begin to express early motor neuron transcription factors islet1 and islet2 (Ericson *et al*. 1992; Tsuchida *et al*. 1994). ARIA immunoreactivity can also be detected along the motor neuron axons as they exit the spinal cord and enter adjacent muscle masses (Loeb *et al*. 1999). ARIA must, therefore, be available at the growing tip of motor axons when neuromuscular contact first occurs. Later in development, between E15 and E17 in the chick, ARIA immunoreactivity increases sharply at the NMJ. This late increase occurs at about the same time as AChR density increases dramatically. A heparan sulphate proteoglycan recognized with a monoclonal antibody increases as well (Goodearl *et al*. 1995; Loeb *et al*., unpublished data). Receptor tyrosine kinases, erbB2, erbB3 and erbB4, some combination of which mediate the effect of neuregulins, have been detected at the neuromuscular junction (Altiok *et al*. 1995; Moscoso *et al*. 1995; Zhu *et al*. 1995). ARIA is also likely to be responsible for switching of AChR isoform from foetal type $\alpha_2\beta\gamma\delta$ to adult type $\alpha_2\beta\epsilon\delta$ in the mammalian muscle. Application of ARIA to the cultured muscle strongly stimulated synthesis of the ϵ subunit of AChR (Chu *et al*. 1995; Martinou *et al*. 1991; Missias *et al*. 1996). AChR containing the ϵ subunit has larger conductance with shorter open time allowing more reliable and precise control of the muscle compared to foetal type. The physiological importance of this isoform switching in the neuromuscular synapse is highlighted by the finding that mutant mice lacking the ϵ subunit of AChR are defective in the development and maintenance of postsynaptic specialization (Missias *et al*. 1997). ARIA also stimulates synthesis of voltage-gated Na^+ channels in cultured myoblasts (Corfas & Fischbach 1993), another protein that is highly concentrated in the postsynaptic membrane at the neuromuscular junction. The fact that these actions of ARIA, increase in overall AChR density, switching of AChR isoforms and increase in voltage-gated Na^+ channel all increase the fidelity of nerve–muscle synaptic transmission strongly supports the conclusion that neuregulin plays an important role in the formation and maturation of the neuromuscular junction.

The most compelling evidence for the role of ARIA at the NMJ came from the studies of neuregulin knockout animals (Sandrock *et al*. 1997). Homozygous mice die at E9.5–E10 before the onset of neuromuscular synapse formation, probably due to heart malformation (Kramer *et al*. 1996; Meyer & Birchmeier 1995). Heterozygous mice survive to adulthood, but careful examination showed that they were much more sensitive to curare. The compound muscle action potential declined in the presence of concentrations of curare that did not affect wild-type animals. Intracellular microelectrode recording showed that the mean amplitude of miniature endplate potentials was reduced by 30–50%. Also the number of

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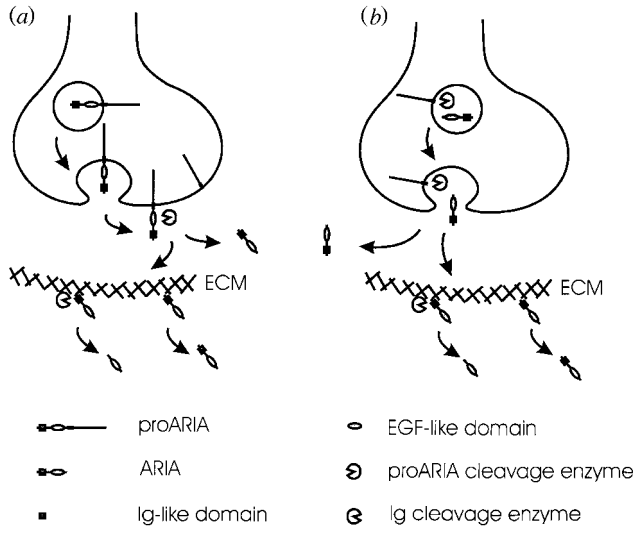

Figure 2. Models for ARIA release. (*a*) Model 1, proARIA is shipped intact to the nerve endings. Cleavage at the plasma membrane in the presynaptic terminus liberates ARIA into synaptic cleft. (*b*) Model 2, proARIA is cleaved intracellularly and ARIA is liberated from the presynaptic terminus via vesicle fusion. The two models may not be mutually exclusive. Indeed, both mechanisms appear to operate in our synaptosome preparation. In both cases, a portion of liberated ARIA may bind to the extracellular matrix (ECM) via its Ig-like domain. Once bound to the ECM, ARIA may be released from the ECM either by simple dissociation or by enzymatic degradation of the Ig domain or the ARIA binding sites.

receptors, assayed by the binding of [^{125}I]-α-bungarotoxin, was decreased by about 50%.

3. SYNAPTOSOMES

To study ARIA release from nerve terminals, we prepared synaptosomes from E19 chick cerebellum. Synaptosomes offer the possibility of understanding the mechanism of ARIA cleavage and release, the possible role of postsynaptic factors, and the regulation of release by ions and other mediators for neurotransmission. Cerebellum was selected for our first choice because the molecular layer, an extremely rich source of synaptic membranes, is also a rich source of neuregulin, presumably synthesized in the granule neurons (Sandrock *et al.* 1995). The cerebellum consists of a limited number of cell types, and is generally considered a 'simple' system. We selected E19 chick embryos because they are convenient to work with. At this stage, however, granule cell migration is not complete, and a substantial external granule cell layer is still evident. The external granule layer disappears shortly after hatching. Thus, we expect that synaptosomes prepared from E19 chick cerebella are derived not only from mature synapses but also from immature synapses and growth cones, and this must be taken into account.

In principle, ARIA might be released in one of two ways (figure 2). First, proARIA may reach the plasma membrane, and the extracellular domain (ARIA) may be cleaved at the cell surface. Second, ARIA may be cleaved from its transmembrane precursor within intracellular vesicles and subsequently released by exocytosis. In this

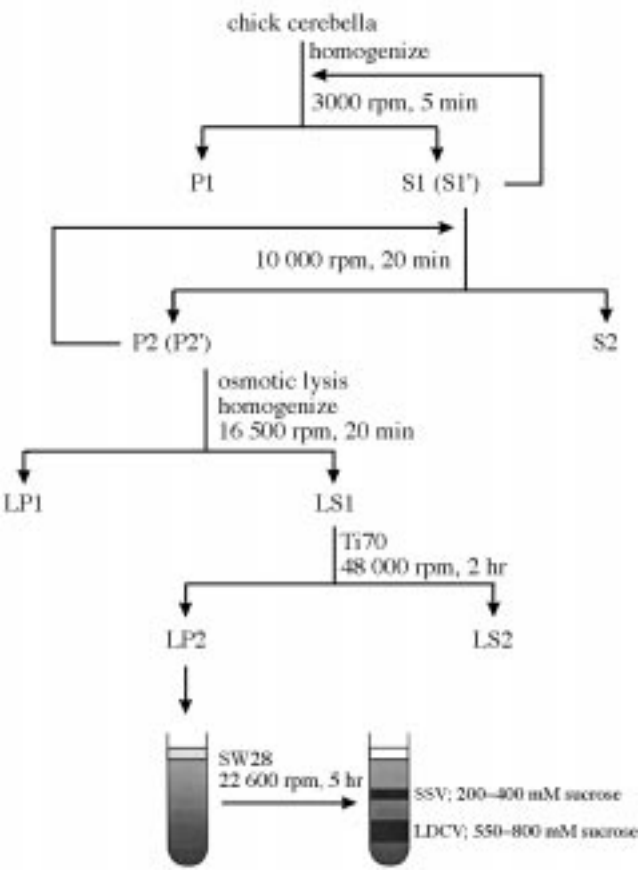

Figure 3. Subcellular fractionation scheme. Crude synaptosomes (P2′) and various subcellular fractions were prepared according to standard protocols as shown schematically.

case, cleavage may occur at all points along the axon during the process of rapid transport, or it may be relatively enhanced in the nerve endings. Our data indicate that both mechanisms contribute to the release of ARIA. Our first experiments also indicate that electrical activity stimulates ARIA release in both calcium-dependent and independent mechanisms.

It is important to be clear about the term 'release' when dealing with a molecule like ARIA that is cleaved from a transmembrane precursor, but that is retained at the cell surface by binding to sites within the extracellular matrix (ECM). ARIA may escape the ECM by dissociation or by enzymatic degradation (of the ARIA Ig domain and/or the matrix binding sites). We use the term 'release' to refer to the appearance of bioactivity free in solution. (This emphasis on diffusable ARIA does not, of course, diminish the importance of juxtacrine actions that may occur in addition to soluble ligand–receptor interactions.)

A low speed pellet (P2′; figure 3) containing synaptosomes was prepared from E19 chick cerebella according to standard procedures (Gray & Whittaker 1962) and resuspended in Krebs buffer at pH 7.4. The presence of sealed synaptosomes was demonstrated by electron microscopy and by analysis of K^+ stimulated release of preloaded [^{3}H]-glutamate.

On incubation of this crude synaptosome preparation at 37 °C, ARIA was released into the medium in a time-dependent manner. In these experiments, ARIA was assayed by tyrosine phosphorylation of a p185 in L6 muscle cells (figure 4). Other experiments have shown

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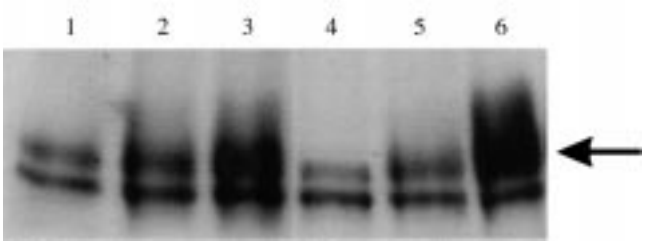

Figure 4. Assay of ARIA release from crude synaptosomes. Lane 1: crude synaptosomes (P2′) were resuspended in cold Krebs buffer then spun to remove membranes. Medium was put on L6 muscle cells to assay ARIA by tyrosine phosphorylation of p185. There was no detectable tyrosine phosphorylation activity in the medium. Lane 2: NaCl (1 M final) was added to synaptosome suspension while on ice to elute ECM bound ARIA. There was a clear increase in p185 phosphorylation activity compared to lane 1, demonstrating a reservoir of ARIA in the ECM. Lane 3: synaptosomes were incubated at 37 °C for 90 min before addition of NaCl. Even higher p185 phosphorylation was observed than in lane 2, demonstrating that more ARIA was liberated into the extracellular space on incubation at 37 °C. Lanes 4–6: controls for p185 phosphorylation assay with purified ARIA at different concentrations; 0 (lane 4), 20 pM (lane 5) and 100 pM (lane 6). Arrow indicates p185 band.

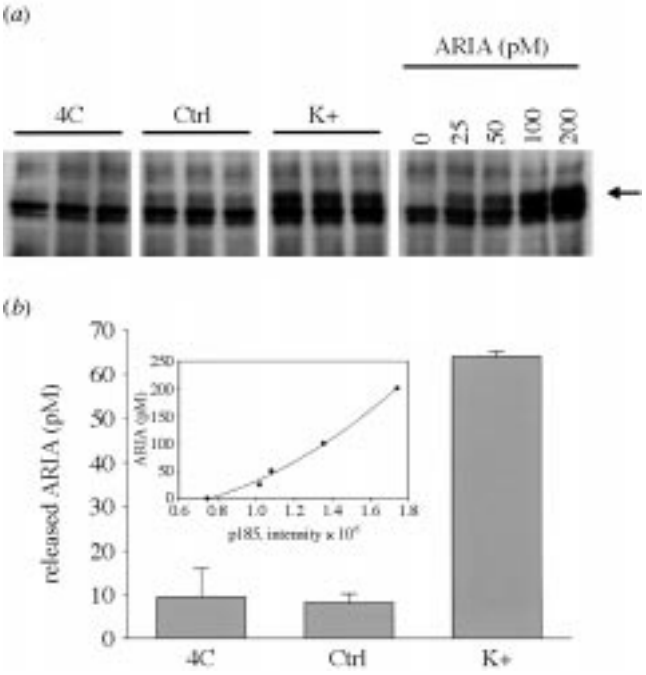

Figure 5. Depolarization by high K^+ stimulated ARIA release from crude synaptosomes (P2′). Crude synaptosomes were incubated at 37 °C for 15 min in Krebs buffer and washed thoroughly. Synaptosomes were then resuspended in cold Krebs buffer and incubated for additional 5 min at 4 °C (4C) or at 37 °C in the absence (Ctrl) or presence (K^+) of 25 mM K_2SO_4. ARIA released into the medium was assayed by p185 phosphorylation (*a*). Arrow indicates p185 band. Quantitation of ARIA was done by comparing intensity of the p185 band with those obtained by known concentrations of purified ARIA (*b*). Error bars indicate standard error of three samples.

that this p185 band contains one or more of the neuregulin receptor tyrosine kinases (erbB2, 3, or 4). L6 p185 phosphorylation was blocked by a soluble erbB4 fragment, a chimeric molecule containing the extracellular domain of erbB4 and the heavy chain of immunoglobulin. Thus, phosphorylation of p185 must be due to the action of ARIA or a closely related molecule. To measure total ARIA (diffusable and bound to the ECM), the membranes were washed with 1 M NaCl. ARIA elutes from heparin columns at NaCl concentrations in excess of 400 mM. The time-course of appearance of total ARIA (released and ECM bound) was best fit by the sum of two exponentials consistent with the existence of two independent pools.

We determined if any of the release of ARIA from the P2′ fraction was 'regulated' by membrane potential and/or by calcium ions. Addition of 25 mM K_2SO_4 to the membrane suspension during the first 5 min of warming P2′ from 4 °C to 37 °C ('fast' phase of ARIA release) did not significantly increase release of ARIA. Cleavage of proARIA is unlikely to be the source of this fast component of ARIA release considering the slow kinetics of proARIA cleavage (see below). We assume that this early release represents 'leakage' from non-sealed compartments as reported in other systems (e.g. Tandon *et al.* 1998).

To examine the 'slow' phase of ARIA release without interference by the fast component, we depleted the fast component by pre-incubating the synaptosomes for 15 min at 37 °C. Based on the observed kinetics, the pool size of the fast component should be reduced to less than 1% (0.6%) of its initial size, while more than 75% of the slow component should remain in place. Thus, the fast component should contribute less than 15% of the amount released in the time interval from 15–20 min.

Raising K^+ to 50 mM produced a 50% increase in release from the 'slow' pool when compared to release at 5 mM K^+. The effect was small, but it was consistent and statistically significant. In these experiments, 50 mM K^+ replaced 50 mM Na^+ so that osmolarity and ionic strength were nearly maintained. The potassium channel blocker, 4-aminopyridine (4-AP), was as effective as the iso-osmotic K^+ depolarization in stimulating ARIA release. Stimulation of ARIA release by two different depolarizing mechanisms strongly supports the model that ARIA can be released by exocytosis from intracellular vesicles via membrane fusion. This result also indicates that depolarization of nerve endings can stimulate ARIA release.

When K^+ concentration was raised by addition of 25 mM K_2SO_4 without substituting for NaCl, there was a much larger increase (more than sixfold) in ARIA release from P2′ membranes (figure 5). We do not yet fully understand the difference between this non iso-osmotic condition and the more nearly iso-osmotic one. It may be simply that our preparation of sealed synaptosomes improved over time. In addition, the small increase in ionic strength may promote some release of ECM-bound ARIA. The effect of added K_2SO_4 in non iso-osmotic conditions was largely insensitive to calcium. Chelation of extracellular Ca^{2+} with 2 mM EGTA decreased the response only slightly. However, insensitivity to calcium does not rule out release of ARIA by exocytosis because K^+-stimulated release of preloaded

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[^{3}H]-glutamate was also largely insensitive to extracellular calcium under the same experimental condition. It remains to be determined if chelation of cytosolic Ca^{2+} can block stimulation of ARIA release by high K^+.

Regulation of release by depolarization and presence of a Ca^{2+}-dependent component suggest that ARIA release involves exocytosis. However, it does not distinguish cleavage of proARIA within vesicles from cleavage at the cell surface. Our first experiments described below, provide evidence for both processes.

The crude synaptosome preparation, P2′, was used as a starting material to separate intraterminal vesicles from plasma membranes by differential centrifugation. P2′ membranes were lysed and homogenized in hypo-osmotic solution, then fractionated to obtain LP1 and LP2 as illustrated in figure 3. More than 90% of the synaptic vesicle marker, SV2, was found in LP2.

Western blots of these membrane fractions with an antibody, SC348 (from Santa Cruz Biotech., Santa Cruz, CA), against the cytoplasmic domain of proARIA showed two bands of approximately 130 kD and 55 kD. Neither band was labelled when the antibody was added in the presence of the cognate peptide. Moreover, a second antibody, 1310, directed against a different intracellular determinant labelled the same two bands. The simplest interpretation of this pattern is that the larger band is proARIA and the smaller band is the remnant (transmembrane plus cytoplasmic domain after cleavage of the extracellular domain). Indeed, the 55 kD band comigrated with the remnant detected in the membranes from CHO or COS cells transfected with proARIA cDNA.

ARIA was released from LP1 membranes maintained at 37 °C over the course of 2 h. The intensity of the 130 kD band labelled with SC348 decreased over the same time-course. Addition of 1 mM $ZnCl_2$ inhibited ARIA release and prevented the decrease in the 130 kD band intensity. These data support the notion that ARIA can be released by enzymes acting at the presynaptic surface membrane, a mechanism that has been shown to operate in CHO cells transfected with proARIA cDNA (Burgess *et al*. 1995; Loeb *et al*. 1998). The inhibitory effect of 1 mM Zn^{2+} is consistent with the action of a zinc-dependent metalloprotease because these proteases are known to be inhibited by high concentration of Zn^{2+}.

In addition to cleavage at the surface, cleavage of proARIA within intracellular vesicles was suggested by the presence of the 55 kD (remnant) band in the LP2 fraction. LP2 was sonicated following freezing and thawing to allow leakage of the contents in the vesicles to the medium and the membranes were removed by centrifugation. A small but significant amount of p185 phosphorylating activity was recovered in the soluble fraction. Brief (<20 min) warming to room temperature before separation of membranes resulted in much higher recovery of ARIA in the soluble fraction. Up to 50% of the total activity, as measured by applying sonicated LP2 directly to the muscle cells, was recovered in the soluble fraction in this way. Cleavage of proARIA in the LP2 was much slower. More than 4 h at 37 °C was necessary to obtain half reduction in proARIA band intensity. Since LP2 is thought not to contain ECM, soluble ARIA in this fraction is considered to be present inside the vesicles.

Judging from studies of peptides and other prohormones, it is unlikely that proARIA is contained in small synaptic vesicles (SVs). This was confirmed by further fractionating LP2 by sucrose density gradient centrifugation. SVs were identified by their sedimentation at 200–400 mM sucrose and by the enrichment of SV2 in Western blots. SVs did not contain detectable amounts of proARIA. proARIA was enriched in, but not restricted to, a fraction that contained large dense core vesicles (LDCV) as identified by enrichment of chromogranin A. However, p185 phosphorylation activity was equally high in SV fraction as in LDCV fraction. Cleavage of proARIA within intracellular vesicles, such as early endosomes, may liberate soluble ARIA within the vesicles. Liberated ARIA may get trapped in clathrin coated pits, which serve as an intermediate step for SV generation. Studies using electron microscopy and colocalization with different marker proteins are currently under way. We conclude that proARIA is present in some type of presynaptic vesicle and that at least some of the molecules are cleaved and the liberated ARIA is stored in this vesicular compartment ready to be released on arrival of nerve impulse. Non-transmembrane forms of NRG exist (β3 isoforms contain a stop codon before the transmembrane domain) and they may be released by exocytosis as well.

Two roles for ARIA in cerebellar development have been suggested in recent studies. During early development, ARIA appears to influence the migration of neurons along glial guides from the external to the internal granule cell layer (Rio *et al*. 1997). Most relevant to our own studies of ACh-gated ion channels, ARIA stimulates synthesis of granule cell NR2C, a relatively rare subunit of NMDA type glutamate receptors (Ozaki *et al*. 1997). ARIA may be responsible for naturally occurring isotype switching of NMDA receptor subunit from NR2A to NR2C, analogous to the isoform switching of AChR from fetal type $\alpha_2\beta\gamma\delta$ to adult type $\alpha_2\beta\varepsilon\delta$ in the mammalian muscle.

4. SUMMARY

In summary, it appears that ARIA can be released from nerve terminals via more than one mechanism (figure 2). Intracellular and cell surface cleavage, each provides several interesting points of regulation. It will be important to determine if the same mechanisms operate along the axon as at the nerve terminal. The effect of steady membrane depolarization suggests that ARIA release can be regulated by ongoing impulse activity. This is an extremely important post-translational mechanism for coupling activity or 'experience' with trophic factor availability.

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