Cerebellar granule cells express a specific isoform of agrin that lacks the acetylcholine receptor aggregating activity

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Abstract **Agrin is a synapse-organizing molecule that mediates nerve-induced aggregation of acetylcholine receptors and other postsynaptic components at the developing and regenerating vertebrate neuromuscular junctions. Several lines of evidence indicate that agrin might play a similar role in directing the organization of postsynaptic specifications of neuron–neuron synapse formation. Here we used immunological methods and polymerase chain reaction to identify the expression of agrin protein and alternatively spliced mRNA isoforms in the culture of rat granule cells. Anti-agrin polyclonal antibody labeled the cultured granule cells and it detected a protein of over 200 kDa in size from the lysate of the cultured cells. Analysis by polymerase chain reaction showed that the granule cells in culture expressed predominantly the B_0 isoform of agrin mRNA. When granule cells were co-cultured with primary chick myotubes, there was no detectable effect on the aggregation of acetylcholine receptors on the surface of the myotubes. These results show that the cerebellar granule cells, similar to motor neurons in vitro, express and secrete agrin but it lacks the acetylcholine receptor aggregating activity.**

Key words: Synapse formation; Extracellular matrix; Acetylcholine receptor; Polymerase chain reaction

1. Introduction

During the development of neuromuscular junctions, there will be the formation of postsynaptic specializations directed by the motor neuron approaching the muscle fiber. These postsynaptic specializations include clustering of acetylcholine receptors (AChRs), acetylcholinesterase (AChE) and other synaptic components [1,2]. Agrin, a synapse-organizing protein, was implicated to be important in directing the formation of these postsynaptic specializations in the developing and regenerating neuromuscular junctions [2–4]. During development, agrin is expressed by motor neurons of the spinal cord [5]; it is released at the sites of contact with developing muscle fibers [6,7] and thus, induces the aggregation of AChRs and other components at the neuromuscular junctions [8]. Anti-agrin antibodies block nerve-induced AChR aggregation in motor neuron–myotube co-cultures [9]. Besides, the co-localization of neural agrin-like molecules and micro-aggregates of AChR at newly formed nerve–muscle contacts in culture [6] further supports the role of agrin in the formation of neuromuscular junctions during synaptogenesis.

Agrin cDNAs had been isolated in ray [10], chick [11] and rat [12]. The predicted size of agrin protein from completed cDNAs in chick and rat is around 200 kDa. The predicted agrin protein has a number of domains that show similarities with regions of other extracellular proteins. These domains include regions homologous to follistatin, a number of epidermal growth factor repeats, repeats of two different domains that share homology with laminin and, in particular interest, three repeated domains with homology to the globular G domain of laminin [13,14]. In chick and rat agrin, different variants of agrin mRNAs due to alternative splicing had been found by polymerase chain reaction (PCR) analysis. These agrin isoforms have previously been shown to differ in AChR aggregating activity [15,16]. The presence of an amino acid insertion, the z site/ B box, near the C-terminus is particularly important for the AChR aggregating activity of agrin; the number of amino acid insertion can be 0, 8, 11 and 19 [15–17]. Isoforms of B_8, B_{11} and B_{19} with biological activities are expressed exclusively in neural tissues [13,18], however, the biological activity of agrin in non-neuronal tissues is still unclear.

The precise role of agrin in central nervous system is not well understood. Brain extracts are capable to induce AChR aggregation and the induction was found to be enhanced if the extracts were affinity enriched with anti-agrin antibodies [19]. Using polymerase chain reaction analysis, most of the brain regions in rat were found to express B_0, B_8 and B_{19} while B_{11} expression was restricted in forebrain [17,20]. In situ hybridization studies indicated that in the adult rat brain, agrin mRNA transcripts were differentially distributed with high intensity in Purkinje cell layer of cerebellum, motor cranial nerve nuclei of brainstem and area postrema [20]. These studies suggested that agrin transcripts are widely distributed in the whole brain including cerebellum while those active isoforms are expressed in particular regions and also they are not restricted to cholinergic neurons [20,21]. This raises the intriguing possibility that agrin may play a role in the formation of neuron-neuron synapse of the central nervous system other than just the AChR aggregation on the surface of muscle fibers. Cerebellar granular cells comprise some 90% of all cerebellar neurons and are the only intrinsic neurons with an excitatory transmitter in cerebellum. These cells follow an intricate and well-characterized pathway of postnatal development and could be cultured to high homogeneity for days, thus offering an excellent system to examine the neuronal phenotype in culture [22,23]. As a step toward understanding the role of agrin in central nervous system, we examined the expression of agrin in cultured granule cell and

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Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor; FITC, fluorescein-5-isothiocyanate; PCR, polymerase chain reaction.

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the AChR aggregating activity of agrin, released by granule cells, in co-culturing with muscle fibers.

2. Materials and methods

2.1. Primary cerebellar granule cell cultures

Cerebellar granule cell cultures were prepared as described by Dutton [23] with some modifications. Briefly, cerebella from 7-day-old pups of Sprague–Dawley rats were minced and dissociated by trypsin treatment (0.25 mg/ml) in solution 1H which contains 25 mM HEPES, pH 7.4, 13.8 mM NaCl, 5 mM KCl, 4.2 mM $NaHCO_3$, 1 mM NaH_2PO_4, 3.2 mM $MgSO_4$. Soybean trypsin inhibitor (30 μg/ml) and DNase I (21 Kunitz Unit/ml) were added and cells were centrifuged for 5 s at 100 × *g*. Cell pellet was resuspended in solution 1H supplemented with soybean trypsin inhibitor (170 μg/ml) and DNase I (130 Kunitz Unit/ml). Cell suspension was triturated 17 times with fire-polished Pasteur pipettes and left to stand for 3 min. Cell suspension of the top layer was sedimented through 4% (wt/vol) bovine serum albumin in solution 1H at 75 × *g* for 4 min. Cell pellet was resuspended in Minimal Essential Medium (MEM) supplemented with 20 mM KCl, 1 mM glutamine, 33 mM glucose, 2.5% (vol/vol) chick embryo extract, 10% heat-inactivated fetal calf serum , 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated at density 1.5×10^6 for each 35 mm culture dish which was pre-coated with poly-L-lysine (100 μg/ml). Ten μM cytosine arabinoside was added after 48 h plating in order to suppress the cell division of non-neuronal cells. Cell cultures were kept at 37°C with 5% CO_2/95% humidified air. Cell homogeneity was monitored by both phase-contrast microscopy and immunocytochemistry using antibodies against neurofilament (a selectable marker for neurons from Sigma, St. Louis, USA).

For primary chick myotube cultures, hind-limb muscles dissected from 11-day-old New Hampshire chick embryo were minced and then dissociated by trypsinization, stirring and centrifugation by the method of Fischbach [24] with minor modifications [8]. Muscle cells were routinely cultured in MEM supplemented with 10% heat inactivated horse serum, 2% (vol/vol) chick embryo extract, 1 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The AChR aggregation assay was performed after 5 to 9 day of culture. In the co-culture experiment, about 1×10^5 cells/ml of rat granule cells were plated on the 4-day-old primary muscle cultures. Normally, they were allowed to co-culture for 3 to 5 days.

2.2. Immunochemical analysis

Anti-agrin polyclonal antibody was raised against recombinant chick agrin CBA-1 as described in [11]. This antibody is specific for agrin as reported in [25] and cross-reacted with rat agrin. For immunofluorescence analysis, the granule cell cultures were washed with phosphate buffered saline (PBS) and fixed for 10 min at room temperature with PBS, pH 7.4, containing 2% (wt/vol) paraformaldehyde, 5% (wt/vol) sucrose. Cells were permeabilised for 3 min with 0.1% (vol/vol) Triton X-100 in PBS followed by washing in PBS for 3 times. Cells were then incubated with the anti-agrin antibodies diluted 1 in 400 in PBS with 10% fetal calf serum for 3 hour at room temperature. The cells were washed with PBS for 3 times, then they were incubated with fluorescein-5-isothiocyanate (FITC)-conjugated anti-rabbit secondary antibodies (Cappel, NC, USA) diluted 1 in 300 in PBS with 10% fetal calf serum, for 2 hours at room temperature. The cells were washed with PBS and mounted with glycerol. The labeled cells were visualized under Zeiss Axiophot fluorescence microscopy.

For Western blot analysis, protein samples were precipitated by 15% (wt/vol) trichloroacetic acid. Protein pellets were then washed with pure acetone for two times and boiled in gel sample buffer of 0.15 mM Tris-HCl, 6% (wt/vol) SDS, 10% (vol/vol) glycerol, 1% dithiothretiol and 8 M urea. They were then subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [26]. Electrophoreted proteins were transferred on nitrocellulose membrane (MSI, MA, USA) as described in [27]. The membrane was blocked with 2.5% (wt/vol) dry milk containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% (vol/vol) Tween 20, for 1 hour at 37°C, followed by incubation with anti-agrin antibodies diluted 1 in 400. Immunoreactivity was detected by ECL Western Blot System (Amersham, UK) using horseradish peroxidase (HRP)-conjugated secondary antibodies against rabbit IgG, and followed the instructions from the supplier.

2.3. PCR analysis of agrin isoforms expression

Granule cell cultures on 100 mm dishes were washed with PBS for 3 times and scraped in PBS. Cells were then pelleted at 150 × *g* for 2 min. Poly $(A)^+$ RNA was prepared with oligo(dT)-cellulose by micro-FastTrack (Invitrogen, CA, USA). Poly$(A)^+$ RNA was then reversely transcribed by AMV reverse transcriptase (Promega, WI, USA) using oligo (dT) as primer [28]. PCR amplification was performed in a programmable thermal controller (MJ Research Inc., MA, USA) with a pair of primers in the following conditions: 1 min denaturing at 94°C, 1 min annealing at 55°C and 1 min elongation at 72°C repeated for 40 cycles. Each reaction contained 10 mM Tris-HCl, pH 8.30, 50 mM KCl, 2 mM $MgCl_2$, 0.8 mM dNTPs, 0.3 μg of each primer and 2.5 U of *Taq* polymerase. The following oligonucleotides flanking the B region were used for amplification : primer A-1 5491–5512 (TTTGATGGGCGGACGTACATCG) and primer B-1 5560–5578 (GCAACCACTTTGAACTGAG), the numbering of nucleotides is according to [12]. The PCR products were subjected to polyacrylamide gel electrophoresis as described [28]. For subcloning of PCR products, amplified DNAs were directly cloned into TA cloning vector pCR according to the manufacturer's instruction (Invitrogen, CA, USA). The identity of cloned PCR products were confirmed by DNA sequencing using Sequenase 2.0 kit (United States Biochemical Corporation, OI, USA).

2.4. Quantification of AChR aggregation

AChR aggregates were visualized and counted as described [8]. Briefly, cell extracts were added to 5-day-old chick myotube cultures for 16–24 h. Culture media were then removed and AChRs were stained by 10^{-7}M rhodamine-conjugated α-bungarotoxin (Molecular Probes, OR, USA) washed with PBS, fixed in ethanol, mounted in glycerol and observed by Zeiss Axiophot fluorescence microscopy. AChR aggregation were counted under a magnification of 400× with phase and fluorescence optics. The mean number of AChR aggregates per myotube segment per field was determined by counting 20 segments in each of the culture dish. The recombinant chick agrin used for AChR aggregating assay was CBA-1 protein as described in [11].

3. Results

The cerebellar granular cells can be cultured up to 2 weeks, however, all the experiments except the co-culture were performed at day 5 after plating. The identity of these cultured granule cells were confirmed by their morphology including the neurite extension, their size and the phase brightness under the light microscope (Fig. 1A). These cultured cells were positively stained with anti-neurofilament-68K antibodies (Fig. 1B). Routinely, over 90% of the cultured cells were granule cells.

As a first step to demonstrate that cultured granule cells express agrin, anti-agrin polyclonal antibody was used to stain the cells. Fig. 2 shows the granule cells express a protein immunologically similar to agrin as visualized by the fluorescence conjugated secondary antibody. Nearly all granule cells in culture show positive staining; both the cell body and the extending neurites were stained by the polyclonal antibody used (Fig. 2B). This specific staining of the granular cells was blocked by pre-incubating the fixed cells with excess amount of recombinant chick agrin (data not shown).

One distinct band of size over 200 kDa was recognized by the anti-agrin polyclonal antibody (Fig. 3, lanes 2 and 3) in the Western blot analysis. The detected minor bands below 200 kDa (Fig. 3, lane 2 and 3) could represent the degradation products of the agrin protein. To test the secretion of agrin by cultured granule cells, the conditioned media depriving serum and chick embryo extracts, after cultured for 2 days, were collected for immunoblotting analysis. These conditioned media, including cultured chick myotubes, granule cells and their co-cultures, gave a band of size over 200 kDa that was

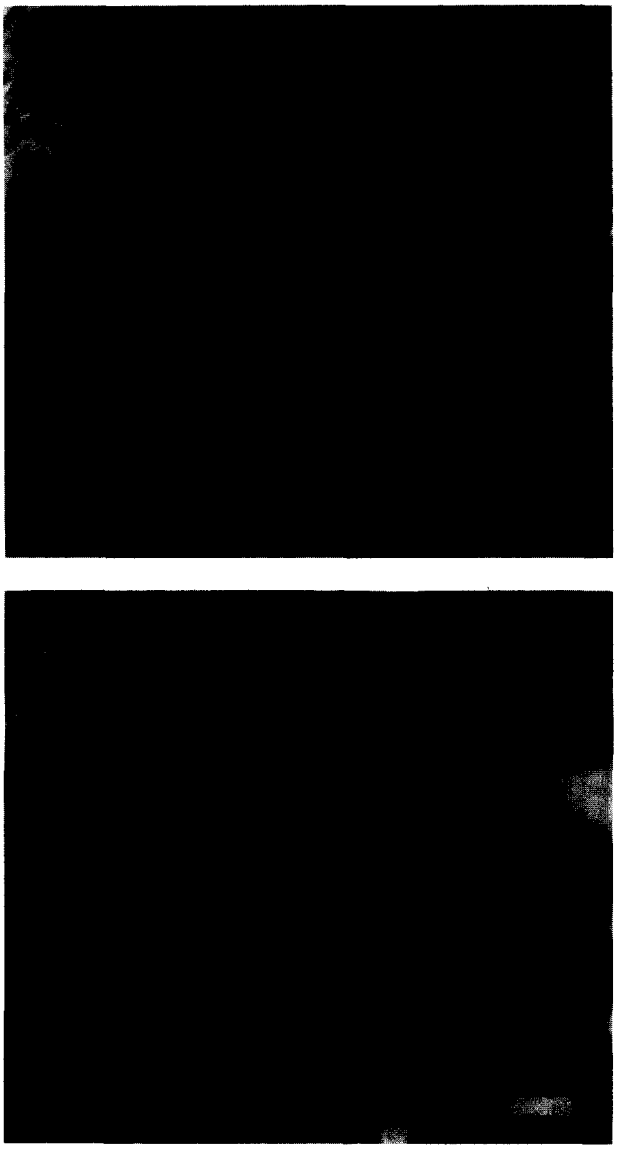

Fig. 1. In vitro culture of granule cells from rat cerebellum. (A) phase-contrast micrograph of 3-day-old culture; (B) fluorescent staining of granule cells recognized by anti-neurofilament antibody. (A) and (B) are the same field. Bar, 20 μm.

similar to the lysates of granule cell (Fig. 3, lane 4, 5 and 6). The ~190 kDa degraded product of agrin detected in the conditioned medium of cultured granule cells (Fig. 3, lane 5) could be due to a specific degradation after 5 days of culture. Moreover, these secreted agrin are not released from dead cells in culture, because the number of cells, either granule cell or myotubes, is not changed significantly through out the period of culture. In this study, the intensity of the recognized agrin band is not quantitative since the amount of protein loaded per sample is different. The protein band recognized in the conditioned medium of granule cell-myotube co-cultures (Fig. 3, lane 6) represents the secreted agrin from both myotubes and granule cells. The ratio of agrin contributing from both parties (myotube and neuron) is not known; it is not able to determine whether the agrin secretion in the neurons could be increased by co-culturing with muscle cells. In addition, the specificity of this 200 kDa protein as agrin was confirmed by blocking the recognition of the antibody by pre-incubating excess amount of recombinant chick agrin (Fig. 3, lane 7).

The poly $(A)^+$ RNA, isolated from 5-day-old cultured granule cells, was reversely transcribed and then underwent the PCR analysis using specific oligonucleotides A-1 and B-1 flanking the B region of agrin (see section 2). The PCR products were separated on a polyacrylamide gel and a single band of 88 base pairs, corresponding to B_0 isoform of agrin, was found (Fig. 4, lane 1); no other bands were detected even after overloading of the samples. A prolonged culture of granule cells did not change the specific expression of B_0 isoform of agrin (Fig. 4, lane 2). The identity of the 88 base pairs PCR product was confirmed as part of the rat agrin sequence by DNA sequencing.

Granule cells were placed on a 4-day-old cultured chick myotubes; these granule cells, after 3 days of co-culture, were found to keep close contact with the myotubes (Fig. 5A). How-

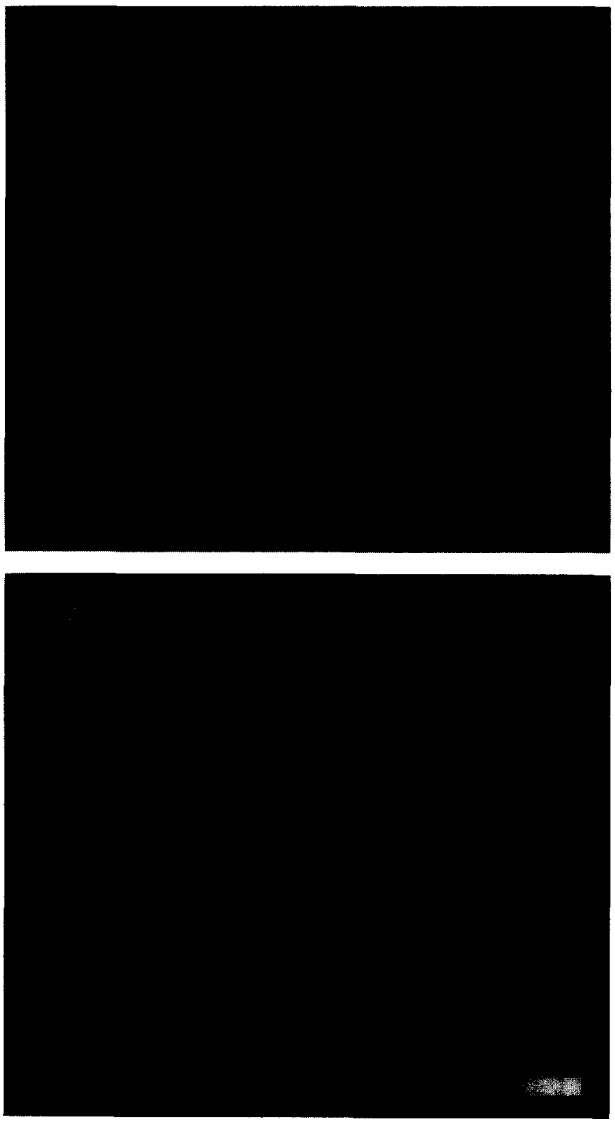

Fig. 2. Immunofluorescence staining of cultured granule cells by anti-agrin polyclonal antibody. (A) phase-contrast micrograph of granule cells; (B) fluorescent staining of granule cells recognized by anti-agrin polyclonal antibody. (A) and (B) are the same field. Bar, 10 μm.

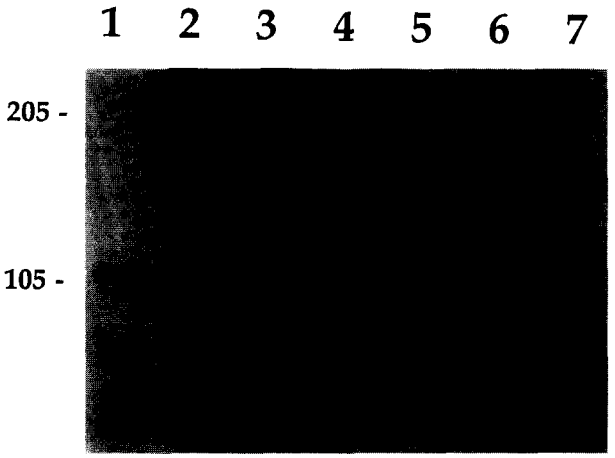

Fig. 3. Western blot analysis of agrin expressed by cultured granule cells. Proteins were loaded onto a 7.5% acrylamide gel and then transferred onto a nitrocellulose paper for the recognition by anti-agrin polyclonal antibody. Lane 1: a positive control of the conditional medium of transfected fibroblast with truncated agrin cDNA, ARP-2, as described in [16]. Lane 2: protein lysate (~2 μg) from 3-day-old cultured granule cells. Lane 3: same as Lane 2 but with double amount of protein. Lane 4: conditioned medium of 7-day-old chick myotube cultures in serum free medium. Lane 5: conditioned medium of 5-day-old cultured granule cells in serum free medium. Lane 6: conditioned medium of co-culture of granule cells and chick myotubes in serum free medium for 4 days. Lane 7: the same amount of protein lysate from cultured granule cells as in Lane 3, however, the anti-agrin antibody was pre-incubated with excess amount of recombinant chick agrin. Molecular weight markers in kDa are shown on the left.

ever, the addition of granule cells were not capable of inducing the aggregation of AChRs on the surface of the myotubes (Fig. 5B), neither did the protein lysate isolated from the cultured granule cells when it was added onto the myotubes (Fig. 5C). To ensure that the chick myotubes were responsive to agrin and that there was no inhibiting agents in the co-cultures, recombinant chick agrin was added to the granule cell-myotube co-cultures. The AChR aggregates were being induced in the presence of recombinant agrin (Fig. 5D). The potency of agrin (5 U) in AChR aggregating activity was not affected in the presence of granule cells or its protein lysate (Fig. 6). Similar results were obtained when a smaller amount of agrin was being used (~1 U) in such inhibition assay. This evidence clearly demonstrated that there was undetectable amount of inhibitory effect by the granular neurons on the activity of agrin.

4. Discussion

Our study provides the first evidence that the primary culture of granular neurons from rat cerebellum express a specific B_0 isoform of agrin. Western blot analysis of the cell lysate of cultured granule cells showed a major band of molecular size around 200 kDa; this size of agrin is consistent with the predicted size of full-length rat agrin cDNA and with the reported size of agrin from the crude protein extract of rat tissues [12]. These staining were specific for agrin as they were extensively blocked when the anti-agrin antibody was pre-incubated with recombinant chick agrin. Other faint bands might represent their degraded products which were commonly encountered in agrin preparation [19,29]. Recent studies have provided evidence that agrin is a major heparan sulfate proteoglycan and shows a size of over 400 kDa from the extracts of embryonic chick brain and vitreous humor in Western blot analysis [25]. This difference of agrin molecular size, as compared to our study, could be due to the cells in culture that may have a different way of post-translational modification of agrin.

When cultured granule cells were stained with anti-agrin antibodies, both cell body and extending neurites showed immunoflourescent staining. The staining of extending neurites indicate that agrin is expressed in the cell body and being transported to the neurites. This was also found in motor neurons of the spinal cord, where agrin-like molecules are synthesized in the cell body and being transported in an anterograde direction [6,7].

Alternative RNA splicing at the B region generates four distinct agrin mRNA isoforms, B_0, B_8, B_{11} and B_{19}, with their level of mRNA expression developmentally regulated. These changes had been reported in chick ciliary ganglion either in vivo or in vitro [18,30], in chick motor neuron at the ventral horn [31], in rat brain and spinal cord [17]. The cultured granule cells expressed predominantly B_0 isoform of agrin; we do not eliminate the possible changes of agrin isoforms due to a longer time of culture either alone or co-culture with myotubes. When we analyzed the agrin isoforms expression from the cerebella of 7-day-old pups, the stage where the granule cells were isolated for cultures, the B_0 isoform of agrin was the only transcript being detected (So et al., unpublished results). Recently, agrin isoform B_0 had been detected by PCR in the cerebellum of adult rat and made up to nearly 60% of total agrin isoforms. Besides, B_0, B_8 and B_{19} but not B_{11} were also expressed [20]. Thus, it is expected that the level of different isoforms of agrin is developmentally regulated in the cerebellum, but so far, there is no direct evidence for which cell types in cerebellum undergoing these changes. This raises the intriguing possibility that these active agrin isoforms (B_8 and B_{19}) were specifically expressed in other neurons especially Purkinje cells because in situ hybridization with agrin showed intense labeling in Purkinje

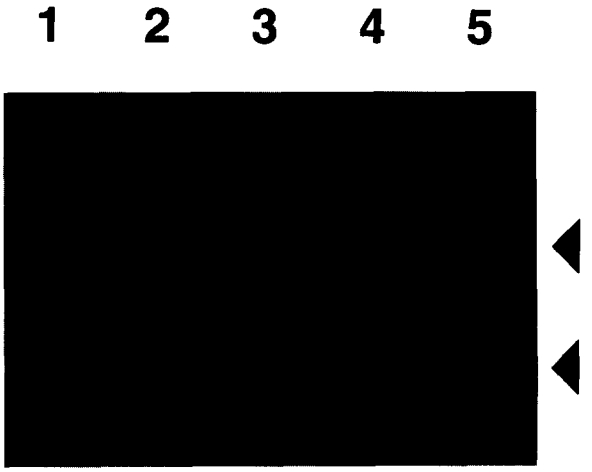

Fig. 4. Cultured granule cells express B_0 isofroms of agrin. Poly $(A)^+$ RNA was reversed transcribed and underwent PCR by using a pair of primers flanking the B insertion of agrin. The PCR product was separated by 9% acrylamide gel. Lane 1: B_0 isoform of agrin expressed by 5-day-old cultured granule cell. Lane 2: B_0 isofrom of agrin expressed by 14-day-old cultured granule cell. Lane 3 and 4 are the positive control of B_{11} and B_0 isoforms of agrin. Lane 5: markers of fX174 DNA/Hae III. Arrowheads indicate the size markers of 72 bp and 118 bp.

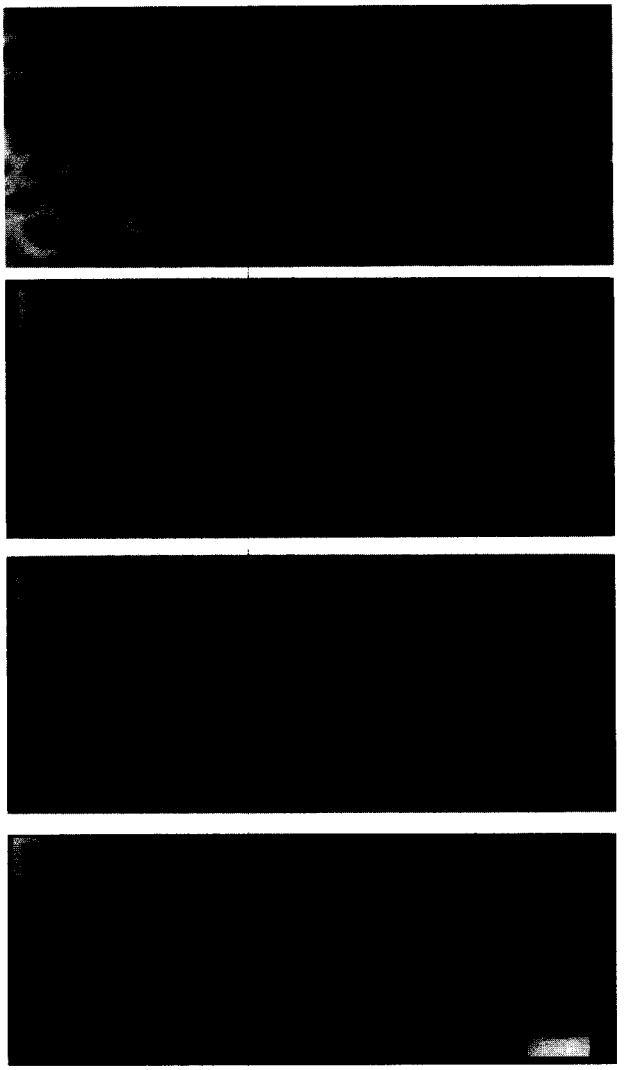

Fig. 5. The AChR aggregating activity of granular neurons when co-cultured with chick myotubes. About 10^5 cells/ml of granule cells were placed onto the 4-day-old chick myotube cultures. After 3 days of co-culture, the AChR aggregates were revealed by rhodamine-conjugated α-bungarotoxin. (A) phase-contrast of co-cultures of granule cells and myotubes. (B) same field as in (A) but viewed with a rhodamine filter after labeled with rhodamine-conjugated α-bungarotoxin. (C) AChR aggregating activity of protein lysate from granule cells on the chick myotubes cultures. (D) AChR aggregates were induced by recombinant chick agrin on the granule cell-myotube cultures. Bar, 20 μm.

cell layer in both chick and rat cerebellum [20,21]. It remains to be determined that this labeling comes from B_8 or B_{19} agrin isoforms.

The co-culturing of granule cells with primary chick myotubes did not cause any increase in the aggregation of AChR on the surface of myotubes. Neither did the addition of cell lysate from cultured granule cells even if the protein lysate content was increased to about 1 mg per dish. The lack of AChR aggregation in our co-culturing system probably does not relate to the fact that the cultured granule cells are not able to secrete agrin to the media or any extensive degradation of agrin. When the conditioned media of co-cultured cells were immunoblotted with anti-agrin antibodies, they showed one major band corresponding to that of agrin. The failure of agrin from granule cells to induce the AChR aggregates might be due to the low AChR aggregating activity of the B_0 isoform of agrin. In transfection studies of rat agrin, Ferns et al. [32] reported that agrin isoforms with B insert of 8, 11 or 19 amino acids were more active in its AChR aggregating activity than with no amino acids insertion (B_0) when the transfected cells were co-cultured with C2 myotubes. The conditioned medium of the transfected cell with B_0 isoform of agrin cDNA showed a 2-fold increase in the number of AChR aggregates, however, the B_8 isoform of agrin was 1000-fold more active than B_0 [32]. Thus, the agrin activity of the B_0 isoform secreted from the cultured granule cells could be too little to be observed, even when we added the cell lysate at a very high concentration. Although we can not exclude the presence of other cells (e.g. fibroblast, Golgi and Purkinje neurons) in our granular cell cultures, they comprised less than 10% of total cells, thus, their influence on our interpretation here is minimal.

The distribution of agrin mRNAs in the brain and its developmental regulation of its isoforms raise some questions about the possible function of agrin in the organization of a neuron–neuron synapse in the central nervous system as it is demonstrated at the developing neuromuscular junctions. Although the agrin expressed in cultured granule cells is not able to induce the AChR aggregates on the surface of myotubes, it could be able to cause the aggregation of others neurotransmitter receptors in the postsynaptic membrane of a neuron–neuron synapse. Another possible function of agrin could be involved in neurite outgrowth or recognition as discussed previously [21]. This proposal is very attractive, in particular, the granular neurons could express predominantly the B_0 isoform of agrin which has very low AChR aggregating activity on the cultured myotubes. Besides, the AChR aggregating activity of agrin only required about 50% of its encoded protein [11,16]. The N-terminal end of agrin, which includes the follistatin-like domain, laminin III-like domain and serine/threonine rich regions

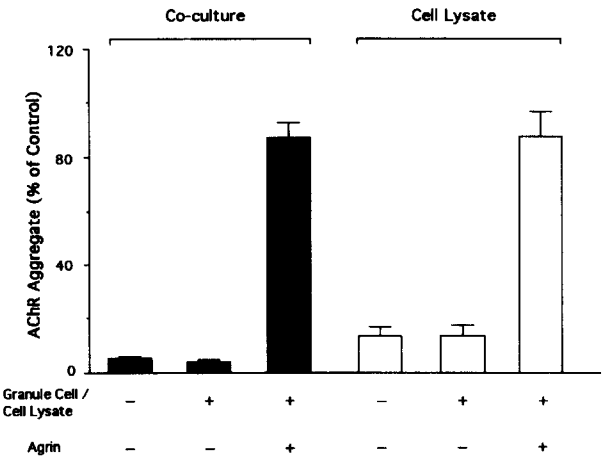

Fig. 6. Lack of inhibitory agents from granule cells on the AChR aggregating activity of agrin. Inhibitory activity was tested both in co-culture and cell lysate of granule cells on the cultured myotubes. AChR aggregates were revealed by rhodamine-conjugated α-bungarotoxin. Blank is the chick myotubes alone. The granule cells (~10^5 cells/ml) were co-cultured for 3 days with myotubes, and the granule cell lysate (1 mg) was added onto myotubes for overnight before assay. Recombinant chick agrin (~ 5 U) was added, either co-cultures or together with granule cell lysate, on myotube cultures. Values are expressed as the percentage of AChR aggregates corresponding to the maximum control of agrin-induced aggregates on myotube cultures, mean ± S.E.M., $N = 3$.

[11,12], is not required for the known agrin function. This N-terminal end could play a role in neurite outgrowth, cell recognition or for binding of growth factors [33]. In addition, the recombinant agrin was shown to be a potent inhibitor of the proteases trypsin, chymotrypsin and plasmin but not thrombin or the plasminogen activators [34]. Thus, it was proposed that agrin isoforms may play a role in the regulation of proteolysis in the extracellular matrix. This diversity of agrin functions, either in the nervous system or other tissues, remains to be determined.

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