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Journal

Biochimica et biophysica acta, 392(1)

ISSN

0006-3002

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Publication Date

1975-05-01

DOI

10.1016/0304-4165(75)90167-1

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BBA 27642

AXOPLASMIC TRANSPORT OF A BRAIN-SPECIFIC SOLUBLE PROTEIN*

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(Received October 28th, 1974)

Summary

The rate and extent of axoplasmic transport of the brain-specific soluble protein (14-3-2 protein) has been investigated in the avian visual system. 1-day-old chicks were injected monocularly with tritiated proline. Incorporation of the isotope into the 14-3-2 protein synthesized within the retina of the injected eye, as well as the appearance of the labeled protein in the optic lobes was determined at 6 h and 6 days. These time periods were chosen to distinguish between the rapid and slow phases of axoplasmic flow. Following preparation of high-speed supernatant fractions, dialysis, chromatography on Sephadex G-150 and immunoprecipitation with specific antiserum, identification of the labeled 14-3-2 protein was carried out by sodium dodecylsulfate-polyacrylamide gel analysis of the radioactive immunoprecipitates. 6 days after isotope administration, approx. 8% of the 14-3-2 protein synthesized in the chick retina had been transported to the contralateral optic lobe. By contrast, at 6 h no labeled 14-3-2 protein was detectable. Thus, transport of this neuronal protein appears to be a relatively slow process with little or no rapid component.

Introduction

It has been well established that a variety of materials are transported along the axon from the primary synthetic site in the nerve cell body to the terminals, as first proposed by Weiss and Hiscoe [1]. The studies of axoplasmic transport have shown that there are two widely differing rates of transport separated by several orders of magnitude [2–5]. Proteins in the fast component are primarily associated with particles whereas those of the slow compo-

* A preliminary report of this work has appeared, (1974) Fed. Proc. 33, 1410.

nent are mainly soluble [6–10]. Recent studies have indicated that the rapid phases of axoplasmic transport may contribute to the formation or maintenance of normal synaptic vesicles and might also provide a communication mechanism linking events in the cell body and synaptic endings [11–14].

Studies in our laboratory are concerned with factors regulating the synthesis of specific brain proteins as well as their functional characterization. One of the nervous system proteins currently being investigated is the 14-3-2 protein originally isolated by Moore and McGregor [15] from beef brain and, more recently, by Bennett and Edelman [16,17] from rat brain. This protein is a soluble highly acidic protein which has been found to be nervous system-specific, species non-specific [18]. At the present time, the function of the 14-3-2 protein is unknown, although it has been shown to be primarily of neuronal localization [19,20] and to be correlated with functional rather than morphological development [21,22]. Since previous studies from our laboratory had demonstrated that the 14-3-2 protein was synthesized primarily on free rather than membrane-bound polysomes of rat cerebral cortex [23], we posed the question of transport of this protein from the site of synthesis in the neuronal perikaryon to the axon terminals. In other tissues, such as liver and pancreas, proteins destined for export are synthesized by membrane-bound polysomes [24–26]. Thus, an analogous situation in the nervous system might be the rapid axoplasmic transport of proteins synthesized on membrane-bound polysomes. If transport of the 14-3-2 protein were found to occur, a slow rate of flow might be expected since this protein is synthesized on free polysomes.

The chick optic system was chosen for our investigation of the 14-3-2 protein since the retinal ganglion cells provide a good experimental preparation for axonal flow studies [8, 27–30] particularly in those birds whose optic fibers completely cross to the contralateral optic tectum of the brain, allowing the ipsilateral tectum to be used as an internal control for background isotope incorporation. Thus, any excess of radioactivity in the contralateral optic lobe, following injection of radioisotope into a single chick eye, can be attributed to axoplasmic transport processes.

Methods

Total soluble protein as well as 14-3-2 protein present in the optic lobe contralateral to the injected eye, relative to that remaining within the retina, were determined in 1-day-old chicks at 6 h and 6 days after isotope administration. 24 chicks were each injected intraocularly with 10 μ l (0.33 mCi) of an aqueous solution of [3 H]proline (40 Ci/mmol) obtained from New England Nuclear Corp. (Boston, Mass.). Tritiated proline was used as a precursor since it has been found to produce more efficient labeling of the transported protein in both the goldfish and the chick optic systems [31,32], with relatively little incorporation into the brain as a whole. Furthermore, the 14-3-2 protein contains 17 residues of proline [33] so that reasonable amounts of isotope incorporation into newly synthesized 14-3-2 protein would be expected. The chicks were lightly anesthetized with halothane and the injection was made into the left eye by means of a 0.5 inch, 28-gauge hypodermic needle. One-half of the chicks were sacrificed at 6 h and the balance at 6 days after isotope administra-

tion. The time periods of 6 h and 6 days were chosen to differentiate between rapid and slow axoplasmic flow [31,34]. After decapitation, the optic lobes from each chick were dissected out on ice and the injected eye was removed. Retinae were peeled from the posterior inner layer of the eye at room temperature since chilling the eye caused the retinae to adhere firmly to the tapetum. The respective tissues from three chicks were pooled for homogenization in 2.5 ml of 10 mM Tris · HCl (pH 7.0). Thus, four samples of the three tissues were obtained at the 6-h and 6-day periods for a total of 24 samples. This procedure was used in order to minimize individual variation as well as to obtain a sufficient number of samples for statistical analysis.

After homogenization, the samples were centrifuged at $100\,000 \times g$, 4°C for 1 h. The supernatant fractions were removed and lyophilized, followed by storage at -20°C prior to determination of the distribution of the labeled 14-3-2 protein as well as total soluble protein.

Identification of the 14-3-2 protein in retinae and optic lobes

The lyophilized chick samples were dissolved in 2 ml of 10 mM Tris/phosphate buffer (pH 7.2) and dialyzed against four changes of the same buffer containing unlabeled proline, followed by dialysis against three changes of distilled water. After dialysis, 20- μl aliquots were taken for measurement of total radioactivity and 10- μl aliquots for Lowry protein determination [35]. Hot trichloroacetic acid-insoluble radioactivity was measured using the Millipore filter assay method with bovine serum albumin as carrier protein [23]. After aliquots were taken, the samples were lyophilized, dissolved in 0.5 ml of Tris/phosphate (pH 7.2) and subjected to Sephadex G-150 (superfine) chromatography in order to eliminate a large proportion of non-(14-3-2)-labeled material. Radioactive fractions corresponding to the position of elution of pure 14-3-2 protein prepared from rat brain [33] were obtained from columns (0.5 $\times$ 20 cm) equilibrated with 10 mM Tris/phosphate (pH 7.2). Elution was carried out with the same buffer. The pooled column fractions were lyophilized and each sample dissolved in 1 ml of sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. 10- μl aliquots were taken for determination of hot trichloroacetic acid-insoluble radioactivity. 10 μg of pure 14-3-2 protein and 50 μl of specific antiserum (diluted 1 : 5 with the phosphate/NaCl buffer) were added to each sample. The carrier 14-3-2 protein had been isolated and purified from rat brain and antiserum was produced in New Zealand white rabbits [33]. The antiserum to the rat 14-3-2 protein produced a single sharp precipitin band by Ouchterlony double diffusion when tested against the high-speed supernatant fractions from chick retinae and optic lobes as well as rat high-speed supernatant and purified rat 14-3-2 protein. After incubating for 2 h at 37°C , followed by 48 h at 4°C , the resulting immunoprecipitates were collected by centrifugation at $10\,000 \times g$ and 4°C for 45 min. The precipitates were washed three times with cold 10 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl and lyophilized.

Gel electrophoresis

The method of Weber and Osborn [36] was used for electrophoretic separation on 0.1% sodium dodecylsulfate-polyacrylamide gels (0.6 cm internal

diameter $\times$ 10 cm). Before electrophoresis, samples were dissolved in a solution containing 0.1 M sodium phosphate buffer (pH 7.2), 1% sodium dodecylsulfate and 1% mercaptoethanol and heated for 1 h at 45°C [37]. Gels were run for 5 h at 8 mA per gel and then sliced and counted for radioactivity after Protosol treatment [38]. Control gels containing pure 14-3-2 protein from rat brain were run simultaneously with radioactive gels. The controls were stained with 0.05% Coomassie blue in methanol/glacial acetic acid/water (20 : 7 : 73, v/v), destained, and the position of 14-3-2 protein determined as reference for the radioactive samples.

Calculation of the relative proportion of 14-3-2 migration

The proportion of 14-3-2 protein synthesized in the retina and transported to the optic lobe contralateral to the injected eye at the 6-day time period was determined by application of the formula $R-L/[E + (R-L)] \times 100$. E , R and L are the total amounts of labeled 14-3-2 protein in the left retina, right optic lobe and left optic lobe, respectively [34].

Results

In Table I are shown the data for the specific activity of the total soluble protein at 6 h and 6 days after monocular injection of tritiated proline into the left eye of 1-day-old chicks. Each of the four determinations for each time period consists of a pool of tissues from three chicks. The level of radioactivity in the soluble protein fraction of retinæ was essentially the same at both time periods. The relative proportion of radioactivity within total soluble protein that migrated distally along the axon was calculated for both the 6-h and 6-day values. By subtracting the counts in the left optic lobes (internal controls) from those present in the right optic lobes, the percentages are independent of the uptake of isotope by the brain from the plasma. Thus, the percent of retinally synthesized soluble protein that is present in the contralateral (right lobe) at 6 h is $3.3 \pm 0.60\%$ and $16.2 \pm 2.2\%$ after 6 days. These values agree well with

TABLE I

PROPORTION OF TOTAL SOLUBLE COUNTS IN 14-3-2 PROTEIN AFTER MONOCULAR INJECTION OF [^3H]PROLINE INTO THE LEFT EYE OF 1-DAY CHICKS

Data are presented as mean $\pm$ S.E. for four samples, each a pool of three chicks.

Source	Time	cpm/mg		Percent of total counts in 14-3-2 protein
		Total soluble protein	14-3-2 protein*	
Left retina	6 h	605 641 $\pm$ 154 216 (4)	5 728 $\pm$ 1080 (4)	0.97 $\pm$ 0.09 (4)
Left retina	6 days	614 053 $\pm$ 120 562 (4)	19 564 $\pm$ 2924 (4)	3.45 $\pm$ 1.36 (4)
Right lobe	6 h	24 204 $\pm$ 4 722 (4)	N.D. (4)	—
Left lobe (control)	6 h	4 669 $\pm$ 478 (4)	N.D. (4)	—
Right lobe	6 days	122 722 $\pm$ 6 210 (4)	1 757 $\pm$ 242 (4)	1.42 $\pm$ 0.15 (4)
Left lobe (control)	6 days	2 809 $\pm$ 492 (4)	47 $\pm$ 7 (4)	1.73 $\pm$ 0.26 (4)

* Refers to counts in 14-3-2 region on sodium dodecylsulfate-polyacrylamide gels of immunoprecipitates per mg of total soluble protein.

N.D., no detectable counts above background on sodium dodecylsulfate-polyacrylamide gels.

data reported in other studies of the axoplasmic flow [34,39]. However, these percentages are subject to upward revision since it is not known what proportion of proteins elaborated from radioactive precursors injected into the eye are synthesized in the retinal ganglion cell bodies rather than other retinal cell types [40,41]. Lasek et al. [42] have recently reported that proteins synthesized in glial cells can be transported into the axon. Thus, it is possible, particularly when considering total soluble protein, that some of the radioactivity found in the retinal soluble protein fractions consists of material synthesized by glial cells. Although the values shown in Table I are minimum values, based on the assumption that all incorporation in the retina is into ganglion cells, it is clear that the major fraction of the total soluble protein synthesized in the nerve cell body is transported along the axon at a slow rate.

After 6 days of labeling, approx. 3% of the radioactivity of the soluble protein of the retina is present in the 14-3-2 protein (Table I). The percentage of label in the 14-3-2 protein in the retina at 6 h is significantly below the corresponding 6-day value (0.97 vs 3.45%). One possible explanation for this is that the 14-3-2 protein is more stable than the average retinal protein. Thus, its concentration in surviving protein counts gradually rises. The 14-3-2 protein may have a long half-life similar to the 16 days reported for another brain-specific soluble protein, the S100 protein [43].

Although the percent of total soluble counts in the 14-3-2 protein is about the same (Table I) for right and left (control) optic lobes, the radioactive 14-3-2 protein found in the optic lobe contralateral to the injected eye must

Fig. 1. Sodium dodecylsulfate-polyacrylamide gel isolation of labeled 14-3-2 immunoprecipitates of extracts of retina and contralateral (right) optic lobe at 6 h after monocular injection of [³H]proline into the left eye of 1-day-old chicks. Procedures for preparation of samples, precipitation with antiserum to 14-3-2 protein and subsequent sodium dodecylsulfate-polyacrylamide gel electrophoresis are described in Methods. ●—●, the pattern of labeling in the left retina of the injected eye; ○—○, the right lobe. The extracts of retina and right lobe, following Sephadex G-150 chromatography, which were used for immunoprecipitation contained 34020 and 9650 counts, respectively.

Fig. 2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis patterns of the labeled 14-3-2 immunoprecipitates from extracts of retina and contralateral (right lobe) 6 days after injection of [³H]proline into the left eye of 1-day-old chicks. Procedures are described in Methods. ●—● the retina; ○—○ the right lobe. The extracts of retina and right lobe, after Sephadex G-150 chromatography, which were used for immunoprecipitation contained 93350 and 59200 counts, respectively.

represent axonal transport and not local synthesis since labeled amino acids *per se* are not transported along the axon [9,31,39]. Thus, it is evident from the data presented in Table I that the 14-3-2 is indeed transported from its site of synthesis in the retinal ganglion cells to the contralateral (right) lobe, but at a slow rate. No radioactivity could be detected in the right lobe at the 6-h time period (Table I, Fig. 1), whereas a high level of radioactivity was present in the 14-3-2 protein in the right optic lobe 6 days after injection of tritiated proline into the left eye (Table I, Fig. 2). The control (left optic lobe) contained only one-fortieth the counts of the right lobe at the 6-day time period. The data for the radioactivity present in the 14-3-2 protein in the retinae and optic lobes are presented in Table I as specific activity (cpm per mg of total soluble protein) in order to standardize the data for statistical analysis. However, the actual radioactivity of the 14-3-2 region on the sodium dodecylsulfate-polyacrylamide gels was much higher, as can be seen in the typical gel patterns of Figs 1 and 2. Furthermore, the level of radioactivity in each of the 6-h soluble fractions from the right lobe which was used for immunoprecipitation was sufficiently high (average 9000 counts) to have permitted the detection of any rapid migration of the 14-3-2 protein.

Application of the formula in Methods for the relative proportion of radioactivity within the 14-3-2 protein that migrated distally along the axon was estimated to be 8%. This value represents the average of four samples, each a pool of three chicks. However, this calculation is an approximation since only two time points are known.

Discussion

Although microtubule protein, a soluble protein in nerve, has been shown to be transported from the nerve cell body by slow axoplasmic flow [7,9,44], this protein is not specific to nervous tissue and is found in a wide variety of other cell types [44-47]. Thus, the present report presents the first evidence for axoplasmic transport of not merely a specific macromolecule, but of a soluble protein which is unique to nervous tissue. Furthermore, since the 14-3-2 protein is made primarily on free polysomes [23] and travels by slow axonal flow, it is possible that proteins transported rapidly along the axon are synthesized primarily on membrane-bound polysomes. Since the levels of the 14-3-2 protein in nervous tissue appear to be correlated with functional rather than morphological development [48], it would be possible, with present techniques, to determine whether axoplasmic flow begins at the time of appearance of the 14-3-2 protein in the nerve cell body.

The fact that the axoplasmic transport of the 14-3-2 protein is a slow process indicates that it is unlikely to be involved in synaptic transmission *per se* which is a very rapid process. However, recent studies in our laboratory [33] on physical and chemical characteristics of the 14-3-2 protein have shown that it is composed of two identical subunits and that it can undergo conformational alterations which may play a role in its function in the nervous system. Thus, one can speculate that the 14-3-2 protein may be involved in slowly developing biological processes which are considered to participate in long-term memory storage [49].

Acknowledgements

The authors express their appreciation to Dr Blake Moore, Washington University, St. Louis, Missouri for samples of beef brain 14-3-2 protein and antiserum used as reference in preparing these materials from rat brain.

S.C.B. is recipient of N.I.H. Research Career Development award NS 49945 and acknowledges support by N.I.H. Grant NS09603.

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