A CALMODULIN-RELATED POLYPEPTIDE RAPIDLY MIGRATES WITHIN THE MAMMALIAN NERVE

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ABSTRACT

After injection of L-[³⁵S]methionine into the guinea pig dorsal motor nuclei, labelled proteins conveyed down the vagal nerve by the fast axoplasmic transport were analysed using one- and two-dimensional gel electrophoreses coupled with fluorography. Two polypeptides with the molecular weights of 26,000 (band A) and 21,000 (band B) were the distinct components of the fast transport in this system. Upon two-dimensional gel electrophoresis, band A gave a single spot with pI at 4.3, while band B remained in the neutral region.

Comparison of methionine-containing peptides of band A and bovine brain calmodulin by tryptic peptide mapping revealed that band A contained eight peptides virtually identical to those found in calmodulin. In addition to these peptides, band A yielded three extra peptides that were not found in calmodulin. It is therefore concluded that band A is a polypeptide larger than but closely related to calmodulin. The possible functional significance of band A either as a calcium-binding protein homologous to calmodulin or as a transit form of the calmodulin molecule is discussed.

KEY WORDS fast axonal transport / calmodulin-related polypeptide / vagal nerve / transit form / peptide map / calcium

In contrast to the slow axoplasmic transport which has been shown to consist mainly of the flow of cytoskeletal proteins (16, 25, 29), identification of protein components in the fast transport has been hampered by a relative complexity of molecular species (24, 27, 34), and also by a much lower radioactive labelling. In the vagal nerve, however, incorporation of radioactive methionine into the fast transport is comparable with or even higher than that into the slow transport. In addition, the protein composition of the fast transport is found to be rather simple in the vagal nerve (*vide infra*), probably due to the relative homogeneity of this system with respect to the extent of myelination and axon diameter.

We have analysed the fast axoplasmic transport in the vagal nerve by one- and two-dimensional gel electrophoreses coupled with fluorography. Of the two major components ob-

served, one was shown to be chemically related to brain calmodulin. This protein with the molecular weight of 26,000 may be another member of a group of calcium-binding proteins, or may play a role as a specialised transport form of calmodulin.

MATERIALS AND METHODS

Axoplasmic Transport in the Vagal Nerve

Guinea pigs weighing 250-300 g were given intraperitoneal injections of atropine (0.05 mg/kg) and chlorpromazine (10 mg/kg), followed by sodium pentobarbitone (28 mg/kg). Chlorpromazine was effective in reducing the time required to attain deep anaesthesia to about 10 min. Using a stereotaxic apparatus, the medulla oblongata was exposed in a horizontal position, and 0.25 μ l of L-[³⁵S]methionine (40-50 μ Ci)

was injected via a glass capillary into the dorsal motor nucleus of the vagus on each side.

At prescribed time intervals after injection, animals were killed with an overdose of pento-barbitone (60 mg/kg). The vagal nerve between the cranium and the diaphragm was dissected out, freed of extraneous tissues, and cut into 6 mm consecutive segments on an ice-cold plastic plate. Segments were kept frozen at -80°C until processed as below.

Each segment was ground in a small teflon-glass homogeniser in 200 μl of the sample buffer, which contained 2% sodium dodecyl sulphate (SDS), 80 mM Tris, 5 mM β -mercaptoethanol, and 10% glycerol, pH 6.8. The suspension was heated at 100°C for 3 min and again homogenised.

For measurement of radioactivity, a 50 μl portion of the homogenate was digested with 0.2 ml of Soluene-350 (Packard Instrument Co.) with heating at 50°C for 2 hr, and counted in a Packard Tricarb 3330 scintillation spectrometer after the addition of 3 ml of toluene-based scintillation fluid.

SDS-Polyacrylamide Gel Electrophoresis

Aliquots of the nerve homogenate were subjected to electrophoresis (37) in 10% or 15% polyacrylamide slab gels, 2 mm thick. Electrophoresis was carried out at 15 mA for 1 hr, then at 25–30 mA for 5 hr. Gels were stained with Coomassie Brilliant Blue R-250 (CBB). In some cases, gels were further processed for fluorography as described below.

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was carried out according to O'Farrell (33), using samples dissolved in SDS (3). Isoelectric focusing (first dimension) was performed at 400 V for 16 hr, and SDS-gel electrophoresis in a 10% or 15% gel (second dimension) as described above. The gel was first stained with CBB and further processed for fluorography.

Isolation of Bands A and B for Peptide Mapping

The labelled homogenate was electrophoresed in a 15% slab gel, and 3 mm-wide strips, with Rf 0.68 (band A) and 0.80 (band B), were cut out from the unstained gel kept on ice. The remaining portion of the gel was stained with CBB, and further fluorographed to ensure that bands A

and B had been cut off correctly.

The gel strips were macerated in a glass to glass microhomogeniser (Radnoti Glass Technology, Inc.) and shaken overnight in 0.05% SDS at 37°C . The suspension was filtered and the residue was shaken for another 3 hr in 0.05% SDS at 37°C and filtered. The combined filtrate was dialysed against distilled water, freeze-dried, and dissolved in 1 ml of distilled water. A 50 μl portion was taken for measurement of radioactivity. The remainder was dialysed against distilled water, then against 0.01 M NH_4HCO_3 , and freeze-dried.

Tryptic Peptide Mapping

Bovine brain calmodulin (1.5 mg), a mixture of calmodulin (1.5 mg) and freeze-dried band A (5,000–6,000 cpm), and freeze-dried band B, were separately dissolved in 3.75 ml of 0.1 M NH_4HCO_3 (pH 8). Trypsin treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone (30 μg ; Worthington Biochemical Corporation) was added to each solution, and digestion was carried out overnight at 37°C .

The digest was dissolved in 30 μl of pyridine: acetic acid: water (1:10:300), pH 3.5 (electrophoresis buffer). A portion, about 8 μl , was spotted on a glass plate coated with cellulose powder (10 $\times$ 10 cm; Avicel; Funakoshi Yaku-hin Co.), sprayed with the same buffer, and subjected to electrophoresis at 5 mA/plate for 40 min. In the second dimension, the plate was subjected to ascending chromatography in *n*-butanol: pyridine: acetic acid: water (15:10:3:12) for 1.5–2 hr. Peptides were stained with ninhydrin. In some cases, dried plates were further processed for fluorography.

Fluorography

Gels were processed according to Bonner and Laskey (6). After being impregnated with 2,5-diphenyloxazole (PPO), gels were dried and exposed at -80°C to RP Royal X-Omat X-ray film (Eastman Kodak Co.), with a brief flashing (26) and with a Cronex intensifying screen (Lightning-Plus; Du Pont Instruments).

In case of thin layer plates for peptide mapping, plates were immersed in 16% PPO in acetone, dried and exposed to X-ray films as above.

Materials

Calmodulin was isolated from the bovine brain

Fig. 1 Fluorographic visualisation of polypeptides migrating in the guinea pig vagal nerve after their resolution by SDS-gel electrophoresis. Nerve segments were obtained 3 hr (a and c) and 7 days (b) after labelling the vagal nuclei with L-[^{35}S]methionine, and processed for electrophoresis and fluorography. Acrylamide concentrations of the gel, 10% in (a) and (b), and 15% in (c). A, 26,000-dalton polypeptide. B, 21,000-dalton polypeptide. Distance zero indicates the point of exit of the nerve from the cranium.

as described (18, 20, 21). Purity of the preparation was confirmed by gel electrophoretic analysis (Fig. 3). L-[^{35}S]Methionine (>600 Ci/mmole) was obtained from Radiochemical Centre, Amersham, U.K., and concentrated by evaporation under a reduced pressure.

RESULTS

Components of Fast Axoplasmic Transport in the Vagal Nerve

Fluorographic visualisation of labelled proteins in the vagal nerve 3 hr after the injection of L-[^{35}S]methionine into the dorsal motor nuclei shows a group of polypeptides conveyed by the fast axoplasmic transport (Fig. 1, a and c).

* This polypeptide pattern of the fast transport is completely different from that of the slow transport (Fig. 1b), which is mainly composed of cytoskeletal proteins (actin, α - and β -tubulin). The vagal system is peculiar in that the triplet polypeptides associated with neurofilaments (30, 31) are virtually undetectable by fluorography, in contrast with the sciatic nerve (16, 29). This seems to be due to the fact that vagal axons are mostly unmyelinated and small in their diameter (2), and do not contain as many neurofilaments.

Two polypeptides with the molecular weights of 26,000 (band A) and 21,000 (band B) are the distinct components*. The speed of fast transport in this system is calculated to be 14–18 mm/hr.

Band A is detected only by fluorography but not by CBB-staining (Fig. 3), indicating a high specific labelling of this band. Band B is also very faintly stained with CBB.

Bands A and B were further characterised by two-dimensional electrophoresis followed by fluorography (Fig. 2). Band A gave a single spot with a highly acidic isoelectric point (pI 4.3), which is close to that of brain calmodulin (pI 4.1). In contrast, band B was detected as a streaked spot in the neutral pH region (pI 6.4–6.8).

Homology of Band A with Brain Calmodulin

The highly acidic nature of the band A protein together with its relatively low molecular weight suggested that it might be related to a group of highly acidic proteins with calcium-binding activity (23). Fig. 3 shows the electrophoretic behaviour of band A (columns 5 and 6) and of brain calmodulin (columns 3 and 4) in the pres-

Fig. 2 Fluorographic visualisation of polypeptides rapidly migrating in the guinea pig vagal nerve after their resolution by two-dimensional electrophoresis. Nerve segments obtained 3 hr after labelling the vagal nuclei with L-[^{35}S]methionine were processed for two-dimensional electrophoresis. First dimension, isoelectric focusing in the pH range from 7 to 4 (from left to right). Second dimension, electrophoresis in 15% polyacrylamide gel. The position for authentic calmodulin is marked with a dotted circle. A, 26,000-dalton polypeptide with pI at 4.3. B, 21,000-dalton polypeptide with pI at 6.4–6.8.

Fig. 3 Electrophoretic comparison of the axonal band A protein with brain calmodulin. Columns 1–4, stained with Coomassie Brilliant Blue. [1] Nerve segment 3 hr after labelling the vagal nuclei with L-[^{35}S]methionine. The nerve sample was processed in the presence of 1 mM CaCl_2 . [2] The same as column 1, except that the sample was processed in the presence of 1 mM EGTA. [3] Brain calmodulin, in the presence of 1 mM CaCl_2 . [4] Brain calmodulin, in the presence of 1 mM EGTA. [5] Fluorogram of column 1. [6] Fluorogram of column 2.

ence and absence of calcium ions. In agreement with a previous observation (14), calmodulin migrated faster in the presence of calcium than in its absence. In contrast, the electrophoretic mobility of band A was not affected by calcium (Fig. 3, columns 5 and 6).

To examine any sequence homology, tryptic peptide mapping was performed with the radio-labelled band A protein isolated from the SDS-gel, and with authentic calmodulin isolated from the bovine brain. Calmodulin contains 9 methionine residues in the total of 148 amino acid residues (20, 36). This high methionine content makes it feasible to compare the [^{35}S]-labelled and [^{35}S]-unlabelled methionine-peptides on the map.

It was possible to collect the pure band A by cutting out the gel portion at Rf 0.68, because there were no other materials stainable by CBB in this region (Fig. 3, columns 1 and 2), and band A is a single molecular species as evidenced by two-dimensional electrophoresis (Fig. 2). Labelled nerve segments from five animals yielded

a band A sample that contained radioactivity amounting to 5,000–6,000 cpm. It was also possible to obtain a band B sample containing similar amounts of radioactivity.

During tryptic digestion, the volume of the incubation mixture should be increased to lower the SDS concentration. This is essential because only a limited digestion occurs when the SDS concentration is high.

The peptide mapping system for calmodulin using the Whatman 3MM paper (21) has been modified to make it applicable to smaller amounts. For this purpose, a glass plate coated

with cellulose powder (10×10 cm) was used, which enabled the analysis of 100–300 μ g of the protein digest, instead of the 1–2 mg required in the original method (21). Peptides can be resolved more satisfactorily with the cellulose-coated plate than with the filter paper, probably because of their weaker adsorption to cellulose powder.

SDS that remained in the dialysed and freeze-dried sample of band A seems to interfere with the peptide resolution, as streaking proves to be more pronounced with calmodulin plus band A (Fig. 4b) than with calmodulin alone (Fig. 4a).

Fig. 4 Tryptic peptide mapping of the axonal band A protein and authentic brain calmodulin. (a) Calmodulin alone, ninhydrin-stained. (b) Calmodulin plus band A, ninhydrin-stained. (c) Fluorogram of plate b. In (d), plates b and c are schematically superimposed. Filled spots, radioactively labelled and ninhydrin-positive. Open spots, ninhydrin-positive, but radio-unlabelled. Dotted spots, radioactively labelled, but ninhydrin-negative. Filled arrows, peptides containing methionine. Open arrows, peptides devoid of methionine (see reference 20).

Still, careful examination of Figs. 4a and 4b indicates that all the ninhydrin-positive spots generated from the calmodulin-band A mixture are from calmodulin, as is expected from a large excess of calmodulin in the mixture. Band A does not give rise to any additional ninhydrin-positive spots.

Fluorography revealed that tryptic digestion of band A produced eleven [^{35}S]methionine-containing peptides (Fig. 4c; spots 2-6, 12-14 and I-III as schematised in Fig. 4d). Among them, eight peptides had corresponding spots in the ninhydrin-stained map (Fig. 4b; spots 2-6 and 12-14 in Fig. 4d). The remaining three peptides (spots I-III) were detected only in the fluorogram. The fluorographic pattern as well as the ninhydrin-stained pattern were highly reproducible.

Ninhydrin-positive spots which have been shown in a previous study (20) to contain methionine are marked with filled arrows (Fig. 4d). It is evident that the fluorographic pattern agrees well with these spots, except for the two spots seen in the basic region (Fig. 4d; spots 9 and 18). These two spots originate from the sequence of Met-Ala-Arg-Lys-Met-Lys [72-77]. Because this sequence is cleaved to varying extents to yield different peptides (20), indefinite and weak spots are visualised when stained with ninhydrin. Lack of radioactivity in this region (Fig. 4c) may be a consequence of such a variability. Spots devoid of methionine (Fig. 4d, open arrows) do not have their counterparts in the fluorogram.

Tryptic digestion of the band B protein yielded six [^{35}S]methionine-containing peptides, none of which overlapped with the tryptic peptides of band A (data not shown).

DISCUSSION

Calmodulin, first described as a calcium-dependent modulator of brain cyclic nucleotide phosphodiesterase (9, 19), has now been recognised to be a ubiquitous calcium-binding protein possessing a regulatory function in many calcium-dependent systems (10, 28). Since calcium plays a key regulatory role in transmitter release (22) as well as in axoplasmic transport (8), it is essential to know the factors that regulate the calcium function within the axon. So far, calmodulin has not been identified in the axon nor in the presynaptic terminal. Its presence has only been shown in the postsynaptic density by biochemical (14) and immunological (38) techniques. Band A may be a candidate for calmodulin-like activity in the axon. On tryptic

digestion, band A yields three extra methionine-containing peptides that are not found in calmodulin, which explains its larger molecular weight compared with calmodulin. In this context, it is of interest to note that a protein with a molecular weight of 23,000 and with pI at 4.1 has been isolated from a preparation enriched with synaptic plasma membrane (32). This protein was shown to bind calcium and to activate phosphodiesterase.

Calmodulin has considerable sequence homologies to muscle troponin C (11, 20, 36). These two proteins and parvalbumin constitute a group of homologous proteins with a common sequence for the calcium-binding site (11). Band A may be another member of this group uniquely present in the axon.

Fast axoplasmic transport of proteins is considered to be associated with membrane materials (1, 7, 12, 13, 24, 27). Recently, a detailed ultrastructural observation has been made on the membrane components anterogradely and retrogradely migrating within the axon (35). A soluble protein like calmodulin should either be bound to the membrane or sequestered inside the organelles to be transported rapidly. If we take into consideration studies on the synthesis and segregation of secretory proteins and membrane proteins (4, 5) it is probable that proteins destined for transport down the axon may also be segregated as they are synthesised, most likely into membraneous or granular structures. The identification of a polypeptide slightly larger than but closely related to calmodulin as one of the major components of the fast transport points to the possible existence of a specialised transit form of calmodulin associated with membrane structures. To prove this idea, functional characterisation of band A as well as the knowledge of its fate at the terminal is needed.

Transport of radioactive calcium at a rate identical with that of the fast protein transport has been reported (15, 17). Iqbal and Ochs (17) have further investigated the compartmentation of labelled calcium and reported that 66% of radioactive calcium was present in the particulate fraction, while 16% was in the soluble fraction. Within the soluble fraction, they found that radioactive calcium was eluted with a 1,100-dalton peptide, and also with a 15,000-dalton polypeptide. Though the molecular weight of 15,000 was close to that of calmodulin, the exact molecular nature of this protein has not been established. The calmodulin-related protein we have shown in the present study differs from the protein reported by Iqbal and Ochs (17) and seems

to belong to the particulate compartment. The abundance of hydrophobic methionine-containing peptides in the band A protein is in accordance with this interpretation.

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