Intracellular Regulators of Neuronal Sprouting: Calmodulin-binding Proteins of Nerve Growth Cones

CAROLYN HYMAN and KARL H. PFENNINGER

Department of Anatomy and Cell Biology, Columbia University, College of Physicians and Surgeons, New York 10032. Dr. Hyman's present address is Cell Biology Department, New York University, 550 First Avenue, New York, 10016.

ABSTRACT The focus of this study is a quantitative biochemical analysis of the calcium-dependent interactions of calmodulin with a nerve growth cone preparation from fetal rat brain (Pfenninger, K. H., L. Ellis, M. P. Johnson, L. B. Freidman, and S. Somlo, 1983, *Cell* 35:573–584). The presence of calmodulin as an integral component of this preparation is demonstrated, and quantitative binding studies are presented. The binding of ^{125}I-calmodulin to nerve growth cone material is shown to be highly specific, calcium dependent, and saturable at nanomolar calmodulin concentrations. Additionally, the growth cones' binding components appear to be membrane proteins. The individual molecular mass species of growth cone proteins displaying calcium-dependent calmodulin binding are also detailed and presented in comparison with those of synaptosomes. This analysis reveals differences between the calmodulin binding proteins of the growth cone preparation and the synaptosome fraction, suggesting the presence in growth cones of a specialized set of components which may be involved in regulatory mechanisms controlling neuritic sprouting.

During development of the nervous system, neurites cease their mitotic phase of growth and undergo sprouting to form axons and dendrites. The leading edge of the neurite, the nerve growth cone, must follow its appropriate path to the target and synapse with its particular target cell. The basic understanding of neurite sprouting depends upon knowledge of growth factors and their corresponding transducer mechanisms.

The best understood growth factor, nerve growth factor is active on peripheral neurons. It has been purified and characterized (for review see reference 5) and a membrane transducer system has been shown to be necessary for the activation of the cellular processes elicited by it (21). However, the mechanism of action and the nature of the secondary messenger system involved remain obscure. Evidence has been presented (18) suggesting that intracellular calcium ion concentration is involved in the control of the growth cone's chemotactic response to nerve growth factor (cf. reference 32). Additionally, nerve growth factor–activated phospholipid transmethylation has been reported to exist on peripheral nerve growth cones (38, cf. reference 40). This activity may be a component in a transmethylation–phospholipase A_2 transducer system (cf. reference 23) allowing calcium-initiated, localized membrane expansion, most likely through exocytotic insertion of preformed plasmalemmal precursor vesicles into the growth cone's plasma membrane (36).

The effects of nerve growth factor on peripheral neurites are likely to be similar in principle to those of growth-promoting activities from a variety of sources (2, 10, 20, 29, 35) on central nervous system neurons. Our hypothesis predicts that growth cones of all neuron classes contain similar transducer mechanisms mediating the growth and chemotactic responses, although the receptors activating the relevant enzyme cascades may be different. Indeed, the presence of phospholipid methyltransferase activity in central nervous system growth cone particles (GCPs)[1] has already been demonstrated (12). Furthermore, Ca^{2+}-dependent kinases are now known to exist in nerve growth cone material isolated from fetal rat brain (26). Therefore, Ca^{2+}-dependent processes are likely to function in a regulatory manner, and calmodulin is almost certain to be an important co-factor.

Studies of the molecular constituents of central nervous system growth cones have been made possible by the successful isolation of GCPs from fetal rat brain (37). A number of biochemical and immunochemical studies have further estab-

[1] *Abbreviations used in this paper:* GCPs, growth cone particles; HRP, horseradish peroxidase.

lished the identity of this fraction (26, 43). The focus of the present study is the biochemical analysis of the calmodulin-binding properties of growth cone membranes. The goal is the identification of calcium-dependent components that may be involved in the growth-regulatory transducer system of the nerve growth cone. Some of these results have been presented in abstract form (25).

MATERIALS AND METHODS

Materials: HEPES, Tris (as Trizma base), EGTA, bovine serum albumin (BSA), bovine brain calmodulin, and polyvinyl pyrrolidone were obtained from Sigma Chemical Co., St. Louis, MO. *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, and protein standards were purchased from Bio-Rad Laboratories, Rockville Center, NY. Additional protein standards and Ficoll 400 were from Pharmacia Fine Chemicals, Piscataway, NJ. Ultrapure sucrose was obtained through Schwarz/Mann Div., Spring Valley, NY. Ultrapure glycine was obtained from Bethesda Research Laboratories, Gaithersburg, MD. *N*-Succinimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl) propionate (Boulton-Hunter reagent) was obtained from Amersham Corp., Arlington Heights, IL. Rabbit anti-rat calmodulin serum was the generous gift of Dr. Linda Van Eldick, Howard Hughes Medical Institute, Nashville, TN. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Nitrocellulose paper was purchased from Schleicher & Schuell, Keene, NH. Maxidens oil was from Nyegaard & Co., obtained through Accurate Chemical & Scientific Corp., Westbury, NY. All other chemicals were purchased from Fisher Scientific Co., Springfield, NJ.

Preparation of GCPs: A modification of the method described by Pfenninger et al. (37) was used to prepare sealed GCPs purified of contaminating supernatant proteins. Briefly, day 17 fetal rat brains were homogenized in 8 vol of 0.32 M sucrose with 1 mM *N*-tris[Hydroxymethyl]methyl 2-amino-ethanesulfonic acid (TES) and 1 mM $MgCl_2$, pH 7.3, containing the following protease inhibitors: pepstatin A (1 μg/ml), soybean trypsin inhibitor (0.1 mg/ml), leupeptin (30 μM), and phenylmethylsulfonyl fluoride (1 mM). After centrifugation at 1,660 g_{max} for 15 min, a low speed supernatant was layered onto a discontinuous sucrose gradient and centrifuged at 248,000 g_{max} for 40 min at 4°C, in a VTi50 rotor (Beckman Instruments, Inc., Palo Alto, CA). Band A at the 0.32 M sucrose (load)/0.75 M sucrose interface was removed and chromatographed over a column of controlled pore glass 3000 (Electro-Nucleonics Inc., Fairfield, NJ) measuring 98 × 1.5 cm. The elution buffer was 1 mM TES, 1 mM $MgCl_2$, 0.65 M sucrose, pH 7.3. Thirty drop fractions were collected at a flow rate of 1.4 ml/min. The excluded peak was collected and dialyzed vs. 1 liter homogenization buffer for 1 h at 4°C. The dialyzed material was then layered onto a 0.5-ml cushion of Maxidens oil and spun at 100,000 *g* for 90 min at 4°C, in a SW55Ti rotor (Beckman Instruments, Inc.). The interface between the load and cushion was collected in a minimal volume and used immediately.

Preparation of EGTA-treated Membranes From GCPs: GCPs were lysed in 8 vol of a solution of 3 mM Tris, 50 mM EGTA, 0.002% saponin, and protease inhibitors as described above (pH 8.1) on ice for 1 h, with stirring. The lysed particles were then layered over a 0.5-ml cushion of 1.0 M sucrose containing 0.5 mM EDTA, and centrifuged for 1 h at 200,000 *g* in a SW55Ti rotor (Ellis, L., I. Wallis, E. Abreu, and K. H. Pfenninger, submitted for publication). The interface was collected and salt washed for 30 min in 8 vol of a solution of 0.3 M $NaSO_4$, 50 mM EDTA, 0.002% saponin, and protease inhibitors at 4°C, with stirring. The material was then centrifuged for 1 h at 200,000 *g* in a SW55Ti rotor. The resulting membrane pellet was washed with ice cold H_2O to remove EGTA and resuspended in the calmodulin-binding (100 mM HEPES, 0.25 mg/ml gelatin, pH 7.3) or homogenization buffers.

Preparation of Synaptosomes: Synaptosomes were prepared from adult rat brain as described by Cohen et al. (9) with modifications. After differential centrifugation of the homogenate, the material was loaded onto a discontinuous sucrose gradient comprised of a 2.6-M cushion overlayered with 15 ml of 1.2 M sucrose containing 1 mM $NaHCO_3$ and 15 ml of 1.0 M sucrose containing 1 mM $NaHCO_3$. The gradiets were centrifuged for 40 min in a VTi50 rotor at 131,700 *g*. The interface between the 1.0/1.2 M sucrose solutions was collected, diluted in 4 vol of a solution of 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and centrifuged for 30 min in a JA17 rotor (Beckman Instruments, Inc.) at 39,000 *g*. The pellets were resuspended in phosphate-buffered saline (PBS) and stored in liquid nitrogen.

Radioiodination of Calmodulin: Bovine brain calmodulin was iodinated with the Boulton-Hunter reagent in a procedure modified after that of Chafouleas et al. (8). Free ^{125}I-reagent was separated from ^{125}I-calmodulin by chromatography on a Sephadex G25 column (18.5 × 1.0 cm) previously equilibrated with 50 mM $NaPO_4$, 0.05% NaN_3, 0.25 mg/ml gelatin, and 0.1 M NaCl, pH 7.5. The peak of radioactivity eluting at the void volume was pooled. There was ~25% incorporation of ^{125}I into calmodulin, and the resulting specific activity was 16.7 μCi/nmol calmodulin.

^{125}I-CM Binding Assay of GCP Membranes: 30–50 μg of GCP membranes were incubated for 90 min at 26°C in a total reaction volume of 100 μl of 100 mM HEPES, pH 7.3, containing 0.25 mg/ml gelatin, 2 mM $CaCl_2$ or 2 mM EGTA, and ^{125}I-calmodulin at the specified concentration. The binding was terminated by the addition of 1.0 ml of binding buffer containing 2 mM $CaCl_2$ or EGTA and centrifugation through a 0.9-ml cushion of 10% BSA at 10,400 *g* in a JS 7.5 rotor (Beckman Instruments, Inc.) for 10 min. The pellets obtained were washed twice with ice cold H_2O, and their radioactivity was determined by gamma counting. Nonspecific binding of ^{125}I-calmodulin to GCP membranes was defined as that binding which occurred in the presence of a 10-fold excess of unlabeled calmodulin. For each concentration of ^{125}I-calmodulin tested, binding was assessed four ways: total binding in the presence of Ca^{2+}, nonspecific binding in the presence of Ca^{2+}, total binding in the presence of EGTA, and nonspecific binding in the presence of EGTA.

Gel Electrophoresis and Electroblotting: SDS PAGE was performed as described (30). Neville PAGE was carried out according to the method of Grab et al. (17). Resolving gels were 5–15% acrylamide gradients (140 × 240 × 0.75 mm) and stacking gels (50 mm) consisted of 3% acrylamide. Samples were electrophoresed 180 mm in the resolving gel. 50-μl sample volumes were loaded per well and contained 25–100 μg protein. Samples intended for electroblotting typically contained 75–100 μg protein. Gels were stained with Coomassie Blue (0.04% in 50% methanol and 7% acetic acid) and destained in 30% methanol and 7% acetic acid. Autoradiography of dried gels was performed using Kodak X-Omat film with or without Dupont Quanta II or III intensifier screens, at −70°C. Electroblotting of gels after electrophoresis was performed using a modification of the procedure described by Towbin et al. (41). Gels were pre-equilibrated in tank buffer twice for 10 min with gentle agitation. Electroblotting was carried out using a 230 ElectroBlot system (E-C Apparatus Corp., St. Petersburg, FL) at 300 mA for the specified time (30–90 min). After blotting, the gel was stained as described above, and the nitrocellulose blot was either stained with Amido black (41) or incubated overnight, at 4°C, with gentle agitation in Tris-buffered saline containing 3% BSA, 2% Ficoll, and 2% polyvinyl pyrrolidone, pH 7.3.

Binding ^{125}I-Calmodulin and Antibodies to GCP Blots: The nitrocellulose blots, prepared and quenched as described above, were incubated for 2 h at room temperature with gentle rocking in Tris-buffered saline containing 3% BSA, 0.1% Nonidet P-40, 3 nM ^{125}I-calmodulin, and 2 mM $CaCl_2$ or 2 mM EGTA (binding buffer). Six 15-min washes in the proper binding buffer were carried out, and the blots were dried and autoradiographed as described. Alternately, blots intended for immunolabeling were prepared according to the method of Van Eldick and Wolchock (42). After electroblotting, the nitrocellulose paper was rinsed in PBS and then incubated in PBS containing 0.2% glutaraldehyde for 45 min at room temperature. After a brief PBS wash, the blots were then quenched at 37°C in PBS containing 2% BSA and 0.1% (vol/vol) gelatin for 1 h. After three 10-min washes in PBS with 0.05% Tween-20 at room temperature, the blots were incubated with rabbit antiserum diluted 1:200, at 37°C for 1 h. After incubation with HRP-conjugated secondary antibody, the development of reaction product was carried out using 4-chloro-1-naphthol (19).

Other Procedures: Protein was determined following the method of Bradford (4) using the Bio-Rad reagent and Bio-Rad bovine serum albumin standard. For electron microscopy, GCP fractions were fixed with buffered glutaraldehyde in suspension and processed as described (37).

RESULTS

Purified GCPs isolated according to Pfenninger et al. (37) exist in a dilute solution after chromatography on controlled pore glass 3000. These particles were determined to be sealed, based on their ability to exclude radiolabeled inulin (Clark, R. and K. H. Pfenninger, unpublished observations). To facilitate biochemical analysis of this preparation, a highly concentrated, sealed preparation was necessary. For this purpose the GCP peak, separated from contaminating supernatant proteins by chromatography on controlled pore glass, was dialyzed briefly to reduce the sucrose concentration, and then centrifuged at 100,000 *g* over a cushion of Maxidens oil. GCPs were collected at the interface in a minimal volume.

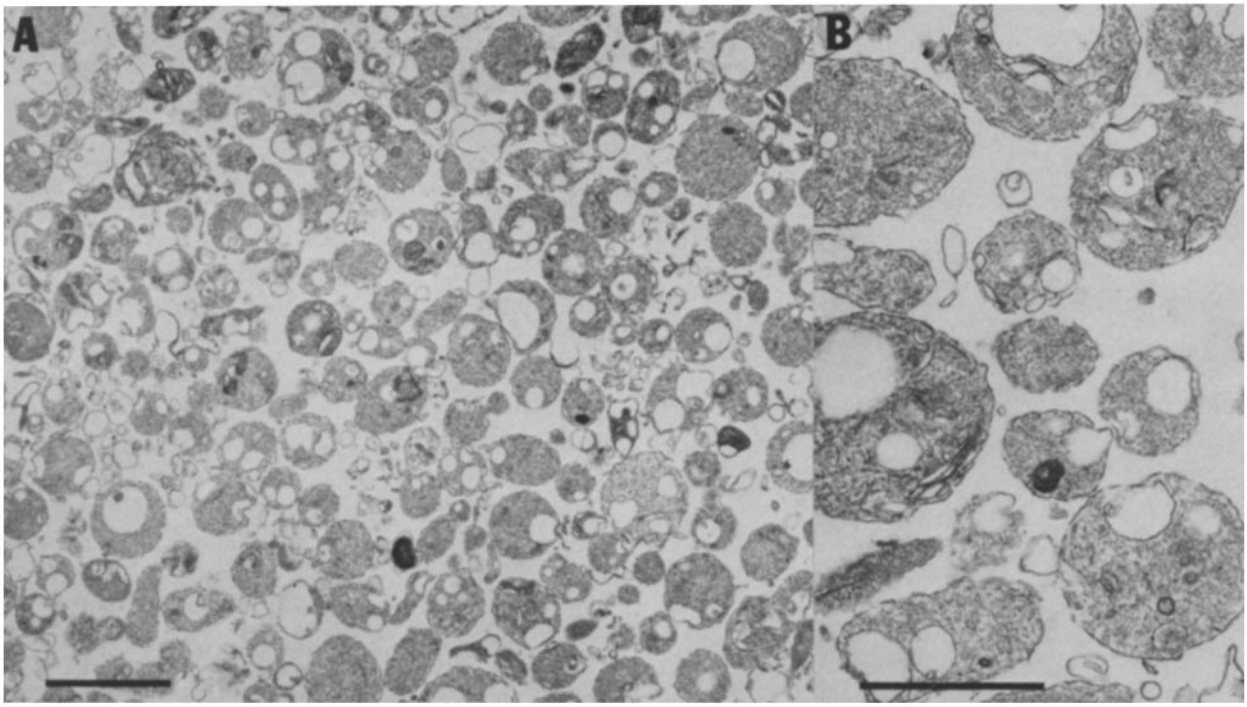

FIGURE 1 Ultrastructure of GCPs pelleted onto Maxidens oil. GCPs were prepared and processed through the final centrifugation over a Maxidens oil cushion as described in Materials and Methods. The sample was then processed for electron microscopic analysis as described in Pfenninger et al. (37). (*A*) Low power survey of GCPs; (*B*) high power view of GCPs. Bar, (*A*) 2 μm; (*B*) 1 μm.

Electron microscopic examination of this preparation (Fig. 1) shows the particles to be bounded by membranes. The appearance of this fraction is not significantly altered from that of GCPs in the "A"-fraction or GCPs collected immediately after chromatography on controlled pore glass. Thus, the GCPs concentrated on Maxidens oil are suitable for the biochemical studies described below.

Intrinsic Calmodulin

Because of the widespread occurrence of calcium-regulated enzyme cascades, calmodulin is likely to be an intrinsic component of the nerve growth cone. To determine if calmodulin is indeed present in GCPs, a Neville PAGE analysis of GCP proteins was carried out. Calmodulin has previously been shown by this technique to exhibit a characteristic shift in electrophoretic mobility in the presence of Ca^{2+} (17). Fig. 2 shows the pattern of GCP protein staining in a 5–15% acrylamide Neville gel in lanes *4* and *5*. In lane *4*, 1.0 mM EDTA was included in the sample applied to the well; in lane *5*, 10 mM $CaCl_2$ was present additionally. A prominent, curved protein band (M_r 16,000) is visible in the presence but not in the absence, of Ca^{2+}. Its spurs can be seen to connect with a sharp band migrating just below a prominent protein of 20 kD. The characteristic shift in the electrophoretic mobility of purified calmodulin is shown for comparison in lanes *2* and *3*. To further examine for the presence of calmodulin in GCPs, immunolabeling experiments were carried out (Fig. 3). GCP proteins were electroblotted onto nitrocellulose paper following separation by SDS PAGE. The blots were incubated in the presence of rabbit anti-rat calmodulin serum (lane *1*) or control rabbit serum (lane *2*). After incubation with HRP-conjugated second antibody and development of the HRP reaction, a single GCP protein with an apparent molecular mass of 16.5 kD was specifically recognized by the anti-calmodulin antiserum.

To carry out a quantitative analysis of ^{125}I-calmodulin binding to growth cone membranes, it was necessary to first remove as completely as possible the intrinsic calmodulin from the GCP fraction. Lanes *6* and *7* of Fig. 2 show the protein staining pattern of GCP membranes which have been treated with EGTA and salt washing as described in Materials and Methods. A significant amount of the protein whose mobility is altered by the presence of Ca^{2+}, previously identified as calmodulin, has been removed from the preparation by this procedure. Comparison of densitometric scans of lanes *5* and *7* shows a reduction of the curved band in the membrane fraction relative to the intact GCP fraction. The densitometric scans show that (*a*) other proteins were also removed from the GCPs by this treatment; (*b*) in addition some proteins were relatively enriched; and finally (*c*) some species were unaffected. These stripped membranes were used in all subsequent calmodulin binding studies.

Calmodulin Binding to GCP Membranes

As a first step, our ^{125}I-calmodulin preparation was examined by electrophoresis in the presence and absence of Ca^{2+} (Fig. 4). For comparison, lanes *1* and *2* show the protein stain of the probe prior to labeling with iodine and its characteristic shift in mobility induced by Ca^{2+}. Lanes *3* and *4* are autoradiograms of the probe radioiodinated as described in Materials and Methods. The Ca^{2+} shift in mobility is evident. Furthermore, no breakdown products due to the iodination or other steps of the procedure were detected, even though gels were overloaded and overexposed.[2]

[2] Previous attempts to radiolabel calmodulin using lactoperoxidase-catalyzed iodination resulted in extensive breakdown of the protein. Calmodulin breakdown of a lesser extent has been reported to occur previously (39).

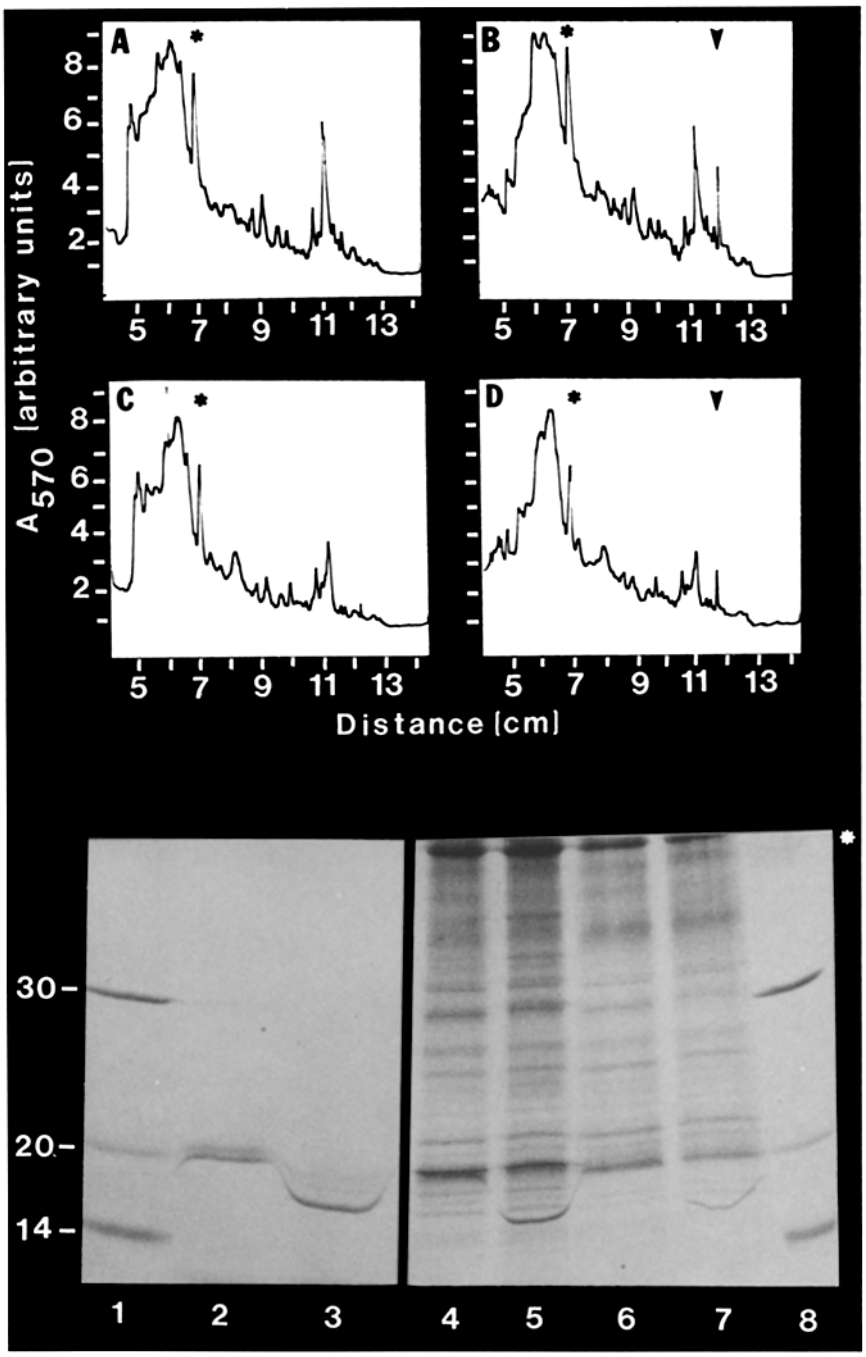

FIGURE 2 Effect of Ca^{2+} on polypeptide mobility in Neville PAGE of GCPs and of EGTA-stripped GCP membranes. GCPs (44 μg, lanes *4* and *5*), EGTA-treated membranes prepared from GCPs (44 μg, lanes *6* and *7*), and purified calmodulin (8 μg, lanes *2* and *3*) were loaded onto a 5–15% Neville gel and electrophoresed as described in Materials and Methods. The samples in lanes *2*, *4*, and *6* contained 1 mM EDTA; samples in lanes *3*, *5*, and *7* contained 1 mM EDTA and 10 mM $CaCl_2$. Lanes *1* and *8* contain 5 μg each of the molecular mass markers carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and lysozyme (14.4 kD). Shown above (*A–D*) are densitometric tracings of lanes *4–7*, respectively. The asterisks indicate the peak corresponding to the top band of the gel. The arrowheads indicate the position of calmodulin in the presence of Ca^{2+}.

The ^{125}I-calmodulin was used to study the calmodulin-binding properties of GCP-derived membranes which had been stripped of their intrinsic calmodulin. For each data point, samples were analyzed as described in Materials and Methods. When calmodulin-stripped GCP membranes were incubated in excess iodinated ligand, the amount of specifically bound, Ca^{2+}-dependent binding was calculated as the difference between specifically bound ^{125}I-calmodulin in the presence of Ca^{2+} and specifically bound ^{125}I-calmodulin in the absence of Ca^{2+}. Nonspecific binding, both Ca^{2+}-dependent and Ca^{2+}-independent, was defined as the binding of ^{125}I-calmodulin in the presence of a 10-fold excess of unlabeled ligand. This binding was found to be directly proportional to the amount of membrane protein present. Comparison of the stripped membranes to those prepared from GCPs by hypotonic lysis alone showed the former to be roughly two-fold enriched in ^{125}I-calmodulin-binding activity (data not shown). Since the additional salt wash resulted in an increase of the calmodulin-binding activity, this suggests a membrane-associated nature of most binding components. Saturation studies were carried out in which a fixed amount of salt-washed, calmodulin-depleted GCP membrane protein was incubated with increasing concentrations of ^{125}I-calmodulin. Fig. 5 shows the specifically bound, Ca^{2+}-dependent binding curve to approach saturation at 40–50 μM ^{125}I-calmodulin. When plotted for Scatchard analysis, the data points yield a curvilinear plot, suggesting the presence of more than one class of binding sites (plot not shown). Since the gel electroblot binding analysis discribed below shows that several calmodulin-binding components exist in GCPs, the Scatchard analysis is of limited applicability to this study. The nonspecifically bound Ca^{2+}-dependent binding comprises a small proportion

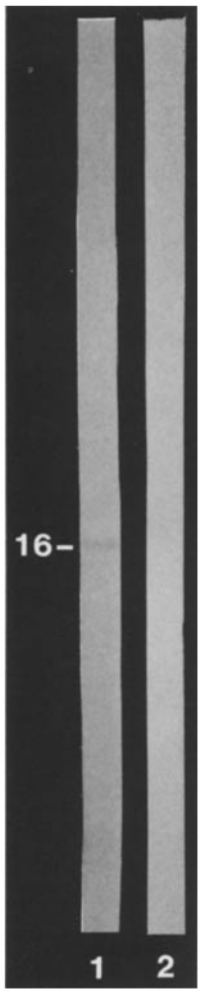

FIGURE 3 Detection of calmodulin in GCPs by immunolabeling of nitrocellulose blots. GCPs (100 μg per lane) were electrophoresed in a 12% Laemmli gel, in the absence of Ca^{2+}, and transferred to nitrocellulose paper as described in Materials and Methods. GCP proteins in lane *1* of the blot were stained with rabbit anti-rat calmodulin serum in a 1:200 dilution. Lane *2* of the blot, containing GCP proteins, was stained with control serum in a 1:200 dilution. Secondary HRP-conjugated antibody incubation and development of reaction product were carried out as described in Materials and Methods. The molecular mass (in kD) of the reactive band in lane *1* is indicated by a dash.

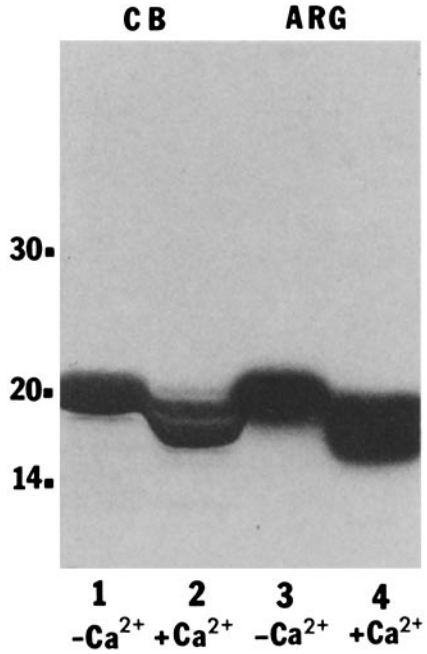

FIGURE 4 Effect of Ca^{2+} on migration in Neville PAGE of ^{125}I-labeled rat brain calmodulin. Samples in lanes *1* and *3* contained 1 mM EDTA, pH 7.8, and samples in lanes *2* and *4* contained 1 mM EDTA and 10 mM $CaCl_2$. The marks on the left show the positions on the gel of the molecular mass standards carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and lysozyme (14.4 kD). Lanes *1* and *2* show a Coomassie Blue stain of 42 μg commercially obtained rat brain calmodulin. The autoradiographic exposure of purified rat brain ^{125}I-calmodulin (60,000 cpm per lane) is shown in lanes *3* and *4*.

of the total Ca^{2+}-dependent binding at all ligand concentrations tested. Calcium-independent binding of ^{125}I-calmodulin to GCP membranes did not comprise a significantly large proportion of the specific ^{125}I-calmodulin binding observed (data not shown). Thus, the binding of ^{125}I-calmodulin to GCP membranes is highly specific, calcium dependent, and saturable.

Calmodulin-binding Proteins

To define more clearly the constituents of GCP membranes that bind calmodulin and to compare them with their synaptosomal counterparts, SDS PAGE was performed using GCP or synaptosome membranes. This was followed by electroblotting the separated polypeptides onto nitrocellulose paper. The blots were then incubated with ^{125}I-calmodulin in the presence and absence of Ca^{2+}, washed, and autoradiograms were prepared. The exposure pattern obtained in the presence of Ca^{2+} is shown in Fig. 6. The binding profiles of the homogenate, GCPs, GCP membranes, and synaptosomes were compared. In the absence of Ca^{2+}, the blots revealed no calmodulin-binding proteins, even with very long exposures. The calmodulin-binding species detected in the various fractions are summarized in Table I.

The autoradiograms of Fig. 6 reveal a family of calmodulin-binding proteins to be present in the GCP membrane. The major calmodulin-binding proteins of the GCP membrane (the 47-, 49-, 57-, and 60-kD species) all have equivalents in synaptosomal membranes. Three of these four synaptosomal

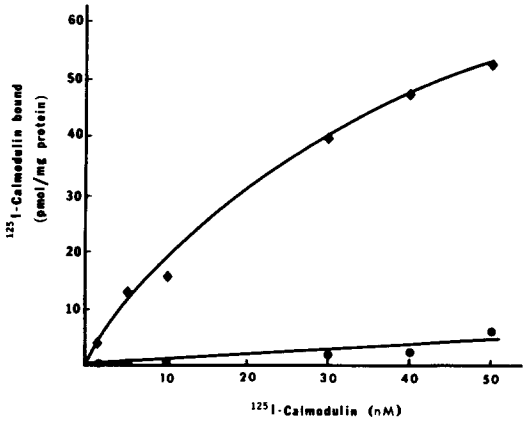

FIGURE 5 Specific calcium-dependent binding of ^{125}I-calmodulin to GCP membranes. Various concentrations of ^{125}I-calmodulin were incubated with 35 μg of calmodulin-depleted GCP membranes at 26°C for 90 min as described in Materials and Methods. The final reaction volume was 0.1 ml, samples contained 100 mM HEPES, 0.25 mg/ml gelatin, 0.002% saponin, and either 2 mM $CaCl_2$ or 2 mM EGTA. At the end of the incubation, 1.0 ml HEPES-gelatin-saponin buffer was added to all samples, the mixture was centrifuged through a BSA cushion and the pellet washed twice before being gamma counted. Nonspecifically bound ^{125}I-calmodulin was determined by preincubation of samples with 0.6 μM calmodulin. Calcium-dependent binding was calculated as the difference between that binding measured in the presence of $CaCl_2$ and calcium-independent binding measured in the presence of EGTA. One pmol ^{125}I-calmodulin represents 1,980 cpm. ◆, calcium-dependent, specific ^{125}I-calmodulin binding; ●, calcium-dependent nonspecific binding.

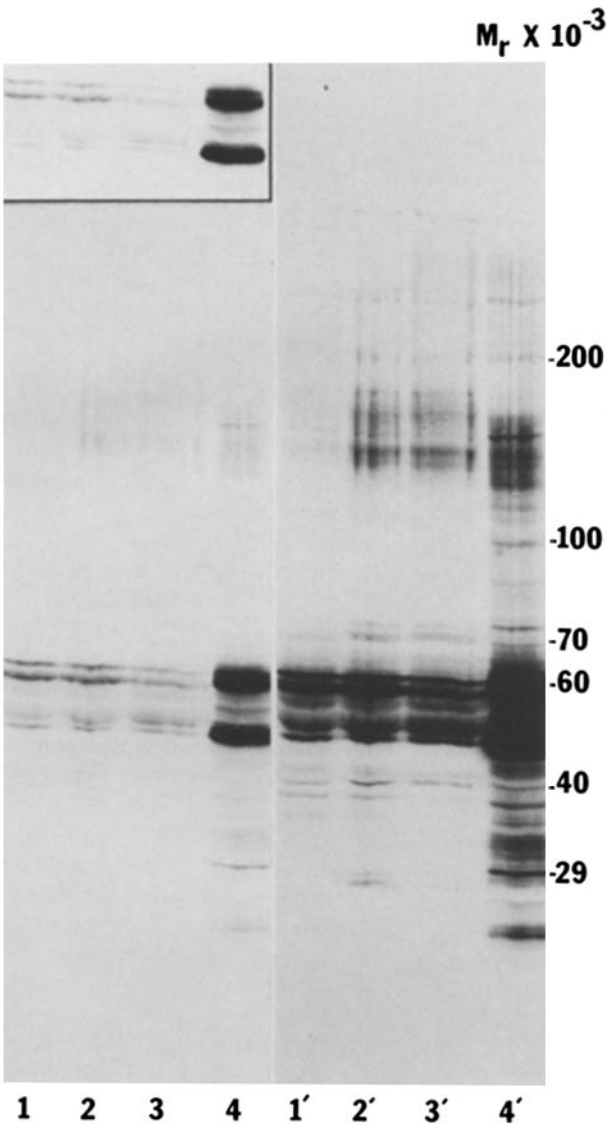

FIGURE 6 Comparison of ^{125}I-calmodulin binding to nitrocellulose blots of synaptosomes and fractions from the GCP membrane preparation. Equal amounts of protein from all samples (100 μg) were applied to wells of a linear 5–15% polyacrylamide gel. A nitrocellulose blot was prepared and then incubated with ^{125}I-calmodulin in the presence of $CaCl_2$ or EGTA, subsequently washed, and autoradiographically exposed as described in Materials and Methods. Shown in lanes *1–4* is an autoradiograph of the nitrocellulose blot labeled in the presence of Ca^{2+}. The fractions shown are as follows: lane *1*, fetal brain homogenate; lane *2*, intact GCPs pelleted onto Maxidens oil; lane *3*, EGTA-stripped membrane from intact GCPs; and lane *4*, synaptosomal membrane. Lanes *1′–4′* show, for facilitated comparison of the patterns, different exposures of lanes *1–4*, respectively. The inset (*top left*) is a very light exposure of lanes *1–4* to show the detail of the 50–60-kD region. The position and molecular mass calibration (in kD's) is indicated by dashes.

species are major calmodulin-binding proteins (the 49-kD protein is a minor component). There are GCP membrane proteins detected by this technique which are unique to GCPs and not found in synaptosomes. Included in this category are the 53-, 70-(doublet), and 347-kD protein. These species are enriched in GCPs relative to the homogenate with the exception of the 53-kD protein. The high molecular mass species which bind calmodulin are also enriched in GCPs relative to the homogenate. The GCP and growth cone membrane calmodulin-binding patterns appear to be very similar. The diversity of the calmodulin binding patterns of the GCP and the synaptosome fractions suggests the presence of a specialized set of components in growth cones.

DISCUSSION

The purpose of this study was to demonstrate the presence of calmodulin and to biochemically characterize its binding sites in nerve growth cones isolated from the central nervous system. Previous analysis of sealed GCPs purified from soluble contaminants were hampered by the relatively low protein concentration of the preparations. To overcome this obstacle we devised the means to prepare a highly concentrated, intact GCP preparation. The excellent morphological preservation observed and the evidence of γ-^{32}P-ATP and ^{3}H-inulin exclusion from this preparation (data not shown) suggest that GCPs isolated and concentrated by the technique described are sealed. Thus, they provide a suitable model system in which to study the regulator mechanisms in nerve growth cones.

Quantitative analyses of calmodulin binding require a membrane preparation derived from sealed GCPs which has been stripped of its intrinsic calmodulin. Osmotic shock of the GCPs followed by salt washing, both in the presence of 50 mM EGTA and 0.002% saponin, produced a GCP mem-

TABLE I. *^{125}I-Calmodulin-binding Proteins*

kD	Fetal rat brain homogenate	Growth cone particles	GCM	Synaptosome
347	–	+	+	–
252	–	+	+	+
202	–	+	+	+
174	–	+	+	+
161	–	+	+	+*
147	–	–	–	+‡
113	–	–	–	+
107	–	–	–	+
99	–	–	–	+
85	–	–	–	+
73	–	+	+	+
70	–	+§	+§	–
68	–	–	–	+
62	–	–	–	+
60	+‡	+‡	+‡	+‡
57	+‡	+‡	+‡	+‡
53	+	+	+	–
51.5	–	–	–	+
49	+‡	+‡	+‡	+
47	+‡	+‡	+‡	+‡
43	–	–	–	+*
40	+	+	+	+
38	+	+	–	–
37	–	–	–	+
35	–	–	–	+
33–30	–	–	–	+‖
29	–	+	–	+‡
27	–	+	–	–
23.5	–	–	–	+
23	–	–	–	+

* Diffuse band.
‡ Major calmodulin-binding protein.
§ Doublet.
‖ 4 bands between 33 and 30 kD.

brane preparation which was depleted of a significant portion of its intrinsic calmodulin. Additionally, a pure ^{125}I-calmodulin probe is necessary. Boulton-Hunter iodination of commercially prepared calmodulin resulted in a probe with the typical electrophoretic mobility and binding properties and a high specific radioactivity (16.7 μCi/nmol). A previous report details the retention of the biological activity of such a preparation of ^{125}I-calmodulin (8).

Endogenous Calmodulin

If a calcium-responsive enzyme cascade were to function in the growth cone, then calmodulin would be expected to be an integral component of GCPs. The demonstration of calmodulin in the sealed GCP preparation fulfills this expectation. It is a reasonable suggestion that calmodulin may exert a regulatory influence in the nerve growth cone by binding to its membrane constituents. The ^{125}I-calmodulin binding data on GCP membranes is suggestive of this. Studies in other systems have shown ^{125}I-calmodulin binding to approach saturation: for adult brain synaptic vesicle membranes, in the range of 1 μM (34) or alternatively, in the range of 30–40 nM (24); for erythrocyte membranes, between 20 and 30 nM ^{125}I-CM (1); for the adrenal chromaffin granule membrane system, between 50 and 100 nM ^{125}I-calmodulin (13, 22); and for the rat adipocyte plasma membrane system, in the range of 10 nM ^{125}I-calmodulin (15). Our finding of saturation of binding near 50 nM ^{125}I-calmodulin is in reasonable agreement with these results. The binding to salt-washed GCP membrane showed a high degree of specificity, calcium dependence, and saturability. All of these characteristics would be expected of elements comprising a regulatory process which is stimulated by changes in intracellular calcium levels. The mechanism of growth regulation at the growth cone is likely to involve Ca^{2+} and to be complex, and other Ca^{2+}-calmodulin–dependent mechanisms may also be present. Thus, one would expect more than one class of calmodulin-binding sites to be present in growth cone membranes.

Calmodulin-binding Components

Further characterization of the binding constituents of GCPs was provided by the analysis of GCP gel blots labeled ^{125}I-calmodulin. Identification of these proteins helps to elucidate the functional role of calmodulin in the growth cone. The comparison of GCP and synaptosomal calmodulin-binding proteins is instructive because the proteins unique to GCPs are candidates for involvement in growth-related mechanisms. The species of synaptosomal calmodulin-binding proteins detected in our study agree well with those described by Carlin et al. (7). The only differences noted are the number and molecular mass calibration of the major calmodulin-binding proteins. Apparent molecular masses in our study were 47, 57, and 60 kD, as compared with the estimates of 51 and 60, respectively, of Carlin et al. (7). Comparisons of GCPs and synaptosomes for the purpose of examining developmental changes in protein expression must be made cautiously. While GCPs constitute a very homogenous fraction, synaptosome preparations show a great deal of ultrastructral heterogeneity. In addition, synaptosomes, unlike GCPs, contain postsynaptic elements. A further caution to be noted here is the possibility that blotting efficiencies may differ for GCP versus synaptosomal material. A comparison of calmodulin-binding profiles of Western blots and those detected by direct labeling of a GCP and synaptosome gel (calmodulin overlay technique; (16, cf. reference 14) (data not shown), however, did not reveal any calmodulin-binding proteins that failed to blot. Many of the GCPs' calmodulin-binding proteins detected appear to have synaptosomal equivalents, such as the 29-, 40-, 47-, 49-, 57-, 60-, 73-, 134-, 141-, 161-, 174-, 202-, and 252-kD proteins. The synaptosomal 47-kD polypeptide and complex between 57 and 60 kD are likely to include the subunits of synapsin I kinase, a calmodulin-dependent, autophosphorylating protein kinase which binds calmodulin on all subunits (3, 27). Other proteins which may exist in this molecular mass range include calcineurin (28), and cyclic nucleotide phosphodiesterase (44). It is unlikely, however, that one of these proteins detected in this range is calcineurin in light of the fact that immunoblotting analysis failed to demonstrate the presence of the 60-kD subunit of this protein in GCPs, but did show it to be present in an adult brain cytosolic preparation (data not shown). The high molecular mass, 347-kD protein may be MAP 2, which has previously been shown to bind ^{125}I-calmodulin in a Ca^{2+}-dependent manner (31). Additionally, fodrin is known to be a major calmodulin-binding protein of the postsynaptic density (6) and may be the 252-kD species detected in our blots, both in growth cones and synaptosomes. The 141-kD calmodulin-binding protein detected in growth cones and synaptosomes may be a proteolytic product of alpha fodrin, which has been identified in postsynaptic density preparations and is known to have calmodulin-binding activity (6). The GCP equivalents to the major calmodulin-binding proteins of synaptosomes are present, but much less prominent when compared with synaptosomes (lanes *1–3* compared to lane *4*). This interpretation is consistent with our previous finding of synapsin I phosphorylation in GCPs (11, 26).

Comparison of growth cone calmodulin-binding proteins with calmodulin-binding proteins detected in coated vesicles also suggests possible equivalent proteins. Photoaffinity labeling experiments with azido-^{125}I-calmodulin revealed calmodulin-binding polypeptides of 110, 73, and 32 kD to be present in coated vesicles (33). The 73-kD protein detected in GCPs and membranes from GCPs may be an equivalent species. Comparison of GCP calmodulin-binding species with those detected in synaptic vesicle preparations also suggests some equivalent proteins to be present in GCPs (24, 34). While a close relationship between calmodulin-binding patterns of synaptosomes and GCPs is evident, there are also several striking differences. First, one of the major binding proteins of GCP membranes (the 49-kD protein) is not a predominant calmodulin-binding protein in synaptosomes. Second, the GCP membrane proteins of 347- and 53-kD and the doublet 70-kD protein do not have synaptosomal counterparts. The differences noted suggest the existence in growth cones of a group of developmentally regulated calmodulin-binding proteins. A possibility is that these proteins are components of a machinery involved in the regulation of growth-related functions.

The possibility that the calmodulin-binding proteins of GCPs are protein kinases will be the subject of future investigation. The role of calmodulin in the control of neuronal sprouting could be a triggering function conveying calcium sensitivity to an intracellular transducer system activated by growth factor binding to the growth cone membrane.

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