Neuromodulin (GAP-43) Can Regulate a Calmodulin-Dependent Target *in Vitro*†

J. Randall Slemmon*,‡,§ and Mark R. Martzen§

Department of Biochemistry and Department of Neurobiology and Anatomy, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, New York 14642

Received January 20, 1994; Revised Manuscript Received March 4, 1994®

ABSTRACT: The calmodulin-binding polypeptide neuromodulin (GAP-43) was tested *in vitro* for its ability to modulate a typical calmodulin target, the enzyme nitric oxide synthase. The titration of enzyme with increasing neuromodulin concentrations demonstrated a concentration-dependent decrease in enzyme activity. Subsequent analysis of the ability of increased calcium concentrations to activate the enzyme was tested in the presence or absence of neuromodulin. The effect of neuromodulin on the calcium-dependent activation of the enzyme was to depress enzyme activity in the range of 0.2 to approximately 6 μM calcium. Treatment of the neuromodulin polypeptide with protein kinase C eliminated its ability to inhibit nitric oxide synthase activation. Subsequent treatment of the phosphorylated neuromodulin with calcineurin (phosphatase 2b) caused it to regain its inhibitory action on the enzyme. The results from these *in vitro* studies have indicated that neuromodulin has the ability to affect the activation of a calmodulin-dependent enzyme at levels of the polypeptide that exist in neurons. They also demonstrated that the regulation occurred within a physiological range of calcium concentrations. Since the inhibition of enzyme activity appeared to be occurring through the interaction of neuromodulin with calmodulin, it seems likely that neuromodulin has a general ability to impede activation of calmodulin-dependent targets.

Many different types of neurons produce an unusual calmodulin-binding polypeptide which displays its highest affinity for calmodulin at low calcium concentrations (Andreasen et al., 1983; Cimler et al., 1985). The binding of this peptide, referred to as neuromodulin (Liu & Storm, 1989), GAP-43 (Skene & Willard, 1981), B-50 (Zwiers et al., 1976, 1980), P-57 (Andreasen et al., 1983), or F1 (Routtenberg & Lovinger, 1985), to calmodulin can be inhibited either by increased calcium concentrations (Alexander et al., 1987) or by the phosphorylation of neuromodulin at serine-41 (Chapman et al., 1991). Neuromodulin has been the subject of intense study in the nervous system for some time, during which it has been implicated as an important component in a broad range of metabolic systems including axonal growth [for reviews, see Skene (1989) and Coggins and Zwiers (1991)] and long-term potentiation (Lovinger et al., 1985, 1986; Routtenberg & Lovinger, 1985). It has also been suggested to be a regulator of calmodulin-mediated target activation [for a review, see Liu and Storm (1990)], although such an action for neuromodulin has never been directly demonstrated. A peptide that is able to bind the calcium-poor or preactivated form of calmodulin would provide an intriguing putative modulator, since it should be capable of effecting target activation in a noncompetitive manner during the early phase of calcium influx into neurons when cytosolic calcium concentrations are still low. This can be compared with the predicted properties of calmodulin regulators which selectively bind the calcium-rich form. Regulators of this type should only be able to invoke significant inhibition after a considerable rise in the intracellular calcium concentration has occurred [e.g., see McIlroy et al. (1991)], and, unlike neuromodulin, they would compete directly with activatable targets for calmodulin binding.

Studying the role of neuromodulin as a potential calmodulin modulator required a prototypical calmodulin-dependent activity. The neuronal form of the nitric oxide-producing enzyme, nitric oxide synthase, requires calcium and calmodulin for activity (Bredt & Snyder, 1990), and some neurons which are rich in the enzyme such as cerebellar granule cells (Kiedrowski et al., 1992a,b) also express neuromodulin in the presynaptic compartment (Rosenthal et al., 1987; Meiri et al., 1988). Nitric oxide synthase can also be readily purified to homogeneity. As a result, this enzyme offered an excellent *in vitro* calmodulin target for studying the possible function of neuromodulin. The system also provided an excellent internal control since the phosphorylation of neuromodulin with protein kinase C has been reported to specifically interrupt its binding to calmodulin (Alexander et al., 1987; Apel et al., 1990). If neuromodulin was able to affect nitric oxide synthase activity through its interaction with calmodulin, then this modulation should itself be regulated by protein kinase C action on the peptide.

As a diffusible activator of guanylate cyclase (Bredt & Snyder, 1989), nitric oxide has itself been implicated both in neuron–neuron signaling [e.g., see Snyder (1992)] and in neuron–astrocyte signaling (Kiedrowski et al., 1992b). Nitric oxide may also be part of the mechanism that promotes long-term depression in the cerebellum [Crepel & Jailard, 1990; Shibuki & Okada, 1991; although see Linden and Conner (1992)] or long-term potentiation in the hippocampus (e.g., Schuman & Madison, 1991; Gribkoff & Lum-Ragan, 1992; Haley et al., 1992; O'Dell et al., 1991; Izumi et al., 1992). Neurons producing nitric oxide synthase are unusual in that they appear less susceptible to degeneration by excitotoxins (Koh & Choi, 1988), to the neurodegenerative effects of quinolinic acid (Koh et al., 1986), to cell death as a result of ischemic damage following stroke (Uemura et al., 1990), or to other neurodegenerative conditions (Ferrante, 1985). This provided an additional impetus to employ nitric oxide synthase as the model target, since the colocalization of neuromodulin

† This work was supported by Grant BNS-9021042 from the NSF and Grant R35 AG09016 from the NIH. M.R.M. was supported by Grant R01 AG01121 from the NIH and by The Markey Foundation Charitable Trust.

* Address correspondence to this author at the Department of Biochemistry, University of Rochester Medical Center.

‡ Department of Biochemistry.

§ Department of Neurobiology and Anatomy.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

0006-2960/94/0433-5653$04.50/0

and this enzyme in some neurons offered an *in vivo* association that could be initially examined *in vitro*.

By titrating the assay with increasing amounts of neuromodulin, it was demonstrated that nitric oxide synthase activity could be totally suppressed over a physiological range of calcium concentrations. The activation of the enzyme by increasing calcium concentrations showed a neuromodulin-dependent displacement of the curve toward a higher concentration of the ion needed for activity. Phosphorylation of the polypeptide with protein kinase C completely reversed the inhibition on the activity of the enzyme, whereas subsequent phosphatase action restored the inhibition. The results from these analyses have indicated that neuromodulin has the potential to modulate calmodulin activity across calcium concentrations that are typical of the neuronal cytosol. With the expanded characterization provided by this study, showing the capability of neuromodulin to affect calmodulin target activation, it will be of special interest to examine the role of neuromodulin on calmodulin function in future experiments employing both cultured cells and *in vivo* models. Additionally, it will be important to compare the properties of neuromodulin to other calmodulin-binding proteins which contain either the "IQ" sequence (such as chicken brain myosin-V; Espreafico et al., 1992) or a PKC-phosphorylatable sequence (such as Neurogranin; Baudier et al., 1991; Watson et al., 1992).

EXPERIMENTAL PROCEDURES

Nitric Oxide Synthase Assay and Purification. The enzyme activity was measured by the method of Bredt and Snyder (1990), with the only change being the substitution of the reaction stop buffer with 20 mM 2-(*N*-morpholino)ethanesulfonic acid sodium salt 2 mM and ethylenediaminetetraacetic acid disodium salt, pH 5.5. All assays were incubated for 10 min at 37 °C. The concentrations of the components in the assay were 4 mM β-nicotinamide adenine dinucleotide phosphate, 0.22 μM bovine calmodulin, and 5.5 μM arginine, in 50 mM tris(hydroxymethyl)aminomethane hydrochloride/5 mM 2-mercaptoethanol, pH 7.4. Calmodulin stock solutions and assay buffer were pretreated with Chelex (sodium form; BioRad Laboratories, Richmond, CA) according to the instructions of the manufacturer in order to remove sufficient calcium to completely inhibit enzyme activity. The nitric oxide synthase activity purified for this study appeared completely calcium/calmodulin-dependent, indicating an absence of other forms of the enzyme. Two millimolar calcium was used in routine assays for enzyme purification. The enzyme was purified according to the protocol of Bredt and Snyder (1990), with no changes. The wash buffer and the enzyme elution buffer for the ADP–agarose affinity column were pretreated with Chelex to avoid contaminating the enzyme preparation with calcium. The purified enzyme was eluted with 10 mM β-nicotinamide adenine dinucleotide phosphate from this column, which both stabilized the enzyme activity and made it unnecessary to add this substrate to the enzyme–substrate cocktail.

Whenever neuromodulin was included in the nitric oxide synthase assay, the enzyme–substrate cocktail (containing the calmodulin and arginine but not calcium) was preincubated for 10 min at 37 °C with the neuromodulin peptide. Following this period, calcium was added to achieve the necessary concentration, and the assay was then started by the addition of enzyme. The preincubation volume was at a 2 times concentration with respect to the substrate components, to which 1 volume of purified enzyme was added for initiation. The final enzyme reaction was carried out in 0.1 mL.

Purification of Neuromodulin. One hundred grams of frozen calf brain was broken into chunks and homogenized on ice in 100 mM sodium borate, pH 10.0, containing 5 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid disodium salt, 1 mM [ethylenebis(oxyethylenenitrilo)tetraacetic acid, and 0.5 mM phenylmethanesulfonyl fluoride. The pH of the homogenate was raised to 11 with 10 N sodium hydroxide for 2 min, and then it was readjusted to 10 with concentrated hydrochloric acid. The homogenate was then centrifuged at 30000*g* for 30 min at 4 °C. The pH of the supernatant was then adjusted to 2.5 by the addition of 86% phosphoric acid. Centrifugation was repeated, and the resultant supernatant was made 2.5% in perchloric acid. After a final centrifugation, the pH of the supernatant was raised to 2.5 by the addition of 10 N sodium hydroxide.

The neuromodulin present in the final supernatant was desalted and concentrated by passing over a 5 × 11 cm column of Bakerbond Wide-Pore Butyl(C4) (J. T. Baker Chemical Co.) preequilibrated in 0.1% trifluoroacetic acid in water. The column was washed with 2 volumes of the same solution, and the polypeptides were eluted with 70% acetonitrile in water (v/v). The eluted material was concentrated to 20 mL in a Savant SpeedVac using the preparative rotor and polypropylene liner. The concentrated sample was chromatographed on a 2.5 × 45 cm column of Sephadex G-50 fine in 0.1% trifluoroacetic acid in water. Polypeptides in the void volume were collected, and the fraction was pH-adjusted to 7.4 by the addition of 1 M tris(hydroxymethyl)aminomethane base. The neutralized sample was made 5 mM in 2-mercaptoethanol, and 1 mM each in [ethylenebis(oxyethylenenitrilo)]tetraacetic acid and ethylenediaminetetraacetic acid disodium salt.

The sample was adsorbed to a 2.5 × 6 cm Sepharose–calmodulin column that had been preequilibrated in 20 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4, containing 5 mM 2-mercaptoethanol and 1 mM each of [ethylenebis(oxyethylenenitrilo)]tetraacetic acid and ethylenediaminetetraacetic acid disodium salt. The column was washed with 20 mL of the same buffer, and protein was eluted first in 30 mL of 50 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4, 5 mM 2-mercaptoethanol, and 4 mM calcium chloride, followed by 10 mL of the same buffer containing 500 mM sodium chloride. The eluted protein was pH-adjusted to 2.5 by the addition of 86% phosphoric acid and injected onto a 1 × 25 cm C4 reverse-phase HPLC column (Vydac; The Separations Group, Hesperia, CA) at 3 mL/min that had been preequilibrated in 0.05% trifluoroacetic acid in water. Proteins were resolved by elution in an increasing gradient of acetonitrile, and the neuromodulin-containing peak was identified by Western blot analysis.

Western Blotting. Protein samples were separated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (12% acrylamide) on a BioRad MiniProtean II, essentially following the directions of the manufacturer. Proteins were transferred to Immobilon P (Millipore) in 10 mM 3-(cyclohexylamino)propanesulfonic acid sodium salt, pH 11, and 10% methanol in a Hoeffer Mighty Small transfer apparatus at 0.5 A for 20 min. Small dots of peroxidase-conjugated antisera were applied with toothpicks to the corners of the blot so that they would be visible both with the protein stain and on the Western blot film image. This served to orient the superimposition of the two images. Proteins were stained with 0.1% Coomassie blue in 50% methanol/10% acetic acid for 5 min and destained in the same solution without the dye. Destaining was stopped by placing the blot in water, which

was followed by blocking the membrane in 5% Carnation nonfat dry milk in 50 mM tris(hydroxymethyl)aminomethane hydrochloride. Anti-B50 antibody (which was a kind gift from Dr. Henk Zwiers, University of Calgary, Calgary, Alberta, Canada) was used at 5000× dilution in blocking solution where the nonfat milk had been replaced with 5 mg/mL ovalbumin. This allowed repeated use of the diluted sera, since the dry milk had a limited life in solution. The blot was incubated with the diluted antisera for 2 h at 23 °C with gentle agitation. The blot was then washed with three 10-min changes of blocking buffer, and then a 20000× dilution of peroxidase-conjugated goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL) in blocking buffer was placed on the blot for 1 h. After a final series of three washes, the blot was rinsed in blocking buffer without the dry milk and then developed in Amersham ECL reagent (Amersham Life Sciences, Buckinghamshire, England). The bands were then visualized by exposure to X-ray film.

Protein Kinase C Phosphorylation of Neuromodulin Peptide. Phosphoneuromodulin was prepared either with nonradioactive adenosine triphosphate (when preparing larger amounts to be used in nitric oxide synthase assays) or with adenosine [γ-^{32}P]triphosphate (when smaller amounts were prepared to monitor protein kinase C activity). The primary purpose of using the radioactive label was to provide analytical phosphorylation control for the protein kinase C stock enzyme and to determine which neuromodulin peaks observed on HPLC were phosphorylated. When nonradioactive phosphopeptide was prepared for use in the nitric oxide synthase assays, 450 μg of neuromodulin was employed. The phosphorylation procedure used the mixed-micelle technique essentially as described in DeVries et al. (1989). Protein kinase C was obtained from Upstate Biotechnologies Inc. (nos. 14–115; Lake Placid, NY), and 0.5 ng of enzyme/1 μg of neuromodulin was employed per phosphorylation. A parallel aliquot of 450 μg of neuromodulin was processed without protein kinase C in order to provide a nonphosphorylated control for the nitric oxide synthase assay. For analytical experiments where phosphorylation was monitored by following radioactive phosphate, protein kinase C was reacted with neuromodulin in the presence of 0.1 mM adenosine triphosphate [tris(hydroxymethyl)aminomethane salt] to which 10^7 dpm of adenosine [γ-^{32}P]triphosphate per reaction was added. The incubation time for both analytical and preparative phosphorylation reactions was increased to 8 h at 30 °C to ensure maximum conversion. The protein kinase C-treated neuromodulin and control neuromodulin were repurified on HPLC using the same system as for the original purification except that the column was a Vydac C18 (0.46 × 25 cm). Integration and graphic analysis of the elution profiles were accomplished with Axxiom 727 software (Axxiom Chromatography, Moorpark, CA), and radioactivity was monitored in-line with a Radiomatic Flo-One/Beta detector (Packard Instrument Co.).

Reaction of Protein Kinase C-Phosphorylated Neuromodulin with Calcineurin (Phosphatase 2b). Calcineurin was purchased from Sigma Chemical Co. (St. Louis, MO), and the protocol for reacting phosphorylated neuromodulin followed that reported by Liu and Storm (1989). One hundred micrograms of protein kinase C-phosphorylated neuromodulin prepared as described above was made 40 mM in *N*-(2-hydroxethyl)piperazine-*N*′-2-ethanesulfonic acid, pH 7.5, 50 mM sodium chloride, 0.5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, and 2.5 mM 2-mercaptoethanol in 50-μL final volume. Seven micrograms of calcineurin was added to start the reaction, and it was allowed to proceed at 30 °C for 30 min. The resulting peptide was repurified on HPLC as described for the protein kinase C-treated peptide. The neuromodulin peptide peak was collected as a 1-mL sample and taken to dryness in a Savant SpeedVac. The resulting sample was sufficiently calcium-free that it could be added to the nitric oxide assays without activating the enzyme.

RESULTS

Neuromodulin Purification. The purification protocol is generally based on the peptide analysis scheme by Slemmon and Flood (1992). This included the extraction of peptides from frozen tissue by dispersion in pH 2.5 buffer, followed by concentration of soluble peptides on a preparative C4 or C18 column. A high-pH homogenization step was added for this study in order to increase the recovery of neuromodulin into the supernatant without the need of using a low ratio of tissue to homogenization buffer (normally 2% w/v). Protein precipitation using 2.5% perchloric acid (Baudier et al., 1989) in the crude supernatant was also exploited in the current study in order to enrich the initial supernatant for neuromodulin. The fractionation of polypeptides recovered from the final supernatant, containing the perchloric acid, on size-exclusion chromatography is shown in Figure 1A. Neuromodulin eluted in the void volume on Sephadex G-50. The material from this fraction was further purified on calmodulin–Sepharose in the presence of excess calcium chelator (Andreasen et al., 1983). The proteins which adsorbed to the column in the absence of calcium were further fractionated on a reverse-phase column (Figure 1B). The prior removal of the smaller molecular weight peptides in the sample for HPLC, which tended to coelute with neuromodulin on reverse-phase HPLC, allowed for the recovery of essentially homogeneous neuromodulin from this step. The entire purification protocol could easily be accomplished within 48 h, with sufficient yield to recover about 2.5 mg of neuromodulin polypeptide per 100 g of frozen calf brain.

As can be seen in Figure 1C, the neuromodulin harvested from the reverse-phase HPLC purification step was the only polypeptide that could be detected in this fraction on polyacrylamide gel electrophoresis in sodium dodecyl sulfate when over 5 μg of protein was analyzed. The protein was sufficiently pure to be considered a homogeneous preparation for its subsequent addition to nitric oxide synthase assays. The protein concentration of neuromodulin was determined using the extinction coefficient at 264 nm reported by Masure et al. (1986), which was found to be in general agreement with the value obtained from amino acid analysis on the purified peptide obtained for the present study.

Titration of Nitric Oxide Synthase Activity with Neuromodulin. The effect of neuromodulin concentration on nitric oxide synthase activity was measured in the presence of excess β-nicotinamide adenine dinucleotide phosphate (Figure 2). The calcium concentration used achieved approximately 70% maximal velocity. Under these conditions, neuromodulin displayed 50% inhibition of nitric oxide synthase activity at a concentration of approximately 1.8 μM. This value is in general agreement with the binding constants of 1.0–3.0 μM reported by Alexander et al. (1987) for P-57 (neuromodulin) and calmodulin when no calcium chelator was present. The two reported values corresponded to neuromodulin binding to calmodulin at low ionic strength and at 150 mM potassium chloride, respectively. The observed change in the affinity of neuromodulin for calmodulin over low to moderate salt concentrations was relatively modest, which has suggested that ionic strength is not likely to be a major factor in regulating

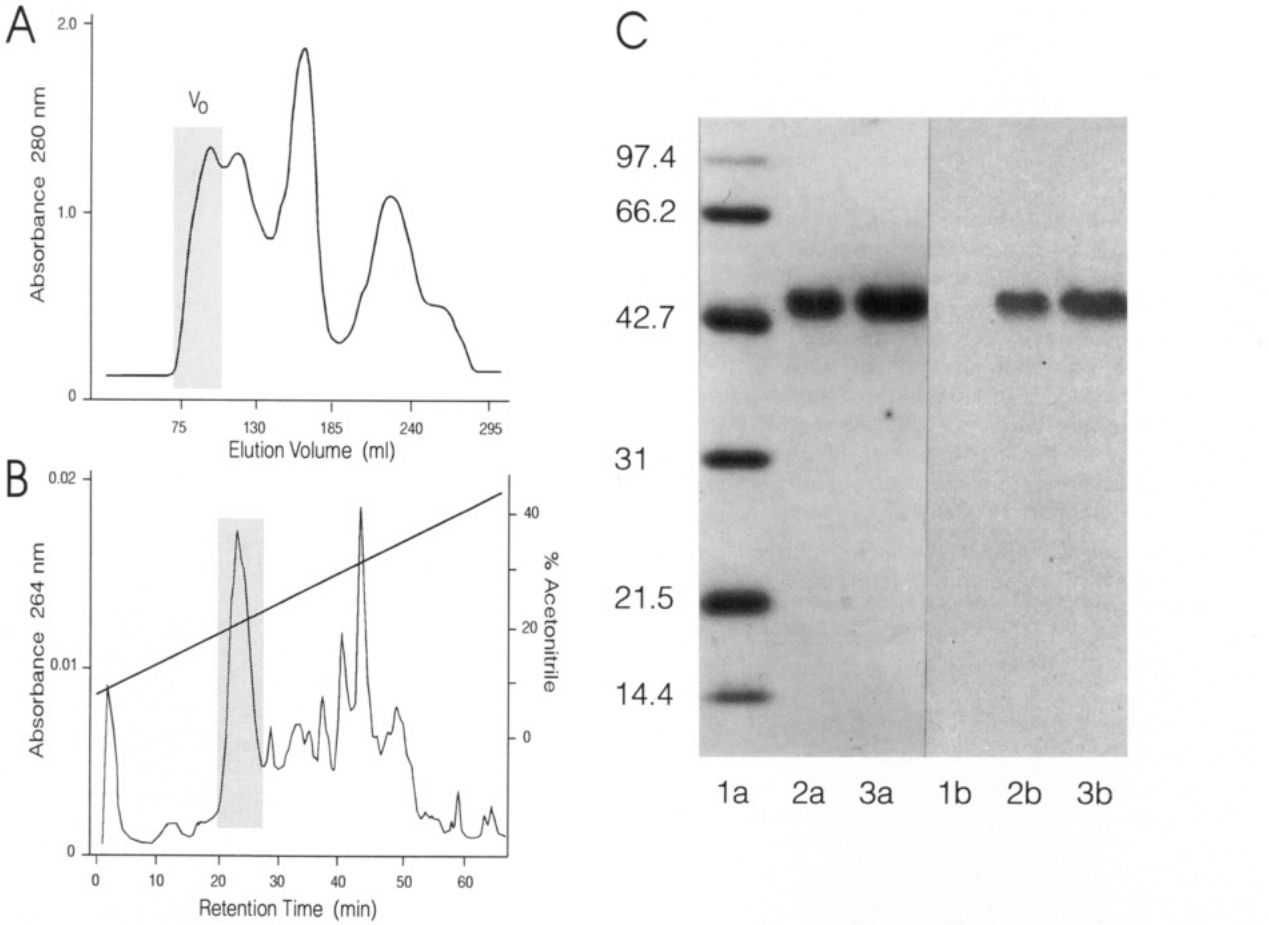

FIGURE 1: Purification of neuromodulin. (A) Size-exclusion chromatography on Sephadex G-50 fine of proteins concentrated from the final crude supernatant. The column (222-mL bed volume + 20 mL of sample) was preequilibrated and developed at room temperature in 0.1% trifluoroacetic acid in water at 2 mL/min. The region of the elution profile which contained the neuromodulin protein is indicated by the shading. All of the neuromodulin eluted in the void volume (V_0) on this matrix as determined by Western blot analysis (data not shown). (B) Purification of neuromodulin by reverse-phase HPLC. The polypeptides from the void volume on Sephadex G-50 separation were brought to neutral pH and then adsorbed to calmodulin–Sepharose in the absence of free calcium. After the column was washed to remove unbound material, the proteins were eluted with calcium, and the pH of the sample was brought to 2.5 by the addition of phosphoric acid. The sample was directly subjected to reverse-phase HPLC separation at 3 mL/min on a Vydac C4 column (1.0 × 25 cm) which was developed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water. Neuromodulin was one of the first components to elute, at a position that is well removed from the bulk of the remaining peptides. Presumably, the other polypeptides in the trace are also capable of binding calmodulin, and could therefore potentially represent other calmodulin regulators. (C) Western blot analysis of purified neuromodulin protein. (1a) One microgram of molecular weight standards visualized with Coomassie blue; (2a) 2.5 μg and (3a) 5 μg of purified neuromodulin visualized with Coomassie blue; (1b, 2b, 3b) film developed from the same blot shown in the "a" lanes after immunodetection for neuromodulin (5000× bovine anti-B50 sera). The molecular weight markers in lane 1b did not visualize, but the purified neuromodulin protein (lanes 2b and 3b) was clearly recognized by the antisera.

the neuromodulin/calmodulin interaction. The amount of neuromodulin needed to achieve the demonstrated effect was consistent with its estimated abundance in brain on the basis of results from this study and others (Skene, 1989; Coggins & Zwiers, 1991; Apel & Storm, 1992).

Neuromodulin and the Calcium Activation Curve for Nitric Oxide Synthase. Nitric oxide synthase showed a characteristic dependence on calcium concentration with an apparent EC_{50} of approximately 0.6 μM (Figure 3). This is similar to the value of 0.2 μM reported by Bredt and Snyder (1990). In both the current study and that study, nitric oxide synthase achieved maximum velocity at 1–2 μM calcium. The shape and span of the calcium activation curves from Bredt and Snyder (1990) and for the current study are in close agreement, which indicated that the kinetic values for the enzyme can be readily recreated. This property of the enzyme makes it an excellent activity for use in studying calmodulin targets.

In contrast to the calcium activation curve in the absence of any neuromodulin, the presence of this polypeptide in the assay clearly shifted the activation curve toward a region where more calcium was needed to activate the enzyme (Figure 3). The curve obtained in the presence of 1.0 μM neuromodulin shifted the calcium concentration needed to achieve half-maximum velocity from approximately 0.6 μM to near 2.0 μM. The same activation in the presence of 4.8 μM neuromodulin raised this value to approximately 4 μM calcium. Regardless of the concentration of neuromodulin present, however, maximum velocity could be achieved, but this required higher calcium concentrations as the amount of neuromodulin was increased. Neuromodulin inhibition of calmodulin signaling could be overcome solely by increased calcium concentration. The range of calcium concentrations over which neuromodulin showed its effect superimposes those which are observed in active growth cones (e.g., Kater & Mills, 1991) and is consistent with calcium concentrations achieved in neuritic compartments by low to moderate calcium currents (e.g., Tsien, 1988; Knight et al., 1989; Müller & Connor, 1991).

Effect of Pretreatment of Neuromodulin with Protein Kinase C. Phosphorylation of neuromodulin with protein kinase C has been reported at serine-41 (Apel et al., 1990; Chapman et al., 1991). Since this position includes the

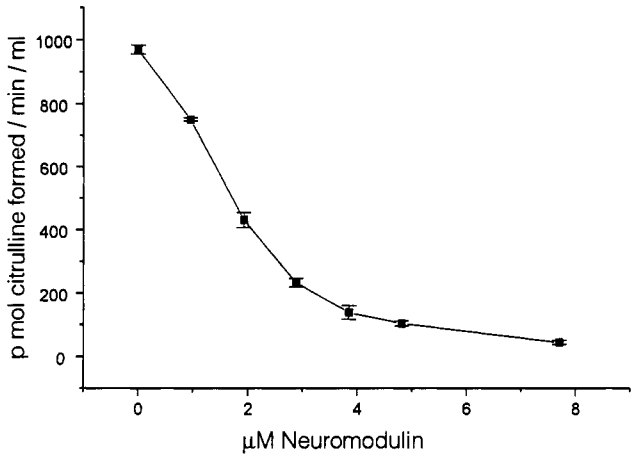

FIGURE 2: Titration of nitric oxide synthase activity with neuromodulin. Enzyme activity was measured in the presence of 0.22 μM calmodulin and excess β-nicotinamide adenine dinucleotide phosphate (4 mM). The calcium concentration employed (0.75 μM) yielded approximately 70% of maximal enzyme velocity. Under these conditions, neuromodulin clearly impeded enzyme activity. Enzyme activity was measured by its ability to produce citrulline in stoichiometric amounts with nitric oxide (Bredt & Snyder, 1990). Error bars are the standard deviation from duplicate determinations.

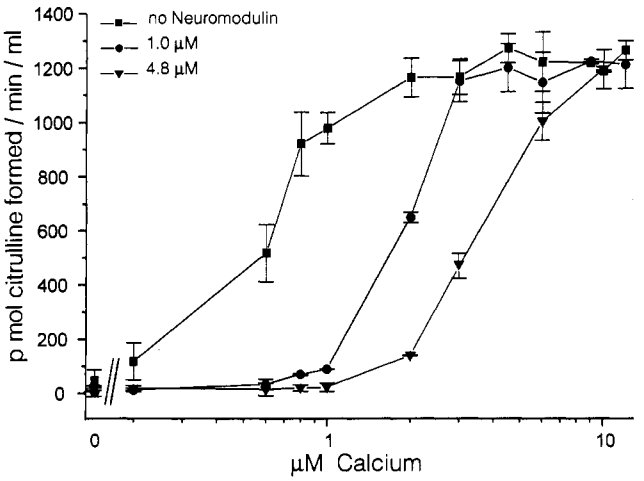

FIGURE 3: Titration of nitric oxide synthase activity with calcium in the presence of different amounts of neuromodulin. The concentration of neuromodulin used to generate each curve is indicated on the figure. Data points are a composite from multiple runs with error bars representing the standard deviation from quadruplicate determinations (no neuromodulin) or duplicate determinations (1 and 4.8 μM neuromodulin). Neuromodulin shifted the calcium concentration needed for activation to a higher value in a neuromodulin concentration-dependent manner.

calmodulin-binding domain, the modification interrupts neuromodulin binding to calmodulin. Because of this, if the effect of neuromodulin on nitric oxide synthase activity seen in Figures 2 and 3 was through calmodulin-dependent activation, then phosphorylation of neuromodulin should reverse its activity. Figure 4A,B provides reverse-phase HPLC analyses of neuromodulin peptide after incubations without and with protein kinase C present. A number of apparent neuromodulin isoforms can be seen to elute as fused peaks, all of which appear to have been phosphorylated by protein kinase C (Figure 4C). Figure 5 demonstrated how the protein kinase C-treated material had lost its ability to inhibit nitric oxide synthase. Conversely, the curve obtained with the nontreated control is essentially identical to that in Figure 2.

Reactivation of Protein Kinase C-Phosphorylated Neuromodulin with Calcineurin (Phosphatase 2b). Since the phosphorylation of neuromodulin appears to correlate with its biological function in paradigms such as the immobilization of growth cones (e.g., Dent & Meiri, 1992) or long-term potentiation in the hippocampus (Lovinger et al., 1985), the function of neuromodulin *in vivo* might also be controlled by the dephosphorylation of the polypeptide. Protein kinase C-phosphorylated neuromodulin was treated with the neuronal phosphatase calcineurin in order to determine if this enzyme could cause reactivation of neuromodulin. Through cycles of

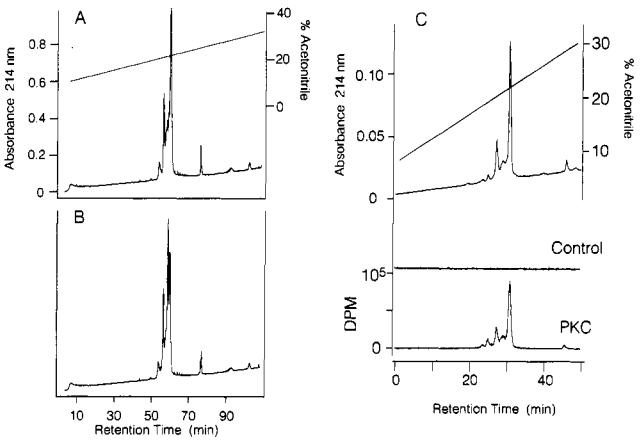

FIGURE 4: Phosphorylation of neuromodulin with protein kinase C. (A) Repurification of control neuromodulin on reverse-phase HPLC; 450 μg of neuromodulin protein that was incubated in parallel with the material shown in (B), but without the kinase being present. (B) Identical aliquot of neuromodulin protein as in (A), but treated with protein kinase C. The total area under each set of peaks differed by less than 5%, indicating quantitative recovery of protein and that both samples contained essentially the same protein concentration. The total material under the peaks was pooled for subsequent analysis. Adenosine [γ-^{32}P]triphosphate was omitted from the reactions used to prepare neuromodulin phosphopeptide for use in the nitric oxide synthase assays. (C) HPLC analysis of neuromodulin peptide after phosphorylation with protein kinase C in the presence of adenosine [γ-^{32}P]triphosphate. Twenty-five micrograms of neuromodulin was simultaneously analyzed on reverse-phase HPLC for isoforms by first monitoring the absorbance at 214 nm and then by detecting ^{32}P. dpm were determined with a Radiomatic Flo-One/Beta in-line scintillation detector (Packard Instruments Co., Meriden, CT) which was connected to the outflow of the UV detector. The dead volume between the detectors was 150 μL. A comparison of the two signals indicated that all of the detectable neuromodulin isoforms incorporated phosphate.

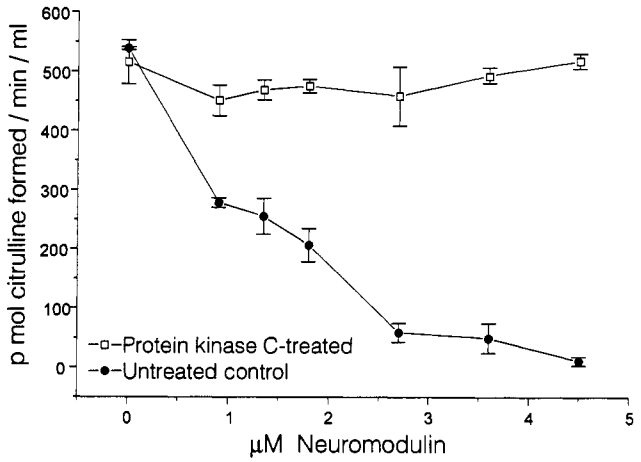

FIGURE 5: Comparison of the effect of untreated and protein kinase C-treated neuromodulin on nitric oxide synthase activity. Neuromodulin protein was reacted with ATP and protein kinase C as described under Experimental Procedures. Kinase-treated and control curves are indicated on the figure. Treatment of neuromodulin with protein kinase C clearly inhibited its activity in the nitric oxide synthase assay over the range of concentrations tested. Error bars are standard deviations of duplicate determinations.

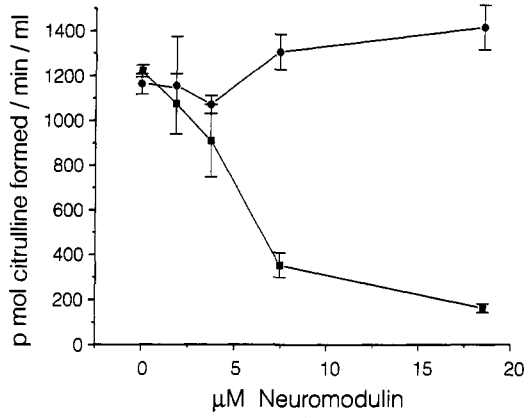

FIGURE 6: Recovery of inhibitory activity by treatment of phosphoneuromodulin with calcineurin (phosphatase 2b). Protein kinase C-produced phosphoneuromodulin was further reacted with calcineurin. This phosphatase-treated peptide was compared to the phosphorylated starting material in order to determine if it had regained its inhibitory activity in the nitric oxide synthase assay. (●) Nitric oxide synthase activity in the presence of protein kinase C-produced phosphoneuromodulin. (■) Nitric oxide synthase activity in the presence of neuromodulin that had been produced by treatment of phosphoneuromodulin with calcineurin. The 30-min reaction time with this phosphatase restored very demonstrable inhibitory activity to phosphorylated neuromodulin. Error bars are standard deviations of duplicate determinations.

protein kinase C phosphorylation followed by dephosphorylation with enzymes such as calcineurin, neuromodulin would become a regulatable inhibitor of calmodulin activation which would itself be under the control of phosphorylation cascades. The data in Figure 6 demonstrated that the dephosphorylation of neuromodulin with calcineurin using the same conditions reported by Liu and Storm (1989) restored considerable activity to protein kinase C-phosphorylated peptide.

DISCUSSION

Neuromodulin as a Putative Endogenous Modulator of Axonal Calmodulin. Calmodulin is present in most cell types (Klee & Vanaman, 1982); however, neuromodulin is largely restricted to axons [for a review, see Skene (1989)]. Teleologically, this might suggest that some neurons evolved molecules such as neuromodulin in order to modify the normal activation by calmodulin of the wide variety of metabolic targets it is known to activate (Means et al., 1991; Ovádi & Orosz, 1992). This would not be unexpected in light of the critical and widespread role calcium signaling plays in neurons (e.g., Kennedy, 1989). If the results demonstrated on nitric oxide synthase activity in this study are a general effect that neuromodulin has on calmodulin activation, then it could be proposed that a major function of neuromodulin is to generally suppress calmodulin-mediated signaling inside any cellular compartment in which it is deployed. To this end, it will be important for future studies to determine if the neuromodulin effect can be reproduced on additional calmodulin-regulated enzymes and calmodulin-dependent processes.

Although initial studies of enzymes and processes which regulate them are traditionally accomplished by *in vitro approaches* such as the one employed for this study, the extrapolation of *in vitro* data to biological systems must always be made with caution. The conditions employed here to study calmodulin-dependent nitric oxide synthase activity in the presence of neuromodulin were designed primarily around conditions known to support nitric oxide synthase stability *in vitro* (Bredt & Snyder, 1990). These conditions were employed both because neuromodulin binding to calmodulin does not appear to be especially dependent on salt concentration over a physiological range (Alexander et al., 1987) and because nitric oxide synthase is purified with a number of noncovalently associated cofactors that can be affected by changing the buffer conditions. For this reason, neuromodulin and calmodulin were allowed to complex in 100 mM tris(hydroxymethyl)-aminomethane hydrochloride, and the nitric oxide synthase assay was performed in 50 mM tris(hydroxymethyl)aminomethane hydrochloride in the presence of thiol reducing agent and excess β-nicotinamide adenine dinucleotide phosphate. Calmodulin concentrations were also chosen so that the nitric oxide synthase added to the assay was maximally active. The present study has demonstrated that under conditions where nitric oxide synthase is active *in vitro*, neuromodulin is able to alter the manner in which it becomes activated. This provided a biological end point for studying possible neuromodulin activity since nitric oxide synthase activity has been characterized *in vivo* (Garthwaite, 1988; Kiedrowski, 1992a,b). It is important to note, however, that the conditions employed to study the effect of neuromodulin in nitric oxide synthase assays were not exhaustive. As the function of neuromodulin in tissue culture and *in vivo* becomes better characterized, it will be necessary to correlate what is known of neuromodulin function *in vitro* with the conditions found *in vivo*.

The nature of the regulation by neuromodulin observed in this study is also of interest, since the presence of this polypeptide did not activate or deactivate the calmodulin-dependent target in an absolute sense. The action of dephosphoneuromodulin on calmodulin activation of nitric oxide synthase was to modify the range of calcium concentrations over which the enzyme could become active. This can be compared to regulation through phosphorylation, which is usually a more defined process that turns activities on or off [for review, see Krebs (1983), Stull et al. (1986), or Hershey (1989)]. Depending on the concentration of neuromodulin at the site of enzyme activation, the inhibition could be totally overcome by increasing the calcium concentration from approximately 3 to 6 times over what was needed with calmodulin alone.

The effect that neuromodulin had on calmodulin activity and the high levels of neuromodulin found in growth cones of axonal processes have made it a good candidate for an endogenous calmodulin regulator. Growth cones are very sensitive to small changes in internal calcium concentration [for discussion, see Kater and Mills (1991)], and at least one mechanism for immobilizing such specializations employs an internally-directed calcium signal with the subsequent activation of calmodulin (Polak et al., 1991). The *in vitro* properties of neuromodulin demonstrated in this study are consistent with the interpretation that the polypeptide could provide at least one mechanism for protecting the growth cone from premature shutdown by suppressing calmodulin activation of targets. Such a mechanism would have the desirable additional property that when the target dendrite was contacted the calcium influx resulting from increased action potentials could then overcome the inhibition and immobilize the growth cone. The effect could be immediate and would not require the time lag necessary for retrograde signaling, induction of new transcription, or additional enzyme activation.

Potential Role for Neuromodulin in Neuronal Signaling. The depression of nitric oxide production observed when neuromodulin was present suggested that neuromodulin could have an effect on neuronal signaling. Nitric oxide synthase produces nitric oxide which then diffuses into nearby cells

(e.g., Garthwaite et al., 1988; Snyder, 1992). If the adjacent cell possesses guanylate cyclase, then a signal originating from the nitric oxide synthase-containing cell will be transduced. On the basis of *in vitro* data, neurons containing significant amounts of neuromodulin would be expected to display a reduced ability to produce and release nitric oxide. Therefore, if neuromodulin functioned *in vivo* as an endogenous modulator of nitric oxide synthase activity, it would have an effect on this signaling process.

To what extent such a system of control may exist in the brain is not clear. The most important prerequisite for control of this type is that nitric oxide synthase and neuromodulin must be in the same presynaptic compartment. Cerebellar granule cells are an example of a type of neuron that both contains messages for neuromodulin (Rosenthal et al., 1987) and expresses nitric oxide synthase (Kiedrowski et al., 1992a; Schmidt et al., 1992; Vincent & Kimura, 1992). As a consequence, the release of nitric oxide from granule cells could be a process that is affected by the level of neuromodulin expression, which in turn could determine the extent to which guanylate cyclase in either astrocytes (Kiedrowski et al., 1992b) or Purkinje cells (Ariano et al., 1982; Nakane et al., 1983; Zwiller et al., 1992) is activated. The ability of neuromodulin to inhibit nitric oxide synthase may also implicate it in the process of long-term depression of parallel-fiber Purkinje cell transmission following climbing fiber stimulation in the cerebellum, since this process appears to require nitric oxide (Shibuki & Okada, 1991).

Neuromodulin is known to undergo increased phosphorylation during long-term potentiation in the hippocampus (Routtenberg & Lovinger, 1985; Lovinger et al., 1985, 1986), a process that may be modulated by nitric oxide signaling (Böhme et al., 1991; O'Dell et al., 1991; Schuman & Madison, 1991; Haley et al., 1992; Izumi et al., 1992; Gribkoff & Lum-Ragan, 1992). Data from this study indicated that the phosphorylation of neuromodulin with protein kinase C reversed the inhibition on nitric oxide synthase activity, thereby promoting the production of nitric oxide. These data, together with information describing long-term potentiation in the hippocampus, have therefore suggested that neuromodulin expression and its subsequent phosphorylation may have a role in this process. To this end, it will be important for future studies to determine if neuromodulin and nitric oxide synthase colocalize to neurons that are responsible for nitric oxide signaling in long-term potentiation.

Neuromodulin as a Putative Calmodulin Modulator in Cytosolic and Membranous Compartments. The data from the present study described neuromodulin activity on calmodulin activation using an entirely soluble system. In the neuron, however, neuromodulin trafficks between the cytosolic and membranous compartments (Cimler et al., 1987; Skene & Virág, 1989; Mangels & Gnegy, 1990; Liu et al., 1991). The extent of association of this polypeptide with membranes was somewhat unexpected, based on its very hydrophilic primary structure (Basi et al., 1987; Wakim et al., 1987). The ability of neuromodulin to become membrane-associated may be related to the observed palmitoylation on cysteine residues at positions 3 and 4 (LaBate & Skene, 1989; Skene & Virág, 1989). It has also been proposed that neuromodulin can ion-pair with charged groups protruding from membranes (Houbre et al., 1991). Membranes are rich in enzymes that require calmodulin for activity (e.g., Klee & Vanaman, 1982; Davis et al., 1991), and calmodulin in membranes is likely to be important in orchestrating responses to stimuli (e.g., Kung et al., 1992; Pirollet et al., 1992). It has also been demonstrated that calmodulin, like neuromodulin, can traffic from membranes to cytosol as a result of many metabolic manipulations including the activation of muscarinic receptors in human neuroblastoma cells (Mangels & Gnegy, 1990) or the binding of dopamine in the striatum and hippocampus of rat brain (Popov & Matthies, 1989). Whatever the mechanism that moves neuromodulin between membrane and cytosolic compartments, the ability of neuromodulin to exist in both places would allow for its potential action on calmodulin regardless of location. One important question for future studies will be to determine if neuromodulin trafficks to and from the membranous compartment in a manner similar to calmodulin. Neuromodulin has already been proposed to serve as a molecule that can sequester calmodulin in membranes (Estep et al., 1990), and this process may have an effect on the ability of calmodulin to be active in this cellular niche. Inactivation of neuromodulin could be accomplished whether it was soluble or membrane-associated since protein kinase C itself is trafficked between the cytosol and the membrane (Kraft & Anderson, 1983; Akers & Routtenberg, 1987).

CONCLUSION

The data presented in this study have indicated that neuromodulin can inhibit nitric oxide synthase activity in a manner that is consistent with its previously characterized ability to bind to calmodulin. The nature of this inhibition is either to shift to a higher concentration the calcium needed to execute calmodulin-mediated signaling or to place it under the regulation of phosphorylation cascades. On the basis of data from this study, neuromodulin levels in cellular compartments where such a regulatory system might exist would need to be approximately 1 μM or greater. This is consistent with estimates of neuromodulin abundance in neurons (Skene, 1989; Coggins & Zwiers, 1991; Apel & Storm, 1992). It appears, therefore, that a function of neuromodulin may be to form a macromolecular complex with calmodulin with the resultant effect that target activation by calmodulin is altered [for additional discussion, see Liu and Storm (1990) and Skene (1990)]. This change in calmodulin activity can be reversed by the action of protein kinase C, thereby providing a system of control that is itself regulated by phosphorylation. The evolution of polypeptides that alter calmodulin-mediated signaling in this manner may provide a class of cell-specific molecules that modulate the activation properties of calmodulin. This would provide proteins that could evolve to suit the needs of a particular cell type without the need to directly mutate calmodulin, a process that could otherwise be lethal.

REFERENCES

Akers, R. F., & Routtenberg, A. (1987) *J. Neurosci. 7*, 3976–3983.

Alexander, K. A., Cimler, B. M., Meier, K. E., & Storm, D. R. (1987) *J. Biol. Chem. 262*, 6108–6113.

Andreasen, T. J., Luetje, C. W., Heiderman, W., & Storm, D. R. (1983) *Biochemistry 22*, 4615–4618.

Apel, E. D., & Storm, D. R. (1992) *Perspect. Dev. Neurobiol. 1*, 3–11.

Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., & Storm, D. R. (1990) *Biochemistry 29*, 2330–2335.

Ariano, M. A., Lewicki, J. A., Brandwein, H. J., & Murad, F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1316–1320.

Basi, G. S., Jacobson, R. D., Virág, I., Schilling, J., & Skene, J. H. P. (1987) *Cell 49*, 785–791.

Baudier, J., Bronner, C., Kligman, D., & Cole, R. D. (1989) *J. Biol. Chem. 264*, 1824–1828.

Baudier, J., Deloulme, J. C., Dorsselaer, A. V., Black, D., & Matthes, H. W. D. (1991) *J. Biol. Chem. 266*, 229–237.
Böhme, G. A., Bon, C., Stutzmann, J.-M., Doble, A., & Blanchard, J.-C. (1991) *Eur. J. Pharmacol. 199*, 379–381.
Bredt, D. S., & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9030–9033.
Bredt, D. S., & Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 682–685.
Chapman, E. R., Au, D., Alexander, K. A., Nicolson, T. A., & Storm, D. R. (1991) *J. Biol. Chem. 266*, 207–213.
Cimler, B. M., Andreasen, T. J., Andreasen, K. I., & Storm, D. R. (1985) *J. Biol. Chem. 260*, 10784–10788.
Cimler, B. M., Giebelhaus, D. H., Wakim, B. T., Storm, D. R., & Moon, R. T. (1987) *J. Biol. Chem. 262*, 12158–12163.
Coggins, P. J., & Zwiers, H. (1991) *J. Neurochem. 56*, 1095–1106.
Crepel, F., & Jailard, D. (1990) *Neuroreport 1*, 133–136.
Davis, E. B., Smith, T. J., Davis, P. J., & Blas, S. D. (1991) *Biochem. J. 277*, 603–606.
Dent, E. W., & Meiri, K. F. (1992) *J. Neurobiol. 23*, 1037–1053.
DeVries, G., Graser, E. D., & Walsh, M. P. (1989) *Biochem. Cell Biol. 67*, 260–270.
Espreafico, E. M., Cheney, R. E., Matteoli, M., Nascimento, A. A. C., DeCamilli, P. V., Larson, R. E., & Mooseker, M. S. (1992) *J. Cell Biol. 119*, 1541–1557.
Estep, R. P., Alexander, K. A., & Storm, D. R. (1990) *Curr. Top. Cell. Regul. 31*, 161–205.
Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D., & Martin, J. B. (1985) *Science 230*, 561–563.
Garthwaite, J., Charles, S. L., & Chess-Williams, R. (1988) *Nature 336*, 385–388.
Gribkoff, V. K., & Lum-Ragan, J. T. (1992) *J. Neurophysiol. 68*, 639–642.
Haley, J. E., Wilcox, G. L., & Chapman, P. F. (1992) *Neuron 8*, 211–216.
Hershey, J. W. B. (1989) *J. Biol. Chem. 264*, 20823–20826.
Houbre, D., Duportail, G., Deloulme, J. T., & Baudier, J. (1991) *J. Biol. Chem. 266*, 7121–7131.
Izumi, Y., Clifford, D. B., & Zorumski, C. F. (1992) *Science 257*, 1273–1276.
Kater, S. B., & Mills, L. R. (1991) *J. Neurosci. 11*, 891–899.
Kennedy, M. B. (1989) *Trends Neurosci. 12*, 417–424.
Kiedrowski, L., Costa, E., & Wroblewski, J. T. (1992a) *J. Neurochem. 58*, 335–341.
Kiedrowski, L., Costa, E., & Wroblewski, J. T. (1992b) *Neurosci. Lett. 135*, 59–61.
Klee, C. B., & Vanaman, T. C. (1982) *Adv. Protein Chem. 35*, 213–321.
Knight, D. E., von Grafenstein, H., & Athayde, C. M. (1989) *Trends Neurosci. 12*, 451–458.
Koh, J.-Y., & Choi, D. W. (1988) *J. Neurosci. 8*, 2153–2163.
Koh, J.-Y., Peters, S., & Choi, D. W. (1986) *Science 234*, 73–76.
Kraft, A. S., & Anderson, W. B. (1983) *Nature 301*, 612–623.
Krebs, E. G. (1983) *Philos. Trans. R. Soc. London, Ser. B 302*, 3–11.
Kung, C., Preston, R. R., Maley, M. E., Ling, K.-Y., Kanabrocki, J. A., Seavey, B. R., & Saimi, Y. (1992) *Cell Calcium 13*, 413–425.
LaBate, M. E., & Skene, J. H. P. (1989) *Neuron 3*, 299–310.
Linden, D. J., & Connor, J. A. (1992) *Eur. J. Neurosci. 4*, 10–15.
Liu, Y., & Storm, D. R. (1989) *J. Biol. Chem. 264*, 12800–12804.
Liu, Y., & Storm, D. R. (1990) *Trends Pharmacol. Sci. 11*, 107–111.
Liu, Y., Chapman, E. R., & Storm, D. R. (1991) *Neuron 6*, 411–420.
Lovinger, D. M., Akers, R. F., Nelson, R. B., Barnes, C. A., McNaughton, B. L., & Routtenberg, A. (1985) *Brain Res. 343*, 137–143.
Lovinger, D. L., Colley, P. A., Akers, R. F., Nelson, R. B., & Routtenberg, A. (1986) *Brain Res. 399*, 205–211.
Mangels, L. A., & Gnegy, M. E. (1990) *Mol. Pharmacol. 37*, 820–826.
Masure, H. R., Alexander, K. A., Wakim, B. T., & Storm, D. R. (1986) *Biochemistry 25*, 7553–7560.
McIlroy, B. K., Walters, J. D., Blackshear, P. J., & Johnson, J. D. (1991) *J. Biol. Chem. 266*, 4959–4964.
McMaster, D., Zwiers, H., & Lederis, K. (1988) *Brain Res. Bull. 21*, 265–276.
Means, A. R., VanBerkum, M. F. A., Bagchi, I., Lu, K. P., & Rasmussen, C. D. (1991) *Pharmacol. Ther. 50*, 255–270.
Meiri, K. F., Willard, M., & Johnson, M. I. (1988) *J. Neurosci. 8*, 2571–2581.
Müller, W., & Connor, J. A. (1991) *Nature 354*, 73–80.
Nakane, M., Ichidawa, M., & Deguchi, T. (1983) *Brain Res. 273*, 9–15.
O'Dell, T. J., Hawkins, R. D., Kandel, E. R., & Arancio, O. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11285–11289.
Ovádi, J., & Orosz, F. (1992) *Curr. Top. Cell. Regul. 33*, 105–126.
Pirollet, F., Margolis, R. L., & Job, D. (1992) *Biochim. Biophys. Acta 1160*, 113–119.
Polak, K. A., Edelman, A. M., Wasley, J. W. F., & Cohan, C. S. (1991) *J. Neurosci. 11*, 534–542.
Popov, N., & Matthies, H. (1989) *Eur. J. Pharmacol. 172*, 205–210.
Rosenthal, A., Chan, S. Y., Henzel, W., Haskell, C., Kuang, W.-J., Chen, E., Wilcox, J. N., Ullrich, A., Goeddel, D. V., & Routtenberg, A. (1987) *EMBO J. 6*, 3641–3646.
Routtenberg, A., & Lovinger, D. M. (1985) *Behav. Neural Biol. 43*, 3–11.
Schmidt, H. H. H. W., Gagne, G. D., Nakane, M., Pollack, J. S., Miller, M. F., & Murad, F. (1992) *J. Histochem. Cytochem. 40*, 1439–1456.
Schuman, E. M., & Madison, D. V. (1991) *Science 254*, 1503–1506.
Shibuki, K., & Okada, D. (1991) *Nature (London) 349*, 326–328.
Skene, J. H. P. (1989) *Annu. Rev. Neurosci. 12*, 127–156.
Skene, J. H. (1990) *Neurosci. Res. Suppl. 13*, s112–125.
Skene, J. H. P., & Willard, M. (1981) *J. Cell Biol. 89*, 86–95.
Skene, J. H. P., & Virág, I. (1989) *J. Cell Biol. 108*, 613–624.
Slemmon, J. R., & Flood, D. G. (1992) *Neurobiol. Aging 13*, 649–660.
Snyder, S. H. (1992) *Science 257*, 494–496.
Stull, J. T., Nunnally, M. H., & Michnoff, C. H. (1986) *Enzymes (3rd Ed.) 17*, 113–166.
Tsien, R. Y. (1988) *Trends Neurosci. 11*, 419–424.
Uemura, Y., Kowall, N. W., & Beal, M. F. (1990) *Ann. Neurol. 27*, 620–625.
Vincent, S. R., & Kimura, H. (1992) *Neuroscience 46*, 755–784.
Wakim, B. T., Alexander, K. A., Masure, H. R., Cimler, B. M., Storm, D. R., & Walsh, K. A. (1987) *Biochemistry 26*, 7466–7470.
Watson, J. B., Sutcliff, J. G., & Fisher, R. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8581–8585.
Zwiers, H., Veldhuis, D., Schotman, P., & Gispen, W. H. (1976) *Neurochem. Res. 1*, 669–677.
Zwiers, H., Schotman, P., & Gispen, W. H. (1980) *J. Neurochem. 34*, 1689–1699.
Zwiller, J., Ghandour, M. S., Revel, M. O., & Basset, P. (1981) *Neurosci. Lett. 23*, 31–36.