Expression of cDNAs Encoding Wild-Type and Mutant Neuromodulins in *Escherichia coli*: Comparison with the Native Protein from Bovine Brain†

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ABSTRACT: Murine cDNA that encodes neuromodulin, a neurospecific calmodulin binding protein, was inserted into the plasmid pKK223-3 for expression in *Escherichia coli*. After being transformed into *E. coli* strain SG20252 (lon^-), the expression vector directed the synthesis of a protein that was recognized by polyclonal antibodies raised against bovine neuromodulin. The recombinant protein expressed in *E. coli* was found to be tightly associated with insoluble cell material and was extractable only with guanidine hydrochloride or sodium dodecyl sulfate. Following solubilization with guanidine hydrochloride, the protein was purified to apparent homogeneity by a single CaM–Sepharose affinity column step with a yield of 0.2 mg of protein/L of *E. coli* culture. The availability of the purified recombinant neuromodulin made it possible to answer several specific questions concerning the structure and function of the protein. Despite the fact that murine neuromodulin is 12 amino acid residues shorter than the bovine protein and the recombinant protein expressed in *E. coli* may lack any posttranslational modifications, the two proteins displayed similar biochemical properties in almost all respects examined. They both had higher affinity for CaM–Sepharose in the absence of Ca^{2+} than in its presence; they were both phosphorylated in vitro by protein kinase C in a Ca^{2+}- and phospholipid-dependent manner; neither form of the proteins was autophosphorylated, and the phosphorylated form of the proteins did not bind calmodulin. The recombinant neuromodulin and neuromodulin purified from bovine brain had similar, but not identical, affinities for calmodulin, indicating that the palmitylation of the protein that occurs in animal cells is not crucial for calmodulin interactions.

Neuromodulin (previously designated P-57) is a neurospecific calmodulin (CaM)[1] binding protein which was discovered and isolated by virtue of its novel CaM binding properties (Andreasen et al., 1981, 1983; Cimler et al., 1985). This protein displays equivalent or greater affinity for CaM in the absence of Ca^{2+} than in its presence. Neuromodulin can be purified by application of CaM-depleted detergent-solubilized bovine membranes to a CaM–Sepharose column in the presence of excess Ca^{2+} chelator. The protein is then eluted from the CaM–Sepharose column with a Ca^{2+}-containing buffer. This property distinguishes it from the majority of CaM proteins which interact with CaM with significantly greater affinity in the presence of Ca^{2+} (Olwin et al., 1984; Olwin & Storm, 1985).

Recent amino acid and nucleotide sequence analyses indicate that bovine and murine neuromodulins correspond to a rat neuronal growth associated protein designated GAP-43, F1, or B-50 (Wakim et al., 1987; Basi et al., 1987; Cimler et al., 1987; Karns et al., 1987). Although neuromodulin has an apparent molecular weight of 57 000 on SDS–polyacrylamide gels, hydrodynamic characterization of bovine neuromodulin (Masure et al., 1986) yielded a molecular weight of 25 700, which is consistent with the amino acid sequence data (Wakin et al., 1987). Fluorescent polarization and sucrose density gradient sedimentation measurements indicated that neuromodulin is an elongated molecule with an axial ratio of approximately 16:1 (Masure et al., 1986). The circular dichroism of neuromodulin revealed that the protein's secondary structure is primarily random coil with only minor portions of α helix or β sheet (Masure et al., 1986). These structural characteristics of neuromodulin distinguish it from other known CaM binding proteins.

Neuromodulin cross-linked with ^{125}I-azido-CaM at a 1:1 molar ratio in the presence of EGTA (Andreasen et al., 1981, 1983). In the absence of added KCl, the dissociation constants for neuromodulin and a fluorescent-labeled CaM derivative were 1 μM in the presence of Ca^{2+} and 0.23 μM in the absence of free Ca^{2+} (Alexander et al., 1987). The neurmodulin·CaM interaction is also regulated by protein kinase C mediated phosphorylation with phosphorylation of neuromodulin promoting dissociation of the complex (Alexander et al., 1987). We have proposed that neuromodulin may function to sequester and concentrate CaM in the vicinity of CaM-activated proteins and release free CaM in response to increased intracellular calcium levels or phosphorylation by protein kinase C. For example, neuromodulin may concentrate CaM at high local concentrations in the growth cones of neurons and release CaM upon phosphorylation by protein kinase C. In this report, we describe the expression of wild-type and mutant murine neuromodulins in *Escherichia coli* and their purification. The recombinant proteins synthesized in *E. coli* were insoluble and associated with inclusion particles. Because of this, it was necessary to use a strong denaturing agent such as guanidine hydrochloride to solubilize the protein. The biochemical properties of the renatured recombinant protein were found

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[1] Abbreviations: CaM, calmodulin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; Tween 20, polyoxyethylenesorbitan monolaurate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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to be similar to those of the native bovine protein in all respects examined. These results demonstrate the feasibility of using this expression system to generate neuromodulin with specific mutations.

Materials and Methods

Materials. *E. coli* strains NM522 (Gough & Murray, 1983) and JM105 (Yanisch-Perron et al., 1985) were generally used for transformations and analyses of plasmid DNA. *E. coli* strain SG20252 (Trisler & Gottesman, 1984), a mutant defective in protein degradation pathways, was used as a host for recombinant neuromodulin expression. The full-length murine cDNA clone encoding neuromodulin has been described previously (Cimler et al., 1987). Plasmids pUC18 and pKK223-3 were obtained from Pharmacia, Inc. The Muta-Gene in vitro mutagenesis kit was purchased from Bio-Rad, and the gel-purified oligonucleotide primers were obtained from Synthetic Genetics (San Diego, CA).

Construction of the Neuromodulin Expression Plasmid. A 1.1-kb *Eco*RI cDNA fragment containing the entire coding region for neuromodulin was cloned into the *Eco*RI site of pUC18. The resulting plasmid, pUC-NEU1 (Figure 1), was digested with *Bam*HI and *Sph*I which had unique cutting sites in the polylinker region of the vector. *Sph*I left a four-base 3′ protrusion that protected the remainder of the vector from exonuclease III attack, allowing unidirectional digestion of the 5′-noncoding cDNA sequence from the 5′-protruding end left by *Bam*HI. After limited *Exo*III digestion of plasmid linearized with *Bam*HI and *Sph*I, the DNA was incubated with mung bean nuclease to remove the protruding ends followed by ligation with T4 DNA ligase. The ligation mixture was used to transform *E. coli* NM522 competent cells. Transformants were selected by plating on LB agar containing 100 μg/mL ampicillin. Plasmids were isolated from small cultures of transformed bacteria. The extent of deletion generated by *Exo*III was determined by DNA sequence analysis. One clone which had all but one noncoding nucleotide removed was obtained.

The deleted cDNA insert was excised from the plasmid pUC-NEU2 (Figure 1) with *Hin*dIII and *Eco*RI followed by mung bean nuclease treatment to create blunt ends. The blunt-ended cDNA fragment was purified from agarose gel electrophoresis and then ligated to the expression vector pKK223-3 which had previously been digested with *Eco*RI and treated with mung bean nuclease. Following transformation into *E. coli* strain JM105, plasmid DNA was prepared from ampicillin-resistant colonies, and the presence and orientation of neuromodulin cDNA were determined by restriction enzyme mapping. One recombinant plasmid, pKK-NEU1, which had the neuromodulin cDNA sequence placed under the control of the tac promoter was obtained. By DNA sequence analysis, the spacing between the Shine–Dalgarno sequence (AGGA) and the translation initiation codon (ATG) was found to be eight nucleotides as expected.

Recombinant DNA Techniques. Plasmid isolation, restriction enzyme digestion, ligation, agarose gel electrophoresis, and transformation were carried out as described (Maniatis et al., 1982; Silhavy et al., 1984). Unidirectional digestion of plasmid DNA with *Exo*III was performed according to the method of Henikoff (1984), except that mung bean nuclease was used instead of S1 nuclease. The nucleotide sequence was determined by the chain termination method of Sanger et al. (1977).

Oligonucleotide-Directed Mutagenesis. A 1.3-kb *Bam*HI fragment containing the entire coding sequence for neuromodulin was excised from pKK-NEU1 and purified by agarose gel electrophoresis. This fragment was then inserted into the *Bam*HI site of M13mp18 such that the coding strand was carried by the single-stranded phage DNA. Phage was amplified in a dut^-ung^- *E. coli* strain CJ236 (Kunkel et al., 1987) to obtain uridylated template DNA. Oligonucleotide site-directed mutagenesis was carried out by using the Bio-Rad Muta-Gene in vitro mutagenesis kit according to the supplier's instructions. Two synthetic oligonucleotides with single base substitution, 5′-CGGAAGCCAGCCTGA-3′ and 5′-GTAGCAGCGCCATCT-3′, were used to prime DNA synthesis and convert the single-stranded template into a double-stranded circular molecule. These oligonucleotides were designed to change Ser-41 and Ser-86, the putative protein kinase C phosphorylation sites of recombinant neuromodulin, separately to Gly-41 and Ala-86. The mutagenesis reaction was used to transform *E. coli* strain NM522 (dut^+ung^+), and mutants were identified by direct nucleotide sequencing. The 1.3-kb *Bam*HI fragment containing either the Gly-41 or the Ala-86 mutation was excised from the replicative form phage DNA and ligated back into pKK-NEU1 which had the 1.3-kb *Bam*HI fragment removed. The resultant plasmids were designated pKK-NEU2 and pKK-NEU3, respectively. To construct a double mutant which had both serine residues altered, the 2-kb *Pvu*II fragment from pKK-NEU2 was ligated to the 3.5-kb *Pvu*II fragment from pKK-NEU3. The recombinant plasmid obtained was designated pKK-NEU4. The retention of the desired mutations in these plasmids was confirmed by nucleotide sequencing.

Expression and Purification of Recombinant Neuromodulin. Two *E. coli* strains (JM105 and SG20252) were used to express neuromodulin upon induction with IPTG. The best result was obtained with a protease-deficient mutant SG20252 (lon^-). The plasmid-harboring cells were grown at 37 °C with shaking in LB medium (Miller, 1972) containing 100 μg/mL ampicillin. At OD_{650} = 0.5–0.7, IPTG was added to a final concentration of 1 mM, and the culture was grown for an additional 2 h. Cells from 6 L of culture were pelleted by centrifugation and resuspended in 100 mL of DB buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, and 0.2 mM PMSF). All manipulations were performed at 4 °C unless otherwise noted. Cell lysis was initiated by addition of lysozyme to a final concentration of 200 μg/mL. After incubation for 30 min on ice, DNase, RNase, and $MgCl_2$ were added to final concentrations of 10 μg/mL, 10 μg/mL, and 4 mM, respectively. After incubation at room temperature for 20 min, the sample was centrifuged at 10000*g* for 20 min. The pellet was resuspended in 75 mL of DB buffer containing 5 M guanidine hydrochloride and then sonicated for 5 min using a Heat System W140 sonicator with the power setting at 30% of maximum. The suspension was stirred on ice for 1.5 h and then centrifuged at 25 000 rpm for 30 min at 4 °C in a Beckman Ti35 rotor. The supernatants were pooled, and Lubrol PX was added to a final concentration of 0.1%. The solubilized extract was dialyzed overnight against 4 L of dialysis buffer (DB buffer containing 0.1% Lubrol PX), and the clear supernatant was collected after centrifugation at 10000*g* for 20 min. This sample was then loaded onto a 40-mL CaM–Sepharose column which had been equilibrated in DB buffer. After the column was washed with 200 mL of DB buffer, recombinant neuromodulin was eluted from CaM–Sepharose with DB buffer containing 4 mM $CaCl_2$ and 1 μg/mL each of the three protease inhibitors (aprotinin, pepstatin, and leupeptin). Fractions containing neuromodulin were pooled and concentrated with an Amicon YM5 filter. Protein concentration was determined by the method of Lowry et al.

(1951) using BSA as standard.

Immunoblot Analyses. Rabbit polyclonal antibodies directed against bovine neuromodulin have been described previously (Cimler et al., 1985). One milliliter of IPTG-induced *E. coli* culture was centrifuged in a microfuge for 2 min, and the cell pellet was lysed in 100 μL of sample buffer according to Laemmli (1970). Samples were boiled for 5 min, and 20 μL per well was loaded onto a 10% SDS–polyacrylamide gel. Proteins separated by SDS–PAGE were electrophoretically transferred onto nitrocellulose paper with the ABN Polyblot Transfer System according to manufacturer's instructions. The electrophoretic transfer was carried out for 1 h at 280 mA. To prevent nonspecific protein binding in subsequent steps, the nitrocellulose paper was blocked by incubation for 1 h at room temperature with TPBS (Tween 20, 5 g/L; KH_2PO_4, 61.25 g/L; NaOH, 10.25 g/L; NaCl, 87.75 g/L) containing 4% (w/v) BSA. The nitrocellulose paper was rinsed 3 times with H_2O and 1 time with TPBS and then incubated overnight at 4 °C with 50 mL of a 1:500 dilution of immune IgG in TPBS. After the nitrocellulose paper was washed 3 times with TPBS, it was incubated with horseradish peroxidase linked goat anti-rabbit IgG for 1 h at room temperature. The nitrocellulose paper was then washed 2 times with TPBS and 1 time with 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl (buffer A), and the antigen–antibody complexes were visualized by incubating the nitrocellulose paper with buffer A containing 20% methanol, 5 mg/mL 4-chloro-1-naphthol, and 0.03% H_2O_2. Staining was terminated by washing the paper several times with buffer A.

Protein Preparations. Bovine neuromodulin was purified according to the method of Masure et al. (1986). Calmodulin was prepared as described by Masure et al. (1984). Calmodulin–Sepharose was prepared from purified CaM and cyanogen bromide activated Sepharose 4B as described by Westcott et al. (1979). Immunoaffinity-purified 5-[[2-[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid labeled CaM (AEDANS-CaM) was prepared by the method of Olwin et al. (1983). Preparation of protein kinase C has been described previously (Alexander et al., 1987). Units for protein kinase C activity are expressed in nanomoles of phosphate transferred per minute per milligram.

Phosphorylation of Neuromodulin. Phosphorylation of bovine and recombinant neuromodulin was assayed in a buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 50 mM ATP + [γ-^{32}P]ATP; 200 μg/mL phosphatidyl-L-serine and 20 μg/mL diolein were added to some samples as indicated. Free calcium was maintained at 400 μM in the +Ca^{2+} samples, while EGTA was added to 5 mM in the +EGTA samples. Total sample volume was 50 μL. Reactions were initiated by the addition of protein kinase C to 0.4 μg/mL. Samples were placed in a 30 °C water bath for 45 min and stopped by the addition of 5× SDS–PAGE sample buffer (0.2 M Tris-HCl, pH 6.9, 10 mM EGTA, 10% glycerol, 5% DTT, 5% SDS, and 0.2% bromophenol blue). Samples were then boiled and subjected to SDS–PAGE (10% acrylamide). The gel was stained with Coomassie Blue, destained, dried, and autoradiographed.

The stoichiometry of phosphorylation of the recombinant wild-type protein was carried out in 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$ 1 mM DTT, 0.5 mM calcium, 1 mM [γ-^{32}P]ATP (200–300 cpm/pmol), 200 μg/mL phosphatidyl-L-serine, 20 μg/mL diolein, and 4 μM neuromodulin. The reaction was initiated with the addition of 0.3 μg/mL protein kinase C and placed in a 30 °C water bath. Five-microliter aliquots were removed at various times and added to 45 μL of 0.1% BSA and 10% cold TCA. Total counts associated with phosphorylated protein in each sample were determined by using phosphocellulose (P81) paper. Greater than 95% of the neuromodulin was absorbed to the phosphocellulose (P81) paper. Each sample was applied onto a small square of P81 paper and allowed to dry. Papers were rinsed with 1% phosphoric acid to eliminate excess free ATP. Total radioactivity associated with each protein sample was then quantitated by using liquid scintillation counting.

Fluorescence Measurements. Fluorescence measurements were made as described by Alexander et al. (1987). The dissociation constant for the neuromodulin·AEDANS-CaM complex was determined in the absence of free Ca^{2+} by measurement of the steady-state anisotropy of AEDANS-CaM fluorescence as a function of added neuromodulin.

Results

Construction of a Neuromodulin Expression Vector. In order to obtain efficient expression of the cDNA in *E. coli*, it was necessary to place it downstream from a strong promoter to generate large quantities of mRNA. In addition, a ribosome binding site must be present immediately upstream from the coding region of the mRNA to initiate translation. A neuromodulin expression vector (Figure 1) was constructed from the cloned neuromodulin cDNA (Cimler et al., 1987) and plasmid pKK223-3 (Amann et al., 1983) as described under Materials and Methods. The expression plasmid pKK-NEU1 contained a hybrid trp-lac (tac) promoter (deBoer et al., 1983) which has been shown to be one of the strongest promoters in *E. coli*. Furthermore, the ATG translation initiation codon was positioned eight bases downstream from the Shine–Dalgarno sequence (Amann et al., 1983). This construct was used in subsequent expression and mutagenesis experiments.

Expression and Purification of Recombinant Neuromodulin. Two *E. coli* strains were transformed with the expression plasmid pKK-NEU1. After induction with IPTG, recombinant neuromodulin was detected only in the whole cell lysate of strain SG20252 (lon$^-$), but not in the lysate of strain JM105 by Western blot analysis. In the absence of the cDNA sequence for neuromodulin, the cells failed to produce detectable levels of neuromodulin. These data indicated that synthesis of the immunoreactive protein was directed by the cloned murine cDNA insert. The recombinant neuromodulins expressed in *E. coli* migrated faster than the bovine protein on SDS–polyacrylamide gels, consistent with the fact that murine neuromodulin is 12 amino acid residues shorter than the bovine protein (Cimler et al., 1987; Wakim et al., 1987).

When cultures of *E. coli* strain SG20252(pKK-NEU1) were lysed and fractionated, recombinant neuromodulin was found in the 10000*g* pellet but not in the supernatant. Neuromodulin could be extracted from the insoluble pellet only with strong denaturants such as guanidine hydrochloride or SDS and was not extractable with the nonionic detergents Triton X-100 or Lubrol PX. To purify the recombinant protein from the insoluble pellet, it was solubilized with 5 M guanidine hydrochloride and renatured as described under Materials and Methods. The soluble extract was then applied to a CaM–Sepharose column in the absence of Ca^{2+}. After the column was washed extensively to remove unbound protein, recombinant neuromodulin was eluted from the column with a Ca^{2+}-containing buffer. SDS–PAGE analysis of the eluted fractions showed the presence of a single protein band with an electrophoretic mobility higher than that of bovine neuromodulin (Figure 2). Starting from 6 L of induced cultures, 1.5 mg of purified protein was routinely obtained. The purified

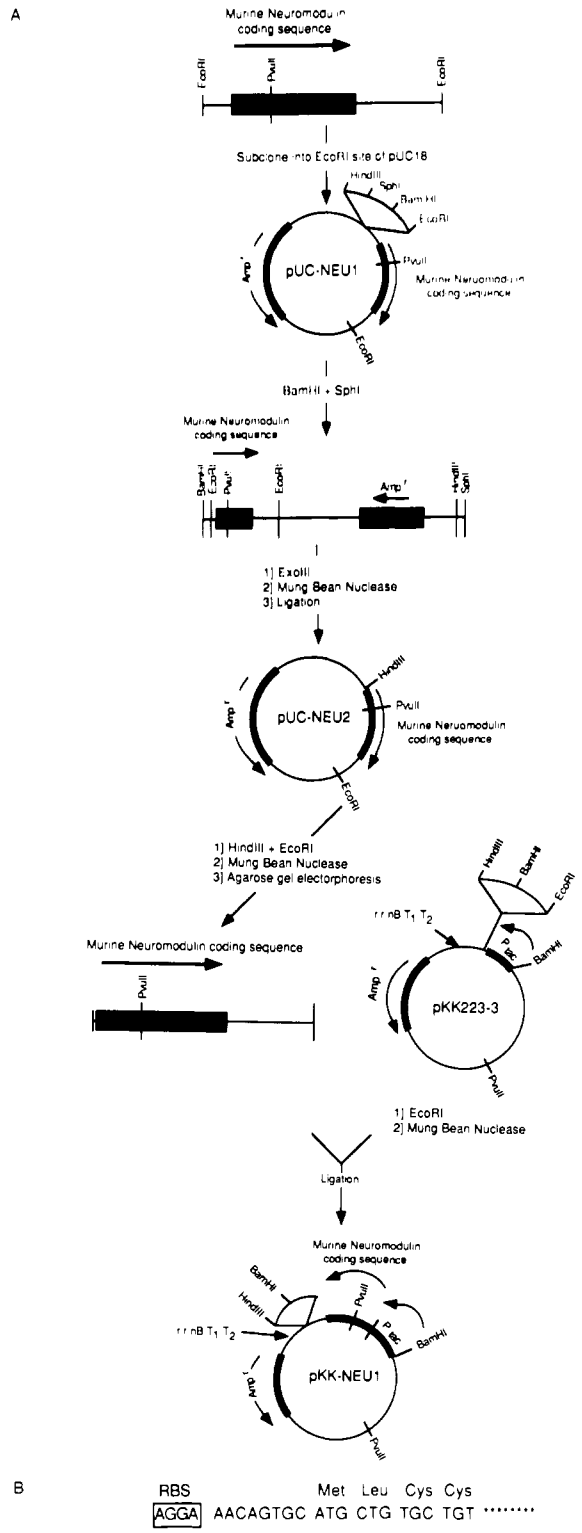

FIGURE 1: Construction of the murine neuromodulin expression vector pKK-NEU1. (A) The murine neuromodulin cDNA fragment containing the entire coding and some 5'- and 3'-noncoding sequence was inserted into the *Eco*RI site of pUC18 to generate pUC-NEU1. After cleavage of this plasmid with *Bam*HI and *Sph*I, the 5'-noncoding sequence was removed by *Exo*III and mung bean nuclease treatment as described under Materials and Methods. The murine neuromodulin coding sequence was excised from the plasmid pUC-NEU2 with *Hin*dIII and *Eco*RI and then treated with mung bean nuclease to create blunt ends. The blunt-ended cDNA fragment was purified from agarose gel and ligated to the expression vector pKK223-3 that had been linearized and treated with mung bean nuclease to remove the sticky ends. The orientation of the neuromodulin cDNA fragment in the final construct pKK-NEU1 was determined by restriction mapping. (B) With the dideoxy chain termination method, the translation initiation codon (ATG) for neuromodulin was found to be eight nucleotides downstream from the ribosome binding sequence (AGGA).

protein was recognized by rabbit polyclonal antibodies raised against purified bovine neuromodulin (data not shown).

Determination of the CaM-Recombinant Neuromodulin Dissociation Constant. Fluorescence anisotropy was used to determine the dissociation constant for the recombinant neuromodulin·AEDANS-CaM complex (Figure 3). When the fractional AEDANS-CaM association with neuromodulin was plotted as a function of recombinant neuromodulin concentration, the resultant curve could be fitted to an equation (Adair, 1925) describing a single class of noninteracting AEDANS-CaM binding sites. In the absence of added Ca^{2+}, the recombinant neuromodulin·AEDANS-CaM dissociation constant was determined to be 0.52 μM, a value comparable to that observed with neuromodulin purified from bovine brain (Alexander et al., 1987).

Phosphorylation of Recombinant Neuromodulin by Protein Kinase C. Bovine neuromodulin is phosphorylated by protein kinase C with the loss of its affinity for CaM (Alexander et al., 1987). The stoichiometry for phosphorylation of the protein purified from bovine brain was 1.3 ± 0.2 P_i/mol of neuromodulin. As reported in Figure 4, the purified preparation of recombinant neuromodulin also served as a substrate for protein kinase C in a Ca^{2+}- and phospholipid-dependent manner. The kinetics for phosphorylation of recombinant neuromodulin by protein kinase C and the neuromodulin concentration dependence for protein kinase C phosphorylation are reported in Figure 5. The protein kinase C K_m's for recombinant neuromodulin and bovine neuromodulin were both approximately 1 μM. The stoichiometry for phosphorylation of the purified recombinant neuromodulin was 0.9 ± 0.2 P_i/mol of neuromodulin.

The ability of the phosphorylated recombinant neurmodulin to interact with CaM–Sepharose was also investigated. Phosphoneuromodulin did not associate with CaM–Sepharose in the presence of calcium or excess EGTA (data not shown). Instead, phosphoneuromodulin eluted in the column void volume, indicating that the affinity of recombinant neuromodulin for CaM was greatly reduced by phosphorylation. This is a property of the protein shared by bovine neuromodulin. It has been previously reported that neuromodulin undergoes autophosphorylation (Meiri & Willard, 1986). However, incubation of recombinant neuromodulin with [γ-^{32}P]ATP and divalent metal ions without protein kinase C present did not result in the incorporation of ^{32}P into the protein (Figure 4). Similar results were obtained with highly purified neuromodulin purified from bovine brain.

Construction of and Expression of Mutant Neuromodulins. Previous studies in this laboratory have demonstrated that protein kinase C phosphorylated neuromodulin contained phosphoserine as the sole phosphoamino acid (Alexander et al., 1987). Preliminary evidence has indicated that neuromodulin can be phosphorylated on Ser-41 and Ser-86 (unpublished results). Three mutants having either or both serine residues mutated were, therefore, constructed by oligonucleotide-directed mutagenesis (Figure 6). The oligonucleotides were designed to alter the AGC and TCT codons for these serine residues to GGC and GCT specifying Gly-41 and Ala-86, respectively. Following transformation of the mutant plasmids into *E. coli* strain SG20252, immunoreactive proteins with the expected molecular weight could be detected in the cell extracts by Western blot analysis. The mutant proteins were purified to homogeneity by the same procedure as employed for the isolation of wild-type recombinant neuromodulin, illustrating that this system is suitable to prepare mutagenized neuromodulins for structure–function relationship studies.

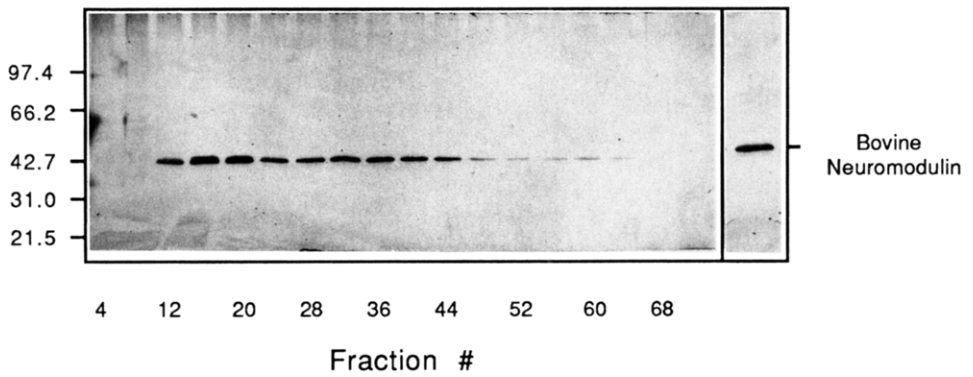

FIGURE 2: Elution of wild-type recombinant neuromodulin from CaM–Sepharose by Ca^{2+}. Recombinant neuromodulin was extracted from the insoluble cell material with guanidine hydrochloride, dialyzed, and loaded into a 40-mL CaM–Sepharose column in the absence of Ca^{2+} as described under Materials and Methods. The column was washed with 200 mL of 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, and 0.2 mM PMSF and eluted with 500 mL of the same buffer containing 4 mM Ca^{2+}. Five-milliliter fractions were collected, and 20-μL aliquots of selected fractions were run on a 10% SDS–polyacrylamide gel. Proteins were visualized by staining the gel with silver stain.

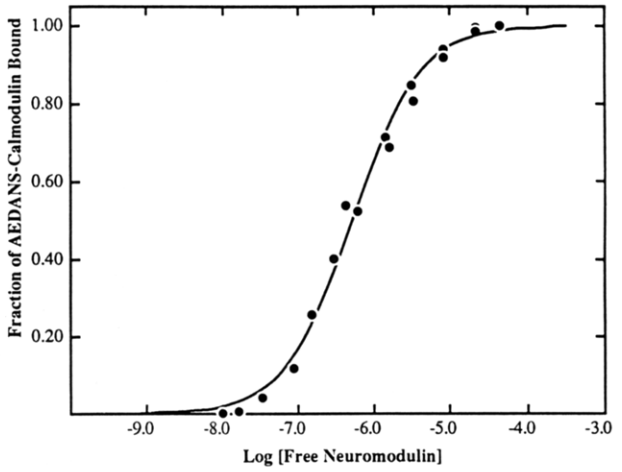

FIGURE 3: Determination of the dissociation constant (K_d) for the recombinant neuromodulin·AEDANS-calmodulin complex. The fraction of immunopurified AEDANS-calmodulin bound is plotted as a function of the log of the free molar neuromodulin concentration. Titration of 93.8 pmol of AEDANS-calmodulin began in 0.375 mL of 10 mM MOPS, pH 7.4, and 5 mM EGTA. Emitted light was filtered through Schott KV 470 filters. The final sample volume did not exceed 0.550 mL, and the blank fluorescence did not exceed 31% of the sample intensity. Two independent titrations were carried out, and the data points shown were generated from the fluorescence measurements as described under Materials and Methods. The average anisotropies of the free (A_f) and bound (A_b) species were 0.029 and 0.053. The average fractional fluorescence intensity (q) was 0.93. The solid line is a nonlinear least-squares fit of the data to the Adair equation for a single class of noninteracting binding sites.

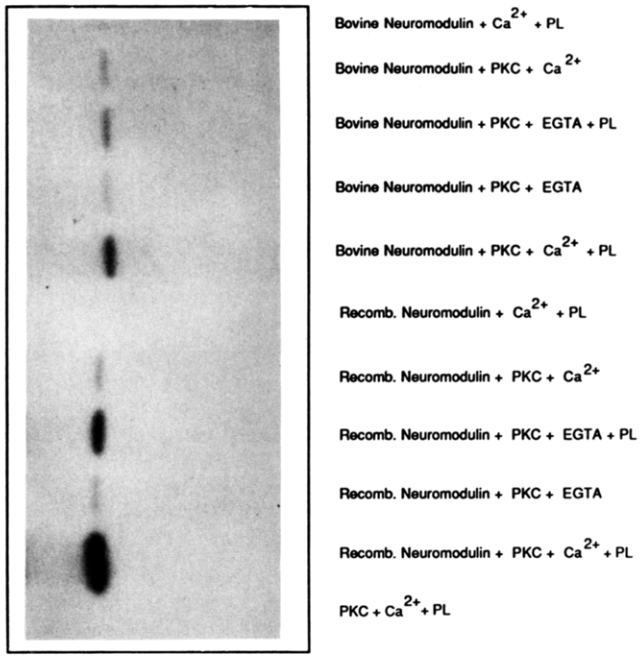

FIGURE 4: Phosphorylation of bovine and recombinant neuromodulins by protein kinase C. One microgram of bovine neuromodulin and 2 μg of recombinant neuromodulin were assayed as substrates for phosphorylation by protein kinase C in a buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 50 mM ATP + [γ-^{32}P]ATP, and 400 μM $CaCl_2$ or 5 mM excess EGTA. 200 μg/mL phosphatidyl-L-serine and 20 μg/mL diolein (designated as PL) were added where indicated. Total sample volume was 50 μL. Reactions were initiated by the addition of protein kinase C to approximately 0.4 μg/mL. Samples were placed in a 30 °C water bath for 45 min and stopped by the addition of 5× SDS–PAGE sample buffer. Samples were then boiled, subjected to SDS–PAGE, and autoradiographed.

Discussion

Neuromodulin was first discovered and purified to homogeneity by virtue of its unique CaM binding properties (Andreasen et al., 1981, 1983). Recent studies have shown that phosphorylation of neuromodulin by protein kinase C strongly inhibits binding of the protein to CaM (Alexander et al., 1987). On the basis of these observations, neuromodulin has been proposed to function to sequester CaM under low-Ca^{2+} conditions at the neuronal cell membrane in the vicinity of CaM-regulated proteins. Phosphorylation of neuromodulin by protein kinase C and/or an increase in intracellular free Ca^{2+} would result in the release of bound CaM, thereby allowing CaM to interact with its target proteins.

A full-length cDNA clone encoding murine neuromodulin has recently been isolated (Cimler et al., 1987). Nucleotide sequence analysis of the murine cDNA clone indicated that neuromodulin is nearly identical with GAP-43, a protein associated with axonal growth and neurite extension (Basi et al., 1987; Cimler et al., 1987; Karns et al., 1987). In order to elucidate the structure–function relationships of this protein, an expression system was developed for the generation of wild-type and mutant neuromodulins with specific modifications. Upon transformation of the neuromodulin expression vector (pKK-NEU1) into an *E. coli* lon$^-$ host, neuromodulin was detected in the whole cell extract by Western blotting using rabbit polyclonal antibodies against bovine neuromodulin.

Recombinant neuromodulin isolated from *E. coli* was shown to be insoluble and could not be extracted with a variety of nonionic detergents including Triton X-100 and Lubrol PX. Solubilization of the protein was achieved by using a strong denaturant, guanidine hydrochloride. Following extensive

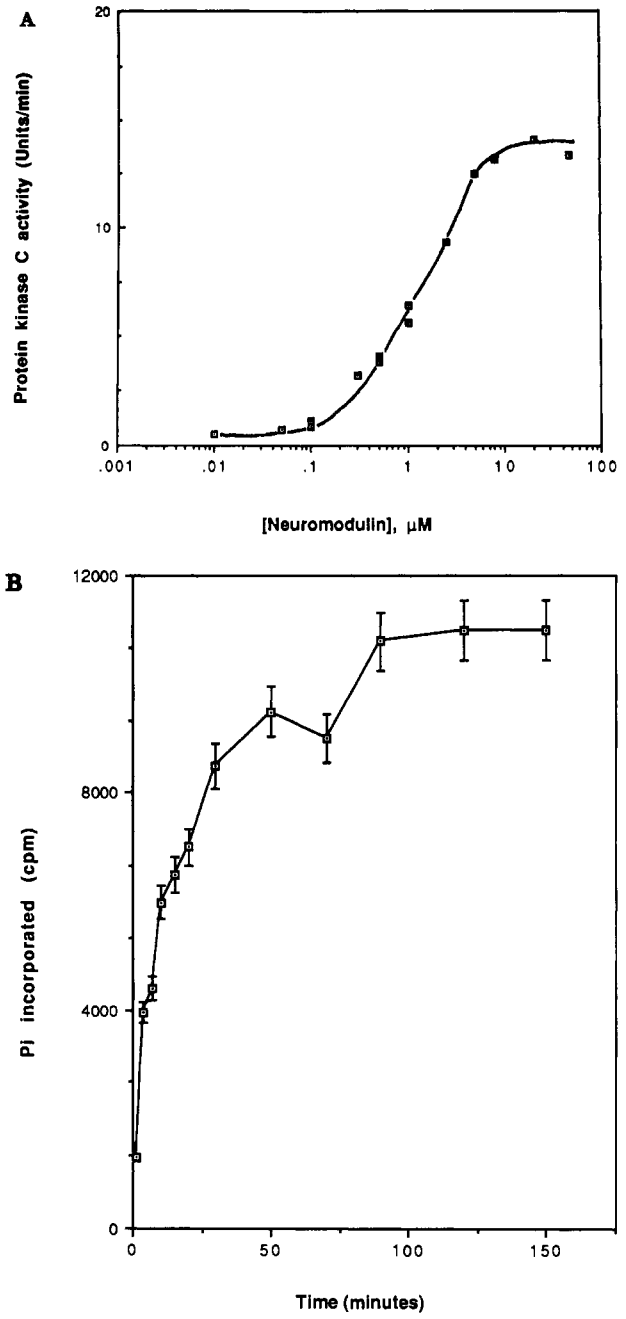

FIGURE 5: Kinetics for phosphorylation of recombinant neuromodulin by protein kinase C. (Panel A) The activity of protein kinase C was determined as a function of the concentration of purified recombinant wild-type neuromodulin. Phosphorylation assays were carried out in a buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 50 μM ATP + [γ-^{32}P]ATP (2000–4000 cpm/pmol), 200 μg/mL phosphatidyl-L-serine, 20 μg/mL diolein, 400 μM free Ca^{2+}, and neuromodulin at the varying concentrations indicated. Reactions were initiated by addition of protein kinase C (approximately 0.2 μg/mL) and allowed to incubate for 15 min at 30 °C. Reactions were stopped by addition of 5× SDS–PAGE sample buffer and immediate boiling. Samples were subjected to SDS–PAGE electrophoresis (10% gels) and stained to identify protein bands, followed by gel slicing and liquid scintillation counting to quantitate the total radioactivity associated with neuromodulin. (Panel B) Recombinant wild-type neuromodulin (4 μM) was treated with protein kinase C and [γ-^{32}P]ATP for various periods of time, and the total amount of [^{32}P]phosphate incorporated was quantitated as described under Materials and Methods. The maximal level of phosphorylation was 1.9 ± 0.2 mol of P_i/mol of neuromodulin.

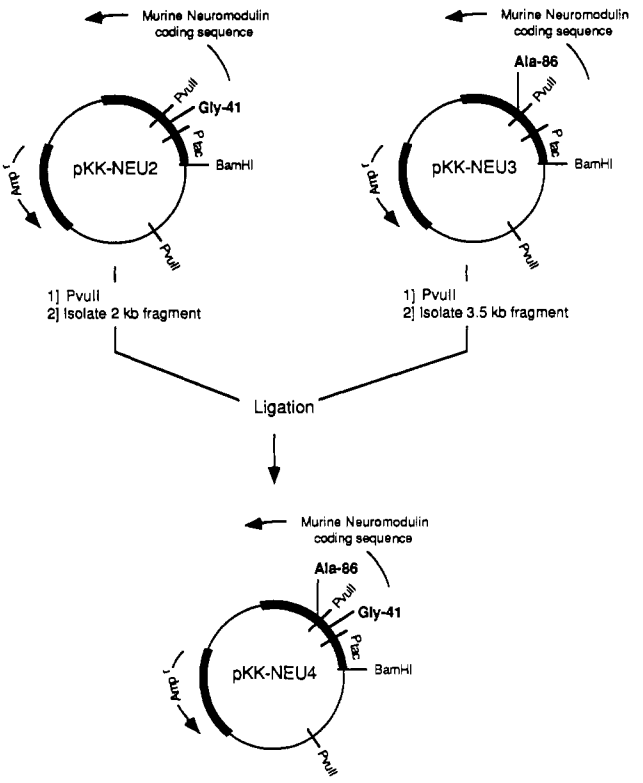

FIGURE 6: Construction of mutant neuromodulin expression plasmids. Plasmids pKK-NEU2 and pKK-NEU3 carrying the Gly-41 and Ala-86 mutations, respectively, were constructed by oligonucleotide-directed mutagenesis as described under Materials and Methods. Plasmid PKK-NEU4 was constructed by ligating the 2-kb *Pvu*II fragment from pKK-NEU2 to the 3.5-kb *Pvu*II fragment from pKK-NEU3.

dialysis to renature the protein, recombinant neuromodulin was purified to apparent homogeneity by CaM–Sepharose affinity chromatography. Recombinant neuromodulin, like bovine neuromodulin, was absorbed to CaM–Sepharose in the absence of Ca^{2+} and eluted from the column upon addition of a Ca^{2+}-containing buffer. This provides evidence that recombinant neuromodulin has greater affinity for CaM in the absence of free Ca^{2+} than in its presence. The apparent molecular weight of the purified recombinant protein was smaller than that of bovine neuromodulin, consistent with the fact that murine neuromodulin is 12 amino acid residues shorter (Cimler et al., 1987; Wakim et al., 1987). Western blot analysis of rat and bovine brain neuromodulins also demonstrated that rat brain neuromodulin had a slightly lower molecular weight compared to the bovine protein (Cimler et al., 1985).

The stoichiometry for protein kinase C catalyzed phosphorylation of neuromodulin purified from bovine brain was 1.3 ± 0.2 P_i/mol of neuromodulin (Alexander et al., 1987). However, we have observed some variability in the stoichiometry for phosphorylation when comparing different preparations of the brain protein. These observations suggested that the protein isolated from rat brain was obtained in a partially phosphorylated form with some variation in the extent of phosphorylation from one preparation to another. The availability of the purified recombinant protein allowed us to unambiguously determine that the stoichiometry for phosphorylation of neuromodulin is 0.9 ± 0.2 P_i/mol of neuromodulin.

Recent studies have indicated that GAP-43 is synthesized in vivo as a soluble protein and becomes attached to membranes posttranslationally via fatty acylation of a short hydrophilic region at the amino terminus of the protein (Skene & Virag, 1989). Although the recombinant protein may lack any posttranslational modifications including fatty acid acy-

lation, all biochemical properties examined were similar between native bovine neuromodulin and the wild-type recombinant protein. Under low ionic strength conditions, the dissociation constant of the recombinant neuromodulin·AEDANS-CaM complex was determined to be 0.52 μM. This value compares favorably with the dissociation constant of 0.23 μM determined under the same conditions for neuromodulin purified from bovine brain (Alexander et al., 1987). The wild-type recombinant protein was phosphorylated by protein kinase C in a Ca^{2+}- and phospholipid-dependent manner. Furthermore, phosphorylation of the recombinant protein by protein kinase C greatly reduced its affinity for calmodulin.

There has been a preliminary report indicating that purified brain neuromodulin (GAP-43) undergoes autophosphorylation, and it was proposed that the protein may be a protein kinase (Meiri & Willard, 1986). The recombinant neuromodulin, described in this study, was not autophosphorylated in the presence of [γ-^{32}P]ATP. Furthermore, highly purified bovine brain neuromodulin was also not autophosphorylated. Autophosphorylations of neuromodulin previously reported were most likely due to minor levels of protein kinase contamination, a problem not encountered with the recombinant protein from *E. coli*.

The results reported in this study demonstrated the feasibility of using this expression system to generate neuromodulin with specific alterations for structure–function relationships of the protein. The availability of these mutant neuromodulins should allow systematic elucidation of the molecular basis of the biochemical and physiological properties of this protein.

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