Identification of the Protein Kinase C Phosphorylation Site in Neuromodulin†

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ABSTRACT: Neuromodulin (P-57, GAP-43, B-50, F-1) is a neurospecific calmodulin binding protein that is phosphorylated by protein kinase C. Phosphorylation by protein kinase C has been shown to abolish the affinity of neuromodulin for calmodulin [Alexander, K. A., Cimler, B. M., Meier, K. E., & Storm, D. R. (1987) *J. Biol. Chem. 262*, 6108–6113], and we have proposed that the concentration of free CaM in neurons may be regulated by phosphorylation and dephosphorylation of neuromodulin. The purpose of this study was to identify the protein kinase C phosphorylation site(s) in neuromodulin using recombinant neuromodulin as a substrate. Toward this end, it was demonstrated that recombinant neuromodulin purified from *Escherichia coli* and bovine neuromodulin were phosphorylated with similar K_m values and stoichiometries and that protein kinase C mediated phosphorylation of both proteins abolished binding to calmodulin–Sepharose. Recombinant neuromodulin was phosphorylated by using protein kinase C and [γ-^{32}P]ATP and digested with trypsin, and the resulting peptides were separated by HPLC. Only one ^{32}P-labeled tryptic peptide was generated from phosphorylated neuromodulin. The sequence of this peptide was IQASFR. The serine in this peptide corresponds to position 41 of the entire protein, which is adjacent to or contained within the calmodulin binding domain of neuromodulin. A synthetic peptide, QASFRGHITRKKLKGEK, corresponding to the calmodulin binding domain with a few flanking residues, including serine-41, was also phosphorylated by protein kinase C. We conclude that serine-41 is the protein kinase C phosphorylation site of neuromodulin and that phosphorylation of this amino acid residue blocks binding of calmodulin to neuromodulin. The proximity of serine-41 to the calmodulin binding domain in neuromodulin very likely explains the effect of phosphorylation on the affinity of neuromodulin for calmodulin.

Neuromodulin (previously designated P-57) is a neurospecific, membrane-associated protein which binds to calmodulin (CaM)[1] with higher affinity in the absence of Ca^{2+} than in the presence of Ca^{2+}. Neuromodulin has been purified to apparent homogeneity from bovine brain membrane preparations using CaM–Sepharose affinity chromatography (Andreasen et al., 1983). The amino acid sequence of the protein has been directly determined (Wakim et al., 1987), and cDNAs encoding the protein have been cloned and sequenced (Karns et al., 1987; Cimler et al., 1987). The availability of sequence data has made it clear that neuromodulin is very similar to, if not identical with, proteins designated GAP-43, B-50, F-1, and pp46 (Cimler et al., 1987). Although neuromodulin migrates with an apparent molecular weight of 57K, its true molecular weight determined from hydrodynamic studies (Masure et al., 1986) and calculated from its amino acid sequence (Wakim et al., 1987) is 24.7K.

Neuromodulin is phosphorylated by protein kinase C in vitro (Alexander et al., 1987; Aloyo et al., 1983; Chan et al., 1986; Nelson & Routtenberg, 1985; Willard et al., 1987), and phosphorylation inhibits binding of CaM to neuromodulin (Alexander et al., 1987). In addition, phosphoneuromodulin is a substrate for the CaM-stimulated phosphatase calcineurin (Liu & Storm, 1989). Neuromodulin is not a substrate for cAMP-dependent protein kinase or calcium–calmodulin kinases type I and II (Chan, 1986; Baudier et al., 1989), but its phosphorylation by casein kinase II has been reported (Pisano, 1988). Phosphoneuromodulin has been detected in nerve growth cone particles in fetal rat brain (Van Hooff et al., 1988), synaptosomal plasma membranes from adult brain (Eichberg et al., 1986; Zwiers et al., 1985; DeGraan et al., 1989), intact hippocampal slices (DeGraan et al., 1989), and the goldfish optic nerve (Larrivee & Grafstein, 1987; Perrone-Bizzozero & Benowitz, 1987). The physiological role of neuromodulin and phosphoneuromodulin is not known, but we have proposed that neuromodulin may bind and concentrate CaM at specific sites within neurons including neuronal growth cones. Free CaM may then be released locally in response to phosphorylation by protein kinase C (Alexander et al., 1987).

In this study, we determined the protein kinase C phosphorylation site of neuromodulin by isolation and sequencing of a single phosphopeptide obtained from the phosphorylated protein. The phosphorylation site is adjacent to the CaM binding domain of the protein and very likely explains why CaM binding is blocked by phosphorylation.

Materials and Methods

Protein Preparations. Wild-type recombinant neuromodulin was expressed in and purified from *Escherichia coli* as previously described (Au et al., 1989). Bovine neuromodulin was purified according to the method of Masure et al. (1986). CaM was prepared as outlined by Gopalakrishna and Anderson (1982), and CaM–Sepharose was prepared from pu-

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[1] Abbreviations: CaM, calmodulin; DTT, dithiothreitol; DPTU, diphenylthiourea; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; PTH, phenylthiohydantoin; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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rified CaM and cyanogen bromide activated Sepharose 4B as described by Westcott et al. (1979).

Protein kinase C was prepared by modifications of previously described methods. Cytosolic extract was prepared from rat brain by the method of LePeuch et al. (1983). The extract was batch-absorbed to preequilibrated DEAE-Sephacel (Pharmacia P-L Biochemicals) and eluted with buffer containing 0.1 M NaCl. The enzyme was then precipitated with ammonium sulfate added to 70% saturation, resuspended in buffer, and applied to a preequilibrated 1.5 × 85 cm Sephacryl S-200 (Pharmacia P-L Biochemicals) column as described (LePeuch et al., 1983). Active fractions were pooled and applied to phenyl-Sepharose (Pharmacia P-L Biochemicals) as described by Wolf et al. (1984). Fractions with peak activity were loaded to a freshly made and equilibrated phosphatidylserine affinity column for further purification (Uchida & Filburn, 1984). The enzyme was stored in 2.5 mM MES, pH 6.5, 100 mM KCl, 1 mM EGTA, 2.5 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/mL leupeptin and pepstatin, and 50% glycerol at −20 °C. The specific activity of this preparation was approximately 2000 nmol of phosphate transferred min^{-1} mg^{-1} using histone III-S as a substrate.

Concentrations of neuromodulin were determined by the method of Lowry (1951), and protein kinase C concentrations were determined by using a modification of the Bradford method (1976).

Phosphorylation Assays. Phosphorylation of recombinant and bovine neuromodulin was assayed in a buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 50–500 μM [γ-^{32}P]ATP (2000–4000 cpm/pmol). Phosphatidyl-L-serine (200 μg/mL) and diolein (20 μg/mL) were added to samples as indicated. Free [Ca^{2+}] was maintained at 400 or 600 μM in the $+Ca^{2+}$ samples, while EGTA was added to 5 mM in the $-Ca^{2+}$ samples. Total volume of the reactions ranged from 50 to 750 μL. Reactions were initiated by the addition of protein kinase C (0.2–0.4 μg/mL) and incubated at 30 °C for the indicated time. Reactions were stopped by addition of 5× SDS–PAGE sample buffer (0.2 M Tris, pH 6.9, 10 mM EGTA, 10% glycerol, 5% DTT, 5% SDS, and 0.2% bromophenol blue) and immediate boiling, or by adding excess EGTA and placing samples on ice, or by the addition of 0.1% BSA and 25% cold TCA. Analysis of phosphorylated neuromodulin was then carried out by the appropriate methods as described below or in figure legends.

For K_m determinations, phosphorylation assays were carried out as described above with neuromodulin at varying concentrations ranging from 0.01 to 50 μM. The activity of protein kinase C was determined as a function of the concentration of purified recombinant wild-type and bovine neuromodulin. These 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 total radioactivity associated with neuromodulin.

For the determination of phosphorylation stoichiometry, phosphorylation conditions were as described above, but aliquots were removed at various time points from 0 to 150 min and added to 45 μL of 0.1% BSA and 25% cold TCA. Total counts associated with phosphorylated protein in each sample were determined by dotting each precipitated sample on glass fiber filters (GF/F) and rinsing with 3 mL of cold 25% TCA with suction filtering, followed by liquid scintillation counting of the filters.

Phosphoneuromodulin Application to CaM–Sepharose. The most convenient assay to determine binding of neuromodulin or phosphoneuromodulin to CaM is to monitor adsorption of the protein to CaM–Sepharose in the absence of free Ca^{2+} (Alexander et al., 1987). Phosphorylations were carried out under the same conditions as described above. Reactions were stopped by the addition of EGTA to 2 mM and immediately placing on ice. Each sample was applied to 5 mL of preequilibrated CaM–Sepharose, and the mixture was incubated overnight at 4 °C. These slurries were then poured into small plastic Bio-Rad "Econo-columns", and the flow-through was collected. The column was washed with 10 mL of buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 1 mM DTT, and 1 mM PMSF) and then eluted with buffer containing 2 mM free Ca^{2+}. Collected fractions were subjected to SDS–PAGE electrophoresis and autoradiography for the detection of phosphorylated neuromodulin.

Preparation of Phosphoneuromodulin for Trypsin Digest. Purified homogeneous recombinant neuromodulin (6–16 nmol) was phosphorylated under conditions described above using 350 μM [γ-^{32}P]ATP. The reaction was terminated by adding excess EGTA and placing on ice. The reaction mixture was dialyzed against Milli Q water for 24 h with several changes to remove free ATP and salts prior to lyophilization. Each sample was resuspended in 100 μL of 6 M guanidine hydrochloride/10 mM KH_2PO_4, pH 6.0, and applied to two 21.5 mm × 600 cm TSK 3000 HPLC columns in tandem with a flow rate of 0.9 mL/min to separate neuromodulin from the occasional minor higher molecular weight contaminating species. The eluted samples were dialyzed for 18 h with three changes against Milli Q water and dried.

Tryptic Digestion and Separation of Peptides. Each phosphoneuromodulin sample (3–10 nmol) was solubilized in 100 μL of 50 mM ammonium bicarbonate, pH 8.0, following the removal of excess ATP and TSK 3000 HPLC separation as described above. Tosylphenylalanyl chloromethyl ketone treated trypsin (Worthington, Inc.) was added at a protease/substrate ratio of 2% by weight, and the mixture was incubated for 1 h at 37 °C. A second identical aliquot of trypsin was added, and the incubation was continued for an additional 30 min. The samples were dried, subsequently resuspended in 0.1% TFA, and injected onto an Aquapore 2.1 mm × 100 mm RP-300 C8 (Pierce) reverse-phase HPLC column to separate the tryptic fragments. The mobile phase was 0.1% aqueous TFA, and the modifier was 80% acetonitrile, 20% water, and 0.08% TFA. The HPLC system components were from Waters. Peptides were optimally separated by using a 0–10% gradient over 5 min after approximately 15 min of isocratic elution with 0.1% TFA, followed by a 10–20% gradient over 50 min and finally to 40% modifier in 20 min. Absorbance was monitored at 206 nm, and the flow rate was 0.3 mL/min. Peptides were collected manually, and total radioactivity associated with each isolated peptide was analyzed by Cerenkov counting. Radioactive peptides were subjected to amino acid sequence analysis on a 477A Applied Biosystems liquid pulse sequenator with an Applied Biosystems 120A PTH analyzer on-line.

Peptide Synthesis and Purification. Standard solid-phase methods were used for synthesis by using an Applied Biosystems 430A peptide synthesizer and Applied Biosystems reagents. The crude mixture of synthetic peptides was purified by reversed-phase HPLC using a SynChropak RP-P column. A 0–18% gradient of acetonitrile + 0.08% TFA, in H_2O +

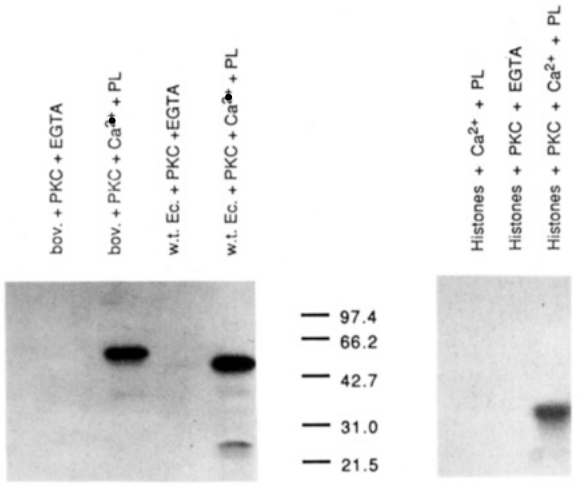

FIGURE 1: Phosphorylation of bovine and recombinant neuromodulin by protein kinase C. The phosphorylation of bovine and recombinant neuromodulins, and histones, by protein kinase C was characterized in a buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (2000–4000 cpm/pmol), and 400 μM free Ca^{2+} or excess EGTA as indicated. Phosphatidyl-L-serine (200 μg/mL) and 20 μg/mL diolein (designated as PL) were added where indicated. Reactions were initiated by addition of protein kinase C (0.4 μg/mL) and allowed to incubate for 45 min at 30 °C. Reactions were stopped by addition of 5× SDS–PAGE sample buffer and immediate boiling. Samples were then subjected to SDS–PAGE electrophoresis and autoradiography. Amounts of protein phosphorylated in each lane were 2 μg of wild-type *E. coli* neuromodulin (w.t. Ec), 2 μg of bovine brain neuromodulin (bov.), and 10 μg of histone III-S.

0.1% TFA, was used to elute purified peptide. Peptide elution from the column was monitored by UV absorption at 206 nm. Peptide peaks were collected and analyzed both for amino acid composition and for sequence. Amino acid composition of the synthetic peptide was determined from a 20-h hydrolysate by reversed-phase HPLC with a Waters Picotag system. The sequence of the synthetic peptide was confirmed by Edman degradation using a Beckman 890C sequencer. Phenylthiohydantoins from the sequencer were identified by HPLC (Glajch et al., 1985).

RESULTS

Phosphorylation of Recombinant Neuromodulin by Protein Kinase C and the Effect of Phosphorylation on CaM Binding. The protein kinase C phosphorylation site of neuromodulin was determined by using recombinant neuromodulin purified from *E. coli* since this protein was readily obtainable in an unphosphorylated form. Before analysis of the phosphorylated recombinant protein was pursued by proteolysis and sequence analysis of phosphorylated peptides, protein kinase C mediated phosphorylation of the recombinant neuromodulin preparation and bovine neuromodulin was compared to ensure that they behaved similarly. As reported in Figure 1, both the recombinant protein used in this study and neuromodulin isolated from bovine brain were phosphorylated by protein kinase C in a calcium- and phospholipid-dependent manner. The recombinant neuromodulin purified from *E. coli* transfected with the murine cDNA migrated somewhat 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). The lack of contaminating kinase activity in the protein kinase C preparation used to phosphorylate neuromodulin was demonstrated by the characteristic protein kinase C calcium- and phospholipid-dependent phosphorylation of histones (Figure 1).

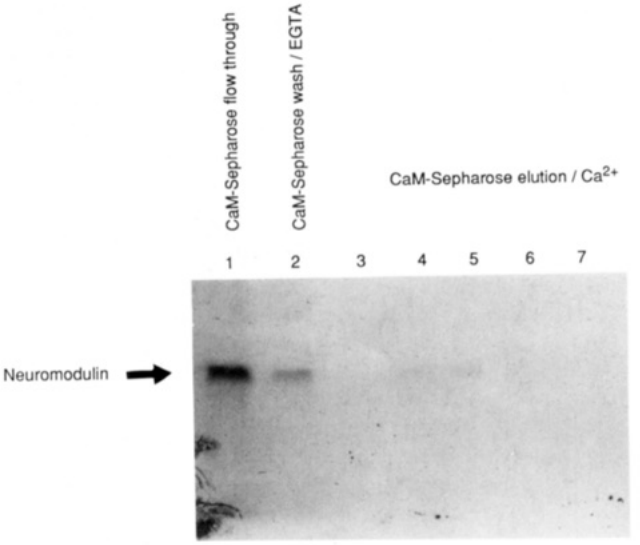

FIGURE 2: Recombinant neuromodulin phosphorylated by protein kinase C does not absorb to CaM–Sepharose. Recombinant neuromodulin (15 μg) purified using CaM–Sepharose was phosphorylated with [γ-^{32}P]ATP as described under Materials and Methods. EGTA was added to a concentration of 2 mM, and the phosphorylated protein was applied to 5 mL of CaM–Sepharose and allowed to incubate overnight at 4 °C. This sample was then poured into a small plastic Bio-Rad "Econo-column", and the flow-through was collected. The column was washed with buffer containing 2 mM EGTA and then eluted with buffer containing 2 mM free Ca^{2+}. Samples from these fractions were subjected to SDS–PAGE electrophoresis and autoradiography to detect phosphorylated neuromodulin. Lanes 1 and 2 are CaM–Sepharose flow-through and wash, respectively, and lanes 3–10 are fractions collected during elution with Ca^{2+}-containing buffer.

Bovine brain and recombinant neuromodulins were phosphorylated by protein kinase C with apparent K_m values of 1 μM. The stoichiometry of phosphorylation should define the number of protein kinase C phosphorylation sites present in neuromodulin; however, values for the phosphorylation stoichiometry reported in the literature range from 0.5 to 1.3 mol of P_i/mol of neuromodulin (Chan et al., 1986; Alexander et al., 1987; Zwiers et al., 1985). The variability of these values reflects the difficulty in unambiguously determining this parameter and the use of different experimental methods for quantitating stoichiometries. In this study, phosphorylation stoichiometry was determined by using a glass fiber filter assay, which gave low background counts and highly reproducible data. Using this method, it was determined that protein kinase C catalyzed the incorporation of 0.8 ± 0.1 mol of P_i/mol of protein into recombinant neuromodulin. This value is consistent with the observation, discussed below, that only one phosphorylated peptide containing a single serine was isolated following a trypsin digest of phosphorylated recombinant neuromodulin. Furthermore, this peptide was phosphorylated with a 1:1 stoichiometry (data not shown). Therefore, we conclude that there is a single protein kinase C phosphorylation site in neuromodulin.

Previous work from this laboratory demonstrated that bovine brain neuromodulin adsorbs to CaM–Sepharose in the absence of free Ca^{2+} and is eluted from CaM–Sepharose with Ca^{2+}-containing buffers (Andreasen et al., 1983). Phosphorylation of bovine neuromodulin by protein kinase C prevents its binding to CaM–Sepharose (Alexander et al., 1987). The recombinant protein phosphorylated by protein kinase C exhibited analogous behavior (Figure 2). Recombinant phosphoneuromodulin did not bind to CaM–Sepharose in the presence of excess calcium chelator. All of the phosphorylated protein was found in the column flow-through and wash

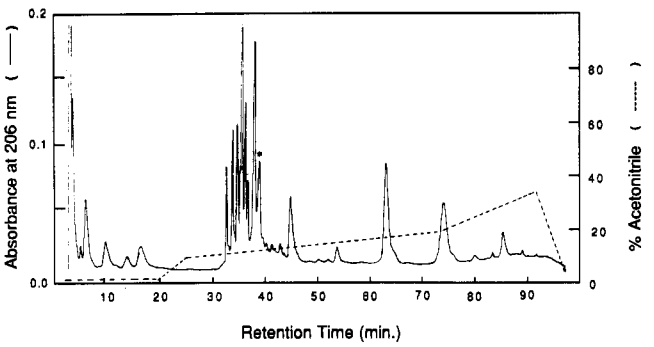

FIGURE 3: Purification of neuromodulin tryptic peptides by C8 reverse-phase HPLC. Recombinant neuromodulin was phosphorylated with [γ-^{32}P]ATP and proteolyzed with trypsin as described under Materials and Methods. The resulting mixture of peptides was resuspended in 100 μL of 0.1% TFA and injected onto an Aquapore RP-300 C8 (Pierce) column. The peptides were separated at a flow rate of 0.3 mL/min with a linear gradient of acetonitrile in 0.1% TFA as shown by the dashed line and described under Materials and Methods. Each peak was collected manually, and the total radioactivity associated with each isolated peptide was determined by Cerenkov radiation. Greater than 95% of the total radioactivity was associated with the peptide labeled with an asterisk in the elution profile.

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                                41
BOVINE          |-------KAHKAATKIQASFRGHITRKKLKGEKKGDAP-------|
RECOMBINANT     |-------KAHKAATKIQASFRGHITRKKLKGEKKGDAP-------|
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FIGURE 4: Protein kinase C phosphorylation site in neuromodulin. The protein kinase C phosphorylation site of recombinant neuromodulin at serine residue 41 is shown in bold type. The corresponding bovine sequence is shown for comparison. The CaM binding domain (Alexander et al., 1988) is underlined with a dark bar.

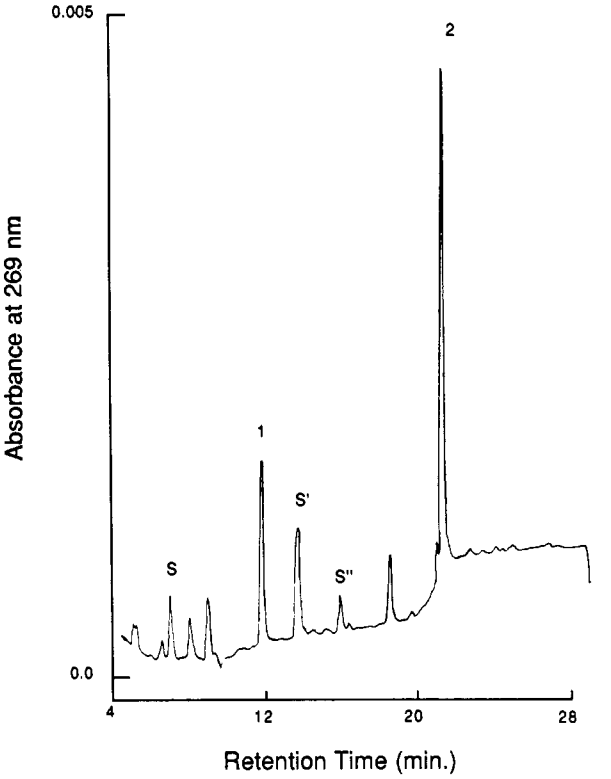

FIGURE 5: Sequence analysis of phosphorylated serine residue. The sequence of the single isolated phosphopeptide was determined to be IQASFR. The chromatogram of the PTH analysis of residue 4 of this peptide is shown. Labeled peaks are (1) PTH-Ala which has diminished in magnitude from the previous turn and (2) DPTU, diphenylthiourea. DPTU is formed during the sequencer chemistry and is a major peak in every chromatogram.

fractions of the column. The phosphorylation of the recombinant neuromodulin, like the bovine protein, abolishes its interactions with CaM.

Identification of the Protein Kinase C Phosphorylation Site. Phosphoamino acid analysis had identified phosphoserine as the sole phosphoamino acid in bovine neuromodulin phosphorylated by protein kinase C (Alexander et al., 1987). The objective of this work was to identify the serine residue(s) phosphorylated by protein kinase C. The recombinant protein was phosphorylated using [γ-^{32}P]ATP and digested with trypsin, and the resulting peptides were separated by HPLC. Initially, trypsin would seem a poor choice of protease to digest a protein containing so much lysine (12%). However, as was noted in the determination of the protein sequence (Wakim et al., 1987), tryptic attack on the protein is greatly limited by the number of lysine residues in otherwise acidic environments and the several Lys–Lys (3) and Lys–Pro (3) bonds. Consequently, a consistent set of peptides were obtained which were easily fractionated by narrow-bore reverse-phase HPLC using an appropriately shallow gradient. A typical HPLC profile showing the separation of the peptides generated during the trypsin digest is shown in Figure 3. Greater than 95% of the total radioactivity was incorporated into a single peptide distinguished by an asterisk in the profile. This peptide was isolated and its sequence determined to be IQASFR from 100–200 pmol of material. The serine in this peptide occupies site 41 in the sequence of bovine neuromodulin (Wakim et al., 1987) and is located adjacent to the CaM binding domain of the protein (Figure 4).

Phosphoserine residues are not amenable to the Edman degradation directly. They tend to appear with low PTH-serine recovery and concomitantly increased recovery of the DTT adduct formed from serine ("ser'"). The latter results from the addition of the thiol to the dehydroalanine residue which is formed in higher yield from phosphoserine than from serine by β-elimination in the sequencer chemistry. This phenomenon was observed during the sequencing of IQASFR (Figure 5), thus indicating that the phosphorylated residue in IQAS(P)FR was indeed phosphoserine.

Since serine-41 in neuromodulin is phosphorylated by protein kinase C, synthetic peptides containing the corresponding sequence from neuromodulin may also be substrates for protein kinase C. Therefore, we examined a peptide with the sequence QA*S*FRGHITRKKLKGEK (designated FP57-Ph) as a substrate for protein kinase C. FP57-Phe corresponds to the sequence of neuromodulin containing serine-41 with the adjacent CaM binding domain. This peptide was phosphorylated by protein kinase C, and it partially inhibited phosphorylation of neuromodulin itself (Figure 6). FP57-Phe was also phosphorylated in a calcium- and phospholipid-dependent manner by protein kinase C in the absence of neuromodulin (unpublished observation). There are two explanations for the lack of complete dependence of the protein kinase C phosphorylations on calcium and phospholipids observed in Figure 6. Proteolysis of protein kinase C, which tends to occur in preparations that are several months old, results in loss of calcium- and phospholipid-dependent enzyme activity. Perhaps more likely is the possibility that trace amounts of calcium which may be present in the assay are sufficient to stimulate the kinase in the presence of high levels of diacylglycerol, which increases dramatically the affinity of calcium for protein kinase C.

Discussion

The major objective of this study was to define the protein kinase C phosphorylation site of neuromodulin. The recom-

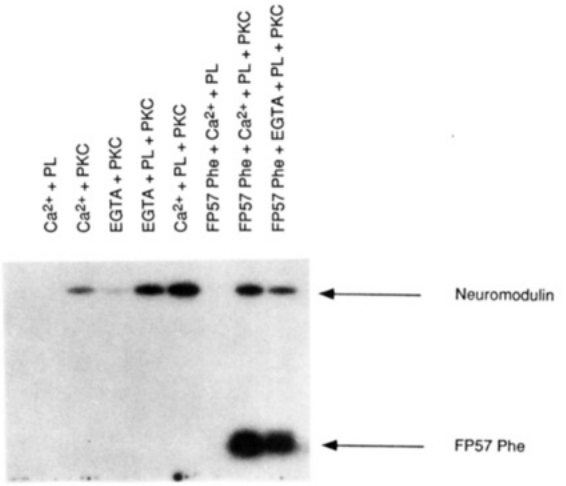

FIGURE 6: Phosphorylation of FP57-Phe by protein kinase C. Bovine neuromodulin (0.5 μg/lane) was phosphorylated by protein kinase C in 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]ATP (2000 cpm/pmol), and 400 μM free Ca^{2+} or excess EGTA as indicated. Phosphatidyl-L-serine (200 μg/mL) and 20 μg/mL diolein (designated as PL) were added where indicated. In lanes 7–9, 10 μg of the peptide having the sequence QASFRGHITRKKLKGEK (designated FP57-Phe) was also added to the reaction mixture. Reactions were initiated by the addition of protein kinase C and allowed to incubate for 45 min at 30 °C. Reactions were stopped by addition of 5× SDS–PAGE sample buffer and immediate boiling. Samples were then subjected to SDS–PAGE electrophoresis (10% gel) and autoradiography.

binant protein was used since significant amounts of the protein were obtainable in an unphosphorylated form, and it is indistinguishable from bovine brain neuromodulin as a substrate for protein kinase C. One of the best known substrates for protein kinase C, histone, is phosphorylated with a K_m of 0.6 μM (Woodgett et al., 1986), and one of the most effective synthetic peptide substrates reported for protein kinase C has a K_m of 0.5 μM (House, 1987). The K_m values for protein kinase C phosphorylation of bovine brain neuromodulin or the recombinant neuromodulin were approximately 1.0 μM. Since the estimated concentration of neuromodulin in brain is 10 μM (Cimler et al., 1987), these K_m values suggest that the interaction of protein kinase C with neuromodulin is functionally significant.

In this study, we report the identification of a single protein kinase C phosphorylation site in neuromodulin located at serine-41. Presently, there is no indication of a simple protein kinase C consensus sequence analogous to the cAMP-dependent protein kinase recognition site. A recent study using synthetic peptide analogues of three known protein kinase C substrates including glycogen synthase, ribosomal protein S6, and the epidermal growth factor receptor indicated that basic residues, particularly arginine, on both sides of the phosphorylation site can have an important influence on the kinetics of phosphorylation and site specificity of protein kinase C (House et al., 1987). Several basic residues are located near serine-41, and the amino acid sequence in this domain of neuromodulin is also rich in lysine.

The location of the protein kinase C phosphorylation site at serine 41-is particularly interesting since it is located adjacent to or within the CaM binding domain of neuromodulin (Alexander et al., 1988). The proximity of this phosphorylation site to the CaM binding domain very likely explains why phosphorylation of neuromodulin by protein kinase C dramatically lowers its affinity for CaM. CaM binding domains of CaM-regulated proteins share a number of common features including clusters of positively charged and hydrophobic amino acids (Buschmeier, 1987). Most CaM binding domains also contain one or more aromatic residues which contribute significantly to the interaction with CaM. There is only one aromatic amino acid in neuromodulin, phenylalanine-42. Phosphorylation of serine-41 not only would introduce multiple negative charges at the CaM binding domain but also could disrupt potential hydrophobic interactions between the adjacent phenylalanine and CaM.

It is noteworthy that serine-41 and the CaM binding domain of neuromodulin are strictly conserved in all vertebrate neuromodulins sequenced to date (Skene, 1989), suggesting that this structural domain is important for the function of the protein. This observation is consistent with the proposal that one of the major functions of neuromodulin in neurons may be to bind and concentrate CaM at specific sites and release CaM locally in response to protein kinase C phosphorylation (Alexander et al., 1987).

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Role of Extracellular Disulfide-Bonded Cysteines in the Ligand Binding Function of the β_2-Adrenergic Receptor†

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ABSTRACT: Evidence is presented for a role of disulfide bridging in forming the ligand binding site of the β_2-adrenergic receptor (βAR). The presence of disulfide bonds at the ligand binding site is indicated by "competitive" inhibition by dithiothreitol (DTT) in radioligand binding assays, by specific protection by β-adrenergic ligands of these effects, and by the requirement of disulfide reduction for limit proteolysis of affinity ligand labeled receptor. The kinetics of binding inhibition by DTT suggest at least two pairs of disulfide-bonded cysteines essential for normal binding. Through site-directed mutagenesis, we indeed were able to identify four cysteines which are critical for normal ligand binding affinities and for the proper expression of functional βAR at the cell surface. Unexpectedly, the four cysteines required for normal ligand binding are not those located within the hydrophobic transmembrane domains of the receptor (where ligand binding is presumed to occur) but lie in the extracellular hydrophilic loops connecting these transmembrane segments. These findings indicate that, in addition to the well-documented involvement of the membrane-spanning domains of the receptor in ligand binding, there is an important and previously unsuspected role of the hydrophilic extracellular domains in forming the ligand binding site.

The β_2-adrenergic receptor (βAR)[1] is an integral membrane glycoprotein coupled, through the guanine nucleotide binding regulatory protein G_s, to adenylyl cyclase and the production of the second-messenger cAMP. The βAR is among the best characterized of the hormone or agonist-responsive G-protein-coupled receptors. On the basis of mechanistic and structural similarities between βAR and the visual pigment rhodopsin, we proposed a topological model of the βAR as having seven membrane-spanning domains, an extracellular glycosylated amino terminus, and cytoplasmic domains which are phosphorylated by the regulatory enzymes β-adrenergic receptor kinase and cAMP-dependent protein kinase (Dixon et al., 1986a). This proposed structure has been partially validated through the use of limited proteolysis to localize these and other functional and structural landmarks (Dohlman et al., 1987a). The physical and regulatory properties of this member of the family of G-protein-coupled receptors have recently been reviewed (Dohlman et al., 1987b; Sibley et al., 1987).

One outcome of the alignment and comparison of the deduced amino acid sequences of the various members of this receptor family has been the identification of a number of highly conserved cysteine residues ($Cys^{106,184,341}$) which may play important structural or functional roles, possibly by forming intramolecular disulfide bonds (Dohlman et al., 1987b). Indeed, the effects of disulfide and sulfhydryl reactive reagents on the adenylyl cyclase coupled βAR system have been the object of extensive study. Pedersen and Ross (1985) have demonstrated the ability of DTT-treated turkey β_1-ad-

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[1] Abbreviations: DTT, dithiothreitol; βAR, β_2-adrenergic receptor; ^{125}I-CYP, 3-[^{125}I]iodocyanopindolol; ^{125}I-pBABC, *p*-(bromoacetamido)-benzyl-1-[^{125}I]iodocarazolol; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; G_s, stimulatory guanine nucleotide binding regulatory protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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