Purification and Characterization of a Brain-specific Protein Kinase C Substrate, Neurogranin (p17)

IDENTIFICATION OF A CONSENSUS AMINO ACID SEQUENCE BETWEEN NEUROGRANIN AND NEUROMODULIN (GAP43) THAT CORRESPONDS TO THE PROTEIN KINASE C PHOSPHORYLATION SITE AND THE CALMODULIN-BINDING DOMAIN

(Received for publication, July 2, 1990)

Jacques Baudier**, Jean Christophe Deloumele†, Alain Van Dorsellaert, Diane Black||, and Hans W. D. Matthes§

From the 3Centre de Neurochimie du Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale Unité 44, 5 rue Blaise Pascal F-67084 Strasbourg Cedex and 4Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique, Unité 184 de Biologie Moléculaire et de Génie Génétique de L’Institut National de la Santé et de la Recherche Médicale, Faculté de Medecine, Institut de Chimie Biologique, 11 rue Humann, F-67085 Strasbourg Cedex, France

Neurogranin, formerly designated p17 (Baudier, J., Bronner, C., Kligman, D., and Cole, R. D.) (1989) J. Biol. Chem. 264, 1824-1828), a brain-specific in vitro substrate for protein kinase C (PKC), has been purified to homogeneity from bovine forebrain. The purified protein has a molecular mass of 7837 ± 0.5 Da, determined by electrospray mass spectrometry. In the absence of reducing agent, dimers and higher oligomers accumulated. On sodium dodecyl sulfate-polyacrylamide gels the protein monomer migrated abnormally with an apparent molecular mass of 15,000-19,000 Da, depending on the percentage of polyacrylamide. The native protein is blocked at its amino terminus. The majority of the primary amino acid sequence was determined following proteolytic and chemical fragmentation. A comparison of the amino acid sequence of neurogranin with that of the brain-specific PKC substrate neuromodulin, revealed a strikingly conserved amino acid sequence AA(X)KIQASF-RGH(X)(X)RRKK(X)K. The two proteins are not related over the rest of their sequences.

Neurogranin was shown to be phosphorylated in hippocampal slices incubated with 32P, and phorbol esters stimulated neurogranin phosphorylation, suggesting that neurogranin is likely to be an in vivo substrate for PKC. In vitro phosphorylation of neurogranin by PKC produced a shift of the isoelectric point of the protein (pI 5.8) to a more acidic value (pI 5.4). Tryptic digestion of the phosphorylated protein yielded a single phosphopeptide having the sequence IQASFR, where the serine residue is the phosphorylated amino acid. This phosphopeptide is part of the conserved sequence shared with neuromodulin and also corresponds to the PKC phosphorylation site on neuromodulin (Apel, E. D., Byford, M. F., Au, D., Walsh, K. A., and Storm, D. R. (1990) Biochemistry 29, 2330-2335). Evidence was obtained suggesting that neurogranin binds to calmodulin in the absence of Ca2+, a feature that also characterizes neuromodulin. We propose that the amino acid sequence shared by neurogranin and neuromodulin reflects a functional relationship between these two proteins and that the consensus sequence represents a conserved PKC phosphorylation site and a calmodulin binding domain that characterizes a class of brain-specific PKC substrates.

Protein kinase C (PKC), a Ca2+-activated phospholipid-dependent kinase, is the receptor for tumor promoting phorbol esters and is thought to play an important role in controlling several cellular processes (1). In vivo, PKC is believed to be activated by diacylglycerol, an intracellular second messenger, generated by an agonist-induced phosphatoinoside metabolism (2). PKC is present ubiquitously in a variety of tissues as multiple species of isozymes and is especially abundant in the brain (3). An understanding of the exact function of the PKC isozymes requires the identification and characterization of their in vivo substrates which represent the first molecular support of the PKC activation signal. Although PKC has been shown to be a key enzyme in mediating specific neural functions in the central nervous system such as neurotransmitter release and neuroplasticity (4-8) in the brain, only two PKC substrates have been formally identified and characterized: neuromodulin (also called GAP 43, B50, F1, p57, pp46) (9-12) and the MARCKS protein (also called p87) (13-15).

Neuromodulin is a calmodulin-binding phosphoprotein of the presynaptic membrane (9, 16). The cellular role of neuromodulin has not been clearly established. Its synthesis is strongly correlated with axon outgrowth suggesting its implication in a general mechanism that controls nerve growth during development and regeneration (17, 18). The correlation between neuromodulin phosphorylation and the persistence of long term potentiation also indicates that neuromodulin may play a role in both normal neurite growth in developing...
brain and neural plasticity at adult synapses (19). Several other functions have been proposed for neuromodulin such as in the sequestration of calmodulin along the cytoplasmic surface of appropriate membrane domains (9), the modulation of neurotransmitter release (20), and the regulation of GTP binding to the G protein (21). MARCKS protein (p87) is a protein of unknown function that has a widespread tissue and subcellular distribution (22).

In bovine brain extracts, we recently identified another low molecular mass in vitro substrate for PKC that we called p17 from its apparent molecular mass on a SDS-polycrylamide gel (23). Immunohistochemical studies in rats showed that p17 is exclusively found in the forebrain and is more specifically concentrated in the perykaria and dendrites of neurons of the cerebral cortex and hippocampus (24). Because p17 immunoreactivity is often found associated with granule-like structures in dendrites of pyramidal cells of the hippocampus seen in electron micrographs, we have called the p17 protein neurogranin. We report here the purification to homogeneity and further characterization of neurogranin from bovine brain. The results demonstrate that neurogranin has structural and functional properties in common with neuromodulin and strongly suggests that neurogranin and neuromodulin belong to the same class of brain-specific PKC substrates. For the first time, a shared amino acid sequence corresponding to a PKC phosphorylation site domain is identified in these two brain-specific proteins.

MATERIALS AND METHODS

The following materials were obtained from the indicated sources: FPLC apparatus and Phast-System Electrophoresis apparatus were from Pharmacia LKB Biotechnology Inc. Immunoblotting and protein determination reagents were from Bio-Rad. The Immobilon blotting membrane was from Millipore. AcA 54 gel was from IBF systems protein sequencer 477A with an on-line 120A PTH analyzer. The following materials were obtained from the indicated sources: FPLC apparatus and Phast-System Electrophoresis apparatus were from Pharmacia LKB Biotechnology Inc. Immunoblotting and protein determination reagents were from Bio-Rad. The Immobilon blotting membrane was from Millipore. AcA 54 gel was from IBF systems protein sequencer 477A with an on-line 120A PTH analyzer.

In Vitro Phosphorylation of Neurogranin by Protein Kinase C—Neurogranin in its monomeric form (in buffer plus 10 mM DTT and 1 mM EDTA) or in its disulfide-linked state was incubated with purified PKC in 40 mM Tris-HCl, pH 7.2, 0.1 mM free Ca++, 0.1 mM phosphatidylinerine, 10 nM phospholipase C, 12-mg millipore 12-micronic-acetate, for 1 min at 35 °C. The phosphorylation reaction was initiated by adding 5 mM MgCl2 and 100 μm [γ-32P]ATP. The total volume of the reaction ranged between 0.1 and 1 ml. The reaction was terminated by addition of an SDS-stop solution (5% SDS, 25% glycerol, 0.5 mM Tris-HCl, pH 6.8) and heated for 5 min at 100 °C. The proteins were separated by 10% SDS, 12.5% PAGE. The gels were dried and the phosphorylated protein detected by autoradiography. The phosphorylated proteins prepared as described (25) for the accurate determination of neurogranin concentration. We also found that the Lowry method was not adequate for the accurate determination of neurogranin concentration.

Mass Spectrometry Measurements—The molecular mass of neurogranin was measured by MALDI (LG B.O.-Q. (UG Bio Tech, Manchester, United Kingdom) mass spectrometer which consists of an electrospray ion source and a quadrupole. The molecular masses were calculated as described (26, 27).

HPLC Purification of Tryptic Phosphopeptides—The phosphorylated neurogranin (11 μg) in 40 mM Tris-HCl, pH 7.5, 10 mM DTT, 1 mM EDTA was incubated with trypsin (1.7 μg of Sequence Grade from Boehringer) at 25 °C. After 20 h, the sample was adjusted to 0.1% trichloroacetic acid and applied to a 2.1 x 300-mm RP-30 Brownlee C8 column. The column was connected to a Hewlett-Packard 1090 liquid chromatograph with an on-line diode-array detector and equipped with a 500-μl loop. Absorbance was monitored at 205 nm (see Fig. 7), and the UV spectra were recorded at any elution time, which was useful for the detection of peptides containing aromatic amino acids or pyridyldithylated cysteines (see below). Before injection, the column had been equilibrated in 0.1% trichloroacetic acid. The flow rate was 0.5 ml/min during the first 5 min and then 0.2 ml/min for the rest of the chromatography. The peptides were separated with a gradient of 0-60% (v/v) acetonitrile in 0.1-0.09% trifluoroacetic acid (v/v) in 50 min. Fractions (0.2 ml) were collected and 5-ml aliquots were subjected to amino acid sequence analysis.

Pyridyldithylation of Neurogranin—Pyridyldithylation was performed according to (28). Neurogranin (10 μg) was dissolved in 6 μl guanidinium hydrochloride, 50 mM EDTA (40 μl). DTT (1 μl, 7 μl) was added, and the mixture was left to react for 10 min. Then, 100 μl of anhydrous acetonitrile was added, and the mixture was centrifuged and the supernatant was transferred to reaction buffer. Neurogranin was precipitated by dot blot assays using neurogranin antibodies or a PKC assay. Neurogranin eluted under a symmetrical protein peak at 260 ml that corresponded to the neurogranin monomer. The pooled samples (40 ml) from the IBF AcA 54 column were split into 4 x 10-ml aliquots. Each aliquot was adjusted to 0.1% trichloroacetic acid, filtered through a Millex 0.45-μm filter (Millipore), and applied separately at 0.5 ml/min to an FPLC ProRPC HR 5 x 10 column equilibrated previously in H2O, 0.1% trifluoroacetic acid. After the column had been equilibrated with 20 mM NaH2PO4, the neurogranin eluted at 0.5 ml/min with a 50-ml linear gradient of 0–90% acetonitrile, 0.1% trifluoroacetic acid. The pH of each fraction was adjusted to 7.5 with aliquots of 2 M Tris-HCl, pH 8.2, as the fractions were collected. Neurogranin elution was followed by SDS-PAGE (see Fig. 1A), a PKC assay or by immunoblotting. After dialysis against 40 mM Tris-HCl, pH 7.2, the protein was stored at ~20 °C.
Speed Vac. Pyridylethylneurogranin eluted slightly earlier than neurogranin and showed an absorption of the pyridylethyl group at 250 nm.

Enzymatic and Chemical Fragmentation of Neurogranin prior to Sequence Analysis—Enzymatic digestion: neurogranin or pyridylethylneurogranin (5 μg) was subjected to digestion with trypsin, Glu-C, or Asp-N endoproteinases according to instructions from the manufacturer. Lys-C endoproteinase digestion was performed in 25 mM Tris-HCl pH 9.3, 1 mM EDTA at 25 °C for 18 h. Partial deblock- ing was obtained by treating pyridylethylated neurogranin (10 μg) with 2 M formic acid for 4 h at 110 °C. A fraction (20%) of this ethylneurogranin Sequence Analysb-Enzymatic digestion: neurogranin or pyridyl derivated was diluted to 500 mM Tris-HCl pH 3-10 ampholines (5%) were used in the first dimensional isoelectric focusing as described above for tryptic phosphopeptides. The endoproteinase-digested or formic acid-treated neurogranin or the pyridylethyl derivative was diluted to 500 μl with 0.1% trifluoroacetic acid and subjected to HPLC purification as described above for trypic phosphopeptides. Major peaks were collected and sequenced.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed essentially as described by O’Farrell (29). pH 3–10 ampholines (5%) were used in the first dimension isoelectric focusing and a 0.1% SDS, 12.5% PAGE followed by autoradiography of the dried gel to identify the anti-neurogranin antibodies. 40 μl of protein A-Sepharose slurry in buffer C was added and incubated for 1 h at room temperature. After centrifugation the supernatants were precipitated with 20% trichloroacetic acid and resuspended in 50 μl of buffer C (50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, pH 7.5). For the immunoprecipitation of 32P-labeled neurogranin, affinity-purified antibodies against neurogranin (200 μl) were incubated overnight at room temperature with primary antibodies
diluted 1:1000 with 1% gelatin in TTBS buffer (2% Tween 20) followed by incubation with alkaline phosphatase-conjugated antibodies in 1% gelatin in TTBS buffer for 2 h. Immunoreactive bands were detected by using 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml) and nitro blue tetrazolium (0.3 mg/ml) in 40 mM sodium carbonate, pH 9.8 and 5 mM MgCl2.

RESULTS

Purification of Neurogranin from Bovine Forebrain

We reported previously a rapid purification method for neurogranin and partial purification of neurogranin from bovine brain taking advantage of their solubility in 2.5% perchloric acid (23). To improve the purification of neurogranin, modifications were introduced into the original method. The 40 mM citric acid buffer used for homogenization brought the crude brain extract to pH 4–4.2. This acidic pH increased the recovery of neurogranin as well as neuromodulin in the supernatant after the first centrifugation and greatly simplified this step. The perchloric acid precipitation step provided a high degree of purification for both neurogranin and neuromodulin, but this step also exposed the proteins to acidic conditions. Nevertheless, perchloric acid treatment of rat brain extract neither modified the electrophoretic mobility nor the immunoreactivity of the proteins as revealed by Western blotting (24), and perchloric acid treatment of the purified proteins did not increase or decrease the ability of PKC to phosphorylate the proteins (data not shown). The final purification step of neurogranin involved FPLC ProRPC chromatography. Neurogranin eluted as a broad protein peak between 22 and 26% acetonitrile (Fig. 1A). Most of the neurogranin eluted as a disulfide-linked dimer and higher oligomers. The oxidized forms of neurogranin could be reduced in the presence of an excess of DTT (10 mM) and EDTA (1 mM) (Fig. 1B). When neurogranin was rechromatographed on the same ProRPC column, separation of the different disulfide-linked species could be achieved. The neurogranin monomer eluted first followed by the dimer and oligomers (data not shown).

Formation of neurogranin oligomers was also observed when we subjected the reduced protein to FPLC chromatography on a Mono Q column at pH 8.6 using a buffer that did not contain a reducing agent or when the protein was stored at −20 °C in 40 mM Tris-HCl buffer, pH 7.4, in the absence of reducing agents and EDTA. Therefore, it was obvious that neurogranin possesses at least two sulfhydryl groups that may oxidize in appropriate conditions to form intermolecular bonds.

This purification procedure yielded 0.5–1 mg of purified neurogranin from 1 kg of bovine forebrain. Comparatively, using the same extraction procedure, the amount of purified neuromodulin was about 20–50 times higher than neurogranin. However, these values do not reflect the true ratios of neuromodulin and neurogranin concentration in vivo. Indeed, we have found that neurogranin, in rat brain extracts, partitions equally in the cytosolic and membrane fractions, whereas neuromodulin exists essentially as a membrane fraction.

Purification and Characterization of Neurogranin

<table>
<thead>
<tr>
<th>Fractlon</th>
<th>Zl</th>
<th>4-4.2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5-1</td>
<td>0.5-1</td>
</tr>
<tr>
<td>2</td>
<td>0.5-1</td>
<td>0.5-1</td>
</tr>
<tr>
<td>3</td>
<td>0.5-1</td>
<td>0.5-1</td>
</tr>
<tr>
<td>4</td>
<td>0.5-1</td>
<td>0.5-1</td>
</tr>
</tbody>
</table>

**Fig. 1.** Purification of neurogranin by reversed-phase chromatography. A, fractions (1 ml) eluted from the FPLC ProRPC column were immediately analyzed by SDS, 10–15% PAGE, showing the presence of neurogranin oligomers in fractions 22–26. B, SDS, 10–15% PAGE of purified neurogranin in 40 mM Tris-HCl, pH 7.5, incubated in the absence (−) or in the presence (+) of 100 mM DTT and 1 mM EDTA for 30 min at 37 °C prior to electrophoresis. In A and B, electrophoresis was performed on a Phast System apparatus from Pharmacia and the gels silver-stained according to the Pharmacia procedure. On the left are the positions of the molecular mass standards. In C and D, purified neurogranin (10 μg of protein) was analyzed by two-dimensional electrophoresis. The resulting gels were stained with Coomassie Blue (C) or transferred to an Immobilon blotting membrane (D) and incubated for 5 h with neurogranin antibodies (1:1000 dilution). The immunoreaction products were visualized as described under "Materials and Methods." The positions of the 14-kDa molecular weight standard and the appropriate isoelectric focusing gradient are indicated. Arrowsheads indicate the position of a more acidic form of a neurogranin-like antigen.
bound protein and can be extracted only with the use of detergent in the homogenization buffer. Western blot analysis, using neurogranin antibodies, of total soluble protein from rat brain homogenized in 100 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM DTT, 100 mM NaCl, and 0.2 mM phenylmethylsulfonyl fluoride revealed a major immunoreactive polypeptide which comigrated with purified bovine brain neurogranin (24). Identical results were obtained when rat brains were first homogenized in buffer containing 1% Triton X-100 and neurogranin was then partially purified by selective solubilization in 2.5% perchloric acid (24). Equally, when rat brains were directly homogenized in 2.5% perchloric acid in the presence of 100 mM NaCl and 1% Triton X-100, a single immunoreactive polypeptide was visualized by Western blot analysis of the solubilized protein which comigrated with the purified bovine neurogranin (not shown). These data, and also the fact that an Asp-Pro sequence, which is known to be the most acid labile peptide linkage, is intact in neurogranin, strongly suggest that the purified protein is not a proteolytic product of a larger molecule generated during purification.

Two-dimensional gel electrophoresis analysis, using in the first dimensional isoelectric focusing ampholines of pH 3–10 and in the second dimension a 0.1% SDS, 15% polyacrylamide gel, revealed a microheterogeneity in the neurogranin preparation. Coomassie Blue staining of the gel (Fig. 1C) showed that neurogranin can be separated essentially into doublet protein spots (indicated by arrows) with molecular mass of 15 kDa and pIs around 5.6. Other minor protein spots can be distinguished. A light but distinguishable protein spot, in the same molecular mass range as that of the major neurogranin doublet and with a more acidic pI of 5.4, was seen during destaining of the gel, the position of which is indicated by an arrowhead. Western blot analysis of the same gel using neurogranin antibodies (Fig. 1D) confirmed the microheterogeneity of the neurogranin preparation and the relationship of the more acidic protein spot (pI 5.4) with neurogranin.

Characterization of Neurogranin

Electrophoretic Mobility—The apparent molecular mass of neurogranin differed from one electrophoretic system to another depending on the percentage of polyacrylamide used in the gel. The apparent molecular mass increased from 15 to nearly 19 kDa when the percentage of polyacrylamide used decreased from 20 to 9% (data not shown). On a 10–15% gradient polyacrylamide gel the protein monomer migrated with an apparent molecular mass of 16.5 kDa (Fig. 1B). Since the true molecular mass of the protein is 7.837 kDa (see below), the apparent molecular mass for the neurogranin monomer determined by gel electrophoresis is overestimated. This has also been observed for other perichloric acid-soluble PKC substrates, including proteins such as neuromodulin, MARCKS protein, and tau proteins (23).

Molecular Size and Amino Acid Sequence Determination—The electrospray mass spectrometry spectrum of the neurogranin monomer is shown in Fig. 2. A major protein species with a molecular mass of 7837.1 ± 0.5 Da, that corresponds to the A series of peaks, predominates. A minor protein component, that corresponds to the B series of peaks, with molecular mass of 7664.3 ± 0.7 Da is also found in the spectrum. The reason for this molecular mass heterogeneity of neurogranin is not yet known.

Initial attempts at sequencing the intact protein were unsuccessful, indicating the presence of a blocked N terminus. Thus, proteolytic and chemical cleavage methods were employed on neurogranin or pyridylethylneurogranin followed by reverse phase HPLC purification to obtain fragments that could be sequenced (see Fig. 3A). Pyridylethylated fragments, containing cysteine residues, could be detected after HPLC by a diode array detector due to their UV absorption at 250 nm. This is important since cysteine is not normally detectable by Edman degradation without derivatization as for example by pyridylethylation. The V8-1 fragment, containing 2 cysteines (see Fig. 3A), was sequenced twice, first as a nonderivatized peptide and then as a pyridylethylated derivative. The nonderivatized V8-1 peptide showed no PTH-derivatives in cycle 3 and 5 (amino acids 7 and 9 in Fig. 3A) which suggested the presence of cysteine in those two positions. The pyridylethylated V8-1 fragment showed pyridylethyl-cysteine-PTH in cycles 3 and 5 in accordance with the standard PTH. In the analysis system used, the pyridylethyl-cysteine-PTH eluted very close to valine-PTH, and we could not distinguish these amino acids with absolute assurance. However, the absence of valine in the derivatized V8-1, as well as in the amino acid analysis, gave us sufficient assurance of the sequence of fragment V8-1 as it is written in Fig. 3A. A further support for the cysteines is the fact that the oligomer formation of neurogranin proves the presence of at least 2 cysteines.

Combining the various sequences of fragments yielded the primary structure of neurogranin. The amino acid sequence of the three N-terminal amino acids could not be determined with great certainty (shown in brackets in Fig. 3A). The N-terminal amino acid sequence was deduced after deblocking of the N-terminal amino acid by mild treatment with 2 M formic acid (30). This treatment unblocked less than 5% of the N-terminal amino acid and therefore uncertainty exists concerning the deduced N-terminal sequence. The N-terminal sequence (Met-Cys-Thr)-Glu was compatible with the fact that methionine is a common N-terminal amino acid (neuromodulin has been shown to have a blocked methionine at the N-terminal (30) and that a single threonine residue was to be expected according to the amino acid analysis (Table I). The fact that N-terminal acetylmethionine is followed invariably by an asparagine, aspartic acid, or glutamic acid is noteworthy (31), in view of the ambiguity in the determination of the cysteine in position 2. An aspartic acid or an asparagine residue in this position would be in better agreement with the amino acid analysis (Table I). The sequence of the C-terminal is also not clearly established, due to the high level of glycine in this region. As glycine is often poorly cleaved during the Edman degradation, this residue gives significant lag (carryover) into the next cycle. Thus, in the sequence of neurogranin, the last few cycles show gradually diminishing quantities of glycine, but it is not quite clear how many of these residues are due to the sequence of the peptide and how many are simply due to the lag from the sequencer.

Two out of three neurogranin preparations showed lysines in the positions noted (Fig. 3A), whereas the third preparation revealed mainly an unidentified residue at the same positions, leading to the conclusion that the unidentified residue is probably a modified lysine. The modified amino acid PTH-derivative eluted close to proline-PTH in our system (acetylated-lysine-PTH elutes in this region). Most amino acid sequencing and other analyses were done on this fraction, including the mass spectrometry measurements. The difference in mass between the theoretical monoisotopic value calculated according to the sequencing results (7243.5 Da) and the determination by mass spectrometry (7837.3 Da) shows a difference of 593.8 Da. This difference probably reflects the mass of the N-lysine.
terminal blocking group as well as the lysine-modifying groups, but could also reflect inaccuracies in the N-terminal and/or carboxyl-terminal sequence. Further investigations on the structure of the post-translational modification on lysine are in progress. Bearing in mind that the amino acid analysis fits reasonably well with the neurogranin sequence within the accuracy of the method (Table I), we believe that the indicated sequence (Fig. 3A) is valid. However, only the cloning of the neurogranin gene will give a final confirmation of the primary structure.

The amino acid sequence is characterized by the presence of a single aromatic amino acid, Phe-35, which gives the protein a very low extinction coefficient at 280 nm. The sequence shows regions of strong clustering of acidic (residues 11-21) and basic (residues 30-45) amino acids and an unusual concentration of glycine residues in the C-terminal half of the protein. It is also noteworthy that proline makes up nearly 10% of the sequence, a feature that also characterizes other PKC substrates including neuromodulin, MARCKS protein, and tau protein (23).

Homology searches between the available neurogranin sequence and other protein sequences in SWISSPROT (version 14, April 1990) and NBRF-PIR (version 25, June 1990) protein databases revealed a sequence homology between neurogranin and the phosphorylation site and calmodulin binding domain of neuromodulin, an abundant neural protein that has been extensively characterized (Fig. 3B). A further sequence homology was found between the glycine-rich C-terminal of neurogranin and collagen. The sequence homology between neurogranin and neuromodulin is of striking interest in the possibility that a consensus phosphorylation site domain for PKC may exist among different brain specific PKC substrates (see below).

**Fig. 2. Electrospray mass spectrometry spectrum of neurogranin monomer.** A major protein appears at ions with 6, 7, 8, and 9 charges (A series) which yields a measured mass of 7837.1 ± 0.5 Da. A minor B series of peaks corresponds to ions also with 6, 7, 8, and 9 charges which yields a mass of 7664.3 ± 0.7 Da.

Incubation of rat hippocampal slices with 32P-labeled orthophosphate for 90 min resulted in the phosphorylation of a number of phosphoproteins soluble in perchloric acid, having a molecular mass above 43 kDa. Two major phosphoproteins soluble in perchloric acid were identified as neuromodulin and MARCKS protein a (p87) after two-dimensional gel electrophoresis (data not shown). Only very faint phosphorylation was visible for proteins that migrated with molecular mass below 43 kDa on autoradiograms after one-dimensional SDS-PAGE and thus did not allow the unambiguous detection of neurogranin. However, immunoprecipitation of neurogranin with affinity-purified antibodies and analysis of the immunoprecipitate by SDS-PAGE, followed by autoradiography of the gel, revealed a phosphoprotein that migrated at the same position as the bovine neurogranin used as a standard (Fig. 4, lane 1). A slight but reproducible stimulation of its phosphorylation occurred in the phorbol ester stimulated hippocampal slices (Fig. 4, lane 2). Because of the very low amount of immunoprecipitated protein, it has not been possible to quantify the increase in incorporated phosphate. Three independent experiments gave similar results.

**In Vitro Phosphorylation of Neurogranin by Protein Kinase C**—We reported previously that the in vitro phosphorylation of neurogranin by PKC was absolutely dependent on the presence of both calcium and phospholipids and that Ca2+/calmodulin-dependent protein kinase II was unable to utilize neurogranin as a substrate (23). We confirmed the relatively specific phosphorylation of neurogranin by PKC by the observation that AMP-dependent kinase or its purified catalytic subunit were not able to phosphorylate the protein in vitro (data not shown). A significant phosphorylation of neurogranin by purified casein kinase II has recently been observed.4 Putative phosphorylation sites for casein kinase II can be predicted on the N-terminal serine residues which are followed by a cluster of acidic residues essential for the recognition by the kinase (32-34).

**Time-course studies on the phosphorylation of the neurogranin monomer and of its oligomeric forms by PKC** are shown in Fig. 5, a-c. The phosphorylation time course of the monomer form is similar to that which we reported previously for another neurogranin preparation (23). There was no significant difference in the phosphorylation kinetics between

---

4 P. Rogue and J. Baudier, unpublished data.
The primary structure of neurogranin, comparison with neuromodulin. A, the primary structure of neurogranin was determined by automatic sequencing following chemical and enzymatic fragmentation and mass spectrometry (for details see “Materials and Methods”). The amino acids shown in brackets are uncertain (see text). The extension of the sequenced fragments are shown below the primary structure. AC, V8, LYS, ASP, and TR, refer to formic acid-, Glu-C-, Lys-C-, Asp-N endoproteinase-, and trypsin-treated fragments, respectively. The serine residue at position 34 is the single residue phosphorylated by PKC in vitro (see text for details). B, identification of a consensus amino acid sequence (underlined amino acids) between neurogranin and neuromodulin. The consensus sequence corresponds to the conserved phosphorylation site and calmodulin binding domain in neurogranin (37, 40).

The phosphorylation site sequence of neurogranin was determined after tryptic digestion of the phosphorylated protein, as evidenced by sequencing the tryptic fragments by HPLC on a reverse-phase column, and automatic sequencing of the major radioactive peptide (see Fig. 7, A and B). Before tryptic digestion the phosphorylated neurogranin eluted as mainly a single protein peak from the reverse-phase column (Fig. 7A). The minor peak eluting in front of the major peak is the nonphosphorylated protein. Cerenkov scintillation counting of the

![Fig. 3. The primary structure of neurogranin, comparison with neuromodulin. A, the primary structure of neurogranin was determined by automatic sequencing following chemical and enzymatic fragmentation and mass spectrometry (for details see “Materials and Methods”). The amino acids shown in brackets are uncertain (see text). The extension of the sequenced fragments are shown below the primary structure. AC, V8, LYS, ASP, and TR, refer to formic acid-, Glu-C-, Lys-C-, Asp-N endoproteinase-, and trypsin-treated fragments, respectively. The serine residue at position 34 is the single residue phosphorylated by PKC in vitro (see text for details). B, identification of a consensus amino acid sequence (underlined amino acids) between neurogranin and neuromodulin. The consensus sequence corresponds to the conserved phosphorylation site and calmodulin binding domain in neurogranin (37, 40).

The phosphorylation site sequence of neurogranin was determined after tryptic digestion of the phosphorylated protein, as evidenced by sequencing the tryptic fragments by HPLC on a reverse-phase column, and automatic sequencing of the major radioactive peptide (see Fig. 7, A and B). Before tryptic digestion the phosphorylated neurogranin eluted as mainly a single protein peak from the reverse-phase column (Fig. 7A). The minor peak eluting in front of the major peak is the nonphosphorylated protein. Cerenkov scintillation counting of the

![Fig. 4. Immunoprecipitation of phosphorylated neurogranin from 32P-labeled hippocampal slices. Hippocampal slices were prelabeled for 90 min with 32P and further labeled for 15 min in the absence (lane 1) or presence (lane 2) of phosphor 12-myristate 13-acetate. After homogenization, neurogranin was immunoprecipitated as described under “Materials and Methods” and the immunoprecipitate analyzed by SDS, 12.5% PAGE followed by autoradiography. p17 indicates the position of the purified bovine neurogranin used as a standard.

**Table I**

Experimentally determined amino acid composition of neurogranin compared with that predicted by the proposed amino acid sequence

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Neurogranin</th>
<th>Neuromodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>8.47</td>
<td>7</td>
</tr>
<tr>
<td>E + Q</td>
<td>3.6</td>
<td>3</td>
</tr>
<tr>
<td>S</td>
<td>4.87</td>
<td>5</td>
</tr>
<tr>
<td>G</td>
<td>17.58</td>
<td>19</td>
</tr>
<tr>
<td>H</td>
<td>0.99</td>
<td>1</td>
</tr>
<tr>
<td>R</td>
<td>4.6</td>
<td>5</td>
</tr>
<tr>
<td>T</td>
<td>1.29</td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>P</td>
<td>6.13</td>
<td>7</td>
</tr>
<tr>
<td>Y</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>V</td>
<td>0.28</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>1.35</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>0.49</td>
<td>3</td>
</tr>
<tr>
<td>I</td>
<td>2.98</td>
<td>4</td>
</tr>
<tr>
<td>L</td>
<td>1.89</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>1.08</td>
<td>1</td>
</tr>
<tr>
<td>K</td>
<td>1.80</td>
<td>5</td>
</tr>
<tr>
<td>W</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Total of 76 amino acids

a Cysteine is generally low when not derivatized.
b Lysine is low, probably due to modification (see “Results”).
c Determined spectrometrically.

**Fig. 4. Immunoprecipitation of phosphorylated neurogranin from 32P-labeled hippocampal slices. Hippocampal slices were prelabeled for 90 min with 32P and further labeled for 15 min in the absence (lane 1) or presence (lane 2) of phosphor 12-myristate 13-acetate. After homogenization, neurogranin was immunoprecipitated as described under “Materials and Methods” and the immunoprecipitate analyzed by SDS, 12.5% PAGE followed by autoradiography. p17 indicates the position of the purified bovine neurogranin used as a standard.**
major protein peak yielded counts of 3300 cpm/11 μg of phosphorylated neurogranin. After tryptic digestion, 90% of the loaded radioactivity was recovered in a single phosphorylated amino acid. Phosphoamino acid analysis by thin layer chromatography of the HC1-hydrolyzed 32P-labeled neurogranin revealed only the presence of phosphoserine (data not shown). The phosphorylated peptide is part of the conserved amino acid sequence found in neurogranin and neuromodulin (see Fig. 3B).

Interaction of Neurogranin with Calmodulin—Analysis of the primary sequence of neurogranin revealed an amino acid sequence shared with neuromodulin, including the proposed calmodulin binding domain of the latter. Therefore, we tested for the possible interaction of neurogranin with calmodulin. A special feature of the neuromodulin-calmodulin interaction is that neuromodulin binds to a calmodulin-Sepharose column in the absence of calcium. We, therefore, also tested neurogranin in this way (Fig. 8). Semi-purified neurogranin (10 μl; 20 μg protein/ml in buffer A), corresponding to the neurogranin-containing fractions eluted from the IBF AcA 54 column (see “Materials and Methods”), was directly applied to a calmodulin-Sepharose column equilibrated with buffer A containing 200 mM NaCl, 10 mM DTT and 2 mM EDTA and washed successively with 3 column volumes of equilibrating buffer and then with buffer containing 3 mM CaCl₂ instead of EDTA. The elution profile shows that the majority of the loaded proteins eluted with buffer containing Ca²⁺ (Fig. 8a).

Dot blot analysis of each eluted fraction using neurogranin antibodies showed that most of the neurogranin eluted with the Ca²⁺ buffer (not shown). No neurogranin immunoreactivity was found associated with the fractions corresponding to the flow-through and the washing step suggesting that the majority of neurogranin molecules are able to bind calmodulin. So as to illustrate the binding of neurogranin to the calmodulin-Sepharose column, SDS-gel electrophoresis and Western blot analysis of the proteins in the flow through fraction 15 and in fraction 40 that eluted with Ca²⁺ buffer are shown in Fig. 8, B and C. Neurogranin was only visualized in the fraction eluted with Ca²⁺. The apparent biphasic elution of the neurogranin peak resolved on the chromatograph might reflect a heterogeneity of the neurogranin preparation and differences in the affinity of neurogranin species for the calmodulin-Sepharose column. A similar behavior has been reported previously for neuromodulin (23). Note that a trace of neurogranin remained bound to the calmodulin-Sepharose column in the presence of Ca²⁺ plus 200 mM NaCl and eluted with a higher salt concentration, i.e., 500 mM NaCl (data not shown). The observed binding of neurogranin to a calmodulin-Sepharose column in the absence of Ca²⁺ might explain why a protein of estimated molecular mass 14 kDa was found to contaminate calmodulin-Sepharose affinity purified neurogranin preparations obtained from crude perchloric acid-soluble bovine brain extracts (23). The binding of neurogranin to a calmodulin-Sepharose column could be exploited to simplify the purification and other manipulations of this protein.

The interaction of neurogranin monomer with calmodulin also resulted in total inhibition of neurogranin phosphorylation by PKC at 5:1 molar excess of calmodulin over the neurogranin monomer (Fig. 5D). Calmodulin alone has no effect on PKC activity in the experimental conditions used (25). The inhibition of neuromodulin phosphorylation by...
FIG. 7. Purification of neurogranin phosphopeptides by C8 reverse phase HPLC chromatography. Phosphorylated neurogranin was prepared as described in legend of Fig. 4. The phosphorylated protein was resuspended in 500 μl of 40 mM Tris-HCl, pH 7.4, 10 mM DTT, 1 mM EDTA, A, 0.3 μg of phosphorylated neurogranin was injected onto a C8 reverse phase column and eluted as described under "Materials and Methods." B, 11 μg of phosphorylated trypsinized neurogranin was injected onto the same column. Fractions were collected at 1-min intervals and the radioactivity of each fraction (counts/min) was determined by Cerenkov scintillation counting. Counts of peptides that proved to be radioactive are indicated.

calmodulin has been observed previously (35). The rather high ratio of calmodulin over neurogranin needed to fully inhibit neurogranin phosphorylation probably stems from the fact that Ca^{2+} is present in the phosphorylation assay and prevents calmodulin-neurogranin interactions.

DISCUSSION

Protein phosphorylation is a major mechanism by which various cell surface receptors, through protein kinases, modulate the activity of key regulatory proteins. Therefore, the elucidation of the functions of these target phosphoproteins is of prime importance. Substrates of PKC in the brain are of particular interest, since this kinase has been implicated in a wide variety of brain-specific processes and in functions such as nerve cell differentiation and maturation, neurotransmitter release, and neuroplasticity (1). In this report, we describe a new purification procedure and the further characterization of one specific in vitro PKC substrate from bovine brain, neurogranin (molecular mass 7.8 kDa), previously called p17 (23).

Phosphorylation studies in intact hippocampal slices revealed that neurogranin is a phosphoprotein in vivo. The observation that phorbol 12-myristate 13-acetate, a PKC activator, stimulated neurogranin phosphorylation in hippocampal slices suggests that neurogranin is an in vivo substrate for PKC. In vitro phosphorylation of neurogranin by PKC produced a shift in the PI of the protein to a more acidic value similar to that observed for a minor neurogranin-like protein component in bovine brain preparations. The primary structure of neurogranin revealed a region of sequence similarity with neuromodulin, a well-characterized brain-specific PKC substrate. The conserved sequence comprised the PKC phosphorylation site domain and the predicted calmodulin binding domain of neuromodulin revealed a region of sequence similarity with neuromodulin and neurogranin to artificial membrane models, liposomes, have shown that these basic residues are involved in the binding of the proteins to negatively charged phospholipids and are also important for the interactions of the proteins with PKC.

Neurogranin has several physico-chemical properties in common with neuromodulin. In addition to being soluble in 2.5% perchloric acid and having abnormal electrophoretic migration, neurogranin also interacted with calmodulin in the absence of calcium (9, 23, 35). The calmodulin binding domain

FIG. 8. Ca^{2+}-independent interaction of neurogranin with a calmodulin-Sepharose column. a, semipurified neurogranin (10 ml; 20 μg protein/ml) eluted from the IBF AcA 54 column (see "Materials and Methods") was applied to a calmodulin-Sepharose column (1 × 5 cm) equilibrated with buffer A, 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl, 10 mM DTT, and 2 mM EDTA. The column was washed with equilibrating buffer (wash I) and then with buffer A containing 200 mM NaCl, 10 mM DTT, and 3 mM CaCl2 (wash II). Fractions (1 ml) were collected, and the elution profile was determined with Cerenkov scintillation counting. Counts of peptides that proved to be radioactive are indicated.

Neurogranin has several physico-chemical properties in common with neuromodulin. In addition to being soluble in 2.5% perchloric acid and having abnormal electrophoretic migration, neurogranin also interacted with calmodulin in the absence of calcium (9, 23, 35). The calmodulin binding domain

D. Hourbe, G. Duportail, and J. Baudier, manuscript in preparation.
of neuromodulin has been determined and corresponds to the sequence shared with neurogranin (9). Therefore, this conserved sequence most probably functions as a calmodulin-binding domain in neuromodulin. The PKC phosphorylation sites of neuromodulin and neurogranin are both within the proposed calmodulin binding domain. It is reasonable to propose that the binding of calmodulin to this domain decreases the accessibility of the phosphorylation site to PKC which explains the observed inhibition of neuromodulin and neurogranin phosphorylation by calmodulin. Recent studies in our laboratory have pointed out that calmodulin inhibits the binding of these substrates to PKC and negatively charged phospholipids, two essential steps in the in vitro phosphorylation process by PKC (39).5

Highly conserved sequences in proteins are frequently sites of binding or interaction with other molecules. The 18-amino acid sequence including the phosphorylation site domain and the calmodulin binding domain is absolutely conserved in all fish and all mammalian neuromodulin sequences known, whereas extensive sequence variation occurs throughout the large carboxy-terminal domain of neuromodulin (40). The strict conservation of this sequence in all vertebrate neuromodulin suggests that the PKC phosphorylation site and the calmodulin binding property are essential to the biological functions of neuromodulin (40). Since neurogranin also contains this conserved 18 amino acid sequence, functional homology may also exist between neurogranin and neuromodulin. Neurogranin and neuromodulin have a distinct subcellular distribution in neuronal cells. Although neurogranin is essentially located postsynaptically (24), neuromodulin is exclusively presynaptic in adult brains (41) and confined to axons (16). It has been proposed that neuromodulin serves to localize calmodulin at the neuronal cell membrane and releases the Ca2+-binding protein in response to increase in intracellular Ca2+ (42) and/or to phosphorylation by PKC (35). It is thus possible that neurogranin also functions to sequester calmodulin in the same manner as neuromodulin, but in different subcellular compartments. Other functions for neuromodulin have been proposed, such as the regulation of phosphatidylinositol-4-phosphate kinase, neurotransmitter release, and modulation of synaptic plasticity. The determination as to whether or not neurogranin has a similar or related function(s) must await further investigation. Many of the questions regarding the similarity of structure and function of these two PKC substrates may be resolved by the molecular cloning of neurogranin and the subsequent comparison of its gene organization with that of neuromodulin. Work is in progress to elucidate the complete sequence of the mouse neurogranin gene.

Acknowledgments—we would like to thank Prof. R. D. Cole (University of California, Berkeley) and Dr. M. Sensenbrenner for their help in initiating this work and critical reading of the manuscript. We also thank Prof. P. Chambron for supporting this project and Drs. S. Ali, R. Hen, M. Leid, T. Luftin, and G. Richards for a critical reading of the manuscript.

Note Added in Proof—After acceptance of this manuscript, the characterization of a rat mRNAs, highly enriched in the cortex, encoding a 78-residue protein called RC3 was reported (Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1986) J. Neurosci. Res. 26, 397–408). The primary structure of bovine neurogranin is identical in at least 73 of 78 residues to that of RC3.

REFERENCES
13. Note Added in Proof—After acceptance of this manuscript, the characterization of a rat mRNAs, highly enriched in the cortex, encoding a 78-residue protein called RC3 was reported (Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1986) J. Neurosci. Res. 26, 397–408). The primary structure of bovine neurogranin is identical in at least 73 of 78 residues to that of RC3.

REFERENCES
13. Note Added in Proof—After acceptance of this manuscript, the characterization of a rat mRNAs, highly enriched in the cortex, encoding a 78-residue protein called RC3 was reported (Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1986) J. Neurosci. Res. 26, 397–408). The primary structure of bovine neurogranin is identical in at least 73 of 78 residues to that of RC3.

REFERENCES
13. Note Added in Proof—After acceptance of this manuscript, the characterization of a rat mRNAs, highly enriched in the cortex, encoding a 78-residue protein called RC3 was reported (Watson, J. B., Battenberg, E. F., Wong, K. K., Bloom, F. E., and Sutcliffe, J. G. (1986) J. Neurosci. Res. 26, 397–408). The primary structure of bovine neurogranin is identical in at least 73 of 78 residues to that of RC3.