Volume 282, number 1, 183–188 FEBS 09650 April 1991

ADONIS 001457939100358U

A rapid purification method for neurogranin, a brain specific calmodulin-binding protein kinase C substrate

Jean Christophe Deloulme, Monique Sensenbrenner and Jacques Baudier

Laboratoire de Neurobiologie Ontogénique, Centre de Neurochimie du CNRS, 5 Rue Blaise Pascal, 67084, Strasbourg Cedex, France

Received 8 February 1991

A rapid purification method is reported for bovine brain neurogranin, a calmodulin-binding protein kinase C (PKC) substrate. This method takes advantage of the fact that the protein remains soluble in 2.5% perchloric acid (PCA) and that it binds to a calmodulin-Sepharose column in the absence of calcium. Other PKC substrate proteins that remain to be identified were also found to share these two properties, suggesting that a class of calmodulin-binding PKC substrates may exist in the brain.

Protein kinase C substrate; Purification; Brain (bovine)

1. INTRODUCTION

Protein kinase C (PKC) has been shown to be a key enzyme in mediating specific neural functions in the central nervous system [1–5]. 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. In the brain, only two PKC substrates have been formally identified and characterized: neuromodulin (also called GAP-43, B50, F1, p57, pp46) [6–9] and the MARCKS protein (also called p87) [10–12]. These two proteins are soluble in 2.5% PCA, suggesting that PCA solubility might be a common property of a class of PKC substrates [13]. In bovine brain extracts, we recently identified another low molecular weight PCA-soluble in vitro substrate for PKC, that we called p17 due to its apparent molecular weight on SDS-polyacrylamide gel [13].

An immunohistochemical study showed that p17 is found exclusively in the rat forebrain and more specifically concentrated in the perikarya and dendrites of neurons of the cerebral cortex and hippocampus [14]. Because p17 immunoreactivity is often found to be associated with granule-like structures in dendrites of pyramidal cells of the hippocampus at the electron microscopical level, we have called this p17 protein 'neurogranin'. The purified bovine brain neurogranin has now been sequenced [15]. Its primary structure was found to be identical, in at least 73 of 78 residues, to that of a new brain-specific protein, called RC3 [16]. Neurogranin (M_r 7.8 kDa, pHi 5.6), like neuromodulin and MARCKS protein, migrated abnormally on SDS-PAGE with an apparent molecular mass of about twice its true value. A comparison of the deduced amino-acid sequence of neurogranin (RC3) with that of neuromodulin revealed a striking conserved amino-acid sequence between the two proteins (Fig. 1). This sequence is the predicted calmodulin-binding domain on neuromodulin [6] and also corresponds to the PKC phosphorylation site [17]. Hence, we demonstrate that neurogranin binds to the calmodulin-Sepharose column in the absence of calcium, a feature that also characterizes neuromodulin, and we took advantage of this last property to develop a new and rapid purification method of neurogranin.

Correspondence and present address: J. Baudier, Laboratoire de Biologie Moleculaire du Cycle Cellulaire, INSERM Unité 309/DBMS, Centre d'Etudes Nucleaires de Grenoble, BP 85X, 38041 Grenoble-Cedex, France. Fax: (33) (76) 88 51 00

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCA, perchloric acid; PKC, protein kinase C; SDS, sodium dodecylsulfate

2. MATERIALS AND METHODS

2.1. *Materials*

The following materials were obtained from the indicated sources: FPLC apparatus and Phast-System Electrophoresis apparatus from Pharmacia; immunoblotting and protein determination reagents from Bio-Rad; the Immobilon blotting membrane from Millipore; phosphatidylserine from Sigma; [γ-^{32}P]ATP (5 mCi/ml) from Amersham. Protein kinase C was a generous gift of Dr F. Rogue and Dr A. Malvyia of the 'Centre de Neurochimie du CNRS'. The calmodulin-Sepharose column was prepared as previously described [18]. Neuromodulin and neurogranin used as references during the purification procedure were purified as previously described [13]. Polyclonal antibodies against neurogranin and neuromodulin were prepared as previously described [14].

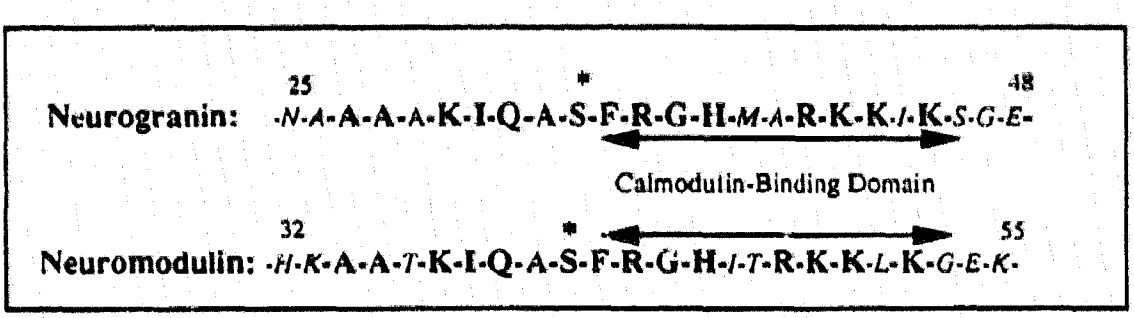

Fig. 1. Identification of a consensus amino-acid sequence (bold amino-acids) between neurogranin and neuromodulin. This consensus sequence corresponds to the conserved phosphorylation site (*) [15,17], and the calmodulin-binding domain on neuromodulin [6].

2.2. *Preparation of perchloric acid-soluble proteins from bovine brain that bind to the calmodulin-Sepharose column*

All the purification steps were performed at +4°C. Seventy bovine brains were homogenized in 40 mM citric acid, 0.5 mM phenylmethylsulfonyl fluoride (1 kg wet tissue/2 liters homogenization buffer) and centrifuged for 15 min at 5000 × *g*. After centrifugation, supernatants were pooled. Solutions of 2.0 M Tris-HCl and of 0.5 M EDTA in 2.0 M Tris-HCl were added to give a final concentration of 60 mM Tris-HCl and 10 mM EDTA. The pH, which was 4.3 in the initial supernatant, was brought to 7.8 with 10 N NaOH. The resulting extract was then passed through a Cybacron Blue column, previously equilibrated with 20 mM Tris-HCl, pH 7.5. This chromatographic step does not improve the purification of neurogranin, but was included to allow the concomitant purification of fibroblast growth factors that remain bound to the Cybacron Blue column [19]. The column flow-through was collected and brought to 70% ammonium sulfate, incubated for 2 h and centrifuged for 30 min at 13000 × *g*. The resulting pellet was resuspended in 50 mM Tris-HCl, pH 6.8, 50 mM NaCl, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride and 10 mM DTT using a Dounce homogenizer. The protein solution was brought to 2.5% PCA and immediately centrifuged for 20 min at 13000 × *g*. The supernatant was collected and made to 20% saturation with trichloroacetic acid for 30 min. A white precipitate formed and was pelletted by centrifugation for 40 min at 13000 × *g*. The pellet was dissolved in 2 M Tris-HCl, pH 7.5, 50 mM EDTA, 10 mM DTT and dialysed overnight against 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5 mM EDTA, 5 mM DTT (Buffer A). Half of the dialysed protein solution was loaded on a calmodulin-Sepharose column (50 mg calmodulin/20 ml Sepharose) equilibrated with buffer A. After extensive washing of the column, the bound proteins were eluted with Ca^{2+}-containing buffer made of 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.5 M NaCl. The elution of the protein was followed by SDS-PAGE using the Phast-System apparatus and silver staining of the gel. After extensive dialysis against 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM DTT, the protein solution (60 ml) was stored at −20°C.

2.3. *Electrophoresis*

Proteins were analyzed on one-dimensional gels containing 12% acrylamide/0.36% bisacrylamide using the buffer system of Laemmli [20]. Gels were stained with Coomassie blue.

2.4. *Phosphorylation assay*

Proteins were incubated with purified PKC in 40 mM Tris-HCl, pH 7.2, 0.1 mM free Ca^{2+}, 0.05 mM phosphatidylserine for 1 min at 35°C. The phosphorylation reaction was initiated by adding 5 mM $MgCl_2$ and 100 μM [γ-^{32}P]ATP. The reaction was terminated by the addition of SDS-stop solution (5% SDS, 25% glycerol, 0.5 M Tris-HCl, 10 mM DTT, pH 6.8) and heated for 5 min at 100°C. The proteins were separated by 0.1% SDS-12% PAGE and submitted to an autoradiographic procedure by using Hyper film HP (Amersham International).

2.5. *Immunoblotting*

Immunoblots were performed as previously described [14].

3. RESULTS

3.1. *Characterization of the PCA-soluble calmodulin-binding proteins*

Fig. 2a shows the electrophoretic pattern of the PCA-soluble proteins that bound to the calmodulin-Sepharose column in the absence of calcium and which were eluted from the column with a buffer containing calcium (lane 1). The major protein which migrated with an apparent molecular mass of 46 kDa (N) can be assigned, without ambiguity to neuromodulin, an abundant brain specific PKC substrate that binds to calmodulin-Sepharose column in the absence of calcium [6,13]. The 46 kDa protein co-migrated with purified neuromodulin (Fig. 2a, lane 2) and showed strong immunoreactivity against neuromodulin antibodies (Fig. 2b, lanes 1,2). Several other Coomassie blue stained proteins, which migrated with lower electrophoretic mobility than neuromodulin, could be seen on the gel (Fig. 2a, lane 1). Most of these proteins reacted positively with neuromodulin antibodies, when used at low dilution (1:500) in Western blot analysis (Fig. 2b, lane 2), suggesting that they might correspond to proteolytic products of neuromodulin generated during the purification procedure, or alternatively that they correspond to other calmodulin-binding proteins that share common antigenic epitopes with neuromodulin. Two other proteins, which cross-reacted with neuromodulin antibodies and which did not bind to the calmodulin-Sepharose column, were also visualized on the Western blot (Fig. 2b, lane 3).

Fig. 2c shows an autoradiogram illustrating the phosphorylation by PKC of the PCA-soluble proteins which bound to the calmodulin-Sepharose column. In the absence of PKC cofactors (phosphatidylserine and Ca^{2+}) most of the Coomassie blue stained proteins (Fig. 2a, lane 1) showed a slight ^{32}P incorporation (Fig. 2c, lane 1), while their phosphorylation was strongly stimulated when phosphatidylserine and Ca^{2+} were present in the phosphorylation assay mixture

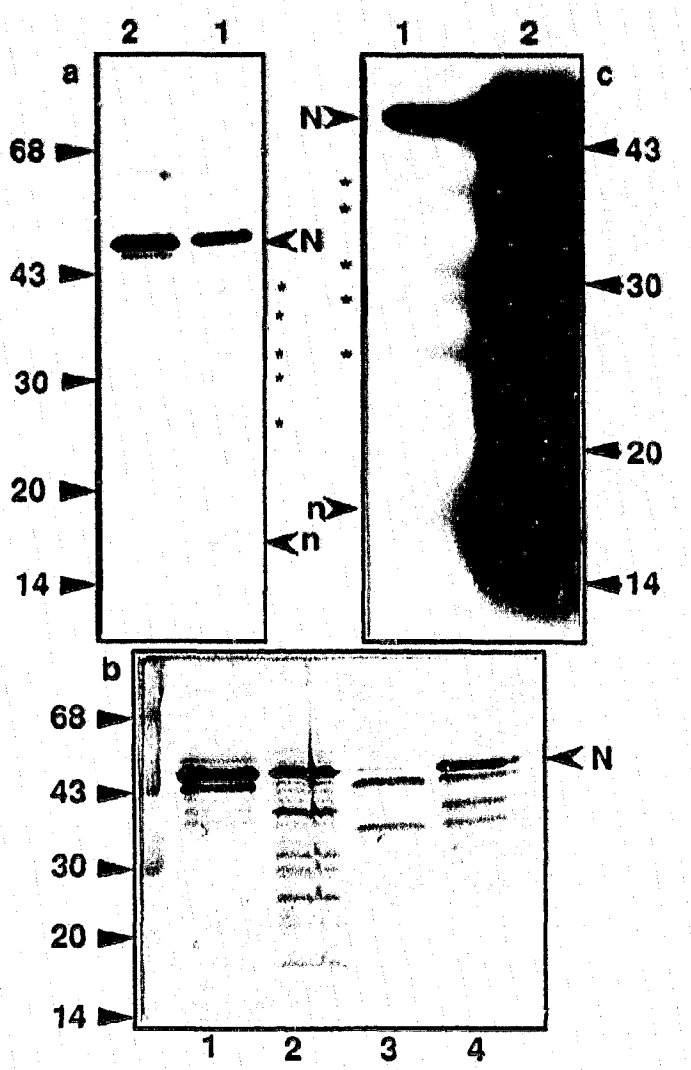

Fig. 2. Characterization of the PCA-soluble calmodulin-binding proteins from bovine brain. (a) Comparison of the electrophoretic mobilities (0.1% SDS-12% PAGE) of the PCA-soluble proteins that bound to the calmodulin-Sepharose column in the absence of Ca^{2+} (lane 1) with that of purified neuromodulin (lane 2). (b) Western blot analysis, using neuromodulin antibodies; purified bovine neuromodulin used as standard (lane 1); proteins that bound to the column (lane 2); proteins that did not bind to the calmodulin-Sepharose column in the absence of Ca^{2+} (lane 3); and total PCA-soluble proteins (lane 4). (c) Autoradiogram showing the phosphorylation by PKC of the proteins that bound to the calmodulin-Sepharose column in the absence of Ca^{2+}. The phosphorylation assay was performed in the absence (lane 1) or presence (lane 2) of Ca^{2+} and phosphatidylserine. N and n indicate neuromodulin and neurogranin, respectively. In a and c, * indicates the position of proteins that cross-reacted with neuromodulin antibodies. In the margins the positions of molecular weight standards are indicated.

(Fig. 2c, lane 2), suggesting that they indeed represent PKC substrates.

3.2. *Purification of neurogranin*

On Fig. 2, 'n' indicates the position of a minor protein that bound to calmodulin-Sepharose column in the absence of calcium and which migrated with an apparent molecular mass of 17 kDa (Fig. 2a). This protein did not react specifically with neuromodulin antibodies on Western blot but was a good substrate for PKC (Fig. 2c). A bovine brain PKC substrate with an apparent molecular mass of 17 kDa, soluble in 2.5% PCA, has been previously described [13] and subsequently called neurogranin [14]. The primary structure and cellular distribution in adult rat brain of neurogranin is identical to those described for RC3, a new brain-specific protein. Its structure was deduced from a sequence of the cDNA of a rat cortex-enriched mRNA [16]. Western blot analysis using neurogranin antibodies revealed that among all the calmodulin-binding proteins present on the Coomassie blue stained gel (Fig. 2a) only the 17 kDa bands reacted with neurogranin antibodies, suggesting that it likely corresponds to neurogranin (Fig. 3b).

The purification to homogeneity of neurogranin was achieved by two further chromatographic steps using the FPLC system. The PCA-soluble calmodulin-binding proteins were first passed onto a Mono Q column equilibrated with 40 mM Tris-HCl buffer, pH 7.5, 2 mM DTT and the proteins were eluted with a NaCl gradient. Fig. 3a shows the Coomassie blue staining of a SDS-PAGE gel used to analyse the eluted protein fractions after 1 h of incubation with 10 mM DTT. Dithiothreitol was used to reduce dimer and higher oligomer formation of neurogranin, occurring spontaneously during the chromatographic procedure. Fig. 3b shows a Western blot analysis of the neurogranin-containing fractions with neurogranin antibodies. Neurogranin (n) was eluted first from the Mono Q column at approximately 0.08–0.11 M NaCl (fractions 24–28) but was still contaminated by other proteins. Neuromodulin (N) was eluted at 0.2 M NaCl (fractions 36–39) and appeared homogeneous. Note, however, the presence of a faint protein band that co-purified with neuromodulin and which migrated just below neuromodulin. This protein band might possibly result from posttraductional modification of the protein. In this line, it is interesting to note that slight differences in SDS-PAGE electrophoresis mobility have been observed between neuromodulin isolated from two different cell types of the rat nervous system [21].

The neurogranin-containing fractions were then pooled together, adjusted to 0.1% trifluoroacetic acid and loaded onto a ProRPC column equilibrated with H_2O and 0.1% trifluoroacetic acid. After washing the column with equilibrating buffer the proteins were eluted with an acetonitrile gradient.

The eluted fractions (1 ml) were immediately neutralized with aliquots of 1 M Tris-base solution and analysed by SDS-PAGE after incubation for 1 h with 10 mM DTT. Fig. 4 shows the Coomassie blue stained gel. The ProRPC column allowed the separation of the

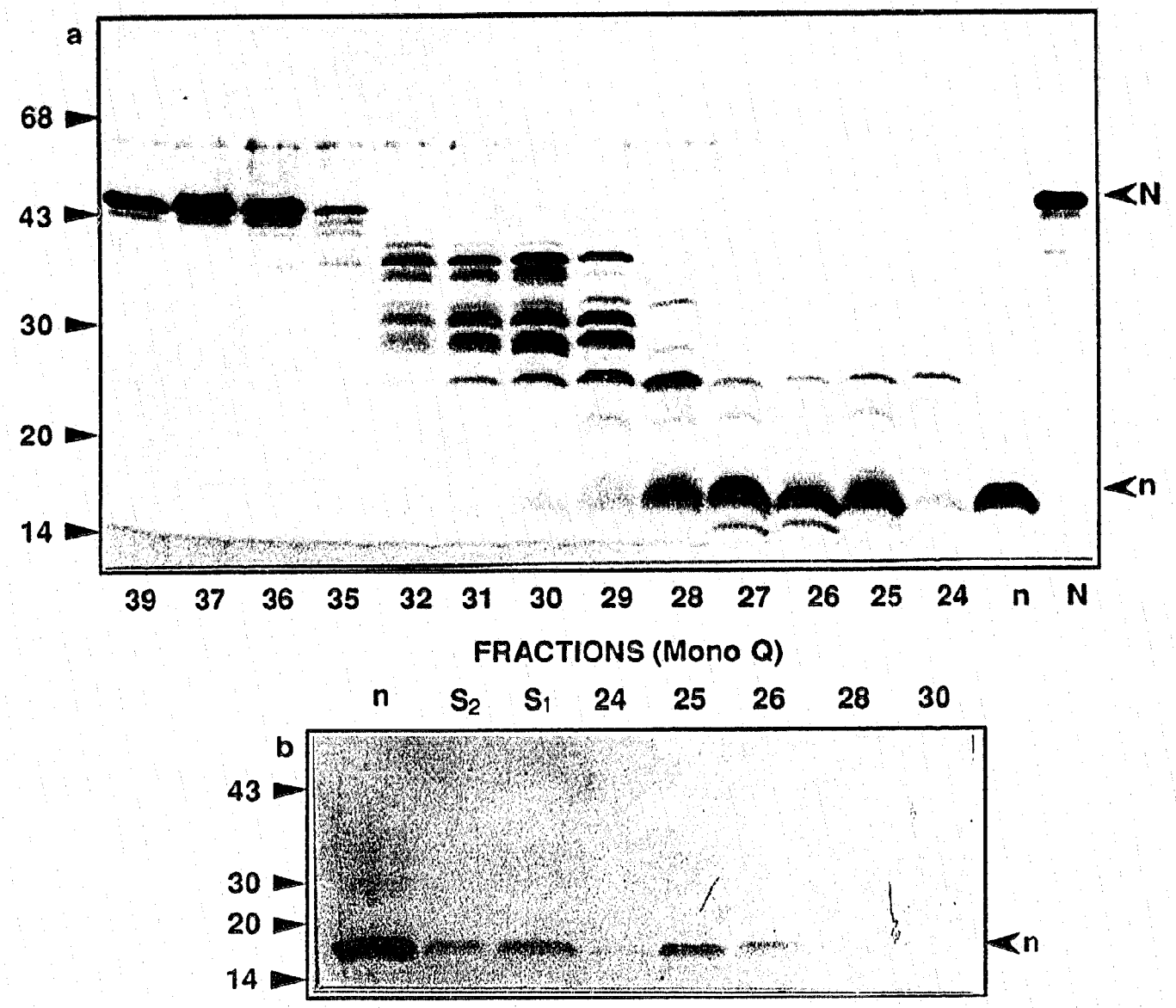

Fig. 3. SDS-PAGE analysis after separation of neuromodulin and neurogranin on a Mono Q column. (a) The dialysed PCA-soluble calmodulin-binding proteins were split into 6 × 10 ml aliquots and loaded separately on a mono Q column equilibrated with 20 mM Tris-HCl, pH 7.5, 2 mM DTT. After washing the column with 4 ml of equilibrating buffer the proteins were eluted with a NaCl gradient from 0 to 0.3 M NaCl within 30 min. The flow rate was 1 ml/min. Fractions (1 ml) were collected and analysed by 0.1% SDS-12% PAGE and Coomassie Blue staining of the gel. In lanes N and n, purified neuromodulin (N) and neurogranin (n) are used as standards. (b) Western blot analysis using neurogranin antibodies (1:1000 dilution) of the neurogranin-containing fractions (fractions 24–26, 28 and 30); n corresponds to purified neurogranin [15] used as standard, S_1 and S_2 correspond to the crude PCA-soluble protein extract and to the loading sample, respectively. In the left margins, the positions of the molecular weight standards are indicated.

protein contaminants from neurogranin that were eluted at approximately 20% acetonitrile (fractions 26–29). The purified neurogranin showed identical immunological and physicochemical properties to that of the neurogranin (p17) protein previously described [13].

4. DISCUSSION

Here, we reported a rapid purification method for neurogranin from bovine brain taking advantage of its solubility in 2.5% PCA [13] and its binding to calmodulin-Sepharose column. The PCA precipitation step provided a high degree of purification for both neurogranin and neuromodulin, but this step also exposed the proteins to acidic conditions. Nevertheless, PCA treatment of rat brain extract modified neither the electrophoretic mobility nor the immunoreactivity of the proteins, as revealed by Western blotting [14]. The PCA treatment of the purified proteins did not increase

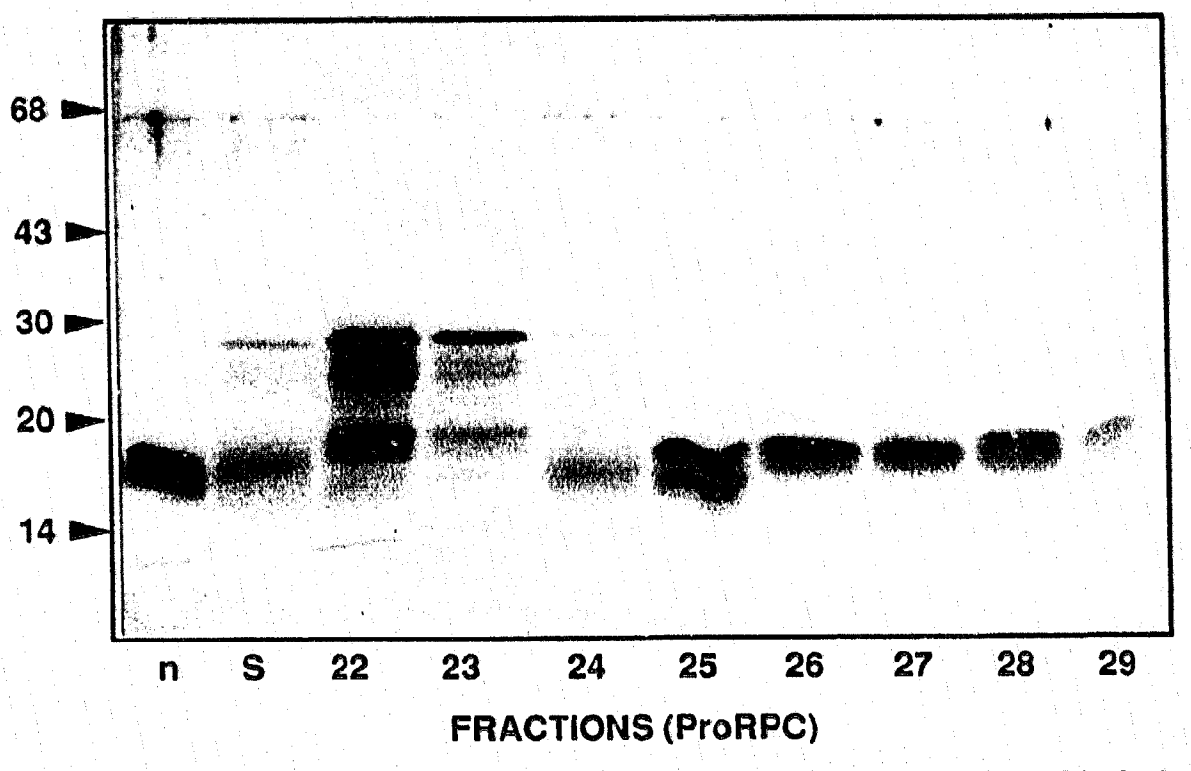

Fig. 4. SDS-PAGE analysis after purification of neurogranin by reversed-phase chromatography. The neurogranin-containing fractions from the mono Q column were pooled together (7 ml), adjusted to 0.1% trifluoroacetic acid, and loaded onto a ProRPC column equilibrated with 0.1% trifluoroacetic acid in H_2O. The column was then washed with 8 ml of equilibrating buffer and the proteins were eluted with an acetonitrile gradient from 0 to 30% acetonitrile within 40 min. The flow rate was 0.5 ml/min. Fractions (1 ml) were collected, immediately neutralized with aliquots of 1.0 M Tris-base solution, and analysed by 0.1% SDS-12% PAGE. n, purified neurogranin used as standard; S, loading sample.

or decrease the ability of PKC to phosphorylate the proteins and did not produce chemical proteolysis of the purified proteins (data not shown). However, the PCA treatment of the proteins did not affect the functional properties of the proteins, since neuromodulin and neurogranin were still able to bind to calmodulin-Sepharose. The calmodulin-Sepharose column step further provided a high degree of purification of the proteins. Most of the neuromodulin (Fig. 2b) and neurogranin (not shown) bound to the column in the absence of calcium, whereas the majority of PCA-soluble proteins did not, including several other PKC substrates, like the MARCKS protein (data not shown). The binding of neurogranin to the calmodulin-Sepharose column was expected because of the existence of a conserved amino acid sequence between neuromodulin and neurogranin (see Fig. 1) that was previously characterized as the calmodulin binding domain on neuromodulin [6]. The amount of purified neuromodulin was about 20 times higher than that of neurogranin. This agrees with the Western blot analysis of rat brain extracts, that showed neuromodulin immunoreactivity to be abundant in the rat brain, whereas neurogranin appeared as minor immunoreactive bands and only in specific regions of the central nervous system [14].

The calmodulin-Sepharose affinity chromatographic step also allowed the identification of other minor proteins on SDS-PAGE, which cross-reacted with neuromodulin antibodies and which were proved to be PKC substrates (Fig. 2c). Whether or not these proteins represent new PKC substrates or proteolytic products of neuromodulin remains to be investigated. One argument for the possibility that some of these proteins are true PKC substrates are the observations that some of these proteins are present at concentrations equivalent to that of neurogranin. Furthermore, the only proteolytic product of neuromodulin so far identified, called B60 [22], which lacks the first 41 amino acids out of 226, but which still possesses the putative calmodulin-binding sequence, has lost its calmodulin binding capacity. This suggests that the native conformation of the protein is probably required for calmodulin affinity. B60 probably corresponds to the major immunoreactive band seen on Fig. 2b in the calmodulin-Sepharose flow-through fraction (lane 3).

The calmodulin-binding domain on neuromodulin also corresponds to the PKC phosphorylation site domain on neuromodulin [17], which is also the case for neurogranin [15]. It is therefore most plausible, that such a consensus sequence represents a conserved amino-acid sequence for PKC phosphorylation site in a

class of brain specific calmodulin-binding PKC substrates.

The possibility that other calmodulin-binding PKC substrates, which belong to the neuromodulin-neurogranin family may also exist in peripheral tissues, is sustained by the recent identification of at least three PKC substrates (M_r 170000, 80000 and <40000 Da) in adipocytes, that selectively bind to calmodulin-Sepharose in the absence of calcium [23].

Acknowledgements: We gratefully acknowledge Jo Wyatt for critically reading the manuscript. This work was supported by grants from 'Fondation pour la Recherche Medicale Française' and INSERM. J.C. Deloulme is a recipient of a fellowship from the 'Association Française contre les Myopathies'.

REFERENCES

[1] Malenka, R.C., Madison, D.V. and Nicoll, R.A. (1986) Nature 321, 174–177.
[2] Madison, D.V., Malenka, R.C. and Nicoll, R.A. (1986) Nature 321, 695–697.
[3] Akers, R.F., Lovinger, D.M., Colley, P.A., Linden, D.J. and Routtenberg, A. (1986) Science 231, 587–589.
[4] Olds, J.L., Anderson, M.L., McPhie, D.L., Staten, L.D. and Alkon, D.L. (1989) Science 245, 866–869.
[5] Malinow, R., Schulman, H. and Tsien, R.W. (1989) Science 245, 862–866.
[6] Alexander, K.A., Wakim, B.T., Doyle, G.S., Walsh, K.A. and Storm, D.R. (1988) J. Biol. Chem. 263, 7544–7549.
[7] Meiri, K.F., Pfenninger, K.H. and Willard, M.B. (1986) Proc. Natl. Acad. Sci. USA 83, 3537–3541.
[8] Karn, L.R., Shi-Chung, N., Freeman, J.A. and Fishman, M.C. (1987) Science 236, 597–600.
[9] Van Hooff, C.O.M., De Graan, P.N.E., Oestreicher, A.B. and Gispen, W.H. (1988) J. Neurosci. 8, 1789–1795.
[10] Albert, K.A., Nairn, A.C. and Greengard, P. (1987) Proc. Natl. Acad. Sci. USA 84, 7046–7050.
[11] Patel, J. and Kligman, D. (1987) J. Biol. Chem. 262, 16686–16691.
[12] Graff, J.M., Stumpo, D.J. and Blackshear, P.J. (1989) J. Biol. Chem. 264, 11912–11919.
[13] Baudier, J., Bronner, C., Kligman, D. and Cole, R.D. (1989) J. Biol. Chem. 264, 1824–1828.
[14] Represa, A., Deloulme, J.C., Sensenbrenner, M., Ben-Ari, Y. and Baudier, J. (1990) J. Neurosci. 12, 3782–3792.
[15] Baudier, J., Deloulme, J.C., Dorsselaer, A.V., Black, D. and Matthes, H.W.D. (1991) J. Biol. Chem. 266, 229–237.
[16] Watson, J.B., Battenberg, E.F., Wong, K.K., Bloom, F.E. and Sutcliffe, J.G. (1990) J. Neurosci. Res. 26, 397–408.
[17] Apel, E.D., Byford, M.F., Au, D., Walsh, K.A. and Storm, D.R. (1990) Biochemistry 29, 2330–2335.
[18] Baudier, J., Mochly-Rosen, D., Newton, A., Lee, S.H., Koshland, D.E. Jr. and Cole, R.D. (1987) Biochemistry 26, 2886–2893.
[19] Pettmann, B., Weibel, M., Sensenbrenner, M. and Labourdette, G. (1985) FEBS Lett. 189, 102–108.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Deloulme, J.C., Janet, T., Au, D., Storm, D.R., Sensenbrenner, M. and Baudier, J. (1990) J. Cell. Biol. 111, 1559–1569.
[22] Coggins, P.J. and Zwiers, H. (1990) J. Neurochem. 54, 274–277.
[23] McDonald, J.R. and Lawrence, J.C. Jr. (1989) J. Biol. Chem. 264, 9611–9618.