Determination of the endogenous phosphorylation state of B-50/GAP-43 and neurogranin in different brain regions by electrospray mass spectrometry

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Abstract Electrospray mass spectrometry coupled to liquid chromatography was utilized to measure two PKC neuronal substrates, B-50/GAP-43 and neurogranin, in single rat brain areas. Aliquots of perchloric acid extracts were directly injected and mass spectra recorded. At elution times of 14.2 and 27.0 min two molecular species of MW 7450 and 23602 Da were observed. These values are in excellent agreement for the expected MW for rat neurogranin and B-50/GAP-43. The presence of molecular species shifted by 80 mass units in both cases indicates that these proteins are present in phosphorylated forms in cortical and hippocampal extracts.

Key words: Synaptic plasticity; Protein kinase C; Hippocampus; Cortex; Phosphoprotein; (Rat)

1. Introduction

In the central nervous system (CNS), protein kinases play a key role in the postreceptor processing of extracellular signals by phosphorylating specific protein substrates [1]. The role played by phosphorylation of individual proteins in these processes has been mainly inferred from in vitro experiments measuring their degree of phosphorylation by procedures involving back-titration to label residual phosphorylatable sites by means of phosphate incorporation from ^{32}P-labelled ATP [1]. The degree of phosphorylation in vitro is therefore thought to represent the number of non-phosphorylated sites in vivo. An alternative to this post-hoc phosphorylation assay, which more closely measures the degree of phosphorylation in vivo, involves in situ labelling of the proteins by incubating intact tissues with ^{32}P and subsequent immunoprecipitation of the protein of interest (for instance, see [2]).

A more recent technique involves the use of antibodies raised against specific phosphopeptides [3]. However, also this approach does not allow for the simultaneous measurement of both phospho and dephospho forms. Therefore, all these methods are indirect and are not able to detect in the same sample and with the same sensitivity each of the two forms of the protein independently.

On the basis of these observations, there is clearly a need for a better method to determine directly the phosphorylation state of a protein, particularly at low concentration levels in complex systems and at present the most suitable technique for this purpose is electrospray mass spectrometry (ES/MS). ES/MS allows for determining the molecular mass of large proteins with a resolution in the order of 10 Da and a precision of less than 1 Da. Therefore, an increase of 80 Da produced by the insertion of a single phosphate group can be easily detected.

We have previously shown that different proportions of phospho and dephospho forms of a small peptide generate linear and proportional changes in the signal intensities obtained within the mass spectrometer, thus allowing quantitative monitoring of changes in the phosphorylation state of the peptide in vitro [4].

The peptide studied corresponded to the aa 39–51 sequence of a large protein, B-50/GAP-43 (226 aa in rat), which contains a phospho site for protein kinase C (PKC) on Ser-41. There is currently much debate on the role of B-50/GAP-43 in CNS function and particularly on its role in synaptic plasticity [5,6]. It has been proposed that in the induction phase of long-term potentiation (LTP, a cellular model for synaptic plasticity underlying learning and memory), an increased phosphorylation state of B-50/GAP-43 is observed and this increase could in turn induce an enhancement in neurotransmitter release [7,8]. Moreover, it has been observed that LTP induction in hippocampal slices increases the phosphorylation state of another PKC substrate, neurogranin (BICKS, RC3) [8]. This protein is composed of only 78 aa in rats and shares a sequence homology with B-50/GAP-43 limited to 15–19 amino acids which includes the PKC phospho site [9,10]. These two PKC substrates, however, show a differential localization in the CNS, B-50/GAP-43 being localized exclusively in the presynaptic compartment [11] whereas neurogranin is localized in the perikarya and dendrites of neurons [12]. Therefore, investigation of changes in the phosphorylation state in vivo of both proteins might provide important clues for understanding the pre- and post-synaptic molecular mechanisms responsible for cognitive functions in normal and pathological conditions.

Previous studies have described ES/MS analysis of B-50/GAP-43 [13] and neurogranin [10]. The proteins were extensively purified from large amounts of bovine brain and their structures characterized. However, the methods described do not allow one to measure in the same sample the ratio of phospho vs dephospho forms of both proteins, mainly because of the extraction procedure utilized, which, in the case

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Abbreviations: ES/MS, electrospray mass spectrometry; HPLC, high-pressure liquid chromatography; LTP, long-term potentiation; PCA, perchloric acid; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid

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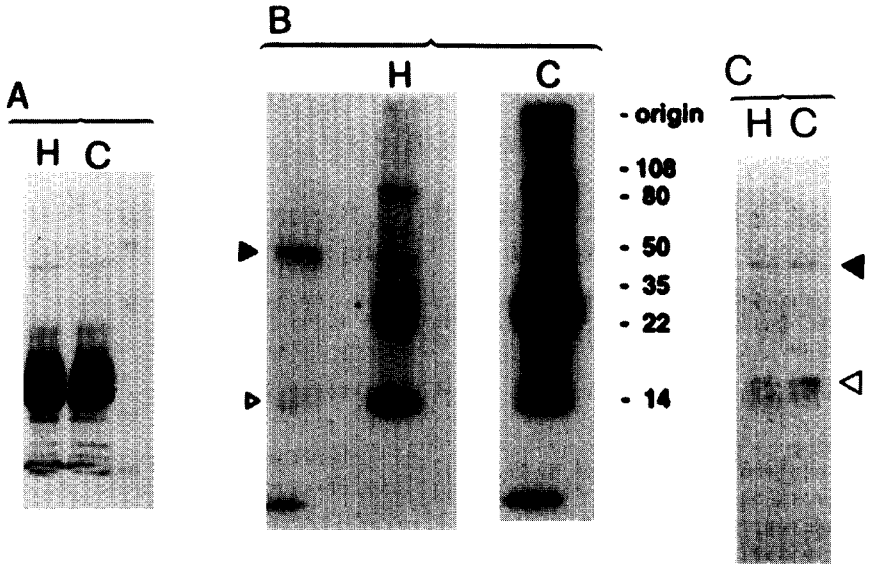

Fig. 1. Proteins in the extracts of rat cortex and hippocampus: silver staining, autoradiograph of SDS-PAGE and immunoblots. A: Proteins present in crude hippocampal (H) and cortical (C) perchloric acid extracts were subjected to SDS-PAGE (15%) and gels silver stained. B: Cortical (C) and hippocampal (H) PCA extracts were incubated in the presence of purified PKC and [γ-^{32}P]ATP, run on 15% polyacrylamide gel, 0.1% SDS and phosphoproteins detected by autoradiography. The migration of molecular weight markers is indicated. Purified and radiolabelled B-50 run in a separated lane (solid arrowhead). Position of neurogranin is indicated by open arrowhead. C: Approx. 1 μg of total proteins from extracts were subjected to 15% SDS-PAGE and transferred on nitrocellulose paper. Hippocampal (H) and cortical (C) extract were immunostained with NM2 monoclonal antibody (B-50, solid arrowhead; neurogranin, open arrowhead).

of B-50/GAP-43, leads to the loss of the major PKC phospho site [13]. Moreover, bovine B-50/GAP-43 and neurogranin show important differences with the rat proteins in their amino acid sequence [10,13].

In this report, we describe a simple extraction procedure based upon the previously reported solubility of B-50/GAP-43 and neurogranin in perchloric acid (PCA) [14,15], thus allowing for their simultaneous detection in individual rat brain areas (cortex and hippocampus) together with their respective phospho forms. Moreover, we show that B-50/GAP-43, bearing numerous phosphorylatable sites, appears to be largely present in its monophosphorylated form in rat brain.

2. Materials and methods

2.1. Materials

Calmodulin agarose gel (phosphodiesterase 3′,5′-cyclic nucleotide activator-agarose), diolein (DL-glycerol 1,2-dioleate), horse heart myoglobin and L-α-phosphatidylserine (PS) from bovine brain were obtained from Sigma (Italy). Gel filtration columns (P6, Bio-Gel DG 10 and P30) were obtained from Bio-Rad (Italy). [γ-^{32}P]ATP (spec. act. 3000 Ci/mmol) was from Amersham Corp. (Italy)

2.2. PCA extraction

Cortex and hippocampus from rat brain were rapidly dissected and homogenized in 2.5% PCA [14] (1 g tissue/5 ml) and centrifuged at 34000×g for 60 min. The pellet was discarded and the supernatant immediately neutralized with a minimal volume of a saturated solution of $(NH_4)_2CO_3$. The supernatants were then desalted on a P6 column and concentrated in a Speed Vacuum Savant centrifuge and stored at −20°C until analyzed by either SDS-PAGE or ES/MS. Protein concentrations in each sample were measured according to Bradford [16], using BSA (Sigma, Italy) as standard.

2.3. Protein phosphorylation assay

To identify PKC substrates in the PCA extracts, proteins were phosphorylated in vitro in a post-hoc assay with purified PKC [17], as previously described [18]. Phosphorylated proteins were then subjected to discontinuous SDS-PAGE (running gel: 15% unless indicated differently in the text). Gels were stained, destained and autoradiographed (Hyperfilm, Amersham, Italy) as previously described. B-50 purified following the method described by De Graan et al. [19] was utilized as control.

2.4. Immunoblotting

Proteins present in PCA extracts were separated in SDS-PAGE on a 15% running gel, transferred electrophoreticallly to nitrocellulose and immunoincubated with the primary antibody (NM2, monoclonal antibody recognizing both B-50 and neurogranin; dilution 1:2000, kind gift of Dr. De Graan) according to Oestreicher et al. [20].

2.5. HPLC-ES/MS

Approx. 20–100 ng of total proteins in the extract were applied to a perfusion microcolumn (Fus-15-R2/H; i.d. 300 μm, length 15 cm, LC-Packings, The Netherlands) coupled to an HPLC (HP 1090, series II, Hewlett-Packard, Palo Alto, USA). The sample was applied in 8 μl of buffer A (H_2O/CH_3COOH 2%/TFA 0.01%/CH_3CN 2%) and eluted with a gradient of buffer B (CH_3CN 98%/CH_3COOH 2%/TFA 0.01%)

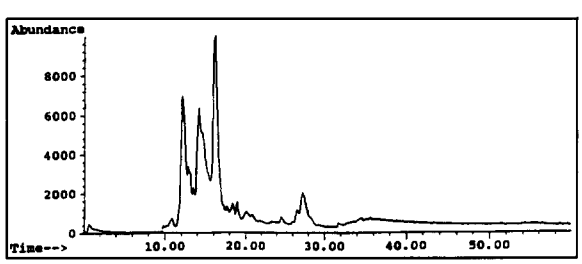

Fig. 2. Total ion chromatogram of a cortical PCA extract. 8 μl of a neutralized and desalted cortical extract were injected into HPLC-ES/MS and the total ion current chromatogram was recorded throughout the elution from the microcolumn. The ordinate indicates the abundance (arbitrary units) of the ions generated by the molecular species present in the mixture. Similar chromatograms were obtained from hippocampal extracts.

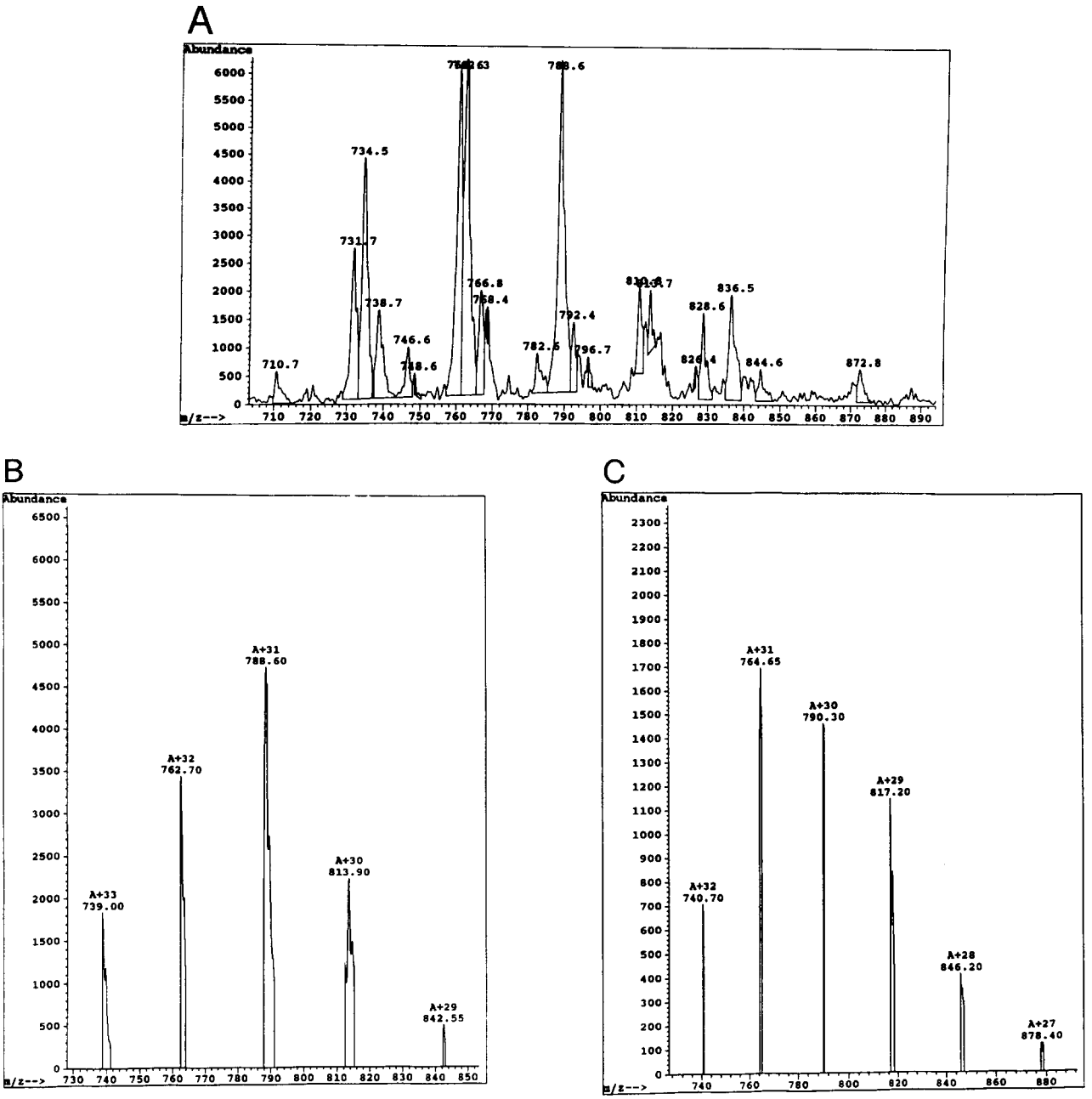

Fig. 3. Electrospray mass spectrum of B-50 in PCA extract. A: Raw mass spectrum was recorded at 27.0 min of elution from the HPLC column. B: Clusters of multiply charged related peaks generated by ionization of a protein of MW 23602.64 ± 2.6, corresponding to dephospho-B-50/GAP-43. C: Cluster of related peaks corresponding to monophospho-B-50/GAP-43: calculated MW 23672.88 ± 10.66.

as follows: isocratic 3 min, 0–5% B 5 min, 5–70% B 20 min, 70–100% B 10 min, and a final isocratic phase 100% B for 40 min, at a flow rate of 50 μl/min. Preconcentration at the head of the column was used in order to allow injection of this volume into the microcolumn. The HPLC column was connected on line with an HP 5989 B mass spectrometer consisting of an electrostatic ion source operating at atmospheric pressure (API/ES/MS 59987 A-HP, Hewlett-Packard, Palo Alto, USA) followed by a quadrupole mass analyzer with a mass range of 0–2000 arbitrary mass units (amu). Calibration was performed using the multiply charged ions from a separate introduction of horse heart myoglobin (16950.5 Da). Deconvolution of charged ions present in raw mass spectra was performed applying an algorithm included into the standard ES/MS software installed on a VECTRA 486 33 U-HP (Hewlett-Packard, Palo Alto, USA) computer system. Values of the molecular masses are given as 'average mass (compound mass)' ± S.D.

3. Results

3.1. PKC subtrates in extracts from brain areas

Proteins in PCA extracts were subjected to SDS-PAGE and the gels were silver stained (Fig. 1A). To identify PKC substrates, aliquots of the extracts were in vitro phosphorylated with the purified kinase. At least four phosphoprotein bands were found in the cortical and hippocampal extracts with

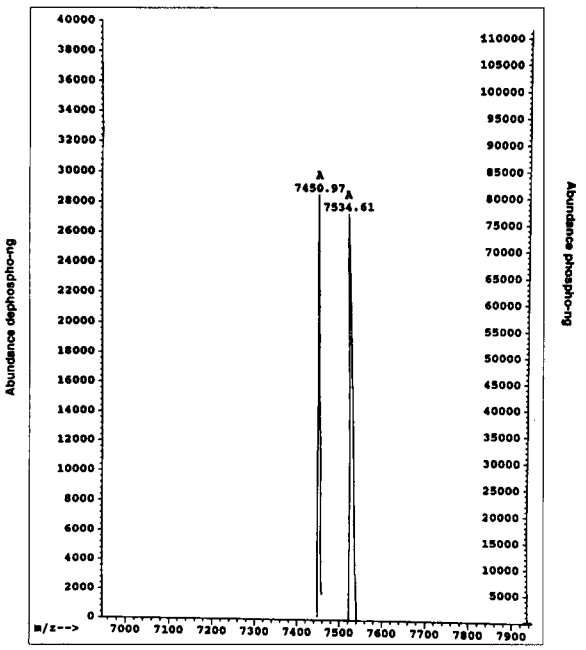

Fig. 4. Electrospray mass spectrum of neurogranin. Deconvolution of clusters present in the mass spectrum recorded at 14.2 min gives two molecular species differing by 83.6 Da. The peak at 7450.97 corresponds to dephosphoneurogranin and that at 7534.61 to the phosphorylated form of the protein. The abundance of the phospho form (right ordinate) is 2.7-fold greater than that of the dephosphorylated form (left ordinate).

estimated M_r values of about 78 000, 47 000, 21 000 and 17 000 (Fig. 1B). Among them, B-50/GAP-43 and neurogranin have been unequivocably identified by western blot analysis of the same gel using a monoclonal antibody (NM2 [20]) recognizing both proteins (Fig. 1C).

The extraction procedure yields 20–25 μg of total proteins from 100 mg of tissue. The recovery of the extraction procedure was evaluated by adding ^{32}P-labelled B-50/GAP-43 in the homogenate. All radiolabelled B-50/GAP-43 (95 ± 3.3%) was found in the extract.

3.2. HPLC-ES/MS analysis of brain extracts

PCA extracts were analyzed with capillary microcolumn HPLC-ES/MS, and mass spectra were continuously recorded throughout the elution from the HPLC column. A representative total ion current chromatogram of a tissue extract is reported in Fig. 2.

The mass spectrum recorded at the elution time of 27.0 min is shown in Fig. 3A. Its deconvolution generates two major clusters of related peaks, corresponding to two molecular species with a calculated average mass of 23 602.64 ± 2.6 mass units (m.u.) and 23 672.88 ± 10.66 m.u. (6 separate experiments using individual cortical and hippocampal extracts of 6 animals each). The expected MW of rat dephospho B-50/GAP-43 is 23 602, which is coincident with that found in the extracts. The MW of the other species differs by about 80 mass units, when taking into account the SD of the calculated value. Considering that it elutes from the HPLC column with the same retention time, we can conclude that it corresponds to the monophospho form of B-50/GAP-43.

The mass spectrum of Fig. 3A shows other clusters of related peaks with small differences in mass units but with lower intensities, suggesting that B-50/GAP-43 obtained from rat brain tissue is present in several post-translational modifications, most likely in several phosphorylation states. However, the low intensity of these signals does not allow for a correct and accurate mass assignment.

Mass spectrometric analysis of the same cortical extract where B-50/GAP-43 is present, also reveals the presence of neurogranin at the elution time of 14.2 min. Deconvolution of the raw mass spectrum (not shown) identifies two clusters of related peaks corresponding to two molecular species of 7450.97 ± 5.3 and 7534.61 ± 4.8 m.u. (Fig. 4). The mass difference of about 80 Da again suggests the presence of dephospho and monophospho forms of neurogranin. No other clusters of peaks can be detected indicating, as expected, only a single post-translational modification. The recorded MW is in good agreement with the theoretical MW of neurogranin calculated from cDNA (7495.0 Da) showing a difference lower than 0.5%. Identical results have been obtained in hippocampal extracts (data not shown).

4. Discussion

In view of the relevant role of phosphorylation processes in the CNS [1], the direct measurement of the phosphorylation state of protein substrates is of great biological importance. We focussed our attention upon two PKC substrates, B-50/GAP-43 and neurogranin, because both proteins have been suggested to be involved in activity-dependent synaptic plasticity and changes in their phosphorylation state might be one of the biochemical mechanisms underlying these processes [7,8]. However, their in vivo phosphorylation state has be inferred through indirect measurements utilizing radioenzymatic procedures involving incorporation of ^{32}P in vitro. We now show that in rat cortex and hippocampus B-50/GAP-43 and neurogranin are present as phosphoproteins in vivo, utilizing a simple extraction procedure and HPLC-ES/MS.

Although previous studies reported ES/MS data for B-50/GAP-43 [13] and neurogranin [10], they failed to show that: (i) phospho-neurogranin is present with its dephospho form in tissue extracts and (ii) B-50/GAP-43 monophospho form is by far the predominant phospho form in rat brain tissue. Indeed, B-50/GAP-43 seems to contain, in addition to the PKC phospho site at Ser-41, additional phosphosites at Ser-96 and at Thr-172 in the rat sequence [21]. The physiological relevance of these phospho sites in vivo is not yet fully characterized. On the other hand the key role played by PKC phospho site Ser-41 has been clarified further in mutant GAP-43 overexpressing mice, where the protein cannot be phosphorylated by PKC and the sprout-promoting activity has been profoundly depressed [22]. Additional experiments, to prove that the predominant monophospho form of B-50/GAP-43 in rat brain is at Ser-41, are required and we are currently testing whether stimulating PKC activity in rat brain, known to phosphorylate Ser-41, is able to increase the peak intensity of the phospho vs that of the dephospho form.

The extraction procedure described here takes advantage of the fact that both B-50/GAP-43 and neurogranin, together with another small number of proteins (Fig. 1A), are soluble in 2.5% vPCA [14,15] and are efficently extracted from brain tissue.

Although we do not have direct proof of the efficency of

extraction for neurogranin under our experimental conditions, it is reasonable to assume that this much smaller protein, with biochemical characteristics similar to those of B-50/GAP-43, is also efficently extracted in both its phospho and dephospho forms. In any event, since the biological activity of the protein is due to changes in the ratio of the phospho vs its dephospho form and since both species are simultaneously detected in the same extract, any in vivo change of this ratio should be detectable by ES/MS. Preliminary experiments in hippocampal slices treated with PKC activators, like phorbol esters, indeed show an increase in the ratio of the phospho vs dephospho form of neurogranin.

Finally, it should be noted that the measured MW of rat B-50/GAP-43 is identical to that expected on the basis of its sequence: the previous study on bovine B-50/GAP-43 showed a difference of more than 380 Da [13]. For neurogranin we have obtained a difference of less than 0.5% in the measured MW vs the expected one, whereas in bovine brain the difference reported is more than 8% [10].

In conclusion, we show that it is possible to detect and measure the ratio of the phosphorylation state of two PKC substrates, B-50/GAP-43 and neurogranin, in single rat brain areas where these proteins seem to exert important biological functions. Limited information is available about the temporal activation of PKC in the two neuronal compartments during LTP. Since B-50 and neurogranin are specific pre- and post-synaptic substrates for PKC in hippocampus, the direct measurement of the phosphorylation state of these two proteins during the induction and maintenance of LTP will contribute to clarification of the relative importance of PKC isoforms and their location in this form of activity-dependent synaptic plasticity.

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