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Dephosphin, a 96 000 Da substrate of protein kinase C in synaptosomal cytosol, is phosphorylated in intact synaptosomes

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A 96 000 dalton phosphoprotein, called dephosphin, is phosphorylated in intact synaptosomes from rat brain and is rapidly dephosphorylated upon depolarisation-dependent calcium entry. A 96 000 dalton phosphoprotein is also a substrate of protein kinase C in synaptosomal cytosol, and the aim of the study was to determine whether the two proteins may be the same. Dephosphin in intact synaptosomes and the 96 000 dalton protein kinase C substrate comigrated on polyacrylamide gels. Both phosphoproteins had identical phosphopeptide maps after digestion with V8 protease. Both phosphoproteins ran on isoelectric focussing gels with a pI of 6.3–6.7 and focussed as a series of 5–6 spots. Both proteins were phosphorylated exclusively on serine. Both proteins could be resolved into a doublet on longer polyacrylamide gels. The two subunits were of 96 and 93 kDa in both phosphorylation conditions and had dissimilar phosphopeptide maps. However, phosphopeptide maps of either the 96 or 93 kDa subunits were identical in intact synaptosomes compared with synaptosomal cytosol. These results show that a phosphoprotein phosphorylated in intact synaptosomes and a 96 000 dalton protein kinase C substrate from rat brain synaptosomal cytosol are the same, and raise the possibility that protein kinase C is the protein kinase responsible for dephosphin phosphorylation in intact synaptosomes.

Protein kinase C; Dephosphin; Synaptosome; Phosphorylation; Phosphoprotein

1. INTRODUCTION

Depolarisation of intact rat brain synaptosomes stimulates calcium influx and the phosphorylation of a variety of synaptosomal proteins [1–5]. Phosphorylation is due to the activation of calmodulin-dependent protein kinase II which phosphorylates synapsin I and other proteins [6,7], and protein kinase C which phosphorylates an 83 kDa protein (now termed the MARCKS protein [2,4,8]) and B-50 (also called GAP-43 [4,9,10]). Depolarisation is also accompanied by the activation of a phosphatase and the rapid dephosphorylation of at least two prominent synaptosomal phosphoproteins termed P96 and P139 [3,11]. P96 in intact synaptosomes is now called dephosphin and its rapid dephosphorylation precedes the phosphorylation increases of other proteins and could play a role in mediating neurotransmitter release [11,12]. Depolarisation-induced dephosphorylation of the protein is not due to proteolysis, as the protein is quickly rephosphorylated after removal of the depolarisation stimulus [11]. In previous studies on the phosphorylation of endogenous proteins in synaptosomes a cytosolic 96000 Da substrate of protein kinase C was described [13]. This phosphoprotein was not phosphorylated by basal kinases, cAMP-dependent protein kinase nor calmodulin-dependent protein kinases in vitro. Based on similar molecular weight and localisation to the cytosolic fraction of synaptosomes it was proposed that this phosphoprotein is dephosphin [12]. The aim of this study was to determine whether these phosphoproteins are the same. It was found that this cytosolic substrate of protein kinase C and dephosphin were indistinguishable.

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Abbreviations: CM, calmodulin; PS, L-phosphatidyl-L-serine

2. EXPERIMENTAL

2.1. *Materials*

Phosphatidylserine (PS), 1,2-diolein, ATP, *Staphylococcus aureus* V8 protease, leupeptin, pepstatin, chymostatin, antipain, phenylmethylsulfonylfluoride, cAMP, calmodulin, phospho-amino acids, and histone III-S were from Sigma. Acrylamide, bis-acrylamide, glycine and SDS were from Bio-Rad, $^{32}P_i$ and [γ-^{32}P]ATP (3000 Ci/mmol) were from New England Nuclear, Percoll was from Pharmacia LKB, and all other reagents were of Analytical Reagent grade or better.

2.2. *Methods*

A purified synaptosomal fraction was freshly isolated from the cerebral cortex of a rat (male Sprague–Dawley, 150–250 g) by differential centrifugation through Percoll [14]. The synaptosomes were either maintained intact in a modified Krebs buffer [3] containing finally 1.2 mM calcium or lysis buffer was added (5 mM Tris-HCl, pH 7.4, containing 15 μg/ml of leupeptin, pepstatin, chymostatin, antipain, and 50 μM phenylmethylsulfonylfluoride), and the synaptosomes homogenised with a motor-driven teflon/glass homogeniser

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and centrifuged at 140000 × g for 75 min to obtain synaptosomal cytosol. The final protein concentration was in the range 2.5–4.0 mg/ml, was determined [15] with bovine serum albumin as the standard and 80 μg protein was added to each assay tube (80 μl final incubation volume). Phosphorylation of intact synaptosomes was performed as described [3,11]. Phosphorylation of synaptosomal cytosol in the presence of [γ-^{32}P]ATP was performed as described [13], the final phosphorylation reaction (80 μl, 37°C) contained 30 mM Tris-HCl, pH 7.4, 1 mM $MgSO_4$, 1 mM EGTA, 40 μM [γ-^{32}P]ATP (4.4 × 10^6 cpm/nmol), 2.5 μg/ml of the inhibitor protein of cAMP-dependent protein kinase [16], and was initiated by the addition of synaptosomal cytosol. Incubations proceeded for 30 s in the presence or absence (as indicated in the figure legends) of 0.2 mM free $CaCl_2$, 25 μg/ml calmodulin, 40 μg/ml PS plus 4 μg/ml 1,2-diolein, and were terminated by the addition of 40 μl of 'SDS stop reagent' [3] followed by rapid freezing. Polyacrylamide slab gel electrophoresis and autoradiography were carried out as described previously [3] using 7.5–15% linear gradients. Two-dimensional gel electrophoresis was performed as described [17] for the first dimension and 7.5–15% gradient gels for the second dimension. Phosphopeptide maps were performed on proteins excised from dried gels as described [7]. Phosphoamino acid analysis was determined as described [18] using proteins excised from dried acrylamide gels.

3. RESULTS

Dephosphin is phosphorylated in intact synaptosomes after 60 min prelabelling with $^{32}P_i$ and is rapidly dephosphorylated after depolarisation with 41 mM K^+ (Fig. 1A, 'Dep' and 'Ctr'), as described previously [3,11]. Dephosphin has been shown to be predominantly cytosolic in intact synaptosomes [11]. A cytosolic protein from synaptosomes that comigrates with dephosphin on polyacrylamide gels is phosphorylated by endogenous protein kinase C (Fig. 1A, 'PS' and 'CM'). Phosphopeptide maps of dephosphin and this 96000 dalton protein kinase C substrate are essentially identical (Fig. 1B), indicating that they are likely to be the same protein and that they are phosphorylated on the same sites. The only difference between the peptide maps from dephosphin from intact or lysed synaptosomes is the presence of a 14 kDa phosphopeptide, more prominent in the lysed

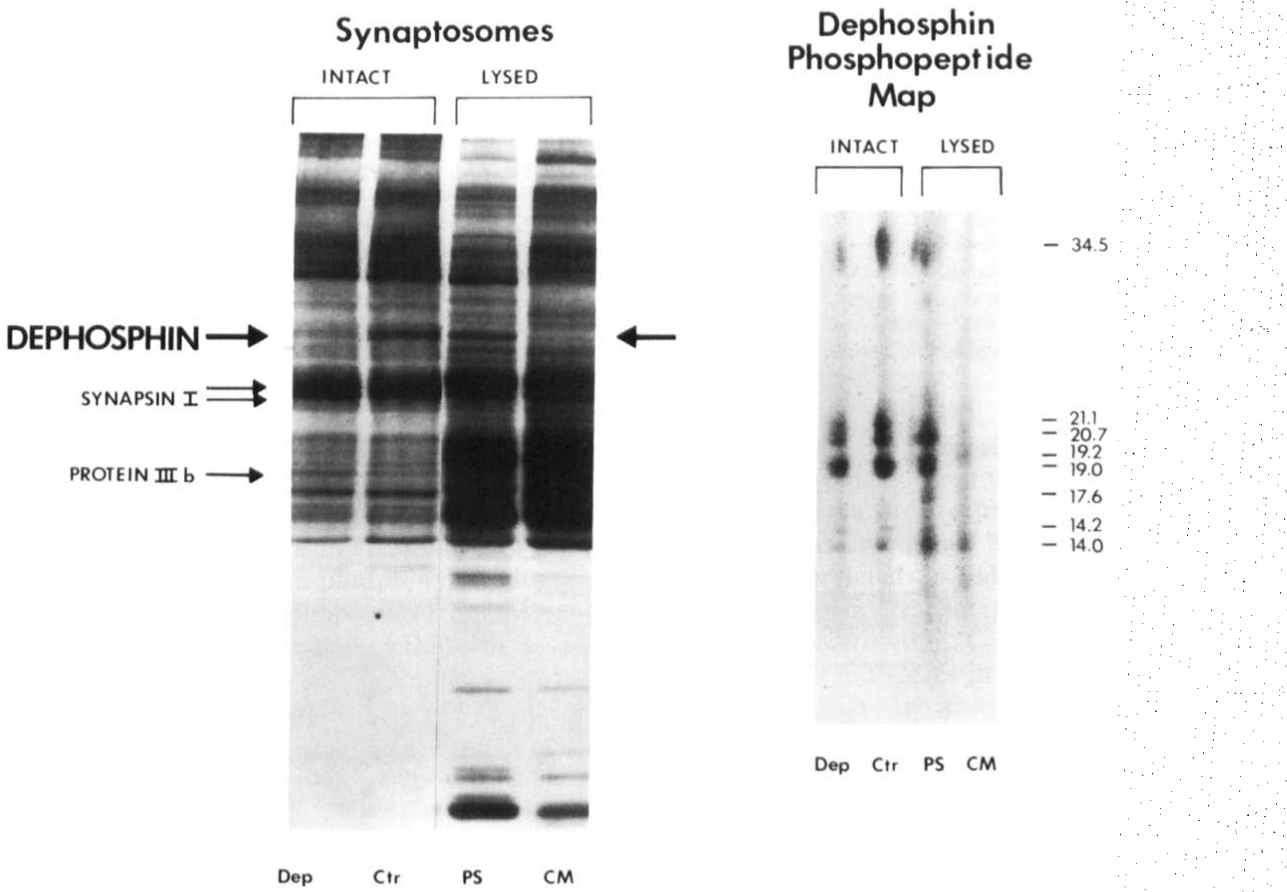

Fig. 1. Phosphorylation of dephosphin in intact synaptosomes and synaptosomal cytosol. (A) Autoradiograph of proteins labelled in intact synaptosomes prelabelled with $^{32}P_i$ for 45 min and stimulated for 5 s with 41 mM K^+ (Dep) or 4.7 mM K^+ (Ctr), or phosphoproteins in synaptosomal cytosol phosphorylated for 30 s in the presence of [γ-^{32}P]ATP and either calcium, PS and 1,2-diolein (PS) or calcium plus calmodulin (CM). Comparisons of intact and lysed synaptosomes were performed on aliquots of the same synaptosomal preparation. The positions of dephosphin, synapsin I and protein III are indicated on the left with arrows. Synapsin I and protein III were identified by criteria previously described [7]. (B) Autoradiograph of phosphopeptides generated by partial digestion of dephosphin from panel A with V8 protease. The molecular weights of certain phosphopeptides are indicated on the right in kDa. The phosphopeptide migrating at 14 kDa is derived from phosphorylase, which comigrates with dephosphin in these gels. Results are representative of 9 independent experiments.

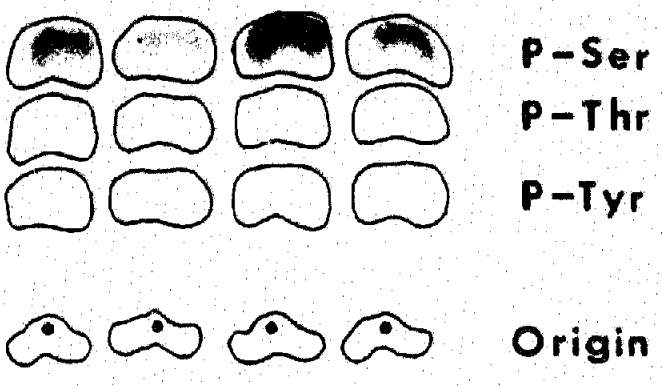

Fig. 2. Phospho-amino acid analysis of dephosphin. The phosphorylated 96 kDa phosphoproteins from Fig. 1, left panel, were excised from the gel, extracted and subjected to partial hydrolysis in 6 N HCl for 2 h at 110°C and the amino acids separated by thin layer electrophoresis and an autoradiograph is presented. The lanes from the left contain dephosphin from intact synaptosomes (control) or depolarised synaptosomes, the 96000 substrate of protein kinase C from cytosol phosphorylated with calcium, PS and 1,2-diolein, or with calcium plus calmodulin. The positions of phospho-amino acid standards are outlined. Results are typical of three experiments.

samples, which derives from phosphorylase and which comigrates with dephosphin. Dephosphin in intact synaptosomes and the 96000 dalton substrate of protein kinase C in soluble fractions are both phosphorylated exclusively on serine (Fig. 2). Isoelectric focusing of phosphoproteins from intact synaptosomes or synaptosomal cytosol shows that dephosphin and the 96 kDa protein kinase C substrate have the same isoelectric point (Fig. 3). Dephosphin focuses as a series of 5–6 spots from pI 6.3–6.7 with the 96 kDa protein kinase C substrate also focusing as 5–6 spots from pI 6.3–6.7 (Fig. 3B and D). In these experiments dephosphin was identified from control gels, run simultaneously, by either depolarisation-dependent dephosphorylation (intact synaptosomes, Fig. 3A) or complete lack of labelling in the presence of calcium-calmodulin (synaptosomal cytosol, Fig. 3C). Dephosphin can be resolved into a doublet on longer acrylamide gels and the 96000 dalton protein kinase C substrate from synaptosomal cytosol also comprises a doublet that comigrates with dephosphin (Fig. 4A). Phosphopeptide maps of the 96 and 93 kDa form of

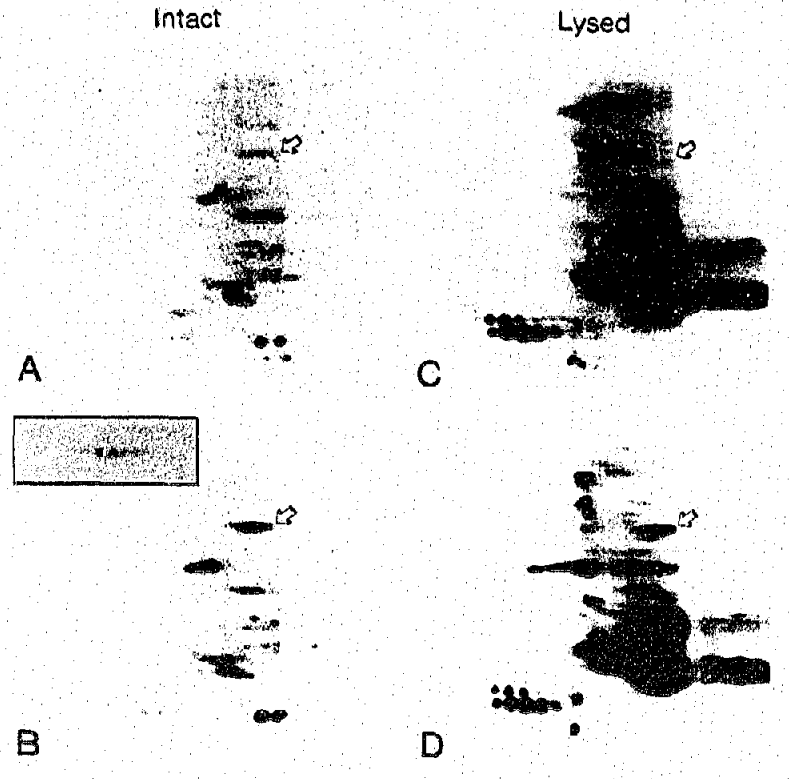

Fig. 3. Isoelectric focusing of synaptosomal phosphoproteins. Dephosphin was labelled in intact control (A) or depolarised synaptosomes (B) or phosphorylated in synaptosomal cytosol by endogenous calmodulin-dependent protein kinase (C) or protein kinase C for 30 s (D) and separated on IEF gels and 7.5–15% gradient polyacrylamide gels. Phosphorylations were performed on the same preparation of synaptosomes and the gels were run simultaneously. The inset in (B) shows an enlargement of dephosphin from a separate experiment to illustrate the multiple isoelectric species in the unstimulated intact synaptosomes. The acidic end of the gel is to the left of each figure.

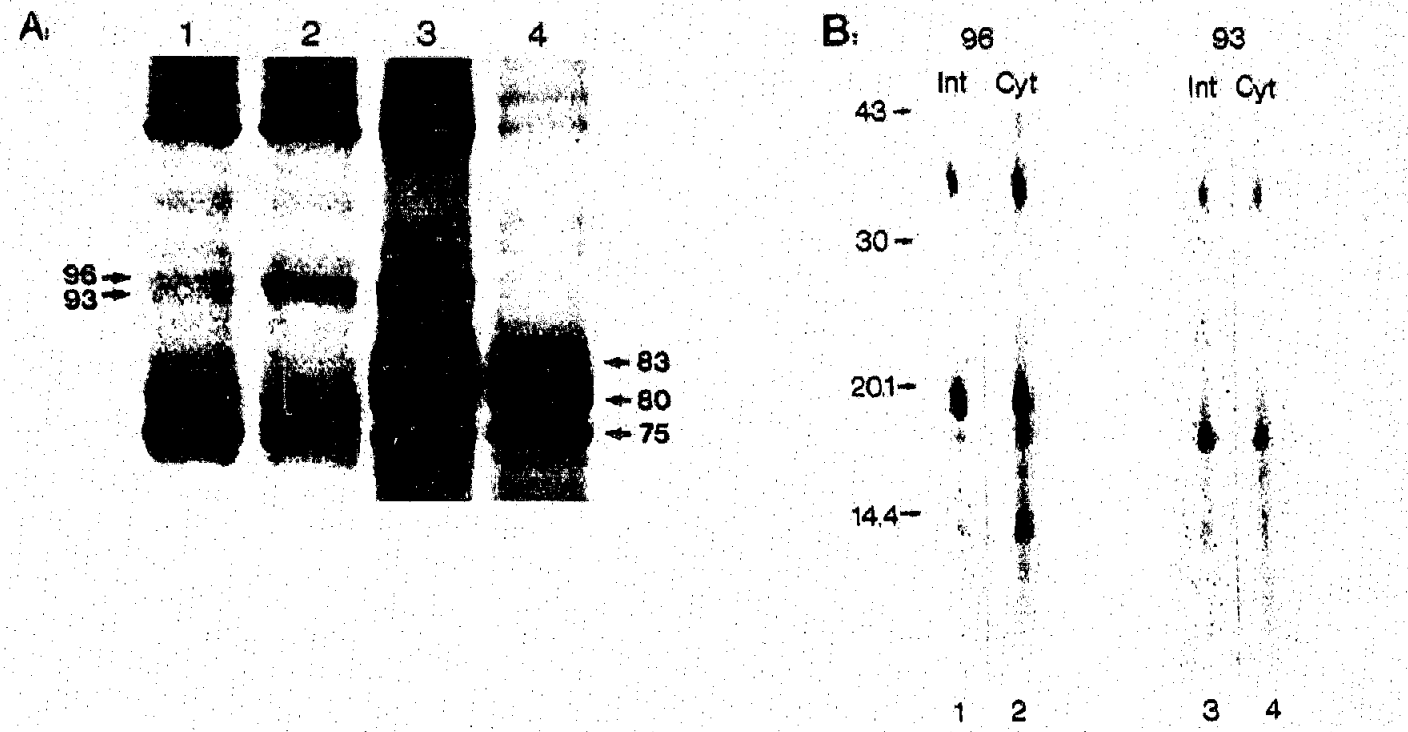

Fig. 4. Dephosphin is composed of a protein doublet. (A) Autoradiograph of dephosphin 96000 and 93000 kDa forms in intact resting synaptosomes (lane 2) or 15 s depolarised synaptosomes (lane 1). Phosphorylation in synaptosomal cytosol in the presence of calcium/PS/Diol (lane 3) or calcium plus calmodulin (lane 4), as described in the legend to Fig. 1. The MARCKS protein is indicated (in kDa) as 83, synapsin Ia and Ib as 80 and 75. (B) Phosphopeptide maps of dephosphin 96 kDa and 93 kDa forms from intact (Int, lanes 1 and 2) synaptosomes or cytosol (Cyt, lanes 3 and 4) from lysed synaptosomes. Each of the two subunits of dephosphin were excised from a gel and subjected to digestion with V8 protease in a second gel. The intact synaptosomes were prelabelled with $^{32}P_i$ for 60 min and were not further stimulated. Cytosol was phosphorylated in the presence of endogenous protein kinase C and calcium/PS/Diol. The peptide maps of intact vs cytosolic proteins were run on the same gel, but the autoradiographic exposure of the subunits from intact synaptosomes was longer since, typically, 1218 ± 186 cpm was loaded for each subunit from cytosol and 390 ± 55 cpm and 306 ± 37 cpm were loaded for dephosphin 96 and 93 kDa forms respectively (determined in separate experiments). Molecular weight marker proteins are indicated on the left in kDa.

dephosphin from intact synaptosomes and synaptosomal cytosol are the same (Fig. 4B), except for a 14 kDa phosphopeptide observed in the 96 kDa form of dephosphin when phosphorylated in cytosol by protein kinase C which is derived from phosphorylase. It is apparent that the dominant phosphopeptide from the 96 kDa form is 20 kDa and that of the 93 kDa form is 19 kDa.

4. DISCUSSION

Dephosphin is a 96000 dalton phosphoprotein doublet phosphorylated in intact synaptosomes. This study identifies a soluble synaptosomal protein kinase C substrate as indistinguishable from dephosphin, based on the following criteria: (i) the two phosphoproteins comigrate on polyacrylamide gels of different acrylamide concentrations; (ii) both are phosphorylated exclusively on serine; (iii) the two phosphoproteins have the same isoelectric point and run as a series of multiple spots on IEF gels; and (iv) both comprise non-idential subunits of 96000 and 93000 daltons with the same phosphopeptide maps. Therefore, dephosphin is an in vitro substrate of protein kinase C and an in vivo phosphorylated protein and henceforth this protein will be termed dephosphin, whether phosphorylated in intact or lysed synaptosomes. Dephosphin has not previously been identified as a protein kinase C substrate in soluble fractions from synaptosomes in other studies [19,20]. This is primarily due to the absence of the protease inhibitor leupeptin from the lysis buffer in those studies (which virtually abolishes dephosphin phosphorylation by protein kinase C [13]) and to the effect of Mg^{2+} on dephosphin phosphorylation. It is common practice to use 10 mM Mg^{2+} for in vitro phosphorylation assays since this is optimal for purified protein kinase C and for phosphorylation of many substrates such as the MARCKS protein. However, 1 mM Mg^{2+} is optimal for dephosphin phosphorylation in synaptosomal cytosol, while 10 mM Mg^{2+} blocks its labelling by protein kinase C (not shown).

The most likely protein kinase to phosphorylate dephosphin in intact synaptosomes is protein kinase C.

Since the in vitro phosphorylated dephosphin is a substrate of protein kinase C, it is probable that the in vivo form of dephosphin is also a substrate of protein kinase C. More direct evidence will be needed to clarify this point. Only two other synaptosomal substrates for protein kinase C have been identified in intact synaptosomes, the MARCKS protein and B-50 [2,4,5,7,9,21], although multiple substrates have been detected in synaptosomal cytosolic fractions [13,22,23]. Both the MARCKS protein and B-50 are phosphorylated in intact synaptosomes after depolarisation-dependent calcium entry [4,5,7,9]. Dephosphin is not phosphorylated under the same stimulus conditions since the dephosphin phosphatase may have a significantly greater affinity or rate of activity towards dephosphin than does protein kinase C. However, dephosphin is subsequently rephosphorylated upon termination of the calcium influx signal [11] and the mechanism of rephosphorylation and the possible role of protein kinase C remain to be determined. The properties of dephosphin are distinct from other characterised synaptosomal phosphoproteins and support a dynamic role for the protein in nerve terminal function.

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