Comparison of Ca^{2+} -dependent phosphorylation in viable dispersed brain cells with calmodulin-dependent protein kinase activity in cell-free preparations of rat brain

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Using two depolarizing agents, veratrine and high concentrations of extracellular KCl, we studied depolarization-stimulated phosphorylations in ^{32}P -labelled dispersed brain tissue in order to identify phosphoprotein substrates for Ca^{2+} - and calmodulin-dependent protein kinase activity at the cellular level, for comparison with findings in cell-free preparations. In intact brain cells, the only prominent depolarization-stimulated phosphorylation was a 77 kDa protein separated on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This phosphorylation was dependent on external Ca^{2+} , since chelation of Ca^{2+} in media with 6 mM-EGTA or the presence of verapamil (a Ca^{2+} -channel blocker) in the incubation media inhibited depolarization-stimulated phosphorylation of the 77 kDa protein. Phosphorylation of the 77 kDa protein also appeared to be dependent on calmodulin, because depolarization-stimulated phosphorylation was significantly decreased ($P < 0.05$) when 100 μM -trifluoperazine was present in the incubation media. Polymyxin B, an inhibitor of Ca^{2+} - and phospholipid-dependent phosphorylation, and 12-*O*-tetradecanoylphorbol 13-acetate, the phorbol ester enhancing Ca^{2+} - and phospholipid-dependent phosphorylation, had no effect on the phosphorylation of the 77 kDa protein. The 77 kDa phosphoprotein was identified as a protein previously named synapsin I [Ueda, Maeno & Greengard (1973) *J. Biol. Chem.* **248**, 8295–8305] on the basis of similar migration of native and proteolytic fragments of the 77 kDa protein with those of authentic synapsin I on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Whereas several studies with cell-free preparations showed that 57 kDa and 54 kDa endogenous phosphoproteins were the most prominent species phosphorylated in a Ca^{2+} and calmodulin-dependent manner, these results indicate that synapsin is the most prominent Ca^{2+} - and calmodulin-dependent phosphorylation in intact cells. The phosphorylations of 54 kDa and 57 kDa proteins may not be as important *in vivo*, but instead occur as a result of the disruption of cellular integrity inherent in preparation of cell-free subfractions of brain tissue.

INTRODUCTION

The increased permeability of neural tissue to Ca^{2+} during depolarization is well documented, and increased intraneuronal Ca^{2+} may be involved in stimulus-coupled secretion of neurotransmitters (Rubin, 1970). The effects of Ca^{2+} on neurotransmission have been shown to be directly dependent on the entry of Ca^{2+} into the presynaptic terminal and not due to secondary effects of Ca^{2+} on the presynaptic action potential (Katz & Miledi, 1970; Miledi, 1973). However, a detailed understanding of the participation of Ca^{2+} in the process of neurotransmitter release has not yet been achieved. Recently there has been interest in the Ca^{2+} -binding protein calmodulin as a possible modulator of the effects of Ca^{2+} on synaptic transmission. Experiments involving synaptosomes, synaptic vesicles and cell-free homogenate preparations of neural tissue have established the presence of several Ca^{2+} - and calmodulin-dependent protein kinase activities, which are hypothesized to be involved in neurosecretion (Schulman & Greengard, 1978; DeLorenzo *et al.*, 1979). When the Ca^{2+} and calmodulin-dependent kinase

activities are assayed in these cell-free preparations for phosphorylation of endogenous protein substrates, proteins of 50–60 kDa are the most prominent ones phosphorylated. Some of these kinase activities have been purified from neural tissue. One activity preferentially phosphorylates tubulin and microtubule-associated proteins (of 50–60 kDa) and may mediate changes in the microtubule system which result in neurotransmitter release (Goldenring *et al.*, 1983). Another kinase activity has been shown to have a protein-substrate preference for synapsin (Bennett *et al.*, 1983), a neurospecific protein that is associated with synaptic vesicles (Ueda *et al.*, 1973), but autophosphorylation of 50–60 kDa kinase subunits was also demonstrated. The functions of this calmodulin-dependent kinase activity and its neurospecific substrate are unknown, but phosphorylation of synapsin in cerebral-cortex slices and synaptosomes occurs with depolarizing events (Krueger *et al.*, 1977; Forn & Greengard, 1978). Hence it is considered that phosphorylation of synapsin by a calmodulin-dependent kinase activity may have a role in neurotransmitter release. Whether other proteins such as tubulin are

Abbreviations used: SDS, sodium dodecyl sulphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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phosphorylated in intact brain cells in response to depolarization-promoted Ca^{2+} influx has not been established.

It is important to establish if the findings obtained with cell-free systems are pertinent to the actions of these kinase activities in intact cells, which presumably more accurately reflect neurophysiology. Therefore we have developed a system using ^{32}P -labelled dispersed brain tissue for the study of Ca^{2+} -dependent phosphorylations. This system permits investigation of protein phosphorylation under conditions more closely approximating to those that occur *in vivo* than has been possible in earlier studies. We have used depolarizing agents, veratrine and KCl, to induce Ca^{2+} -dependent changes in phosphorylated proteins in the dispersed brain cells for comparison with Ca^{2+} - and calmodulin-dependent protein phosphorylation in cell-free preparations.

MATERIALS AND METHODS

Materials

Sprague–Dawley rats (100–250 g) were obtained from the breeding colony of the Washington University Department of Pediatrics. The calmodulin used in protein kinase assays was prepared by a method using fluphenazine–agarose chromatography (Landt & McDonald, 1984). D-[U- ^{14}C]Glucose, [γ - ^{32}P]ATP and [^{32}P]P_i (carrier free) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Synapsin I (Protein I) from rat brain was kindly given by Dr. P. Greengard, Rockefeller University, New York, NY, U.S.A. Trifluoperazine was given by Smith Kline and French Laboratories, Philadelphia, PA, U.S.A. *Staphylococcus aureus* V8 protease was purchased from Miles Scientifics, Naperville, IL, U.S.A.; polymyxin B was purchased from U.S. Biochemical Corp., Cleveland, OH, U.S.A. All other reagents, including veratrine, verapamil, TPA and isobutylmethylxanthine, were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Isolation of tissue

Sprague–Dawley rats were killed, the cerebral hemispheres were removed and the tissue was placed immediately in ice-cold Hepes-buffered saline (HBS), containing 130 mM-NaCl, 4 mM-KCl, 2.5 mM- CaCl_2 , 1 mM-magnesium acetate, 10 mM-glucose and 20 mM-Hepes/NaOH, pH 7.4. Dispersal of the tissue was accomplished by mincing the cerebral hemispheres with a razor blade in ice-cold HBS and then by mechanical dispersion with a pipette with a 1 mm opening. The minced tissue was filtered through silk screening cloth, and the filtrate was centrifuged at room temperature at 500 *g* for 1 min. The pellet was resuspended in 4 ml of ice-cold HBS. Examination of the dispersed tissue with a haemocytometer and light microscope showed that most of the tissue was dispersed as clusters containing up to 100 cells per cluster.

The viability of the dispersed brain tissue was assessed by measuring production of $^{14}\text{CO}_2$ from [^{14}C]glucose oxidation. Portions (200 μl) of dispersed brain tissue were incubated in buffer containing 2 μmol of glucose and 1 μCi of [U- ^{14}C]glucose for up to 3 h. Reactions were stopped at 30 min intervals by the addition of 3M- H_2SO_4 . $^{14}\text{CO}_2$ production was measured by scintillation counting of the $^{14}\text{CO}_2$ trapped on Hyamine hydroxide-soaked filter

paper suspended above the media present in each reaction vial. These experiments showed that, after an initial 30 min lag, $^{14}\text{CO}_2$ production was linear over a period of 3 h at a rate of 18.9 ± 3.9 nmol of $^{14}\text{CO}_2$ /h per mg of protein. Somewhat lower rates (2–6 nmol/h per mg) have been reported for trypsin-dispersed brains cells, but [$6\text{-}^{14}\text{C}$]glucose was used in those experiments (Tildon *et al.*, 1983).

Labelling phosphorylated proteins in dispersed tissue

^{32}P labelling of endogenous intracellular phosphoproteins of dispersed brain tissue (2–4 mg of protein/ml) was accomplished by incubating the tissue with 2 mCi of [^{32}P]P_i (carrier-free) in a total volume of 2 ml of HBS at 37 °C in a water bath oscillating at 60 cycles/min. A labelling period of 90 min was chosen after timed ^{32}P incorporation studies showed linear incorporation of [^{32}P]P_i into precipitable protein and into a specific 54 kDa phosphoprotein band over a 3 h period. After labelling, dispersed brain tissue was pelleted by centrifugation for 1 min at 500 *g* at room temperature. The supernatant was discarded and the labelled tissue was resuspended in 2 ml of ice-cold HBS. Portions of the suspension (100 μl) were added to various media (50 μl) and incubated for 0–60 s at 37 °C. Incubations were stopped by the addition of 150 μl of ice-cold trichloroacetic acid (final concn. 10%, w/v). The precipitated protein was pelleted in an Eppendorf Microfuge, washed with 150 μl of ice-cold deionized water and dissolved in 150 μl of 100 mM-NaOH. A portion of dissolved protein (100 μl) was combined with 50 μl of stop solution [45% (w/v) sucrose, 500 mM-Tris, 6% (v/v) β -mercaptoethanol, 9% (w/v) SDS, pH 6.7] and was incubated for 2 min in a 70 °C water bath to complete denaturation of protein. Gradient polyacrylamide gels (7.5–15% acrylamide; 16 cm $\times$ 32 cm) were used to separate electrophoretically phosphoproteins in the SDS-denatured solubilized samples. Changes in phosphorylation induced by incubation with various agents were quantified by liquid-scintillation counting of incorporated radioactivity in protein bands, which were excised from polyacrylamide gels, hydrated and digested before counting. Autoradiograms of the polyacrylamide gels were used to locate the phosphoprotein bands for excision.

Protein kinase assay

Briefly, these assays employed homogenate of rat brain, which served as a source of endogenous protein kinase and endogenous protein substrate (Landt & McDonald, 1984). In several assays, synapsin was added to serve as protein substrate. The 100 μl assays were conducted at pH 7.0 in 50 mM-Pipes/NaOH/10 mM- MgCl_2 /0.2 mM-EGTA with or without 700 nM-calmodulin/1 mM- CaCl_2 . After a 2 min preincubation at 37 °C, the reaction was initiated by the addition of 25 μM -[γ - ^{32}P]ATP (10–20 Ci/mmol) and terminated (usually at 5 s of incubation) by the addition of stop solution. Phosphoproteins were separated by SDS/polyacrylamide-gel electrophoresis by using an autoradiogram as a guide for excision of specific phosphoprotein bands; quantification of ^{32}P incorporated into these bands was accomplished by scintillation counting. Ca^{2+} - and calmodulin-dependent protein kinase activity was subsequently computed from the difference in phosphorylation in control and Ca^{2+} /calmodulin incubations.

Proteolytic analysis of phosphorylated proteins

Digestions of authentic synapsin and the 77 kDa brain endogenous protein were performed with the method of Huttner & Greengard (1979). Endogenous brain proteins, which had been prelabelled with ³²P in the dispersed-tissue phosphorylation studies, and purified synapsin labelled in protein kinase assays (27 µg of synapsin and 35 µg of brain homogenate protein were incubated under conditions maximally stimulating for calmodulin-dependent phosphorylations), were subjected to SDS/polyacrylamide-gel electrophoresis. The appropriate phosphoprotein bands were excised from the gels and transferred to the slots of a second gel after light staining, destaining, and autoradiography. Digestion of the phosphoprotein bands with V8 protease and separation of digestion products were accomplished simultaneously by electrophoresis on 15% (w/v) acrylamide in the presence of SDS.

Other procedures

Protein content was measured by the method of Lowry *et al.* (1951). *M_r* values of phosphorylated proteins were determined by their relative migration in 10%-polyacrylamide-gel electrophoresis in comparison with standards ranging from 94 to 24 kDa (Weber & Osborn, 1969). Data shown in Figures and Tables represent means ± 1 s.d. Statistical comparisons were calculated by Student's *t* test for unpaired data.

RESULTS

Effect of veratrine on phosphorylation of dispersed brain tissue

Veratrine, a plant alkaloid mixture whose active component is veratridine, increases membrane permeability to Na⁺, causing depolarization in neural tissue (Ulbricht, 1969). Veratrine was used to study depolarization-stimulated phosphorylation changes in ³²P-labelled dispersed brain tissue. The concentrations of veratrine used in the present studies were calculated by using the *M_r* of its active component veratridine.

Phosphoprotein bands of 83, 77, 64 and 47 kDa in ³²P-labelled dispersed brain cells had visibly increased phosphorylation after exposure to veratrine. A similar pattern of phosphorylation changes was seen with K⁺ (Fig. 1: see below). However, the 77 kDa band had clearly the most prominent change in phosphorylation and was the only phosphorylation which increased significantly (44 ± 15%, *P* < 0.05) in the presence of 100 µM-veratrine (Fig. 2). A 93 kDa protein showed a statistically significant decrease in phosphorylation (*P* < 0.05) after veratrine exposure, but, because this decrease in phosphorylation was not likely to be due to a calmodulin-dependent protein kinase activity, it was not studied further. The concentration of veratrine required for half-maximal activation of phosphorylation of the 77 kDa protein was 2.7 ± 1.5 µM. Veratrine-stimulated phosphorylation of this protein appeared to reach a plateau at concentrations of veratrine greater than 50 µM. Stimulation of phosphorylation of the 77 kDa protein by veratrine was maximal at 45 s of exposure (Fig. 3). At time periods beyond 60 s, veratrine-stimulated phosphorylation of the 77 kDa protein decreased slowly (results not shown).

Veratrine-stimulated phosphorylation of the 77 kDa protein was observed to be dependent on the Ca²⁺

Fig. 1. Autoradiogram of Ca²⁺-dependent phosphoproteins in ³²P-labelled dispersed brain tissue and in [γ -³²P]ATP-labelled brain homogenate

(a) Phosphoprotein pattern of labelled brain tissue in the absence and presence of extracellular K⁺. ³²P-labelled dispersed brain tissue was incubated with either the usual media (C) or with a medium containing 40 mM-KCl instead of an equal concentration of NaCl (K⁺). The phosphoproteins were separated by electrophoresis as described in the Materials and methods section. The autoradiogram of the resulting gel is shown. A prominent phosphoprotein of 77 kDa, and a minor 54 kDa phosphoprotein seen after depolarization, are indicated. (b) Phosphoprotein pattern of [γ -³²P]ATP-labelled cell-free brain homogenate in the absence and presence of Ca²⁺ and calmodulin. Brain homogenate was incubated in a standard 5 s protein kinase assay in the absence (C) or presence of Ca²⁺ and calmodulin (CM), as described in the Materials and methods section. Phosphoproteins were separated by electrophoresis on a 7.5–15%-acrylamide gradient gel. The prominent Ca²⁺ and calmodulin-dependent 54 kDa phosphoprotein and the minor 77 kDa phosphoprotein are indicated on an autoradiogram of a representative gel.

concentration in the incubation media. Extracellular Ca²⁺ concentrations of 1.0 mM were required for maximal veratrine stimulation of the 77 kDa-protein phosphorylation, which occurs under standard incuba-

Fig. 2. Effect of veratrine on phosphorylation of the 77 kDa protein

^{32}P -labelled dispersed brain tissue was incubated with veratrine (0–100 μM) for 1 min. Incorporation of [^{32}P] P_i into the 77 kDa protein was quantified by scintillation counting of gel slices as described in the Materials and methods section. Control phosphorylation was defined as the incorporation of ^{32}P phosphate that occurred in the absence of veratrine. The effect of veratrine on incorporation of ^{32}P is expressed as a percentage of control phosphorylation. The data represent means $\pm$ S.D. for three experiments.

Fig. 3. Effect of time of incubation with veratrine on phosphorylation of the 77 kDa protein

^{32}P -labelled dispersed brain tissue was incubated with 50 μM -veratrine for time intervals of 0–60 s. Incorporation of ^{32}P into the 77 kDa protein was quantified by scintillation counting of gel slices as described in the Materials and methods section. The veratrine-stimulated effect on phosphorylation at various time intervals is expressed as a percentage of the incorporation of ^{32}P at zero time. The data represent means $\pm$ S.D. of three experiments.

tion conditions at 2.5 mM- CaCl_2 . When Ca^{2+} was absent from the incubation media, veratrine-stimulated phosphorylation of the 77 kDa protein was $63.4 \pm 6.2\%$ of that in standard veratrine-stimulated incubations. Higher concentrations of extracellular Ca^{2+} (5 mM) did not result in any increase in veratrine-stimulated phosphorylation of the 77 kDa protein compared with control incubations. The presence of EGTA in excess of Ca^{2+} concentration in the media completely inhibited the ability of veratrine to stimulate phosphorylation of the 77 kDa band (Table 1). The presence of 100 μM -trifluoperazine, an agent known to bind to and prevent the action of calmodulin, significantly decreased veratrine-stimulated phosphorylation ($P < 0.05$) of the 77 kDa protein (Table 1), suggesting that the Ca^{2+} -dependent veratrine-stimulated phosphorylation of this protein may also be calmodulin-dependent. Neither EGTA nor trifluoperazine altered basal phosphorylation of the 77 kDa band or that of other proteins visible on autoradiograms of the electrophoretically separated samples.

Effect of K^+ on phosphorylation of proteins in dispersed brain tissue

Because of the known efficacy of K^+ as a depolarizing agent, the effect of high concentrations of extracellular KCl on phosphorylation in ^{32}P -labelled dispersed brain cells was evaluated. Like veratrine, increased extracellular K^+ significantly increased the phosphorylation of the 77 kDa phosphoprotein ($P < 0.05$) without pronounced or reproducible effects on phosphorylations of other proteins (Fig. 1). The increase in K^+ -induced phosphoryl-

ation of the 77 kDa protein was approximately linear over a range of K^+ concentrations from 4.0 to 40 mM (Fig. 4). At the extracellular concentration of 40 mM-KCl, phosphorylation of the 77 kDa protein was increased by 120% above basal (4.0 mM-KCl). The apparent concentration of external K^+ required for half-maximal activation of phosphorylation of the 77 kDa protein was 22 ± 4 mM. A study of time-dependency of K^+ -induced changes in 77 kDa-protein phosphorylation demonstrated that maximal phosphorylation of this protein occurred after 30 s. K^+ -stimulated phosphorylation remained maximal at 30–60 s, but declined in incubations of 120 s (results not shown).

K^+ -stimulated phosphorylation of the 77 kDa protein was also found to be Ca^{2+} -dependent (Table 1). The presence of EGTA in excess of the Ca^{2+} concentration in the media during incubation of dispersed brain tissue with 40 mM-KCl prevented any significant increase of phosphorylation of the 77 kDa protein above basal phosphorylation (4.0 mM-KCl). The Ca^{2+} -channel blocker verapamil significantly inhibited K^+ -stimulated phosphorylation of this protein ($P < 0.05$) presumably by preventing the entry of extracellular Ca^{2+} into the depolarized cells. In the presence of 40 mM-KCl, 100 μM -trifluoperazine significantly decreased (Table 1) the amount of K^+ -stimulated phosphorylation of the 77 kDa protein ($P < 0.05$), suggesting a role for calmodulin in mediating KCl-stimulated phosphorylation of that protein. In addition, 50 μM -polymyxin B, a cyclic polycationic peptide antibiotic, which is a potent inhibitor of Ca^{2+} - and phospholipid-activated kinases (Wrenn & Wooten, 1984), had no effect on the

Table 1. Effect of various agents on veratrine-stimulated and K⁺-stimulated phosphorylation of the 77 kDa protein

To test the Ca²⁺-dependence of veratrine-stimulated and K⁺-stimulated phosphorylations, ³²P-labelled dispersed brain tissue was incubated with basal media in the presence or absence of 50 μ M-veratrine or 40 mM-KCl and in the presence of 100 μ M-trifluoperazine, 6.0 mM-EGTA or 100 μ M-verapamil (K⁺-stimulated experiment only) for 1 min. Samples were processed and incorporation of [³²P]P_i into the 77 kDa protein was quantified as described in the Materials and methods section. The results are expressed as percentages of the control phosphorylations, which were the amounts of [³²P]P_i incorporated into the 77 kDa protein in the absence of veratrine (first two columns) or of 40 mM-KCl (last two columns). The data represent means $\pm$ s.d. for three experiments with 50 μ M-veratrine and five experiments with 40 mM-KCl. *Statistically significant comparisons ($P < 0.05$, Student *t* test for unpaired data) of stimulated phosphorylation without additions versus stimulated phosphorylations in the presence of trifluoperazine, EGTA or verapamil.

	Phosphorylation (% of control)			
	Control (no veratrine)	50 μ M- Veratrine	Control (4.0 mM-KCl)	40 mM- KCl
No additions	100	194 $\pm$ 11	100	162 $\pm$ 17
100 μ M-Trifluoperazine	92 $\pm$ 13	109 $\pm$ 9*	99 $\pm$ 11	126 $\pm$ 4*
6 mM-EGTA	89 $\pm$ 9	101 $\pm$ 11*	101 $\pm$ 20	112 $\pm$ 9*
100 μ M-Verapamil	—	—	103 $\pm$ 11	128 $\pm$ 10*

K⁺-stimulated phosphorylation of the 77 kDa protein (185 $\pm$ 6% at 40 mM-KCl, 181 $\pm$ 13% with polymyxin + 40 mM-KCl; $n = 3$). Similarly, the tumour-promoting phorbol ester TPA (200 ng/ml) did not enhance the phosphorylation of the 77 kDa phosphoprotein (100% at 4 mM-KCl, 107 $\pm$ 8% with TPA; $n = 3$), implying that it is not phosphorylated by a Ca²⁺- and phospholipid-dependent kinase (Castagna *et al.*, 1982).

Identification of the 77 kDa phosphoprotein and comparison of the phosphorylation pattern in intact cells with the pattern in cell-free preparations

The KCl- and veratrine-stimulated 77 kDa phosphoprotein of dispersed brain tissue was identified as synapsin I. When a partially purified preparation of synapsin I from rat brain was added to a Ca²⁺ and calmodulin-dependent protein kinase assay, with the phosphorylated proteins then being separated by SDS/polyacrylamide-gel electrophoresis, Ca²⁺- and calmodulin-dependent phosphorylation of synapsin I was readily detected. Comparison of the migration rate of synapsin phosphorylated in the protein kinase assay with the phosphorylated 77 kDa protein of ³²P-labelled dispersed brain tissue showed that these proteins migrated identically. When homogenate of rat brain (without addition of exogenous synapsin) was analysed in the endogenous protein kinase assay for Ca²⁺ and calmodulin-dependent phosphorylation of endogenous substrate, phosphorylation of synapsin I was minor in intensity compared with that of the principal Ca²⁺- and calmodulin-dependent 50–60 kDa phosphoproteins (Fig. 1).

The identity of the 77 kDa band as synapsin was confirmed by subjecting phosphorylated 77 kDa protein to proteolytic digestion with V8 protease, as described previously for purified synapsin (Huttner & Greengard, 1979). The radioactive band of 77 kDa protein was excised from a SDS/polyacrylamide gel and digested with V8 protease during electrophoresis alongside identically treated authentic synapsin which had been phosphorylated by calmodulin-dependent activity in brain homogenate (Fig. 5). The principal phosphorylated peptide from digestion of the 77 kDa species migrated on SDS/

Fig. 4. Effect of KCl on phosphorylation of the 77 kDa protein

³²P-labelled dispersed brain tissue was incubated with KCl (4.0–46.5 mM) for 1 min. Incorporation of ³²P was quantified by scintillation counting of gel slices as described in the Materials and methods section. K⁺-stimulated changes in phosphorylation are expressed as percentage of control phosphorylation, which is defined as the amount of ³²P incorporated into the 77 kDa band at 4.0 mM-KCl (standard conditions). The data represent means $\pm$ s.d. for three experiments.

polyacrylamide gel electrophoresis with an apparent molecular mass of 37 kDa, which is similar to that of the major product reported for synapsin digestion (Huttner & Greengard, 1979), and was coincident with the major peptide product of phosphorylated synapsin Ib. Minor peptides from digestion of the 77 kDa species had counterparts of similar electrophoretic mobility in the digest of authentic synapsin. The two species of synapsin

Fig. 5. Proteolytic digestion of the 77 kDa band and synapsin Ib by V8 protease

Gel slices containing the 77 kDa band or synapsin were transferred to a 15% (w/v)-acrylamide gel containing SDS. Each slice was overlaid with 100 μ l of 125 mM-Tris/HCl (pH 6.8), containing 0.1% SDS, 100 μ g of *S. aureus* V8 protease/ml and 15% glycerol. Digestion and separation of products occurred during electrophoresis. The autoradiograph comparing the radioactive products of digestion of the 77 kDa band (77K) with the faster-migrating (Ib) of the two synapsin species is shown. The major phosphorylated peptide in each protein is of approx. 37 kDa. Owing to high efficiency of synapsin labelling in kinase assays, the synapsin digestion products were more intensely labelled and appear more prominent on the autoradiograph.

present in the purified authentic sample (Ia and Ib) gave qualitatively and quantitatively similar V8-protease-digestion patterns, except that peptides from synapsin Ib ran slightly faster than the analogous peptides of synapsin Ia (results not shown). The digestion peptides of the 77 kDa band had migration rates similar to those from synapsin Ib digestion (Fig. 5).

DISCUSSION

There is growing evidence for a role of protein phosphorylation in the modulation of neurotransmitter

release (Greengard, 1979; DeLorenzo, 1981). The role of protein phosphorylation in neurosecretion has primarily been characterized by using subcellular preparations of neural tissue (Schulman & Greengard, 1978; DeLorenzo *et al.*, 1979; Goldenring *et al.*, 1983; Bennett *et al.*, 1983). Though studies with cell-free systems have provided a wealth of information, secretion of neurotransmitter appears to involve a complex array of intracellular components which are likely to be dissociated or disrupted by the homogenization that is used to produce these cell-free systems. In order to understand better the physiological role of phosphorylation in neurosecretion, it is desirable to have an intact cell system for investigation and characterization of the kinases and their protein substrates. The results of this investigation have demonstrated that phosphorylation of proteins in intact brain cells labelled with [32 P]P_i may be readily detected and studied.

Our principal aim in developing the intact cell system was to characterize the Ca²⁺- and calmodulin-dependent phosphorylation activity in these preparations, in order to confirm and clarify the findings of previous cell-free studies concerning the potential involvement of Ca²⁺- and calmodulin-dependent protein kinase activities in neurosecretion. Depolarization of these cells significantly increased phosphorylation of a single protein, synapsin I, implying a specific involvement of phosphorylation of this protein in depolarizing events. Experiments involving alterations of extracellular Ca²⁺ concentration and alteration of Ca²⁺ influx during depolarization indicated that the phosphorylation of synapsin was Ca²⁺-dependent. Studies using polymyxin B and TPA undertaken to characterize further the nature of the Ca²⁺-dependent phosphorylation imply that it was not mediated by a Ca²⁺- and phospholipid-dependent protein kinase (Wrenn & Wooten, 1984; Castagna *et al.*, 1982). However, studies with the phenothiazine trifluoperazine suggested that this phosphorylation was catalysed by a calmodulin-dependent protein kinase. Although phenothiazines are reported to decrease ATP concentrations independently of calmodulin and to act as α -adrenergic antagonists (Corps *et al.*, 1982; Blackmore *et al.*, 1981), phenothiazines remain useful for presumptively classifying whether or not Ca²⁺-dependent processes are mediated by calmodulin (Levin & Weiss, 1978). The principal differences between the phosphorylation patterns observed in cell-free and intact cell systems were the relative intensities of phosphorylation of synapsin and proteins of 50–60 kDa. Synapsin had the most prominent Ca²⁺- and calmodulin-dependent phosphorylation in 32 P-labelled dispersed brain tissue. However, in the standard Ca²⁺- and calmodulin-dependent protein kinase assay, the proteins most prominently phosphorylated in a Ca²⁺- and calmodulin-dependent manner ranged from 50 to 60 kDa. Synapsin phosphorylation in the protein kinase assay gave a band of minor intensity (Fig. 1). The relative difference in phosphorylation may be due to preferential availability of endogenous protein substrate for a Ca²⁺- and calmodulin-dependent protein kinase activity which exists in intact cells (e.g. association of substrate and kinase as a complex or with a subcellular organelle), which was disrupted during preparation of subcellular fractions. Decreased availability of an endogenous substrate may be reflected as increased phosphorylation of less avidly phosphorylated proteins, such as autophosphorylation. Other studies indicate that the Ca²⁺- and

calmodulin-dependent kinase activities of brain have autophosphorylated subunits of 50–60 kDa (Goldenring *et al.*, 1983; Bennett *et al.*, 1983). Alternatively, relative phosphorylation differences may result from the very disparate assay conditions existing inside whole cells as compared with the cell-free assay media.

Comparison of phosphorylation of synapsin in cell-free systems and in intact cells showed that there were differences in the ability of certain agents to stimulate phosphorylation of synapsin. In cell-free systems, synapsin phosphorylation has been shown to be regulated by Ca²⁺/calmodulin as well as by cyclic AMP (Krueger *et al.*, 1977; Ueda & Greengard, 1977). The effect of cyclic AMP on synapsin phosphorylation was not consistently demonstrable when cell-free preparations such as synaptosomes were incubated with cyclic AMP or its analogues (Krueger *et al.*, 1977; Kennedy & Greengard, 1981). Another study demonstrated an increase in phosphorylation of synapsin when brain slices were incubated with the phosphodiesterase inhibitor isobutylmethylxanthine (Forn & Greengard, 1978). In the present study with the intact cell system, incubation with 0.2–4.0 mM-dibutyl cyclic AMP and 300 μ M-isobutylmethylxanthine for time periods of up to 5 min failed to alter the phosphorylation of synapsin (results not shown). The absence of an effect with the dispersed tissue system and the difficulty that others have experienced in observing the effects in cell-free systems (Krueger *et al.*, 1977; Huttner & Greengard, 1979) raise the possibility that cyclic AMP has a minor role in modulating synapsin phosphorylation.

The presence of significantly decreased phosphorylation of a 93 kDa protein was also observed during depolarization of the ³²P-labelled dispersed brain cells. The decrease in phosphorylation of this protein may be due to the action of a Ca²⁺-dependent phosphatase, though inhibition of a calmodulin-independent protein kinase is also possible. Large amounts of a Ca²⁺- and calmodulin-dependent protein phosphatase have been demonstrated in cell-free preparations from brain (Stewart *et al.*, 1982). Because the focus of the present study has been to characterize Ca²⁺-stimulated phosphorylations mediated by Ca²⁺- and calmodulin-dependent protein kinases, no further investigation of the phosphorylation of the 93 kDa species was undertaken.

The purification and characterization of two apparently distinct Ca²⁺- and calmodulin-dependent kinase activities from brain have been reported previously (Bennett *et al.*, 1983; Goldenring *et al.*, 1983). They share a similar *K_m* for ATP, similar calmodulin requirements and similar autophosphorylating subunits. One of these kinase activities had a preference for synapsin as a substrate (Bennett *et al.*, 1983), but the other activity phosphorylated tubulin subunits and not synapsin (Goldenring *et al.*, 1983). It is not evident from cell-free studies which phosphorylation may be the more important or principal result of Ca²⁺-sensitive depolarization of intact brain cells. The present study indicates that the kinase activity which preferentially phosphorylates synapsin may be modulated by depolarization in the neuroconduction process.

The results of our studies using ³²P-labelled intact brain cells extend the findings of other studies (Krueger *et al.*, 1977; Forn & Greengard, 1978) which employed

subcellular synaptosomes and brain slices to demonstrate that synapsin phosphorylation was increased by depolarizing agents. Those studies were aimed specifically at synapsin phosphorylation, however. They did not determine if synapsin was the principal depolarization-sensitive phosphorylation or if other, previously unrecognized, species were also sensitive. Other, potentially important, Ca²⁺- and calmodulin-dependent phosphorylations include the subunits of tubulin and autophosphorylation of the 50–60 kDa subunits of Ca²⁺- and calmodulin-dependent protein kinases, which have been demonstrated in cell-free preparations (Goldenring *et al.*, 1983; Bennett *et al.*, 1983). It is clear from the present findings however, that phosphorylation of synapsin should be the major focus of effort in understanding how depolarization promotes neurotransmitter release.

This investigation was supported by U.S. Public Health Service Grant AM28233.

REFERENCES

- Bennett, M. K., Erondy, N. E. & Kennedy, M. B. (1983) *J. Biol. Chem.* **258**, 12735–12744
- Blackmore, P. F., El-Refai, M. F., DeHaye, J., Strickland, W. G., Hughes, B. P. & Exton, J. H. (1981) *FEBS Lett.* **123**, 245–248
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Corps, A. N., Hesketh, T. R. & Metcalfe, J. C. (1982) *FEBS Lett.* **138**, 280–284
- DeLorenzo, R. J. (1981) *Cell Calcium* **2**, 365–385
- DeLorenzo, R. J., Freedman, S. D., Yohe, W. B. & Maurer, S. C. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1838–1842
- Forn, J. & Greengard, P. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5195–5199
- Goldenring, J. R., Gonzales, B., McGuire, J. S. & DeLorenzo, R. J. (1983) *J. Biol. Chem.* **258**, 12632–12640
- Greengard, P. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2208–2217
- Huttner, W. B. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5402–5406
- Katz, B. & Miledi, R. (1970) *J. Physiol. (London)* **207**, 789–801
- Kennedy, M. B. & Greengard, P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1293–1297
- Krueger, B. K., Forn, J. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 2764–2773
- Landt, M. & McDonald, J. M. (1984) *Int. J. Biochem.* **16**, 161–169
- Levin, R. M. & Weiss, B. (1978) *Biochim. Biophys. Acta* **540**, 197–204
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Miledi, R. (1973) *Proc. R. Soc. London Ser. B* **183**, 421–425
- Rubin, R. P. (1970) *Pharmacol. Rev.* **22**, 389–428
- Schulman, H. & Greengard, P. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5432–5436
- Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B. & Cohen, P. (1982) *FEBS Lett.* **137**, 80–84
- Tildos, J. T., Merrill, S. & Roeder, L. M. (1983) *Biochem. J.* **216**, 21–25
- Ueda, T. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 5155–5163
- Ueda, T., Maeno, H. & Greengard, P. (1973) *J. Biol. Chem.* **248**, 8295–8305
- Ulbricht, W. (1969) *Ergeb. Physiol. Biol. Chem. Exp. Pharmacol.* **61**, 18–71
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wrenn, R. W. & Wooten, M. W. (1984) *Life Sci.* **35**, 267–276