Calcium-dependent protein phosphorylation and dephosphorylation in intact brain neurons in culture

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Preincubation of intact fetal rat brain neurons in culture with $^{32}P_i$ results in the incorporation of $^{32}P_i$ into about 20 specific proteins. Upon stimulation by electrical field stimulation or by K^+-induced depolarization, highly significant calcium-dependent increase in phosphorylation of a protein of app. M_r 43000 and decrease in phosphorylation of an app. M_r 55000 protein occur. These changes can be attributed to the entry of Ca^{2+} into the cellular cytoplasm since they can occur upon selective permeabilization of the cell membrane to Ca^{2+} by the Ca^{2+}-ionophore A23187 and are not observed upon stimulation of the cells in the presence of the Ca^{2+} channel blocker D-600. These data suggest that these phosphoproteins may be involved in the regulation of processes underlying neurotransmitter release.

Phosphorylation *Brain* *Neuron* *Neurotransmitter release*

1. INTRODUCTION

Neurotransmitter release is triggered by an elevated level of free Ca^{2+} in the cytoplasm of the nerve endings [1,2]. However, the biochemical mechanisms underlying the coupling of Ca^{2+} stimulation to neurotransmitter release are unknown. Protein phosphorylation is now recognized as a ubiquitous regulatory mechanism in enzymatic and cellular activities, as well as in synaptic events [3–6]. The cellular heterogeneity and complexity of brain tissue restricts the possibilities of studying the function of phosphorylation in neurons alone, without interfering with their cellular integrity. The development of methods for culturing embryonic brain neurons which exhibit neuronal properties [7] opened the way to such investigations in intact neuronal cells.

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Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; EBSS, Earle's balanced salt solution

It has been shown that neural brain cells can survive and mature under conditions in which growth of glia and other supportive cells is curtailed [8,9]. The mature cells accumulate [^{3}H]dopamine and are also able to release it in a calcium-dependent process upon elevation of the extracellular potassium concentration [9] or upon electrical stimulation [10].

Here, we report on the regulation of phosphorylation in intact fetal brain neurons by Ca^{2+} under conditions leading to neurotransmitter release.

2. MATERIALS AND METHODS

2.1. *Culture and phosphorylation of brain neurons*

Fetal rat brain neurons were grown in culture as in [9]. Cells attached to the plastic Petri dish were pre-incubated with Ca^{2+}-free EBSS solution (116 mM NaCl, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 5.5 mM glucose) for 10 min. [^{32}P]Phosphoric acid (carrier-free, 50 μCi/ml) (New England Nuclear, Chicago

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IL) was added and the mixture was allowed to stand for a further 30 min at 37°C. The preincubation medium was removed and the cells were subjected to stimulation under conditions which induce dopamine release [9,10] as follows:

(i) Depolarizing isotonic solution (containing 53 mM KCl, 58 mM NaCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$ and 5 mM glucose) was added in the presence and absence of methoxyverapamil (D-600) 100 μM (gift of Professor M. Sokolovsky) and in the presence and absence of 1 mM Ca^{2+} for 5 min [9]. D-600 belongs to a group of compounds which specifically inhibit transmembrane Ca^{2+} fluxes [11].

(ii) Fresh EBSS was added in the absence and in the presence of 1 mM Ca^{2+}. Two parallel platinum electrodes were immersed, 2 cm apart, in the Petri dish contents. The electrodes were connected to a stimulator (Grass model SD9) and a set of stimuli (75 V, 10 Hz, 0.8 ms pulse duration, for 90 s) was applied [10].

(iii) Fresh EBSS containing the Ca^{2+}-specific ionophore A23187 (19 μM) (Sigma) was added in the absence and in the presence of 1 mM Ca^{2+} for 5 min. Phosphorylation was terminated by the addition of 0.1 ml of 100% trichloroacetic acid at 4°C, and the precipitated proteins were collected by centrifugation. The pellet was dissolved in

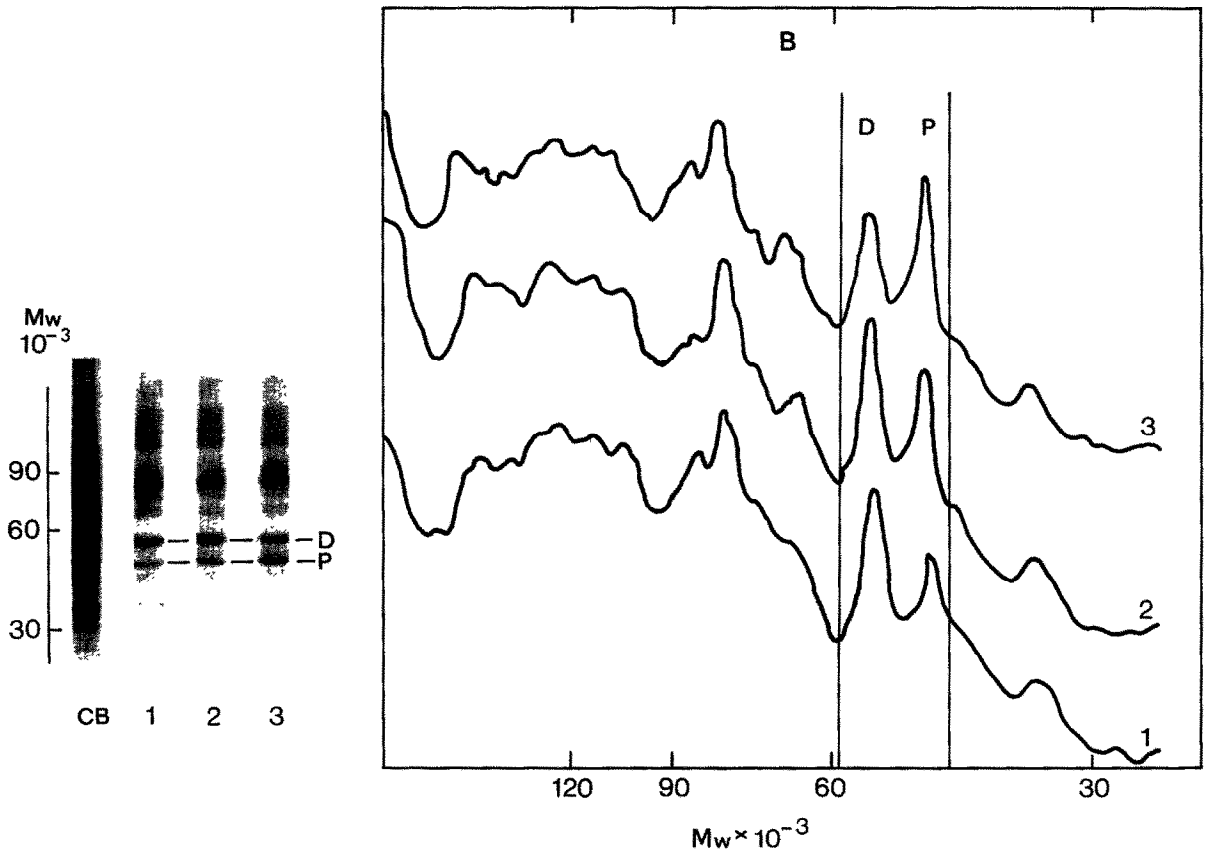

Fig.1. Effect of K^+-depolarization in the presence and absence of Ca^{2+} on the endogenous phosphorylation of proteins in cultured fetal rat brain neurons. The cells, 12 days in culture, were preincubated with [^{32}P]phosphate and then incubated for 5 min in: (1) Ca^{2+}-free EBSS; (2) high K^+ buffer in the absence of Ca^{2+}; or (3) as before but in the presence of Ca^{2+} (1 mM). The reaction was terminated by the addition of trichloroacetic acid. The samples were solubilized and subjected to SDS–polyacrylamide gel electrophoresis and autoradiography. Coomassie blue staining (CB) and autoradiograms of the gels are depicted in (A). Densitograms of the same autoradiograms are shown in (B). The M_r 43000 and 55000 bands are indicated by P and D, respectively.

sodium dodecyl sulfate (SDS) sample buffer (containing 36 mM Tris–HCl (pH 6.8), 3% SDS, 5% β-mercaptoethanol and 10% glycerol) and subjected to polyacrylamide gel electrophoresis on 7–17% polyacrylamide slab gels [12]. The gels were subsequently stained with Coomassie brilliant blue, dried and subjected to autoradiography on Kodak no-screen X-ray film. Films were scanned on a Helena quick scan densitometer to give quantitative profiles of ^{32}P-labeled polypeptides.

2.2. *Characterization of $^{32}PO_4$ incorporated into protein*

Precipitated phosphorylated proteins were extracted 3 times with acetone to remove trichloroacetic acid. Pellets (~100 μg protein) were then suspended in 100 μl KH_2PO_4 (1 mM) containing either:

(i) Pronase (10 μg/ml) (Sigma), ribonuclease A (10 μg/ml (Sigma) or deoxyribonuclease (10 μg/ml) (Sigma) for 10 min at 37°C; or

(ii) 0.5 M NaOH or 5% trichloroacetic acid and boiled for 1 min.

The samples were then solubilized in sample buffer and subjected to SDS–polyacrylamide gel electrophoresis and autoradiography. Total protein was determined by a modification of the Lowry method [13] with bovine serum albumin (Sigma) as a protein standard.

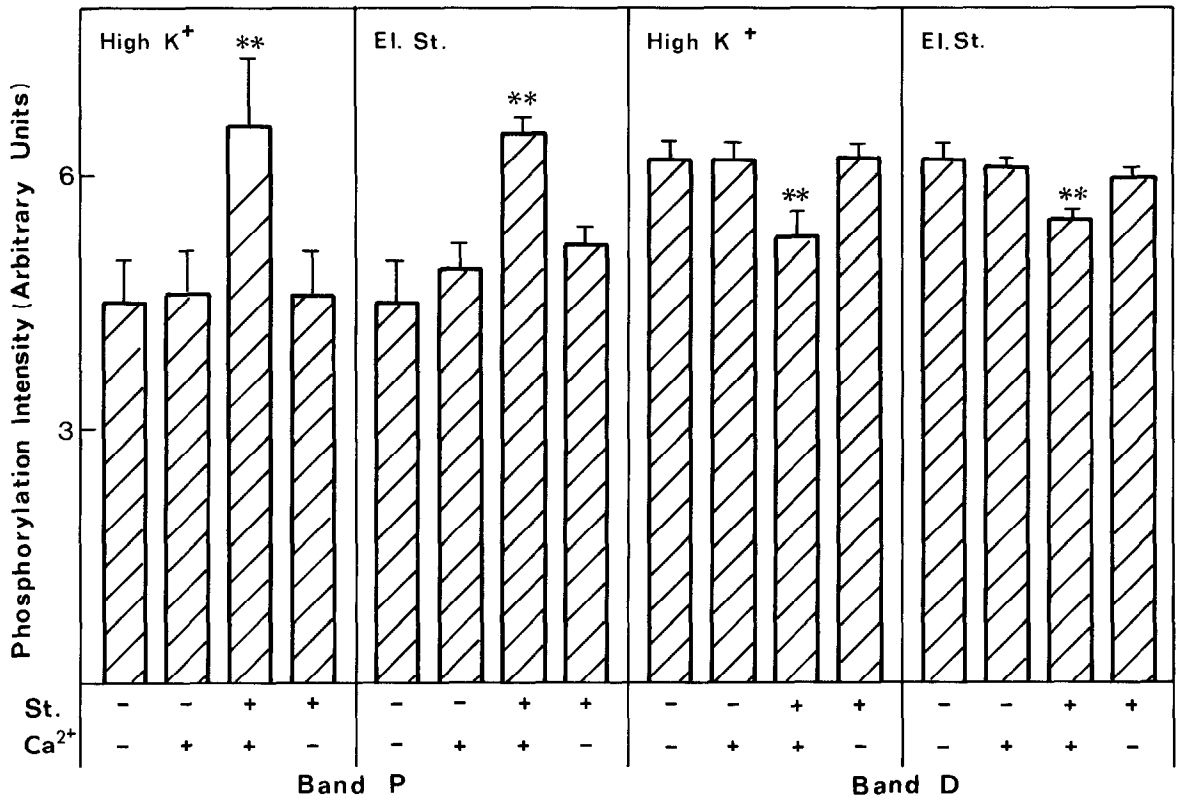

Fig.2. Regulation of the state of phosphorylation of bands P and D by Ca^{2+} and stimulation. Brain neurons, 12 days in culture, preincubated with [^{32}P]phosphate, were subjected to stimulation (st.) by either high K^+ or electrical field (el.) in the absence or presence of Ca^{2+}. The amount of ^{32}P incorporated into bands P and D was estimated from densitograms obtained from autoradiograms of the polyacrylamide gels which were used to resolve the proteins. The results are expressed as the percentage of the areas of the phosphorylated bands P and D from the total area of the densitogram.

** $p < 0.005$, Student's t-test.

Table 1

Effect of A23187 and of D-600 on the state of phosphorylation of proteins P and D

Compound	Buffer	Phosphorylation intensity (arbitrary units)		Phosphorylation ratio P/D
		P	D	
A23187	EBSS + Ca	6.4 ± 1.0	5.3 ± 0.3	1.2 ± 0.2
A23187	EBSS	4.7 ± 0.5	6.2 ± 0.2	0.7 ± 0.2
–	EBSS + Ca^{2+}	4.6 ± 0.5	6.2 ± 0.2	0.7 ± 0.1
D-600	High K^+ + Ca^{2+}	4.6 ± 0.7	6.1 ± 0.2	0.7 ± 0.2
D-600	EBSS + Ca^{2+}	4.6 ± 0.5	6.2 ± 0.2	0.7 ± 0.1
–	High K^+ + Ca^{2+}	6.6 ± 1.0	5.3 ± 0.3	1.2 ± 0.2

Brain neurons 12 days in culture, preincubated with [^{32}P]phosphate were subjected to stimulation by either Ca^{2+}-selective ionophore A23187 or by high K^+ in the absence or presence of Ca^{2+} (1.8 mM) or of the Ca^{2+} uptake blocker, D-600. Phosphorylation intensity was calculated as in fig.2

3. RESULTS

When intact fetal rat brain neurons grown in tissue culture were preincubated with [^{32}P]phosphoric acid, ^{32}P was incorporated into about 20 specific proteins (fig.1a). Stimulation by a high [K^+] failed to affect protein phosphorylation significantly in calcium-free medium (fig.1b). Depolarization of the cells by using a high [K^+] buffer (fig.1c) in the presence of Ca^{2+} resulted in neurotransmitter release; it also resulted in a significantly increased incorporation of ^{32}P into a protein with apparent subunit M_r 43000 and decreased incorporation of ^{32}P into a protein with an app. M_r 55000. Similar results were observed when the cells were subjected to electrical field stimulation.

Ribonuclease A, deoxyribonuclease and cold or hot trichloroacetic acid had little or no effect on ^{32}P incorporation into the phosphorylation bands. Hot NaOH and pronase removed most of the protein staining and ^{32}P, except for small components which migrated with the tracking dye (not shown). The data indicate that the [^{32}P]phosphate associated with the bands was indeed incorporated into proteins.

Since the total area of the other peaks was unaltered, the percentage of the areas under peaks P (M_r 45000) or D (M_r 55000) from the total area was employed as an arbitrary unit with which the extent of $^{32}P_i$ incorporation was quantitated.

Both modes of stimulation (i.e., K^+ depolarization or electrical field stimulation in the presence of Ca^{2+}), yielded a highly significant ($P < 0.005$) 50% increase in the phosphorylation of band P and about a 15% decreased incorporation into band D, as compared to that obtained under non-stimulating conditions in the presence or absence of Ca^{2+}. Ca^{2+} by itself increased the phosphorylation of band P only slightly (fig.2). In the presence of the verapamil derivative D-600, the effect of depolarization with high K^+ in the presence of Ca^{2+} on the phosphorylation of the M_r 43000 and 55000 proteins was inhibited (table 1). However, in the presence of the Ca^{2+} ionophore A23187 and Ca^{2+}, the pattern of phosphorylation resembled that obtained upon stimulation of the cells in the presence of Ca^{2+} (table 1).

When the data from the individual experiments were expressed in terms of the ratio between the areas under the M_r 43000 and 55000 bands in the scans of the autoradiograms, highly reproducible mean values of 0.7 for non-stimulating and 1.2 for stimulating conditions were obtained (table 1). However, further experimentaiton is necessary before coupling between the increase in the phosphorylation of bands P and the decrease in D can be assumed.

4. DISCUSSION

Our results indicate that conditions which cause Ca^{2+}-dependent release of neurotransmitters also lead to changes in the state of phosphorylation of specific neuronal proteins, namely, enhanced phosphorylation of a protein with an apparent subunit M_r 43000 and reduced phosphorylation of an M_r 55000 protein. These changes may be attributed to the entry of Ca^{2+} into the cellular cytoplasm since they can occur upon selective permeabilization of the cell membrane to Ca^{2+} by the Ca^{2+}-ionophore and are not observed upon stimulation of the cells in the presence of the Ca^{2+} channel blocker D-600.

Ca^{2+}-dependent phosphorylation of an M_r 43000 protein by [^{32}P]ATP has been observed in ruptured synaptosomes [14]. Phosphorylation of a specific M_r 43000 protein in the crude mitochondrial pellet of rat cerebral cortex has been shown to be stimulated by glutamate, and was suggested to be a component of the pyruvate dehydrogenase complex of mitochondria [15]. It has been shown that the state of phosphorylation of a protein with app. M_r 40000 changes under the influence of high frequency stimulation of the rat hippocampus [16]. However, from these results, no clear connection between all these phosphorylated M_r 40000–43000 proteins can be inferred.

The endogenous phosphorylation of an M_r 55000 protein identified as tubulin occurs in a Ca^{2+}- and calmodulin-dependent manner in synaptic vesicles prepared from rat cerebral cortex [6]. Another protein of similar M_r is enriched in the cerebellum, and its phosphorylation is induced by calcium and calmodulin [17]. An M_r 55000 protein present in mammalian brain was shown to become phosphorylated by depolarizing agents in the presence of Ca^{2+} [18]. Our results showed that stimulation of the cultured brain neurons causes reduction and not enhancement of phosphorylation in an M_r 55000 band. A phosphorylated protein of M_r 55000 has been identified as a regulatory subunit of a cAMP-dependent protein kinase [19]. Increase in the cellular level of cAMP upon stimulation may result in the dissociation of the regulatory and catalytic subunits of the enzyme and, consequently, to reduced phosphorylation of the regulatory component by the catalytic subunit as observed in rat skeletal muscle cytosol [20]. The latter possibility is under investigation. It remains to determine whether a causal relationship exists between changes in the phosphorylation pattern and neurotransmitter release.

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