Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6075–6079, October 1982
Neurobiology

Regulation of phosphorylation of proteins I, III_a, and III_b in rat neurohypophysis *in vitro* by electrical stimulation and by neuroactive agents

(calcium/cAMP/dopamine/fluphenazine)

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Contributed by Paul Greengard, June 1, 1982

ABSTRACT **The state of phosphorylation of proteins I, III_a, and III_b—neuron-specific phosphoproteins—was studied in neurosecretory endings of the neurohypophysis *in vitro*. Brief periods (a few seconds) of electrical stimulation caused large increases in the state of phosphorylation of all three proteins. The three proteins were dephosphorylated within 1 min after termination of the stimulation. High potassium, 8-bromo-cAMP, and dopamine also stimulated the phosphorylation of the three proteins. The effect of dopamine was blocked by the dopamine antagonist fluphenazine. Peptide mapping of protein I revealed that electrical stimulation or high potassium increased the state of phosphorylation of two regions of the molecule, whereas 8-bromo-cAMP and dopamine increased the state of phosphorylation of only one of these regions.**

Protein I is a neuron-specific protein present in most and probably all presynaptic terminals, where it is at least partially associated with neurotransmitter vesicles (1–5). It is a major endogenous substrate for both cAMP-dependent (1) and calcium/calmodulin-dependent (6, 7) protein kinases. Neurotransmitters (8, 9), apparently acting through cAMP, and depolarizing agents (8–10), apparently acting through calcium, have been shown to increase the state of phosphorylation of protein I in intact preparations of the central and peripheral nervous system. Recently, it has been shown that brief periods of nerve impulse conduction increase the state of phosphorylation of protein I in the rabbit superior cervical ganglion (11). These and other findings suggest that protein I may have a functional role in the nerve terminal (12). Proteins III_a and III_b are also neuron-specific proteins that have cellular and subcellular distributions and certain other properties similar to those of protein I (13, 14).

The neurohypophysis is a neuroendocrine organ of quite homogeneous composition, the nervous structure of which consist entirely of nerve terminals without any postsynaptic neurons. Vasopressin and oxytocin release from the neurohypophysis can be detected *in vitro*, and the rate of release of these neurohormones can be manipulated easily. The state of phosphorylation of proteins I, III_a, and III_b can be determined in a single neurohypophysis of the rat. Therefore, we have studied the regulation of the state of phosphorylation of these proteins in the neurohypophysis as a step towards understanding their possible role in the function of nerve terminals.

METHODS

Isolation, Incubation, and Stimulation of Neurohypophysis. Sprague–Dawley rats (150–175 g) were decapitated, and their skulls were opened through the sagittal suture. The brainstem was lifted from behind so that the pituitary stalk could be seen, and the stalk was cut underneath the basal hypothalamus. The brain was then removed. After dissecting away the covering sheath, the whole pituitary gland was removed from the base of the skull with a moistened brush and placed in a Petri dish containing standard buffer (Krebs–Ringer bicarbonate buffer containing 125 mM NaCl/5.0 mM KCl/25 mM $NaHCO_3$/1.0 mM $CaCl_2$/1.5 mM Na_2HPO_4/1.5 mM $MgSO_4$/10 mM glucose). The neurohypophysis with its stalk and attached fragments of the pars intermedia was separated from the anterior pituitary with a brush. A half cut along the posterior midline of the neurohypophysis was made to facilitate penetration of drugs into the gland.

The neurohypophysis was incubated and stimulated as described in the physiological studies of Lightman *et al.* (15) on vasopressin release. The pituitary stalk was drawn into the tip of a platinum wire suction electrode. The gland was then placed in a glass vial containing 15 ml of standard buffer and gassed continuously with 95% O_2/5% CO_2. The gland was preincubated at 37°C for 45 min before stimulation or treatment with neuroactive agents. The stimulation parameters studied were similar to those examined in an investigation of the effect of impulse conduction on the state of phosphorylation of protein I in the rabbit superior cervical ganglion (11). Stimulation was carried out with pulses shown in preliminary experiments to be supramaximal: a pulse amplitude of 40 V and a pulse width of 2 msec.

Extraction of Proteins I, III_a, and III_b and Assay of Their Dephosphorylated Forms. The technique of back-phosphorylation (10) was used to quantitate the amounts of dephosphoproteins I, III_a, and III_b. The principle of this technique is to homogenize the tissue in the presence of Zn^{2+}, which prevents changes in the state of phosphorylation of phosphoproteins by inhibiting endogenous protein kinase and protein phosphatase activities. The proteins are then extracted, and the dephosphorylated forms are phosphorylated with [γ-^{32}P]ATP by the exogenous catalytic subunit of cAMP-dependent protein kinase.

After electrical stimulation or treatment with neuroactive agents, each neurohypophysis was transferred into a glass homogenizer containing 2 ml of ice-cold Zn acetate (5 mM) and homogenized with a Teflon pestle. The homogenate was centrifuged at 4,000 × *g* for 10 min, and the pellet was resuspended in 220 μl of 0.01 M citric acid (pH 3.0) at 4°C. The suspension was centrifuged at 23,000 × *g* for 15 min, and a 200-μl aliquot of the supernatant was adjusted to pH 6 with 0.5 M Na_2HPO_4 and centrifuged at 15,000 × *g* for 10 min. A 60-μl aliquot of the

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Abbreviation: kDal, kilodaltons.

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supernatant was used for protein assay. All pH 6 supernatants from one experiment were then diluted to equal protein concentration with 10 mM Na citrate/phosphate buffer, pH 6.

The standard back-phosphorylation assay mixture (final volume, 100 μl) contained 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 3 μM [γ-^{32}P]ATP (specific activity 50–100 cpm $fmol^{-1}$), 60 μl of tissue extract, and a low concentration (7.5 nM) of the purified catalytic subunit of cAMP-dependent protein kinase. (This enzyme was prepared from bovine heart and supplied by A. C. Nairn of this laboratory.) The assay was carried out for 30 min at 30°C, the reaction was terminated by boiling the assay mixture in "$NaDodSO_4$ stop solution," and the samples were then subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography as described (10). The bands containing proteins I, III_a, and III_b were cut from each lane of the slab gels, and radioactivity was determined by liquid scintillation spectrometry. Under the assay conditions used, phosphorylation of proteins I, III_a, and III_b was proportional to the amount of tissue extract over a 10-fold concentration range of extract.

The standard assay conditions, involving a low concentration of catalytic subunit, were chosen in order to provide selective phosphorylation of the site in protein I that is phosphorylated under physiological conditions by both a cAMP-dependent and a calcium/calmodulin-dependent protein kinase (6, 16). This site is located in the 10-kilodalton (kDal) fragment obtained upon digestion of protein I with *Staphylococcus aureus* protease (see below). Under standard back-phosphorylation assay conditions, this site accounted for 95% of the total phosphate incorporated into protein I (data not shown).

The total amounts of proteins I, III_a, and III_b were not altered by any of the experimental procedures used in this study. Therefore, changes in the amount of the dephosphorylated forms of proteins I, III_a, and III_b reflect changes in the states of phosphorylation of those proteins.

Assay of Individual Phosphorylation Sites in Protein I. In experiments in which the state of phosphorylation of individual sites in protein I was to be determined, the conditions of the standard back-phosphorylation procedure were used except for a 10 times higher concentration (75 nM) of catalytic subunit. The use of this higher amount of catalytic subunit in these peptide mapping experiments was necessary in order to incorporate sufficient [^{32}P]phosphate into the 30-kDal region of the molecule (see below) to analyze its state of phosphorylation. One-dimensional peptide mapping of protein I was then carried out by the method of Cleveland *et al.* (17) as described (6). For this purpose, gel slices containing ^{32}P-labeled holoprotein I were soaked in 2 ml of 125 mM Tris, pH 6.7/0.1% $NaDodSO_4$ for 10 min at room temperature and then placed into the sample wells of a second polyacrylamide gel. Each gel slice was overlaid with 5 μg of *Staphylococcus aureus* V8 protease (Miles) in 50 μl of 125 mM Tris, pH 6.7/0.1% $NaDodSO_4$/10% glycerol with pyronin Y as a marker. The concentration of acrylamide in the lower gel was 15% with a ratio of acrylamide to *N,N'*-methylenebisacrylamide of 30:0.8. The gels were electrophoresed at 60 V. At the completion of electrophoresis, the gels were dried, and the ^{32}P-labeled fragments of protein I were identified by autoradiography. Two major phosphopeptide fragments of 10 and 30 kDal are generated when protein I is digested by this technique (6, 16).

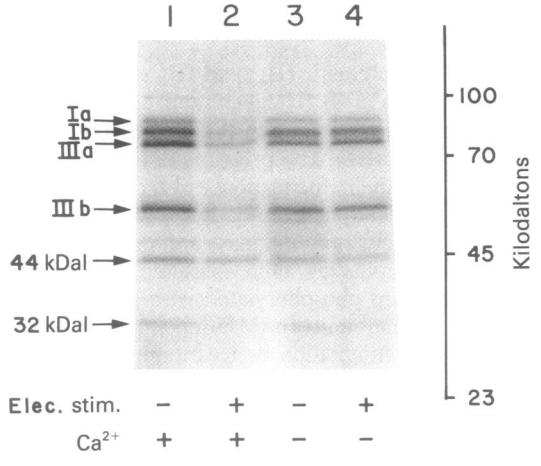

FIG. 1. Autoradiograph of a $NaDodSO_4$/polyacrylamide gel showing the effect of electrical stimulation on the phosphorylation of proteins I, III_a, and III_b in the rat neurohypophysis and the dependence of this phosphorylation on extracellular calcium. The neurohypophysis was mounted through its stalk in a platinum wire suction electrode and was preincubated at 37°C for 45 min in standard Krebs–Ringer buffer (1 mM Ca^{2+}) (lanes 1 and 2) or in Krebs–Ringer buffer in which calcium was replaced by 0.3 mM EGTA (lanes 3 and 4). The pituitary stalk was then electrically stimulated (lanes 2 and 4) at 10 Hz for 20 sec or served as an unstimulated control (lanes 1 and 3).

RESULTS

Effect of Electrical Stimulation. When the pituitary stalk was stimulated supramaximally at 10 Hz for 20 sec, there was a pronounced decrease in the amounts of the dephosphorylated forms of proteins I, III_a, and III_b in the neurohypophysis (Fig. 1). This effect was dependent on the presence of extracellular calcium ions. Because the total amounts of proteins I, III_a, and III_b determined by radioimmunoassay were not altered by electrical stimulation (data not shown), the results demonstrate that electrical stimulation of the pituitary stalk increased the state of phosphorylation of proteins I, III_a, and III_b.

Tables 1 and 2 show the state of phosphorylation of proteins I, III_a, and III_b in the neurohypophysis as a function of the number of pulses delivered to the pituitary stalk. In one series of experiments (Table 1), the pituitary stalk was stimulated for 20 sec at various frequencies, and the amounts of the dephosphorylated forms of proteins I, III_a, and III_b present upon the termination of electrical stimulation were determined. The state of phosphorylation of all three proteins was increased upon stimulation of the pituitary stalk. A near-maximal effect was produced by stimulation at 2 Hz (40 pulses). In another series of experiments (Table 2), the pituitary stalk was stimulated at 10 Hz for various periods of time, and the amounts of the dephosphorylated forms of proteins I, III_a, and III_b present 30 sec after the initiation of stimulation were determined. Under these conditions, 2 sec of pituitary stalk stimulation (20 pulses) sig-

Table 1. Effect of frequency of stimulation during a constant interval (20 sec) on phosphorylation of proteins I, III_a, and III_b in rat neurohypophysis

Frequency, Hz	Pulses, no.	Dephosphorylated form, % of unstimulated control		
		I	III_a	III_b
1	20	87 ± 5 (3)	95 ± 2 (3)	99 ± 1 (3)
2	40	57 ± 10 (3)*	59 ± 12 (3)*	63 ± 11 (3)*
5	100	48 ± 11 (3)*	68 ± 14 (3)	64 ± 12 (3)*
10	200	44 ± 5 (20)*	49 ± 5 (20)*	58 ± 7 (20)*
30	600	57 ± 7 (7)*	54 ± 12 (7)*	59 ± 11 (7)*

The amount of [^{32}P]phosphate incorporated into proteins I, III_a, and III_b in the stimulated glands was calculated as a percentage of the amount incorporated into control glands and is shown as the mean ± SEM for the number of experiments shown in parentheses.
* Two-tailed *t* test, $P < 0.05$.

Table 2. Effect of duration of stimulation at constant frequency (10 Hz) on phosphorylation of proteins I, III_a, and III_b in rat neurohypophysis

Duration of stimulation, sec	Pulses, no.	Dephosphorylated form, % of unstimulated control		
		I	III_a	III_b
0	0	100 ± 9	100 ± 8	100 ± 11
2	20	45 ± 8*	74 ± 11	69 ± 14
5	50	34 ± 3*	63 ± 4*	65 ± 10*
10	100	27 ± 9*	57 ± 7*	44 ± 10*
20	200	37 ± 2*	56 ± 10*	59 ± 12*

The amount of [^{32}P]phosphate incorporated into proteins I, III_a, and III_b in the stimulated glands was calculated as a percentage of the amount incorporated using control glands and is shown as the mean ± SEM for four experiments.
* Two-tailed t test, $P < 0.05$.

nificantly increased the state of phosphorylation of protein I, and 5 sec of stimulation (50 pulses) significantly increased the state of phosphorylation of proteins III_a and III_b. Ten seconds of pituitary stalk stimulation (100 pulses) appeared to produce a maximum phosphorylation of all three proteins.

Fig. 2 shows the changes in the amounts of the dephosphorylated forms of proteins I and III_a, observed in response to pituitary stalk stimulation at 10 Hz for 20 sec, as a function of the time after termination of the stimulation. A substantial decrease in the amounts of dephosphoproteins I and III_a was observed when the glands were extracted "immediately"—i.e., within 20 sec after termination of the stimulation. The amounts of the dephosphorylated forms of proteins I and III_a returned to control levels within 1 min after termination of stimulation. Results obtained with protein III_b (data not shown) in these experiments were erratic but similar to those shown in Fig. 2 for proteins I and III_a.

Effect of High K^+, 8-Bromo-cAMP, and Dopamine. High K^+ concentration (60 mM for 30 sec) and 8-bromo-cAMP (4 mM for 10 min) reduced the amounts of the dephosphorylated forms of proteins I, III_a, and III_b in the rat neurohypophysis (Table 3). The effect of high K^+ was abolished in calcium-free medium (not shown). Dopamine has been shown to be present in the rat neurohypophysis (18, 19) and to affect vasopressin release *in vitro* (15). When the neurohypophysis of the rat was incubated for 3 min in the presence of 100 μM dopamine, a significant decrease in the amounts of the dephosphorylated forms of proteins I, III_a, and III_b was observed (Table 3). The effect of dopamine was abolished in calcium-free medium (not shown). The effect of dopamine also was abolished by the dopamine antagonist fluphenazine at 1 μM (Table 3). In contrast to the effect of dopamine, the α-agonist phenylephrine (100 μM) and the β-agonist isoproterenol (10 μM) failed to alter substantially the state of phosphorylation of proteins I, III_a, and III_b. These results suggest that the effect of dopamine is not mediated by α- or β-adrenergic receptors but through a dopamine receptor.

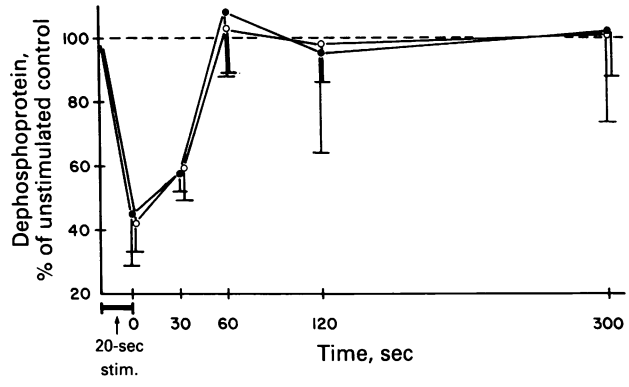

FIG. 2. Effect of electrical stimulation on the amounts of dephosphoprotein I and dephosphoprotein III_a in rat neurohypophysis as a function of time after termination of stimulation. The pituitary stalk of each neurohypophysis was stimulated by means of a suction electrode at 10 Hz for 20 sec. The amount of [^{32}P]phosphate incorporated into protein I and protein III_a was calculated as the percentage of the amount incorporated using control glands. Each datum point represents the mean ± SEM for four experiments. ●, Protein I; ○, protein III_a.

Analysis of Phosphorylation Sites in Protein I. Protein I contains a serine residue that is phosphorylated both by a cAMP-dependent and by a calcium/calmodulin-dependent protein kinase in the collagenase-insensitive region (10-kDal fragment) of the molecule and two serine residues that are phosphorylated by a second calcium/calmodulin-dependent protein kinase in the collagenase-sensitive region (30-kDal fragment) of the molecule (6, 16). One-dimensional peptide mapping of phosphorylation sites in protein I revealed that the decrease in the amount of dephosphorylated holoprotein I seen in response either to electrical stimulation or to high K^+ concentration (Table 3) was reflected in similar changes in both the 10-kDal fragment and the 30-kDal fragment of the molecule (Figs. 3 and 4). In con-

Table 3. Regulation of the state of phosphorylation of proteins I, III_a, and III_b in rat neurohypophysis

Agent	Duration of treatment, sec	Dephosphorylated form, % of unstimulated control		
		I	III_a	III_b
1. Electrical stimulation, 10 Hz	20	46 ± 7*	58 ± 7*	49 ± 7*
2. High K^+, 60 mM	30	28 ± 9*	42 ± 11*	33 ± 9*
3. 8-Bromo-cAMP, 4 mM	600	28 ± 5*	25 ± 4*	27 ± 9*
4. Dopamine, 100 μM	180	60 ± 6*	54 ± 9*	67 ± 11*
5. Dopamine, 100 μM, and fluphenazine, 1 μM	180	104 (98–110)	102 (89–115)	89 (78–100)
6. Phenylephrine, 100 μM	180	98 (88–108)	106 (101–111)	82 (77–87)
7. Isoproterenol, 10 μM	180	122 (94–150)	125 (122–128)	104 (99–109)

Neurohypophyses were incubated under the conditions shown. All test agents were present in standard buffer except for dopamine, which was present in standard buffer containing 1 mM ascorbic acid to prevent oxidation. The amount of [^{32}P]phosphate incorporated into proteins I, III_a, and III_b in the experimental glands was calculated as a percentage of the amount incorporated using control glands and is shown as the mean ± SEM for six experiments with each agent 1–4 and as the mean and range for two experiments with each agent 5–7.
* Two-tailed t test, $P < 0.05$.

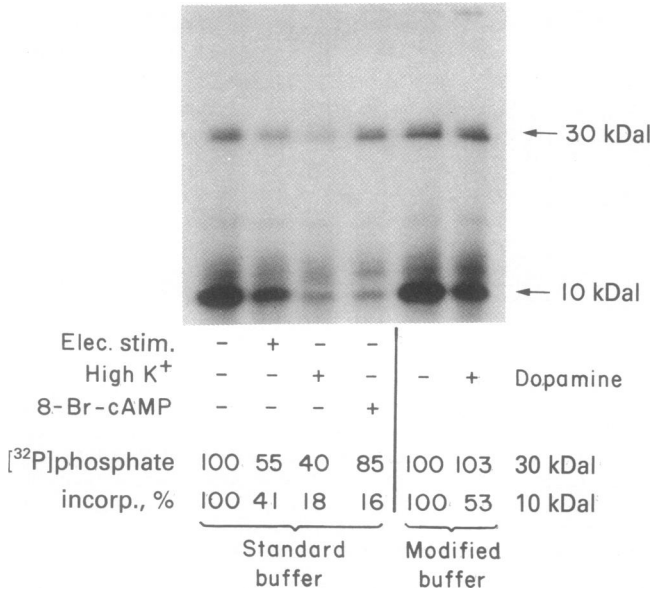

FIG. 3. Autoradiograph showing the effect of electrical stimulation and of neuroactive agents on phosphorylation of individual sites in protein I. Rat neurohypophyses were subjected to electrical stimulation at 10 Hz for 20 sec or to high K^+ (60 mM, 30 sec), 8-bromo-cAMP (4 mM, 10 min), or dopamine (100 μM, 3 min), as indicated. [Dopamine was dissolved in a modified buffer (standard Krebs–Ringer buffer with 1 mM ascorbic acid added to prevent its oxidation).] Then, protein I was extracted from the gland and subjected to proteolytic digestion with *Staphylococcus aureus* protease. Autoradiography illustrates the resultant phosphopeptide pattern obtained. The arrows indicate the 30-kDal and 10-kDal fragments. The amount of [^{32}P]phosphate incorporated into each of the two fragments under various test conditions in this experiment was calculated as a percentage of the amount incorporated under control conditions. incorp., Incorporation.

trast, 8-bromo-cAMP and dopamine had significant effects only on the 10-kDal fragment.

DISCUSSION

It seems likely that proteins I, III_a, and III_b, studied in the present investigation, were located in the neurosecretory endings of the neurohypophysis rather than in some other structure of this gland. The neurohypophysis is a relatively homogeneous system, the nervous structure of which is composed entirely of nerve terminals. Moreover, the nerve terminals are predominantly secretory endings, although nonneurosecretory terminals containing γ-aminobutyric acid or dopamine have been reported to be present (18–20). Peptides found in the neurohypophysis, like enkephalins (21) and dynorphin (22), have been reported to exist with either vasopressin or oxytocin in the neurosecretory terminals. Although our neurohypophyseal preparations were contaminated with pars intermedia fragments, it has been demonstrated by immunocytochemistry (3) that the intermediate lobe has very little protein I in contrast to its abundance in the nerve terminals of the neurohypophysis. Thus, it seems likely that the phosphorylation changes observed in protein I in this study occurred in the neurosecretory endings. Proteins III_a and III_b are neuron-specific proteins, which have a cellular and subcellular distribution similar to that of protein I (13, 14); therefore, it seems likely that the phosphorylation changes observed in proteins III_a and III_b also occurred in the neurosecretory endings of the neurohypophysis.

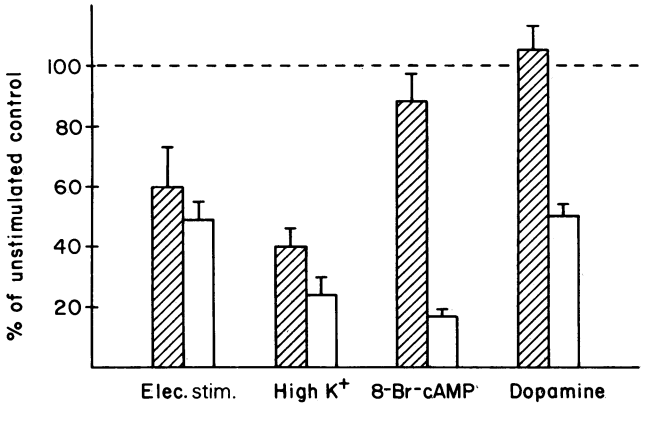

FIG. 4. Effect of electrical stimulation and of neuroactive agents on phosphorylation of individual sites in protein I in rat neurohypophysis. Experimental conditions were as described in the legend to Fig. 3. The amount of [^{32}P]phosphate incorporated into the 30-kDal (▨) and 10-kDal (□) fragments under various test conditions was calculated as a percentage of the amount incorporated under control conditions and is shown as the mean ± SEM for four experiments.

The results of the present study indicate that the state of phosphorylation of proteins I, III_a, and III_b in the neurohypophysis is regulated by neuroactive agents in a manner similar to that demonstrated earlier for these phosphoproteins in slices of rat cerebral cortex (10), in slices of rat facial motonucleus (8), in slices of bovine superior cervical ganglion (9), and in intact rabbit superior cervical ganglion (11, 23). In addition, there are several similarities between the effects of electrical stimulation on protein I phosphorylation in the neurohypophysis (present study) and the rabbit superior cervical ganglion (11). However, a significantly greater reduction (>70%) in the amount of dephosphoprotein I was observed in the neurohypophysis, which contains only nerve terminals, than in the other four preparations examined, where the maximal decrease in dephosphoprotein I was about 50% (8–10, 23). This may be explained by the presence of both presynaptic and postsynaptic elements in these latter preparations because postsynaptic protein I is apparently not responsive to various electrical or chemical stimuli (23).

Proteins I, III_a, and III_b have been shown to be enriched in purified preparations of synaptic vesicles (2, 5, 14, 24). These and other observations (12) suggest that these phosphoproteins may be involved in regulation of release from nerve terminals. The present results indicate that large changes occur in the state of phosphorylation of these proteins in the nerve endings of the neurohypophysis in response to procedures that affect the release of hormones from these nerve endings (15, 25). For instance, the changes in state of phosphorylation of proteins I, III_a, and III_b seen in the neurohypophysis in response to electrical stimulation and to high K^+ depended on extracellular calcium. Vasopressin release in response to electrical stimulation and high K^+ also showed a dependence on extracellular calcium (26). The optimal stimulus frequency for the phosphorylation of proteins I, III_a, and III_b in the present work was about 10 Hz, in agreement with the optimal stimulus frequency of 10–20 Hz for vasopressin release *in vitro* (25) and with the discharge frequency of magnocellular neurons observed *in vivo* in water-deprived rats (27). Thus, the neurohypophysis should be an excellent system in which to study the role of protein phosphorylation in general and of proteins I, III_a, and III_b in particular in the regulation of release from nerve endings.

This work was supported by U.S. Public Health Service Grants MH-17387 and NS-08440 and by a grant from the McKnight Foundation.

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