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Depolarizing agents and cyclic nucleotides regulate the phosphorylation of specific neuronal proteins in rat cerebral cortex slices

(protein phosphorylation/protein I/cyclic AMP/veratridine)

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ABSTRACT **The regulation of the state of phosphorylation of two specific neuronal proteins, designated protein Ia and protein Ib, has been studied in slices of rat cerebral cortex incubated *in vitro*. For this purpose, a method was developed that prevents dephosphorylation of these proteins during their extraction. When the slices were incubated in a standard Krebs–Ringer solution, proteins Ia and Ib were present almost entirely in the dephosphorylated form. Incubation with cyclic AMP, 8-bromo cyclic AMP, N^6-monobutyryl cyclic AMP, or with a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, increased the phosphorylation of proteins Ia and Ib in the slices. Depolarization of neuronal membranes by high K^+ or by veratridine was also associated with an increased phosphorylation of proteins Ia and Ib. The effect of depolarizing agents, but not that of cyclic nucleotides or 3-isobutyl-1-methylxanthine, required the presence of external Ca^{2+} in the incubation medium. Tetrodotoxin blocked the stimulation of the phosphorylation of proteins Ia and Ib induced by veratridine but not that induced by the other agents tested. Incubation of the brain slices with 8-bromo cyclic AMP, 3-isobutyl-1-methylxanthine, high K^+, or veratridine also increased the state of phosphorylation of two other neuronal proteins found in extracts of the slices.**

Calcium (1) and cyclic nucleotides (2, 3, 4) appear to play important roles in the functioning of the nervous system. Evidence has been reviewed (5) indicating that several of the effects of calcium and the cyclic nucleotides on the functioning of the nervous system may be mediated through protein phosphorylation.

When subcellular fractions of brain enriched in synaptic membranes are incubated in the presence of [γ-^{32}P]ATP, there is a cyclic AMP (cAMP)-dependent phosphorylation of several endogenous substrate proteins, catalyzed by endogenous protein kinase (6–8). Two of the most prominent of these substrate proteins, protein Ia and protein Ib, have been solubilized and purified to apparent homogeneity (7). Proteins Ia and Ib have remarkably similar structural properties (7). They have been found only in neuronal tissue (6, 7), and within neurons are associated primarily, and perhaps exclusively, with synaptic vesicles, synaptic membranes, and postsynaptic densities (unpublished observations).

In preparations of intact synaptosomes (isolated nerve ending particles), depolarizing agents, including high K^+ and veratridine, through an influx of calcium, stimulate the phosphorylation of two specific proteins that have the same apparent molecular weights as proteins Ia and Ib, as determined by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis (9). Recent studies (10) indicate that these two synaptosomal proteins are similar, if not identical, to proteins Ia and Ib, whose phosphorylation is stimulated by cAMP in synaptic membrane fractions. Interestingly, cAMP, 8-bromo-cAMP, and 3-isobutyl-1-methylxanthine (iBuMeXan) fail to stimulate phosphorylation of these proteins in intact synaptosomes (9).

The development of a method for the selective extraction of proteins Ia and Ib (collectively referred to as protein I) from nervous tissue (7), together with the finding that homogenization of brain tissue in the presence of Zn^{2+} prevents the dephosphorylation of phosphorylated proteins Ia and Ib, has allowed us to study the regulation of the phosphorylation of these neuronal proteins in intact slices of brain.

METHODS

Materials. cAMP, 8-bromo-cAMP, N^6-monobutyryl-cAMP, cyclic GMP (cGMP), 8-bromo-cGMP, 2-mercaptoethanol, Coomassie blue, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), Tris base, EDTA, and ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Sigma, iBuMeXan from Aldrich, and tetrodotoxin from Calbiochem. 8-Azido-[^{32}P]cAMP was prepared (11) and kindly supplied by Ulrich Walter. [γ-^{32}P]ATP was prepared by the method of Post and Sen (12). Protein was determined by the method of Lowry *et al.* (13), with bovine serum albumin as standard.

Preparation and Incubation of Brain Cortex Slices. Sprague–Dawley rats (200–250 g) were killed by decapitation and the brains were removed. Both cerebral cortex hemispheres were dissected, the white matter was removed, and slices 0.26 × 0.26 mm were prepared with a McIlwain tissue chopper. Slices were suspended in standard (Krebs–Ringer bicarbonate) buffer (composition in mmol/liter: NaCl, 124; KCl, 5.0; $NaHCO_3$, 25; $CaCl_2$, 1; Na_2HPO_4, 1.5; $MgSO_4$, 1.5; and glucose, 10), which was equilibrated with 95% O_2/5% CO_2, and had a final pH of 7.4. The slices were preincubated in a shaking water bath for 60 min at 37° with two changes of medium. In most experiments, aliquots (500 μl) containing 30–40 mg of tissue (wet weight) were transferred to glass tubes (13 × 100 mm) containing various test substances dissolved in 50 μl of standard buffer. The tubes were flushed with 95% O_2/5% CO_2 and capped; the slices were then incubated in a shaking water bath at 37° for the indicated periods of time. In experiments in which high KCl was used, a sufficient volume of a "129 mM KCl buffer" was added to the standard buffer to obtain the final desired concentration of K^+. This 129 mM KCl-buffer had the same composition as the standard buffer, except that 129 mM KCl was substituted for 124 mM NaCl/5 mM KCl. The incu-

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Abbreviations: iBuMeXan, 3-isobutyl-1-methylxanthine; cAMP, cyclic AMP; cGMP, cyclic GMP; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

bation was terminated by rapidly adding 6 ml of an ice-cold solution of 5 mM Zn acetate, quickly transferring the slice suspension to a cold glass homogenizer, and homogenizing the slices with a motor-driven Teflon pestle (six strokes at 1500 rpm). The homogenate was centrifuged at 150,000 × *g* for 30 min at 4° to obtain the total particulate fraction. In more recent experiments we have found that for samples treated with Zn^{2+}, which causes aggregation of membranes, a 10-min centrifugation at 10,000 × *g* gives the same recovery of protein I.

Extraction of Protein I. Protein I was extracted from the particulate fraction by modification of the acid extraction procedure (7). All steps in the extraction procedure were performed at 4°. The pellet was resuspended in 2 ml of 10 mM citric acid, transferred to a Teflon/glass homogenizer, and rehomogenized by hand. A 1-ml aliquot was then transferred to a plastic centrifuge tube, the pH was checked and, if necessary, adjusted to pH 3.0 with 100 mM citric acid, and the tube was centrifuged at 23,500 × *g* for 15 min. The supernatant was adjusted to pH 6.0 by addition of 0.2 M Na_2HPO_4, and centrifuged at 4000 × *g* for 10 min. This pH 6.0 supernatant, referred to as the "extract," was used for assay of protein I. An important aspect of the extraction procedure used was that it inactivated any protein I kinase and protein I phosphatase activity that might have been extracted along with protein I (data not shown).

Preparation of cAMP-Dependent Protein Kinase and Protein Phosphatase. cAMP-dependent protein kinase present in a synaptic membrane fraction of calf cerebral cortex was solubilized and purified through step 3 of the procedure of Uno *et al.* (14). Heart protein phosphatase was purified through step 4 of the procedure of Chou *et al.* (15).

Assay of Amount of Dephospho-protein I. Dephospho-protein I was assayed by determining the maximum amount of radioactive phosphate incorporated into it in the presence of [γ-^{32}P]ATP and added cAMP-dependent protein kinase. The assay mixture (final volume, 100 μl) contained 50 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 10 μM cAMP, 6.5 units of protein I kinase, and 30 μl of the extract. The presence of EDTA in the final assay mixture was necessary in order to chelate the Zn^{2+} carried over from the original homogenate which otherwise would have inhibited the activity of the exogenous protein kinase. The phosphorylation reaction was initiated by the addition of 100 pmol of [γ-^{32}P]ATP (4–5 × 10^7 cpm/nmol) in a volume of 10 μl. Incubation was carried out at 30° for 30 min, which caused maximal phosphorylation of proteins Ia and Ib. The reaction was terminated by the addition of 50 μl of a "NaDodSO$_4$-stop solution" containing 9% NaDodSO$_4$, 6% mercaptoethanol, 15% glycerol, and 0.01% bromphenol blue dye in 186 mM Tris·HCl (pH 6.7). Aliquots (80 μl) were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis as described (16), except that the final concentration of acrylamide in the separating gel was 8%. The slab gels were stained for protein with 0.025% Coomassie blue, destained, and dried, and autoradiography was carried out as described (6). The resulting autoradiograph was used to locate those proteins into which ^{32}P had been incorporated. A band containing proteins Ia plus Ib was cut from each slot of the slab gels, and radioactivity was determined in a liquid scintillation spectrometer. Under the conditions described for homogenization of brain slices and for extraction and assay of protein I, the amount of phosphorylated protein I present in the extract was proportional to the amount of total protein used for the extraction.

RESULTS

Quantitative Estimation of Dephospho-protein I and Phospho-protein I. An autoradiograph showing the incorporation of ^{32}P from [γ-^{32}P]ATP into proteins in extracts of brain cortex slices is shown in Fig. 1. Of the many protein bands revealed by protein staining, only four were significantly phosphorylated under the assay conditions used. Two of these proteins were identified as protein Ia (M_r 86,000) and protein Ib (M_r 80,000), as judged by their extraction with acid, by their mobility in the gel electrophoresis system used, and by their proteolysis, in the presence of a highly purified preparation of collagenase, to the same peptide fragments as are observed (7) when highly purified proteins Ia and Ib are digested with collagenase. The other two phosphorylated proteins seen in Fig. 1, with apparent M_r of 74,000 and 55,000, will be discussed below.

When slices of rat cerebral cortex were incubated in the presence of 4 mM 8-bromo-cAMP, prior to acid extraction in the presence of Zn acetate, there was a decrease in the amount of radioactive PO_4 subsequently incorporated from [γ-^{32}P]ATP into proteins Ia and Ib. For instance, in the experiment of Fig. 1, there were 62,000 cpm in protein I in the control channel (lane 1), but only 26,900 cpm in protein I in the 8-bromo-cAMP channel (lane 2). When tissue slices were extracted with water rather than Zn^{2+}, so that endogenous protein phosphatases were not inhibited, there was no difference between radioactivity in protein I in the control channel (59,000 cpm; lane 3) and that in the 8-bromo-cAMP channel (58,400 cpm; lane 4). In some experiments, extracts were incubated with heart protein phosphatase prior to phosphorylation with [γ-^{32}P]ATP and added protein kinase. (The amount of protein phosphatase added was sufficient to fully dephosphorylate [^{32}P]phospho-protein I added as substrate.) In such phosphatase-treated extracts, no difference was observed in the amount of radioactivity incorporated into protein I, whether the slices had been incu-

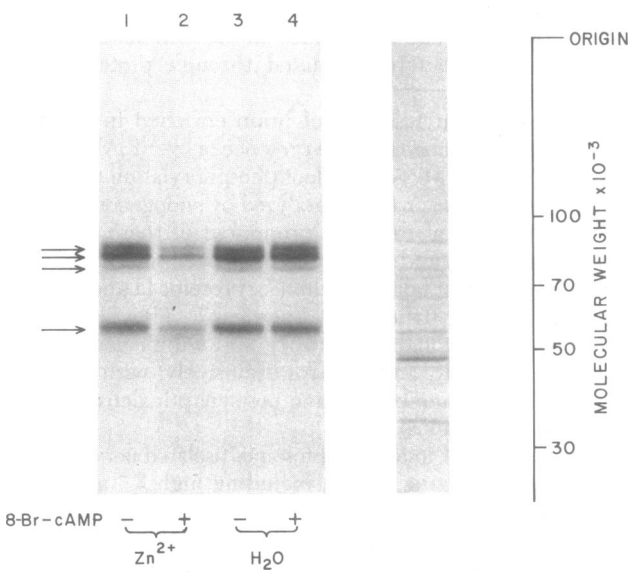

FIG. 1. Phosphorylation of proteins in extracts prepared from rat cerebral cortex slices that had been incubated *in vitro* in the absence (−) or presence (+) of 8-bromo-cAMP (8-Br-cAMP). Slices were incubated for 10 min and were then homogenized in 6 ml of 5 mM Zn acetate or 6 ml of H_2O. Proteins were extracted, phosphorylated with [γ-^{32}P]ATP and exogenous protein kinase, and subjected to NaDodSO$_4$-polyacrylamide gel electrophoresis, protein staining (*Right*) and autoradiography (*Left*). (*Right*) Pattern shown corresponds to lane 1. (Arrows) Positions of proteins, with molecular weights 86,000 (protein Ia), 80,000 (protein Ib), 74,000, and 55,000, whose phosphorylation was affected by 8-bromo-cAMP.

bated in the absence or presence of 8-bromo-cAMP and whether the slices had been homogenized in Zn acetate or H_2O. Moreover, the amount of radioactive protein I observed in these extracts treated with protein phosphatase was the same, within experimental error, as that observed in extracts from control tissue homogenized in Zn acetate (Fig. 1, lane 1) or H_2O (Fig. 1, lane 3) but not treated with added protein phosphatase.

The fact that treatment of extracts with added protein phosphatase did not detectably increase the amount of phosphate incorporated into protein I in extracts from control brain slices indicates that the protein I in these slices was present almost entirely as dephospho-protein I. The results with added protein phosphatase also indicate that there was no change in the total amount of protein I (dephospho-protein I plus phospho-protein I) when brain slices were incubated with 8-bromo-cAMP. Rather, the data demonstrate that, in brain slices incubated with 8-bromo-cAMP, there was a decrease in the amount of dephospho-protein I and a corresponding increase in the amount of phospho-protein I.

From the specific activity of the [γ-^{32}P]ATP used, it was possible to calculate the amount of phosphate that had been incorporated into proteins Ia and Ib in the brain slices. The amount of protein I phosphate present in slices of rat cerebral cortex was calculated to be 9.2 ± 1.1 pmol (± SEM) per mg of total protein. If 1 mol of phosphate is incorporated per mol of protein Ia or Ib (see ref. 7), this value would also represent the concentration of protein Ia plus protein Ib per mg of total protein. Since the total amount of protein I appears not to change during the experiment, we have, in the remainder of this paper, equated the experimentally determined decrease in dephospho-protein I with the formation of phospho-protein I. The formation of phospho-protein I in the slices is expressed as the ratio of phospho-protein I to total protein I.

In some experiments, slices were preincubated in glucose-free standard buffer, containing 0.1 mM 2,4-dinitrophenol, in an effort to deplete intracellular pools of ATP. In such slices, the effect of 8-bromo-cAMP on protein I phosphorylation was abolished. The amount of [^{32}P]phosphate that could be incorporated into protein I in extracts of such slices was the same as in extracts from control slices incubated in standard buffer.

Effects of 8-Bromo-cAMP and iBuMeXan on Protein I Phosphorylation. The effects of various concentrations of 8-bromo-cAMP and of the phosphodiesterase inhibitor iBuMeXan on phospho-protein I levels in slices of rat cerebral cortex are shown in Fig. 2. A half-maximal increase in the amount of phospho-protein I occurred with about 1 mM 8-bromo-cAMP or 0.1 mM iBuMeXan. The maximal effect produced was similar for the two agents, and amounted to phosphorylation of about 65% of the total protein I present in the slices. In the presence of a maximally effective concentration of either agent, no further increase in the phosphorylation of protein I could be produced by the addition of the other agent. N^6-Monobutyryl-cAMP gave results similar to those obtained with 8-bromo-cAMP, whereas cAMP at the highest concentration tested (4 mM) had a smaller effect (about 40% of the maximal effect of 8-bromo-cAMP).

The effects of 8-bromo-cAMP and of iBuMeXan on phospho-protein I levels are shown as a function of time of incubation in Fig. 3. The relatively high concentration of 8-bromo-cAMP required to increase phospho-protein I levels and the slow time course observed with this substance are probably attributable to a low permeability of the plasma membranes to cyclic nucleotides. High concentrations of cyclic nucleotides are also required in other intact cell preparations to observe physiological effects (17) as well as effects on protein phosphorylation (18). The lower activity of cAMP itself may be due to a greater difficulty in crossing plasma membranes or to its

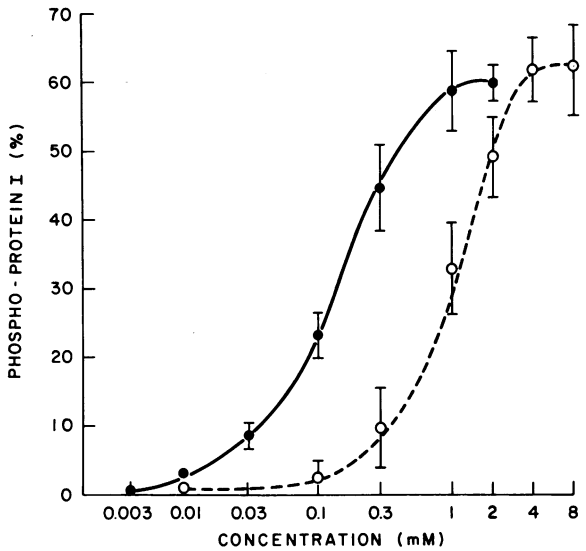

FIG. 2. Formation of phospho-protein I from dephospho-protein I in rat cerebral cortex slices incubated in the presence of various concentrations of 8-bromo-cAMP (○) or iBuMeXan (●) for 10 min. Data are expressed as 100 × phospho-protein I/total protein I. Each point represents the mean ± SEM of determinations on five (8-bromo-cAMP) or eight (iBuMeXan) samples incubated, extracted, and assayed separately.

greater susceptibility to hydrolysis by phosphodiesterase. cGMP and 8-bromo cGMP, in concentrations up to 4 mM, were without effect on protein I phosphorylation. The increase in phospho-protein I induced by iBuMeXan occurred more rapidly than that induced by the cyclic nucleotides (Fig. 3), probably due to a more rapid entrance of iBuMeXan into the cells. In slices incubated with iBuMeXan under the same conditions as were used for the study of protein phosphorylation, both the dose-response curve and the time-course for cAMP formation were similar to those observed for protein I phosphorylation (data not shown).

Effects of K^+ and Veratridine on Protein I Phosphorylation. Two depolarizing agents, K^+ and veratridine, both of

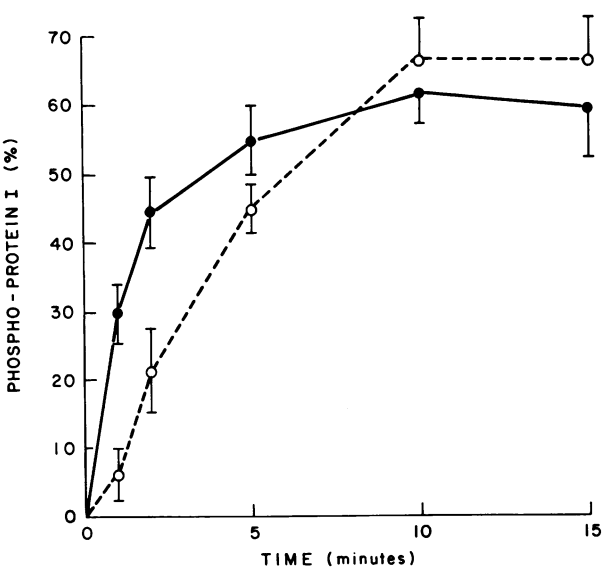

FIG. 3. Formation of phospho-protein I from dephospho-protein I in rat cerebral cortex slices incubated for various times in the presence of 4 mM 8-bromo-cAMP (○) or 1 mM iBuMeXan (●). Data are expressed as 100 × phospho-protein I/total protein I. Each point represents the mean ± SEM of determinations on six samples incubated, extracted, and assayed separately.

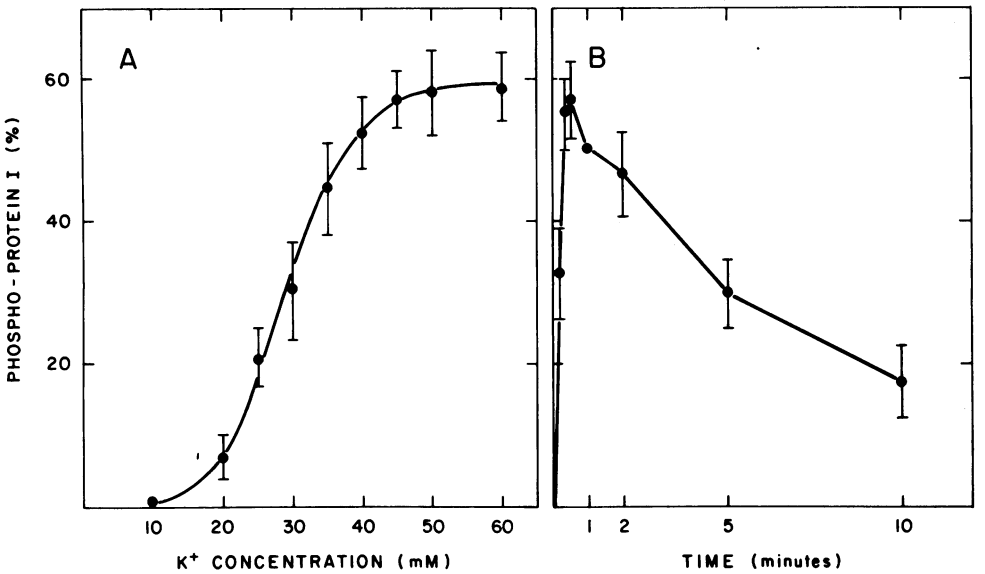

FIG. 4. Formation of phospho-protein I from dephospho-protein I in rat cerebral cortex slices incubated (*A*) in the presence of various concentrations of K^+ for 30 sec and (*B*) in the presence of 60 mM K^+ for various times. Data are expressed as 100 × phospho-protein I/total protein I. Each point represents the mean ± SEM of determinations on six samples incubated, extracted, and assayed separately.

which cause calcium influx into, and neurotransmitter release from, presynaptic nerve terminals, as well as calcium-dependent accumulation of cAMP in brain slices (2, 4), were tested for their ability to affect the state of phosphorylation of protein I in slices of rat cerebral cortex. Both depolarizing agents caused large increases in the amount of phospho-protein I in such slices. The effect of various concentrations of K^+ on phospho-protein I levels is shown in Fig. 4*A*; a half-maximal increase in protein I phosphorylation occurred in the presence of 25–30 mM K^+. A maximally effective concentration of K^+ caused the phosphorylation of about 60% of the total protein I present in the slices. The increase in phosphorylation of protein I in the presence of high K^+ was rapid; the effect was more than half-maximal in the shortest time studied (10 sec), and a maximal effect was observed within 20 sec of incubation (Fig. 4*B*). Despite the continuous presence of high K^+ in the incubation medium, protein I became dephosphorylated rapidly. This effect may be related to the observation that calcium entry into presynaptic terminals in response to maintained depolarization induced by K^+-rich solutions also declines rapidly with time (1). It was possible to carry out successive cycles of phosphorylation and dephosphorylation of protein I simply by successive periods of exposure to high and low concentrations of K^+ (Fig. 5), provided that the slices were exposed to the high K^+ for only brief periods.

Veratridine (100 μM) also produced a rapid increase in protein I phosphorylation, which reached a maximal level in 30 sec (data not shown). Despite the continuous presence of veratridine, protein I was dephosphorylated and returned to control values in about 5 min (data not shown). The maximal level of phosphorylation produced by veratridine was about 55–65% of the maximal level of phosphorylation produced by high K^+, 8-bromo-cAMP, or iBuMeXan (see Table 1). A half-maximal effect was obtained with about 0.03 mM veratridine, and a maximal effect with about 0.1 mM veratridine.

Effects of Calcium-Free Solution and of Tetrodotoxin on Phosphorylation of Protein I. When brain slices were incubated in a buffer solution modified from the standard buffer by substitution of 0.1 mM EGTA for the 1 mM $CaCl_2$, the effects of 60 mM K^+ and of 0.1 mM veratridine on the phosphorylation of protein I were abolished (Table 1). The absence of calcium had no influence on the effects of 8-bromo-cAMP or iBuMeXan on phosphorylation of protein I. Tetrodotoxin, which selectively inhibits the depolarizing effects of veratridine and the subsequent entry of calcium, abolished the effect of veratridine on phosphorylation of protein I (Table 1). The effect of K^+ on protein I phosphorylation was unaffected by tetrodotoxin, as might be expected since this drug prevents neither the K^+ depolarization nor the subsequent entry of Ca^{2+} through voltage-dependent channels. Similarly, tetrodotoxin did not

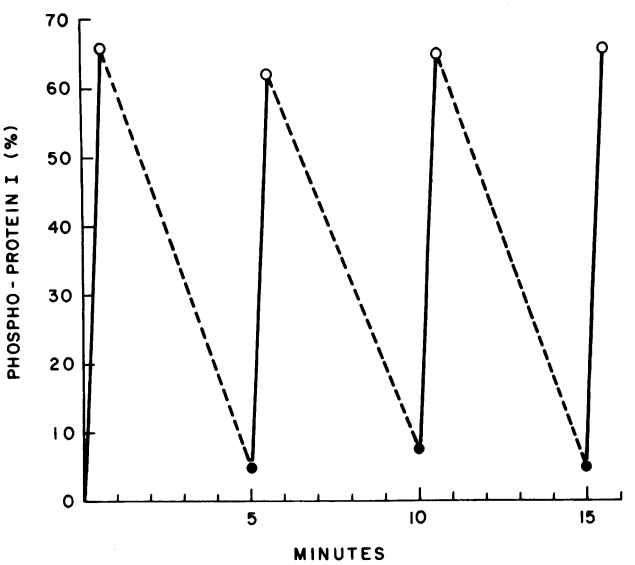

FIG. 5. Successive cycles of interconversion of dephospho-protein I and phospho-protein I in rat cerebral cortex slices. Slices were incubated alternately in high (60 mM) and low (5 mM) K^+. At zero time, a 5-ml suspension of slices in low K^+ (standard) buffer was adjusted to a final KCl concentration of 60 mM by addition of 129 mM KCl buffer; after mixing, enough incubation medium was removed to reach the 5-ml volume. After 30 sec of incubation in the high K^+ buffer, a 0.5-ml aliquot was removed and rapidly homogenized in 5 mM of Zn acetate for analysis of protein I. To the remaining 4.5 ml of slices, 15 ml of low K^+ buffer was immediately added and the suspension was shaken. After the slices had settled, the supernatant was removed and the slices were washed twice with 15 ml of low K^+ buffer. Low K^+ buffer was then added to a final volume of 4.5 ml. At 5 min, another 0.5-ml aliquot was removed for analysis. The 4-ml suspension of slices remaining was adjusted to a final KCl concentration of 60 mM by addition of 129 mM KCl buffer; after mixing, enough incubation medium was removed to reach the 4-ml volume. After 30 sec of incubation in the high K^+ buffer, another 0.5-ml aliquot was removed for analysis. This cycle was repeated twice more, as indicated. Data are expressed as 100 × phospho-protein I/total protein I. Each point (○, 60 mM K^+; ●, 5 mM K^+) represents the average of determinations on two samples incubated, extracted, and assayed separately.

Table 1. Effect of Ca^{2+} and of tetrodotoxin on formation of phospho-protein I from dephospho-protein I in response to high K^+, veratridine, 8-bromo-cAMP, and iBuMeXan

Test substance	Standard buffer	Ca^{2+}-free standard buffer	Standard buffer + tetrodotoxin
High K^+ (60 mM)	59 ± 6	4 ± 1	56 ± 4
Veratridine (100 μM)	38 ± 5	3 ± 1	4 ± 1
8-Bromo-cAMP (4 mM)	68 ± 7	65 ± 4	68 ± 10
iBuMeXan (1 mM)	64 ± 6	66 ± 6	59 ± 8

Cerebral cortex slices were preincubated for 5 min in standard buffer, in Ca^{2+}-free standard buffer, or in standard buffer plus 5 μM tetrodotoxin. Slices were then incubated with high (60 mM) K^+ for 20 sec, 100 μM veratridine for 30 sec, 4 mM 8-bromo-cAMP for 10 min, or 1 mM iBuMeXan for 10 min. Data are expressed as 100 × phospho-protein I/total protein I. Each point represents the mean ± SEM for determinations on five samples incubated, extracted, and assayed separately.

alter the increase in protein I phosphorylation that occurred in the presence of 8-bromo-cAMP or iBuMeXan.

Other Phosphorylated Proteins. As in the case of proteins Ia and Ib, the 74,000 and 55,000 M_r proteins (Fig. 1) appear to be specific to nervous tissue, since they have not been found in extracts prepared, under the same experimental conditions, from various non-neuronal tissues (heart, liver, spleen, kidney, testicle, and adrenal). Furthermore, within the nervous system they seem to be located in neurons, since the injection of kainic acid, a compound that destroys neuronal cell bodies without affecting glia cells, decreased by more than 70% the amount of these phosphoproteins in the extracts (data not shown). The 55,000 M_r protein has an apparent M_r similar to that of the phosphorylated regulatory subunit of type II cAMP-dependent protein kinase (11). However, these two proteins could be distinguished from each other by several criteria: (*i*) the two proteins have slightly different mobilities in polyacrylamide gels; (*ii*) the regulatory subunit of the protein kinase is not extracted by acid; and (*iii*) the 55,000 M_r protein does not bind 8-azido-[^{32}P]cAMP, a photoaffinity reagent that is highly specific (11) for cAMP-dependent protein kinase. Under all the experimental conditions described in this article, we have observed changes in the level of phosphorylation of the 74,000 and 55,000 M_r proteins qualitatively similar to those observed for protein Ia and protein Ib.

Other Brain Regions. In some experiments we have observed that the effects of high K^+, veratridine, 8-bromo-cAMP, and iBuMeXan on phosphorylation of protein I in slices of caudate nucleus and cerebellum were similar to those observed in slices of cerebral cortex.

Other Agents. Various putative neurotransmitters were tested, alone and in combination, for possible effects on the phosphorylation of protein I. These included dopamine, norepinephrine, carbachol, serotonin, γ-aminobutyric acid, histamine, and adenosine at 0.01 and 0.1 mM as well as glycine and glutamate at 0.1 and 1 mM. Slices were incubated with these agents for 0.5, 1, and 10 min, in the absence and presence of iBuMeXan (0.03 and 0.1 mM). None of these treatments had a detectable effect on the phosphorylation of protein I. Work is still in progress to determine whether experimental conditions can be found in which putative neurotransmitters will affect the state of phosphorylation of protein I.

DISCUSSION

The development of a procedure (7) for isolating proteins Ia and Ib, as well as the methodology described in this paper for preventing their dephosphorylation during the isolation procedure, has allowed us to study the regulation of the state of phosphorylation of these proteins in intact slices of rat brain cerebral cortex incubated *in vitro*. The present studies demonstrate that high potassium and veratridine, in the presence of external calcium, as well as 8-bromo-cAMP and iBuMeXan, independently of external calcium, stimulate the phosphorylation of proteins Ia and Ib in brain slices. These agents stimulated protein I phosphorylation to a similar extent; potassium, 8-bromo-cAMP, and iBuMeXan each converted maximally about 50–70% of proteins Ia and Ib from the dephospho- to the phospho-form.

Studies with kainic acid, which selectively destroys nerve cells, have demonstrated that protein I is located in neurons (19). In addition, subcellular fractionation studies and immunocytochemical studies indicate that protein I is associated with synaptic vesicles and synaptic membranes. Within the synaptic membrane complex, proteins Ia and Ib appear to be concentrated in the postsynaptic density (unpublished observations). It is not yet possible to ascribe a particular subcellular localization to the fraction of protein I that was converted from the dephospho- to the phospho-form by the depolarizing agents, cAMP derivatives, and iBuMeXan.

The most important aspect of the present study lies in the demonstration that agents that are capable of affecting neuronal function and metabolism are also capable of altering the state of phosphorylation of proteins Ia and Ib in intact cells. Taken together with evidence on the localization of protein I in those regions of the nervous system associated with synaptic transmission, namely, synaptic vesicles and postsynaptic membranes, the data suggest that protein I may be involved in the physiology of synaptic transmission.

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