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Ergopeptine-Sensitive Calcium-Dependent Protein Phosphorylation System in the Brain

*Carol A. Stratford, †Stephen K. Fisher, and *‡Tetsufumi Ueda

**Department of Pharmacology; †Neuroscience Laboratory; and ‡Mental Health Research Institute, The University of Michigan, Ann Arbor, Michigan, U.S.A.*

Abstract: We studied a protein phosphorylation system that is regulated by the dopamine-mimetic ergot bromocriptine. Bromocriptine was found to inhibit selectively the endogenous phosphorylation of a threonine residue(s) in 50,000- and 60,000-dalton proteins in a synaptosome fraction. The bromocriptine-sensitive phosphorylation is stimulated by calcium and by calmodulin, and occurs predominantly in the brain. The inhibitory effect of bromocriptine was not mimicked by 3,4-dihydroxyphenylethylamine or by any of the neurotransmitters and related agents tested, but was mimicked, although less effectively, by other ergots that contain peptide moieties. In the hippocampus, the brain region with the highest content of the 50,000- and 60,000-dalton proteins, the ergopeptine-sensitive protein phosphorylation appears to be localized to interneurons or cell bodies whose axons synapse outside the hippocampus. The results raise the possibility that some of the bromocriptine- and ergopeptine-induced pharmacological effects in the CNS may be mediated by the inhibition of the calcium/calmodulin-dependent phosphorylation of these specific proteins. **Key Words:** Bromocriptine—Calcium—Calmodulin—Ibotenate—Protein phosphorylation. **Stratford C. A. et al.** Ergopeptine-sensitive calcium-dependent protein phosphorylation system in the brain. *J. Neurochem.* **42,** 842–855 (1984).

Protein phosphorylation is involved in the regulation of enzymatic and cellular activities in a variety of biological systems, including the nervous system, where it has been proposed to serve as a mediator of at least some of the physiological effects of calcium (see Krebs and Beavo, 1979; Glass and Krebs, 1980; Greengard, 1981; Cohen, 1982, for reviews). A number of calcium-dependent protein phosphorylation systems in brain tissue have been described (DeLorenzo, 1976; Schulman and Greengard, 1978; Takai et al., 1979; Wrenn et al., 1980); however, a direct relationship between calcium-dependent phosphorylation and neuronal function has yet to be established. In the present study, we investigated interactions between neuroactive agents and calcium-dependent phosphorylation in a synaptosome fraction of rat brain. We report here that the 3,4-dihydroxyphenylethylamine (dopamine)-mimetic ergot bromocriptine (2-Br-α-ergocriptine) and other peptide-containing ergots selectively inhibit the calmodulin-stimulated phosphorylation of 50,000- and 60,000-dalton proteins which are highly enriched in the brain. Lesion studies on the guinea pig hippocampus are also presented, and indicate that these phosphoproteins are predominantly localized to interneurons or cell bodies whose axons synapse outside the hippocampus.

These bromocriptine-sensitive phosphoproteins have electrophoretic mobilities in sodium dodecyl sulfate (SDS) similar to those of diphenylhydan-

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Address correspondence and reprint requests to Tetsufumi Ueda, Mental Health Research Institute, 205 Washtenaw Pl., The University of Michigan, Ann Arbor, MI 48109, U.S.A.
The present address of C.A.S. is Department of Anatomy and Cell Biology, The University of Michigan, Ann Arbor, MI 48109, U.S.A. The present address of S.K.F. is Department of CNS Research, Medical Research Division of American Cyanamid, Lederle Laboratories, Pearl River, NY 10965, U.S.A.

Abbreviations used: BrC_{50}, $BrC\text{-}P_{60}$, Bromocriptine-sensitive phosphoproteins with molecular weights of 50,000 and 60,000, respectively; ChAT, Choline acetyltransferase; Dopamine, 3,4-Dihydroxyphenylethylamine; DPH, Diphenylhydantoin; DPH-L, DPH-M, Diphenylhydantoin-sensitive proteins; GABA, γ-Aminobutyric acid; GAD, Glutamate decarboxylase; KRB, Krebs-Ringer bicarbonate buffer; SDS, Sodium dodecyl sulfate; Serotonin, 5-Hydroxytryptamine.

toin (DPH)-sensitive phosphoproteins termed DPH-L and DPH-M (DeLorenzo, 1976), the major Ca^{2+}/calmodulin-dependent phosphoproteins in a synaptosomal membrane fraction (Schulman and Greengard, 1978), and the 51,000- and 62,000-dalton phosphoproteins in the postsynaptic density fraction (Grab et al., 1981). DPH-L and DPH-M have been shown to be present in synaptic vesicle, synaptic junctional complex, postsynaptic density, and synaptic membrane fractions (DeLorenzo, 1980). DeLorenzo and coworkers (DeLorenzo et al., 1979; DeLorenzo, 1980) have provided evidence that Ca^{2+}/calmodulin-dependent phosphorylation of DPH-L and DPH-M is correlated with depolarization-dependent calcium uptake and neurotransmitter release. The bromocriptine-sensitive phosphorylation system is discussed in relation to the above Ca^{2+}/calmodulin-dependent phosphoproteins as well as with respect to a possible association with a particular neurotransmitter system.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (126–150 g) were obtained from Charles River. Male guinea pigs (350–400 g) were obtained from Buckberg Animals (Tomkins Cove, NY). [γ-^{32}P]ATP (>5000 Ci/mmol), [1-^{14}C]L-glutamic acid (50 Ci/mmol), and [^{14}C]acetyl-coenzyme A (56.6 Ci/mmol) were purchased from Amersham. Acrylamide, *N,N,N′,N′*-tetramethylene ethylenediamine, and X-ray film (XAR-5) were from Eastman Kodak. *N,N′*-Methylene bisacrylamide (electrophoresis grade) was from Polysciences. Ibotenic acid was obtained from Regis Chemical (Morton Grove, IL). Molecular weight standards for SDS gel electrophoresis were from Bio-Rad. SDS, ATP, α-chymotrypsin, ergotamine tartrate, α-ergocriptine, ergonovine, 2-mercaptoethanol, phosphoserine, and phosphothreonine were from Sigma Chemical. All other chemicals were of reagent quality.

The following were gifts, generously provided by the person or company indicated: bromocriptine (Sandoz Pharmaceuticals, East Hanover, NJ); (+)- and (−)-butaclamol (Ayerst Laboratories, New York, NY); chlorpromazine (Smith, Kline and French, Philadelphia, PA); clonidine HCl (Boehringer Ingelheim, Ridgefield, CT); dihydroergocriptine (Sandoz Pharmaceuticals); fluphenazine HCl (E. R. Squibb and Sons, Princeton, NJ); haloperidol (Dr. R. Katz, University of Michigan); metoclopramide HCl (A. H. Robins, Richmond, VA); molindone (Abbott Laboratories, N. Chicago, IL); phentolamine mesylate (Ciba Pharmaceuticals, Summit, NJ); quipazine (Dr. J. Woods, University of Michigan); spiroperidol (Janssen Pharmaceutica, New Brunswick, NJ); sulpiride (Delagrange Intl., Paris, France); thioridazine HCl (Sandoz Pharmaceuticals); trifluoperazine dihydrochloride (Smith, Kline and French); yohimbine HCl (Dr. C. B. Smith, University of Michigan). Purified calmodulin was the gift of Dr. M. E. Gnegy (University of Michigan), and phosphotyrosine was the gift of Dr. S. Cohen (Vanderbilt University). All other chemicals used were of reagent quality.

Krebs-Ringer bicarbonate buffer (KRB) (Daly et al., 1980) was prepared fresh each day and contained the following components (millimolar concentrations): NaCl (122); $NaHCO_3$ (25); D-glucose (10); KCl (3.0); KH_2PO_4 (1.0); $MgSO_4$ (1.2); $CaCl_2$ (1.3). The solution was gassed with 95% O_2-5% CO_2 to pH 7.4 prior to use.

Preparation of a synaptosome fraction

A crude syaptosome fraction (P_2) was prepared from rat cerebral cortex (unless otherwise indicated) using a method similar to that described by Daly et al. (1980). Rats were killed by decapitation. Brains were removed and cortical regions were dissected free of striatal regions and excess white matter. The cortices were then roughly chopped with a scalpel, immersed briefly in ice-cold KRB, drained on a small nylon mesh screen, and weighed. Homogenization was carried out in three volumes of KRB in a teflon-glass homogenizer (five or six strokes, 900 rpm). The homogenate was centrifuged at 1,000 g_{max}, 15 min. The resulting supernatant was centrifuged at 20,000 g_{max}, 20 min. The resulting pellet (P_2) was resuspended in three volumes of KRB (based on original wet weight) by gentle homogenization by hand.

Standard assay for phosphorylation of the P_2 fraction

Aliquots (50 μl, 60–80 μg protein) of the P_2 suspension were transferred to tubes on ice containing 35 μl KRB and 5 μl test agent. The mixtures were preincubated at 37°C for 2 min in a shaking water bath. The phosphorylation reaction was initiated by addition of 10 μl [γ-^{32}P]ATP (40 μ*M*, 5–10 Ci/mmol). After a 2-min incubation period, the reaction was terminated by addition of 50 μl SDS stop solution containing 0.186 *M* Tris-HCl, pH 6.7; 9% SDS; 15% (vol/vol) glycerol; 12% 2-mercaptoethanol; and a trace of bromphenol blue (Ueda and Greengard, 1977) followed by immediate placement in a boiling water bath. Modifications to this procedure, when applicable, are indicated in the text and/or figure legends.

Lesions of the guinea pig hippocampus

Three unilateral infusions of 10 μg ibotenic acid, each in 4 μl phosphate-buffered saline (pH 7.4), were administered to seven guinea pigs, as previously described (Fisher et al., 1981). The contralateral side of the hippocampus served as a control. Anodal electrolytic lesions were placed in the fornix-fimbria of six guinea pigs as described by Fisher et al. (1980) to effect bilateral ablation of this pathway. Unoperated animals served as controls for the fornix-fimbria-lesioned animals.

Preparation and phosphorylation of guinea pig hippocampal homogenates

Ibotenate-treated guinea pigs were sacrificed 13 days postinjection, and those with lesions of the fornix-fimbria, 7–10 days postlesion. Guinea pigs were killed by stunning and exsanguination, followed by removal of the cerebrum and dissection of the hippocampal formation (dentate gyrus and hippocampus proper) from the surrounding tissue. Tissues were weighed and homogenized in three volumes KRB (five or six strokes in a Teflon-glass homogenizer, 900 rpm). An aliquot of this homogenate was then diluted in six and one-half volumes 0.32 *M* sucrose for all enzyme assays. Immediate preparation and use of the tissues was essential to obtain consistent

control phosphorylation levels between animals. Twenty microliters of tissue suspension were added to 75 μl KRB in test tubes resting in an ice bath. Phosphorylation of samples in the presence of bromocriptine (25 μM, final concentration) or carrier was carried out as described for the P_2 fraction. All tissue samples were assayed in triplicate.

SDS polyacrylamide gel electrophoresis and autoradiography

Phosphorylated samples (100-μl aliquots) were applied to discontinuous SDS polyacrylamide slab gels. The composition of these gels was identical to that described by Ueda and Greengard (1977), except that a 6.9% acrylamide resolving gel was used unless otherwise indicated. Electrophoresis was carried out at constant voltage (40 V, overnight). Gels were stained, dried, and placed on X-ray film for autoradiography for 2–5 days. In some cases, Cronex intensifying screens (DuPont) were employed.

Quantification of ^{32}P-phosphate incorporation into protein bands was performed as described by Ueda et al. (1973). Darkness of the autoradiograph was measured by a scanning densitometer and expressed as arbitrary units. The arbitrary units were converted to fmol of incorporated ^{32}P-phosphate by determining the radioactivity present in representative protein bands from the same gel in a liquid scintillation counter.

Peptide mapping

Phosphorylated samples were subjected to SDS-polyacrylamide gel electrophoresis as described above. Following electrophoresis, the unstained gels were soaked in several changes of acetic acid-methanol-water (1:5:4), dried, and autoradiographed. With the autoradiograph as a guide, the pieces of gel containing phosphoproteins were excised, scraped of paper backing, and the proteins were eluted as described by Huttner and Greengard (1979). Elution was carried out at 37°C for 20 h in 50 mM ammonium bicarbonate-1 mM dithiothreitol containing 75 μg/ml *each* trypsin and chymotrypsin (1 ml/protein band) plus a trace of phenol red. The eluate was removed and fresh elution buffer added for further incubation at 37°C for 6 h. (Concentration of enzymes was reduced to 25 μg/ml during the second elution.) The eluates were pooled and lyophilized in a rotary evaporator (Savant). Samples were dissolved in 10–50 μl of high-voltage electrophoresis buffer composed of 10% acetic acid and 1% pyridine (vol/vol), pH 3.5. Samples were then spotted on cellulose thin-layer plates (Eastman Kodak 13255), and subjected to high-voltage electrophoresis (1,000 V, 30 min). The plates were air-dried and subjected to ascending chromatography in the second dimension using a solution of *n*-butanol-pyridine-acetic acid-water (37.5:25:7.5:30). Dried thin-layer plates were placed on X-ray film for autoradiography.

Phosphoamino acid analysis

The P_2 fraction was phosphorylated in the absence or presence of 25 μM bromocriptine, as described above, except that the specific activity of [γ-^{32}P]ATP was increased to 20 Ci/mmol. Samples were electrophoresed at 180 V for 18 h on 27-cm long SDS gels (6.9%). The gels were rinsed briefly with distilled water, immediately dried, and subjected to autoradiography. Those portions of the gel corresponding to the positions of the bromocriptine-sensitive 50,000- and 60,000-dalton phosphoproteins were excised, using the autoradiograph as a guide. The proteins were extracted from the gels with 0.1% SDS-0.05 M NH_4CO_3, precipitated with 20% trichloroacetic acid, and the precipitates washed successively with ethanol and ethanol-ether (1:1) at −10°C, as described by Beemon ad Hunter (1978). The washed precipitates were dissolved in 1 ml of 98% formic acid, lyophilized, redissolved in 1 ml of 6 M HCl, and hydrolyzed *in vacuo* at 110°C for 2 h, as described by Eckhart et al. (1979). The samples were then lyophilized and dissolved in 60 μl of pH 2 electrophoresis buffer [glacial acetic acid-formic acid (88%)-water, 78:25:897] containing 5 mM each authentic phosphoserine, phosphothreonine, and phosphotyrosine. Ten microliters of each sample were applied to separate thin-layer cellulose plates (Eastman Kodak 13255) and electrophoresed toward the anode (1,000 V, 90 min). The plates were then dried and chromatographed in the second dimension in isobutyric acid-0.05 M NH_4CO_3 (5:3). Phosphoamino acid markers were detected by ninhydrin staining. Autoradiography was carried out at −70°C, using intensifying screens.

Marker enzyme assays

Choline acetyltransferase (EC 2.3.1.6, ChAT) activity was measured in guinea pig hippocampal homogenates by the method of Fonnum (1975). Glutamate decarboxylase (EC 4.1.1.15, GAD) activity was measured in the homogenates as described previously (Fisher et al., 1980) with the exceptions that the final concentration of sodium L-glutamate was 20 mM and incubations were terminated after 60 min. All tissue samples were assayed in triplicate. Paired Student's *t*-tests were used to compare means of data from ibotenate-treated guinea pigs, since both control and treated tissues were taken from each animal. Unpaired Student's *t*-tests were employed for comparisons between groups of animals used in the fornix-fimbria lesion studies.

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

RESULTS

Effects of neurotransmitters and agonists on synaptosome protein phosphorylation

Figure 1 shows the results of an experiment in which a number of neurotransmitters and neurotransmitter agonists were tested for effects on phosphorylation of proteins in a crude synaptosome (P_2) fraction of rat cerebral cortex. Bromocriptine (25 μM), an ergot with dopamine-mimetic properties (Fuxe et al., 1978; Kebabian and Calne, 1979), caused a marked inhibition of the phosphorylation of 50,000- and 60,000-dalton proteins, and a slight inhibition of the phosphorylation of a 150,000-dalton protein (P_{150}). However, neither dopamine nor any of the other neurotransmitters or agonists tested significantly altered the phosphorylation of these or other proteins in the P_2 preparation under the conditions employed in this study. In other ex-

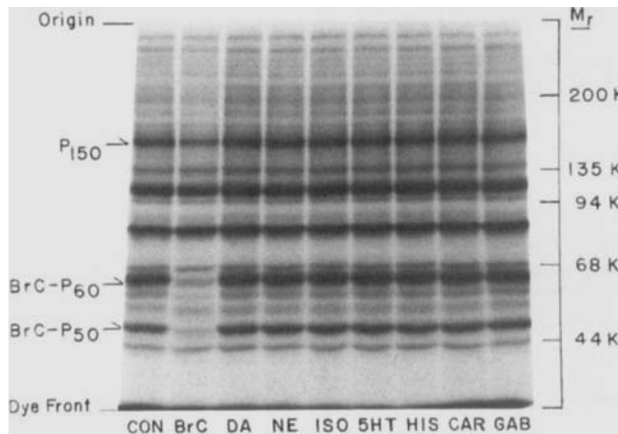

FIG. 1. Effects of various neurotransmitters and agonists on protein phosphorylation in the P_2 fraction of rat cortex. Aliquots of the P_2 fraction (60 μg protein) were subjected to phosphorylation for 2 min in the absence (control; CON) or presence of 25 μ*M* bromocriptine (BrC), dopamine (DA), norepinephrine (NE), isoproterenol (ISO), serotonin (5HT), histamine (HIS), carbachol (CAR), or GABA (GAB), as described in Materials and Methods. Aliquots (40 μg protein) of the phosphorylated samples were subjected to SDS-gel electrophoresis (40 V, 16 h; followed by 100 V, 1 h) and autoradiography as described in Materials and Methods. BrC-P_{50} and BrC-P_{60}, Bromocriptine-sensitive phosphoproteins with approximate molecular weights of 50,000 and 60,000, respectively. 200K, 200,000; 135K, 135,000; 94K, 94,000; 68K, 68,000; 44K, 44,000.

periments not shown here, the potent dopamine agonist apomorphine (25 μ*M*) caused only a slight inhibition of the phosphorylation of these proteins. Moreover, the bromocriptine-induced inhibition was not prevented by any of the dopamine antagonists tested (data not shown). These observations suggest that the inhibitory effect of bromocriptine is not mediated through dopamine receptors.

The major bromocriptine-sensitive phosphoproteins with molecular weights of 50,000 and 60,000 will hereafter be referred to as BrC-P_{50} and BrC-P_{60}, respectively. Experiments were carried out to determine the nature of the bromocriptine-induced reduction of phosphorylation of these proteins. Addition of bromocriptine to the reaction mixture after preincubation of the P_2 fraction with [γ-^{32}P]ATP, followed by additional incubation, did not result in a change in the state of phosphorylation of these proteins as compared with control (data not shown). This experiment suggests that the effect of bromocriptine on BrC-P_{50} and BrC-P_{60} phosphorylation does not reflect activation of a specific protein phosphatase. The possibility that bromocriptine causes the disappearance of these phosphoproteins via activation of a specific phosphoprotein protease was tested by subjecting the phosphorylated samples to electrophoresis on SDS-polyacrylamide (15%) gels. No appearance of new lower molecular weight phosphoprotein species in bromocriptine-treated samples was observed. In addition, cyclic AMP had no effect on the state of phosphorylation of BrC-P_{50} and BrC-P_{60} (data not shown). On the basis of these observations, we conclude that bromocriptine directly inhibits the protein phosphorylation reaction.

Time course of phosphorylation of BrC-P_{50} and BrC-P_{60}

To characterize further the bromocriptine-sensitive phosphorylation, the time course of net phosphorylation of BrC-P_{50} and BrC-P_{60} was determined in the presence of 4 μ*M* ATP, both in the absence and in the presence of 25 μ*M* bromocriptine. As shown in Fig. 2, maximal phosphorylation of both proteins occurred within 15 s after addition of ATP to the reaction mixture. Bromocriptine decreased both the rates and the final levels of net phosphorylation of BrC-P_{50} and BrC-P_{60}. When a saturating concentration of ATP (500 μ*M*) was used, bromocriptine had little effect on the final levels, although it reduced the rates of phosphorylation substantially (data not shown). The final levels of phosphorylation of both proteins were approximately 25-fold higher under the ATP-saturating conditions than under standard conditions. Also apparent from these experiments is the relatively slow turnover of ^{32}P-phosphate on these phosphoproteins. Thus, after an incubation period of 10 min, only a slight decrease in their phosphorylation levels was observed (Fig. 2), indicating that the P_2 preparation contains relatively low levels of BrC-P_{50} and/or BrC-P_{60} phosphatase.

Effect of ATP concentration on phosphorylation of BrC-P_{50} and BrC-P_{60}

The phosphorylation of BrC-P_{50} and BrC-P_{60} as a function of ATP concentration is shown in Fig. 3. In this experiment, the incubation was carried out for 5 s in an effort to obtain initial rates. Maximal phosphorylation of both proteins occurred with ATP concentrations >50 μ*M*. From the Hanes plots of these data shown in the insets of Fig. 3, apparent K_m's of the phosphorylation of BrC-P_{50} and BrC-P_{60} for ATP were determined to be 17.6 μ*M* and 15.5 μ*M*, respectively. Apparent V_{max}'s of the reactions were determined to be 131 fmol · s^{-1} and 122 fmol · s^{-1}, respectively.

Ion requirements for phosphorylation of BrC-P_{50} and BrC-P_{60}

Under standard conditions, phosphorylation of the P_2 fraction was carried out in the presence of a KRB solution approximating the ionic composition of the extracellular fluid in the brain (Dingledine et al., 1980). In experiments designed to determine ion requirements of the bromocriptine-sensitive phosphorylation reaction, it was found that addition of EGTA to the reaction mixture caused a marked decrease in the phosphorylation of both BrC-P_{50} and

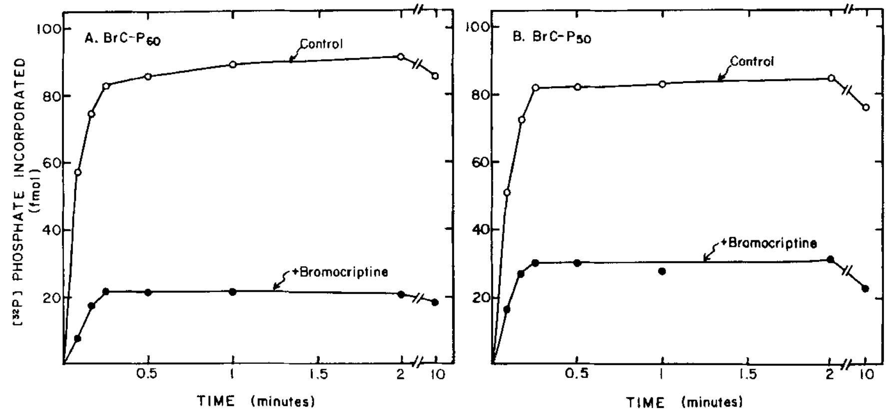

FIG. 2. Time course of phosphorylation of BrC-P_{50} and BrC-P_{60}. Aliquots (50 μg protein) of the P_2 fraction were incubated with 4 μ*M* ATP (final concentration) for various periods of time in the absence (○) or presence (●) of 25 μ*M* bromocriptine, as described in Materials and Methods. Aliquots (50 μg protein) of the reaction mixture were subjected to SDS-gel electrophoresis and autoradiography. Quantitation of ^{32}P-phosphate into protein bands was carried out as described in Materials and Methods. **A:** BrC-P_{60}. **B:** BrC-P_{50}.

BrC-P_{60} (data not shown). Therefore, calcium dependence of the reaction was investigated further. Figure 4 shows the effect of varying the calcium concentration in the presence of 3 m*M* EGTA on the phosphorylation of BrC-P_{50} and BrC-P_{60} in the presence of 0 m*M*, 1.2 m*M*, and 10 m*M* magnesium. From these data, it is clear that calcium markedly stimulates the phosphorylation of BrC-P_{50} and BrC-P_{60}. The free calcium concentrations required for one-half maximal stimulation of phosphorylation, calculated as described by Nanninga and Kempen (1971), were in the range of 2–7 μ*M*. Figure 4 also demonstrates the magnesium requirement of the protein phosphorylation.

Addition of purified calmodulin caused a substantial increase in the phosphorylation of BrC-P_{50} and BrC-P_{60} (Table 1). The calmodulin-stimulated activity was also inhibited by bromocriptine. These results suggest that the bromocriptine-sensitive phosphorylation of BrC-P_{50} and BrC-P_{60} is catalyzed by calcium/calmodulin-dependent protein kinase(s).

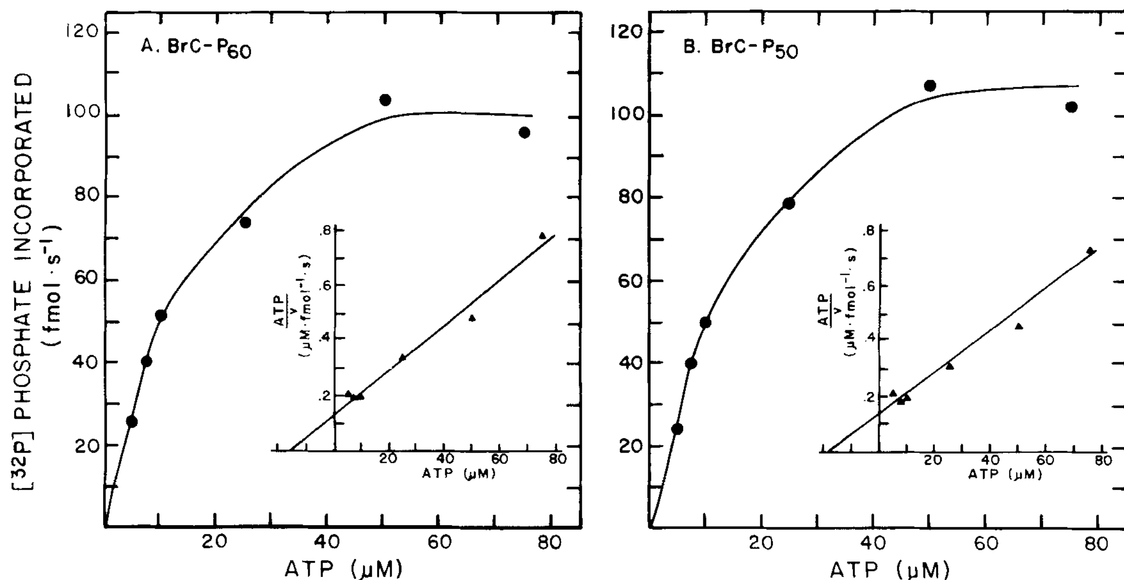

FIG. 3. Effects of various concentrations of ATP on phosphorylation of BrC-P_{50} and BrC-P_{60}. Aliquots (69 μg protein) of the P_2 fraction were preincubated at 37°C for 2 min. [γ-^{32}P]ATP was added to each sample to yield the final concentrations indicated, and the reaction terminated after 5 s incubation time. SDS-gel electrophoresis, autoradiography, and quantitation of ^{32}P-phosphate incorporated into protein bands were carried out as described in Materials and Methods. Insets show the data plotted as the Hanes transformation (Cornish-Bowden, 1979) of the Michaelis-Menten equation: $(S/v) = (K_m/V_{max}) + (S/V_{max})$. The *x*-intercept represents $-K_m$(app); the *y*-intercept represents $-K_m(\text{app})/V_{max}(\text{app})$, and the slope of the line equals $1/V_{max}$(app). Lines drawn are from linear regression by least squares analysis of the data. **A:** BrC-P_{60}, K_m(app) = 15.5 μ*M*; V_{max}(app) = 122 fmol • s^{-1}. **B:** BrC-P_{50}, K_m(app) = 17.6 μ*M*; V_{max}(app) = 131 fmol • s^{-1}.

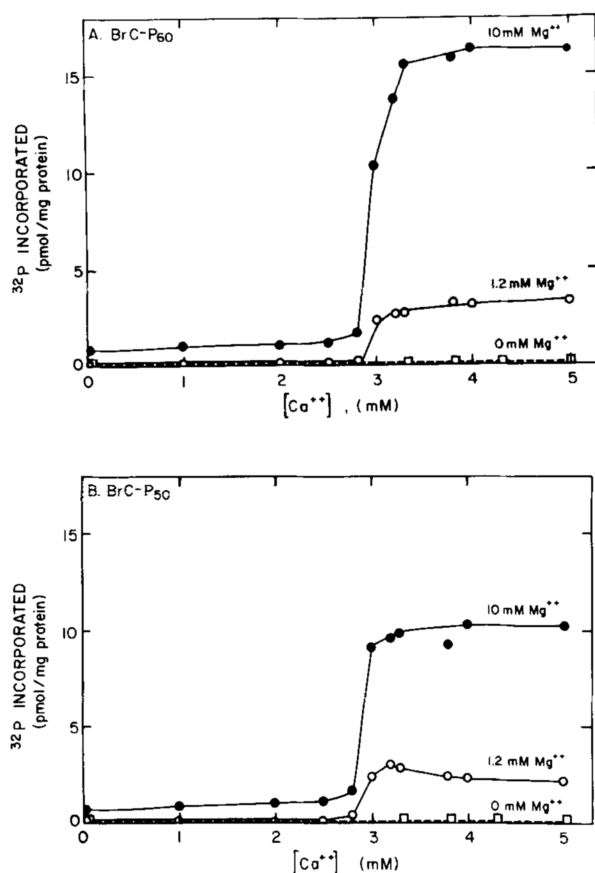

FIG. 4. Effects of various concentrations of calcium ions on phosphorylation of BrC-P_{50} and BrC-P_{60}. The P_2 fraction was resuspended in calcium- and magnesium-free KRB. Aliquots (40 μg) were incubated with [γ-^{32}P]ATP in the presence of 3 m*M* EGTA and of the final concentrations of magnesium and calcium indicated, as described in Materials and Methods. Phosphorylation of samples, autoradiography, and quantitation of ^{32}P-phosphate incorporation into protein bands were carried out as described in Materials and Methods. **A:** BrC-P_{60}. **B:** BrC-P_{50}. (□), 0 m*M* Mg^{2+}; (○), 1.2 m*M* Mg^{2+}; (●), 10 m*M* Mg^{2+}.

Phosphoamino acid analysis of BrC-P_{50} and BrC-P_{60}

To determine the ^{32}P-phosphoamino acid residues in BrC-P_{50} and BrC-P_{60}, phosphorylated BrC-P_{50} and BrC-P_{60} were separated from other phosphoproteins by SDS-gel electrophoresis, eluted from gels, and subjected to partial acid hydrolysis. The hydrolysates were then analyzed for [^{32}P]phosphoserine, [^{32}P]phosphothreonine, and [^{32}P]phosphotyrosine by electrophoresis in the first dimension and chromatography in the second dimension. As shown in Fig. 5, bromocriptine-sensitive phosphorylation occurs on a threonine residue(s) of both BrC-P_{50} and BrC-P_{60}. Although to lesser degrees, both of the proteins were also phosphorylated on a serine residue(s); however, the serine phosphorylation was not affected by bromocriptine. Whether the phosphoserine resides in BrC-P_{50} and BrC-P_{60} or in contaminating proteins with similar molecular weights is not known at present.

Phospho-peptide mapping of BrC-P_{50} and BrC-P_{60}

The similarities in the phosphorylation of BrC-P_{50} and BrC-P_{60} noted in the experiments described above indicated that the proteins themselves might be similar. Therefore, to compare further BrC-P_{50} and BrC-P_{60}, the phosphoproteins were subjected to analysis by peptide mapping as described in Materials and Methods. Figure 6 shows the phosphorylated peptide fragments produced after the tryptic/chymotryptic digestion. It appears that BrC-P_{50} and BrC-P_{60} share many identical or highly similar phosphopeptides (labeled 1–6). Although precise quantitative comparisons could not be made between plates due to differential recovery of samples from the original gel, a close examination of the changes in the phosphopeptide patterns indicates that bromocriptine inhibited ^{32}P-phosphate incorporation into most, but not all, of the phosphopeptides. Bromocriptine had relatively small effects on the phosphorylation of the peptide fragments labeled 8 and 9 of BrC-P_{60} and of the fragments labeled 10 and 11 of BrC-P_{50}. It is therefore likely that these phosphopeptides contain phosphoserine residue(s).

Tissue distribution of bromocriptine-sensitive protein phosphorylation

Various tissue homogenates were examined for bromocriptine-sensitive phosphorylation of 50,000- and 60,000-dalton proteins. As shown in Fig. 7A and B, the bromocriptine-sensitive phosphorylation system is most highly concentrated in the brain. It was not detected in nonnervous tissues such as heart, liver, kidney, lung, and spleen. Although each of these tissues has a phosphoprotein whose electrophoretic mobility is indistinguishable from that of BrC-P_{50}, its phosphorylation is not bromocriptine-sensitive. It may be noted, however, that adrenal tissue exhibits bromocriptine-sensitive phosphorylation of a 50,000-dalton protein.

The distribution of BrC-P_{50} and BrC-P_{60} within some brain regions is shown in Fig. 8. The bromocriptine-sensitive BrC-P_{60} phosphorylation system is present in all of the brain regions tested; however, there are regional differences in their specific contents. Thus, the phosphorylation of BrC-P_{60} is the highest in the hippocampus, followed by the cortex and striatum, and is lowest in the hypothalamus. With the exception of the cerebellum, the distribution of BrC-P_{50} is roughly parallel to that of BrC-P_{60}. The cerebellum has a relatively much lower level of BrC-P_{50}; instead, a bromocriptine-sensitive 56,000-dalton phosphoprotein is prominent. This phosphorylation system is much more abundant in the cerebellum than in the other regions examined. In the hypothalamus, the 56,000-dalton protein is the most prominent phosphoprotein component. The pituitary, which is not part of the brain proper,

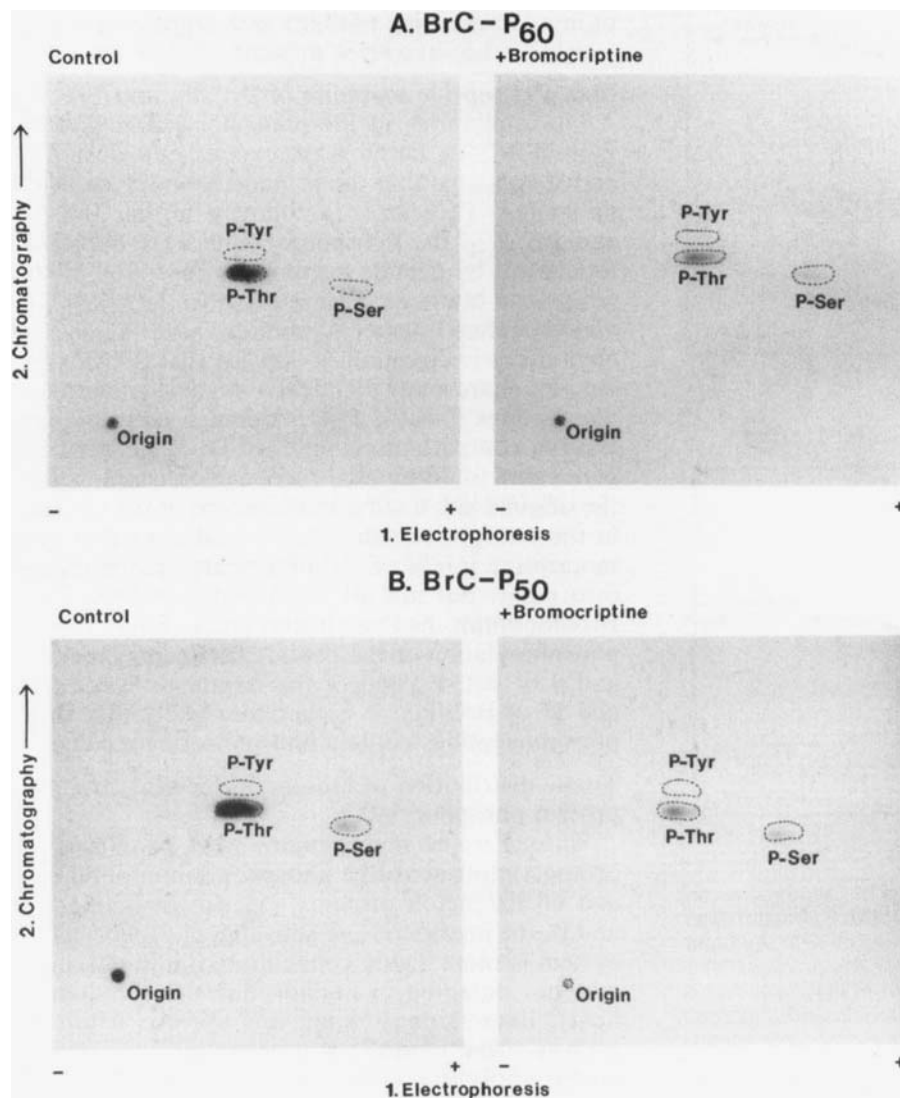

FIG. 5. Analysis of phosphoamino acid residues in BrC-P_{50} and BrC-P_{60} by electrophoresis and chromatography on thin-layer cellulose. Aliquots of P_2 fraction prepared from rat cortex (150 μg protein) were subjected to phosphorylation in the absence or presence of 25 μ*M* bromocriptine, in a final volume of 0.2 ml, as described in Materials and Methods. Following electrophoresis (180 V, 20 h) on 27-cm long gels, the BrC-P_{50} and BrC-P_{60} bands were identified by autoradiography; the portions of the gels containing BrC-P_{50} and BrC-P_{60} were excised and extracted from the gel with 0.1% SDS-0.05 *M* NH_4CO_3, followed by acid hydrolysis, as described in detail in Materials and Methods. The lyophilized samples were dissolved in a pH 2 electrophoresis buffer containing 5 m*M* each authentic phosphoserine, phosphothreonine, and phosphotyrosine, spotted on TLC plates, and analyzed by electrophoresis in the first dimension and by chromatography in the second dimension, as described in Materials and Methods. P-Ser, Phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. +, anode; −, cathode.

does not appear to possess a bromocriptine-sensitive phosphorylation system.

Cellular localization of BrC-P_{50} and BrC-P_{60}

To investigate the cellular localization of BrC-P_{50} and BrC-P_{60} within the brain, we carried out lesion studies on the guinea pig hippocampus. In these studies ibotenic acid, a toxin that selectively destroys neuronal cell bodies but spares nerve terminals and axons of passage (Schwarcz et al., 1979; Kohler et al., 1979; Fisher et al., 1981), was injected into one side of the guinea pig hippocampus. Thirteen days postlesion, the two sides were compared for bromocriptine-sensitive protein phosphorylation and marker enzymes as described below. In addition, the effects of electrolytic lesions of the fornix-fimbria on protein phosphorylation and marker enzyme activities in the hippocampus were investigated. In both series of experiments, hippocampal homogenates were used for the protein phosphorylation assays.

Ibotenate lesions of the hippocampus. Following unilateral intrahippocampal injection of ibotenate, the lesioned side of the hippocampus was shrunken in appearance and weighed 26% less than the control side. In homogenates obtained from the lesioned hippocampus, the specific activity of GAD, a marker for intrinsic γ-aminobutyric acid (GABA)-ergic neurons (Storm-Mathisen, 1972), was reduced by 40%. However, ChAT activity was not significantly reduced following ibotenate treatment (Table 2), confirming the selective toxicity of this agent. The concentration of protein/mg wet weight was also not altered by the lesion (Table 2).

Incubation of guinea pig hippocampal homogenates in the presence of [γ-^{32}P]ATP resulted in the

TABLE 1. *Effect of calmodulin on phosphorylation of BrC-P_{50} and BrC-P_{60}*

Bromocriptine (μM)	Phosphorylation (fmol ^{32}P-phosphate)			
	Control		+Calmodulin	
	BrC-P_{60}	BrC-P_{50}	BrC-P_{60}	BrC-P_{50}
0	74	63	295	359
100	24	23	91	77

Phosphorylation was carried out under standard conditions as described in Materials and Methods, except that purified calmodulin (1 μg) was added to some samples prior to the addition of carrier or bromocriptine. Quantitation of ^{32}P-phosphate was carried out as described in Materials and Methods. 40 μg of protein were present in each sample.

rapid appearance of label in several phosphoproteins, the most prominent being BrC-P_{50} and BrC-P_{60} (Fig. 9). Inclusion of bromocriptine in the assay resulted in a selective inhibition of the phosphorylation of these two proteins by 57–69% (Table 2). (Differences between groups were not statistically significant.) Ibotenate lesion of the hippocampus resulted in a marked reduction in the basal phosphorylation of BrC-P_{50} (55%) and BrC-P_{60} (51%) (Table 2). However, the percentage inhibition of phosphorylation resulting from addition of bromocriptine to homogenates prepared from the lesioned side of the hippocampus was comparable to that obtained from the control side (Table 2). Thus, the structures remaining undestroyed by the neurotoxin retain a functional bromocriptine-sensitive protein phosphorylation system.

Fornix-fimbria lesions. Transection of the fornix-fimbria resulted in a 71% reduction of ChAT activity, an indicator of the structural integrity of this pathway (Lewis et al., 1967). However, the lesion had no effect on hippocampal wet weight, protein content, or GAD activity (Table 2). Similarly, neither the basal phosphorylation of BrC-P_{50} or BrC-P_{60} nor the magnitude of bromocriptine inhibition was affected by the lesion (Table 2; Fig. 9).

Effects of other ergots and centrally active agents on BrC-P_{50} and BrC-P_{60}

Since dopamine (Fig. 1) and the potent dopamine agonist apomorphine (data not shown) failed to mimic the inhibitory effect of bromocriptine on the protein phosphorylation, it is unlikely that the inhibitory effect can be ascribed to the dopaminergic properties of the ergot. Structurally, bromocriptine

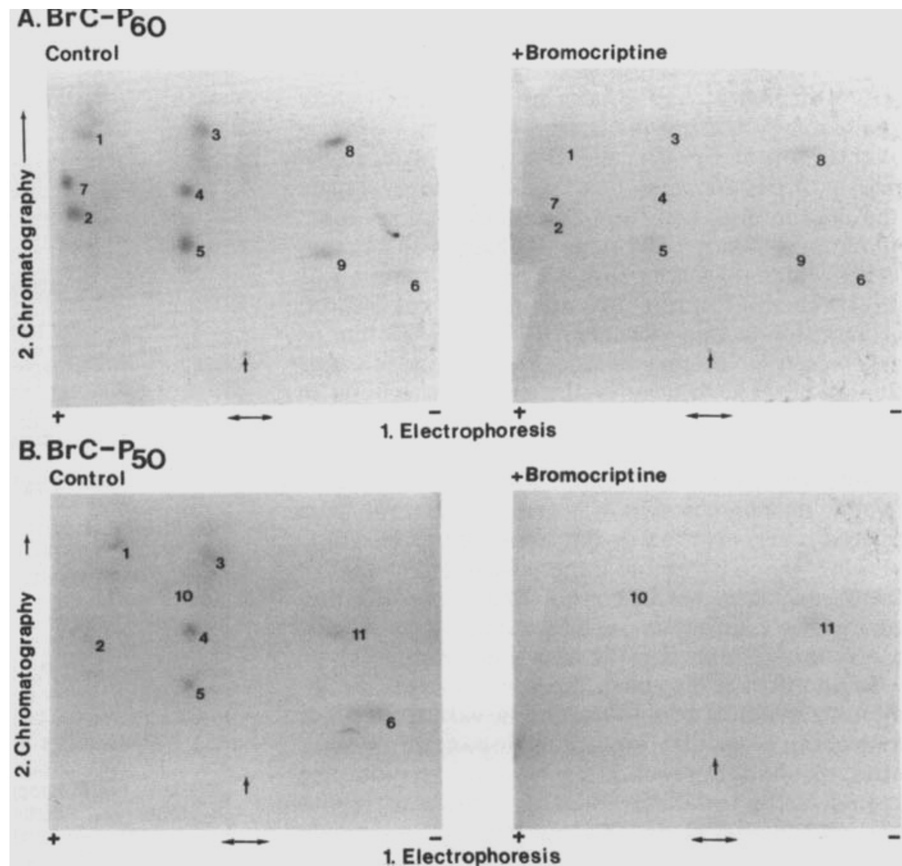

FIG. 6. Peptide mapping of BrC-P_{50} and BrC-P_{60}. The P_2 fraction was prepared from rat cortex and aliquots phosphorylated in the absence or presence of 25 μM bromocriptine. Phosphorylated BrC-P_{50} and BrC-P_{60} were extracted from SDS-gels and subjected to exhaustive trypsin/chymotrypsin digestion as described in Materials and Methods. Samples were applied to cellulose thin-layer plates at small arrows, and phosphorylated peptides were separated by electrophoresis followed by ascending chromatography in dimensions indicated in the figure, as described in Materials and Methods. Shown here are autoradiographs of thin-layer plates. **A:** Phosphopeptides of BrC-P_{60} phosphorylated in the absence (left) and presence (right) of bromocriptine. **B:** Phosphopeptides of BrC-P_{50} phosphorylated in the absence (left) and presence (right) of bromocriptine.

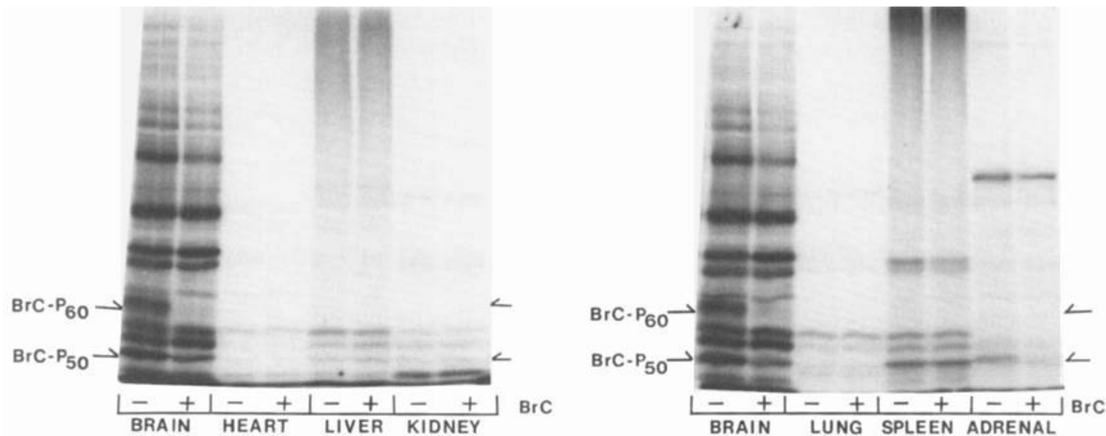

FIG. 7. Tissue specificity of bromocriptine-sensitive phosphorylation of BrC-P_{50} and BrC-P_{60}. Various tissues as indicated were homogenized in three volumes of KRB, and a subcellular fraction was prepared in the same manner as for the P_2 fraction of rat cortex. Aliquots of the fraction (brain, 56 μg protein; heart, 70 μg protein; liver, 88 μg protein; kidney, 100 μg protein; spleen, 113 μg protein; and adrenal gland, 20 μg protein) were subjected to phosphorylation in the absence or presence of 100 μ*M* bromocriptine (BrC), followed by SDS-gel electrophoresis (40 V, 18 h) and autoradiography, as described in Materials and Methods. **A (left):** Comparison of brain, heart, liver, and kidney. **B (right):** Comparison of brain, lung, spleen, and adrenal.

is classified in a group of ergots termed ergopeptines, which contain peptide moieties substituted on the ergoline ring structure (Rall and Schleifer, 1980). To determine whether the inhibitory effect of bromocriptine is attributable to the ergoline ring structure, the peptide moiety, or to both, various ergot compounds were tested for their capacities to inhibit the phosphorylation of BrC-P_{50} and BrC-P_{60}. As shown in Fig. 10 and Table 3, those ergots that possess peptide moieties—α-ergocriptine, dihydroergocriptine, and ergotamine—were slightly less potent than bromocriptine in inhibiting phosphorylation of BrC-P_{50} and BrC-P_{60}. Those ergots that lack peptide moieties, such as methysergide and ergonovine, had little or no effect. The dopamine-mimetic ergot lergotrile (Kebabian and Calne, 1979), which does not possess a peptide moiety, was relatively ineffective in causing the inhibition; bromocriptine was more than 10 times as potent as was lergotrile. It appears that the presence of a particular peptide moiety in the ergots is crucial in causing a potent and specific inhibition of the phosphorylation of BrC-P_{50} and BrC-P_{60}. Thus, although bromocriptine was the most potent of all agents tested, its potency was not greatly different from that of α-ergocriptine or dihydroergocriptine, both of which have a peptide moiety identical to that of bromocriptine. On the other hand, ergotamine, which has a different peptide moiety, was significantly less potent than the above ergopeptines.

In an effort to establish the specificity of the inhibitory effect of bromocriptine, a variety of other neuroactive agents, including dopamine antagonists, α- and β-adrenergic agents, and serotonergic agents, were tested for their capacities to inhibit phosphorylation of BrC-P_{50} and BrC-P_{60}. As shown in Table 3, none of these agents caused as potent an inhibition as did bromocriptine. Among all the nonergot agents tested, spiroperidol, a potent dopamine antagonist, was the most potent inhibitor. However, the IC_{50} values are several orders of mag-

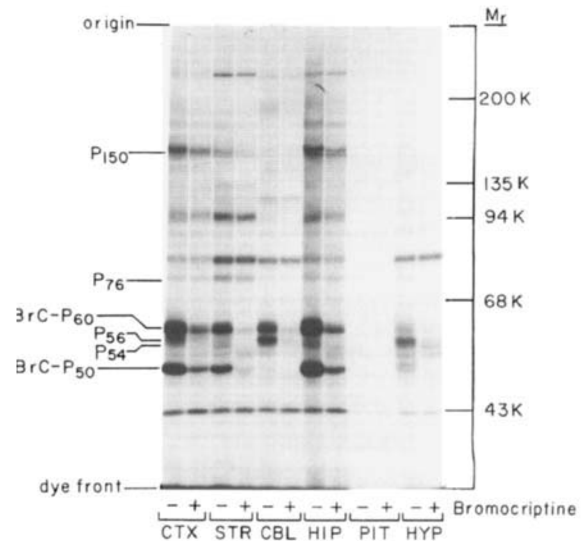

FIG. 8. Effects of bromocriptine on phosphorylation of proteins in various regions of rat brain and pituitary. Tissues were pooled from three rats, P_2 fractions prepared, and subjected to phosphorylation as described in Materials and Methods. SDS-gel electrophoresis was carried out at 120 V, 18 h, followed by 150 V, 3 h on a 27-cm long SDS-gel (6.9% acrylamide). BrC-P_{50} and BrC-P_{60} are indicated on left. Also indicated are other proteins whose phosphorylation appears to be affected by bromocriptine (+, 25 μ*M*) (P_{150}, P_{76}, P_{56}, P_{54}). Abbreviations and amounts of protein (μg) applied to gel: CTX, cortex (53); STR, striatum (106); CBL, cerebellum (67); HIP, hippocampus (73); PIT, pituitary (46); HYP, hypothalamus (36). Positions of molecular weight standards are indicated at right: 200K, 200,000; 135K, 135,000; 94K, 94,000; 68K, 68,000; 43K, 43,000.

TABLE 2. *Effects of ibotenate and fornix-fimbria lesions on guinea pig hippocampal wet weight, protein content, marker enzymes, and bromocriptine-sensitive protein phosphorylation*

	Ibotenate (n = 7)		Fornix-Fimbria (n = 6)	
	Control	Lesion	Control	Lesion
A. Wet weights, protein content, and marker enzymes				
Hippocampus wet weight (mg)	113 ± 1	84 ± 6[a]	215 ± 7[e]	208 ± 12
Protein	115 ± 5	111 ± 5	96 ± 2	93 ± 3
GAD	1.48 ± 0.14	0.89 ± 0.12[b]	1.17 ± 0.09	1.12 ± 0.07
ChAT	1.26 ± 0.15	1.31 ± 0.14	0.84 ± 0.03	0.24 ± 0.04[d]
B. Protein phosphorylation				
BrC-P_{50}				
Basal	3.20 ± 0.35	1.45 ± 0.30[b]	2.89 ± 0.26	2.77 ± 0.43
Bromocriptine inhibition (%)	57 ± 2	46 ± 5	61 ± 4	56 ± 5
BrC-P_{60}				
Basal	2.60 ± 0.15	1.28 ± 0.14[c]	3.01 ± 0.16	2.71 ± 0.2
Bromocriptine inhibition (%)	69 ± 6	65 ± 8	66 ± 3	64 ± 4

Ibotenate was injected into one side of the hippocampus, and the hippocampus divided into control and lesion sides for analysis as described in Materials and Methods. Fornix-fimbria lesions were bilateral, and control tissue was taken from separate animals; hence, the entire hippocampal formation was employed in these studies. Marker enzymes, protein content, and phosphorylation of BrC-P_{50} and BrC-P_{60} were measured as described in Materials and Methods. Values shown are means ± SEM of six or seven experiments, as indicated (n). All tissue samples were assayed in triplicate. Paired Student's *t*-tests were used to compare values from ibotenate-treated and control samples. Unpaired Student's *t*-tests were used to compare values from fornix-fimbria lesion and control groups. Units of measurement are as follows: Protein, mg/g wet weight; GAD and ChAT, nmol/mg protein/min; protein phosphorylation, pmol ^{32}P-phosphate/mg protein/2 min.

[a] $p < 0.05$, compared with control, paired analysis.

[b] $p < 0.01$, compared with control, paired analysis.

[c] $p < 0.005$, compared with control, paired analysis.

[d] $p < 0.001$, compared with control, unpaired analysis.

[e] In fornix-fimbria lesion experiments, entire hippocampal formations were employed, whereas unilateral formations were used in ibotenate treatment experiments.

nitude higher than the concentrations required for eliciting other dopamine antagonistic effects (Clement-Cormier et al., 1974; Iversen, 1975; Seeman et al., 1976; Creese et al., 1978). Moreover, (+)-butaclamol, another potent dopamine antagonist, was not significantly different in its capacity to inhibit the phosphorylation of BrC-P_{50} and BrC-P_{60} than was the inactive enantiomer (−)-butaclamol. These results are in agreement with the notion that the inhibition of BrC-P_{50} and BrC-P_{60} phosphorylation is not mediated through dopamine receptors.

DISCUSSION

In the present study, we have shown that bromocriptine and other ergopeptines selectively inhibit phosphorylation of 50,000- and 60,000-dalton proteins in a crude synaptosome fraction. These bromocriptine-sensitive phosphoproteins are highly enriched in the brain, particularly in the hippocampus, cerebral cortex, and caudate nucleus, and are not detectable in nonnervous tissues.

The results of the studies directed at characterizing the phosphorylation of BrC-P_{50} and BrC-P_{60} indicate that the reactions are very much alike. The time course of phosphorylation of BrC-P_{50} and BrC-P_{60} and the apparent Michaelis constants (K_m's) for ATP are similar (Figs. 2 and 3). Phosphorylation of both proteins requires the presence of magnesium and calcium ions at approximately the same optimal concentrations (Fig. 4). In addition, phosphorylation of both BrC-P_{50} and BrC-P_{60} is stimulated by calmodulin (Table 1). The amino acid residue that undergoes bromocriptine-sensitive phosphorylation is threonine in both proteins. Peptide mapping analyses of BrC-P_{50} and BrC-P_{60} indicate that the two proteins are digested to several identical or very similar phosphopeptide fragments, suggesting a homology between the two proteins in the primary structure in the vicinity of the phosphorylation site(s). Taken together, these observations suggest that the bromocriptine-sensitive phosphorylation of both BrC-P_{50} and BrC-P_{60} is catalyzed by a single Ca^{2+}/calmodulin-dependent protein kinase.

Although bromocriptine has been characterized as a dopaminergic agonist in other systems (Kebabian and Calne, 1979), dopamine and other dopaminergic agonists had little or no effect on the phosphorylation of BrC-P_{50} and BrC-P_{60}. Dopaminergic

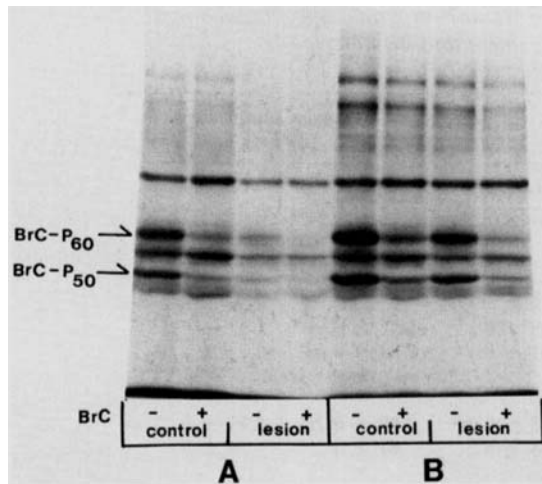

FIG. 9. Phosphoproteins in guinea pig hippocampus: effects of ibotenate-induced lesion and lesion of the fornix-fimbria on phosphorylation of proteins in homogenates of guinea pig hippocampus. Lesions were made and phosphorylation of homogenates carried out as described in Materials and Methods in the absence (−) or presence (+) of 25 μM bromocriptine (BrC). Ibotenate control and lesion samples **(A)** are from the same animal. Fornix-fimbria control and lesion samples **(B)** are from two separate animals. BrC-P_{60} and BrC-P_{50} are indicated by arrows.

antagonists (fluphenazine, haloperidol, sulpiride) did not block the bromocriptine effect (data not shown); in fact, some dopamine antagonists mimicked the inhibitory effect of bromocriptine on BrC-P_{50} and BrC-P_{60}. The effects of these agents were not, however, stereospecific. These results, and the observation that the highest specific content of the bromocriptine-sensitive phosphorylation system is in the hippocampus, a region of relatively low dopamine content (Scatton et al., 1980), militate against a dopaminergic association of the bromocriptine-sensitive protein phosphorylation system. The central effects of ergot compounds have also been attributed to their actions on α-adrenergic and serotonergic systems (Loew et al., 1978; Rall and Schleifer, 1980). Agonists and antagonists of these systems, as well as of agonists of β-adrenergic, GABAergic, cholinergic, and histaminergic receptor systems, failed to mimic the bromocriptine-induced inhibition of phosphorylation of BrC-P_{50} and BrC-P_{60} (Fig. 1 and Table 3). These results and the observation that the peptide-containing ergots are the most potent of all the agents tested in the selective inhibition of the phosphorylation of BrC-P_{50} and BrC-P_{60} raise the possibility that the BrC-P_{50} and BrC-P_{60} phosphorylation systems may be associated with a specific receptor for ergopeptines or for a particular peptide identical or analogous to the peptide moiety of bromocriptine. Whether there is an endogenous substance that produces the inhibitory effect of bromocriptine on the protein phosphorylation is an interesting question that remains to be investigated.

In studies directed at elucidating the cellular localization of the bromocriptine-sensitive protein kinase system, we found that transection of the fornix-fimbria, which carries cholinergic, serotonergic, noradrenergic, histaminergic, and dopaminergic inputs to the hippocampus (Lewis et al., 1967; Storm-Mathisen and Guldberg, 1974; Barbin et al., 1976; Scatton et al., 1980), did not affect the phosphorylation of BrC-P_{50} and BrC-P_{60}, indicating that

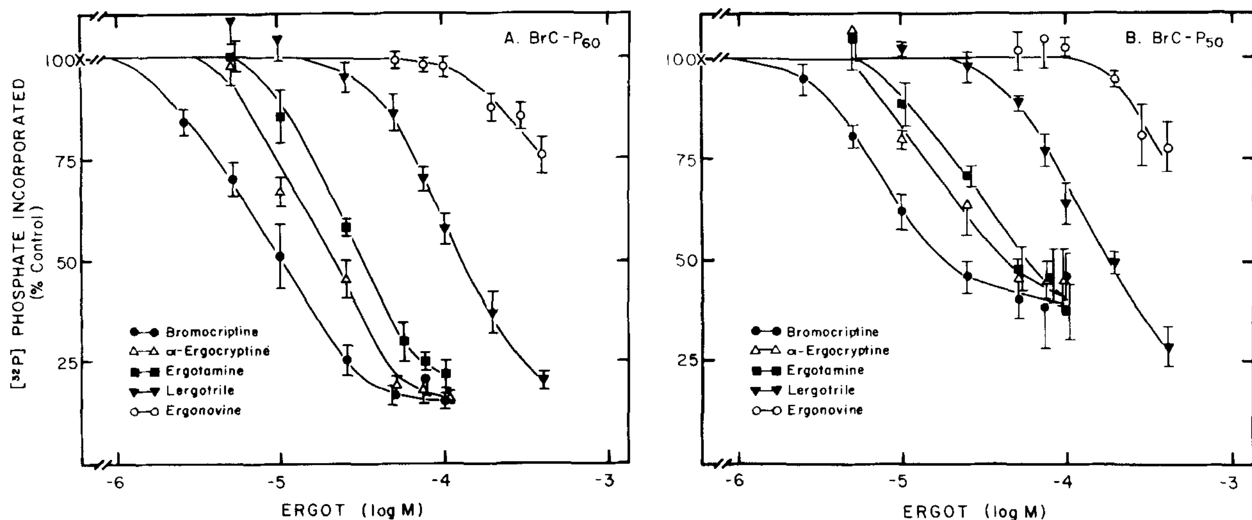

FIG. 10. Effects of various ergot derivatives on phosphorylation of BrC-P_{50} and BrC-P_{60}. Aliquots of a P_2 fraction of rat cortex were phosphorylated in the presence of various concentrations of ergot derivatives as indicated on abscissa (log M). Relative incorporation of ^{32}P-phosphate into BrC-P_{60} **(A)** and BrC-P_{50} **(B)** was determined by scanning densitometry of autoradiographs, and is expressed as % control (X). Data are from four separate experiments; error bars represent ± SEM. (●), Bromocriptine; (△), α-ergocriptine; (■), ergotamine; (▼), lergotrile; (○), ergonovine.

TABLE 3. *IC_{50} values for various agents on phosphorylation of Brc-P_{50} and BrC-P_{60}*

Agent	IC_{50} (μM)	
	BrC-P_{60}	BrC-P_{50}
Ergot derivatives		
Bromocriptine	8	9
Dihydroergocriptine	14	12
α-Ergocriptine	14	22
Ergotamine	26	31
Lergotrile	103	115
Methysergide	>400	>400
Ergonovine	>400	>400
Dopaminergic antagonists		
Spiroperidol	36	32
Haloperidol	83	87
Fluphenazine	83	79
Trifluoperazine	105	93
Thioridazine	87	200
Chlorpromazine	100	110
(+)-Butaclamol	151	200
(−)-Butaclamol	120	158
Metoclopramide	>400	>400
Molindone	>400	>400
Sulpiride	—	—
α-Adrenergic agents		
Yohimbine	151	145
Phentolamine	>400	210
Clonidine	—	—
Phenylephrine	—	—
β-Adrenergic antagonist		
Propranolol	100	158
Serotonergic agents		
Methysergide	>400	>400
Quipazine	>400	>400

Aliquots of P_2 fraction prepared from rat cortex were phosphorylated in the presence of several concentrations of each agent listed using standard procedures described in Materials and Methods. IC_{50} values for each agent were determined graphically from plots of % control phosphorylation vs. log concentration agent as the concentration of the agent required to cause 50% of the maximal inhibition produced by 200 μM bromocriptine (see Fig. 10). BrC-P_{60} maximal inhibition = 85% of total; BrC-P_{50} maximal inhibition = 62.5% of total. IC_{50} values are listed as micromolar (μM) concentrations. >400 signifies that some inhibition of phosphorylation was observed at 400 μM test agent, but was <50% of maximal inhibition; (−) signifies that no effect on phosphorylation was observed at maximum concentration of agent tested (400 μM).

nerve terminals derived from these tracts do not contribute significantly to the phosphorylation of the two phosphoproteins. However, phosphorylation of BrC-P_{50} and BrC-P_{60} and a number of other hippocampal proteins was significantly reduced following intrahippocampal injection of ibotenate. The observed effects of ibotenate on protein phosphorylation are qualitatively similar to those of Sieghart et al. (1980), who reported that injections of a similar toxin, kainic acid, into the rat striatum resulted in a general reduction of calcium-dependent protein kinase activity due to the destruction of neuronal cell bodies. Moreover, we have recently found that the bromocriptine-sensitive protein phosphorylation system is highly enriched in a postsynaptic density fraction prepared from rat brain according to the procedures described by Cohen et al. (1977) (unpublished results). Taken together, these results suggest that the reduced phosphorylation of BrC-P_{50} and BrC-P_{60} following ibotenate treatment results from the destruction of neuronal cell bodies in the hippocampus that contain the bromocriptine-sensitive protein phosphorylation system. A glial cell association of the system appears unlikely, since a general proliferation of glial cells is observed following ibotenate treatment (Fisher et al., 1981). Nor is it likely that a major association exists between the bromocriptine-sensitive phosphorylation system and the ventral (perforant) pathway, since this fiber tract would be spared the effects of ibotenate (Schwarcz et al., 1979).

Calcium- and calmodulin-stimulated protein phosphorylation in brain tissues has been described by a number of investigators (DeLorenzo, 1976; Schulman and Greengard, 1978; Grab et al., 1981; Kennedy et al., 1983). In the course of our studies, we attempted to compare the bromocriptine-sensitive 50,000- and 60,000-dalton phosphoproteins with the calcium-dependent phosphoproteins of similar electrophoretic mobilities described by these authors. We found that BrC-P_{50} and BrC-P_{60} comigrated on one-dimensional SDS gels with the 51,000- and 62,000-dalton Ca^{2+}/calmodulin-stimulated phosphoproteins in crude synaptosomal preparations reported by Schulman and Greengard (1978) and with those in isolated postsynaptic densities reported by Grab et al. (1981). Bromocriptine also inhibited phosphorylation of the proteins in these preparations (data not shown). Recently, these phosphoproteins have been shown to copurify with calcium-dependent synapsin I kinase activity (Kennedy et al., 1983). We also prepared a brain homogenate according to the procedure described by DeLorenzo and coworkers (1977) and tested the effects of bromocriptine on phosphorylation of proteins in this fraction. We found that BrC-P_{50} and BrC-P_{60} comigrated with prominent phosphoproteins termed DPH-M and DPH-L in the DeLorenzo preparation; however, phosphorylation of these proteins was only slightly inhibited by the presence of bromocriptine (100 μM) in the phosphorylation assay carried out as described by DeLorenzo et al. (1977). Under the conditions of our standard phosphorylation assay, bromocriptine exhibited no effect on the phosphorylation of DPH-L or DPH-M. DPH (400 μM), which inhibits phosphorylation of DPH-L and DPH-M, caused only a slight inhibition of phosphorylation of BrC-P_{50} and BrC-P_{60} (unpublished observations). Moreover, DPH-L and DPH-M have been reported to be phosphorylated mainly on serine residues (69–75%), with the remainder occurring on threonine residues (DeLorenzo et al., 1977). Interestingly, BrC-P_{50} and BrC-P_{60} exhibited

almost exactly the opposite proportions. That is, phosphorylation was predominantly on threonine residues (approximately 75%), with the remainder on serine residues. However, only the threonine phosphorylation was bromocriptine-sensitive (Fig. 5). Burke and DeLorenzo (1981) have reported that the 50,000- and 60,000-dalton phosphoprotein regions are comprised of a number of phosphoproteins, including α- and β-tubulin and their kinases. We compared BrC-P_{50} and BrC-P_{60} with purified tubulin and found that they did not comigrate on one-dimensional SDS gels (unpublished observations). Kennedy and coworkers (1983) reported that tubulin is distinct from the components of the synapsin I kinase system. Thus we conclude that although BrC-P_{60} and BrC-P_{50} have molecular weights in SDS in the same range as DPH-L and DPH-M described by DeLorenzo and coworkers, they are not identical to the DPH-sensitive phosphoproteins. However, it is possible that these phosphoproteins may account for some of the bromocriptine-insensitive phosphoserine residues present in our preparations.

The functional significance of the state of phosphorylation of BrC-P_{50} and BrC-P_{60} is not at present known. Bromocriptine, like other neurotransmitter-related agents, is believed to elicit pharmacological effects by acting on a receptor on the cell surface (Kebabian and Calne, 1979). Compatible with this notion is recent evidence, obtained using brain slices and neuronal cell cultures, which suggests that bromocriptine-sensitive phosphorylation of BrC-P_{50} and BrC-P_{60} may occur on the cell exterior (unpublished observations). Since there is good evidence that ATP is released from nerve terminals in response to membrane depolarization (McIlwain, 1972; Kuroda and McIlwain, 1974; White, 1977; Burnstock, 1981), it is conceivable that BrC-P_{50} and BrC-P_{60} phosphorylation is involved in mediating certain of the synaptic effects of released ATP, which are yet to be defined.

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