ISOLATION OF A CYCLIC AMP DEPENDENT PROTEIN KINASE FROM BOVINE HYPOTHALAMUS AND ITS INTERACTION WITH HYPOTHALAMIC SUBSTITUENTS

Jeffrey F. McKelvy

Department of Anatomy
University of Connecticut Health Center
Farmington, Connecticut 06032

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Summary: A soluble cyclic AMP-dependent protein kinase has been partially purified from bovine stalk-median eminence tissue. Its activity was stimulated maximally by low concentrations of cyclic AMP. The kinase catalyzed the phosphorylation of histones and of neurosecretory granules (NSG), prepared from bovine posterior pituitary glands, but not of other subcellular fractions. The kinase also catalyzed the phosphorylation of a deamidase, from porcine hypothalami, that inactivates thyrotropin releasing hormone. Monoamines known to be present in the hypothalamus (dopamine, norepinephrine and 5-hydroxytryptamine) inhibited protein kinase activity at concentrations of 0.1 and 1.0 mM. These results suggest the participation of protein kinases in mechanisms of neuroendocrine regulation in the hypothalamus.

Recently, evidence has been presented that regulatory events in the mammalian nervous system may be mediated by cyclic AMP-dependent protein kinases (1, 2). While reports have appeared on the purification of a soluble cyclic AMP-dependent protein kinase from whole brain (3, 4, 5), the isolation of the enzyme from specific brain regions has not yet been reported. This would be of interest since it is presently unclear whether soluble cyclic AMP-dependent protein kinases are identical in all tissues or whether there exist variations in forms of the enzyme which might subserve the needs of various specialized tissues or regions.

Because of our interest in biochemical mechanisms operative in neuroendocrine regulation, we have partially purified and characterized a cyclic AMP-dependent protein kinase from bovine stalk-median eminence fragments. The median eminence of the hypothalamus is an important site of regulatory activities in neuroendocrine integration, comprising a point of convergence of sets of neurosecretory neurons which produce the hypothalamic releasing hormones (6), which regulate anterior pituitary secretion,

and the neurohypophysial hormones which regulate whole body osmotic balance (7). In this communication we report on the partial purification and properties of a cyclic AMP-dependent protein kinase isolated from bovine stalk-median eminence tissue and on the interaction of this enzyme with hypothalamic substituents known to be involved in neuroendocrine regulatory phenomena in the hypothalamus.

MATERIALS AND METHODS

The protein kinase was purified from 200 bovine stalk-median eminence fragments (Pel Freez Biologicals). The crude extract was prepared as described by Miyamoto et al. (3) for the enzyme from whole brain. Subsequently, the enzyme activity was collected in the 0 - 25% (w/v) ammonium sulfate precipitate, which was dialyzed against 5 mM potassium phosphate buffer, pH 7.0, and then chromatographed on a column (1.5 x 5 cm) of Whatman DEAE cellulose (DE-52), equilibrated in 5 mM potassium phosphate, pH 7.0, which was washed with 0.1 M potassium phosphate and eluted with a linear gradient (200 ml) of from 0.1 - 0.5 M potassium phosphate, pH 7.0. The fractions showing both phosphotransferase and cyclic AMP binding activity were concentrated by Amicon PM-30 diafiltration and then chromatographed on a column (2.5 x 95 cm) of Sephadex G-200 equilibrated in 50 mM potassium phosphate buffer, pH 7.0.

Assay of protein kinase activity was carried out using [γ-^{32}P]ATP in the incubation mixture described by Miyamoto et al. (3) and determination of protein-bound phosphate as described by Tao and Hackett (8). Cyclic AMP binding was assayed by a modification (9) of the method of Gilman (10). Protein concentration was determined by a micro-Folin procedure (11). Na dodecyl sulfate acrylamide gel electrophoresis was carried out as described by Maizel (12) on 5% gels.

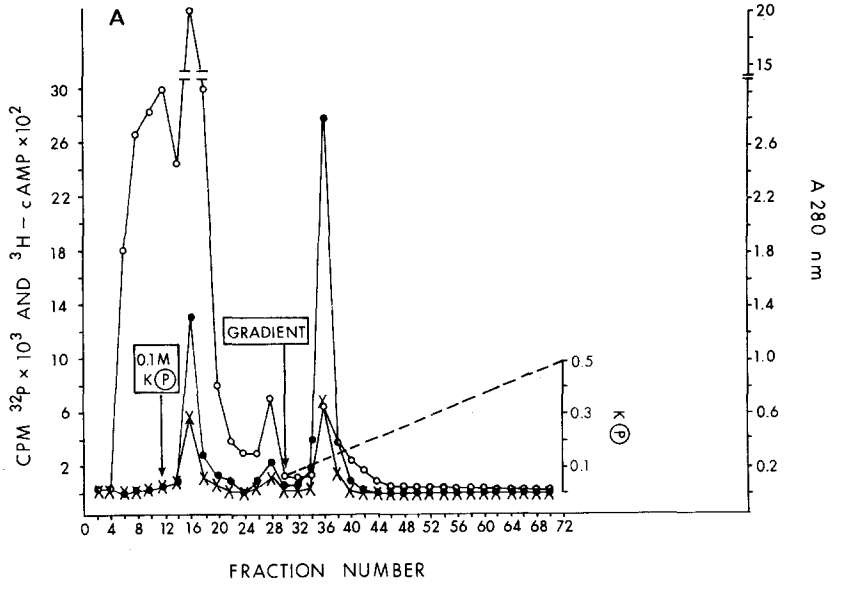
Subcellular fractionation of fresh bovine posterior pituitary glands was carried out by differential centrifugation by a modification of the method of Ishii (13).

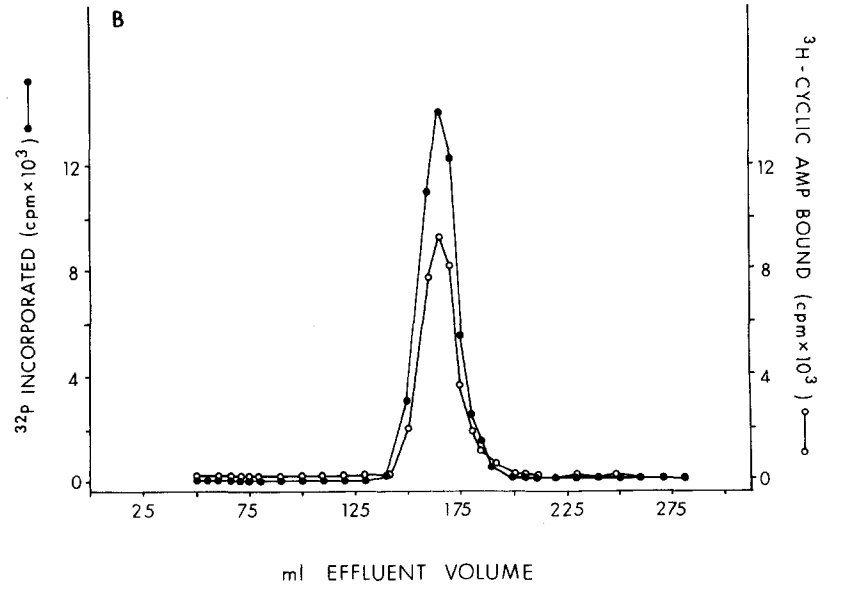
ATP, GTP and all cyclic nucleotides were obtained from Sigma, as were calf thymus histone, lysine-rich histone, arginine-rich histone and casein. Bovine serum albumin was from Pentex. Dopamine, norepinephrine, 5-hydroxytryptamine (creatinine sulfate salt) were from Regis Chemicals. Synthetic oxytocin and arginine vasopressin were the kind gifts of Dr. Victor Hruby. Synthetic thyrotropin-releasing hormone (TRH) was from Peninsula Laboratories and synthetic luteinizing hormone-releasing hormone (LRH) was the gift of Dr. Roger Guillemin. Purified TRH deamidase (14) was kindly provided by Dr. Karl Bauer, and bovine brain S-100 protein by Dr. Steven Pfeiffer. Bovine neurophysins I and II were purified from posterior pituitary powder as previously described (15). [γ-^{32}P]ATP and cyclic [^{3}H]AMP were from New England Nuclear.

RESULTS

Isolation of Cyclic AMP-Dependent Protein Kinase. Fig. 1A shows the elution profile resulting from DE-52 anion exchange chromatography of the stalk-median

eminence protein kinase activity, subsequent to precipitation with 25% ammonium sulfate. Two peaks of phosphotransferase and cyclic AMP-binding activity emerged in the 0.1 M potassium phosphate wash. The first peak comprised 30% of the total





phosphotransferase and 41% of the total cyclic AMP-binding activities, and the second peak comprised 5% of the total phosphotransferase and 8% of the total cyclic AMP binding activities. These peaks were pooled and saved for further characterization, and the peak which eluted at 0.15 M potassium phosphate in the gradient, comprising 65% of the total phosphotransferase and 49% of the total cyclic AMP-binding activities, was pooled, concentrated by Amicon PM-30 diafiltration, and subjected to gel filtration on Sephadex G-200. As seen in Fig. 1B, a single, symmetrical peak of overlapping phosphotransferase and cyclic AMP binding activities was observed. This peak was pooled and concentrated and used for the studies subsequently described. The specific activity of this peak was 3820 pmoles $[^{32}P]$ transferred from $[\gamma\ ^{32}P]$ATP to recovered protein in 5 min. at 30° per milligram protein and was 100 fold increased over that of the crude extract. 20% of the phosphotransferase activity and 0.19% of the protein present in the crude extract were recovered in this fraction.

Kinetic Propeties of the Stalk-Median Eminence Protein Kinase. The phosphotransferase activity of the protein kinase was dependent on ATP as a phosphoryl donor. In experiments in which UTP, CTP, GTP or dTTP were (separately) added to incubation mixtures together with $[\gamma^{32}P]$ATP in molar ratios of from 20 - 100, XTP/ATP, no inhibition of phosphorylation of calf thymus histone was detected. Moreover, negligible

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Figure 1. A. DE-52 chromatography of the 25% (w/v) ammonium sulfate ammonium sulfate precipitate from the 18,000 g supernatant of an homogenate of 200 bovine stalk-median eminence fragments. o—o, A 280 nm; •—•, protein kinase activity with calf thymus histone as substrate; x—x, $[^{3}H]$ cyclic AMP binding.

B. Sephadex G-200 chromatography of the major peak of protein kinase activity from A. •—•, protein kinase activity; o—o, cyclic AMP binding.

amounts of radioactivity were incorporated when [γ^{32}P]GTP was used as phosphoryl donor over a range of from 5 - 50 μM GTP. The Km for ATP was 1.0×10^{-5} M in the presence and 3.9×10^{-5} M in the absence of 5×10^{-6} M cyclic AMP. Mg^{2+} ion was required for activity (Km 10 mM at 5 μM cyclic AMP). Cyclic nucleotide titration (range 0.025 - 50 μM) revealed that cyclic GMP, cyclic CMP and cyclic UMP exhibited sigmoidal saturation curves with concentrations for half-maximum stimulation in excess of 5×10^{-5} M. Cyclic TMP had little effect on activity. Both cyclic AMP (Km 5.9×10^{-8}M) and cyclic IMP (Km 3.0×10^{-7} M) exhibited hyperbolic saturation curves. Under appropriate assay conditions (5 μg enzyme, 5 μM cyclic AMP, 10' at 22°) cyclic AMP exhibited a 40 fold stimulation of enzyme activity. Both calf thymus histone (Km = 0.48 mg/ml) and $F2_a$ histone (Km = 0.5 mg/ml) were good substrates for the enzyme while arginine rich histone and casein were much less active. The pH optimum, using calf thymus histone as substrate, was broad with a maximum at 7.0.

Phosphorylation of Macromolecules Present in Stalk-Median Eminence Region of Hypothalamus. As shown in Table 1, certain macromolecules known to be present in the stalk-median eminence region - neurosecretory granules, S-100 protein and TRH deamidase - served as substrates for the stalk-median eminence protein kinase with significant degrees of effectiveness, based on comparison to calf thymus histone, the most effective exogenous substrate for the enzyme. The major protein constituents of the neurosecretory granules, neurophysins I and II, did not serve as substrates, while 4 protein species of the neurosecretory granule membrane underwent phosphorylation, as revealed by Na dodecyl sulfate gel electrophoresis of solubilized granule membranes (data not shown).

Effect of Stalk-Median Eminence Substituents on the Activity of the Hypothalamic Protein Kinase. The median eminence of the hypothalamus is a termination site both for hypothalamic peptidergic neurons, and for monoaminergic neurons implicated in the

Table 1

PHOSPHORYLATION OF MACROMOLECULES PRESENT IN HYPOTHALAMIC STALK-MEDIAN EMINENCE BY HYPOTHALAMIC CYCLIC AMP DEPENDENT PROTEIN KINASE

Macromolecule* Posterior Pituitary Subcellular Fractions		[^{32}P] Incorporated, % of Equal Concentration of Calf Thymus Histone
Neurosecretory Granules**	50 µg protein	63
" "	100 µg protein	72
Mitochondria	300 µg protein	1
Crude Nuclei	300 µg protein	1
Synaptosomes	300 µg protein	3
Neurophysin I	50 - 500 µg protein	2
Neurophysin II	50 - 500 µg protein	1
S-100 Protein	50 µg protein	30
	100 µg protein	28
TRH Deamidase	50 µg protein	116
	100 µg protein	121

* All substrates incubated in the standard assay mixture in the presence of 5 µM cyclic AMP and 5 µg hypothalamic protein kinase. Comparison made to incubations with an equivalent amount of calf thymus histone as substrate.

** Corrected for endogenous phosphorylation.

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regulation of release of hypothalamic peptide hormones (16). At the present time, it is not known whether monoamines and hypothalamic releasing hormones occur together in the same neuron in this region of the brain. To examine the possibility of intracellular sites of action of neurohormones in the modulation of peptidergic neuronal activity, the effect of monoamines and hypothalamic peptide hormones on the activity of the hypothalamic protein kinase was tested. As shown in Table 2, the monoamines norepinephrine, dopamine and 5-hydroxytryptamine inhibited the phosphorylation of calf thymus histone by the enzyme at concentrations of 0.1 and 1.0 mM. Although not shown here, both cyclic AMP stimulated and nonstimulated activity was inhibited, and the presence of the monoamines lowered the V_{max} for

Table 2

EFFECT OF CERTAIN MONOAMINES AND HYPOTHALAMIC PEPTIDE HORMONES ON PHOSPHORYLATION OF CALF THYMUS HISTONE BY HYPOTHALAMIC PROTEIN KINASE*

Addition		% Activity
none		100
norepinephrine	0.1 mM	80
	1.0 mM	66
dopamine	0.1 mM	72
	1.0 mM	51
5-hydroxytryptamine	0.1 mM	60
	1.0 mM	43
TRH	10 nM - 1 uM	98
LRH	10 nM - 1 uM	99
oxytocin	1 - 10 uM	101
arginine vasopressin	1 - 10 uM	93

* All additions made to the standard assay mixture in 25 µl or less and preincubated for 5 min in the presence of enzyme and 5 µM cyclic AMP.

ATP, but did not change the Km. Neither the neurohypophysial peptide hormones, oxytocin and vasopressin, nor the hypothalamic releasing hormones TRH and LRH, had any effect on enzyme activity at concentrations of from 10^{-8} -10^{-5} M.

DISCUSSION

The hypothalamic cyclic AMP dependent protein kinase described here was of the same specific activity as the enzyme purified from whole bovine brain, using

the same assay incubation mixture (3), but the kinetic properties of the two enzymes were not identical. For the hypothalamic enzyme, there was a much smaller effect of cyclic AMP on the Km for ATP, a broader pH optimum and a difference in the nature of the response of the enzyme to cyclic nucleotides. A fuller understanding of possible differences between whole brain and hypothalamic enzymes awaits the completion of studies on molecular properties of the hypothalamic enzyme, now in progress.

The present studies have shown that macromolecules and organelles operative in neuroendocrine processes in the hypothalamus can serve as substrates for a hypothalamic protein kinase. Further studies on the effect of phosphorylation on these structures may implicate cyclic nucleotides in the regulation of the packaging, secretion and turnover of hypothalamic peptide hormones.

The ability of certain monoamines present in the hypothalamus to influence the activity of the hypothalamic protein kinase may point to the possibility of intracellular sites of action of monoamines in the modulation of the function of the endocrine hypothalamus. While high concentrations of monoamines (0.1 - 1.0 mM) were required for significant inhibition of enzyme activity, it should be noted that studies on single invertebrate neurons (17) imply that monoamine concentrations can be of the order of from 1 - 5 mM in the cell body and from 10 - 100 times greater in nerve terminals. An extravesicular pool of from only 1 - 10% of the total could provide concentrations of monoamines sufficient to influence protein kinase activity. Moreover, a recent report (18) has suggested that the cyclic AMP activation of tyrosine hydroxylase may mediated by a cyclic AMP dependent protein kinase. The ability of monoamines to inhibit protein kinases could serve as an additional negative signal in the regulation of monoamine biosynthesis.

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