A CYCLIC AMP-DEPENDENT PROTEIN KINASE FROM FROG BLADDER EPITHELIAL CELLS

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SUMMARY. A protein kinase which catalyzes a cyclic AMP-dependent phosphorylation of histone was found in extracts prepared from frog bladder isolated epithelial cells. The enzyme content of these cells is high as compared to the contents reported for several other types of tissue. About 30 % of the total activity present in crude extracts is associated with a particulate fraction; 70 % is recovered in the cytoplasmic fraction. The characteristics of the cytoplasmic enzyme are very similar to those of protein kinases purified from mammalian tissues. This enzyme's apparent Km for cyclic AMP (10^{-8} M) is in the range of the intracellular concentrations measured in the bladder; this suggests that a protein kinase could be involved in the two cyclic AMP-mediated effects of neurohypophysial hormones on this epithelium, namely, increased osmotic water permeability and stimulation of active sodium transport.

A cyclic adenosine 3'-5'-monophosphate (cyclic AMP)-dependent protein kinase was found in rabbit skeletal muscle which catalyzes the phosphorylation of phosphorylase kinase.[1] It was suggested that this enzyme acts as the link between the epinephrine stimulation of adenyl-cyclase and the activation of phosphorylase kinase which occurs in skeletal muscle in vivo.[2] The protein kinase from muscle is not highly specific since it is also able to catalyze the phosphorylation of several other proteins. Later experiments showed it was possible, using artificial protein substrates, of which histone is one of the best substituants, to demonstrate the presence of cyclic AMP-dependent protein kinase(s) in several other structures (liver[3], fat cells[4], brain [5-6] and micro-organisms [7]) despite the fact that the natural substrates of the protein kinase(s) and the sequence of events leading up to the cyclic AMP-mediated final effect are not entirely known. In all the structures so far studied, cyclic AMP is involved in the regulation of metabolic processes or protein synthesis. Cyclic AMP is also involved in the hormonal regulation of cellular permeability. Thus, it mediates the two biological effects of neurohypophysial hormones on the epithelial cells of the amphibian urinary bladder[8] and skin[9], i.e. increased osmotic permeability of the epithelium resulting from structural modification of the apical plasma membrane, and stimulation of transepithelial active sodium transport. The mechanism of

this last effect is not yet clearly established; it could either result from the apical membrane's increased passive permeability to sodium ions[10] or from a direct effect on an active transport system located at the basal or the apical membrane.[11] The aim of this study is to ascertain whether a cyclic AMP-dependent protein kinase could also be involved in the regulation of the amphibian bladder's permeability to water and sodium. As a preliminary, we describe some of the characteristics of a protein kinase present in extracts prepared from frog bladder isolated epithelial cells. Taking into account the intracellular localization of the final cyclic AMP effects, special attention was devoted to studying the distribution of protein kinase activity between the cytoplasmic and particulate fractions.

METHODS

The protein kinase activity was measured by the incorporation of ^{32}P into histone, using $\gamma^{32}P$-ATP as a precursor. The incubation medium (final volume 0.5 ml) contained TRIS-HCl pH 7.4 25mM, $MgCl_2$ 10mM, NaF 10mM, β mercaptoethanol 20mM, ouabain 0.1mM, histone 500 µg, theophylline cyclic AMP or other nucleotides in various amounts, as indicated in the legends of the figures. After thermic equilibration at 30°C, the reaction was initiated by the addition of $\gamma^{32}P$-ATP (250.000 ipm, final concentration 5×10^{-5}M) and terminated by adding 0.5 ml of a 25 % CCl_3COOH solution containing ATP (1 mM) and PO_4H_2K (0.1 mM). The precipitation was allowed to proceed for 15 minutes at 0°C. The protein precipitate was then separated by filtration on a 25 mm HAWP millipore and washed with 50 ml of a 5 % CCl_3COOH solution containing PO_4H_2K (0.1 mM). Radioactive phosphate on the millipore was determined by liquid scintillation. A 5 µl aliquote of the $\gamma^{32}P$-ATP solution was counted under the same conditions. Each experimental series included a blank without enzyme.

For the preparation of the extracts, the bladders of 30 frogs (Rana esculenta) were excised, rinsed in TRIS-HCl pH 7.4 100 mM, gently blotted on gauze and congelated. After decongelation, the tissue was cut into small pieces and incubated for 30 minutes at 35°C in 5 ml of a medium containing TRIS-HCl pH 7.4 100 mM, $CaCl_2$ 5 mM, and collagenase 40 µg/ml. After this treatment, epithelial cells were released by vigorous stirring. The supporting connective tissue was removed by filtration on gauze and the isolated epithelial cells collected by centrifugation (500 g for 15 minutes). The cells were then disrupted by incubation for 60 minutes at 0°C in TRIS-citrate pH 7.4 100 mM, E.D.T.A. 1.5 mM and by overnight dialysis against a hypotonic medium (TRIS-E.D.T.A. 1.5 mM pH 7.4). The resulting crude extract contained about 1 mg protein/ml. Soluble and particulate fractions were

separated by centrifugation for 60 minutes at 100.000g. Some of the preparations were treated with sodium deoxycholate before centrifugation. The supernatant was used directly as the source of protein kinase. The pellet was washed with TRIS-HCl pH 7.4 10 mM, E.D.T.A. 1.5 mM and dissolved in the same buffer to a final concentration of about 0.5 mg protein/ml.

The total ATPase activity of crude extracts was measured by the release of ^{32}P from $\gamma^{32}P$-ATP. Inorganic phosphate was extracted using the method described by POST [12] and radioactive phosphate measured in the butylester phase. Under the experimental conditions used for measuring the protein kinase activity, it was found that a maximum of 40 % of the added ATP was hydrolyzed. Phosphodiesterase activity was measured by adding ^{3}H-cyclic AMP (250.000 ipm, final concentration 10^{-6} M) and separating cyclic AMP from its metabolites by thin layer chromatography, using the C_2H_5OH, $CH_3COO(NH)_4M$, 70/30 V/V system. In the presence of Theophylline 10^{-4} M, the phosphodiesterase activity of crude extracts was found to be negligible. Protein was estimated by the method of LOWRY et al [13]. The following products were used: $\gamma^{32}P$-ATP 1 Ci/mM (C.E.A. France), ^{3}H-cyclic AMP 1.5 Ci/mM (SCHWARTZ BIORESEARCH), Collagenase type IIIA (SIGMA), Histone type IIA from calf thymus (SIGMA), Theophylline (MANN), Ouabain (MERK), cyclic AMP (SCHWARTZ BIORESEARCH), and Cyclic 3'-5'-Inosine monophosphate (cyclic IMP), cyclic 3'5'-Cytidine monophosphate (cyclic CMP), cyclic 3'-5'-Uridine monophosphate (cyclic UMP), cyclic 3'-5'-Guanosine monophosphate (cyclic GMP), and cyclic 3'-5'-Thymidine monophosphate (cyclic TMP), all from BOEHRINGER.

RESULTS

Distribution of protein kinase activity in crude extracts: The phosphorylation of histone by a crude extract of frog bladder epithelial cells is shown in Fig. 1. The phosphorylation rate is almost linear with time between 0 and 10 minutes: Cyclic AMP(10^{-6} M) has a 2.5-fold stimulatory effect on the reaction. The phosphorylation measured in the absence of histone is negligible, so that the results were not corrected for the phosphorylation of the proteins contained in the enzymatic preparation. After a 100.000 g centrifugation, 70 % of the total protein kinase activity of the crude extract is recovered in the supernatant, and 30 % remains associated with the particulate fraction. As indicated in Table 1, the specific activity ($\mu\mu M^{32}P$ incorporated per mg of enzyme) is higher and the stimulatory effect of cyclic AMP more pronounced in the soluble than in the particulate fraction. Previous treatment of the crude extract with sodium deoxycholate slightly increases both the specific activity and the total amount of the protein kinase present in the supernatant.

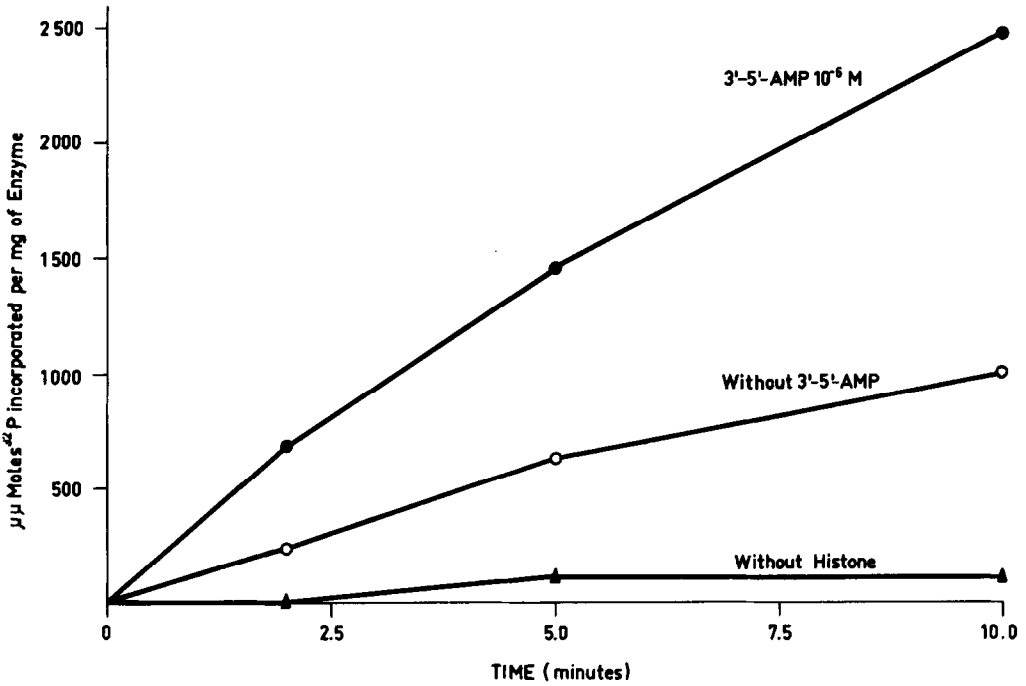

Fig. 1 : Time course of protein phosphorylation catalyzed by a crude extract of frog bladder epithelial cells. The incubation medium contained enzyme 37 μg and Theophylline 10^{-4} M. Other conditions are described in the text.

Table 1

Distribution of protein kinase activity in crude extracts

ENZYME	100.000 g SUPERNATANT			100.000 g PELLET			SUPERNATANT / PELLET	
	^{32}P incorporated		%	^{32}P incorporated		%		
	CONTROL	3'5'AMP	INCREASE	CONTROL	3'5'AMP	INCREASE	CONTROL	3'5'AMP
1	1086+	5135	473	587	1147	195	1.85	2.23
2	1405	5527	393	636	1992	313	2.21	2.77
3	1562	6062	388	696	1604	230	2.24	3.78

Phosphorylation of histone was measured as described in the text, in the absence or presence of cyclic AMP (10^{-6} M). The incubation medium contained theophylline 10^{-4} M. Enzymes 2 and 3 were prepared from the same crude extract. Enzyme 3 was treated with sodium deoxycholate (0.04 %).
+ Values are μμM ^{32}P incorporated for 10 minutes and per mg of enzyme.

Characteristics of the protein kinase activity in the 100.000 g supernatant fraction: 1) It was found that the cyclic AMP-dependent phosphorylation of

histone was not strictly proportional to the amount of enzyme introduced; in the range studied (6 to 40 µg), the specific activity of the enzyme decreases with dilution. 2) As indicated in Fig. 2, the enzyme shows a smooth pH dependence (optimum: 7.6). 3) Magnesium ions are necessary for the reaction. Maximal activity is obtained with 10 mM; higher concentrations are inhibitory. In the presence of 1.5 mM E.D.T.A., half the maximum stimulation is obtained with 1.5 mM (see Fig. 2). 4) Sodium ions have no effect in the range of 3 to 10 mM; high concentrations produce slight inhibition (35 % inhibition for 100 mM).

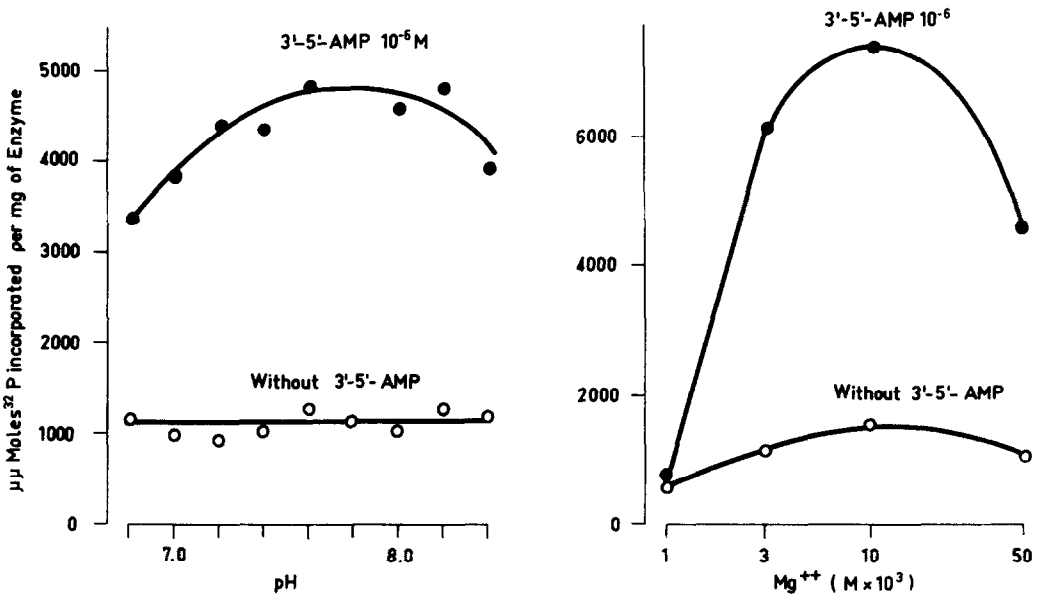

Fig. 2 : Magnesium and pH dependences of histone phosphorylation in the presence or absence of cyclic AMP. The 100.000 g supernatant fraction was the source of enzyme (52 µg). Incubation was performed for 10 minutes in the absence of theophylline. Other conditions are described in the text.

Stimulation by cyclic AMP and other cyclic nucleotides: Maximum stimulation of histone phosphorylation by cyclic AMP is obtained at a concentration of 10^{-7} M. The apparent Km is 1.1 10^{-8}M (Fig. 3). As indicated in Table 2, the protein kinase exhibits a high degree of specificity towards cyclic AMP. Significant stimulation is obtained with cyclic IMP, but the apparent Km for this nucleotide (5 X 10^{-7}M) is 50 times higher than the Km for cyclic AMP. At high concentrations (10^{-5}M) cyclic GMP, cyclic IMP and to a lesser extent cyclic CMP exert a slight stimulatory effect. Cyclic TMP has no effect.

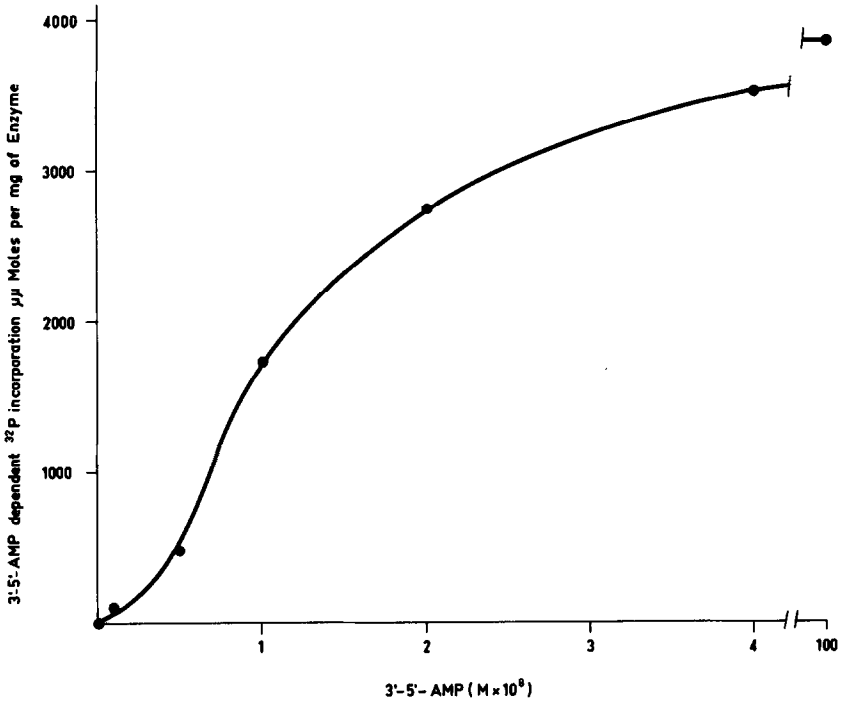

Fig. 3 : Activation of histone phosphorylation as a function of cyclic AMP concentration in the medium. The 100.000 g supernatant fraction was the source of enzyme (52 μg). Incubation was performed for 10 minutes in the presence of theophylline 10^{-4} M. Other conditions are described in the text.

Table 2

Stimulation of histone phosphorylation by different cyclic nucleotides.

Concentration (M)	3'5'IMP	3'5'GMP	3'5'UMP	3'5'CMP	3'5'TMP
10^{-7}	18.8	1.5	3.9	2.5	5.6
10^{-6}	75.2	4.3	5.0	1.7	4.3
10^{-5}	88.0	54.2	45.6	25.2	6.5

The 100.000 g supernatant fraction was the source of enzyme (41 μg). Incubation was performed for 10 minutes in the absence of theophylline. Other conditions are described in the text. Stimulation by the different cyclic nucleotides is expressed as a % of the maximal stimulation produced by cyclic AMP on the same preparation.

DISCUSSION:

A high content in protein kinase phosphorylating histone was found in frog bladder isolated epithelial cells. The specific activity of crude

extracts from these cells is about 100 times higher than that of crude extracts from rabbit brain and muscle (5 - 1). The larger part of the protein kinase activity is soluble and recovered in the 100.000 g supernatant fraction. The sum of the activities recovered in the supernatant and the particulate fractions is equal to the activity measured in the crude extract; this observation indicates that the centrifugation does not lead to isolation of an inhibitor within one of the two fractions similar to the inhibitors encountered during the purification of protein kinase from brain [5-6] or Escherichia coli. [7] The soluble protein kinase from frog bladder epithelial cells shows properties similar to those of cytoplasmic protein kinases purified from mammalian tissues. It requires the presence of magnesium ions; the apparent Km for cyclic AMP (10^{-8}M) is in the range of the different values reported (1-3-4-5), and its specificity towards cyclic nucleotides is identical to that reported by CORBIN and KREBS [4] for protein kinase from rat fat cells.

The high content of the frog bladder epithelial cells in protein kinase activated by concentrations of cyclic AMP in the range of intracellular concentrations [14] raises the possibility that the two cyclic AMP-mediated effects of neurohypophysial hormones on these cells imply a phosphorylation step. However, since the natural substrate(s) of the protein kinase(s) have not been determined, it is not at present possible to exclude the possibility that the protein kinase activity detected is not exclusively involved in the regulation of metabolic enzymes. An increase in glycogenolysis in response to stimulation of the toad bladder by vasopressin or exogeneous cyclic AMP was reported. [15-16] As previously indicated, the final effects of cyclic AMP on the sodium and water permeabilities of the frog bladder epithelial cells are located at the membrane level. If these effects imply a phosphorylation step, it can reasonably be assumed that the natural substrate(s) for the protein kinase(s) are membrane components. Such localization of the substrates is not incompatible with the presence of the protein kinase in the soluble cytoplasmic fraction. However, it is of great interest in this respect to note that about 30 % of the total protein kinase activity of frog bladder epithelial cells is associated with a particulate fraction. Unfortunately one cannot be sure that at least part of the particulate activity is linked with the plasma membranes, since, as reported by PINNA et al [17], a protein kinase activity is most probably present in microsomes.

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