Cyclic 3′,5′-AMP-Dependent Protein Kinase Activity in Toad Bladder Epithelium[1] (36524)

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The urinary bladder of the toad has been utilized extensively as a model system for investigating the antidiuretic action of neurohypophyseal hormones in the distal tubule and collecting duct of the mammalian kidney. The effects of arginine vasotocin (AVT), the naturally occurring amphibian water balance principle, and other neurohypophyseal peptides, have been studied in broken cell preparations as well as intact preparations of toad bladder epithelium; AVT and its congeners have been found to increase adenylate cyclase activity (1–3) and to elevate the intracellular concentration of adenosine 3′,5′-monophosphate (cyclic AMP) (4, 5). Cyclic AMP in turn has been implicated in the activation of phosphorylase kinase kinase in rabbit muscle *in vitro* (6). Subsequently, many other cyclic AMP-dependent protein kinases have been demonstrated *in vitro* in vertebrate and invertebrate tissues [(7) and refs. therein], bacteria (8), frog urinary bladder epithelial cells (9) and rat renal medullary cells (10); in the latter two systems a possible effector role for protein kinase in the induction of permeability changes has been suggested.

In this communication we report the presence and partial purification of a cyclic AMP-dependent protein kinase derived from epithlial cells of the toad urinary bladder, and describe some of its properties.

Materials and Methods. Toad bladder cyclic AMP-dependent protein kinase was partially purified according to the method of Miyamoto, Kuo and Greengard (11). Toads (*Bufo marinus* from National Reagents, Inc., Bridgeport, CT), after pithing, were deblooded by ventricular perfusion. Urinary bladders were removed quickly, and epithelial cells were scraped from the serosa with the aid of glass slides and collected in 4 m*M* EGTA, pH 6.5, at 0°. Scraped material from 10 whole bladders was used for each enzyme preparation. The cells were then vigorously homogenized in a Dounce homogenizer and centrifuged at 27,000*g* for 30 min. The supernatant was adjusted to pH 4.8 with 1 *M* acetic acid. After standing on ice 10 min the precipitate was removed by centrifugation and the hydrogen ion concentration of the clear supernatant was readjusted to pH 6.5 with potassium phosphate buffer. Protein kinase activity was precipitated from the neutralized supernatant by the addition of 325 mg $(NH_4)_2SO_4$/ml; the precipitate was separated from the supernatant by centrifugation and dissolved in 0.5 ml of 5 m*M* potassium phosphate buffer containing 2 m*M* ethylene glycol bis(2-aminoethyl)tetraacetate (EGTA) The dissolved enzyme preparation was dialyzed twice for several hours against 100 ml each of the same buffer, and centrifuged at 27,000*g* for 30 min. Enzyme preparations were used immediately, or after storage at —50° for 2–3 days; there was no detectable loss of activity or sensitivity to the cyclic nucleotide after storage.

The assay mixtures were those described by Kuo and Greengard (12) and Maeno, Johnson and Greengard (13): 50 m*M* sodium acetate buffer (pH 6.5), 200 μg histone mixture (Sigma, Type IIA), 10 m*M* $MgCl_2$, 2.5 μM ATP labeled in the γ position with ^{32}P

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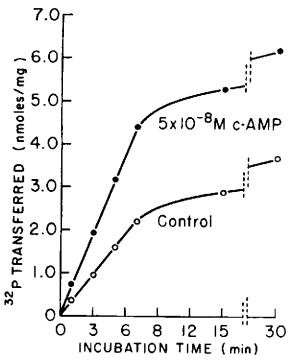

FIG. 1. Time course of histone phosphorylation (^{32}P transferred to histone from γ-labeled ATP) catalyzed by partially purified toad bladder epithelial protein kinase in the absence (control) and presence of 5×10^{-8} *M* cyclic AMP. Standard assay conditions were employed (see text) unless otherwise noted.

(International Chemical and Nuclear Corp., Irvine, CA, and Sigma), protein kinase extract 20-50 μg protein/ml, measured according to the method of Lowry *et al.* (14), and nucleotide [cyclic AMP (Nutritional Biochemical Corp.), or 5′-adenosine monophosphate (5′-AMP) (Sigma)], in a total volume of 0.2 ml. All incubations were performed at 30° for 5 min in a rotatory metabolic shaker. The conditions just described will be referred to as standard assay conditions. Experiments to test the effects of theophylline (Mann Research Laboratories) or EGTA on bladder protein kinase activity were performed under the above conditions in the presence and absence of 0.1 and 1.0 m*M* concentrations of these agents. Reactions were stopped by the addition of 200 μl of a solution containing 25% trichloroacetic acid (TCA), 10 m*M* ATP, and 1 m*M* KH_2PO_4, and by placing the mixture on ice for 15 min. TCA-insoluble protein was collected on 25 mm (0.45 μ) HAWP Millipore filters (presoaked in distilled water for at least 0.5 hr) and washed with 50 ml of 10% TCA–0.1 m*M* KH_2PO_4. Filters containing the washed protein precipitate were transferred to scintillation vials containing 10 ml of a dioxane scintillation fluid and counted.

Protein kinase activity, unless otherwise indicated, is expressed as picomoles of phosphate transferred from γ-labeled ATP to histone per milligram of enzyme preparation protein per minute. All data have been corrected for nonspecific phosphorylation of histone in the absence of added protein kinase and for autophosphorylation of the protein kinase in the absence of histone.

Results and Discussion. Data obtained with different protein kinase preparations of toad urinary bladder show that enzyme activity increases linearly with time for at least 6 min both in the absence (control) and presence of cyclic AMP (5×10^{-8} *M*). The results of a typical experiment are shown in Fig. 1. Maximum protein kinase activity attainable in the presence of cyclic AMP was consistently two to three times the activity obtained under control conditions using the histone mixture as a substrate. Casein, the only other phosphate-acceptor protein tested, was found to be poorly phosphorylated by bladder protein kinase.

Figure 2 shows control and cyclic AMP-dependent protein kinase activities as a function of amounts of enzyme protein in the test system. All enzyme preparations tested showed a linear response in the range of 1 μg to at least 12 μg of extract protein/sample, which corresponds to 5 to 60 μg/ml of incubation mixture. Enzyme activity was studied as a function of hydrogen ion concentration in three buffers: Tris–maleate, sodium cacodylate, and sodium acetate; in all three, maximal protein kinase activity was obtained at pH 6.5, both in the absence and presence of 5×10^{-8} *M* cyclic AMP (Fig. 3). At this

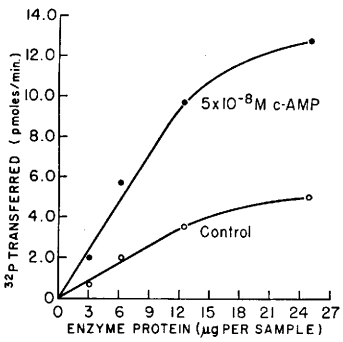

FIG. 2. The effect of different amounts of protein kinase enzyme (μg protein/sample) on the activity of the enzyme.

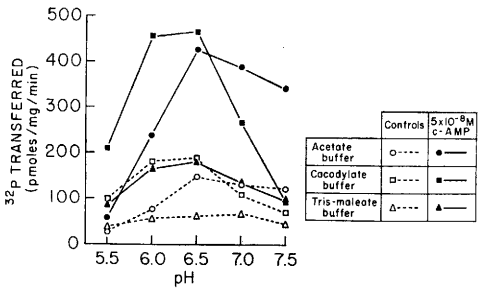

FIG. 3. The effect of pH on control (○,□,△) and cyclic AMP-dependent (●,■,▲) protein kinase activity expressed as ^{32}P transferred to histone using acetate (○,●), cacodylate (□,■), and Tris–maleate buffers (△,▲).

pH, the protein kinase activity was about the same with the sodium acetate or the sodium cacodylate buffers, but the activity was somewhat lower when Tris–maleate buffer was used.

Figure 4 shows protein kinase activity in response to increasing concentrations of cyclic AMP. Statistically significant stimulation was found at concentrations as low as 5×10^{-10} M, maximal stimulation at 5×10^{-8} M. Higher concentrations of cyclic AMP resulted in a diminished stimulation, and at a concentration of 5×10^{-4} M the enzyme activity fell below control values. Half-maximal stimulation was obtained at a nucleotide concentration of 1.5×10^{-9} M; this apparent K_m for cyclic AMP has been estimated from a Lineweaver-Burk plot. As shown in Fig. 4, the difference in activity between unstimulated and maximally cyclic-AMP-stimulated protein kinase amounts to about 450 pmoles/mg/min; this value may be

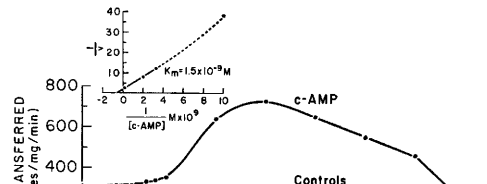

FIG. 4. Effects of cyclic AMP concentration on protein kinase activity. The insert shows a Lineweaver-Burk plot.

considered the specific activity of the cyclic AMP-dependent protein kinase.

In order to examine the specificity of the nucleotide-stimulated protein kinase activity, we tested the effect of 5′-AMP under identical experimental conditions. No stimulation was found at concentrations ranging from 5×10^{-10} to 5×10^{-5} M; however, like the cyclic analog, high concentrations of 5′-AMP were associated with a depression of kinase activity.

The effects of Mg^{2+}, Ca^{2+}, EGTA and theophylline on the activities of the protein kinase preparation were evaluated in the absence and presence of cyclic AMP. Maximal activities are obtained at a Mg^{2+} concentration of approximately 10 mM (Fig. 5). No

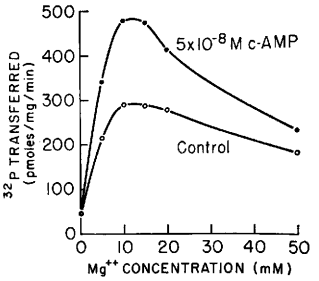

FIG. 5. The effect of increasing concentrations of Mg^{2+} on protein kinase activity in the absence (control) and presence of 5×10^{-8} M cyclic AMP.

cyclic AMP stimulation was observed in the complete absence of Mg^{2+}. As shown in Figs. 3 and 5, the activity of the unstimulated enzyme and that of the cyclic nucleotide-stimulated enzyme appeared to vary analogously. This parallel sensitivity of unstimulated and cyclic nucleotide-stimulated enzyme activity is also evident in Fig. 6, which shows the effects of increasing concentrations of Ca^{2+}. No effect of added Ca^{2+} was detected at concentrations lower than 0.5 mM, but at concentrations of 0.5 to 25 mM Ca^{2+} there was a progressive decline in both control and cyclic nucleotide-stimulated enzyme activity. Neither theophylline nor EGTA produced any enhancement of bladder kinase activity; theophylline in fact inhibited slightly both control and cyclic AMP-dependent protein kinase activity.

The above results, which demonstrate the

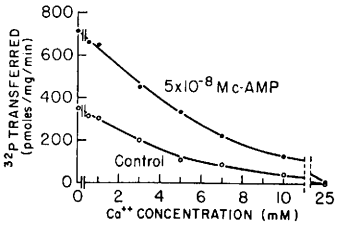

FIG. 6. The effect of increasing concentrations of added Ca^{2+} on protein kinase activity in the absence (control) and presence of 5×10^{-5} *M* cyclic AMP.

presence of a cyclic nucleotide-stimulated protein kinase in toad urinary bladder epithelium, may form a basis for further studies to define the cellular events triggered by antidiuretic hormone. Preliminary experiments indicate that a 1000*g* membrane fraction, probably a heterogeneous mixture of cellular membranes, may serve as a phosphate acceptor for cyclic AMP-dependent kinase. The plasma membrane has been suggested as a possible phosphate acceptor for frog bladder kinase (9) and the phosphorylation of a plasma membrane fraction derived from rat renal medulla has been reported (10). Similarly, cyclic AMP-stimulated protein kinases have been found to catalyze the phosphorylation of the endoplasmic reticulum of adrenal cortex (15) and the plasma membranes of rat liver cells (16).

Further investigations are needed to relate cyclic AMP-dependent protein kinase activity to the hormone-induced permeability change, and to identify the natively phosphorylated protein—which could be a regulatory enzyme located within cellular membranes of the epithelium in the toad urinary bladder and mammalian distal nephron.

Summary. A cyclic AMP-dependent protein kinase was partially purified from toad urinary bladder epithelium. Cyclic AMP-stimulated protein kinase activity as well as the unstimulated (control) activity of the enzyme was maximal at pH 6.5 using Tris–maleate, sodium cacodylate or sodium acetate buffers. The apparent K_m value of cyclic AMP for the stimulation of the enzyme (in sodium acetate buffer and in the presence of 10 m*M* Mg^{2+}) was 1.5×10^{-9} *M*. The acyclic nucleotide 5′-AMP (in the concentration range 5×10^{-10} to 5×10^{-5} *M*) failed to activate bladder protein kinase. Ca^{2+} was found to be inhibitory at concentrations higher than 5×10^{-4} *M*. A role for cyclic AMP-dependent protein kinase in neurohypophyseal hormone-induced permeability changes is discussed.

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