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Inhibition by diuretics of cyclic 3′, 5′-AMP-dependent protein kinase from toad bladder epithelium

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Summary

1. A cAMP-dependent protein kinase enzyme has been isolated from toad bladder epithelium.

2. This enzyme catalyses the phosphorylation of histones; a reaction stimulated by 0·1 μM cAMP.

3. Activity of this enzyme in the presence of 0·1 μM cAMP was significantly inhibited by 100 μM mercuderamide, bumetanide and frusemide. A small, though statistically insignificant inhibition was seen with the same concentrations of hydrochlorothiazide and ethacrynic acid.

4. The half maximal inhibition was achieved with mercuderamide, 5 μM, and with frusemide and bumetanide 600 μM.

Introduction

The urinary bladder of the toad (*Bufo marinus*) has been used extensively as a model for investigating the actions of neurohypophysial hormones on active sodium transport and osmotic water flow. It has also been used for studying the actions of diuretic drugs on the same phenomena (Ferguson, 1966; Bentley, 1968, 1969; Handler, Preston & Orloff, 1972; Ferguson & Smith, 1972).

Neurohypophysial hormones increase the transport of sodium from the mucosal to the serosal surface (Leaf, Anderson & Page, 1958) and also the osmotic flow of water in the same direction (Bentley, 1958). These actions of neurohypophysial hormones are thought to be mediated by cyclic 3′, 5′-adenosine monophosphate (cAMP) as an intracellular second messenger (Orloff & Handler, 1962; Bär, Hechter, Schwartz & Walter, 1970). Cyclic AMP has been shown to be necessary for the activation of phosphorylase kinase in rabbit muscle *in vitro* (Walsh, Perkins & Krebs, 1968). Subsequently, many other cAMP-dependent protein kinases have been demonstrated including a cAMP-dependent protein kinase from mucosal cells of toad urinary bladder (Kirchberger, Schwartz & Walter, 1972).

It has been suggested that certain diuretics might act on cAMP systems at a cellular level (Robison, Butcher & Sutherland, 1971). We have used the urinary bladder of the toad as a model to test this hypothesis, and in this paper report the inhibition of a cAMP-dependent protein kinase from the toad bladder by certain diuretics.

Methods

Preparation of cAMP-dependent protein kinase and the assay procedure were similar to those used by Kirchberger *et al.* (1972). *Bufo marinus*, obtained from Pet Farms Inc., Miami, Florida, were stored on damp peat at 30° C until use.

To prepare the protein kinase, ten toads were pithed, their urinary bladders excised and blood and mucus removed. Transporting epithelial cells were scraped from the stroma with the edge of a glass microscope slide and collected in 5 ml of 4 mM EDTA, pH 6·5 at 0° C. The cells were homogenized using an Ultra Turrax homogenizer for 1 min and centrifuged at 27,000*g* for 30 minutes. The supernatant was adjusted to pH 4·8 with 1 M acetic acid. After standing on ice for 10 min, the precipitate was removed by centrifugation. The supernatant was readjusted to pH 6·5 with 1 M potassium phosphate. Protein was precipitated from the supernatant overnight in the cold room by addition of 325 mg ammonium sulphate/ml. This precipitate was removed by centrifugation and resuspended in 0·5 ml of 5 mM potassium phosphate buffer containing 2 mM EDTA, pH 7·0. The dissolved enzyme was dialysed twice against 100 ml of the same buffer for several hours, then centrifuged at 27,000*g* for 30 minutes. The supernatant was divided into 1 ml volumes and stored at −50° C until use.

In the enzyme assay, incorporation of ^{32}P into histones with labelled [^{32}P]-ATP as substrate was measured. The assay mixture was that described by Kirchberger *et al.* (1972); 50 mM sodium acetate buffer pH 6·5, 200 μg histone mixture (Sigma, Type IIA), 100 mM magnesium chloride, 2·5 μM ATP (γ labelled with ^{32}P), protein kinase 30–50 mg protein/ml (Lowry, Rosebrough, Farr & Randall, 1951) and where necessary, 0·1 μM cAMP in a final volume of 200 μl.

Incubations were performed in polystyrene test tubes in a Gallenkamp metabolic shaker at 30° C. The reaction was started by adding [^{32}P]-ATP, continued for 10 min and stopped by adding 20 μl of a mixture of 25% TCA, 10 mM ATP and 1 mM potassium phosphate. The tubes were placed on ice for 15 minutes. TCA-insoluble protein was collected on 25 mm (0·45) HAWP Millipore filters, pre-soaked in distilled water for at least 30 minutes. The filters were washed with 50 ml of 10% TCA and 0·1 mM potassium phosphate which had been cleared of any sediment by pre-filtration through an Acropor AN450 (0·45) acrylonitrite polyvinyl chloride filter. The filters were placed in counting vials and dried at 90° C for 30 minutes. One ml dioxane was then added followed by 20 ml of toluene scintillation fluid. Vials were counted in a Packard Tri-Carb liquid scintillation spectrometer. An aliquot of the [^{32}P]-ATP solution was counted under the same conditions. Each experimental series included a blank of boiled enzyme. Diuretics were dissolved in assay medium at the concentrations indicated.

Protein kinase activity is expressed as (pmole phosphate/mg enzyme protein)/10 min transferred from ATP to histone.

Materials

ATP labelled in the γ position with ^{32}P was obtained from the Radiochemical Centre, Amersham, specific activity 1,350 mCi/mmol, cAMP was supplied by Boehringer Mannheim GmbH and ATP (disodium salt) by the Sigma Chemical Co.

Ethacrynic acid and hydrochlorothiazide were kindly donated by Merck, Sharp & Dohme, Ltd., Hoddesdon, Hertfordshire, mercuderamide by May & Baker Ltd., Dagenham, Essex, frusemide by Farbwerke Hoechst A. G., Frankfurt, and bumetanide by Leo Laboratories Ltd., London.

Results

Table 1 shows the activity of cAMP-dependent protein kinase in the presence and absence of 0·1 μM cAMP. Enzyme activity in the presence of cAMP is about three times the basal activity. Table 2 shows the effect of diuretic drugs, 100 μM, on the enzyme activity in the presence of 0·1 μM cAMP. All drugs were tested at this high concentration; those that showed a significant effect on enzyme activity

TABLE 1. *The effect of* 0·1 μM *cAMP on the activity of cAMP-dependent protein kinase*

cAMP concentration	Number of observations	(pmole phosphate/mg enzyme protein)/ 10 min*
0	4	354±27
0·1 μM	4	1206±24

* Mean ±S.E.

TABLE 2. *Effect of diuretic drugs on cAMP-dependent protein kinase activity*

Diuretic	Concn. (μM)	Activity % of control	*n*	*P*
Ethacrynic acid	100	95±6	4	N.S.
Mercuderamide	100	0	4	<0·01
Hydrochlorothiazide	100	93±3	4	N.S.
Frusemide	100	91±3	4	<0·05
Bumetanide	100	87±3	4	<0·05

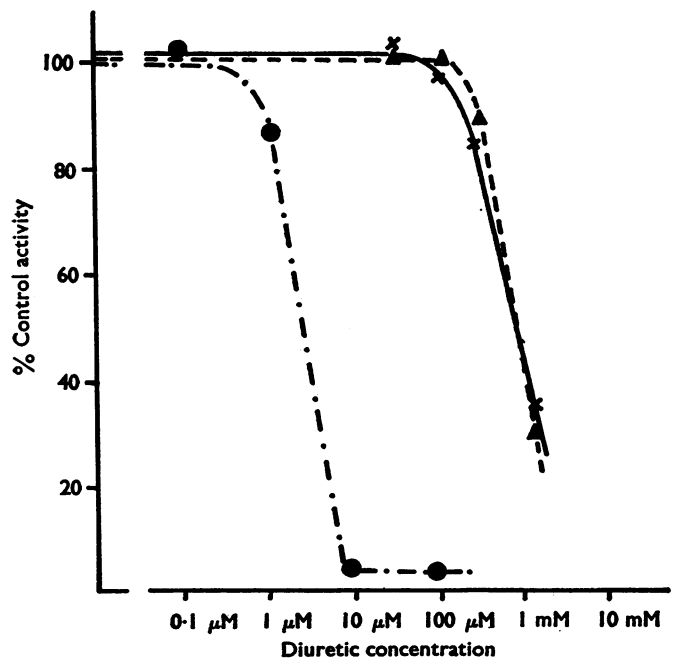

FIG. 1. Inhibition of cAMP-dependent protein kinase by increasing concentrations of mercuderamide (●---●), frusemide (×——×), and bumetanide (▲----▲) in the presence of 0·1 μM cAMP.

were studied further. Fig. 1 shows protein kinase activity in presence of varying concentrations of mercuderamide, frusemide and bumetanide. Half-maximal inhibition occurred at 5 μM for mercuderamide and 600 μM for frusemide and bumetanide.

Discussion

Frusemide inhibits vasopressin-stimulated sodium transport in the intact toad bladder at 7·6 μM (Ferguson, 1966). Both frusemide and bumetanide however, showed half-maximal inhibition of protein kinase activity at 600 μM. This particular reaction is unlikely to be of direct relevance to the biochemical mechanisms of sodium transport, though the phosphorylation of plasma membrane fractions such as has been demonstrated in rat renal medulla by Dousa, Schwartz, Sands, Hechter & Walter (1971) may prove to be more significant. Until the relationship of cAMP-dependent systems to sodium transport has been further elucidated we have to rely on demonstrating effects of diuretics on probably similar, but less sensitive systems.

Mercuderamide showed half-maximal inhibition of protein kinase activity at 5 μM. This concentration is similar to that which produces inhibition of the Na, K-dependent ATPase of the kidney (Yoshitoshi, Oda, Maeda & Kume, 1966). It has been suggested that inhibition of Na, K-dependent ATPase is the *in vivo* mode of action of organic mercurial diuretics, but this hypothesis has been challenged (Nechay, Palmer, Chinoy & Posey, 1967). Organic mercurial diuretics do reach the intracellular cytoplasm of kidney cells (Bowman & Landon, 1967). These results suggest that mercurial diuretics may have actions on several biochemical systems.

Ethacrynic acid and hydrochlorothiazide at 100 μM produced a small, but statistically insignificant reduction in protein kinase activity. Bentley (1969) showed that ethacrynic acid, 100 μM, inhibited basal short circuit current across the toad bladder and the natriferic and hydro-osmotic effects of vasopressin. Mendoza, Handler & Orloff (1970) showed that an analogue of ethacrynic acid inhibited both basal and vasopressin stimulated short circuit current, but had no effect on cAMP stimulated transport. These results suggest that ethacrynic acid has no effect on the site of action of cAMP within the toad bladder cell; a conclusion supported by the present results.

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