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INHIBITION BY CYCLIC AMP AND DIBUTYRYL CYCLIC AMP OF TRANSPORT OF ORGANIC ACIDS IN KIDNEY CORTEX

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SUMMARY

1. Cyclic adenosine 3′,5′-monophosphate and N^6-2′-*O*-dibutyryl cyclic adenosine 3′,5′-monophosphate decrease the initial entry rate and the steady-state uptake of *p*-aminohippurate and uric acid by rabbit kidney cortex slices.

2. N^6-2′-*O*-Dibutyryl adenosine 3′-5′-monophosphate inhibits the tubular transport of *p*-aminohippurate competitively.

3. Isoproterenol, known to increase cyclic nucleotide concentration of the cortical tubules by activation of adenyl cyclase, decreases *p*-aminohippurate transport. Antidiuretic hormone which is known to stimulate only medullary adenyl cyclase has no effect on *p*-amino-hippurate uptake by cortical slices.

4. Theophylline, which inhibits cyclic nucleotide phosphodiesterase and, therefore, enhances the cellular accumulation of endogenous cyclic nucleotide, depresses *p*-aminohippurate transport.

INTRODUCTION

Recently, it has been suggested that cyclic adenosine 3′,5′-monophosphate (cyclic AMP) might be involved in the transport of amino acids [1, 2] and sugars [3] by the kidney. This view was supported by the observation that cyclic AMP and N^6-2′-*O*-dibutyryl cyclic adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) in the external bath stimulated transport of the two classes of organic substances by renal cortical cells from several mammalian species. The mechanism by which this stimulation occurs is not known. In addition, some of the characteristics of the cyclic AMP stimulation of amino acids were the opposite of those observed for sugars. In order to obtain further insights concerning the action of cyclic nucleotides on the transport of organic substances by renal tissue, we have studied the influence of these compounds on organic acid uptake by renal cortical cells. We observed that both cyclic nucleotides and compounds known to increase cyclic AMP cellular concentration inhibited transport of *p*-aminohippurate and uric acid by renal cortical cells. This observation forms the basis of the present report of some of the characteristics concerning cyclic nucleotides induced inhibition of organic acids transport by rabbit kidney cortex slices.

METHODS

Tissues

White male rabbits weighing 2–4 kg, were anesthetized with sodium pentobarbital. The kidneys were removed and perfused with acetate–Ringer solution. Slices of cortex approximately 0.2–0.4 mm thick were prepared with a Stadie–Riggs microtome.

Incubation procedure

All incubations were performed at 27 °C in special incubation flasks [4] with O_2 bubbling from below. The bathing medium consisted of 135 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.5 mM CaH_4 $(PO_4)_2$, 10 mM sodium acetate, 20 mM Tris–Cl (pH 7.4 at 27 °C) and 5 mM glucose. In preliminary experiments, tissues were first incubated for 120 min with or without various nucleotides (cyclic AMP, dibutyryl cyclic AMP, AMP, or ATP). Following this incubation period, *p*-amino-[1-^{14}C]hippuric acid and [methoxy-^{3}H]inulin were added and the incubation continued for 60 min. Since it was observed that such a preincubation period did not influence the nucleotide effect, it was deleted. Incubation was started by addition of 2–5 slices to 5 ml of the acetate–Ringer solution containing the isotopes ([*methoxy*-^{3}H]inulin and either *p*-amino-[1-^{14}C]hippuric acid or [2-^{14}C]uric acid) and the investigated chemical compound. Unless otherwise stated, the final concentration of *p*-aminohippurate or uric acid in the bathing medium was 0.15 mM.

Determination of organic acid uptake and electrolyte and water content

After the completion of incubation, slices were removed, blotted on filter paper, weighed and dried overnight in an oven at 70 °C. Dried tissues were extracted in 3 ml of 0.18 M trichloroacetic acid with a sonifier (Sonifier B12 Branson, Conn., U.S.A.), and the resulting extracts were centrifuged 10 min at $5000 \times g$. Determinations of ^{14}C and ^{3}H were performed with some modifications as described by Amiel et al. [5]: 50 μl aliquots of the tissue extracts, and of the incubation media diluted 1: 30 with trichloracetic acid (0.18 M), were deposited in duplicate on fiber-glass discs Whatman G.F.C. and evaporated under an infrared lamp. The discs were covered with 5 ml of solution containing 4 g of PPO and 0.3 g of POPOP per liter of toluene. The radioactivities of ^{14}C and ^{3}H were measured with a Nuclear Chicago Mark I scintillation counter. The settings were such that the spillover of ^{14}C into ^{3}H was 10 % and ^{3}H into ^{14}C less than 0.05 %. The counting rates were carried out for sufficient length of time to achieve a statistical accuracy of at least 3 %.

Cellular water was calculated for each sample by the difference between wet and dry weight after correction for the extracellular water, as previously described [6]. For each sample, tissue ^{14}C-labeled organic acids radioactivity was corrected for that portion of radioactivity trapped along with extracellular water. This contamination was calculated as the product of extracellular ^{14}C concentration times the trapped fluid volume measured by the [^{3}H]inulin space. The distribution ratio of the organic acid concentration between cellular water and extracellular water was then calculated. In some experiments, the initial rate of *p*-aminohippurate accumulation in the slices (expressed in mmoles/kg dry wt per 30 min) was determined as the amount of *p*-aminohippurate entering the cells during a 30 min incubation period. In preliminary

experiments, it was verified that ^{14}C radioactivity in tissue extracts, as assessed by the method of Sørensen [7] was at least 90 % [^{14}C]uric acid.

Tissue content of Na^+ and K^+ were determined as previously described [6].

In all cases, kidneys from the same animal were used to prepare tissues for control and experimental studies.

Results were expressed as the mean $\pm$ standard deviation (S.D.). The significance of the difference between two means has been assessed on the basis of the Student's *t* test.

MATERIALS

All nucleotides except ATP, a product of Sigma Chemical Co., were supplied by Calbiochem. Lysine–vasopressin (antidiuretic hormone) and isoproterenol were purchased from Sigma Chemical Co. [*Methoxy*-^3H]Inulin, *p*-amino-[1-^{14}C]hippuric acid were purchased from New England Nuclear. [2-^{14}C]Uric acid was obtained from Amersham, and recrystallized as described by Sørensen [7] to a specific activity of approximately 10 μCi/mg. All chemicals were of reagent grade.

RESULTS

Inhibition of organic acid transport

All the nucleotides tested inhibited the uptake of *p*-aminohippurate by rabbit kidney cortex slices (Table I). However, for equal concentrations, the dibutyryl derivative is approximately twice as active as cyclic AMP, and about three times as active as AMP or ATP.

Incubation of kidney slices in media containing from 0.1 to 2.4 mM of dibutyryl cyclic AMP caused a progressive decrease of *p*-aminohippurate accumulation (Fig. 1). On the other hand, there is no requirement for prior incubation of the tissue with cyclic nucleotide in order to see the inhibitory effect. Exposing tissues to dibutyryl cyclic AMP for 2 h or not did not change significantly the degree of inhibition (Table II). To inhibit the transport of *p*-aminohippurate, dibutyryl cyclic AMP must be present during the uptake period. Tissues exposed to dibutyryl cyclic AMP for 2 h and subse-

TABLE I

EFFECT OF NUCLEOTIDES ON *p*-AMINOHIPPURATE UPTAKE BY KIDNEY CORTEX SLICES

Slices were incubated for 120 min with or without 0.5 mM nucleotide as indicated. Labeled *p*-aminohippurate subsequently added to a concentration of 0.15 mM, and continued incubation for 60 min. Each value represents the mean $\pm$S.D. from six individual experiments.

Nucleotide	Distribution ratio
None (control)	21.44 ± 3.61
Dibutyryl cyclic AMP	11.27 ± 0.90 ($P < 0.005$)
Cyclic AMP	15.49 ± 1.35 ($P < 0.01$)
AMP	18.34 ± 0.60 ($P < 0.05$)
ATP	18.39 ± 1.38 ($P < 0.05$)

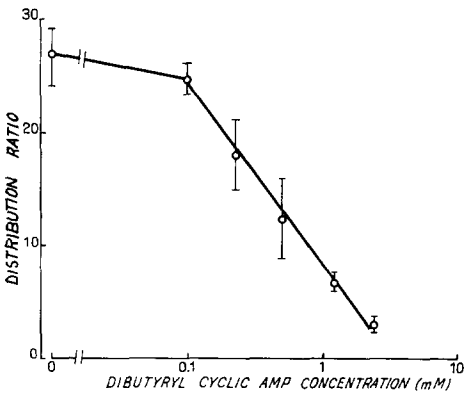

Fig. 1. Effects of various concentrations of dibutyryl cyclic AMP on *p*-aminohippurate uptake by kidney slices. Preparations initially incubated for 120 min with or without dibutyryl cyclic AMP at indicated concentrations. Labeled *p*-aminohippurate was then added to give a concentration of 0.15 mM and the incubation continued for 60 min. Each point represents the mean of five experiments and the vertical bars indicate ± 1 S.D.

quently transferred in a medium devoid of this nucleotide failed to inhibit *p*-aminohippurate transport (Table II). For these reasons, no intervening time was allowed between adding the cyclic nucleotide and the test solute in the subsequent studies.

TABLE II

EFFECT OF PRIOR EXPOSURE TO DIBUTYRYL CYCLIC AMP AND ITS DELETION DURING *p*-AMINOHIPPURATE INCUBATION

Tissues were initially incubated for 120 min with or without 0.5 mM dibutyryl cyclic AMP. The slices were washed twice and resuspended in the appropriate incubation solution. Labeled *p*-aminohippurate was then added to give a concentration of 0.15 mM and the incubation continued for 60 min. Each result represents the mean $\pm$S.D. from six individual experiments. N.S., not significant.

Preincubation	Incubation	Distribution ratio
Control	Control	19.31 ± 3.28
Control	Dibutyryl cyclic AMP	12.62 ± 1.62 ($P < 0.002$)
Dibutyryl cyclic AMP	Dibutyryl cyclic AMP	11.66 ± 0.67 ($P < 0.002$)
Dibutyryl cyclic AMP	Control	17.67 ± 1.24 (N.S.)

The greater effectiveness of the dibutyryl derivative observed was unchanged when nucleotide pretreatment was suppressed. As compared to control tissues, incubation of kidney slices for 60 min with 0.5 mM dibutyryl cyclic AMP induces a mean inhibition of *p*-aminohippurate uptake of 43.12 ± 6.03 % and of only 27.20 ± 8.37 % with 0.5 mM cyclic AMP. Each value represents the mean $\pm$S.D. of six paired observations. As shown in Table III, dibutyryl cyclic AMP and to a lesser degree cyclic AMP inhibit urate uptake by rabbit kidney slices. However, AMP is ineffective.

Effects of isoproterenol, antidiuretic hormone and theophylline

The effects of isoproterenol, antidiuretic hormone and theophylline on *p*-aminohippurate uptake by cortical slices are shown in Table IV. Isoproterenol, a

TABLE III

EFFECT OF VARIOUS NUCLEOTIDES ON URIC ACID UPTAKE

Tissues were incubated with or without 0.5 mM nucleotide at 27 °C for 60 min. [^{14}C]Uric acid concentration in the medium was 0.15 mM. Each value represents the mean ±S.D. from six individual experiments. N.S., not significant.

Nucleotide	Distribution ratio
None	5.82±0.62
Dibutyryl cyclic AMP	3.34±0.60 ($P < 0.002$)
Cyclic AMP	4.73±0.64 ($P < 0.01$)
AMP	6.14±0.84 (N.S.)

β-adrenergic stimulating agent, known to enhance the production of cyclic AMP in cortical tissue [8] inhibits *p*-aminohippurate uptake. In contrast, antidiuretic hormone which stimulates specifically medullary adenylcyclase [9] does not affect *p*-aminohippurate uptake. On the other hand, theophylline, a well known inhibitor of the cyclic nucleotide phosphodiesterase, inhibits *p*-aminohippurate transport.

TABLE IV

EFFECT OF ISOPROTERENOL, ANTIDIURETIC HORMONE AND THEOPHYLLINE ON *p*-AMINOHIPPURATE UPTAKE BY KIDNEY CORTEX SLICES

Slices incubated for 60 min at 27 °C with 0.15 mM of labeled *p*-aminohippurate. Each experiment corresponds to a different animal. Values are means ±S.D. Within parentheses are the numbers of experiments. N.S., not significant.

Number of experiment	Compound	Distribution ratio
1	None	21.96±1.31 (6)
	Isoproterenol (1 mM)	18.01±0.87 (6) ($P < 0.0005$)
2	None	23.84±2.13 (10)
	Antidiuretic hormone (50 μg/ml = 4.5 units/ml)	24.57±1.93 (10) (N.S.)
3	None	22.78±1.25 (5)
	Theophylline (0.1 mM)	16.28±1.53 (5) ($P < 0.0005$)
	Theophylline (1 mM)	10.40±0.69 (5) ($P < 0.0005$)

Time course of effect of dibutyryl cyclic AMP on p-aminohippurate uptake

Dibutyryl cyclic AMP inhibits both the initial entry rate and the cumulative uptake of *p*-aminohippurate (Fig. 2). The uptake of *p*-aminohippurate was significantly depressed after a 15 min incubation period ($P < 0.01$). This inhibitory effect was maintained during a 3 h incubation period.

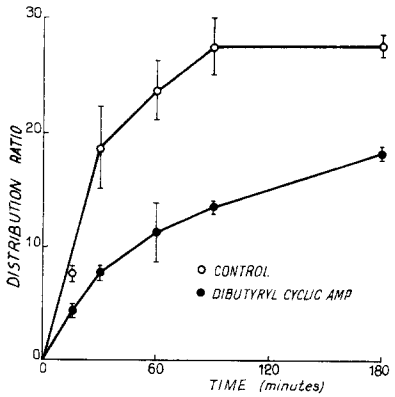

Fig. 2. Time course of the effects of dibutyryl cyclic AMP on *p*-aminohippurate uptake by kidney slices. Each preparation incubated with labeled *p*-aminohippurate for specified periods in absence of and presence of 0.5 mM dibutyryl cyclic AMP. Each point represents the mean of three individual experiments and the vertical bars indicate ± 1 S.D.

Effect of substrate concentration

The effects of dibutyryl cyclic AMP on the accumulation of *p*-aminohippurate which is a saturable process, were studied at concentrations of external *p*-aminohippurate varying from 0.09 to 2.7 mM (Fig. 3). In the presence of the nucleotide the mean value for the apparent K_m has more than doubled (control, 0.49 ± 0.16 mM; dibutyryl cyclic AMP, 1.15 ± 0.47 mM; $P < 0.05$) whereas V was not significantly affected by this agent (control, 12.16 ± 2.53 mmoles/kg dry wt per 30 min; dibutyryl cyclic AMP, 13.65 ± 5.40 mmoles/kg dry wt per 30 min; $P > 0.4$).

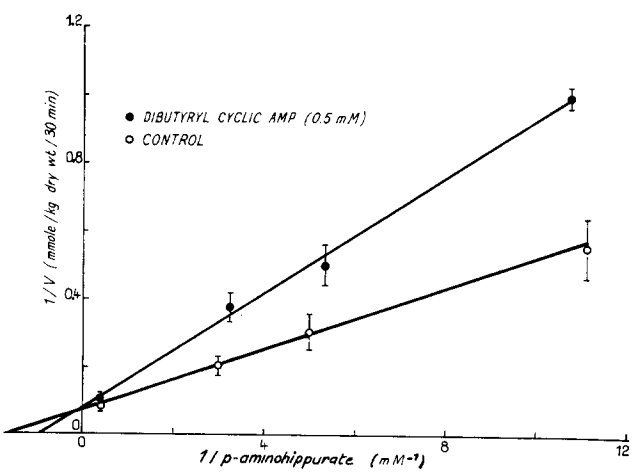

Fig. 3. Lineweaver–Burk plots of the inhibition of *p*-aminohippurate uptake in kidney slices by dibutyryl cyclic AMP. Ordinate is the reciprocal of *p*-aminohippurate uptake (mmoles/kg dry wt per 30 min) and abscissa is the reciprocal of medium concentration of *p*-aminohippurate (mM^{-1}). Each point is the mean $\pm$S.D. from four individual experiments. Lines were drawn by the least squares method.

Influence of dibutyryl cyclic AMP on electrolytes and water content

Incubation of kidney cortex slices for 60 min with 0.5 mM of dibutyryl cyclic AMP does not significantly affect the tissue content of Na^+, K^+ and water (Table V).

TABLE V

EFFECT OF DIBUTYRYL CYCLIC AMP ON ELECTROLYTE AND WATER CONTENT OF KIDNEY CORTEX SLICES

Tissues were incubated for 60 min at 27 °C. Each value represents the mean ± S.D. from six individual experiments. N.S., not significant.

Addition	Tissue ion and water content		
	Water (kg/kg dry wt)	Na^+ (mequiv/kg dry wt)	K^+ (mequiv/kg dry wt)
None (control)	1.76±0.11	122±11	303±11
Dibutyryl cyclic AMP (0.5 mM)	1.79±0.07 (N.S.)	131±7 (N.S.)	294±6 (N.S.)

DISCUSSION

The present observations add to the list of the numerous cellular processes already known to be influenced by cyclic AMP. Our investigation shows that cyclic AMP and its dibutyryl derivative inhibit initial rate of entry and cumulative uptake of organic acids in the rabbit kidney cortex.

It has been shown recently that cyclic AMP penetrates with relative facility the peritubular membrane of renal tubular cells [10]. It has also been claimed that probenecid inhibits cyclic AMP uptake by the isolated perfused rat kidney [10]. It also diminishes urinary excretion of the cyclic nucleotide in humans without altering the plasma levels [11]. These findings suggest that the nucleotide enters the cell by the organic acid transport system. The present finding that dibutyryl cyclic AMP acts competitively to inhibit *p*-aminohippurate entry into renal cells is a strong argument in favour of this hypothesis. In view of these data, it is reasonable to assume that intracellular concentration of dibutyryl cyclic AMP decreases when external *p*-aminohippurate increases. Hence, the observed interference of dibutyryl cyclic AMP with *p*-aminohippurate in the concentration dependence studies might reflect an interaction with the membrane or might instead be a consequence of the decreased cellular concentration of dibutyryl cyclic AMP. This latter interpretation of the kinetic data would be consistent with the observation that *p*-aminohippurate uptake by renal cortical cells was inhibited by compounds known to enhance cellular concentration of cyclic AMP. Indeed, isoproterenol known to stimulate cortical adenyl cyclase inhibits *p*-aminohippurate uptake by kidney cortex, whereas antidiuretic hormone known to stimulate medullary adenyl cyclase has no effect. Uptake of *p*-aminohippurate is also depressed by theophylline. However, the present results do not exclude the possibility that the effect of theophylline was not mediated by an increased cellular concentration of cyclic AMP. Indeed, theophylline which is structurally related to uric acid may act competitively to inhibit the entry of organic acid into renal cells.

It has been claimed recently that methylxanthines inhibit the renal uptake of both cyclic AMP and *p*-aminohippurate [10].

At equimolar concentrations, AMP and ATP cause a smaller effect than cyclic AMP on *p*-aminohippurate uptake, whereas AMP is ineffective on urate transport. Since our results suggest that endogenous cyclic AMP is active as an inhibitor, it might be that these effects of ATP and AMP on *p*-aminohippurate transport are secondary to an enhanced formation of this cyclic nucleotide [12].

It has been recently reported that the uptake of amino acids [1, 2] and sugars [3] was stimulated by cyclic nucleotides after a time lag. Kinetic studies concerning sugars indicated that the stimulation was noncompetitive. Therefore, the effect of cyclic nucleotides and its major characteristics on the transport of these two classes of organic substances are the opposite of those observed for organic acids, using the same tissue preparation. One plausible explanation for this divergence is that our observations reflect events occurring at the basal or blood surface of the cell, whereas studies with amino acids and sugars may be concerned with transport processes occurring at the apical or urinary surface of the proximal tubules. This is in line with the major known sites of proximal tubule transport of these different substances. With regard to uric acid there is now good evidence that its tubular transport occurs in both directions [13–15]. In vitro, however, tubular uptake of uric acid is inhibited not only by pyrazinamide, a urate secretion depressor, but also by probenecid, a urate reabsorption depressor [16].

The importance of Na^+ and K^+ for the transport of organic acids by mammalian kidney cortex has been well documented [16–21]. In other respects, it has been reported that cyclic AMP inhibited the (Na^+–K^+)-dependent ATPase obtained from several mammalian tissues [22, 23]. One theoretical possibility could have been that the observed interference with organic acid accumulation was secondary to depression of cation transport. This possibility, however, seems unlikely since the intracellular concentration of water and electrolytes is unchanged with dibutyryl cyclic AMP.

The findings that organic acid transport was influenced not only by high external concentration of cyclic nucleotides but also by compounds known to enhance endogenous cellular cyclic AMP concentration, suggest that the observed effects are not merely pharmacological. It is, therefore, likely that cyclic AMP might be involved in the regulation of renal cortical transport of organic acids. In view of the effects of cyclic AMP on uric acid transport, we might speculate further that this nucleotide would play a role in the actions of uricosuric and antiuricosuric agents.

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