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# EFFECTS OF DIBUTYRYL CYCLIC AMP ON THE TRANSPORT OF α-METHYL-D-GLUCOSIDE AND α-AMINOISOBUTYRIC ACID IN SEPARATED TUBULES AND BRUSH BORDER MEMBRANES FROM RABBIT KIDNEY

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## Summary

The effect of dibutyryl cyclic AMP on the transport of α-methyl-D-glucoside and α-aminoisobutyric acid in separated tubules and purified brush border membranes from rabbit kidney was investigated using a rapid filtration procedure. Dibutyryl cyclic AMP stimulated the uptake of α-methyl-D-glucoside and α-aminoisobutyric acid by separated renal tubules in agreement with prior studies utilizing renal slices (Rea, C. and Segal, S. (1973) Biochim. Biophys. Acta 311, 615—624; Weiss, I.W., Morgan, K. and Phang, J.M. (1972) J. Biol. Chem. 247, 760—764). However, in contrast to previous reports, no preincubation of the tissue with dibutyryl cyclic AMP was required for stimulation of transport to be manifest. Dibutyryl cyclic AMP stimulated oxygen consumption by separated tubules suggesting that stimulation of transport may occur by a linkage with renal oxidative metabolism. Dibutyryl cyclic AMP increased the uptake of α-aminoisobutyric acid into purified renal brush border membranes. However the uptakes of α-methyl-D-glucoside, proline, leucine and phosphate into brush border membranes were significantly inhibited.

## Introduction

Numerous prior studies have determined that cyclic AMP and its dibutyryl derivative can influence renal metabolism [1] and transport [2—5]. In partic-

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ular, these cyclic nucleotides have been shown to affect the renal cortical transport of the amino acid, α-aminoisobutyric acid [6,7] and the sugar, α-methyl-D-glucoside [8,9].

Weiss et al. reported that dibutyryl cyclic AMP stimulated the uptake of α-aminoisobutyric acid in renal cortical slices from the rat [7]. Characteristics of the response were a delay in stimulation of transport and a blockade of expression of the effect by inhibitors of protein synthesis. These investigators hypothesized that dibutyryl cyclic AMP exerted its effect by stimulating the synthesis of an intermediate involved in amino acid transport. Segal et al. determined that dibutyryl cyclic AMP stimulated uptake of α-methyl-D-glucoside in renal cortical slices from the rat and rabbit [8,9]. Stimulation of transport by dibutyryl cyclic AMP required 90 min of preincubation, however the effect was not abolished by inhibitors of protein synthesis.

In preliminary experiments in our laboratory, we also found stimulation of α-aminoisobutyric acid and α-methyl-D-glucoside transport by dibutyryl cyclic AMP in separated renal tubules of the rabbit. However, it was not required to preincubate the tissue with dibutyryl cyclic AMP in order to obtain the effect on transport. In further exploring the possible mechanisms of the effect of dibutyryl cyclic AMP on renal transport, we examined its relationship to renal oxygen consumption. In addition, to distinguish between metabolic effects and effects at the membrane level, we studied effects of dibutyryl cyclic AMP on sugar and amino acid transport by purified renal brush border membranes.

## Materials and Methods

Separated renal tubules were prepared from New Zealand White rabbits (2—3 kg) by a modification [10] of procedures originally devised by Burg and Orloff [11] and Nagata and Rasmussen [12]. The buffer solution used in experiments with separated renal tubules had the following composition: $NaH_2PO_4/Na_2HPO_4$ buffer, pH 7.4, 10.0 mM; NaCl, 120 mM; KCl, 16.2 mM; $MgSO_4 \cdot 7H_2O$, 1.2 mM and $CaCl_2$, 1.0 mM. Uptake solutions contained 0.1 mM α-methyl-D-glucoside or α-aminoisobutyric acid (unlabelled plus tracer) and dibutyryl cyclic AMP as indicated. Solutions were adjusted to pH 7.4 after addition of all constituents.

Uptake was initiated by addition of 100 μl of tubule suspension (4—8 mg protein) to 1 ml of uptake medium in a 16 ml polypropylene test tube. The tubes were gassed with 100% oxygen for 30 s, capped, and shaken gently in a water bath at 37°C. After the appropriate time, a 0.5 ml aliquot was removed and filtered under vacuum through a Nuclepore filter (8.0 μ pore size). The filter was washed with 4 ml of ice cold buffer and placed in a liquid scintillation vial with 10 ml of Aquasol (New England Nuclear). The length of time for filtering and washing each sample was under 10 s. The vials were then counted in a liquid scintillation spectrometer. Tubule protein was determined by the Biuret method [13].

Oxygen consumption by separated renal tubules was determined with an oxygen monitor (Yellow Springs Instrument Company) equipped with a Clark type oxygen electrode fitted to a 1.6 ml water-jacketed cell. To 1.44 ml of uptake buffer were added 160 μl of tubule suspension containing approx.

10 mg of tubule protein. The suspension was stirred with a magnetic stirrer and the temperature maintained at 37° C by a circulating water bath.

Highly purified renal brush border membranes were prepared by a procedure recently developed in this laboratory [14] utilizing differential and density gradient centrifugation. Membrane purity was determined routinely by assay of alkaline phosphatase [15] and trehalase [16], markers for the renal brush border, $(Na^+ + K^+)$-ATPase [17], marker for basal-lateral membranes, and succinic dehydrogenase [18], a marker for mitochondria. Protein was determined by the BioRad protein assay (BioRad Laboratories). We consistently obtained a 10-fold increase in the specific activity of alkaline phosphatase in the brush border membranes as compared to the initial homogenate, whereas the specific activity of $(Na^+ + K^+)$-ATPase was always reduced as compared with the initial homogenate, indicating substantial purification of brush border membranes with respect to basal-lateral membranes.

In the experiments with brush border membranes, the uptake buffer was 1 mM Tris-Hepes (1 mM Hepes adjusted to pH 7.5 with Tris). The buffer contained either 300 mM mannitol or 100 mM mannitol plus 100 mM NaCl and, in addition, 0.1 mM α-aminoisobutyric acid or α-methyl-D-glucoside (including tracer amounts of the corresponding $^{14}C$-labelled compound) and the dibutyryl cyclic AMP concentration indicated. We also examined the effects of dibutyryl cyclic AMP on the uptake of 0.1 mM L-alanine, L-proline, L-leucine, L-glutamic acid and phosphate. All solutions were re-adjusted to pH 7.5 after addition of all constituents.

Uptake into brush border membranes was initiated by addition of 50 μl of the brush border membrane suspension containing 0.4—0.6 mg of membrane protein to 100 μl of uptake buffer in a 6 ml polystyrene test tube. The tube was shaken gently at 22° C until termination of uptake by addition of 850 μl of ice cold 154 mM NaCl in 1 mM Tris-Hepes, pH 7.5 [19]. The suspension was then rapidly filtered through a Millipore filter (HAWP 0.45 μ) and washed with 4 ml of the stop buffer. The filter was then placed in 10 ml of Aquasol and counted by liquid scintillation spectrometry. The stopping, filtration and washing procedures took less than 10 s. Correction for non-specific binding to the membranes and filters was done by subtracting from all data the value of a blank prepared routinely by adding membranes to a tube to which stop buffer had already been added.

Radioactive isotopes were obtained from New England Nuclear and were as follows: α-methyl-D-[*glucose*-U-$^{14}C$]glucoside, 184 Ci/mol; α-amino[1-$^{14}C$]isobutyric acid, 52 Ci/mol; L-[U-$^{14}C$]alanine, 175 Ci/mol; L-[U-$^{14}C$]proline, 248 Ci/mol; L-[U-$^{14}C$]glutamic acid, 298 Ci/mol; L-[U-$^{14}C$]leucine, 289 Ci/mol; monosodium phosphate carrier free. All other chemicals were of reagent grade purity and were purchased from Sigma.

## Results

The time courses of accumulation of α-methyl-D-glucoside and α-aminoisobutyric acid into separated renal tubules have been presented separately [20] and are hyperbolic up to at least 60 min. Steady-state distribution ratios of approx. 6 for α-methyl-D-glucoside and 2 for α-aminoisobutyric acid are

generally obtained in this laboratory. In the current series of experiments, we examined the effects of dibutyryl cyclic AMP on substrate uptake into the tubules at a single time point, 15 min. Data from a previous study [20] indicate that, at least for α-methyl-D-glucoside, uptake into the separated renal tubules occurs primarily across the brush border membrane.

The data shown in Fig. 1 indicate that the stimulation of α-methyl-D-glucoside and α-aminoisobutyric acid uptake into renal tubules by dibutyryl cyclic AMP increases in an approximately linear manner with respect to time of preincubation prior to the 15 min accumulation period. However, we obtained significant stimulation of uptake by dibutyryl cyclic AMP without any preincubation of the tubules. That is, if the dibutyryl cyclic AMP was present only during the 15 min uptake period, stimulation of α-methyl-D-glucoside and α-aminoisobutyric acid uptake occurred. This result differs from those of Weiss et al. for the amino acid [7] and Rea and Segal for the sugar [8], where preincubation of renal slices for at least 90 min with dibutyryl cyclic AMP was required to produce significant stimulation of uptake.

The data presented in Fig. 2 show that stimulation of uptake of α-methyl-D-glucoside and α-aminoisobutyric acid is also linear with respect to dibutyryl cyclic AMP concentration, up to at least 1 mM. Maximum stimulation of uptake occurred at 10 mM dibutyryl cyclic AMP.

We have previously shown that a number of substrates for renal oxidative metabolism can stimulate the renal transport of sugars and amino acids [20] as well as *p*-aminohippuric acid and uric acid [21]. Since dibutyryl cyclic AMP is rapidly metabolized by renal tubules [22], it appeared possible that the stimulation of transport of α-methyl-D-glucoside and α-aminoisobutyric acid by dibutyryl cyclic AMP might occur by way of its effects on oxidative metabolism. To further explore this possibility, we examined the effect of dibutyryl

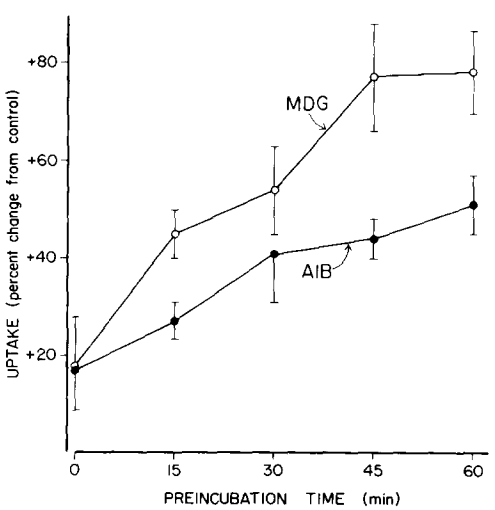


**Fig. 1. Effect of various times of preincubation with dibutyryl cyclic AMP (1 mM) on the uptake of α-methyl-D-glucoside and α-aminoisobutyric acid into separated renal tubules. Data are given as percent change from control ± S.E.M. for uptake at 15 min in six experiments done in duplicate. Zero preincubation indicates that the dibutyryl cyclic AMP was present only during the 15 min uptake period.**

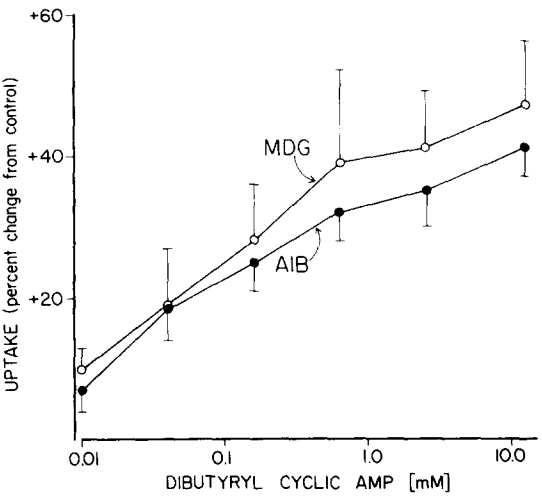


Fig. 2. Effect of various concentrations of dibutyryl cyclic AMP on the uptake of α-methyl-D-glucoside and α-aminoisobutyric acid into separated renal tubules. Data are 15 min uptakes from three experiments done in duplicate.

cyclic AMP on oxygen consumption in separated tubules. As shown in Fig. 3, dibutyryl cyclic AMP stimulated renal oxygen consumption in a concentration dependent manner, however the shape of the curve was non-linear.

Since our data indicated that the effects of dibutyryl cyclic AMP on sugar and amino acid transport might be mediated via oxidative metabolism, it was of particular interest to determine if dibutyryl cyclic AMP affected the transport of α-methyl-D-glucoside and α-aminoisobutyric acid in the absence of cellular metabolism. To that end, we next examined the effects of dibutyryl cyclic AMP on the transport of the sugar and amino acid in purified renal brush border membranes. We first ran a time course of the uptake of α-methyl-D-glucoside and α-aminoisobutyric acid to insure that the time courses of uptake

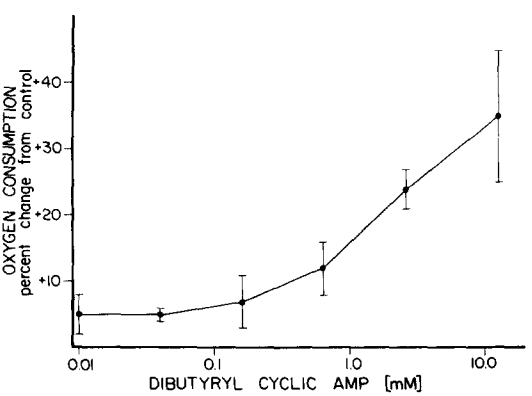


Fig. 3. Effect of various concentrations of dibutyryl cyclic AMP on the oxygen consumption of separated renal tubules measured as described in the text. Data are the mean of three experiments done in duplicate.

into our membranes were comparable to what had been reported previously. Shown in Fig. 4 are the time courses of uptake in the presence and absence of a NaCl gradient. The data for uptake of α-methyl-D-glucoside are similar to those previously obtained for the uptake of D-glucose by rabbit renal brush border membranes [14,19]. The initial rate of uptake was increased approx. 30-fold in the presence of a gradient of NaCl directed into the membrane vesicles. The uptake of α-aminoisobutyric acid was doubled in the presence of a NaCl gradient, however no overshoot was obtained in these experiments.

Using a 1 min time point to estimate the uptake rates, we examined the effects of various concentrations of dibutyryl cyclic AMP on the uptake of α-methyl-D-glucoside and α-aminoisobutyric acid into the brush border membrane vesicles. As shown in Fig. 5, the uptake of α-methyl-D-glucoside into the membranes was inhibited whereas the uptake of α-aminoisobutyric acid was increased.

The finding that dibutyryl cyclic AMP increased the uptake of the amino acid but not the sugar into the brush border membranes was surprising to us. We thus investigated the effects of dibutyryl cyclic AMP on the uptake into the brush border membranes of several other amino acids and also phosphate. Shown in Fig. 6 are the effects of dibutyryl cyclic AMP on the 1 min uptakes of L-alanine, L-proline, L-leucine, L-glutamic acid and phosphate (all at 0.1 mM). Dibutyryl cyclic AMP slightly inhibited the uptake of L-alanine and L-glutamic acid and markedly inhibited the uptake of L-proline, L-leucine and phosphate. The effects of dibutyryl cyclic AMP on amino acid transport in brush border membranes have not previously been reported to our knowledge. Evers et al. [23] have previously shown that $10^{-6}$ M dibutyryl cyclic AMP does not affect phosphate transport by renal brush border membranes, a finding supported by the present results. However, it is clear that higher concentrations of dibutyryl

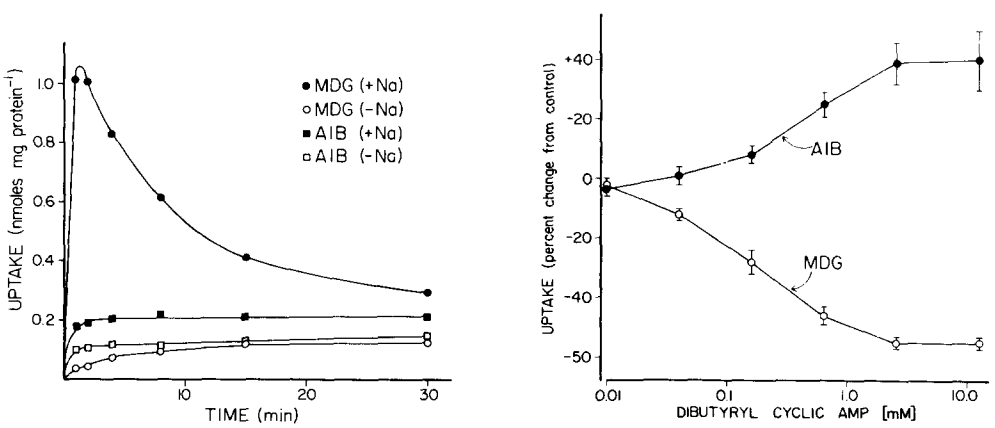


**Fig. 4. Time courses of uptake of 0.1 mM α-methyl-D-glucoside and α-aminoisobutyric acid into purified renal brush border membranes in the presence (+Na) and absence (—Na) of an externally imposed gradient of 100 mM NaCl. Data are from a single typical experiment.**

**Fig. 5. Effect of various concentrations of dibutyryl cyclic AMP on the uptake of α-methyl-D-glucoside and α-aminoisobutyric acid into brush border membranes in the presence of a NaCl gradient. Data are the 1 min uptakes from three experiments done in duplicate.**

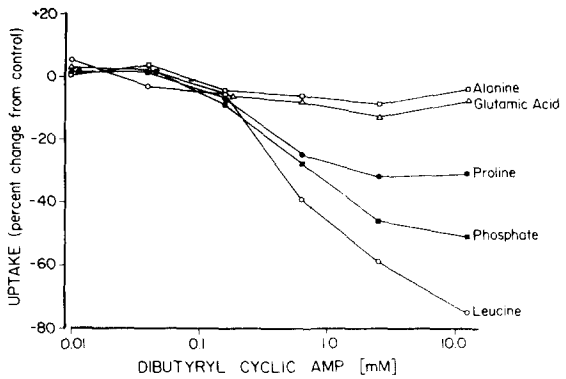


Fig. 6. Effect of various concentrations of dibutyryl cyclic AMP on the uptake of 0.1 mM L-alanine, L-proline, L-leucine, L-glutamic acid and phosphate into renal brush border membranes in the presence of a NaCl gradient. Data are the mean of three experiments done in duplicate.

cyclic AMP substantially inhibit the uptake of phosphate into these membranes.

Finally, we compared the effects on the 1 min uptake of α-methyl-D-glucoside and α-aminoisobutyric acid of 10 mM concentrations of dibutyryl cyclic AMP, cyclic AMP, adenosine-5′-monophosphate, inosine and sodium butyrate (Table I). Uptake of α-methyl-D-glucoside was inhibited 42% by dibutyryl cyclic AMP and 22% by sodium butyrate indicating that the butyryl moiety of the dibutyryl cyclic AMP probably imparts a significant fraction of the inhibitory potency of the molecule. Dibutyryl cyclic AMP stimulated the uptake of α-aminoisobutyric acid by 37% whereas none of the other compounds tested had a significant effect.

TABLE I

EFFECTS OF 10 mM DIBUTYRYL CYCLIC AMP AND RELATED COMPOUNDS ON THE UPTAKE OF α-AMINOISOBUTYRIC ACID AND α-METHYL-D-GLUCOSIDE

Uptake was determined from 1 min time points in the presence of a 100 mM NaCl gradient. Uptake data is given as pmol mg protein$^{-1}$ · min$^{-1}$. Data are the mean of 3 experiments ± S.E.

| Compound | α-Aminoisobutyric acid | | | α-Methyl-D-glucoside | | |
|---|---|---|---|---|---|---|
| | Uptake | Percent change from control | $P$ * | Uptake | Percent change from control | $P$ |
| Control | 192 ± 27 | — | — | 763 ± 153 | — | — |
| Dibutyryl cyclic AMP | 263 ± 40 | +37 | <0.05 | 446 ± 74 | −42 | <0.05 |
| Cyclic AMP | 177 ± 21 | −8 | n.s. ** | 838 ± 154 | +10 | n.s. |
| Adenosine-5′-monophosphate | 166 ± 23 | −14 | n.s. | 845 ± 164 | +11 | n.s. |
| Inosine | 173 ± 23 | −10 | n.s. | 745 ± 141 | −2 | n.s. |
| Sodium butyrate | 203 ± 24 | +6 | n.s. | 598 ± 101 | −22 | 0.05 |

* $P$ determined by Student's $t$ test for paired data.
** n.s., not significant.

## Discussion

The data presented indicate that dibutyryl cyclic AMP stimulated the uptake of α-methyl-D-glucoside and α-aminoisobutyric acid in respiring separated renal tubules. Of particular significance was the finding that preincubation of the tubules with dibutyryl cyclic AMP was not required to obtain stimulation of transport, in disagreement with previous studies utilizing renal slices rather than separated tubules [7,8]. Weiss et al. [7] reported that cycloheximide, an inhibitor of protein synthesis could block the effect of dibutyryl cyclic AMP on the transport of α-aminoisobutyric acid. Thus, it was proposed that the preincubation period necessary to observe the stimulation of transport was due to a time requirement for synthesis of an intermediate involved in amino acid transport. In studies in this laboratory, we have been unable to show any significant effect of cycloheximide on the stimulation of α-aminoisobutyric acid transport in separated tubules by dibutyryl cyclic AMP.

Rea and Segal [8] also reported that preincubation of renal slices with dibutyryl cyclic AMP was required to obtain stimulation of transport of α-methyl-D-glucoside, however cycloheximide was without effect on this stimulation. They hypothesized that a likely reason for the preincubation requirement was slow penetration of dibutyryl cyclic AMP into the renal cells. On the basis of the present studies, it would seem that this hypothesis is probably correct. Thus, in separated renal tubules where accessibility of the dibutyryl cyclic AMP to each cell would be far more rapid than in renal slices, the stimulatory effects would become manifest earlier.

In considering various possibilities for the mechanisms of the stimulation of sugar and amino acid transport by dibutyryl cyclic AMP, it seemed of particular interest to investigate the role of oxidative metabolic processes. A number of compounds which are metabolized by the renal tubule have previously been found to stimulate the transport of sugars and amino acids by separated renal tubules [20]. Furthermore it has recently been shown that dibutyryl cyclic AMP is rapidly metabolized by the renal tubule [22]. Thus we investigated the effect of dibutyryl cyclic AMP on renal oxygen consumption with the finding (Fig. 3) that dibutyryl cyclic AMP stimulated renal oxygen consumption in a concentration dependent manner. The shapes of the curves for the concentration dependence of the stimulation of transport and the concentration dependence for the stimulation of oxidative metabolism did not correspond exactly; at lower concentrations of dibutyryl cyclic AMP the effect on transport was relatively greater than that on oxygen consumption. However, since our current understanding of the linkage between metabolic processes and transport is limited, there is no way of determining if the shapes of the two curves must correspond identically in order to indicate a connection between the stimulation of transport and the stimulation of oxidative metabolism.

In order to further explore the possible mechanisms of the stimulation of transport by dibutyryl cyclic AMP it was of interest to examine its effects on the transport of α-methyl-D-glucoside and α-aminoisobutyric acid across purified renal brush border membranes. In these membranes, the cellular metabolic apparatus has been essentially completely removed. Thus it is possible to examine the effects of dibutyryl cyclic AMP at the membrane level in the absence

of complicating metabolic effects. Furthermore, one can examine transport under conditions where the main driving force for transport, the electrochemical potential difference for sodium [19] is well controlled.

Our experiments showed that dibutyryl cyclic AMP inhibited the uptake of α-methyl-D-glucoside across renal brush border membranes, a finding not surprising to us since we had previously noted (data not shown) that it inhibits the uptake of D-glucose in these membranes. However, the uptake of α-aminoisobutyric acid was increased by dibutyryl cyclic AMP. We thus examined the effect of dibutyryl cycic AMP on the uptake of L-alanine, L-proline, L-glutamic acid and L-leucine, and found inhibition of uptake of all of these amino acids. It should also be pointed out that the uptake of α-aminoisobutyric acid into brush border membranes differed from that of other amino acids reported in previous studies in that no sodium gradient-dependent overshoot was seen (Fig. 4) in contrast to the overshoot seen in the uptakes of L-proline [24], L-alanine [25], L-phenylalanine [26] and glutamic acid [27]. Therefore the use of α-aminoisobutyric acid as a 'model' amino acid in renal transport studies should possibly be reconsidered.

The experiments presented suggest the following overall interpretation. Stimulation of α-methyl-D-glucoside uptake into respiring renal tubules by dibutyryl cyclic AMP could well be mediated via oxidative metabolism. One possible sequence of events is the following: As dibutyryl cyclic AMP enters the cell, the metabolic rate increases (Fig. 3). The increased metabolic rate leads to increased activity of the $(Na^+ + K^+)$-ATPase, causing greater extrusion of sodium from the cell and thus increasing the electrochemical potential difference for sodium across the brush border membrane, increasing the driving force for the sugar uptake. The fact that stimulation of α-methyl-D-glucoside uptake by dibutyryl cyclic AMP does not take place in the absence of metabolism would support the above interpretation.

With respect to the transport of α-aminoisobutyric acid, the situation is more complicated. Dibutyryl cyclic AMP increased the uptake of this amino acid in both respiring tissue and in the brush border membranes. Thus it is not possible, at this time, to know whether the increased uptake in the respiring tissue was due to the increased metabolic rate or to an effect at the membrane level. Furthermore, the reason for the difference between the effects of dibutyryl cyclic AMP on the transport of α-aminoisobutyric acid and the other amino acids examined is not clear. There are at least four systems for amino acid transport in plasma membranes and the degree to which each system contributes to the uptake of each amino acid in the brush border membrane is not yet known. Presumably, inhibition of the transport of the amino acids other than α-aminoisobutyric acid (and also of α-methyl-D-glucoside) occurs through an interaction with brush border membrane components, however kinetic studies are required to further describe the mechanisms of the interactions occurring. Also, our data do not yet provide insight as to why the uptake of α-aminoisobutyric acid into the brush border membranes was increased by dibutyryl cyclic AMP. We cannot, at this point, distinguish between alterations in the driving forces for transport of this compound and alterations at the membrane level, e.g. changes in membrane permeability.

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