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INVOLVEMENT OF CELLULAR CYCLIC AMP IN THEOPHYLLINE-INDUCED SUGAR ACCUMULATION IN CHICKEN INTESTINAL EPITHELIAL CELLS

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We have studied the possible involvement of cellular cyclic AMP in theophylline-induced sugar gradient enhancement in isolated chicken enterocytes. Theophylline increases 3-*O*-methylglucose accumulation 3-fold after 30 min incubation. Exogenous cyclic AMP enhances sugar accumulation by 48%. Adenylyl cyclase inhibitor RMI 12330A reduces theophylline-induced sugar gradients by 22% and theophylline-induced cyclic AMP levels by 24.5%. At the concentration used, RMI 12330A has no effect on 3-*O*-methylglucose accumulation or basal cellular cyclic AMP. Since theophylline has a rapid inhibitory effect on Na^+-independent sugar permeability, we conclude that the effects of the drug on sugar gradients are the result of its acting by both direct – surface membrane – and indirect – cyclic AMP mediated – mechanisms. The effect of theophylline and exogenous cyclic AMP on sugar accumulation is independent of external chloride.

Introduction

Theophylline enhances the accumulation of some sugars and amino acids in the intestinal epithelium [1]. For sugars, this effect of theophylline has been observed for galactose in rabbit ileum [2], glucose in rat jejunum [3] and 3-*O*-methylglucose in isolated chick enterocytes [4], among other epithelia.

The effect of theophylline on sugar accumulation is explained by a drug-induced reduction in serosal permeability to sugars [2,4]. Theophylline has no effect on coupled sodium and sugar bidirectional fluxes across the brush-border membrane [2]. Since this drug is an effective inhibitor of phosphodiesterase, it has been suggested that the permeability of the serosal border to sugars is affected by the tissue levels of cyclic AMP [5] and, indeed, it has been shown that exogenous cyclic AMP mimics the effect of theophylline in the rabbit ileum [2]. However, Randles and Kimmich [4], using isolated chick enterocytes, found no evidence to support any dependence of serosal permeability on cellular cyclic AMP levels, and the effect of theophylline was ascribed entirely to a direct action of the drug on the Na^+-independent transport pathway located at the basolateral membrane. For these reasons, it was considered relevant to investigate further the mechanism of theophylline effect on intestinal sugar accumulation. Some of the results of this study have been reported in abstract form [6].

Methods and Materials

Cell isolation. All experiments were carried out with intestinal epithelial cells obtained from 4–7-week-old Leghorn chickens, according to the method described by Kimmich [7]. The composi-

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tion of incubation medium was: 80 mM NaCl/100 mM mannitol/3 mM K_2HPO_4/1 mM $MgCl_2$/2.5 mM $CaCl_2$/20 mM Tris-HCl (pH 7.4)/0.1 mM EGTA/1 mg per ml bovine serum albumin. Isolation medium contained 1 mg per ml of hyaluronidase in addition to the above components. Osmolarity was 300 mosM.

Sugar determination. Measurement of unidirectional influx and steady-state accumulation of ^{14}C-labelled substrates was determined by removing 200-μl samples of the cell suspension. These samples were added to 1.8 ml of ice-cold medium and centrifuged. The pellets were washed twice with ice-cold medium to eliminate the extracellular radioactivity prior to extraction of cellular sugar with 3 ml per dl perchloric acid. The quantitation of labelled sugar was by scintillation counting.

Protein content was determined by the Biuret method [8]. The results are expressed in nanomoles of sugar accumulated per milligram of cell protein. Studies were done without preincubation interval for all the agents studied.

Cyclic AMP determination. 2-ml samples from the incubation medium were rapidly centrifuged. The cellular pellets were frozen in solid CO_2/acetone and resuspended in 4 mM EDTA in order to minimize cyclic AMP degradation. Protein was precipitated in warm oil (105°C). After centrifugation, 50-μl samples were taken from the supernatant and assayed for cyclic AMP in triplicate (Amersham Cyclic AMP Assay Kit, code TRK.432).

Materials. 2-Deoxy-D-glucose, 1-*O*-methyl α-D-glucopyranoside (α-methylglucoside), theophylline, phloridzin and $N^6O^{2'}$-dibutyryl adenosine 3',5'-(cyclic)monophosphate were purchased from Sigma Chemical Co., St. Louis, U.S.A.; 3-*O*-methylglucose was purchased from Koch-Light, Colnbrook, Bucks, U.K.; RMI 12330A (*N*-(*cis*-2-phenylcyclopentyl) azacyclotridecan-2-imine hydrochloride) was a gift from Richardson-Merrell Inc., Cincinnati, U.S.A.; 2-deoxy-D-[U-^{14}C]glucose, 3-*O*-methyl-D-[U-^{14}C]glucose and 1-*O*-methyl α-D-[U-^{14}C]glucopyranoside were purchased from Amersham, U.K. The other reagents were obtained from commercial suppliers.

Statistics. When appropriate, data were analyzed by analysis of variance and were considered statistically significant at $P < 0.05$.

Results

We first verified the main theophylline effects described by Randles and Kimmich [4] in similar experimental conditions to those used by these authors. The influx of 2-deoxyglucose (1.5 mM), a sugar that is not a substrate for the Na^+-dependent transport system, is shown in Fig. 1. During the short incubation period used, uptake of 2-deoxyglucose is linear with time and is a good indicator of the permeability of the Na^+-independent sugar pathway. The figure also shows that theophylline inhibits 2-deoxyglucose influx by 65% after 60 s incubation. The inhibition is already observed after 20 s incubation, which indicates that the drug has a rapid effect on cell membrane permeability. In longer incubation periods, and using 3-*O*-methylglucose as substrate, theophylline enhances sugar accumulation 3-fold compared to controls (in min 30) as shown in Fig. 2. Phloridzin induces a marked inhibition on sugar accumulation, as expected. Accumulation ratios in control and theophylline-treated cells in min 30 were 6 and 16, respectively. As a whole, this set of results seems to show that theophylline behaves as a rather specific inhibitor of the Na^+-independent sugar transport system, agreeing with the results obtained by Randles and Kimmich [4].

We then tested the effect of exogenous cyclic AMP on sugar accumulation. Fig. 2 shows the effect of 2 mM dibutyryl-cyclic AMP on the steady-state 3-*O*-methylglucose gradient. Dibutyryl-cyclic AMP increases sugar accumulation

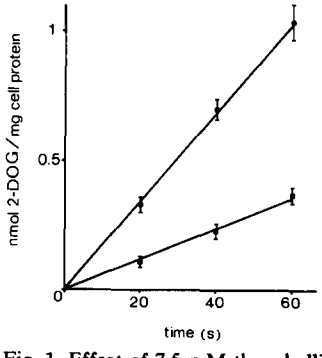

Fig. 1. Effect of 7.5 mM theophylline (■) on Na^+-independent unidirectional influx of 2-deoxyglucose (DOG) (1.5 mM) into isolated intestinal epithelial cells. ●, Control. Mean ± S.E. (n = 3).

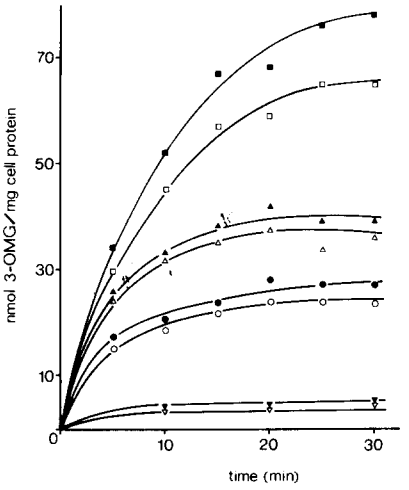

Fig. 2. Effect of 7.5 mM theophylline (■, □) or 2 mM dibutyrylcyclic AMP (▲, △) on steady-state gradients of 3-*O*-methylglucose (OMG) (1.5 mM) maintained by isolated intestinal epithelial cells. Phloridzin (▼, ▽) was used at a concentration of 0.15 mM. ●, ○, Control. Each point is the mean of duplicate results from two separate experiments, as indicated by filled and blank symbols.

by 48% in min 30. To test the hypothesis that the effect of theophylline in long-term experiments involves cellular cyclic AMP, we used the drug RMI 12330A. This drug has been shown to prevent intestinal secretion induced by cholera toxin by inhibiting adenylyl cyclase activity [9]. RMI 12330A also inhibits the increase in tissue levels of

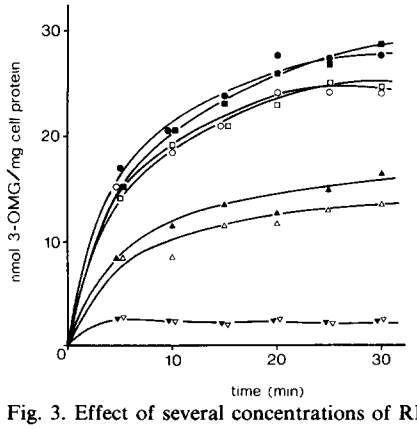

Fig. 3. Effect of several concentrations of RMI 12330A (▼, ▽, 0.5 mM; ▲, △, 0.05 mM; ■, □, 0.005 mM) on steady-state gradients of 3-*O*-methylglucose (OMG) (1.5 mM) maintained by isolated intestinal epithelial cells. ●, ○, Control. Each point is the mean of duplicate results from two separate experiments, as indicated by filled and blank symbols.

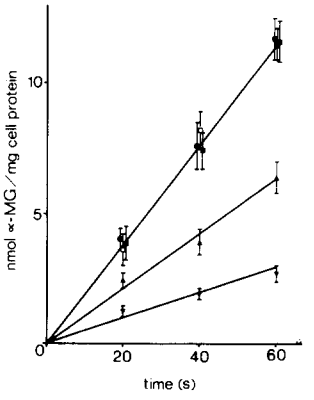

Fig. 4. Effect of RMI 12330A (●, 0.005 mM; ▲, 0.05 mM), 7.5 mM theophylline (□) and 0.15 mM phloridzin (▼) on unidirectional influx of α-methylglucoside (α-MG) (1.5 mM) into isolated intestinal epithelial cells. ●, Control. Mean ± S.E. (n = 3).

cyclic AMP, induced by theophylline in rabbit ileum [10]. We first tested the effect of several concentrations of RMI 12330A on 3-*O*-methylglucose accumulation by the isolated enterocytes. Fig. 3 shows that 0.5 and 0.05 mM RMI 12330A inhibit net sugar accumulation, the effect of 0.5 mM being similar to that of 0.15 mM phloridzin, but 5 μM RMI 12330A does not affect the sugar gradient in the cells (Fig. 3). However, the possibility of an effect of the drug on the Na^+-dependent pathway was studied using α-methylglucoside as specific substrate. The results (Fig. 4) show that the unidirectional influx of α-methylglucoside is

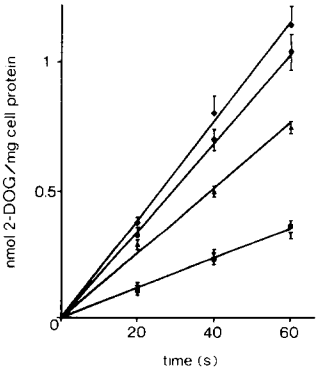

Fig. 5. Effect of RMI 12330A (◆, 0.01 mM; ▲, 0.05 mM), 7.5 mM theophylline (■) and 0.01 mM RMI 12330A plus 7.5 mM theophylline (▼) on unidirectional influx of 2-deoxyglucose (DOG) (1.5 mM) into isolated intestinal epithelial cells. ●, Control. Mean ± S.E. (n = 3).

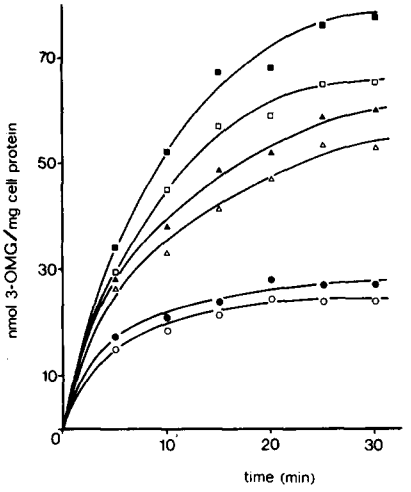

Fig. 6. Effect of 7.5 mM theophylline with (▲, △) or without (■, □) 5 μM RMI 12330A on steady-state gradients of 3-*O*-methylglucose (OMG) (1.5 mM) maintained by isolated intestinal epithelial cells. ●, ○, Control. Each point is the mean of duplicate results from two separate experiments, as indicated by filled and blank symbols.

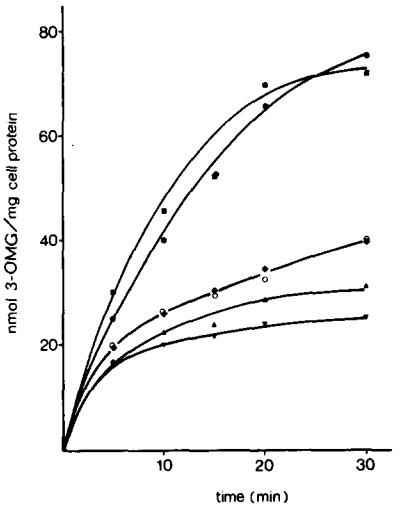

Fig. 7. Effect of 7.5 mM theophylline or 2 mM dibutyryl-cyclic AMP on 3-*O*-methylglucose (OMG) accumulation by isolated intestinal epithelial cells incubated in media containing Cl^- or SO_4^{2-} as only anion. In case with SO_4^{2-} incubation medium contained (in mM): 40, Na_2SO_4; 120, mannitol; 3, K_2SO_4; 2.5, $CaSO_4$; 1, $MgSO_4$; 20, Tris-SO_4 (pH 7.4); 0.1, EGTA; 1.5, 3-*O*-methylglucose; and 1 mg/ml bovine serum albumin. ▼, Cl^- control; ▲, SO_4^{2-} control; ◆, Cl^- dibutyryl-cyclic AMP; ○, SO_4^{2-} dibutyryl-cyclic AMP; ■, Cl^- theophylline; ●, SO_4^{2-} theophylline. The results represent typical data obtained from three separate experiments.

not affected by a low concentration of RMI 12330A. The next step was to study whether RMI 12330A was capable of affecting theophylline effects in sugar accumulation and the results are shown in Figs. 5 and 6. Fig. 5 shows that 0.05 mM RMI 12330A inhibits 2-deoxyglucose influx by 30% after 60 s incubation, while a lower concentration (0.01 mM) has no effect on Na^+-independent sugar entry either in the presence or absence of theophylline. However, 5 μM RMI 12330A significantly inhibits the sugar gradient enhancement induced by theophylline (Fig. 6). This inhibition was 22% in min 30.

To ascertain whether 5 μM RMI 12330A is capable of inhibiting the increase in cytosolic cyclic AMP induced by theophylline, we determined cellular cyclic AMP levels after 40 min incubation either with the drug alone or with the drug plus theophylline. Taking cyclic AMP levels in control incubated cells as 100, those in cells incubated with theophylline were 1433 ± 261 (mean ± S.E.; $n = 7$), whereas cyclic AMP levels in cells incubated with theophylline plus RMI 12330A were 1082 ± 221 (24.5% reduction; $P < 0.05$). Cyclic AMP concentration in cells incubated with RMI 12330A alone did not differ from controls (87 ± 8; $P < 0.05$). The above results support the view that steady-state sugar gradients induced by theophylline are dependent, in part, on cyclic AMP cellular levels.

Finally, the effect of theophylline and exogenous cyclic AMP on steady-state sugar accumulation was studied in the absence of Cl^-, in order to know if the sugar gradient enhancement could be ascribed to a change in cellular Na^+ due to cyclic AMP-mediated inhibition on coupled NaCl entry. The results (Fig. 7) indicate, first, that the effects of both theophylline and dibutyryl-cyclic AMP are independent of exogenous Cl^- and, second, that Cl^- substitution by SO_4^{2-} allows the establishment of greater gradients in both control and dibutyryl-cyclic AMP-treated cells, but not in cells incubated with theophylline.

Discussion

One way to control sugar accumulation by intestinal epithelium is the regulation of serosal permeability to the substrate. The addition of drugs that reduce serosal sugar efflux such as theophyl-

line, cytochalasin B and some flavonoids [4,11,12], increases sugar gradients more than predicted for a 1 : 1 Na^+/sugar stoichiometry [13].

The primary purpose of this study was to characterize the mechanism by which theophylline enhances the accumulation of sugars by intestinal tissue. Theophylline can interfere with the function of the Na^+-independent transport system regardless of the direction of net flux occurring through the system [2,4]. Thus, the inhibition of unidirectional 2-deoxyglucose influx (Fig. 1) is interpreted as an indication that the drug restricts the sugar exit through that pathway. This is confirmed in long-term incubation of the cells with theophylline, which results in the establishment of greater concentration gradients, as shown in Fig. 2, also reported by Randles and Kimmich [4]. The time lag of the theophylline effect is short, as 2-deoxyglucose influx is already inhibited by 66% after 20 s incubation. Consequently, the rapid effect of theophylline on serosal permeability may be interpreted as a direct interaction of the drug with the serosal surface of the membrane. Theophylline does not affect sugar permeability at the brush border [2,4] (Fig. 4).

The incubation of a wide variety of tissues with theophylline results in an increase in cellular cyclic AMP levels, since the drug inhibits phosphodiesterase. Table I lists the effect of theophylline on cyclic AMP concentration in intestinal tissue from four species. The table shows that there is great variability in the response, even in epithelia (or cells) from the same species. The marked discrepancy in cyclic AMP levels obtained in the present study after theophylline treatment (14-fold) as compared to those reported by Hyun and Kimmich [17] (2-fold) is noteworthy.

TABLE I

EFFECT OF THEOPHYLLINE ON CYCLIC AMP LEVELS IN INTESTINAL TISSUE FROM SEVERAL ANIMAL SPECIES

Epithelium	Theophylline concentration in the medium (mM)	Increase in cellular [cAMP] compared to controls (-fold)	Time elapsed from the addition of theophylline (min)	Ref.
Rabbit ileum	5	11	5	14
Rabbit ileum	10	7	30	10
Trout intestine	10	5	15	15
Rat jejunum	7	2–3	40	16
Chick enterocytes	5	2	40	17
Chick enterocytes	7.5	14	40	present study

Since cellular cyclic AMP enhancement is a consequence of theophylline treatment, a possible role of the nucleotide in the maintenance of high sugar gradients in the presence of the drug cannot be excluded a priori. The study of the effects of exogenous cyclic AMP on sugar accumulation has yielded conflicting results. Holman and Naftalin [2] found that 1 mM dibutyryl-cyclic AMP has effects on transmural galactose flux and tissue accumulation equivalent to the maximal response elicited by 5 mM theophylline, whereas Randles and Kimmich [4] found no alteration on cellular sugar transport capability by added exogenous cyclic AMP. Our results, using 2 mM dibutyryl-cyclic AMP, indicate that the nucleotide increases sugar accumulation by 48% after 30 min incubation, the increase being statistically different from both control and theophylline-treated cells (Fig. 2).

In an attempt to further correlate cyclic AMP levels and the effect of theophylline, cells were incubated with the adenylyl cyclase inhibitor RMI 12 330A, and this resulted in a significant inhibition in both sugar gradient and cellular cyclic AMP content. This supports the view that steady-state sugar gradients induced by theophylline are dependent, at least in part, on cellular cyclic AMP levels.

A possible explanation of cyclic AMP effects is that increased sugar accumulation results from an increase in the Na^+ gradient across the membrane. In this way, Holman and Naftalin [2] suggested a correlation between the control of NaCl-coupled entry and the effect of theophylline. This effect has been confirmed neither by Randles and Kimmich [4] nor by the findings reported here, since Cl^- substitution by SO_4^{2-} does not prevent the increase in sugar gradient induced by either theophylline or exogenous cyclic AMP. An alternative possibility is that the nucleotide regulates an un-

coupled Na^+ entry, as shown in experiments by Hyun and Kimmich [17], in which a 8–12-fold increase in cellular cyclic AMP reduces by 60% the total Na^+ influx in chick enterocytes, an effect also shared by exogenous cyclic AMP.

In conclusion, our results show that theophylline affects sugar accumulation in enterocytes by both direct – surface membrane – and indirect – cyclic AMP mediated – mechanisms. The precise role of cyclic AMP in this process awaits further clarification.

Acknowledgement

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