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Calcium-dependence of sugar transport in rat small intestine

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The involvement of Ca^{2+} in the theophylline action on sugar transport was investigated in isolated rat small intestinal mucosa. Theophylline significantly increased cell water free sugar accumulation and reduced mucosal to serosal sugar fluxes both in the presence and absence of calcium, but the effects of theophylline were significantly less in calcium free media. In theophylline untreated tissues, calcium-deprived bathing solutions decreased tissue galactose accumulation and increased mucosal to serosal sugar flux. The calcium-channel blocker verapamil produced similar effects on intestinal galactose transport to those induced by low extracellular calcium activity. RMI 12330A and the calmodulin antagonist trifluoperazine abolished the theophylline-effects on intestinal galactose transport. Both drugs also affected sugar transport in basal conditions. These studies suggest that calcium might modulate sugar permeability across the basolateral boundary of rat enterocytes, and that its effect may be mediated by calmodulin.

Introduction

Theophylline has been shown to affect different aspects of the small intestinal function: (i) it reverses the normal direction of net NaCl movement (see Ref. 1 for recent review), (ii) it increases amino acid accumulation in intact rat small intestine [2,3], and (iii) it enhances sugar accumulation in intact rat jejunum [4], in intact rabbit ileum [5] and in isolated chicken enterocytes [6,7]. Theophylline-dependent intestinal secretion appears to result from an increase in Cl^- conductance across the mucosal border of the intestinal epithelia [8]. On the other hand the effect of theophylline on intestinal organic solutes transport seems to result from a reduction in their permeability across the basolateral cell border [5,6].

It has been suggested that activation of intestinal secretion by theophylline is mediated by an increase in intracellular free calcium concentration [9–11], which after binding to the calcium-binding protein, calmodulin, would increase mucosal Cl^- conductance [9].

Recently, it has been reported that theophylline reduces basolateral sugar permeability in isolated chicken enterocytes by both a direct effect at the surface membrane and by increasing intracellular cAMP levels [7].

Since theophylline appears to interfer with different intestinal transport processes, it was considered of interest to investigate whether all the theophylline effects on intestinal epithelia were mediated by the same intracellular messenger, i.e. Ca-calmodulin complex.

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Methods and Materials

Animals, incubations solutions

Male albino Wistar rats weighing 150–200 g were anesthetized with ether and killed by ether overdose. A segment of distal small intestine was removed and rinsed free of intestinal contents with ice-cold Ringer's solution. The tissue was then stripped of its serosal and external muscle layers using the method of Powell and co-workers [12]. The Ringer's solution contained, in mM: 140 NaCl, 10 $KHCO_3$, 0.4 KH_2PO_4, 2.4 K_2HPO_4, 1.2 $CaCl_2$ and 1.2 $MgCl_2$, and was continuously bubbled with 95% O_2/5% CO_2. In experiments where Ca^{2+}-free conditions were required, Ca^{2+} was omitted from the bathing solutions and 0.5 mM EGTA was added to remove interstitial calcium.

Transepithelial flux measurements

The stripped mucosa was mounted as a flat sheet in Ussing-type chambers. The bathing solutions on the mucosal and serosal surfaces of the tissue were maintained at 37°C using a circulating water bath as described previously [13]. Both solutions contained 2 mM D-galactose. Mucosal to serosal sugar fluxes were measured by placing the ^{14}C-labelled galactose in the mucosal side. Samples were removed from the not radioactively labelled side at 10-min intervals for 30 min, after a 20-min preincubation period. One sample only was taken for counting from the radioactively labelled side. Samples of the radioactive solution were counted using a liquid scintillation counter.

Extracellular space determinations

Pieces of isolated mucosa were incubated in Ringer's solution at 37°C containing 0.2 μCi/ml ^3H-labelled poly(ethylene glycol) (mol. wt. 4000, PEG 4000, New England Nuclear) for 20 min. The pieces of mucosa were then gently blotted on filter paper and weighed, then extracted in 1 ml 0.1 M HNO_3 overnight. Aliquots of the extracts were then counted together with aliquots of the bathing solutions. Following extraction the tissues were dried at 80°C for 24 h, then reweighed. Tissue water was calculated as the difference between wet and dry weights.

The test agents (0.1 mM trifluoperazine, 0.1 mM RMI 12330A and 3 mM theophylline) were present in the bathing solutions from the start of the incubation. None of the modifiers caused a significant effect neither on the extracellular space (ranging from 0.19 ± 0.01 to 0.22 ± 0.03 ml/g wet weight), nor on tissue water fraction (ranging from 0.83 ± 0.01 to 0.85 ± 0.01 ml/g wet weight), nor on cell water fraction (ranging from 0.62 ± 0.02 to 0.66 ± 0.01 ml/g wet weight). The extracellular space was also measured in the tissue, mounted in the Ussing-type chamber as described above, in control conditions and in the presence of 3 mM theophylline. 0.2 μCi/ml ^3H-PEG 4000 was added to the serosal bathing solution. After a 30-min incubation period the chambers were opened and the exposed circle (0.5 cm^2) of tissue was cut out. Then the tissue was extracted as described above. The extracellular space, expressed as $\mu l \cdot cm^{-2}$, measured under control conditions (18 ± 0.10) was not significantly modified by theophylline (17 ± 0.10).

Sugar uptake measurements

Pieces of distal small intestine, weighing about 50 mg, were incubated at 37°C in Ringer's solution containing D-galactose labelled with ^{14}C for 20 min. At the end of the experiment the tissues were washed with gentle shaking in ice-cold Ringer's solution and blotted carefully on both sides to remove excess moisture. The tissue was weighed wet and extracted by shaking for 15 h in 1 ml 0.1 M HNO_3. Samples were taken from the bathing solutions and from the extracts of the tissues for radioactivity counting.

All the modifiers were added to the incubation solution at the beginning of the incubation period.

In a few experiments the concentration of free sugar within the cells, in control tissues and in the presence of the modifiers used in the current study, was estimated by precipitating the phosphorylated sugar from the tissue, using the $Ba(OH)_2$ and $ZnSO_4$ method [14]. It was found that the amount of phosphorylated derivatives in the tissue ranged from 10% to 13% of the total sugar measured with ^{14}C.

The results, after being corrected for the phosphorylated galactose and extracellular space, are expressed as the cell/medium ratio.

Materials

D-Galactose and theophylline were obtained from Sigma (St. Louis, MO). Trifluoperazine was a gift from Smith, Kline and French Laboratories. R.M.I. 12330A was a gift from Merrel Dow Pharmaceuticals INC (Cincinnati). DL-Verapamil was supplied by Knoll AG, (Ludwigshafen, F.R.G.).

Statistics

Results are expressed as mean ± S.E. Statistical significance was evaluated by the two-tailed Student's *t*-test.

Results

1. Effect of theophylline on the steady-state cell water D-galactose accumulation

Under control conditions the C/M ratio (cell water free sugar concentration/sugar concentration in the bathing solution) was about 2.14 at 0.5 mM galactose and 2.46 at 1 mM galactose (see Table I). 3 mM theophylline caused a 2-fold increase in the C/M ratios. 5 mM theophylline also increased C/M ratio, but the effect was even lower than that induced by 3 mM theophylline.

2. Effect of theophylline on the transepithelial mucosal to serosal D-galactose fluxes

The first row of results summarized in Table II shows that theophylline (3 mM) significantly ($p < 0.001$) reduced mucosal to serosal galactose movement. This finding agrees with those reported by Holman and Naftalin [5] in rabbit ileum.

TABLE II

TRANSMURAL MUCOSAL TO SEROSAL D-GALACTOSE FLUXES ACROSS SHEETS OF RAT SMALL INTESTINE

Sugar concentration in the bathing solutions was 2 mM. Values presented are the means ± S.E. of the number of experiments indicated between brackets.

	J_{ms}^{Gal} (μmol·cm^{-2}·h^{-1})	
	Control	Theophylline (3 mM)
No addition	0.70 ± 0.017(38)	0.39 ± 0.02(41) [a]
RMI 12330A (0.1 mM)		0.71 ± 0.04(18)
Trifluoperazine (0.1 mM)		0.75 ± 0.04(27)

[a] $p < 0.001$ test compared with control (column 1).

3. Effect of bathing solution calcium on tissue D-galactose accumulation and on mucosal to serosal D-galactose fluxes

In theophylline untreated tissues, low extracellular Ca^{2+} activity significantly ($p < 0.001$) decreased tissue galactose accumulation and increased mucosal to serosal sugar flux (see Table III). Furthermore Ca^{2+}-removal from the bathing solutions significantly reduced, but did not abolish, the theophylline effects on both tissue sugar accumulation and sugar fluxes. The calcium-dependent decrease in the theophylline effects on tissue sugar accumulation and sugar fluxes did not differ significantly.

These results are consistent with the view that sugar efflux across the serosal boundary might be affected by changes in intracellular Ca^{2+} concentration.

TABLE I

EFFECT OF DIFFERENT CONCENTRATIONS OF THEOPHYLLINE ON THE CELL TO MEDIUM SUGAR CONCENTRATION RATIO (C/M)

The concentration of sugar in the incubation solutions was either 0.5 mM or 1 mM. Values presented are the means ± S.E. Between brackets the number of independent determinations.

	C/M		
Theophylline (mM):	0	3	5
Galactose (0.5 mM)	2.14 ± 0.08(14)	4.73 ± 0.27(12) [a]	3.62 ± 0.3 (12) [a]
Galactose (1 mM)	2.46 ± 0.12(17)	5.06 ± 0.30(17) [a]	4.12 ± 0.36 (6) [a]

[a] $p < 0.001$ test compared with control (column 1).

TABLE III

EFFECT OF CALCIUM-DEPRIVED BATHING SOLUTIONS ON BOTH (A) CELL TO MEDIUM SUGAR CONCENTRATION RATIO (C/M) AND (B) MUCOSAL TO SEROSAL GALACTOSE FLUXES

Values presented are the means ± S.E.; the figure in parentheses is the number of independent estimates of the mean. Cont: tissues incubated in the absence of theophylline; theo.: tissues incubated in the presence of 3 mM theophylline. p, comparations between tissues exposed to calcium deprived and normal Ringer bathing solutions; p^*, theophylline-treated tissues compared with non-treated tissues.

Ca^{2+} (mM)	C/M		p^*	J_{ms}^{Gal} (μmol·cm^{-2}·h^{-1})		p^*
	Cont	Theo.		Cont	Theo.	
1.2	2.02 ± 0.08(29)	4.08 ± 0.21(27)	< 0.001	0.74 ± 0.04(15)	0.35 ± 0.03(16)	< 0.001
0 + 0.5 mM EGTA	0.81 ± 0.03(44)	1.20 ± 0.05(37)	< 0.001	0.92 ± 0.04(18)	0.67 ± 0.03(18)	< 0.001
p	< 0.001	< 0.001		< 0.001	< 0.001	

TABLE IV

EFFECT OF 0.2 mM DL-VERAPAMIL ON TRANSEPITHELIAL MUCOSAL TO SEROSAL GALACTOSE (2 mM) FLUXES

Values presented are the means ± S.E. between brackets the number of independent determinations. p^*, theophylline-treated tissues compared with non-treated tissues; p, verapamil-treated tissues compared with non-treated tissues.

	J_{ms}^{Gal} (μmol·cm^{-2}·h^{-1})		p^*
Theophylline (mM):	0	3	
No addition	0.52 ± 0.02(15)	0.16 ± 0.01(15)	< 0.001
Verapamil	0.65 ± 0.03(15)	0.49 ± 0.01(15)	< 0.001
p	< 0.005	< 0.001	

TABLE V

EFFECTS OF RMI 12330A AND TRIFLUOPERAZINE ON CELL TO MEDIUM SUGAR CONCENTRATION RATIO (C/M)

Sugar concentration in the bathing solutions was 0.5 mM. Values presented are the means ± S.E. Between brackets the number of independent determinations.

	C/M		
Theophylline (mM):	0	3	5
No addition	2.50 ± 0.08(16)	5.15 ± 0.13(13) [a]	4.66 ± 0.095(18) [a]
RMI 12330A (0.1 mM)	2.09 ± 0.04(26) [b]	2.19 ± 0.08(10) [b]	2.19 ± 0.08 (16) [b]
Trifluoperazine (0.1 mM)	2.02 ± 0.08(19) [b]	2.12 ± 0.13 (9) [b]	2.11 ± 0.20 (10) [b]

[a] $p < 0.001$ test compared with control (column 1).
[b] $p < 0.001$ comparisons between tissues treated with either RMI 12330A or trifluoperazine and untreated tissues (row 1).

4. Effects of DL-verapamil on transepithelial mucosal to serosal D-galactose fluxes

To supplement the evidence obtained from the studies with low extracellular calcium activity we determined the effects of the 'calcium-channel' blocker DL-verapamil [15,16] on mucosal to serosal sugar movement.

The addition of verapamil (0.2 mM) to the bathing solutions produced a similar pattern of results to those obtained in the absence of extracellular calcium (see Table IV). Mucosal to serosal sugar flux was increased in theophylline untreated tissues and the theophylline effect on sugar flux was not completely abolished in the presence of verapamil.

5. Effects of trifluoperazine and RMI 12330A on intestinal D-galactose transport

Trifluoperazine is a phenothiazine which binds with high affinity and specificity to the Ca-

calmodulin complex [17], preventing the calmodulin from activating a wide variety of cellular processes [17,18].

RMI 12330A has been shown to prevent intestinal secretion by inhibiting secretagogues-induced adenylyl cyclase activity [10,19] and to compete with [^{3}H]trifluoperazine for the Ca^{2+}-dependent binding site of calmodulin [10].

Both drugs, RMI 12330A and trifluoperazine, prevented the theophylline effects on intestinal galactose transport (see Tables II and V), having a small but significant effect ($p < 0.001$) on tissue sugar accumulation in the absence of theophylline (see Table V).

Discussion

Sugar efflux across the basolateral boundary of the intestinal epithelial cells has been shown to take place via a Na^+-independent, facilitated diffusion system [6,20]. When this process is inhibited by theophylline the cells are allowed to establish much greater sugar concentration gradients [6,7].

The experimental results presented in the current study show that theophylline decreased mucosal to serosal D-galactose fluxes (see Table II) and enhanced tissue sugar accumulation (see Table I). These findings suggest that the drug may act as an inhibitor of sugar exit across the basolateral enterocytes border, as reported earlier in isolated chicken enterocytes [6,7].

Calcium has been implicated as a possible intracellular mediator of the theophylline action on intestinal electrolytes transport [9–11,21]. The results summarized in Table III show that Ca^{2+}-free bathing solutions significantly reduced, but did not abolish, the changes in intestinal galactose transport induced by theophylline. This partial inhibition could be due to a decrease in intracellular Ca^{2+}. This explanation, however, seems unlikely inasmuch as preincubations of 45 min or longer in Ca^{2+}-free bathing solutions are needed to reduce available intracellular Ca^{2+} [21]. On the other hand theophylline has been reported to rise passive Ca^{2+} permeability in rabbit ileum [9].

Low extracellular calcium activity also affected galactose transport in theophylline untreated tissues (see Table III). The calcium-channel-blocker verapamil (see Table IV) produced a similar pattern of results to those obtained in the absence of extracellular calcium. Furthermore, it has been reported that the Na^+-linked sugar transport across the brush-border is not impaired by lowering extracellular Ca^{2+} activity [11,22].

Taken together these findings suggests that intracellular Ca^{2+}, linked to extracellular Ca^{2+}, may be involved in the control of sugar efflux across the serosal border of the tissue, in basal conditions and in theophylline treated tissues. That is, lowering intracellular Ca^{2+} by exposure to Ca^{2+}-free bathing solutions or verapamil appears to increase serosal sugar efflux.

Many intracellular effects of Ca^{2+} require binding to calmodulin [24], which has been shown to be present in the intestinal epithelial cells [25]. That the Ca^{2+}-calmodulin antagonists, trifluoperazine and RMI 12330A [10,17,18], prevented the theophylline-effects on intestinal galactose transport (see Table II and V) suggests a role for calmodulin in controlling intestinal sugar transport.

These findings also indicate that the effect of theophylline which remained after the calcium was prevented from coming into the cells, may be as well mediated by an increase in cytosolic Ca^{2+}. It has been suggested that theophylline may release Ca^{2+} from intracellular stores via an increase in cAMP [23]. Therefore, it appears that both external and internal sources of Ca^{2+} are involved in the response to theophylline.

The observation that both, trifluoperazine and RMI 12330A decreased tissue sugar accumulation in theophylline untreated tissues, might suggest that under basal conditions there would be enough intracellular Ca^{2+} to activate calmodulin and hence to reduce serosal sugar exit.

Though RMI 12330A has been said to inhibit Ca-calmodulin, the possibility that the drug may also act by preventing the cAMP-dependent release of Ca^{2+} from intracellular stores can not be ruled out.

Though the contribution of the muscularis mucosa to the overall changes in intestinal galactose transport has not been determined, previous reports [6,7,20] and the extracellular space measurements (see Methods and Materials) support the view that the changes in galactose transport reported in the current study might be due, as

well, to changes in sugar permeability at the basolateral enterocyte border.

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