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Role of Calcium in the Phloretin-Effects on Sugar Transport in Rat Small Intestine

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The effect of phloretin on D-galactose transport in rat small intestine has been investigated. Phloretin enhanced tissue sugar accumulation and reduced mucosal to serosal D-galactose fluxes. Calcium-deprived bathing solutions and verapamil significantly reduced, but did not abolish, the phloretin-effects on intestinal galactose transport. Furthermore in the presence of the anticalmodulin drugs, RMI 12330A and trifluoperazine, phloretin was without effect on D-galactose transport. These findings suggest that phloretin may reduce serosal sugar permeability via an increase in Ca^{2+}-calmodulin complex.

Key words: Phloretin, Galactose, Intestine, Calcium.

Sugar exit across the basolateral cell boundary has been shown to occur via a Na^+ —independent facilitated diffusion system. When this process is inhibited by either theophylline or phloretin the cells established much greater sugar concentration gradients (see reference 12 for recent review).

In previous papers it was reported (10) that theophylline reduces serosal sugar permeability in rat small intestine and that this effect appears to be Ca^- dependent.

The present work was designed to investigate the effect of phloretin on galactose transport in rat small intestine. In addition to examining the general features of phloretin action, the role of Ca^{2+} in the phloretin-effects on intestinal sugar transport has also been studied.

Materials and Methods

Male albino Wistar rats weighing 150-200 g, were anaesthetized with ether and killed by ether overdose. A segment of

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distal small intestine was rapidly 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 *et al.* (15).

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.

Pieces of distal small intestine, weighing about 50 mg, were incubated at 37 °C in Ringer's solution, containing 0.5 mM D-galactose and tracers of ^{14}C-D-galactose, 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 N HNO_3. Samples were taken from the bathing solutions and from the extracts of the tissues for radioactivity counting. The test agents were present in the incubation solutions from the start of the incubation period. The results after being corrected for the extracellular space are expressed as μmol galactose $\times$ ml^{-1} cell water.

In a few experiments the extracellular space was estimated by using ^{3}H-labelled poly-(ethylene-glycol) (mol. wt. 4000 NEN) as previously described (10). None of the modifiers, used in the current study, caused a significant effect in cell water content (total water-extracellular water) (ranging from 0.60 ± 0.02 to 0.65 ± 0.02 ml $\times$ g^{-1} w.w., n = 8).

The stripped mucosa was mounted as a flat sheet in Ussing-type chambers. The bathing solutions on the mucosal and serosal surfaces of the tissues were maintained at 37 °C using a circulating water-bath as described previously (14). Both solutions contained 2 mM D-galactose. Mucosal to serosal fluxes were measured by placing the ^{14}C-labelled D-galactose in the mucosal bathing solution. Samples were removed from the cold side at 10 min intervals for 30 min following addition of the isotope. Only one sample was taken from the hot side. Samples of the radioactive solution were counted using a liquid scintillation counter.

Results are expressed as mean $\pm$ S.E. Statistical significance was evaluated by the two-tail Student's t-test for paired variates.

D-galactose and phloretin were obtained from Sigma. Trifluoperazine was a gift from Smith, Kline and French Laboratories. RMI 12330A was a gift from Merrel Dow Pharmaceuticals INC. dl-Verapamil was supplied by Knoll AG.

Results

Effect of phloretin on D-galactose transport in rat small intestine. — In the absence of exogenous modifiers tissue D-galactose accumulation was about 1.07 ± 0.03 μmol · ml^{-1} cell water and mucosal to serosal sugar flux was $0.59 \pm \pm 0.01$ μmol · cm^{-2} · h^{-1}. Phloretin significantly increased tissue sugar accumulation (table I, first row) in a concentration-dependent manner. Also mucosal to serosal galactose fluxes were significantly reduced by 0.1 mM phloretin (table II).

In the presence of 0.1 mM phloretin tissue sugar accumulation (2.07 ± 0.05 μmol · ml^{-1} cell water) did not differ significantly from values measured either in the presence of 3 mM theophylline alone (2.28 ± 0.02 μmol · ml^{-1} cell water) or with theophylline and phloretin together (2.00 ± 0.03 μmol · ml^{-1} cell water).

Effect of RMI 12330A and Trifluoperazine on intestinal sugar transport. —

Table I. *D-Galactose uptake (μmol D-galactose/ml cell water) into stripped rat small intestine under different experimental conditions.*
Sugar concentration in the bathing solution was 0.5 mM. Values are the means ± S.E. of the number of estimates indicated between brackets. TFP: Trifluoperazine.

	Phloretin (mM)			
	0	0.001	0.05	0.1
No addition	1.07 ± 0.03 (23)	1.56 ± 0.032 (28)[a]	1.62 ± 0.04 (23)[a]	1.88 ± 0.05 (22)[a]
RMI 12330A (0.1 mM)	0.91 ± 0.02 (28)[b]		0.94 ± 0.03 (28)[b]	0.95 ± 0.04 (27)[b]
TFP (0.1 mM)	0.89 ± 0.02 (20)[c]		0.88 ± 0.03 (20)[b]	0.87 ± 0.05 (21)[b]

[a] $p < 0.001$ Test compared with control (column 1); [b] $p < 0.001$; [c] $p < 0.005$ Comparisons between tissues treated with either RMI 12330A or trifluoperazin and untreated tissues (row 1).

Table II. *Mucosal to serosal 2 mM D-galactose flux (μmol D-galactose · cm^{-2} · h^{-1}).*
The concentration of the test agents in the bathing solutions was 0.1 mM. TFP: Trifluoperazine. Values are the means ± S.E. Between brackets the number of independent determinations.

Control	Phloretin	Phloretin + RMI 12330A	Phloretin + TPF
0.59 ± 0.01 (40)	0.31 ± 0.02 (19)*	0.55 ± 0.02 (19)	0.57 ± 0.03 (22)

* $p < 0.001$ *vs* control.

Table III. *Effect of Ca-deprived bathing solutions on D-galactose transport in phloretin (0.1 mM) untreated and treated tissues.*
Values are the means ± S.E. Between brackets the number of independent estimates of the mean. Comparisons between tissues exposed to calcium deprived and normal Ringer bathing solutions: $p < 0.001$.

$[Ca^{2+}]$	J_{ms}^{cal} (nmol · cm^{-2} · h^{-1})		μmol · ml^{-1} cell water	
mM	Control	Phloretin	Control	Phloretin
1.2	0.76 ± 0.02 (19)	0.29 ± 0.01 (18)*	1.12 ± 0.03 (27)	1.97 ± 0.03 (28)*
0 + 0.5 EGTA	0.91 ± 0.03 (18)	0.62 ± 0.03 (18)*	0.65 ± 0.03 (28)	0.96 ± 0.04 (28)*

* Phloretin treated tissues compared to phloretin untreated tissues: $p < 0.001$.

The effects of the adenylate cyclase inhibitor RMI 12330A (19) and of the calmodulin antagonist trifluoperazine (13, 21) on intestinal galactose transport are shown in table I and II. Both drugs significantly reduced tissue sugar accumulation and prevented the phloretin-dependent effects on both tissue sugar accumulation and on transepithelial mucosal to serosal galactose flux.

Effect of Ca-deprived bathing solutions on basal intestinal sugar transport and on phloretin action on intestinal sugar transport. — Intestinal galactose transport was measured under normal conditions and in Ca^{2+}-free bathing so-

Table IV. *Effect of 0.2 mM dl-Verapamil on intestinal D-galactose (0.5 mM) accumulation and transmural mucosal to serosal D-galactose (2 mM) fluxes.*
Values are the means ± S.E. Between brackets the number of independent determinations.

	μmol D-galactose/ml cell water		J_{sm}^{Gal} (μmol D-galactose · cm^{-2} · h^{-1})	
dl-Verapamil (mM)	Control	Phloretin (0.1 mM)	Control	Phloretin (0.1 mM)
0	1.27 ± 0.04 (8)	2.45 ± 0.06 (10)*	0.52 ± 0.05 (15)	0.17 ± 0.03 (15)*
0.2	1.01 ± 0.05 (10)	1.45 ± 0.05 (10)*	0.72 ± 0.07 (16)	0.48 ± 0.05 (15)*
p	< 0.005	< 0.001	< 0.05	< 0.001

* Phloretin treated tissues compared with nontreated tissues ($p < 0.001$).
p = Comparisons between tissues exposed to dl-Verapamil and normal Ringer's bathing solutions.

lutions, which contained 0.5 mM EGTA, with or without 0.1 mM phloretin. Table III shows that low extracellular Ca^{2+} activity significantly increased transepithelial mucosal to serosal galactose fluxes and decreased tissue sugar accumulation. Furthermore, in the absence of extracellular Ca^{2+} the effects of phloretin on both tissue sugar accumulation and sugar fluxes were significantly reduced.

Effect of dl-Verapamil on intestinal galactose transport. — To supplement the evidence obtained from studies with Ca^{2+} - free conditions, the effect of verapamil, an agent thought to block the Ca^{2-} - channels of cell membranes (3, 4), was determined.

The addition of verapamil (0.2 mM) to the fluid bathing the tissue produced a similar pattern of results to those obtained in the absence of extracellular Ca^{2+} (table IV). That is, phloretin untreated tissues verapamil increased mucosal to serosal galactose fluxes and decreased tissue sugar accumulation. Furthermore the phloretin-effects on sugar transport were significantly reduced in the presence of verapamil.

Discussion

Some sugars have been shown to cross the basolateral enterocytes boundary by a carrier-mediated system, which is Na^+ - independent and inhibited by theophylline and phloretin (12).

The results presented in the current study indicate that phloretin produced similar effects on D-galactose transport in rat small intestine to those previously found in isolated chicken enterocytes (12), i.e., phloretin increased tissue sugar accumulation (table I). Moreover phloretin reduced mucosal to serosal sugar movement (table II). The fact that the phloretin and theophylline-effects on tissue sugar accumulation were similar to each other and not synergistic, suggests that both drugs may interfere with the same intestinal process. All together these findings are consistent with the view that phloretin may act by reducing sugar permeability across the serosal border of rat small intestine.

Phloretin has been reported (1) to inhibit the Na^+ - energized D-glucose transport process. However, if that were the only effect of phloretin, tissue galactose accumulation would have been reduced by addition of the drug. Furthermore, that phloretin affected tissue sugar accumulation (1.8 - fold increase) and mucosal to serosal sugar fluxes (1.9-fold decrease) to a comparable degree, is consistent with an effect of phloretin on

serosal sugar efflux rather than on mucosal sugar uptake.

The current study further shows that Ca-deprived bathing solutions significantly reduced, though did not abolish, the effects of phloretin on intestinal galactose transport (table III). Low extracellular Ca-activity also increased intestinal galactose absorption in basal conditions.

Since the integrity of the epithelial tight junctions is Ca-dependent (5), the effects on sugar transport caused by Ca^{2+} removal from both bathing solutions simultaneously may have occurred because of tissue damage. However this seems unlikely inasmuch as the Ca-channel blocker verapamil (table IV) produced a similar pattern of results to those produced by low extracellular Ca-activity.

The findings discussed above suggest that high intracellular Ca^{2+} might reduce serosal sugar efflux. The effects observed when Ca^{2+} was prevented from coming into the cells, may be thought to be due to a decrease in available intracellular calcium rather than to a decrease in Ca-influx into the tissue. However, preincubations of 45 min or longer have been demonstrated to be necessary to partially deplete intracellular Ca^{2+} stores (22). Therefore external Ca^{2+} appears to be involved in the control of sugar transport in basal conditions and in the response to phloretin in the rat small intestine.

Many of the intracellular effects of Ca^{2+} appears to be mediated by calmodulin (7), which has been shown to be present in the intestinal epithelial cells (8). Most of the evidence for this role of calmodulin is indirect and based on studies using drugs that bind to and inhibit the Ca-calmodulin actions. Both trifluoperazine (13, 21) and RMI 12330A (11) have been described as anticalmodulin drugs. The fact that both trifluoperazine and RMI 12330A prevented the phloretin-effects on intestinal sugar transport is consistent with the view that calmodulin might be involved in the intestinal sugar transport response to phloretin.

It is not at first sight apparent how phloretin could lead to an increase in intracellular free Ca^{2+} concentration. Phloretin has been reported to possibly inhibit Cl^- transport in red blood cells (6, 17, 18) and increase K^+ conductance in lipid membranes (2) by altering the intramembrane dipole potential. Ca-permeability could also be postulated to be modified by this mechanism. On the other hand, RANDLES and KIMMICH (16) found that Phloretin induces a 23 % decrease in total cellular ATP. Since low cytoplasmatic Ca^{2+} concentration is maintained by the action of (Ca^{2+}, Mg^{2+})-ATPases which actively extrude Ca^{2+} at the expense of ATP hydrolysis, a decrease in ATP supply would also lead to an increase in intracellular Ca-concentration. At least in lymphocytes a reduction in ATP levels caused a slow rise in intracellular Ca^{2+} (20).

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Resumen

Se estudia el efecto de la floretina sobre el transporte de D-galactosa por intestino delgado de rata. La floretina aumenta la acumulación tisular de galactosa y disminuye el flujo del azúcar desde mucosal a serosal; este efecto es inhibido tanto por la ausencia de Ca^{2+} del medio de incubación como por la presencia de verapamilo. En presencia de RMI 12330A o de trifluoperazina, inhibidores de la acción biológica de la calmodulina, el transporte intestinal de galactosa no se modifica por la floretina. Todos estos resultados parecen sugerir que la floretina reduce la permeabilidad serosal al azúcar vía la formación del complejo Ca-calmodulina.

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