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The effect of glucose on the activity of phosphofructokinase in the mucosa of rat small intestine

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In common with other phosphofructokinase isoenzymes, phosphofructokinase in the epithelial cells of rat small-intestinal mucosa is activated by fructose 2,6-bisphosphate. However, fructose 2,6-bisphosphate was found not to be present in mucosa as judged by three criteria: (1) chromatography on Sephadex G-25 of crude mucosal extracts from fed rats did not result in a decrease, or indeed any change, in the activity of phosphofructokinase under suboptimal conditions at pH7; (2) ultrafiltrates of mucosal extracts did not possess any acid-labile activating activity when tested against chromatographed liver phosphofructokinase; (3) phosphofructokinase-2 activity was not detectable in mucosal extracts. Furthermore, the perfusion *in vitro* of isolated loops of jejunum or the incubation of mucosal scrapings from either fed rats or rats starved for 48h showed that the activity of mucosal phosphofructokinase is not subject to short-term regulation by glucose. These observations are consistent with the view that phosphofructokinase is the rate-limiting enzyme of glycolysis in intestinal mucosa and account for the fact that the rate of glucose utilization by rat small intestine is not very responsive to changes in the concentration of glucose in the lumen.

In previous work we have presented evidence consistent with the view that phosphofructokinase is the rate-limiting enzyme of glycolysis in the epithelial cells of rat small-intestinal mucosa and that its activity under suboptimal conditions at pH7 is diminished in starved or streptozotocin-diabetic rats (Jamal & Kellett, 1983*a*,*b*). A similar diminution in the activity of liver phosphofructokinase (Nieto & Castano, 1980) is mediated by the effector fructose 2,6-bisphosphate, the concentration of which is controlled by glucose and glucagon (Van Schaftingen *et al.*, 1980*a*,*b*; Uyeda *et al.*, 1981; Pilkis *et al.*, 1981; Hers & Van Schaftingen, 1982). Thus the incubation of hepatocytes from starved rats with glucose results in a large increase in the concentration of fructose 2,6-bisphosphate and activation of phosphofructokinase, whereas the converse is true when hepatocytes from fed rats are incubated with glucagon (Van Schaftingen *et al.*, 1980*a*,*b*; Claus *et al.*, 1980; Hue *et al.*, 1981; Richards *et al.*, 1981; Hue *et al.*, 1982).

In contrast with liver, the rates of glucose utilization and lactate production by rat small intestine are known not to be very responsive to changes in the concentration of glucose in the lumen (Hanson & Parsons, 1976; Windmueller & Spaeth, 1980; Nicholls *et al.*, 1983). We have therefore investigated whether fructose 2,6-bisphosphate and glucose play a significant role in the regulation of mucosal phosphofructokinase.

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Materials and methods

The preparation of extracts of jejunal mucosa and the assay of phosphofructokinase activity were as described previously (Jamal & Kellett, 1983*a*). The regulatory properties of phosphofructokinase were expressed in terms of the activity ratio, v/V, where v is the activity observed at pH7.0 in the presence of 2.5mM-ATP and V is the maximal activity at pH8.0. One unit of activity is defined as the formation of 1μmol of fructose 1,6-bisphosphate/min at 27°C. Liver extracts were prepared and assayed in the same way as mucosal extracts. Phosphofructokinase-2 activity was assayed as described by Van Schaftingen & Hers (1981).

Chromatography of mucosal or liver extracts to separate fructose 2,6-bisphosphate from phosphofructokinase was performed on a column

(22cm × 2.5cm) of Sephadex G-25 equilibrated with extraction buffer minus trypsin inhibitor. Mucosal or liver ultrafiltrates for the assay of fructose 2,6-bisphosphate were then prepared as follows. Tissue samples were homogenized in 100mM-imidazole buffer, pH7.0, containing 30mM-KF, 1mM-EDTA and 5mM-2-mercaptoethanol, and centrifuged at 75000*g* for 30min at 4°C. The resulting supernatant was then centrifuged in a Centriflo (Amicon) membrane cone with M_r cut-off limit 25000. The ultrafiltrate was tested for the presence of fructose 2,6-bisphosphate by the extent that the addition of 50μl to the assay cuvette increased the activity ratio at 2.0mM-fructose 6-phosphate, $v_{2.0}/V$, of liver phosphofructokinase that had been previously freed of fructose 2,6-bisphosphate by chromatography on Sephadex G-25. The K_a of the liver enzyme for fructose 2,6-bisphosphate under the conditions of the assay was approx. 1.8×10^{-8}M, so that initial concentrations of fructose 2,6-bisphosphate as low as 0.1μM could be determined. The acid-lability of the activating activity, characteristic of fructose 2,6-bisphosphate, was determined by adjusting the pH of the ultrafiltrate to pH2 for 30min at 22°C, followed by neutralization and re-assay (Van Schaftingen *et al.*, 1980*a*,*b*). When fructose 2,6-bisphosphate was added to mucosal extracts immediately after homogenization at concentrations of 0.2–1.0μM, the average recovery by the procedures described was 68±4%. Fructose 2,6-bisphosphate was stable when added to mucosal extracts for at least 3h.

The effect of glucose on phosphofructokinase activity was studied in isolated loops of whole intestine *in vitro* by the method of Fisher & Parsons (1949). Jejunal loops were bathed on the serosal side in a medium of Krebs–Henseleit buffer only and perfused luminally at 37°C, with a gas-lift apparatus, with a recirculated medium of Krebs–Henseleit buffer containing glucose at a concentration of 0, 5 or 28mM. At the end of the perfusion mucosal extracts were prepared and assayed as above. Control extracts were prepared from mucosal scrapes taken directly from control rats without perfusion.

The effect of glucose on phosphofructokinase activity and fructose 2,6-bisphosphate content of mucosa was also determined by incubation of suspensions of mucosal scrapings with glucose. In these experiments, approx. 150–200mg of mucosal scrapings were added to 5ml of Krebs–Henseleit buffer containing glucose at a concentration of 0, 5 or 28mM. The 25ml polypropylene flask was quickly gassed and stoppered, shaken manually for a few seconds to disperse the mucosa, and the resulting suspension incubated for 10min at 37°C in a shaking water bath (80 strokes/min). The suspension was then centrifuged at 800*g* for 30s, the supernatant fluid all but decanted, so that the firm mucosal pellet was just covered with incubation buffer, and the pellet frozen in liquid N_2 while still in the plastic centrifuge tube. Samples were stored overnight in liquid N_2 and then homogenized directly in 4vol. (v/w) of extraction buffer without first thawing, before assay of phosphofructokinase activity and fructose 2,6-bisphosphate content.

Mucosal samples for the determination of intracellular glucose were first frozen in liquid N_2; tissue glucose was then determined as described by Leese (1974).

Fructose 2,6-bisphosphate and other biochemicals were obtained from Sigma.

Results

Mucosal extracts from fed rats were first examined for the presence of fructose 2,6-bisphosphate by looking for a decrease in activity ratio of phosphofructokinase after chromatography of extracts on Sephadex G-25, in the same way as reported for the separation of fructose 2,6-bisphosphate from the liver isoenzyme (Van Schaftingen *et al.*, 1980*a*). In contrast with liver, no change in the fructose 6-phosphate saturation curve of mucosal phosphofructokinase was observed (Fig. 1*a*); $v_{0.5}/V$ was 0.45±0.03 and 0.40±0.02 ($n = 5$) before and after chromatography respectively. Because it was possible that fructose 2,6-bisphosphate might bind much more strongly to the mucosal than to the liver isoenzyme, tests were performed to determine whether chromatography on Sephadex G-25 was sufficient to separate fructose 2,6-bisphosphate from mucosal phosphofructokinase.

Fructose 2,6-bisphosphate was first added to mucosal extracts from a fed rat to a concentration of 10μM, when a large activation was observed and the usual sigmoidal fructose 6-phosphate saturation curve became hyperbolic (Fig. 1*a*). When this augmented extract was chromatographed on Sephadex G-25, a large decrease in activity of phosphofructokinase was observed, the fructose 6-phosphate saturation curve became sigmoidal once again and, indeed, was indistinguishable from that for phosphofructokinase either in the original crude extract (not augmented) or after the chromatography of the latter on Sephadex G-25 (Fig. 1*a*).

Despite the fact that fructose 2,6-bisphosphate added to mucosal extracts from fed rats could be readily separated from phosphofructokinase, it was still possible that mucosal phosphofructokinase might possess a distinct class of tight-binding sites for fructose 2,6-bisphosphate, that could be responsible for the difference in $v_{0.5}/V$ between fed rats and rats starved for 48h, and from which

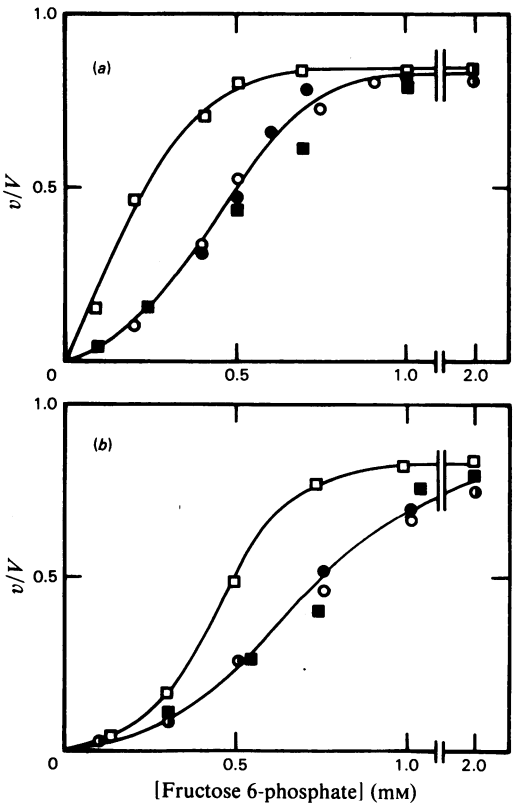

Fig. 1. *Effect of Sephadex G-25 chromatography on the fructose 6-phosphate saturation curves of mucosal extracts*
The fructose 6-phosphate saturation curves were determined at pH 7.0 in the presence of 2.5 mM-ATP as described by Jamal & Kellett (1983*a*). (*a*) Mucosal extract from a fed rat before (○) and after (●) chromatography on Sephadex G-25; extract from a fed rat, augmented with 10 μM-fructose 2,6-bisphosphate, before (□) and after (■) chromatography. (*b*) As for (*a*), but with extracts prepared from rats starved for 48 h. Overlapping points are denoted by half-filled symbols. For experimental details see the text.

fructose 2,6-bisphosphate could not be readily removed. This was not the case, however, for fructose 2,6-bisphosphate could be completely separated from phosphofructokinase after its addition at a concentration of 10 μM to extracts prepared from starved rats. The fructose 6-phosphate saturation curve of the augmented extract was less sigmoidal than for the crude extract, though it was not completely hyperbolic. Chromatography of augmented extract resulted in a large decrease in phosphofructokinase activity and in a fructose 6-phosphate saturation curve that was identical within experimental error with that of the crude extract (Fig. 1*b*). When the latter was chromatographed on Sephadex G-25, no change in the fructose 6-phosphate saturation curve was observed (Fig. 1*b*); $v_{0.5}/V$ was 0.22 ± 0.02 and 0.20 ± 0.03 ($n = 5$) before and after chromatography respectively.

By using extracts that had been prepared from the mucosa of fed rats and that had been previously chromatographed on Sephadex G-25, the K_a of phosphofructokinase was determined to be 0.6 μM at 0.5 mM-fructose 6-phosphate and 2.5 mM-ATP. These concentrations correspond closely to the midpoint of the fructose 6-phosphate saturation curve (Fig. 1*a*), where the enzyme activity is most sensitive to changes in the concentrations of other effectors. The K_a confirms that fructose 2,6-bisphosphate does not bind unusually strongly to mucosal phosphofructokinase.

Mucosal extracts from fed rats were also tested for the presence of fructose 2,6-bisphosphate by determining the acid-labile activating activity of ultrafiltrates by using chromatographed liver phosphofructokinase as the test system. The behaviour of the liver system shown in Table 1 is similar to that described by Van Schaftingen *et al.* (1980*a*). Thus, when liver extracts from fed rats were chromatographed on Sephadex G-25, a large decrease in $v_{2.0}/V$ of liver phosphofructokinase was observed. The addition of 50 μl of liver ultrafiltrate to the assay medium restored $v_{2.0}/V$ for chromatographed extract to that for the extract, but the activating activity of the ultrafiltrate was completely abolished by prior exposure of the ultrafiltrate to acid, confirming that it was caused by fructose 2,6-bisphosphate. By using this working test system for the assay of fructose 2,6-bisphosphate, it was found that 50 μl of ultrafiltrate derived from mucosal extracts prepared from fed rats caused a significant activation of chromatographed liver phosphofructokinase, though to a much lesser extent than liver ultrafiltrate. However, in contrast with the latter, the prior exposure of mucosal ultrafiltrate to acid did not abolish the activation or even diminish its extent. Thus the activating activity of mucosal ultrafiltrates was acid-stable and could not be ascribed to fructose 2,6-bisphosphate.

The time taken between the removal and homogenization of a mucosal scrape was approx. 2 min. It was possible in principle then mucosa contained fructose 2,6-bisphosphate, but that it was degraded before homogenization. This seemed most unlikely, for we have previously examined extracts prepared within 10 s from small amounts of mucosa (approx. 20 mg wet wt.), removed from intestine *in situ* by using a rapid sampling device, and shown that the regulatory properties of phosphofructokinase are the same as those in samples obtained after 2 min (Jamal & Kellett,

1983*a*). Nevertheless the possibility was investigated with mucosal suspensions incubated at 37°C for 10min with glucose (0, 5 and 28mM) and then frozen in liquid N_2 in the centrifuge tube while the pellet was still covered with a little incubation buffer, so that effectively no time elapsed between incubation and homogenization. No acid-labile activating activity was detectable in extracts prepared in this way from fed rats, confirming the absence of fructose 2,6-bisphosphate from mucosa (Table 1).

The activity of phosphofructokinase-2 in crude liver extracts was determined to be 1.6 ± 0.2nmol/min per g wet wt. ($n = 5$). This value is the same as that reported by Kuwajima & Uyeda (1982), but smaller than that reported by Van Schaftingen & Hers (1981). Phosphofructokinase-2 activity was not detectable in mucosal extracts.

Because fructose 2,6-bisphosphate is an intracellular signal for glucose (Hers & Van Schaftingen, 1982), glucose-perfusion studies of whole intestine *in vitro* were performed to determine whether or not mucosal phosphofructokinase activity was subject to regulation by glucose. Table 2 shows that, when isolated loops of jejunum from rats starved for 48h were luminally perfused with glucose at either 5 or 28mM for either 15 or 60min, no change in $v_{0.5}/V$ was observed compared with control samples from mucosal scrapes obtained directly from starved rats without any perfusion. The same was true when loops were perfused in the absence of glucose for up to 60min. Table 2 also shows that, when similar perfusions of isolated loops of jejunum from fed rats were performed, no change in $v_{0.5}/V$ was observed compared with control samples from fed rats, irrespective of whether glucose was present or not.

It is not a simple matter to determine the

Table 1. *Acid-labile activating activity of ultrafiltrates prepared from extracts of liver and mucosa from fed rats*

Extracts were prepared from fed rats by using either tissue samples obtained directly (liver and mucosa) or mucosal suspensions after incubation with glucose for 10min at 37°C. The acid-labile activating activity of ultrafiltrates prepared from these extracts was then assayed by determining the effect that 50μl of ultrafiltrate had on $v_{2.0}/V$ of chromatographed liver phosphofructokinase. The values of $v_{2.0}/V$ for liver extracts prepared from fed rats were 0.82 ± 0.03 and 0.44 ± 0.03 before and after chromatography on Sephadex G-25 respectively. For full experimental details see the text. Values of $v_{2.0}/V$ are given as means ± S.E.M. for three or four experiments each. Significant differences before and after exposure of an ultrafiltrate to acid are denoted: $^*P < 0.001$.

	$v_{2.0}/V$ of phosphofructokinase in chromatographed liver extracts	
Ultrafiltrate source	+ Untreated ultrafiltrate	+ Acid-exposed ultrafiltrate
Liver (tissue)	0.84 ± 0.03	0.46 ± 0.02*
Mucosa (tissue)	0.62 ± 0.03	0.60 ± 0.03
Mucosal suspension in:		
0mM-glucose	0.59 ± 0.04	0.59 ± 0.04
5mM-glucose	0.61 ± 0.02	0.63 ± 0.02
28mM-glucose	0.60 ± 0.04	0.58 ± 0.02

Table 2. *Effect of glucose on the activity ratio of mucosal phosphofructokinase in isolated loops of jejunum from fed and starved rats*

For experimental details see the text. Values of $v_{0.5}/V$ are given as means ± S.E.M. for the numbers of rats in parentheses. The values of the activity ratio for control scrapes are taken from the data of Jamal & Kellett (1983*a*). No significant differences between perfusions with and without glucose were observed. The difference between any starved or fed sample was highly significant ($P < 0.001$).

	Perfusion				
Status of rat	Glucose (mM)	Time (min)	$v_{0.5}/V$	$v_{0.5}/V$ (average)	Control scrape $v_{0.5}/V$
Starved for 48h	0	15	0.18 ± 0.02 (3)	0.18 ± 0.02 (3)	
	5	15	0.24 ± 0.02 (4)		0.21 ± 0.01 (14)
	28	15	0.21 ± 0.02 (4)	0.22 ± 0.01 (11)	
	28	60	0.19 ± 0.02 (3)		
Fed	0	15	0.42 ± 0.04 (4)		
	0	30	0.43 ± 0.03 (4)	0.42 ± 0.02 (11)	
	0	60	0.42 ± 0.02 (3)		
					0.45 ± 0.02 (23)
	5	15	0.37 ± 0.02 (4)		
	28	15	0.48 ± 0.04 (5)	0.44 ± 0.02 (12)	
	28	60	0.45 ± 0.02 (3)		

Table 3. *Effect of glucose on the activity ratio of mucosal phosphofructokinase in suspensions of mucosa from fed and starved rats*

Mucosal suspensions were incubated with glucose for 10min at 37°C. For experimental details see the text. Values of $v_{0.5}/V$ are given as means ± S.E.M. for the numbers of rats in parentheses. No significant differences between samples with or without glucose are observed. The difference between any starved or fed sample was highly significant ($P<0.001$).

Status of rat	Glucose (mM)	$v_{0.5}/V$	$v_{0.5}/V$ (average)
Starved for 48h	0	0.26±0.01 (3)	0.26±0.01 (3)
	5	0.27±0.01 (4)	0.26±0.01 (7)
	28	0.26±0.02 (3)	
Fed	0	0.39±0.02 (4)	0.39±0.02 (4)
	5	0.42±0.03 (4)	0.42±0.02 (8)
	28	0.42±0.03 (4)	

intracellular concentration of glucose in mucosa when loops of intestine are perfused luminally with glucose, because of the inevitable contamination from the lumen. However, a glucose concentration of 28mM is sufficient to saturate the glucose-transport system of the brush border, and the data of Davidson & Leese (1977) and of Bronk & Ingham (1979) show that under such conditions the intracellular concentrations of glucose in isolated loops lacking vascular clearance will be in excess of 20mM. When isolated loops of jejunum are perfused in the absence of glucose, intracellular glucose is depleted very rapidly. As a consequence, determination of mucosal glucose in samples taken from such perfusions with jejunum from both fed and starved rats showed that in every sample the intracellular glucose concentration was so low that it could not be measured. Hence perfusions of jejunum from both fed and starved rats were performed over a range of intracellular mucosal glucose concentrations from effectively 0 to over 20mM.

When the effect of glucose on phosphofructokinase activity was studied with mucosal suspensions incubated with glucose (0, 5 and 28mM), results similar to those from perfusion experiments were obtained (Table 3).

Discussion

The chromatography on Sephadex G-25 of extracts prepared from the mucosa of fed rats does not result in a decrease in the activity of mucosal phosphofructokinase under suboptimal conditions at pH7; neither do ultrafiltrates of mucosal extracts possess acid-labile activating activity when tested against chromatographed liver phosphofructokinase, nor does mucosa possess phosphofructokinase-2 activity. By these criteria, therefore, fructose 2,6-bisphosphate is not present in mucosa, a conclusion consistent with our finding that the perfusion of whole intestine or the incubation of suspensions of mucosa from both fed rats and rats starved for 48h did not affect the activity of mucosal phosphofructokinase over a wide range of glucose concentration. The mucosal enzyme, which is a distinct isoenzyme of phosphofructokinase (Khoja & Kellett, 1983), was nevertheless strongly activated by fructose 2,6-bisphosphate (K_a 0.6μM). In this respect, it is similar to erythrocyte phosphofructokinase, which is also activated by fructose 2,6-bisphosphate, even though the latter and phosphofructokinase-2 are not present in erythrocytes (Heylen *et al.*, 1982; Kuwajima & Uyeda, 1982).

When rats are starved for 48h, glucose utilization is diminished by some 60% (Hanson & Parsons, 1978; Windmueller & Spaeth, 1978). We have reported evidence supporting the idea that phosphofructokinase is the principle rate-limiting enzyme of glycolysis in small-intestinal mucosa, rather than hexokinase, and have shown that the combination of a decrease in total activity and an increase in the susceptibility of phosphofructokinase to inhibition by ATP is sufficient in principle to account for the observed diminution in glucose utilization on starvation (Jamal & Kellett, 1983*a*). Our present observations are in keeping with this view. Thus a particular feature of glycolysis in rat small intestine is that the rates of glucose utilization and lactate production are not very responsive to changes in the concentration of glucose in the lumen. For example, the rate of lactate production is enhanced less than 2-fold when the concentration of glucose in the lumen is varied from 0 to 28mM at a fixed concentration of vascular glucose (Tables 2 and 6 of Hanson & Parsons, 1976; Table 2 of Nicholls *et al.*, 1983). Furthermore, intraluminal loading of glucose at a concentration of 100mM does not significantly alter the concentrations of glycolytic intermediates determined after 10min in freeze-clamped whole intestine from fed rats (Lamers & Hulsmann, 1973). Similarly, luminal glucose at a concentration of 70mM, 10

times higher than in blood, does not significantly enhance glucose utilization in starved rats (Windmueller & Spaeth, 1980). Such relative unresponsiveness of the rate of glycolysis by rat small intestine to the concentration of glucose in the lumen is explained by our finding that fructose 2,6-bisphosphate is absent from intestinal mucosa and that mucosal phosphofructokinase activity is therefore not controlled by glucose.

The present data show that the inhibition of mucosal phosphofructokinase that occurs on starvation cannot be explained by changes in the concentration of fructose 2,6-bisphosphate. This conclusion is perhaps most clearly demonstrated by the observation that the differences in regulatory properties of phosphofructokinase between the fed and starved states are preserved after chromatography of extracts on Sephadex G-25 and by the observation that perfusion of intestinal loops or the incubation of suspensions of mucosa from fed rats without glucose did not result in the diminution of phosphofructokinase activity to the value in starved animals. Alternative explanations for the mechanism of regulation of mucosal phosphofructokinase must therefore be sought.

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