Journal of Physiology (1992), **453**, *pp.* 449–459
With 5 figures
Printed in Great Britain

A SUBMUCOSAL MECHANISM OF ACTION FOR PROSTAGLANDIN E_2 ON HEXOSE ABSORPTION AND METABOLISM IN MOUSE INTESTINE

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(*Received 9 April 1991*)

SUMMARY

1. The involvement of prostaglandin E_2 (PGE_2) in hexose absorption and metabolism was studied in mouse small intestinal villus cells.

2. Phlorizin-sensitive, Na^+-dependent α-methyl-D-glucoside (α-MG) uptake (0·8 mM) during 2 min cell incubations (37 °C) was 74 ± 4 nmol (mg protein)$^{-1}$. Maximal uptake was 110 ± 8 nmol (mg protein)$^{-1}$, representing an accumulation of 50-fold. Metabolism of D-glucose (5 mM) to L-lactate was 38 nmol min^{-1} (mg protein)$^{-1}$.

3. Incubation of isolated cells with indomethacin or PGE_2 did not affect α-MG uptake or D-glucose metabolism. By including indomethacin during cell isolation from whole intestine, α-MG uptake was inhibited dose dependently (50–250 μM) by up to 70% ($P < 0·001$). PGE_2 present during both isolation and incubation inhibited by 85% ($P < 0·001$) at 1 μM and by 27% ($P < 0·05$) at 0·1 μM, with no effect at lower concentrations. α-MG uptake was reduced to 38% ($P < 0·01$) when 1 μM-PGE_2 and 250 μM-indomethacin were presented in combination. When present during cell isolation and incubation, 1 μM-PGE_2 inhibited lactate production by 24% ($P < 0·05$), except when present in combination with 250 μM-indomethacin. Indomethacin, itself, had no effect on lactate production.

4. A submucosal mechanism is proposed to account for the observed inhibitory effects of PGE_2 on brush-border uptake of α-MG and cellular lactate production. Indomethacin appears to exert not only an effect of its own, possibly via PG-independent actions within the submucosa, but at high concentrations also disrupts the effects of exogenously applied PGE_2.

INTRODUCTION

Prostaglandins (PGs) cause important alterations in blood flow, motility, secretion and glucose absorption in the gastrointestinal tract (Whittle & Vane, 1987), thus acting on endothelial cells, muscle cells, and mucosal epithelium, possibly directly at the enterocyte or via the enteric nervous system. The best identified action of PGs in the intestine is the local regulation of fluid and electrolyte transport (Field, 1981;

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Rask-Madsen & Bukhave, 1982; Whittle & Vane, 1987). Although effects on mucosal transport have usually been reported with pharmacological doses (10^{-7}–10^{-4} M), *in vitro* studies (Bukhave & Rask-Madsen, 1980) showed that when endogenous PG formation was first inhibited by indomethacin, exogenous PGE_2 had major effects at very low doses (10^{-9}–10^{-10} M).

There have been few studies in the small intestine in which attempts have been made to differentiate secretory effects at the crypt cell level with anti-absorptive effects on the villus cells. The evidence to date couples secretory actions of PGs with anti-absorptive effects (Pierce, Carpenter, Elliott & Greenough, 1971; Matuchansky & Bernier, 1973; Mize, Wu & Whulen, 1973; Coupar & McColl, 1975), but specific roles in absorption remain unclear (Al-Awquati & Greenough, 1972; Coupar & McColl, 1972). However, since PGs are released into the lumen, they come into contact with absorptive processes located at the brush-border membrane and an action on absorption would, therefore, not be surprising. There have been few investigations on PG effects on absorption, but Coupar & McColl (1975) and Balint, Kiss, Varkonyi, Wittman & Varro (1979) suggest PGs are inhibitory. This study investigates the effects of PGE_2 and indomethacin both on the uptake of α-methyl-D-glucoside (α-MG) and on the metabolism of D-glucose in epithelial villus cells isolated from whole small intestinal loops. Using this technique, the location of PGE_2 action is established. The results show inhibition of α-MG uptake and lactate production by PGE_2 which occurs via submucosal mechanisms.

METHODS

The operative procedure for cell isolation

Male Balb/c mice (25–30 g) were killed by cervical dislocation. The abdomen was quickly opened and the intestine removed from the Ligament of Treitz to a few centimetres above the caecum. A syringe was inserted into the proximal end of the segment and the intestine was flushed out with ice-cold Krebs–Henseleit bicarbonate buffer (A) (118 mM-NaCl; 25 mM-$NaHCO_3$; 4·7 mM-KCl; 1·2 mM-KH_2PO_4; 3·5 mM-$MgSO_4.7H_2O$; 0·5 mM-dithiothreitol (DDT), Ca^{2+}-free) to remove food debris and mucus. All buffers were pre-warmed to 37 °C and gassed (19:1 O_2:CO_2) for at least 30 min to pH 7·4 before use. The intestine was then ligated at one end and filled to distension with buffer A plus 5 mM-EGTA (ethyleneglycol tetraacetic acid) and 0·4 mM-mannitol (buffer B). (Mannitol was used as a mechanical buffer during isolation in place of bovine serum albumin, since the latter binds arachidonic acid and may interfere with prostaglandin action.) The free end was tied. The resultant intestinal loop was placed gently in a conical flask of buffer A and shaken (100 cycles min^{-1}) at 37 °C for 15 min. After incubation, the intestine was drained and flushed gently with buffer C (buffer A plus 1·2 mM-$CaCl_2.2H_2O$, 0·5 mM-β-hydroxybutyrate and 0·8 mM-HEPES) at room temperature. The loop was filled with buffer C, gently rubbed to loosen the epithelial cells and the detached cells were emptied into a plastic centrifuge tube. This was repeated to increase the yield. The cells were washed twice by centrifuging (3000 r.p.m, 3 min) and resuspended in fresh buffer C, and then in final buffer. Cell density was estimated by pellet size and the cell suspension diluted appropriately. Protein concentration was, therefore, fairly similar for each cell batch and assayed later. Tests of cellular integrity were carried out as described by Towler, Pugh-Humphreys & Porteous (1978).

Cell incubation with α-methyl-D-glucoside for uptake studies

The isolated cells were incubated in plastic conical flasks, replicated for each experimental condition with 0·8 mM-α-methyl-D-glucoside (α-MG), ^{14}C-labelled with 0·2 μCi(5 ml buffer C)$^{-1}$ (at 0·4 mg cell protein ml^{-1}) at 37 °C for up to 15 min. Eppendorf microcentrifuge tubes were set up on ice containing 250 μl oil (mixture 3:2 volume ratio of di-*n*-phthalate:dinonyl phthalate), below 400 μl buffer C. During cell incubation, 500 μl aliquots were removed at time points of 1, 2, 5, 10

and 15 min. Incubation was terminated by quenching the sample in ice-cold buffer in the Eppendorf tubes and pelleting below the oil by centrifugation at 13000 r.p.m. for 10 s. After removing the supernatant and most of the oil, the pellet was lysed by vortexing with 0·5% Triton X-100 (500 μl). After addition of 30% trichloroacetic acid (100 μl), the resultant precipitate was spun (13000 r.p.m., 45 s) and a supernatant aliquot (300 μl) was added to optiphase (3 ml) in a scintillation vial, thoroughly mixed and counted for ^{14}C emissions using an LKB MiniBeta scintillation counter. Phase separation effects were not observed. The counts were adjusted for dilution of the sample or standard aliquot and expressed as nanomoles per milligram cell protein.

Cell incubation with D-glucose for lactate production studies

The isolated cells were incubated (2 mg cell protein per flask of 5 ml buffer C) as described above, but containing 5 mM-D-glucose as uptake substrate. Eppendorf centrifuge tubes were set up on ice containing 50 μl 30% perchloric acid. Each flask was incubated for 30 min, and 500 μl aliquots were removed at time points of 5, 10, 15, 20 and 30 min. Incubation was terminated by addition of the aliquot to the acid and vortexing immediately. Zero time points evaluated initial cellular lactate concentrations. The samples were centrifuged (13000 r.p.m., 60 s) and the supernatant (400 μl) was added to 5 μl universal indicator in Eppendorf tubes. The samples were neutralized with 30% KOH and finely adjusted to pH 6·5–7·5 with 2 M-KH_2PO_4. After freezing overnight, the supernatants (300 μl) were assayed for lactate using a lactate dehydrogenase reaction (Leese & Bronk, 1972). The lactate assayed was adjusted for dilution and expressed as nmoles per milligram cell protein.

Cellular protein assay

Spun homogenates of the original cell suspension and bovine serum albumin (BSA) samples were assessed by a microtannin assay. A 1 ml:1 ml mix of diluted sample or BSA to tannin reagent (196 ml 1 N-HCl (80 °C)+4 ml phenol+20 g tannic acid, cooled and filtered), were incubated in test tubes at room temperature for 10 min. The reaction was terminated by adding 1 ml 0·2% (w/v) gum arabic and vortexing. Absorbances were read at 500 nm and the sample protein was estimated from a calibration graph of the BSA standard.

Na^+–K^+-ATPase assay

Activity of Na^+–K^+-ATPase in cellular homogenates was assessed in the presence and absence of 1 mM-ouabain, as described by Kellett & Barker (1989).

Incubation with hormones and drugs

Cells were either incubated with agents subsequent to isolation, or were isolated and incubated in the presence of the agents. In all experiments, $n = 6$ replicates × number of cell isolations, as stated. The effects of cell incubation with indomethacin (50–250 μM) and PGE_2 (10^{-3}–10^{-9} M) present or absent throughout cell isolation on α-MG (0·8 mM) uptake and lactate production from D-glucose (5 mM) were determined. Carbachol (10^{-3}–10^{-8} M), a more stable analogue of acetylcholine, and noradrenaline (5×10^{-5}–10^{-7} M, including 10^{-4} M-pargyline, an inhibitor of monoamine oxidase) were added directly to the isolated cells at the start of incubation in the presence of 0·8 mM-α-MG.

All chemicals were obtained from Sigma unless otherwise stated and were of highest grade. All results are presented as the mean values ± S.E.M. for n experiments. Statistical significances were assessed by Student's t test unless otherwise stated.

RESULTS

The isolated enterocytes were examined under light microscopy with Trypan Blue exclusion dye. Around 80% of the cell suspension revealed evidence of brush borders. Cell concentration was approximately 4×10^6 cells (ml suspension)$^{-1}$ (= 1 mg ml^{-1}) and consisted mainly of small epithelial sheets rather than single cells. Lactate dehydrogenase loss never exceeded 20% of the total activity during 15 min incubations under the given experimental conditions.

Since the non-metabolizable-D-glucose analogue α-methyl-D-glucoside (α-MG) is not a substrate for the Na^+-independent aldohexose carrier on the basolateral membrane (Kimmich & Randles, 1981), cellular uptake of ^{14}C-labelled α-MG represented brush-border hexose absorption in the absence of serosal efflux. Uptake

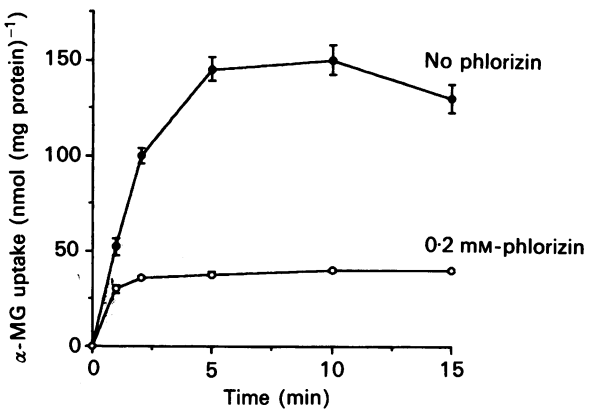

Fig. 1. Uptake of α-methyl-D-glucoside (α-MG, 0·8 mM) by isolated mouse enterocytes in the absence and presence of phlorizin (0·2 mM). Values are the means ± S.E.M. for $n = 48$.

of α-MG from 0·8 mM medium concentration (Velasco, Dominguez, Shears & Lazo, 1986) in the absence and presence of 0·2 mM-phlorizin is shown in Fig. 1. An intra- to extracellular hexose gradient was established by the isolated cells and maintained for up to 15 min. Initial (2 min) and maximum (10 min) phlorizin-sensitive Na^+-dependent α-MG uptake rates were 74 ± 4 and 110 ± 8 nmol (mg cell protein)$^{-1}$ respectively. All further uptake data were expressed as the phlorizin-sensitive, active component of hexose absorption. Incubation (5 min) of cells with ^3H_2O or [^{3}H]-inulin (1 μCi (ml buffer)$^{-1}$) and assessment of the counts associated with the pellet yielded:

$$(\text{Pellet volume } (^3H_2O) = 10{\cdot}85 \pm 0{\cdot}54) - (\text{extracellular volume } ([^3H] \text{ inulin}) = 8{\cdot}05 \pm 0{\cdot}48) = \text{intracellular volume} = 2{\cdot}80 \pm 0{\cdot}72 \ \mu l) \text{ mg protein})^{-1}.$$

Subtraction of the phlorizin data also automatically corrected for counts associated with the extracellular volume of the cell pellet. With a medium concentration of 0·8 mM (i.e. 0·8 nmol μl^{-1}), the amount of intracellular α-MG would be 2·24 nmol mg^{-1}. The maximal phlorizin-sensitive α-MG uptake of 110 ± 8 nmol (mg protein)$^{-1}$, therefore, represents an accumulation of 50-fold. Kinetic evaluation of initial phlorizin-sensitive α-MG uptake using incubations with 0·5–5 mM substrate yielded a Michaelis–Menten hyperbola with J_{max} (maximal uptake rate) and K_t (transport constant) values calculated by non-linear least-squares regression as 53 nmol min^{-1} (mg protein)$^{-1}$ and 1·0 mM respectively. In other studies, rabbit jejunal cells produced a $J_{max} = 6$ nmol min^{-1} (mg protein)$^{-1}$ and $K_t = 0{\cdot}6$ mM (Brown & Sepulveda, 1985), and chicken intestinal cells produced $J_{max} = 10$ nmol min^{-1} mg^{-1} and $K_t = 2{\cdot}4$ mM (Kimmich & Randles, 1984). In whole preparations of mouse small intestine, monosaccharide transport was also high (Diamond & Karasov, 1984; J. A.

Dempster & G. L. Kellett, unpublished observations) in comparison with other species. The medium concentration used during the experiments, 0·8 mM, is below the K_t so that shifts in either K_t or V_{max} (maximum velocity) induced by addition of drugs can be observed.

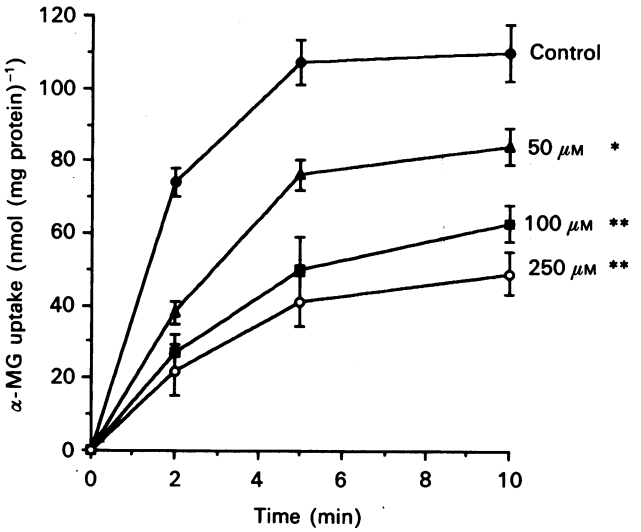

Fig. 2. Effects of indomethacin, present during both cell isolation and incubation at the concentrations indicated, on phlorizin-sensitive α-methyl-D-glucoside (α-MG) uptake (0·8 mM) by isolated mouse enterocytes. Values are the means ± S.E.M. for $n = 24$. $*P < 0·05$, $**P < 0·001$ as significantly different to the control, determined by two-way analysis of variance.

The isolated cells maintained a linear production of lactate from 5 mM-D-glucose in the uptake medium. Regression analysis of 0–15 min incubations yielded a lactate production rate of 38 nmol min^{-1} (mg cell protein)$^{-1}$ (data not shown), in good agreement with data from rat intestinal cells (Watford, Lund & Krebs, 1979) and jejunal loop perfusions of rat (Kellett & Barker, 1989) and mouse (J. A. Dempster & G. L. Kellett, unpublished observations).

Addition of indomethacin to isolated cells at the start of incubation had no effect on either α-MG uptake or lactate production. When the intestinal loop was pre-incubated with the drug during both cell isolation (15 min) and incubation, cellular metabolism of glucose to lactate remained unaltered (data not shown), but phlorizin-sensitive α-MG uptake was dose-dependently inhibited by indomethacin, as shown in Fig. 2. Initial 2 min uptake of α-MG was inhibited by 49, 64 and 70% ($P < 0·001$) with 50, 100 and 250 μM-indomethacin, respectively. Maximum accumulation of α-MG (at 10 min) was inhibited by 24, 43 and 55% ($P < 0·001$) by these concentrations of indomethacin respectively.

Addition of PGE_2 or PGD_2 (10^{-5}–10^{-8} M) at the start of incubation and following cell isolation in the absence or presence of 50 μM-indomethacin, had no effect on initial α-MG uptake or metabolism of glucose to lactate (data not shown). When

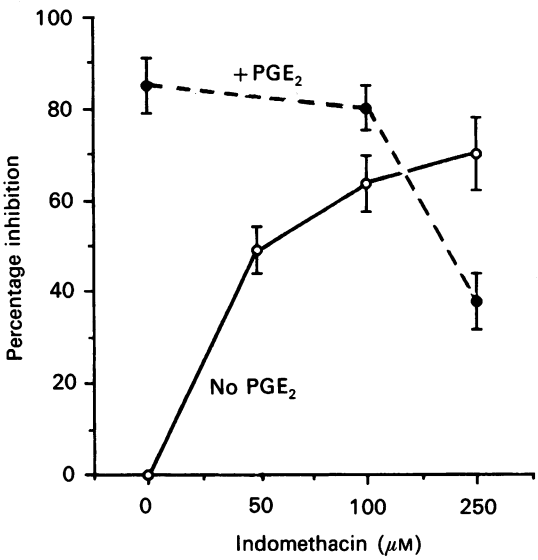

Fig. 3. Interaction between the effects of indomethacin (at the concentrations indicated) and prostaglandin (PGE_2, 1 μM) both present during cell isolation and incubation on α-methyl-D-glucoside uptake (0·8 mM) by isolated mouse enterocytes. The data, calculated from phlorizin-sensitive uptakes at 2 min, are expressed as the percentage inhibition of control uptake (74 ± 4 nmol (mg protein)$^{-1}$), the means ± S.E.M. for $n = 24$.

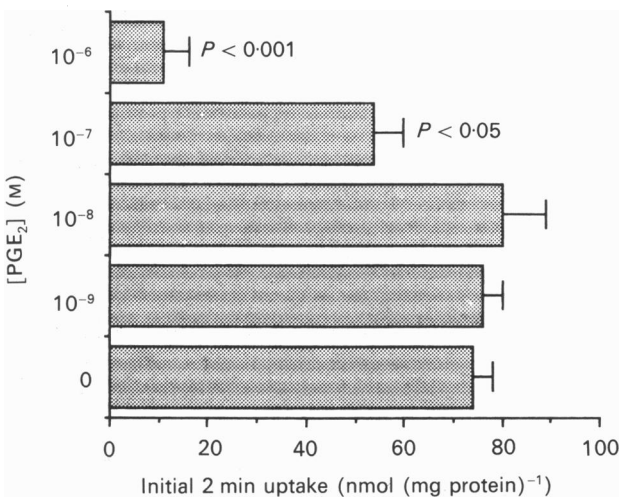

Fig. 4. Effect of concentration of prostaglandin (PG) E_2 presented during cell isolation and incubation on the 2 min phlorizin-sensitive uptake of α-methyl-D-glucoside (0·8 mM) by isolated mouse enterocytes. Bars show the means ± S.E.M. for $n = 18$.

included during both cell isolation from the intestinal loop and incubation, 1 μM-PGE_2 inhibited initial α-MG uptake by 85% ($P < 0·001$, Figs 3 & 4), with a significant reduction over 0–15 min ($P < 0·001$ as assessed by two-way analysis of variance).

Presented in this way, 1 μM-PGE_2 also inhibited lactate production rate by 24% ($P < 0.05$), except when 250 μM-indomethacin was also present (data not shown). Following from this, 1 μM-PGE_2 present in combination with indomethacin (0–250 μM) throughout cell isolation and incubation, produced interesting results

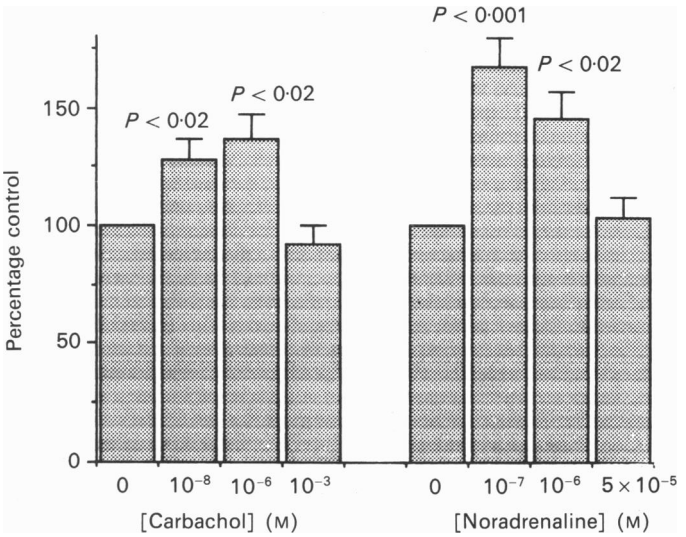

Fig. 5. Effects of carbachol and of noradrenaline (in the presence of 10^{-4} M)monoamine oxidase) added directly to incubating cells at the concentrations indicated, on the 2 min phlorizin-sensitive uptake of α-methyl-D-glucoside (0·8 mM) by isolated mouse enterocytes, expressed as a percentage of the control (74 $\pm$ 4 nmol (mg protein)$^{-1}$). Bars show the means $\pm$ S.E.M. for $n = 12$.

(Fig. 3). PGE_2 had no significant effect on the 100 μM-indomethacin-induced inhibition of uptake, but reduced the 250 μM-indomethacin-induced inhibition of 70% to 38%, i.e. a 44% reversal ($P < 0.01$, Fig. 3). PGE_2 inhibited initial α-MG uptake at 10^{-6} and 10^{-7} M by 85% ($P < 0.001$) and 27% ($P < 0.05$) respectively, with no significant effects by 10^{-8} or 10^{-9} M-PGE_2 (Fig. 4).

Activity of Na^+–K^+-ATPase in cellular homogenates prepared and incubated in the presence of 250 μM-indomethacin or 1 μM-PGE_2 was unchanged, although indomethacin inhibited ouabain-insensitive ATPase activity by 30% ($P < 0.01$, data not shown).

Due to the general lack of direct effects of PGE_2 and indomethacin on cellular α-MG uptake and glucose metabolism, a submucosal mechanism of action, possibly via neural activities, was proposed. Sensitivity of α-MG uptake to carbachol and noradrenaline, added directly to incubating cells, i.e. absent during cell isolation, is shown in Fig. 5. At 10^{-6} and 10^{-8} M, carbachol enhanced initial (2 min), phlorizin-sensitive uptake by 37 and 28% ($P < 0.05$) respectively, with no significant effect at 10^{-3} M. At 10^{-7} and 10^{-8} M, noradrenaline (in the presence of 10^{-4} M-pargyline) enhanced initial uptake by 67 and 45% ($P < 0.001$) respectively, with no significant effect at 5×10^{-5} M or concentrations less than 10^{-7} M.

DISCUSSION

The structural and functional integrity of the isolated mouse enterocytes was established. Since light microscopic study with Trypan Blue revealed the presence of brush borders on more than 80% of the cell suspension, the origin was concluded to be mid to upper villus. Cellular lactate dehydrogenase (LDH) loss never exceeded 20% of the total activity during cell treatment. Lactate production from D-glucose was found to proceed linearly up to a 15 min incubation period under the experimental conditions used. Finally, the cells were able to accumulate both lactate and α-methyl-D-glucoside (α-MG) against a concentration gradient from the external medium. α-MG could be accumulated intracellularly to a concentration some fifty times that in the medium. The substantial accumulation of α-MG by our enterocyte preparation may result from a Na^+:hexose coupling ratio greater than one in the mouse, (e.g. via a high capacity/low affinity hexose carrier system), or from the maintenance of ion gradients and membrane potential.

The lack of any direct effect of indomethacin on sugar accumulation by isolated cells and the finding of concentration-dependent effects of indomethacin when presented during both cell isolation and incubation indicates its site of action of PG inhibition to be within the subepithelium, in agreement with Smith, Warhurst & Turnberg (1982) and Lawson & Powell (1987).

When included during both isolation and incubation of cells, indomethacin (50–250 μM) and PGE_2 (0·1–1 μM) inhibited α-MG uptake significantly. The inhibition in the presence of PGE_2 (85%) was greater than that induced by the same treatment with indomethacin (maximally 70%). In combination with 250 μM-indomethacin, the induced inhibition was reduced (to 38%), but this is not believed to occur by a simple protection against the effects resulting from decreases in endogenous PGE_2 in the presence of indomethacin. A biphasic effect of PGE_2 with inhibitory actions at both high ($> 10^{-7}$ M) and low ($< 10^{-9}$ M) is unlikely since there were no significant effects of PGE_2 at concentrations less than 10^{-7} M or in the presence of 50 μM-indomethacin. Bearing in mind that indomethacin inhibits all prostanoid formation, a role for individual prostanoids exerting interactional effects cannot be excluded. It is more likely, however, that 250 μM-indomethacin exerts non-specific, PGE_2-independent effects. This is corroborated by the observation that in the presence of 100 μM-indomethacin, the effect of 1 μM-PGE_2 was still evident. More specific inhibitors of PG synthesis are clearly needed. On the other hand, indomethacin had no effect on glycolysis in cells isolated and incubated with the drug, while 1 μM-PGE_2 significantly inhibited cellular lactate production by 24%. In the presence of 250 μM-indomethacin, PGE_2 (1 μM) was without effect, indicating again some non-specific interaction of indomethacin.

The effects of PGE_2 ultimately involve changes at the brush-border membrane of the enterocyte, since α-MG can only utilize the apical membrane hexose carrier (Kimmich & Randles, 1981). Rapid adaptation (i.e. within a few hours) of hexose absorption has been shown to involve alterations at the basolateral membrane (Cheeseman & Maenz, 1989) by modulation of carriers already in the membrane and subsequent changes in carrier site density. Faster responses within minutes, such as insulin (Wollen & Kellett, 1988) involve direct action on basolateral membrane

receptors (Fernandez-Moreneo, Senano-Rios & Prieto, 1987; Gingerich, Gilbert, Comens & Gavin, 1987). Very rapid changes in brush-border glucose absorption may occur via an alteration of the number of functional hexose carriers in the membrane (affecting J_{max}) or of the intrinsic activity of pre-existing transporters (affecting the 'apparent' K_t) which can be investigated with kinetic studies. Alternatively, changes in absorption and metabolism may take place via an alteration in the Na^+–K^+-ATPase activity, thus influencing the Na^+ gradient-forming capacity of the apical membrane. Although the Na^+–K^+-ATPase activity measured in this study in cellular homogenates from mouse jejunum was unaffected by indomethacin or PGE_2, the possibility of alteration of pump activity by PG-responsive neuromodulators cannot be excluded.

Although PGE_2 receptors are believed to exist on the enterocytes themselves, no effect on α-MG uptake or lactate production could be observed when PGE_2 was presented directly. Significant effects were only seen when PGE_2 was included during both cell isolation and incubation. This suggests that PGE_2 may regulate hexose absorption and metabolism by influencing submucosal release of neurotransmitters from the enteric neural network. Spontaneously active neurons from the submucosal plexus are thought to have a direct or indirect inhibitory influence on the mucosa (Andres, Bock, Bridges, Rummel & Schreiner, 1985; Ahsan, Naftalin & Smith, 1988). Certainly, we observed that α-MG uptake by isolated epithelial cells in the present study was enhanced by carbachol and noradrenaline. Although we have not attempted to characterize these effects further, the observations are in agreement with work on ion transport with carbachol (Tapper, Powell & Morris, 1978) and on glucose and ion transport with noradrenaline (Aulsebrook, 1965; Tapper, Bloom & Lewand, 1981).It is not known whether such effects on intestinal glucose absorption result indirectly, e.g. from changes in membrane potential. Our work leads to the idea of a set-point of spontaneous neurotransmitter release and functional modulation of this by PGs. Independent of PG synthesis, indomethacin appears to interact non-specifically with the mechanism of PGE_2 action on enteric neurotransmitter release. More specific inhibitors of PG synthesis, blocking submucosal neurons with tetrodotoxin or measuring neurotransmitter release, in the presence and absence of PGE_2, should help the elucidate the mechanism(s) of action of PGE_2 on hexose absorption and metabolism.

J.A.D. was supported by the Science and Engineering Research Council. The work was funded by the Wellcome Trust.

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