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EFFECTS OF CYCLOHEXIMIDE ON INFLUX ACROSS THE BRUSH BORDER OF RABBIT SMALL INTESTINE

RAYMOND A. FRIZZELL, HUGH N. NELLANS, LOUISE S. ACHESON AND STANLEY G. SCHULTZ

Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pa. 15213 (U.S.A.)

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SUMMARY

1. Unidirectional influxes of Na^+, Cl^-, alanine, 3-*O*-methylglucose and mannitol across brush border of ileum and of iron across brush border of duodenum were determined in normal and cycloheximide-pretreated rabbits.

2. Unidirectional influx of all solutes, with the exception of mannitol, was significantly reduced by cycloheximide administration. Mannitol influx was unaffected.

3. The inhibitory effect of cycloheximide on alanine influx was attributable, almost exclusively, to a 70% reduction in maximal alanine influx.

4. These results are consistent with the notion that cycloheximide inhibits the synthesis of protein(s) directly or indirectly involved in carrier-mediated transport across the brush border of mature, villus absorptive cells.

INTRODUCTION

Absorptive cells formed in the crypts of the small intestine have been thought to retain a stable complement of protein through their migration up the villi until the time at which they are shed from the villus tips[1,2]. However, James *et al.*[3] have recently demonstrated a turnover of radioactive leucine associated with total brush border proteins and disaccharidases that was too rapid to be attributed to the loss of labeled intestinal cells through exfoliation. Their results strongly suggest that brush border proteins undergo constant synthesis and degradation throughout the life cycle of the mature enterocyte.

Cycloheximide is a potent inhibitor of intestinal protein synthesis[4] and, at higher concentrations, of mitotic activity in rat[5] and rabbit[6] small intestine. The purpose of the present investigation was to examine the effects of cycloheximide pretreatment on the unidirectional influxes of Na^+, Cl^-, Fe^{2+}, L-alanine, 3-*O*-methyl-D-glucose and mannitol across the brush border of rabbit small intestine. The influxes of all of these solutes, with the exception of mannitol, appear to involve brush border carrier mechanisms[7–11]. Further, compelling autoradiographic evidence has been presented that the carrier-mediated influxes of Fe^{2+}, sugars and amino acids are largely confined to the mature villus absorptive cells[12,13].

METHODS

Male white rabbits (2–3 kg) were injected with 20 mg/kg cycloheximide (Sigma Chemical Corp.) 3 h, and in some experiments at 6 h and again at 3 h, prior to sacrifice with intravenous pentobarbital. Uninjected animals from the same colony served as controls.

Verbin *et al.*[4] have shown that injection of rats with 0.8–1.5 mg/kg cycloheximide inhibits intestinal protein synthesis more than 90% and that this effect is associated with a disappearance of mitotic activity of the crypt cells. However, Serebro *et al.*[6] observed no effects on crypt cell mitosis of rabbit small intestine at cycloheximide doses of less than 20 mg/kg. This dose did not elicit other morphologic alterations in the intestinal mucosa. Differences in species sensitivity to the drug may be involved; nevertheless, in the present studies, a dose of 20 mg/kg was employed since preliminary experiments using 5 mg/kg resulted in inconsistent effects on solute influx.

The methods employed for the determinations of the unidirectional influxes of Na^+, Cl^-, sugar and amino acid across the brush border of rabbit ileum[7] and the influx of Fe^{2+} across the brush border of rabbit duodenum[8] were identical to those described previously. Briefly, the procedure involves exposure of defined areas of the mucosal surface of the tissue to a mucosal solution containing radioactive solute *plus* [^{3}H]-inulin for a precisely timed period of 30–45 s. The zero-time rate of uptake of the solute across the brush border alone is calculated from the tracer content of the tissue after correction for the inulin space.

RESULTS

The effect of cycloheximide on solute influx across the brush border of rabbit small intestine is given in Table I. Pretreatment with 20 mg/kg cycloheximide 3 h prior to sacrifice significantly inhibits the influxes of Na^+, Cl^-, alanine and 3-*O*-methyl-D-glucose across the brush border of rabbit ileum and of Fe^{2+} across the brush border of rabbit duodenum. In contrast, mannitol influx is unaffected by cycloheximide administration. Although control data were obtained on different animals from the same colony, the control influxes given in Table I are in excellent agreement with those

TABLE I

EFFECT OF CYCLOHEXIMIDE PRETREATMENT ON SOLUTE INFLUX IN RABBIT SMALL INTESTINE

All values represent the unidirectional solute influx across the brush border and are expressed as μmoles/cm^2 per h except those for Fe^{2+} which are in nmoles/cm^2 per h. Mucosal solutions employed in the determination of alanine and 3-*O*-methyl-D-glucose influxes contained 140 mM Na^+. All errors are S.E.; number of determinations is indicated in parentheses.

Solute	J_{mc}	
	Control	*Cycloheximide*
Na^+ (140 mM)	16.5 ± 1.0 (12)	12.5 ± 0.8 (16)
Cl^- (20 mM)	7.6 ± 0.5 (16)	4.8 ± 0.4 (16)
Alanine (5 mM)	4.1 ± 0.1 (7)	1.7 ± 0.2 (37)
3-*O*-Methylglucose (5 mM)	1.6 ± 0.2 (6)	0.8 ± 0.1 (38)
Mannitol (5 mM)	0.10 ± 0.02 (8)	0.11 ± 0.06 (4)
Fe^{2+} (1 mM)	37 ± 6 (18)	20 ± 3 (8)

reported in previous studies[8,9,11], so that comparison between values obtained using tissues from different animals appears justified.

The effects of two injections of cycloheximide, the first 6 h prior to sacrifice and the second 3 h prior to sacrifice, on Na^+, Cl^- and Fe^{2+} influxes are illustrated in Fig. 1; the data are expressed relative to those obtained from untreated control animals*. These results suggest an exponential decline of solute influx following cycloheximide administration and, assuming a rapid onset of drug action, are consistent with half-times of 8, 4.4 and 3.9 h for inhibition of Na^+, Cl^- and Fe^{2+} influxes, respectively.

In several experiments, the mucosal surface of tissues from control animals were exposed to solutions containing $5 \cdot 10^{-4}$ M cycloheximide *in vitro*. The presence of the agent during the brief test period (30–45 s) or in the preincubation solution for 20 min prior to the influx measurement had no effect on solute influxes. These observations indicate that cycloheximide *per se* does not directly affect the brush border transport mechanisms studied**.

Alanine influx across the ileal mucosal border of rabbits that had received cycloheximide 3 h prior to sacrifice is plotted as a function of the alanine concentration

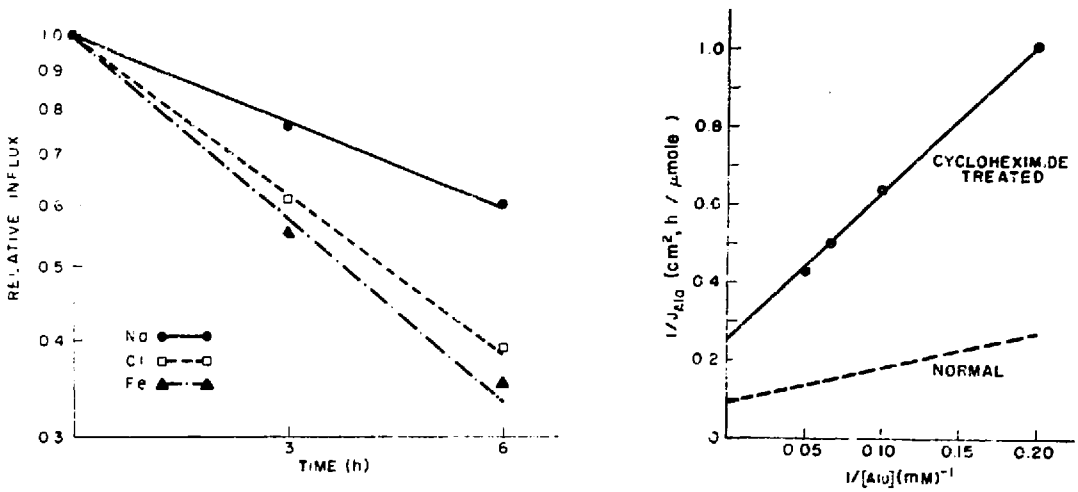

Fig. 1. Time-course of the effect of cycloheximide pretreatment on Na^+, Cl^- and Fe^{2+} influxes. Influx was determined either 3 or 6 h after an injection of cycloheximide (20 mg/kg) at zero time. In the latter case, an additional injection was given 3 h after the first. The values are expressed relative to those obtained using untreated control animals. Each point is the average of at least 8 determinations.

Fig. 2. Lineweaver–Burk plots of alanine influx as a function of mucosal alanine concentration in normal[14] and cycloheximide-treated rabbits. A single injection of cycloheximide was administered 3 h prior to the influx determination. Each point is the average of 8 experimental values.

* The inhibition of mitotic activity in rat small intestine produced by a single injection of cycloheximide is completely reversed 5 h after its administration[4]. Thus, in the present studies, a supportive injection was given 3 h after the first. Subsequent experiments indicate that a single injection of 20 mg/kg at zero time results in a similar exponential decline in Na^+ influx, so that the decrease observed at 6 h (Fig. 1) cannot be attributed to the fact that these animals received twice the dose of cycloheximide as those used 3 h after a single injection.

** Determinations of Na^+ influx performed using animals that had received 20 mg/kg cycloheximide 30 min prior to sacrifice resulted in a 10% inhibition of Na^+ influx compared with control values. Inasmuch as these experiments were not internally controlled, it may not be reasonable to suggest that this marginal effect falls on the time course for inhibition of Na^+ influx by cycloheximide shown in Fig. 1, but serves to emphasize the lack of a rapid, pronounced effect of the drug on solute influx.

in the mucosal solution according to the method of Lineweaver and Burk in Fig. 2. Clearly, alanine influx after cycloheximide treatment is a saturable process that conforms to Michaelis–Menten kinetics. The dashed line is representative of data obtained for the kinetics of alanine influx in normal animals from previous studies[14] and is included for comparison. The good agreement between alanine influxes in normal rabbits obtained in this and previous studies[11,14] (*i.e.* approximately 4 μmoles/cm^2 per h in the presence of 5 mM alanine) justifies this comparison. The data indicate that cycloheximide pretreatment results in a marked reduction in maximal alanine influx from a normal value of 12.5 μmoles/cm^2 per h to 3.9 μmoles/cm^2 per h in the treated animals. In contrast, there is little or no effect of cycloheximide on the alanine concentration required to elicit a half-maximal influx (K_t) (10 mM in control animals *versus* 13 mM in cycloheximide-treated animals). Thus, the inhibitory effect of cycloheximide pretreatment on alanine influx appears to be attributable, almost exclusively, to a 70% reduction in the maximal influx.

DISCUSSION

Previous studies have shown that carrier-mediated processes are implicated in the influxes of Na^+ (refs 7, 11 and 15), Cl^- (ref. 9), alanine[7,14,15] and 3-*O*-methyl-D-glucose[10,11] across the brush border of rabbit ileum. Sodium influx is subject to marked inhibition by Li^+, K^+ and guanidinium in the mucosal solution and the influxes of Cl^-, alanine and 3-*O*-methyl-D-glucose display classical Michaelis–Menten kinetics and are subject to competitive inhibition; all of these findings are characteristic of carrier-mediated transport processes. Further, evidence has been presented that the movement of Fe^{2+} across the duodenal brush border is a carrier-mediated process[8,16]. Finally, autoradiographic evidence has directly implicated the villus absorptive cells in the carrier-mediated transport of amino acids, sugars and Fe^{2+} (refs 12 and 13). Thus, the present data indicate that pretreatment with cycloheximide inhibits a variety of brush border carrier mechanisms some of which are localized to the so-called "mature" villus enterocyte. In contrast, cycloheximide did not significantly affect the unidirectional influx of mannitol, a molecule that appears to traverse the mucosal surface by simple diffusion[10,11]. Further, the effect of cycloheximide is far too rapid to be reasonably attributed to the loss of villus absorptive cells through normal exfoliation and the inhibition of cell renewal[3,17].

Recently, MacDonald and Ellis[18] have demonstrated that incubation of red beet discs with cycloheximide resulted in a stimulation of oxygen uptake similar to that elicited by 2,4-dinitrophenol. These authors suggested that some of the effects of cycloheximide may not be a direct consequence of the inhibition of protein synthesis but may be the result of the inhibition of energy-yielding metabolic processes. However, the effects observed in the present study need not be attributed to an impairment in the availability of metabolic energy since Chez *et al.*[19] have demonstrated that alanine and Na^+ influxes across the brush border of rabbit ileum are not affected by metabolic inhibitors.

Instead, our observations are more readily reconciled with the role of cycloheximide as an inhibitor of protein synthesis and with the recent observations of James *et al.*[3]. These authors demonstrated an 18-h half-life of brush border protein and an

11.5-h half-life of purified brush border disaccharidases from villus absorptive cells after maximal labelling of these fractions with radioactive amino acids. These half-lives are far too rapid to be attributed to cell turnover and renewal, and these authors concluded that a constant synthesis and degradation of brush border proteins is characteristic of the villus absorptive cell. There is abundant evidence from studies of bacterial transport systems that membrane proteins are involved in the binding of transported solutes and are, thereby, implicated in at least the "first step" in the overall translocation process[20]. It seems reasonable to assume that membrane proteins are similarly involved in transport across animal cell membranes although direct evidence on this point is lacking. Thus, it is possible that our results are attributable to the inhibition of the synthesis of membrane components that are in a dynamic state of turnover and are directly involved in the carrier-mediated processes. Alternatively, these results could be attributed to the inhibition of the synthesis of membrane (or, perhaps, cytoplasmic) components that indirectly influence a variety of carrier processes (*e.g.* structural membrane proteins). The finding that the inhibition of alanine influx is attributable to a decrease in the maximal influx, a capacitative parameter that appears to be directly proportional to the concentration of a brush border component that is directly involved in the influx process[15], favors the former alternative but is not conclusive.

Greenberger and Ruppert[16] had previously demonstrated that cycloheximide treatment inhibits the enhancement of Fe^{2+} transport by rat duodenum resulting from hemorrhagic anemia or dietary iron deficiency. These authors concluded that cycloheximide inhibits the synthesis of a protein intimately involved in Fe^{2+} transport. The present observations corroborate the findings of Greenberger and Ruppert[16] and extend the effect of cycloheximide to a variety of other carrier-mediated transport processes.

Finally, these observations may have important implications regarding the recent report of Serebro *et al.*[6] that pretreatment of rabbits with 20 mg/kg cycloheximide inhibited the fluid production by isolated intestinal loops challenged with cholera toxin. Since the predominant change in small intestinal morphology following this dose of cycloheximide is a decrease in mitotic figures in the undifferentiated crypt cells, these authors suggested that the crypt cells are responsible for the secretory response to cholera toxin and that continuing protein synthesis is required for this response. The finding that cycloheximide markedly inhibits influx processes that are, for the most part, localized to the mature villus absorptive cells indicates that its action cannot be restricted to the crypt cell population and that the implication of the crypt cells on the basis of morphological evidence alone may be unjustified.

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