Biochemical Pharmacology, Vol. 25, pp. 2727–2731. Pergamon Press, 1976. Printed in Great Britain.

CYCLIC AMP-DEPENDENT AMINO ACID UPTAKE IN INTESTINE—

THE IMPORTANCE OF β-ADRENERGIC AGONISTS

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(*Received* 26 *December* 1975; *accepted* 21 *May* 1976)

Abstract—Cyclic AMP (cAMP)-dependent stimulation of neutral and dibasic amino acid uptake by intestinal mucosa was investigated by the use of everted intestinal rings. β-adrenergic receptor sites on the mucosal cell itself were implicated, since epinephrine and isoproterenol stimulated uptake *in vitro*, which was blocked by propranolol. Glucagon, vasopressin and norepinephrine had no effect on tissue cyclic AMP levels or on amino acid uptake but cholera toxin given *in vivo* increased both parameters tested *in vitro*. Theophylline and isoproterenol caused an increase in cyclic AMP levels in the villus tip cells, where these drugs also stimulated amino acid uptake. Neither drug increased cAMP in crypt cells. Thus, the cyclic AMP-stimulated uptake of amino acids *in vitro* is a function of the villus tip cells. Moreover, when metabolic products, such as cAMP, are measured in whole intestinal mucosa, it cannot be assumed that the change is uniform throughout the tissue, but may involve only one type of cell on the intestinal villus.

Cyclic 3′5′-AMP specifically and significantly stimulates the uptake of amino acids into a wide variety of tissues and nucleated cells, including bone [1], kidney cortex [2], myometrium [3] and liver [4], mostly in response to physiological doses of specific hormones. Previous work in this laboratory [5] established the presence of a cAMP-stimulated uptake of neutral amino acids in intestinal mucosa. The stimulated uptake correlated with the tissue level of cyclic AMP (cAMP), and a similar effect could be produced by incubation with cyclic AMP or dibutyryl cyclic AMP, or by elevating the tissue cAMP level by exposure *in vivo* to cholera toxin. The present study was undertaken to define possible natural mediators of this response, and to see whether chemicals which elevate cAMP levels in other tissues had the same effect in intestinal mucosa.

MATERIALS AND METHODS

[^{3}H]L-valine (4.3 mCi/μmole) and [4, 5-^{3}H]L-leucine (5 mCi/μmole) were purchased from New England Nuclear Corp. (Boston, Mass.). L-Norepinephrine and isoproterenol bitartrate were obtained from Winthrop Laboratories (New York, N.Y.), epinephrine from Wyeth Laboratories (Philadelphia, Pa.), glucagon from Lilly Laboratories (Indianapolis, Ind.), and vasopressin from Parke-Davis & Co. (Detroit, Mich.) as sterile solutions for injection, and were diluted in an isotonic lactate buffer just prior to use where necessary. Theophylline was a product of Sigma Corp. (St. Louis, Mo.). DL-Propranolol was the product of Ayerst Pharmaceuticals (New York, N.Y.). Cholera toxin (Crude, Wyeth Lot No. 001) was obtained through the courtesy of Dr. John Seale of the National Institutes of Health Cholera Advisory Committee.

Studies in vitro *of amino acid uptake*

Rats (150–200 g) were stunned and decapitated. The jejunum was removed, rinsed, everted and cut into rings (10–15 mg) or sacs (5–6 cm). Tissue was kept in oxygenated Krebs–Ringer bicarbonate buffer at 4° for no longer than 30 min before use.

For each experiment, rings from three rats were randomly selected and placed in 2 ml Krebs–Ringer buffer with and without test substance and/or 7 mM theophylline, and gassed with 95% O_2/5% CO_2. After a 20-min preincubation in a 37° shaking water bath, [^{3}H]-leucine or [^{3}H]valine was added to a final concentration of 0.065 mM and incubation was continued for 40 min as previously reported [5]. The medium concentration of amino acid (0.065 mM) was chosen because it was far below the K_m for each amino acid. A steady state equilibrium for distribution ratio was always reached by 40 min using the intestinal ring preparation. Although the rings include a full thickness of intestine (mucosa, lamina propria and muscle), only the mucosal cells take up amino acids [6]. Moreover, by cell volume over 80 per cent of the ring is comprised of mucosal cells. The rings were removed, rinsed in normal saline containing the appropriate unlabeled amino acid, blotted and frozen for cAMP determination by radioimmunoassay as described by Steiner *et al.* [7], or boiled in distilled water for 7 min. Since cAMP values for mucosal scrapings were about ten times that of the underlying muscle, whole tissue slices were routinely used. Specific activity of media and tissue water was determined by liquid scintillation counting in Bray's solution [8] or Triton–toluene [9].

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The distribution ratio (counts in intracellular fluid/extracellular fluid) was calculated, applying correction for the total tissue water and [^{14}C]insulin space [10]. Each value reported is the mean ± one standard deviation of four separate flasks, each one containing three jejunal rings. For studying the localization of cAMP along the intestinal villus, 5-cm everted rat jejunal sacs [11] were filled with 0.5 ml of Krebs–Ringer bicarbonate buffer with and without 7 mM theophylline, plus 8 μM isoproterenol, and incubated in 9.5 ml buffer containing the same additives for 25 min, the time by which theophylline had achieved its maximum effect on amino acid uptake. The sacs were removed, rinsed, blotted and opened logitudinally. The intestinal strip was mounted on the block of a tissue planing device [12] and kept at 4° for the entire slicing procedure. The successive fractions obtained at 125-μm intervals from the villus tip were frozen immediately for subsequent cAMP assay. The entire rinsing and slicing procedure took 5 min. Values for control or theophylline- or isoproterenol-treated tissue obtained in this manner were similar to those obtained by freezing tissue immediately after incubation (11.6 ± 1.8 vs 18.2 ± 0.9 pmoles/mg of protein). Villus tip and crypt fractions were identified (a) by histological examination of punch biopsies taken from the tissue remaining after each slice was removed, and (b) by sucrase activity. Crypt fraction contained less than 5 per cent of sucrase specific activity of villus tip fractions. Because of the variability inherent in this technique, only the most extreme fractions (villus tip and crypt) were studied.

Effect of cholera toxin. Rats (200 g) were fasted and anesthetized with pentobarbital (30 mg/kg), the abdomen was opened, and two 7-cm loops in the proximal jejunum were isolated with ligation of both ends. Five mg of either cholera toxin or boiled cholera toxin in 0.5 ml of 0.85% NaCl was injected into the loop via a 27-gauge needle. Two and one half hr later, when the maximum effect of cholera toxin on fluid secretion had been achieved [5], the abdomen was re-opened and tied off segments were removed without clamping the vascular pedicle. The sacs were opened, rinsed with cold saline, everted, cut into rings, and incubated as described above.

RESULTS

Effect of β-adrenergic agents. Because β-adrenergic agents are known to stimulate amino acid transport in salivary gland [13, 14], an entodermally derived tissue, and appear to mediate their actions via cAMP [15, 16], we investigated the effects of these agents on isolated intestinal slices. Table 1 shows that pre-incubation with isoproterenol results in a significant increase in L-valine uptake, but the effect was not additive to that concentration of theophylline (7 mM) which maximally stimulated valine uptake [5]. Blockade of the β-receptors with propranolol completely eliminated the isoproterenol effect, but propranolol had no effect on valine uptake. Phentolamine, an α-blocker, was without effect in the presence or absence of isoproterenol. The response to isoproterenol and its complete blockade by propranolol suggested that β-adrenergic receptors were present on the mucosal cells and that they mediated the stimulated uptake. When isoproterenol concentration was varied from 2 to 10 μM, the effect was maximal at 8 μM. At a lower concentration (0.8 μM), it could be potentiated by submaximal (1 mM) concentration of theophylline (Table 1), again suggesting that its effects were mediated by a β-receptor via cyclic AMP. When hormonal agents which have been reported to modify water and solute flux in the intestine (including glucagon [17] and vasopressin [18, 19]) were used, all were found to be without significant effect on leucine uptake *in vitro* either in the presence or absence of theophylline (Table 2). Leucine was used in these experiments since it shares a common carrier mechanism with valine, and should be similarly affected. Similar results were obtained using [^{14}C]L-valine. Epinephrine [20, 21] caused a significant stimulation of leucine uptake at a higher concentration, but had no detectable effects at less than 10 μM. Its effect was probably due to its β-activity, since the slight increase in uptake was not blocked by phentolamine, an α-blocker. Propranolol did inhibit the increase in uptake by epinephrine (Table 2). L-Norepinephrine was ineffective in stimulating leucine uptake.

Cholera toxin has been demonstrated to stimulate adenylate cyclase in a manner which is not additive to epinephrine [22] and is known to increase the level of cyclic AMP in intestinal cells [23, 24]. As we have reported previously [5], tissue from closed rat jejunal loops 2 hr after injection of potent cholera toxin had a significantly increased uptake of leucine when compared to that taken from control loops (Table 2). In contrast to control, theophylline elicited no additional increment in uptake from the toxin-treated tissue,

Table 1. Effect of isoproterenol and inhibitors on valine uptake *in vitro* by rat jejunum*

Agent added	Concn (μM)	Distribution ratio		
		Buffer	+Theophylline (7 mM)	+ Theophylline (1 mM)
None		13.4 ± 2.26	23.3 ± 4.5	16.1 ± 1.1
Isoproterenol	8	18.6 ± 2.1†	24.9 ± 3.7	
Isoproterenol	0.8	14.8 ± 1.2		20.2 ± 1.8†
Propranolol	8	13.6 ± 1.3	23.0 ± 4.1	
Phentolamine	20	12.8 ± 1.8	23.7 ± 3.1	
Isoproterenol + propranolol	8 + 8	13.6 ± 2.0	21.2 ± 2.3	
Isoproterenol + phentolamine	8 + 20	18.8 ± 1.7†	23.3 ± 3.9	

* Jejunal rings were prepared from three rats and incubated as outlined in Materials and Methods. [^{3}H]valine (uniformly labeled) was added after a 20-min pre-incubation with the appropriate drug. The distribution ratio is the mean of four separate flasks, ± one standard deviation, each containing three jejunal rings.

† Statistically significant differences compared with control values, using Student's *t*-test ($P < 0.05$).

Table 2. Effect of adrenergic agonists and inhibitors on leucine uptake *in vitro* by rat jejunum*

Agent added	Concn (μM)	Distribution ratio	
		Buffer	+ Theophylline
None		7.0 ± 0.9	11.9 ± 3.0
Epinephrine	20	10.8 ± 1.3†	12.0 ± 2.5
Epinephrine + propranolol	20 + 8	7.3 ± 0.6	11.6 ± 2.1
Epinephrine + phentolamine	20 + 20	10.8 ± 1.4†	13.2 ± 1.7
Vasopressin	4.7	7.5 ± 0.4	12.8 ± 3.2
Norepinephrine	60	7.0 ± 2.0	11.1 ± 1.0
Glucagon	0.15	8.0 ± 0.6	12.0 ± 2.5
Boiled cholera toxin control	1 mg/ml	5.1 ± 1.2	9.5 ± 1.3
Cholera toxin	1 mg/ml	8.5 ± 1.6†	8.4 ± 2.1

* All experiments excepting those involving cholera toxins were performed as described in Table 1. [^{3}H]leucine was the amino acid used. Pretreatment with cholera toxin was performed *in vivo*, as outlined in Materials and Methods.

† Statistically significant differences compared with control values, using Student's *t*-test ($P < 0.05$).

suggesting that the tissue levels of cAMP produced by cholera toxin are sufficient to fully stimulate leucine uptake. Similar observations have been made with regard to ion secretion in the ileal mucosa [25].

Relationship of stimulated uptake to cAMP levels. Both isoproterenol and epinephrine caused significant elevations in the mucosal level of cyclic AMP in incubated tissue (Table 3) when compared with controls, and both of these agents stimulated amino acid uptake. Similar elevations were observed when mucosal scrapings were used as the source of cAMP. Actually, the addition of isoproterenol and epinephrine prevented the fall in cAMP levels which occurs with time in intestinal slices *in vitro* [5]. cAMP levels remained fairly constant throughout the incubation period. Incubation with glucagon or vasopressin at a variety of concentrations did not affect the concentration of cAMP in jejunal slices, while exposure *in vivo* to cholera toxin markedly increased the cyclic AMP concentration. As was observed with amino acid uptake (Table 2), theophylline further stimulated cAMP levels in all cases except where cholera toxin was used.

Although jejunal elevations of cAMP were associated with increased amino acid uptake, the intestine is a heterogeneous tissue, comprised of relatively undifferentiated crypt cells and villus cells specialized for digestion and transport. Amino acid uptake has been shown to occur in the cells near the villus tip [26]. It seemed of interest to see whether theophylline and isoproterenol elevated cAMP levels in crypt as well as villus cells. To test the relationship of cAMP to amino acid uptake, we studied both parameters in the absorbing cells at the end of the villus and in the crypt cells.

Sacs of everted rat jejunum were incubated with buffer, 7 mM theophylline or 8 μM isoproterenol in the presence or absence of [^{14}C]valine and then emptied and mounted on a tissue-planing apparatus as described by Imondi *et al.* [12]. Fractions corresponding to villus tips and crypts were obtained by taking serial horizontal slices at 126-μm intervals, the first being made at 540 μm from the base of the block, which results in a relatively pure preparation of villus tips. The appropriate fractions were weighed and assayed for distribution ratio or for cyclic AMP, expressed per mg of protein. When sacs incubated in 7 mM theophylline were fractionated, the concentration of cyclic AMP in the villus tip cells was doubled and distribution ratio increased nearly 2-fold (Table 4). The increase after isoproterenol was about 50 per cent and similar for valine uptake and cAMP

Table 3. Effect of adrenergic agonists on rat jejunal cAMP concentration*

Agent added	Concn (μM)	cAMP (pmoles/mg wet weight)	
		Buffer	+ Theophylline
None		0.39 ± 0.04 (11)	0.96 ± 0.08 (15)
Isoproterenol	8	0.60 ± 0.08† (4)	1.11 ± 0.1† (4)
Isoproterenol + propranolol	8 + 8	0.42 ± 0.05 (4)	1.02 ± 0.11 (4)
Epinephrine	20	0.74 ± 0.2† (4)	1.22 ± 0.12† (4)
Glucagon	0.15	0.36 ± 0.4 (4)	0.70 ± 0.07† (4)
Boiled cholera toxin control	1 mg/ml	0.42 ± 0.06 (3)	1.02 ± 0.13 (3)
Cholera toxin	1 mg/ml	1.41 ± 0.09† (3)	1.81 ± 0.18† (4)

* Jejunal rings were prepared, incubated for 40 min, extracted and assayed for cAMP as described in Materials and Methods. Pretreatment with cholera toxin was performed *in vitro*, as in Table 2. The figure in parentheses refers to the number of individual flasks tested. One flask of three jejunal slices was tested for each separate experiment utilizing intestine from three rats. Reproducibility of the cAMP radioimmunoassay is ±5 per cent.

† Statistically significant differences ($P < 0.05$) reported as the mean ± one standard deviation.

Table 4. Effect of theophylline and isoproterenol on cAMP and valine uptake in intestinal crypts and villi*

	Crypt		Villus	
Agent added	Valine uptake (D.R.)	cAMP (pmoles/mg protein)	Valine uptake	cAMP (pmoles/mg protein)
None	1.4 ± 0.6	15.8 ± 2.3	11.3 ± 2.1	11.6 ± 1.8
Theophylline	1.8 ± 0.9	13.2 ± 2.7	20.9 ± 2.7†	23.4 ± 1.6†
Isoproterenol	1.3 ± 0.7	16.3 ± 3.7	17.3 ± 2.0†	18.3 ± 4.6†

* Jejunal sacs were incubated, processed and assayed for cAMP or for amino acid uptake, as outlined in Materials and Methods. Concentrations of drugs added were 7 mM (theophylline) and 8 μM (isoproterenol). Each valine uptake and cAMP value is the mean of three determinations from three separate experiments ± one standard deviation.

† Statistically significant differences compared with control values ($P < 0.05$).

levels. Since villus cells account for roughly 80 per cent of the total mucosal cell population, it is not surprising that the data in villus cells mimic those found in whole tissue (Tables 1 and 3). The fact that the differences are somewhat less than those found in whole tissue is probably due to the fact that we have analyzed only the cells on the outer half of the villus. These results suggest that the absorptive cells at the villus tip were stimulated by theophylline and isoproterenol, and that the increased amino acid uptake depends in large part on the level of cAMP in these mature cells.

DISCUSSION

Cyclic AMP-dependent or -mediated amino acid transport systems have been demonstrated in a variety of mammalian tissues. In some cases, the cyclic AMP-associated amino acid response has been elicited by hormonal stimulation of specifically responsive adenylate cyclases, notably in the liver [4] and the kidney [2]. The mechanism by which cyclic AMP stimulates amino acid uptake is unknown, but in some tissues, the uptake may merely reflect the altered intermediary metabolism of amino acids. Thus, in liver slices, the uptake of some amino acids is actually decreased while the uptake of others [4] is increased. A second possibility, especially in an actively transporting epithelium, is that the changes observed are related to enhanced electrolyte transport, and merely reflect the altered electrochemical gradients across cells [27].

In mammalian intestine, Na^+ and amino acid transport are interrelated [28], and stimulation of one transport system may be responsible for diverse effects on others. However, it seems likely that cyclic AMP-stimulated amino acid transport is a specific effect in the rat jejunal mucosa, because it is a saturable process specific for one class of amino acids [5].

β-adrenergic agents have been shown to act via the cyclic AMP "second messenger" in many systems in which their mechanism has been defined [15, 16, 29–31]. Amino acid transport stimulated by pure β-agonists is demonstrable in several glandular derivatives of entodermal origin, salivary glands [13, 14] and liver [4]. The report of Gorman and Bitensky [22] that effects of cholera toxin and epinephrine are not additive in liver slices is interesting in this regard because cholera toxin was the only agent tested other than β-adrenergic agonists which produced enhanced amino acid uptake. Our incubation data *in vitro* showing a rise in cAMP with isoproterenol are evidence suggesting the presence of a catecholamine-sensitive adenylate cyclase in small intestinal mucosa. Other workers [31] have previously reported negative results in isolated intestinal membrane preparations. This may reflect the insensitivity of the assay procedures used, for the increases in amino acid uptake and tissue cAMP were modest, but significant.

Epinephrine may not always mediate its effect through cAMP, however. The effect of epinephrine on ileal ion transport is opposite to that of cAMP in respect to short circuit current and Na and Cl fluxes [32]. Isoproterenol, a β-stimulator, had no effect in the rabbit ileum used by Field and McColl, whereas phentolamine, an α-blocker, inhibited the effect of epinephrine. Since the effects of epinephrine and isoproterenol reported here were blocked by propranolol, a β-blocker, the two studies may not be comparable. Moreover, the ileum and jejunum are not identical tissues, especially in regard to electrolyte transport.

The finding of elevated amino acid uptake in villus tip, but not crypt cells, was not surprising (see Table 4). However, the lack of increase in cAMP levels in crypt cells after theophylline or isoproterenol was unexpected and is as yet unexplained. This finding suggests: (1) that the poorly differentiated crypt cells have no surface receptor for these drugs, (2) that they have an adenylate cyclase unresponsive to β-adrenergic stimulation, (3) that they do not allow the drug to cross the membrane, or (4) that the drugs do not reach the crypt cells in sufficient concentration to be effective. Even if the last more trivial reason is correct, the fact remains that addition of a drug known to stimulate at least two cellular functions does not do so uniformly throughout the intestinal mucosa. Since this tissue is composed of two major types (crypt and absorptive cells), the addition of a modifier of cell function cannot be assumed to act equally on both cell types. In the case of isoproterenol, the elevation of cAMP and stimulation of amino acid uptake both take place in the mature cells along the villus.

Acknowledgements—We would like to thank Miss Dottie Kinscherf for excellent technical assistance and Mrs. Lillian Hwang Peiper for excellent secretarial help. We are especially grateful to Dr. James Ferrendelli, in whose laboratory the cyclic AMP measurements were performed.

This work, which was supported in part by grants AM-14038 and AM-05280 from the United States Public Health Service, was presented in part at a meeting of the American Society for Clinical Investigation, Atlantic City, N.J., on April 29, 1973.

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