PROPERTIES OF GUANYLATE CYCLASE AND LEVELS OF CYCLIC GMP IN RAT SMALL INTESTINAL VILLOUS AND CRYPT CELLS

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1. Introduction

A number of studies on the mechanism of action of bacterial enterotoxins [1,2], prostaglandins [2] and the recently discovered gastrointestinal hormone vasoactive intestinal polypeptide (VIP)* [3] on small intestinal epithelium stress the role of cyclic AMP as a regulator of water and electrolyte transport in this tissue. The function of cyclic GMP in the small intestine is however entirely unknown. Its level as well as the activity of the particulate form of the enzyme guanylate cyclase in rat small intestinal epithelium are relatively high as compared to most other mammalian tissues [4–7]. The soluble form of guanylate cyclase which, at least in rat heart [6] and lung [8], clearly differs from the particulate enzyme in kinetic and physical properties, is nearly absent in small intestinal mucosa [4,6,7]. Our previous work [7] showed that the major part of the intestinal enzyme activity is associated with the microvillous membrane of the brushborder, the remaining part being localized in the basal-lateral plasma membranes. Accordingly, proliferative crypt cells displayed a much lower guanylate cyclase activity than the mature villous cells [7]. Since intestinal brushborders, which are easily obtained in a relatively pure state, provide one of the richest sources of particulate guanylate cyclase in the rat, the kinetic and other properties of this enzyme may be measured accurately even in the absence of an activating detergent used in other studies [6,8].

In the present investigation some characteristics of the brushborder-localized guanylate cyclase are compared with those of microsomal enzyme preparations from isolated villous and crypt cells, mainly representing enzyme bound to basal-lateral plasma membranes. A variety of hormones or hormone-like agents reported to raise cyclic AMP levels in rat small intestine, to elevate cyclic GMP in other tissues or to affect intestinal transport functions, were also tested for their ability to alter the intestinal guanylate cyclase activity in vitro or to regulate cyclic GMP levels in isolated villous and crypt cells and mucosal scrapings. The apparent insensitivity of the particulate enzyme to hormones and the possible function of cyclic GMP in the intestinal mucosa are discussed.

2. Materials and methods

Upper villous and crypt cells from the whole length of rat small intestine were harvested separately as described earlier [9,10]. Brushborders were isolated and purified in principle according to Harrison and Webster [11] as described before [12]. Microsomal preparations from villous and crypt cells were obtained by homogenizing the cells in 0.25 M sucrose–1 mM EDTA–1 mM Tris–HCl (pH=7,6) in a tightly fitting Potter-Elvehjem homogenizer, followed by differential centrifugation [10]. Mucosal scrapings were isolated by rinsing the intestinal lumen with icecold saline followed by gently scraping

* *Abbreviations:* Cyclic GMP, cyclic 3′, 5′-guanosine monophosphate. Cyclic AMP, cyclic 3′, 5′-adenosine monophosphate. GTP, guanosine 5′-triphosphate. PEP, phosphoenolpyruvate, tricyclohexylammonium salt. PTH, parathyroid hormone. VP, vasopressin. CP, creatinephosphate.

the superficial mucosal layer with a microscope slide. The cell sheets were taken up in saline and centrifuged for 2 min at 850 *g*. This procedure was repeated twice. Brushborders, cell preparations and microsomes were taken up in 0.10 M Tris–HCl (pH=7.6; final protein concentration: about 1 mg/ml) and homogenized in a Potter-Elvehjem homogenizer prior to enzyme assay.

Assay of guanylate cyclase activity and purification of the labeled product were done essentially as before [7]. The concentration of free Mn^{2+} was calculated from $[Mn^{2+}_{total}] = [Mn^{2+}_{free}] + [MnCP^-] + [MnGTP^{2-}]$. The stability constant of $MnCP^-$ used for calculating $[MnCP^-]$ was 110 M^{-1} [13]. Conversion of creatine-phosphate during the assay was less than 15 per cent. In view of the high binding constants of Mn^{2+}-nucleoside triphosphates [14] it was assumed that all GTP was present as the $MnGTP^{2-}$ complex. Binding of Mn^{2+} to cyclic GMP and the possible binding of GTP to albumin were neglected.

Cyclic GMP concentration was measured according to Dinnendahl [15], using a cyclic GMP-binding protein from lobster tail muscle [16]. Cyclic AMP concentration was measured according to Tovey et al. [17], using a cyclic AMP-binding protein isolated

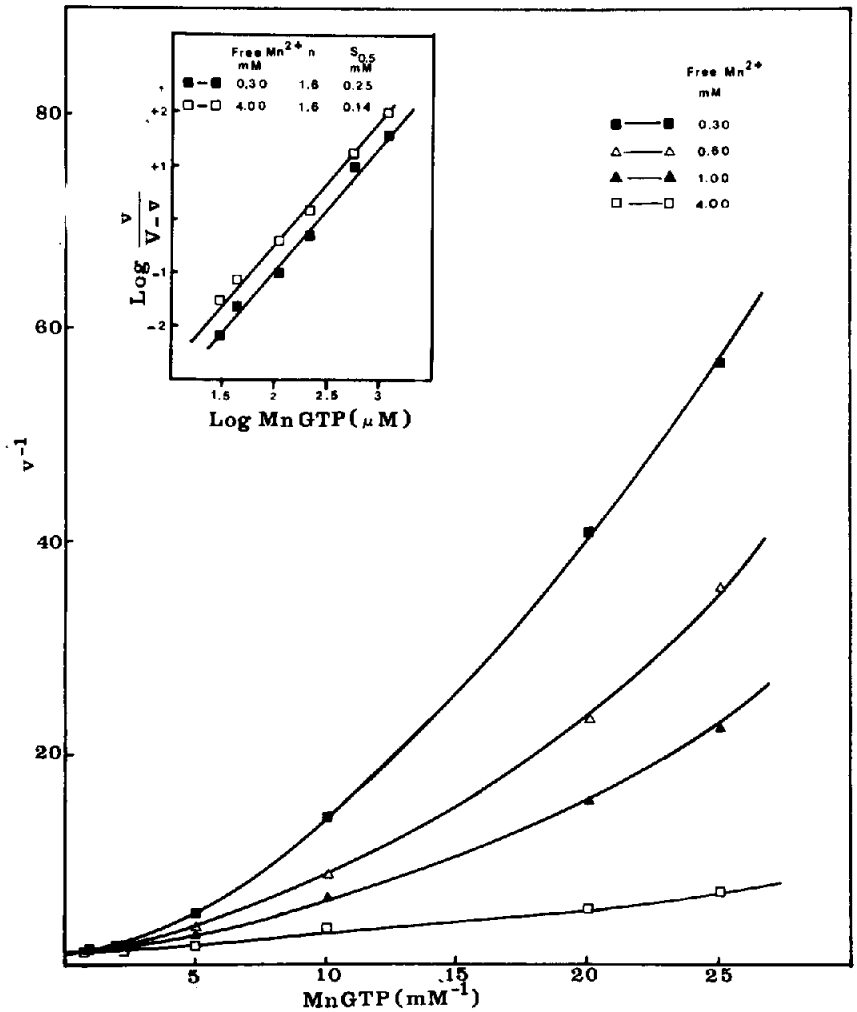

Fig. 1. Double reciprocal plots of cyclic GMP formation as a function of the $MnGTP^{2-}$ concentration measured with the brushborder preparation. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein. Inset: Hill plots.

from bovine skeletal muscle [18]. After further purification of the cyclic nucleotide samples by thinlayer chromatography on silica plates or chromatography on Dowex $50H^+$-columns, essentially the same results were obtained.

Protein was determined according to Lowry et al. [19], using bovine serum albumin as a standard.

Biochemicals were usually from Boehringer, Mannheim. Radioactive materials were from the Radiochemical Centre, Amersham. Arginine-vasopressin (synthetic, grade IV) was from Sigma, PTH (TCA-powder) from Inolex Corp. (Chicago), secretin from the Boots Company (Nottingham), concanavalin A (3 times crystallized) from Miles Labs., insulin (pure) from Boehringer, pentagastrin (Peptavlon) from ICI (Maclesfield), bradykinin (synthetic) from Sandoz and glucagon from Lilly (Indianapolis). The ionophore A-23187 was a gift from the Eli Lilly Company; it was dissolved in ethanol prior to use. Prostaglandins were gifts from the Unilever Research Lab. (Vlaardingen). Purified choleratoxin was kindly supplied by Dr R. S. Northrup, SEATO Cholera Research Program, and was prepared under contract for NIAID by R. A. Finkelstein, Ph.D., the University of Texas, Southwestern Medical School, Dallas, Texas.

3. Results

Some kinetic properties of the brushborder-bound guanylate cyclase are shown in fig.1 and 2. Lineweaver-Burk plots for different concentrations of free Mn^{2+} (fig.1) showed a positive co-operative behaviour indicating multiple binding sites for

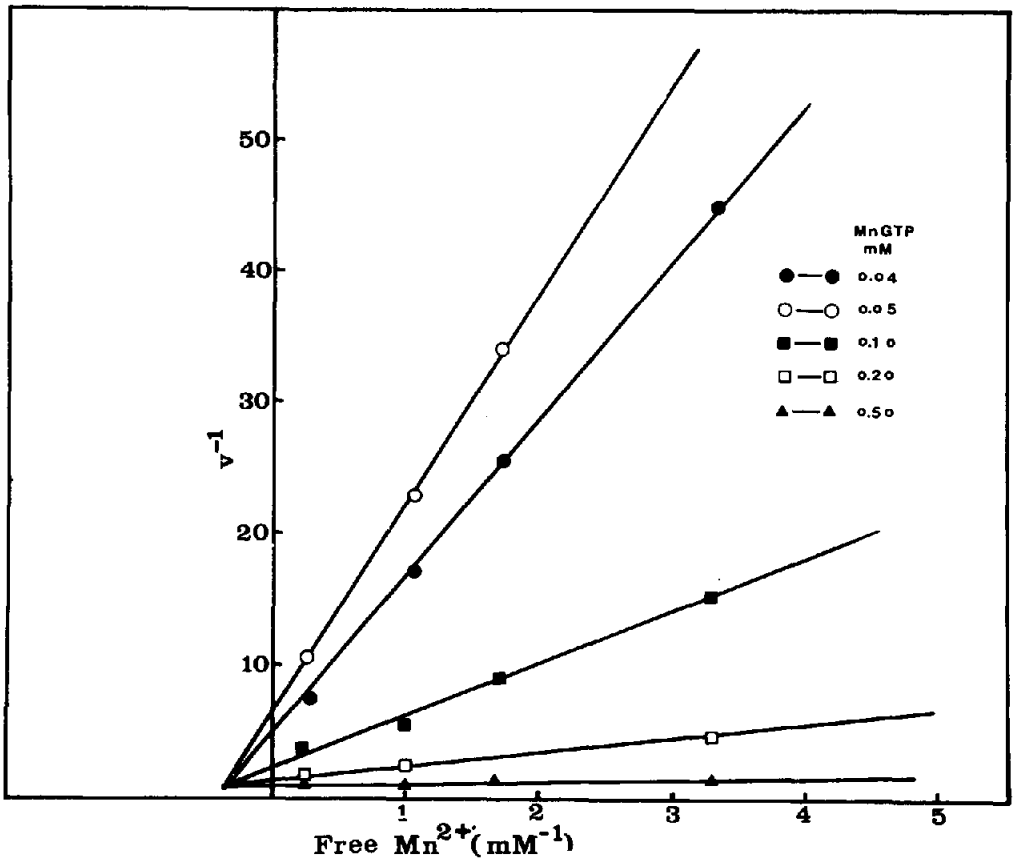

Fig.2. Double reciprocal plots of cyclic GMP formation as a function of excess Mn^{2+} measured with the brushborder preparation. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein.

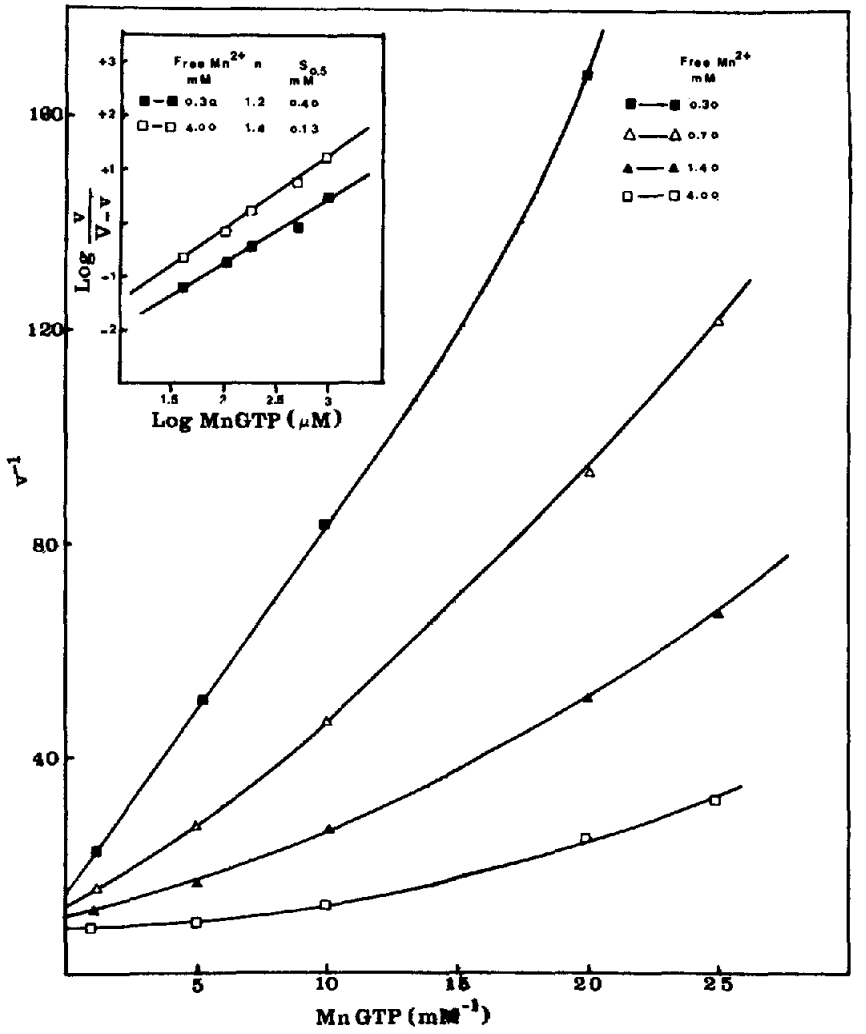

Fig.3. Double reciprocal plots of cyclic GMP formation as a function of $MnGTP^{2-}$ concentrations measured with the microsomal preparation from upper villous cells. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein. Inset: Hill plots.

$MnGTP^{2-}$. The Hill plot (fig.1, inset) gave a Hill coefficient (n) of 1.6. The $S_{0.5}$ for $MnGTP^{2-}$ decreased from 0.25 to 0.14 mM when Mn^{2+}_{free} was changed from 0.3 to 4.0 mM. Double reciprocal plots of cyclic GMP formation as a function of excess Mn^{2+} at fixed levels of $MnGTP^{2-}$ gave straight lines with a common point of intersection (fig.2). The dissociation constant for excess Mn^{2+}, calculated from the position of this point, was 2.5 mM. At $[MnGTP^{2-}] > 0.5$ mM the slopes of the plots approached zero, indicating that at saturating substrate concentrations the guanylate cyclase no longer needs excess Mn^{2+} for optimum activity.

The kinetic patterns of particulate guanylate cyclase from upper villous cell microsomes, presumably only slightly contaminated with brushborder material [10], clearly showed different characteristics (figs.3 and 4). There was a marked loss of co-operativity with respect to $MnGTP^{2-}$, especially at low $[Mn^{2+}_{free}]$. At 0.3 mM Mn^{2+}_{free} the Hill coefficient approached 1.2 and the value for $S_{0.5}$ was increased (fig.3). Even at near saturating concentrations of $MnGTP^{2-}$ the

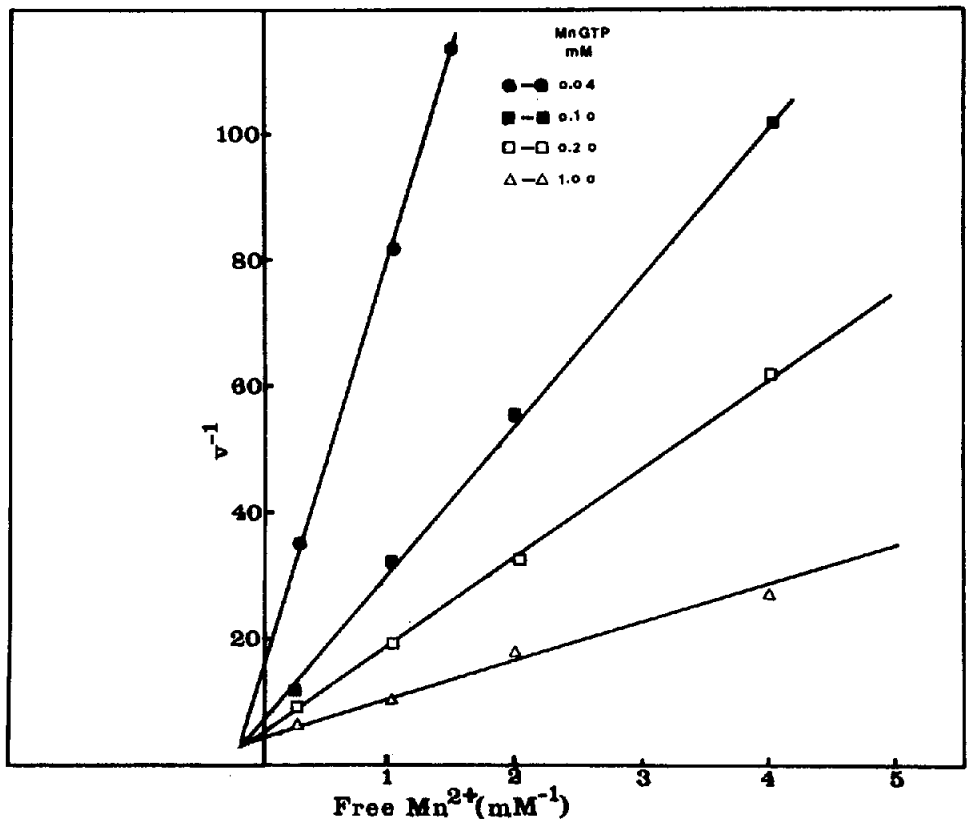

Fig.4. Double reciprocal plots of cyclic GMP formation as a function of excess Mn^{2+} measured with the microsomal preparation from upper villous cells. v is expressed as nmoles cyclic GMP · min^{-1} · mg^{-1} protein.

slope of the reciprocal plots of enzyme activity versus $[Mn^{2+}_{free}]$ did not approach zero. At 0.10 mM $MnGTP^{2-}$ the apparent K_m for Mn^{2+}_{free} was 2.7 mM and decreased to 0.7 mM at 1.0 mM $MnGTP^{2-}$ (fig.4).

Guanylate cyclase from crypt microsomes displayed an intermediate type of kinetic behaviour showing n-values for $MnGTP^{2-}$ between 1.3 and 1.4 (results not shown). These preparations, however, are seriously contaminated with luminal membranes (compare [20]) and thus contain a mixture of guanylate cyclase activities localized in basal-lateral membranes and partially developed microvilli.

Some regulatory properties of guanylate in vitro were tested with homogenates of isolated villous and crypt cells, mucosal scrapings, microsomes and brush-borders. Activities were measured at 0.1 mM $MnGTP^{2-}$ and 0.3 or 4.0 mM Mn^{2+}_{free}. Table 1 summarizes our results with the brushborder preparation. In agreement with Ishikawa et al. [4] we found that incubation of the enzyme in the presence of 0.1% Triton X-100 gave a two-fold rise in activity. 1.4 mM $CaCl_2$ slightly inhibited the enzyme at 0.3 mM Mn^{2+}_{free} but stimulated 1.2 fold at 4 mM Mn^{2+}_{free}. Strong inhibitory effects were found with PEP, oxaloacetate and ATP or ADP. Similar effects of metabolites have been reported earlier for the soluble enzyme from rat lung [21]. Alloxan, a strong inhibitor of intestinal adenylate cyclase (H. R. de Jonge, unpublished results), slightly depressed guanylate cyclase activity, possibly due to an in vitro oxidation of SH-groups (compare [22]). Aminoacids in physiological concentrations had a small stimulatory effect (table 1). D-isomers were ineffective in this respect. No significant differences from control values were obtained by addition of NaF, glucose, choleragen, prostaglandin E_1, $F_{2\alpha}$ and A_1, concanavalin A and a number of catechol-

Table 1
Guanylate cyclase activity in brushborder preparations from rat small intestinal epithelium

Agents	Cyclic GMP formation at 0.1 mM GTP (% of basal rate)	
	0.3 mM Mn^{2+}_{free}	4.0 mM Mn^{2+}_{free}
–	100	100
Triton X-100 (0.1%)	190	210
NaF (10 mM)	100	100
D (+)glucose (1–50 mM)	100	100
$CaCl_2$ (1.4 mM)	71	117
PEP (1 mM)	74	82
PEP (10 mM)	56	62
Oxaloacetate (0.5 mM)	52	61
ATP (1 mM)	26	29
ADP (1 mM)	32	36
D-alanine (1 mM)	100	99
L-alanine (1 mM)	119	120
L-aspartate (1 mM)	116	114
L-arginine (1 mM)	118	115
Glycine (1 mM)	109	110
Alloxan (5 mM)	70	75

The following agents, tested in the presence of 1.4 mM $CaCl_2$, had no effect as compared to $CaCl_2$ alone: Concanavalin A (0.2–1 μM), choleratoxin (10–100 μg/ml), prostaglandin E_1, A_1 and $F_2\alpha$ (1–50 μM), the catecholamines adrenalin, noradrenalin and serotonin-creatinesulphate (10–100 μM), the cholinergic agents carbamylcholine, acetylcholine and pilocarpine (0.5–50 μM) and the peptide hormones insulin (1–100 μg/ml), glucagon (1–10 μM) ± pentagastrin (10–50 μM), VP (4–50 mU/ml), PTH (0.04–2 U/ml) and bradykinin (50 μM).

amines, cholinergic agents and peptide hormones specified in the legend of table 1. These agents have been reported to alter intestinal transport of water and electrolytes (Na^+, $K^+$and HCO_3^- or Ca^{2+}) [2,3], to stimulate the soluble form of guanylate cyclase in liver in vitro (secretin, [23]) or to raise cyclic GMP levels in other tissues (compare [24]). Similar negative results were obtained using villous and crypt cell homogenates, microsomes or mucosal scrapings. Also after addition of Mg^{2+} (1 mM) or Ca^{2+} (1.4 mM), the insensitivity of the guanylate cyclase for these agents was maintained.

Because in other tissues cyclic GMP levels may be raised by hormones that are unable to stimulate the guanylate cyclase activity in vitro, our studies were extended with measurements of cyclic nucleotide levels in isolated villous and crypt cells during incubation at 37°C. Because hormone action may be disturbed by isolation of cells in EDTA-containing media, these studies were repeated with mucosal scrapings, isolated in saline.

As shown in table 2, the basal levels of cyclic GMP, like the cyclic AMP levels [25], decreased from crypt to villous cells. In contrast, the guanylate cyclase activity of cell homogenates increased about three-fold [7]. This apparent discrepancy may indicate a concomitantly raised cyclic GMP degradation rate in the villous cells. Moreover the assay conditions in vitro (concentrations of substrates and inhibitors) may considerably differ from the enzyme environment in the intact cells. The cyclic AMP/cyclic GMP ratio was relatively low as compared to a number of other tissues [4,26]. $CaCl_2$ (1.4 mM) alone caused a slight but statistically insignificant depression of basal cyclic GMP levels. Also the ionophore A-23187, known to mimic hormone actions in other tissues by stimulating Ca^{2+} transport through the plasma

Table 2
Levels of cyclic GMP and cyclic AMP in upper villous and crypt cells and mucosal scrapings from rat small intestinal epithelium

Additions	1.4 mM $CaCl_2$	pmoles/mg protein*						cyclic AMP / cyclic GMP ratio
		Cyclic GMP			Cyclic AMP			
		Villous cells	Crypt cells	Scrapings	Villous cells	Crypt cells	Scrapings	Villous cells
–	–	0.97 ± 0.20 (5)	1.90 ± 0.36 (4)	0.80 ± 0.15 (4)	4.3 ± 0.7 (7)	12.8 ± 1.1 (5)	5.1 ± 0.8 (6)	4.3
–	+	0.75 ± 0.10 (8)	1.35 ± 0.22 (6)	0.70 ± 0.10 (5)	5.0 ± 0.6 (7)	11.9 ± 1.0 (5)	5.0 ± 0.7 (5)	6.7
Ionophore A-23187 (1 μg/ml)	+	0.79 ± 0.21 (5)	1.78 ± 0.41 (4)	0.88 ± 0.17 (4)	5.2 ± 0.9 (3)	–	4.8 ± 1.0 (3)	6.6
Theophylline (5 mM)	+	0.76 ± 0.25 (7)	–	0.76 ± 0.20 (5)	8.5 ± 1.3 (6)***	–	9.6 ± 1.7 (4)***	11.2
Alloxan (5 mM)	+	2.20 ± 0.36 (4)***	–	2.05 ± 0.40 (4)***	5.5 ± 0.9 (4)	–	4.5 ± 1.2 (3)	2.5
PGE_1 (20 μM)	+		no effect		10.7 ± 1.1 (6)***	–	–	
PG $F_{2\alpha}$ (20 μM)	+		no effect		7.1 ± 0.9 (3)***	–	–	
Adrenalin (20 μM)	+		no effect		7.8 ± 0.7 (4)***	–	–	
Cholinergic agents **								
Choleratoxin	+		no effect		no effect			
Peptide hormones								

Freshly isolated villous and crypt cells or mucosal scrapings were gently dispersed in a Krebs-Ringer HCO_3^- buffer (pH=7.4) containg 20 mM glucose to a final protein concentration of 10–20 mg/ml. 1 ml portions of the suspension were preincubated in plastic vials for 5 min at 37°C, with gentle agitation, followed by a further incubation period of 1 min in the presence of the additions shown. $CaCl_2$, ionophore and theophylline were already present during preincubation. The incubation was rapidly stopped with 1 ml trichloroacetic acid (20% v/v) containing 0.01 μCi [^{3}H]cyclic AMP (0.5 pmol) or [^{3}H]cyclic GMP (0.7 pmol) immediately followed by ultrasonic disruption. Trichloroacetic acid was removed by ether extraction and most of the non-cyclic nucleotides were bound to Al_2O_3-columns as described previously [7]. The eluate was concentrated under a stream of N_2 and the residue was taken up in water prior to measurements of recovery and cyclic nucleotide-binding assays as described in Materials and methods. Overall recovery cyclic GMP: 60–80%, cyclic AMP: 50–70%.

* Cyclic nucleotide levels are expressed as means ± S.E.M., with the number of animals in parentheses. Similar results (not shown) were obtained after 5 min incubation (10 min in the presence of A-23187).

** A specification of these agents and their concentration range tested is given in the legend of table 1.

*** $p < 0.05$. p-values were calculated from the Student t-test.

membrane or releasing Ca^{2+} from intracellular stores, had no effect of intestinal cyclic GMP concentrations. Again, no significant changes of cyclic GMP were found in a Ca^{2+}-containing medium in the presence of choleratoxin, prostaglandins, cholinergic agents, concanavalin A or the hormones mentioned above.

Unexpectedly, incubation for 6–10 min in the presence of the phosphodiesterase inhibitor theophylline (5 mM) with or without hormones, did not raise the cyclic GMP content whereas cyclic AMP was significantly elevated (table 2, [25]). A possible depression of net formation of cyclic GMP by cyclic-AMP-elevating agents would not be unique for intestinal epithelium (compare [26]). It is also possible that theophylline induced a selective leakage of cyclic GMP to the medium where it may be rapidly metabolized by the residual activity of phosphodiesterases released from damaged cells.

Alloxan (5 mM) strongly elevated cyclic GMP in the isolated cells but had no influence on cyclic AMP levels (table 2). Apparently, the interaction of alloxan with cyclases in the intact cell is opposite to its in vitro effect, or else alloxan possesses an additional inhibitory effect on intracellular cyclic nucleotide degradation. In either case this compound could be a useful tool in future studies of the relationship between elevated cyclic GMP levels and intestinal functions.

4. Discussion

A clear difference was found between the kinetic behaviour of guanylate cyclase in brushborder and microsomal preparations from rat small intestinal villous cells. Whereas the brushborder enzyme had many characteristics in common with the particulate form of guanylate cyclase from sea urchin sperm [27] and rat lung [8] (positive substrate co-operativity, independency from free Mn^{2+} at saturating $MnGTP^{2-}$ concentrations), the kinetics of the microsomal enzyme, mainly localized in the basal-lateral plasma membranes, resembled more the soluble form of the enzyme in rat heart [6] and lung [8] (loss of co-operativity, activation by free Mn^{2+} at high $MnGTP^{2-}$ levels). Maybe the nearly complete absence of a soluble guanylate cyclase in rat small intestine is due to a high affinity of this type of enzyme for the basal-lateral plasma membranes. An alternative interpretation, assuming a rapid release of guanylate cyclase from membranes in other tissues during homogenization, has been suggested previously [26]. Also an alteration of kinetic behaviour during preparation of the microsomes cannot be entirely excluded yet.

The activity of the particulate guanylate cyclase in vitro or the levels of cyclic GMP in isolated small intestinal epithelial cells appeared insensitive to a large number of hormones or hormone-like agents under our experimental conditions. This insensitivity may be interpreted in different ways:

1) The epithelium lacks receptors for the hormones. Consequently the reported hormonal effects on transport rates should be ascribed to effects on intestinal smooth muscle or blood vessels.

2) Hormone effects are mediated by factors different from cyclic GMP.

3) Hormone receptors or coupling systems between receptors and guanylate cyclase are damaged during cell isolation or homogenization. Moreover, 'permissive' serum factors needed for full expression of hormone action may be lost in the in vitro system. The determination of cyclic GMP in samples of intestine, obtained by freeze-clamping the tissue in situ, would better reflect the physiological situation but lacks any tissue specificity.

4) Cyclic GMP levels are indeed altered by some hormones but the changes were too small, transient or delayed to be measured in our experiments. Small effects may be expected particularly if only the guanylate cyclase outside the brushborder region responds to hormones.

5) The reported action of cholinergic agents [24, 26], insulin [24] and the ionophore A-23187 [28, 29] on cyclic GMP levels in other tissues could be mediated by the soluble form of guanylate cyclase which is almost completely missing from the intestinal epithelium.

To our knowledge, a hormone effect on particulate guanylate cyclase is only well-established in fibroblasts where cyclic GMP levels may be elevated by a specific growth factor, a peptide hormone secreted by the pituitary gland [30]. It is not known if a similar growth factor for proliferative crypt cells, regulating the guanylate cyclase activity in their basal-lateral plasma membranes exists. However, in mature villous cells, at least 70 per cent of the total

guanylate cyclase activity has been found in the microvillous structure of the brushborder, making a direct coupling to hormone receptors at the antiluminal plasma membranes highly unlikely.[7]. Therefore, a possible role of cyclic GMP, generated by this enzyme, as a second messenger of hormone action must be seriously questioned.

The universal occurrence of specific cyclic GMP-dependent protein kinases in rat tissues and the high levels of these enzymes in arthropod tissues, where the particulate guanylate cylase is usually very active [31], strongly suggest that the biological effects of cyclic GMP are mediated by this class of proteins. In membranes from intestinal smooth muscle, and endogenous cyclic GMP-dependent protein kinase has been detected in association with two distinct substrate proteins [32]. It would be interesting to look for a similar system in the intestinal brushborder. Should the endogenous substrates be transport proteins, the slight stimulation of gunaylate cyclase activity in the brushborder by aminoacids (table 1) might be of physiological significance. The striking resemblance between the tubular structure of microvilli and flagellae [33], the high activity of guanylate cyclase in flagellar membranes from sea urchin sperm [27,34] and in platelets [35], and the possibility that the microfilaments within the core of the intestinal microvilli are actin-like [33,36], lend some support to the idea that cyclic GMP has a role in the assembly or regulation of contractile systems.

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