THE LOCALIZATION OF GUANYLATE CYCLASE IN RAT SMALL INTESTINAL EPITHELIUM

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1. Introduction

The distribution of guanylate cyclase activity among particulate and soluble fractions of broken cell preparations is strongly tissue-dependent. In scrapings from rat small intestinal epithelium virtually all activity has been found to be particulate [1,2]. Mucosal scrapings consist of a heterogeneous population of absorptive villous cells, proliferative and differentiating crypt cells and fragments of lamina propria. In our present study the distribution of guanylate cyclase among these various cell types and its intracellular localization was further explored.

The results show that guanylate cylase activity strongly increases from the depth of the crypt to the tip of the villus. The major part of its activity in the villous cells resides in the microvillous structure of the intestinal brushborder, the remaining part appears to be localized principally in the basal-lateral plasma membranes of the epithelial cell. In contrast, adenylate cyclase activity is nearly absent in the brushborder and predominates in the anti-luminal cell borders [3–5]. Possible implications of the distribution pattern of guanylate cyclase for the role of the particulate enzyme as a potential regulator of intestinal functions will be discussed.

2. Materials and methods

Upper villous, total villous and crypt cells from the whole length of rat small intestine were isolated separately as described earlier [5,6]. A crypt cell fraction enriched in proliferative cells was selected from the total crypt population by preferential binding to concanavalin A. We followed a slight modification of the procedure described by Podolsky and Weiser [7], using con A covalently bound to Sepharose (Pharmacia) instead of nylon fibers and filtration through screening nylon (porosity 110 μm) to separate con A-Sepharose from the unbound cells.

Fractionation of purified brushborders into membrane and fibrillar components was performed according to Forstner et al. [8] by means of hypotonic swelling followed by density gradient centrifugation.

For cellular fractionation studies, villous cells isolated from two rats were suspended in 0.25 M NaCl and 1 mM Tris–HCl pH 8.1, and homogenized in the Parr pressure bomb (Parr Instrument Co., Moline, Ill., U.S.A.) exactly as described by Douglas et al. [9]. Subfractionation of the homogenates was also performed according to the Douglas-method [9]. Brushborders, cell preparations and subfractions were finally taken up in 0.10 M Tris–HCl pH 7.6, and homogenized in a tightly fitting Potter-Elvehjem homogenizer prior to enzymic assay.

The assay system for guanylate cyclase (total vol 50 μl) contained 40 mM Tris–HCl, pH 7.6, 100 μg fatty acid poor bovine serum albumin (Fluka), 15 mM creatine phosphate, 20 μg creatine phosphokinase (approx. 25 U/mg), 0.5 mM cyclic GMP, 0.1 mM [α-^{32}P]GTP (sodium salt, 1 μCi), 2 mM theophylline and 0.5 or 5.0 mM free Mn^{2+}. The free Mn^{2+} concen-

Abbrevations: Cyclic GMP, cyclic 3′, 5′-guanosine monophosphate. Cyclic AMP, cyclic 3′, 5′-adenosine monophosphate. GTP, guanosine 5′-triphosphate.

tration was calculated from the total Mn^{2+} concentration by subtracting the amounts of Mn^{2+} sequestrated in creatinephosphate and GTP [10]. Samples of 2–10 μg protein were preincubated at 30°C for 2 min and incubated in the presence of substrate for 10 min. The reaction was stopped by adding 0.1 ml of a solution containing 5 mM GTP, 0.5 mM cyclic [^{3}H]GMP (approx. 20 000 dpm) and 6 mM pyrophosphate, pH 7.6. The final mixture was applied to Al_2O_3 columns (1 g dry Al_2O_3 (Merck) in Pasteur pipettes) and eluted with 2 ml 10 mM Tris–HCl, pH 7.6. The eluate was acidified with 30 μl 5 M HCl and applied to a Dowex 50H$^+$-column (200-400 mesh, 4 × 1 cm). During elution with 0.05 M HCl 80-85 per cent of the applied cyclic [^{3}H]GMP was recovered in the 4th through 8th ml and directly counted in a Packard liquid scintillation spectrometer (Tri-Carb 3380) for measurements of ^{3}H and ^{32}P activities using Dimilume (Packard) as the liquid scintillation mixture. Overall recovery of cyclic [^{3}H]GMP: 75–80%. Reaction blanks (complete system minus enzyme) were approximately 0.01 per cent of the initially added ^{32}P activity. About 95% of the ^{32}P-labelled product could be identified as cyclic GMP by means of a cyclic 3′, 5′-phosphodiesterase degradation procedure followed by rechromatography on Dowex 50H$^+$-columns. Conversion of cyclic GMP during incubation was less than 5% with all preparations tested. In all experiments the production of cyclic GMP was linear with time and protein concentration.

Alkaline phosphatase was determined at 37°C as described by Iemhoff et al. [11], non-specific esterase activity was measured according to Higgins and Lapides [12] at 37°C, sucrase was tested for according to Dahlqvist [13], rotenone-insensitive NADPH-cytochrome *c* reductase, succinate dehydrogenase and adenylate cyclase were determined as described earlier [5,14]. Ouabain-sensitive (Na$^+$-K$^+$)-ATPase was measured spectrophotometrically at 30°C according to Ferard et al. [15], using 1 mM ouabain. Protein was determined by the Lowry procedure [16], using bovine serum albumin as standard.

Biochemical products, if not stated, were obtained from Boehringer, Mannheim. Radioactive materials were obtained from the Radiochemical Centre, Amersham.

3. Results

By measuring guanylate cyclase activities in 100 000 *g* supernatants of isolated villous and crypt cells after homogenization, using Potter-Elvehjem (Teflon-glass), Polytron homogenizers or a pressure bomb (table 3) it could be confirmed that over 95% of the total activity was particulate in both cell types. The stability of the enzyme at 0°C as well as at 30°C [6] appeared of much advantage in prolonged fractionation studies.

Table 1 summarizes the specific enzyme activities measured in cell homogenates and brushborders from rat small intestine. On a protein base, sucrase, a marker enzyme of the microvillous membrane [17] is 5.4 times enriched in the purified brushborders as compared to upper villous cells. Alkaline phosphatase, localized in both microvilli and basal-lateral plasma membranes [18] appeared 4.2 times enriched in the brushborder preparations. However, the specific activities of (Na$^+$-K$^+$)-ATPase, a plasma membrane marker [19] and NADPH-cytochrome *c* reductase or non-specific esterase, both mainly localized in the endoplasmic reticulum [20,21] are similar or slightly diminished in the brushborders. The same holds for adenylate cyclase, also generally accepted as a basal-lateral plasma membrane marker in small intestinal epithelium [3–5]. In contrast, guanylate cyclase activity, measured at two different Mn^{2+} concentrations, is increased by 4.6-fold in the brushborders as compared to upper villous cells. The strongly increased specific activity is not due to a removal of enzyme inhibitors or release of activators during brushborder purification for combined assays of upper villous cell and brushborder samples gave completely additive results.

The finding of a preferential localization of guanylate cyclase in the brushborder region agrees with the rise of its activity during the development of the epithelial cell from the proliferative stage (represented by the crypt-con A cells) via its differentiating phase (present in total crypt cell preparations) into mature villous cells (table 1). The activities of other brushborder-bound enzymes (sucrase, alkaline phosphatase) also increase together with the development of intestinal microvilli (table 1). However, the presence of an activity jump from

Table 1
Specific enzyme activities in various cell preparations and brushborders isolated from rat small intestinal epithelium

Enzyme	Upper villous cells	Brush-borders	Crypt cells (total)	Crypt cells (con A-selected)
Guanylate cyclase*				
0.5 mM Mn^{2+}_{free}	16.1 ± 1.0 (9)	75.6 ± 15.2 (6)	6.1 ± 0.6 (5)	2.2 ± 0.3 (3)
5.0 mM Mn^{2+}_{free}	48.3 ± 2.4 (9)	218 ± 18 (6)	16.5 ± 1.0 (5)	7.4 ± 0.4 (3)
Adenylate cyclase (fluoride-stimulated)*	26.3 ± 1.2 (4)	24.9 ± 1.5 (4)	34.5 ± 1.0 (4)	–
(Na^+-K^+)-ATPase (ouabain-sensitive)**	70.7 ± 5.5 (4)	83.1 ± 4.8 (4)	28.3 ± 2.7 (3)	–
NADPH-cytochrome *c*-reductase (rotenone-insensitive)**	6.3 ± 0.4 (4)	4.1 ± 0.5 (3)	2.4 ± 0.3 (3)	–
Esterase**	2780 ± 70 (4)	1610 ± 40 (4)	980 ± 30 (4)	570 (2)
Alkaline phosphatase**	410 ± 16 (8)	1710 ± 42 (6)	40 ± 4 (5)	26 ± 2 (3)
Sucrose**	92.7 ± 4.3 (3)	495 ± 24 (4)	12 ± 3 (4)	–

* Specific activity expressed as picomoles cyclic GMP or cyclic AMP formed per min mg of protein.
** Specific activity expressed as nanomoles substrate converted per min per mg of protein.
Number of preparations tested is given in parentheses.

Table 2
Relative specific activities and distribution of enzymes after subfractionation of purified brushborders according to Forstner et al. [8].

Brushborder fractions**	Relative specific activity*				
	Sucrase	Alkaline phosphatase	NADPH-cytochrome *c* reductase (rotenone-insensitive)	Esterase	Guanylate cyclase
Membrane fraction I (microvillous)	1.33 (24)	1.72 (31)	3.70 (65)	3.33 (60)	1.40 (25)
Membrane fraction II (microvillous)	1.54 (63)	1.28 (52)	0.68 (27)	0.84 (34)	1.61 (66)
Fibrillar fraction	0.26 (10)	0.26 (10)	0.13 (5)	0.11 (4)	0.29 (11)
Recovery (%)	97	93	97	98	103

* The relative specific activity represents the ratio of the specific activity of the enzyme in each fraction and its specific activity in the whole brushborder preparation (see table 1).
** Terminology derived from the paper of Forstner et al. [8].
The percentage of total brushborder activity recovered in the subfractions is given in parentheses.
Mean values of two experiments are shown.

crypt to villus does not give sufficient proof for a typical localization in the brushborder region because marker enzymes of the endoplasmic reticulum (e.g. esterase, table 1 and [21]) and of basal-lateral plasma membranes ((Na^+-K^+)-ATPase, table 1 and [22]) also rise in activity from crypt to upper villus.

After separation of the isolated brushborders into microvillous and fibrillar fractions (table 2) the distribution of guanylate cyclase followed that of the microvillous enzymes sucrase and alkaline phosphatase and was clearly different from the endoplasmic reticulum markers. The distribution pattern also excludes a possible fibrillar origin of the enzyme.

Finally, homogenates of isolated villous cells were subfractionated according to the fractionation scheme of Douglas et al. [9] and the distribution of guanylate cyclase and a variety of marker enzymes among these fractions was studied.

The distribution of alkaline phosphatase and guanylate cyclase followed the same pattern and was clearly different from that of the other membrane-bound markers tested (table 3). The highest relative specific activity of both enzymes was found in the so-called M_1 fraction, enriched in the basal-lateral plasma membrane marker (Na^+-K^+)-ATPase and nearly devoid of mitochondrial and microvillous membranes. The conclusions drawn from the previous experiments with isolated brushborders are strengthened by the observation that the major part of the guanylate cyclase activity is recovered in the 1500 *g* pellet (fraction B) that mainly consists of brushborders, nuclei and whole cells. The data from table 3 strongly suggest that most, if not all of the guanylate cyclase outside the brushborder is

Table 3
Relative specific activities and distribution of enzymes in subcellular fractions prepared from rat small intestinal villous cells according to Douglas et al. [9]

Cell fraction**	(Na^+-K^+)-ATPase ouabain-sensitive	Alkaline phosphatase	NADPH-cytochrome *c* reductase (rotenone-insensitive)	Sucrase	Guanylate cyclase	Succinate dehydrogenase
Fraction B	1.37	2.53	0.94	2.35	2.76	0.34
	(38)	(71)	(26)	(66)	(77)	(9)
Sorbitol layer	1.22	0.07	4.06	0.26	0.26	0.68
	(12)	(1)	(41)	(3)	(3)	(7)
Fraction D	3.02	3.00	1.23	1.44	2.32	7.70
	(30)	(30)	(12)	(14)	(23)	(77)
Fraction M_1	7.69	3.69	2.79	0.11	3.93	0.50
	(10)	(6)	(4)	(0)	(5)	(1)
Fraction M_2	2.43	1.26	5.64	0.04	1.60	0.42
	(6)	(3)	(14)	(0)	(4)	(1)
Supernatant	–	0.06	–	–	0.08	–
(105 000 *g*)	(0)	(2)	(0)	(0)	(3)	(0)
Recovery	87	113	97	83	115	95

* The relative specific activity compares the specific activity of each fraction (on a protein base) with that of the whole homogenate obtained by disruption of the villous cells in a pressure bomb.

** Terminology used by Douglas et al. [9].

The M_1 and M_2 fractions were isolated by dextran gradient centrifugation at barrier density of 1.075 exactly as described by Douglas et al. [9].

The percentage of total homogenate activity recovered in the subcellular fractions is given in parentheses.

Fraction B mainly contains brushborders, nuclei and whole cells, the sorbitol layer is enriched in endoplasmic reticulum, fraction D contains a mixture of mitochondria, basal-lateral plasma membranes and some brushborder and endoplasmic reticulum activities, fraction M_1 consists mainly of basal-lateral plasma membranes and fraction M_2 is enriched in endoplasmic reticulm membranes.

localized in the anti-luminal membranes of the villous cell, together with (Na^+-K^+)-ATPase, alkaline phosphatase and adenylate cyclase.

4. Discussion

Our results indicate that the bulk of guanylate cyclase is present in the apical region of the villous cell together with other microvillous enzymes like sucrase and alkaline phosphatase. A much smaller part of the enzyme content is integrated in the basal-lateral plasma membrane as has been shown too for alkaline phosphatase [18]. The different distribution pattern of membrane-bound guanylate cyclase and adenylate cyclase together with their different properties [1,10] argues strongly against the hypothesis [23] that the same enzyme system would be responsible for the synthesis of both cyclic GMP and cyclic AMP.

The preponderance of guanylate cyclase in the brushborder presumably corresponds to the greater surface area of the microvilli. In proliferating crypt cells, lacking the microvillous structure [24], the enzyme activity is about 15% of the upper villous cell activity (table 1). The preferential localization in the brushborder may explain the high specific activity of this particulate enzyme in mucosal scrapings as compared to most other tissues of the rat in which microvillous structures are less well developed or nearly absent (compare [2]). Leakage of cyclic GMP from the brushborder region into the lumen could also account for the considerable amounts of this nucleotide found in the intestinal contents [1].

It is tempting to speculate about the function of this brushborder-bound guanylate cyclase. Regulation of its activity by direct interaction with hormone receptors in the basel-lateral membranes of the epithelial cell seems very unlikely. Regulation by changes of the free Ca^{2+} concentration within the cell [25] seems equally questionable because Ca^{2+} has very little influence on the activity of the particulate enzyme in vitro [10].

Maybe the unique localization of guanylate cyclase in this cell type is related to the transport function of the microvillous membrane. In rabbit ileum in virto, addition of cyclic GMP caused an alteration of ion fluxes in the same direction as evoked by cyclic AMP [26]. A possible role of cyclic nucleotides in regulation of transport processes has been brought forward earlier in studies correlating cyclic AMP levels and transport rates of Na^+, K^+ and HCO_3^- [5,27], Ca^{2+} [28] and amino acids [29] in small intestinal epithelium.

Up till now no physiological conditions are known in which intestinal cyclic GMP levels undergo significant changes [10,27]. This might indicate that constant levels of cyclic GMP are necessary to maintain normal cellular functions and that deviations from this level, as in the case of cyclic AMP accumulation [5,27], soon lead to pathological states. However, physiological alterations of cellular cyclic GMP levels could be very small, especially if confined to a certain compartment within the cell. In other tissues these are usually very rapid and transient phenomena. Such changes may therefore easily be missed in an experimental approach, particularly as long as the role of cyclic GMP in the small intestinal epithelium is a matter of mere speculation.

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References

[1] Ishikawa, E., Ishikawa, S., Davis, J. W. and Sutherland, E. W. (1969) J. Biol. Chem. 244, 6371–6376.
[2] Kimura, H. and Murad, F. (1974) J. Biol. Chem. 249, 6910–6916.
[3] Parkman, D. K., Ebel, H., Dibona, D. R. and Sharp, G. W. G. (1972) J. Clin. Invest. 51, 2292–2298.
[4] Grand, R. J., Torti, F. M. and Jaksina, S. (1973) J. Clin. Invest. 52, 2053–2059.
[5] de Jonge, H. R. (1975) Biochim. Biophys. Acta 381, 128–143.
[6] Webster, H. L. and Harrison, D. D. (1969) Exp. Cell Res. 56, 245–253.

[7] Podolsky, D. K. and Weiser, M. M. (1973) J. Cell Biol. 58, 497–500.
[8] Forstner, G. G., Sabesin, S. M. and Isselbacher, K. J. (1968) Biochem. J. 106, 381–390.
[9] Douglas, A. P., Kerley, R. and Isselbacher, K. J. (1972) Biochem. J. 128, 1329–1338.
[10] de Jonge, H. R., manuscript in preparation.
[11] Iemhoff, W. G., van den Berg, J. W. O., de Pijper, A. M. and Hülsmann, W. C. (1970) Biochim. Biophys. Acta 215, 229–241.
[12] Higgins, C. and Lapides, J. (1947) J. Biol. Chem. 170, 467–482.
[13] Dahlqvist, A. (1964) Anal. Biochem. 7, 18–25.
[14] de Jonge, H. R. and Hülsmann, W. C. (1972) Eur. J. Biochem. 32, 356–364.
[15] Ferrard, G., Sall, I. and Metais, P. (1975) Enzyme 19, 38–47.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[17] Eichholz, A. (1967) Biochim. Biophys. Acta 135, 475–482.
[18] Clark, S. L. (1961) Amer. J. Anat. 109, 57–83.
[19] Fujita, M., Ohta, H., Kawai, K., Matsui, H. and Nakao, M. (1972) Biochim. Biophys. Acta 274, 336–347.
[20] Clark, M. L., Lanz, H. C. and Senior, J. R. (1969) Biochim. Biophys. Acta 183, 233–235.
[21] Deimling, O. V. and Madreiter, H. (1972) Histochemie 29, 83–96.
[22] Charney, A. N., Gots, R. E. and Giannella, R. A. (1974) Biochim. Biophys. Acta 367, 265–270.
[23] Illiano, G., Tell. G. P. E., Siegel, M. I. and Cuatrecasas, P. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2443–2447.
[24] de Both, N. J., van Dongen, J. M., van Hofwegen, B., Keulemans, J., Visser, W. J. and Galjaard, H. (1974) Dev. Biol. 38, 119–137.
[25] Goldberg, N. D., O'Dea, R. F. and Haddox, M. K. (1973) in: Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G. A. eds) Vol. 3, pp. 155–223. Raven Press, New York.
[26] Field, M. (1971) Amer. J. Physiol. 221, 992–997.
[27] Field, M. (1974) Gastroenterology 66, 1063–1084.
[28] Neville, E. and Holdsworth, E. S. (1969) FEBS Lett. 2, 313–316.
[29] Kinzie, J. L., Ferrendelli, J. A. and Alpers, D. H. (1973) J. Biol. Chem. 248, 7018–7024.