Calmodulin independence of human duodenal adenylate cyclase

J A Smith, M Griffin, S E Mireylees, R G Long

Abstract

The calmodulin and calcium dependence of human adenylate cyclase from the second part of the duodenum was assessed in washed particulate preparations of biopsy specimens by investigating (a) the concentration dependent effects of free $[Ca^{2+}]$ on enzyme activity, (b) the effects of exogenous calmodulin on enzyme activity in ethylene glycol bis (b-aminoethyl ether)*N,N'*-tetra-acetic acid (EGTA) washed particulate preparations, and (c) the effects of calmodulin antagonists on enzyme activity. Both basal ($IC_{50}=193.75$ (57.5) nmol/l (mean (SEM))) and NaF stimulated ($IC_{50}=188.0$ (44.0) nmol/l) adenylate cyclase activity was strongly inhibited by free $[Ca^{2+}]$ greater than 90 nmol/l. Free $[Ca^{2+}]$ less than 90 nmol/l had no effect on adenylate cyclase activity. NaF stimulated adenylate cyclase activity was inhibited by 50% at 2.5 mmol/l EGTA. This inhibition could not be reversed by free Ca^{2+} . The addition of exogenous calmodulin to EGTA (5 mmol/l) washed particulate preparations failed to stimulate adenylate cyclase activity. Trifluoperazine and *N*-(8-aminohexyl)-5-iodo-1-naphthalene-sulphonamide (iodo 8) did not significantly inhibit basal and NaF stimulated adenylate cyclase activity when measured at concentrations of up to 100 μ mol/l. These results suggest that human duodenal adenylate cyclase activity is calmodulin independent but is affected by changes in free $[Ca^{2+}]$.

Adenylate cyclase is a basolateral membrane enzyme which catalyses the formation of adenosine 3'-5' monophosphate (cyclic AMP) from adenosine triphosphate (ATP). In the intestinal epithelium adenylate cyclase and cyclic AMP are involved in the regulation of water and electrolyte transport, particularly Cl^- secretion and Na^+ absorption. Changes in intracellular free calcium have also been shown to be important in the regulation of intestinal electrolyte transport.¹ Activation of adenylate cyclase is thought to be important in a number of gut disorders such as the secretory diarrhoea associated with cholera and vasoactive intestinal peptide secreting tumours.^{2,3} Calmodulin is an ubiquitous intracellular calcium binding protein which influences many enzymic processes. The possible calmodulin dependence of intestinal adenylate cyclase has potential clinical implications as a large number of drugs which are calmodulin antagonists are now well characterised and available – for example, chlorpromazine, trifluoperazine, haloperidol, α -adrenergic antagonists, antimalarials, anticholinergics, and antihistamines.⁴ The use of calmodulin antagon-

ists as antidiarrhoeal agents, however, has had only limited success in humans.²

Ca^{2+} and calmodulin in some tissues, such as brain, are known to influence adenylate cyclase activity and therefore cyclic AMP production. Several investigators have shown that Ca^{2+} inhibits the activity of adenylate cyclase at concentrations greater than 1 μ mol/l while at submicromolar Ca^{2+} concentrations activation of the enzyme occurs. It is thought that this activation of adenylate cyclase is mediated by calmodulin but the inhibition of the enzyme by Ca^{2+} is calmodulin independent.³⁻⁷ There is now evidence to suggest that some rat,⁸ guinea pig,⁹ and rabbit¹⁰ intestinal adenylate cyclase activity is Ca^{2+} and calmodulin dependent.

In brain tissue both calmodulin dependent and calmodulin independent forms of adenylate cyclase have been identified. The calmodulin dependent component can be inhibited by micromolar concentrations of the calcium chelating agent ethylene glycol bis (b-aminoethyl ether)*N,N'*-tetra-acetic acid (EGTA).³ Furthermore, fluoride ions, which stimulate adenylate cyclase activity by direct activation of the regulatory guanine nucleotide binding proteins, may also help to distinguish the two forms of adenylate cyclase. In vitro the calmodulin dependent form of the enzyme is more responsive to fluoride stimulation than the independent form.¹¹

The objective of this study was to assess the importance of Ca^{2+} and calmodulin in the regulation of adenylate cyclase in the second part of the human duodenum. This was accomplished by assessing (a) the concentration dependent effects of free $[Ca^{2+}]$ on enzyme activity, (b) the effects of adding exogenous calmodulin to the adenylate cyclase assay system, and (c) the effects on enzyme activity of the calmodulin antagonists trifluoperazine and *N*-(8-aminohexyl)-5-iodo-1-naphthalene-sulphonamide (iodo 8), a new naphthalene sulphonamide calmodulin antagonist of improved potency and specificity.¹² All these parameters were tested on basal and NaF stimulated adenylate cyclase activity. The results cast doubt on the role of calmodulin in the regulation of human duodenal adenylate cyclase.

Methods

COLLECTION OF BIOPSY SPECIMENS

Patients presenting at the clinic with diarrhoea or iron deficiency anaemia were biopsied as part of diagnostic investigations to exclude malabsorption. Biopsy specimens of the second part of the duodenum were collected as previously described.¹³ Two specimens were placed in formalin for histological examination to exclude villous

Medical Research
Centre, City Hospital,
Nottingham NG5 1PB
J A Smith
R G Long

Department of Life
Sciences, Nottingham
Polytechnic, Nottingham
NG11 8NS
M Griffin
S E Mireylees

Correspondence to:
Dr R G Long.

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abnormalities. Those for adenylate cyclase studies were immediately placed in liquid nitrogen. Only specimens from patients subsequently found to have normal histology, who had no underlying upper gastrointestinal disease, and who were not taking any medication were used for these studies. Ethical permission for the study was obtained from the Nottingham City Hospital Ethical Committee and patients gave informed written consent.

TISSUE PREPARATIONS

The presence of extracellular and intracellular fluids in tissue homogenates has been shown to interfere with adenylate cyclase activity.¹⁴⁻¹⁶ We therefore washed biopsy homogenates with a series of homogenisation and centrifugation steps. Four or five biopsy specimens from one patient were homogenised in 2 ml ice cold 2 mmol/l HEPES buffer, pH 7.5, containing 5 mmol/l $MgCl_2$ and 5 mmol/l EGTA (MEH) using 10 strokes of a Potter S homogeniser (B Braun) at 1200 rpm. The homogenate was made up to 12 ml and was centrifuged at 2500 g for 20 minutes at 4°C. The pellet was resuspended in 2 ml fresh MEH and the homogenisation/centrifugation procedure repeated twice more.

Any large tissue fragments remaining after the second homogenisation were removed. Microscopic examination showed them to be underlying muscularis mucosa and not epithelium.¹⁷ The third pellet was resuspended in 2.4 ml MEH and homogenised a fourth time using an Ultra-Turrax (Janke & Kunkel, Ika-Werk) at maximum speed setting for 15 seconds. Calmodulin may be removed from membrane preparations by washing them in EGTA buffers.³ Therefore, in experiments in which no attempt was made to remove endogenous calmodulin EGTA was omitted from the tissue buffer. All buffers were made up in double distilled deionised water or HiperSolv (high performance liquid chromatography grade water).

Cerebral cortex from 2 male Wistar rats were combined and treated as for duodenal biopsy specimens. This washed particulate preparation was used as a positive control to ensure that the assay conditions used were capable of detecting calmodulin dependent adenylate cyclase activity.

ASSAY OF ADENYLATE CYCLASE

Adenylate cyclase was assayed by the procedure of Salomon *et al.*^{18,19} The assay measures the formation of [^{32}P] cyclic AMP from [α - ^{32}P]ATP. In a total volume of 100 μ l the standard reaction mixture contained: an ATP regenerating system of 13 mmol/l creatine phosphate and 0.1 mg/ml creatine phosphokinase, 1 mmol/l ATP labelled with [α - ^{32}P]ATP to give approximately 1×10^6 cpm, and 40 μ l adenylate cyclase assay buffer which consisted of 125 mmol/l tris-HEPES, 0.25% bovine serum albumin, 5 mmol/l $MgCl_2$ and 2.5 mmol/l of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine, at pH 7.5. For NaF stimulated activity, reactions also contained 10 mmol/l NaF (originally dissolved in 1 mmol/l acetic acid). When calmodulin was

added to the assay it was dissolved in the adenylate cyclase assay buffer. Calmodulin antagonists (trifluoperazine and IODO 8) were dissolved in dimethyl sulphoxide before addition to the reaction system. Control reaction mixtures contained dimethyl sulphoxide only and final dimethyl sulphoxide assay concentrations were never allowed to exceed 1% (v/v).

Reactions were initiated by the addition of approximately 30 μ g of particulate protein and carried out for 30 minutes at 37°C. Adenylate cyclase activity was linear up to the maximum conditions tested for time (60 minutes) and protein concentration (3.9 mg/ml). Reactions were terminated by the addition of 100 μ l of stopping solution containing: 10 mmol/l ATP, 1 mmol/l [^3H]cyclic AMP (25 000 cpm/100 μ l) (to estimate [^{32}P] cyclic AMP recovery which was 70–90%), 2% sodium dodecylsulphate (w/v), and 50 mmol/l Tris-HCl at pH 7.5. Tubes were placed in a boiling water bath for 2 minutes, allowed to cool, and made up to 1 ml with distilled water. [^{32}P] cyclic AMP was purified, before liquid scintillation counting, by the double column procedure of Salomon *et al.*¹⁸

CALMODULIN ASSAY

The calmodulin content of the particulate preparations was assayed according to the phosphodiesterase activation method of Thompson *et al.*^{20,21}

SUCRASE DETERMINATION

The brush border enzyme, sucrase, was assayed by the method of Dahlqvist.²²

PROTEIN DETERMINATION

The protein content of the tissue preparation was determined by Lowry's method²³ and was normally found to be approximately 1 mg/ml. Bovine serum albumin was used as standard.

CALCULATION OF FREE Ca^{2+} CONCENTRATIONS

Contamination of buffered solutions by Ca^{2+} (up to 20 μ mol/l) necessitates the use of the divalent chelator EGTA in controlling and estimating the free [Ca^{2+}].^{24,25} For each calcium chloride addition the free [Ca^{2+}] was computed for ambient pH 7 in the presence of Mg^{2+} , EGTA, and ATP using an updated version of the ligand-metal binding program of Feldman *et al.*^{26,27}

STATISTICAL ANALYSIS

Where appropriate the Wilcoxon two sample test and the Wilcoxon signed rank tests were used to estimate probability values. Probability values of <0.05 were taken as significant.

MATERIALS

All chemicals were from Sigma Chemical with the exception of [^3H]cyclic AMP and [α - ^{32}P]ATP which were from Amersham (UK) and HiperSolv from BDH (Aterstone). The IODO 8 was kindly given by Ian Coutts and Pam

TABLE 1 Relative specific activity of sucrase and adenylate cyclase activity in pooled supernatants and particulate preparations of human duodenal biopsy specimens. Results are mean (SEM) from three separate preparations assayed twice

Fraction	Sucrase	Adenylate cyclase	
		Basal	NaF stimulated
Homogenate	1	1	1
Pooled supernatant	6.60 (0.87)	0.67 (0.25)	0.95 (0.20)
Pellet	0.83 (0.02)	1.69 (0.32)	2.53 (0.09)

O'Donnell of Nottingham Polytechnic, Nottingham.

Results

CHARACTERISTIC OF PARTICULATE PREPARATIONS FROM HUMAN DUODENAL BIOPSY SPECIMENS

Table I gives the relative specific activity of the brush border enzyme, sucrase, compared with the basolaterally located adenylate cyclase in pooled supernatants and particulate preparations from three independent tissue preparations. Sucrase activity was enriched in the pooled supernatants and adenylate cyclase activity was enriched in the particulate fractions.

The calmodulin content of the EGTA washed particulate preparations was compared with that of preparations washed in the absence of EGTA to assess the effectiveness of the EGTA washing procedure at removing calmodulin. In brain tissue there was a 25% (7.49 µg/mg protein to 5.61 µg/mg protein) reduction in calmodulin after washing with EGTA containing buffer. The calmodulin content of the particulate preparation from human duodenal biopsy speci-

mens, however, was approximately 4–6 µg/mg protein irrespective of whether the preparation had been washed in EGTA containing buffer or not.

THE EFFECTS OF Ca^{2+} , AND EGTA ON ADENYLATE CYCLASE ACTIVITY

Stimulation of adenylate cyclase activity at low free $[\text{Ca}^{2+}]$ has been reported to be a good indication of the enzyme's calmodulin dependence.⁵ Therefore, the concentration dependent effects of Ca^{2+} on adenylate cyclase activity in particulate preparations of human duodenal biopsy specimens was measured. Both basal ($\text{IC}_{50}=193.75$ (57.5) nmol/l (mean (SEM))) and NaF stimulated ($\text{IC}_{50}=188.0$ (44.0) nmol/l) adenylate cyclase activities were strongly inhibited by free $[\text{Ca}^{2+}]$ greater than 90 nmol/l (Fig 1). Free $[\text{Ca}^{2+}]$ less than 90 nmol/l neither stimulated nor inhibited adenylate cyclase activity. These data indicate a dramatic reduction in adenylate cyclase activity over a narrow free $[\text{Ca}^{2+}]$ range.

An alternative way of assessing the importance of Ca^{2+} in our assay system was to measure the concentration dependent effects of the Ca^{2+} chelating agent EGTA on adenylate cyclase activity (Fig 2). NaF stimulated adenylate cyclase activity was inhibited by EGTA in a concentration dependent manner with 50% inhibition at 2.5 mmol/l. Attempts to restore EGTA inhibited adenylate cyclase activity with Ca^{2+} did not result in a recovery of the enzyme's activity, indicating direct inhibition of adenylate cyclase activity by EGTA. A slight but highly significant increase in basal adenylate cyclase

Figure 1: Adenylate cyclase concentration response to free Ca^{2+} . NaF stimulated (A) and basal (B) adenylate cyclase activity was assayed in particulate preparations of duodenal biopsy specimens from four patients. Activity was in the presence of 2.5 mmol/l ethylene glycol bis (b-aminoethyl ether) N,N'-tetra-acetic acid (EGTA) and CaCl_2 at concentrations which gave calculated $[\text{Ca}^{2+}]$ free as indicated on the abscissa. Each line represents one patient and each data point is the mean of four replicates. Controls were in the absence of added CaCl_2 and in the presence of EGTA.

Figure 2: Adenylate cyclase concentration response to EGTA. Particulate preparations of duodenal biopsy specimens were assayed for NaF stimulated (■) and basal (□) adenylate cyclase activity in the absence of added CaCl_2 and in the presence of EGTA at the concentrations indicated. Data are the means (SEM) from three experiments – that is, three patients – each with four replicates.

TABLE II Effect of exogenous calmodulin on adenylate cyclase activity in particulate preparation from human duodenal biopsy specimens. Data are the results of three separate experiments each with four replicates

Added $[\text{Ca}^{2+}]$ (mmol/l)	[calmodulin] ($\mu\text{mol/l}$)	[EGTA] (mmol/l)	Calculated free $[\text{Ca}^{2+}]$ (pCa)	pmol cAMP/min/mg protein (SEM)	
				Basal	NaF stimulated
0.16	0.15	2.5	7.76	3.20 (0.29)	19.88 (1.07)
0.16	0	2.5	7.62	2.85 (0.24)	20.77 (0.76)
1.16	0.15	2.5	6.52	0.54 (0.04)	4.22 (0.32)
1.16	0	2.5	6.52	0.51 (0.05)	3.50 (0.32)
0	0	2.5	–	3.40 (0.17)	20.60 (0.49)
0.80	3.13	2.5	6.78	3.72 (0.03)	14.98 (0.28)
0.80	0	2.5	6.78	3.04 (0.08)	13.33 (0.45)
0	0	2.5	–	4.25 (0.04)	14.74 (0.51)
0.40	3.73	1.0	6.63	1.36 (0.06)	11.74 (0.12)
0.40	0	1.0	6.63	1.88 (0.03)	9.90 (0.26)
0	0	1.0	–	2.34 (0.05)	18.73 (0.72)

TABLE III Control experiments in which the effect of exogenous calmodulin on adenylate cyclase activity in particulate preparation of rat cerebral cortex was measured. Data are the results of two separate experiments each with duplicate determinations

Added $[\text{Ca}^{2+}]$ (mmol/l)	[calmodulin] ($\mu\text{mol/l}$)	[EGTA] (mmol/l)	Calculated free $[\text{Ca}^{2+}]$ (pCa)	pmol cAMP/min/mg protein (SEM)	
				Basal	NaF stimulated
2.30	0.61	2.5	5.40	9.93 (4.71)	53.37 (10.53)
2.30	0	2.5	5.40	6.32 (0.25)	33.48 (3.25)
0	0	2.5	–	5.93 (0.09)	17.32 (0.26)
0.90	0.61	1.0	5.51	18.57 (1.32)	51.83 (2.61)
0.90	0	1.0	5.51	4.73 (1.18)	37.35 (3.47)
0	0	1.0	–	6.72 (0.26)	23.94 (0.04)

activity was observed at 0.25 mmol/l EGTA compared with controls; thereafter the curve plateaued up to the maximum concentration tested (5 mmol/l) (Fig 2).

EFFECTS OF EXOGENOUS CALMODULIN ON ADENYLATE CYCLASE ACTIVITY

The removal of calmodulin by washing membranes with EGTA buffers and the subsequent stimulation of adenylate cyclase activity by adding calmodulin back to the assay system has been shown for several tissues.⁵ No stimulation could be shown in our results from similar experiments using human duodenal biopsy particulate preparations. The addition of calmodulin failed to stimulate adenylate cyclase activity in the presence of various combinations of free calcium, calmodulin, and EGTA (Table II). None of the assay conditions shown resulted in a stimulation of adenylate cyclase activity over controls in the presence or absence of 10 mmol/l NaF.

The possibility existed that our assay methods were incapable of detecting calmodulin dependent adenylate cyclase activity. We therefore measured adenylate cyclase activity in a tissue well recognised to have a highly active calmodulin dependent component, rat cerebral cortex.³ The preparation and conditions of assay for rat cerebral cortex were conducted in exactly the same way as for human duodenal biopsy specimens. This tissue clearly showed the following characteristics: (a) the calmodulin content of the preparation was reduced by 25% by the EGTA washing procedure used, (b) a biphasic response to calcium (activation followed by inhibition) with a maximum requirement for free $[\text{Ca}^{2+}]$ of 1–10 $\mu\text{mol/l}$, (c) the addition of 10 $\mu\text{g/ml}$ calmodulin to EGTA wash particulate preparations stimulated adenylate cyclase activity by 30–60% in the presence and absence of 5 mmol/l NaF (Table III). These findings are fully compatible with what is known for brain calmodulin dependent adenylate cyclase.³ Verification of the assay methods used was thus established.

EFFECTS OF CALMODULIN ANTAGONISTS ON ADENYLATE CYCLASE ACTIVITY

The dose dependent effects of trifluoperazine and IODO 8 were examined to establish at what concentration these calmodulin antagonists exerted an influence on adenylate cyclase activity and further to compare the effects of the relatively non-specific trifluoperazine to that of the more specific IODO 8.¹² By analysis of variance trifluoperazine (Fig 3) and IODO 8 (Fig 4) did not significantly inhibit basal and NaF stimulated adenylate cyclase activity up to a concentration of 100 $\mu\text{mol/l}$. The IC_{50} for trifluoperazine of NaF stimulated adenylate cyclase activity was 225 $\mu\text{mol/l}$ and for basal activity 125 $\mu\text{mol/l}$. Corresponding IC_{50} values for IODO 8 were 450 and 175 $\mu\text{mol/l}$, however, indicating that the NaF stimulated adenylate cyclase activity was inhibited less potently than basal activity. This also shows that IODO 8 is less potent than trifluoperazine at directly inhibiting adenylate cyclase activity. These data

Figure 3: Adenylate cyclase concentration response to trifluoperazine. Biopsy particulate preparations were assayed for NaF stimulated (■) and basal (□) adenylate cyclase activity in the presence of background Ca^{2+} and the trifluoperazine concentrations indicated. No attempt was made to remove endogenous calmodulin from these preparations. Data are the means (SEM) for three separate patients with four replicates for each trifluoperazine concentration.

Figure 4: Adenylate cyclase concentration response to N-(8-aminohexyl)-5-iodo-1-naphthalene-sulphonamide (IODO 8). Biopsy particulate preparations were assayed for NaF stimulated (■) and basal (□) adenylate cyclase activity in the presence of background Ca^{2+} and the IODO 8 concentrations indicated. No attempt was made to remove endogenous calmodulin from these preparations. Data are the means (SEM) for three separate patients with four replicates for each IODO 8 concentration.

suggest that inhibition of basal as well as stimulated adenylate cyclase activity at concentrations of this magnitude represent the non-specific effects of these calmodulin antagonists.

Discussion

Data presented in this paper suggest that human duodenal adenylate cyclase activity was affected by small changes in free $[\text{Ca}^{2+}]$. Unlike the intestinal adenylate cyclase from rat,⁸ guinea pig,⁹ and rabbit,¹⁰ it was calmodulin independent. Evidence to support this comes from a number of our findings. Firstly, we were unable to detect any consistent Ca^{2+} stimulation of adenylate cyclase activity of a magnitude previously reported in other systems.^{5,8,10} The detection of Ca^{2+} stimulation of adenylate cyclase is notoriously difficult because it occurs at very low Ca^{2+} concentrations. Therefore, careful manipulation of free ion concentrations by EGTA buffers is necessary.^{3,4,24,25}

The inhibition of adenylate cyclase activity by EGTA implies a requirement of the enzyme for Ca^{2+} . This effect is only apparent on the NaF stimulated adenylate cyclase activity which initially suggests that Ca^{2+} /calmodulin may only be important in the regulation of the stimulated enzyme. The effects of EGTA, however, could not be reversed by Ca^{2+} . This suggests that the chelator may have been inhibiting the enzyme directly and not in a way mediated by Ca^{2+} chelation and calmodulin inactivation.³⁴ The slight increase in basal adenylate cyclase activity at low EGTA concentrations may be the result of the chelation of an unknown inhibiting ion.²⁸ The mechanism for Ca^{2+} inhibition of adenylate cyclase has not been elucidated. Some evidence indicates specific Ca^{2+} inhibitory sites,²⁹ while other evidence implies Ca^{2+} competition for Mg^{2+} binding sites.³⁰ There is no evidence that the inhibition of adenylate cyclase by Ca^{2+} is mediated through calmodulin.³⁴

Secondly, we were unable to stimulate adenylate cyclase activity in EGTA washed particulate preparations with exogenous calmodulin. This may be explained by the inadequate removal of the endogenous membrane bound calmodulin by buffers containing EGTA. Indeed, the calmodulin content of our particulate preparations from duodenal biopsy specimens was not significantly reduced by the EGTA washing procedure. The levels of calmodulin in our assay system were comparable to those of other workers using animal intestinal membranes and some have shown calmodulin dependence in the presence of endogenous calmodulin at concentrations similar to those in our assay system.^{8,9} The effective removal of calmodulin from different tissues has been reported to vary considerably.³⁻⁵ Our data suggest there may also be species differences between the same tissue. This observation has been noted for other tissues, such as heart sarcoplasmic reticulum,³¹ kidney,³² and pancreatic islets.³³

Recent evidence on the distribution of calmodulin in enterocytes indicated that in the human duodenum calmodulin was confined to the soluble fractions and to the brush border membrane; none was detected in the basolateral

membrane.³⁴ This provides indirect support for our findings in that the distribution of human duodenal adenylate cyclase (a basolateral membrane enzyme) is not coincidental with the distribution of membrane bound human duodenal calmodulin. It may be that in our assay system, in which both brush border and basolateral membranes are present, the tightly bound brush border membrane calmodulin becomes available to influence the basolateral membrane adenylate cyclase. But if this were so, and we had been measuring a fully calmodulin stimulated adenylate cyclase system, we would have expected to be able to block the calmodulin effect with calmodulin antagonist (at concentrations specific to calmodulin) and this was not the case. The IC₅₀ for trifluoperazine and IODO 8 were well in excess of those reported for other calmodulin dependent enzymes (6–50 and 3–10 μmol/l respectively).^{12 35 36} Interestingly, the distribution of calmodulin in the membranes of rat enterocytes seems to be different from that of human enterocytes. Charpin *et al*³⁷ reported that in rat duodenal basolateral membranes fractions calmodulin could be detected.

Although further investigations are necessary to confirm our findings, these preliminary data indicate that adenylate cyclase in the human duodenal mucosa is calmodulin independent. This implies that increasing the specificity of calmodulin antagonists for use as anti-diarrhoeal drugs, which operate through the adenylate cyclase system, would have little pharmacological benefit. It would be interesting to see if human jejunal and ileal tissue adenylate cyclase activity behaves in a similar way.

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