Mucosal (Na⁺-K⁺)-ATPase and Adenylate Cyclase Activities in Children with Toddler Diarrhea and the Postenteritis Syndrome

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Summary

Assays for (Na⁺-K⁺)-ATPase and basal, fluoride stimulated, and percentage of activation of adenylate cyclase have been established for small portions of human jejunal biopsies. Control means and ranges have been established for each activity in a group of children with failure to thrive but no gastrointestinal disease. Activities of (Na⁺-K⁺)-ATPase and percentage of activation and basal activity of adenylate cyclase are increased in children with toddler diarrhea. (Na⁺-K⁺)-ATPase activity is reduced in children with active postenteritis syndrome, and recovery from this syndrome is associated with an increase in this activity and of percentage of activation and basal activity of adenylate cyclase.

Speculation

Assay of mucosal (Na⁺-K⁺)-ATPase and adenylate cyclase activities in jejunal biopsies will enhance knowledge of the pathophysiologic mechanisms operating in diarrheal states. Alterations in the activities of these enzymes in some children with "toddler diarrhea" provide a pathophysiologic basis to explain their symptoms, and the similar changes found in children who have recovered from "postenteritis syndrome" suggest a link between the two conditions.

In recent years, the pathophysiology of diarrheal states has been the center of intensive investigations, and it has become apparent that the mucosal enzyme, adenylate cyclase (located at the basolateral membranes of the enterocyte), is of fundamental importance in the genesis of certain diarrheal states such as cholera (5, 8). Sodium-potassium activated adenosine triphosphatase [(Na⁺-K⁺)-ATPase] is similarly located in the enterocyte, and there is good evidence that it plays an important role in water and electrolyte absorption (21, 24). Moreover, it has been suggested that depressed (Na⁺-K⁺)-ATPase activity may be of pathophysiologic importance in some diarrheal states (11, 14, 16, 27, 28).

In this paper, assays to determine the specific activities of these two enzymes in small portions of small intestinal mucosa have been validated and applied to patients with toddler diarrhea and postenteritis syndrome and a group of control children. No similar studies have previously been reported.

SUBJECTS AND METHODS

CONTROLS

Controls consisted of 22 children (12 boys and 10 girls aged 0.7 to 14 years with a median age of 4.8 years) who were investigated for short stature but who had no gastrointestinal symptoms. All were receiving a normal diet at the time of biopsy. Biopsies were

performed to exclude asymptomatic coeliac disease and were normal under light microscopy. Full investigations including hemoglobin, white cell count, differential, erythrocyte sedimentation rate, microscopy, culture of urine, plasma electrolytes, urea, calcium, magnesium, phosphate, proteins, transaminases, alkaline phosphatase, immunoglobulins, urinary vanillin mandelin acid, amino acids, sweat tests and, where clinically indicated, barium contrast studies were all within normal limits. None were hypothyroid or had growth hormone deficiency by standard criteria.

TODDLER DIARRHEA

In this group, 23 biopsies were carried out in 22 children (14 boys and 8 girls aged 1.5 to 9 years with a median age of 3 years) who had intermittent (*n* = 7) or continuous (*n* = 15) diarrhea and fell within the clearly defined syndrome of toddler (chronic non-specific) diarrhea (6). The children had chronic diarrhea dating from preschool years which was not associated with food intolerances, a histologically abnormal biopsy, or an abnormal growth velocity. One patient, on a gluten-free diet at the time of initial biopsy, was rebiopsied later after a formal 3-month challenge with a normal diet. The age of onset varied from 0.25 to 4 years with a median of 0.75 years, and the duration of symptoms from 0.8 to 4 years with a median of 2 years. Although the majority (*n* = 12) had heights and weights that were on or above the 50th centiles for age, eight children had heights or weights that were on or just below the third centile. This distribution of heights and weights is not representative of our clinical experience of patients with toddler diarrhea, but reflects our policy to perform jejunal biopsies in children of short stature when there is no other apparent cause.

POSTENTERITIS SYNDROME

Twenty-six biopsies were performed on the 22 patients in this group, (12 boys and 10 girls aged 0.25 to 1.9 years with a median age of 1 year). These patients had persistent diarrhea after an acute episode of diarrhea ("gastroenteritis") associated with failure to thrive (*i.e.*, weight static or falling), or an histologically abnormal jejunal biopsy, or both (Fig. 1). Nineteen patients were biopsied during the active (group 1) phase of the disease, and seven (group 2) were biopsied during recovery (*i.e.*, no gastrointestinal symptoms and gaining weight). Nine of group 1 were receiving normal diets at the time of biopsy, and 10 were on exclusion diets: eight cow's milk-free, and two clear fluid regimes. Five of group 2 patients were receiving milk-free diets. In some of the patients, gluten had been excluded on an empirical basis, but none were shown to have coeliac disease on subsequent formal gluten challenge. Investigations excluded a diagnosis of cystic fibrosis and immunodeficiency in all patients, and in most cases, anatomical abnormalities of the gastrointestinal tract were excluded by barium studies. In group 1, eight of the biopsies were morphologically normal, nine showed varying degrees of partial

Figure 1 Subdivision of patients with the post-enteritis syndrome

Fig. 1. Subdivision of patients with postenteritis syndrome. Numbers in parentheses, number of patients.

villous atrophy, and two showed subtotal villous atrophy. All biopsies were normal in group 2. In four patients, sequential biopsies were performed.

Jejunal Biopsies. Jejunal biopsies were obtained by standard methods (19) using a modified Crosby capsule (13) and divided for light microscopy and snap freezing for enzyme assays after storage at -70°C for periods of up to 1 year.

Homogenization. Homogenization was carried out within 1 min of the start of the assays using 20 strokes of a Dounce homogenizer in the following medium: (mM concentrations) ice cold Tris/HCl (20):Na₂EDTA (1):MgSO₄ (1) at pH 7.4. Final protein concentration was less than 1.5 mg/ml.

Adenylate Cyclase. Adenylate cyclase activities were assayed in the absence and presence of 3.75 mM NaF which gave maximal (8 to 13 times basal) activation in a total volume of 150 μl of medium which contained the following: Tris/HCl buffer (40):MgSO₄ (5):dithiothreitol (1):bovine serum albumin (1 mg/ml):1-methyl-3-isobutyl xanthine (1):disodium ATP (2) at pH 7.6; the concentration of Mg⁺⁺ and pH was optimal. Ten-min incubations were terminated by transfer of 50 μl aliquots to 50 μl of acetate buffer (400) containing approximately 1.8×10^5 dpm of [2,8-³H]cyclic adenosine 3':5'-monophosphate (cyclic AMP) (Amersham, Radiochemical Centre, TRK 498) at pH 4.5. Reactions were linear to 10 min and 2 mg/ml of homogenate protein. The generated cyclic AMP was measured by the protein kinase binding method of Gilman (10) preparing the protein by modifications of the methods of Walsh *et al.* (29) and Miyamoto *et al.* (17). The partially purified protein was diluted for use in 2 mg/ml of bovine serum albumin when the association constant for [³H]cyclic AMP was 2.0×10^{-6} M in the presence of cyclase assay medium. [³H]ATP had an association constant of >1 M, and NaF (3.75) had no effect on binding. Standards were made up using cyclic AMP in the cyclase buffer giving a linear standard curve (free/bound *versus* standard) over the range 0.1 to 20 pmoles cyclic AMP per 50 μl with a correlation coefficient of >0.997 and a coefficient of variation of $<2\%$. Nonspecific binding of cyclic AMP by homogenate was minimal (80 to 100 dpm) and was subtracted as a blank. Recovery of cyclic AMP added before enzyme assay was $108 \pm 10\%$ ($n = 12$). A quality control of pooled rat intestinal mucosa was included at the beginning and end of each assay run and, on 24 occasions over a 2-year period, gave coefficients of variation of 12.1 and 9.8% for basal and fluoride-stimulated adenylate cyclase activities, respectively.

(Na⁺-K⁺)-ATPase. The activity of (Na⁺-K⁺)-ATPase was estimated by the subtraction of the magnesium-stimulated ATPase from the total (magnesium, sodium, and potassium stimulated) ATPase activity. The total activity was assayed in a medium consisting of imidazole buffer (at mM concentrations) (30), Na₂EDTA (0.15), MgCl₂ (6.5), NaCl (140), KCl (13), and disodium

ATP (4.4) at pH 6.6. The Mg-ATPase activity was measured in a similar medium which included ouabain (1) and omitted the KCl. Reactions were initiated by adding 25 μl of homogenate to 550 μl of medium and were run for 30 min at 37°C before being stopped by the addition of 100 μl of trichloroacetic acid (25% w/v). The mixture was then mixed and spun at 4°C , and the inorganic phosphate liberated was estimated in the supernatant by an automated modification of the method of Fiske and SubbaRow (9). The assays were optimised for pH and the concentrations of inorganic ions. The K_m for (Na⁺-K⁺)-ATPase in human small intestinal mucosa was found to be 0.33 mM. The reaction was linear for greater than 60 min and with protein concentrations less than 3 mg/ml. A quality control (as used for the cyclase assay) was run on 28 occasions over a 2-year period and gave a coefficient of variation of 12.5%.

Protein. Protein concentrations were estimated using an automated modification of the method of Lowry *et al.* (15).

STATISTICS

Means, standard deviations, and standard errors were calculated for grouped data and groups compared by Student's *t* tests. Separate variance estimates were used in the calculation where *F* tests showed a significant difference in the variances of the groups being compared. *P* values of <0.05 were taken as indicating a significant statistical difference. Results were also analysed by nonparametric methods (Mann-Whitney *U* test), and the *P* values were similar in every instance.

RESULTS

CONTROLS

Figure 2 shows the specific activities of (Na⁺-K⁺)-ATPase and adenylate cyclase in control biopsy material. All the values fell within the ranges calculated as means ± 2 S.D. The distribution of the control values was approximately Gaussian for each of the enzymes, and statistical analysis confirms that there is no significant skewness or kurtosis.

TODDLER DIARRHEA

In patients with toddler diarrhea, the mean activities of (Na⁺-K⁺)-ATPase ($P < 0.05$) and of percentage of activation ($P < 0.01$) and basal ($P < 0.02$) adenylate cyclase were increased as compared to controls (Table 1). Approximately one-third of the individual values were increased to levels above the control ranges for each of these activities. There was some correspondence between patients with increased enzyme activities in that, of the seven patients with raised (Na⁺-K⁺)-ATPase activities, five also had increased

percentage of activation, and four had increased basal activity of adenylate cyclase, with an increase in both parameters of cyclase activity in three. There was, however, no significant correlation between any of the parameters of adenylate cyclase and $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ in either group as a whole. Inspection of the clinical

details of those with increased activity of one or more measured activity as a subgroup and comparison with the remainder did not reveal any differences in age, sex, type of diarrhea, or duration of symptoms.

POSTENTERITIS SYNDROME-GROUP 1

In the patients in group 1, the mean $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ activity was significantly reduced ($P < 0.002$) compared with the controls (Table 2), and this difference was further accentuated if only the morphological abnormal biopsies were compared with the controls (1.81 ± 0.31 ; $P < 0.001$). The mean for those with normal morphology was 2.45 ± 0.29 , which is not significantly less than the controls or more than the subgroup with abnormal morphology. There were no significant differences in any of the measured parameters of adenylate cyclase activity between group 1 and the controls or between those on normal and those on exclusion diets. The three patients who were thriving, although having histologically abnormal jejunal biopsies (Fig. 1) had $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ activities (0.92, 1.08, and $1.69 \mu\text{moles Pi per mg protein per hr}$) similar to those of group 1 (mean activity, 2.08) and dissimilar to those of group 2 (mean activity, 3.46). Adenylate cyclase activities were no different to either group 1 or 2.

Fig. 2. Activities of $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ and adenylate cyclase in control biopsies. See Table 1 for 1, 5, and 7. —, mean; ===== ± 1 S.E. Bars, ± 2 S.D.

Table 1. $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ and adenylate cyclase activities in controls and toddler diarrhea

	Controls	<i>P</i>	Toddler diarrhea
$(\text{Na}^+-\text{K}^+)\text{-ATPase}^1$	3.05 ± 0.17^2 (20) ³	$<0.05^4$	3.75 ± 0.34 (23)
Adenylate cyclase basal ⁵	13.1 ± 0.6 (22)	$<0.02^4$	16.3 ± 1.2 (23)
Fluoride stimulated ⁶	135 ± 6 (20)	NS ⁶	132 ± 9 (23)
Percentage of activation ⁷	9.9 ± 0.5 (20)	$<0.01^4$	13.2 ± 1.1 (23)

¹ $\mu\text{moles inorganic phosphate} \cdot \text{mg protein}^{-1} \cdot \text{hr}^{-1}$.

² Mean ± 1 S.E.

³ Numbers in parentheses, number of biopsies assayed.

⁴ Separate variance estimate of Student's *t* test.

⁵ $\mu\text{moles cyclic AMP} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$.

⁶ NS, not significant.

⁷ $\frac{\text{Basal activity}}{\text{Fluoride-stimulated activity}} \times 100$.

POSTENTERITIS SYNDROME-GROUP 2

Comparison of the enzyme activities of patients in the recovery phase with those in the active phase (Table 2) reveals a significant increase in $(\text{Na}^+-\text{K}^+)\text{-ATPase}$, percentage of activation and basal activity of adenylate cyclase. In four patients where serial biopsies were taken during both the active and the recovery phases (Fig. 3), there was a significant mean increase in $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ ($+1.38 \pm 0.42$; $P < 0.05$) and in each of the measured parameters of adenylate cyclase (basal: $+5.1 \pm 1.6$, $P < 0.025$; fluoride stimulated: $+28 \pm 8$, $P < 0.025$; and percentage of activation, $+2.5 \pm 1.0$, $P < 0.05$). There were no significant differences for any of the measured enzyme activities between the subgroups on normal or exclusion diets.

DISCUSSION

In this paper, we report the establishment and validation of methods to reproducibly assay the specific activities of $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ and basal, fluoride-stimulated, and percentage of activation of adenylate cyclase in small portions of jejunal biopsies obtained during the clinical investigation of children. Mean values for each of the measured activities for a population of control children have been established allowing comparison of data from children in different clinical groups and obviating the need for

Table 2. $(\text{Na}^+-\text{K}^+)\text{-ATPase}$ and adenylate cyclase activities in controls and postenteritis syndrome¹

	Adenylate cyclase			
	$(\text{Na}^+-\text{K}^+)\text{-ATPase}^2$	Basal ³	Fluoride stimulated ²	% Activation ⁴
Controls	3.05 ± 0.17^5 (20) ⁶	13.1 ± 0.6 (22)	135 ± 6 (20)	9.9 ± 0.5 (20)
<i>P</i>	<0.002	NS ⁷	NS	NS
Postenteritis syndrome				
Group 1	2.08 ± 0.22 (19)	11.9 ± 1.1 (18)	143 ± 12 (18)	8.9 ± 0.9 (18)
<i>P</i>	<0.006	<0.04	NS	<0.05
Group 2	3.46 ± 0.46 (7)	16.1 ± 1.6 (7)	151 ± 24 (7)	12.6 ± 1.9 (7)

¹ *P*'s compare adjacent values.

² $\mu\text{moles inorganic phosphate} \cdot \text{mg protein}^{-1} \cdot \text{hr}^{-1}$.

³ $\mu\text{moles cyclic AMP} \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$.

⁴ $\frac{\text{Basal activity}}{\text{Fluoride-stimulated activity}} \times 100$.

⁵ Mean ± 1 S.E.

⁶ Numbers in parentheses, number of biopsies assayed.

⁷ NS, not significant.

within-patient controls which are often not available from clinical material. A close temporal relationship has been documented between adenylate cyclase activation, accumulation of tissue levels of cyclic AMP, and secretion of fluid and electrolytes into the small intestinal lumen (8). In view of our findings, it is of interest to note that the effect of secretagogues such as bacterial toxins, vasoactive intestinal peptide, and prostaglandins on the basal activity of adenylate cyclase is by increasing activation of the enzyme, fluoride-stimulated activity (a measure of total enzyme protein) remaining unchanged. We have previously assayed (Na⁺-K⁺)-ATPase and adenylate cyclase in the jejunal mucosa of patients with active coeliac disease and shown that (Na⁺-K⁺)-ATPase activity was reduced whereas, in contrast to the present study, all three measured parameters of adenylate cyclase activity were increased (28). On the basis that adenylate cyclase activity is higher in crypt cells (20, 22) and (Na⁺-K⁺)-ATPase is higher in villous cells (3, 4), we postulated that our findings in coeliac disease resulted from an increased epithelial population of immature, crypt-like cells secondary to the increased cell turnover rate which occurs in the active phase of this disease (30). A similar explanation has been proposed for the findings of reduced ouabain-inhibited sodium efflux [equivalent to (Na⁺-K⁺)-ATPase activity] in mucosa with partial villous atrophy obtained from piglets infected by transmissible gastroenteritis virus (12).

TODDLER DIARRHEA

It is unlikely that our findings in toddler diarrhea are related to changes in cell turnover rates because the morphologic appearances of the mucosa were not consistent with abnormal turnover rates, and the activities of (Na⁺-K⁺)-ATPase and adenylate cyclase were both increased rather than inversely related. Dodge (7) has reported that approximately 30% of a group of children with toddler diarrhea had raised serum levels of prostaglandins and that the diarrhea in the patients responded to aspirin, a prostaglandin synthetase inhibitor. Although we did not determine serum prostaglandins in our patients, it is possible that excessive synthesis of prostaglandins could account for the increased basal activity and activation of adenylate cyclase. The gastrointestinal hormone VIP might be expected to induce similar changes in adenylate cyclase, but to our knowledge it has not been measured in the serum or stools of patients with toddler diarrhea.

Oddsson *et al.* (18) studied adults with the irritable bowel syndrome (continuous or intermittent diarrhea in whom standard investigations were normal), demonstrated an increased sensitivity of the small intestine to the secretagogue glycochenodeoxycholic acid, and suggested that their findings were consistent with a prostaglandin mediated stimulus. They did not, however, assay mucosal adenylate cyclase.

It is also possible that stimuli such as prostaglandins or peptide hormones might account for the observed increase in (Na⁺-K⁺)-ATPase activity, although we are not aware of any experimental data to support the former possibility. However, glucocorticoid administration to rats has been shown to stimulate (Na⁺-K⁺)-ATPase activity to a degree that reverses secretion-induced by cholera toxin (2). Thus, it is conceivable that in certain situations increased (Na⁺-K⁺)-ATPase activity might represent a compensatory mechanism to counteract the action of secretagogues. Moreover, it is known that (Na⁺-K⁺)-ATPase activity is increased in the colon by potassium loading (1, 23), a better stimulus to the enzyme than salt-deficiency states (26).

Our findings of abnormal enzyme activities in morphologically normal small intestinal mucosa together with those of Dodge (7) and Oddsson *et al.* (18) suggested that toddler diarrhea should not necessarily be regarded as a variant of normal stool patterns, but rather as a self-limiting and probably harmless disorder.

POSTENTERITIS SYNDROME

The activities of (Na⁺-K⁺)-ATPase in group 1 patients were similar to those which we have previously reported in active coeliac disease (27, 28) in that mean activity was depressed with

Fig. 3. Activities of (Na⁺-K⁺)-ATPase and adenylate cyclase in serial biopsies in four children who recovered from postenteritis syndrome. See Table 1 for 1, 5, and 7. ○, ●, normal morphology; ■, partial villous atrophy; ▣, subtotal villous atrophy. ►, ■, ●, presence of diarrhea; ○, no diarrhea. Difference between groups 1 and 2 each significant at *P* < 0.05 level using paired tests.

the greatest reduction in those with morphologically abnormal biopsies. The fact that (Na⁺-K⁺)-ATPase activity was depressed in the three patients who were thriving in group 1 suggests that the morphologic abnormality *per se* is more important in the mechanisms of reduced activity rather than any effects of semi-starvation (25, 31).

In contrast to our findings in coeliac disease (22, 28), the activity of adenylate cyclase was not increased, suggesting that enterocyte immaturity is unlikely to be the explanation for the reduction in (Na⁺-K⁺)-ATPase activity in the active phase of postenteritis syndrome.

The mean activities of (Na⁺-K⁺)-ATPase, basal-, fluoride- and percentage-activated adenylate cyclase were all increased in patients recovering from the postenteritis syndrome (group 2) compared to controls, but only percentage of activation was statistically significant (*P* < 0.03). However, compared with patients with active postenteritis syndrome (group 1), activities of (Na⁺-K⁺)-ATPase and each parameter of adenylate cyclase were increased although the fluoride-stimulated adenylate cyclase activity did not reach significance (Table 2). The results of the serial biopsies confirmed these differences, all reaching significance at *P* < 0.05 level (Fig. 3). We conclude that during the recovery phase there is an increase in all measured enzyme activities. Moreover, the activities during recovery closely approximated those found in patients with toddler diarrhea, suggesting a possible link between the patient with recovering postenteritis syndrome and toddler diarrhea, and it is of interest that the parents of our patients not infrequently dated the onset of toddler diarrhea to an acute episode of diarrhea and vomiting.

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