

Calmodulin content and activity in normal and coeliac duodenum

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Abstract

Calmodulin is an important modulator of intracellular calcium processes and may be implicated in the calcium malabsorption of coeliac disease. The calmodulin content in extracts of duodenal biopsy specimens from 48 normal control subjects and 28 patients with coeliac disease was determined. Radioimmunoassay was used to measure immunoreactive calmodulin while a cyclic adenosine 3',5'-monophosphate phosphodiesterase activity assay was used to measure biologically active calmodulin. Calmodulin values measured by both assays were similar for control and disease groups. Mean (SEM) immunoreactive calmodulin values were 1.68 (0.09) $\mu\text{g}/\text{mg}$ protein for controls and 1.67 (0.15) and 1.45 (0.15) $\mu\text{g}/\text{mg}$ protein for partial and total villous atrophy respectively. These values were not significantly different. Biologically active calmodulin values were 2.77 (0.21), 1.82 (0.34), and 3.24 (0.33) $\mu\text{g}/\text{mg}$ protein for control, partial, and total villous atrophy subjects respectively. The biologically active calmodulin values in the partial villous atrophy group were significantly lower than in controls and total villous atrophy subjects. In the phosphodiesterase assay, the calmodulin antagonist trifluoperazine inhibited the activity stimulated by purified calmodulin and by the extracts to the same extent. These results show that calmodulin values are normal in coeliac disease and provide no evidence that changes in calmodulin account for the abnormal calcium absorption in these patients.

Osteomalacia, osteoporosis, and secondary hyperparathyroidism are complications that may occur in coeliac disease because of calcium malabsorption.¹ In untreated coeliac disease, the upper small intestine is characterised by villous atrophy and crypt hyperplasia with relatively immature enterocytes.² Intestinal transcellular calcium absorption involves calcium entry at the brush border, intracellular translocation, and active transport of calcium out of the cell at the basolateral membrane. This occurs predominantly in the duodenum and upper jejunum.³

Calmodulin is a major calcium binding protein that mediates many of the intracellular actions of calcium. It is a heat stable, acidic protein with a molecular weight of 16 700 Daltons and is found in all eukaryotic cells.³ Calmodulin is involved in cyclic nucleotide metabolism, hormone secretion, neurotransmitter release, cell motility, intestinal secretion, and plasma membrane calcium transport.^{4,5} In enterocytes, calmodulin is found both in the cytosol and as a structural component of the microvilli, where it is also

thought to act as a calcium buffer.^{6,7} There is conflicting evidence as to whether calmodulin is present in the basolateral membrane.^{8,9} Jejunal calmodulin values measured by radioimmunoassay (RIA) are similar in normal subjects and in those with coeliac disease.⁶ However, several studies have shown that there may be differences between calmodulin, measured by RIA, and the biological activity of calmodulin, determined using a calmodulin dependent cyclic adenosine monophosphate (AMP) phosphodiesterase activity assay.¹⁰⁻¹² The aim of this study was to determine calmodulin values and activity in duodenal mucosa from control and coeliac subjects, since differences may partially explain the changes in calcium absorption observed in coeliac disease.

Patients and methods

PATIENTS

Biopsy specimens were obtained from the second part of the duodenum using an Olympus GIF IT endoscope with 3.7 mm forceps. Two specimens were placed in 10% formalin for histological assessment and others were immediately stored in liquid nitrogen.

Forty eight control subjects with normal duodenal histology undergoing investigation for diarrhoea or anaemia, or both, and 28 adult coeliac disease subjects with villous atrophy were studied. Subjects with coeliac disease were subdivided into those with partial villous atrophy (PVA) (n=8) and those with subtotal villous atrophy (TVA) (n=20). Two TVA patients were investigated by bone biopsy: both had histological criteria for osteomalacia and one also had osteoporosis.

CALMODULIN EXTRACT PREPARATION

One biopsy specimen from each patient was homogenised in 1.3 ml sample buffer (40 mmol/l Tris-HCl pH 7.2, 100 $\mu\text{mol}/\text{l}$ CaCl₂, 50 mg/l phenylmethylsulphonyl fluoride, 50 $\mu\text{l}/\text{l}$ pepstatin A) with 30 strokes at 1200 rpm in a Potter homogeniser at 4° C.¹⁰ Aliquots were taken for total protein determination by the method of Lowry *et al.*¹³ The homogenates were heated to 90° C for six minutes, rapidly cooled, and centrifuged (3000 rpm, 10 minutes). Supernatants were passed through 0.22 μM sterile Millipore filters and the extracts stored in aliquots at -70° C.

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Extracts of three biopsy specimens in 1.5 ml

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sample buffer were made using the method described above. The extracts were lyophilised and diluted in a small volume of electrophoresis sample buffer (2.5% sodium dodecyl sulphate, 5% 2-mercaptoethanol, 5% glycerol, 60 mmol/l Tris, and 0.005% bromophenol blue as a marker). Discontinuous SDS-PAGE, utilising the Laemmli methodology, was performed.¹⁴ Fifteen per cent acrylamide with 5% cross linking slab gels were used. Thirty μ l of extracts from the three groups of subjects were applied to the gels as were pure calmodulin and molecular weight markers. The gels were stained with Coomassie blue and double stained with silver.¹⁵ The double staining procedure is a method for selectively detecting calmodulin in a mixture of proteins.¹⁶

CALMODULIN ASSAYS

Radioimmunoassay of calmodulin

The calmodulin content of the extracts was measured using a standard RIA kit (Du Pont NEN Research Products). The sensitivity of the assay kit was 0.16 ng and the intra- and inter-assay coefficients of variation were 3.42% and 8.51% respectively. The extracts were diluted with the assay buffer (0.125 mol/l borate, pH 8.4, 0.075 mol/l NaCl, 0.2% bovine serum albumin, 1 mmol/l ethyleneglycol-bis (beta-aminoethylether) N,N,N',N'-tetra-acetic acid, 0.1% sodium azide) and measured in duplicate in two assays.

Cyclic AMP phosphodiesterase activity assay of calmodulin

This assay measured biological calmodulin activity in the extracts by detecting the conversion of [³H] cyclic adenosine 3',5'-monophosphate to [³H]5' adenosine monophosphate by calmodulin stimulated cyclic adenosine 3', 5'- monophosphate phosphodiesterase. The resultant [³H] 5' adenosine monophosphate was converted by *Crotalus atrox* snake venom (a source of 5' nucleotidase) to [³H] adenosine and inorganic phosphate. The [³H] adenosine was separated with anion exchange resin (Dowex AG1X 8) and quantified by liquid scintillation counting. The methodology has previously been described by MacNeil *et al.*¹⁷

The final reaction volume was 400 μ l and contained 40 mmol/l Tris-HCl, pH 7.2, 4 mmol/l 2-mercaptoethanol, 25 μ mol/l CaCl₂, 5 mmol/l MgCl₂, and 100 μ mol/l cyclic adenosine 3',5'-monophosphate with 1×10^5 cpm [³H] cyclic adenosine 3',5'-monophosphate/tube and 100 μ l diluted extract or various dilutions of purified bovine brain calmodulin. Extracts were measured in triplicate in two assays.

Calmodulin antagonist

Trifluoperazine is a phenothiazine antipsychotic drug that is commonly used to investigate calmodulin dependent cellular processes.⁵ Calmodulin antagonism by trifluoperazine was used to inhibit the phosphodiesterase activity stimulated by extracts. Final assay concentrations of 9 μ mol/l and 90 μ mol/l trifluoperazine

in 10% dimethylsulphoxide (DMSO) or 10% DMSO only as control were added to normal histology (n=14), partial villous atrophy (n=4), and total villous atrophy (n=9) extracts and to pure calmodulin (n=3). The final DMSO concentration was 0.25%.

STATISTICS

Data are presented as mean (SEM). Comparison between the different groups of patients was analysed by Mann-Whitney U test for unpaired data.

CHEMICALS

Calmodulin deficient beef heart cyclic adenosine 3',5'-monophosphate phosphodiesterase was purchased from Boehringer Mannheim, London. [³H] cyclic adenosine 3',5'-monophosphate and purified bovine brain calmodulin were from Amersham Int plc, Amersham. All other chemicals were from Sigma Co Ltd, Dorset.

Results

On SDS-PAGE the extracts showed several bands, including a definite band that ran identically with purified calmodulin and gave an apparent molecular weight of approximately 16600 Dalton. In all the extracts tested the 'calmodulin' band remained blue on double staining with silver. The double staining procedure is a method for selectively detecting calmodulin in a mixture of proteins.¹⁶

In both RIA and phosphodiesterase assays the extracts diluted in parallel with pure calmodulin. The calmodulin values measured by RIA were 1.68 (0.09), 1.67 (0.15), and 1.45 (0.15) μ g calmodulin/mg protein for normal duodenal histology, partial villous atrophy, and subtotal villous atrophy groups respectively. These differences were not statistically significant ($p > 0.05$) (Fig 1). Aliquots of the same extracts measured by phosphodiesterase activity assay gave values of 2.77 (0.21), 1.82 (0.34), and 3.24 (0.33) μ g calmodulin/mg protein for normal duodenal histology, partial villous atrophy, and subtotal villous atrophy groups. The partial villous atrophy value was statistically significant when compared with both normal duodenal histology ($p = 0.026$) and subtotal villous atrophy ($p = 0.020$) (Fig 2). The difference between normal duodenal histology and subtotal villous atrophy was not significant ($p > 0.05$). The subject with osteomalacia only had an immunoreactive calmodulin value of 1.33 μ g/mg protein and 3.45 μ g/mg protein of biologically active calmodulin. The corresponding values for the subject who also had osteoporosis were 1.13 and 6.88 μ g calmodulin/mg protein. The mean biologically active calmodulin values of the extracts to which the antagonist had been added were 3.13 (0.35) for normal duodenal histology (n=14), 2.47 (0.47) for partial villous atrophy (n=4), and 3.82 (0.26) μ g calmodulin/mg protein for subtotal villous atrophy (n=8). With 9 and 90 μ mol/l trifluoperazine, phosphodiesterase activity stimulated by pure calmodulin and by the extracts from all three groups was inhibited to

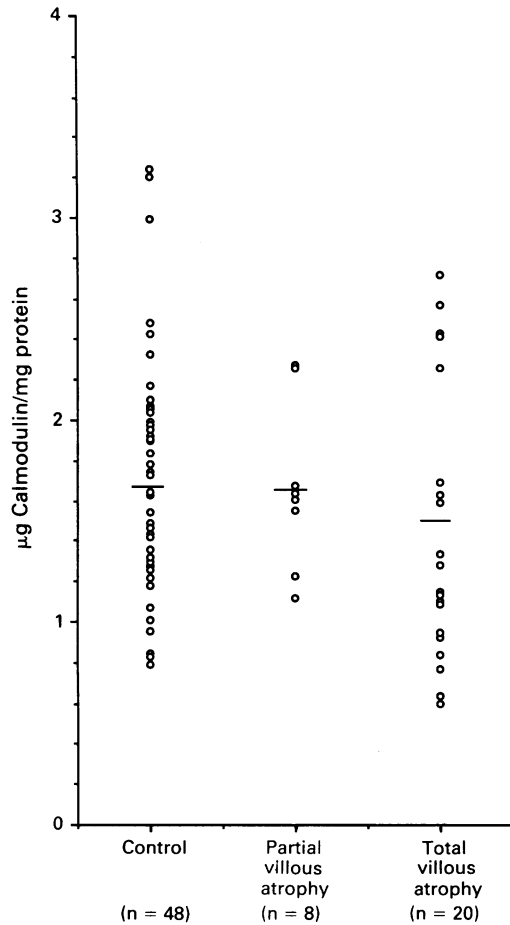


Figure 1: Immunoreactive calmodulin values in control, partial villous atrophy, and total villous atrophy subjects measured by radioimmunoassay. Individual data and the means of the groups are shown.

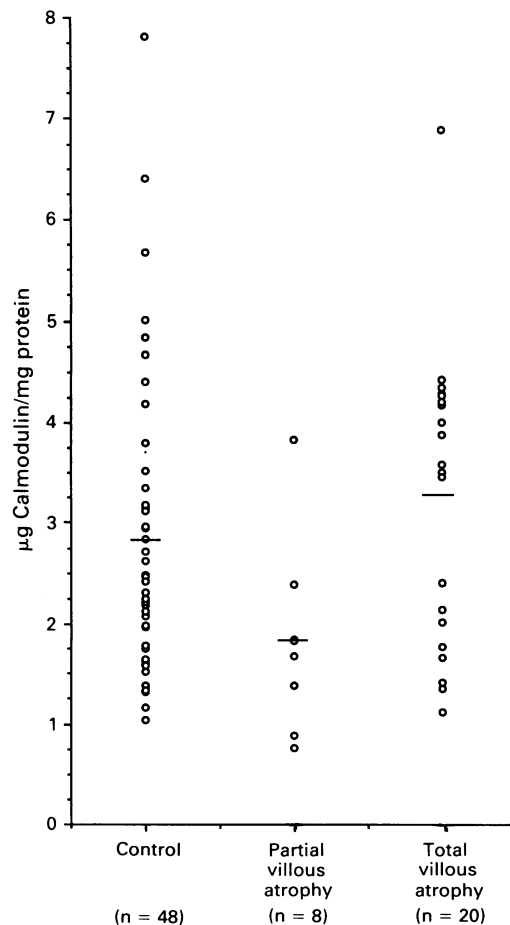


Figure 2: Biologically active calmodulin values in control, partial villous atrophy, and total villous atrophy subjects. Individual data and the means of the groups are shown. The difference between means for partial villous atrophy and normal histology is statistically significant, $p=0.026$. Partial villous atrophy v total villous atrophy is also significant, $p=0.020$.

a similar extent (Fig 3). The trifluoperazine results added further evidence that the phosphodiesterase stimulation by the extracts was due to the presence of calmodulin.

Discussion

In this study, the presence of calmodulin in heat treated extracts of normal and coeliac disease duodenal biopsy specimens was shown by the inhibition of calmodulin stimulated phosphodiesterase activity and the staining characteristics of the 'calmodulin' bands in the extracts which were comparable with pure calmodulin. Control subjects and those with coeliac disease, both partial villous atrophy and subtotal villous atrophy, had similar concentrations of immunoreactive calmodulin. The latter finding is consistent with the work of McPherson *et al*, who found no difference between jejunal immunoreactive calmodulin values in coeliac disease in relapse or remission and controls.⁶

Calmodulin is a major calcium binding protein that modulates many calcium dependent cellular processes. In the small intestine, the calcium/calmodulin complex is involved in the regulation of intracellular coupled NaCl transport across the rabbit microvillus membrane.¹⁸ Calcium uptake by human brush border membrane vesicles is reduced in the presence of calmodulin inhibitors.⁹ ATP-dependent calcium transport is stimulated by calmodulin in both rat and

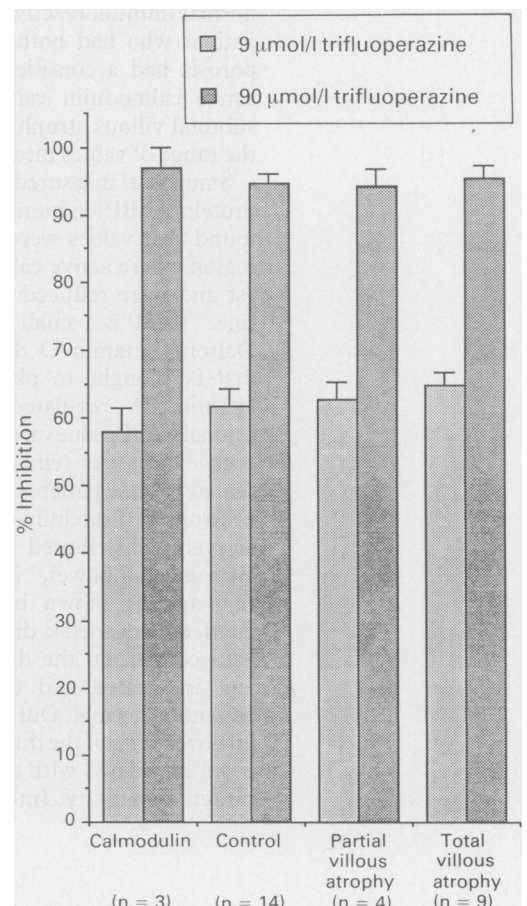


Figure 3: The percentage inhibition (mean (SEM)) by trifluoperazine of phosphodiesterase activity stimulated by pure calmodulin and by extracts from control, partial villous atrophy, and total villous atrophy subjects.

human basolateral membrane preparations.^{19,20} Calmodulin might therefore be implicated in the calcium malabsorption associated with coeliac disease that can lead to osteomalacia, osteoporosis, and secondary hyperparathyroidism.

Calmodulin has also been shown to be important in DNA synthesis and cellular proliferation in several tissues.²¹ In hyperproliferative tissue, such as the skin in psoriasis, both the immunoreactive calmodulin content and calmodulin activity measured by phosphodiesterase assay are raised.¹⁰ It could be hypothesised that the crypt hyperplasia and increased cell turnover associated with coeliac disease leads to an increase in calmodulin content. Our results do not support this hypothesis. Biologically active calmodulin values were similar in control and subtotal villous atrophy subjects, and in contrast to the immunoreactive values, were reduced in subjects with partial villous atrophy. In a study on submandibular glands from patients with cystic fibrosis, biologically active calmodulin values were raised in the disease group while immunoreactive values were not. The difference was attributed to the presence of a regulator of calmodulin activity.¹¹ This is unlikely to be an explanation in this instance, as only the partial villous atrophy values were different. It is possible that since both subtotal villous atrophy and control groups show quite a wide range of interindividual variation in biologically active calmodulin values, an increased partial villous atrophy sample would have a similar larger spread. Both subjects with osteomalacia had normal immunoreactive calmodulin values. The patient who had both osteomalacia and osteoporosis had a considerably higher biologically active calmodulin value than the rest of the subtotal villous atrophy group but it was within the range of values measured in control subjects.

Staun *et al* measured another calcium binding protein (CaBP) in human intestinal epithelia and found that values were highest in the proximal region where active calcium absorption is greatest and were reduced in the distal small intestine.²² CaBP is a small (molecular weight 10 000 Daltons) vitamin D dependent acidic protein, that is thought to play an important role in vitamin D regulated calcium absorption.²³ Jejunal CaBP values are very low in patients with coeliac disease in remission and are negligible in coeliac disease patients in relapse compared with controls.²⁴ Calmodulin content on the other hand is evenly distributed along the length of the human small bowel,²⁵ is independent of vitamin D,²⁶ and as shown by the present study, is unaffected in coeliac disease.

In conclusion the duodenal calmodulin content in treated and untreated coeliac disease patients is normal. Our results show that the high turnover state of the duodenum in coeliac disease is not associated with an increase in calmodulin content or activity. Intestinal calmodulin abnor-

malities are not implicated in the calcium malabsorption of coeliac disease.

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