Alimentary tract and pancreas

Calmodulin in normal and cystic fibrosis human intestine at different developmental stages

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SUMMARY Calmodulin concentrations and localisation have been analysed as a function of development in human intestinal epithelial cells from normal and cystic fibrosis individuals. In normal fetuses up to eight weeks of gestation intestinal epithelial cells which were still undifferentiated were not immunoreactive and their calmodulin content was low. From eight weeks onwards there was a significant overall increase in calmodulin content concomitant with its segregation to the apical side of epithelial cells. At 14 weeks of gestation calmodulin concentrations and localisation closely resembled those of adults. The developmental pattern of calmodulin appeared to parallel the morphological and functional maturation of brush borders which occurs during the first trimester of pregnancy. In the intestinal epithelial cells from a 19 weeks cystic fibrosis fetus and a cystic fibrosis newborn infant neither calmodulin concentration, nor its localisation were affected. Similarly, brush border calmodulin binding proteins and enzymatic activities were similar in normal subjects and the cystic fibrosis intestine.

Calmodulin is an ubiquitous calcium binding protein^{1,2} which in the brush borders of intestinal epithelial cells^{3,4} serves as the predominant calcium buffer.⁵ In addition, it regulates brush border contractility through the cytoskeleton associated calmodulin dependent myosin light chain kinase system⁶ and a number of cytoskeletal proteins with actin and calmodulin binding capacities.⁷

The developmental pattern of calmodulin has been already studied in rat brain⁸ and by ourselves in the developing rat intestine.⁹ Nothing is known about calmodulin ontogenesis in the human intestine, however, in spite of numerous studies concerning morphogenesis¹⁰ and maturation of brush borders hydrolases.¹¹⁻¹³ In human fetal small intestine, villus morphogenesis occurs proximodistally between eight and 14 weeks, the first villi appearing around eight weeks in the duodenum and around 11 weeks in the ileum.¹² Furthermore the existence of a clear parallel between morphological, ultrastructural, and enzymatic differentiation has been shown: the first outgrowth of villi coincides with the progressive differentiation of true microvilli and with the appear-

ance of digestive enzyme activities. The major maturation events proceed up to 14 weeks. At that stage, the level of enzyme activities is very comparable with that of adults.¹² Therefore, it appeared of interest to investigate whether there is a correlation between the developmental pattern of calmodulin and the differentiation of brush borders in human intestine.

A second interesting aspect on which we focused our attention concerned the analysis of calmodulin concentrations and localisation in the intestinal epithelial cells of cystic fibrosis (CF) patients. Indeed, in recent reports, increased calmodulin concentrations have been shown in CF lung,¹⁴ skin fibroblasts,¹⁵ and blood.¹⁶ Cystic fibrosis is an autosomal recessive disorder characterised by a generalised epithelial dysfunction, involving a decreased chloride permeability in sweat gland duct and airway epithelia.¹⁷⁻²¹ This defect in CF was found to be caused not by abnormal conductive properties of chloride channels but by an altered regulation of these channels²⁰ eventually implicating calcium ions and/or calmodulin.²²

At the intestinal level a meconium ileus resulting in intestinal obstruction has been observed between 17 and 20 weeks of gestation²³ concomitant with a

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marked decrease of microvillar enzymes in amniotic fluid.²⁴ This latter change makes possible early prenatal diagnosis of CF^{25,26} and subsequent therapeutic abortion. Therefore, because CF is a single gene locus disease,²⁷⁻²⁸ it would be of interest to know whether this mutation might affect the synthesis and/or the function of calmodulin in intestinal epithelial cells.

The aim of the present study was first to analyse both calmodulin concentrations and immunocytochemical localisation, in human intestine, at various stages of fetal development and second, to investigate whether these parameters are affected in CF intestine.

Methods

HUMAN INTESTINAL SPECIMENS

Fetal human intestines were obtained between six and 14 weeks of gestation after legal or therapeutic induced abortions with the informed consent of the mothers. The specimens were dissected out from abortion material and only healthy and undamaged intestinal tracts were used. Fetal age was determined by the developmental pattern of hand morphology.²⁹ The specimens analysed were from 6, 8, 9, 10, 11, 12, 14, and 19 week-old fetuses and were dissected into small fragments (10–20 mg) from duodenum to colon.

The intestine of two preterm infants of 27–28 weeks of gestational age, who died from hyaline membranes disease with severe asphyxia were obtained with parental consent within three hours of death. These newborns survived for 10 days and were fed intravenously.

Fragments (10–20 mg) from human adult duodenum and jejunum without malabsorption or histological abnormalities, were obtained by endoscopic biopsy. Healthy areas of colon mucosa were from surgical resection for cancer.

CYSTIC FIBROSIS

Cystic fibrosis intestinal specimens were from a 19 week old fetus and a newborn infant. The former was obtained after early prenatal diagnosis of CF²⁶ and subsequent therapeutic abortion; the disease was confirmed by the observation of a meconium ileus at the intestinal level. The latter was obtained after surgical resection for obstructed ileum with meconium ileus.

REAGENTS

Calmodulin was purified as described by Rochette-Egly *et al*³⁰ and iodinated using [¹²⁵I] Bolton and Hunter reagent (2000 Ci/ μ mol, Amersham); the specific radioactivity of [¹²⁵I] calmodulin thus obtained ranged from 40 to 80 μ Ci/ μ g.

Benzamidin, antipain, pepstatin A and poly (ethylene oxide) 20 sorbitan monolaurate (Tween 20) were obtained from SIGMA chemicals (St Louis, MO, USA). Leupeptin and aprotinin were from Boehringer (Mannheim, W. Germany).

Calmodulin antibodies, raised in rabbits, were generously provided by Dr J De Mey (Beerse, Belgium). Normal goat serum and fluorescein conjugated goat antirabbit IgG were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands).

Sodium dodecyl sulphate (SDS), acrylamide, N, N' methylenebisacrylamide were obtained from Bio-Rad laboratories (Richmond, CA, USA), Nitrocellulose (NC) membranes (0.45 μ m pore size) were purchased from Schleicher and Schuell (Dassel, W Germany).

DETERMINATION OF CALMODULIN CONTENT

Up to 12 weeks of gestation calmodulin concentration was determined on full thickness intestinal fragments. In 14 and 19 week old fetuses as well as in newborns, measurements were done on epithelial cells isolated according to Bjerknes and Cheng.³¹ In adults the whole mucosal biopsies were used. Samples were homogenised in calcium-magnesium free (CMF) Hanks buffer (20 mM Hepes, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 140 mM NaCl, 5.4 mM KCl pH 7.3), containing 1 mM EGTA and a cocktail of protease inhibitors (pepstatin 1 μ g/ml, antipain 1 μ g/ml, benzamidin 15 μ g/ml, leupeptin 10 μ g/ml and aprotinin 10 μ g/ml).

The homogenized fragments were treated at 80°C for five minutes, rapidly cooled on ice and centrifuged at 8000 g for five minutes. Then the supernatants were assayed for calmodulin content using a commercially available [¹²⁵I] calmodulin radioimmunoassay kit from New England Nuclear (Frankfurt, Germany). Results are expressed as the mean of four replicates in ng/mg protein. In the NEN calmodulin kit the sensitivity of the radioimmunoassay was determined to be 0.16 ng and the reproducibility ranged from 2–10%.

IMMUNOFLUORESCENCE

Specimens were rinsed in iced saline buffer and immediately fixed with 2% paraformaldehyde in 0.1 M Pipes buffer at pH 7.0 containing 5% sucrose for two hours at 4°C.

After three washes in buffer, the specimens were frozen in melting freon and then stored in liquid nitrogen before cryostat sectioning (4–5 μ m sections). Immunofluorescence staining of calmodulin was done as previously described.⁹ After a 20 min pretreatment with 5% normal goat serum in Tris buffered saline (TBS), the sections were

Fig. 1 Intestinal calmodulin concentrations (ng/mg protein) as a function of age and its proximodistal distribution. □ duodenum; ▨ proximal jejunum; ▩ distal jejunum; ▤ ileum; ■ colon. The intestines of the youngest fetuses (eight weeks) were subdivided into two parts. From the 10th week onwards, the whole small intestine was divided into five to eight segments which were assayed separately in triplicate. Presumptive duodenum and colon were plotted separately (1st and 5th columns), whereas the intermediate segments were pooled into three groups corresponding respectively to the presumptive proximal and distal jejunum and to the ileum (2nd, 3rd, and 4th columns). At 10 and 12 weeks, full thickness intestines were tested whereas at 14 and 19 weeks, calmodulin was measured in isolated epithelial cells. For adults, whole mucosal biopsies were assayed. For each stage, two to five fetal intestines were assayed and the results presented are the mean (SD) of two to four fragments from a representative intestine and assayed in triplicate. Concerning the adults, the values presented herein are the mean (SD) of three to 10 fragments assayed in triplicate.

incubated overnight with the calmodulin antibodies diluted in TBS containing 0.1% bovine serum albumin. After washing with TBS, the sections were incubated for one hour with fluorescein labelled goat antirabbit serum, extensively washed with TBS and mounted in glycerol containing paraphenylenediamine as described by Schmit *et al.*³²

DETECTION OF CALMODULIN BINDING PROTEINS

Intestinal fragments were processed for brush borders preparation according to MacKenzie *et al.*³³ Brush borders were then homogenised in Hanks

buffer and proteins were separated by electrophoresis in 5–15% linear gradient polyacrylamide gels (0.75 mm thickness) in the presence of 0.1% SDS using the buffer system previously described.³⁴ Upon completion of electrophoresis the proteins were electrophoretically transferred to NC membranes in 0.025 M Tris-HCl, 0.192 M glycine pH 8.2 containing 20% v/v methanol. An LKB transblot electrophoretic cell was used, at a constant current of 220 mA (40 to 50 V) for 16 hours at +4°C.

The calmodulin binding proteins bound to nitrocellulose membranes were visualised as described by Flanagan and Yost.³⁵ The nitrocellulose membranes

were incubated for one hour at room temperature in buffer A 0.05 M Tris HCl pH 7.6, 0.2 M NaCl; 1 mM CaCl_2 , and 0.05% Tween 20) and then overlaid with [^{125}I] calmodulin (4 $\mu\text{Ci/ml}$) diluted in buffer A for five hours at room temperature and on a rocker platform. After three washes (30 min each), with buffer A, membranes were air dried and [^{125}I] calmodulin binding was visualised by autoradiography on Fuji x-ray film. Molecular weights were calculated by using prestained standards (Bethesda Research Laboratories) in the blots used to produce the autoradiograms.

ENZYMATIC ASSAYS

The brush border membranes were isolated from homogenised samples according to Schmitz *et al.*³⁶ Enzyme activities were determined according to the methods of Messer and Dahlqvist³⁷ for the disaccharidases, of Garen and Levinthal³⁸ for alkaline phosphatase and of Maroux *et al.*³⁹ for aminopeptidase. Proteins were estimated according to Lowry *et al.*⁴⁰ All enzyme activities were expressed as milliunits per mg of protein, 1 unit of activity equalling 1 μmol of product formed per min at 37°C.

Results

CALMODULIN CONCENTRATIONS IN THE DEVELOPING HUMAN INTESTINE

Figure 1 shows the pattern of calmodulin content in different intestinal segments extending from the duodenum to the colon, at different representative stages of intestinal development. At eight weeks of gestation, calmodulin concentrations were low; they ranged around uniform values from the proximal to the distal part of the intestine, with a mean level of 20.85 ± 3.66 ng/mg protein. Then, from eight weeks onwards, calmodulin concentrations increased progressively until 12 to 14 weeks. Indeed, at 10 weeks, calmodulin had increased four times in the proximal part of the jejunum ($p < 0.01$), but remained low in the other parts of the intestine. At 12–14 weeks, calmodulin had increased significantly over the whole length of the intestine ($p < 0.001$), but the highest concentrations (10 fold higher than in eight weeks fetuses) were still observed in the jejunum and ileum. Finally as early as 14 weeks, in these parts of the intestine, calmodulin concentrations were similar to those of the adult. In adults, no clear cut gradient was visible all along the intestinal tube down to the colon.

IMMUNOCYTOCHEMICAL LOCALISATION OF CALMODULIN IN THE DEVELOPING HUMAN INTESTINE

At eight weeks of human gestation, although villi start to protrude in the proximal intestine, the

endodermal tube is still undifferentiated. At this stage, calmodulin was undetectable by immunofluorescence all along the intestine (Fig. 2a, b).

At 12 weeks of gestation, villi have increased in height over the whole length of the intestine. Calmodulin displayed a linear fluorescence at the apical side of epithelial cells, in the proximal intestine (Fig. 2c), while this staining only started to be obvious in the distal one (Fig. 2d).

At 14 weeks of gestation, the proximodistal gradient of intestinal morphogenesis is achieved.¹² Concomitantly, the intensity of the apical calmodulin fluorescence had increased markedly all along the intestine (Fig. 2e, f), and did not differ from that found in the adult (Fig. 2g, h).

CALMODULIN IN THE CF INTESTINE

First, calmodulin concentrations and calmodulin localisation were determined in the different intestinal segments of a 19 weeks CF fetus. From the data presented in Figure 1, it is obvious that all along the CF intestine, from duodenum to colon, calmodulin concentrations were in a similar range to those found in a normal 14 weeks fetus and in adults. Furthermore, as in the normal specimens, calmodulin was localised at the apical side of the epithelium (Fig. 3). Such an absence of discrimination between CF and normal intestine was also observed in the ileum of a newborn infant: both calmodulin concentrations (Table 1) and localisation (not shown) in the CF ileum were similar to those observed in control preterm infants.

Cell cultures originating from amniotic fluids were used as an additional criterion: calmodulin concentrations were similar in normal and CF cells at 18–19 weeks of gestation (Table 1).

We also investigated calmodulin binding proteins in the brush borders of normal and CF small bowel. Figure 4 shows that normal human intestinal brush borders do contain three major calmodulin binding proteins, as assessed by electrophoresis and incubation of the nitrocellulose replicas with [^{125}I] calmodulin in the presence of calcium. Their apparent molecular weights are respectively 240, 140, and 110 Kdaltons (Fig. 4, lanes 1–3). In a parallel study^{42,43} immunoblotting experiments revealed that these calmodulin binding bands are labelled by specific antibodies reactive with fodrin, caldesmon, and the 110 KDa protein, respectively. A minor band with a molecular weight of 160 KDa is also detectable, and corresponds to a breakdown product of fodrin.^{43,44,45} These proteins are the major calmodulin binding proteins presently known to be associated with the brush borders cytoskeleton in either avian,⁷ rat,^{7,45} or human^{42,43} intestines. The specificity of the [^{125}I] calmodulin binding to these

Fig. 2 Immunofluorescence staining of calmodulin at different stages of development in the proximal (a,c,e,g) and distal (b,d,f,h) intestine. (a–b ($\times 157$): eight week-old fetus; c ($\times 157$); d ($\times 190$): 12 week-old fetus, e–f ($\times 220$): 14 week-old fetus; g ($\times 157$); h ($\times 440$): adult. At 14 weeks (f), certain epithelial cells show some amorphous material which is a non-specific reaction shown by mucous cells. In the lamina propria from adult intestine, numerous mast cells show autofluorescence of histamine which is easily distinguished from the greenish immunoreactions by its yellowish colour.⁴¹

Fig. 3 Immunofluorescence staining of calmodulin in the proximal (a) and distal (b) intestine of a 19 weeks CF fetus.

bands was assessed by its inhibition in the presence of unlabelled calmodulin (1 mM) or trifluoperazine (50 μ M) during the radioactive calmodulin binding assay (Fig. 4, lanes 4, 5). In the 19 weeks CF-fetus as in the CF newborn infant, neither the number nor the intensity of these calmodulin-binding bands appeared to be affected (Fig. 4, lanes 6 and 7).

Table 1 Calmodulin levels in control and CF intestine and amniotic cells

	Calmodulin concentrations (ng/mg proteins)	
	Control	Cystic fibrosis
Ileal epithelial cells of newborn infants	309.8 (11.4) [2]	322.7 (13.6) [1]
Amniotic cells from 18–19 week-old fetuses	15.5 (5.2) [11]	12.2 (1.5) [1]

The numbers in square parentheses represent the number of cases tested. In the case of ileal epithelial cells from the CF newborn infant, the values are the mean (SD) of three fragments assayed in triplicate. Amniotic fluids were obtained by amniocentesis, centrifuged, and the cell pellet was seeded in culture flasks in a medium composed of 80% MEM and 20% fetal calf serum (Flobio). Calmodulin assays were done on confluent cells scrapped mechanically from the dishes after two to six passages. The results are the mean (SD) of triplicate assays carried out on two dishes per case.

Finally, as an indication of the state of functional maturation of CF small bowel, we studied the concentrations of brush border digestive enzymes in the CF fetus and neonate.

First, in the 19 week-old fetus, the proximodistal gradient of the different enzymes¹³ has been shown to be unaffected (Fig. 5): maximal activity in the proximal jejunum and decreasing activity towards the distal ileum for sucrase, maltase and alkaline phosphatase, and an inverse gradient for aminopeptidase. Moreover, Table 2 shows that at least for this CF 19 week old fetus, digestive enzyme activities ranged around values similar to those observed in controls of an equivalent developmental stage. It is worth noting that in this 19 weeks CF fetus, enzymatic activities were present in the colon as already described in normal fetuses^{11,46} and that sucrase activity ranged around values expressed as mean (SD) [260 (39) mU/mg protein] previously reported for control tissue [199 (59) mU/mg protein].

Concerning the CF newborn infant, lactase and alkaline phosphatase appeared increased (Table 2). In addition, the CF fragments from proximal ileum were compared with midjejunum controls, and according to the decreasing proximodistal gradient, the overall enzyme activities seem to be increased in CF specimens compared with the control ones. Such differences might be attributed to individual variations which are important at the late gestational stages and around birth (M. Kedinger *et al.*, unpublished observations).

Discussion

From the present study, two main points appear to be of interest. First, we have shown that there is a clear parallel between the developmental pattern of calmodulin and the morphological differentiation of human fetal intestine. As early as the eighth week of fetal life, when epithelial cells are still undifferentiated, calmodulin is already detectable by radioimmunoassay, but not yet by immunocytochemistry. We have previously shown such a discrepancy between the biochemical and cytochemical observations at early fetal stages, in the rat,⁹ which reflects the low amount of this protein and its presence in the whole epithelial cell rather than associated with a specialised brush border. Of interest was the existence, from eight weeks to 14 weeks, of a significant overall increase in calmodulin concentration, which coincides with the main morphogenetic events in the developing intestine.^{10–13} Moreover, at the same time as brush border maturation occurs, calmodulin segregates to the apical side of epithelial cells, as visualised by the appearance of an intense fluorescence lining the luminal surface of the epithelium.

Fig. 4 Autoradiograms of calmodulin binding proteins in normal human jejunal brush borders at different stages of development (lanes 1 to 5) and in cystic fibrosis (lanes 6 and 7). Brush borders proteins were resolved by electrophoresis, transferred to nitrocellulose, overlaid with [125 I] calmodulin in the presence of 1 mM CaCl_2 (lanes 1,2,3,6,7) and autoradiographed. Controls were obtained by overlaying the NC membranes with [125 I] calmodulin in the presence of 1 mM calmodulin (lane 4) or 50 μM Trifluoperazine (lane 5). 1: 14 week-old fetus; 2: preterm infant; 3–4–5: adult; 6: 19 week-old CF fetus; 7: CF newborn infant. The lower molecular weight labelled bands which are visible, correspond to degradation products generated during sample preparation.

Indeed, the first segregation of calmodulin is obvious in the proximal intestine and proceeds in parallel to the proximodistal gradient of brush border assembly. In the colon, this recruitment of calmodulin is slightly delayed as is the brush border organisation^{11,12} but is

Fig. 5 Sucrase (—) and aminopeptidase (....) activities (mU/mg protein) in brush borders purified from different intestinal segments of the 19 week-CF fetus: D, duodenum; J, jejunum; I, ileum. The results are the mean (SD) of three determinations.

very similar to that of the small intestine. Finally at 14 weeks of gestation, calmodulin concentrations and localisation resemble closely those of the adult. Concomitantly, during the first trimester of pregnancy, the major calmodulin binding proteins are also progressively inserted into the microvillus core.^{42,43}

The second interesting finding is that no major changes in calmodulin concentrations and properties could be found in CF intestine either in the fetus or neonate. Several authors have described calmodulin abnormalities in different cell types originating from CF patients,^{14–16,22} thus raising three possibilities: (1) an increase or a defect in calmodulin

Table 2 Enzyme activities in a brush border enriched fraction from normal (N) and cystic fibrosis (CF) intestines

	Sucrase	Maltase	Lactase	Amino-peptidase	Alkaline phosphatase
19 week-old N ¹	598 (22)	1365 (1.5)	51 (1)	571 (68)	570 (15)
fetus CF ¹	426 (40)	1293 (133)	63 (19)	272 (33)	458 (258)
Newborn N ¹	765 (233)	1761 (605)	352 (52)	909 (128)	3798 (789)
infant CF ²	944 (4)	2323 (70)	893 (24)	917 (77)	8799 (96)

Each value corresponds to the mean (SD) of three assays carried out on different fragments from a single intestine. Results are from mid jejunal (1) or proximal ileal (2) segments.

concentrations, (2) a modification of calmodulin localisation or (3) alterations of calmodulin binding proteins. In the present study, none of these hypothesis was supported by our results: the amount of calmodulin and the intracellular localisation of this protein were similar in control and CF intestines and finally the number and the nature of calmodulin binding proteins were unaltered. It must be stressed that the functional maturation of brush borders also appeared to be unaffected, as no quantitative alteration nor modification in the proximodistal gradient of brush border enzymatic activities was obvious between control and CF intestines. Similarly, a lack of changes in the synthesis and in the molecular properties of one digestive brush border hydrolase, sucrase, in CF intestines, had already been reported by Sips *et al.*⁴⁷

In conclusion, from the results of our developmental study, it appears that calmodulin is expressed in undifferentiated human intestinal cells. At such an early stage, calmodulin is not associated with ultrastructurally recognisable cytoskeletal structures but acts as an ubiquitous Ca^{++} acceptor protein, mediating the action of Ca^{++} , and thus acting as a cytosolic regulator of cellular function.^{1,2} As soon as brush border assembly occurs, however, calmodulin segregates to the apex of the cells. There, calmodulin is known to serve as a calcium buffer,⁵ to activate the actomyosin contractile system and to interact with a number of actin binding proteins.⁷ Finally, the developmental pattern of calmodulin appears to parallel not only the morphological differentiation of brush borders but also its functional and enzymatic maturation.

Furthermore, from our study neither an abnormal concentration nor an altered subcellular localisation of calmodulin were found in CF absorptive intestinal cells at two developmental stages. These observations are in agreement with the recent finding that calmodulin concentrations and properties are normal in CF submandibular glands as assessed by radio-immunoassay. In fact, as there is no abnormality in the calmodulin gene itself,⁵⁰ but increased intracellular calcium concentrations^{22, 48, 49} have been reported in CF cells, some functional properties of calmodulin, such as its affinity for calcium ions or for target proteins, could be altered.⁵⁰ Although the major calmodulin binding proteins found in the brush border cytoskeleton do not appear to be disturbed, an alteration of a specific modulator of calmodulin function in CF cells thus disturbing the pathways mediating membrane transport of chloride cannot be excluded. These aspects remain to be explored.

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