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The ontogeny of rat gastric H^+/K^+-ATPase

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The ontogeny of rat H^+/K^+-ATPase was studied between foetal day 18 and neonatal day 18, using a specific monoclonal antibody (95–111 mAb). The H^+/K^+-ATPase content of gastric subcellular membranes was assayed and the ATPase subunits were characterized by Western blot. The epithelium density in parietal cells was measured by immunohistochemistry. H^+/K^+-ATPase was present in the 18-day-old foetuses and parietal cells were detected on foetal day 19. The H^+/K^+-ATPase concentration remained stable from foetal day 18 to neonatal day 1, while the parietal cell density increased 2.5-fold. The H^+/K^+-ATPase concentration increased by 2.5-fold on day 6, then remained constant up to day 18. The parietal cell density remained unchanged during this period, suggesting that the concentration increase on day 6 was due to an increase in parietal cell ATPase content. The 95–111 mAb recognized a 95 kDa single band on foetal day 18 and a doublet at all the other stages of development. Previous studies had demonstrated that acid secretion drops critically at day 12 post partum in the rat and that H^+/K^+-ATPase activity is lost. The present study demonstrates that the H^+/K^+-ATPase is, however, present on day 12.

Introduction

Gastric acid secretion by the foetal rat begins during late gestation on day 20 [1], and its regulatory mechanisms develop around the same time [2]. H^+/K^+-ATPase activity can be detected in 18-old-day foetuses by an in vitro assay [3]. Acid secretion increases steadily after birth, up to postnatal day 10. However, between postnatal days 10 and 14, there is a lack of secretagogue stimulation [4] and H^+/K^+-ATPase activity can no longer be detected in vitro [3]. This 'silent' period is transient and all secretory functions are recovered after day 14 [4–9].

To further characterize this ontogenic period, a specific monoclonal antibody to H^+/K^+-ATPase (Ref. 10, unpublished data) was used to characterize H^+/K^+-ATPase changes during rat ontogeny, especially around day 12 post partum, and to study the development of parietal cells.

Material and Methods

Animals. Wistar rate foetuses were obtained as previously described [1]. Pups (20 neonates per mother) were allowed to suckle from birth (day 0) to day 18 of life. All animals were killed by cervical dislocation. The stomachs were removed, opened and washed free of all content.

Immunohistochemical localization. Tissue blocks were frozen, embedded in OCT compound (Tissue-Tek, Division Miles Scientific) and cryostat sections (4 μm) were cut through the epithelium perpendicular to the mucosal surface. These sections were rinsed for 10 min in 50 mM Tris (pH 7.6), 150 mM NaCl, 1/1000 normal rabbit serum, then incubated overnight at 0–4°C with the 95–111 mAb. Rabbit immunoglobulins to mouse immunoglobulins (DAKOPATTS, Denmark, Ref Z.259) were then added for 1 h at room temperature. Slides were rinsed in 50 mM Tris (pH 7.6), 150 mM NaCl and incubated for 15 min in PAP complex (DAKOPATTS, Denmark, Ref P850). Peroxidase activity was revealed by a 5–8 min incubation in diamino benzidine (Sigma, MO. U.S.A., Ref D.8001) H_2O_2 1/1000. Slides were mounted in EUKITT. Epithelium height was measured under the microscope using a calibrated ocular grid. The stained cells were counted (each count covered a 240 μm length of epithelium). The means ± S.E. of five counts were made to calculate the cell density as the

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Abbreviations: BSA, bovine serum albumin: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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number of stained cells per mm^2. The counted area was calculated as 0.24 mm × epithelium height in mm.

Subcellular fractions. The gastric fundus were prepared by removal of the rumen (foetuses) or both the rumen and antrum (neonates and adults), and subcellular fractions were prepared as previously described [3]. Fundic tissues were homogenized in 5 mM Tris-HCl (pH 7.5), 1 mM EGTA, 250 mM sucrose, and centrifuged at 4°C for 10 min at 1000 × *g*. The supernatants were collected and centrifuged at 20000 × *g* for 10 min (Sorvall RC5C) at 4°C, resulting in a mitochondrial pellet (P2 fraction) and a supernatant. This was finally centrifuged at 100000 × *g* for 60 min at 4°C to pellet the microsomes (P3 fraction). The P2 and P3 fractions were either pooled or used separately.

Proteins. Proteins were measured by the Bradford's Coomassie blue staining method using bovine serum albumin (Fraction V) as standard [11].

Immunotitration of H^+/K^+-ATPase

H^+/K^+-ATPase was quantified using a hog gastric fraction as a reference. This standard fraction contained 0.36 μg of 95 kDa polypeptides per μg of protein and had a specific activity of 80 μmol P_i per h per mg protein for K^+-stimulated ATPase (unpublished data).

Antigenic fractions (100 μl) were incubated overnight at 0–4°C in the wells of polyvinyl microplates (Flow laboratory (Lab System, Les Ulis, France)). The solutions were decanted and the wells were rinsed four times with 200 μl of phosphate-buffered saline plus 0.1% BSA. Monoclonal antibody (100 μl of 95–111) was incubated into each well for 1 h at room temperature. The plates were rinsed four times and ^{125}I anti-mouse IgG (H + L) was added for 1 h. Unbound radioactivity was decanted and the wells were washed three times, cut out and radioactivity counted (1275 mini-gamma LKB).

Western blots of H^+/K^+-ATPase

Membrane proteins were separated by SDS-PAGE on a 5–20% polyacrylamide separating gel. Proteins were transferred to nitrocellulose sheets [12] and stained with Ponceau S [13]. The nitrocellulose sheets were incubated for 30 min in 50 mM Hepes, 1% BSA, 0.5% Tween 20 (pH 7) to saturate non-specific sites and incubated in the same buffer containing 0.4 μg/ml of mAb 95–111 for 1 h. The sheets were rinsed four times and incubated with 0.5 μg/ml peroxidase-labelled sheep antimouse IgG (H + L), (Biosys S.A. France, Ref BI 2413MN) for 1 h, rinsed (four times) and stained with diamino benzidine.

Results

Localization of foetal and neonatal H^+/K^+-ATPase

Rat foetuses and neonates grew normally as indicated by their increase in body weight (Table I). The gastric epithelium developed: the epithelium of 18-day-old foetuses was a very thin (16–21 μm) monolayer of undifferentiated cells. No immunoreactive cells were detected. Small gastric glands appeared between foetal days 18 and 19, and, the epithelium was 29–46 μm thick on day 19. The 19-day epithelium contained 630 ± 120 immunoreactive cells per mm^2. The mean epithelium thickness had reached 75μm by foetal day 21 and immunoreactive cells were present throughout the epithelium (1520 ± 112 cells mm^2), although the adult gland organization was not yet apparent.

TABLE I

Titration of H^+/K^+-ATPase during the ontogenic development of rat

The number of rats at each ontogenic stage is given. Each animal was weighed and the mean weights ± S.E. calculated. Rat gastric P2 + P3 fractions were prepared as described in Materials and Methods. The ATPase was assayed on microplates using 1, 2 and 4 μg/100 μl rat (P2 + P3) fractions, 300 ng/ml 95–111 ascite and 200000 cpm ^{125}I-labelled rabbit anti-mouse IgG. The bound ^{125}I was used to calculate the concentration of H^+/K^+-ATPase by reference to the bound radioactivity of a standard which contained 364 μg ATPase per mg protein. H^+/K^+-ATPase μg/mg protein is the μg of ATPase per mg of protein in the P2 + P3 fraction; μg/P2 + P3 means the μg of ATPase in the total P2 + P3 fraction. The specific activities of H^+/K^+-ATPase are from Ref. 3.

Stage	No. of rats	Animal weight (g)	Protein P2 + P3 (μg/animal)	H^+/K^+-ATPase (μg/mg protein)	H^+/K^+-ATPase (μg/P2 + P3)	ATPase spec. act. (from Ref. 3)
F18	48	1.4 ± 0.1	22	20 ± 2	0.44	1.4
F19						2.4
F21	38	4.7 ± 0.1	56	18 ± 2	1.0	2.2
N1	16	5.9 ± 0.1	39	20 ± 1	0.80	2.1
N6	22	9.9 ± 0.2	132	47 ± 3	6.2	3.8
N12	33	23.7 ± 0.2	433	51 ± 6	22	0.1
N14	6	27.5 ± 0.4	532	45 ± 4	24	3.5
N18	10	37.5 ± 1.8	660	58 ± 5	39	3.7
Adults		200	10000	147 ± 11	1470	11.4

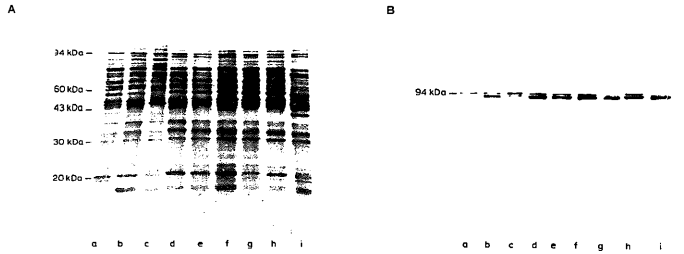

Fig. 1. Ponceau S staining and Western blot of P2 SDS-PAGE. Rat P2 fractions were prepared from foetal, neonatal and adult stomachs as described in Materials and Methods. SDS-PAGE gel (20 μg protein per lane) was run overnight [13]. Proteins were transferred to a nitrocellulose sheet and stained with 0.4% ponceau S (Fig. 1A). Destained sheet was incubated with 400 ng/ml 95–111 mAb in 40 mM Tris (pH 7) and immune complexes were revealed with diaminobenzidine-H_2O_2 using a peroxidase-coupled antimouse IgG (Fig. 1B). For all figures: lane a, foetus 18 days; lane b, foetus 19 days; lane c, foetus 21 days; lane d, neonate day 1; lane e, neonate day 6; lane f, neonate day 12; lane g, neonate day 14; lane h, neonate day 18; lane i, adult. The standard molecular mass and their positions in the gel are shown on the left.

The density of immunoreactive cells remained at 1632 ± 69 cells per mm^2 1 day after birth. The gastric glands of 5-day-old rats had begun to acquire their adult organization, with mucous cells in the apical region of the gland and immunoreactive parietal cells at the middle and base of the gland (1707 ± 79 immunoreactive cells per mm^2). The mean epithelium thickness in 12-day-old neonates was 160 μm, and there were 1830 ± 51 immunoreactive cells per mm^2. As acid secretion is inhibited on day 12, the parietal cells were examined by electron microscopy. No morphological alterations which could be associated with cell malfunction were detected.

Titration of foetal and neonatal rat H^+/K^+-ATPase

The protein content of the P2 + P3 fraction increased steadily throughout the perinatal period, except between foetal day 21 and neonatal day 1. There was an apparent decrease in the protein content between these 2 days. This, however, could be because the foetus stomachs were very small and were used as a whole after rumen removal to prepare P2 + P3, while the fundic mucosa was scraped off the larger stomachs of neonates and adults.

A 95–111 mAb-positive antigen was detected by radioimmunoassay as early as day 18 post-coïtum in contrast with the lack of detection by immunohis-

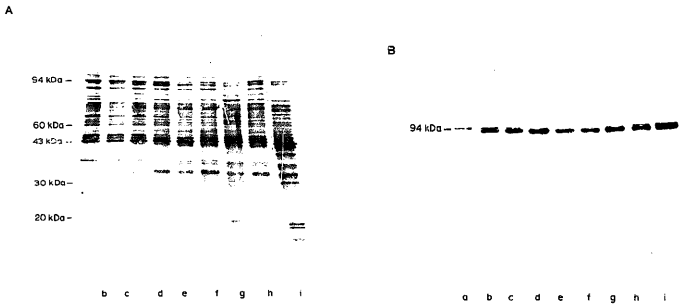

Fig. 2. Ponceau S staining and Western blot of P3 SDS-PAGE. The results are presented as in Fig. 1, but P3 fractions were used instead of P2. (A) Ponceau S staining. (B) Western blots.

tochemistry. The H^+/K^+-ATPase concentration of P2 + P3 fractions was close to 20 μg ATPase per mg protein at this time, and remained constant up to 1 day after birth, suggesting that the ATPase synthesis was proportional to overall tissue protein synthesis. The ATPase concentration more than doubled between neonatal days 1 and 6, and remained at that level up to day 18 when it was only 39% of the adult rat ATPase concentration, indicating that a further increase occurs after day 18 (Table I). The specific H^+/K^+-ATPase activity doubled between neonatal days 1 and 6, as did the concentration and, in 18-day-old neonates, was also only 32% of the adult animal activity (Table I).

The total ATPase content of the P2 + P3 fractions increased progressively from foetal day 18 to neonatal day 18 (Table I).

Special attention was paid to the ATPase content in 12-day-old neonates, because, the ATPase activity disappeared on this day, return to normal on day 14. ATPase concentration, as measured with 95–111 mAb, was the same on days 6, 12 and 14, strongly suggesting that the drop in activity was not due to loss of the enzyme itself.

Characterization of foetal and neonatal 95–111 mAb antigen

Western blots using the 95–111 mAb (400 ng/ml) and rat gastric P2 and P3 demonstrated that the epitope was carried by two very closely migrating bands (95a, upper band; 95b, lower band) at 95 kDa. Only 95a was present in both P2 and P3 on foetal day 18. The other band, 95b, appeared on foetal day 19 and became the major antigen until neonatal day 18 (Fig. 1B (except lane c), and Fig. 2B). The two bands were almost undissociated in the gastric fractions of adults. There was no change in the Western blot profiles of 12-day-old neonates, suggesting that the loss of ATPase activity was not due to ATPase proteolysis.

Discussion

We have previously demonstrated that H^+/K^+-ATPase activity can be detected in 18-day-old foetuses, i.e., 3 days before birth [3]. The results of the present study confirm the presence of the enzyme in 18-day-old foetuses, demonstrating that H^+/K^+-ATPase subunits are 95a bands. The parietal cells are detected 1 day later when the 95b band of the ATPase appears.

Maximum radioimmunoassay sensitivity was obtained by assaying the membrane fractions (P2 + P3), which as we previously demonstrated (Ref. 3, and unpublished data), contain more than 80% of the total homogenate ATPase activity. P2 + P3 ATPase can therefore be regarded as a reasonable estimate of the H^+/K^+-ATPase content of the animal stomachs. Western blot analysis indicated that the ATPase occurred as two protein bands close to 95 kDa, the larger form is present on foetal day 18 and the smaller one, 1 day later. These data provide no explanation for the presence of two bands, and whether they are due to RNA or protein processing or even to protein glycosylation remains to be elucidated. The specific activity of each band is also unknown, as is their relationship to the two bands described by Karlsson A. et al. in adult hog membranes [14] after staining with autoimmune serum from gastritic patients with pernicious anaemia. However, the lack of immunohistochemical reactions on day 18 suggests that the 95a band is not detected by this method.

The morphological features of the developing fundic epithelium have been clearly described by Helander [15]. In the present study, the 95–111 mAb is used to quantify parietal cells during the ontogenic development. The density of immunoreactive cells in 12-day-old gastric epithelium is in agreement with the density of parietal cells which can be calculated from a previous study (1899 cells per μm^2 [16] as compared to 1830 cells per μm^2). The plot of the 95–111 immunoreactive cells per unit area as a function of days appears to be hyperbolic (unshown plot): parietal cells more than doubled between foetal days 19 and 21 and then plateaued towards a density of 2040 cells per mm^2. When further plotted as 1/cell density vs. 1/development day, the linearization is obtained when the zero on the hyperbola y-axis is postulated to be 1/3 of a day prior to day 19, suggesting that parietal cells start appearing at that date. This plot further suggests that half-maximal parietal cell density is reached on foetal day 19.5. As the density of parietal cells plateaued after birth, any important change in the tissue ATPase concentration after that date should result from an increase in parietal cell ATPase content. This should account for the 2.5-fold increase in H^+/K^+-ATPase concentration between days 1 and 6. It should also be remembered that the 18-day-old neonates have not reached the adult secretory capacities, so that a further increase in parietal cell ATPase content must occur.

It was previously demonstrated that the acid secretion of 12-day-old neonates is decreased, and that the activity of H^+/K^+-ATPase is lost [3,4]. A decrease of the number of H^+/K^+-ATPase molecules was suggested to explain the phenomenon. However, the present results demonstrate that H^+/K^+-ATPase molecule is present on day 12, that its concentration expressed as μg of 95 kDa polypeptides per mg protein is not different on day 6, 12 and 14, and furthermore that the protein which is titrated by the 95–111 mAb is not proteolysed, because the molecular mass of the antigen remains constant. The morphology of 12-day-old neonate parietal cells is normal, suggesting no major toxic process. Therefore another explanation than the disappearance of H^+/K^+-ATPase should be looked for

to explain the decreased acid secretion on day 12.

In a separate work, we demonstrated that the concentration of H^+/K^+-ATPase in the rat gastric epithelium (mg of ATPase per g tissue) is similar to those of other species such as hog, rabbit and man (unpublished data). This is contradictory to the finding that the in vitro ATPase activity is often low in rats, due to enzyme lability. The reasons for the lability of rat H^+/K^+-ATPase have not been totally elucidated, but phospholipases have been implicated [17]. The loss of ATPase activity on day 12 might therefore result from very active phospholipases at that stage.

References

1 Garzon, B., Ducroc, R. and Geloso, J.P. (19881) Pediatr. Res. 15, 921–925.
2 Garzon, B., Ducroc, R. and Geloso, J.P. (1982) J. Dev. Physiol. 4, 195–205.
3 Hervatin, F., Moreau, E., Ducroc, R., Garzon, B., Millet, P. and Geloso, J.P. (1987) Am. J. Physiol. 252, G28–G32.
4 Hervatin, F., Moreau, E. and Geloso, J.P. (1989) J. Pediatr. Gastr. Nutr., in press.
5 Takeuchi, K., Peitsch, W. and L.R. Johnson (1981) Am. J. Physiol. 240, G163–G169.
6 Ackerman, S.H. (1982) Science 217, 75–77.
7 Ikezaki, M. and Johnson, L.R. (1983) Am. J. Physiol. 224, G165–G170.
8 Seidel, E.R. and Johnson, L.R. (1984) Am. J. Physiol. 246, G550–G555.
9 Johnson, L.R. (1985) Annu. Rev. Physiol. 47, 199–215.
10 Benkouka, F., Bayle, D., Péranzi, G., Lewin, M.J.M. and Soumarmon, A. (1989) Diabètes et Métabol. 15, XXXV.
11 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254, 1976.
12 Burnette, N. (1981) Anal. Biochem. 112, 195–203.
13 Coudrier, E., Reggio, H. and Louvard, D. (1983) EMBO J. 2, 469–475.
14 Karlsson, F.A., Burman, P., Lööf, L. and Mardh, S. (1988) J. Clin. Invest. 81, 475–479.
15 Helander, H.F. (1969) Gastroenterology 56, 35–52.
16 Puccio, F. and Lehy, T. (1988) Digestion 40, 157–167.
17 Im, W.B. and Blakeman, D.P. (1982) Biochem. Biophys. Res. Commun. 108, 635–639.