J exp Biol. 158, 531–537 (1991)
Printed in Great Britain © The Company of Biologists Limited 1991

DISTRIBUTION OF CARBONIC ANHYDRASE IN THE UTERUS OF LATE-TERM PREGNANT SPINY DOGFISH (*SQUALUS ACANTHIAS*)

BY CASSANDRA FLÜGEL AND ELKE LÜTJEN-DRECOLL

Department of Anatomy, University of Erlangen-Nürnberg, Krankenhausstraße 9, 8520 Erlangen, Germany

Accepted 31 January 1991

Summary

Carbonic anhydrase (CA) activity was localized in the late-term pregnant spiny dogfish uterus using the modified histochemical staining method of Hansson. Staining was found in the cytoplasm of red blood cells and in the membranes of the endometrial epithelial cells. Under the electron microscope, reaction products were seen in the basolateral membranes of the superficial cells, whereas the apical membranes were unstained. The cells of the underlying layer contacting the stromal capillaries showed staining in all their membranes. This staining pattern is similar to that found in other epithelia noted for active ion transport.

Introduction

The pregnant late-term spiny dogfish, *Squalus acanthias*, is known to establish special environmental conditions for the pups lying in the uterus and completing the period of gestation. These are characterized by an acidic uterine fluid with high ammonia concentration and low CO_2 content (Kormanik and Evans, 1986; Kormanik, 1988). The carbonic anhydrase (CA) inhibitor acetazolamide has been shown to prevent uterine seawater acidification and extraction of HCO_3^- (Kormanik and Kremer, 1986; Kormanik and Maren, 1988). However, it is not known which structures or cells are responsible for the acidification. During the sampling and investigation of developing embryos by Kormanik (1989) we were able to obtain uterine tissue from late-term pregnant spiny dogfish taken from Frenchman Bay at the Mount Desert Island Biological Laboratory (Salsbury Cove, Maine). We investigated the tissue histochemically to localize the carbonic anhydrase.

Materials and methods

Endometrial tissue from a late-term pregnant spiny dogfish uterus (about 6 kg,

Key words: carbonic anhydrase, late-term pregnant spiny dogfish uterus, histochemistry, *Squalus acanthias*.

eighteenth month of gestation) was prepared by stripping the endometrium from the underlying muscle. The specimens also contained the branches of the posterior oviducal artery, which lie in the endometrial stroma between mucous membrane and muscle. The specimens were fixed for 3 h in 2.5 % glutaraldehyde solution buffered with 0.1 mol l^{-1} cacodylate buffer, pH 7.4, then washed in the same buffer and embedded according to Ridderstråle (1976) in hydroxypropylmethacrylate (JB4, Polysciences, Pennsylvania). For the light microscopical localization of CA, semithin sections 2 μm thick were stained according to the method of Hansson (1967) modified by Ridderstråle (1976, 1980). In the incubation medium the concentration of $CoSO_4$ was 1.75 mmol l^{-1} and that of KH_2PO_4 was 11.7 mmol l^{-1}. After being incubated for 1–9 min, the sections were washed in 0.9 % NaCl, pH 5.9, and floated in 0.5 % $(NH_4)_2S$ for 3 min. The sections were washed three times for 1 min in distilled water, dried on glass slides and mounted in Eukitt (Kindler GmbH, Freiburg, FRG). Some of the sections were counterstained with Haematoxylin and Eosin before mounting in Eukitt.

Control sections were stained as described above, but with the incubation medium modified by the addition of 10 μmol l^{-1} acetazolamide (Diamox; Cyanamid-Novalis GmbH, Wolfratshausen, FRG). [For a discussion of the specificity of the method, see Lönnerholm (1974, 1980), Maren (1980) and Sugai and Ito (1980).] Other sections cut at 2 μm were stained with Richardson's stain (Richardson *et al.* 1960) to compare them with the sections stained for CA.

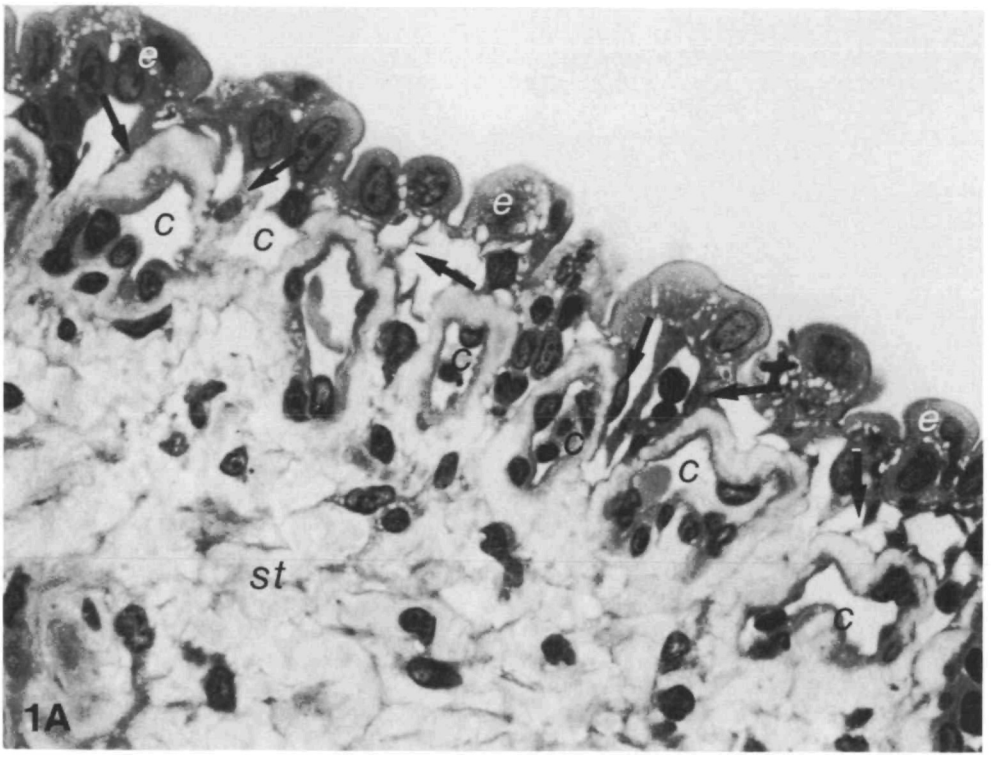
For the electron microscopical demonstration of CA, 2 μm thick methacrylate sections were stained as described above with periods of incubation ranging from 5 to 9 min. Those sections that showed clear staining without artificial precipitations were put on Thermanox™ tissue culture cover slips (Miles, Naperville, IL) and embedded in Epon. Ultrathin sections were cut and viewed with a Zeiss 902 EM either without counterstaining or after staining with uranyl acetate and lead citrate.

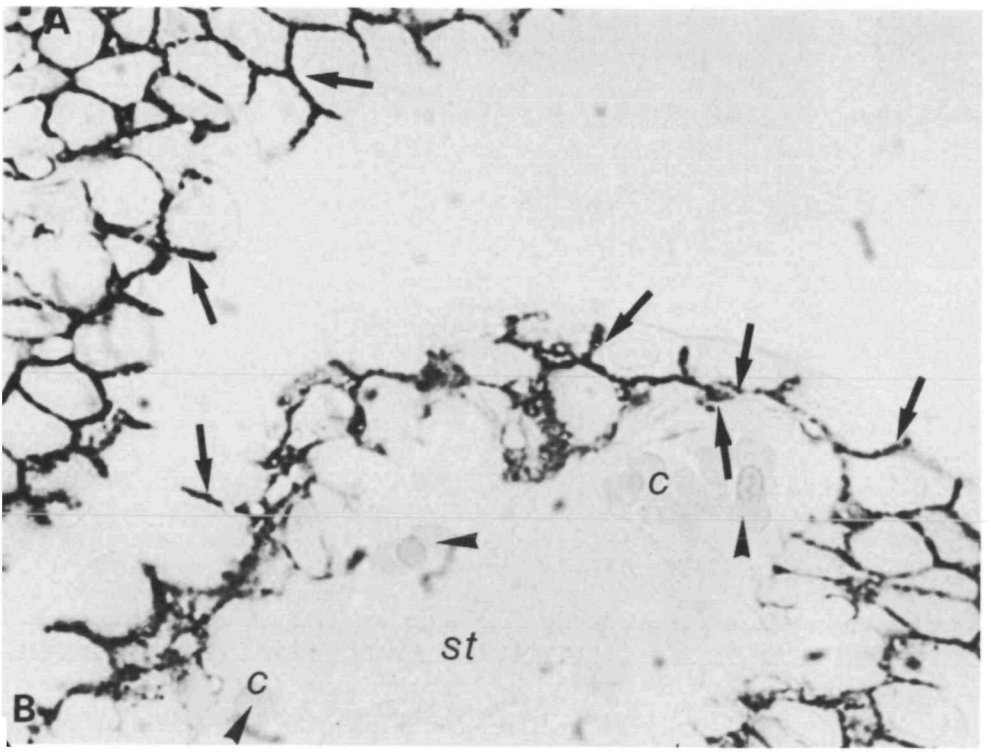
Results

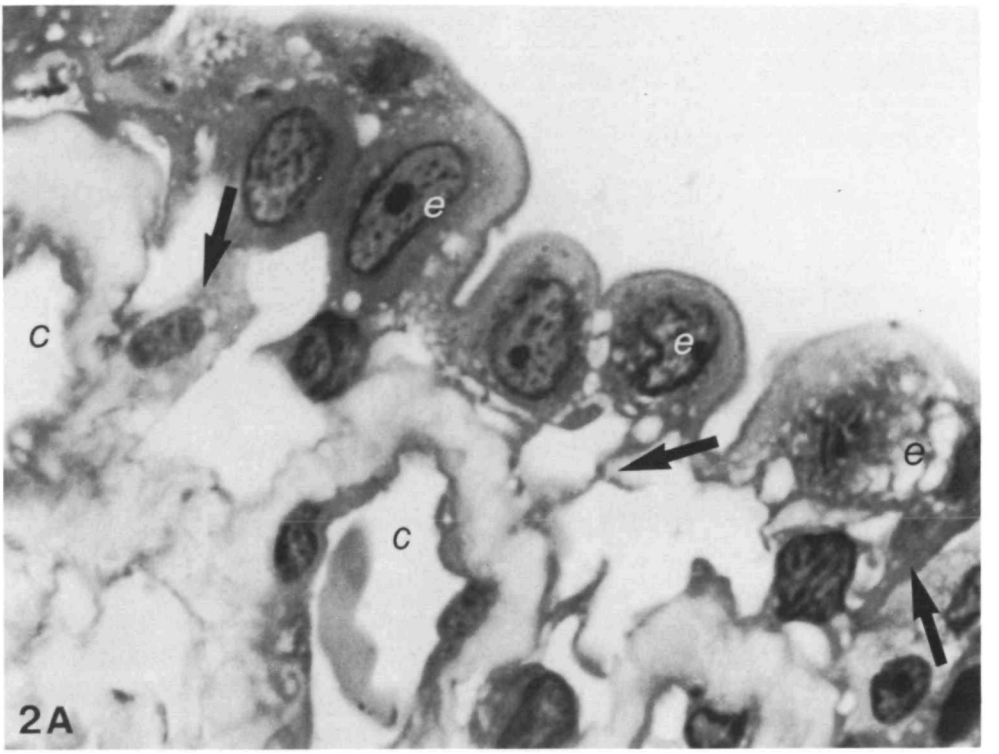
Light microscopy

The mucosal epithelium consists of partly single-layered but mostly two-layered cuboidal cells (Figs 1A, 2A). The underlying stromal tissue is loosely arranged and contains many free cells. A large number of capillaries form a dense network lying

Fig. 1. (A) Endometrium (*e*) and underlying stromal tissue (*st*) with capillaries (*c*). Note the foot-like extensions of the endometrial cells (arrows) which seem to be connected to the underlying capillaries (Richardson's stain; ×200). (B) Light micrograph of the uterus endometrium after staining for carbonic anhydrase. The reaction products are seen in the basal and lateral membranes of the endometrium (arrows). The apical cell membrane, facing the lumen of the uterus, and the stromal tissue (*st*) with capillaries (*c*) are not stained. Within the capillaries, red blood cells show faint cytoplasmic staining at this incubation time (arrowheads). On the left, the endometrium is cut tangentially, therefore a honeycomb-like pattern is seen (incubation time 4 min, ×200).


e
c
c
e
c
c
c
e
st
c
1A


A
c
st
c
B


e
e
e
c
c
2A

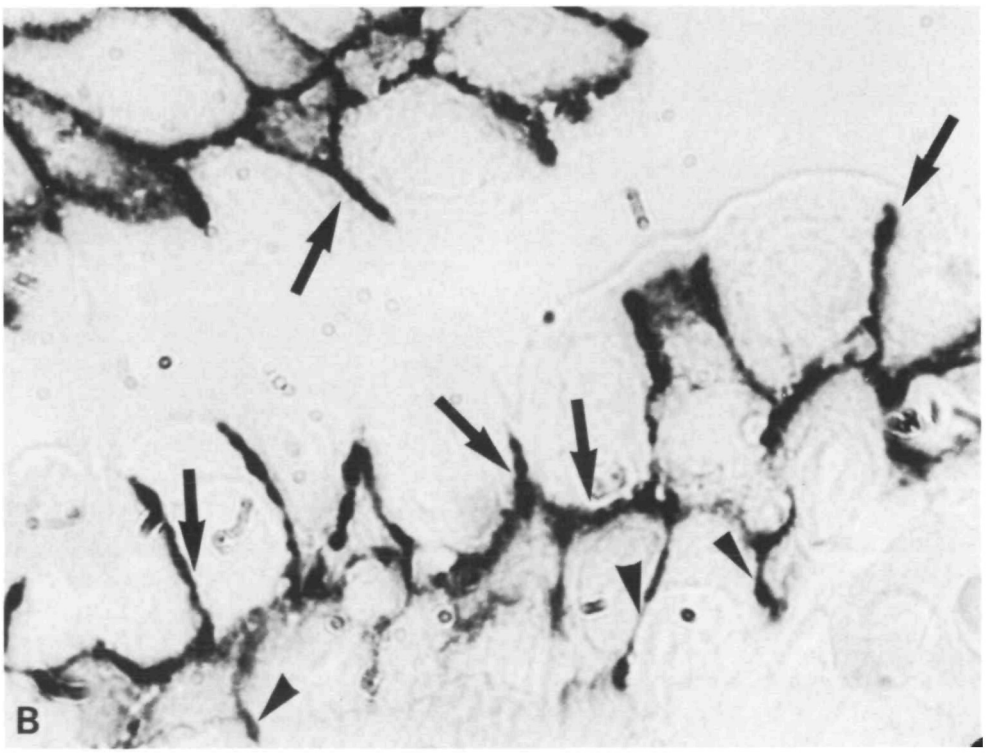

B

Fig. 2. (A) Higher magnification of the endometrial cells (*e*) and their basal extensions (arrows) towards the capillaries (*c*) (Richardson's stain, ×500). (B) Higher magnification of the endometrial cells stained for carbonic anhydrase. The basal and lateral cell membranes (arrows) as well as the basal protrusions towards the capillaries (arrowheads) are clearly stained (incubation time 4 min, ×500).

directly underneath the mucosal epithelium. The epithelial cells are connected to the capillaries by foot-like cytoplasmic extensions (Figs 1A, 2A).

Histochemistry

Light microscopy

After staining for CA, reaction products are found only in the mucosal epithelial cells (Figs 1B, 2B). The staining appears after 4 min of incubation. No staining is seen in the endothelium of the capillaries or in any stromal cell (Fig. 1B). The red blood cells within the capillaries show faint cytoplasmic staining (Fig. 1B).

Electron microscopy

In the mucosal epithelial cells the lateral portions, including the foot-like extensions towards the stromal capillaries, are stained, whereas the luminal surface is not. With the electron microscope one can see that the reaction products are localized in the basolateral membranes, which have numerous invaginations. If two layers of epithelial cells are present, all the cell membranes of the round cells lying between the superficial cells and stromal cells are also stained. The cytoplasm near the stained membranes shows abundant mitochondria (Fig. 3).

Controls

The control sections treated with $10\,\mu mol\,l^{-1}$ acetazolamide in the incubation medium show total inhibition of staining.

Discussion

In this study we demonstrate that the enzyme CA in the pregnant spiny dogfish uterus is localized not only in the red blood cells but also in the epithelium itself. In the surface epithelium, the basolateral cell membranes stain in the same way as epithelia performing active ion transport, e.g. intestine, choroid plexus, etc. (for a review, see Lütjen-Drecoll *et al.* 1985).

In places where the uterus epithelium consists of two cell layers, the abluminal cells also show membrane-bound staining. This staining pattern is similar to that of the stria vascularis of the inner ear (Hsu and Nomura, 1985; E. Lütjen-Drecoll, unpublished observations) and the ciliary epithelium in the eye (Hansson, 1968; Lütjen-Drecoll and Lönnerholm, 1981), where all the cells between the capillary and the lumen are stained.

The presence of abundant mitochondria adjacent to the stained membranes as well as the close contact of the stained foot-like extensions with the stromal

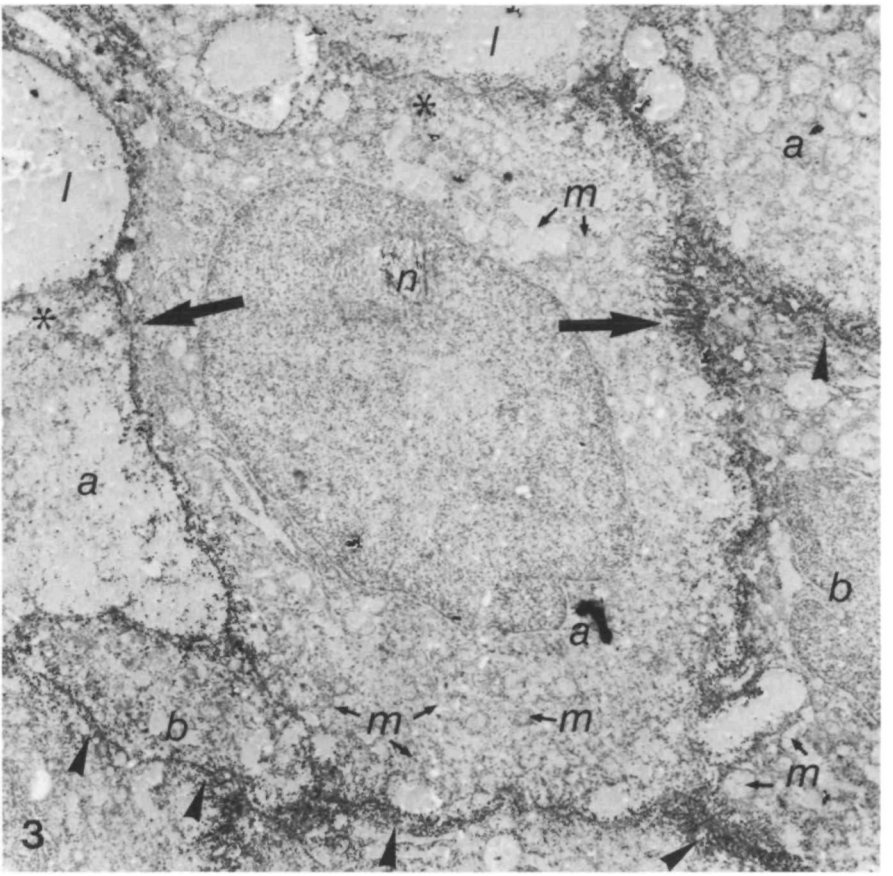


Fig. 3. Electron micrograph of the mucosal epithelium stained for carbonic anhydrase. Intense staining is seen at the basolateral membranes (arrows) of the luminal cells (*a*), whereas the apical cell membranes are unstained (*). In the underlying epithelial cells (*b*) all the membranes are stained (arrowheads). *m*, mitochondria; *l*, lumen; *n*, nucleus (incubation time 5 min, ×5400).

capillaries support the suggestion that both cell layers are involved in active transport.

Cytoplasmic staining for CA was not observed in the epithelium. However, lack of staining does not prove the absence of an enzyme. It is well known that during the processing for histochemical staining some enzymes can be lost from the cytoplasm (Ridderstråle, 1980; Hyyppä, 1968). In contrast, the intense staining of the mucosal epithelial cells indicates real abundance of the enzyme in this location. Inhibition of the epithelial CA by acetazolamide could explain the changes in the uterine lumen described by Kormanik and Kremer (1986).

We are most grateful to J. Gehr and K. Göhler for their expert technical assistance and to M. Gößwein for his photographic assistance.

References

HANSSON, H. P. J. (1967). Histochemical demonstration of carbonic anhydrase activity. *Histochemie* **11**, 112–128.

HANSSON, H. P. J. (1968). Histochemical demonstration of carbonic anhydrase activity in some epithelia noted for active transport. *Acta physiol. scand.* **73**, 427–434.

HSU, C. J. AND NOMURA, Y. (1985). Carbonic anhydrase activity in the inner ear. *Acta Otolaryngol.* **418**, 1–42.

HYYPPÄ, M. (1968). The effects of fixations on carbonic anhydrase activity. *Histochemie* **12**, 184–188.

KORMANIK, G. A. (1988). Time course of the establishment of uterine seawater conditions in late-term pregnant spiny dogfish (*Squalus acanthias*). *J. exp. Biol.* **137**, 443–456.

KORMANIK, G. A. (1989). Nitrogen budget in developing embryos of the spiny dogfish *Squalus acanthias*. *J. exp. Biol.* **144**, 583–587.

KORMANIK, G. A. AND EVANS, D. H. (1986). The acid–base status of prenatal pups of the dogfish, *Squalus acanthias*, in the uterine environment. *J. exp. Biol.* **125**, 173–179.

KORMANIK, G. A. AND KREMER, W. (1986). Effect of acetazolamide on the establishment of uterine seawater conditions in pregnant dogfish (*Squalus acanthias*). *Bull. Mt Desert Isl. biol. Lab.* **26**, 142–144.

KORMANIK, G. A. AND MAREN, T. H. (1988). Carbonic anhydrase in the uterine sea water acidification process in *Squalus acanthias*: localization and the effect of various inhibitors. *Bull. Mt Desert Isl. biol. Lab.* **27**, 25–27.

LÖNNERHOLM, G. (1974). Carbonic anhydrase histochemistry, a critical study of Hansson's cobalt–phosphate method. *Acta physiol. scand.* (Suppl.) **418**, 1–43.

LÖNNERHOLM, G. (1980). Carbonic anhydrase in the rat liver and rabbit skeletal muscle; further evidence for the specificity of the histochemical cobalt–phosphate method of Hansson. *J. Histochem. Cytochem.* **28**, 427–433.

LÜTJEN-DRECOLL, E., EICHHORN, M. AND BARANY, E. H. (1985). Carbonic anhydrase in epithelia and fenestrated juxtaepithelial capillaries of *Macaca fascicularis*. *Acta physiol. scand.* **124**, 295–307.

LÜTJEN-DRECOLL, E. AND LÖNNERHOLM, G. (1981). Carbonic anhydrase distribution in the rabbit eye by light and electron microscopy. *Invest. Ophthalmol. vis. Sci.* **21**, 782–797.

MAREN, T. H. (1980). Kinetics, equilibrium and inhibition in the Hansson histochemical procedure for carbonic anhydrase: a validation of the method. *Histochem. J.* **12**, 183–190.

RICHARDSON, K. C., JARETT, L. AND FINKE, E. H. (1960). Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35**, 313–323.

RIDDERSTRÅLE, Y. (1976). Intracellular localization of carbonic anhydrase in the frog nephron. *Acta physiol. scand.* **98**, 465–469.

RIDDERSTRÅLE, Y. (1980). Intracellular localization of carbonic anhydrase in some vertebrate nephrons. Thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

SUGAI, N. AND ITO, S. (1980). Carbonic anhydrase, ultrastructural localization in the mouse gastric mucosa and improvements in the technique. *J. Histochem. Cytochem.* **28**, 511–525.