Localization of Carbonic Anhydrase in Reproductive Organs

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Abstract

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This thesis is a comparative study on localization of carbonic anhydrase (CA) in male and female reproductive organs. CA functions in pH and bicarbonate homeostasis and catalyzes the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. At present 15 different isozymes (CA I-XIV) have been characterized, three of them without catalytic activity. Only active isozymes are of physiological interest and a method demonstrating CA activity in tissues was used. Furthermore, a study on knockout mice deficient in CA II has been undertaken since these are used as model animals for the inherited human CA II deficiency syndrome.

Tissues from testis and epididymis were taken from sexually mature pig, rabbit and mouse males; and from the ovary, oviduct and uterus from females of the same species and also cat. Corresponding tissues were taken from CA II-deficient mice. Tissues from other species were included when available from other studies. The tissues were fixed and processed for histochemical demonstration of CA activity and on mouse tissue also for immunohistochemical localization for CA II and CA IV. Spermatozoa were collected from testis, caput, corpus and cauda epididymidis and labelled for CA IV for scanning electron microscopy.

Testis: The seminiferous epithelium was unstained except for the Sertoli cells in pig and acrosomes in hamster and lemming. The Leydig cells were CA positive in rat and pig. The capillaries were stained for CA activity in the testis of all species.

Epididymis: The apical cells in caput showed CA activity in all species. The principal cells had basolateral membrane staining in cauda. The stereocilia/microvilli were stained in some regions in the species but not in pig. The membrane-bound CA IV was found to be transferred to the spermatozoa during passage through the duct. The capillaries were unstained.

Ovary: The oocyte was unstained except for the rabbit cell membrane. The granulosa cells were stained only in the mouse and the theca interna was negative in all species. The luteal cells of corpus luteum were always unstained. The capillaries were variably stained for CA activity and unstained in mouse.

Uterus: The surface epithelium was stained during at least some stage of the oestrus cycle in all species except pig. CA disappeared from the epithelium during the non-luteal phase in rabbit, while the glands were negative during the entire cycle. In the other species the glands were stained for CA activity. Stained capillaries were present in all species, except during the luteal phase in rabbit.

CA II-deficient mouse: Membrane-bound CAs were more predominant than CA II in males, which may explain why the CA II-deficiency does not affect male fertility in the mouse. In the control females CA II was present in the uterine epithelium and glands as well as membrane-bound CA. The deficient females, however, often produce stillborn pups, which might be related to the lack of CA II in the uterine mucosa.

Conclusions: The CA present in the epididymis is thought to be involved in the gradual acidification and bicarbonate resorption taking place in the duct. The envi-

ronment in cauda must have a low pH to keep the spermatozoa immotile during storage. The CA in the female is thought to have a function in the micro milieu in the oviduct and uterus during transport of spermatozoa and ovum and later for embryonic implantation in the uterus. However, the cyclic variation affects CA activity, which together with a significant species variation warrant further studies.

Keywords: Carbonic anhydrase, Histochemistry, Immunohistochemistry, Testis, Epididymis, Ovary, Oviduct, Uterus, CA II-deficient mice

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Swedish summary

Svensk populärvetenskaplig sammanfattning

Reproduktion är en fascinerande och komplicerad process där spermier och äggceller genomgår olika mognadsprocesser innan befruktningen kan ske en och en ny individ utvecklas. Spermierna bildas i testikeln, men måste passera bitestikeln för att mogna och lagras sedan i slutet av denna. Äggcellen bildas i äggstocken och är mogen vid ägglossningen då den fångas upp av äggledaren där befruktning senare kan ske.

Sammansättningen på vätskan som omger både spermier och äggcell har stor betydelse för funktionen. Spermierna genomgår mognadsprocesser under transporten i bitestikeln som gör att de får förmåga att senare kunna befrukta en äggcell. Hos husdjuren tar denna transport ca 12 dagar. Spermierna rörlighet beror bland annat på pH och under lagring i bitestikeln då spermierna måste vara orörliga är pH något surare. Enzymet karbanhydras (CA) har en viktig roll vid syra-bas reglerande processer i kroppen. Genom att studera den mikroskopiska lokalisationen av CA kan man få en uppfattning om i vilka celler dessa processer sker. Enzymfamiljen CA består av 15 genetiskt olika isoenzymer som skiljer sig mellan de olika djurslagen. I avhandlingen har i huvudsak en metod använts som bygger på att det bildas en mikroskopiskt synlig fällning som visar var samtliga CA isoenzymer arbetar (histokemisk metod). Därför var det av intresse att undersöka lokalisationen av CA i reproduktionsorganen och till en början studerades testikel och bitestikel. Främst har gris, kanin och mus undersökts men även andra djurslag ingår. Dessutom har en studie utförts på en knockout-mus som saknar ett av de viktigaste cytoplasmatiska isoenzymerna, CA II.

Resultaten visar att det finns stora artskillnader. Exempelvis är gris det enda djurslag som har CA aktivitet i testikelns Sertoliceller. Man vet att dessa celler utsöndrar bikarbonatjoner vilket ofta kräver CA, men tydligen inte hos alla djurslag. Könscellerna har i regel ingen CA aktivitet medan alla undersökta djurslag har CA i testikelns kapillärer där CA underlättar transporten av CO_2. Hos alla djurslag förekommer CA aktivitet i bitestikelns apikalceller som utsöndrar vätejoner. I bitestikelsvansen, där spermierna lagras, finns membranbundet CA hos alla djurslag och man tror att det bidrar till att sänka pH för att hålla spermierna orörliga, ett sätt att spara energi på. Ett intressant fynd här är att hos mus överförs ett isoenzym (CA IV) från epitelcellerna och fästs på spermiemembranets utsida. Spermierna är därefter försedda med aktivt CA hela vägen som kan påverka den omkringliggande vätskan, vilket kan ha betydelse vid befruktningen i äggledaren.

Även honliga reproduktionsorgan har undersökts, främst gris men även mus, kanin och katt. Hos hondjur varierar förekomsten av CA i äggledare och livmoder betydligt, både under brunstcykeln som är hormonellt reglerad och mellan arter. Detta är inte färdigutrett ännu. Som exempel kan dock nämnas att livmoderslemhinnan har stark CA aktivitet hos kanin under gulkroppsfasen, men under tidigare skeende i brunstcykeln saknas CA aktivitet i livmoderslemhinnan. CA stimuleras av hormonet progesteron, som bildas av gulkroppen. Det förkommer också CA i

äggstocken, t ex hos mus i de växande granulosacellerna som omger äggcellen. Denna typ av lokalisation av CA finns endast hos mus, vilket visar på stora artskillnader.

Jämförelsen mellan CA II-knockout-musen och normal mus visar att cytoplasmatiskt CA II spelar mindre roll än membranbundet CA hos hannar, medan skillnaden inte är lika stor hos honor. CA II tros därmed ha olika stor betydelse för fertiliteten hos könen.

Genom jämförande studier kan man få ökade kunskaper om vad som är grundläggande likheter mellan arter för att därmed kunna dra mer generella slutsatser om funktionen. Studien visar också att det förekommer artskillnader och att det är viktigt att beakta dessa för att öka förståelsen för de grundläggande mekanismer som har betydelse för reproduktion. Olikheterna har betydelse för att korsning mellan arter förhindras.

Contents

Appendix

This thesis is based on the following papers (I-IV). Papers I and II were included in manuscript form in my licentiate thesis (1989).

I. Ekstedt, E., Ridderstråle, Y., Plöen, L. & Rodriguez-Martinez, H. 1991 Histochemical localization of carbonic anhydrase in the testis and epididymis of the boar. *Acta Anatomica* 141, 257-261.

II. Ekstedt, E. & Ridderstråle, Y. 1992 Histochemical localization of carbonic anhydrase in the testis and epididymidis of the rabbit. *Acta Anatomica* 143, 258-264.

III. Rodriquez-Martinez, H., Ekstedt, E. & Ridderstråle, Y. 1991 Histochemical localization of carbonic anhydrase in the female genitalia of pigs during the oestrous cycle. *Acta Anatomica* 140, 41-47.

IV. Ekstedt, E., Holm, L. & Ridderstråle, Y. 2004 Carbonic anhydrase in mouse testis and epididymis; transfer of isozyme IV to spermatozoa during passage. *Journal of Molecular Histology* 35, 16-173.

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To My Family

Introduction

Reproduction in mammals is a remarkably complex process leading to new individuals. The starting points are the male and female germ cells, which undergo their own individual maturation processes before the moment of fertilization (Yanagimachi, 1994). The sperm cells are continuously produced in large amounts in the testes in a complex process called spermatogenesis. Mammalian spermatozoa are immature when leaving the testis. They then pass through the efferent ductules into the epididymal duct where they undergo a transformation during which they acquire capacity for motility and part of their fertilising ability. The composition of the fluid in the epididymis is of great importance, especially with regard to the pH and bicarbonate concentration during the maturation process of the spermatozoa.

In the females only a restricted number of mature oocytes are released at each cycle. The oocytes are developed in the ovary, delivered to the oviduct at ovulation and transported towards the uterus. Once the spermatozoa have entered the female reproductive organs, a number of processes take place such as capacitation and acrosome reaction, which prepare the spermatozoa for the fertilization in the oviduct. These processes are dependent on an optimal production of bicarbonate ions i.e. at the right time and place (Carr & Acott, 1984; Lee & Storey, 1986; Yanagimachi, 1994). This means that changes in pH and bicarbonate concentration are of outmost importance as the spermatozoa traverse both the male and female reproductive tract. Carbonic anhydrase (CA) is a key enzyme in acid-base balance and bicarbonate-carbon dioxide homeostasis, since it catalyzes the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. This makes it of interest to study the localization of CA in reproductive organs of different mammalian species, which is the main focus of this thesis.

Background

Reproductive organs

Testis and efferent ductules; structure and function

The testis consists of the seminiferous tubules surrounded by the interstitium. The seminiferous tubules consist of spermatogonia, developing germ cells and Sertoli cells. During development the germ cells are in intimate contact with the fluid secreting and supportive Sertoli cells. Cytoplasmic processes of the Sertoli cells are connected to each other with tight junctions in such a way that the tubule is divided into a luminal and a basal compartment. This is the structural basis for the blood-testis barrier. The immotile testicular spermatozoa are shed from the Sertoli cells and are passively transported via rete testis to the efferent ductules. The Leydig cells are situated in the interstitial tissue and are testosterone producing cells. Part of the testosterone is converted to oestrogen by the Sertoli cells (de Krester & Kerr, 1994; Setchell *et al.,* 1994).

The efferent ductules connect the testis to the initial part of the epididymis. The epithelium is columnar and consists of ciliated and non-ciliated cells (Ilio & Hess, 1994). The major function of the efferent ductules is reabsorption of luminal fluid increasing the concentration of spermatozoa (Clulow *et al.,* 1998). Reabsorption of bicarbonate also takes place in the efferent ductules (Newcombe *et al.,* 2000).

Epididymis; structure and function

The first recorded description of the epididymis was made in the middle of the 4th century BC by Aristotle in the "Historia Animalium", but the first detailed description of a dissected epididymis was not made until 1668 (De Graaf, in Jocelyn & Setchell, 1972). In 1926 Benoit published a landmark paper describing the epididymal duct of different mammalian species. Shortly after Young (1931) showed that guinea-pig spermatozoa from the proximal part of the epididymis were much less fertile than the spermatozoa taken from the distal part of the duct when these were used for insemination of females.

The mammalian epididymis is a single, long and extremely coiled duct strictly organized in specific regions. By tradition, mostly because of the anatomical shape of the human epididymis, the different regions are called caput, corpus and cauda. The shape of the epididymis varies considerably depending on species and different terminologies have evolved. Some of these have been described in more detail in functional and structural aspects (Nicander, 1957; Glover & Nicander, 1971; Hoffer & Greenberg, 1978; Sorano *et al.,* 1982; Goyal, 1985; Robaire & Hermo, 1988; Axner *et al.,* 1999).

The surface epithelium of the epididymal duct is pseudostratified with tall, columnar cells and smaller basal cells situated close to the basal lamina. The most frequent columnar cells are the principal cells with non-motile stereocilia/microvilli at the apical cell surface (Jones *et al.,* 1979; Abe *et al.,* 1983; Robaire & Hermo, 1988). Scattered among the principal cells, preferentially in the caput, are the apical/narrow cells (Sun & Flickinger, 1980; Adamali & Hermo, 1996; Martinez-Garcia et al., 1995). The clear cells are found in the corpus and cauda epididymidis in hamster, rat and mouse (Robaire & Hermo, 1988). Extensive descriptions of the ultrastructure of the different cell types has been published for the rat (Hoffer *et al.,* 1973), rabbit (Jones *et al.,* 1979; Nicander & Plöen, 1979), mouse (Abe *et al.,* 1983; Abou-Haila & Fain-Maurel, 1984) and pig (Stoffel & Friess, 1994).

The epididymis plays a major role in the development of mammalian spermatozoa (Cooper, 1986). Orgebin-Crist (1967) showed in rabbits that if the spermatozoa are prevented from passing along the epididymal duct and are retained in the proximal part, they remain viable but do not acquire full fertilizing capacity. It appeared that the maturation is the result of sequential events in the different epididymal regions and this has not been possible to mimic under *in vitro* conditions. Some of the major changes to the spermatozoa involve substantial remodelling of the cell membrane (Jones, 2002; Olsson, 2002). It has been shown that several proteins, linked by so called GPI-anchors (glycosylphosphatidylinositol), are transferred from the epididymal epithelium to the spermatozoa (Yeung *et al.,* 1997; Zhang & Martin-DeLeon, 2003). The spermatozoa become motile during the passage through the epididymis, but this ability has to be depressed during storage in the last part before ejaculation.

The last part, cauda epididymidis, is specialised to keep the spermatozoa in a quiescent state (Turner, 1991; Amann *et al.,* 1993; Setchell *et al.,* 1993). Several factors have been proposed to be of importance for inhibition of sperm motility such as high fluid viscosity, decreased Na^+ concentration, increased K^+ concentration and by pH (McGrady & Nelson, 1972, Mann & Lutwak-Mann, 1982). It has been shown that the fluid surrounding the spermatozoa is acidified during the transit through the epididymis (Levine & Marsh, 1971; Levine & Kelly, 1978; Rodriguez *et al.,* 1990). Acidification inhibits motility of spermatozoa from several different species (Acott & Carr 1984; Carr & Acott, 1984; Holm & Wishart, 1998). The most favoured hypothesis is, therefore, that the spermatozoa are kept quiescent by the acidic pH created in the epididymal cauda.

Ovary, oviduct and uterus; structure and function

In the ovary the primordial follicles are embedded in the connective tissue in the cortex. During development the oocyte increases in size and becomes surrounded by several layers of granulosa cells, which are in turn enclosed by the theca interna cells. The granulosa cells produce a follicular fluid and the antrum is formed. The granulosa cells together with the theca interna cells have endocrine functions as well. The mature follicle ruptures at ovulation and the granulosa cells and theca interna cells form the corpus luteum.

The funnel-shaped beginning of the oviduct, infundibulum, reaches towards the ovary at ovulation and picks up the ovum, which is transported through the oviduct to the uterus. The oviduct is divided into a proximal part, the ampulla and a more distal part, the isthmus. The epithelium consists of secretory and ciliated cells. In the uterus the endometrium consists of a surface columnar epithelium and deeper situated uterine glands. These layers undergo large variations due to endocrine changes during the reproductive cycle.

It is within the oviduct that mammalian spermatozoa complete the physiological processes required to fertilise an egg. The oviducal fluid is a complex mixture with specific ionic composition, pH, osmolarity and macromolecular content (Leese *et al.,* 2001). Variations between species and during the reproductive cycle are also prominent. The pH profile in the pig oviduct differs between regions during the cycle (Nichol *et al.,* 1997) and in rabbit, estimates of bicarbonate concentrations are significantly higher in the oviduct than in blood plasma (Vishwakarma, 1962; Maas *et al.,* 1987). Bicarbonate stimulates sperm motility (Taimja *et al.,* 1987). Furthermore, several experiments have shown that capacitation is bicarbonate-dependent (Lee & Story, 1986; Boatman & Robbins, 1991; Neill & Olds-Clarke, 1987; Gadella & Gestel, 2004) and that defective bicarbonate secretion in the female genital tract causes impaired sperm capacitation and decreased fertility (Wang *et al.,* 2003b).

A sperm reservoir at the uterotubal junction (UTJ) of mammals was first discovered in hamsters by Yangimachi and Chang (1963) and has later been reported to exist in several other mammals such as rabbit, pig and mouse (Harper, 1973; Oversteet *et al.,* 1978; Hunter, 1981; Suarez, 1987). Small amounts of spermatozoa colonize the specialised mucosal furrows and can be stored, protected by the epithelium. Storage of viable spermatozoa for long periods is well known also in many non-mammalian species such as birds (reviewed in Bakst, 1998).

Carbonic anhydrases

The enzyme CA was first discovered by Meldrum & Roughton (1933) and Stadie & O´Brien, (1933) as the catalytic factor present at the rapid transfer of CO_2 to HCO_3^- in erythrocytes. In the early 1960s it was found that the CA in erythrocytes in fact was two different isozymes catalyzing the same reaction: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. At present there are 15 different isozymes numbered CA I-XIV. They are all zinc metalloenzymes showing different rates of catalytic activity and varying sensitivity to inhibition by sulfonamides. The effects of different sulfonamides and anions on the CA isozymes have recently been reviewed (Supuran *et al.,* 2003). Three of them are without catalytic activity and therefore named carbonic anhydrase-related proteins (CA-RPs VIII, X, XI).

Since the reaction catalyzed by CA is so fundamental in animals, it does not astonish that one or more of the isozymes are present in almost all organs. CAs take part in a variety of physiological processes such as respiration, acid-base balance, ion and fluid transport, bone remodelling and calcification. Furthermore CAs are involved in ureagenesis, fatty acid synthesis and gluconeogenesis, as well as cell growth with implications for cancer. The isozymes differ in localization. Some are soluble in the cytoplasm, others are membrane-bound or present in mitochondria.

A short presentation of the different isozymes is given below, summarized from the following reviews: Maren (1967), Tashian (1989, 2000), Sly & Hu (1995), Henry (1996) and Chegwidden & Carter (2000). Although the localization of CAs in different tissues and species is far from complete, it is possible to present a few examples.

Cytoplasmic isozymes: CA I, CA II, CA III, CA VII and CA XIII

CA I, next to haemoglobin, is the most common protein in erythrocytes, where it is co-localized with CA II except in erythrocytes of ruminants and felids (Deutsch, 1987) where it is absent. It is also present in the epithelium of colon and caecum in several species and in the rumen epithelium (Carter 1971). In individuals having a deficiency of CA I no abnormalities are seen.

CA II is one of the fastest enzymes ever known, having a turn over number of 1.4×10^6 s^{-1} under physiological conditions. CA II appears to be almost universally expressed in some cell types of all major mammalian tissues, such as erythrocytes, kidney tubules, the digestive system, eye and central nervous system to mention a few. In humans the inherited CA II deficiency syndrome causes disorders like osteopetrosis, renal tubular acidosis and cerebral calcification due to a non-functional CA II protein.

CA III is a low activity isozyme with a catalytic rate about 1% of that of CA II. It is relatively resistant to acetazolamide inhibition. CA III is present in high amounts in the cytoplasm of skeletal muscle type I fibres and adipose tissue. The isozyme is found in male rat livers but not in the female. CA III has been ascribed a function in facilitated CO_2 diffusion in muscle tissue and a role in cell protection during oxidative stress.

CA VII is known from its gene expression. The protein has been cloned and characterized, but it has not been localized in any tissues so far.

CA XIII is the most recently discovered cytoplasmic isozyme of the high catalytic type. It has been immunohistochemically localized in human and mouse tissues including the kidney, colon and endometrium (Lehtonen *et al.,* 2004).

Membrane-bound isozymes: CA IV, CA IX, CA XII, CA XIV

Membrane-bound CA was first demonstrated by histochemical studies that showed intense CA activity in association with cell membranes in kidneys (Ridderstråle 1976, Lönnerholm & Ridderstråle, 1980). This was later shown to be CA IV, which is the only CA isozyme linked to the extra-cellular surface of the cell membrane by a GPI anchor. CA IV has been found in kidney, lung, gastrointestinal tract and also in the endothelial cells of the capillary beds in many organs such as the brain, skeletal and heart muscle. CA IV is a high activity isozyme, with only slightly less activity than CA II.

CA IX, CA XII and CA XIV are trans-membrane proteins with the catalytic domain facing the extra-cellular space. They are present in many different organ systems including the gastrointestinal tract, kidney and brain, although with many variations among species. In addition, CA IX and CA XII have been found in tumour cells. (For review see Karhumaa, 2002).

Mitochondrial isozymes: CA VA and CA VB

The mitochondrial isozyme CA V is of importance for delivering the bicarbonate needed in ureagenesis and gluconeogenesis. It is present in mitochondria of liver and kidney proximal tubules. CA V was found to be two different isoforms, CA VA and CA VB, with the latter specifically located in the human heart and skeletal muscle (Fujikawa-Adachi *et al.,* 1999).

Secreted isozyme: CA VI

CA VI is the only isozyme known to be secreted and was initially found in salivary glands and saliva. Later it was found in milk. Besides having a protective role in preventing acid damage to dental surfaces it acts as a trophic factor on taste buds and in the intestinal tract (Karhumaa, 2002).

Carbonic anhydrase-related proteins, CA-RPs: CA VIII, CA X and CA XI

CA VIII, CA X and CA XI have been described from cDNA libraries and so named because the proteins have CA-like domains. However, they lack CA activity and have been found mainly in brain tissue.

Nuclear carbonic anhydrase

The presence of CA activity in the cell nucleus has long been a controversial question. Several histochemical studies have shown CA activity in the cell nucleus of different cell types in many organs (Hansson, 1967; Ridderstråle, 1991, Ridderstråle & Sperber, 1991). Recently a new CA has been purified which, after amino acid sequencing, was found to be similar to an earlier described nuclear protein, nonO/p54^{nrb} (Karhumaa, 2002). NonO/p54^{nrb} was shown to have CA activity and immunological cross-reactivity between CA and nonO/p54^{nrb} was also found.

Methodology

There are many techniques available for studying the different stages between the expression of a gene and the final working enzyme, let us say a CA isozyme, in its position in the cell. All the different techniques have specific advantages and disadvantages, which have to be taken into account when interpreting the results.

The concentration of mRNA produced during gene transcription can be measured in tissue homogenates by solution hybridization using specific probes and localized in tissue sections by in situ hybridization. However, the mRNA undergoes splicing, which can lead to several different proteins being translated.

Proteins in solution can be separated and identified with specific antibodies with Western blotting technique and concentration determined by immunoassays. With the help of antibodies and immunohistochemistry, the isozyme can be localized in tissue sections, but none of these techniques can identify catalytic activity in the isozyme.

There are several different techniques for biochemical measurements of CA activity in tissue homongenates. By determining the rate of substrate breakdown and/or product formation an estimation of the enzyme concentration can be made. However, it is not always possible to separate connective tissue and muscle tissue from epithelia and glandular cells when doing a homogenate from an organ, thus making it difficult to interpret the physiological reason for the presence of the enzyme.

This can be done with enzyme histochemistry, where tissue sections are incubated on a solution to activate the enzyme and then transform the reaction products into a visible precipitate, which can be studied in the microscope. However, none of the activity-based techniques can separate low- and high-activity isozymes.

The histochemical method, described by Ridderstråle (1976, 1991), was used in the present study. The incubation medium is a modification of the Hanson cobalt-phosphate precipitation method (1967). However, the main difference from earlier methods is the embedding of the tissue in a water-soluble resin before incubation instead of using cryo sections. This improves the morphology significantly and facilitates the localization of CA by keeping the formed precipitate closer to the enzyme molecules. The new method gave a more detailed localization of CA and for the first time membrane-bound CA activity was visible and CA activity in capillaries was demonstrated (Ridderstråle, 1976). However, when several isozymes are present this technique cannot separate them and low concentrations cannot be detected. A combination of the histochemical and immunohistochemical methods can solve most of such problems.

A group of specific CA inhibitors have been developed, the sulfonamides, which mainly differ with regard to membrane permeability and therefore with different accessibility to the different isozymes depending on the subcellular location. These have been extensively used giving invaluable information about the physiological function of the CAs.

CA in reproductive organs

Males

CA in the male reproductive tract was first demonstrated in a homogenate of the rat dorso-lateral prostate by Mawson and Fisher (1952). This was confirmed by several other research groups (Fisher *et al.,* 1955; Waldeyer & Häusler, 1959; Miyake &Pincus, 1959; Leiter, 1964; McIntosh, 1969) and CA activity in rat prostate was shown to be regulated by androgens (Miyake & Pincus, 1959; Pincus & Bialey, 1963). Further studies have revealed that the CA of rat prostate is CA II, which is regulated by androgens but is also sensitive to estrogens (Härkönen & Väänänen, 1988, Härkönen *et al*., 1991). Ductus deferens, seminal vesicles, ampulla, prostate and bulbourethal glands have been investigated immunohistochemically in humans, stallions and bulls in varying combinations of CA I, II, III IV and VI. The results show considerable differences between species (Kaunistao *et al.,* 1990; Parkkila *et al.,* 1993a; Asari *et al.,* 1996; Ishihara *et al.,* 1997; Wilhelm *et al.,* 1998).

In the testis, histochemical localisation of CA activity revealed enzyme activity only in the interstitial tissues of the rat (Cohen *et al.,* 1976) and in no structure of the bull (Goyal *et al.,* 1980). CA II has been reported in spermatids of mouse and human (Lentonen *et al.,* 2004) and from ejaculated sperm cells of rat and human (Parkkila *et al.,* 1991). Faint labelling of the membrane-bound CA IX and XII has been detected in spermatids of the mouse (Hilvo *et al.,* 2004; Halmi *et al.,* 2004). The cytoplasmic CA XIII has been found in the developing germ cells of humans (Lentonen *et al.,* 2004). CA III has been found in stallion Sertoli cells (Asari *et al.,* 1996), the only report together with the present study on pig (paper I) on CA in Sertoli cells.

In the efferent ductules CA activity has been localised histochemically in the rat and bull (Waldeyer & Häusler, 1959; Cohen *et al.,* 1976; Goyal *et al.,* 1980). Using immunohistochemistry, cells of efferent ductules were found to be positive for XII and IX in humans (Karhumaa *et al.,* 2001; Ivanov *et al.,* 2001) and for CA II in the mouse (Zhou *et al.,* 2001).

In histochemical investigations on rat epididymis CA activity has been demonstrated in apical cells in caput, principal cells in corpus and clear cells in cauda (Cohen *et al.,* 1976; Membre *et al.,* 1985). Similar results were obtained in the bull except for clear cells, which are not present in the bull (Goyal *et al.,* 1980).

Immunohistochemical labelling has shown that the apical/narrow cells are positive for CA II in the rat, mouse, bull, stallion and human epididymis (Brown *et al.,* 1992; Kaunisto *et al.,* 1995; Asari *et al.,* 1996; Ichihara *et al.,* 1997; Hermo *et al.,* 2000; Karhumaa *et al.,* 2001).

The principal cells in corpus and cauda show immunohistochemical labelling for CA II in rats (Kaunisto *et al.,* 1995) and the stereocilia/microvilli are stained for CA IV in the same region of the rat and human (Kaunisto *et al.,* 1995; Parkkila *et al.,* 1993a). In the mouse epididymis the membrane-bound CA XII shows faint staining of basolateral membranes (Hilvo *et al.,* 2004).

Clear cells in corpus and cauda show positive labelling for CA II in investigations performed by Brown et al. (1992), while others have failed to show labelling for CA II in these cells (Kaunisto *et al.,* 1995; Hermo *et al.,* 2000).

Females

CA has been found in the female reproductive tract in a variety of mammalian species. Using a biochemical method CA was first found in the oviduct homogenate of sheep and cow, and in the endometrial mucosa homogenate of rabbit and sheep (Lutwak-Mann, 1955; Lutwak-Mann, 1957). This was later confirmed by other investigations in mouse, rat, rabbit, human and guinea-pig (Pincus & Bialey, 1963; Korhonen *et al.,* 1966; McIntosh, 1970; Hodgen & Falk, 1971; Falk & Hodgen, 1972; Hetherington, 1973).

The biochemical CA activity has been reported to respond to hormonal regulation and varies during the oestrus cycle and pregnancy with profound differences between species. Oestrogen increases uterine CA concentration in the mouse and decreases it in the rat (Pincus & Bialey, 1963). In the rabbit progesterone induces a marked increase of CA concentration in the uterus (Lutwak-Mann, 1955; Pincus & Bialey, 1963; Hodgen & Falk, 1971).

No changes in CA localisation or histochemical staining intensity during the oestrus cycle were detected in the species studied by Friedely & Rosen (1975). The histochemical technique used, on cryo sections, demonstrates the presence of CA in the ovarian surface epithelium of all examined species; the granulosa cells in the mouse and the oocyte of the rabbit. The uterus showed staining of the surface and glandular epithelium and the oviduct some staining in the basal part of the epithelium in all studied species.

Immunohistochemical localization in the mouse ovary shows the presence of CA I, CA II and CA III in the follicular wall, interstitial cells and in the corpus luteum (Ge & Spicer, 1988); and a few cells express CA XII (Hynninen *et al.,* 2004).

In the oviduct of the mouse the labelling for CA II varied being most intense in the isthmus. Both rat and mouse expressed CA III in the infundibulum (Ge & Spicer, 1988). In the uterus the surface and glandular epithelium of the mouse show CA II, which could not be detected in the rat but both species are positive for CA III in the uterus (Ge & Spicer, 1988).

Recent findings have shown that mouse and human endometrium contain CA XII in the basolateral membrane in both surface and glandular epithelium and CA XIII in the cytoplasm of the mouse endometrium and human cervix (Karhumaa *et al.,* 2000a; Ivanov *et al.,* 2001; Hynninen *et al.,* 2004; Lethonen *et al.,* 2004).

Aims

General aims

The starting point for my licentiate thesis was the histochemical method recently developed at the department, which made it possible to localize CA with improved resolution and morphology. Since pH and bicarbonate homeostasis are of such vital importance for reproduction, a comparative study on males was initiated (papers I, II) and later extended to include females (paper III). When I returned to science after a considerable time I found that the area had exploded with characterisation of a number of new isozymes. This gave valuable new

information and in addition our histochemical results on membrane-bound CA have now been confirmed with immunohistochemical techniques. However, the lack of antibodies against the most recently discovered isozymes for most species except mouse, rat and human excludes most species of interest for veterinarian immunohistochemical analysis. The histochemical method is thus to be preferred when an overview of the distribution of CA activity is needed to understand function. It may also lead to hitherto undiscovered isozymes.

It was of interest to undertake a comparative study because extensive species variation exists in reproduction and could be expected as regards the activity of CA. The pig was chosen since it is used as a model animal for both human and veterinary medicine and is bred for animal production. The mouse was used as a reference species and was of special interest since a knockout mouse for CA II had been produced. The rabbit is a common laboratory animal and is therefore of interest. Finally the cat was chosen because it is one of our most popular pet animals, which justifies performing more basic studies. Male reproductive organs from some additional species have also been studied when it has been possible to obtain access to them from other investigations or from the slaughterhouse.

Specific aims

- to compare the histochemical localisation of CA in the male reproductive tract of pig, rabbit and mouse
- to further specify the localisation of the two isozymes CA II and CA IV in the male reproductive tract of the mouse by using immunohistochemistry and knockout mouse deficient in CA II
- to study the histochemical localisation of CA in the female reproductive tract of pigs and to study if CA localisation is altered in pigs during the oestrus cycle or by insemination
- to initiate a comparative study of histochemical CA localisation in the female reproductive tract of mouse, rabbit and cat

Materials and methods

For information on the animals used for studies in the published papers (I-IV), see the section “Materials and methods” in the respective paper.

Preliminary study on female

Animals

Mice: Three female mice (C57BL/6) were obtained from Charles River, Uppsala, as control animals and three CA II-deficient female mice from an ongoing project were used for the study. The CA II-deficient animals are described in paper IV. The investigation was approved by the Local Ethics Committee in Uppsala.
Rabbits: Three adult female rabbits, two New Zealand white rabbits and one dwarf breed, were used in the preliminary study. The animals were euthanized for other reasons and the organs were taken with permission from the animal owner.

Cat: One female cat was used. The animal was euthanized for other reasons and the organs were taken with permission from the animal owner.
Tissue from ovary, oviduct and uterus was taken from all animals. Tissues from the rabbit and cat were immersion fixed by 2.5% solution of glutaraldehyde in 0.067 M phosphate buffer (pH 7.2). The mouse tissues were prepared according to paper IV.

Processing of tissue samples

Processing of tissue samples for histochemical demonstration of CA activity and immunohistochemistry is described in paper IV.

Results

Localization of carbonic anhydrase

The histochemical incubation results in a black precipitate at sites of active CA and all active isozymes are thus visualized. The controls run with the CA inhibitor acetazolamide in the incubation medium showed no significant staining. The results are summarized in Tables 1 and 2.

The immunohistochemical method results in a brown staining showing the presence of the cytoplasmic isozyme CA II and the membrane-bound CA IV. The immunohistochemical controls were consistently negative except for a general background staining in the connective tissue. Antibodies were available only for mouse tissues. No immunolabelling of CA II could be detected in any tissue of CA II-deficient mice.

Testis (for details see paper I, II and IV)

The spermatogonia and developing germ cells were unstained in all species studied. The most striking difference between the species was seen in the Sertoli cells. In the pig these cells showed both cytoplasmic and intense nuclear staining, but they were completely without staining in rabbit and mouse. In addition, some of the pig Leydig cells showed cytoplasmic staining of varying intensity, which once more could not be seen in rabbit or mouse.

The endothelium of capillaries and postcapillary venules from all three species showed staining for CA activity. The staining was often intense and clearly membrane associated and present in both the apical and basal cell membranes. In the mouse the capillary endothelium showed a positive immunolabelling for CA IV.

In the efferent ductules CA activity was localized to the basolateral cell membranes in the pig, while the non-ciliated cells showed staining in the cytoplasm and nucleus of the rabbit. Only weak cytoplasmic staining of the epithelium was found in the mouse, with some inconsistency between animals.

Epididymis (for details see paper I, II, IV)

Below follows a summary of the main results from the three different species studied. For comparative reasons the epididymis is divided into caput, corpus and cauda according to the common terminology based on anatomical features. A terminology more suited for the respective species is used in the separate papers. In paper I and II the Glover & Nicander (1971) terminology has been used which is

based on structural and functional characteristics and paper IV follows the terminology of Robaire & Hermo (1988). In brief this means that the initial and middle segments belong to caput and the terminal segment to corpus and cauda.

Caput: The principal cells of the pig were unstained. In rabbit, the principal cells showed stained stereocilia/microvilli and more distally in the duct the principal cells in addition contained vacuoles with stained cell membranes. The principal cells were unstained in the mouse proximally, but started to show staining in the distal part of caput.

The apical/narrow cells were scattered among the principal cells in the caput and showed both cytoplasmic and nuclear staining in all three species. In the control mouse this cytoplasmic staining was labelled for CA II by immunohistochemistry and was lacking in the CA II-deficient mouse. The basal cells showed membrane-bound staining in the pig and were unstained in the rabbit and mouse.

Table 1**.** *Histochemical localization of CA in the testis and epididymis (paper I, II and IV)*

	Pig	**Rabbit**	**Mouse**
Testis			
Germ cell	0	0	0
Sertoli cell	**cyt**	0	0
Leydig cell	cyt	0	0
Capillaries	**mb**	**mb**	**mb**
Efferent ductules	mb	cyt	cyt[1]
Caput epididymidis			
Principal cell	0	**mb**[a], cyt [2]	0
Apical cell	**cyt**	**cyt**	**cyt**[1]
Basal cell	mb	0	0
Spermatoza	0	0	0
Capillaries	0	0	0[3]
Corpus epididymidis			
Principal cell	**mb**[b]	cyt	**mb**[ab], cyt[1]
Basal cell	0	**cyt**	0
Spermatozoa	0	0	**mb**
Capillaries	0	0	0
Cauda epididymidis			
Principal cell	**mb**[b]	**mb**[b]	**mb**[ab]
Clear cell			mb[ab], cyt
Basal cell	0	**cyt**	0
Spermatozoa	0	0	**mb**
Capillaries	0	0	0

mb = strong membrane-bound staining, mb = weak membrane-bound staining
[a] = stereocilia/microvilli, [b] = basolateral cell membrane
cyt = strong cytoplasmic staining, cyt = weak cytoplasmic staining
0 = no staining,
[1] not present in the CA II-deficient mouse
[2] stained vacuoles in the principal cells
[3] stained capillaries in the initial segment

Corpus: The principal cells in the pig showed stained basolateral cell membranes and in the rabbit occasional principal cells contained weak cytoplasmic activity. In the mouse, the principal cells showed intensely stained stereocilia/microvilli and somewhat weaker staining in the basolateral cell membranes. Using antibodies against CA IV it was found that only the CA activity seen in the microvilli was due to CA IV. Numerous vesicles were seen in the apical part of the principal cells and these structures were also visualised with antibodies against CA IV. No apical cells were found in this part.

The basal cells were unstained in the pig and mouse, but showed intense cytoplasmic and nuclear staining in the rabbit.

Cauda: In the principal cells basolateral membrane-bound staining was present in all species and in the mouse membrane-bound staining was found also in the stereocilia/microvilli. The earlier described stained vesicles were present even here but decreased in number towards the distal cauda epididymidis.

The basal cells were unstained in the pig and mouse, but continued to show intense cytoplasmic and nuclear staining in rabbit.

Clear cells are present only in the mouse and showed membrane-bound staining as well as cytoplasmic and nuclear histochemical staining. No labelling for CA II was detected.

General structures: The capillary endothelium was generally unstained in the epididymis except for a short part in the initial segment of mouse caput. Smooth muscle cells surrounding the duct in the mouse showed a weak membrane-bound staining which could not be detected in the pig and rabbit.

Spermatozoa (paper I, II and IV)

The maturing spermatozoa in the epididymal duct in pig and rabbit showed no detectable CA activity, which was also the case for mouse spermatozoa in caput. However, in the corpus the mouse spermatozoa started to show strong membrane-bound staining, which remained throughout the duct. This staining pattern was seen in both control and CA II-deficient mice. The spermatozoa stained positive for CA IV in tissue sections from these regions. In preparations for scanning electron microscopy (SEM) of whole spermatozoa collected from corpus and cauda, strong immunolabelling for CA IV was present all over the spermatozoa, but most densely packed over the middle piece of the tail. No labelling for CA IV was observed in the SEM preparations of whole spermatozoa collected from testis and caput epididymidis. In addition, no cytoplasmic staining or CA II-reactivity was observed in the spermatozoa of either CA II-deficient or control mice at any location.

Table 2. *Histochemical localization of CA in the ovary, oviduct and uterus (paper III and preliminary results on mouse, rabbit and cat)*

	Pig	**Rabbit**		**Mouse** [3]	**Cat**
		N	L		
Ovary					
Oocyte	0	**mb**	**mb**	0	0
Granulosa cell	0	0	0	**mb**[ab]	0
Theca interna	0	0	0	0	0
Capillaries	**mb**	**mb**	**mb**	0	**mb**
Corpus luteum					
Lutein cell	0	0	0	0	0
Capillaries	**mb**	0	0	0	**mb**
Oviduct					
Ampulla					
Surface epithelium	cyt	0	-	cyt	0
Capillaries	**mb**	0	-	0	mb
Ishmus					
Surface epithelium	mb	0	-	mb[b], cyt	0
Capillaries	**mb**	0	-	0	mb
UTJ					
Surface epithelium	0	-	-	-	-
Furrows	**mb**[b]	-	-	-	-
Capillaries	**mb**	-	-	-	-
Uterus					
Surface epithelium	0	0	**cyt**	mb[b]**, cyt**	mb[a]
Glandular epithelium	**cyt**	0	0	mb[b]**, cyt**	**mb**[ab]
Capillaries	**mb**	**mb**	0	**mb**	**mb**

mb = strong membrane-bound staining, mb = weak membrane-bound staining
[a] = apical cell membrane, [b] = basolateral cell membrane
cyt = strong cytoplasmic staining, cyt = weak cytoplasmic staining
0 = no staining
– = not investigated
N = non luteal phase
L = luteal phase
[3] CA II-deficient mouse showed similar staining as the normal mouse except that cytoplasmic staining was absent
Smooth muscle cells in the myosalpinx and myometrium showed membrane-bound staining in the pig, no staining in the other animals

Ovary, oviduct and uterus (paper III and preliminary results)

In the pig neither the intensity nor the localisation patterns of CA activity seemed to change with the phase of the oestrus cycle in any of the areas investigated (see paper III).

Ovary: The ovary surface epithelium showed membrane-bound staining only in the rabbit. The oocyte was unstained in pig, mouse (Fig. 1a,) and cat (Fig. 1e) but in the rabbit the cell membrane of the oocyte was strongly stained (Fig. 1c).

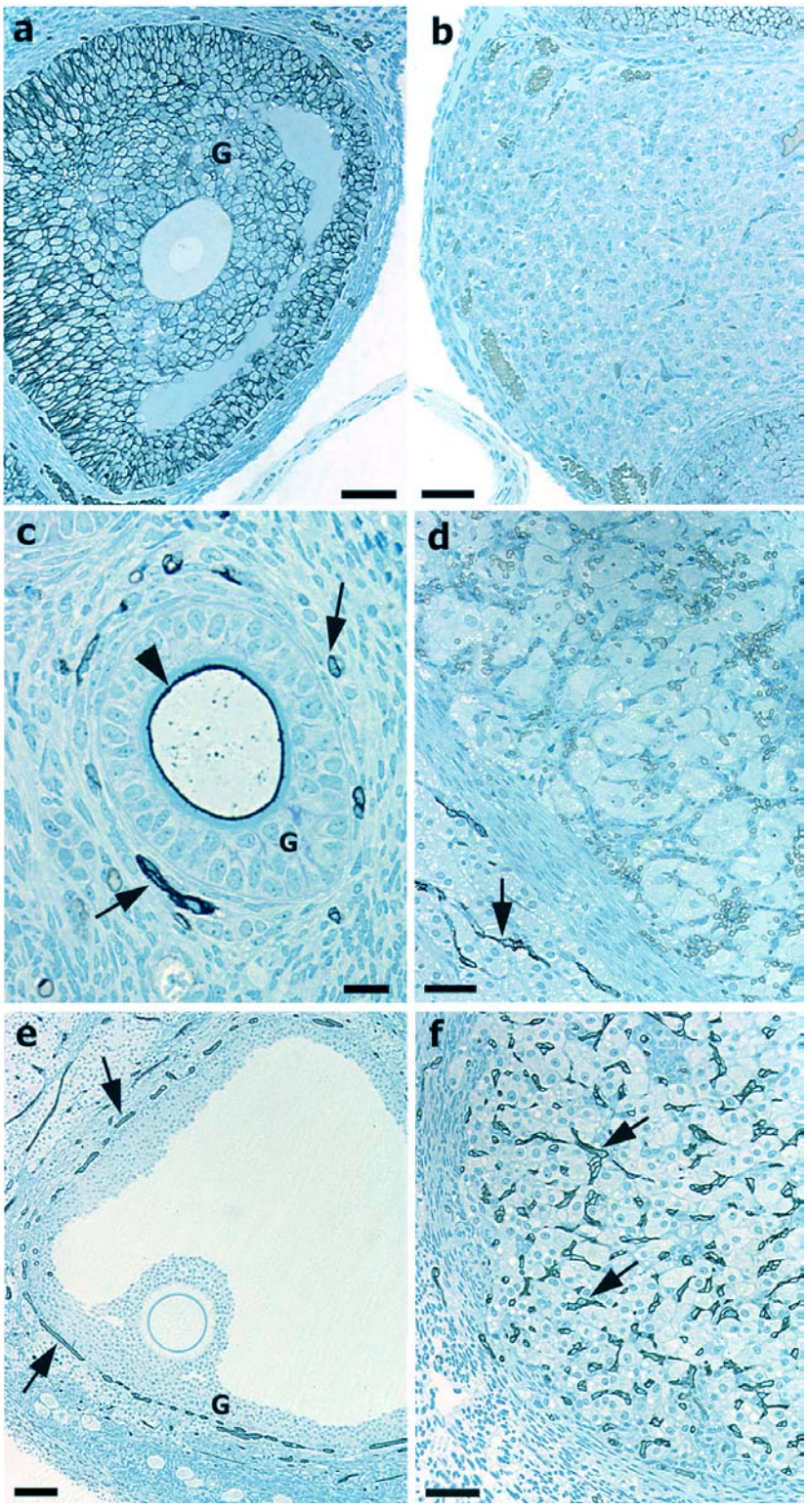
The granulosa cells were unstained in the pig, rabbit (Fig. 1c) and cat (Fig. 1e). In mouse, the granulosa cells of secondary and growing follicles showed strong membrane-bound staining for CA activity (Fig. 1a) and a similar staining was present in the CA II-deficient mouse. No immunohistochemical labelling for CA II and CA IV was detected in the ovary of the control and the CA II-deficient mouse. The theca interna cells were unstained in all species (Fig. 1a, 1c, 1e).

The endothelium of capillaries located around the follicles and in the surrounding tissue was intensely stained for CA activity in the pig, rabbit (Fig. 1c) and cat (Fig. 1e), while the capillaries were unstained in the mouse. In the corpus luteum of the cat this endothelial activity was very prominent (Fig. 1f), whereas it was undetectable in the pig, rabbit (Fig. 1d) and mouse (Fig. 1b). The luteal cells of corpus luteum in all species were unstained (Fig. 1b, 1d, 1f).

Oviduct: In the pigs the epithelial lining of the oviduct was stained, but with variations in activity between the ampulla and isthmus. In the ampulla secretory cells showed cytoplasmic staining, while the ciliated cells were unstained. In the isthmus CA activity was confined to the lateral cell membranes of secretory cells, whereas CA was absent in ciliated cells.

At the uterotubal junction in pigs, the cells lining the deep furrows showed marked membrane-bound basolateral activity, but the surface epithelium was negative. In pigs slaughtered after artificial insemination clusters of spermatozoa were seen with their heads pointing towards the bottom of these furrows. The presence of spermatozoa did not alter CA activity.

Figure 1. Localisation of carbonic anhydrase activity (black precipitate) in the ovary of mouse (a, b), rabbit (c, d) and cat (e, f) after 6 min incubation. Azure blue counterstain.
a) Follicle of the mouse ovary with membrane-bound staining of granulosa cells (G). Capillary endothelial cells are unstained but erythrocytes show cytoplasmic activity. Bar = 50 µm
b) Corpus luteum of mouse ovary showing weak cytoplasmic activity of erythrocytes only. Bar = 50 µm
c) Rabbit ovary showing intense staining of the oocyte cell membrane (arrowhead) and staining of endothelial cell membranes in capillaries surrounding the follicle (arrows). G = granulosa cells. Bar = 20 µm
d) Corpus luteum of rabbit ovary showing weak cytoplasmic activity of erythrocytes only. Capillaries in the surrounding tissues show membrane-bound staining of endothelial cells (arrow). Bar = 50 µm
e) Follicle of the cat ovary with membrane-bound staining of endothelial cells in capillaries around the follicle and in surrounding tissues (arrows). G = granulosa cells. Bar = 50 µm
f) Corpus luteum of cat ovary with intensely stained membranes of capillary endothelial cells (arrows) and similarly stained capillaries in the surrounding tissue. Bar = 50 µm


a
G
b
c
G
d
e
G
f

In the mouse the epithelial cells in the ampulla showed cytoplasmic staining and in isthmus a basolateral membrane-bound staining was present in addition to weak cytoplasmic activity. The oviduct was not studied in the CA II-deficient mouse. The oviducal epithelium was unstained in the rabbit (non-luteal phase) and cat.

Uterus: The surface epithelium was unstained in the uterus of the pig during all stages of the oestrous cycle. In the rabbit the epithelium was clearly different depending on stage. Thus, in non-luteal phase (two animals) the epithelium was unstained (Fig. 2c) and during luteal phase (one animal) intense cytoplasmic and nuclear staining was present (Fig. 2d). In the mouse cytoplasmic and weak membrane-bound CA activity was present in the surface epithelium (Fig. 2a). The cytoplasmic staining was shown by immunohistochemistry to be CA II, but no CA IV was detected. The CA II-deficient mouse showed membrane-staining of varying intensity (Fig. 2b). The surface epithelium in cat was unstained (Fig. 2e) with only faint staining in the apical cell membrane.

The glandular cells of the endometrium showed cytoplasmic CA activity in the pig and both cytoplasmic and membrane-bound staining in the mouse (Fig. 2a, 2b). The cytoplasm stained positively for CA II, but no CA IV was detected. The glands in the rabbit were unstained in both non-luteal and luteal phase (Fig. 2c, 2d). Some luminal content showed CA staining in the rabbit in luteal phase (Fig. 2d). In the cat strong apical and moderate basolateral staining was present in the glands and the luminal contents showed CA activity (Fig. 2e).

Generally the subepithelial capillaries of the uterus in all species showed membrane-bound staining of endothelial cells (Fig. 2a-c, 2e) except in the rabbit in luteal phase. Vascular smooth muscle cells were generally negative in all species except the smooth muscle cells of the myosalpinx and myometrium of the pigs, which showed membrane-bound activity. Moderate cytoplasmic staining was seen in erythrocytes in all animals.

Figure 2. Localisation of carbonic anhydrase activity (black precipitate) in the uterus of control mouse (a), CA II-deficient mouse (b), rabbit (c, d), cat (e) and testis of hamster (f) after 6 min incubation. Azure blue counterstain.
a) Uterus of control mouse with staining in the surface and glandular (arrowheads) epithelium. The capillaries (arrow) are stained in the endothelium. L = lumen. Bar = 30 µm
b) CA II-deficient mouse showing strong basolateral membrane-bound staining in the surface and glandular epithelium (arrowhead). Capillaries with weakly stained erytrocytes in the lumen, show membrane-bound staining in the endothelial cells (arrow). L = lumen. Bar = 30 µm
c) Uterus of rabbit in non-luteal phase, showing unstained surface and glandular epithelium (arrowhead) and stained endothelial cells of the capillaries (arrow). L = lumen. Bar = 30 µm
d) Rabbit uterus in luteal phase, with stained surface epithelium, strong cytoplasmic and nuclear staining. The deep folding of the surface epithelium with stained secreted content. The glandular epithelium (arrowhead) is unstained and capillaries are unstained. L = lumen. Bar = 30 µm
e) Uterus of cat with unstained surface epithelium and weak membrane staining in the glandular epithelium (arrowheads). Luminal content is stained for CA activity. Capillary endothelial cells are stained (arrow). L = lumen. Bar = 20 µm
f) Testis of hamster. The acrosome of the round (arrows) and elongated spermatids are showing strong staining for CA activity. The rest of the seminiferous tubules are unstained. L = lumen. Bar = 20 µm

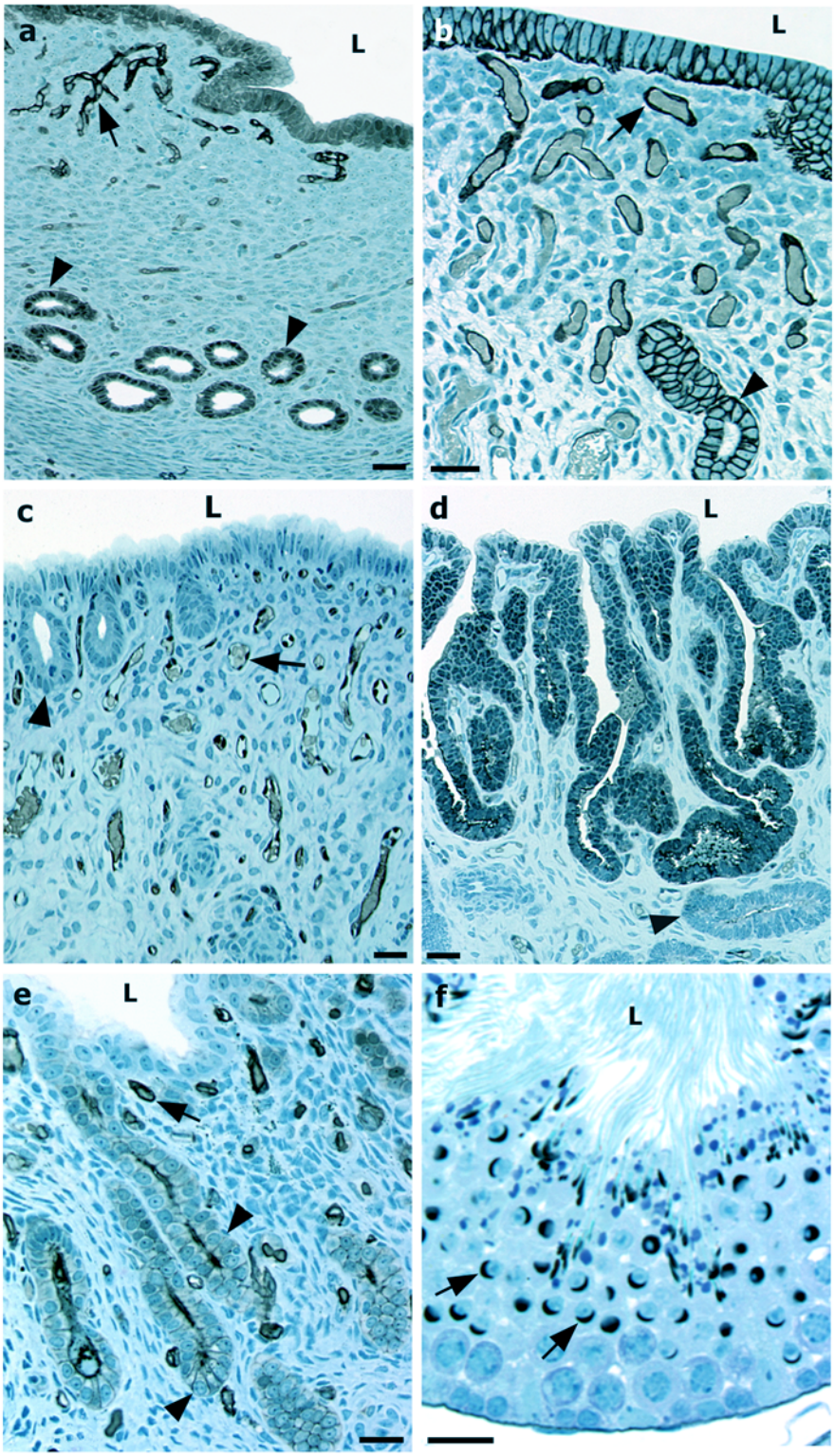

a
L
b
L
c
L
d
L
e
L
f
L

Table 3. Histochemical localization of CA in the testis and epididymis
(results from Ekstedt, 1989)

		Cat	Dog	Hamster	Lemming	Goat	Bull	Rat
Testis	Germ cell	0	0	**acr**	**acr**	0	0	0
	Sertoli cell	0	0	0	0	0	0	0
	Leydig cell	0	0	0	0	0	0	**cyt**
	Efferent ductules	cyt	cyt	-	-	cyt, mb	-	-
	Capillaries	**mb**	**mb**	**mb**	**mb**	**mb**	**mb**	**mb**
Caput epididymidis								
	Principal cell	0	0	mb[b]	0	0	0	0
	Apical cell	**cyt**	**cyt**	**cyt**	**cyt**	**cyt**	**cyt**	**cyt**
	Basal cell	0	0	0	0	0	0	0
	Spermatoza	0	0	**acr**	**acr**	0	0	0
	Capillaries	0	0	0	0	0	0	mb
Corpus epididymidis								
	Principal cell	**mb[b]**	0	0	cyt	0	0	mb[ab], cyt
	Apical cells	0	cyt	0	0	cyt	cyt	0
	Basal cell	0	0	0	0	0	0	0
	Spermatozoa	0	0	**acr**	**acr**	0	0	0
	Capillaries	0	0	0	0	0	0	mb
Cauda epididymidis								
	Principal cell	**mb[b]**	mb[b]	mb[b]	-	mb[b]	-	mb[b]
	Clear cell	-	-	-	-	-	-	mb[b]
	Basal cell	0	0	0	-	0	-	0
	Spermatozoa	0	0	**acr**	-	0	-	0
	Capillaries	0	0	0	-	0	-	mb

Acr = acrosome
mb = strong membrane-bound staining, mb = weak membrane-bound staining
[a] = stereocilia/microvilli, [b] = basolateral cell membrane
cyt = strong cytoplasmic staining, cyt = weak cytoplasmic staining
0 = no staining,
– = not investigated

Discussion

Can we draw any general conclusions regarding the localization of CA in male and female reproduction organs from the species studied here together with some of our earlier results (Ekstedt, 1989 and Table 3) and other reports available in the literature? It will become evident that it is difficult to find a common denominator for the localization of CA in different species. However, this may not be surprising in view of the fact that there are subtle but important species differences in the reproductive organs which prevents mismatches. In the following sections we discuss our findings in relation to the present knowledge of the importance for pH and bicarbonate homeostasis in the different parts of the reproductive organs.

CA during germ cell development

Testis

There was generally no staining for CA activity in spermatogonia and early developing germ cells in testis of the species in the present study, which is in agreement with other studies on rat, bull, stallion and human (Cohen *et al.*, 1976; Goyal *et al.,* 1980; Kaunisto *et al.,* 1990; Ichihara *et al.*, 1997; Asari *et al.*, 1996; Parkkila *et al.*, 1993a).

However during late development we found cytoplasmic CA activity in the acrosomes of hamster (Fig. 2f) and lemming (see discussion below). Parkkila *et al.* (1991) have found CA II in the rat acrosome and Lehtonen *et al*. (2004) in human and mouse spermatids. The latter also found the cytoplasmic CA XIII in human spermatids. Furthermore, a faint immunolabelling for CA IX and CA XII is present in mouse spermatids as shown by Hilvo *et al.* (2004) and Halmi *et al.* (2004).

However, we failed to detect histochemical CA activity or CA II in the mouse spermatids in the testis. At low concentrations of protein, immunohistochemical techniques can detect lower levels of protein than activity based methods such as histochemistry. This might explain the discrepancy between the results. The lack of CA II protein in our study could also be the result of different fixatives used, which might affect the antigenicity of the protein.

The interstitial Leydig cells in the testis showed cytoplasmic and nuclear CA staining in the rat and some also in the pig. In the rat our results confirm the findings by Cohen *et al.* (1976), but there is no obvious task for CA during steroid synthesis.

The Sertoli cells were negative for CA activity except in the pig, which had strong cytoplasmic and nuclear staining. A weak labelling for CA III is present also in the horse (Asari *et al.*, 1996), but to our knowledge there are no other reports on CA in Sertoli cells. Sertoli cells fulfil a number of important functions for the regulation of spermatogenesis and secrete a bicarbonate containing fluid to the lumen. The reason why the pig had such strong CA activity in the Sertoli cells, besides the production of bicarbonate, is difficult to understand. However, one prominent feature of the pig testis is the unusually high number of Leydig cells in the interstitium surrounding the seminiferous tubules, thus separating the luminal

parts of the seminiferous epithelium from the capillary bed. The distance for CO_2 diffusion from the epithelium to the capillaries is therefore long in the pig. However, the Leydig cells do have some CA and this, together with the rich amount of CA activity in the Sertoli cells, may facilitate CO_2 diffusion (Enns, 1967; Swensson & Maren, 1984).

The cytoplasmic CA staining visualizes the Sertoli cells very effectively. Thus CA can be used as a morphological marker in studies on pigs where effects of *e.g.* toxins and drugs on the Sertoli cell population will be studied. This is possible also during foetal development since CA has been shown to be present in Sertoli cells during early development (Rodriguez-Martinez *et al.*, 1990b).

Ovary

The oocytes in the ovarian follicles in our material were unstained, except in the rabbit where CA activity was present in the cell membrane of developing oocytes. This confirms the findings of Friedly & Rosen (1975).

Both the granulosa and theca interna cells of the follicular epithelium were negative in all species except the mouse, which had strong membrane-bound CA activity in the granulosa cells. This location was not positive for CA IV and according to Hynninen *et al.* (2004) neither CA IX nor CA XII are present at these sites. Thus, the only known candidate for the membrane-bound staining that we found is the newly characterized CA XIV, but this needs to be confirmed by immunohistochemistry.

Ge & Spicer (1988) used antibodies and found the cytoplasmic CA I in mouse granulosa cells and Friedley & Rosen (1975) found histochemical CA activity in the cytoplasm when incubating cryo sections. With this method the reaction product formed at the membranes diffuses easily into the cytoplasm, which makes it difficult to separate nuclear from cytoplasmic CA. This diffusion is much less when using resin sections, as in our study, which can explain why we found only membrane-bound CA staining at this location.

The theca interna cells were unstained for CA activity in the present study and no CA II or CA IV was seen by immunohistochemistry in the mouse, but CA I, II and III have been demonstrated in the mouse theca cells by Ge & Spicer (1988). Different fixatives and antibodies used could be an explanation for the diverging results, but hormonal influences on enzyme levels during different stages of the oestrus cycle cannot be excluded.

The luteal cells in corpus luteum were always negative in our studies, labelled for CA I, II and III in the mouse according to Ge & Spicer (1988) and a few cells weakly for CA XII (Hynninen *et al.*, 2004). Thus, a similar discrepancy between studies is present as for the theca interna cells described above.

CA and the micromilieu during transport and storage

Efferent ductules and epididymis

From the rete testis the spermatozoa are transported to the efferent ductules, which empty into the epididymal duct. Cytoplasmic staining for CA activity was present in mouse and rabbit efferent ductules and membrane-bound CA in the pig. Cytoplasmic staining has also been found in the rat (Cohen *et al.*, 1976), bull (Goyal *et al.*, 1980) and CA IX and CA XII in basolateral membranes in human (Karhumaa *et al.*, 2001). Fluid and bicarbonate reabsorption takes place in this region (New-

come *et al.*, 2000) resulting in an increasing sperm concentration. This process is under oestrogen control in the mouse according to Zhou *et al.* (2001). They found a decrease in the expression of the Na^+/H^+ exchanger in the efferent ductules of oestrogen-receptor-alpha-knockout-mice and as a secondary effect to this, a decrease in immunohistochemical staining for CA II occurred. The oestrogen comes with the fluid from the Sertoli cells, where testosterone is converted to oestrogen by aromatase.

The epididymal duct presents considerable morphological variations between species. This has turned out to be true also for the distribution of CA. However, CA activity in the apical cells are found in all species studied so far (Cohen *et al*., 1976; Goyal *et al.*, 1980; Membre *et al.*, 1985). They are interspersed in the epithelium of caput and proximal corpus. This activity was shown to be CA II when studied with immunohistochemical techniques (Brown *et al.,* 1992; Kaunisto *et al*., 1995, Ichihara *et al*., 1997, Breton *et al*., 1999; Karhumaa *et al.,* 2001).

Another location with CA activity in most species was the cauda epididymidis, the sperm reservoir, where the basolateral cell membranes of principal cells were stained. This location has so far not been recognized by antibodies.

CA IV was present in stereocilia/microvilli in corpus and cauda of the mouse epididymis, but not in the basolateral membranes. This is also described for the rat and for the entire duct in human (Parkkila *et al.*, 1993a; Kaunisto *et al.*, 1995). We also found that the basal cells, so called because they are located basally in the epithelium with no part reaching the lumen, have CA activity in the pig and rabbit. The location within the duct differed between the two species and basal cells in other species lack CA activity.

What could the physiological implications be of the distribution of CA? Regulation of sperm motility is dependent on pH with acidification inhibiting motility (Acott & Carr, 1984, Carr *et al*., 1985, Holm & Wishart, 1998) and increasing pH stimulating sperm motility (Babcock *et al*., 1983, Okamura *et al*., 1985, Tajima *et al,*. 1987, Gatti *et al*., 1993). Acidification processes are known to take place in the epididymal duct (Levine & Marsh 1971 Levine & Kelly 1978, Rodriguez-Martinez *et al.,* 1990a) with the lowest pH in the caudal part where the spermatozoa are kept immotile during storage. CA is thought to be involved since both intraluminal and intravenous infusions of the CA inhibitor acetazolamide depress the luminal acidification in rat epididymis when measured as bicarbonate resorption (Au & Wong, 1980). However, Caflisch & DuBose (1990) found no effect of acetazolamide on pH after intravenous infusion. This could, according to the authors, be explained by the different experimental setup, by the different sites in the duct used for the measurements and by the presence of the membrane-bound CA IV. This isozyme has its active site facing the luminal fluid. It makes it less available for intravenously infused acetazolamide contra intraluminally, since acetazolamide is not fully membrane permeable.

The most proximally located acidification in the epididymis is supposed to be provided by the proton secreting apical cells. The proton secretion depends on a vacuolar H^+ATPase co-localised with CA II (Brown *et al.*, 1992; Breton *et al.*, 1999; Hermo *et al*., 2000). The present study and several earlier works on different species show the presence of cytoplasmic CA in these cells (see above) with a function to supply the hydrogen ions needed for the acidification. The clear cells, located more distally in the duct, are supplied with a similar proton pump as

the apical cells and do often have CA activity (Brown *et al.*, 1992; Breton *et al*., 1999; Pushkin *et al*., 2000).

The bicarbonate concentration and pH decrease significantly in the duct towards cauda as shown in the pig by Rodriguez-Martinez *et al.* (1990a). As mentioned above, CA was present in the basolateral cell membranes at this location in all studied species and may thus have a function in keeping the spermatozoa immotile during storage. In order to regulate the pH both protons and bicarbonate have to move across membranes, but they are not freely permeable and transporters are therefore needed. The newly-described functional complex, the so called metabolon is of great interest with regard to the functions of CA (Vince *et al.*, 2000). It is a complex of transport proteins and enzymes that exert their effects in a sequential order. A physical co-localization between CA and the metabolons enhances the transporting capacity considerably. Thus, such a metabolon has been shown to exist between CA II and the so called band 3 anion exchanger (Vince & Reithmeier, 1998) and between the same anion exchanger and CA IV (Sterling *et al.*, 2002). The ubiquitously expressed Na^+/H^+ exchanger is coupled to CA II (Li *et al.*, 2002) and the capacity of the exchanger is reduced by the CA inhibitor acetazolamide (Wu *et al.*, 1998).

In the epididymis a Na^+/H^+exchanger has been localized in the apical cell membrane of principal cells (Chew *et al.*, 2000; Pushkin *et al.*, 2000; Bagnis *et al.* 2001; Kaunisto *et al.*, 2001) and an anion exchanger, Cl^-/HCO_3^-, is present in the basolateral membranes (Jensen *et al.,* 1999b; Pushkin *et al.*, 2000) as well as Na^+/HCO_3^- co-transporters (Jensen *et al*. 1999a). These different transport proteins exist in several isoforms some of which are co-localized with some of the CA isozymes, although not yet shown to be metabolons. Thus, it is obvious that there exists an effective organization of transporters and CA in the epididymis which must be important for the acid-base regulating processes taking place here and which are needed for normal development of the spermatozoa.

Transfer of CA IV to mouse spermatozoa: A remarkable change in CA activity of mouse spermatozoa was found at the transition between caput and corpus where the spermatozoa gained CA activity. Preparations of spermatozoa collected from testis and caput studied with SEM after labelling for CA IV had just a few gold particles attached to them. In contrast the spermatozoa from corpus and cauda were intensely labelled demonstrating the presence of CA IV on their extracellular surface.

Since no protein synthesis is known to take place in the spermatozoa after leaving testis (Cooper 1986, Robaire & Hermo 1988) it appears likely that the enzyme is synthesised by the epididymal epithelium and transferred to the passing spermatozoa. To our knowledge such a transfer of CA has not been described earlier. CA IV is linked to the membrane by a GPI-anchor (Waheed *et al.* 1992) and other investigations have reported transfer of similarly linked proteins from the epididymal epithelium to the spermatozoa, e.g. the sperm adhesion molecule 1 found in the mouse (Zhang & Martin-DeLeon, 2003). This is secreted in small vesicles, epididymosomes, in a form which can bind to the spermatozoa (Yeung *et al.,* 1997; Zhang & Martin-DeLeon, 2003). It is interesting to note that CA IV appeared in the apical cell membrane and vesicles of the principal cells shortly before it occurred in spermatozoa. It is tempting to assume that these structures may be the source for the CA IV in spermatozoa. This is, so far, exclusive for the

mouse, since rat (Kaunisto *et al.* 1995) and human (Parkkila *et al.* 1993a) have no CA IV in the spermatozoa although CA IV is present in their microvilli. Protein transfer has also been described to occur in the human prostate where so called prostasomes adhere to the spermatozoa (Ronquist & Nilsson, 2004).

Interestingly the sperm motility pattern changes into a progressive movement according to Soler *et al*. (1994) at the site where we found CA IV to be transferred to the spermatozoa in the mouse. The intracellular pH of the spermatozoa is of importance for sperm motility (Babcock *et al*., 1983, Acott & Carr 1984, Gatti *et al*., 1993) and the capacitation processes (Lee & Storey, 1986; Gadella & van Gestel, 2004). Several studies show the participation of HCO_3^- transporters in mouse spermatozoa (Zeng *et al*. 1996; Demarco *et al.,* 2003) and also in rat and human spermatozoa (Parkkila *et al*. 1993b). These findings point to a function for CA IV in mouse spermatozoa in several events where HCO_3^- is needed.

CA in acrosomes: Cytoplasmic CA was also present in the acrosome of hamster (Fig. 2f) and lemming in the present study. CA II has earlier been shown in the rat acrosome by Parkkila *et al*. (1991). The acrosome contains hydrolytic enzymes which are needed for the penetration of the egg at fertilisation and the acrosome reaction has been shown to be HCO_3^- dependant in both mouse (Lee & Storey, 1986) and hamster (Visconti *et al.*, 1999). Moreover the acrosomal content has an acidic pH in hamster (Meizel & Dreamer, 1978). This may explain the presence of CA, but gives no explanation to why most species lack CA here.

Oviduct and uterus

In the pig and mouse the oviduct epithelium showed CA activity. The uterine surface epithelium contained CA activity in the mouse and cat while the epithelium pig did not. In the rabbit the epithelium showed staining only during the non-luteal phase. The glandular cells expressed CA activity in the pig, cat and mouse with prominent staining of apical membranes and also in the glandular lumen of cats. The stained luminal content indicates a secreted form of CA, as demonstrated in salivary and mammary glands (Karhumaa, 2002) and in mucus from the gastrointestinal tract (Kleinke *et al.*, 2005). This has not been described for the uterus earlier.

Our preliminary results on mouse agree with previous findings of CA II present in both surface and glandular epithelium (Ge & Spicer, 1988). The isozymes CA XIII and CA XII have also been found in the uterus (Karhumaa *et al.*, 2000a; Hynninen *et al.*, 2004; Lehtonen *et al.*, 2004). However, it is not possible at the moment to draw any conclusion from the comparative results since the staining for CA activity varied both with regard to localization and intensity during the oestrus cycle (see below) and between species and further research is needed.

The uterine and oviducal luminal fluid make up the micro environment surrounding the spermatozoa during important processes such as capacitation and fertilization. Changes in the ionic composition of the uterine fluid and a high HCO_3^- content in the lumen have been observed (Vishwakarma, 1962; Maas *et al.*, 1987; Nichol *et al.*, 1997). The mechanisms involved in the transport of ions across the epithelium include a Na^+/H^+-exchanger (Wang *et al.*, 2003a) and a basolaterally located Na^+/HCO_3^- -co-transporter in the endometrium of the mouse (Wang *et al.*, 2002). It is tempting to assume the presence of a similar physical coupling of transporters and CA as the above described metabolons in the

epididymis. This could be of importance for the precise regulation of the luminal fluid during the transport of spermatozoa through uterus, for both spermatozoa and ovum passing the oviduct and later for an optimal environment for embryonic implantation in the uterus.

Sperm storage in the female

The furrows of the utero-tubal junction in pigs function as a sperm reservoir (Hunter, 1981) where spermatozoa may be arrested for as long as 48 hours (Austin, 1975). The furrows show staining for CA activity in the basolateral cell membranes, while the surface epithelium is unstained. CA may have a similar function for keeping spermatozoa immotile here as was suggested for cauda epididymis. A sperm reservoir has been found in several other mammalian species such as hamster (Yanagimachi & Chang, 1963), rabbit (Harper, 1973; Overstreet *et al.*, 1978) and mouse (Suarez, 1987). Sperm storage is also well known in female birds where the spermatozoa may be stored in special glands for several weeks. CA is present in these glands (Holm *et al.*, 1996; Holm & Ridderstråle, 1998) and the motility of spermatozoa can be modulated by changes in extracellular pH *in vitro* (Holm & Wishart, 1998).

CA and hormonal regulation

It appears that CA activity can be regulated by both oestrogen and progesterone but the field is yet largely unexplored probably due to the marked differences between species in their reproductive cycles. For example, studies on rabbit have shown an increase in CA concentration in endometrial homogenate under progesterone influence (Lutwak-Mann, 1955, Hodgen & Falk, 1971) while a similar increase is caused by oestrogen in mouse (Pincus & Bialey, 1963) and guinea-pigs (Hodgen & Falk, 1971). In rat Pincus & Bialey (1963) and Korhonen *et al.* (1966) obtained opposite results on CA activity during diestrus. However, it is not clear from their reports if the rats were mated or not. This could be of importance since only the mated female develops a fully active corpus luteum.

In the present study the staining intensity for CA activity increased considerably in the surface epithelium of the endometrium during the luteal phase in rabbits, while the glandular epithelium was unaffected. This confirms the biochemical results obtained by Lutwak-Mann (1955) and Hodgen & Falk (1971). However, Friedley and Rosen (1975) reported no differences with respect to the oestrus cycle in rabbit, but it is unclear which stages that were investigated. Moreover they used cryo sections for the histochemical localization which is not optimal for quantitative studies. In the present study on pig uterus using resin sections the histochemical staining for CA activity did not change during the oestrus cycle. There are obviously large species variations regarding hormonal regulation of CA activity during the sexual cycle. Furthermore, the isozymes may be regulated differently. This, taken together with methodological considerations, makes it necessary to be careful when interpreting the differences between studies regarding the amount and localization of CA.

Male reproductive organs are also a target for oestrogens with effects on CA activity. As mentioned earlier CA II is reduced by oestrogen treatment in mouse efferent ductules and enhanced by disruption of oestrogen receptors (Lee *et al.*, 2001; Zhou *et al.*, 2001). Härkönen & Väänänen (1988) have shown that cytoplasmic CA II is regulated by testosterone in the lateral prostate of the rat and by

oestrogen in the dorsal (Härkönen *et al.*, 1991). According to Kaunisto *et al.* (1999) CA IV and CA II mRNAs decrease by 80-90% in the corpus of rat epididymis after bilateral castration. One of the mice in the present study showed aberrantly strong cytoplasmic CA activity in corpus. This has to be further investigated, but it could be due to hormonal regulation. Thus, also in males the hormonal influence on CA activity appears species dependent.

CA II-deficient mouse

When comparing the control mouse and the CA II-deficient mouse it was obvious that the main part of CA activity in the male organs was not due to CA II, but to CA IV and in addition some unidentified isozymes. This lack of CA II appears thus to be of minor importance since the homozygote deficient male functions in breeding. The female homozygotes are, however, poor breeders often producing stillborn pups. CA II was present in the uterine epithelium, inconsistently in the oviduct and not at all in the ovary. This points to an important role for CA II in the uterus, which may be worth further investigations. In humans the inherited CA II deficiency syndrome causes disorders like osteopetrosis, renal tubular acidosis, cerebral calcification and in some cases mental retardation (Sly & Hu, 1995).

CA in cell nuclei

Recently the nuclear protein nonO/p54^{nrp} was shown to have CA activity and to be present in Leydig cells and uterine epithelium (Karhumaa *et al.*, 2000b). This seems to explain the histochemical CA activity found in the cell nuclei of several different cells and tissues. If this staining represents CA has long been a controversial question, as discussed by Ridderstråle (1991), and which has now been clarified.

An interesting observation is that when the nucleus in a cell expresses CA activity the cytoplasm most often does as well. In the present study this was the case in the Sertoli cells in the pig, the apical cells, in the non-ciliated cells of efferent ductules in the rabbit, Leydig cells in rat and pig and the uterine surface epithelium of rabbit and mouse.

The nonO/p54^{nrp} binds to both RNA and DNA and is implicated in transcriptional regulation and splicing (Karhumaa, 2002), which might indicate a need for CA activity during these processes. It is interesting to note that strong CA activity was present in the nuclei and cytoplasm of epithelial cells during the luteal phase in rabbit uterus when the epithelium is well developed but not during the non-luteal phase. However, some more specific function for the nuclear CA must be at hand since it is present in specific cells and transcription is a more general cell function.

Capillaries in reproductive organs

The capillaries were stained for CA activity in the testis of all studied species, and in the mouse it was shown to be CA IV. On the other hand the capillaries of epididymis were regularly unstained. The endothelial CA is generally supposed to be needed for the interconversion of CO_2 – HCO_3^- in blood plasma (Swenson, 2000) and is needed in organs with high production of CO_2. This would rather speak for the presence of CA in the epididymis than the opposite, which therefore remains unexplained.

In the female reproductive organs the staining varied with no obvious pattern. Therefore, many more comparative studies are needed to clarify the importance of CA in the capillaries of these organs in females.

Conclusions

Males

- The seminiferous tubules in testis showed in most cases no CA activity except in acrosomes of hamster and lemming. I had expected the Sertoli cells, these bicarbonate secreting cells, to have CA activity and I found so in only one species, the pig. However, the capillaries were stained for CA activity in all species studied and are supposed to have a function in carbon dioxide diffusion.
- In epididymis the proton secreting apical cells were stained for CA activity in all species. The principal cells in cauda epididymidis showed CA activity in the basolateral cell membranes. CAs are coupled to bicarbonate transporters and proton pumps and supply the ions needed for the acidification of the fluid taking place in the duct and for the decreased pH in cauda which is needed for sperm storage. The capillaries were unstained in epididymis.
- An interesting new finding was the transfer of CA IV to the cell membrane of the mouse spermatozoa during passage through the epididymal duct.

Females

- The uterine epithelium varied in CA staining between species and accumulated evidence shows that large variations occur during the oestrus cycle. In the pig there was no change in CA activity during the cycle. In the rabbit, on the other hand, the uterine surface epithelium was intensely stained during the luteal phase but showed no activity in the non-luteal phase. The glandular epithelium was unstained.
- The oocyte membrane showed CA activity only in the rabbit. The granulosa cells in the ovarian follicle showed membrane-bound CA activity in the mouse only. The capillaries in corpus luteum were stained in the cat only. This cannot be explained without further studies carried out with a well-defined hormonal status.

CA II-deficient mouse

- By comparing normal and CA II-deficient mice it was found that membrane-bound CAs were more common than CA II in males, which may explain why the CA II-deficiency does not affect male fertility. In the normal females CA II was present in the uterine epithelium and glands as well as membrane-bound CA. Since CA II is absent from the uterus of the deficient animal it might explain why these often have stillborn pups.

Reproduction follows a basic concept in animals and still the species variation is considerable. What we call variations are in fact specific adaptations needed for each species to ensure that healthy offspring are produced during the right season for that species to achieve optimal survival. Furthermore it is essential that cross-breeding be avoided. This thesis describes the localization of one enzyme family, of the importance for pH and bicarbonate homeostasis, and will hopefully provide additional facts that lead to a better understanding of the whole process.

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