# CARBONIC ANHYDRASES IN THE REPRODUCTIVE SYSTEM

With special emphasis on isoenzymes VI, IX, XII, and a novel nuclear nonclassical form

*PEPE KARHUMAA*

Department of Anatomy and Cell Biology,
University of Oulu

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### *Abstract*

Carbonic anhydrases (CAs) are a group of zinc-containing metalloenzymes that catalyze the interconversion of carbon dioxide and bicarbonate ($CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$). They are present in almost all organs and are implicated in various biological functions, the most important of which is participation in the regulation of ion, water, and acid-base balance. Recently, some members of the CA gene family have been suggested to promote cell proliferation and to act as trophic growth factors.

The present study was undertaken to examine the distribution of CA isoenzymes in the reproductive system, to attain a more detailed view on their linkage to the reproductive processes and to neonatal development.

The expression of membrane-bound CA IX and CA XII was studied in the female and male reproductive tracts by immunohistochemistry and western blotting. CA XII was found to be expressed in the basolateral plasma membrane of luminal and glandular epithelia in human uterus. In human efferent ducts, it was located in the basolateral plasma membrane of luminal epithelium, where it coexpressed with Aquaporin-1. In epididymal duct, CA XII was only expressed in occasional epithelial cells. These cells coexpressed CA II, suggesting that they represent apical mitochondria-rich cells (AMRC). CA IX was also expressed in the basolateral plasma membrane of luminal epithelium in human efferent ducts, but its expression was not uniform among the tubules. These findings suggest that basolateral plasma membrane-associated CA IX and CA XII contribute, along with CA II and CA IV, to the regulation of acid-base balance and water transport in the reproductive tract.

Western blotting of rat Leydig tumor cells and testis for CA II revealed an unidentified 66-kDa polypeptide band. The polypeptide was successfully purified from several rat tissues using CA inhibitor affinity chromatography. The amino acid sequence of the polypeptide showed it to be identical to NonO/p54$^{nrb}$, a non-POU domain-containing octamer-binding protein previously implicated in transcriptional regulation. The recombinant NonO/p54$^{nrb}$ was shown to display CA activity, and the antibody to it predominantly immunostained the nuclei in lymphocytes, where CA activity was also detected histochemically. Accordingly, the nuclear Leydig cell CA immunoreactivity represents NonO/p54$^{nrb}$. It is classified as a novel, nonclassical CA, and it may participate in pH-related events in the nucleus.

Human and rat milk was found to contain CA VI by immunohistochemistry and western blotting. The enzyme purified from human milk by CA inhibitor affinity chromatography was confirmed by PNGase F digestion and amino acid sequence as CA VI. The CA VI concentrations in human colostral milk were approximately eight times higher than those in mature milk (34.7 mg/l vs. 4.5 mg/l). Secretion of CA VI into milk is suggested by its localization in the alveolar epithelium of the rat mammary gland. The structural and functional stability of CA VI in an acidic milieu, its suggested growth-supporting function in taste bud stem cells, and its high concentration in colostrum suggest that it is an essential factor for the growth and development of the newborn alimentary canal.

*Keywords:* efferent ducts, milk, testis, uterus, carbonic anhydrases

***To my family***

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Oulu, March 2002 Pepe Karhumaa

# Abbreviations

| | |
|---|---|
| αGST | α-glutathione S-transferase |
| AE | Anion exchanger |
| AMRC | Apical mitochondria-rich cell |
| AQP1 | Aquaporin-1 |
| Asp | Aspartic acid |
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| CA | Carbonic anhydrase |
| cAMP | Cyclic adenosine 3´,5´ monophosphate |
| CA-RP | Carbonic anhydrase-related protein |
| cDNA | Complementary deoxyribonucleic acid |
| CHIP28 | Channel-forming integral protein of 28 kDa |
| Cys | Cysteine |
| DAB | 3,3'-diaminobenzidine tetrahydrochloride |
| CHO | Chinese hamster ovary |
| DNA | Deoxyribonucleic acid |
| EGF | Epidermal growth factor |
| Eu | Europium |
| FITC | Fluorescein isothiocyanate |
| HHMI | Howard Hughes Medical Institute |
| His | Histidine |
| IEF | Isoelectric focusing |
| IgA | Immunoglobulin A |
| IGF | Insuline-like growth factor |
| IgG | Immunoglobulin G |
| kDa | KiloDalton |
| MALDI-MS | Matrix-assisted laser desorption ionization mass spectrometry |
| mRNA | Messenger ribonucleic acid |
| NBC | Sodium bicarbonate cotransporter |
| NGF | Nerve growth factor |
| NHE | Sodium/proton exchanger |

| | |
|---|---|
| NonA | No-on-transient A |
| NonO | Non-POU (Pit-Oct-Unc) domain-containing octamer-binding protein |
| $p54^{nrb}$ | Nuclear RNA-binding protein, 54 kDa |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| pI | Isoelectric point |
| PMSF | Phenylmethylsulphonyl fluoride |
| PNGase F | Endoglycosidase F |
| PSF | Polypyrimidine tract-binding protein-associated splicing factor |
| PVDF | Polyvinylidene difluoride |
| Q | Glutamine |
| RPTP | Receptor-type protein tyrosine phosphatase |
| RT-PCR | Reverse transcriptase-polymerase chain reaction |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| TBST | Tris-buffered saline with Tween-20 |
| TGF | Transforming growth factor |
| TR-IFMA | Time-resolved fluoroimmunoassay |
| TRITC | Tetramethylrhodamine isothiocyanate |
| TSH | Thyroid-stimulating hormone |
| VHL | Von Hippel-Lindau |

# List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Karhumaa P, Parkkila S, Türeci Ö, Waheed A, Grubb JH, Shah G, Parkkila A-K, Kaunisto K, Tapanainen J, Sly WS & Rajaniemi H (2000) Identification of carbonic anhydrase XII as the membrane isozyme expressed in the normal human endometrial epithelium. Mol Hum Reprod 6:68-74.

II Karhumaa P, Kaunisto K, Parkkila S, Waheed A, Pastoreková S, Pastorek J, Sly WS & Rajaniemi H (2001) Expression of the transmembrane carbonic anhydrases, CA IX and CA XII, in the human male excurrent ducts. Mol Hum Reprod 7:611-616.

III Karhumaa P, Parkkila S, Waheed A, Parkkila A-K, Kaunisto K, Tucker PW, Huang C-J, Sly WS & Rajaniemi H (2000) Nuclear NonO/p54$^{nrb}$ protein is a nonclassical carbonic anhydrase. J Biol Chem 275:16044-16049.

IV Karhumaa P, Leinonen J, Parkkila S, Kaunisto K, Tapanainen J & Rajaniemi H (2001) The identification of secreted carbonic anhydrase VI as a constitutive glycoprotein of human and rat milk. Proc Natl Acad Sci USA 98:11604-11608.

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# 1 Introduction

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that participate in the regulation of ion, water, and acid-base balance by catalyzing the reversible hydration of carbon dioxide in a reaction: $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$ (Sly & Hu 1995). CAs include three evolutionary unrelated gene families termed alpha-, beta-, and gamma-CAs. In the animal kingdom, all of the heretofore identified CAs belong to the alpha-CA family (Hewett-Emmet & Tashian 2000). Eleven enzymatically active isoenzymes in the alpha family have been identified so far, including four cytoplasmic, two mitochondrial, four membrane-associated, and one secreted form (Fujikawa-Adachi *et al.* 1999).

Before this study, two carbonic anhydrase isoenzymes, CA II and CA IV, had been identified and localized in human reproductive organs. The expression of CA IV was found in the apical plasma membrane of epithelial cells in epididymis and proximal vas deferens, and CA II in the cytoplasm of epithelial cells in seminal vesicle, vas deferens, and sporadically in epididymis (Kaunisto *et al.* 1990, Parkkila S *et al.* 1993a). Various histochemical studies in human and animal reproductive organs suggest, however, that other CA isoenzymes are possibly also expressed (Korhonen *et al.* 1966, Friedley & Rosen 1975, Ekstedt *et al.* 1991, Ekstedt & Ridderstråle 1992).

The exact physiological role of these isoenzymes in the reproductive tract is not fully understood. CA II and CA IV probably participate in the acidification of epididymal fluid, whereas CA II in the seminal vesicle may promote alkalinization of the ejaculate (Kaunisto *et al.* 1990, Parkkila s et al. 1993a). CA I and CA II are expressed in human placenta and foetal membranes, suggesting that they are implicated in the regulation of acid-base balance in amniotic fluid and the developing foetus (Mühlhauser *et al.* 1994). After birth, breast milk is the primary food for the newborn. In addition to nutrients, it contains a number of bioactive factors, including growth factors and host defence agents, which contribute to the growth and development of the newborn (Kunz *et al.* 1999). Salivary CA VI has been implicated as a developmental factor in taste bud growth (Thatcher *et al.* 1998). During the early postnatal period, however, saliva secretion by the newborn infant is minimal due to the immaturity of the salivary glands (Davidson 1982, Scott 1979), and milk as a good saliva substitute (Herod 1994) may compensate for this low secretion during the neonatal period.

In the present study, the distribution of different CA isoenzymes was investigated in the reproductive system, to achieve a more comprehensive view of their role in reproductive functions and neonatal development.

# 2 Review of the literature

## 2.1 Carbonic anhydrase gene families

To date, three independent CA gene families have been discovered: α-CA, β-CA and γ-CA (Hewett-Emmett & Tashian 1996, Hewett-Emmett 2000). The earlier postulation that α-CAs would be restricted to the animal kingdom and plant green algae (Chlamydomonas), the β-CAs to plants and eubacteria, and the γ-CAs to archaebacteria and eubacteria has been disproven, since the plant Arabidopsis has homologues of all the three families (Hewett-Emmett & Tashian 1996). In addition, recent findings show that both α- and β-CA genes are present in many plants, lower eukaryotes and invertebrates, but α-CAs are clearly dominant in vertebrates (Hewett-Emmett 2000).

Based on X-ray crystallographic studies, the active center of α-CAs contains one zinc ion that is surrounded by three conserved histidine residues and one water molecule (Kannan *et al.* 1977, Eriksson & Liljas 1991). In addition, the active center of α-CAs consists of 33 other residues, whose homology varies between the isoenzymes (Fujikawa-Adachi *et al.* 1999). The structural studies on β-CAs and γ-CAs are less numerous. The active center of γ-CAs also contains three histidines, as in α-CAs, but with different spacing in the linear sequence (Hewett-Emmett & Tashian 1996). Mitsuhashi *et al.* (2000) suggested that zinc in the active center of β-CAs is coordinated by a Cys-Asp-His-Cys tetrad, which is strictly conserved in the gene family. They found no water molecule in a zinc-liganding radius in β-CAs, indicating the existence of distinct catalyzing sites for the same $CO_2$ hydration as in α-CAs and probably also in γ-CAs. Kimber & Pai (2000) suggested that zinc in the active center of β-CAs is surrounded by Cys-His-Cys, and that α-CAs and β-CAs are likely to share a common $CO_2$ hydration mechanism.

### *2.1.1 Mammalian CA isoenzymes*

Carbonic anhydrase was first purified from red blood cells (Meldrum & Roughton 1932, 1933). Thereafter, numerous biochemical studies have demonstrated the presence of CA

in different tissue or cell homogenates. The localization of CA in tissues became possible in 1953, when Kurata (1953) reported a novel histochemical technique for demonstrating CA activity in tissues. However, this technique does not differentiate between isoenzymes, which has, in turn, been facilitated by isoenzyme-specific antibodies and immunohistochemical techniques. To date, eleven active CA isoenzymes have been characterized in the animal kingdom, including four cytoplasmic (CA I, CA II, CA III and CA VII), two mitochondrial (CA VA and CAVB), one secreted (CA VI), and four membrane-associated (CA IV, CA IX, CA XII and CA XIV) forms (Fig. 1).

CA I is a moderate-activity isoenzyme present in erythrocytes and, at lower levels, in epithelial cells of the gastrointestinal tract, capillary and corneal endothelium, lens of the eye, islets of Langerhans, placenta and fetal membranes (Lönnerholm *et al.* 1985, Venta *et al.* 1987, Mühlhauser *et al.* 1994, Parkkila *et al.* 1994). CA II has the highest activity of all isoenzymes, and it is present in certain cell types of almost all tissues. It contributes, e.g. to $H^+$ secretion by gastric parietal cells, renal tubular cells, and osteoclasts and to $HCO_3^-$ secretion by pancreatic duct cells, ciliary body epithelium, choroid plexus, salivary gland acinar cells, and distal colonic epithelium (Sly & Hu 1995). In large intestine and gallbladder, CA II is involved in NaCl and water absorption (Swenson 1991, Parkkila & Parkkila 1996). CA II also promotes $CO_2$ exchange in erythrocytes, lung, and kidney. It has also been suggested to take part in fatty acid and amino acid synthesis (Sly & Hu 1995). CA III has the lowest activity of the isoenzymes, and it is present abundantly in slow-twitch (type I fiber) red skeletal muscle and, in lesser amounts, in certain other tissues (Carter *et al.* 1979, Jeffery *et al.* 1980, Shima *et al.* 1983, Väänänen *et al.* 1985, Väänänen & Autio-Harmainen 1987, Nishita & Matsushita 1989, Spicer *et al.* 1990). It has been suggested to protect cells from oxidative damage, and thus also to affect growth-signaling pathways (Räisänen *et al.* 1999). CA VII mRNA has been detected in baboon salivary glands, rat lung, and mouse brain (Montgomery *et al.* 1991, Ling *et al.* 1994, Lakkis *et al.* 1997). The recombinant protein of CA VII has been shown to be a high-activity isoenzyme, but tissue expression of the protein has not been described yet (Earnhardt *et al.* 1998). Mitochondrial CA was first identified by Dodgson *et al.* (1980), and its function was linked to gluconeogenesis and ureagenesis and the regulation of insulin secretion (Dodgson *et al.* 1983, Metcalfe *et al.* 1985, Sly & Hu 1995, Parkkila *et al.* 1998). Recently, it has become clear that there are two mitochondrial CAs, termed CA VA and CA VB (Fujikawa-Adachi *et al.* 1999, Shah *et al.* 2000). The expression of human CA VA has been demonstrated in liver (Fujikawa-Adachi *et al.* 1999). The mRNA of CA VB has been shown in normal human heart and skeletal muscle by northern blotting. In human pancreas, salivary glands, kidney, and spinal cord, the mRNA of human CA VB could be detected using reverse transcription-PCR (Fujikawa-Adachi *et al.* 1999). CA VI is the secretory CA, which exclusive secretion has been found so far only into saliva by salivary glands. CA IV is a membrane-anchored CA expressed in the apical surface of epithelial cells. It is expressed in, e.g. certain segments of nephron and gastrointestinal epithelial cells, the endothelial cells of certain capillary beds, and the epithelium of epididymis and vas deferens (Brown *et al.* 1990, Hageman *et al.* 1991, Ghandour *et al.* 1992, Fleming *et al.* 1993, Parkkila S *et al.* 1993a, Sender *et al.* 1994, Fleming *et al.* 1995, Kaunisto *et al.* 1995, Parkkila *et al.* 1996). CA IX and CA XII are recently characterized transmembrane proteins, which have been linked to oncogenesis. However, their presence in non-malignant tissues has been demonstrated as well. The

third transmembrane isoform, CA XIV, has recently been identified and cloned from mouse and human tissues (Mori *et al.* 1999, Fujikawa-Adachi *et al.* 1999). Its mRNA has been demonstrated in human heart, brain, liver, and skeletal muscle using the northern blotting technique and in colon, small intestine, urinary bladder, kidney, and spinal cord using more sensitive techniques (Fujikawa-Adachi *et al.* 1999). *In situ* hybridization in mouse kidney showed that CA XIV mRNA is expressed in the proximal convoluted tubule and in the outer border of the inner stripe of the outer medulla (Mori *et al.* 1999). Kaunisto *et al.* (2002) have recently described immunohistochemical localization of CA XIV protein in mouse and rat kidney. It was found to be present in the proximal tubules and thin limbs of Henle. A study by Parkkila *et al.* (2000a) demonstrated the presence of CA XIV protein in neuronal membranes and axons in different parts of human and mouse brain. The mammalian CA gene family also contains three CA-related proteins (CA-RP VIII, X, and XI) and two subtypes of receptor-type protein tyrosine phosphatases (RPTP-β and γ). These proteins share the CA-like domain but lack CA activity (Tashian *et al.* 2000). CA-RPs have been found from cDNA libraries and named in the order of their discovery. mRNAs of CA-RP VIII, X and XI have been detected mainly in brain but also in a variety of other tissues (Kato 1990, Skaggs *et al.* 1993, Ling *et al.* 1994, Lakkis *et al.* 1997a, Lakkis *et al.* 1997b, Fujikawa-Adachi *et al.* 1999, Tashian *et al.* 2000). The localization and function of these proteins are so far unknown. The CA-like domain has also been shown in RPTPs (Krueger & Saito 1992, Levy *et al.* 1993, Barnea *et al.* 1993). The extracellular region of RPTPβ has been shown to be identical to a chondroitin sulphate proteoglycan, phosphacan (Maurel *et al.* 1994), which provides a binding site for a cell surface signal-transducing molecule, contactin (Peles *et al.* 1995). The exact functions of RPTPs are, however, unknown.

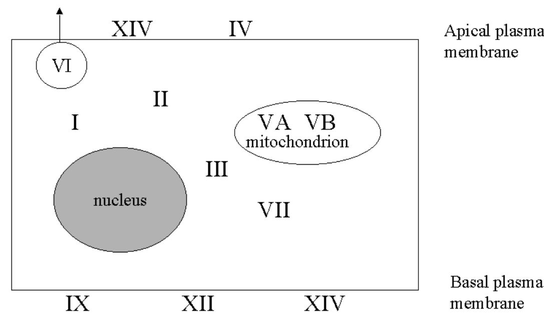


**Fig. 1. Subcellular localization of the active CA isoenzymes. CA I, II, III, and VII are cytoplasmic, CA VA and VB are mitochondrial, CA VI is secreted, CA IV is associated with the apical plasma membrane, and CA IX and XII are associated with the basolateral plasma membrane. CA XIV is located in both apical and basolateral plasma membranes.**

### *2.1.1.1 CA VI*

CA VI is so far the only known secretory isoenzyme of the CA gene family. It was first found in ovine parotid gland and saliva by Fernley *et al.* (1979). Feldstein and Silverman (1984) purified it from rat saliva and determined its amino acid composition. Murakami and Sly (1987) and Kadoya *et al.* (1987) purified it independently from human saliva. The amino acid sequence of sheep CA VI was reported by Fernley *et al.* (1988), and the cDNA sequence of human CA VI was determined by Aldred *et al.* (1991). The molecular weight of CA VI polypeptide is 39-46 kDa (Feldstein & Silverman 1984, Kadoya *et al.* 1987, Murakami & Sly 1987, Fernley 1991a,b, Parkkila *et al.* 1991a, Ogawa *et al.* 1992). The molecule contains two 3-kDa N-linked oligosaccharide chains, which can be cleaved off by endo-β-N-acetylglucosaminidase F but not by endo-β-N-acetylglucosaminidase H, indicating that the oligosaccharides are of a complex type (Murakami & Sly 1987). CA VI has two cysteine residues that form an intramolecular disulfide bond, which probably enables the enzyme to retain its enzymatic activity in an acidic milieu (Fernley *et al.* 1988, Aldred *et al.* 1991, Parkkila *et al.* 1997). The CA domain of CA VI is highly homologous to four other CAs (CA IV, CA IX, CA XII and CA XIV), and they form together a cluster of "extracellular" CAs (Fujikawa-Adachi *et al.* 1999, Mori *et al.* 1999). The gene for bovine CA VI reported a few years ago showed some similarity to the membrane-bound CA (CA IV and CA IX) gene structures (Jiang & Gupta 1999). The human CA6 gene is located in the distal short arm of chromosome 1 at a position where some human malignancies and genetic diseases frequently exhibit deletions, rearrangements, or single mutations (Sutherland *et al.* 1989, White *et al.* 1998).

CA VI is secreted into saliva by the serous acinar cells of the parotid and submandibular glands (Kadoya *et al.* 1987, Parkkila *et al.* 1991a, Ogawa *et al.* 1992). Its secretion is controlled by the autonomic nervous system (Fernley 1991). It has been estimated that human CA VI represent about 3 % of total protein in parotid saliva, the mean +/- SD concentration being 47.0 +/- 39.2 mg/ml in radioimmunoassay (Fernley *et al.* 1995). Fluoroimmunoassays of total human saliva have demonstrated mean +/- SD concentrations of 6.8 +/- 4.3 mg/ml (Parkkila S *et al.* 1993b). The CA VI concentration also shows circadian periodicity, being very low during sleep and higher during the daytime and after meals (Parkkila *et al.* 1995).

In addition to its salivary expression, CA VI has been shown to be present in serum by fluoroimmunoassay and western blotting, in occasional acinar cells of lacrimal gland by immunohistochemical methods, and in pancreas by the RT-PCR method (Kivelä *et al.* 1997, Ogawa *et al.* 1995, Fujikawa-Adachi *et al.* 1999).

The exact physiological functions of CA VI have remained undefined. It has been proposed to neutralize locally the protons produced by cariogenic bacteria, thus protecting teeth from caries (Leinonen *et al.* 1999). Low CA VI concentrations have been shown to be associated with increased caries prevalence, particularly in subjects with poor oral hygiene (Kivelä *et al.* 1999). It has also been suggested to neutralize excess acid in the upper gastrointestinal tract and pancreas (Parkkila *et al.* 1997, Fujikawa-Adachi *et al.* 1999). In fact, patients with verified oesophagitis or oesophageal, gastric, or duodenal ulcer have reduced salivary CA VI concentrations compared to patients with non-acid peptic disease (Parkkila *et al.* 1997). A novel role for CA VI was discovered by Thatcher *et al.* (1998), who demonstrated that gustin, a salivary factor involved in taste function, is

CA VI. Low salivary CA VI concentrations have been shown to be associated with the loss and distortion of taste and smell after an influenza-like illness with apoptotic-like changes in taste buds (Henkin *et al.* 1999a). Treatment with zinc normalized the CA VI concentrations and the senses of taste and smell in some cases (Henkin *et al.* 1999b). The taste bud morphology was also normalized in these patients, suggesting that CA VI might function as a trophic factor for taste bud stem cells (Henkin *et al.* 1999b). CA VI has also been shown to have characteristics similar to the nerve growth factor (NGF) (Henkin *et al.* 1988). In addition, CA VI activates calmodulin-dependent bovine brain cAMP phosphodiesterase, which is a factor involved in taste function (Law *et al.* 1987).

Recently, a novel stress-inducible intracellular form of CA VI (CA VI type B) has been identified. It has been suggested to participate in intracellular pH changes induced by stress (Sok *et al.* 1999).

#### *2.1.1.2 CA IX*

Both cDNA (Pastorek *et al.* 1994) and the gene for CA IX (Opavský *et al.* 1996) have been cloned and characterized. The predicted protein has been shown to consist of a signal peptide, a proteoglycan-related sequence, a carbonic anhydrase domain, a transmembrane segment, and a short intracellular tail (Pastorek *et al.* 1994). CA IX (initially termed MN) was discovered in HeLa cells, and its participation in oncogenesis was initially suggested by the facts that its expression correlated with the tumorigenity of HeLa x fibroblast hybrids and its expression was also detected in immunoblots in various carcinomas but not in corresponding normal tissues (Závada *et al.* 1993). In 1996, a renal cell carcinoma-related antigen, G250, was sequenced and found to be homologous to CA IX (Oosterwijk *et al.* 1996).

CA IX is a glycoprotein of 54 and 58 kDa mass expressed at the basolateral plasma membrane of epithelial cells and, in some cases, also in the nucleus (Pastoreková *et al.* 1992). Its binding to DNA has also been demonstrated (Pastorek *et al.* 1994). The finding by Závada *et al.* (1993) that CA IX is expressed in various neoplasms but not in corresponding normal tissues has been confirmed by several groups (Liao *et al.* 1994, Uemura *et al.* 1997, Turner *et al.* 1997, Liao *et al.* 1997, Saarnio *et al.* 1998, Vermylen *et al.* 1999). However, CA IX has been found in certain non-malignant cells, including the epithelium of stomach, small intestine, colon, and gallbladder, the basal layer of oesophageal epithelium, and occasional cells in uterine cervical epithelium (Pastorek *et al.* 1994, Liao *et al.* 1994, Pastoreková *et al.* 1997, Turner *et al.* 1997). CA IX has also been found in the ductal cells of liver and pancreas (Pastoreková *et al.* 1997) and, recently, in occasional acinar cells in pancreas (Kivelä *et al.* 2000). In a recent extensive study, Ivanov *et al.* (2001) identified several other normal tissues expressing CA IX. Among the reproductive organs, they reported its expression in efferent ducts, rete testis, and ovary.

Although the exact function of CA IX is still unknown, its role in non-malignant cell proliferation and intercellular communication has been suggested based on two independent observations. First, the expression of CA IX in intestine is mainly restricted to the rapidly proliferating area, i.e. crypts of Lieberkühn (Saarnio *et al.* 1998). Second,

its expression is regulated by cell density. CA IX expression is induced in dense cultures of HeLa cells but not in rapidly growing sparse cultures. (Pastoreková *et al.* 1992, Závada *et al.* 1993, Pastoreková *et al.* 1997, Saarnio *et al.* 1998). It has also been shown to contribute to cell adhesion properties via its proteoglycan-related domain (Závada *et al.* 1997, 2000). Its role in malignant cell transformation has also been suggested by the finding that transfection of NIH 3T3 fibroblasts with CA IX causes typical morphological features of *in vitro* malignant transformation, including cell proliferation and anchorage independence (Pastorek *et al.* 1994). The transcriptional regulators of CA IX identified so far include von Hippel-Lindau and p53 tumor suppressor proteins (Ivanov *et al.* 1998, Kaluzová *et al.* 2000). In addition, a novel silencer element within the CA IX promoter has been characterized (Kaluz *et al.* 1999). The use of CA IX as a biomarker of certain carcinomas has been suggested, and it has been shown to be an excellent target for immunotherapy in renal cell carcinoma (Steffens *et al.* 1997, Uemura *et al.* 1997, 1999).

#### *2.1.1.3 CA XII*

CA XII was cloned and characterized by two different groups independently (Türeci *et al.* 1998, Ivanov *et al.* 1998). Its cDNA sequence predicted a 354-amino acid polypeptide with a molecular mass of 39,448 Da, and it had potential sites for asparagine glycosylation (Türeci *et al.* 1998, Ivanov *et al.* 1998). The enzyme has features of the type I membrane protein, and its sequence includes a 29-amino acid signal sequence, a 261-amino acid CA domain, an additional short extracellular segment, a 26-amino acid hydrophobic transmembrane domain, and a 29-amino acid C-terminal cytoplasmic tail that contains two potential phosphorylation sites. The CA domain has three zinc-binding histidine residues found in active CAs and shows 30-42 % homology with the other CAs. When expressed in COS cells, the cDNA produced a 43- to 44-kDa protein in membranes, and PNGase F digestion reduced its molecular mass to 39 kDa, consistent with the removal of two oligosaccharide chains (Türeci *et al.* 1998). The recombinant CA XII protein is an active isoenzyme, whose catalytic properties resemble those of a high-activity membrane-associated CA IV (Ulmasov *et al.* 2000). Its 4.3- to 4.5-kb mRNA has been demonstrated in various tissues, including kidney, colon, prostate, pancreas, ovary, testis, lung, and brain using the northern blotting technique (Türeci *et al.* 1998, Ivanov *et al.* 1998). However, RT-PCR analysis showed a much wider tissue distribution pattern (Türeci *et al.* 1998). Similarly to CA IX, CA XII is over-expressed in certain cancers and tumor cell lines (Türeci *et al.* 1998, Ivanov *et al.* 1998, Kivelä *et al.* 2000, Parkkila *et al.* 2000b,c, Ivanov *et al.* 2001), and it is involved in von Hippel-Lindau (VHL)-mediated carcinogenesis. Wild-type VHL protein clearly down-regulates the over-expression of CA XII in parental renal cell carcinoma cell lines (Ivanov *et al.* 1998). Recent studies have further demonstrated the presence of CA XII protein in many normal tissues, including reproductive organs (efferent ducts, prostate gland, uterus, ovary, and breast) (Ivanov *et al.* 2001). In addition, the expression of both CA XII and CA IX is induced under hypoxic conditions in tumors and cultured tumour cells (Ivanov *et al.* 2001).

The function of CA XII in normal and malignant tissues is so far unknown. Recently, Ivanov *et al.* (1998) suggested that, in carcinogenesis, it may acidify the immediate

extracellular milieu surrounding the cancer cells and thus create a microenvironment conducive to tumor growth and spread.

## 2.2 Sperm maturation and transport in the male reproductive tract

The spermatozoa are formed within the seminiferous tubules of the testes in a complex process called spermatogenesis. The continuous maintenance of this process is enabled by androgens secreted mainly by testicular Leydig cells located in the interstitial tissue outside the seminiferous tubules. In addition, the epididymal maturation of spermatozoa is also an androgen-dependent process (Robaire & Hermo 1988). Once formed within the seminiferous tubules, the immotile spermatozoa are released into luminal fluid and transported to the epididymis, where they gain the ability to move and fertilize the ovum (Yanagimachi 1994).

The testicular spermatozoa are transported passively to the rete testis, which is a branched reservoir of the openings of the seminiferous tubules. From the rete testis, the transport of spermatozoa to the epididymis takes place via the efferent ducts, whose number varies between studies and individuals (Stieve 1930, Holstein 1969, Jonté & Holstein 1987, Saitoh *et al.* 1990). The epithelium lining these ducts is columnar and consists of two cell types called ciliated and nonciliated cells (Robaire & Hermo 1988). Both cell types are capable of performing endocytosis, and ciliated cells also maintain the movement of luminal fluid and sperms. Nonciliated cells are mainly responsible for the absorption of water and ions. The efferent ducts absorb most of the fluid discharged from the testis with spermatozoa, thus increasing the epididymal sperm concentration (Clulow *et al.* 1994).

The epididymis can be divided into three parts, called caput, corpus, and cauda. In the human epididymis, the caput is mainly filled by efferent ducts, which open to the epididymal duct near the border between the caput and corpus (Saitoh *et al.* 1990, Yeung *et al.* 1991). The human ductus epididymis contains four types of epithelial cells called principal cells, basal cells, apical mitochondria-rich cells (AMRC), and halo cells (Reid & Cleland 1957, Martan *et al.* 1964, Robaire & Hermo 1988, Palacios *et al.* 1991). Principal and basal cells are the main cell types, the former being involved in secretion and absorption, while the latter probably participate in detoxification processes or act as scavenger cells (Robaire & Hermo 1988, Veri *et al.* 1993, Yeung *et al.* 1994). AMRC are most abundant in the proximal epididymis, and their number declines towards the cauda (Palacios *et al.* 1991). These cells correspond to rat narrow and clear cells, which are involved in the acidification of epididymal fluid (Brown *et al.* 1992, Martìnez-Carcìa *et al.* 1995, Adamali & Hermo 1996). Halo cells are considered intraepithelial macrophages or lymphocytes (Hoffer *et al.* 1973, Dym & Romrell 1975, Wang & Holstein 1983).

In mammals, the transit of spermatozoa through the epididymis usually takes 10-13 days, whereas in humans the estimated transit time is 2-6 days (Amann & Howards 1980, Johnson & Varner 1988, Robaire & Hermo 1988). The epididymal segment where most spermatozoa attain their full fertilizing capacity appears to be the proximal cauda. The spermatozoa from that region are capable of moving progressively, which is characteristic of spermatozoa preceding fertilization, and bind to zona-free hamster ova *in vitro* at a

higher percentage than spermatozoa obtained from more proximal locations (Yanagimachi 1994, Turner 1995). To attain the capacity to fertilize, sperm undergoes many maturational changes during its transit in the epididymal duct (Yanagimachi 1994). These include, for instance, changes in plasma membrane lipids, proteins and glycosylation, alterations in the outer acrosomal membrane, gross morphological changes in acrosome in some species, and cross-linking of nuclear protamines and proteins of the outer dense fiber and fibrous sheath. Numerous studies have questioned whether the human epididymal and efferent ducts are necessary for sperm maturation. In these studies, sperms that have bypassed the epididymis partly or completely or have also passed the efferent ducts and rete testis have still been able to fertilize eggs (Schyosman & Bedford 1986, Silber *et al.* 1988, Silber 1988, 1989, Schyosman 1993). However, these studies have been conducted on subjects with an abnormal reproductive tract, and spermatozoa aspirated from these subjects are not comparable to sperm aspirated from the corresponding segment in normal subjects. In addition, after surgical bypassing of the proximal excurrent ducts, vas deferens may become able to substitute the bypassed segments. Thus, these studies may, misleadingly, underevaluate the importance of the human epididymis and efferent ducts in sperm maturation (Cooper 1993, Bedford 1994, Turner 1995, Jones 1999). Alternatively, the requirement for post-testicular sperm maturation is not so essential in men as in other mammals (Yanagimachi 1994, Jones 1999), or this maturation may be faster and require only a brief exposure to some part of the post-testicular tract (Bedford 1994, Turner 1995). The cauda epididymidis (and proximal ductus deferens) are the regions where spermatozoa are stored before ejaculation (Turner 1995, Jones 1999). When ejaculation occurs, the stored spermatozoa with the surrounding fluid are mixed with the alkaline secretions of the male accessory sex glands and deposited to the vagina.

## 2.3 Sperm transport in the female reproductive tract

Spermatozoa are actively transported from the vagina via the cervical canal and the uterine cavity to the ampulla of the oviducts, where fertilization occurs. In the human vagina, the ejaculated semen is deposited near the external cervical opening where the environment is very acidic due to lactic acid and thus hostile to spermatozoa (Harper 1988). The alkaline pH of the ejaculate protects spermatozoa in this acidic environment (Speroff *et al.* 1994). This protection is, however, temporal, and most spermatozoa only remain motile in the vagina for a few hours (Fordney-Settlage 1981, Speroff *et al.* 1994). The spermatozoa are transported into the cervical canal by the pressure alterations in the vagina due to the female orgasm assisted by the normal motility of sperm (Fox *et al.* 1970, Speroff *et al.* 1994). During their transit in the female reproductive tract, the spermatozoa are stored in cervical crypts (Harper 1988). The passage of spermatozoa through the cervix is thought to maintain the muscle contractions of the reproductive tract wall and some properties of the spermatozoa. The interactions between sperm and mucus and the motility of spermatozoa during the transport are important, and one cause of infertility is presumably the impaired sperm movement through cervical mucus (Speroff

*et al.* 1994). The change in the composition of cervival mucus at mid-cycle also affects the passage (Fordney-Settlage 1981, Harper 1988, Barratt & Cooke 1991).

The release of spermatozoa from human cervical crypts may continue for several days (Yanagimachi 1994). Motile sperm has been recovered in the uterine cavity up to 24 h after intercourse (Rubinstein *et al.* 1951, Moyer *et al.* 1970), occasionally even at 85 h (Harper 1988). The transport of spermatozoa from the cervix to the uterotubal junction is mainly attributable to uterine wall contractions (Yanagimachi 1994). The human endometrium prepares for ovulation by secreting a unique kind of fluid to the uterine lumen. The fluid has a different protein pattern, ionic composition, and volume than at the other stages of the cycle (Casslén & Nilsson 1984, Casslén 1986, Harper 1988). This fluid serves to suspend spermatozoa and to keep them viable during the transport process, and it also contains macrophages that remove dead and nonviable spermatozoa.

The uterotubal junction and the lower isthmus in some animals act as a site of storage for spermatozoa before the arrival of the ovum to the site of fertilization (Suarez 1998). During their storage, spermatozoa remain weakly motile and attached to the mucosal surface of luminal epithelial cells, especially in specific epithelial crypts (Cooper *et al.* 1979, Yanagimachi 1994, Suarez 1998). Whether the human oviduct has this function is, however, not fully determined (Harper 1988, Williams *et al.* 1993, Baillie *et al.* 1997).

The ampulla of the oviduct is the site of fertilization, and motile spermatozoa can be found there up to 85 h after intercourse. The transport of spermatozoa through the oviducts is a combination of sperm motility, fluid flow, and contractive movements of the oviduct walls (Harper 1988).

During its transport in the female reproductive tract, spermatozoa first undergo a maturational change called capacitation. Capacitation includes a variety of changes in the sperm plasma membrane, intracellular ions, metabolism, adenylate cyclase-cAMP system, nucleus, and acrosome (Yanagimachi 1994, Fraser 1995). Human spermatozoa do not need to experience the uterus or the isthmic region of the oviduct to became capacitated, and it has been proposed that capacitation is initiated and possibly already completed in the cervix (Speroff *et al.* 1994, Yanagimachi 1994).

Capacitation is followed by an acrosome reaction, which occurs when spermatozoa come into close contact with the ovum in the ampulla of the oviduct. The acrosome reaction enables spermatozoa to penetrate through the zona pellucida and fuse with the egg plasma membrane. In this reaction, the plasma membrane and the outer acrosomal membrane fuse, enabling release of the acrosomal content. Acrosome contains certain enzymes, e.g. hyaluronidase and acrosin, important for the events preceding fertilization (Yanagimachi 1994).

Sperm hyperactivation occurs before the acrosome reaction (Yanagimachi 1994). Hyperactivation takes place in the oviduct and helps the spermatozoa to swim in the viscous oviduct fluid and to penetrate the zona pellucida (Stauss *et al.* 1995, Suarez 1996).

If fertilization occurs, the fertilized ovum enters the uterine lumen at the morula stage 4 days after ovulation. Approximately one day later, the morula develops into a blastocyst. Implantation begins about 7 days after ovulation. The implantation process involves apposition and adhesion of the blastocyst to the uterine surface, followed by invasion of the trophoblast. During apposition, uterine fluid is pinocytosed by microprotrusions of the apical surface of the luminal endometrial epithelium, resulting in

a close association of the blastocyst with the uterine epithelium. Apposition is followed by adhesion of the outer layer of the trophoblast cells to the uterine epithelium, which initiates the invasion of trophoblasts. In humans, the invasion process is called interstitial invasion, since the syncytiotrophoblasts invade first between uterine epithelial cells and thereafter through the basal lamina (Klentzeris 1997).

## 2.4 pH regulation in the male reproductive tract

The pH regulation of luminal fluid in the male reproductive tract has been studied only in animals, since its examination in men is difficult. In rats, approximately 96 % of the fluid discharged from testis with spermatozoa is reabsorbed in the efferent ducts (Clulow *et al.* 1994). This is thought to be important for the completion of sperm maturation and storage in the epididymis (Ilio & Hess 1994, Eddy *et al.* 1996, Hess *et al.* 1997). The fluid absorption is driven by sodium (Ilio & Hess 1994). Hansen *et al.* (1999) have shown that amiloride, an inhibitor of $Na^+/H^+$ exchanger (NHE), reduces fluid reabsorption by 70 %. Bicarbonate is reabsorbed from the efferent duct lumen at the same percentage as the fluid (96 %). The concentration of this ion is approximately the same at the both ends of the efferent ducts, and pH remains almost unaltered along the ducts (Newcombe *et al.* 2000). Acidification of epididymal fluid is a common feature among species, and it probably also occurs in men (Wales *et al.* 1966, Levine & March 1971, Levine & Kelly 1978, Au & Wong 1980, Rodriguez-Martinez *et al.* 1990, Caflisch & DuBose 1990).

Although pH regulation in the male reproductive tract is a complex process, recent immunohistochemical studies have shed light on the role of different ion transport proteins in this process. The proteins in efferent duct epithelium that may participate in pH regulation include cytoplasmic and/or membrane-bound carbonic anhydrases in nonciliated (and ciliated) cells (Cohen *et al.* 1976, Goyal *et al.* 1980), apical $Na^+/H^+$ exchanger isoform 3 (NHE-3) in nonciliated cells (Bagnis *et al.* 2001, Kaunisto & Rajaniemi 2002), apical and cytoplasmic $H^+$ATPase in nonciliated cells (Herak-Kramberger *et al.* 2001), and basolateral $Cl^-/HCO_3^-$ exchanger (AE2) in ciliated epithelial cells (Jensen *et al.* 1999). Epididymal duct epithelium also expresses various ion transporters capable of participating in pH regulation. Jensen *et al.* (1999a,b) demonstrated intense basolateral expression of both AE2 and $Na^+/HCO_3^-$ cotransporter (NBC1) in the epithelium of rat proximal epididymal duct and faint expression in distal epididymal duct epithelium. In rats, CA IV is expressed in the apical plasma membrane of the principal cells mainly in corpus, distal caput, and proximal cauda epididymidis and in a few cells in the other parts of cauda (Kaunisto *et al.* 1995). NHE3 is abundantly present in the apical plasma membrane of the principal cells in the epididymal epithelium (Pushkin *et al.* 2000, Kaunisto *et al.* 2001, Bagnis *et al.* 2001). Evidence of its involvement in sodium-dependent acidification has also been obtained in *in vitro* functional studies using inhibitors to NHE (Bagnis *et al.* 2001). NHE1 is expressed in basolateral plasma membrane throughout rat epididymal epithelium and NHE2 in the apical plasma membrane of the principal cells of caput, corpus, and cauda (Chew *et al.* 2000). Narrow and clear cells of rat epididymal epithelium and the epithelium of proximal vas deferens express both vacuolar $H^+$ATPase and cytoplasmic CA II (Brown *et*

*al.* 1992, Breton *et al.* 1996, Kaunisto *et al.* 1995, Brown *et al.* 1999, Hermo *et al.* 2000). These cells also express apical NBC3 (Pushkin *et al.* 2000). In addition, narrow cells also express basolateral NBC1 and AE2 (Jensen *et al.* 1999a,b).

Of the ion transport proteins expressed in the human reproductive tract, only carbonic anhydrase has been demonstrated. CA II is expressed in occasional cells in epididymal duct epithelium (Kaunisto *et al.* 1990) and CA IV at the apical plasma membrane of the epithelial cells in epididymal duct and in proximal ductus deferens (Parkkila *et al.* 1993).

## 2.5 pH regulation in the female reproductive tract

The pH of the human vagina is acidic throughout the menstrual cycle. However, Fox *et al.* (1973) have shown that, during coitus, seminal plasma increases vaginal pH rapidly from 4.3 to 7.2 due to its high bicarbonate concentration. In human cervical mucus, pH varies during the menstrual cycle, the highest values being found at the time of ovulation (Lamar *et al.* 1940, Moghissi 1966, McDonald & Lumley 1970). Breckenridge *et al.* (1950) found, however, no cyclic variation in pH. The pH appears to vary from 6.5 to 7.5 during the cycle (McDonald *et al.* 1970, Breckenridge *et al.* 1950), although higher values have also been measured (Meaker & Glaser 1929, Miller & Kurzrok 1932, Moghissi 1966). The pH of human uterine fluid appears to vary around 7.0, depending on the stage of the cycle (pH 6.6-7.6), and to drop transiently to slightly acidic at the time of ovulation (Fox *et al.* 1982, Maas *et al.* 1983). Intercourse increases the pH of uterine fluid by 0.2 to 0.95 pH units for about 30 minutes, and this increase is not attributable to the bicarbonate ions of seminal plasma (Fox *et al.* 1982). Thus, the female reproductive tract epithelium may provide an alkaline environment for maintaining the motility of sperm after coitus (Fox *et al.* 1982). The pH of normal human oviduct fluid is not known, but the pH of hydrosalphinx fluid ranges within 7.2-7.7, and its bicarbonate concentration is 20 +/-7.22 mM (David *et al.* 1973, Strandell *et al.* 1998). The pH of human follicular fluid has been estimated to vary between 7 and 8 (Shalgi *et al.* 1972, Imoedembe *et al.* 1993, Dale *et al.* 1998).

The proteins so far identified that may participate directly in the pH regulation of uterine fluid are carbonic anhydrase and vacuolar $H^+$ATPase, which are found in both glandular and luminal epithelia (Korhonen *et al.* 1966, Friedley & Rosen 1975, Ge & Spicer 1988, Skinner *et al.* 1999). The expression of the latter has not been examined in human uterus. Endometrial epithelial cells in animals have been shown to secrete bicarbonate *in vitro* in response to various stimulants (Kyriakides & Levin 1973, Levin & Scargill 1987, Chan *et al.* 1997, Fong *et al.* 1998). Proteins that participate in pH regulation in cervix uteri and oviducts have not been extensively studied. In human, CA has been shown to be expressed in cervical and oviductal epithelia (Korhonen *et al.* 1966, Friedley & Rosen 1975).

## 2.6 Bicarbonate in reproductive physiology

During the sperm transport, bicarbonate plays an important role, first in cauda epididymidis, where spermatozoa are stored in a quiescent state (Carr & Acott 1984, Carr *et al.* 1985). Low intraluminal pH acidifies the cytoplasm of spermatozoa, suppressing their motility (Acott & Carr 1984, Carr *et al.* 1989, Carr & Acott 1989). It has also been demonstrated that bicarbonate directly stimulates sperm motility through activation of adenylate cyclase (Okamura *et al.* 1985, Tajima *et al.* 1987). Accordingly, the low bicarbonate concentration in the epididymis is attributable to the suppressed motility of spermatozoa during storage (Okamura *et al.* 1985, 1988).

The motility of spermatozoa increases when they come into contact with seminal plasma during ejaculation (Lindholmer 1974). The bicarbonate present in that fluid is known to enhance sperm motility, which crucially facilitates their entry into the cervical canal (Okamura *et al.* 1985, Speroff *et al.* 1994). The alkaline seminal fluid also buffers the low pH of the vaginal milieu, providing protection to the spermatozoa against acidity (Speroff *et al.* 1994). In addition, the lowered levels of bicarbonate in semen are at least partly responsible for the poor sperm motility in some infertile patients (Okamura *et al.* 1986).

Low endocervical pH may impair the sperm-mucus interaction and lead to reduced fertility (Zavos *et al.* 1980, Jenkins *et al.* 1989). Douching of vagina with sodium-bicarbonate improves the penetration of sperm into the cervical canal (Ansari *et al.* 1980, Everhardt *et al.* 1990).

In addition to its role in the regulation of sperm motility, bicarbonate also participates in other processes occurring in the female reproductive tract. Bicarbonate has been shown to be the key factor in capacitation and also to participate in the acrosome reaction (Harrison 1996, Sabeur & Meizel 1995). Moreover, bicarbonate has been shown to induce hyperactivated motility of sperm (Stauss *et al.* 1995). All these processes are known to involve cAMP, whose synthesis is stimulated by bicarbonate. cAMP increases sperm protein phosphorylation through cAMP-dependent protein kinase (Yanagimachi 1994, Harrison 1996, Chen *et al.* 2000). Bicarbonate also increases intracellular pH, which may be essential in these processes (Yanagimachi 1994). One goal of bicarbonate action is to cause sperm plasma membrane alterations during and after capacitation, possibly due to the increase of cAMP (Purohit *et al.* 1998, Harrison 1996, Harrison & Miller 2000, Gadella & Harrison 2000). Bicarbonate also acts as a dispersing factor for cumulus and corona cells and thereby facilitates the penetration of sperm to zona pellucida (Stambaugh *et al.* 1969, Boatman & Robbins 1991).

After fertilization, bicarbonate/$CO_2$ is required for embryo cleavage and embryonic formation, particularly at the blastocyst stage (Boatman 1997). Strandell *et al.* (1998) demonstrated that proper pH is important and that low levels are detrimental for embryonic development in hydrosalphinx fluid. Bicarbonate also helps the human embryo to recover from acidosis (Phillips *et al.* 2000). Böving (1959, 1965) has hypothesized that $CO_2$ is an important signal for implantation. Accordingly, the $CO_2$ produced as a metabolic by-product of the developmentally active blastocyst that has entered the uterus results in a local pH increase in the uterine epithelium after its hydration by carbonic anhydrase. $CO_2$ has also been suggested to be involved in eliciting the decidual reaction (Hetherington 1968a,b). Moreover, bicarbonate has been shown to

be an important ion in *in vitro* fertilization media (Bhattacharyya & Yanagimachi 1988, Suzuki *et al.* 1994).

## 2.7 Carbonic anhydrase in the male reproductive tract

The presence of carbonic anhydrase in the male reproductive organs was first demonstrated about 50 years ago. Mawson and Fisher (1952) showed biochemically that rat dorsolateral prostate homogenate contains a large amount of CA activity. Many investigators have later confirmed the finding (Fisher *et al.* 1955, Pincus & Bialy 1963, Leiter 1964, McIntosh 1969). Biochemical studies have also demonstrated that human and rat seminal vesicle and rat coagulating gland contain CA activity (Miyake & Pincus 1959, Pincus & Bialy 1963, Leiter 1964, Maren 1967). The CA activity in rat prostate and seminal vesicle was found to be regulated by androgens (Miyake & Pincus 1959, Pincus & Bialy 1963). Interestingly, Leiter (1964) did not find CA activity in human prostate. Some years later, Hodgen *et al.* (1969, 1971) suggested the presence of a testis-specific isoenzyme. The identity of this isoenzyme has remained obscure, however.

Histochemical studies have demonstrated CA activity in testis, efferent ducts, epididymis, ductus deferens, seminal vesicle, and prostate in some species (Waldeyer & Häusler 1959, Cohen *et al.* 1976, Goyal *et al.* 1980, Ridderstråle *et al.* 1985, Rodriguez-Martinez *et al.* 1990, Ekstedt *et al.* 1991, Ekstedt & Ridderståle 1992). In testis, CA activity has been located in Sertoli and Leydig cells and in interstitial tissue in rat and boar (Cohen 1976, Ridderstråle *et al.* 1985, Ekstedt *et al.* 1991). Endothelial cells of capillaries and some larger vessels in testis have been shown to contain CA activity in many species, including human (Cohen 1976, Ridderstråle *et al.* 1985, Ekstedt *et al.* 1991, Ekstedt *et al.* 1992). However, Goyal *et al.* (1980) failed to demonstrate CA activity in bovine testicular seminiferous epithelium or interstitial tissue. Late spermatids in some species showed CA activity (Ridderståle *et al.* 1985, Ekstedt *et al.* 1991). In efferent ducts, mainly nonciliated, and in boar also ciliated cells, expressed CA activity in apical and basolateral plasma membranes, nucleus, and cytoplasm (Waldeyer & Häusler 1959, Cohen *et al.* 1976, Goyal *et al.* 1980, Ekstedt *et al.* 1991, Ekstedt & Ridderståle 1992).

Immunohistochemical studies have shown that spermatozoa contain CA II (Parkkila *et al.* 1991), but no isoenzymes have been reported in other cell types in human testis. It is noteworthy that the mRNA of CA II in chicken, mouse, and human testis diverges from the somatic CA II mRNA in the 5′ and 3′ untranslated regions (Mezquita *et al.* 1994, Mezquita *et al.* 1999). Immonohistochemical studies have also shown CA II to be expressed in rat dorsolateral prostate epithelium, and its expression is regionally controlled in a complex way by sex steroids. (Härkönen & Väänänen 1988, Härkönen *et al.* 1991). CA II is also expressed in rat seminal vesicle epithelium, where it is under androgen control (Härkönen & Väänänen 1988). There are also studies demonstrating CA II expression in rat epididymal (principal cells mainly in corpus and narrow and clear cells elsewhere) and coagulating gland epithelium (Härkönen & Väänänen 1988, Kaunisto *et al.* 1995, Wilhelm *et al.* 1998, Brown *et al.* 1999). However, the expression of

CA II in clear cells is still controversial (Kaunisto *et al.* 1995, Brown *et al.* 1999, Hermo *et al.* 2000). CA IV is coexpressed with CA II in the principal cells of rat corpus epididymidis (Kaunisto *et al.* 1995), and this expression has been shown to be under testosterone control (Kaunisto *et al.* 1999).

Both CA II and CA IV have also been detected in the human reproductive tract, and their expression pattern is different from that in rats. The seminal vesicle epithelium and occasional epithelial cells in epididymis express CA II (Kaunisto *et al.* 1990). The epithelium in the ampulla and the distal parts of vas deferens contain both CA II and CA IV (Kaunisto *et al.* 1990, Parkkila *et al.* 1993). All segments of the human epididymis express CA IV (Parkkila *et al.* 1993). CA XII mRNA has been demonstrated in human testis and prostate using the northern blot and RT-PCR techniques (Ivanov *et al.* 1998, Türeci *et al.* 1998). In a more recent study, Ivanov *et al.* (2001) demonstrated the expression of CA IX in the epithelia of efferent ducts and rete testis. They also found that CA XII is expressed in efferent duct and prostate epithelia. Moreover, the expression of CA III in testis has been demonstrated (Tashian 1989), and the sequence of CA-RP VIII has been identified from the cDNA library of human testis (Skaggs *et al.* 1993). A summary of CAs in the human male reproductive tract is shown in Table 1.

## 2.8 Carbonic anhydrase in the female reproductive tract

Carbonic anhydrase activity was first demonstrated biochemically by Common (1941) in hen oviduct homogenate. Later, its presence in human and other mammalian oviducts (Lutwak-Mann 1955) and uterus has been detected (Lutwak-Mann 1955, Matsuda 1964, Nicholls & Board 1967). The CA activity has been shown to vary during the estrous cycle, pregnancy, and in response to hormonal treatment. It is notable that estrogen stimulates CA activity in some species, whereas progesterone is the stimulant in others, including human (Lutwak-Mann 1955, Pincus & Bialy 1963, Matsuda 1964, Nicholls 1967, Maren 1967, Hodgen & Falk 1971, Falk & Hodgen 1972). Biochemical CA activity has been also demonstrated in cervical mucus (Chantler *et al.* 1977) and in the ovary of guinea pig and rabbit (Lutwak-Mann 1955, Friedley & Rosen 1975). The problem inherent in the biochemical measurements of CA activity in tissue homogenates is blood contamination. Erythrocytes contain high concentrations of active isoenzymes, CA I and CA II, which may mask tissue-specific CA activity.

The existence of a uterus-specific isoenzyme has been proposed in human uterus, which could be mainly responsible for the rise in CA activity in response to hormonal stimulation (Hodgen & Falk 1971, Falk & Hodgen 1972, Ganguly *et al.* 1978). However, no such isoenzyme has been identified so far.

Histochemical studies have confirmed the presence of CA activity in reproductive tissues. These studies have shown marked differences in CA expression between species. In humans, CA staining was found in the surface epithelium of ovary and in the granulosa cells of maturing follicles. Oviduct epithelium showed occasional basal staining, while smooth muscle was constantly stained for CA activity (Friedley & Rosen 1975). The staining distribution in uterine luminal and glandular epithelial cells was variable and diffuse, except in the luteal phase, when it was confined more basally, as in oviducts (Korhonen *et al.* 1966, Friedley & Rosen 1975). No alteration was, however, seen in the

overall staining intensity during the cycle (Friedley & Rosen 1975). In the vaginal part of portio uteri, CA activity was located basally in squamous epithelium (Korhonen et al 1966). Pig oviduct epithelium has been shown to contain membrane-bound CA in the sperm-storing region and cytoplasmic CA in the ampulla (Rodriquez-Martinez *et al.* 1991).

Three immunohistochemical studies identifying CA isoenzymes in the female reproductive tract have been published (Ge & Spicer 1988, Liao *et al.* 1994, Ivanov *et al.* 2001). The study by Ge & Spicer (1988) showed that almost all cells in mouse and rat ovary express one or all of the isoenzymes I, II and III. In mouse oviduct, CA II expression was most intense in close proximity to the ovary, while CA II was absent in rat oviduct. CA III was expressed in the oviducts of both species. In murine uterus, CA II was expressed in the glandular and luminal epithelia extending into endocervical epithelium, whereas rat uterine luminal epithelium was devoid of the enzyme. The expression of CA II appeared to vary during the estrous cycle only in mouse uterine glandular epithelium. CA III was expressed in luminal and glandular epithelia in both species. In this animal study, however, Ge and Spicer (1988) used antibodies against human CA isoenzymes. CA IX expression has been detected in occasional epithelial cells in cervix uteri (Liao *et al.* 1994, Ivanov *et al.* 2001). In addition, Ivanov *et al.* (2001) demonstrated CA IX expression in the surface epithelium and rete ovarii of human ovary. The mRNA of CA XII has also been demonstrated in ovary by northern blotting and in ovary, uterus, and breast using RT-PCR (Ivanov *et al.* 1998, Türeci *et al.* 1998). In an extensive study, Ivanov *et al.* (2001) demonstrated the expression of CA XII in some cells of human cervix epithelium, in uterine glandular epithelia at the proliferative phase, and in the surface coelomic epithelium of ovary. A summary of CAs in the human female reproductive tract is shown in Table 1.

*Table 1. Histochemical and immunohistochemical localization of CAs in human reproductive system.*

| Organ | Localization of CAs |
|---|---|
| Cervix | CA activity in epithelium [a,b], CA IX and XII in reserve cells of the glands (rare)[c,d], CA XII in basal cells of squamous mucosa (focal)[c] |
| Endometrium | CA activity in luminal and glandular epithelium[a,b], CA XII in proliferative glandular epithelium (focal)[c] |
| Oviduct | CA activity in luminal epithelium and smooth muscle[b] |
| Ovary | CA activity in surface epithelium and granulosa cells[b], CA IX (diffuse) and CA XII (focal) in surface coelomic epithelium and CA IX in rete ovarii (diffuse)[c] |
| Testis | CA activity in endothelial cells of capillaries and some large vessels[e], CA IX in rete testis (diffuse)[c], CA II in spermatozoa[f] |
| Efferent ducts | CA IX and CA XII in epithelium (diffuse)[c] |
| Epididymis | CA II in occasional epithelial cells[g], CA IV in epithelium and subepithelial smooth muscle layer[h] |
| Vas deferens | CA II in epithelium[g] and CA IV in epithelium and subepithelial smooth muscle layer[h] |
| Seminal vesicle | CA II[g] and CA XII (focal)[c] in epithelium |
| Breast | CA XII in lobular and ductal units (focal)[c] |
| Prostate | CA XII in epithelium (focal)[c] |

[a] Korhonen *et al.* 1966, [b] Friedley & Rosen 1975, [c] Ivanov *et al.* 2001, [d] Liao *et al.* 1994, [e] Ridderstråle *et al.*1985, [f] Parkkila *et al.* 1991, [g] Kaunisto *et al.* 1990, [h] Parkkila *et al.* 1993
Diffuse, ≥40% of cells within a field stain positively; Focal, <40% of cells within a field stain positively; Rare, <5% of cells within a field stain positively

## 2.9 Effects of CA inhibitors on reproduction

Harris & Goto (1984) demonstrated that inhibition of CA activity in fowl testis and ductus deferens with acetazolamide resulted in a reduced volume of spermatozoa and seminal plasma per collection. Similarly, Setchell & Waites (1967) demonstrated a marked reduction in the rate of testicular fluid secretion in ram after administration of acetazolamide. The role of CA in the acidification of epididymal fluid is still controversial. Au & Wong (1980) reported that systemic administration of acetazolamide inhibits acidification of epididymal fluid in rats, while Caflisch & DuBose (1990) found no effect. In a more recent study, Breton *et al.* (1998) showed marked inhibition in *in vitro* acidification of rat proximal vas deferens fluid with acetazolamide.

The effects of CA inhibition in the female reproductive tract have also been studied. It has been shown that carbonic anhydrase is involved in rat and mouse endometrial bicarbonate secretion. (Kyriakides & Levin 1973, Fong *et al.* 1998). Acetazolamide has been shown to abolish the disaggregating effect of tubal fluid on the coronal cells of the ovum (facilitates the penetration of spermatozoa into zona pellucida), which is known to be a bicarbonate-dependent process (Stambaugh *et al.* 1969). Acetazolamide also reduces the number of implantations, according to Böving (1963), and terminates the pregnancy if administered directly into uterine lumen (Pincus and Bialy 1963). Systemic administration of acetazolamide does not, however, have this effect (Edgren *et al.* 1971).

## 2.10 Carbonic anhydrase in mammary gland and milk protein composition and functions

CA activity has been located histochemically in the alveolar capillaries of goat mammary gland, and this activity correlates with the milk yield (Cvek *et al.* 1998). Recently, focal expression of CA XII was reported in the lobular and ductal units of human breast (Ivanov *et al.* 2001, Table 1).

The protein content of total milk is highest in colostrum (during the first few postpartal days) and decreases thereafter until mature milk is secreted (over three weeks after parturition). (Emmet & Rogers 1997, Kunz *et al.* 1999).

The human milk protein fraction contains nutrients, enzymes, growth factors, hormones, and host defence agents, most of which are glycosylated (Kunz *et al.* 1999). These components are present as soluble forms in the whey fraction, as casein micelles, or bound within the fat globule membrane. The major proteins in the human whey fraction are lactoferrin, secretory IgA, serum albumin, and α-lactalbumin. The predominant caseins are β-casein and κ-casein. The concentration of whey proteins tends

to decrease and the concentration of caseins to increase during the first few days of lactation.

Both nutritive and non-nutritive properties of milk proteins are important for the infant (Kunz *et al.* 1999). As nutritive factors, milk proteins are sources of peptides, amino acids, and nitrogen. The non-nutritive effects of milk proteins are also numerous, and their major role is to promote infantile growth and development. These proteins act as, for example, anti-microbial factors (e.g. secretory IgA, lactoferrin, and lysozyme), anti-inflammatory agents (e.g. secretory IgA, lactoferrin, lysozyme, platelet-activating factor acetylhydrolase, and epidermal growth factor (EGF)), transporters (e.g. lactoferrin), and digestive enzymes (e.g. bile-salt stimulated lipase) (Hamosh 1998, Xanthou 1998, Kunz *et al.* 1999). In addition, proteins are involved in the maturation and development of the gastrointestinal tract. These include, for instance, growth factors, such as EGF, transforming growth factor (TGF), nerve growth factor (NGF), and IGF I and II, hormones, such as insulin, thyroxin and cortisol, and other proteins, such as bombesin, lactoferrin, and neurotensin (Kunz *et al.* 1999, Xu 1996).

Many milk proteins are multifunctional. For example, lactoferrin, a 692-amino-acid glycoprotein, has bactericidal, antiviral, bacteriostatic, antiadhesive, immunomodulating, and anti-inflammatory activities. In addition, lactoferrin contributes to growth promotion in the intestine and liver and to transcriptional regulation (Hamosh 1998).

# 3 Aims of the present study

Expression of cytoplasmic CA I and II and membrane-bound CA IV has previously been detected in the male and female reproductive organs. Their function is linked to sperm motility, fertilization, and embryonic development through acidification of epididymal fluid, alkalinization of seminal plasma and uterine fluid, and regulation of acid-base balance in amniotic fluid and the developing foetus. The wide distribution of CA activity in histochemical studies of the reproductive organs suggests, however, that other isoenzymes are also expressed in these organs and that CAs have a much broarder role in the regulation of reproductive functions than has been thought earlier. The overall aim of this study was to provide a more comprehensive view of the expression of different CA isoenzymes in the male and female reproductive systems and of their linkages to reproductive functions.

The specific aims were:

1. to determine the expression of recently identified membrane-bound CA IX and CA XII in the male and female reproductive tracts,
2. to identify the CA isoenzyme(s) present in testicular Leydig cells, and
3. to elucidate the presence of CA VI in mammary gland and milk.

# 4 Materials and methods

## 4.1 Collection of human samples (I-IV)

Samples of human uterus, testis, efferent ducts, and epididymis were obtained alongside routine histopathological specimens taken during surgical operations. The specimens were fixed immediately in Carnoy's fluid (absolute ethanol + chloroform + glacial acetic acid 6:3:1) for 6 h at 4°C, dehydrated, and embedded in paraffin wax in a vacuum oven at 58°C. Sections of 5 µm were placed on microscope slides and used for immunohistochemistry. Western blotting analyses of efferent ducts and epididymis were performed using the same paraffin-embedded specimens. They were first deparaffinized in xylene for 5 minutes, washed in 100% ethanol for 5 minutes, and extracted with SDS-PAGE sample buffer according to Conti *et al.* (1988).

Colostral milk samples were obtained from 9 mothers on the 2-4th days postpartum, and saliva samples were obtained at the same time from two 3-day-old infants before nursing. Mature milk samples were obtained from four mothers on the 90th day postpartum. The milk samples were centrifuged (15000 x g) at 4°C for 10 minutes and the supernatants were subjected to SDS-PAGE. Informed consent was obtained from each mother, and the research was carried out according to the provisions of the Declaration of Helsinki.

## 4.2 Collection of rat samples (III, IV)

Rat milk samples were collected from fentanyl-fluanisone anesthetized (3ml/kg, Janssen Pharmaceutica, Beerse, Belgium) rats of the Sprague-Dawley strain on the second day postpartum. Mammary gland specimens were taken from adult female rats and from rats two days before and after parturition. Other tissues were taken from adult rats of the same strain. The tissue samples were fixed in Carnoy´s fluid and embedded in paraffin or homogenized in ice-cold 0.1 M Tris-$SO_4$ buffer, pH 8.7, containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, and 1 mM o-

phenanthroline as protease inhibitors and used for western blotting or CA purification. For CA purification, the samples were sonicated after homogenization and centrifuged at 100,000 x g for 30 min. Thereafter, the supernatants were collected and subjected to CA purification.

## 4.3 Cell culture (I, III)

Leydig tumor cells (LC-540; CCL-43; American Type Culture Collection, Rockville, USA) were grown for 3 days to confluency, trypsinized and centrifuged at 1000 RPM for 10 minutes, after which the cell pellet was subjected to western blotting. For immunohistochemistry, CHO cells transfected with CA XII cDNA and LC-540 cells were grown on plastic chamber slides and fixed in 4 % neutral-buffered formaldehyde before immunostaining.

## 4.4 Chinese hamster ovary (CHO) cells expressing CA XII (I)

The cell lines expressing CA XII were produced in the laboratory of Dr. William S. Sly at St Louis University School of Medicine, St Louis, MO. cDNAs of either wild-type or truncated human CA XII were ligated into the mammalian expression vector pCXN (Türeci *et al.* 1998). To produce a secretory form of CA XII, a stop codon was introduced at Q260. These gene constructs were used to transfect CHO-K1 cells by electroporation. After selection in 400 µg/ml G418 for 10 days, colonies were isolated and cultured. Clones secreting high levels of human CA XII into the medium were identified by CA activity assay (Sundaram *et al.* 1986).

## 4.5 Antibodies and immunoreagents (I-IV)

The polyclonal antiserum against the secretory form of human CA XII was raised in the laboratory of Dr. William S. Sly. The CA XII secreted into the medium by transfected CHO cells was affinity-purified using a sulfonamide-agarose resin and used to prepare antibody as previously described (Zhu & Sly 1990, Waheed *et al.* 1996). The polyclonal rabbit antisera to human (CA I, II, IV, V, VI, histidine-tagged CA XII fusion protein) and rat (CA II and VI) carbonic anhydrases and the monoclonal antibody to human CA IX (M75) have been produced and characterized previously (Parkkila *et al.* 1990, 1993, 1996, Saarnio *et al.* 1999, Türeci *et al.* 1998, Kaunisto *et al.* 1995, Leinonen *et al.* 2001, Pastoreková *et al.* 1992). The generation and use of rabbit antiserum against αGST-nonO fusion protein was described by Yang *et al.* (1993). The antibodies against aquaporin-1 (AQP1) (Delporte *et al.* 1996) and rat P450c17 were generous gifts from Dr. Bruce Baum (NIH, Bethesda, MD) and Dr. Michael R Waterman (Vanderbilt University, Nashville,

TN), respectively. The polyclonal antibody to human Ki-67 was commercialy available (Zymed Laboratories, San Francisco, CA).

The following secondary antibodies were used for immunohistochemistry: Fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark), FITC-conjugated goat anti-rabbit IgG (Dakopatts), FITC-conjugated goat anti-guinea pig IgG (Sigma, St. Louis, MO), tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit IgG (Dakopatts), biotinylated swine anti-rabbit IgG (Dakopatts), and biotinylated goat anti-mouse IgG (Dakopatts).

The alkaline phosphatase-conjugated goat anti-rabbit IgG, alkaline phosphatase-conjugated goat anti-mouse IgG, and peroxidase-conjugated goat anti-rabbit IgG for western blottings were from Bio-Rad Laboratories (Richmond, CA).

## 4.6 Immunohistochemistry (I-IV)

The localization of carbonic anhydrase isoenzymes, nonO/p54$^{nrb}$, P450c17, aquaporin-1, and Ki-67, was examined using the immunoperoxidase or immunofluorescence technique. The immunoperoxidase staining of Carnoy-fixed tissue sections was performed using the biotin-streptavidin complex method (Guesdon *et al.* 1979) with the following steps: (1) Pre-treatment of sections with undiluted cow colostral whey (Hi-Col, Oulu, Finland) for 40 min and rinsing in PBS. (2) Incubation for 1 h or overnight with primary antibody diluted in 1% BSA-PBS. (3) Treatment with cow colostral whey for 40 min and rinsing in PBS. (4) Incubation for 1 h with secondary antibody diluted in 1% BSA-PBS. (5) Incubation for 30 min with peroxidase-conjugated streptavidin (Dakopatts) diluted in PBS. (6) Incubation for 2 min in DAB solution containing 9 mg 3,3'-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 15 ml PBS + 5 µl 30% $H_2O_2$. The sections were properly washed in PBS after the steps 2, 4, and 5. Step 3 was only employed in (I).

Immunofluorescence staining was used for paraffin-embedded endometrium sections and neutral-buffered formaldehyde-fixed cells. For endometrium sections, the steps were briefly: (1) Pre-treatment of sections with 1% BSA-PBS for 40 min. (2) Incubation for 1 h with primary antibody diluted in 1% BSA-PBS. (3) Incubation for 1 h with FITC-conjugated secondary antibody diluted in 1% BSA-PBS. The sections were washed thoroughly after both incubations.

When cultured cells were stained, the steps were otherwise the same but 0.1% BSA-PBS was used instead of 1% BSA-PBS, and 0.05% saponin was present in all incubations and washings to permeabilize the cells.

## 4.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (I-IV)

The electrophoreses were performed in a Mini-Protean electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) under reducing conditions according to Laemmli (1970), using a 10% acrylamide separating gel and a 4% acrylamide stacking gel. The protein

concentrates exposed to the gels were measured spectrophotometrically using a Bio-Rad protein assay kit (Bio-Rad Laboratories). The molecular mass standards were purchased from Bio-Rad. The proteins were visualized from the gels with Colloidal Coomassie staining (Novex, San Diego, CA).

## 4.8 Western blotting (I-IV)

The separated proteins were transferred electrophoretically from the gels on to a PVDF membrane (Millipore Corporation, Bedford, MA) in a Novex Blot Module (Novex). The membranes were first incubated with TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 10% cow colostral whey (Hi-Col) for 30 min at room temperature followed by primary antibody diluted in TBST buffer for 1 h at room temperature or overnight at 4°C. The membrane was washed five times for 5 min with TBST buffer and incubated with secondary antibody diluted in TBST buffer for 30-60 min at room temperature. After washing four times for 5 min in TBST buffer, the polypeptides were visualized by chemiluminescence substrates (Bio-Rad Laboratories; Pierce, Rockford, IL).

## 4.9 Isoelectric focusing (I)

Isoelectric focusing (IEF) was carried out using Novex Pre-Cast vertical IEF gels (pH 3-10) (Novex) containing 5% polyacrylamide and 2% ampholytes, following the procedure recommended by Novex. The electrophoreses were performed in a Xcell II Mini-Cell unit (Novex) at a constant power of 2 watts per gel for 2 h with a voltage limit of 500 V.

## 4.10 Northern blotting (I)

Northern blotting was performed in the laboratory of Dr. Özlem Türeci at the University of Saarland, Homburg, Germany. The procedure has been described in detail previously (Türeci *et al.* 1998). Briefly, RNA was extracted from renal tumor, normal kidney, and uterus using guanidium thiocyanate-phenol-chloroform (Chomczynski & Sacchi 1987). Gels containing 10 µg of RNA per lane were blotted onto nylon membranes (Hybond N, Amersham, Buckinghamshire, UK). After prehybridization, the membranes were hybridized with the specific $^{32}$P-labeled CA XII cDNA probe (Türeci *et al.* 1998). The membranes were washed at progressively higher stringencies, followed by autoradiography.

## 4.11 Inhibitor affinity purification of CAs (I, III, IV)

Human endometrium specimens, rat tissue specimens, and cultured cells were homogenized and/or sonicated in ice-cold 0.1 M Tris-$SO_4$ buffer, pH 8.7, containing 1 mM PMSF, 1 mM benzamidine, and 1 mM o-phenanthroline as protease inhibitors. The homogenates were centrifuged at 100000 x g for 30 min, and the supernatants were subjected to affinity purification. The particle pellets were suspended in 0.1 M Tris-$SO_4$ buffer containing 1% TritonX-100 and incubated for 30 min on ice. The suspensions were centrifuged (13000 x g) for 10 min, and the supernatants were subjected to affinity purification. Human colostrum (15 ml) was centrifuged (35000 x g) for 30 min, and the clear supernatant (10 ml) was collected and mixed with 30 ml of ice-cold 0.1 M Tris-$SO_4$ buffer, pH 8.7, containing 1 mM benzamidine as a protease inhibitor, and subjected to affinity purification. Inhibitor affinity chromatography was performed using the CM Bio-Gel A coupled to p-aminomethyl benzenesulfonamide as described (Parkkila *et al.* 1990). Briefly, samples were mixed with the buffer and the affinity gel in a rotatory shaker for 1h to overnight at +4°C. The affinity gel was collected on to a cellulose filter in a funnel by gravitation and washed, after which the enzyme was eluted with elution buffer.

## 4.12 Protein sequence analysis (III, IV)

The trypsin-digested polypeptides were sequenced in the HHMI Biopolymer/W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Their protocol included matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) followed by analysis with ProFound and PeptideSearch programs.

## 4.13 Production and purification of recombinant nonO (III)

Recombinant nonO was obtained from Dr. Philip W. Tucker at the University of Texas, Austin, TX. Production and purification included the following steps: The coding region of nonO with a six-histidine tag at the N-terminus was amplified by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA) and cloned into the transfer vector of the BAC – BAC system (Gibco BRL, Life Technologies, Inc., Rockville, MD). Baculoviral DNA-containing nonO integrated into the polyhedron gene was isolated from Ecoli strain DH10BAC and transfected into sf9 insect cells (9 x $10^5$ cells). Large-scale protein expression was generated by infecting the nonO-containing virus into insert cells incubated at 27 °C for 72 h. Infected cells were harvested, resuspended in HK buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 15 mM imidazole, and protease inhibitor; Roche Molecular Biochemicals, Indianapolis, IN), lysed by sonication, and centrifuged at 5500 rpm for 30 min. The supernatant was incubated with Ni-NTA beads (Qiagen, Valencia, CA) for 1 h at 4ºC. The beads were washed 3 times with HK buffer and then eluted with elution buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 250 mM imidazole,

protease inhibitor). The eluted protein was dialyzed against buffer D and stored at –70ºC in aliquots. The purity of the preparations was estimated by SDS-PAGE to exceed 90%.

## 4.14 Carbonic anhydrase assay (III)

The CA activity of recombinant nonO protein was determined in the laboratory of Dr William S. Sly as previously described (Maren 1960, Sundaram *et al.* 1986). This method is based on the rapid pH change due to CA activity in the test solution. Briefly, the A test tube (containing phenol red (indicator color) in distilled water) is placed next to a pH standard tube (phenol red + water + phosphate, pH 7.2). $CO_2$ is continuously bubbled to saturate the test solution. After 60 seconds of uninterrupted $CO_2$ flow, diluted enzyme and thereafter barbital buffer, pH 7.9, are added, and timing by a stopwatch is begun. The reaction reaches the end point when the indicator color matches that of the 7.2 pH standard. Uncatalyzed time represents the time required for spontaneous return of the test solution to acidity due to the uncatalyzed hydration of $CO_2$ when only water instead of enzyme is added prior to the association of the barbital buffer. The CA activity unit was calculated as follows: (uncatalyzed time – catalyzed time)/catalyzed time. Specific activity was represented as CA activity units/mg of purified protein.

## 4.15 Histological staining of CA activity and electron microscopy (III)

The rat lymph nodes were stained for CA activity by the method of Hansson (1967), in which cobalt and phosphorous precipitate at sites of carbonic anhydrase activity, and ammonium sulphide converts this complex into a visible CoS precipitate. Briefly, the sections were floated on the surface of the incubation medium for 6 min. This medium was prepared by adding a freshly prepared solution of 0.75g $NaHCO_3$ in 40 ml distilled water to a solution containing 1 ml of 0.2 M $CoSO_4$, 6 ml of 0.5 M $H_2SO_4$ and 10 ml of 67 mM $KH_2PO_4$. Before mixing the $NaHCO_3$ solution, $CO_2$ was blown over the surface of the medium for 10 minutes. Thereafter, the sections were placed into a rinsing solution (9 ml of 67 mM $KH_2PO_4$ and 1 ml of 67 mM $Na_2HPO_4$ in a litre of physiological saline) followed by visualization in the blackening solution (0.5% $(NH_4)_2S$ in distilled water), rinsing in distilled water, and embedding in Epon. Sections of 66 nm were taken and studied using transmission electron microscopy. Control stainings were performed in incubation medium containing sodium acetazolamide (Diamox, Lederle Parenterals, Inc., Carolina, Puerto Rico) at a final concentration of 10 mM.

## 4.16 Binding of nonO protein and CA II to the p-amino methyl benzene sulfonamide-Affigel 10 column (III)

The binding studies were carried out in the laboratory of Dr. William S. Sly. Briefly, Sulfonamide-Affigel 10 resin (200 µl) was washed and equilibrated with 10 mM Tris-$SO_4$ buffer (pH 7.5). 7 µg of nonO protein alone or together with 7 µg of purified human CA II was mixed with equilibrated affinity resin in the absence or presence of 2 mM acetazolamide at 4°C for 30 min. Unbound protein was recovered after centrifugation. After two washes with 0.5 ml of 10 mM Tris-$SO_4$ buffer (pH 7.5), the bound protein was eluted with 1 ml of 0.5 M sodium perchlorate in 0.1 M sodium acetate (pH 5.6). The eluted proteins were concentrated and the perchlorate was dialyzed out on Centricon tubes using 10 mM Tris-$SO_4$ buffer (pH 7.5). Unbound and bound proteins were analyzed by SDS-PAGE, followed by western blotting.

## 4.17 Deglycosylation studies (IV)

Purified milk and salivary CA VI (1 µg) were digested with PNGase F digestions as described earlier (Petäjä-Repo *et al.* 1991). The deglycosylated and nondeglycosylated proteins were subjected to SDS-PAGE followed by Colloidal Coomassie staining (Novex, San Diego, CA).

## 4.18 Fluoroimmunoassay (IV)

The CA VI concentrations in human milk samples (colostrum n=9, mature milk n=4) were quantified using competitive time-resolved fluoroimmunoassay (TR-IFMA) as described in detail earlier for saliva samples (Parkkila *et al.* 1993). Briefly, purified human salivary CA VI was labelled with Eu labelling reagent according to the manufacturer´s instructions (Wallac, Turku, Finland). Sheep anti-rabbit microtitration strips (Wallac) were washed with 200 µl of Delfia wash solution (Wallac). Anti-human CA VI diluted 1:25000 in Neo hTSH Assay Buffer (Wallac) was applied to the microtitration wells (200 µl/well). After incubation for 4 h at room temperature with continuous shaking, the wells were washed six times with the wash solution. The labelled salivary CA VI specimens (100000 counts/well), standards, or milk samples (50 µl of 1:100 diluted milk) were added to the wells, and the incubation was brought to 200 µl/well (all dilutions in Neo hTSH Assay Buffer). The mixture was incubated in a shaker at room temperature for 20 h, followed by washing of the wells six times with the wash solution and shaking for 5 min with enhancement solution (200 µl/well, Wallac). Thereafter, fluorescences were measured with a 1234 Delfia Research Fluorometer (Wallac).

# 5 Results

## 5.1 Expression of transmembrane CAs in the human male and female reproductive tracts

### *5.1.1 CA IX (II)*

Human male efferent ducts were found to express CA IX (Table 2). This expression was confined to ductal epithelium but varied in the tubules. In some tubule sections, virtually all epithelial cells were stained for CA IX, including both ciliated and nonciliated cells. The presence of CA IX in efferent ducts was confirmed by western blotting. Since the function of CA IX has been linked to cell proliferation, its colocalization with a proliferation marker, Ki-67, in efferent ducts was also studied. The cellular or tubular expression patterns of CA IX had no correlation with that of Ki-67, suggesting another role for CA IX in efferent ducts. No staining of CA IX was found in epididymal duct or testis (Karhumaa *et al.* unpublished observation).

### *5.1.2 CA XII (I ,II)*

The two antibodies to CA XII employed here recognized a major 45-kDa and a minor 40-kDa band on the western blots of the CHO cells expressing wild-type CA XII, as reported earlier (Türeci *et al.* 1998). Subjection of the transfected CHO cells to immunocytochemistry in conjunction with these antibodies confirmed the distinct plasma membrane expression.

Human uterus was found to express CA XII. The expression was confined to the basolateral plasma membrane of luminal and glandular epithelia. Staining intensity was slightly stronger in the deep part of uterine glandular epithelia. In cervix uteri, only occasional epithelial cells were faintly positive. In one oviduct sample tested, epithelium showed a faint reaction for CA XII (Karhumaa *et al.* unpublished observation). The

expression of CA XII in uterus was confirmed by northern blotting. pI (6.3) was determined by isoelectric focusing, followed by western blotting.

CA XII was also found to be expressed in human efferent ducts. Virtually all cells in efferent duct epithelium were stained for CA XII. Pre-treatment of anti-human CA XII serum with recombinant CA XII protein totally abolished the immunohistochemical reaction. The enzyme was predominantly located in the basolateral plasma membrane of nonciliated cells. These cells also coexpressed aquaporin-1 (AQP1), a water channel protein. The presence of CA XII in efferent ducts was confirmed by western blotting. CA XII was also expressed in some epithelial cells in epididymal ducts. These cells coexpressed CA II, suggesting that they represent apical mitochondria-rich cells (AMRC). Table 2 summarizes the immunohistochemical findings of CA XII presented above.

*Table 2. Summary of the immunohistochemical results of CA IX and CA XII in the reproductive tract.*

| Localization | CA IX | CA XII |
|---|---|---|
| Cervix | Nd | OEC + |
| Endometrium | | |
| LE | Nd | + |
| GE | Nd | + |
| Oviductal epithelial cells | Nd | (+) |
| Epididymal epithelial cells | - | AMRC + |
| Epithelial cells of efferent duct | + | + |

Nd, not defined; OEC, occasional epithelial cells; LE, luminal epithelium; GE, glandular epithelium; AMRC, apical mitochondria-rich cells

## 5.2 Identification of a novel nonclassical carbonic anhydrase and its expression in human and rat Leydig cells (III)

Subjection of human and rat testicular sections and rat Leydig tumor cells to immunohistochemical stainings with CA II antibody revealed a specific cytoplasmic and nuclear reaction in Leydig cells and Leydig tumor cells. In western blots, however, the antibodies repeatedly recognized only an unidentified 66-kDa polypeptide in Leydig tumor cell homogenates. This polypeptide was successfully purified from several rat tissues using CA inhibitor affinity chromatography, suggesting that it represents a novel CA isoenzyme. The amino acid sequence of the polypeptide (33 % coverage) showed it to be identical to NonO/p54$^{nrb}$, a non-POU domain-containing octamer-binding protein previously implicated in transcriptional regulation. The recombinant NonO/p54$^{nrb}$ was shown to display CA activity ($CO_2$ hydration activity; 25 units/mg of protein), and it specifically bound to the CA inhibitor affinity column and showed immunological similarity to CA II on western blotting. In addition, the antibody to rat CA II revealed specific staining in the nuclei and cytoplasm and the antibody to rat NonO/p54$^{nrb}$ in the

nuclei of lymphocytes. Furthermore, CA activity was demonstrated histochemically in the nuclei of lymphocytes.

## 5.3 CA VI in milk (IV)

The subjection of human colostrum to western blotting for CA VI revealed a major 42-kDa polypeptide band identical in size to purified human salivary CA VI. Similarly, identical-sized 42-kDa and 36-kDa polypeptide bands were also obtained from rat milk and rat purified salivary CA VI samples subjected to immunoblotting with specific antibody to rat salivary CA VI. The 42-kDa polypeptide was purified from human colostrum on CA inhibitor affinity chromatography. PNGase F digestion of the purified human milk and salivary CA VI reduced their molecular size from 42 kDa to 36 kDa, indicating that both glycopolypeptides have a similar-sized polypeptide backbone. To confirm that the human milk CA is identical to the salivary isoenzyme VI, the purified colostral isoenzyme was isolated from a SDS gel, trypsin-digested, and sequenced with MALDI-MS. The analysis of the sequence data with the ProFound and PeptideSearch databases revealed 100% identity with human salivary CA VI. The sequenced polypeptides covered 40% of the full-length CA VI.

The mean concentrations of CA VI were 34.7 mg/l (range 10.0-78.4 mg/l, n=9) in colostrum and 4.5 mg/l (2.6-6.9 mg/l, n=4) in mature milk. The CA VI levels in the saliva of two infants were 1.9 and 3.6 mg/l, representing about half of the concentration in adults (Parkkila et al. 1993).

The resting mammary gland and the glands of adult female rat two days before and after parturition showed a faint positive reaction for CA VI in alveolar epithelia. Alveolar milk, however, showed a strong reaction. Rat mammary gland homogenates subjected to western blotting showed the most intense band for CA VI in the lactating gland, a moderate band in glandular tissue two days before parturition, and only a faint band in the resting gland.

# 6 Discussion

## 6.1 CA IX and CA XII in the human reproductive tract

Although histochemical studies have demonstrated a wide distribution of CA activity in the male and female reproductive tracts (Friedley & Rosen 1975, Rodriquez-Martinez *et al.* 1991, Ekstedt *et al.* 1991, Ekstedt & Ridderståle 1992), only three isoenzymes have been identified earlier in human. Cytoplasmic CA II has been demonstrated in seminal vesicle epithelium and in occasional cells in the ductal epithelium of epididymis (Kaunisto *et al.* 1990). CA IV is expressed in the apical plasma membrane of all epithelial cells throughout the epididymal duct and proximal vas deferens (Parkkila *et al.* 1993). The expression of CA IX has been demonstrated in occasional epithelial cells of the uterine cervix, where its function is unclear (Liao *et al.* 1994). In the seminal vesicle, CA II has been suggested to participate in the alkalinization of the ejaculate, and thereby in the activation of sperm motility (Kaunisto *et al.* 1990). In the epididymis, CA II and CA IV are probably involved in the acidification of epididymal fluid, thereby helping to keep the spermatozoa immotile during storage (Kaunisto *et al.* 1990, Parkkila S *et al.* 1993a).

The results of the present study show that two plasma membrane-bound isoenzymes, CA IX and CA XII, are expressed in the basolateral plasma membrane of the epithelia in the uterus and male excurrent ducts.

CA XII was present in the glandular and luminal epithelia of the endometrium during the proliferative and luteal phases and in occasional cervical epithelial cells. Recently, Ivanov *et al.* (2001) reported CA XII expression in glandular epithelium during the proliferative phase and in the basal cells of cervical epithelium.

In the human male reproductive tract, CA XII and CA IX were expressed in excurrent duct epithelium. CA IX expression was restricted exclusively to certain segments of the efferent ducts, while CA XII expression was detected in all segments, predominantly in nonciliated cells. CA XII was also expressed in certain cells of the epididymal duct. These cells also expressed CA II, suggesting that they represent AMRCs. These cells correspond to rat narrow and clear cells, which are known to be involved in the acidification of epididymal fluid (Brown *et al.* 1992, Martìnez-Carcìa *et al.* 1995,

Adamali & Hermo 1996). Similar distribution of CA IX and CA XII in efferent ducts has been reported later by Ivanov *et al.* (2001), although the cell types remained undefined.

The functional role of CA XII is possibly linked to the water and bicarbonate absorption in efferent ducts and the acidification of luminal epididymal fluid in AMRC. There are several lines of evidence to support this role of CA XII. First, CA XII is colocated with a water channel protein, AQP1, in nonciliated cells, which display a marked water-absorbing capacity (Brown *et al.* 1993). Water is absorbed from the lumen driven by sodium (and chloride) ions through AQP1 water channels (Brown *et al.* 1993, Ilio & Hess 1994, Clulow *et al.* 1994, Fisher *et al.* 1998). Second, CA XII is expressed in the proximal convoluted tubule of the kidney, where AQP1 is also expressed (Nielsen *et al.* 1993, Parkkila *et al.* 2000b). Third, CA XII is colocated with AQP2 in the same epithelial cells in the kidney collecting tubule (Parkkila *et al.* 2000b). Fourth, CA XII is also expressed in other water-reabsorbing epithelia, such as colon and gallbladder (Kivelä *et al.* 2000, S. Parkkila, unpublished observation). Moreover, Tsuruoka *et al.* (2001) have reported that the inhibition of basolateral CA in the kidney proximal tubule decreases bicarbonate (60%) and fluid (30%) absorption.

Ion and fluid transport in the male reproductive tract is a complex system involving several ion transport proteins, many of which are still unidentified. The sodium-dependent water absorption in efferent ducts is probably driven by $Na^+K^+$-ATPase, which is located at the basolateral plasma membrane of luminal epithelia (Ilio & Hess 1994). The apical entry of sodium into the epithelial cell is possibly mediated by the apically located $Na^+/H^+$ exchanger, NHE-3 (Bagnis *et al.* 2001, Kaunisto & Rajaniemi 2002). Hansen *et al.* (1999) have shown that amiloride reduces fluid reabsorption by 70 %, probably by inhibiting the $Na^+/H^+$ exchanger. Water can be transported via the water channel protein isoform, AQP1, which is located in nonciliated epithelial cells (Brown *et al.* 1993). Recently, Herak-Kramberger *et al.* (2001) demonstrated the expression of $H^+$ATPase at the apical pole and the cytoplasm of nonciliated cells, providing another mechanism for protons to exit the cell. In the renal proximal tubule, basolateral CA has been suggested to eliminate $CO_3^{2-}$ gradients across the membrane (Muller-Berger *et al.* 1997). In efferent ducts, basolateral CA XII could promote the elimination of excess $HCO_3^-/CO_3^{2-}$ from the extracellular space transported by a so far unidentified bicarbonate transporter, thus facilitating bicarbonate and water absorption (Fig. 2). CA XII would act effectively enough, since it has high catalytic activity that is only slightly lower than in the most efficient isoenzyme CA II (Ulmasov *et al.* 2000).

The narrow and clear cells of epididymal epithelium and proximal vas deferens contain both vacuolar $H^+$ATPase and cytoplasmic CA II. These cells also contain apical NBC and basolaterally located NBC and AE2. The acidification of luminal fluid in proximal vas deferens has been studied using inhibitors of these proteins. The vacuolar $H^+$ATPase inhibitor, bafilomycin, decreased acidification by 80 %, and acetazolamide inhibited proton secretion by 46 % (Breton *et al.* 1998). Acidification was also markedly decreased when basolateral bicarbonate transport was inhibited. Thus, CA II may produce the protons secreted into the lumen by vacuolar $H^+$ATPase, and CA XII may facilitate the elimination of bicarbonate from extracellular space, as in the nonciliated cells of efferent ducts.

It is noteworthy that all above cell types where CA XII is expressed shows morphological changes in estrogen receptor-$\alpha$ knockout mice (Hess *et al.* 2000). This

receptor is essential for fluid reabsorption in efferent ducts, and without its expression the male is infertile (Hess 2000). Therefore, it would be important to study in the future whether CA XII is under estrogen control and whether it thus participates in the events leading to infertility in animals missing estrogen receptor-α.

There is only limited information of the presence of ion transporters in endometrial epithelium, where the functional role of CA XII remains obscure. However, endometrial epithelium shows some similarities to efferent duct epithelium and AMRC. Like the epithelium of efferent ducts, endometrial epithelium absorbs sodium and transports water (uterine fluid volume is highest at ovulation and decreases before implantation), expressing $Na^{+}$-$K^{+}$-ATPase at its basolateral surface (Casslén & Nilsson 1984, Casslén 1986, Ge & Spicer 1988, Deachapunya *et al.* 1999). Cytoplasmic CA and vacuolar $H^{+}$-ATPase are expressed in endometrial epithelium (Korhonen *et al.* 1966, Friedley & Rosen 1975, Skinner *et al.* 1999), and CHIP28 (AQP1) mRNA is expressed in uterus (Li *et al.* 1994). In addition, the pH of uterine fluid changes during the menstrual cycle and coitus (pH is lowest at mid-cycle and rises thereafter and during coitus) (Maas *et al.* 1983, Fox *et al.* 1982). As a high-activity isoenzyme, CA XII may be involved in the regulation of water transport and the acid-bace balance of uterine fluid and, thus, participate in the maintenance of proper pH and fluid volume during fertilization and implantation. The finding that acetazolamide decreased the number of implantations in rabbits (Böving 1963) is still somewhat controversial but accordant with this hypothesis.

The endometrium undergoes extensive growth and morphological organization during the menstrual cycle. This process is dependent on proteolytic enzymes and, thus, on coordinated regulation of extracellular pH. The function of CA XII in endometrium is possibly linked to this pH regulation, since CA XII has been suggested to be involved in the acidification of the extracellular space, which facilitates tumor spread (Ivanov *et al.* 1998), and CA XII is strongly expressed in the basal part of the endometrium, where endometrial repair takes place. Knudsen *et al.* (1969) found no acetazolamide inhibition of the proliferation of endometrium in rabbits. However, the influence of the drug on the proliferation of human endometrium has not been studied and, on the other hand, the expression of CA XII has not been studied in rabbit endometrium.

The functional role of CA IX in the reproductive tract remains undetermined here. It is expressed in epithelial cells of some normal tissues, shows high expression in neoplastic cells (Liao *et al.* 1994, Pastorek *et al.* 1997, Turner *et al.* 1997, McKiernan *et al.* 1997, Saarnio *et al.* 1998, Vermylen *et al.* 1999, Pastoreková *et al.* 1997, Kivelä *et al.* 2000), and its function has been linked to cell proliferation (Pastoreková *et al.* 1992, Závada *et al.* 1993, Pastoreková *et al.* 1997, Saarnio *et al.* 1998). This role in the epithelium of efferent ducts is, however, unlikely, since its expression does not correlate with that of Ki-67, a well-known marker of cell proliferation. CA IX has also been suggested to participate in intercellular communication and cell adhesion (Pastoreková *et al.* 1997, Závada *et al.* 2000). Whether CA IX has these functions in efferent ducts remains to be established.

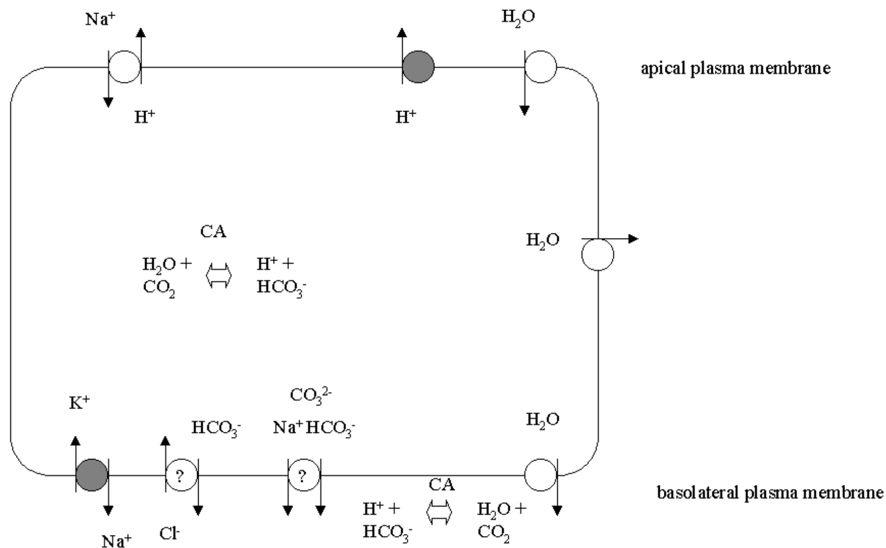


**Fig. 2. Hypothetical model illustrating the suggested role of CA XII in the nonciliated cells of efferent ducts. See text for details.**

## 6.2 Identification of a novel nonclassical CA, NonO/p54$^{nrb}$-protein

A novel CA of 66 kDa was purified in the present study from several rat tissues, using inhibitor affinity chromatography developed for CA purification. Amino acid sequencing of the polypeptide revealed that it represents the previously cloned and characterized nuclear protein, nonO/p54$^{nrb}$, a non-POU (Pit-Oct-Unc) domain-containing octamer-binding protein, which is homologous to the nuclear 54 kDa RNA-binding protein (Yang *et al.* 1993, Dong *et al.* 1993). Conventional octamer-binding proteins are a family of transcription factors that contain a DNA-binding motif, the POU domain. NonO also has homology with PSF, an essential mammalian splicing factor, and NonA, a Drosophila optomotor protein of unknown function (Yang *et al.* 1993). NonO lacks the POU domain, but it binds both RNA and DNA and is implicated in transcriptional regulation (Yang *et al.* 1993, Dong *et al.* 1993, Yang *et al.* 1997, Kamat *et al.* 1995, Lamb *et al.* 1992, Basu *et al.* 1997, Ladias *et al.* 1992, Hallier *et al.* 1996, Xin *et al.* 1992). NonO was also shown to possess CA activity. This is a novel and interesting finding, because no other class of mammalian proteins except CAs has been shown to bind specifically to the CA inhibitor affinity chromatography matrix and to contain CA catalytic activity. It is noteworthy that the catalytic activity of nonO is higher than that determined for CA III, for example (Jeffery & Carter 1980). Significant immunological cross-reactivity between CA II and nonO was also shown. The reason for this cross-reactivity is not known, but one explanation is the minor sequence homology found between CA II and nonO. The predicted amino acid sequence of nonO shares no structural elements required for

conventional CA activity. It lacks all conserved histidines, which are involved in zinc binding and have heretofore been considered essential for CA activity (Kannan *et al.* 1977, Eriksson & Liljas 1991). Nor did it show structural similarity to β or γCAs (Dr. R. Tashian, personal communication). Because the CA activity and zinc binding capacity of CA II are retained when His119 is substituted with glutamine (Lesburg *et al.* 1997), a polyglutamine stretch, Q29-Q38, in the nonO protein is a potential site for Zn binding and could also be a potential site for the CA activity of nonO. However, the tertiary structure of nonO could give more information of the CA catalytic site in nonO. CA catalytic activity in nonO explains at least part of the nuclear CA activity seen in histochemical CA stainings. It is also possible that CA activity is an important factor in transcriptional regulation, which would represent a novel function for CA. In addition, whether this CA activity in nonO affects steroid synthesis in Leydig cells will warrant attention in the near future.

## 6.3 Secretion of CA VI in milk

The present results show that CA VI is secreted into human and rat milk. The CA VI concentration in human colostral milk is approximately eightfold compared to mature milk. The concentration of CA VI in mature milk corresponds to the mean level reported earlier for adult saliva (Parkkila S *et al.* 1993b). The CA VI concentrations in saliva were found to be much lower in newborns than in adults (Parkkila S *et al.* 1993b). In addition, the saliva secretion rate of a newborn is low (Davidson 1982). Therefore, it is conceivable that the high concentrations of CA VI in colostrum possibly compensate for the low salivary CA VI and thus play an important functional role in the newborn gastrointestinal canal during the early postnatal period.

Recently, Thatcher *et al.* (1998) demonstrated that gustin, a salivary factor involved in taste function, is CA VI. Thereafter, CA VI has been suggested to function as a trophic factor that promotes the growth and development of taste buds through its action on taste bud stem cells (Thatcher *et al.* 1998, Henkin *et al.* 1999a,b). This view has been supported by the clinical disorder demonstrated by Henkin *et al.* (1999a), who described the association of a decreased concentration of salivary CA VI with pathological changes in taste buds anatomically consistent with apoptosis in a group of patients with loss and distortion of taste and smell after an influenza-like illness. Zinc treatment caused the recovery of taste and smell in patients who showed an increase in synthesis/secretion of CA VI (Henkin *et al.* 1999b). The zinc treatment also normalized the taste bud morphology. CA VI has also been shown to have characteristics similar to the nerve growth factor (NGF) (Henkin *et al.* 1988). It also specifically displaces NGF from its binding sites on purified taste bud membranes (Lum & Henkin 1977). CA VI activates calmodulin-dependent bovine brain cAMP phosphodiesterase, which is a factor involved in taste function (Law *et al.* 1987). In addition, CA inhibitors used for different clinical purposes, cause taste distortions (Hansson 1961, Graber & Kelleher 1988, Miller & Miller 1990). These observations together with the present results suggest that the high concentration of CA VI in colostrum may be linked to the development of the taste and smell function in a newborn. Interestingly, CA VI retains its structural and functional

stability in the acidic milieu of gastric secretions (Parkkila *et al.* 1997), which enables it to act as a trophic factor even in the lower gastrointestinal tract.

CA VI may be a multifunctional protein, similarly to many other glycoproteins present in milk (Kunz *et al.* 1999, Rodriquez-Palmero *et al.* 1999). As a glycoprotein and acid-base balance-modulating protein, it may have antimicrobial (Hooper *et al.* 1995), anti-inflammatory, immunomodulating, and mucosa-protecting effects (Parkkila *et al.* 1997). In fact, the results from our laboratory have shown that CA VI may bind to IgA and IgG (Parkkila *et al.* 1990, Kivelä *et al.* 1997), and it may thus be implicated in the known antimicrobial and anti-inflammatory functions of these immunoglobulins (Xanthou 1998). It will be challenging in the future to clarify the functions in which CA VI participates and to determine the possible defects resulting from its absence in milk. The presence or absence of CA VI in different milk formulas is another unsolved question, and this information would be important for the development of new formulas.

# 7 Conclusions

1. CA XII is expressed in the surface and glandular epithelial cells in the human endometrium and in only occasional epithelial cells in the uterine cervix. It probably regulates the extracellular pH on the basolateral surface of epithelial cells.
2. CA IX and CA XII are both expressed in the human excurrent ducts. CA XII is expressed in the nonciliated cells of the efferent ducts and the apical mitochondria-rich cells (AMRC) in the epididymal duct. CA IX is expressed in almost all cells in some tubule sections of the efferent ducts and is almost absent in the others. In the efferent ducts where CA XII is expressed in the same cells as AQP1, CA XII may be involved in ion-coupled water absorption from testicular fluid. In AMRC, it may participate in the acidification of epididymal fluid. The function of CA IX in the efferent ducts remains unclear.
3. NonO/p54$^{nrb}$ is a novel nuclear nonclassical CA with no amino acid sequence homology to the other known CAs. It has CA activity and immunological similarity to CA II. In the reproductive tract, it is expressed at least in testicular Leydig cells and the uterus.
4. CA VI is present in human and rat milk. Its concentration in human colostrum is several times higher than that in mature milk or saliva, suggesting that it is an essential regulating factor in the development of a newborn.

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