Histol Histopathol (2001) 16: 141-154

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Isoforms of Na^+, K^+-ATPase in human prostate; specificity of expression and apical membrane polarization

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Summary. The cellular distribution of Na^+, K^+-ATPase subunit isoforms was mapped in the secretory epithelium of the human prostate gland by immunostaining with antibodies to the α and ß subunit isoforms of the enzyme. Immunolabeling of the α1, ß1 and ß2 isoforms was observed in the apical and lateral plasma membrane domains of prostatic epithelial cells in contrast to human kidney where the α1 and ß1 isoforms of Na^+, K^+-ATPase were localized in the basolateral membrane of both proximal and distal convoluted tubules. Using immunohistochemistry and PCR we found no evidence of Na^+, K^+-ATPase α2 and α3 isoform expression suggesting that prostatic Na^+, K^+-ATPase consists of α1/ß1 and α1/ß2 isozymes. Our immunohistochemical findings are consistent with previously proposed models placing prostatic Na^+, K^+-ATPase in the apical plasma membrane domain. Abundant expression of Na^+, K^+-ATPase in epithelial cells lining tubulo-alveoli in the human prostate gland confirms previous conclusions drawn from biochemical, pharmacological and physiological data and provides further evidence for the critical role of this enzyme in prostatic cell physiology and ion homeostasis. Na^+, K^+-ATPase most likely maintains an inwardly directed Na^+ gradient essential for nutrient uptake and active citrate secretion by prostatic epithelial cells. Na^+, K^+-ATPase may also regulate lumenal Na^+ and K^+, major counter-ions for citrate.

Key words: Na^+, K^+-ATPase, Prostate, Immunohistochemistry, Membrane polarization, Apical membrane, Polymerase chain reaction

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Introduction

It is established that an important physiological function of the prostate gland is the synthesis, accumulation and secretion of citrate (e.g. Kavanagh, 1985; Farnsworth, 1990; Costello and Franklin, 1997). However, relatively little is known about some basic aspects of prostate cell physiology. In particular, the membrane transport mechanisms involved in the formation and secretion of prostatic fluid are not fully understood. It is well known that citrate secretion depends upon the accumulation of the amino acid aspartate, an important precursor that is further metabolized by transamination to oxaloacetate for citrate synthesis (Franklin and Costello, 1984). Work by Franklin et al., (1990) has shown that the concentration of aspartate is up to 43 times higher in prostate epithelial cells than serum. Two kinetically distinct Na^+-dependent aspartate transport systems maintain this large concentration gradient for aspartate. Continuous supply of aspartate to the prostate is essential for rapid secretion of citrate and ensures high-capacity aspartate uptake from plasma and prostatic secretory fluid. These Na^+-dependent aspartate transporters are inhibited *in vitro* when steroidal Na^+, K^+-ATPase inhibitors (e.g. ouabain) are present indicating the absolute dependency of aspartate accumulation, and consequently citrate secretion, on an inward Na^+ gradient generated and maintained by the plasma membrane Na^+, K^+-ATPase. Thus, the available data supports a model of citrate secretion dependent on the activity of Na^+, K^+-ATPase. The active uptake of several other amino acids and sugars by the prostate gland and the activity of the Na^+, K^+-ATPase are androgen dependent (Farnsworth, 1970, 1972, 1990). Despite the large body of pharmacological, biochemical and anecdotal evidence supporting its

function in the prostate (Farnsworth, 1993), however, the precise cellular distribution and patterns of isoform expression of Na^+, K^+-ATPase have remained unexplored. Accordingly, the goals of the present investigation were to determine the cell type specific and subcellular expression pattern of Na^+, K^+-ATPase isoforms in the adult human prostate gland.

Materials and methods

Tissues

Prostate tissues were obtained from 16 patients aged between 55 and 78 years undergoing transurethral resection of the prostate for benign prostatic hyperplasia (BPH). All prostate samples were obtained from patients of Greenwich District Hospital (Greenwich, UK), St. Thomas's Hospital (King's College London, UK) and East Surrey Hospital (Redhill, UK). Local Ethics Committee approval was obtained at the outset and proper institutional guidelines were followed throughout this study. Samples were fixed in neutral buffered formalin for at least 48 hours and subsequently embedded in paraffin wax prior to sectioning at 5-7 μm and mounting on Vectabond glass slides (Vector Laboratories, Peterborough, UK). Sections of human kidney, obtained from necropsies were used as positive controls for the detection of Na^+, K^+-ATPase expression in basolateral membranes of renal tubules.

Chemicals

All chemicals were molecular biology grade and purchased from Sigma/Aldrich and Sigma Biosciences (Poole, Dorset, UK). Peroxidase kits were purchased from Sigma/Aldrich and Vector Laboratories (Peterborough, UK). Molecular biology and PCR kits were obtained from Advanced Biotechnologies (Epsom, Surrey, UK). PCR primers were synthesized by Amersham-Pharmacia (Bucks, UK), and Sigma-Genosys (Dorset, UK).

Panel of isoform specific antibodies

Immunohistochemical studies were conducted utilizing a panel of well-characterized monoclonal and polyclonal isoform specific antibodies against known Na^+, K^+-ATPase isoforms (see Table 1 for codes, sources, dilutions and references). In addition, a monoclonal antibody to prostate specific antigen (Vector Laboratories/Novocastra, Peterborough, UK) was used as phenotypic marker for the prostatic epithelium (Fig. 1B,C).

α antibodies

(1) Monoclonal antibodies 6F and α620 against the α1-subunit isoform of the Na^+, K^+-ATPase were obtained from Dr. D.M. Fambrough (Biology Department, Johns Hopkins University, Baltimore, USA; Takeyasu et al., 1988) and Dr. Michael Caplan (Yale University, New Haven, U.S.A.; Gottardi and Caplan, 1993a,b) respectively. Antibody 6F was also purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, USA). Antibodies 6F and α620 have been demonstrated to exhibit a broad mammalian species specificity, which includes binding to epitopes of the human α1 subunit. (2) A commercial monoclonal antibody specific for the α1 isoform (designated UBIα1) was purchased from Upstate Biotechnology Inc. (Lake Placid, New York, USA). This antibody originally developed by Dr. Michael Caplan (Gottardi and Caplan, 1993a,b) was used at a final titre of 1:100. (3) Monoclonal antibody McB2 against the α2 subunit of the Na^+, K^+-ATPase was a gift from Dr. K.J. Sweadner (Massachusetts General Hospital, Harvard Medical School, Boston, USA; Urayama et al., 1989). McB2 was raised against Na^+, K^+-ATPase derived from rat brainstem axolemma and is specific for the α2 subunit, and not the ß subunit or any other isozyme of the Na^+, K^+-ATPase (Urayama et al., 1989). McB2 has been mapped to the peptide epitope sequence GREYSPAATTAENG near the N terminus of the α2 subunit isoform (Peng et al., 1997). McB2 cross reacts with many avian and mammalian species including human and recognizes α3 subunits of the Na^+, K^+-ATPase whether native, denatured or fixed. (4) Monoclonal antibody XVIF9-G10 against the α3 isoform of the Na^+, K^+-ATPase originally developed by Dr. K.P. Campbell (University of Iowa, Iowa City, USA;

Table 1. Isoform specific antibodies used in this study to detect isoforms of the Na^+, K^+-ATPase subunits in immunohistochemistry.

ANTIBODY	ISOFORM	WORKING DILUTION	SOURCE	REFERENCE
6F* (Monoclonal)	α1	Neat Supernatant	Developmental Studies Hybridoma Bank	Takeyasu et al., 1988
α620* (Polyclonal)	α1	1:100	M. Caplan	Gottardi and Caplan, 1993a,b
UBIα1 (Monoclonal)	α1	1:100	Upstate Biotechnology	Gottardi and Caplan, 1993a,b
McB2 (Monoclonal)	α2	Neat Supernatant	K.J. Sweadner	Urayama et al., 1989
XVIF9G10 (Monoclonal)	α3	Neat Supernatant	K.J. Sweadner	Arystarkhova and Sweadner, 1996; Peng et al., 1997
SpETß1 (Polyclonal)	ß1	1:600	P. Martín-Vasallo	Gonzalez-Martinez et al., 1994
SpETß2 (Polyclonal)	ß2	1:600	P. Martín-Vasallo	Gonzalez-Martinez et al., 1994

*: used as cocktail in most experiments.

Peng et al., 1997) was a gift from Dr. K.J. Sweadner. XVIF9-G10 was used at a concentration of $3\mu g.ml^{-1}$ or as neat hybridoma supernatant. XVIF9-G10 localizes the Na^+, K^+-ATPase in a variety of mammalian species expressing the α3 isoform.

ß antibodies

Polyclonal isoform specific antibodies against the ß1 and ß2 subunits of Na^+, K^+-ATPase (antisera SpETß1 and SpETß2, respectively) were developed in rabbits against fusion proteins of human ß1 and ß2 isoforms produced by over-expression in *E.coli* (González-Martínez et al., 1994). The ß-specific antisera were both diluted 1:600 for immunohistochemistry. These antibodies are highly specific for ß isoforms and have been used successfully in determining the polarized localization of Na^+, K^+-ATPase in the apical membrane of the rat inner ear and choroid plexus (González-Martínez et al., 1994).

Secondary antibodies

An affinity isolated anti-mouse IgG (whole molecule) peroxidase conjugate developed in goat was purchased from Sigma (product A-4416) and used at a working dilution ranging between 1:1000 and 1:5000. An affinity isolated anti-rabbit IgG (whole molecule) peroxidase conjugate developed in goat was purchased from Sigma (product A-0545) and used at a working dilution ranging between 1:1000 and 1:10000. Both antibodies were pre-adsorbed with human IgG to ensure minimal cross-reactivity with human proteins in tissue sections.

Immunohistochemistry

Paraffin embedded prostate sections (5-7 μm) were cleared in 100% xylene for 10 minutes before immersing in 100% ethanol. Immersing the sections in 3% (v:v) H_2O_2 in absolute methanol for a minimum of 1 hour quenched endogenous peroxidase activity. The sections were then briefly taken to water and equilibrated in phosphate buffered saline (PBS) consisting of 0.01M sodium and potassium phosphate buffer (pH 7.4), containing 27mM potassium chloride and 0.137M sodium chloride for 5 minutes before antigen retrieval. Two antigen retrieval techniques were compared and utilized in this study. The first involved microwave heating of prostate sections in a solution of 1-2% zinc sulfate for 10 minutes (Shi et al., 1991). The second antigen retrieval technique used involved incubating prostate sections in a 1% solution of sodium dodecyl sulfate (SDS) in PBS for 5-10 minutes (Brown et al., 1996). This technique gave superior results to microwave heating and was adopted as the preferred method of antigen retrieval. Prostate sections were immersed in 10% (v:v) normal goat serum (NGS) in PBS for at least one hour at room temperature to block non-specific antibody binding. Thereafter excess serum was removed and prostate sections were incubated overnight at 4 °C with the primary monoclonal and polyclonal antibodies diluted in PBS containing 1-2% NGS (see Table 1). Subsequently the sections were rinsed 3x15 min in PBS before incubation with the secondary peroxidase conjugated antibodies diluted in PBS containing 3-5% NGS for 2 hr at room temperature. Prostate sections were rinsed (4x15 min) in PBS before developing the peroxidase product. Sigma FAST DAB (3,3'-Diaminobenzidine tetrahydrochloride) tablets or Vector Laboratories DAB were used in immunohistochemistry as the precipitating agent in this reaction. For some antibodies, particularly SpETß1 and SpETß2, which exhibited extraordinary affinities for their respective antigens in the prostate sections, the Vector Laboratories DAB substrate kit for peroxidase (SK-4100) was preferred. Sections were lightly stained with haematoxylin following immunohistochemistry to reveal nuclei and mounted in DPX .

Microscopy

Slides were viewed and photographed using a Zeiss

Table 2. Sequences of primers used for the specific amplification of α isoforms. Isoform specific primers were specially designed to anneal to areas that show minimal homology and thus amplify only one isoform under high stringency PCR conditions (Mobasheri et al., 1997). Before primer synthesis, all primer sequences were checked by submitting the sequences using BLAST to NCBI (http://www.ncbi.nlm.nih.gov/). In all cases the isoform specific primers retrieved cDNA sequences of the specific isoform. Also shown is the sequence for the gene ß actin used as an internal PCR control.

PRIMER SPECIFICITY AND DIRECTION	SEQUENCE	EXPECTED PRODUCT (bp)
α1 Forward	5' CTTAGCCTTGATGAACTTCATC 3'	349
α1 Reverse	5' TGATTACAACGGCTGATAGCAC 3'	349
α2 Forward	5' CAGAAGGAGAAGGAACTG 3'	279
α2 Reverse	5' TTGTCGTTGGATGGTTCATCCTC 3'	279
α3 Forward	5' ATGACCTCAAGAAGGAGGTGGC 3'	307
α3 Reverse	5' GTTGTCAGCAGAGGGGT 3'	307
α4 Forward	5' TAACCTTGGAAGAGCTGAGCA 3'	494
α4 Reverse	5' TCCTTGTGCAGAGATAAGCC 3'	494
ß-actin Forward	5' TTCAACTCCATCATGAAGTGTGACGTG 3'	310
ß-actin Reverse	5' CTAAGTCATAGTCCGCCTAGAAGCATT 3'	310

Axioskop microscope fitted with an MC 100 SPOT 35 mm microscope camera with automatic exposure control. Kodak TMAX professional black and white film and Kodak Ektachrome 64 Tungsten color film were used throughout this study.

Human prostate cDNA library

A Human prostate 5'-stretch plus cDNA library was purchased from Clontech laboratories (Palo Alto, California, USA). The cDNA library was prepared from whole prostates of 10 caucasians, aged between 14-60 with healthy prostates. The library was amplified once in XL-1-Blue and was found to contain $3.0x10^6$ independent clones with an average cDNA size of 1.7kb.

Polymerase Chain Reaction (PCR)

PCR was employed to confirm data obtained using the α isoform specific monoclonal antibodies. Oligonucleotide primers, complementary to nucleotide sequences in the known human α isoform genes (α1, α2, α3 and α4) were synthesized (Amersham-Pharmacia, UK and Sigma-Genosys, UK). The sequences for the isoform specific primers used are shown in Tables 2 and 3 (Mobasheri et al., 1997; Stengelin and Hoffman, 1997). The Reddy-Load PCR Master Mix (Advanced Biotechnologies, Surrey, UK) was chosen as the PCR reagent of choice due to the high fidelity and efficiency of its constituent Taq DNA polymerase. PCR was carried out for 40 cycles. PCR products were loaded directly onto 1% horizontal agarose gels buffered with Tris-borate EDTA (TBE), separated electrophoretically over 45 min and stained for 15 min with ethidium bromide before visualization under UV light. In cases where high-resolution electrophoretic separation was desired 12% TBE buffered polyacrylamide gels were used instead of agarose gels. Gels were photographed digitally using a Pulnix digital video camera equipped with a dedicated macro-lens attached to an IBM PC running DT-Acquire (Data Translation, Wokingham, UK) and Scion Image analysis software (Scion Corporation, USA).

Results

All the prostate tissue used in this study was obtained from normal subjects but the majority of the tissues exhibited classic morphological features of BPH (Fig. 1A). The tubulo-alveoli of the prostate gland varied enormously in size and form. The appeared as alveoli, as alveoli with branches, tubes or tubes with branches. Many of the individual secretory glands appeared distended or collapsed. The epithelial cell layer consisted mainly of columnar cells, although some basal cells were present. For this reason, the epithelium is often classified as pseudostratified columnar. Many epithelial cells possessed apical cytoplasmic protrusions that appeared to break off into the lumen. The stroma surrounding the glands consisted of smooth muscle cells, neuroendocrine cells and blood vessels (blood vessels not visible in Fig. 1A). Immunohistochemical studies using an antibody to prostate specific antigen (PSA) revealed abundant expression in the prostatic epithelium. (Fig. 1 A,B).

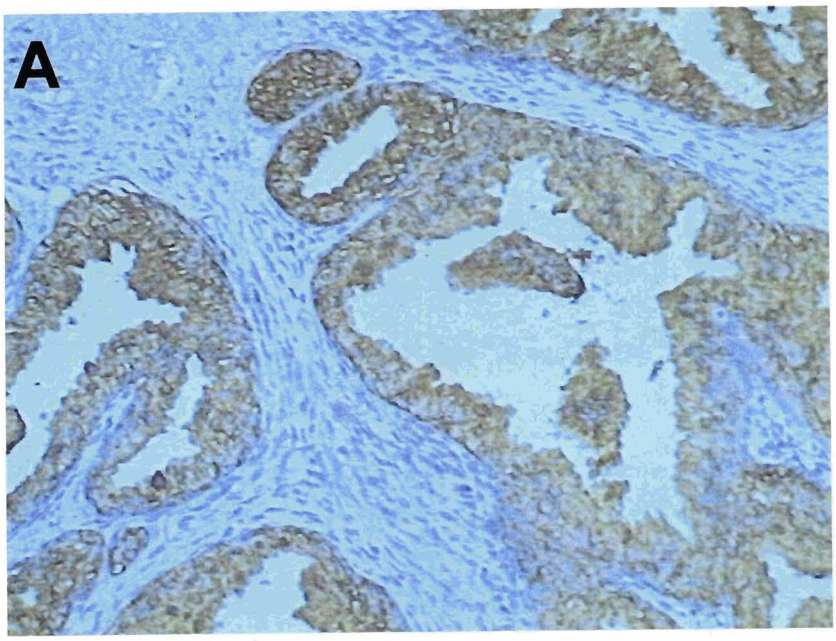
The most striking feature of the immunohistochemical data presented in this study was that Na^+, K^+-ATPase α1, ß1 and ß2 isoforms were abundantly expressed in the prostatic epithelial cells (Figs. 2-4). In contrast, antibodies specific for the α2 and α3 isoforms (McB2 and XVIF9-G10, respectively) gave no immunostaining in paraffin sections whether or not subjected to antigen retrieval (Fig. 5). The α1 isoform of Na^+, K^+-ATPase was found in the epithelial cell layer of prostate tubulo-alveoli varying in size and shape. Data shown in Figs. 2, 3 (panels A,B) summarize the results of experiments in which a cocktail of two α1 specific monoclonal antibodies was used on prostate sections and human kidney sections (used as controls). Although some staining was observed in vascular smooth muscle (Fig. 2 panels A-C) the strongest immunostaining was observed in epithelial cells (Fig. 2 panels A-C). The α1 immunostaining in the epithelial cell layer was clearly apical and lateral in most tubulo-alveoli examined.

Table 3. Sequences of alternative primers for amplification of Na^+, K^+-ATPase α isoforms and the ß isoforms (Stenelin and Hoffman, 1997).

PRIMARY SPECIFICITY AND DIRECTION	SEQUENCE	EXPECTED PRODUCT (bp)
α1 Forward	5' ATGGGGAAGGGGGTTGGACGTGATAA 3'	530
α1 Reverse	5' TTCTCACCATTTCGAATCACAAGGGCTT 3'	530
α2 Forward	5' GGCGGCAAGAAGAAACAGAAGGAG 3'	612
α2 Reverse	5' TGGGGCTCCGACTCTCCTGTTAAG 3'	612
α3 Forward	5' ATGGAGATACACGACAAGAAAGATGACAAGGAC 3'	540
α3 Reverse	5' CCCGACCACCACCTCCTCAGCGTTCACCTGCAT 3'	540
ß1 Forward	5' ATGGCCCGCGGGAAAGCCAAGGAGGAGGGCAGC 3'	441
ß1 Reverse	5' CTTTCGCTCTCCTCGTTCATGATTAAAGTCTCC 3'	441
ß2 Forward	5' ATGGAGTCCTCAACTACCCCAAACTGGCCT 3'	455
ß2 Reverse	5' TCAGGTTTTGTTGATGCGGAGTTTGAAGGCCAC 3'	455
ß3 Forward	5' AAGAAGTCCCTCAACCAGAGCCTGGC 3'	414
ß3 Reverse	5' TGCAACATAAACTGGACCCTTCTGT 3'	414

Intense peroxidase activity was detected in the apical "domes" and lateral membranes of the epithelial cells. In contrast in human kidney sections the α1 and ß1 subunit isoforms were detected exclusively in the basolateral domain of convoluted tubules (Fig. 3 panels C, D). Negative controls consisting of prostate sections incubated exclusively with peroxidase labeled secondary antibodies (omitting the primary monoclonal) gave no discernible immunoreactivity and the background staining was negligible (Fig. 2 panel D). Experiments in which antigen retrieval was not performed prior to routine immunolabeling produced significantly lower immunostaining (results not shown). From a methodological viewpoint, the results obtained following SDS antigen retrieval are consistent with previous reports of increased α1 isoform detection in the kidney and cultured MDCK cells (Brown et al., 1996).

Prostate sections incubated with the ß isoform specific polyclonal antisera gave intense immunostaining of both ß1 and ß2. As with the pattern of α1 expression, both ß1 and ß2 were expressed by the epithelial cells (Fig. 4 panels A, C) and concentrated in the apical and lateral membranes of the cells (Fig. 4 panels B,D). Portions of the smooth muscle layer were immuno-



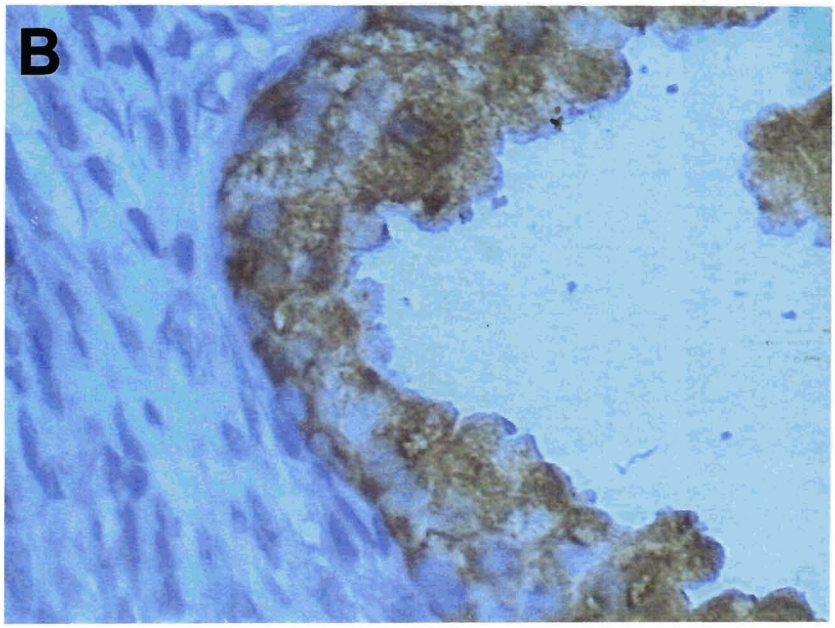


Fig. 1. Low **(A)** and high **(B)** power immunohistochemical digital photomicrographs showing expression of prostate specific antigen (PSA) in the prostatic epithelium. The majority of sections examined exhibited classic morphological features of benign prostatic hyperplasia (BPH) (panel A). A, x 400; B, x 1,000

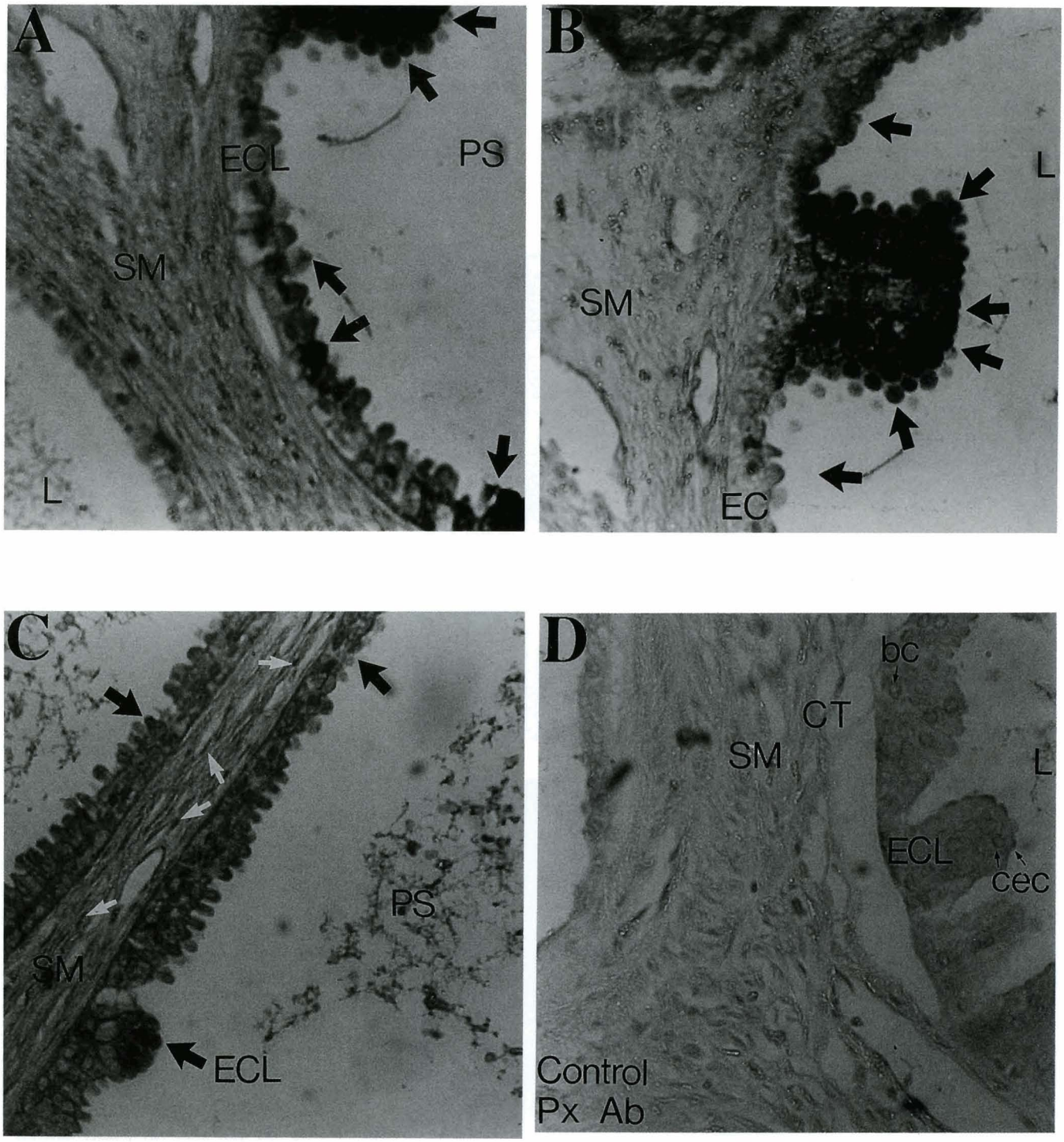


Fig. 2. Immunolocalization of Na^+, K^+-ATPase $\alpha 1$ isoform in the human prostate gland. **A.** Light micrograph demonstrating abundant expression in epithelial cells (ECL) and to a lesser extent in the smooth muscle cells (SM). The most abundant immunostaining is related to the apical membranes and significantly enhanced in a subset of cells. Expression of the $\alpha 1$ isoform appears more pronounced in apical and lateral membranes of epithelial cells of hyperplastic nodules **(B)** but is less marked in non-hyperplastic zones of larger tubulo-alveoli **(C)**. Sections incubated without primary antibody (secondary peroxidase-conjugated antibody only) produced no staining and background staining is negligible **(D)**. PS: prostatic secretion; L: lumen; CT: connective tissue; EC/ECL: epithelial cell layer; Px Ab: peroxidase antibody; cec: columnar epithelial cell; bc: basal cell. x 400

reactive, particularly for ß2. The apical and lateral membrane domains of the epithelial cells demonstrated by far the strongest peroxidase staining compared to basolateral surfaces. Overall there was no significant cellular heterogeneity in Na^+, K^+-ATPase expression in ducts or glands. The fact that Na^+, K^+-ATPase localized to different membrane domains in the prostate and kidney indicated differential plasma membrane sorting and targeting in these two tissues.

PCR using Na^+, K^+-ATPase α isoform specific primer pairs was used as described previously (Mobasheri et al., 1997; Stengelin and Hoffman, 1997). Specific DNA fragments corresponding to the α1 isoform were amplified from prostate cDNA libraries pooled from subjects with normal prostates (Fig. 6A,B). The 349 bp and 530 bp DNA fragments amplified using α1 specific primer sets confirmed that normal human prostate contained mRNA only for the α1 subunit of Na^+, K^+-ATPase. Absence of DNA fragments from lanes corresponding to α2, α3 and α4 confirmed the absence of mRNA encoding these polypeptides (Fig. 6B). PCR products corresponding to ß1 and ß2 were detected but

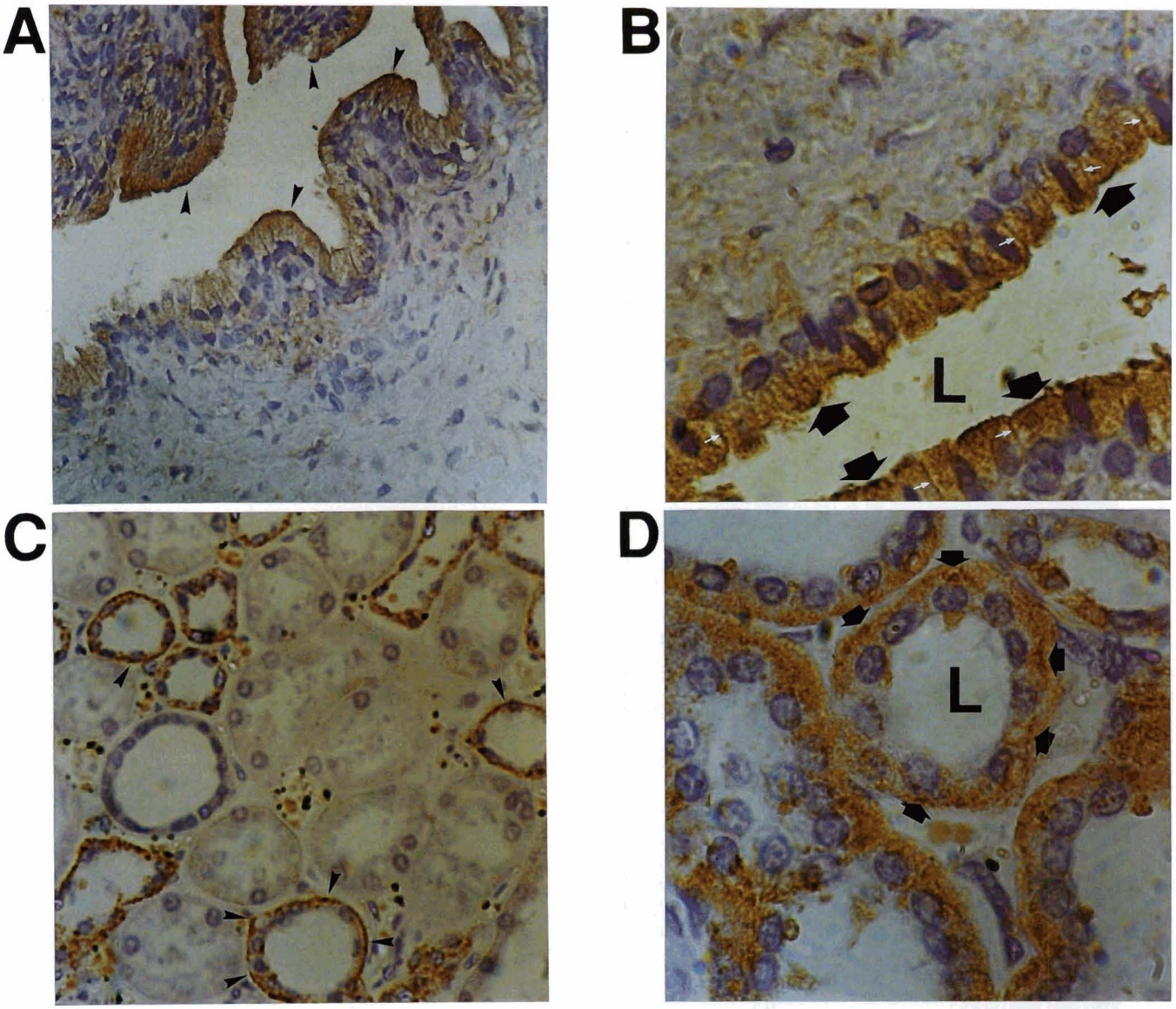


Fig. 3. Expression of the α1 isoform of Na^+, K^+-ATPase in different membrane domains of human prostate and kidney sections. **A and B.** Low and high power immunohistochemical photomicrographs of human prostate stained for the α1-subunit using monoclonal antibodies 6F/α620 showing intense staining of the apical (large black arrows) and lateral (white arrows) domains of epithelial cells. Immunohistochemical preparations of human kidney localized the α1 isoform of Na^+, K^+-ATPase to basolateral membranes of cells (black arrows in panel D) lining the convoluted tubules (panels C and D). A, C, x 400; B, D, x 1,000

ß3 expression was not detected (Fig. 6A).

Discussion

Na^+, K^+-ATPase is an integral membrane enzyme responsible for regulating intracellular Na^+ and K^+ concentrations using ATP as an energy source (Mobasheri et al, 2000). It maintains a low intracellular Na^+:K^+ ratio in face of an inward concentration gradient for Na^+ and an outward gradient for K^+ (see Sweadner, 1989, 1995). The enzyme is composed of a 110 kDa catalytic α subunit and a 35 kDa regulatory ß subunit (45-55 kDa including N-linked sugars) (Sweadner, 1995). The α subunit contains the binding sites for Na^+, K^+, ATP and cardiac glycosides, a class of steroid compounds, which serve as specific inhibitors of Na^+, K^+-ATPase. The ß subunit is required for biogenesis (Ueno et al., 1997) and activity of the enzyme complex which has been proposed to be a heterodimeric protomer $(\alpha\text{-}ß)_2$ (Brotherus et al., 1983; Fambrough et al., 1994). Thus far four α, and three ß subunit isoforms of Na^+, K^+-ATPase have been identified in mammals (Martín-Vasallo et al., 1989; Shamraj and Lingrel, 1994; Malik et al., 1996). The α1 isoform serves as the 'housekeeping' isoform, as judged by its abundance and ubiquitous cellular distribution. It is believed to be the dominant α isoform in absorptive epithelia (Farman, 1996). The remaining isoforms exhibit restricted patterns of tissue

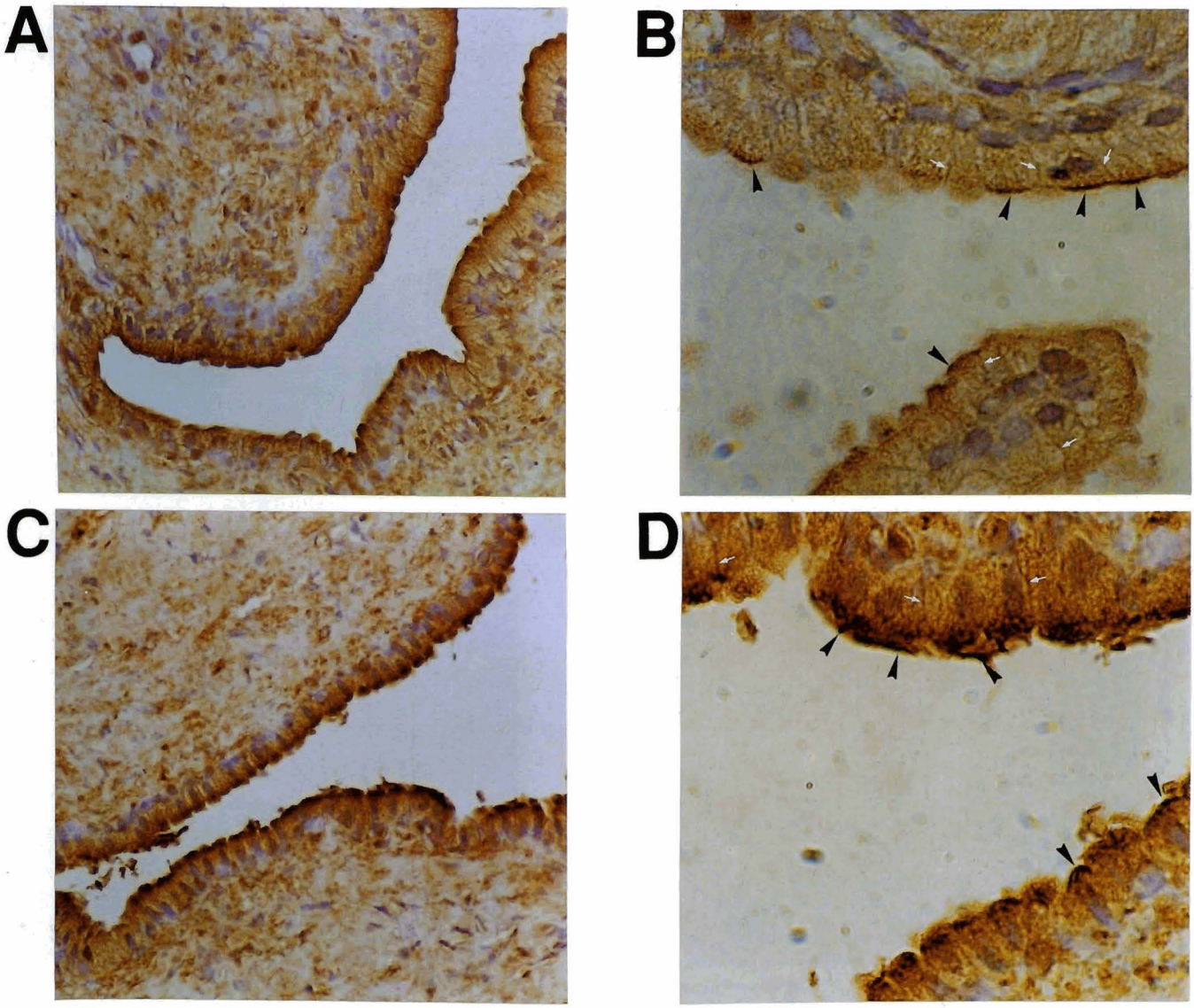


Fig. 4. Immunolocalization of Na^+, K^+-ATPase ß1 and ß2 isoforms in the human prostate gland. **A and C.** Low power light micrographs demonstrate abundant expression of ß1 **(A)** and ß2 **(C)** in epithelial cells. **B and D.** High power light micrographs demonstrate abundant expression of ß1 **(B)** and ß2 **(D)** in apical (indicated by black arrows) and lateral membranes (shown by white arrows) of epithelial cells. A, C, x 400; B, D, x 1,000

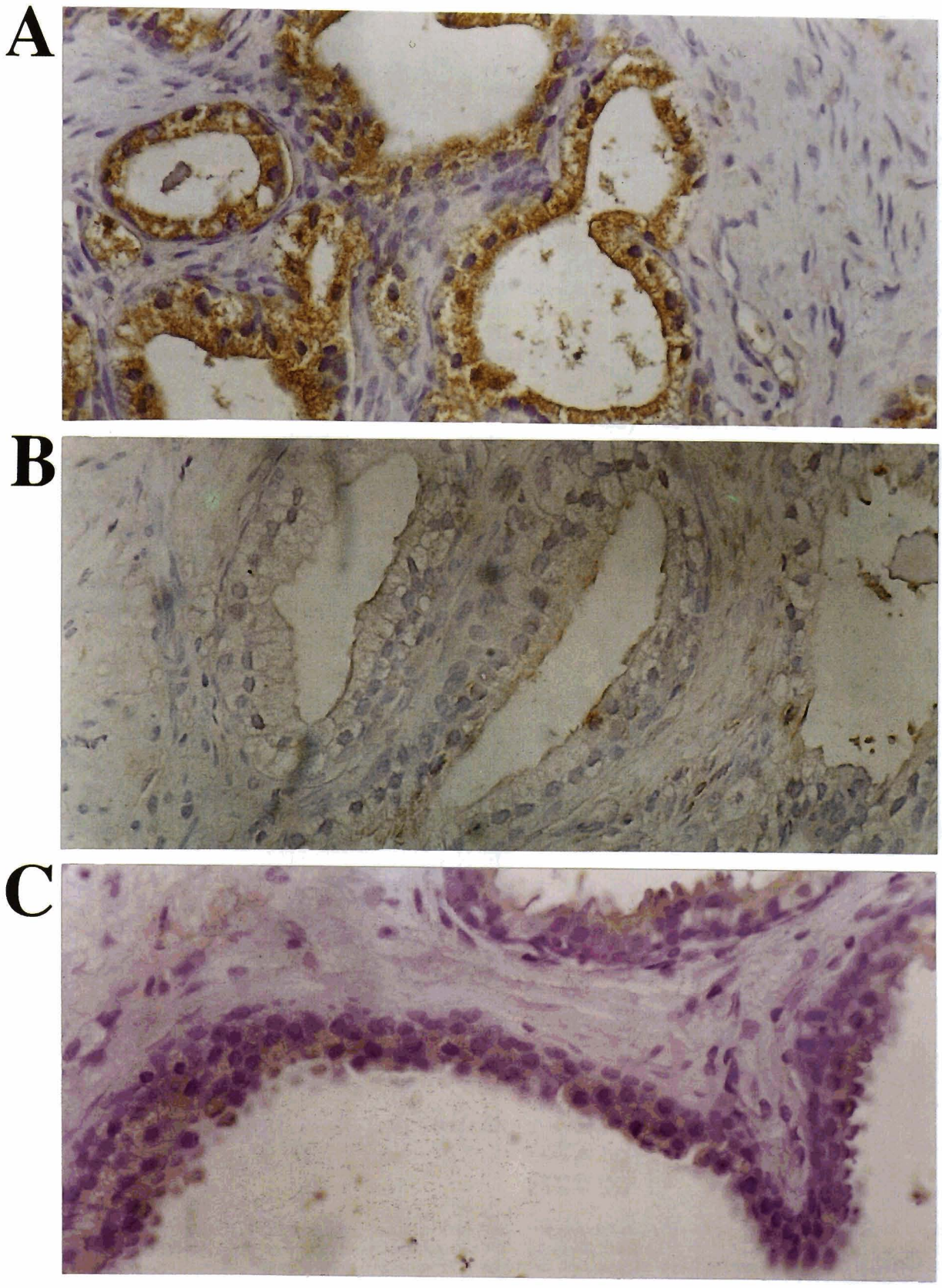


Fig. 5. Photomicrographs comparing relative immunolabeling of Na^+, K^+-ATPase $\alpha 1$, $\alpha 2$ and $\alpha 3$ in the human prostate gland. The $\alpha 1$ isoform was expressed abundantly in the epithelial cells **(A)** but no staining was observed for $\alpha 2$ **(B)** and $\alpha 3$ **(C)** in the epithelial cells. x 1,000

specificity and developmental expression (Mobasheri et al., 2000). The α2 isoform is expressed most abundantly in cardiac muscle, skeletal muscle and adipose tissue (Sweadner et al., 1994). The α3 isoform is found in high concentrations in neuronal cells in the central nervous system (Sweadner, 1995). The α4 isoform appears to be specific to the testis (Shamraj and Lingrel, 1994). The ß1 isoform is ubiquitously expressed whereas ß2 appears to be concentrated in nervous tissue (Martín-Vasallo et al., 1989; Lecuona et al., 1996; Peng et al., 1997); ß2 is an adhesion molecule on glial cells (AMOG) specifically involved in mediating interactions between neuronal cells and glia (Gloor et al., 1990). The ß3 isoform is the most recently described member of the ß isoform gene family and is expressed predominantly in testis but also in the brain, kidney, lung, spleen, liver and intestines (Malik et al., 1996; Arystarkhova and Sweadner, 1997; Peng et al., 1997).

Uptake and accumulation of citrate precursors such as aspartic acid by the prostate epithelium have been shown to be Na^+ dependent and abolished by ouabain treatment (Lao et al., 1993), which also has dramatic effects on the vascular tone of the prostate (Guh et al., 1996). Thus, Na^+-dependent aspartate transport and subsequent metabolic steps leading to citrate secretion critically depend on the activity of Na^+, K^+-ATPase. Although much is known about amino acid transport and metabolism in the prostate (Franklin et al., 1990; Lao et

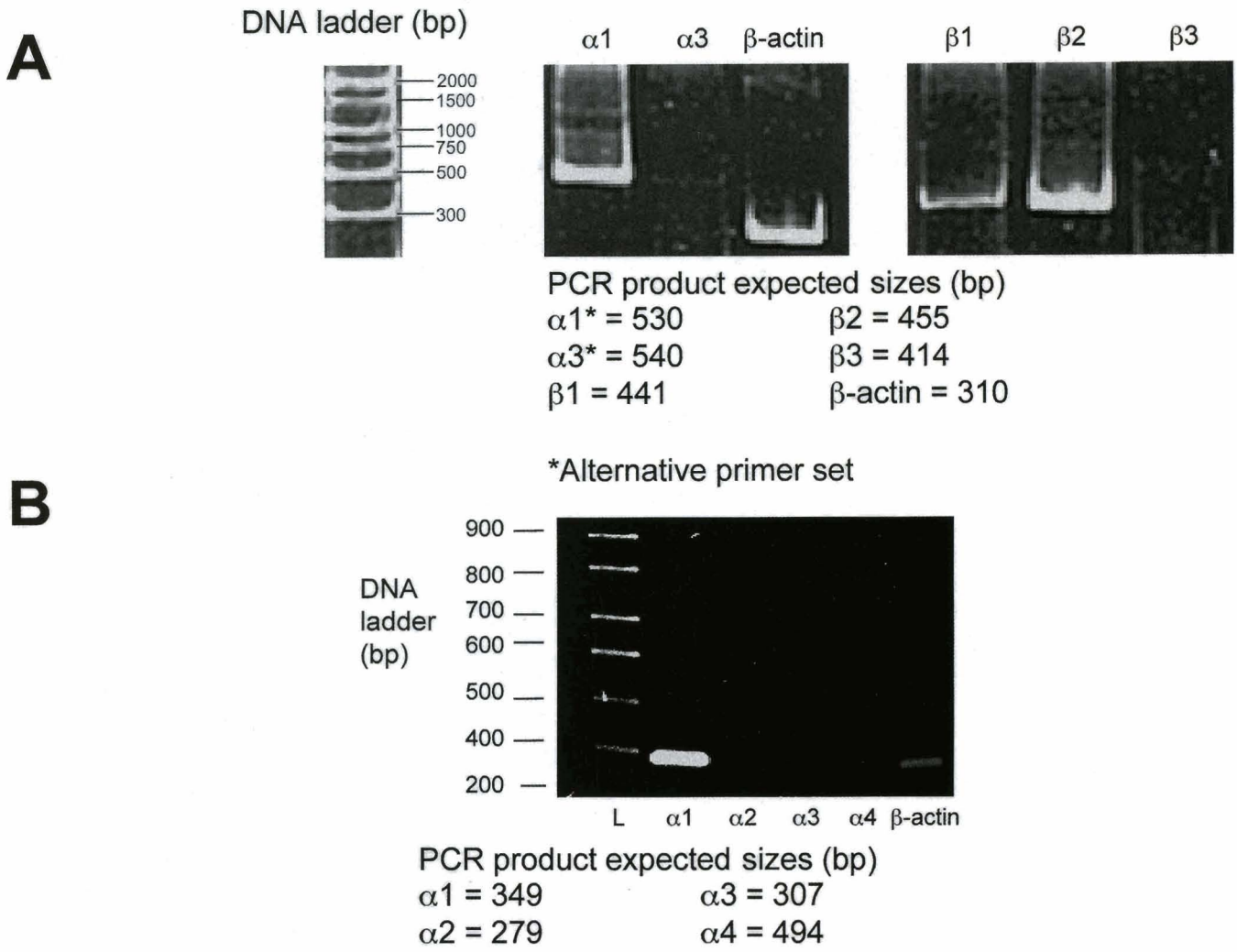


Fig. 6. Evidence for expression of α1, ß1 and ß2 isoforms of Na^+, K^+-ATPase in human prostate. PCR using human α and ß isoform specific primers (Mobasheri et al., 1997; Stengelin and Hoffman, 1997; see tables 2 and 3) established the presence of α1, ß1 and ß2 and the absence of α2, α3, α4 and ß3 mRNA. **A.** Using oligonucleotide primer sets originally developed by Stengelin and Hoffman (1997) PCR products of approximately 530 bp, 441 bp and 455 bp corresponding to α1, ß1 and ß2 were amplified from a prostate cDNA library. Expected products of 540 bp and 414 bp corresponding to α3 and ß3 were not detected. **B.** In a separate experiment using oligonucleotide primers originally described by Mobasheri et al. (1997),a 349 bp PCR product corresponding to the α1 isoform was detected in the human prostate cDNA library but products corresponding to α2, α3 and α4 were not detected. In all PCR experiments expression of ß-actin (310 bp product) was used as internal positive control.

al., 1993), less is known about Na+, K+-ATPase. Reports of Na+, K+-ATPase function in the human prostate gland have been limited to biochemical and pharmacological studies (Wilson and Villee, 1975; Hirano et al., 1994). It has been hypothesized that Na+, K+-ATPase may serve as a plasma membrane receptor for androgen in the fully developed prostate where its hormonal activation serves as a metabolic pacemaker (Farnsworth, 1990, 1993). However, no one has been able to provide any experimental data to support this hypothesis so far. Recent *in vitro* studies employing prostate cancer cell lines have demonstrated that expression of the ß1 subunit of Na+, K+-ATPase is androgen responsive and both ß1 mRNA and protein are down-regulated in its presence (Blok et al., 1998, 1999). Thus, there is indirect evidence that androgen influences Na+, K+-ATPase expression. Despite the insensitivity of Na+, K+-ATPase α1 subunit expression to androgen in prostate cancer cell lines the plasma membrane density of the enzyme complex appears to be significantly down-regulated in the presence of androgen indicating regulation of this transport system by androgen (Blok et al., 1999). It has also been proposed that Na+, K+-ATPase is involved in regulating an important physiological function of the prostate gland, namely citrate synthesis and secretion, a Na+ dependent process (Farnsworth, 1993). Therefore, Na+, K+-ATPase plays a pivotal role in the normal physiology of the prostate gland by generating steep transmembrane concentration gradients for Na+ that is utilized by a variety of other Na+ dependent transport systems for the Na+ dependent uptake of metabolites (amino acids and sugars; Rossier et al., 1987), regulation of prostatic fluid pH and control of citrate secretion. Thus far, evidence for the expression and localization of

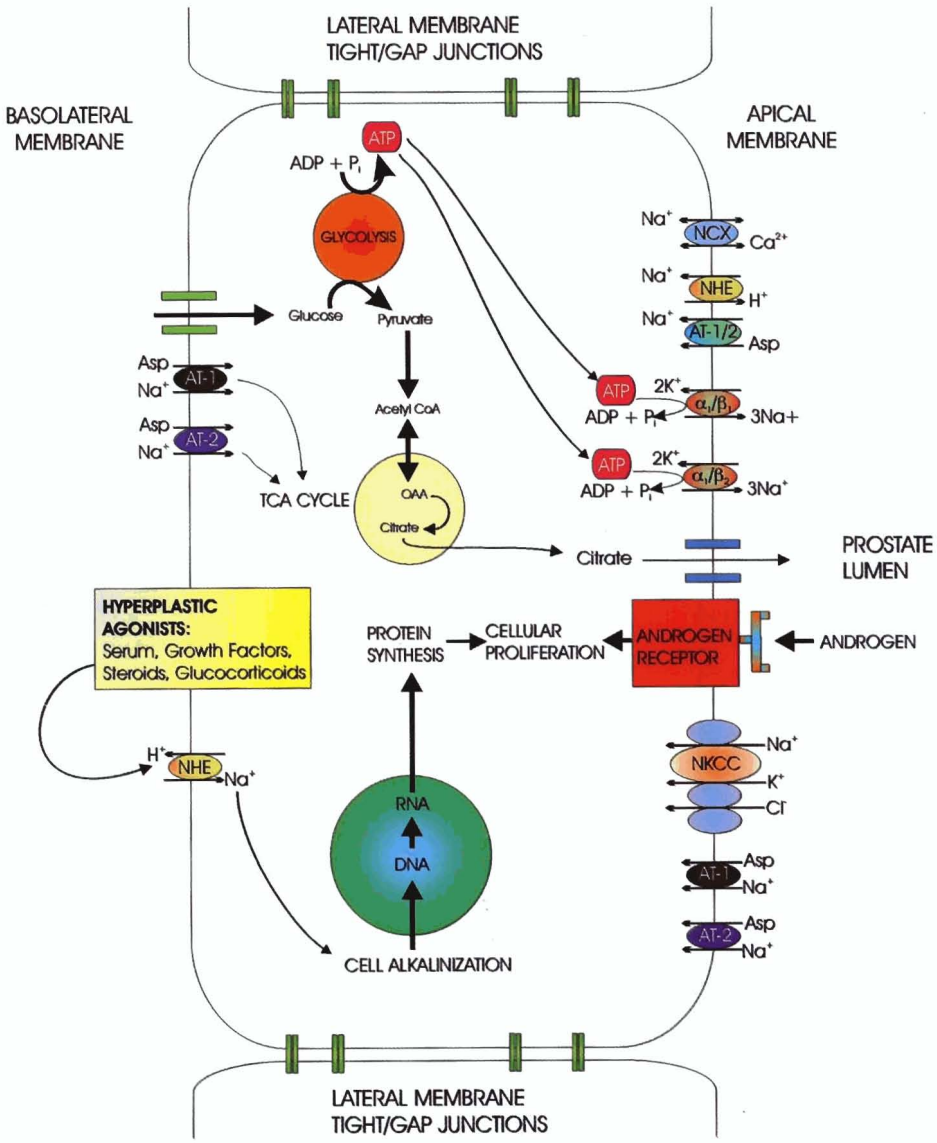


Fig. 7. Model of physiological ion transport in the prostate secretory epithelium. This proposed model is a revision of the original model proposed by Farnsworth (1993). The new model incorporates the data of Franklin et al. (1990), Lao et al. (1993), the data presented in this study as well as some of our unpublished observations. Glycolysis and oxidative phosphorylation provide the ATP required by ATPase pumps. The molecular composition of the individual transport proteins shown, and their membrane localization, is based on data presented in this article and unpublished immunohistochemical data. The proposed model places Na+, K+-ATPase consisting of α1/ß1 and α1/ß2 isozymes predominantly in the apical membrane domains where their function is determined by the intracellular Na+:K+ ratio, transmembrane gradients of Na+ and K+ and ultimately availability of intracellular ATP (Farnsworth, 1991, 1993). The presence of Na+ dependent aspartate transport systems and the cation exchangers (NHE) in basolateral and apical membrane domains of secretory epithelial cells (Franklin et al., 1990; Lao et al., 1993) identifies major entry routes for Na+. Na^+/Ca^{2+} (NCX) exchanger and $Na^+/K^+/Cl^-$ (NKCC) co-transporter mechanisms represent two alternative entry routes for Na+ also involved in intracellular Ca^{2+} regulation and volume homeostasis respectively. The identities of the citrate transporter(s) in the apical membrane and the glucose transporter(s) in the basolateral membrane are not known. Na+, K+-ATPase in the apical membrane is abundantly expressed and is responsible for active ejection of Na+ ions that gain access to the prostate cell cytoplasm via Na+ dependent transport mechanisms shown and others that remain to be identified.

Na^+, K^+-ATPase in specific membrane domains of prostatic epithelial cells has been indirect and anecdotal. Based on biochemical data, Lao and co-workers (1993) and Farnsworth (1993) have suggested that Na^+, K^+-ATPase is localized in the apical membrane of the columnar epithelial cells in the prostate. Although the apical localization of Na^+, K^+-ATPase perfectly fits the plasma membrane receptor model of prostatic secretory function proposed by Farnsworth (1993; see Fig. 7), no immunohistochemical evidence has been provided to support this notion until now. To our knowledge there are no published reports of the cellular localization, molecular composition and isoform distribution of Na^+, K^+-ATPase in the human prostate gland. Therefore, we employed a well-characterized panel of isoform specific antibodies to unambiguously identify the domain localization of prostatic Na^+, K^+-ATPase, and its isoform composition and relative abundance.

Expression of the catalytic α1 isoform and the regulatory ß1 and ß2 isoforms suggests that the system may be composed of α1-ß1 and α1-ß2 isozymes. Previous evidence for the domination of α1 containing Na^+, K^+-ATPase complexes comes from pharmacological studies in which 10^{-5} M ouabain has been shown to inhibit less than 30% of Na^+, K^+-ATPase enzyme activity (Hirano et al., 1994). Thus, 70% or more of the enzyme activity remains suggesting that the prostatic Na^+, K^+-ATPase is relatively resistant to ouabain. This high level of insensitivity to ouabain is indirect evidence for the dominance of α1 isoforms. Thus, based on this collateral evidence, it is unlikely that prostatic Na^+, K^+-ATPase contains ouabain sensitive α2 and α3 isoforms characteristic of excitable tissues (Jewell and Lingrel, 1991). Our immuno-histochemical data support this notion. Furthermore, the polymerase chain reaction provides firm evidence for the presence of transcripts encoding α1 and the absence of mRNA for α2 and α3 from normal human prostate.

Interestingly, the α1-ß1 complex is the most ubiquitous combination and contributes significantly to Na^+ reabsorption in absorptive epithelia such as kidney where Na^+, K^+-ATPase is confined to the basolateral membrane of cells lining the proximal tubule (Farman, 1996). Thus, dominance of α1 expression in the prostate was expected as this isozyme combination is ubiquitous (Mobasheri et al., 2000). The apical/lateral Na^+, K^+-ATPase cannot be involved in Na^+ reabsorption in the prostate. It is far more likely that it regulates the concentration of lumenal Na^+ and K^+ ions. Indeed, reports of Na^+, K^+-ATPase expression in other secretory epithelia have shown a particularly enhanced expression of ß subunit isoforms of Na^+, K^+-ATPase in apical membranes of cuboidal cells of the choroid plexus and the ampullary crests of the inner ear's semicircular ducts (González-Martínez et al., 1994). Smith (1969) demonstrated that Na^+ and K^+ are dominant counter-ions for secreted citrate in prostatic fluid; thus it may be speculated that apical Na^+, K^+-ATPase finely regulates the concentration of counter-ions according to the citrate levels present in prostatic fluid. Na^+, K^+-ATPase in lateral membrane domains probably plays a housekeeping homeostatic role. This model fits with a number of previous studies that have reported the range of ion concentrations found in prostatic and seminal fluids. Sodium (153 mM), potassium (48 mM) and chloride (38 mM) have been reported for normal prostatic fluid (Huggins et al., 1942) although the range of ion concentrations may vary widely according to age, secretory activity or the presence of prostatic disease (Kavanagh, 1985). Of particular importance is the finding that increased prostatic citrate secretion is accompanied by increased concentrations of Na^+, K^+, Ca^{2+}, Mg^{2+} and Zn^{3+}, and by decreased Cl^- (Kavanagh, 1985). Citrate will have low membrane permeability so that its secretion results in movement of diffusible ions to maintain the new Donnan equilibrium conditions. Citrate has a high affinity for the divalent cations Ca^{2+}, Mg^{2+} and Zn^{3+} (Sillen and Martel, 1971), which may be expected to be co-transported across the prostate secretory epithelium as a citrate complex. However, this is unlikely for unbound Na^+ and K^+, whose concentrations are tightly regulated in prostatic secretions, and likely to be achieved by the specific action of Na^+, K^+-ATPase and other ion transport mechanisms (i.e. epithelial Na^+ channels, K^+ channels, $Na^+/K^+/2Cl^-$ co-transporter, Na^+/H^+ exchanger) some of which are present in prostate epithelial cells (Mobasheri, unpublished observations).

Polarized expression of α1, ß1 and ß2 isoforms to apical and lateral membranes of prostatic epithelial cells would suggest a specific role in contrast to many other absorptive epithelia in which Na^+, K^+-ATPase is primarily targeted to basolateral membrane domains. Furthermore, apical localization of the Na^+, K^+-ATPase is concordant with the role of prostatic epithelial cells in prostatic fluid production: ouabain-sensitive Na^+, K^+-ATPase-mediated transport is implicated in prostatic fluid formation since Na^+ and K^+ are major counter-ions for citrate which, together with Cl^-, represent a large component of prostatic fluid osmolarity (Smith, 1969). Our immunohistochemical observations taken together with other published information allows us to propose a revision of the prostate plasma membrane receptor model originally proposed by Farnsworth (1993). In the new model, active Na^+, K^+-ATPase located in apical and lateral membrane domains of prostatic epithelial cells maintain an inwardly directed Na^+ gradient essential for uptake of aspartate (and other nutrients) as well as secretion of citrate (Fig. 7). Na^+, K^+-ATPase probably regulates the concentration of lumenal Na^+ and K^+, major counter-ions for citrate. Although the molecular identity of other transport systems responsible for reabsorption of excess counter-ions from the prostate lumen remains unknown, preliminary data (Mobasheri, A., unpublished data) suggest that the $Na^+/K^+/2Cl^-$ co-transporter and Na^+/H^+ exchanger are likely to be involved.

It is clear that the plasma membrane is the most

obvious target for drugs. Identification of ion transport systems involved in the process of cellular proliferation may enhance the strategy for future pharmacological intervention aimed at suppressing accelerated proliferation of prostate cells observed with aging in benign prostatic hyperplasia and invasive carcinomas of the prostate. This is particularly consistent with recent observations of Laniado et al. (1997) and Smith et al. (1998) demonstrating that voltage activated Na^+ channels are upregulated in highly metastatic cells derived from invasive carcinomas of the prostate and that inhibition of these Na^+ channels with highly specific natural toxins abolishes invasive behavior *in vitro*. Ion channel expression is altered during neoplastic transformation (Caffrey et al., 1987) and this will affect the expression and abundance of other transport systems. Future studies in this area will be aimed at identification of Na^+, K^+-ATPase isozymes and other Na^+ dependent transport systems in human prostate cancer. Where ion transport systems play indispensable physiological functions in the prostate, existing or novel pharmacological agents may target them making it possible to control and thus modulate the biosynthetic activity and proliferative capability of prostate cells (Foster et al., 1999). Therefore, our results suggest that Na^+, K^+-ATPase plays a key role in ion homeostasis in the prostate gland. Whether Na^+, K^+-ATPase is an active or passive participant in pathologies of the prostate gland or male infertility in general, remain open but challenging questions.

Acknowledgments. This study was inspired by the original work of Professor Leslie Costello (University of Maryland) and Professor Wells Farnsworth (Northwestern University Medical School). Their early hypotheses and their proposed models for citrate secretion in the prostate motivated this study. Research supported by grants from The Wellcome Trust (UK), The University of Westminster, Intersep Ltd. (Wokingham, UK) and The Teaching Company Directorate (Department of Trade & Industry, UK, Programme No. 2733) to A. Mobasheri. We are also grateful to the Association for International Cancer Research (M.B.A. Djamgoz) and FIS, Spain (P. Martín-Vasallo). Artwork in figures 6 and 7 courstesy of M. Batuwangala (King's College, London).

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Accepted September 29, 2000